Application of high performance hydrogels in tissue engineering and supercapacitors

By

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A Thesis submitted to the Faculty of Graduate Studies of
The University of Manitoba
In partial fulfilment of the requirements of the degree of

MASTER OF SCIENCE

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University of Manitoba
Winnipeg

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Abstract

Hydrogels, gels that mainly consist of water, have found their way in numerous applications. Hydrogels are currently under extensive research in the research fields such as: tissue engineering, conductive/unconducive biosensors, bio-actuators, and energy storage devices. In the present text, the application of hydrogels in 3D bioprinting for tissue engineering as well as application of thin film hydrogels in energy storage devices are investigated. To this end, a specific 3D bioprintable hydrogel for fabrication of tissue engineering scaffolds and thin film electrodes for use in supercapacitors were developed.

The first chapter in the present text is dedicated to hydrogels and their various applications. In chapter two, recent trends and advances in 3D bioprinting are reviewed. 3D bioprinting, an additive manufacturing based technology for precise 3D construction, is currently widely employed to enhance applicability and function of cell laden scaffolds. Research on novel compatible biomaterials for bioprinting is an essential prerequisite toward advancing 3D printing applications in tissue engineering. Printability and cell encapsulation and support are the main factors to be considered in development of 3D bioprinting. An effort has been made to include high performance bioinks and studies with diverse types of crosslinking methods such as photo, chemical and ultraviolet (UV) in this chapter.

In chapter three, a 3D printable bioink based on chitosan is developed that exhibits fast gelation, suitable mechanical properties to print 3D structures, biodegradability, and chondrocyte cells support. This novel bioink is employed by calcium physical crosslinking to avoid toxicity-related difficulties attributed to chemical crosslinking. Furthermore, unlike other physically crosslinked biomaterials, such as alginate, it promotes cell attachment and proliferation. Herein, chitosan is
modified by ethylenediaminetetraacetic acid (EDTA) to provide more carboxyl groups for physical crosslinking and increase the stability of the hydrogel. Dynamic mechanical analysis was carried out by rheology testing with concern to evaluate viscoelastic properties. Chondrocyte cells’ viability and proliferation were evaluated by live/dead, EdU assay, rt-PCR, respectively. Based on these results, we can deduce that the presented chitosan-based bioink qualifies as a candidate for 3D printable bioink capable of bioprinting tissue-like structures.

In chapter four, application of thin film hydrogels in supercapacitors has been studied. To this end, flexible electrodes were prepared by vacuum filtration of blends of chitosan (CS) and graphene oxide (GO). Reduction of GO/CS electrodes was carried out by Hydroiodic acid (HI) to obtain rGO/CS membranes. GO reduction was then followed by multiple in situ polymerization of polyaniline (PANI). One benefit of using CS in our method is to prevent the formation of highly stacked graphene sheets and therefore enhancing the ionic conductivity. Through utilization of this combination, we have combined the benefits of each material by fabricating a mechanically robust and flexible electrode offering outstanding energy storage capabilities.

Finally, in chapter five, a brief summary of the present text as well as future outlook of the studied research fields is presented.
Acknowledgments

I would like to express my sincere gratitude to my advisor Dr. Malcolm Xing for the continuous support of my studies. His guidance helped me in research and writing of this thesis. Besides my advisor, I would like to thank the rest of my thesis committee: Dr. Zhong and Dr. Wu for their insightful comments and encouragement.

I thank my fellow lab mates, Dr. khosrozadeh, Dr. Liu, Dr. Darabi, and Dr. Mbeleck for their constructive advices and trainings. Last but not least, I would like to thank my mother for her continuous support and encouragement and unconditional love throughout my life.

This research was supported in part by the University of Manitoba, Graduate Enhancement of Tri-Council Stipends (GETS) program.
# Contents

Abstract .................................................................................................................. i

Acknowledgments .................................................................................................... iii

Contents ...................................................................................................................... iv

List of Tables ............................................................................................................. viii

List of Figures ............................................................................................................ ix

List of abbreviations ............................................................................................... xiii

Chapter 1 ..................................................................................................................... 1

1 Introduction ............................................................................................................. 1

1.1 Applications of hydrogels .................................................................................. 1

1.2 Hydrogels in tissue engineering and biomedical applications ......................... 2

1.3 3D bioprinting of hydrogels ............................................................................... 3

1.4 Hydrogels in supercapacitors ........................................................................... 15

1.5 Objectives .......................................................................................................... 15

Chapter 2 ..................................................................................................................... 16
2 3D bioprinting for biomedical devices and tissue engineering: a review of recent trends and advances ................................................................. 16

2.1 Introduction .................................................................................. 16

2.2 Extrusion-based bioprinting ............................................................ 17

2.3 Inkjet bioprinting ........................................................................... 27

2.4 Stereolithography-based bioprinting .............................................. 29

2.5 Laser-assisted bioprinting .............................................................. 33

2.6 High performance bioink ............................................................... 35

2.7 Challenges, applications and future perspective ............................ 39

2.8 Summary and conclusions ............................................................ 41

Chapter 3 .......................................................................................... 43

3 Characterization and application of an Engineered Carboxymethyl chitosan-based Bioink ..... 43

3.1 Introduction .................................................................................. 43

3.2 Experimental Section ..................................................................... 47

3.2.1 Materials .................................................................................. 47

3.2.2 Mechanical characterization ....................................................... 47

3.2.3 Hydrogel Preparation Methods ................................................... 48
3.2.4 Printing Method................................................................................................................. 48
3.2.5 Rabbit Chondrocyte Isolation and Cell Seeding ............................................................. 50
3.2.6 Proliferation Assays ........................................................................................................ 50
3.2.7 Cell Viability Assessment ............................................................................................... 50
3.2.8 Quantitative Real-Time Polymerase Chain Reaction (PCR) Analysis ...................... 51
3.3 Results and discussion........................................................................................................ 52
  3.3.1 FT-IR and NMR analysis ............................................................................................... 52
  3.3.2 Specification of the Bioinks ......................................................................................... 54
  3.3.3 Swelling ratio .............................................................................................................. 60
  3.3.4 Chondrocyte viability evaluation ............................................................................... 61
  3.3.5 Chondrocyte proliferation evaluation ......................................................................... 62
  3.3.6 Chondrogenic markers expression .............................................................................. 63
3.4 Conclusion......................................................................................................................... 64

Chapter 4 ................................................................................................................................ 65

4 Fabrication of PANI-coated flexible membranes based on chitosan and graphene ........... 65
  4.1 Introduction ..................................................................................................................... 65
  4.2 Experimental section ...................................................................................................... 70
4.2.1 Materials............................................................................................................................................. 70

4.2.2 Preparation of GO/CS composite membranes ........................................................................... 70

4.2.3 Coating of PANI on the membranes ......................................................................................... 70

4.2.4 Characterization ......................................................................................................................... 71

4.2.5 Electrochemical measurements ............................................................................................... 71

4.3 Results and discussion .................................................................................................................. 72

4.4 Conclusions ...................................................................................................................................... 78

Chapter 5 .................................................................................................................................................. 80

5 Summary and future Works ............................................................................................................... 80

6 Bibliography ........................................................................................................................................ 81
List of Tables

Table 1-1. A brief review of common bioprinting techniques .......................................................... 4

Table 1-2. A short summary of outstanding recent bioprinting studies ............................................... 7

Table 3-1. A short summary of outstanding bioprinting studies ............................................................ 45

Table 4-1. Composition of three types of electrodes fabricated. ............................................................ 73

Table 4-2. Comparison of maximum areal capacitance in references with similar composition ...... 78
List of Figures

Figure 2-1. Schematic diagram of common extrusion-based bioprinting methods: (A) pneumatic, (B) Piston-driven, and (C) screw-driven dispensing method. In pneumatic dispensing air pressure provides the driving force while in piston and screw-driven dispensing, mechanical displacement and rotation are utilized to drive a continuous flow of biomaterial through the nozzle. ........................ 20

Figure 2-2. Schematic diagram of drop-on-demand inkjet printing method using A) Thermal, and B) Piezoelectric actuators. A thermal printing head employs a heating element that raises the temperature locally and creates a bubble that drives droplets through the nozzle. A piezoelectric head is utilized with a material that changes shape upon voltage application and pushes droplets out. ........................................................................................................................................... 28

Figure 2-3. Schematic diagram of stereolithography using beam projector. Focused light beams allow for precise photopolymerization of layers of light-sensitive polymer to apply any desired pattern to the bioink................................................................. 30

Figure 2-4. Schematic diagram of laser-assisted bioprinting. A nozzle-free technique using pulsed laser source to deposit microdroplets of bioink with/without cells on a substrate......................... 34

Figure 2-5. 3D printed constructs of conductive and nonconductive bioinks. A) A typical chitosan-based extrusion bioprinted mesh structure, B-D) a conductive 3D printed sensor based on chitosan and acrylic acid, sealed in PDMS. The resistance response at various bending angles from testing the hydrogel as a sensor in strip form (left) and in 3D printed mesh form (right) is displayed. © 2017 Reprinted with permission of John Wiley and Sons [119].................................................. 37
Figure 3-1. Schematic diagram of hydrogel preparation and printing: (A) First step: Chitosan reacting with EDTA, unreacted carboxyl groups (green) take part in the next step. (B) Second step: additional Chitosan is added to the solution and crosslinked with CaCl₂ solution after printing to form hydrogel. (C) Hydrogel printing method. 49

Figure 3-2. Schematic diagram of chemical synthesis of hydrogel. 52

Figure 3-3. FTIR spectra of (A) water soluble chitosan without any modification, (B) modified chitosan (chitosan-EDTA), and (C) freeze-dried hydrogel (after gelation by adding 1 M calcium solution). Chitosan/CE conjugate ratio is 90:10 in (B) and (C). 53

Figure 3-4. ¹H NMR spectrum of the EDTA modified chitosan hydrogel network at 300 MHz. Chitosan/CE conjugate ratio of the sample tested is 90:10. 54

Figure 3-5. Printed samples with different chitosan:CE ratios. Ratios outside of a certain range result in poor and inaccurate printing. (A) Highly viscous bioink resulting in a discontinuous print. (B) Highly viscous bioink printed by using a large diameter needle resulting in an inaccurate print. (C) Low viscous bioink incapable of holding its shape after printing. (D) An accurate printed structure with chitosan:CE ratio of 90:10. The mesh size is 25 x 25 mm and the diameter of the printing needle is 0.5 mm. 55

Figure 3-6. Micrographs of a printed five-layer hydrogel. (A) Bonding of two layers in junctions. (B, C) Layers of the 3D printed hydrogel. Chitosan/CE conjugate ratio of the sample shown is 90:10. 56

Figure 3-7. (A) Storage and (B) loss modulus comparison of chitosan-based hydrogel with NFC/Alginate hydrogel [61]. Four Chitosan/CE conjugate ratios tested. (C) Storage modulus, G’ and loss modulus, G” of the bioink as a function of crosslinking time. Solid lines represent 45 minutes of
crosslinking and dashed lines represent 30 minutes of crosslinking. 1M CaCl2 solution is used as the crosslinking agent (45 minutes) …………………………………………………………………………………… 57

Figure 3-8. Study of crosslinker concentration effect on gel retraction and appearance. Images of hydrogel discs crosslinked with (A) 0.1M, (B) 0.5M, (C) 1M, and (D) 2M CaCl2 solution. Top images in each set represent gel precursor before final crosslinking and bottom images represent the resulting gel after crosslinking. Chitosan/CE conjugate ratio of the samples shown is 90:10 and the crosslinking time is 45 minutes for all samples. …………………………………………………………………………………… 58

Figure 3-9. (A, B) Study of crosslinking concentration effect on compressive strength of the hydrogel and comparison with NFC/Alginate hydrogel14. (C) Storage modulus, G’ and loss modulus, G” of the bioink as a function of crosslinking concentration. Solid lines represent storage modulus and dashed lines represent loss modulus. (D) Study of shear thinning properties of four Chitosan/CE conjugate ratios. (E) Study of swelling ratio in samples crosslinked with four concentrations of calcium solution ranging from 0.1M to 2M. In (A), (B), (C) and (E), Chitosan/CE conjugate ratio of the samples tested is 90:10 and the crosslinking time is 45 minutes for all samples. …………………………………………………………………………………………………………………………………………………………………………………… 59

Figure 3-10. (A) Live/Dead staining of chondrocytes. (B) Flow cytometry result of cell viability in the control group. (C) Flow cytometry result of cell viability in the hydrogel mesh group. (D) Quantification of cell viability in both groups. Scale bar = 100um. ……………………………………………………… 62

Figure 3-11. (A) EdU staining of chondrocytes in both group. (B) Quantification of chondrocytes proliferation rate in both group. Scale bar =100 um. …………………………………………………………………………………… 63

Figure 3-12. Chondrogenic markers expression. ……………………………………………………………………………………………… 64
Figure 4-1. Schematic showing the effect of chitosan on enlarged interlayer spacing and enhanced PANI coating ................................................................. 69

Figure 4-2. Photographs of freestanding and flexible film of graphene oxide and chitosan. .......... 72

Figure 4-3. SEM images of GC95 after first (A, B), second (C, D), and third (E, F) PANI coating. Magnification is 10K for the images on the left column and 20K for the images on the right .......... 73

Figure 4-4. SEM images of (A, B) GC90 and (C, D) GC95 after thrice PANI coatings. Magnification is 5K for the images on the left column and 50K for the images on the right .......... 74

Figure 4-5. Nyquist plots for three weight ratios of GO/CS. Right image shows higher magnification of the semicircular part of the graphs. The samples with 95 and 97% GO display the minimum charge-transfer resistance in this group ................................................................. 75

Figure 4-6. GCD plots of the sample with 95% Go and trice PANI coatings at various current densities. Higher magnification of the higher current densities are shown in the right image .......... 76

Figure 4-7. (A) mass-specific and (B) areal-specific capacitance versus current density for SCs with GO weight ratios ranging from 90-100% ......................................................... 77

Figure 4-8. (A) mass-specific and (B) areal-specific power density versus energy density for SCs with GO weight ratios ranging from 90-100% ......................................................... 77
# List of abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>SC</td>
<td>Supercapacitor</td>
</tr>
<tr>
<td>GO</td>
<td>Graphene oxide</td>
</tr>
<tr>
<td>CS</td>
<td>Chitosan</td>
</tr>
<tr>
<td>rGO</td>
<td>Reduced graphene oxide</td>
</tr>
<tr>
<td>HI</td>
<td>Hydroiodic acid</td>
</tr>
<tr>
<td>PANI</td>
<td>Polyaniline</td>
</tr>
<tr>
<td>ELD</td>
<td>Electrical double layer</td>
</tr>
<tr>
<td>AM</td>
<td>Additive manufacturing</td>
</tr>
<tr>
<td>EDTA</td>
<td>(Ethylenedinitrilo)tetraacetic</td>
</tr>
<tr>
<td>APS</td>
<td>Ammonium persulfate</td>
</tr>
</tbody>
</table>
Chapter 1

1 Introduction

1.1 Applications of hydrogels

Hydrogels have been studied in various applications for more than a century. Due to the advances in chemistry and polymer science in the past few decades, hydrogels have gained enormous attention and found specific applications in diverse fields in medical sciences and engineering. Development of hydrogels with properties such as elasticity/rigidity, conductivity, PH/temperature/humidity/light sensitivity, hydrophilicity/hydrophobicity, and self-healing has paved the way for the employment of hydrogels in distinct applications such as scaffolds for tissue engineering, biosensors, and energy storage devices. Since essential properties for biomedical applications such as biodegradability and biocompatibility was achievable in hydrogels, biomaterial scholars gained interest in hydrogels since 1970s [1-3]. Application of hydrogels based on synthetic or natural polymers in development of scaffolds for tissue engineering is currently under extensive research. In this regard, 3D printing has been employed as a relatively precise fabrication method for scaffolds. For the cell studies in tissue engineering, the main advantage of using hydrogels is the very high water content of hydrogels which is similar to human body. Also, hydrogels could be loaded with nutrition and growth factors to enhance cell viability and proliferation. Therefore, combining benefits of using hydrogels and precise distance between the cells by 3D printing, tissue engineering applications of hydrogels have now caught more attention than before. Also, conductive hydrogels achievable by
using conductive polymers or addition of conductive carbon derivatives have been used in biosensor
and electrical skin applications [4-7].

1.2 Hydrogels in tissue engineering and biomedical applications

Every year, thousands of patients suffer from organ or tissue damage resulted from diseases or
accidents. As the main process involved in cell growth and reconstruction of organs, tissue
regeneration is currently under extensive study. Organ transplantation, replacement and repair are
the options for patients with damaged organs depending on the situation and intensity of the
damage. Extensively long waiting lists for organ transplantation exist all around the world.
According to U.S. Department of Health & Human Services, as of June 2017, around 120000
patients were in need of lifesaving organ transplant in the United States while only about 5200
donors were available. Also, while the number of transplants performed every year since 2003 has
been somehow constant, the number of patients waiting at the year-end has been growing
([https://optn.transplant.hrsa.gov](https://optn.transplant.hrsa.gov)). Costly treatments and surgeries are also another reason that has
led to more interest in new methods in tissue engineering research. Under these circumstances,
scientists are eager to find alternative ways to compensate for this shortage of organ. Tissue
engineering, on the other hand, has been considered as an effective method to help save lives and
improve the quality of life. Since proposed in 1993 [8], tissue engineering has been intended to
develop practical replacements for damaged tissue by means of applying biology and engineering
principles. Hydrogels are of interest in the field of tissue engineering as templates or scaffolds for
repair and regeneration of various tissues and organs. Scaffolds have found their place in tissue
engineering as templates for cell interaction, providing physical support to the afresh developed
tissue [9]. Also, scaffolds can function as delivery vehicles to incorporate essential growth factors to control and enhance tissue growth [10].

Hydrogels are required to possess certain properties and meet specific design criteria to be beneficial in tissue formation. These properties can be categorized as physical and biological parameters [11]. Physical category includes properties such as degradation and mechanics while biological category includes properties such as cell adhesion and biocompatibility. Biocompatibility is the ability of the hydrogels to interact with body tissue and cells without causing any damage or inflammatory response. Biocompatibility can be a concern with synthetic polymers while natural polymers are often more likely to be biocompatible. Some of the most common natural polymers include collagen, gelatin, alginate, agarose, and chitosan while poly(acrylic acid), poly(ethylene oxide), poly(vinyl alcohol), and polypeptides are among the common synthetic polymers employed in hydrogels. Frequently used crosslinking mechanisms are physical (ionic) and chemical (covalent) crosslinking. Ionic crosslinking is simple and effective method for hydrogel gelation. However, ions could engage in other reactions when the hydrogel comes in contact with other ionic solutions and result in uncontrolled degradation of the hydrogel. Covalent crosslinking, on the other hand, offers much better control on the degradation of the hydrogel. However, non-degradability and toxicity concerns are often attributed to chemical crosslinking.

1.3 3D bioprinting of hydrogels

As the main process involved in cell growth and reconstruction of organs, tissue regeneration is currently under extensive study. Organ transplantation, replacement and repair are the options for patients with damaged organs depending on the situation and intensity of the damage. Extensively long waiting lists for organ transplantation exist all around the world. According to U.S. Department
of Health & Human Services, as of June 2017, around 120000 patients are in need of lifesaving organ transplant in the United States while only about 5200 donors are available. Also, while the number of transplants performed every year since 2003 has been somehow constant, the number of patients waiting at the year-end has been growing (https://optn.transplant.hrsa.gov). Under these circumstances, scientists are eager to find alternative ways to compensate for this shortage of organ. Tissue engineering, on the other hand, has been considered as an effective method to help save lives and improve the quality of life. Since proposed in 1993 [8], tissue engineering has been intended to develop practical replacements for damaged tissue by means of applying biology and engineering principles. Scaffolds have found their place in tissue engineering as templates for cell interaction, providing physical support to the afresh developed tissue [9]. Also, scaffolds can function as delivery vehicles to incorporate essential growth factors to control and enhance tissue growth [10]. A combination of cells and biomaterials is often employed as the printing precursor in 3D bioprinting of scaffolds. 3D Bioprinting is an actively studied method in tissue engineering since it shows effective control over scaffold fabrication and cell distribution. Printing resolution of 3D bioprinting techniques is 10-10000 μm which is a wide range showing flexibility of bioprinting compared to other assembly methods such as molding and porous scaffolds [12, 13].

**Table 1-1. A brief review of common bioprinting techniques**

<table>
<thead>
<tr>
<th>Extrusion</th>
<th>Inkjet</th>
<th>Stereolithography</th>
<th>Laser-assisted</th>
</tr>
</thead>
<tbody>
<tr>
<td>Advantages</td>
<td>Simple, capable of printing various</td>
<td>Ability to print low viscosity biomaterials, fast fabrication speed,</td>
<td>Nozzle-free technique, printing time independent of complexity [14, 15],</td>
</tr>
</tbody>
</table>
Introduction

<table>
<thead>
<tr>
<th>biomaterials</th>
<th>low cost, high resolution</th>
<th>high accuracy and cell viability</th>
<th>solid or liquid phase</th>
</tr>
</thead>
<tbody>
<tr>
<td>, ability to print high cell densities</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Drawbacks**

- Only applicable for viscous liquids
- Inherent inability to provide a continuous flow [16], poor functionality for vertical structures, low cell densities
- UV light source and near-UV blue light’s toxicity to cells [17, 18], lack of printing multicells, and damage to cells during photo curing [19]
- High cost, thermal damage due to nanosecond/femtosecond laser irritation [20]

|-------|---------------|---------------|-----------|-------------|

|------|-------------------|-------------|-------------|---------------|

|---------------------------|-----------|-----------|-----------|-------------|
Introduction

<table>
<thead>
<tr>
<th>Cell viability</th>
<th>89.46±2.51% [24]</th>
<th>80-95% [25, 26]</th>
<th>&gt;90% [27, 28]</th>
<th>&lt;85% [20]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell density</td>
<td>High [29]</td>
<td>Low [29]</td>
<td>Medium [29]</td>
<td>Medium [29]</td>
</tr>
<tr>
<td>Resolution</td>
<td>100 µm [16]</td>
<td>50 µm [16]</td>
<td>100 µm [27, 28]</td>
<td>10 µm [30]</td>
</tr>
</tbody>
</table>

As an additive manufacturing technique, 3D bioprinting is based on deposition of biomaterials, either encapsulating cells or loaded with cells later on, in micrometer scale to form subtle structures comparable to tissue. In most cases, a three-axis mechanical platform controls the movements of extruders printing the bioink in the required algorithm and shape. This platform’s movement is governed by coordinates created by the designer and saved in a file format such as g-code that could be easily followed by the printer. Due to vantages such as precise deposition, cost-effectiveness, simplicity, and cell distribution controllability, 3D bioprinting development and application has been increasing constantly over the past few years. As a result, need for new bioinks providing required properties for successful printing, such as printability, printing fidelity, and mechanical properties has been rising leading to extensive work to develop new materials. In the present review, an account of the most recent and functional research studies on bioinks and bioprinting developments is presented. To this end, first outstanding works in major bioprinting methods, including extrusion-based, inkjet, stereolithography-based, and laser-assisted bioprinting methods, are reviewed. Also, a brief review of the above mentioned bioprinting techniques is presented in Table 1-1 and a short
summary of recent outstanding bioprinting studies is tabularized in Table 1-2. Next, the most fundamental recent studies in bioink development and applications are cited in “High performance bioink” section. Later on, challenges in bioink development and bioprinting, as well as applications and future perspective of bioprinting is discussed. Finally, a short summary of the present article is presented.

**Table 1-2. A short summary of outstanding recent bioprinting studies**

<table>
<thead>
<tr>
<th>Ref.</th>
<th>Material</th>
<th>Method</th>
<th>Commercial</th>
<th>Application</th>
<th>Research summary</th>
</tr>
</thead>
<tbody>
<tr>
<td>[31]</td>
<td>Nanocellulose</td>
<td>Extrusion</td>
<td>Y</td>
<td>Wound dressing</td>
<td>Development of 3D porous structures</td>
</tr>
<tr>
<td>[32]</td>
<td>Alginate</td>
<td>Extrusion</td>
<td>N</td>
<td>Bioprinting of tissue/organ</td>
<td>New micro-fabrication technique to create tissue strands as a “bioink”</td>
</tr>
<tr>
<td>[9]</td>
<td>Collagen/gelatin/alginate hydrogel</td>
<td>Extrusion</td>
<td>N</td>
<td>Tissue engineering (general)</td>
<td>Printing cell-laden hydrogel to study cell proliferation</td>
</tr>
<tr>
<td>[12]</td>
<td>Gamma-irradiated alginate, poly(ε-caprolactone) (PCL) fibers</td>
<td>Extrusion</td>
<td>Y</td>
<td>Whole bone organ engineering</td>
<td>Biofabrication and in vitro and in vivo analysis of mechanically reinforced cartilaginous template</td>
</tr>
<tr>
<td>Study Reference</td>
<td>Bioinks/Techniques</td>
<td>Bioprinting Method</td>
<td>Success Rate</td>
<td>Tissue Regeneration and Therapy</td>
<td>Printing/Object</td>
</tr>
<tr>
<td>-----------------</td>
<td>--------------------</td>
<td>--------------------</td>
<td>--------------</td>
<td>---------------------------------</td>
<td>-----------------</td>
</tr>
<tr>
<td>[33]</td>
<td>M13 phages and alginate</td>
<td>Extrusion</td>
<td>N</td>
<td>Regeneration of various tissues</td>
<td>Printing 3D cell-laden matrices using genetically engineered M13 phage</td>
</tr>
<tr>
<td>[34]</td>
<td>Collagen, alginate, human adipose stem cells (hASCs)</td>
<td>Extrusion</td>
<td>N</td>
<td>Tissue regeneration and cell therapy</td>
<td>Fabrication and study of cell-laden 3D printed core-sheath structure</td>
</tr>
<tr>
<td>[35]</td>
<td>Alginate, carboxymethyl-chitosan, and agarose</td>
<td>Extrusion</td>
<td>Y</td>
<td>Neural tissue</td>
<td>Direct-write printing of cell-laden bioink to engineer a novel functional 3D neural mini-tissue construct</td>
</tr>
<tr>
<td>[37]</td>
<td>Gelatin-based bioinks</td>
<td>Extrusion</td>
<td>N</td>
<td>A referable template for designing new</td>
<td>Study of printing parameters effect on cell survival rate and printability</td>
</tr>
<tr>
<td>Reference</td>
<td>Bioinks</td>
<td>Technique</td>
<td>Crosslinked</td>
<td>Application</td>
<td></td>
</tr>
<tr>
<td>-----------</td>
<td>------------------------------------------------------------------------</td>
<td>-------------------</td>
<td>-------------</td>
<td>-----------------------------------------------------------------------------</td>
<td></td>
</tr>
<tr>
<td>[38]</td>
<td>Poly(ethylene glycol) diacrylate (PEGDA), gelatin methacrylate (GelMA), eosin Y based photoinitiator</td>
<td>Stereolithography</td>
<td>N</td>
<td>Development of a low-cost printing system for visible light stereolithography solution</td>
<td></td>
</tr>
<tr>
<td>[39]</td>
<td>Alginate, PCL/alginate mesh</td>
<td>Extrusion</td>
<td>N</td>
<td>Regeneration of hard tissue Fabrication and in vitro study of mechanically reinforced cell-laden scaffolds</td>
<td></td>
</tr>
<tr>
<td>[40]</td>
<td>Methacrylated gelatin (GM) and mature adipocytes</td>
<td>-</td>
<td>-</td>
<td>Adipose tissue engineering Evaluation of photo-crosslinkable (GM) and mature adipocytes as for 3D fatty tissue constructs</td>
<td></td>
</tr>
<tr>
<td>[41]</td>
<td>Hyaluronic acid</td>
<td>Extrusion</td>
<td>Y</td>
<td>Tissue engineering (general) Development of a dual-crosslinking hyaluronic acid hydrogel as a bioink</td>
<td></td>
</tr>
<tr>
<td>[42]</td>
<td>Polyurethane (PU), c2c12 cells, NIH/3T3 cells, hyaluronic acid,</td>
<td>Extrusion</td>
<td>N</td>
<td>Muscle–tendon unit Development of a complex tissue construct for use in muscle–tendon tissue</td>
<td></td>
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<td></td>
<td>Gelatin, fibrinogen</td>
<td>Extrusion</td>
<td>N</td>
<td>Liver tissue engineering</td>
<td>Development and evaluation of 3D printed constructs for liver tissue engineering</td>
</tr>
<tr>
<td>---</td>
<td>---------------------</td>
<td>-----------</td>
<td>---</td>
<td>--------------------------</td>
<td>--------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>43</td>
<td>PCL, collagen, and three different types of cells</td>
<td>Extrusion</td>
<td>Y</td>
<td>Tissue engineering (general)</td>
<td>Development of bioinks suitable for freeform fabrication</td>
</tr>
<tr>
<td>44</td>
<td>Gelatin, polyethylene oxide (PEO), HEK293 cells, human umbilical vein endothelial cells (HUVECs)</td>
<td>Extrusion</td>
<td>Y</td>
<td>Tissue engineering (general)</td>
<td>Finding a way to use pluronic as a biocompatible ink for 3D printing</td>
</tr>
<tr>
<td>45</td>
<td>Acrylated, pluronic F127</td>
<td>Extrusion</td>
<td>Y</td>
<td>Tissue engineering (general)</td>
<td>Introduction of a new cell dispensing method using a core-shell nozzle</td>
</tr>
<tr>
<td>46</td>
<td>Alginate in phosphate-buffered saline (PBS), hASCs</td>
<td>Extrusion</td>
<td>N</td>
<td>Hepatogenic differentiation of hASCs -embedded mesh structures</td>
<td>Introduction of a new cell dispensing method using a core-shell nozzle</td>
</tr>
<tr>
<td>47</td>
<td>Collagen/extracellular matrix (ECM) and alginate,</td>
<td>Extrusion</td>
<td>-</td>
<td>Tissue engineering (general)</td>
<td>Introduction of a strategy for obtaining highly bioactive alginate-based ink</td>
</tr>
</tbody>
</table>
**Introduction**

<table>
<thead>
<tr>
<th>Reference</th>
<th>Material/Cell Type</th>
<th>Technique</th>
<th>Success Rate</th>
<th>Application</th>
</tr>
</thead>
<tbody>
<tr>
<td>[48]</td>
<td>Hyaluronic acid and gelatin</td>
<td>Extrusion</td>
<td>N</td>
<td>Primary liver constructs with high viability</td>
</tr>
<tr>
<td>[49]</td>
<td>Type I collagen and chitosan–agarose blends, human bone marrow derived mesenchymal stem cells (hMSCs)</td>
<td>Extrusion</td>
<td>N</td>
<td>3D printed mesenchymal tissues</td>
</tr>
<tr>
<td>[50]</td>
<td>Decellularized adipose tissue (DAT) matrix bioink, hASCs</td>
<td>Extrusion</td>
<td>N</td>
<td>Soft tissue regeneration</td>
</tr>
<tr>
<td>[51]</td>
<td>Alginate, GelMA, HUVECs</td>
<td>Extrusion</td>
<td>N</td>
<td>Tissue engineering (general)</td>
</tr>
<tr>
<td>[52]</td>
<td>Spider silk protein, human fibroblasts</td>
<td>Extrusion</td>
<td>Y</td>
<td>Tissue engineering (general)</td>
</tr>
</tbody>
</table>

Development of stable printable bioink

Study of purpose-driven printing and the parameters affecting printing quality

Devising a biomimetic approach for printing adipose tissue constructs employing decellularized adipose tissue

Development of a versatile 3D bioprinting technique and a novel low viscosity alginate-based bioink

Development of a novel bioink without the need for post processing and better shear
<table>
<thead>
<tr>
<th>Reference</th>
<th>Description</th>
<th>Method</th>
<th>Y/N</th>
<th>Detailed Information</th>
</tr>
</thead>
<tbody>
<tr>
<td>[53]</td>
<td>Poly(N-isopropylacrylamide), poly(N-isopropylacrylamide) grafted hyaluronan (HA-pNIPAAM), methacrylated hyaluronan (HAMA)</td>
<td>Extrusion</td>
<td>Y</td>
<td>Thinning properties compared to alginate</td>
</tr>
<tr>
<td>[54]</td>
<td>Sodium alginate, sodium periodate, Arginylglycylaspartic acid (RGD) peptides</td>
<td>Extrusion</td>
<td>Y</td>
<td>3D printing at physiological temperature of a range of biopolymer solutions Improving glycosaminoglycan-based hydrogels’ printing by blending</td>
</tr>
<tr>
<td>[55]</td>
<td>Fibroblasts, sodium alginate, polystyrene microbeads and</td>
<td>Droplet-based</td>
<td>Y</td>
<td>Controlled degradation of oxidized alginates in bioprinting Evaluation of alginate hydrogels with varied oxidation percentages and concentrations as bioinks</td>
</tr>
<tr>
<td></td>
<td>Tissue engineering (general)</td>
<td>Study of droplet formation and inkjet printing quality of a cell-laden alginate-based bioink</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Reference</td>
<td>Cells &amp; Materials</td>
<td>Fabrication Method</td>
<td>Success Rate</td>
<td>Field of Study</td>
</tr>
<tr>
<td>-----------</td>
<td>------------------</td>
<td>--------------------</td>
<td>--------------</td>
<td>----------------</td>
</tr>
<tr>
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<td>3T3 cells</td>
<td>Droplet-based</td>
<td>Y</td>
<td>Tissue engineering (general)</td>
</tr>
<tr>
<td>[57]</td>
<td>Gelatin, methacrylamide, gellan gum</td>
<td>Extrusion</td>
<td>N</td>
<td>Tissue engineering (general)</td>
</tr>
<tr>
<td>[58]</td>
<td>MG63 cells, alginate, PCL electrospun scaffold,</td>
<td>Laser-assisted</td>
<td>N</td>
<td>Tissue engineering (general)</td>
</tr>
<tr>
<td>[59]</td>
<td>Polylactic acid, gelatin methacrylamide-gellan gum, Mesenchymal stem cells (MSCs)</td>
<td>Extrusion</td>
<td>Y</td>
<td>Living tissues constructs</td>
</tr>
<tr>
<td>Reference</td>
<td>Materials</td>
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<td>Success</td>
<td>Field</td>
</tr>
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<td>-----------</td>
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<td>------</td>
</tr>
<tr>
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<td>Alginate, gelatin, hydroxyapatite, hMSCs</td>
<td>Extrusion</td>
<td>Y</td>
<td>Tissue engineering (general)</td>
</tr>
<tr>
<td>[61]</td>
<td>Nanofibrillated cellulose (NFC), alginate</td>
<td>Extrusion</td>
<td>Y</td>
<td>Bioprinting of living tissues and organs</td>
</tr>
<tr>
<td>[62]</td>
<td>Various natural and synthetic materials such as PEG and gelatin</td>
<td>Extrusion</td>
<td>Y</td>
<td>Tissue engineering (general)</td>
</tr>
<tr>
<td>[63]</td>
<td>Silk fibroin, gelatin, Human turbinate mesenchymal stromal cells (hTMSCs)</td>
<td>Extrusion</td>
<td>N</td>
<td>Tissue engineering (general)</td>
</tr>
<tr>
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<td>Decellularized adipose (adECM), cartilage (cdECM), and heart (hdECM) tissue, PCL</td>
<td>Extrusion</td>
<td>N</td>
<td>Tissue engineering (general)</td>
</tr>
</tbody>
</table>
1.4 **Hydrogels in supercapacitors**

Recent advances in electronics and material science has opened new horizons in electronics research field. Ability to make stretchable and/or flexible electronics is currently a hot topic with potential applications in various industries such as wearable gadgets and portable electronics. Stretchable devices such as strain sensors, pressure sensors, and transistors have been developed [65-67]. Energy storage devices are one of the important applications for flexible/stretchable electronics. Among energy storage devices, supercapacitors have advantages such as fast charging/discharging and long cycle life over batteries. Current supercapacitor research is mostly based on development of membranes or films based on conductive polymers and carbon derivatives. Development of stretchable hydrogels as ionic conductors or electrolytes, contributes to fabrication of stretchable supercapacitors. Hydrogels in the form of membranes are fabricated by methods such as vacuum filtration of solutions containing conductive particles with the ability to store energy by electrical double layer (EDL) or pseudocapacitance.

1.5 **Objectives**

This work was carried out to study applications of hydrogels in diverse applications of tissue engineering and supercapacitor. Several characteristics were taken into account for each application. To introduce a thorough insight, this work is presented in the following chapters. In chapter two, various bioprinting methods as well as recent advances in 3D bioprinting and development of bioinks are cited. Later, in chapter three, a 3D bioprintable bioink developed based on chitosan is presented and characterized. In chapter four, a thin film supercapacitor fabricated by blending graphene oxide, polyaniline (PANI), and chitosan is presented and analyzed. Finally, in chapter five, summary and future perspective of the research in the current field is discussed.
2 3D bioprinting for biomedical devices

and tissue engineering: a review of recent trends and advances

2.1 Introduction

3D printing, an additive manufacturing based technology for precise 3D construction, is currently widely employed to enhance applicability and function of cell laden scaffolds. Research on novel compatible biomaterials for bioprinting exhibiting fast crosslinking properties is an essential prerequisite toward advancing 3D printing applications in tissue engineering. Printability to improve fabrication process and cell encapsulation are two of the main factors to be considered in development of 3D bioprinting. Other important factors include but are not limited to printing fidelity, stability, crosslinking time, biocompatibility, cell encapsulation and proliferation, shear-thinning properties, and mechanical properties such as mechanical strength and elasticity. In this review, we recite recent promising advances in bioink development as well as bioprinting methods. Also, an effort has been made to include studies with diverse types of crosslinking methods such as photo, chemical and ultraviolet (UV). We also propose the challenges and future outlook of 3D bioprinting application in medical sciences and discuss the high performance bioinks.
2.2 Extrusion-based bioprinting

Extrusion-based methods have been widely employed in recent years to provide researchers with alternative methods for scaffold fabrication. The extensive popularity of extrusion-based methods mostly relies on clear-cut processing method leading to simplicity, diversity and predictability of this technique. Bioinks having viscosity in the range of $30-6 \times 10^7$ mPa.s are reported to be printable via extrusion printing [21]. In comparison with inkjet bioprinting, extrusion-based bioprinting offers higher cell densities but lower speed and resolution [21]. Wide range of printable biomaterials and inexpensive equipment are among extrusion bioprinting advantages. Many researchers have simply modified conventional commercial 3D printers to print biomaterials or developed their printing machines in-house to reduce the costs [9, 32, 37, 39, 41-43, 46, 49-51, 57, 63, 64]. On the other hand, due to the need for development of bioprinters, commercial bioprinters have become widely available and employed by researchers [13, 31, 35, 45, 52-54, 59-62], focusing on enhancing the printing quality and suitability for printing wider range of biomaterials. A review of the outstanding research works using extrusion-based techniques is presented in this section. Moreover, Figure 2-1 illustrates common extrusion-based printing methods categorized into pneumatic, piston-driven, and screw-driven dispensing. In pneumatic dispensing, air pressure provides the required driving force, while in piston and screw-driven dispensing, vertical and rotational mechanical forces initiate printing, respectively.

There are three main factors to take into account toward printability via extrusion bioprinting: 1) adjustability of the viscosity, 2) bioink phase prior to extrusion, and 3) material-specific biofabrication window [19]. Viscosity can be a function of temperature or shear thinning and therefore, needs to be adjusted for different printing methods. Also, bioink needs to be in liquid phase to avoid nozzle clogging. Finally, not all biomaterials are printable and those which are
printable may not be printable in a wide range of processing parameters. To illustrate the current state of the art, the most recent extrusion bioprinting studies are cited in the following paragraphs.

To begin with, Rees et al. considered two types of oxidized nanocellulose 3D printed structures as wound dressings [31]. First type was prepared by (2,2,6,6-tetramethylpiperidin-1-yl) oxidanyl (TEMPO) mediated oxidation and the second type was prepared by carboxymethylation and periodate oxidation combined. The produced nanocellulose bioink was then used to print 3D porous structures, studied for bacterial growth support, and shown to have the potential to carry and release antimicrobial components while not supporting bacterial growth. Yu and Ozbolat utilized a coaxial nozzle system to print tissue strands as a bioink for organ printing [32]. Alginate-based bioink developed in this work showed mouse TC3 cell viability close to 90%. Also, human umbilical vein smooth muscle cells were incorporated in the bioink to fabricate structures similar to pancreatic tissue to further demonstrate the applicability of their method. In another study, a hydrogel based on gelatin, alginate, and collagen was used for cell-laden 3D printed tissue constructs [9]. One integral part of this work was to control the degradation rate of the hydrogel by changing the mole ratio of sodium citrate present in the medium to the sodium alginate present in the hydrogel. High cell proliferation rate indicated the possibility to improve the alginate bioink by utilizing the method used in this work.

Although bioprinting has been developing extensively in recent years, but the current technologies implemented in bioprinting are mostly incapable of printing functional solid organs. Researches have approached this issue by developing templates that could be used in vivo to support the development of vascularized solid organs such as bones [12]. Stem cells were encapsulated in a gamma-irritated alginate-based bioink that was further reinforced by adding PCL fibers. RGD
peptides were also incorporated to improve osteogenesis for bone tissue engineering applications. In this work, a cartilaginous construct similar to vertebral body was fabricated and shown to support vascularized bone development in vivo. In most cases, researchers use a combination of multiple biomaterials to achieve the required properties by the application. For instance, in one study, alginate and gellan were used along with BioCartilage (clinical product) to prepare a new-fashioned bioink for printing cartilage grafts proved to support chondrocytes’ proliferation [13]. Furthermore, a cation-loaded polymer was also utilized to stabilize overhanging structures in this strong but ductile bioink.

While alginate is a common biomaterial employed as bioink, most studies are based on native alginates with limited degradation. In a study, oxidized alginate hydrogels with various degrees of oxidization were studied as bioinks with controlled degradation [54]. Effect of viscosity and density of the alginate solutions on their printability was studied. Furthermore, alginate solutions with various biodegradability were loaded with hASCs and were shown to provide the ability to control proliferation and spreading of the cells.

Also, alginate-based bioinks often exhibit low cell-activating properties. Lee at al. tried to overcome this weakness by printing porous 3D constructs with a novel bioink consisted of collagen/ECM and alginate [47]. Cell studies showed that the developed bioink in this study displays decent cell viability and higher osteogenic activity compared to conventional bioinks based on alginate.
Figure 2-1. Schematic diagram of common extrusion-based bioprinting methods: (A) pneumatic, (B) Piston-driven, and (C) screw-driven dispensing method. In pneumatic dispensing air pressure provides the driving force while in piston and screw-driven dispensing, mechanical displacement and rotation are utilized to drive a continuous flow of biomaterial through the nozzle.

The biomaterials chosen for the bioink development play pivotal role in the research. For this reason, researchers prefer to use biomaterials previously proven to be compatible with cells in commercial or non-commercial products or devices. Hence, the variables in the projects are decreased and the outcome is more likely to be predicted. As an example, RGD-phage solution was employed in one study to develop a versatile bioink with cell printing ability [33]. In particular, M13 phages were shown to provide good blending properties with alginate. Also, the proliferation of MC3T3-E1 cells were shown to improve proportionally with concentration of phages. In another study, Pati et al., devised a new method to print cell-encapsulating DAT bioink [50]. Porous dome-shaped structures were prepared and tested in vitro and in vivo for cell viability and differentiation.
of hASCs. The DAT 3D printed constructs were found to express more adipogenic lineages than that of non-printed DAT gel.

Integrated organ printing (IOP) is a technology focused on tissue-like structures which is especially useful in systems with local differences in cell types and mechanical properties. Merceron et al. employed an IOP system to fabricate a muscle-tendon unit construct composed of four different elements [42]. Thermoplastic PU and PCL were used to provide the structure with elasticity for muscle development and stiffness for tendon development, respectively. These constructs showed above 80% cell viability one week after printing.

3D printing is considered as a new concept in tissue engineering. Previously, cell studies took place using 2D structures, but with the introduction of 3D printing to the tissue engineering, it became possible for researchers to use 3D scaffolds instead of 2D ones. Lee et al., for instance, worked on the development of 3D structures with improved mechanical properties for liver tissue regeneration [43]. A multi-head tissue printing system was employed to print PCL as a framework material to provide proper mechanical properties. Also, three types of cells were printed in the PCL canals to study liver cells’ proliferation. Results of this work suggested that the employed co-cultured microenvironment promoted heterotypic cellular interaction within a 3D construct.

In another study, gelatin was employed in free form fabrication of 2D and 3D cell encapsulating constructs [44]. PEO was also utilized with gelatin to enhance printing precision. Printed hydrogels showed support for cell proliferation and spreading. Skardal et al., in another study, considered a blend of hyaluronic acid and gelatin to prepare liver-specific bioink with the ability to be further exploited for other tissue types [48]. PEG crosslinkers with various molecular weights were utilized to facilitate bioprinting. A 2-crosslinker, 2 stage polymerization method was employed to improve
bioink properties. This research outcome showed high cell viability for the proposed bioink. Kesti et al. also employed a tandem gelation mechanism (thermally and photo-triggered) to crosslink a blend of poly(N-isopropylacrylamide) grafted hyaluronan (HA-pNIPAAM) with methacrylated hyaluronan (HAMA) [53]. The proposed bioink displayed good printing fidelity as well as fast gelation and proper mechanical stability. Although no direct toxicity was observed in cells cultured on the surface of the 3D constructs, encapsulating cells in the bioink led to high cell fatality. However, cell death decreased significantly by removing HA-pNIPAAM in a brief washing step.

Any material has its own properties which may or may not be suitable for 3D printing of scaffolds. Pluronic, for example, is a thermo-sensitive polymer that has been in use for applications such as drug delivery [68, 69] and wound dressing [70]. Block copolymer Pluronic is known to have good printing properties but weak cell-culture properties. However, Muller et al. proposed a method to improve the biocompatibility of Pluronic [45]. This goal was achieved through blending acrylated with unmodified Pluronic F127 followed by UV crosslinking.

Recently a new extrusion-based method has emerged. This method utilizes a core-shell nozzle and a crosslinking agent is being printed at the same time the bioink is being dispensed from the core of the nozzle. In one study, core-shell printing was employed for rapid printing and gelation of cell-laden alginate 3D constructs [46]. The printed mesh structures, showed cell viabilities of 93% and 92% for preosteoblasts and hASCs. Yeo et al. also, employed this method to print cell-laden bioink based on alginate and collagen [34]. Cell encapsulating collagen bioink was loaded in the core barrel and alginate was loaded in the shell to improve cell viability during printing and crosslinking as well as enhancing overall printing fidelity. An aerosol crosslinking method was used to achieve multi-
layered mesh constructs. 3D constructs prepared using core-shell method in this study, showed noticeable higher cell viability compared to regular alginate-based bioinks.

Silk protein is another material exploited in bioinks for 3D printing of tissue-like constructs. Its potential as a bioink has been evaluated alone and in blends with other biomaterials. Studies suggest that silk fibers are biocompatible, possess unique mechanical properties, and allow for decoration with growth and adhesion factors due to their diverse side chain chemistries [71]. In one study, Schacht et al. fabricated cell encapsulating three-dimensional spider silk structures [52]. Robotic dispensing was employed for printing the constructs without any crosslinking additives. Different cell lines were cultivated on the hydrogels and cell adhesion and proliferation were studied. Furthermore, unlike common biomaterial as a bioink, no post print crosslinking is not needed. Good cell viability and proliferation for at least one week was reported. Also, a blend of silk fibroin and gelatin was utilized to print 3D tissue constructs in another study [63]. Mushroom tyrosinase and physical crosslinking via sonication were employed as crosslinking mechanisms. In vitro studies were taken place on the bioink encapsulating human nasal inferior turbinate tissue-derived mesenchymal progenitor cells. The 3D printed structures were shown to support multilineage differentiation of encapsulated stem cells. Furthermore, a blend of silk, gelatin, and glycerol was employed to enhance the printing resolution to meet patient-specific needs for soft tissue regeneration [72]. In vitro and in vivo studies showed that the developed material is stable and biocompatible while supporting tissue integration.

Properties and parameters

Engineering bioinks directly affects mechanical and biological properties. Even by slightly changing the concentration of the components, the gelation, printability, and properties of the resulting 3D
constructs can be affected significantly. Thus, it is of great importance to design the bioink based on the requirements of the application. As an example, Gu et al. developed a novel bioink for neural tissue construction based on alginate, carboxymethyl-chitosan, and agarose [35]. Fast gelation, stable crosslinking, and porous surface of the cell-encapsulating bioink was shown to be promising in human neural development and may also be applicable to other types of cells. Also, in another study, 3D printing technique was utilized to mimic liver tissue [73]. HepG2 cells were encapsulated in alginate bioink and multilayer three-dimensional constructs were fabricated. Stable cell proliferation and enhanced gene expression profiles were observed.

Printing parameters, such as printing speed and temperature, could also affect cell survival and printability. For this reason, some researchers focus on evaluation of these potential dependencies. To do so, Zhao et al. studied the effect of composition, concentration, temperature and holding time on printability and also, cell survival after extrusion printing [37]. In this study, cell survival rate was found to decrease when viscoelasticity of the gelatin-based bioinks were increased. Also, in a recent study, different compositions of alginate with low and high molecular weight were loaded with NIH 3T3 fibroblast cells and effects of alginate molecular weight on printability and cell viability were studied [74]. It was concluded that 3 wt% alginate composed of a blend of high and low molecular weight alginate with the ratio of 2:1 offers the optimum results with respect to printability and cell response.

To achieve required mechanical properties for any specific application, it’s necessary to reinforce the scaffolds in some cases. Different methods have been used to accomplish this goal. In one study, for example, PCL/alginate struts were coated with alginate-based bioink to reinforce the structure [39]. The ratio of alginate crosslinking agent was also varied to find the optimum conditions for cell-
coating. Using this method, multi-layered reinforced cell-laden scaffolds were constructed. Moreover, dual-crosslinking is another method employed to improve the quality and stability of printed constructs. Ouyang et al. utilized this method to prepare a printable hydrogel ink based on hyaluronic acid [41]. Guest-host assembly and covalent crosslinking were used to include self-healing and shear-thinning properties in the bioink. They were able to prepare structures with more than 16 layers which were stable over a month without loss of mechanical properties. The developed bioink was later functionalized to improve cell adhesion.

In an interesting study, effects of stiffness and 3D structure of the printed constructs on cell differentiation were studied [49]. Collagen type I, agarose, and chitosan blends were employed to study human mesenchymal stromal cells differentiation. Among the studied blends, osteogenesis was shown to be more likely in anisotropic soft collagen-rich substrates while adipogenesis was more likely in isotropic stiff agarose-rich matrices. Different ratios of collagen type I and agarose blend were concluded to suit wide range of mesenchyme-based applications.

Effective blend of alginate and GelMA has also been employed as a bioink in literature [51]. GelMA is used because of its ability to form stable hydrogels via UV crosslinking above alginate physical crosslinking. A coaxial extrusion printing system was used to print 3D constructs using low viscosity bioink with high cell viability in vitro. GelMA has also been shown to promote cell adhesion and migration [75]. In another work, a highly concentrated bioink consisting of alginate and polyvinyl alcohol was developed and studied for co-printing with bovine serum albumin (BSA) and bone morphogenetic 2 (BMP-2) [76]. It was shown that the release profiles of BSA and BMP-2 were strongly dependent on the micropores in the scaffolds which was related to the polyvinyl alcohol (PVA) sols.
Specific applications

Another application of bioprinting is in bone and cartilage tissue engineering. In one study, a bioink mainly consisted of alginate sulfate and nanocellulose encapsulating bovine chondrocytes was developed and printed via extrusion printing [77]. However, it was shown that printing cell laden bioink resulted in lower cell proliferation compared to non-printed samples. Heo et al., also, developed a new bioink consisting of alginate and bone formation peptide-1 to enhance bone regeneration [78]. In vitro and in vivo studies showed that the developed bioink provided a stable environment for the cells to proliferate. In another study, bioprinted calcium sulfate hydrate (CSH)/mesoporous bioactive glass (MBG) scaffolds were loaded with human bone marrow-derived mesenchymal stem cells (hBMSCs) and studied in vitro and in vivo [79]. Results revealed that CSH/MBG scaffolds promoted cell adhesion and proliferation, enhancing new bone formation. In a similar study, printed mesoporous silica/calcium phosphate cement porous scaffolds were fabricated and loaded with recombinant human bone morphogenic protein-2 (rhBMP-2) and studied in vitro and in vivo [80]. It was concluded that this blend is able to eliminate tissue necrosis issues during regeneration process. Yang et al., in one study, employed extrusion printing to print novel PCL scaffold with spiral struts encapsulating MG63 cells [81]. In vitro studies indicated that novel spiral-like struts did improve cell attachment, proliferation and differentiation with respect to normal struts. 3D bioprinting has also been used to print GelMA scaffolds on titanium implant surface, triggering mineral deposition of MG63 osteoblasts and human osteoblasts [82]. It was shown that while directly grafting on titanium alloy within a groove system, the hydrogel can survive from shear forces in a marrow implantation model.
Another application for 3D bioprinting is in fabrication of human bilayered skin. Cubo at al., for instance, utilized an extrusion-based technique to print bioinks containing human plasma as well as primary human fibroblasts and keratinocytes [83]. In vitro and in vivo studies revealed that the printed skin was very similar to human skin and was indistinguishable from handmade dermo-epidermal equivalents.

### 2.3 Inkjet bioprinting

Inkjet printing application in 3D bioprinting has been limited compared to extrusion-based studies. The main reason for that is the inherent inability of printing head to provide a continuous flow which limits its application in bioprinting [16]. Bioinks with viscosities lower than 10 mPa s have been reported printable via inkjet printing. In comparison with other methods, inkjet printing offers fast fabrication speed but low cell densities [21]. Inkjet printing methods could be classified into three groups: continuous-inkjet bioprinting, electro-hydrodynamic jet bioprinting, and drop-on-demand inkjet bioprinting. The latter category happens to be the largest and the most common one consisting of thermal, piezoelectric, and electrostatic inkjet bioprinting [84]. Thermal and piezoelectric inkjet printing are shown schematically in Figure 2-2. A few outstanding studies in this area are reviewed in this section.
Figure 2-2. Schematic diagram of drop-on-demand inkjet printing method using A) Thermal, and B) Piezoelectric actuators. A thermal printing head employs a heating element that raises the temperature locally and creates a bubble that drives droplets through the nozzle. A piezoelectric head is utilized with a material that changes shape upon voltage application and pushes droplets out.

Although currently extensive research is being done on bioink development, not all of these works are likely to be commercialized. For a new bioink to become commercially available, it has to be cost effective and show the potential to be standardized according to industrial environments and requirements. To this end, Rimann et al. developed an all-in-one printing method for soft tissue construction [36]. In this work, a PEG-based bioink was developed and used along with a commercial 3D discovery inkjet bioprinter. Printing took place in sterile environment. To verify the applicability of their work, long-term culture of the printed structures was carried out. The results approved the human primary dermal fibroblasts viability and proliferation up to seven weeks.

In another study, droplet formation process during inkjet printing of cell-laden bioink consisting of fibroblasts and alginate with different cell concentrations was studied [55]. Breakup time, droplet size, droplet velocity, and satellite formation were among the parameters studied in this work. It was reported that increasing cell concentration, decreases velocity and droplet size while increasing
breakup time. Also, the process was compared to polystyrene microbead-laden suspension inkjet printing to illustrate the effect of particle physical properties on the droplet formation. Furthermore, double chemical functionalization of gelatin was undertaken in another novel work to control its physical and chemical properties for bioprinting [56]. This was achieved by methacrylation and acetylation of free amino groups to gain control over viscosity and mechanical properties of the bioink. The resulting soft hydrogels were printed by drop-on-demand inkjet printing and shown to be cytocompatible and suitable to print viable mammalian cells.

It is worth mentioning that thermal inkjet printing is not common in tissue engineering due to activity loss resulting from very high temperatures which may reach above 200 °C. For instance, Setti et al. reported 15% activity loss while printing b-galactosidase (GAL) [85]. However, some studies do employ piezoelectric inkjet bioprinting in their research. As an example, a piezoelectric inkjet printing system was utilized in one study to print breast cancer cell suspensions [86]. Preparing neutrally buoyant suspensions using Ficoll PM400, it was shown that nozzle clogging was eliminated and dispensing accuracy was enhanced. Through this work, improved dispensing by rheological manipulation was studied. Furthermore, Xu et al. proposed a novel 3D bioprinting system capable of scaffold-free printing of 3D cellular tubes [87]. Cell viability of constructed cell-based tubes was reported as high as 82% even after 3 days of incubation. Gudapati et al. have well expanded the droplet-based bioprinting methods including inkjet printing, common biomaterials, and cells employed [84].

### 2.4 Stereolithography-based bioprinting

Stereolithography printing is based on polymerization of light-sensitive polymers by precisely controlled light glinted from digital micromirrors. In comparison with other methods,
Stereolithography is a technique with high printing quality, speed, and cell viability. However, drawbacks have been reported resulting from using this method. For instance, UV light source which is the common polymerization method, has been reported to be harmful for DNA cells and even cause skin cancer \cite{17, 18}. To address this issue, visible light stereolithography bioprinting systems have gained attention. As an example, Wang et al. employed a bioprinting system consisted of a beam projector and blends of PEGDA, GelMA, and erosin Y based photoinitiator as bioinks \cite{38}. This work’s results of NIH 3T3 cell bioprinting indicated that the proposed low cost system is capable of printing and visible-light curing of hydrogels with 50 μm resolution and relatively high cell viability. Figure 2-3 schematically shows stereolithography using a beam projector.

![Figure 2-3. Schematic diagram of stereolithography using beam projector. Focused light beams allow for precise photopolymerization of layers of light-sensitive polymer to apply any desired pattern to the bioink.](image)

Versatility, controllability, and precision of stereolithography has been studied by Melchels and colleagues \cite{88}. Porous scaffolds were designed and fabricated with either a poly(\text{D,L}-lactide)-based resin or a poly(\text{D,L}-lactide-co-\text{ε}-caprolactone)-based resin. It was shown that by varying the composition of the macromeres and the pore architecture, mechanical properties of the scaffolds can be controlled. Elomaa et al. also prepared scaffolds by employing a photocrosslinkable PCL-based
resin with high gel content networks [89]. Porous scaffolds were prepared by stereolitography using the resin prepared by Irgacure 369 photoinitiator, inhibitor and dye. The fabricated scaffolds matched the design and proved the suitability of the prepared resin for fabrication of tissue engineering scaffolds.

In a novel study, a projection stereolitography (PSL) platform was proposed to design 3D tissue scaffolds based on computer aided design [27]. Various structures and concentrations of GelMA were employed to control the mechanical properties of the scaffolds. Complex porous constructs were seeded with HUVECs and were studied in vitro. Precisely fabricated scaffolds with interconnected pores were shown to support cell growth resulting in high cell densities.

Melchels et al., in another work, employed stereolitography to fabricate porous constructs from a resin based on a 2-armed poly(D,L-lactide), ethyl lactate, photoinitiator, inhibitor and dye [90]. Good pre-osteoblast adherence and comparable proliferation to high-molecular weight poly(D,L-lactide) and tissue culture polystyrene were reported. Shie at al. employed a commercial 3D printer using blue light digital stereolitography to prepare polyurethane with hyaluronic acid for cartilage repair [91]. The printable photosensitive material developed in this work was shown to be non-toxic, supporting high resolution printing, cytocompatible, and promote cell adhesion, proliferation, and differentiation. In another study, fumaric acid monoethyl ester-functionalized poly(D,L-lactide)/N-vinyl-2-pyrrolidone resins were prepared and used with stereolitography to fabricate scaffolds [92]. Mouse pre-osteoblasts were shown to adhere and spread well onto the material. Also, a resin composed of Poly(propylene fumarate) (PPF), diethyl fumarate (DEF), and bisacrylphosphrine oxide (BAPO) has been utilized to fabricate scaffolds by stereolitography [93]. Fabrication of constructs with controlled microstructure by optimizing resin composition and laser parameters was
studied in this work. To optimize the microstructure and achieve high porosity, sugar particles have also been used in a projection-based stereolithography [94]. This method was shown to increase the porosity of the scaffolds by two times in comparison with the current stereolithography method.

Application of PEG hydrogels with stereolithography have also been reported in literature [95]. Due to presence of the photoreactive groups, UV light can crosslink PEG into a hydrogel in the presence of a photoinitiator. Complex multilayer 3D PEG hydrogel constructs were prepared by using stereolithography and two different molecular weight of PEG. Effects of factors such as photoinitiator, photopolymer concentration, and energy dose on the gel properties as well as effects of stereolithography parameters on in vitro cell studies were investigated. Use of Poly(trimethylene carbonate)-Based Resins in stereolithography has been also reported [96]. Results of this work approved attachment, diffusion, and differentiation of bovine chondrocytes, providing evidence for applicability of the proposed resin for cartilage tissue engineering.

Scaffolds made by stereolithography have been also used for heart valve tissue engineering [97]. A blend of a thermoplastic elastomer, a poly-4-hydroxybutyrate (P4HB) and a polyhydroxyoctanoate (PHOH) was used to form the resin. Direct pressure measurements of the sample heart valves revealed synchronous opening and closing of the valves in a pulsatile flow bioreactor. Another application of stereolithography is to prepare sacrificial moulds for scaffold preparation. Chopra et al., for instance, were able to control the architecture of gel-cast glass-ceramic tissue scaffolds [98]. Similarly, Bian et al. employed ceramic stereolithography and gel casting to fabricate beta-tricalcium phosphate/collagen scaffolds for osteochondral tissue engineering [99]. Using this method, high resolution scaffolds ideal for bone tissue engineering were developed. Furthermore, development and application of epoxy/hydroxyapatite in stereolithography made scaffolds has been
3D bioprinting for biomedical devices and tissue engineering

reported [100]. Prepared scaffolds were kinetically characterized and importance of factors such as weigh percentage of ceramic powder and viscosity of the suspensions for fabrication was studied. Green ceramic bars fabricated in this work were reported to offer good mechanical properties. Zheng et al. also used stereolithography to fabricate very precise and complex moulds using 3D models designed based on Computed tomography (CT) images of rat mandible [101]. A silicon tissue transformation mould was prepared using the prepared precise moulds by stereolithography and bone formation was observed by X-ray. The applicability of this method for in vivo tissue transformation for vascularized bone reconstruction was reported. For further reference, a comprehensive review of the materials processed using stereolithography has been presented by Skoog et al. in 2014 [102].

2.5 Laser-assisted bioprinting

Laser-induced forward transfer (LIFT) is technique presented more than 30 years ago by Bohandy et al. [103]. This technique allows high resolution deposition of material in solid or liquid phase. While several versions of this technique exists, a solid phase material printing version is illustrated schematically in Figure 2-4. In one study, Matrix-assisted pulsed-laser evaporation direct-write, one variation of LIFT technique, was employed for cell printing [104]. Sodium alginate loaded with NIH 3T3 mouse fibroblast cells was used as the bioink along with calcium chloride as the crosslinking agent. Effects of alginate gelation and concentration, gelation time, and laser fluence on cell viability were studied. It was observed that longer gelation time decreases the cell viability after 24 hours of incubation due to the reduced nutrition and oxygen transfer through the thick gel wall.
Figure 2-4. Schematic diagram of laser-assisted bioprinting. A nozzle-free technique using pulsed laser source to deposit microdroplets of bioink with/without cells on a substrate.

Although cell transfer using LIFT technique has been successful but, cell survival rate is often below 85% [20]. Thermal damage due to nanosecond lase irritation was recognized as the main cause of cell death. To decrease the damage to the cells, femtosecond lasers were employed. Particularly, absorbing film-assisted LIFT (AFA-LIFT) method, which is an improved LIFT method, was studied by Hopp et al. as a method of controlled living cell transfer onto various acceptor surfaces [20]. However, experimental results of this work revealed that femtosecond AFA-LIFT caused higher fatality rates in cells compared to nanosecond AFA-LIFT which was mainly attributed to the strong photomechanical influences of laser pulse. Laser-assisted bioprinting by LIFT technique was further investigated to print cell-laden three-dimensional structures [105]. Collagen encapsulating fibroblasts and keratinocytes was employed to print 3D skin tissue like structures. These lines of cells were previously proven to be resistant to damage during laser-
assisted printing process [106]. Proliferation of cells over a period of 10 days was studied and the ability of 3D printed cells to form real tissue was demonstrated.

In general, cells could be either printed onto/in the depth of ECM layer or printed as encapsulated particles in an ECM-like printable biomaterial. It is important to know the effects of different printing parameters on cell viability. In one study, effects of laser pulse energy, ECM thickness, and viscosity of the bioink on the cell viability was studied [107]. Cell viability 24 hours post-printing was measured to compare different printing settings. It was concluded that while higher laser energy leads to more cell fatality, increasing film thickness as well as bioink viscosity results in increased cell viability. Furthermore, effects of bioink viscosity, laser energy, and printing speed on printing resolution was studied by Guillotin et al. [108]. It was shown that microscale resolution and 5 kHz printing speed are within reach. This work is another proof for applicability of printing blends of cells and ECM via laser-assisted bioprinting to fabricate soft free form tissue able to host a high cell density in vivo.

### 2.6 High performance bioink

Among all the research works on bioinks, there are some studies that stand out by the benefits they offer. Specific applications, new methods, and spectacular properties are some of the reasons making these type of studies inspiring.

Application specific studies engineer the bioink based on the requirements of the application. In certain biomedical devices, for instance, conductivity can be of great importance while in scaffolds, cell support is essential. In a novel study, a special bioink was developed for cardiac tissue regeneration [109]. This bioink was developed to provide proper conductivity and avoid delayed electrical coupling in cardiac cells. This new gold nanorod-integrated gelatin methacryloyl-based
bioink was shown to be accurately printable, cytocompatible, and enhance cardiac cells functionality. Nerve [110], kidney [111], and cartilage [13] regeneration and repair as well as bionic ear [112] are other specific applications studied for bioink development. Flexible electronics for bioelectronic interfaces are also under extensive research currently [113, 114]. In one study, a new method for fabrication of inkjet-printed flexible gold electrodes was demonstrated [114]. Fabricated gold electrode arrays were shown to be mechanically and electrically promising for bioimpedance and biopotential measurements. Also, to increase the survival time of the bioprinted tissue, development of bioinks for vascularized bioprinted tissue has been studied [115, 116]. Sensing applications such as tactile sensors are also focus of many studies. Guo et al., for example, employed a multifunctional bioprinting method for fabrication of stretchable tactile sensors [117]. Fabricated sensors in this work, were shown to be able to measure finger motions and pulse. Furthermore, inks have been developed to fabricate strain sensors within structures guiding the self-assembly of cardiac tissue [118]. This versatile fabrication approach was claimed to be applicable to a wide range of instrumented micro-physiological devices, further expanding in vitro tissue engineering. A typical image of a 3D printed hydrogel in the form of a mesh structure as well as a 3D printed conductive sensor is displayed in Figure 2-5.
Figure 2-5. 3D printed constructs of conductive and nonconductive bioinks. A) A typical chitosan-based extrusion bioprinted mesh structure, B-D) a conductive 3D printed sensor based on chitosan and acrylic acid, sealed in PDMS. The resistance response at various bending angles from testing the hydrogel as a sensor in strip form (left) and in 3D printed mesh form (right) is displayed. © 2017 Reprinted with permission of John Wiley and Sons [119].

Many research works could be found in the literature that focus on specific properties of the 3D bioprinted constructs, properties such as high strength structures. Zhu et al., for instance, employed polyion to prepare ultratough hydrogels by extrusion printing [120]. Also, Qin et al. combined 3D bioprinting and computational modelling to evaluate mechanical behavior of elastomeric webs mimicking spider webs [121]. This work’s results suggest that loading pattern governs the material distribution in the spider web. Based on that and computational modelling, authors showed that
mechanical functions of 3D printed Polydimethylsiloxane (PDMS) webs are controllable by material distribution.

Development of new methods is also vital for the expansion of bioprinting. Enhancing the printing resolution and versatility of the current methods as well as development of new ones is an ongoing research. As an example, self-healing hydrogels were shown to provide support for direct printing of high resolution 3D constructs by utilizing shear-thinning hydrogels, providing the ability to print in any direction [122]. In another study, a new approach to print nonviscous photo-crosslinkable bioinks was introduced [123]. In this method, light is introduced to the hydrogel via a photopermeable capillary immediately before deposition, allowing for high resolution and uniform filaments with high cell viability. Also, Colosi et al. developed a low viscosity bioink based on mixing alginate and gelatin methacroyl during the extrusion process and crosslinking of alginate just prior to the deposition [124]. Using this versatile approach, printing highly functional tissue-like structures with high resolution was demonstrated. Furthermore, significant enhancements in microdrop bioprinting have been reported by Pataky et al [125]. Compatibility of this method with alginate and collagen printing as well as the ability to print resolutions comparable to industrial prototyping was demonstrated. In another study, Rutz et al, proposed a bioprinting method capable of extruding various natural and synthetic gel-phase bioinks [62]. Authors proved the versatility of this approach by designing and printing 35 formulations of bioinks. Overcoming vascularization in tissue-like structures by implementing fluidic channels is another method receiving a great deal of attention recently. Gao et al., for instance, employed extrusion bioprinting to fabricate multilayer macro-channel embedded alginate-based structures loaded with two type of cells [126]. These printed multilayer constructs with multilevel fluidic channels were reported to be biocompatible and have acceptable mechanical strength. In yet another interesting and recent study, programmable
structures capable of changing to complex 3D morphologies were studied [127]. Inspired by botanical systems, Gladman et al. printed composite hydrogel constructs using four-dimensional printing pathways which are capable of changing shape upon localized swelling due to water absorption. This approach can potentially lead to new shape transforming structures and find applications in tissue engineering and biomedical devices.

2.7 Challenges, applications and future perspective

Despite all the progress over the years in tissue engineering, many challenges still remain unsolved. Challenges fall into two main categories: 1) biomanufacturing which involves 3D fabrication of the cells and biomaterials and 2) in vivo integration which involves post-implantation functionality and integration. One challenge in fabrication process is nozzle clogging in nozzle-based fabrication methods. Depending on the application, fabrication time can take several hours. To avoid nozzle clogging in these cases, printing precursor needs to be homogenous and have proper viscosity and shear thinning properties. Another challenge is that the 3D constructs need to be sufficiently stable and mechanically rigid to ensure successful transplantation. For example, in the case of hard tissue repair, elastic modulus of the scaffolds needs to be high enough to maintain its designed structure and porosity while implanted to support natural cell growth [128]. If the scaffold is not capable of maintaining its structure and provide mechanical support, any newly formed tissue will probably fail as a result of scaffold deformation [129].

Bioprinted constructs for tissue engineering, being ultimately implanted in body, need also to support vascularization in vivo to provide the cells with sufficient nutrition, growth factors, oxygen and remove waste. In vivo, capillaries are found within a distance of 100 µm from most cells so that there is sufficient diffusion for the cells to survive [130]. For distances more than that, such as thick
tissues in printed organs, additional means for diffusion may be needed. To overcome this challenge, Hutmacher et al. suggested an artificial vascular system to enhance transportation of nutrients and removal of waste products [131].

3D bioprinting is currently expanding swiftly toward a large industry due to its diversity and potential applications. 3D printing market size is predicted to reach $10.8 billion in 2021 from $2.2 billion in 2012 [132]. Currently, several companies are working on 3D bioprinting products for tissue engineering applications such as cartilage, liver tissue, breast, and bone [133]. Tissue Regeneration Systems is among the companies that have already produced commercially available bioprinted products. This company develops bioprinted PCL-based solutions customized for individual patients to repair skeletal defects [134]. This solution was approved by the food and drug administration in 2013 as the first implant for skeletal reconstruction and bone regeneration prepared by 3D bioprinting. Furthermore in 2014, Organove introduced bioprinted human liver tissue, named exVive3D™ Liver, designed to evaluate drug toxicity [135]. While this product offers in vitro drug screening, a commercially available liver tissue has not been successfully developed yet. Generally, bioprinting applications can be categorized into two major groups: 1) tissue regeneration and regenerative medicine and 2) biomedical applications. The first group is about applications of bioprinted constructs such as vascular grafts, skin, neuron, bone, and liver while drug discovery and biopreservation fall under the second category [133].

Needless to say, bioprinting has been constantly evolving over the past decade and this trend seems to be continuing. As more research is done on bioprinting techniques, printing resolution and quality will eventually improve, providing capability to print more complex 3D constructs. Natural organs are often very complex structures consisting of different types of tissue, ligaments, etc., each having
their specific functions. By further advancement of bioprinting, biofabrication of complex constructs accurately mimicking natural organs becomes practical. Structural complexity of the bioprinted products can also be improved by precise fabrication of multi-material 3D constructs. Simultaneous deposition of materials with different physical and chemical properties is also a useful approach to fabricate organs with various properties in different regions. Multimaterial bioprinting provides the ability to adjust factors such as concentration of growth factors, cell adhesion, and degradation rate in different regions of the printed object. Capability to load different type of cells in different zones and on compatible biomaterials is another benefit of this approach allowing for closely mimicking of natural cellular diversity and activity.

Future development of bioprinting can also potentially overcome vascularization challenge which is among the most important factors limiting bioprinting applications in tissue engineering \[136\]. Biofabrication of microstructures within scaffolds by employing technologies such as microfluidic systems \[137\] and layer-by-layer assembly \[138\]. Furthermore, it is predictable that advances in biofabrication, will also benefit related fields such as imaging and diagnostic applications.

### 2.8 Summary and conclusions

In this text, recent research on development of bioinks, 3D bioprinting methods, as well as current state of art is discussed. Extensive research on 3D bioprinting over the past decade is a sign of its wide applications and promises in tissue engineering. However, to overcome challenges such as vascularization, biomanufacturing issues, and unfit properties, more research on bioink development and 3D bioprinting techniques is required. Further expansion of multimaterial hydrogels, development of more accurate bioprinting methods, and combining different printing techniques are some of the most important areas that can help advance the applications of bioprinting in tissue
engineering. A few bioprinting products have been already introduced and are commercially available in the market. Given the swift development of this industry over the past years, it is predictable that more bioprinting products will eventually become available in the market to help patients suffering from a wide range of diseases and 3D bioprinting will continue to be a strong fabrication tool for tissue engineering and development of biomedical systems.
Chapter 3

3 Characterization and application of an Engineered Carboxymethyl chitosan-based Bioink

3.1 Introduction

Tissue Engineering is currently an attractive and fast developing research field focusing on restoration and regeneration of damaged tissues and organs. Researchers often consider cells, scaffolds and growth factors as the main components in tissue engineering. A controlled 3D structure loaded with cells allows for specific distribution of cells and therefore results in improved cell proliferation and tissue regeneration. To this end, additive manufacturing (AM) has been used to construct 3D structures mimicking nature of tissue [139]. The importance of this matter resulted in a new subcategory field called 3D bioprinting [62] which is now one of the most attractive research topics in biomedical and tissue engineering fields. The importance of bioprinting arises from providing biomedical end-users the ability to print scaffolds in required size and configurations with manipulated physical and chemical properties. These scaffolds could be either pre-loaded with cells before bioink deposition or after printing and gelation process. Also, biomaterial-based biomedical devices and sensors are another proposed application for 3D bioprinting [140]. In our group’s previous work, for instance, a conductive self-healing sensor was fabricated by 3D bioprinting[7].
Currently, available 3D printable and cell compatible bioinks are very limited and offer weak ability to tune materials properties \[139, 141\]. It has been proved previously that material properties such as stiffness and degradation, affect tissue formation and cell behavior \[142-144\]. Therefore, developing new tunable 3D printable materials and techniques can lead to significant advances in tissue engineering. In most cases, a polymer solution is printed through printer nozzle and gelation occurs rapidly outside or on the printing substrate. Polymer concentration in the solution plays an important role in the properties of the bioink, as well as printing quality and cell migration and proliferation \[145, 146\].

Although chemical crosslinking is a diverse method, many of the chemical crosslinking agents are toxic and result in unwanted reactions in the hydrogel \[147\]. For instance, glutaraldehyde, formaldehyde, and carbodiimide are recognized for their cytotoxicity in gelatin-based hydrogels \[148, 149\]. Therefore, physical crosslinking is a preferred alternative.

Previously, it has been shown that alginate-NFC (Nano fibrillated cellulose) bioink can be used as a cell compatible bioink combining fast gelation properties of alginate and good shear thinning properties of NFC\[61\]. However, alginate hydrogels are considered to have relatively poor cell adhesion\[150\].

Other than alginate, chitosan is a prominent candidate as a biodegradable and biocompatible polymer suitable for scaffold preparation \[151\]. Also, hydrogels are attractive for biofabrication thanks to their several features of the natural extracellular matrix and allow cell encapsulation in hydrated environment with good mechanical support \[139\]. Therefore, development of 3D printable chitosan based hydrogel was investigated in the present work that could be used to print 3D templates for \textit{in vitro} and \textit{in vivo} cell studies as well as, potentially, for biosensing applications.
### Table 3-1. A short summary of outstanding bioprinting studies

<table>
<thead>
<tr>
<th>Bioink</th>
<th>Printing method</th>
<th>Research summary</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>Suspended cells in Dulbecco’s Modified Eagle Medium (DMEM) supplemented with alginate and glycerol.</td>
<td>Laser-assisted bioprinting</td>
<td>Study of viscosity, laser energy, and printing speed on the printing resolution.</td>
<td>[152]</td>
</tr>
<tr>
<td>Decellularized adipose tissue (DAT) matrix encapsulating human adipose-derived stem cells (hASCs)</td>
<td>Extrusion bioprinting</td>
<td>Designing and printing dome-shaped structures with engineered porosity.</td>
<td>[50]</td>
</tr>
<tr>
<td>Cell loaded Alginate-Gelatin hydrogel</td>
<td>Extrusion bioprinting</td>
<td>Extrusion bioprinting using novel heated syringe tips.</td>
<td>[60]</td>
</tr>
<tr>
<td>Poly(N-isopropylacrylamide) grafted hyaluronan (HA-pNIPAAM) with methacrylated hyaluronan (HAMA)</td>
<td>Extrusion bioprinting</td>
<td>Preparation and evaluation of bioink for encapsulated bovine chondrocytes.</td>
<td>[53]</td>
</tr>
<tr>
<td>Cell-laden silk fibroin–gelatin hydrogel</td>
<td>Extrusion bioprinting</td>
<td>Development of tissue analogs by 3D bioprinting.</td>
<td>[63]</td>
</tr>
<tr>
<td>Oxidized Alginate hydrogel loaded with human adipose-derived stem cells (hADSCs)</td>
<td>Extrusion bioprinting</td>
<td>Study of the effects of viscosity and density of alginate solutions on their printability.</td>
<td>[54]</td>
</tr>
<tr>
<td>Nanocellulose–Alginate bioink loaded with human chondrocytes</td>
<td>Extrusion bioprinting</td>
<td>Study of printability and use of Nanocellulose–Alginate Bioink for 3D printing of living tissues and organs.</td>
<td>[61]</td>
</tr>
<tr>
<td>Mesenchymal stem cells (MSC)-laden polylactic acid microcarriers encapsulated in gelatin methacrylamide-gellan gum bioinks</td>
<td>Extrusion bioprinting</td>
<td>Study of printability and cell viability.</td>
<td>[59]</td>
</tr>
<tr>
<td>Novel microgel suspension in a surfactant-containing tissue culture medium</td>
<td>Inkjet printing</td>
<td>Study of the printability and stability of cell suspension.</td>
<td>[153]</td>
</tr>
<tr>
<td>Cell loaded alginate-gelatin bioink</td>
<td>Extrusion bioprinting</td>
<td>Study of printability and cell viability.</td>
<td>[154]</td>
</tr>
<tr>
<td>Double chemical functionalized gelatin hydrogel</td>
<td>Extrusion bioprinting</td>
<td>Study of cell viability and control of the hydrogel properties by double chemical functionalization.</td>
<td>[56]</td>
</tr>
<tr>
<td>Decellularized extracellular matrix (dECM) bioink</td>
<td>Extrusion bioprinting</td>
<td>Study of cell viability and functionality of the printed dECM structures.</td>
<td>[64]</td>
</tr>
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### Various hydrogel bioinks

<table>
<thead>
<tr>
<th>Bioink Type</th>
<th>Printing Method</th>
<th>Description</th>
<th>Reference</th>
</tr>
</thead>
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<td>Cell loaded spider silk hydrogel</td>
<td>Extrusion bioprinting</td>
<td>Study of silk proteins as a new bioink.</td>
<td>[52]</td>
</tr>
<tr>
<td>Alginate bioink loaded with MG63 cell line</td>
<td>Laser-assisted bioprinting</td>
<td>In vivo and in vitro study of layer by layer printed alginate bioink.</td>
<td>[58]</td>
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Hydrogel 3D printing methods are divided into multiple categories such as, laser-assisted bioprinting, inkjet printing (including thermal and piezoelectric actuated), and robotic dispensing (including pneumatic, piston, and screw driven)[139]. The printing method used in current work is a piston driven method (piston-pneumatic) which falls into robotic dispensing category. In robotic dispensing methods, achievable resolution is generally lower than the other categories but, printing speed is much higher instead. A short summary of outstanding research on bioprinting over the past few years is tabularized in Table 3-1. Also a schematic of the printing method is illustrated in Figure 3-1c.

Chitosan has been shown to be cell friendly and support attachment and proliferation of different cell type such as cartilage and bone cells [155, 156]. Herein, carboxymethyl chitosan, water soluble at neutral pH values, was employed due to the simplicity of working with water rather than acidic solvents. Moreover, chitosan was modified by EDTA to further increase carboxyl groups and enhance stability and mechanical properties of the hydrogel by physical crosslinking via calcium solution.

Cartilage is an avascular tissue consisting of a small number of chondrocytes (10% to 15%) with limited self-regenerative properties [157, 158] and there is a critical need for tissue engineering to generate a scaffold to serve as a matrix for new cartilage formation. The aim of this study was to 3D
print a chondral bioscaffold using modified chitosan and assess the chondrocytes viability, proliferation and differentiation on the scaffold. To this end, chitosan was modified by (Ethylenedinitrilo)tetraacetic (EDTA) acid to enhance gelation and crosslinking by introducing more carboxyl groups to the hydrogel precursor. Furthermore, while many hydrogels are prepared by chemical crosslinking, physical crosslinking by calcium solution was administrated in this work to avoid concerns over cytotoxicity associated with chemical crosslinking. It is important to mention that although alginate is also used with calcium solution as a crosslinker but, alginate has been shown to have poor cell adhesion properties. Inspired by physical crosslinking of carboxyl groups in alginate by calcium, we have crosslinked a modified chitosan by calcium while cell adhesion properties are improved compared to alginate. This study was undertaken to evaluate the effect of chitosan modification on cartilage cells viability and proliferation as well as the improvement of mechanical support.

3.2 Experimental Section

3.2.1 Materials

Carboxymethyl chitosan, ultra-pure grade EDTA free acid (292.25 g/mol), and EDC-HCl (191.7 g/mol) were purchased from CLEARSYNTH, AMRESCO, and PROTEOCHEM, respectively. 3D bioprinter was from HKable 3D.

3.2.2 Mechanical characterization

The Discovery HR-1 hybrid rheometer (TA Instruments) with a Peltier plate was used to evaluate the rheological properties of the bioink. Shear rate was swept from 0.1-100 s\(^{-1}\) for shear viscosity measurement and frequency was swept from 0.1-200 rad.s\(^{-1}\) for storage and loss evaluation. The
Characterization and application of engineered chitosan bioink

Instron 5965 dual column tabletop testing system was used to evaluate compressive strength of the hydrogel. All measurements were performed at 22 °C.

### 3.2.3 Hydrogel Preparation Methods

200 mg of water soluble chitosan was dissolved in 10 ml of double-distilled water. 240 mg of EDTA free acid was added to the solution. 160 mg of EDC-HCl was added as a carboxyl activating agent to form amine bonds in the solution. The reaction mixture was incubated at room temperature under constant stirring overnight. The solution was later purified using dialysis tubing for two days. The resulting solution was then freeze dried for 72 hours. The final puffy powder produced, referred to as CE, was used as the first component used to prepare printing precursor. To prepare the printable hydrogel precursor, 80 mg of CE powder was dissolved in 5 ml of double-distilled water. 670 mg of water soluble chitosan was added to the solution under constant stirring for 2 hours to prepare the final printable precursor. The final precursor was centrifuged at 3000 rpm for 5 minutes to remove the bubbles followed by printing using a piston-pneumatic customized printer. calcium solutions with concentrations ranging from 0.1M to 2M were used as crosslinking agents for the 3D printed products. The ratio of chitosan added in the final step before printing to the CE powder used in the precursor (chitosan:CE) could be slightly changed to tune the mechanical properties of the hydrogel.

### 3.2.4 Printing Method

The method used for printing the bioink developed in the present work is a combination of pneumatic and piston driven methods. The bioink goes through an extrusion process in order to print 3D constructs by layer-by-layer deposition of biomaterial. The thickness and the width of each layer can be tuned by tuning printing speed, extruder needle size, and air pressure applied to the piston.
The printed structure could be composed of as many layers as needed. A schematic presentation of the hydrogel preparation and printing is depicted in Figure 3-1.

Figure 3-1. Schematic diagram of hydrogel preparation and printing: (A) First step: Chitosan reacting with EDTA, unreacted carboxyl groups (green) take part in the next step. (B) Second step: additional Chitosan is added to the solution and crosslinked with CaCl$_2$ solution after printing to form hydrogel. (C) Hydrogel printing method.
3.2.5 Rabbit Chondrocyte Isolation and Cell Seeding

Rabbit chondrocytes were used to investigate the effects of the chitosan scaffold on cell viability, proliferation and differentiation. To obtain primary chondrocytes, macroscopically intact rabbit cartilage was harvested, minced, and soaked 1 hour with 2 mg/ml protease, followed by overnight incubation with 1.5 mg/ml type II collagenase (Catalog :17101015, Thermo Fisher Scientific, US) in a 37°C incubator. After centrifugation and filtration, primary chondrocytes were harvested.

For cell seeding, prepared chitosan scaffolds were placed in 12 well cell culture cluster. After that, 1 ml of complete media containing the cells (1 x 10^5) was directly pipetted onto each scaffold and cultured at 37 °C in 5% CO2 in a humidified atmosphere.

3.2.6 Proliferation Assays

After seeding the cells on the scaffolds for 36 h, cellular proliferation was measured using the Cell-Light EdU Apollo 567 in vitro Imaging Kit (RiboBio, Guangzhou, People’s Republic of China) according to the manufacturer’s instructions. 4′,6-diamidino-2-phenylindole (DAPI) was used to stain the cellular nuclei. Proliferation indicator 5-Ethynyl-2′-deoxyuridine (EdU) incorporated into the DAPI of chondrocytes was detected by fluorescence microscopy (Nikon Corp.). The chondrocyte proliferation rate was assessed by counting the percentage of EdU–labeled cells in DAPI–labeled cells in 5 fields of each scaffold.

3.2.7 Cell Viability Assessment

Seeding cells viability was assessed by live/dead viability/cytotoxicity kit (Catalog number: L3224, Invitrogen, UK) quantified by flow cytometry measurement. After 24 h incubation on chitosan scaffold, cells were collected and stained with the following fluorescein isothiocyanate-conjugated anti-human mouse antibodies: anti-CD31, anti-CD34, and anti-CD45 (BD Biosciences,
San Jose, Calif.). Multicolor flow cytometry was performed using an LSR II (BD Biosciences) flow cytometer, and changes in cell composition were calculated according to surface marker expression profiles.

### 3.2.8 Quantitative Real-Time Polymerase Chain Reaction (PCR) Analysis

RNA was isolated from seeding chondrocytes at different time points using Trizol Reagent (Invitrogen Life Technologies, Carlsbad, Calif.) according to the manufacturer’s protocol, followed by reverse transcription. Quantitative real-time PCR was carried out according to the TaqMan method with previously designed primers (Thermo Fisher Scientific) and was used for expression analysis. Relative expressions were calculated by cycle threshold method using ACTB as an endogenous reference gene. The primers used to amplify messenger RNA sequences were:

- **Collagen II** forward AGCGGTGACTACTGGA and reverse: CTGCTCCACCAGTTCTTCTT;
- **Sox9** forward CCACCTCTCTTACCTCTCTCAT and reverse: GGACAGCTTACAAGGTTTCT

Statistical Analysis

Data were expressed as mean ± SEM analyzed using IBM SPSS Version 21.0 software (IBM Corp., Armonk, N.Y.). A repeated measures analysis of variance was used to analyze the results. Furthermore, an independent t test was used to compare two groups at a single time point. Meanwhile, one-way analysis of variance was used to compare groups at all time points. A value of p < 0.05 was considered significant.
3.3 Results and discussion

Figure 3-2. Schematic diagram of chemical synthesis of hydrogel.

3.3.1 FT-IR and NMR analysis

Three FTIR spectra (Figure 3-3) show the strong absorption peaks around 3450 cm\(^{-1}\) which are attributed to the presence of OH stretching vibrations. The peak around 2924.60 cm\(^{-1}\) is due to the presence of C-H the stretching vibrations. The spectrum of carboxymethyl chitosan shows two peaks around 1657 and 1587 cm\(^{-1}\) which are attributed to N-H bending vibration and C=O groups of anionic carboxylates respectively. The second spectrum shows the appearance of peak around 1710 cm\(^{-1}\) which is characteristic of C=O vibration of carboxylic acid and the strong peak at 1652 cm\(^{-1}\) which is attributed to the carbonyl stretching of amide vibrations. These bands suggest that ethylenediaminetetraacetic acid (EDTA) has coupled with the carboxymethyl of chitosan. The third spectrum show the disappearance of peak at 1710 cm\(^{-1}\) and the appearance of band 1633 cm\(^{-1}\). These observations confirm the chelation of calcium to EDTA via O-H of the carboxylic acid functionality. The appearance of band 1633 cm\(^{-1}\) is in fact the shift of peak 1652 cm\(^{-1}\) that suggest the presence of
calcium in the form of metal-complex with chitosan-EDTA. In addition, the three spectra show the peak at 1410 cm\(^{-1}\) are assigned to the bending vibrations of the methylene protons of the CH2COOH groups. The peaks range from 1156.8-1068.38 cm\(^{-1}\) are attributed to C-O-C of the ring and the C-O stretching vibrations respectively. The FT-IR spectra confirm the successful coupling between carboxymethyl chitosan (CMC) and ethylenediaminetetraacetic acid (EDTA) and the formation of calcium-complex with CMC-EDTA.

![FTIR spectra](image)

Figure 3-3. FTIR spectra of (A) water soluble chitosan without any modification, (B) modified chitosan (chitosan-EDTA), and (C) freeze-dried hydrogel (after gelation by adding 1 M calcium solution). Chitosan/CE conjugate ratio is 90:10 in (B) and (C).

The \(^1\)H NMR spectrum at 300 MHz was carried out in D\(_2\)O. The results (Figure 3-4) show that the resonance proton at 7.90 ppm is belonged to the amide proton. The proton H\(_1\) appeared at 4.65 ppm, and the protons resonance H\(_3\) to H\(_6\) range from 3.6-3.9 ppm. The presence of the proton H\(_a\) at 2.3 ppm and the proton H\(_b\) at 3.40 ppm suggest the successful coupling of the
ethylenediaminetetraacetic acid (EDTA) with the carboxymethyl chitosan (CMC). The $^1$H NMR confirms the coupling between EDTA and CMC.

![Figure 3-4. $^1$H NMR spectrum of the EDTA modified chitosan hydrogel network at 300 MHz. Chitosan/CE conjugate ratio of the sample tested is 90:10.](image)

### 3.3.2 Specification of the Bioinks

The very first step to prepare a 3D printable bioink is to find the proper range of the concentrations of the components in the precursor solution. In the present work hydrogel precursor is made of two main components, CE powder and additional chitosan added afterward. Additional chitosan, provides more polymer chains with carboxyl groups for enhanced mechanical properties and adjusts the viscosity of the bioink for extrusion bioprinting. Later, by adding calcium solution, the additional carboxyl groups on different chains will form ionic bonding among themselves as well as with carboxyl groups left unreacted from the CE powder. Therefore, the amount of these two
main components have been modified to find the proper viscosity and concentration for a successful printing and gelation of the precursor. As the amount of these two components are changed, the bioink printability and gelation quality is affected. A few pictures of samples printed with different mixture ratios and the final printed sample with high printing precision are shown in Figure 3-5 to illustrate this step. It is important to note that since the focus of this work was to develop a bioink suitable for 3D bioprinting, the bioink ratio found to be accurately printable (90:10) was evaluated specifically in all plots.

Another essential factor to be considered is the ability to print multiple layers in order to construct complex structures. Figure 3-6 displays the optical microscope images of printed multilayered structures showing the ability of the present bioink to print complex structures consisted of multi-layer straight and arced printed filaments.
Rheology, as the study of flow of matter under external forces, is notably important in bioprinting and biofabrication. Various rheological parameters, such as viscosity and shear thinning, influence the biofabrication process and therefore are needed to be investigated. To begin with, viscosity, as the resistance of a hydrogel precursor under external forces, is determined primarily by the solution concentration and the molecular weight of the polymers. Higher concentrations generally mean higher viscosity and denser polymer networks that could potentially hinder favorable cell proliferation and tissue formation. On the other hand, low concentrations negatively affects shape fidelity after deposition of the hydrogel and the printed strands will spread out on the printing substrate. Therefore, in the present work, rheology results are presented and compared for four bioprintable concentrations of the developed bioink while chitosan:CE ratio of 90:10 is selected for cell studies due to its optimum shape fidelity.
To investigate the change of properties due to changes in the composition, four bioinks were formulated and investigated with regards to rheological and mechanical properties. Four chitosan:CE ratios (namely 93:07, 90:10, 87:13, and 85:15) were studied and compared. The frequency oscillation measurements presented in Figure 3-7 showed increased storage and loss modulus for higher proportions of CE in the bioink.

In these samples, the weight of the CE powder in the solution is kept constant while the weight of additional chitosan is slightly changed. As depicted in Figures 3-7a and b, higher weight ratio of CE in the solution results in higher storage and loss modulus. Results are compared to NFC/Alginate...
hydrogel developed by Markstedt et al[61]. Also, the effect of crosslinking time on storage and loss modulus is illustrated in Figure 3-7c. As expected, longer crosslinking time results in tighter polymer networks and higher storage and loss modulus.

Furthermore crosslinking effect is investigated by measuring shrinkage of the gels due to crosslinking. To this end, four concentrations of calcium ranging from 0.1M to 2M were considered. As the concentration of crosslinking agent is increased, reduction in the diameter of the hydrogel disc is also increased (Figure 3-8) introducing denser intertwined networks. Also, gelation patterns on the hydrogel appear to be smoother in higher concentrations of calcium due to fast gelation.

![Figure 3-8. Study of crosslinker concentration effect on gel retraction and appearance. Images of hydrogel discs crosslinked with (A) 0.1M, (B) 0.5M, (C) 1M, and (D) 2M CaCl₂ solution. Top images in each set represent gel precursor before final crosslinking and bottom images represent the resulting gel after crosslinking. Chitosan/CE conjugate ratio of the samples shown is 90:10 and the crosslinking time is 45 minutes for all samples.](image)

To further study the effect of the crosslinking concentration, compressive strength of hydrogels crosslinked with same four concentrations of calcium solution was extracted.
Figure 3.9. (A, B) Study of crosslinking concentration effect on compressive strength of the hydrogel and comparison with NFC/Alginate hydrogel14. (C) Storage modulus, $G'$ and loss modulus, $G''$ of the bioink as a function of crosslinking concentration. Solid lines represent storage modulus and dashed lines represent loss modulus. (D) Study of shear thinning properties of four Chitosan/CE conjugate ratios. (E) Study of swelling ratio in samples crosslinked with four concentrations of calcium solution ranging from 0.1M to 2M. In (A), (B), (C) and (E), Chitosan/CE conjugate ratio of the samples tested is 90:10 and the crosslinking time is 45 minutes for all samples.
To evaluate the strength of the bioink after crosslinking, compression tests were carried out. The stress-strain curves and young’s modulus of the four groups were extracted and presented in Figure 3-9a and b. As expected, higher crosslinking concentrations lead to higher stiffness and lower elasticity which could be attributed to more restricted polymer networks and shifted the peak stresses to the right to happen in higher strain. Also, a six-fold increase in the peak stress is observed as calcium concentration is increased from 0.1M to 2M. It can also be concluded from figure 3-9a that the stiffness of the present bioink is tunable to provide the proper support and host various type of cells. Furthermore, the effect of crosslinking concentration is studied by testing the same four samples for storage and loss modulus (Figure 3-9c). This figure also shows the tunability of viscoelastic properties of the hydrogel by adjusting the crosslinking concentration. As expected, higher crosslinking concentration leads to stronger polymer networks which in turn increases the storage and loss modulus.

Shear thinning, the reduction of viscosity by increased shear rate, is observed in Figure 3-9d that verifies the non-Newtonian behavior of the hydrogel precursor. This phenomenon is often observed in solutions containing high molecular weight polymers. It is observed that for higher concentrations, relative viscosity reduction is greater. The significant change of the shear thinning plots indicates the importance of proportions of CE and chitosan as well as change in concentration.

### 3.3.3 Swelling ratio

Due to high water content, hydrogels are considered as biodegradable soft materials being able to mimic soft tissue. Being biodegradable and biocompatible, hydrogels have found important applications in wound dressing[160], biomedical implants[161], cell studies, etc. To find out the suitability of any specific hydrogel for these type of applications, it is required to study the amount of water they can absorb over time. Swelling behavior of the hydrogel crosslinked by various
crosslinker concentrations were studied and presented in Figure 3-9e. Hydrogels were weighed before immersion in double distilled water and weighed multiple times over a period of 22 days. As it could be inferred from this figure, hydrogels crosslinked with 1M and 2M calcium solution lose weight slightly in the first day and start to absorb water from the second day. The small de-swelling observed in higher concentrations could be attributed to PH or ionic strength change. The overall swelling trend is to reach a stable state which fall in the range of 14 to 24 % of weight increase.

3.3.4 Chondrocyte viability evaluation

The potential harmful effects of the hydrogel mesh on cell viability was investigated. The samples used for all cell studies were prepared with a chitosan:CE ratio of 90:10 and crosslinked by 0.5 M calcium chloride solution . Chondrocyte viability was evaluated with The LIVE/DEAD Viability/Cytotoxicity Kit after 36 h of seeding on the 3D printed scaffold. Live cells emit green fluorescence in the cytoplasm, whereas nuclei of dead cells emit red fluorescence. Only a small amount of apoptotic cells can be seen under fluorescence microscopy, indicating low cytotoxicity of the hydrogel. The cell viability was further evaluated by flow cytometry. Quantification analysis revealed that the hydrogel mesh did not affect the cell viability.
Figure 3-10. (A) Live/Dead staining of chondrocytes. (B) Flow cytometry result of cell viability in the control group. (C) Flow cytometry result of cell viability in the hydrogel mesh group. (D) Quantification of cell viability in both groups. Scale bar = 100um.

3.3.5 Chondrocyte proliferation evaluation

After seeding on the scaffold for 36 h, chondrocyte proliferation status was assessed by EdU staining. EdU positive chondrocytes were detected in both groups, which indicates the proliferation of chondrocytes. Quantification of the chondrocytes proliferation status revealed a similar proliferation rate between hydrogel mesh group and the control, indicating the hydrogel scaffold did not impair the proliferation of chondrocytes.
Figure 3-11. (A) EdU staining of chondrocytes in both group. (B) Quantification of chondrocytes proliferation rate in both group. Scale bar =100 um.

### 3.3.6 Chondrogenic markers expression

Chondrogenic markers expression were evaluated at different time points, including the ECM marker Collagen II and the chondrogenic transcription factor Sox 9. As extracellular matrix protein of cartilage, collagen II expression reached its peak at day 6 in mesh group and was higher than that in the control group between day 6 and day 12. The expression of chondrogenic gene Sox 9 reached its peak at day 6 and the relative expression of Sox 9 was higher in mesh group than that in the control during the same period.
3.4 Conclusion

This study presents novel chitosan-based bioink mainly composed of chitosan and EDTA using physical crosslinking by calcium solution. Multilayer 3D mesh structures were 3D printed showing stability and high printing fidelity. Based on the presented results, the newly developed bioink exhibits suitable stability and mechanical properties as well as fast gelation and high printing precision. According to the rheology and mechanical testing results, the bioink viscoelastic properties and mechanical strength are tunable by adjustment of the proportions of the components which provides a platform to expand the application of the bioink in tissue engineering. Furthermore, cell studies with chondrocytes show that the bioink is biocompatible and supports cell proliferation and also helps cells to retain their chondrogenic phenotype. Our results illustrate that the developed bioink has the potential to be employed for 3D bioprinting of scaffolds for tissue engineering and therefore, is a suitable candidate as a bioink.
Chapter 4

4 Fabrication of PANI-coated flexible membranes based on chitosan and graphene

4.1 Introduction

In electrochemical capacitors, marketed first by NEC as supercapacitors (SCs) in 1971, energy may be delivered by oxidation-reduction (redox) reactions or formation and release of electrical double layers (ELDs). Fast charging/discharging, long charge/discharge cycles, and high power density are among the SCs’ strengths [162]. SCs employ double-layer capacitance and pseudocapacitance rather than solid dielectrics in conventional capacitors. In double-layer capacitors, charge is stored electrostatically while in pseudocapacitors it is stored via electrochemical reactions. In the past few years, research on materials that might be able to combine high energy density, which is attributed to batteries, and fast charging, attributed to supercapacitors, has been extensively expanded. To this end, composite structures consisting of carbon-based materials, such as graphene, and pseudocapacitance materials, such as polyaniline, has been utilized [163-165].

Pseudocapacitors are considered to potentially be able to achieve higher capacitance in comparison with electrical double layer capacitors (EDLs) by reversible faradic reactions of redox-active
materials [166]. A great deal of effort has been dedicated to find the ideal compact pseudocapacitor offering high power and volumetric capacitance by using fibers, films, and wires in nanoscale [167-169].

Polyaniline (PANI) is an inexpensive and easy to synthesis polymer which has been extensively studied due to its rich chemistry, high specific capacitance, high doping level, and potential for large pseudocapacitance. Mass specific capacitance of PANI in sulfuric acid has been theoretically estimated to be about 2000 Farads per gram while practically reaching this number is in doubt due to unlikeliness of full doping and low charge transfer rate [170]. However, slow charge transfer reactions of PANI, which are responsible for pseudocapacitance, are known to offer poor rate capability in comparison with electrical double layer reactions [166]. Furthermore, making a mechanically strong freestanding membrane with PANI alone is not feasible due to its brittleness. Unfavorable cyclic stability of PANI, due to cracking of the polymer during doping and dedoping of ions in pseudocapacitive reactions, is another factor that limits its application.

Therefore, a composite structure of PANI and another conductive or nonconductive support material has been considered in the literature [171, 172]. Blends of PANI and carbon structures, for instance, combine properties of pseudocapacitance and ELD capacitance.

A composite of graphene and polyaniline (PANI) is a common route to couple the benefits of both materials [173-185]. Graphene, a well-known nanostructure, is a 2D carbon structure offering unique mechanical, electrical, and thermal properties that has been studied extensively in various applications such as supercapacitors, fuel cells, and Li-ion batteries [186]. Graphene, a EDL capacitive material, offers appealing characteristics such as thermal stability, beneficial functional groups, large surface area, and superior electrical conductivity [187]. However, there are known
drawbacks attributed to graphene. Although it is possible to make highly conductive graphene oxide (GO) free standing membranes by vacuum filtration or drop-casting, but such membranes may be unfit for supercapacitor application due to low porosity and surface area. Moreover, the maximum applicable current density for graphene-based supercapacitors is generally lower than composite supercapacitors. Also, densely stacked GO sheets can lead to low surface area and small pore sizes which hinder ion transfer through the membranes in supercapacitor applications. In one study, for example, densely stacked graphene sheets were observed in a graphene film prepared by vacuum filtration \[^{188}\]. Although such film was reported to offer high electrical conductivity, it is not suitable as an EDL electrode in a supercapacitor due to its low porosity which limits electrolyte ions penetration into the lower graphene sheets.

Therefore, to exploit graphene’s great potential, often a composite structure is employed. In the case of supercapacitors, PANI/graphene composite has been studied extensively as mentioned earlier. However, it is of great importance to take into account details such as nano-architecture, porosity, electrical conductivity, and ionic conductivity to achieve high overall performance. The structure of electrodes directly affect properties such as specific capacitance, rate capability, and power density. For instance, bulk PANI has very small pores that hinders its ion-transfer conductivity. Small pores mean low porosity and accessible surface area which does not allow for proper penetration of electrolyte ions into active materials. Limited ion-transfer conductivity results in poor capacitance, rate capability, and power density. On the other hand, electronic conductivity could be deteriorated by very large pores \[^{189}\].

It is important to know that low surface area can negatively affect the capacitance and also, low ionic conductivity can adversely affect the specific capacitance, rate capability, and power density.
Studies have been undertaken to address the aforementioned parameters affecting overall performance of supercapacitors. Meng et al., for instance, successfully fabricated high performance flexible graphene/polyaniline supercapacitors with interconnected porous structure [185]. It was reported that the high rate performance in this work could be achieved due to fast ion transfer through the film via interconnected pores. In another work, cellulose fibers were employed as a flexible backbone for a graphene/PANI/cellulose/AgNPs supercapacitor binding components together [181]. It was observed that the weight ratios between the components of the supercapacitor could be adjusted to achieve high cyclic stability and tune power and energy densities. Furthermore, in a novel study, Wang et al. reported a graphene/polyaniline electrode that was prepared by vacuum filtration and controlled capillary compression. It was concluded that rather than pore size, pore connectivity is the substantial factor in pseudocapacitance.

In the present work, we have employed blends of chitosan (CS) and GO along with vacuum filtration to fabricate flexible membranes as electrodes for supercapacitors. After filtration, the GO/CS electrodes were reduced by Hydroiodic acid (HI) to obtain rGO/CS membranes. GO reduction was then followed by multiple in situ polymerization of PANI. It is important to mention the advantages of our method. One benefit of using CS is to prevent the formation of highly stacked graphene sheets and therefore enhancing the ionic conductivity and PANI coating by enhancing the interlayer gaps as schematically shown in Figure 1-1. Also, adding even very low amounts of CS to graphene, dramatically decreases the filtration time. Furthermore, chitosan could potentially play a role as vibration isolator supporting PANI during repeated expansion and shrinkage during cycling similar to polyurethane role in the work by Khosrozadeh et al.[172]. In addition, since chitosan is relatively soft compared to graphene, a composite substrate of graphene and chitosan could
potentially mitigate the stress due to PANI volume change and enhance overall cycling performance of the SC much better than a graphene substrate.

![Diagram](image)

**Figure 4-1.** Schematic showing the effect of chitosan on enlarged interlayer spacing and enhanced PANI coating.

Herein, we report fabrication of composite membranes by vacuum filtration of blends of chitosan and reduced graphene oxide loaded with PANI via in situ polymerization. Through utilization of this combination, we have combined the benefits of each material by fabricating mechanically robust and flexible electrodes providing outstanding energy storage capabilities.
4.2 Experimental section

4.2.1 Materials

Graphene oxide and chitosan were purchased from Suzhou Tanfeng Graphene Technology Co and Shanghai Zhanyun Chemical, respectively. Also, nylon filter papers were purchased from Tianjin Jinteng Experiment Equipment and 57 wt% Hydroiodic acid (HI) and Aniline were purchased from Sigma-Aldrich. Moreover, Sulfuric acid, reagent alcohol, and sodium hydroxide used were from Anachemia, VMR ANALYTICAL, and Fisher Scientific, respectively. Unless stated otherwise, all materials were used without further purification.

4.2.2 Preparation of GO/CS composite membranes

A solution of chitosan with a concentration of 20 mg/ml in 2 % acetic acid was prepared. Graphene oxide was dispersed in deionized water with a concentration of 0.5 mg/ml. The GO solution was then stirred for 5 minutes and bath sonicated for an hour. CS solution was added dropwise while stirring to obtain different GO/CS ratios followed by addition of sodium hydroxide solution (0.05 molar) to enhance the dispersion and prevent aggregation of the blend in acidic environment. The suspension was then bath sonicated for 45 minutes followed by vacuum filtration using nylon filters. Hydroiodic acid was then introduced to the membrane to reduce GO (overnight) and rGO/CS membrane was obtained.

4.2.3 Coating of PANI on the membranes

As prepared rGO/CS membranes were washed with distilled water repeatedly to remove HI residues and then were immersed in a solution of 160 µl Aniline dissolved in a blend of 1 molar sulfuric acid (30 ml) and reagent alcohol (5 ml) for 30 minutes at room temperature. Also, 0.133 gram of
Ammonium persulfate (APS) was dissolved in 5 ml of 1 molar sulfuric acid and kept at 4 degrees for 30 minutes. APS solution was then mixed with aniline solution and the polymerization took place over a period of 4 hours and 30 minutes. The PANI coated rGO/CS membrane was then removed and washed with deionized water. Similarly, PANI coating step was repeated to increase the mass loading of PANI on the membrane.

4.2.4 Characterization

A scanning electron microscope (ZEISS) was used to study the surface morphology of the membranes. A Thermo Scientific Nicolet iS10 FT-IR Spectrometer was used to extract the Fourier transform infrared (FTIR) spectra. Electrochemical measurements were conducted by an electrochemical workstation (CS350, CorrTest Instruments Inc.).

4.2.5 Electrochemical measurements

The Galvanostatic charge-discharge data was recorded from 0 to 0.8 V at currents from 2 to 75 mA. Moreover, the electrochemical impedance spectroscopy (EIS) tests were performed at 10 mV and 20 mA current range over a frequency span from 0.01 to 100000 Hz.

To assemble supercapacitors for electrochemical testing, two alike rectangular strips (2 cm × 1.5 cm) were cut out of each membrane (electrodes) and assembled symmetrically with a cellulose membrane separator. Both electrodes and the separator were soaked in 1 M sulfuric acid for 10 minutes before being sandwiched between two stainless steel foils (current collectors). The assembled SCs were then sealed tightly with Parafilm, placed between two glass slides, and clipped.

Discharge slope of the charge-discharge curves, excluding the potential drop, is used to extract the capacitance of the supercapacitor. The equation $C = I \Delta t / (\Delta V - V_{IR})$ was employed to calculate the
capacitance, where $I$ is the current, $\Delta V$ is potential change after full discharge, $\Delta t$ is the time of discharge, and $V_{IR}$ is the initial potential drop during discharge. The calculations of power density (P) and energy density (E) are carried out using $P = E/\Delta t$ and $E = \frac{1}{2}C(\Delta V - V_{IR})^2$, respectively. Dividing C, P, and E by the total mass of the electrodes, specific capacitance, power density, and energy density are calculated. Also, dividing C, P, and E by area of the supercapacitor, the areal specific capacitance, power density, and energy density are calculated.

### 4.3 Results and discussion

Electrodes fabricated by vacuum filtration of various blends of Graphene oxide and chitosan were studied in the present work. Based on the trends in the results, three compositions were selected for detailed characterisation by electrochemical testing (Table 4-1). Figure 4-1 displays photographs of the freestanding and flexible composite film of graphene oxide and chitosan. High flexibility of the film is attributed to proper bonding between chitosan and graphene oxide functional groups.

SEM images of different samples comparing surface morphology of samples with various compositions with or without PANI coating and also morphology change during repeated PANI coatings are presented in figures 4-2 to 4-4. Figure 4-2 shows morphology change of one sample after each step of PANI coating. Increment of the density as well as the change in grain size of PANI particles after each repetition of polymerization is noticeable in these images.

*Figure 4-2. Photographs of freestanding and flexible film of graphene oxide and chitosan.*
Table 4-1. Composition of three types of electrodes fabricated.

<table>
<thead>
<tr>
<th>sample</th>
<th>Graphene oxide (mg)</th>
<th>Chitosan (mg)</th>
<th>GO %</th>
</tr>
</thead>
<tbody>
<tr>
<td>GC90</td>
<td>63.5</td>
<td>7</td>
<td>90</td>
</tr>
<tr>
<td>GC95</td>
<td>67</td>
<td>3.5</td>
<td>95</td>
</tr>
<tr>
<td>GC100</td>
<td>70.5</td>
<td>0</td>
<td>100</td>
</tr>
</tbody>
</table>

Figure 4-3. SEM images of GC95 after first (A, B), second (C, D), and third (E, F) PANI coating. Magnification is 10K for the images on the left column and 20K for the images on the right.
Moreover, chitosan addition affects the amount and pattern of the PANI coated on the membranes. Figure 4-3 displays effect of chitosan on patterns of PANI coating on electrodes with 10 and 5 % chitosan after thrice in situ PANI coatings. From these images, it can be concluded that higher amounts of chitosan limit mass loading of PANI on the electrodes during polymerization which is also confirmed later by electrochemical testing of the electrodes.

![SEM images of (A, B) GC90 and (C, D) GC95 after thrice PANI coatings. Magnification is 5K for the images on the left column and 50K for the images on the right.](image_url)
Figure 4-5. Nyquist plots for three weight ratios of GO/CS. Right image shows higher magnification of the semicircular part of the graphs. The samples with 95 and 97% GO display the minimum charge-transfer resistance in this group.

The electrochemical performance of the supercapacitors was investigated by Galvanostatic charging/discharging (GCD) and Electrochemical Impedance Spectroscopy (EIS) using a two-electrode system. Nyquist plots from EIS tests of samples with three PANI coatings are displayed in Figure 4-4. Based on the diameter of the semicircles, GC95 displays the lowest charge-transfer resistance in this group with 2.1 ohms. It is noteworthy to discuss the reasons for the tremendous difference in the height of GC100 and the other two SCs in Figure 4-4. In GC100, the highly compact graphene sheets that form without the presence of chitosan, limit PANI coating between the layers. Also, in GC90, excessive amount of chitosan hinders optimum PANI polymerization on the electrodes as observed in Figure 4-3.
From this difference in the heights, it can also be concluded that GC95 stores much more energy in comparison with the other two SCs which is also confirmed by capacitance and power density results.

Figure 4-6. GCD plots of the sample with 95 % Go and trice PANI coatings at various current densities. Higher magnification of the higher current densities are shown in the right image.

The GCD plots of the sample with 95 % Go and trice PANI coatings at various current densities is shown in Figure 4-5. The potential drop occurring before discharging is attributed to the resistance of the connections between the SCs and electrochemical workstation, electrolyte resistance, and the SC electrodes resistance. It is noteworthy that potential drop decreases slightly after each step of repeated PANI coatings. Mass and areal-specific capacitance are compared in Figure 4-6 for three samples with rGO weight ratios ranging from 90 to 100 %. Among these plots, calculated from GCD data, GC95 shows the highest capacitance. The areal and mass specific power density versus energy density at various currents (Ragone plots) are presented in figure 4-7. GC95 GO exhibits
superior performance in these plots as well. As it could be inferred from figures 4-6 and 4-7, GC95 exhibits significantly higher capacitance at any given current density and higher energy density compared to the other SCs. Based on these results, GC95 with only 5% chitosan shows the best performance among these SCs with similar composition.

Figure 4-7. (A) mass-specific and (B) areal-specific capacitance versus current density for SCs with GO weight ratios ranging from 90-100%.

Figure 4-8. (A) mass-specific and (B) areal-specific power density versus energy density for SCs with GO weight ratios ranging from 90-100%.
To investigate the benefit of this method, maximum areal capacitance has been compared to references with similar compositions in Table 4-2. It is shown that this strategy leads to much higher areal capacitance at similar current densities.

Table 4-2. Comparison of maximum areal capacitance in references with similar composition

<table>
<thead>
<tr>
<th>Ref</th>
<th>Composition</th>
<th>Maximum areal capacitance (F/cm²)</th>
<th>Current density at max capacitance (mA/cm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>This work</td>
<td>GO/Chitosan/PANI</td>
<td>1.6</td>
<td>1.7</td>
</tr>
<tr>
<td>[190]</td>
<td>GO/PANI</td>
<td>0.7</td>
<td>2.2</td>
</tr>
<tr>
<td>[191]</td>
<td>GO/PANI/eCFC</td>
<td>0.4</td>
<td>1.2</td>
</tr>
<tr>
<td>[185]</td>
<td>GO/PANI</td>
<td>0.2</td>
<td>1.3</td>
</tr>
<tr>
<td>[172]</td>
<td>PU/CNT/PANI</td>
<td>0.3</td>
<td>1.7</td>
</tr>
<tr>
<td>[184]</td>
<td>GO/PANI</td>
<td>0.2</td>
<td>1.3</td>
</tr>
</tbody>
</table>

4.4 Conclusions

In conclusion, a high performance, scalable, and low-cost supercapacitor has been developed by vacuum filtration of GO and chitosan and in situ coating of PANI on the flexible filtered films. The amount of each component as well as the methods employed were optimized to maximize the electrochemical performance of the developed composite electrodes. Tuning the ratio of GO/CS plays an important role in both mechanical properties [192] and electrochemical performance of the electrodes. The findings of this work indicate that the optimum ratio of GO/CS is 95/5 wt%. Higher amount of CS were found to deteriorate the electrochemical performance and lower amount of CS were observed to increase the filtration time, hinder PANI coating on the electrodes, and impair the feasibility of electrode fabrication. The high capacitance, adequate rate capability, rapid fabrication,
and excellent cycle life of the developed flexible electrodes, make them good candidates for many current and future applications such as portable and wearable electronics.
Chapter 5

5 Summary and future Works

The objective of this work was to study the application of high performance hydrogels in tissue engineering and energy storage devices. To this end, 3D bioprinting techniques and advances where reviewed in chapter two as trending tools for utilization of hydrogels in tissue engineering. Employing appropriate bioprinting tool, in chapter three, a novel engineered bioink based on chitosan was developed and studied as a template for rabbit chondrocyte cells. It was shown that the proposed bioink is not toxic for cells and promotes cell proliferation. Later, in chapter four, application of thin film hydrogels in supercapacitors as energy storage devices was sought. Composite supercapacitors based on chitosan, graphene oxide and polyaniline was developed and evaluated. These flexible supercapacitors were shown to offer short fabrication time and proper stability and performance.

The presented projects in this text are currently under extensive study due to their significant applications. Tissue engineering is very important due to health benefits that offers and supercapacitors can play a substantial role in future of wearable electronics and move the industry forward. There is plenty of room on both topics for future works. Just to mention a few, self-healing platforms in normal environment and high flexibility are among the most important factors that need to be addressed for further developments in both topics.
6 Bibliography


89


