PREPARATION AND SYNTHESIS OF CELLULOSE-BASED FUNCTIONAL MATERIALS

By

NUSHENG CHEN

A DISSERTATION PRESENTED TO THE GRADUATE SCHOOL OF THE UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY UNIVERSITY OF FLORIDA 2016
To all the people have helped me
ACKNOWLEDGMENTS

I would like to express my deep gratitude to my advisor and mentor, Dr. Zhaohui Tong for her continuous support for my Ph.D. study, for her patient guidance, enthusiastic encouragement and useful critiques on my research. I would also like to thank Dr. Pratap Pullammanappallil, Dr. Bin Gao, Dr. Eric Mclamore and Dr. Jiangeng Xue for serving on my dissertation committee. Their advices and assistances are keeping my progress on schedule. My grateful thanks are also extended to Dr. Anthony B. Brennan, Dr. Weihua Yang and Dr. Chen Lin for their direction in doing related thermal, rheological characterization and cytotoxicity test.

I would also like to extend my thanks to the technicians in Major Analytical & Particle Analysis Instrument Centers (MAIC) at the University of Florida for their help in offering me the resources to conduct materials characterization, which facilitate my research.

Additionally, I do appreciate the help from my research peers. Their detailed and accurate answers to my questions and confusions through e-mail promoted my research; I do enjoy the scientific spirit of discussion and sharing.

Finally, I wish to thank my families for their selfless love and full support throughout my life. Without them, I could not have achieved this goal.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACKNOWLEDGMENTS</td>
<td>4</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>8</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>9</td>
</tr>
<tr>
<td>LIST OF SCHEMES</td>
<td>11</td>
</tr>
<tr>
<td>LIST OF ABBREVIATIONS</td>
<td>12</td>
</tr>
<tr>
<td>ABSTRACT</td>
<td>14</td>
</tr>
<tr>
<td>CHAPTER</td>
<td></td>
</tr>
<tr>
<td>1  CURRENT APPLICATIONS AND TRENDS OF CELLULOSE BASED BIOMATERIALS</td>
<td>16</td>
</tr>
<tr>
<td>Global Perspective</td>
<td>16</td>
</tr>
<tr>
<td>Cellulose</td>
<td>18</td>
</tr>
<tr>
<td>Nanocellulose</td>
<td>19</td>
</tr>
<tr>
<td>Cellulose-Based Biocomposite</td>
<td>22</td>
</tr>
<tr>
<td>The Modification of Cellulose</td>
<td>24</td>
</tr>
<tr>
<td>Advanced Applications of Cellulose</td>
<td>27</td>
</tr>
<tr>
<td>Summaries and Potential Opportunities</td>
<td>29</td>
</tr>
<tr>
<td>2  FABRICATION OF MICROFIBRILLATED CELLULOSE GEL FROM WASTE PULP SLUDGE</td>
<td>35</td>
</tr>
<tr>
<td>VIA MILD MACERATION COMBINED WITH MECHANICAL SHEARING</td>
<td></td>
</tr>
<tr>
<td>Introduction</td>
<td>35</td>
</tr>
<tr>
<td>Materials and Methods</td>
<td>38</td>
</tr>
<tr>
<td>Materials</td>
<td>38</td>
</tr>
<tr>
<td>Gel Preparation by a Disc Refining (DR) Method</td>
<td>39</td>
</tr>
<tr>
<td>Gel Preparation by an Ultrasonication plus Homogenization (UH) Process</td>
<td>39</td>
</tr>
<tr>
<td>Optical Microscopy Characterization</td>
<td>40</td>
</tr>
<tr>
<td>Scanning Electronic Microscopy (SEM) Characterization</td>
<td>40</td>
</tr>
<tr>
<td>Water Retention Value (WRV) Measurement</td>
<td>40</td>
</tr>
<tr>
<td>Specific Surface Area (SSA) Measurement</td>
<td>41</td>
</tr>
<tr>
<td>Dynamic Rheology Characterization</td>
<td>41</td>
</tr>
<tr>
<td>Results and Discussion</td>
<td>42</td>
</tr>
<tr>
<td>Chemical Composition Analysis of the Waste Pulp Sludge before and after Maceration</td>
<td>42</td>
</tr>
<tr>
<td>Water Retention Values (WRVs) of MFC Hydrogels</td>
<td>42</td>
</tr>
<tr>
<td>Specific Surface Areas (SSAs) of MFC Hydrogels</td>
<td>43</td>
</tr>
</tbody>
</table>
Materials and Methods

Introduction

BIOCOMPOSITES WITH TUNABLE PROPERTIES FROM POLY (LACTIC ACID)-BASED COPOLYMERS AND CARBOXYMETHYL CELLULOSE VIA IONIC ASSEMBLY

Introduction ........................................................................................................................................ 61
Materials and Methods ................................................................................................................... 65
Materials ........................................................................................................................................... 65
Synthesis of Poly (lactic acid) (PLA-OH) ......................................................................................... 66
Synthesis of Macroinitiator PLA-Br .................................................................................................. 67
Synthesis of Copolymer PLA-b-PDMAEMA ................................................................................. 67
Quaternization of PLA-b-PDMAEMA Copolymer ........................................................................ 68
Preparation of Block Copolymer Micelles in Water ........................................................................ 68
Preparation of Biocomposite ........................................................................................................... 68
Characterization Methods ................................................................................................................ 69
NMR spectrum .................................................................................................................................. 69
Morphology studies ........................................................................................................................... 69
Hydrodynamic radius analysis .......................................................................................................... 69
Fourier transform infrared spectrometry (FTIR) analysis ............................................................. 69
Thermal stability analysis ................................................................................................................ 70
Dynamic mechanical analysis (DMA) ............................................................................................ 70
Results and Discussion ..................................................................................................................... 70
Preparation of Cationic Copolymer PLA-b-PDMAEMA ............................................................. 70
NMR results of PLA-b-PDMAEMA copolymer ............................................................................ 70
Dynamic particle radii and morphologies of copolymer micelles .................................................. 71
Preparation of CMC/PLA Biocomposite Suspension .................................................................... 72
Optical observation of morphology .............................................................................................. 72
Scanning electron microscopic analysis ......................................................................................... 73
FTIR results ....................................................................................................................................... 73
Preparation and Characterization of CMC/PLA Biocomposite Films .......................................... 74
Thermogravimetric analysis (TGA) ................................................................................................. 74
Dynamic mechanical analysis (DMA) ........................................................................................... 75
Summary .......................................................................................................................................... 76

CELLULOSE-BASED INJECTABLE HYDROGEL COMPOSITE FOR PH-RESPONSIVE DRUG DELIVERY ......................................................................................................................... 87

Introduction ........................................................................................................................................ 87
Materials and Methods ................................................................................................................... 90
Materials ........................................................................................................................................... 90
Preparation of Copolymer Micelles ................................................................................................. 91
The synthesis of PEO macroinitiator ............................................................................................... 91
The synthesis of PEO-b-PDPA copolymer ..................................................................................... 92
Preparation of PEO-b-PDPA copolymer micelles ....................................................................... 92
# LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-1</td>
<td>The main chemical composition before and after maceration</td>
<td>50</td>
</tr>
<tr>
<td>3-1</td>
<td>Hydrodynamic radius and the number average molecular weight of PLA and PLA-b-PDMAEMA with different chain length of PDMAEMA segment</td>
<td>79</td>
</tr>
</tbody>
</table>
**LIST OF FIGURES**

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-1</td>
<td>Chemical association in the plant cell wall</td>
<td>30</td>
</tr>
<tr>
<td>1-2</td>
<td>Interconversion of the polymorphs of cellulose</td>
<td>31</td>
</tr>
<tr>
<td>1-3</td>
<td>The categories of nanocellulose</td>
<td>32</td>
</tr>
<tr>
<td>1-4</td>
<td>The most applied mechanical treatment processes used in the fabrication of microfibrillated cellulose (MFC): the homogenizer, the microfluidizer and the grinder</td>
<td>33</td>
</tr>
<tr>
<td>1-5</td>
<td>Examples of cellulose derivatives through esterification and etherification</td>
<td>34</td>
</tr>
<tr>
<td>2-1</td>
<td>The waste pulp sludge before and after maceration treatment</td>
<td>51</td>
</tr>
<tr>
<td>2-2</td>
<td>The morphology of cellulose after maceration: optical micrograph and differential interference contrast micrograph</td>
<td>52</td>
</tr>
<tr>
<td>2-3</td>
<td>The water retention values (WRVs) of MFC gels after disc refining (DR) and ultrasonication plus homogenization (UH)</td>
<td>53</td>
</tr>
<tr>
<td>2-4</td>
<td>Specific surface areas (SSAs) of freeze-dried MFC gels after disc refining (DR) and ultrasonication plus homogenization (UH), where 5 min ultrasonication was applied</td>
<td>54</td>
</tr>
<tr>
<td>2-5</td>
<td>Differential interference micrographs of DR-treated MFC and UH-treated MFC at different processing conditions</td>
<td>55</td>
</tr>
<tr>
<td>2-6</td>
<td>The SEM images of freeze-dried MFC gels after different shear force treatments</td>
<td>58</td>
</tr>
<tr>
<td>2-7</td>
<td>The storage moduli and the loss moduli as functions of frequency for all MFC gels</td>
<td>59</td>
</tr>
<tr>
<td>2-8</td>
<td>The Influence of shear rate on the viscosity of different MFC gels</td>
<td>60</td>
</tr>
<tr>
<td>3-1</td>
<td>$^1$H NMR spectra of synthesized products: PLA, macroinitiator PLA-Br and PLA-b-PDMAEMA</td>
<td>80</td>
</tr>
<tr>
<td>3-2</td>
<td>Hydrodynamic radii of quaternized PLA-b-PDMAEMA copolymer micelle particles in water</td>
<td>81</td>
</tr>
<tr>
<td>3-3</td>
<td>Morphologies in different suspensions and composite film</td>
<td>82</td>
</tr>
<tr>
<td>3-4</td>
<td>SEM images of CMC, and the mixture of CMC and quaternized copolymer PLA-b-PDMAEMA (copolymer 2)</td>
<td>83</td>
</tr>
</tbody>
</table>
Fourier transformed infrared spectra (FTIR) of copolymer PLA-b-PDMAEMA, CMC and the resultant composite ................................................................. 84

Thermogravimetric analysis (TGA) biocomposite films with different molecular weight of PDMAEMA segment................................................................. 85

Temperature dependence of storage modulus and tan δ of CMC and biocomposites with different molecular weight of PDMAEMA segment........ 86

$^1$H NMR spectrum of PEO-Br and PEO-b-PDPA.................................................. 111

$^1$H NMR spectrum of CMC-CHO ...................................................................... 112

$^1$H NMR spectrum of CMC and CMC-NH$_2$............................................................ 113

Study of the effects on gelation time .................................................................. 114

SEM images of hydrogels through different combinations................................. 115

Elastic moduli ($G'$) of various injectable hydrogels at 2% strain using a rheometer with a parallel geometry ............................................................ 116

Storage moduli ($G'$) of hydrogels prepared under different pH values........... 117

Swelling ratios and physical appearance of hydrogels ....................................... 118

Fluorescence emission spectra of Nile Red in the presence or absence of PEO-b-PDPA at pH 7.4 ................................................................................. 119

Normalized fluorescence intensity versus pH (the insert shows the fluorescence spectra of micelles at different pH) ............................................. 120

Fluorescence emission spectra of Nile Red in different systems....................... 121

Hydrodynamic radius change after loading of doxorubicin in the copolymer micelles and the morphology of copolymer micelles under TEM ............. 122

In vitro doxorubicin release profiles from doxorubicin suspension, doxorubicin loaded micelles and injectable hydrogel composite................................. 123

Effects of hydrogel precursors and synthesized hydrogels on cell viability...... 124

Cell viability of HeLa cells after adding different concentrations of micelle suspension ........................................................................................................ 125
<table>
<thead>
<tr>
<th>Scheme</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-1</td>
<td>Schematic overview of two procedures to prepare MFC gels</td>
<td>49</td>
</tr>
<tr>
<td>3-1</td>
<td>The schematic diagram for the construction of biocomposite using cationic</td>
<td>78</td>
</tr>
<tr>
<td></td>
<td>poly(l-lactide)-block-poly(N,N-dimethylamino-2-ethyl methacrylate) (PLA-b-</td>
<td></td>
</tr>
<tr>
<td></td>
<td>PDMAEMA) and anionic carboxymethyl cellulose (CMC)</td>
<td></td>
</tr>
<tr>
<td>4-1</td>
<td>The schematic diagram for the fabrication of drug loaded injectable hydrogel</td>
<td>110</td>
</tr>
<tr>
<td></td>
<td>composite</td>
<td></td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
<td></td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
<td></td>
</tr>
<tr>
<td>AGU</td>
<td>Anhydroglucose</td>
<td></td>
</tr>
<tr>
<td>ATRP</td>
<td>Atom-transfer radical polymerization</td>
<td></td>
</tr>
<tr>
<td>BNC</td>
<td>Bacterial nanocellulose</td>
<td></td>
</tr>
<tr>
<td>Br-BuBr</td>
<td>α-Bromoisobutyryl bromide</td>
<td></td>
</tr>
<tr>
<td>CMC</td>
<td>Carboxymethyl cellulose</td>
<td></td>
</tr>
<tr>
<td>CNT</td>
<td>Carbon nanotube</td>
<td></td>
</tr>
<tr>
<td>CRP</td>
<td>Controlled radical polymerization</td>
<td></td>
</tr>
<tr>
<td>CuAAC</td>
<td>Copper(I)-catalyzed Huisgen 1,3-dipolar cycloaddition of azide and alkyne</td>
<td></td>
</tr>
<tr>
<td>DOX</td>
<td>Degree of oxidation</td>
<td></td>
</tr>
<tr>
<td>DP</td>
<td>Degree of polymerization</td>
<td></td>
</tr>
<tr>
<td>DR</td>
<td>Disc refining</td>
<td></td>
</tr>
<tr>
<td>DS</td>
<td>Degree of substitution</td>
<td></td>
</tr>
<tr>
<td>EDC</td>
<td>1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride</td>
<td></td>
</tr>
<tr>
<td>HA</td>
<td>Hyaluronan</td>
<td></td>
</tr>
<tr>
<td>HOBt</td>
<td>Hydroxybenzotriazole</td>
<td></td>
</tr>
<tr>
<td>HPMC</td>
<td>Hydroxypropyl methyl cellulose</td>
<td></td>
</tr>
<tr>
<td>MC</td>
<td>Methyl cellulose</td>
<td></td>
</tr>
<tr>
<td>MFC</td>
<td>Microfibrillated cellulose</td>
<td></td>
</tr>
<tr>
<td>NCC</td>
<td>Nanocrystalline cellulose</td>
<td></td>
</tr>
<tr>
<td>NMP</td>
<td>Nitroxide-mediated polymerization</td>
<td></td>
</tr>
<tr>
<td>PEO</td>
<td>Polyethylene glycol</td>
<td></td>
</tr>
<tr>
<td>PEO-b-PDPA</td>
<td>Poly(ethylene oxide)-block-poly(2-(diisopropylamino)ethyl methacrylate)</td>
<td></td>
</tr>
<tr>
<td>PLA</td>
<td>Poly lactic acid</td>
<td></td>
</tr>
<tr>
<td>PLA-b-PDMAEMA</td>
<td>Poly lactic acid block poly (dimethylaminoethyl methacrylate)</td>
<td></td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
<td></td>
</tr>
<tr>
<td>----------</td>
<td>--------------------------------------------------</td>
<td></td>
</tr>
<tr>
<td>RAFT</td>
<td>Reversible addition fragmentation</td>
<td></td>
</tr>
<tr>
<td>ROP</td>
<td>Ring opening polymerization</td>
<td></td>
</tr>
<tr>
<td>sulfo-NHS</td>
<td>N-hydroxysuccinimide-succinimide</td>
<td></td>
</tr>
<tr>
<td>TEMPO</td>
<td>2,2,6,6-Tetramethyl-1-piperidinyloxy,</td>
<td></td>
</tr>
<tr>
<td>UH</td>
<td>Ultrasonication plus homogenization</td>
<td></td>
</tr>
<tr>
<td>WPCs</td>
<td>Wood-plastic composites</td>
<td></td>
</tr>
</tbody>
</table>
PREPARATION AND SYNTHESIS OF CELLULOSE-BASED FUNCTIONAL MATERIALS

By

Nusheng Chen

May 2016

Chair: Zhaohui Tong
Major: Agricultural and Biological Engineering

Cellulose, as the most abundant natural polymer on earth, has been a very promising renewable feedstock to fabricate various materials due to its excellent mechanical properties, low density, good biocompatibility, and versatile modification potential. Current applications of cellulose can be far beyond traditional pulp, paper or board. However, the use of cellulose has been greatly hindered by energy intensive processes to extract cellulose from lignocelluloses, its hydrophilicity, and insufficient exploration of possible applications. To tackle these challenges, the objective of this research is to seek a cost-effective method to prepare cellulose and further fabricate cellulose-based biocomposites with value-added functionality.

First, a facile route was designed, which combined mild maceration of waste pulp sludge and a mechanical shearing process, to prepare microfibrillated cellulose (MFC) with high storage modulus reaching 4000 Pa at 2% concentration. This maceration method could effectively extract cellulose from residues and the mechanically treated MFC will have a great potential to work as low-density reinforcing fillers for biocomposites or as a template facilitating further surface modification, for example, it is known that cellulose can be easily modified to carboxymethyl cellulose (CMC).
In the second part, we successfully prepared biocomposites with tunable properties through ionic assembly between anionic CMC and cationic copolymers (quaternized poly (l-lactide)-block-poly N, N-dimethylamino-2-ethyl methacrylate) (PLA-b-PDMAEMA). The resultant biocomposites could form transparent and uniform films with tailored storage moduli and maximum degradation temperature by adjusting the chain length of inclusive PDMAEMA segment. These composites with tunable features have potential for food packaging and coating, biosensors, and other applications.

Cellulose is also a good feedstock to fabricate biomedical materials due to its excellent biocompatibility and versatile modification capability. Thus, in the third part of this study, we attempted to synthesize a cellulose-based injectable hydrogel encapsulated with pH responsive poly (ethylene oxide)-block-poly (2-(diisopropylamino)ethyl methacrylate) (PEO-b-PDPA) copolymer micelles for localized, stimuli-triggered drug delivery. Covalently in situ generated hydrogels were designed by mixing hydrazide-modified carboxymethyl cellulose (CMC-NH₂) with oxidized carboxymethyl cellulose (CMC-CHO). The entrapment of PEO-b-PDPA facilitated the loading of hydrophobic substances and the pH triggered slow release of drugs was also achieved.
CHAPTER 1
CURRENT APPLICATIONS AND TRENDS OF CELLULOSE BASED BIOMATERIALS

Global Perspective

The excessive consumption of petroleum products has already led to numerous economic, social, and environmental issues. Petroleum products including fuels, asphalt, and the feedstocks used to make chemicals, plastics, and synthetic materials are obtained from crude oil through refining. Crude oil is a fossil fuel deriving from large quantities of dead organisms under intense heat and high pressure for several million years (Speight, 2014). The prolonged processing time determines the non-renewable feature of petroleum products. Based on BP’s (British Petroleum) annual report at the end of 2013, global oil reserves will only last for about 53 years (Tully, 2014). Along with the depletion threat by fast consumption of non-renewable petroleum products, the resultant environmental pollution has also become a great concern in recent decades. For example, the exhaust generated by burning petroleum as fuel has caused severe greenhouse gas emission and air pollution. At the same time, solid wastes like plastics have invaded our homeland. According to the data from Environmental Protection Agency (EPA), 33 million tons of plastic wastes were generated in 2013. However, only 9 percent of wastes were recovered for recycling (EPA, 2014). The petroleum-based plastics are not biodegradable, so it takes hundreds of years for their complete degradation. Meanwhile, waste burning and piling processes can release various hazardous chemicals into the environment. These issues have led to increasing exploration of renewable and environmentally-friendly substitutes.

Recently, bio-based materials have attracted significant attention due to their superior features. First, the feedstocks to produce bio-based materials are renewable,
which means they can be replenished at a sufficient rate, so we don’t need to worry about the depletion of raw materials. Second, since bio-based materials are carbon neutral, the released carbon can be offset with little contribution to greenhouse gas emission. In addition, biodegradability and low toxicity are other advantages of using bio-based materials. Thanks to these benefits, there have already been many applications of bio-based materials in people’s daily life. For example, agricultural products like corn and potato were fermented to produce lactic acid for the synthesis of biodegradable plastics (Reddy, Altaf, Naveena, Venkateshwar, & Kumar, 2008). Natural polymers such as chitosan and cellulose have been widely used as additives to construct composites with improved properties (Bledzki & Gassan, 1999; Pighinelli & Kucharska, 2013).

Although, the advantages of bio-based materials have been greatly recognized, several challenges still exist hindering their broad applications. 1) Most bioplastics cannot compete economically with dominating petroleum-based plastics (L. Liu, Liu, Fishman, Hicks, & Liu, 2005). 2) The performance of bio-based materials is still inferior to that of petroleum-based materials, which limited their application area. For example, starch based polymers often own poor mechanical property, moisture sensitivity, and weak thermal stability (Kuusipalo, 2001). 3) Some feedstocks used to prepare biomaterials have conflicts with food commodities, which has already caused a fierce controversy. For instance, most currently marketed biopolymers are made from edible starch.

To tackle these challenges, the identification of an abundant raw material that can be easily modified to fabricate products with high value-added functionality is of
great importance. Moreover, a sustainable design that increases product yield and reduces waste is also indispensable. Based on these needs, a natural polymer like cellulose is a very promising feedstock. Cellulose is a non-edible raw material with wide availability. Agricultural wastes like corncob, straw, and pulp industry residues all can be sources to extract cellulose. Besides, various modification potential of cellulose through chemical or physical methods can further broaden the application of resultant materials.

**Cellulose**

Cellulose is the most abundant organic compound on earth with an estimated annual yield around 1.5×10^{12} tons. It can be considered as an almost inexhaustible renewable resource for the production of bio-based materials (Kaplan, 2013). Cellulose is the main structural component of plants and also exists in bacteria, fungi, algae, and even animals (Osullivan, 1997). Among them, lignocellulosic materials like wood is the main source of cellulose. In addition, agricultural residues, water plants, and grasses can also provide abundant raw materials.

Cellulose has its own unique molecular structure compared with other polysaccharides. Acellulosic molecule is a linear carbohydrate polymer consisting of repeating β-D-glucopyranose units by covalent links between equatorial hydroxyl groups of C4 and C1 carbon atoms (Figure 1-1). In order to allow the full conformation of β-1, 4 glycosidic bond, every second anhydroglucose (AGU) is rotated 180° in the plane, which stabilizes the chair structure by minimizing its flexibility. In each AGU unit, there exist three hydroxyl groups. The abundant hydroxyl groups result in plentiful intra- and intermolecular hydrogen bonds, which enable native cellulose to become a very stable polymer. There are six polymorphs of cellulose (Figure 1-2) and they can be
interconverted through certain processes (Osullivan, 1997). Among them, cellulose I is the native cellulose, a form that can be found in nature and it is the origin for other types. Additionally, as a linear polymer, the length of cellulose is defined as the number of AGU unit (degree of polymerization, DP). Generally, 300 and 1700 DP can be found in wood pulp, while for cotton and other plant fibers, the values are in the range of 800 to 10000 (Klemm, Heublein, Fink, & Bohn, 2005).

Cellulose is the main building block of plants’ cell wall and the majority of cellulose can be found in the secondary cell wall. Cellulose is organized as a fine cellular hierarchical structure with other substances including hemicellulose, lignin, and pectin. These components interact with each other to form cell wall composite. Cellulosic fiber has a particularly elaborate bottom-up structure, which is made up of cellulosic molecules, elementary fibril, microfibril, and macrofibril (Meier, 1962). Figure 1-1 shows the detailed construction of cellulose. In this structure, the adjacent linear cellulosic chains compact together to form a framework of water-insoluble aggregates, which are called elementary fibrils. Several elementary fibrils with dimensions around 3-4 nm further bundle together to generate the units called crystallites (Fengel & Wegener, 1983). These crystallites are held by a monolayer of hemicellulose yielding a natural composite referred as cellulose microfibril. Then, microfibrils further evolve to macrofibril and cellulosic fibers. According to different patterns in how these fibril elements pack, two distributed regions can be found in cellulose: crystalline (high order) and amorphous (low order) regions (Osullivan, 1997).

**Nanocellulose**

Nanocellulose is a type of cellulose with nano structure. In a general definition, cellulosic materials with at least one dimension in nano range are sorted into
nanocellulose (Klemm et al., 2011). Nano-sized cellulose can be obtained either from wood and agricultural feedstock through fibrillation or from specific bacteria by bottom-up synthesis. According to the dimension difference and cellulosic resource, nanocellulose can be further categorized into three groups: nano-/microfibrillated cellulose (NFC/MFC), nanocrystalline cellulose (NCC) and bacterial nanocellulose (BNC) (Figure 1-3).

Microfibrillated cellulose (MFC) is the nano-scale cellulose with high aspect ratio and is normally produced from wood pulp by mechanical delamination. In practice, MFC exhibits a network structure containing micro-sized fibrils and microfibrils; the latter has a nano-dimension diameter (Herrick, Casebier, Hamilton, & Sandberg, 1983). Due to high aspect ratio, MFC can entangle with each other forming a network structure, which contributes to its gel-like behavior. In order to fabricate MFC, various mechanical treatments providing high shear force were used to treat cellulosic fibers (Figure 1-3). In order to facilitate the production, many efforts have been made. It was found that adding hydrophilic polymers is beneficial during the delamination (Turbak, Snyder, & Sandberg, 1982). Moreover, pretreatments using chemical 2,2,6,6-Tetramethyl-1-piperidinyloxy (TEMPO) (Saito, Kimura, Nishiyama, & Isogai, 2007) or enzyme (M. Henriksson, G. Henriksson, L. A. Berglund, & T. Lindström, 2007) could reduce the energy input compared with solely mechanical treatment. The application of MFC is very versatile. For example, as MFC owns good thickening property and biocompatibility, it is a useful additive in food products, cosmetics, and paints. In addition, due to its light weight and high aspect ratio, MFC has been widely used in packaging materials.
Nanocrystalline cellulose (NCC), also called whisker, is a rod like particle with a dimension of 5-70 nm in diameter and 100-250 nm in length. NCC is obtained through mineral acid hydrolysis of native cellulose and following mechanical treatment. During the hydrolysis, amorphous regions are broken, while more resistant crystalline regions are reserved. After appropriate mechanical processes like sonication, uniformly dispersed suspension of NCC will form.

Bacterial nanocellulose (BNC) is produced by several species of ubiquitous fermentation bacteria, most importantly *Gluconacetobacter xylinus*. BNC is biosynthesized from low molecular weight carbon source inside bacteria and excreted to the aqueous medium as nano fibers. Different from MFC and NCC, BNC is very pure, so there is no need for complicated purification processes. Moreover, one of the great advantages of BNC is its easiness to design the shape and supramolecular structure by directly controlling culture conditions during the synthesis of cellulose (Klemm et al., 2011).

Nano-sized cellulose inherits the properties from cellulose and also possesses its own unique features by small dimension. Due to the abundant hydroxyl groups on its repeat units, cellulosic chains are very hydrophilic and have a high potential for chemical modification. At the same time, the dramatically increased surface area facilitates the interaction between cellulose and surrounding species, which can significantly improve the retention of water, the contact efficiency with polymers, and the fixation ability of nanoparticles (Gardner, Oporto, Mills, & Samir, 2008). Moreover, other characteristics like nanoporosity and transparency have also been shown in the dispersions and composite materials using nano-scale cellulose. Finally, when cellulose
reaches nano level, mechanical properties like elastic modulus can be further enhanced (Bledzki and Gassan 1999). In summary, nanocellulose greatly expands the application of traditional cellulose by the introduction of new features from nano-sized materials.

**Cellulose-Based Biocomposite**

Due to its superior mechanical properties, renewability, biodegradability, low cost, low density, and abundance, cellulose has been widely introduced as reinforcing fillers in composite. Compared with traditional modifiers such as calcium carbonate, mica, and glass or carbon fibers, cellulosic fibers are cheaper materials to prepare composites (John & Thomas, 2008). In United States, wood fibers have been successfully incorporated with traditional plastics like polyethylene or polypropylene to fabricate wood-plastic composites (WPCs) (Clemons, 2002). This combination brings the advantages from both wood fiber and plastics. The resultant composites can be worked like wood and processed like plastics, and they also have better durability and water resistant features. Currently, various applications of WPCs can be found in commercial products, such as automotive interior material and building material. In addition to wood fibers, annual plants are also good fiber sources. Their fibers have a higher aspect ratio, high degree crystallinity, and small lumen giving them the potential to be more effective as reinforcement for composites (Madsen & Gamstedt, 2013).

However, several challenges exist in the fiber-based composites. The major issue is how to maximize the mixing between wood fibers and plastic polymers, while minimize the damage to fibers (Bledzki, Letman, Viksne, & Rence, 2005). Since cellulosic fibers have low degradation temperature, the processing temperature that can be applied is very limited. Moreover, aggregation cannot be avoided during processing, because long chain fibers tend to entangle with each other, which may lead to their
inhomogeneous dispersion. This phenomenon usually results in the poor performance of corresponding composites. Finally, fibers are easy to absorb moisture due to their hydrophilic nature, so they could cause the swelling of fibers and impeded the performance of composites (Bledzki & Faruk, 2004).

Due to better dispersibility, large surface area and high theoretical stiffness, the introduction of nano-sized cellulose in composites might solve some of the problems mentioned above. The early report of using nanocellulose as reinforcing fillers can date back to 1987, in which, nano-sized celluloses were added into various traditional thermoplastics including polypropylene, polystyrene and high density polyethylene (Boldizar, Klason, Kubat, Näslund, & Saha, 1987). The reinforcing effects could be found for all three types of nanocellulose. For instance, compounding nano crystal cellulose (NCC) with styrene and butyl acrylate copolymer latex could significantly improve the mechanical property even with the small amount of whiskers (Favier, Chanzy, & Cavaille, 1995; Gauthier, 1994). Additionally, the applications of MFC and BNC to construct composites with enhanced properties were extensively described in recent reviews (Shah, Ul-Islam, Khattak, & Park, 2013; Siró & Plackett, 2010).

Along with the combination of nano-scale cellulose and traditional plastics, in recent years, “green” composites containing nanocellulose and biodegradable polymers have drawn increasing interests (Khalil, Bhat, & Yusra, 2012). These bioplastics or bioresins are promising materials for their renewable and biodegradable features. However, their drawbacks - brittleness, low thermal stability, and poor barrier property - greatly reduce their ability to compete with traditional materials. In order to overcome these disadvantages, nanocellulose was imported to improve performance. Bio-based
polymers like poly lactic acid (PLA) (Iwatake, Nogi, & Yano, 2008), poly-hydroxy butyrate (PHA) (Grunert & Winter, 2002) and starch (Cao, Chen, Chang, Muir, & Falk, 2008; Grande et al., 2008; Sreekala, Goda, & Devi, 2008) have been compounded with different types of nano-sized cellulose and the resultant composites exhibited better properties than those of original materials. Meanwhile, these obtained composites are completely bio-derived and biodegradable, so they almost have no negative impact on the environment.

**The Modification of Cellulose**

Cellulose has a large number of hydroxyl groups on its molecular chains and the –OH group can react with various chemicals for different purposes of modification. These modifications can not only change certain physical properties of cellulose, but also further expand its application by introducing various functional groups. There are two major types of reaction to modify cellulose: esterification and etherification (Figure 1-4).

Appropriate modification of cellulose can improve its performance in composites. Cellulose has been widely used as reinforcing fillers due to its high aspect ratio and high mechanical strength. However, most polymers are hydrophobic, while cellulose is hydrophilic. This incompatibility hinders the uniform dispersion of cellulose in a polymer matrix and leads to a poor-performance composite. To resolve this problem, hydrophilic cellulose can be hydrophobilized to increase its compatibility in a hydrophobic environment by esterification. Cellulose has been reacted with acid anhydride (Berlioz, Molina-Boisseau, Nishiyama, & Heux, 2009; Ifuku et al., 2007; Philippe Tingaut, Zimmermann, & Lopez-Suevos, 2009), acyl chlorides (Berlioz et al., 2009) or carboxylic acid to increase the hydrophobicity on the surface. In order to maintain the structure of
cellulose, these heterogeneous reactions usually happened in a non-swelling media. In addition, since there are three theoretical hydroxyl groups in each AGU unit and the degree of substitution (DS) for hydroxyl groups varies under different reaction conditions, the hydrophobicity can be well tuned through the control of DS in the reaction.

The esterification of cellulose imports the hydrophobicity, while the etherification usually increases the solubility of cellulose in an aqueous medium. In cellulose, due to the β-1,4 glucosidic bonds, the linear polymer chains are aligned side by side, favoring the formation of inter chain hydrogen bonds. This conformation leads to the exceptional strength of cellulose and its insolubility in water. However, after etherification, hydroxyl groups are substituted by other groups, so the hydrogen-bonding network is broken and cellulosic chains easily disassemble from original structure. Currently, many etherified cellulose derivatives have already been commercialized such as methylcellulose (MC), carboxymethyl cellulose (CMC) and (Hydroxypropyl) methyl cellulose (HPMC). These water-soluble cellulose derivatives carry the possibility for further modification under mild conditions. Take CMC as an example, the carboxyl groups on CMC can be specifically reacted with amine groups mediated by 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC). This reaction is very promising because of its high conversion efficiency, mild reaction condition and excellent biocompatibility (J. Sheehan, Cruickshank, & Boshart, 1961; J. C. Sheehan, Preston, & Cruickshank, 1965).

Graft copolymerization is another option for cellulose modification, which introduces oligomer or polymer onto cellulose. Depending on the oligomers or polymers
grafted on cellulose, it is possible for modified cellulose to obtain various new properties such as temperature responsiveness and bacterial resistance. Generally, the graft copolymerization techniques can be categorized into three major approaches (Odian, 2004): “graft from”, “graft to” and “graft through”. In “graft from”, a polymer containing functional groups is used to initiate the polymerization, while “graft to” is related to the reaction between functional groups on two different polymers. As to “graft through”, cellulose is modified to become a macromonomer containing vinyl groups which initiate the polymerization.

Free radical reaction is widely applied to mediate the graft modification of cellulose. In the cases of “graft from” and “graft through”, conventional free radical polymerization was conducted by various initiation methods (Bhattacharyya & Maldas, 1984). Free radical reaction can achieve high graft density due to the effective diffusion of monomers, but grafted polymers often have a broad molecular weight distribution and homopolymers also exit as byproducts. In order to obtain graft polymers with narrow polydispersity, controlled radical polymerization (CRP) and ring opening polymerization (ROP) are promising alternatives. In the CRP method, either atom transfer molecular polymerization (ATRP), nitroxide-mediated polymerization (NMP) or reversible addition fragmentation (RAFT) could be used on cellulose (Roy, Semsarilar, Guthrie, & Perrier, 2009). Through these reactions, polymers with various controlled architectures can be obtained. In the ROP technique, hydroxyl groups on cellulose acted as initiators mediating the polymerization of cyclic monomers, which led to the uniform grafting polymers on cellulose (Carlmark, Larsson, & Malmström, 2012). ROP can be applied to modify both native cellulose and cellulose derivatives.
“Graft to” technology allows cellulose to be covalently linked with already synthesized oligomers or polymers. Over the past few years, the copper (I)-catalyzed Huisgen 1, 3-dipolar cycloaddition of azide and alkyne (CuAAC) has provided a reliable way for this link to occur (Thakur, 2015). In this method, pre-click modification of cellulose is required to introduce azide or alkyne groups for the following reaction. Then various branch chains can be grafted onto cellulose through this efficient reaction (Joubert, Musa, Hodgson, & Cameron, 2015; Li, Kang, & Liu, 2012; Peng et al., 2012; Xu, Zhang, & Kadla, 2012). In addition, other alternative linking methods like photochemical thiol-ene reaction (Zhao, Hafrén, Deiana, & Córdova, 2010) was also reported. However, “graft-to” approach is the least useful method, because it is inherently limited by the crowding of chains on the surface, which hinders the diffusion of chain ends to the surface for reaction.

**Advanced Applications of Cellulose**

Due to the unique features of cellulose and its derivatives, their applications are far broader than only being used as reinforcing fillers for traditional plastics. Instead of simply blending plastics and cellulose, new cellulose-based composites can be elaborately designed with precisely tuned properties and novel functions. Biocompatible cellulose is also the ideal feedstock to fabricate biomedical materials, which will greatly boost the value of cellulose. Furthermore, cellulose is an excellent support material for versatile applications such immobilization, coating.

Several unique cellulose-based composites have been fabricated. For example, biomimetic composites with high stiffness and toughness were obtained by imitating soft-hard structure in natural materials. Natural material like nacre contains soft proteins between hard inorganic sheets. The soft part enables energy dissipation under a high
force load, which significantly improves the resistance to external force. Based on this structural concept, genetically engineered protein with hydrophobic head and cellulose binding domain was expressed to act as a bridge between stiff graphene and NFC (Laaksonen et al., 2011). The obtained composite films exhibited much improved stiffness and toughness. This soft-hard combination concept was also applied to fabricate composites using a synthetic copolymer (M. Wang et al., 2011). Moreover, cellulose-based functional materials like shape memory composites were fabricated by percolating a nano-whisker network in an elastomeric thermoplastic polyurethane (TPU) matrix (Zhu et al., 2012). This composite material appeared to have water-sensitive shape memory property, which provides the possibility of producing breathable clothing and medical devices.

Cellulose and its derivatives are excellent resources to create biocompatible materials for tissue engineering and drug delivery purposes (Miyamoto, Takahashi, Ito, Inagaki, & Noishiki, 1989). Plenty of research has been done to explore the potential of fabricating cellulose-based biomedical materials. For example, one of cellulose derivatives, MC was blended with hyaluronan (HA) for localized drug delivery in an injured spinal cord (Gupta, Tator, & Shoichet, 2006). Due to MC’s thermal gelling characteristic, fast *in situ* gelation after injection was realized. The generated hydrogel could not only increase the biocompatibility of drugs, but also form a drug depot for its slow release. In another example, cellulose-based hydrogels could also promote three-dimensional live cell culture by providing scaffold support (Bhattacharya et al., 2012; Rederstorff et al., 2015).
Cellulose also has its role in the area of conductive materials. Novel cellulose nanocomposites were fabricated by incorporating NFC aerogel and carbon nanotube (CNT) (M. Wang et al., 2013). The final product possessed both mechanoresponsive conductivity and pressure sensing features. Moreover, other forms of bulk cellulose such as cellulose paper, cotton, and textile were introduced as templates to support CNT or graphene, forming conductive electrodes (Weng et al., 2011; G. Yu et al., 2011). Furthermore, the “soak and polymerization” method has been reported to fabricate polypyrrole coated paper, which was used as an electrode for supercapacitors (Yuan et al., 2013). Cellulose/polypyrrole conductive composite aerogels were also reported to have the function for nerve regeneration (Shi et al., 2014).

**Summaries and Potential Opportunities**

In summary, cellulose is a peerless raw material for the production of bio-based materials. The development and application of cellulose-based materials meet the requirements for reducing reliance on petroleum-based products and, at the same time, maintain minimum impact on environment. Though, much research has been done on cellulose processing and many cellulose related products have already been created, there is still more room for development including cost-effective process to extract cellulose, construction of smart cellulosic composites and exploration of other unknown applications. In this dissertation, low-value waste pulp residues were used as raw materials to prepare MFC with high storage modulus. Then, one of cellulose derivatives, CMC was explored its potential in the fabrication of tunable composite with PLA, and injectable hydrogel composite system for localized drug delivery and pH-triggered release.
Figure 1-1. Chemical association in the plant cell wall: (1) the cellulose backbone, with an indication the length of its basic structural unit, cellobiose; (2) frame work of cellulose chains in the elementary fibril; (3) cellulose crystalline; (4) microfibril cross section, showing stands of cellulose molecules embedded in a matrix of hemicellulose and proto lignin (Ramos, 2003).
Figure 1-2. Interconversion of the polymorphs of cellulose (Osullivan, 1997).
<table>
<thead>
<tr>
<th>Type of nanocellulose</th>
<th>Selected references and synonyms</th>
<th>Typical sources</th>
<th>Formation and average size</th>
</tr>
</thead>
<tbody>
<tr>
<td>microfibrillated cellulose (MFC)</td>
<td>microfibrillated cellulose[^1],</td>
<td>wood, sugar beet, potato tuber, hemp, flax,</td>
<td>delamination of wood pulp by mechanical pressure before and/or after chemical or enzymatic treatment</td>
</tr>
<tr>
<td></td>
<td>nanofibrils and microfibrils,</td>
<td>flax</td>
<td>diameter: 5-60 nm</td>
</tr>
<tr>
<td></td>
<td>nanofibrillated cellulose</td>
<td></td>
<td>length: several micrometers</td>
</tr>
<tr>
<td>nanocrystalline cellulose (NCC)</td>
<td>cellulose nanocrystals, crystallites[^2], whiskers[^3], rodlike cellulose microcrystals[^4]</td>
<td>wood, cotton, hemp, flax, wheat straw, mulberry bark, ramie, Avicel, tunicin, cellulose from algae and bacteria</td>
<td>acid hydrolysis of cellulose from many sources diameter: 5-70 nm length: 100-250 nm (from plant cellulosederived materials)</td>
</tr>
<tr>
<td>bacterial nanocellulose (BNC)</td>
<td>bacterial cellulose[^5], microbial cellulose[^6], biocellulose[^7]</td>
<td>low-molecular-weight sugars and alcohols</td>
<td>100 nm to several micrometers (fromcellulosederived materials) bacterial synthesis diameter: 20-100 nm; different types of nanofiber networks</td>
</tr>
</tbody>
</table>

Figure 1-3. The categories of nanocellulose (Klemm et al., 2011).
Figure 1-4. The most applied mechanical treatment processes used in the fabrication of microfibrillated cellulose (MFC): the homogenizer, the microfluidizer and the grinder (Lavoine, Desloges, Dufresne, & Bras, 2012).
Figure 1-5. Examples of cellulose derivatives through esterification and etherification.
CHAPTER 2
FABRICATION OF MICROFIBRILLATED CELLULOSE GEL FROM WASTE PULP SLUDGE VIA MILD MACERATION COMBINED WITH MECHANICAL SHEARING

This chapter describes a facile route, which combines mild maceration of waste pulp sludge and a mechanical shearing process, to prepare microfibrillated cellulose (MFC) with high storage modulus. In the maceration, the mixture of glacial acetic acid and hydrogen peroxide was used to extract cellulose from never-dried waste pulp sludge. Then, two different mechanical processes including disc refining (DR) and ultrasonication plus homogenization (UH) were applied to the cellulose after maceration and resulted in MFC with a highly tangled fibril network. All of the resultant cellulosic suspensions (2% w/w) exhibited a gel-like and shear-thinning behavior with storage moduli (G') ranging from 200 to 4000 Pa. Among them, 30-min DR treated MFC gels had the maximum G', which was much higher than previously reported MFC gels at the same concentration. Additionally, after mechanical processing, specific surface areas (SSAs) and water retention values (WRVs) of MFC were accordingly increased with the enhancement of shear force, but the storage moduli (G') were not consistently increased. Finally, a strong MFC gel was successfully prepared using never-dried waste pulp sludge, cost-effective chemicals, and one-step disc refining process. The obtained hydrogels will have the potential as low-density reinforcing fillers and a template for further surface modification.

Introduction

Microfibrillated cellulose (MFC) has attracted significant interests due to its biocompatibility, biodegradability, strong mechanical properties, and highly porous network structure (Lavoine, Desloges, Dufresne, & Bras, 2012). Various applications of MFC can be found in composite materials (S Iwamoto, Nakagaito, & Yano, 2007;
Leitner, Hinterstoisser, Wastyn, Keckes, & Gindl, 2007; Medeiros et al., 2008; Siró & Plackett, 2010), fire barrier materials (A. D. Liu, Walther, Ikkala, Belova, & Berglund, 2011), nanopapers (Henriksson, Berglund, Isaksson, Lindstrom, & Nishino, 2008; W. Wang, Sabo, et al., 2015), and hydrogels (Czaja, Young, Kawecki, & Brown, 2006; Nair, Zhu, Deng, & Ragauskas, 2014). Cellulosic fiber, the most abundant natural resource to produce MFC, has an elaborately hierarchical structure composed of cellulose chains, elementary fibrils, microfibrils, and macrofibrils (Meier, 1962). In order to obtain MFC, cellulosic fibers are usually unraveled to expose smaller fibrils and microfibrils; the latter have the diameter from 10 to 100 nm as observed by scanning electronic microscope (Herrick et al., 1983; Q. Wang, Zhu, Gleisner, et al., 2012). When cellulosic material reaches micro- or nano- scale, its surface area will be dramatically increased. The augmented surface area can significantly improve the interaction between cellulose and surrounding species, which will facilitate its chemical modification or dispersion in other polymer matrices. Thus, fibrillating cellulose to MFC has considerably expanded the application of cellulose materials.

Various methods including chemical, biological, mechanical approaches or their combinations have been attempted to fibrillate cellulose into nano or micro scale. For example, cellulose nanocrystals (CNCs) were produced by strong mineral acid hydrolysis and following mechanical treatments (Chen et al., 2015; Huntley, Crews, Abdalla, Russell, & Curry, 2015; Pan, Zhou, & Chen, 2013; Q. Wang, Zhu, Reiner, et al., 2012). In cellulose, there exist ordered and disordered regions (Osullivan, 1997), and the latter is much easier to be hydrolyzed by strong acid. During hydrolysis, amorphous cellulosic fibers are easily broken yielding cellulose nanocrystals (CNC) with
low aspect ratio, which may not be favorable for polymer reinforcement. Strong acid could also result in the corrosion of devices. In addition, solely mechanical shearing process was typically used to prepare MFC, which could yield final products with thixotropic viscosity and stable gel properties due to strong entanglement among relatively high aspect ratio fibrils or microfibrils. However, it has been greatly impeded by extensive energy consumption for solely mechanical fibrillation (Q. Wang, Zhu, Gleisner, et al., 2012). To reduce energy input, various chemical or biological pretreatments like TEMPO-mediated oxidation (Saito & Isogai, 2004), ionic liquid treatment (J. Li et al., 2012), and enzyme hydrolysis (M. Henriksson, G. Henriksson, L. Berglund, & T. Lindström, 2007; W. Wang, Mozuch, et al., 2015) have been applied. For example, mild enzyme treatment followed by homogenization was reported to not only preserve long fibrils, but also reduce total energy consumption (M. Paakko et al., 2007). The resultant MFC gels had high storage moduli (10^5 Pa at a 5.9% w/w concentration and 10^3 Pa at a 2% w/w concentration) and an extensively tangled network structure. Zhu et al. further studied the effects of different types of enzyme and the results showed that the fiber length strongly depended on the enzyme type (W. Wang, Mozuch, et al., 2015). Although the introduction of appropriate pretreatments does assist the fibrillation, the cost of chemicals or enzymes needs to be considered.

In addition, currently reported MFC processes mainly used expensive bleached wood fibers as the feedstock. Therefore, it is important to seek an alternative and cost-effective feedstock for the preparation of MFC. In contrast to these processes, this study used never-dried waste fiber residues, which were treated by a mild maceration process and following two different mechanical shearing processes. These residues were
gathered from the waste stream to manufacture specialty wood cellulose or fluffy pulp. They had higher lignin content than bleached wood fibers. In order to remove lignin, we introduce a mild maceration method, which was originally used in wood anatomy to characterize wood structure (Chamberlain, 1905). Our rational is that maceration using a mixed solvent containing acetic acid and hydrogen peroxide under mild condition can effectively remove lignin from waste fiber sludge to extract cellulose and also minimize the size reduction of cellulosic fibers. The mixture of acetic acid and hydrogen peroxide yields peracetic acid (PAA), which has high delignification selectivity (Nada, Ibrahim, Fahmy, & Abo-Yousef, 1999). After maceration, we applied two different mechanical shearing processes containing one-step disc refining (DR) and a typical ultrasonication plus homogenization (UH) process (Scheme 2-1). We investigated the effects of various mechanical shearing parameters such as refining time, pass number of homogenization, and ultrasonication time on the properties of corresponding MFC gels. The mild maceration method plus one-step disc refining is expected to prepare strong MFC hydrogels with tunable storage moduli by controlling mechanical shearing conditions.

Materials and Methods

Materials

The waste pulp sludge (32.42% dry weight) was kindly provided by Georgia-Pacific Inc. and served as the feedstock. It was collected from the last screening step of the process to manufacture specialty wood cellulose or fluffy pulp. The residues were delignified by a modified maceration method (Franklin, 1945). Briefly, the waste pulp sludge with 5% dry weight was added into the solvent containing equal volume of glacial acetic acid (Acros 99.7%) and hydrogen peroxide (Ricca 30%). Then, the mixture was
transferred to an incubator shaker (Innova 4000 Benchtop Incubator Shaker, New Brunswick Scientific, USA) and the incubating condition was set at 60 °C, 150 rpm for 48 hours. After maceration, the product was washed by deionized water for several times until neutral pH. The chemical composition of residues before and after maceration was analyzed according to the standard from National Renewable Energy Laboratory (NREL) (Sluiter et al., 2008). Purified cellulose was directly used for the DR process, while further size reduction using a hammer mill (Model 4, Wiley laboratory, USA) equipped with a 0.5mm mesh sieve was applied before the UH process to avoid the blocking of homogenizer by long fibers.

**Gel Preparation by a Disc Refining (DR) Method**

Macerated cellulose was dispersed in deionized (DI) water at 2% (w/w) consistency and then this suspension was refined using a Supermasscolloider ultra-fine grinder (disc refiner, model: MKZA6-2, Disk model: MKGA6-80#, Masuko Sangyo CO., Ltd, Japan) at 1500 rpm. When cellulose suspension was forced to pass through the gap between the rotor and stator discs, repeated cyclic stress was applied to fibers for fibrillation. The gap setting was -100 microns after loading of fibers. Two discs were not in contact even with negative settings due to the presence of fibers. Three different disc refining time periods of 30, 60, and 120 minutes were used.

**Gel Preparation by an Ultrasonication plus Homogenization (UH) Process**

Cellulose after milling was prepared to 2% (w/w) suspension and further treated by high intensity ultrasonication at 80% of system’s maximal power under a continuous mode for 10, 20, and 30 min. The ultrasonic processor (Model 1500 W, 20 kHz, Sonic, Newtown, CT) was directly applied to the suspension in a 250-ml beaker. After sonication, cellulosic suspensions were passed through a high-pressure homogenizer.
(Model APV-1000, Spx Flow Technology Rosista GMBH, Germany) at 80 MPa internal pressure. The passing numbers were set at 10, 20, 30, and 40, respectively.

**Optical Microscopy Characterization**

An optical microscope (Leica DMR, Leica microsystems, Germany) at differential interference contrast mode was used to characterize morphological changes of cellulose after maceration and mechanical processes, respectively. The testing sample was prepared by diluting 1 ml MFC suspension in 4 ml deionized water and then mixed by a vortex mixer (Standard vortex mixer, Fisher Scientific, USA). After that, one or two drops of suspension were transferred to a glass slide covered with a micro cover glass and observed under the microscope at a magnification of 400.

**Scanning Electronic Microscopy (SEM) Characterization**

The SEM (FEI XL 40 FEG SEM, FEI instruments, USA, operating voltage 30 KV) was employed to examine the morphology of MFC at a higher magnification. First, MFC suspension was diluted to 0.02% (w/w), and then one drop of diluted suspension was placed on a SEM mount covered with a carbon tab. Samples were frozen by liquid nitrogen and immediately vacuum dried by a freeze dryer (Freeze dryer 8, Labconco, USA). The lyophilization process was applied to avoid possible aggregation during a normal drying process, which preserved the morphology of MFC in a wet state (e.g. suspension or gel). Finally, freeze-dried samples were coated with Au-Pd (~10 nm) before SEM imaging.

**Water Retention Value (WRV) Measurement**

Water retention value (WRV) is defined as the ratio of water remained in sample after centrifugation to its dry weight. In this study, water retention values (WRVs) of MFC at different mechanical shearing conditions were measured according to the
Method UM256 from Technical Association of the Pulp and Paper Industry (TAPPI) with a slight modification. In brief, a stainless steel centrifuge holder was prepared in our lab, which had a membrane filter thimble with filter paper and 0.5 mm metal mesh located on the perforated bottom. First, the holder was pre-weighed ($W_1$). Then, MFC suspension was uniformly poured into the holder, which was placed inside a 50 ml centrifuge tube and subjected to centrifugation for 30 min at 2600 rpm. After centrifugation, each holder containing sample was re-weighed ($W_2$) immediately to prevent water evaporation. Finally, the holder containing the sample was dried in an oven at 105 °C overnight and the dry weight ($W_3$) was measured. The WRV of each sample was calculated according to the following equation (Equation 2-1).

$$WRV = \frac{(W_2 - W_3)}{(W_3 - W_1)} \quad (2-1)$$

A total three replicates were performed for each sample.

**Specific Surface Area (SSA) Measurement**

The specific surface areas (SSAs) of MFC gels were determined by $N_2$ sorption isotherms using a NOVA 1200 series volumetric gas adsorption instrument (Quantachrome, FL, USA) according to the Brunauer-Emmett-Teller (BET) theory (Brunauer, Emmett, & Teller, 1938). In a typical procedure, lyophilized MFC gels were first degassed in an outgas station at 40 °C overnight to remove moisture. Then, samples were loaded in a specific glass cell, which was submerged in liquid nitrogen, and the BET analysis was carried out in a relative vapor pressure range from 0.01 to 0.3 $P/P_0$ at −196 °C. The SSA was calculated from 6-recorded values.

**Dynamic Rheology Characterization**

The rheological properties of different MFC gels were characterized by a controlled strain rheometer (AR 2000, TA instrument, USA) equipped with a 40 mm
steel plate geometry. The gap was set at 1 mm between plate and plate geometry. First, the linear viscoelastic region of each sample was determined by a 0.1-100 % strain sweep at 1Hz and 2% strain was chosen for all tested samples. Under this condition, frequency sweeps were carried out in a range of 0.1-100 Hz and the corresponding moduli were recorded. In addition, the effect of shear rate on viscosity was also examined by a stepped flow method with the shear rate ranging from 0.1 to 100 1/s.

**Results and Discussion**

**Chemical Composition Analysis of the Waste Pulp Sludge before and after Maceration**

Waste pulp sludge was treated by a modified maceration method to remove lignin. After maceration, a dramatic color change could be easily distinguished (Figure 2-1) and composition analysis showed that the lignin was radically dropped from 8.47% to 1.61% (Table 2-1). The cellulosic content was increased from 80.35% to 94.81%, while the hemicellulose content was reduced from 10.37% to 3.48% (the standard deviation of each data was listed in Table 1). After maceration, as shown in Figure 2-2, cellulosic fibers are composed of long fibers with a width ranging from 20 to 50 μm and a length of several hundred micrometers. In the maceration process, the mixture of acetic acid and hydrogen peroxide could yield peracetic acid (PAA). The above results indicated that the yielded PAA could effectively remove lignin, partially hydrolyze hemicelluloses and meanwhile retain the original cellulose morphology.

**Water Retention Values (WRVs) of MFC Hydrogels**

Typically, WRV is an important parameter to characterize the degree of cellulose fibrillation (Hu, Zhao, Li, Zhu, & Gleisner, 2015). In the present study, the WRVs of DR-treated MFC hydrogels were plotted as a function of refining time in Figure 2-3 (a). It
was demonstrated that 120-min DR-treated MFC could retain water almost 16 times its own weight. We also found its WRV was 2.5 times higher than that of the 30-min DR-treated sample. Figure 2-3 (b) shows the effects of homogenizer pass numbers on WRVs of UH-treated MFC gels under three different ultrasonication time periods. Ultrasonication is a mechanical technique that applies cavitation effects to generate intense shear force for the rupture of materials, such as fibers (Suslick, 1990). In the UH process, ultrasonication was expected to break down fibers to facilitate subsequent homogenization process. However, it is found that the duration of ultrasonication has almost no influence on WRVs, while the pass number through homogenizer is a dominant factor. Taking all samples under 5-min ultrasonication as an example, the WRV of MFC was increased from 1.72 g/g without homogenization to 5.15 g/g after 40 passes. In addition, the WRVs of MFC after DR (Figure 2-3 (a)) were greater than those after UH processes (Fig 2-3 (b)). Even the minimum WRV of DR-treated MFC (6.3 g/g, 30 min DR treatment) was greater than the maximum WRV of UH-treated MFC (5.15 g/g, 5-min ultrasonication plus 40 passes). Therefore, we can conclude that the DR process is capable of preparing MFC with higher water retention value than the UH process. The water retention feature of cellulose is closely related to its surface area. The greater surface area of cellulose, the more available hydroxyl groups could be exposed to form more hydrogen bonds with water (Herrick et al., 1983; Spence, Venditti, Rojas, Habibi, & Pawlak, 2010).

**Specific Surface Areas (SSAs) of MFC Hydrogels**

The specific surface areas (SSAs) of MFC hydrogels from the two different aforementioned mechanical processes as well as solely macerated cellulose were investigated. For all UH-treated samples, the ultrasonication time was set for 5 min. As
shown in Figure 2-4, the SSA of cellulosic fiber after maceration was only 0.4 m$^2$/g, while the SSAs of MFC gels were significantly enhanced to the range from 10 to 55 m$^2$/g. The SSAs of MFC gels in this study were comparable to those from previously reported cellulose aerogels (Marjo Paakko et al., 2008) and foams (Sehaqui, Salajkova, Zhou, & Berglund, 2010), which possessed 20–66 and 10–40 m$^2$/g specific surface area respectively. In all MFC gels, 120-min DR-treated sample possessed the highest SSA, while 10-pass UH sample had the lowest value. Moreover, it is worth noting that, although the SSAs of MFC after 30- and 40- pass homogenization (40 m$^2$/g for 40 passes and 38 m$^2$/g for 30 passes) were slightly greater than that by a 30-min DR treated sample (27 m$^2$/g), the latter had a higher WRV. This inconsistency implies that besides SSA, some other factors such as entanglement degree, hydrogen bonding numbers, and aspect ratio may also affect the WRV of MFC (Herrick et al., 1983).

**Morphologies of MFC Hydrogels**

The morphologies of MFC hydrogels were examined by both differential optical microscopy and scanning electron microscopy (SEM). Figure 2-5A (a) shows that the 30-min DR sample is mainly composed of fibrils and fibril bundles. With the increase of refining time, the number of large bundles are gradually reduced and a more uniform size distribution can be observed from Figure 2-5A (b-c). The 60 min and 120 min DR treated samples include a majority of entangled fibrils and a small portion of fibril bundles. Among them, 120-min DR treated sample has a greater number of short fibrils, indicating a higher surface area. Figure 2-5B also shows the micrographs of MFC gels after different UH treatments (5-min ultrasonication and various homogenizer pass numbers). The 10-pass UH sample mainly consisted of large fibers with the diameter ranging from 10 to 40 μm and a few disintegrated fibrils (Figure 2-5B (a)). With the
increase of homogenization passes, large fibers were substantially fibrillated. In the 30- and 40-pass treated UH samples, there exist a number of fibril bundles and some fibrils with an entangled network. It is worth mentioning that when the pass number was increased to 40, the suspension became very viscous, which resulted in the blocking problem of homogenizer. In a word, both DR and UH mechanical processes can effectively reduce the size of cellulosic fibers and the DR process is more efficient than the UH process, because DR leads to smaller and more uniform fibrils with less steps.

In order to elucidate the morphologies of MFC hydrogels at a higher resolution, we further characterized these samples by SEM. Figure 2-6 shows the structures of corresponding MFC gels after 30-min, 120-min DR treatment, and 5-min ultrasonication plus 40-pass homogenization. The SEM image shows that the 30-min DR sample is mainly composed of a highly entangled network of fibrils and fibril bundles (Figure 2-6 (a)), which agrees well with the previous optical microscopy result. With a significant extension of refining time, the 120 min DR treatment led to numerous short microfibrils with an approximate average diameter of 60 nm (Figure 2-6 (b)), indicating a high degree of fibrillation. However, the 120-min DR sample possesses a lower degree of entanglement. This phenomenon can be ascribed to the over cutting of fibers in transverse dimension by extensive mechanical processing, which results in the formation of fibrils with low aspect ratio. Thus, a high-intensity mechanical treatment does lead to better fibrillation, but greatly reduce the entanglement degree by producing more fibrils with low aspect ratio. In addition, UH-treated sample shown in (Figure 2-6 (c)) has a looser fiber matrix and some large fibril bundles.
Rheology Properties of MFC Hydrogels

The rheological properties of MFC hydrogels prepared under different mechanical shearing conditions were characterized at 2% w/w consistency. Figure 2-7 shows the plot of storage moduli ($G'$) and loss moduli ($G''$) as the function of frequency. It is observed that all MFC aqueous suspensions are relatively independent of the angular frequency. For a typical viscous fluid, $G'$ and $G''$ are dependent on frequency and $G' \ll G''$, while in an ideal gel, $G'$ and $G''$ are relatively independent and $G' \gg G''$ (Tenijenhuis & Mijs, 1998). Based on this rational, all tested MFC samples exhibited a gel-like behavior, even for the 10-pass suspension, since its $G'$ ($10^3$ to $10^4$ Pa) were much greater than $G''$ ($10^2$ to $10^3$ Pa) and both $G'$ and $G''$ were relatively insensitive to frequency. The entangled network formed by topological interaction of cellulose contributes to the gel-like behavior for all suspensions (M. Paakko et al., 2007; Picout & Ross-Murphy, 2003).

As shown in Figure 2-7, the 30-min DR-treated MFC had the maximum storage modulus, which was greater than $G'$ of the 60-min and 120-min DR-treated MFC gels as well as all UH-treated samples. Regarding UH-treated MFC, the storage moduli of 20 and 30-pass MFC gels were greater than those of the 40-pass sample. The 10-pass MFC gel exhibited the weakest mechanical property, which could be attributed to the least entanglement of large fibers. It is worth noting that excessive shear force (60-min DR, 120-min DR, 40-pass UH) did not facilitate the fabrication of strong hydrogel, but reduced the $G'$ instead. Under intensive shear force, fibers were more susceptible to be broken down in the transverse dimension yielding fibrils with lower aspect ratio, which are detrimental to form entangled networks, so MFC gels with low $G'$ were obtained (Liimatainen, Visanko, Sirvio, Hormi, & Niinimaki, 2012; Stelte & Sanadi, 2009). This
phenomenon agrees well with the morphological observation in previous SEM images, in which a large number of fibrils with low aspect ratio exist in the 120-min DR-treated sample (Figure 2-6). The G’ value of the MFC gel by 30-min DR treatment is almost 4 times that of previously reported MFC gels at the same concentration (M. Paakko et al., 2007). In addition, the values of storage moduli of the entire MFC gels prepared in this study are higher than previously reported nanoscale cellulose crystallites (Ono, Shimaya, Sato, & Hongo, 2004; Rudraraju & Wyandt, 2005; Tatsumi, Ishioka, & Matsumoto, 2002).

Moreover, the viscosities of MFC gels were investigated as a function of shear rate and plotted in Figure 2-8. The viscosities of all MFC gels decreased with the rise of shear rate indicating a pseudoplastic (shear thinning) behavior. The shear thinning occurred when the rate of imposed motion by external shear force was gradually higher than the rate needed for the formation of new entanglement (Picout & Ross-Murphy, 2003). MFC gels presented a viscous property at low shear rate, and a fluid characteristic at high shear rate, which was in accordance with Herrick’s report (Herrick et al., 1983). The shear viscosities provide the evidence of structure variance among differently fibrillated MFC hydrogels. For example, at the shear rate of 0.1 1/s, the viscosity of highly entangled 30-min DR-treated MFC hydrogel was about 1000 Pa•s, which was 1 order of magnitude larger than 10-pass UH-treated sample. The degree of entanglement plays an important role on shear viscosity. With the increase of external shear force, weakly entangled fibrils with low aspect ratio were easily driven apart, while more entangled fibril networks could maintain higher viscosity. The result indicates that
the higher entanglement degree of MFC hydrogel, the more difficult it is to disrupt MFC hydrogel by external shear force.

**Summary**

This study presented the production of strong microfibrillated cellulosic (MFC) gels through a mild maceration followed by a one-step disc refining process, which was compared with an ultrasonication plus homogenization process, for the aspects of SSAs, WRVs, morphologies, and rheological properties. It was demonstrated that the mild maceration (1:1 v/v of acetic acid and hydrogen peroxide) effectively removed lignin from never-dried waste pulp sludge. The macerated product had a cellulosic content up to 95% and the most remained original cellulose morphology. In addition, the morphological results illustrated that maceration plus one-step disc refining could produce MFC with a high degree of entanglement. Furthermore, the rheological characterization results indicated that all prepared MFC suspensions at low concentration (2% w/w) exhibited a typical gel-like and shear thinning behavior. Particularly, the 30-min DR treatment resulted in a strong MFC gel with G’ as high as 4000 Pa, which was much higher than previously reported MFC gels. The strong MFC gel in this study may open a door for future widespread use as reinforcing fillers for low density polymer composites, templates for further functionalization, and immobilization.
Scheme 2-1. Schematic overview of two procedures to prepare MFC gels.
Table 2-1. The main chemical composition before and after maceration *

<table>
<thead>
<tr>
<th>Material</th>
<th>Lignin (%)</th>
<th>Cellulose (%)</th>
<th>Hemicellulose (%)</th>
<th>Ash (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Before maceration</td>
<td>8.47 ± 0.00</td>
<td>80.35 ± 2.36</td>
<td>10.37 ± 0.60</td>
<td>0.80 ± 0.02</td>
</tr>
<tr>
<td>After maceration</td>
<td>1.61 ± 0.05</td>
<td>94.81 ± 0.11</td>
<td>3.48 ± 0.18</td>
<td>0.10 ± 0.02</td>
</tr>
</tbody>
</table>

*error bar was represented by standard deviation
Figure 2-1. The waste pulp sludge before (a) and after (b) maceration treatment.
Figure 2-2. The morphology of cellulose after maceration: (a) optical micrograph and (b) differential interference contrast micrograph.
Figure 2.3. The water retention values (WRVs) of MFC gels after disc refining (DR, a) and ultrasonication plus homogenization (UH, b).
Figure 2-4. Specific surface areas (SSAs) of freeze-dried MFC gels after disc refining (DR) and ultrasonication plus homogenization (UH), where 5 min ultrasonication was applied.
Figure 2-5. Differential interference micrographs of (A) DR-treated MFC and (B) UH-treated MFC at different processing conditions in (A) a: 30 min, b: 60 min and c: 120 min; in (B) a: 10 passes, b: 20 passes, c: 30 passes, d: 40 passes.
Figure 2-6. The SEM images of freeze-dried MFC gels after different shear force processing: (a) 30-min DR, (b) 120-min DR, (c) 40-pass UH.
Figure 2-7. The storage moduli (G’s) (a) and the loss moduli (G”s) (b) as a function of frequency for all MFC gels.
Figure 2-8. The Influence of shear rate on the viscosity of different MFC gels.
Biocomposites with tunable properties were successfully prepared through ionic assembly between anionic carboxymethyl cellulose (CMC) and cationic copolymers (quaternized poly(l-lactide)-block-polyN,N-dimethylamino-2-ethyl methacrylate) (PLA-b-PDMAEMA). The quaternized PDMAEMA segment not only works as a compatibilizer between hydrophilic CMC and hydrophobic PLA, but also acts as a lubricant between these two rigid biopolymers. The $^1$H NMR (nuclear magnetic resonance) spectra demonstrated successful synthesis of PLA-b-PDMAEMA with controlled molecular weight of PDMAEMA segment. The results from scanning electronic microscopy (SEM) and Fourier transform infrared spectrometry (FTIR) verified the interaction between quaternized copolymer micelles and anionic CMC networks. The resultant biocomposite could form a transparent and uniform film after casting. Both storage moduli and maximum degradation temperature of PLA/CMC composites were increased with the reduction of molecular weight of PDMAEMA segments. It suggests that the properties of biocomposite materials can be tailored by adjusting the chain length of inclusive PDMAEMA segment.

**Introduction**

Both industry and academia show great interests in the production of biocomposites materials, due to environmental issues originating from petroleum-based materials and the impacts of petroleum shortages. The synthesis elements of biocomposites are predominantly derived from biomass (Weiss et al., 2012), which embraces great application potential in many areas like food packaging and coating.
Cherian et al., 2013; Lagarón & Fendler, 2009), biosensor (Ruan et al., 2012), and orthopedic operations (Boroujeni, Zhou, Luchini, & Bhaduri, 2013). Cellulose, as a natural fiber has been widely introduced for the fabrication of biocomposites since cellulose owns relatively high strength and stiffness, while maintains lightweight. The elastic modulus of a cellulose fiber through a chemical pulping process is approximately 40 GPa and its subdivided microfibrils can reach as high as 140 GPa (Shinichiro Iwamoto, Kai, Isogai, & Iwata, 2009). Besides, cellulose is the most abundant resource in the world and possesses excellent optical transparency and biocompatibility. These favorable features contribute to the prevalence of cellulose as reinforcing components for various composites (Bledzki & Gassan, 1999). Since native celluloses are hydrophilic, they are easy to be incorporated with water-soluble matrix such as poly (vinyl alcohol), ethylene vinyl copolymers, polyurethane, starch, poly (ethylene oxide), chitosan, and amylopectin (Cheng et al., 2013; Siró & Plackett, 2010). However, main obstacles that limit the applications of cellulose include its incompatibility with commonplace hydrophobic plastics and poor solubility in an organic phase. For example, solution casting (Dalmas, Cavaillé, Gauthier, Chazeau, & Dendievel, 2007; Malainine, Mahrouz, & Dufresne, 2005) by blending cellulose with polymer latex and solvent casting by dispersing lyophilized cellulose into organic solvent before hybridization with polymer solution were used to mix cellulose with hydrophobic polymer matrix (Sanchez-Garcia & Lagaron, 2010; Schroers, Kokil, & Weder, 2004; Tang & Weder, 2010). Nevertheless, they required a large quantity of organic solvent and the dispersion of cellulose in polymer matrix was not uniform (T. Wang & Drzal, 2012). To resolve this incompatible issue, chemicals such as acetic anhydride (P. Tingaut,
or silane reagents (Goffin et al., 2011; C. Goussé, Chanzy, Cerrada, & Fleury, 2004; Cécile Goussé, Chanzy, Excoffier, Soubeyrand, & Fleury, 2002) have been successfully grafted on cellulose (micro- and nano-size), resulting in homogeneous dispersion of cellulose in organic solvents. However, multiple steps and a large quantity of chemicals were required.

Polylactic acid (PLA) is one of a few commercially available biopolymers. It is a hydrophobic thermoplastic by polymerization of lactic acid from renewable feedstock (e.g. corn starch) (Lunt, 1998). Its tensile strength is comparable to petroleum-based plastics such as polystyrene (PS) and low-density polyethylene (LDPE) (Jamshidian, Tehrany, Imran, Jacquot, & Desobry, 2010). However, the application of PLA is restricted by its brittleness, low thermal stability, and high cost. Cellulosic fibers are promising reinforcing fillers to reduce material cost or improve certain properties (Huda, Mohanty, Drzal, Schut, & Misra, 2005). However, there are many issues for the fabrication of cellulose reinforced PLA composite containing the incompatibility between hydrophilic cellulose and hydrophobic PLA, low mass fraction of cellulose (less than 20%), and a low strain-in-failure of resultant composite due to the brittleness of both cellulose and PLA. A control of a self-assembly by using a soft compatible segment between hydrophilic cellulose and hydrophobic PLA in an under-micron size may represent a promising approach.

It is known that in nature, composites under the micron size with excellent mechanical properties such as nacre, mollusk shell, and wood cell wall, are synthesized through self-assembling approaches (Meyers, Chen, Lin, & Seki, 2008). In these
structures, synergistic features are created by self-assembly of each component, usually comprising ordered distribution of hard segments in soft polymer matrix (Braun, 2004; Espinosa, Rim, Barthelat, & Buehler, 2009; Fratzl, Burgert, & Keckes, 2004). For example, in the wood, crystalline cellulosic macrofibril bundles are embedded in the network consisting of hemicelluloses and lignin that are covalently bonded together. All three components are then tangled together to form a complex crosslinking structure in the cell wall, which enables wood to have high stiffness and toughness (Bowyer, Shmulsky, Haygreen, & Lilley, 2003). A few researchers have applied this biomimetic concept to prepare nanocomposite. Laaksonen et al. prepared a recombinant protein with cellulose binding domain and hydrophobic head, which could connect nanofibrillated cellulose and flakes of graphene to form biomimetic nanocomposites. However, this self-assembling process required a very complicated protein preparation process. Wang et al. (M. Wang et al., 2011) reported a biomimetic nanocomposite consisting of nanocellulose and commercially available polybutadiene through ionic assembly, but the synthesized composite contained a large mass of petroleum-based polybutadiene.

In the present work, we propose a biomimetic concept by constructing a soft polymer segment PDMAEMA (poly N, N-dimethylamino-2-ethyl methacrylate) to link hydrophilic carboxymethyl cellulose (CMC) network and hydrophobic PLA hard core through ionic self-assembly in a colloid system, which is expected to avoid macroscale dispersion problem and generate biocomposite with tunable thermal and mechanical properties. This is the first time to use under-micron ionic self-assembly approach to prepare biocomposite with controllable properties from abundant cellulose and PLA, a
commercially available biopolymer on a large scale. Scheme 3-1 displays the entire concept. A copolymer PLA-b-PDMAEMA with controlled molecular weight of PDMAEMA segment was synthesized and the following quaternization provides cationic charges on the PDMAEMA segment. This amphiphilic copolymer was dispersed in an aqueous medium to form a micelle structure with a hydrophobic PLA core surrounded by hydrophilic PDMAEMA chains. When mixed with anionic CMC, cationic charges on the copolymer micelles could adsorb CMC through ionic interaction. In this system, PDMAEMA provides the compatibility between hydrophilic CMC and hydrophobic PLA. Besides, PDMAEMA may behave like a soft lubricant between these two rigid segments, because its glass transition temperature is less than 19 °C (Mirous, 2006), which makes it a rubbery material at room temperature.

In the next, we will describe the detailed methods containing the synthesis of the copolymer with controllable molecular weight and its modification, formation and characterization of the micelle structure; and the self-assembling process to form CMC/PLA bio-composite complex under the micron size. We will also study the resultant composite films after casting and how their properties are tuned with the inclusive molecular weight of soft PDMAEMA segments.

**Materials and Methods**

**Materials**

N, N-dimethylaminoethyl methacrylate (DMAEMA, Aldrich, 98%) was passed through a column of aluminum oxide (Aldrich, activated) to remove stabilizing agents before use. Copper (I) bromide (CuBr, Aldrich, 98%) was washed by glacial acid three times and rinsed with methanol and diethyl ether, then vacuum dried before use. 3,6-dimethyl-1,4-dioxane-2,5-dione (Lactide), α-Bromoisobutyryl bromide (Br-iBuBr), tin(II)
2-ethylhexanoate (95%), triethylamine (99%), and 1,1,4,7,10,10-
hexamethyltriethylenetetramine (HMTETA, 97%) were purchased from Aldrich and used
without further purification. Toluene (Fisher, 95%), benzyl alcohol (Aldrich, 99%), and
tetrahydrofuran (THF, Acros, 99%) were dried by the calcium hydride and then distilled
under normal pressure. Iodomethane (Acros, 99%) and carboxymethyl cellulose (CMC,
Acros, M.W.=250,000) were used as received.

**Synthesis of Poly (lactic acid) (PLA-OH)**

PLA was synthesized using tin (II) 2-ethylhexanoate (stannous octoate) as the
catalyst. In a typical run, monomer (20 g lactide, 0.139 mol), initiator (dried benzyl
alcohol, 3.97 mmol), and solvent (dried toluene, 150 mL) were charged into a 250 mL
three-neck flask with a magnetic stir bar. An azeotropic distillation device (a distilling
receiver and a condenser) was set in the middle neck of flask and the other two necks
were fitted with rubber septa. The mixture was heated to an appropriate temperature for
azeotropic distillation to remove trace water. After distillation, temperature was adjusted
to 100 °C. One drop of stannous octoate was dispersed in 5 mL dried toluene and
transferred into the flask through rubber septum using a syringe with a stainless steel
needle. Under positive nitrogen pressure, polymerization was carried out overnight at
100 °C and then terminated by adding several drops of aqueous HCl solution (1 mol/L).
The polymer was precipitated in hexane, filtrated, and dried under reduced pressure
until constant weight. The obtained white solid was then dissolved in chloroform and
washed by EDTA buffer (0.1 mol/L) once and distilled water twice to remove the
catalyst. The organic layer was then poured into methanol to recover PLA through
precipitation. Finally, the synthesized PLA-OH was vacuum dried and ready for use.
Synthesis of Macrorinitiator PLA-Br

The PLA-OH was converted into PLA-Br macro-initiator through terminal modification by Br-i-BuBr according to a known procedure (Spasova et al., 2009). PLA-OH (2 g, 0.55 mmol) and excess triethylamine (2.68 mL, 19.25 mmol) were dissolved in 10 mL of dichloromethane solution. Then, the solution was stirred in an ice bath for half an hour and Br-i-BuBr (2.38ml, 19.25mmol) was added dropwise at 0 ºC. The reaction was carried out for 3 h at room temperature under normal pressure. After that, small amount of charcoal was added to the flask and the mixture was stirred for 5 h to purify the solution. Finally, charcoal was removed through filtration and PLA-Br solid was obtained by precipitation in methanol and then vacuum dried for the subsequent use.

Synthesis of Copolymer PLA-b-PDMAEMA

PLA-b-PDMAEMA copolymer was synthesized via macro-initiated atom-transfer radical polymerization (ATRP) reaction (Matyjaszewski & Xia, 2001). Briefly, in a typical run, the macro-initiator PLA-Br (2.117 g, 0.478 mmol) and CuBr (68 mg, 0.474 mmol) were placed in a Schlenk tube, which was pre-purged with nitrogen. Ligand HMTETA (259.8μl, 0.955mmol), DMAEMA (2.41ml, 14.302 mmol), and 18 mL THF were injected into the tube, followed by three times freeze-pump-thaw degassing cycles. Then, polymerization was carried out in a silicon oil bath at 60 ºC overnight with positive pressure nitrogen. After reaction, the mixture was diluted with THF and the copper catalyst was removed by passing the copolymer in THF through an alumina column. After that, hexane was added into solution to recover the purified copolymer through precipitation. The precipitates were filtrated and vacuum dried at room temperature to obtain final products.
Quaternization of PLA-b-PDMAEMA Copolymer

PDMAEMA chain in the copolymer was quaternized by reaction with iodomethane (MeI) based on a previously reported method (Mori, Walther, Andre, Lanzendorfer, & Muller, 2004). PLA-b-PDMAEMA (1 g) was dissolved in 20 mL acetone. Then five-fold excess MeI, compared to the nitrogen content in PDMAEMA was added into solution. The mixture was stirred for 24 h in a 50 °C water bath and a yellow precipitate was collected after reaction through filtration. The precipitate was further washed with acetone, hexane, and THF to remove excess MeI. Finally, purified solid was vacuum dried at room temperature to constant weight.

Preparation of Block Copolymer Micelles in Water

In a typical procedure, quaternized copolymer (0.2 g) was dissolved in 20 mL DMF solvent (99.8%, Fisher). The solution was then filled in a dialysis tube (Spectra/Por 3500 Da MWCO, Canada) and dialyzed against nano water (18.2 MΩ, Barnstead) in a 2 L beaker for 72 h (M. Wang et al., 2011). During the dialysis, nano water was changed twice. After dialysis, copolymer micelles with hydrophobic PLA core surrounded by anionic PDMAEMA chains were obtained.

Preparation of Biocomposite

The CMC was dissolved in nano water to obtain 5 g/L suspension. The pH of both CMC and PLA-b-PDMAEMA suspension was adjusted to 8.3 for ionization. Then, CMC and the copolymer with a mass ratio of 5:1 were mixed and then treated by a homogenizer for 5 min (Biospec Products Inc. OK, USA) to obtain uniform complex suspension. The mixture was then centrifuged at 2500 rpm for 5 min to remove air bubbles. After centrifugation, it was cast on an aluminum tray and transferred to an
oven at 100 °C until most of water was removed to form a paste-like gel. The paste was further vacuum dried in a desiccator at room temperature over night to form a film.

**Characterization Methods**

**NMR spectrum**

The obtained products were dissolved in CDCl₃ (30 mg/0.6 mL) and their ¹H NMR spectra were recorded by using a Varian Mercury 300 MHz apparatus at room temperature.

**Morphology studies**

SEM was carried out on a FEI XL 40 FEG SEM (FEI instruments, OR, U.S.A.) at an operation voltage of 15 keV. The concentration of CMC and CMC/quaternized copolymer suspension were diluted to about 0.02%. Then one droplet of each sample was loaded on a carbon tab and lyophilized using a freeze dryer (Labconco, MO, USA). After that, the dried samples were coated with Au/Pd (~10 nm) before scanning. TEM was conducted on a JEOL 200CX instrument (JEOL instruments, MA, USA) with 100 keV accelerating voltage and using carbon type A grids. One drop of the copolymer colloid suspension was loaded on TEM grids and then dried at room temperature.

**Hydrodynamic radius analysis**

Dynamic light scattering (DLS) (Zetasizer ZS 3600 with a noninvasive back scatter under 500 mw and a 532 nm laser, Malven, U.K.) was used to estimate the size and size distribution of copolymer micelles. Micellar solution was diluted into 0.2 g/L before test.

**Fourier transform infrared spectrometry (FTIR) analysis**

FTIR spectra were recorded on a Magna System 560 from Nicolet Instrument (WI, U.S.A.). Samples were prepared in pellet form by mixing with potassium bromide
(KBr) (0.5% w/w to KBr) before test. 16 scans were taken for each sample at a resolution of 4 cm⁻¹.

**Thermal stability analysis**

Thermogravimetric analysis was carried out in a thermal analyzer (Mettler Toledo, OH, USA) to determine thermal stability of corresponding biocomposites. All samples were heated from 50 to 400 °C at a heating rate of 10 °C/min in air.

**Dynamic mechanical analysis (DMA)**

Films were cut into a rectangle shape with a 7 mm width and a 20 mm length and stored in a desiccator for about 48 h to avoid the effect of moisture on films. DMA tests were carried out using a dynamic mechanical analyzer (D110, Seiko Instruments, USA). Samples were heated from -50 °C to 150 °C at a heating rate of 2 °C/min, a frequency of 0.1 Hz, and a strain rate of 0.1%.

**Results and Discussion**

**Preparation of Cationic Copolymer PLA-b-PDMAEMA**

The ATRP reaction has been recently used to construct the copolymer with controlled molecular weight. In the present study, copolymer PLA-b-PDMAEMA was synthesized using a three-step procedure. Ring opening polymerization (ROP) of L-lactide using tin (II) 2-ethylhexanoate as the catalyst led to the formation of PLA-OH, which was followed by esterification to generate macro-initiator (PLA-Br). After that, PLA-b-PDMAEMA was synthesized via ATRP reaction using PLA-Br as the initiator.

**NMR results of PLA-b-PDMAEMA copolymer**

Figure 3-1 (a) displays the ¹H-NMR spectra of each product from each step that are marked as spectrum 1, 2, and 3, respectively. Spectrum 1 shows the characteristic peaks of PLA. The multiple peaks at 4.35 ppm could be assigned to terminal methine
proton and the strong peaks around 5.15 ppm could be contributed to the methine proton in the repeat units. The corresponding number average molecular weight (Mn) of PLA was calculated by comparing the relative intensity of proton in these two types of methine groups (Table 3-1). The esterification of PLA was confirmed by spectrum 2. After esterification with Br⁻-BuBr, the characteristic peak of terminal methine proton for PLA disappeared and instead, new chemical shift appeared at 1.9 ppm corresponding to the methyl in macroinitiator PLA-Br.

The macroinitiator PLA-Br was subsequently used for ATRP reaction to produce PLA-b-PDMAEMA. CuBr and HMTETA were used as the catalyst and ligand respectively. The corresponding NMR result of the copolymer was shown in the spectrum 3 in Figure 3-1 (a). The appearance of two single peaks at δ = 4.00 ppm and δ = 2.56 ppm could be ascribed to two methylene groups in PDMAEMA repeat units. Theoretically, the Mn of PDMAEMA in copolymer can be determined by the molar ratio of DMAEMA monomers to PLA-Br. According to this rationale, three copolymers with various molecular weight of PDMAEMA were synthesized and their NMR spectra were displayed in Figure 3-1 (b). The intensity of methylene group of PDMAEMA (δ = 4.00 ppm) in the copolymer was increased accordingly from spectrum 1 (bottom) to spectrum 3 (top). As the Mn of PLA was already known from spectrum 1 in Figure 3-1 (a), the Mn of the copolymer could be calculated by comparing the relative intensity of methylene group in PDMAEMA (δ = 4.00 ppm) and methine group (δ = 5.15 ppm) in PLA. The corresponding Mn of PLA-b-PDMAEMA copolymers was listed in Table 3-1.

**Dynamic particle radii and morphologies of copolymer micelles**

To increase the compatibility between anionic cellulose and the copolymer, cationic copolymer was prepared via quaternization of PLA-b-PDMAEMA. When this
quaternized PLA-b-PDMAEMA was dispersed in water, the micelle particles were formed due to the amphiphilicity of the copolymer. This micellar structure contains a hydrophobic spherical PLA core surrounded by a cationic polyelectrolyte PDMAEMA chains. Figure 3-2 shows the distribution of hydrodynamic radii of quaternized copolymer micelles with different PDMAEMA segment lengths and its TEM image. The results from DLS indicated that the micelles were spherical particles with an average radius of approximately 100 nm (Table 3-1), which was in agreement with TEM image (Figure 3-2(b)). Besides, the micelle particles had a broad particle size distribution and the average radius of copolymer micelles were increased from 87 nm to 113 nm accordingly with the increase of the PDMAEMA chain length.

**Preparation of CMC/PLA Biocomposite Suspension**

**Optical observation of morphology**

In order to form a uniform biocomposite suspension, the compatibility between hydrophobic PLA and hydrophilic CMC must be attained. The synthesized quaternized copolymer PLA-b-PDMAEMA provides both hydrophilicity and cationic ions for PLA. It is known that cellulose can be modified to become polyelectrolytes with either cationic or anionic groups on the surface. These modified ionic cellulose polyelectrolytes have been broadly used to construct ionic interaction (Aulin, Johansson, Wågberg, & Lindström, 2010; Karabulut, Pettersson, Ankerfors, & Wågberg, 2012; Tripathy & Raichur, 2013). In this study, carboxymethyl cellulose with myriad anionic carboxyl groups was utilized to bind cationic PLA-b-PDMAEMA micelle particles via colloidal ionic self-assembly. The pH of CMC suspension was adjusted to 8.3, which was higher than its pKa to ensure the sufficient ionization of carboxyl groups (M. Wang et al., 2011). Meanwhile, the dialyzed PLA copolymer micellar solution was also adjusted to
pH=8.3 before mixing. Figure 3-3 (a) and (b) represents a transparent CMC suspension and a uniform copolymer colloid individually. After mixing CMC and PLA-b-PDMAEMA at a mass ratio of 5:1, the flocculation was observed immediately and the suspension had opaque color due to ionic complexion (Figure 3-3(c)). The long chain CMC with negative charge could bridge cationic copolymer micelles through ionic attraction. However, at the aid of homogenization, the flocculi were broken down evenly and a stable colloid was achieved (Figure 3-3 (d)). In this colloid, there existed a network structure consisting of dominant long chain cellulose with pending cationic PLA copolymer micelle particles.

**Scanning electron microscopic analysis**

The micro-morphologies of CMC and its biocomposite suspension were further characterized by SEM. As shown in Figure 3-4 (a), commercial CMC had a diameter ranging from 100 nm to 700 nm and a length in several micrometers. It was also observed that in CMC suspension, cellulosic fibrils were fully extended as a relatively loose network. When cationic copolymer PLA-b-PDMAEMA was added into CMC suspension (Figure 3-4 (b)), fibrils were tangled together and a denser structure compared with CMC was formed. Some spherical particles either coated on the surfaces of cellulosic nanofibers or bound them together as a bridge. This phenomenon verifies the interaction between anionic cellulose and cationic copolymers. Besides, the colloidal ionic assembly only occurred at the nanometer scale since we observed that both macroscopical dispersion and the obtained film were still uniform (Figure 3-3).

**FTIR results**

To further elucidate the interaction between cationic copolymers and anionic cellulose networks, the chemical bonding of CMC, quaternized copolymer, and
CMC/PLA biocomposites were investigated by FTIR (Figure 3-5). The peaks at approximately 3500 cm\(^{-1}\) and 3000 cm\(^{-1}\) observed in all three samples could be assigned to the stretching vibrations of O-H from residue water and stretching vibration of C-H groups individually. Strong characteristic peaks observed at 1595 cm\(^{-1}\) and 1726 cm\(^{-1}\) were ascribed to the stretching vibration of C=O in carboxyl and ester individually. In carboxymethyl cellulose, all the C=O bonds existed in carboxyl form, therefore, only 1595 cm\(^{-1}\) peak was detected. While in the copolymer, there was a strong intensity peak at 1726 cm\(^{-1}\) because it contained C=O bonds in ester groups. In biocomposite, due to the coexistence of two components, both peaks for C=O bonds in carboxyl and ester form were observed.

**Preparation and Characterization of CMC/ PLA Biocomposite Films**

The CMC/PLA biocomposite films with three different molecular weight of PDMAEMA were prepared through vacuum dry. Our concept is to use soft PDMAEMA segment in the copolymer as a lubricant to dissipate energy between rigid cellulose network and hard PLA core. The mass ratio of CMC to PLA copolymer could reach to 5:1 and this ratio was used for all biocomposite films. Figure 3-3 demonstrates that a uniform and transparent film was achieved from biocomposite colloid after water evaporation. The properties of these films were further characterized.

**Thermogravimetric analysis (TGA)**

TGA was used to study the effect of different molecular weight of PDMAEMA segment on thermal stability of corresponding composites. Figure 3-6 shows thermograms (a) and the maximum degradation temperature (obtained through first derivative TGA curve) (b) of CMC, CMC/PLA composites 1, 2, and 3. The weight degradation curve obeys the usual sigmoid like shape and there are three main weight
loss regions found in the given temperature range. At the very beginning, an initial weight loss from 70 °C to 180 °C was observed for all the samples, which could be contributed to water loss. The CMC film had the maximum water loss that might be related to its good hydrophilicity (Toğrul & Arslan, 2004). The second degradation region occurred in the temperature range from 210 °C to 300 °C and with a maximum weight loss of approximately 40%. When the temperature was above 300 °C, the rate of decomposition was significantly decreased.

It was observed from TGA curve that in the addition of the copolymer, both onset and maximum degradation temperatures shifted towards lower values. The maximum degradation temperature of the CMC film was approximately 278 °C, which fitted well with the previous report (de Britto & Assis, 2009). Besides, with the increase of PDMAEMA segment in the copolymer, both temperatures decreased accordingly. It is reasonable since thermal stability of cellulose is superior than the copolymer, and in the copolymer, PDMAEMA has much less thermal stability than PLA segment. These results demonstrate that thermal stability of biocomposite films could be tailored by adjusting the molecular weight (chain length) of soft PDMAEMA segment.

**Dynamic mechanical analysis (DMA)**

DMA technique was introduced to explore the viscoelastic behavior of composites with varied temperature under a sinusoidal stress. Figure 3-7 presents the storage modulus (E´) and corresponding tan δ for CMC and all biocomposite films. In Figure 3-7 (a), three regions were observed including glass state (below 25 °C), the glass transition (approximately between 20 and 60 °C) and the rubbery plateau (after 60 °C).
As shown in Figure 3-7, the addition of the copolymer has a significant influence on the viscoelastic response of biocomposites. In glassy region, the storage modulus gradually decreased with the increase of the molecular weight of PDMAEMA segment for all the prepared composites. It is noticed that the composite with the least molecular weight of PDMAEMA had the highest storage modulus, even higher than that of pure CMC. This can be explained that the high stiffness of PLA played the major role in the copolymer that resulted in higher storage modulus of the biocomposite. With the further increase of PDMAEMA segment, the soft segment PDMAEMA had significant influence on the storage moduli of the composites. In the glass transition range, the peak and damping intensity were very similar among CMC, composites 1, and 2 (Figure 3-7(b)), while for composite 3, it had an increasing intensity and higher glass transition temperature, Tg. The rise of Tg in composite 3 may be associated with a restriction in molecular motion and a higher degree of crosslinking (Pötschke, Fornes, & Paul, 2002). The presence of maximum amounts of cationic groups in composite 3, the interaction between the cellulose chains and micelles would be very strong, which led to the reduction of macromolecular chain mobility. However, this effect is not significant for the composites containing smaller portion of PDMAEMA (composite 1 and 2) due to insufficient interaction between cellulose and the copolymer. Besides, the greater tan δ peak was observed for composite 3 as well. This further provides the evidence that strong interaction between the copolymer and cellulose and the existence of the soft segment in the network may facilitate the dissipation of energy in the composite.

Summary

For the first time, ionic assembly method is designed to prepare the biocomposite from hydrophobic PLA and hydrophilic cellulosic fibers in an aqueous medium. The
compatibility between colloidal CMC and the PLA copolymer at nanoscale level was achieved through a colloid suspension consisting of anionic carboxymethyl cellulose network with appending cationic copolymer PLA-b-PDMAEMA micellar particles. As a result, a transparent and uniform biocomposite film was obtained and its thermal and mechanical properties could be tuned by adjusting the molecular weight of PDMAEMA segments. This biomimetic assembly provides many possibilities for future biocomposites’ synthesis such as widely tuned properties by adjusting the mass fraction (molecular weight) of each inclusive component, and improved compatibility when combining biopolymers with different hydrophobicity and fracture toughness.
Scheme 3-1. The schematic diagram for the construction of biocomposite using cationic poly(l-lactide)-block-poly(N,N-dimethylamino-2-ethyl methacrylate) (PLA-b-PDMAEMA) and anionic carboxymethyl cellulose (CMC).
<table>
<thead>
<tr>
<th>Sample</th>
<th>Mn of PLA (g/mol)</th>
<th>Mn of PDMAEMA (g/mol)</th>
<th>Mn of PLA-b-PDMAEMA (g/mol)</th>
<th>Hydrodynamic radius (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Copolymer 1</td>
<td>3636</td>
<td>3144</td>
<td>6780</td>
<td>87.2</td>
</tr>
<tr>
<td>Copolymer 2</td>
<td>3636</td>
<td>7546</td>
<td>11182</td>
<td>95.7</td>
</tr>
<tr>
<td>Copolymer 3</td>
<td>3636</td>
<td>11948</td>
<td>15584</td>
<td>113</td>
</tr>
</tbody>
</table>
Figure 3-1. NMR spectra of synthesized products: (a) $^1$H NMR spectra of PLA (spectrum 1), macroinitiator PLA-Br (spectrum 2), and PLA-b-PDMAEMA (spectrum 3); (b) $^1$H NMR spectra of PLA-b-PDMAEMA with different molecular weight of PDMAEMA segment.
Figure 3-2. Hydrodynamic radii of quaternized PLA-b-PDMAEMA copolymer micelle particles in water: (a) particle size distribution; (b) TEM image of quaternized PLA-b-PDMAEMA copolymer micelles (copolymer 2).
Figure 3-3. Comparison of different suspension and composite film. Left: The suspension of copolymer PLA-b-PDMAEMA, CMC, and their mixture in different conditions: (a) 10 mg/mL quaternized copolymer, (b) 5 mg/mL CMC, (c) the mixture of CMC and quaternized copolymer, (d) the mixture of CMC and quaternized copolymer after 5 min homogenization. Right: Biocomposite film of CMC and copolymer PLA-b-PDMAEMA after water evaporation (copolymer 2).
Figure 3-4. SEM images of (a) CMC, and (b) the mixture of CMC and quaternized copolymer PLA-b-PDMAEMA (copolymer 2).
Figure 3-5. Fourier transformed infrared spectra (FTIR) of copolymer PLA-b-PDMAEMA, CMC and the resultant composite.
Figure 3-6. Thermogravimetric analysis (TGA): (a) thermo degradation curve, and (b) summary of maximum degradation temperature of CMC and biocomposite with different molecular weight of PDMAEMA segment.
Figure 3-7. Temperature dependence of storage modulus (a), and tan δ (b) of CMC and biocomposites with different molecular weight of PDMAEMA segment.
CHAPTER 4
CELLULOSE-BASED INJECTABLE HYDROGEL COMPOSITE FOR PH-RESPONSIVE DRUG DELIVERY

Localized drug delivery with prolonged, stimuli-driven release function can maximize the applicability of drugs while minimizing their side effects. This study developed a new cellulose-based injectable hydrogel composite system containing pH responsive poly (ethylene oxide)-block-poly (2-(diisopropylamino) ethyl methacrylate) (PEO-b-PDPA) copolymer micelles for localized delivery and pH-triggered release. First, covalently in situ generated hydrogels were constructed by mixing hydrazide-modified carboxymethyl cellulose (CMC-NH$_2$) with oxidized carboxymethyl cellulose (CMC-CHO). The resultant hydrogels could be well tuned in terms of gelation time, degradation profile, and mechanical properties. Then, the pH-responsive copolymer PEO-b-PDPA was introduced to enhance the loading of hydrophobic substances and endow the system with a pH-triggered release feature, which was demonstrated by fluorescent spectra using Nile Red as a model drug. In addition, hydrogel itself could act as a second diffusion barrier to reduce the drug release rate. The cell viability tests showed that both CMC-NH$_2$ and hydrogels possessed excellent biocompatibility, while CMC-CHO was only cytotoxic at a relative high concentration.

Introduction

An ideal drug delivery system requires active drugs available in targeted sites of action at a desired time (Langer, 1998). Hydrogels are becoming promising carriers for drug delivery because of their unique features (Hoare & Kohane, 2008). Drug-loaded hydrogels can create a depot for the slow elution of drugs while, maintaining a high local concentration over an extended period. In addition, due to their high water content and network structure, hydrogels resemble living tissues more than any other synthetic
materials. By entrapping drugs inside, hydrogels can improve their compatibility with surrounding tissues. It is also possible to tune the pore size of hydrogels to control the loading and release rate of drugs (Barbucci, Leone, & Vecchiullo, 2004). Last but not least, hydrogels possess good deformability, which can conform shape to adapt to local environment. This is very beneficial to the in vivo application, because some areas may have complicated structures. Based on these advantages, a variety of studies have been done to explore the potential of hydrogels for smart drug delivery purposes.

Recently, hydrogels with injectability have attracted a lot of interest due to their in situ gelation ability (L. Yu & Ding, 2008). Injectable hydrogels can replace invasive surgical operation, because they form inside the body. Specifically, flowable components are extruded from the syringe and locally gelate shortly after injection through either physical or covalent crosslinking. This in situ gelation allows hydrogels to better integrate with local environment. Additionally, the covalent bond induced hydrogels exhibit better mechanical performance and design flexibility (Hiemstra, van der Aa, Zhong, Dijkstra, & Feijen, 2007). This type of hydrogels are suitable for long-term biomedical applications or in the bodily area with a load-bearing requirement (Patenaude, Campbell, Kinio, & Hoare, 2014; Yang, Bakaic, Hoare, & Cranston, 2013). For injectable gels, rapid gelation after injection is a critical factor, since it can prevent diffusion of hydrogel components in the body. A variety of fast, covalently bonded reactions have been applied to form injectable hydrogels under physiological conditions such as Michael addition reaction (Gao et al., 2010; R. Jin et al., 2010), Diels-Alder reaction (Wei et al., 2011) and Schiff base reaction (Wu, He, Wu, & Chen, 2016; Yang et al., 2013). Among them, hydrazine bonding by Schiff base reaction is of particular
interest, because hydrazine groups possess hydrolytic ability that can facilitate the formation and degradation of hydrogels (Ito, Yeo, Highley, Bellas, & Kohane, 2007). However, one important issue for the use of injectable hydrogels is that their hydrophilicity could greatly inhibit the quantity and homogeneity of hydrophobic drug loading, which may finally lead to rapid drug release.

The introduction of amphiphilic copolymer micelles as drug carriers is an effective approach to solve incompatibility between hydrophobic drugs and hydrophilic gels (Adams, Lavasanifar, & Kwon, 2003; Gaucher et al., 2005). In an aqueous medium, amphiphilic copolymers form core-shell structure with hydrophobic core regions serving as reservoirs for hydrophobic drugs. In addition, some copolymers are designed to achieve a stimuli-triggered drug release profile. Various copolymers with environmental responsiveness such as pH-responsive (Giacomelli et al., 2011; N. Jin et al., 2013), thermo-responsive (Chung, Yokoyama, & Okano, 2000) or enzyme-triggered properties (Guo et al., 2014) have been extensively reported. The stimuli-responsive polymeric micelles enable smart and localized release of drugs (Barbucci et al., 2004). For example, inflamed pathological tissue is more acidic than healthy tissue (Friese et al., 2007), so surrounding environment can trigger the release of drugs in pH-responsive micelles. Furthermore, the embedment of copolymer micelles into hydrogel matrix can also increases the biocompatibility of micelles and prevents their migration from targeted sites. However, to the best of our knowledge, there was no report on constructing an injectable hydrogel system encapsulated with pH-responsive copolymer micelles for controlled delivery of hydrophobic drugs. The ideal system is expected to
have a pH-triggered, localized drug release and a tunable, prolonged release rate by using injectable hydrogel as the second barrier.

Recently, cellulose has been widely used to fabricate hydrogels due to its abundance, biocompatibility, good mechanical properties, and versatile modification potential. Cellulose derivatives like carboxymethyl cellulose (CMC), methyl cellulose (MC) have already been introduced as partial components for the fabrication of injectable hydrogels (Hudson, Langer, Fink, & Kohane, 2010; Ito et al., 2007; Yang et al., 2013; Zhang et al., 2014). In this research, we attempted to create a solely cellulose-based injectable hydrogel, which was embedded with pH-responsive copolymer micelles to accomplish hydrophobic drug delivery and pH-triggered release. Injectable hydrogels were constructed by the modified CMC as precursors. A pH-responsive copolymer PEO-b-PDPA (poly (ethylene oxide)-block-poly (2-(diisopropylamino) ethyl methacrylate) was synthesized and rendered to micelle suspensions. Then, two hydrogel precursors were dissolved in micelle suspensions separately and coextruded from a double-barrel syringe (Scheme 4-1). The gel formed by hydrazine bonds after the mixing of two hydrogel components under normal physiological conditions. A series of injectable hydrogel properties like gelation time, swelling ratio, degradation rate, cross-liking density, and mechanical properties were investigated. Finally, the loading efficiency, pH-triggered release profile, and cytotoxicity of synthesized injectable hydrogel composite system were also examined.

**Materials and Methods**

**Materials**

Poly (ethylene oxide) (PEO, MW = 5000 g/mol), α-Bromoisobutyryl bromide (Br-BuBr), triethylamine (99%), and 1,1,4,7,10,10-hexamethyltriethylenetetramine
(HMTETA, 97%), carboxymethyl cellulose (DS=1.2, CMC) were purchased from Aldrich and used without further purification. Copper (I) bromide (CuBr, Aldrich, 98%) was washed by glacial acid three times and rinsed with methanol and diethyl ether, then vacuum dried before use. 2-(Diisopropylamino) ethyl methacrylate (DPA, Aldrich, 97%) was passed through a column of aluminum oxide (Activated, Aldrich) to remove stabilizing agents. Tetrahydrofuran (THF, Fisher, 99.9%) was dried by the calcium hydride and then distilled under normal pressure. Dichloromethane, dimethylsulfoxide, sodium periodate and adipic dihydrazide were purchased from Acros and directly used without further processing. 1-Hydroxybenzotriazole hydrate (HOBt) and N-(3-Dimethylaminopropyl) -N-ethylcarbodiimide (EDC) were obtained from Fisher Scientific.

**Preparation of Copolymer Micelles**

**The synthesis of PEO macroinitiator**

Macroinitiator was prepared based on a previous method (S. Liu, Weaver, Save, & Armes, 2002) with slight modifications. Briefly, PEO$_{113}$-OH (10 g, 0.002 mol) was dissolved in 80 mL of methylene chloride in a 250 mL single-neck flask. Then, triethylamine (2.79 mL, 0.02 mol) was added and the solution was stirred in an ice bath (0 °C). After that, α-bromoisobutyryl bromide (2.47 ml, 0.02 mol) was added dropwise for 30 min at 0 °C and the reaction mixture was stirred overnight at room temperature. The solution was stirred with charcoal for 5 h at room temperature. Through filtration, the charcoal was removed and most of the methylene chloride was evaporated by a rotary evaporator. After precipitation using excess ether, the precipitate was filtrated and dried under vacuum. Further purification was performed by dissolving the product in sodium carbonate solution (pH 8-9), and then extracted with dichloromethane. The
organic layers were collected and dried over MgSO$_4$. After removal of the solvent, the purified macroinitiator (PEO$_{113}$-Br) was obtained.

**The synthesis of PEO-b-PDPA copolymer**

Typically, PEO$_{113}$-Br (1 g, 0.194 mmol), 1,1,4,7,10,10-hexamethyltriethylene tetramine (HMTETA, 111.74 mg, 0.485 mmol), CuBr (55.68 mg, 0.388 mmol), DPA (1.62 g, 7.59 mmol), and tetrahydrofuran (THF) (15 ml) were added into a 50 ml reaction tube. The mixture was degassed through four freeze-pump-thaw cycles. After degas, the flask was transferred to a 60 ºC oil bath. The reaction was performed for 24 h under N$_2$. Then, the tube was cooled down to room temperature and diluted by extra volume of THF. The copper complex was removed by passing diluted product through a basic alumina column using THF as solvent. The obtained solution was concentrated by the rotary evaporator and then dissolved in acetone at room temperature. The copolymer was obtained by the precipitation in acetone/dry ice bath as described before (N. Jin et al., 2013). This process was conducted for three times. The purified copolymer was vacuum dried at room temperature until constant weight. Then, it was dissolved in CDCl$_3$ (30 mg/0.6 mL) and its $^1$H NMR spectrum was recorded by using a Varian Mercury 300 MHz apparatus at room temperature.

**Preparation of PEO-b-PDPA copolymer micelles**

The solvent-switching method was used to prepare PEO-b-PDPA micelles in aqueous solution (M. Wang et al., 2011). In brief, 200 mg PEO-b-PDPA was dissolved in DMA to a 2 g/L solution. The mixture was stirred overnight to ensure the complete dissolution. Then, it was transferred to a 3500 MWCO dialysis tubing (Thermo Scientific, USA) and dialyzed against pH 7.4 PBS buffer for 3 days. The buffer was changed every
After dialysis, the concentration of PEO-b-PDPA was adjusted to 10 mg/g for future use.

**Preparation of Injectable Hydrogel Precursors**

**Modification of carboxymethyl cellulose with hydrazide (CMC-NH$_2$)**

The modification of CMC with hydrazine groups was carried out according to a previous method (Bulpitt & Aeschlimann, 1999). Briefly, 1 g CMC was dissolved in 200 ml deionized water and then 3 g of adipic dihydrazide was added. The suspension was stirred at room temperature to form a well-dispersed mixture. Then, HOBt (258 mg suspended in 2 ml of dimethylsulfoxide: water (1:1) mixture) and EDC (262 mg in 2 ml of a dimethylsulfoxide: water (1:1) mixture) were added respectively. The pH was kept at 6.8 by using 0.1 M sodium hydroxide when necessary. After overnight reaction, the suspension was dialyzed against nano water (18.2 MΩ, Barnstead) directly using a 3,500 MWCO (SnakeSkin dialysis tubing, Thermo Scientific) membrane. The final modified product was obtained through lyophilization. The degree of modification was calculated by element analysis using an elemental analyzer (Carlo-Erba NA-1500 CNS analyzer, Thermo Scientific).

**Preparation of aldehyde modified carboxymethyl cellulose (CMC-CHO)**

CMC with aldehyde groups was obtained through oxidation. Briefly, 1.5 g CMC was dissolved in 150 ml water and then 750 mg sodium periodate was added. The mixture was stirred for 2 h until the reaction was stopped by adding 200 µl ethylene glycol. The obtained product was further purified by dialysis against water. After freeze dry, the final product was obtained and kept at 4 °C for future use. The degree of oxidation (DOX) was determined by a iodometric titration method, as described by previous researchers (Gomez, Rinaudo, & Villar, 2007). Typically, after different time-
period oxidation, instead of using ethylene glycol to stop reaction, 5 ml sample was transferred to a 50 ml beaker and neutralized by adding 10 ml of 10% NaHCO₃. Then, 2 ml 20% potassium iodide was added and the mixture was stirred in the dark for about 15 min. The unreacted sodium periodate could oxidize iodide ions to iodine. When excess potassium iodide was added, iodine could dissolve in the iodide-containing solution to give triiodide ions with a dark brown color. The triiodide ion solution was titrated with a standard thiosulphate solution to produce iodide again and the brown color will disappear at the endpoint. Two types of CMC-CHO with different DOX were prepared in this research.

**Characterization of Hydrogel Properties**

**Gelation time and morphology**

The aldehyde and amine modified CMC was dissolved in a 0.1 M phosphate buffered saline (PBS buffer) with pH of 7.4 at room temperature, individually. Then, two suspensions were added to a double barrel syringe. Through co-extrusion by slowly pushing the plunger in syringe, the precursors were mixed uniformly. To determine the generation time of a stable gel, the mixture was extruded to a glass vial (3.7 ml volume), and an inversion test was applied. The gelation time was recorded as the time that hydrogels could completely stick on the bottom of vial. Three replicates were performed for each assay and standard deviation was shown as error bar in corresponding figure.

In addition, the morphologies of different hydrogels were characterized by FEI XL 40 FEG SEM (FEI instruments, OR, USA) at an operation voltage of 15 keV. Hydrogels were loaded on the SEM mount containing carbon tab on the surface, and lyophilized using a freeze dryer (Labconco, MO, USA). Then, Au/Pd (~10 nm) coating was performed on all freeze-dried samples.
Swelling and degradation

The swelling and degradation properties of hydrogels were characterized as follows. Disc-like hydrogels were prepared by co-extrusion of hydrogel precursor solutions into silicon models (8-9mm diameter, 1.8mm depth, Grace Bio-labs), which were covered with two micro glass slides to form a sandwich structure. The hydrogels were left at room temperature for approximately 5 h to ensure the complete gelation. After that, hydrogels were transferred to cell cultural inserts (12-well format, BD Falcon) and immersed into a 12-well cell culture plate containing pH 7.4 PBS buffer. The plate was stored in an incubator at 37 °C. At every measured time point, hydrogels were weighed after drawing off excess (unbound) water using Kimwipe. The swelling ratio Q, was determined gravimetrically at predetermined time points according to the previous research (Ito et al., 2007). Q = W_s/W_i, where W_s is the weight of hydrogel after submergence in PBS buffer at determined time point (the unbound water were drew off using Kimwipe), and W_i is the initial weight of hydrogel after gelation. A total n=4 replicates were performed for each sample.

Mechanical properties

The storage moduli (G') of hydrogels were measured by a rheometer (ARES LS1, Texas Instrument, TX). Before test, hydrogels were left on the bench for about 5 h at room temperature to ensure complete gelation. A parallel plate geometry with a diameter of 8 mm was chosen. Hydrogels were carefully loaded in the center of the bottom plate and the gap between two plates was carefully adjusted until the force reached 0.5 g·cm. An initial strain sweep (0.1-100%) was conducted to determine the linear viscoelastic region of samples at a frequency of 1Hz and a 2% strain was chosen for all hydrogel samples. Then, the storage moduli (G') of hydrogels were determined by
the frequency sweep ranging from 1 to 100 rad/s. A total n=3 replicates were performed for each sample.

**Hydrophobic Dye Loading Study**

Nile Red was used as a model hydrophobic substrate to examine the loading ability of prepared copolymer micelles. At first, 100 µl Nile Red in acetone at a concentration of 0.2 mg/ml was transferred to empty glass vials. The acetone was completely vacuum evaporated at 50 °C for 5 hours. Then, the micellar solutions at different concentrations (0.75, 0.5 and 0.25 mg/ml) were added to these vials. The mixture was sonicated for 15 min to encapsulate dye into the micelles. The fluorescence spectra of Nile Red were recorded using a PerkinElmer LS 45 fluorescence spectrometer equipped with a pulsed xenon discharge lamp at room temperature. The excitation wavelength was set at 370 nm and the emission spectra were collected in the range from 700 to 800 nm.

**pH-Induced Dissociation of PEO-b-PDPA Micelles in Aqueous Buffer Solutions**

0.75 mg/ml micelle suspension containing Nile Red dye was further used to study the pH responsive feature. The pH was adjusted by adding 1 M HCl solution dropwise into dye-loaded micelle suspension. At different pH levels, samples were taken out to measure the emission intensity. Moreover, the dynamic release profile was also studied. 2 ml suspension was transferred into two cuvettes and then, 1 ml pH 7.4 PBS and 1 ml pH 4.0 citric acid–Na$_2$HPO$_4$ buffer were added into cuvettes respectively. The emission intensity of each cuvette was recorded at determined time points.
Triggered Release of Nile Red from Injectable Hydrogels Embedded With Nile Red-Loaded PEO-b-PDPA Micelles

Nile Red-loaded 0.75 mg/ml PEO-b-PDPA suspension was used to dissolve CMC-CHO and CMC-NH₂ separately. Then, these two hydrogel precursor suspensions containing dye loaded micelles were mixed uniformly by a double barrel syringe. The mixture was co-extruded to a cuvette. After gelation, the obtained hydrogels were stored in the refrigerator overnight before use. In release test, 1 ml pH 4.0 buffer was added into the cuvette containing hybrid hydrogel and meanwhile, the same amount of pH 7.4 PBS buffer was added in another cuvette as control. The corresponding emission intensities were recorded as the function of time according to aforementioned method.

Preparation of Doxorubicin Loaded PEO-b-PDPA Micelles and Hydrogel Composite

Doxorubicin was loaded into polymeric micelles using solvent evaporation method (Diao et al., 2011). Typically, doxorubicin hydrochloride was dissolved in methylene chloride with the addition of excess triethylamine. The mixture was stirred for 4 h to neutralize doxorubicin solution. Then, 10 mg of copolymer PEO-b-PDPA was dissolved in this doxorubicin solution. Afterwards, 10 mM PBS (pH 7.4) buffer was added and the mixture was vigorously stirred overnight to allow slow evaporation of organic solvent. The methylene chloride residue was removed by rotary evaporation. After that, the dialysis against 10 mM PBS buffer was applied. The final volume of micelle solution was adjusted to 10 ml and vigorous ultrasonication was performed to ensure the encapsulation of doxorubicin in micelles. The product was then filtrated by passing a 0.22 µm filter to eliminate the residual copolymer and doxorubicin aggregates. All the processes were operated under light protection. The particle size was determined at room temperature using a zetasizer (Zetasizer Nano S, Malvern).
Instrument Ltd, UK) with a He-Ne laser at 633nm excitation. Finally, the hydrogel composite containing doxorubicin was prepared by following the aforementioned method.

**In Vitro Release of Doxorubicin**

*In vitro* drug release tests were conducted via a dialysis method. At first, various concentration of doxorubicin solutions were prepared and their absorptions at 461 nm wavelength were used for a calibration curve (Kim & Chu, 2000). Then, two types of hydrogel composites containing doxorubicin with different crosslinking density were prepared by mixing 3% (w/w) CMC-CHO (23% DOX) with 1.5% and 4.5% (w/w) CMC-NH₂ respectively, which were named 1.5% hydrogel and 4.5% hydrogel. In order to compare the release profile, doxorubicin, doxorubicin loaded copolymer micelles, and two injectable hydrogels containing doxorubicin loaded copolymer micelles were placed in four individual dialysis bags with 14,000 molecular weight cut off (MWCO). The introduction of high MWCO membrane could ensure the unrestricted diffusion of the released drug (Missirlis, Kawamura, Tirelli, & Hubbell, 2006). A 10 mM PBS buffer containing 0.1% tween-80 together was used as dialysis solvent, which met the sink condition of a typical drug release test (Diao et al., 2011). A drug release study was performed at 37 °C in an incubator shaker at a rate of 100 rpm. At predetermined time intervals, the solution outside the dialysis bag was withdrawn and its absorbance was measured by a UV-Vis spectrometer (DU 800, Beckman Coulter, USA).

**Cell Viability Test**

Human cervical adenocarcinoma cells (HeLa cells) from American Type Culture Collection (Manassas, VA) were used to test the cytotoxicity of hydrogel precursors and obtained hydrogels. HeLa cells were seeded in a 96 well plate with complete Dulbecco’s
Modified Eagle’s Medium (DMEM, Sigma-Aldrich) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS, Sigma-Aldrich), 100 µg/ml penicillin, and 100 µg/ml streptomycin (P/S; Lonza, Walkersville, MD) at 37 °C in humidified air containing 5% (v/v) CO₂. The density of cells was controlled at 5 × 10³ cells/well. After 24 h seeding of cells, test materials were added. 50 µl precursor solution was added to the wells and for cross-linked hydrogels, 40 mg gel was placed in each well and floated in the culture medium. The incubation was carried out for another 24 h, and then, medium containing test materials was removed by replacing fresh cultural medium. The cell viability was assessed by using a Cell Count Kit-8 (CCK-8; Sigma-Aldrich). 10 µL CCK-8 reagent was added to wells and the optical density at 450 nm was measured on a SpectraMax M5 microplate reader. All tests were replicated three times and the mean absorbance of non-exposed cells was used as the reference of 100% cellular viability.

Results and Discussion

Synthesis of PEO-b-PDPA

In the present study, pH-responsive copolymer PEO-b-PDPA was synthesized through atom transfer radical polymerization (ATRP) reaction. PEO was first modified to macroinitiator (PEO-Br) through esterification of end hydroxyl group by α-bromoisobutryl bromide. Then, ATRP reaction was conducted to yield PEO-b-PDPA using HMTETA/CuBr as the catalyst under air-free condition. Figure 4-1 (a) shows the NMR spectrum of PEO-Br after esterification. The characteristic peak at 1.95 ppm is ascribed to the terminal methyl group which was introduced by Br-iBuBr. The NMR spectrum of obtained diblock copolymer was present in Figure 4-1 (b) verifying the chemical structure of PDPA. For example, peaks appeared at 2.97 and 2.61 ppm correspond to the proton in -NCH₂- and -N(CH)₂- structure from PDPA repeat units.
respectively. In addition, peak at 3.82 ppm is assigned to the methylene next to the carboxylic acid ester group. Since the molecular weight of PEO was already known, the degree of polymerization (DP) of PDPA can be calculated by comparing the integral values of peaks at 3.63 ppm (-CH₂CH₂-, repeat units in PEO) and 2.97 ppm (-NCH₂-, repeat units in PDPA). The DP of PEO and PDPA in the copolymer were 113 and 48 individually.

**Modification and Characterization of CMC-CHO and CMC-NH₂**

Periodate oxidation has been widely used to analyze the molecular structure of carbohydrates because of its mild reaction condition and rapid, quantitative characteristics (Dryhurst, 2013). In this study, different from previous research, this reaction was introduced to cleave the vicinal glycols and create aldehyde groups on cellulose. The addition of aldehyde groups on cellulose was confirmed by ¹H NMR (Figure 4-2). A peak at 9.2 ppm with low intensity can be assigned to the proton of aldehyde groups. The weak signal of aldehyde is because of its instability. The aldehyde group is easy to be converted to hemiacetals by reacting with hydroxyl groups from either water or CMC by oxidation (Ishak & Painter, 1978; R. J. Yu & Bishop, 1967). However, the equilibrium between aldehyde and hemiacetal does not seem to impair the reaction with hydrazide (Maia, Ferreira, Carvalho, Ramos, & Gil, 2005).

CMC was modified by adipic dihydrazide through EDC mediated reaction to introduce amine groups. The water soluble EDC allows the reaction in aqueous media. The by-product is also water-soluble, so it is easy to purify the final product through simple dialysis or gel filtration (J. Sheehan et al., 1961; J. C. Sheehan et al., 1965). Figure 4-3 shows the NMR spectra of CMC and CMC-NH₂. In comparison with the spectrum of unmodified CMC, new peaks at 2.23, 2.13 (4H, -COCH₂-), and 1.13 ppm
(4H, -CH₂CH₂-) were derived from grafted adipic hydrazide. Additionally, the elemental analysis was applied to determine the degree of modification. The results showed that the weight percentage of carbon and nitrogen were 37.7% and 7.2% in CMC-NH₂, respectively. When we define the degree of amine modification equals to the molar ratio of CMC-NH₂ units to the sum of CMC-NH₂ units and unmodified CMC units, the modification degree was estimated to be 4.7%.

**Gelation Time**

An appropriate gelation time for injectable hydrogels is very important. Slow gelation will lead to the loss and delocalization of hydrogel due to the diffusion phenomenon, but on the other hand, rapid gelation may generate clog in the syringe needle (Lee, Chung, & Kurisawa, 2009). In the preparation of hydrogel, we found that when the concentration of CMC-NH₂ is higher than 4.5%, the high viscosity will impede injection, so three concentrations of CMC-NH₂ (1.5, 3 and 4.5%) were prepared. Figure 4-4 (a) shows the gelation time of hydrogels in various precursor combinations. When CMC-CHO was set at 3%, it is found that with the increase of CMC-NH₂ concentration, the gelation time declined drastically. For example, for 23% oxidized CMC-CHO, the time needed for gelation dropped from 450 s to about 175 s when the concentration of CMC-NH₂ increased from 1.5% to 4.5%. Moreover, the higher DOX (64%) of CMC-CHO led to a faster rate of gelation. Taking 4.5% CMC-NH₂ precursor as an example, the use of CMC-CHO with 64% DOX could reduce the gelation time to about 1 min, compared with 175 s by CMC-CHO with 23%. It indicates that more aldehyde groups presented in cellulose could accelerate Schiff base reaction.

The pH value also plays an important role in the Schiff base mediated gelation process. Figure 4-4 (b) shows the effects of pH on gelation time. Three buffer solutions...
with pH of 7.4, 6.0 and 4.0 were used to prepare hydrogel precursor suspensions. It was found that the lower pH of prepared buffer, the shorter gelation time needed. For instance, the gelation of 3% CMC-NH₂ and 3% CMC-CHO at pH 4.0 occurred almost immediately after extrusion from syringe. This phenomenon can be explained by the fact that H⁺ acted as a catalyst for the nucleophilic addition of amine to carbonyl group (Wade Jr, 2006). Low-pH induced fast gelation is very beneficial to the rapid localization of hydrogels in the acidic environments such as tumor tissue and inflamed pathological tissue (Fründer, 1949; J. Liu et al., 2014). In addition, the increase of CMC-NH₂ concentration could shorten gelation time at different pH levels.

**The Morphology of Hydrogels**

The SEM images of various hydrogels are shown in Figure 4-5. All hydrogels exhibit porous structures. The hydrogel made by 1.5% CMC-NH₂ and 3% CMC-CHO (23% DOX) had the largest and deepest pores (Figure 4-5 (a)), which can be attributed to the least number of CMC-NH₂ molecules and less aldehyde groups on oxidized CMC. When the oxidized degree of CMC-CHO was increased from 23% to 64%, the pore sizes were greatly reduced (Figure 4-5 (b)). It indicates that extra aldehyde groups on cellulose could generate more crosslinking points. Figure 4-5 (c) and Figure 4-5 (d) show the increase of crosslinking density of hydrogels when more concentrated CMC-NH₂ suspensions (3 and 4.5%) were used. Thus, the pore size of injectable hydrogels could be easily tuned by controlling the concentration of precursor and the DOX of CMC-CHO. Hydrogels with adjustable porous structure is expected to tune the release rate of encapsulated substances, since large pores allow easier solvent uptake and content diffusion, while high crosslinking density could lead to the slow release (Kelner & Schacht, 2005).
**Rheological Properties of Injectable Hydrogels**

Rheology characterization provides a quick, sensitive method to characterize the mechanical properties of hydrogels. The high stiffness of injectable hydrogel enables loaded drugs to retain locally against *in vivo* biological forces (Yan & Pochan, 2010). In Figure 4-6 (a), storage moduli (G’) of hydrogels made by 64%, 23% oxidized CMC-CHO and CMC-NH$_2$ with different concentrations were plotted. It was found that when the mass content of CMC-NH$_2$ was increased from 1.5% to 4.5%, the corresponding hydrogels became stiffer. The improved stiffness can be associated with the growth of crosslinking points by increasing the concentration of CMC-NH$_2$. In addition, the DOX had a similar function as concentration on G’. The G’ of hydrogel using 64% oxidized CMC was almost 3 times higher than that using 23% oxidized CMC (Figure 4-6 (b)). These results agreed well with the morphologies observed by the SEM images. The resultant hydrogels had a wide range of G’ from 100 Pa to over 2000 Pa, which indicated that the stiffness of hydrogels can be well tuned through varying the concentration of precursor suspensions and the DOX.

The effect of pH on G’ was also studied and the results were shown in Figure 4-7. 3% CMC-CHO (64% DOX) and 3% CMC-NH$_2$ precursors were dissolved in three different buffer solutions (pH 7.4, 6.0 and 4.0). The stiffness of obtained hydrogels had little difference among each other. This phenomenon indicated that although low pH environment promoted the gelation, it had almost no influence on the mechanical property of corresponding hydrogels.

**Swelling Ratio and Degradation Profile of Injectable Hydrogels**

The swelling and degradation of hydrogels were studied in pH 7.4 PBS buffer at 37 °C, the physiological conditions of human body. Cellulose-based hydrogel had
abundant hydroxyl groups that could adsorb a large quantity of water and meanwhile, their crosslinking structure protected hydrogels from dissolution. The swelling ratio was plotted as a function of time in Figure 4-8 (a). In the first 20 days, hydrogels prepared by 23% DOX CMC-CHO possessed relatively high swelling ratios. Among them, hydrogel made up of 4.5% CMC-NH₂ and 3% CMC-CHO (23% DOX) achieved the highest value during this period. It is believed that hydrogels formed by CMC-CHO with low DOX had low crosslinking density, which provided more space for the dwelling of water. In addition, the introduction of highly concentrated CMC-NH₂ (4.5%) offered more hydroxyl groups to form hydrogen bonds with water. In contrast, CMC with higher DOX (64%) contains more short chains and aldehyde groups, which were favorable to generate high density matrix structure (Lü, Liu, & Ni, 2010). Hydrogels generated by 64% DOX CMC-CHO were relatively stable and possessed low swelling ratios in the very beginning (McBath & Shipp, 2010). However, this trend changed after 30 days. A sharp increase of swelling ratio was observed, which can be ascribed to the partial hydrolysis of crosslinking bonds. Hydrogels always exhibit an inhomogeneous crosslinking density distribution and this spatial inhomogeneity increases with crosslinking density (Okay, 2009). The sites with less crosslinking bonds were easier to be hydrolyzed, while in crosslinking rich areas, the highly crosslinked structure could maintain the morphology for a long time. This partial hydrolysis resulted in more accessible space for water retention, so the volume of hydrogel was expanded several times (Figure 4-8 (b)). The highest swelling ratio was obtained by the mixing of 4.5% CMC-NH₂ and 3% CMC-CHO (64% DOX).
Hydrogels’ degradation accompanied with their swelling. The hydrolysis of hydrazone bonds is the main factor for the degradation of hydrogels generated by Schiff-base reaction (Patenaude & Hoare, 2012). In the initial stage, the swelling of water was dominant compared with the rate of hydrolysis, so the mass weight kept increasing until it reached a peak. Then, the weight started to decline, since the crosslinking structure collapsed by hydrolysis and hydrogels could not maintain the same quantity of water. Figure 4-8 (a) shows that the degradation rate can be controlled by tuning DOX and the concentration of CMC-NH$_2$. Compared with 23% oxidized CMC generated hydrogels, hydrogels made by 64% oxidized CMC could form more hydrazone bonds among cellulosic chains. As a result, the latter had a longer time for complete degradation. For example, the complete degradation of hydrogels composed of 4.5% CMC-NH$_2$ and 3% CMC-CHO (64% DOX) almost took about 75 days.

**Nile Red Encapsulation and pH-Triggered Release Study**

Nile Red was used as a hydrophobic fluorescence probe (Jiang & Zhao, 2008) to study the encapsulation of hydrophobic substance and pH-induced release profile. In most polar solvent, such as water, Nile Red will not fluoresce, while in a hydrophobic environment, it can be intensely fluorescent. Compared with control, the emission intensities of suspensions containing copolymer micelles were much higher (Figure 4-9). This is because the micelle structure could provide a hydrophobic inner core to encapsulate Nile Red and enable it to have fluorescent emission. It was also observed that the fluorescence intensity increased dramatically upon enhancing the concentration of micelles PEO-b-PDPA. This is a strong evidence that Nile Red can be efficiently incorporated into micelles.
The fluorescent emission spectra of Nile Red at different pH levels were recorded and shown in the insert of Figure 4-10. It is clear that with the decrease of pH, the emission intensity drops accordingly. The emission intensity was further normalized by plotting \((F-F_{\text{min}})/(F_{\text{max}}-F_{\text{min}})\) versus pH, from which, a sharp transition was found around pH 6.2. With the further drop of pH, the fluorescence became stable and approached to zero which is an indicator of complete dissociation of micelles. The protonation of PDPA block in acidic environment reduced the hydrophobicity of micelle core and resulted in a dramatic decrease of emission intensity. The pH responsiveness of synthesized PEO-b-PDPA copolymer observed in this study agreed well with previous research (N. Jin et al., 2013; H. Yu et al., 2013).

Furthermore, the dynamic release of dye in micelles and micelle embedded hydrogels were characterized. Figure 4-11 (c) shows the spectrum after addition of 1 ml pH 7.4 buffer. There was a slight drop of intensity compared with original 0.75mg/ml micelle suspension due to the dilution of suspension. The emission intensity was kept comparatively stable even after 3 days, which indicated micelles formed by PEO-b-PDPA could work effectively as a barrier to inhibit the release of Nile Red at physiological conditions. However, a dramatic reduction of intensity was observed immediately after adding 1 ml pH 4.0 buffer in Figure 4-11 (b). The decreasing propensity continued until 24 h, after which, the emission intensity became constant, since almost all the Nile Red was released. However, the sharp intensity drop was effectively impeded by introducing hydrogel network (Figure 4-11 (a)). In the hydrogel composite system, the degree of fluorescence gradually declined. Hydrogels worked as a barrier to slow down the penetration of low pH buffer, so micelle structure can
maintain longer at acidic environment. Furthermore, even the Nile Red was liberated from core shell structure, the biocompatible hydrogel could act as a second barrier to inhibit its release to external solution. Thus, if applied in vivo, this hybrid hydrogel-micelle system has a double barrier for hydrophobic substances. Finally, the maximum emission intensity at each time point was plotted as a function of time in Figure 4-11 (d) and the differences of fluorescent profiles could be more easily distinguished.

**Drug Encapsulation and Release Study Using Doxorubicin**

The hydrodynamic radius of micelles and doxorubicin loaded micelles were measured by DLS (Figure 4-12 (a)). It clearly shows the loading of doxorubicin increased the radius by physical entrapment of doxorubicin into hydrophobic core. In addition, a broader size distribution was observed for doxorubicin encapsulated micelles, which may indicate the existence of micelles without doxorubicin. The morphologies of micelles without doxorubicin were shown in Figure 4-12 (b).

*In vitro* drug release study was conducted in the environment mimicking physiological conditions. The cumulative drug released at determined time points was calculated and plotted in Figure 4-13. Micelles and hydrogels worked effectively as barriers to slow down the drug release. In the suspension containing only doxorubicin, a significant burst release occurred in the initial stage. Within the first hour, almost 55% drug was freed from dialysis bag, while for micelle trapped doxorubicin, a much slower release rate was observed. Moreover, the introduction of hydrogel could further decrease the release rate by offering a second barrier. Among all hydrogel composites, the release delay was more significant in 4.5% hydrogel than 1.5% hydrogel. This phenomenon can be ascribed to the higher crosslinking density, which could further impede the diffusion of doxorubicin molecules. The double barrier system consisting of
micelles and hydrogels can eliminate the burst release and prolong the release of doxorubicin.

**Cell Viability Test**

In order to assess the availability of using hydrogel composite containing copolymer micelles, hydrogel precursors and hydrogels for *in vivo* application, cell viability test was performed using a CCK-8 kit from Sigma.

Figure 4-14 shows the cell viability for the hydrogel precursors (CMC-NH₂ and CMC-CHO) and hydrogel on HeLa cells. There was no significant cytotoxicity shown for CMC-NH₂, CMC-CHO (23% OX) and CMC-CHO (64% OX) when the concentration was below 1 mg/ml. Major decrease in cell viability was observed for the CMC-CHO with higher DOX. When its concentration was higher than 1 mg/ml and the cell viability was reduced to 20% at the concentration of 10 mg/ml. This phenomenon can be associated with aldehyde groups on polymers (Ito et al., 2007). Aldehydes are highly active and they can readily react with various nucleophilic entities like amino and thiol groups, which will lead to the irreversible crosslinking of proteins (Deneer, Seinen, & Hermens, 1988; Schauenstein, Esterbauer, & Zollner, 1977). CMC-CHO with 64% DOX contains more active aldehyde groups, so higher cytotoxicity was observed. In contrast, CMC-NH₂ exhibited almost no effect on HeLa cell viability within the tested concentrations. Moreover, the biocompatibility was also examined for the synthesized copolymer micelles (Figure 4-15), which confirmed the noncytotoxic property of micelles as reported previously (N. Jin et al., 2013).

Cell viability of HeLa cells in the presence of hydrogels was also examined by placing certain amount of hydrogels into cultural wells. No significant toxicity was found among all the prepared hydrogels, whereas there was a slight decrease of cell viability
for the hydrogels containing the least CMC-NH₂. This could be attributed to the existence of excessive aldehyde groups not participating Schiff-base reaction with inadequate amines. When the concentration of CMC-NH₂ was increased, the corresponding hydrogels showed almost no influence on the growth of HeLa cells. The obtained data can be used as a guidance to prepare hydrogels with minimum cytotoxicity.

**Summary**

Cellulose-based injectable hydrogel composite containing embedded pH-responsive copolymer micelles was successfully fabricated. It possessed localized delivery ability and pH-triggered slow release feature. The introduction of PEO-b-PDPA micelles provided enhanced loading ability of hydrophobic substance, as well as sensitive pH-stimuli response to surrounding environment. Besides, injectable cellulose-based hydrogels could further improve the biocompatibility by hiding micelles inside and slow down the drug release as a second barrier. The gelation time, mechanical properties, drug release rate and degradation rate could be well tuned by adjusting the concentration of hydrogel precursor and the degree of modification. In addition, the low pH condition contributed to the faster gelation of hydrogels, which is a very useful feature applied in the tissue with acidic environment. Results from cell viability test showed that both gel precursors and obtained hydrogel exhibit minimal cytotoxicity. This constructed novel injectable hydrogel composite provides an effective approach for the localized delivery of hydrophobic drug and has the potential to achieve long-term, environment-triggered release of drugs.
Scheme 4-1. The schematic diagram for the fabrication of drug loaded injectable hydrogel composite.
Figure 4-1. $^1$H NMR spectrum of PEO-Br (a), and PEO-b-PDPA (b).
Figure 4-2. $^1$H NMR spectrum of CMC-CHO.
Figure 4-3. $^1$H NMR spectrum of CMC (a), and CMC-NH$_2$ (b).
Figure 4-4. Effects on gelation time: (a) concentration of CMC-NH₂ and DOX of CMC-CHO; (b) pH condition.
Figure 4-5. SEM images of hydrogels: (a) 1.5% CMC-NH$_2$ + 3% CMC-CHO (23%DOX); (b) 1.5% CMC-NH$_2$ + 3% CMC-CHO (64%DOX); (c) 3% CMC-NH$_2$ + 3% CMC-CHO (64%DOX); (d) 4.5% CMC-NH$_2$ + 3% CMC-CHO (64%DOX).
Figure 4-6. Elastic moduli ($G'$) of various injectable hydrogels at 2% strain using a rheometer with a parallel geometry: (a) the effects of precursor concentration on $G'$; (b) the effects of oxidation degree on $G'$.
Figure 4-7. Storage moduli (G') of hydrogels prepared under different pH values.
Figure 4-8. Swelling ratios and physical appearance of hydrogels: (a) swelling ratios of various hydrogel expressed as average ± standard deviation (N=3); (b) physical appearance of hydrogels by the combination of CMC-NH$_2$ (4.5%) and CMC-CHO (3%, 64% DOX): 1. Initial hydrogels; 2. Hydrogels after immersion for about 60 days.
Figure 4-9. Fluorescence emission spectra of Nile Red in the presence or absence of PEO-b-PDPA at pH 7.4.
Figure 4-10. Normalized fluorescence intensity versus pH (the insert shows the fluorescence spectra of micelles at different pH).
Figure 4-11. Fluorescence emission spectra of Nile Red: (a) adding 1 ml pH 4.0 buffer in hydrogel composite system at 0 h; (b) adding 1 ml pH 4.0 buffer in PEO-b-PDPA micelle suspension at 0 h; (c) adding 1 ml pH 7.4 buffer in PEO-b-PDPA micelle suspension at 0 h; (d) the plot of maximum emission intensity against time.
Figure 4-12. Hydrodynamic radius change after loading of doxorubicin in the copolymer micelles (a); morphology of copolymer micelles under TEM (b).
Figure 4-13. *In vitro* doxorubicin release profiles from doxorubicin suspension, doxorubicin loaded micelles and injectable hydrogel composite.
Figure 4-14. Effects of hydrogel precursors (a) and synthesized hydrogels (b) on cell viability.
Figure 4-15. Cell viability of HeLa cells after adding different concentrations of micelle suspension.
CHAPTER 5
OVERALL CONCLUSIONS AND RECOMMENDATIONS

Summary

There is an increasing demand for cellulose-based products over the past decades due to the concern on resource depletion and environment issues. This PhD dissertation investigated the possibility of utilizing waste pulp residues as raw materials to obtain cellulose and further explore the potential of cellulose in the fabrication of value-added functional materials.

Chapter 2 demonstrates the applicability of using low-value, never-dry waste pulp residues as the feedstock to prepare MFC with high storage modulus via mild maceration and following mechanical treatments. Solvent used in the maceration could selectively degrade lignin and high purity cellulose with intact morphology was gained. By investigating two mechanical treatments: disc refining (DR) and ultrasonification plus homogenization (UH) under various parameters, it was found that 30 min DR could produce the hydrogel with the highest storage modulus. Our results indicate that the combination of maceration method and short-time DR is appropriate to prepare strong cellulosic hydrogels from waste pulp residues.

Chapter 3 describes the synthesis and characterization of a novel biocomposite by incorporating PLA and carboxymethyl cellulose (CMC) through ionic self-assembly. Cationic quaternized PLA-b-PDMAEMA micelles were prepared through dialysis, which were successfully used to solve the incompatible issue between hydrophobic PLA and hydrophilic cellulose. The introduction of soft PDMAEMA in block copolymer was proved to be able to tune the mechanical and thermal properties of final composite films. This tunability was realized by adjusting the content of PDMAEMA in copolymer through
ATRP reaction. This novel cellulose-based biocomposite provides a new route to fabricate cellulosic composites with tunable properties.

Chapter 4 presents a cellulose-based injectable hydrogel system embedded with copolymer micelles for the application of drug delivery. Modified cellulosic hydrogel precursors containing aldehyde and amine functional groups successfully formed injectable hydrogel through efficient Schiff-base reaction. At the same time, pH responsive amphiphilic copolymer micelles in hydrogels tackled the loading issue of hydrophobic drugs and introduced pH triggered release property. Furthermore, micelles and hydrogels worked together as double barriers to prevent burst release of encapsulated drugs. Through cell viability test, it was also demonstrated that the injectable hydrogel composites exhibited excellent biocompatibility. This new cellulose-based hydrogel composite greatly extends the application of low-valued cellulose in the biomedical areas.

**Future Work**

Studies in this research successfully explore the potential application of cellulose, but the CMC used in the tunable composite films and injectable hydrogel composites was directly purchased from the company. There is a gap of knowledge using the MFC obtained in Chapter 2 as starting materials. Thus, appropriate methods can be introduced to modify MFC to CMC and the effects on final products will be further studied. In addition, anti-cancerous efficacy of injectable hydrogel loaded with doxorubicin can be investigated using mice as a model. Also, as proposed in chapter 4 that the amphiphilic copolymer PEO-b-PDPA is supposed to be a ubiquitous carrier for hydrophobic drugs, other hydrophobic drugs will be tested on their efficacy using this novel injectable hydrogel composite. In a word, cellulose is a fabulous natural polymer
and its infinite possibilities open a door for our sustainable future. There are numerous applications of cellulose waiting us to explore.
LIST OF REFERENCES


133


BIOGRAPHICAL SKETCH

Nusheng Chen was born in Zhuji, Zhejiang, China in August, 1987. He enrolled in Northwest A&F University in the fall of 2006. In 2010, he received his B.S. degree in biological engineering. Based on his academic achievement, he was recommended to graduate school of Northwest A&F University majoring in biochemistry and molecular biology in 2010. He joined Dr. Zhaohui Tong’s group at the University of Florida in August, 2011 to pursue his Ph.D. degree, and started his research on cellulose-based materials. In the spring of 2016, he received his Ph.D. in agricultural and biological engineering from the University of Florida.