NANOCELLULOSE EXTRACTION AND SURFACE MODIFICATION
TOWARD ACTIVE PACKAGING APPLICATIONS

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YING WU

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DEDICATION

I would like to dedicate my work to my family for their support and encouragement.
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I would like to express my sincere gratitude to all those who have provided support and encouragement to me during my graduate study.

I would like to express my genuine appreciation to my advisor, Dr. Caixia Wan, for the support, guidance, and mentorship she provided to me throughout my graduate study. Dr. Wan has always been patient with me and guided me through the graduate study. I would not have been able to finish my graduate study without her support.

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ABSTRACT

Cellulose nanocrystals (CNCs) have attracted growing interest as a renewable nanomaterial. The main objective of this study was to explore the extraction of CNCs from switchgrass and surface modification of CNCs into antimicrobially active nanomaterials for active packaging application. Switchgrass was first fractionated via different pretreatment/pulping methods (i.e., acid-chlorite pretreatment, organosolv pretreatment, and deep eutectic solvent (DES) pretreatment), yielding high cellulose-enriched pulp (> 75.53% cellulose). Acid-chlorite pretreatment was the most effective for delignification, removing 97.80% lignin from switchgrass. In contrast, DES pretreatment was more effective for hemicellulose removal (> 79.55% xylan). Fourier transform infrared (FTIR) spectra also showed significant lignin and hemicellulose removal and enrichment of cellulose after pretreatment. Post-treatment with NaOH and H2O2 bleached organosolv and DES pulp, making them more suitable for CNCs extraction via sulfuric acid hydrolysis. The yields of CNCs extracted from the resulting pulps ranged from 30.52 to 35.82% (based on the dry mass of pulp loaded) via sulfuric acid hydrolysis with the highest yield observed with mildly post-treated ChCl: FA pulp. The surface charge of the prepared CNCs ranged from -20.30 to -26.70 mV. And the average particle size ranged from 63.55 to 222.20 nm.

Surface modification by grafting polyethyleneimine (PEI) onto the surface of CNCs with carboxylic groups endowed CNCs with antimicrobial activity, especially toward Gram-positive bacteria Bacillus megaterium. The modified CNCs (CNCs-PEI) showed positive surface charge, indicating successful cationization. FTIR also confirmed the presence of PEI on surface modified CNCs. Incorporation of CNCs-PEI by 5% into PVA film improved its mechanical strength remarkably. This study demonstrated successful
extraction of CNCs from switchgrass and development of antimicrobially active CNCs via surface modification toward active packaging applications. Antimicrobially active CNCs have great potential to be used as a multifunctional nanomaterial for advanced applications.
Chapter 1 Introduction

Growing concerns over the depletion of petroleum sources and global warming are the driving force to find renewable alternatives to petroleum sources. In this context, biopolymer has attracted extensive interests in replacing petroleum source-based counterparts (Kallel et al., 2016). Cellulose, a naturally occurring biopolymer with glucose as the building block, is the most abundant organic polymer on earth. Cellulose consists of long molecular chains with β-1,4-glycosidic linkages and has unique hierarchical structures. Individual molecular chains are assembled into elementary fibrils (also called microfibrils), which are aggregated into microfibrillated cellulose and further cellulose fiber (Figure 2.2) (Lavoine et al., 2012). Cellulose has tightly packed crystalline regions and ill-ordered amorphous regions. In addition, cellulose at nanoscale, also called nanocellulose, upon size reduction, is of increasing interest to industry, especially in the fields relevant to material science, food processing, and biomedical engineering.

Cellulose is a major cell wall component of lignocellulosic biomass. It can also be produced by bacteria. While bacterial cellulose is pure cellulose with delicate nanostructure, lignocellulosic biomass is the primary source for the manufacturing of cellulose fiber and subsequent nanocellulose since it is cheap and abundant. Cellulose-enrich pulp is widely applied in paper industry and cellulosic glucose-based biorefinery. Due to the heterogeneous structure and recalcitrant nature of lignocellulosic biomass, various pulping methods have been developed to fractionate lignocellulosic biomass for cellulose-enriched paper and pulp manufacture. Traditional methods, such as kraft pulping, soda pulping, and acidified sodium chlorite pretreatment, are cost-effective and have been adopted by industry. However, adverse environmental impacts of these traditional methods, especially
the use of hazardous chemicals and extensive post-treatment of liquid waste streams, have driven the development of environmentally benign fractionation methods. Organosolv pretreatment mainly utilizes low boiling point of organic solvents, such as methanol and acetone, to simultaneously degrade, solvate, and solubilize of non-cellulosic components in the lignocellulosic biomass. It is an environmentally benign pulp making method, featured with clean fractionation and solvent recycling (Katahira et al., 2014). In addition to highly enriched organosolv pulp, organosolv pulping also delivers high-quality lignin and hemicellulose-enriched liquid, which can be used in many industrial applications. This method has thus become popular in recent years. Deep eutectic solvents (DESs) are composed of hydrogen bond acceptors (HBAs) and hydrogen bond donors (HBDs) that form a eutectic liquid with a melting point lower than individual components. DESs can be facilely synthesized from natural and renewable components, are designer solvents and an analog to ionic liquids. DESs have not been explored for biomass pretreatment until recent few years. DESs, especially with carboxylic acids as hydrogen bond donors, are effective for fractionating lignocellulosic biomass (Kumar et al., 2016). Ultrafast DES pretreatment assisted by microwave was reported to be as short as 45 s while markedly improving cellulose digestibility (Chen & Wan). As an emerging pretreatment, DES pretreatment is worthy to be further explored not only for cellulose digestion but also for pulp manufacture. From the perspective of pulp preparation for CNCs extraction, different pretreatments endow pulp with varying properties, which would affect the properties of CNCs. However, it lacks of comparison studies concerning the relationship of biomass fractionation process (especially emerging methods) and properties of pulp/CNCs.
Nanocellulose has two primary categories: cellulose nanofiber (CNF) and CNCs. They differ in dimensions and presence of amorphous regions. CNF has a width of 10 - 100 nm and length of 1 - 100 μm and contains both crystalline and amorphous cellulose, while CNCs are crystalline cellulose and have a width of 3 - 50 nm and length of 30 - 1000 nm. The molecular weight of CNCs is lower than 14000 Da (Usov et al., 2015), while the molecular weight of CNF could go as high as 52000 Da. In addition, CNCs have high length-diameter ratio (around 70) and large specific surface area (around 150 m²/g) as well as high mechanical strength (around 7500 MPa) (Fan & Li, 2012). CNCs also act as liquid crystals, showing chiral nematic structures (Habibi et al., 2010; Zhang et al., 2012). Various methods, such as acid hydrolysis, enzymatic hydrolysis, and 2,2,6,6-tetramethylpiperidin-N-oxyl (TEMPO) oxidation, can be used to extract CNCs from pulp. Despite the fact that pristine CNCs possess some unique properties, surface modification can endow new properties to CNCs, which makes CNCs more useful. One example is to modify inert, hydrophilic CNCs into active, hydrophobic ones. Such modified CNCs can be particularly useful for active packaging materials, which may eliminate the addition of antimicrobial agents while improving the processability of CNCs other than their typical function in mechanical reinforcement. In other words, surface-modified CNCs have the potential to serve as both antimicrobial and reinforcement agents.

This study aimed at investigating CNCs extraction and their surface modification toward active packaging application. It involved biomass fractionation, CNCs extraction, surface modification, fabrication of CNCs nanocomposite film, evaluation of antimicrobial activities, as well as material characterization. Switchgrass was used as a lignocellulosic
feedstock for pulp preparation and CNCs extraction. The research tasks were broken into four specific objectives as outlined below:

1) To investigate three biomass fractionation methods, namely, acid-chlorite pretreatment, organosolv pretreatment, and DES pretreatment for fractionating switchgrass into cellulose-enriched pulp. The compositions and surface functional groups of pulps were characterized.

2) To extract CNCs via sulfuric acid hydrolysis from fractionated switchgrass-based pulp and characterize CNCs especially for particle size distribution and dimension.

3) To extract CNCs with carboxylic groups by TEMPO oxidation and to further modify the CNCs by grafting polyethylenimine (PEI) onto their surface. The antimicrobial activities of the resultant CNCs-PEI were evaluated using agar diffusion zone test and bacterial killing kinetics test.

4) To fabricate PVA/ CNCs-PEI nanocomposite films. CNCs-PEI were loaded at varying contents to the films. The resultant films were characterized and the reinforcement effects of CNCs-PEI on the films were evaluated.
Chapter 2 Literature review

2.1. Introduction

Cellulose is the most abundant organic polymer on earth with renewability, biodegradability and non-toxicity. It is estimated that over $1.5 \times 10^{12}$ tons of cellulose are produced by photosynthesis per year (Klemm et al., 2005; Nechyporchuk et al., 2016). Cellulose is mainly produced from lignocellulosic biomass. Other sources include bacteria (e.g., *Gluconacetobacter xylinus* (Sherif, 2005), algae (Imai & Sugiyama, 1998), fungi (Morita et al., 2005), invertebrates, and marine animals (tunicate) (Varshney & Naithani, 2011). Recently, cellulosic biofuels and bioproducts (Isogai, 2009; Sun & Cheng, 2002) have attracted considerable interest due to the depletion of fossil fuels.

Cellulose has multiple structural levels as shown in Figure 2.1. An individual cellulose chain is a homo-polymer of glucose units (Habibi, 2014) linked through $\beta$-1,4-glycosidic bond. The chemical formula of cellulose is $(C_6H_{11}O_5)_n$. It has a reducing end and a non-reducing end (Klemm et al., 2005). The chain length of cellulose can be determined by the number of glucose $(C_6H_{12}O_6)$, expressed as a degree of polymerization. Individual cellulose chains are assembled into larger unit elementary fibrils through van der Waals forces and hydrogen bonding (Juntao, 2016). These unit fibrils further aggregate into microfibrillated cellulose. A bunch of microfibrillated cellulose are compiled into a cellulose fiber, as shown in Figure 2.1 (Lavoine et al., 2012). At the same time, van der Waals forces and hydrogen bonding differ in different area of cellulose resulting in crystalline regions and amorphous regions. In the crystalline regions, cellulose chains are tightly packed, while in amorphous regions, cellulose chains are assembled in disorder. Different origins of cellulose as well as different isolation methods lead to different
structures of cellulose (Nechyporchuk et al., 2016). For example, the crystallinity of cellulose from some plants (e.g., corncob (Liu et al., 2016)) is around 50%, while the crystallinity of cellulose produced from Acetobacter xylinum Y22 can reach 98% (Lu & Jiang, 2014).

Figure 2.1. Schematic diagram of the structural levels of cellulose (Lavoine et al., 2012).

With the rapid development of nanoscience and nanotechnology, much interest is directed to the isolation, characterization, modification, and application of nanocellulose. Nanocellulose has at least one dimension less than 100 nm, mainly including cellulose nanofibers and cellulose nanocrystals. Cellulose with a width longer than 100 nm is usually referred as microfibrillated cellulose. Due to its intrinsic properties (e.g., high aspect ratio and high tensile strength), bio-renewability, non-toxicity, and biodegradability. Nanocellulose has been extensively investigated in multiple applications, especially in the application of food packaging.

2.2. Biomass fractionation for making pulp

Lignocellulosic biomass contains about 30 - 50 wt% cellulose and regarded as the main source of cellulose. Other primary components of lignocellulosic biomass are hemicellulose (19 - 45 wt%) and lignin (15 - 30 wt%) (Metzger & Hüttermann, 2009; Mood
et al., 2013). The common lignocellulosic biomass and their primary cell wall compositions are shown in Table 2.1. Lignocellulosic biomass has a complex hierarchical structure where hemicellulose is covalently linked with cellulose, and lignin is the outermost layer. In addition to its structural function, lignin acts as the protective layer helping cellulose and hemicellulose against microbial attacks (Lee et al., 2014). However, the complex hierarchical structure hinders the full application of lignocellulosic biomass.

Biomass fractionation has been a focus for the utilization of lignocellulosic biomass, and various strategies have been developed (Sannigrahi & Ragauskas). The goal of biomass fractionation is to break down lignocellulosic complex. The schematic diagram of pulp making from lignocellulosic materials is shown in Figure 2.2. This section focuses on pulp making, including acid-chlorite pretreatment, organosolv pretreatment, and designer solvents-based pretreatment.

<table>
<thead>
<tr>
<th>Types of biomass</th>
<th>Lignocellulosic biomass</th>
<th>Cellulose (%)</th>
<th>Hemicellulose (%)</th>
<th>Lignin (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Agricultural waste</td>
<td>Corn stover</td>
<td>39 - 42</td>
<td>22 - 28</td>
<td>18 - 22</td>
</tr>
<tr>
<td></td>
<td>Wheat straw</td>
<td>34 - 40</td>
<td>21 - 26</td>
<td>11 - 23</td>
</tr>
<tr>
<td>Energy crops</td>
<td>Switchgrass</td>
<td>29 - 36</td>
<td>23 - 27</td>
<td>16 - 20</td>
</tr>
<tr>
<td></td>
<td>Miscanthus</td>
<td>38 - 41</td>
<td>18 - 20</td>
<td>21 - 22</td>
</tr>
<tr>
<td></td>
<td>Poplar wood</td>
<td>38 - 48</td>
<td>18 - 38</td>
<td>23 - 27</td>
</tr>
<tr>
<td>Woody biomass</td>
<td>Hardwood</td>
<td>40 - 55</td>
<td>24 - 40</td>
<td>18 - 25</td>
</tr>
<tr>
<td></td>
<td>Softwood</td>
<td>45 - 50</td>
<td>25 - 30</td>
<td>25 - 35</td>
</tr>
<tr>
<td>Industrial waste</td>
<td>Waste paper from chemical pulps</td>
<td>60 - 70</td>
<td>10 - 20</td>
<td>10 - 15</td>
</tr>
</tbody>
</table>

*The information obtained from prior studies (Carrier et al., 2011; Keshwani & Cheng, 2009; Khan & Mubeen, 2012; Lee et al., 2014; Mirmohamadsadeghi et al., 2016).
2.2.1. Acid-chlorite pretreatment

Acid-chlorite pretreatment is one of the most popular methods for cellulose fractionation from lignocellulosic biomass (Hubbell & Ragauskas, 2010). Acid-chlorite pretreatment mainly utilizes acidified sodium chlorite solution and alkaline solution to remove lignin and hemicellulose. The hydronium ions in the acid solution can attack and break down the covalent bond linked between cellulose, hemicellulose, and lignin in the lignocellulosic complex (Lee et al., 2014). It has been proven that dilute acid was efficient to remove hemicellulose in biomass (Cara et al., 2008; Kapoor et al., 2017). Ahlgren found that chlorite was a strong oxidant that could selectively remove over 60% lignin with 2 - 5% of hemicellulose (Ahlgren & Goring, 1971; Kahar, 2013). In contrast, cellulose remained integrate without changing the degree of polymerization after chlorite pretreatment (Hubbell & Ragauskas, 2010), which was favorable for further utilization of cellulose.
To make acid-chlorite pretreatment more effective, organic solvents, usually toluene or ethanol, were used first to remove extractives including wax, pectin, and pigments followed by the acid-chlorite treatment. An additional alkaline treatment was utilized to further remove the non-cellulosic parts (Oun & Rhim, 2016). The compositions of lignocellulosic biomass after acid-chlorite pretreatment are shown in Table 2.2. The cellulose content after acid-chlorite pretreatment is over 70%. However, it is time-consuming and labor-intensive. In addition, the process requires large quantity of solvents (organic solvents and water) and extensive post-processing of liquid waste streams.

Table 2.2. Chemical compositions of lignocellulosic biomass after acid-chlorite pretreatment.

<table>
<thead>
<tr>
<th>Lignocellulosic biomass</th>
<th>Cellulose (%)</th>
<th>Hemicellulose (%)</th>
<th>Lignin (%)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bleached garlic straw pulp</td>
<td>86.00</td>
<td>-</td>
<td>-</td>
<td>(Kallel et al., 2016)</td>
</tr>
<tr>
<td>Bleached sugarcane bagasse pulp</td>
<td>72.25 ± 0.53</td>
<td>16.31 ± 0.42</td>
<td>2.54 ± 0.32</td>
<td>(Zhang et al., 2016)</td>
</tr>
<tr>
<td>Bleached birch pulp</td>
<td>80.20 ± 0.30</td>
<td>19.60 ± 0.60</td>
<td>≤ 0.10</td>
<td>(Li et al., 2015)</td>
</tr>
<tr>
<td>Bleached sugarcane bagasse pulp</td>
<td>83.60 ± 1.60</td>
<td>0.87 ± 0.50</td>
<td>3.25 ± 0.70</td>
<td>(Slavutsky &amp; Bertuzzi, 2014)</td>
</tr>
<tr>
<td>Bleached soy hulls pulp</td>
<td>84.60 ± 4.00</td>
<td>11.20 ± 4.00</td>
<td>3.67 ± 0.33</td>
<td>(Neto et al., 2013)</td>
</tr>
<tr>
<td>Bleached kenaf fiber pulp</td>
<td>91.00 ± 0.20</td>
<td>6.00 ± 0.20</td>
<td>≤ 0.50</td>
<td>(Kargarzadeh et al., 2012)</td>
</tr>
</tbody>
</table>

2.2.2. Organosolv pretreatment

Organosolv pretreatment involves simultaneous degradation, solvation, and solubilization of non-cellulosic components using organic solvents. These organic solvents are usually low boiling point alcohols, such as methanol, acetone, and ethanol (Lee et al., 2014). Typical reactions or processes in the organosolv pretreatment include (1) hydrolysis
of internal lignin bonds and bonds between lignin and hemicellulose (α-ether linkages, β-ether linkages, and 4-O-methylglucuronic acid ester bonds linked to the α-carbons of the lignin units) and (2) hydrolysis of the glycosidic bonds in hemicellulose and less frequently in cellulose (Sannigrahi & Ragauskas; Zhao et al., 2009). Addition of catalysts, including inorganic (HCl or H$_2$SO$_4$) or organic acids (oxalic or salicylic acid), can facilitate the hydrolysis process resulting in the removal of non-cellulosic components under relatively less severe conditions.

Several studies have proven that organosolv pretreatment is an effective method to extract the cellulose. Organosolv pretreatment of wheat straw with 50:50% w/w acetone-water at 205 °C for 1 h resulted in 82% hemicellulose removal, 79% delignification, and 93% cellulose recovery (Huijgen et al., 2010). Cellulose content of 97% with only 1.5% hemicellulose and 2.8% lignin was obtained after pretreating Aspen (*Populus tremuloides*) with 16/34/50 ratio of methyl isobutyl ketone/ethanol/H$_2$O at 140 °C for 56 min (Bozell et al., 2011). Also, the pretreatment process is very fast. It usually could be finished in a couple of hours. More importantly, lignin and hemicellulose as well as organic solvents used for pretreatment can be recovered for further valorization. Thus, this method is extensively investigated for clean fractionation of biomass (Huijgen et al., 2010; Katahira et al., 2014; Zhao et al., 2017b).

### 2.2.3. Designer solvents-based pretreatment

Developing new solvent for sustainable chemistry processes is one of the key subjects in Green Chemistry (Espino et al., 2016). The designer solvents refer to the solvents which properties (e.g., melting point, polarity, viscosity, hydrophobicity, and hydrogen-bonding capability) can be adjusted by changing the ratio or structures of the component ions to suit
the specific reactions (Bhaskar, 2012). Ionic liquids (ILs) and deep eutectic solvents (DESs) are two major types of designer solvents. The designs of ILs and DESs for effective lignocellulosic biomass pretreatment have been extensively investigated recently.

Ionic liquids were found to be a very efficient method for the removal of non-cellulosic components. Over 95% of lignin can be removed after pretreating the bagasse with \([C_2\text{mim}][\text{ABS}]\) (Tan et al., 2009). In addition, the structure of cellulose was preserved without swelling and degradation (Hou et al., 2017). However, the high cost of the ILs and the intensive energy consumption of the process may bring a series of economic, engineering, and environmental challenges into practice (Hou et al., 2017).

DESs, analog to ILs, are mixtures of chemicals containing hydrogen bond acceptors (HBAs) and hydrogen bond donors (HBDs) that form a eutectic liquid with a melting point that is lower than that of their individual components. However, DESs are more economic and environmentally friendly. The major components of DESs are biodegradable and bio-renewable, such as betaine, choline, and amino acids, and organic acids. One of the major types of DESs are mixtures of quaternary ammonium salts as hydrogen bond acceptors (HBAs) and other chemicals as hydrogen bond donors (HBDs) such as carboxylic acids (Abbott et al., 2007). DES pretreatment was found to be effective for lignocellulosic biomass pretreatment. Over 50% of lignin was found to be solubilized in lactic acid/betaine (at molar ratio 2:1) and lactic acid/chlorine chloride (at molar ratio 5:1) (Kumar et al., 2016). Formic acid/chlorine chloride (at molar ratio 2:1) was successfully applied for the corn stover pretreatment for the butanol fermentation, indicating its low toxicity and high pretreatment efficiency (Xu et al., 2016). In addition, the process is fast, and the
fractionated lignin can be recovered for further valorization, favoring the whole process (Kumar et al., 2016; Yang et al.).

2.3. Extraction of nanocellulose from biomass

2.3.1. Nanocellulose

Nanocellulose refers to cellulose with at least one dimension less than 100 nanometers in size (Hindi, 2017). Depending on its particle dimension, nanocellulose can be divided into two main categories: (1) cellulose nanofiber (CNF), which is also named as nanofibrillated cellulose (NFC), or nanocellulose fiber (NCF). It has a diameter of 1 - 100 nm and a length of several micrometers; and (2) Cellulose nanocrystals (CNCs), also known as nanocrystalline cellulose (NCCs), cellulose whiskers, or cellulose nanowhiskers (Hindi, 2017; Nechyporchuk et al., 2016). Individual CNC has a diameter of 3 - 50 nm and a length of 30 - 1000 nm (Moon et al., 2011).

Cellulose nanofiber is typically produced via mechanical disintegration methods including homogenization (Iwamoto et al., 2005; Stelte & Sanadi, 2009), grinding (Iwamoto et al., 2007), refining (Karande et al., 2011), and ball milling (Zhang et al., 2015). Due to intensive energy consumption of mechanical process, chemical treatments, such as 2,2,6,6-tetramethylpiperidinyl-1-oxyl (TEMPO) oxidation and enzymatic hydrolysis are often conducted to reduce the energy requirement.

CNF contains both crystalline regions and amorphous regions. The crystallinity of CNF varies depending on the sources and production methods (Lavoine et al., 2012). Bacterial cellulose (regarded as CNF) usually has higher crystallinity compared to CNF from plants (Cybulaska et al., 2011). Other remarkable properties of CNF involve the high specific surface area (100 - 349 m²/g) (Lavoine et al., 2012; Saito et al., 2011), high aspect
ratio (length to width ratio, up to 100) (Juntao, 2016), low axial thermal expansion coefficient ($10^{-7} \text{ K}^{-1}$) (Nishino et al., 2004), and extraordinary elastic modulus (120 - 167 GPa) (Iwamoto et al., 2009). Such properties make CNF a suitable candidate for a variety of applications including film, hydrogel, and aerogel.

CNCs correspond to defect-free rod-like colloidal nanoparticles (Ramires & Dufresne, 2011). It was first introduced and described as “cellulose micelles” by sulfuric acid hydrolysis (Ranby, 1949), which is still commonly used today. Depending on the type of CNCs that are desired, other chemical treatments have been developed to prepare CNCs from cellulose fiber, as detailed in section 2.3.

Unlike CNF, CNCs only contain crystalline regions and present a rod-like shape (Lee et al., 2014). The crystallinity of CNCs ranges from 54% to 100%, depending on the cellulose sources and extraction methods (Moon et al., 2011). Compared to CNF, CNCs have larger surface area (250 - 500 m$^2$/g) making it more attractive for surface modification (Klemm et al., 2011). In addition, CNCs have high mechanical strength with tensile strength of 7500 MPa and Young’s modulus of 100 - 140 GPa (Juntao, 2016). CNCs have been widely investigated and applied as reinforcement filler in nanocomposite materials (Azeredo et al., 2016; HPS et al., 2016).

The most unique property is their self-assembly ability. When CNCs suspensions reach a critical concentration, CNCs will self-organize into spectacular liquid crystalline arrangements, resulting in a chiral nematic phase with iridescent birefringence pattern, as shown in Figure 2.3. More strikingly, the chiral nematic structure can be preserved after complete evaporation of water, resulting in the iridescent films of CNCs (Habibi et al., 2010; Zhang et al., 2012), which can potentially be used as security papers since the optical
properties cannot be reproduced by printing and photocopying (Habibi et al., 2010). Such unique property can also be of great interest in coating and sensor manufacturing (Chen et al., 2016; Juntao, 2016).

Figure 2.3. Aqueous CNCs suspension (A) 0.63% (w/w, right); (B) 5.40% (w/w, left) observed under polarized-light microscope; (C) Image of iridescence from white light illumination of a solid CNCs film (Araki & Kuga, 2001; Araki et al., 2000).

In addition to the above mentioned physical properties, much attention has been paid to the surface functional groups of CNCs, such as hydroxyl groups and carboxylic groups. Such functional groups can be modified to endow CNCs with new properties. The common methods used for surface modification of CNCs will be reviewed in section 2.4.

2.3.2. Extraction of CNCs

CNCs are extracted from cellulose by cleaving cellulose chain and removing amorphous regions while leaving crystalline regions behind as shown in Figure 2.4.
2.3.2.1. Acid hydrolysis

CNCs were first introduced and described by Randy using sulfuric acid hydrolysis (Ranby, 1949). Until today, acid hydrolysis is still the most common method used for processing of CNCs (Dong et al., 2016; Kallel et al., 2016). Typically, cellulose fiber/pulp was subjected to the concentrated acid for hydrolysis first followed by adding cold DI water to quench the reaction. Afterwards, the suspension underwent a series of washing steps (centrifuging and dialysis). Ultrasound was applied to facilitate the dispersion of CNCs in the suspension. CNCs in the form of powder can be obtained after freeze drying.

The proposed mechanism for CNCs extraction via acid hydrolysis is that the protons of acid directly attack glycosidic bonds in cellulose chain, resulting in the scission of C-O bonds and the separation of conjugated acid into cyclic carbonium ions. After adding a water, the proton is liberated, leading to the cleavage of the cellulose chain (Figure 2.5) (Moon et al., 2011). Overall, the acid preferentially hydrolyzes the amorphous regions in cellulose chain because of their relatively low density, and leaves individual crystallites (known as CNCs).
Acids, such as sulfuric acid (Wang et al., 2014), hydrochloric acid (Yu et al., 2013), phosphoric acid (Camarero Espinosa et al., 2013), and organic acids (e.g. citric acid, malic acid, and malonic acid (Spinella et al., 2016)) have been used for CNCs extraction. Among all the acids, sulfuric acid is the most extensively investigated for the CNCs preparation (Jonoobi et al., 2015). During the hydrolysis, sulfuric acid reacts with the hydroxyl groups on the surface of CNCs via esterification, resulting in the formation of anionic sulfate ester groups (-OSO$_3^-$) (Mariano et al., 2014). These negative charged sulfate ester groups on the surface of CNCs improve the electrostatic repulsion between individual nanoparticles, leading the high stability of CNCs suspension. However, the thermal stability of sulfuric acid prepared CNCs were relatively low compared with the ones hydrolyzed with hydrochloric acid and phosphoric acid (Camarero Espinosa et al., 2013; Roman & Winter, 2004). One-pot hydrolysis/Fischer Esterification was also used for preparing CNCs with carboxylic groups using mixed acids including hydrochloride acid, citric acid, malic acid, and malonic acid (Spinella et al., 2016). In addition to different types of acids, reaction
conditions (acid concentration, hydrolysis time, and acid fiber ratio) as well as cellulose source will also cause different characteristics of CNCs. The most obvious differences are the particle size and surface charge. Longer hydrolysis time, more concentrated acid or higher acid/fiber ratio usually lead to shorter CNCs and higher surface charge (Azizi Samir et al., 2005; Dong et al., 1998). However, if the reaction time is too long, the acid would completely hydrolyze cellulose fiber into glucose. On the contrary, if the reaction time is too short, it may lead to incomplete removal of amorphous regions of cellulose chain (Beck-Candanedo et al., 2005; Siqueira et al., 2010). The impurities present on the surface of CNCs may also have an effect on their surface charge (Eyley & Thielemans, 2014).

2.3.2.2. Enzymatic hydrolysis

Cellulase have been used for saccharification of cellulose to produce sugar for the following fermentation (Mirmohamadsadeghi et al., 2016). Enzymatic hydrolysis was found to be another method used to prepare CNCs (Cui et al., 2016), which utilizes cellulase to remove amorphous regions in cellulose chain.

Cellulases can be divided into three classes: (1) endoglucanases, also named endocellulases, which can selectively hydrolyze internal 1,4-\(\beta\)-glycosidic linkages at amorphous sites; (2) cellobiohydrolases, also called exoglucanases, which act on the end of the cellulose chain; and (3) \(\beta\)-glucosidases, also termed cellobiases, which hydrolyze the exoglucanases products into glucose, as shown in Figure 2.6 (Lynd et al., 2002; Nechyporchuk et al., 2016; Zhou & Ingram, 2000). Endoglucanase alone was found to work more effectively for CNCs production than a mixture of the hydrolysis enzymes (An et al., 2016; Teixeira et al., 2015). Higher yield and less depolymerization of CNCs was obtained with endoglucanase hydrolysis (Nechyporchuk et al., 2015).
In spite of the greenness, CNCs obtained from enzymatic hydrolysis do not have surface charge (Teixeira et al., 2015), resulting in unstable suspensions. In addition, the yield of CNCs is low, less than 20% (Cui et al., 2016). Combined enzymatic hydrolysis with other pretreatments, such as disk milling (Teixeira et al., 2015) and ultrasonication (Cui et al., 2016), was found to be more effective for CNCs production than enzymatic hydrolysis alone.

2.3.2.3. 2,2,6,6-tetramethylpiperidin-N-oxyl (TEMPO) oxidation

TEMPO oxidation is considered as an efficient method for selectively conversion of hydroxyl groups to aldehydes, ketones, and carboxyl groups under mild conditions (Fukuzumi, 2012). Specifically for cellulose, the primary hydroxyl groups on the surface of cellulose chain can be selectively oxidized to carboxyl groups by TEMPO oxidation (Nechyporchuk et al., 2016). Researchers found that the depolymerization of cellulose occurred during the oxidation reaction led to the formation of CNCs. In the oxidation systems, cellulose materials were suspended in the solution containing TEMPO and

Figure 2.6. Schematic diagram of cellulases acting on cellulose (Lynd et al., 2002).
sodium bromide at pH 10 - 11. Sodium hypochlorite (NaClO) solution, which acted as an oxidant, was added to the suspension to start the oxidation. After the reaction, the oxidation was stopped by adding ethanol followed by a series of separation and washing steps. Additional mechanical treatment (ultrasonication or homogenization) is normally applied to get the final CNCs with high density of carboxyl groups on their surface (Rohaizu & Wanrosli, 2017; Zhang et al., 2016).

The oxidation of cellulose fiber is mainly due to the nitrosonium ion (’N=O) generated through the reaction of TEMPO radical with the oxidant (NaClO), which can selectively oxidize the primary alcohol groups on the surface of cellulose into aldehydes, and further into carboxyl groups as shown in Figure 2.7 (Fukuzumi, 2012; Nechyporchuk et al., 2016). The more NaClO added to the system, the more carboxylic groups will be formed (Lavoine et al., 2012).

Contrary to other CNCs preparation methods, TEMPO oxidation can be conducted in room temperature. However, additional treatments, such as mechanical treatment (Rohaizu & Wanrosli, 2017) or acid hydrolysis (Fraschini et al., 2017; Li et al., 2015; Lin et al., 2012a; Yu et al., 2016) are usually required to obtain the final CNCs.
2.3.2.4. Ammonium persulfate (APS) oxidation

Different from the above three methods, APS oxidation can be used for directly extracting CNCs from lignocellulosic biomass (Leung et al., 2011). The prepared CNCs have high crystallinity and high density of carboxylic groups on their surface.

Two major mechanisms were proposed to explain the production of CNCs with carboxylic groups from lignocellulosic biomass by APS oxidation: (1) Both SO$_4^{2-}$ free radical ions ($S_2O_8^{2-} + \text{heat} \rightarrow 2\text{SO}_4^{2-}$) generated by the cleavage of peroxide bond when APS solution was subjected to heat treatment, and hydrogen peroxide formed ($S_2O_8^{2-} + 2\text{H}_{2}\text{O} \rightarrow 2\text{HSO}_4^{+} + \text{H}_2\text{O}_2$) under acidic conditions, were able to penetrate into the amorphous regions to hydrolyze the 1,4-β bond of cellulose chain as well as opening the aromatic rings of lignin (Leung et al., 2011), resulting in simultaneously removing non-cellulosic components and amorphous regions of cellulose to form CNCs (Lam et al., 2012); and (2) mono peroxysulfuric acid (Caro’s acid, H$_2$SO$_5$), an effective delignification agent, was also formed under acidic condition (> 0.5 M) (Mascheroni et al., 2016) to remove the
non-cellulosic components (Springer & Minor, 1991). Free radicals and hydrogen peroxide generated during the oxidized process were responsible for the cellulose depolymerization and oxidation to form carboxylated CNCs.

The direct CNCs extraction from raw biomass by APS oxidation avoids the tedious pretreatment and washing steps for removing the non-cellulosic contents (mainly lignin and hemicellulose) (Leung et al., 2011). Moreover, almost no hazardous chemicals were found after the oxidation reaction. However, the yield of CNCs prepared from APS oxidation is low (Leung et al., 2011). Long reaction time is required, indicating its low effectiveness (Mascheroni et al., 2016; Zhang et al., 2016). Different CNCs extraction methods are compared and summarized in Table 2.3. The produced CNCs with distinct surface chemical properties are summarized in Figure 2.8.

Table 2.3. Comparison of different CNCs extraction methods.

<table>
<thead>
<tr>
<th>Preparation methods</th>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acid hydrolysis</td>
<td>High suspension stability of CNCs, short reaction time, energy-efficient</td>
<td>Low thermal stability, highly oxidative and corrosive environment, high equipment cost, high work safety demand, high operation cost</td>
</tr>
<tr>
<td>Enzymatic hydrolysis</td>
<td>Green process, no pollution</td>
<td>Low suspension stability, Low CNCs yield, long reaction time, expensive</td>
</tr>
<tr>
<td>TEMPO oxidation</td>
<td>CNCs with carboxylic groups, energy-efficient</td>
<td>Not efficient enough to break down the amorphous domain to produce CNCs, long oxidation time, toxic reagents</td>
</tr>
<tr>
<td>APS oxidation</td>
<td>One step procedure for producing CNCs from lignocellulosic biomass, CNCs with carboxylic groups</td>
<td>Long reaction time, low efficiency</td>
</tr>
</tbody>
</table>

21
2.3.2.5. Other methods

ILs have been exploited for producing CNCs. 1-Butyl-3-methylimidazolium hydrogen sulfate ([Bmim]HSO₄ is the most studied IL for the fabrication of CNCs (Man et al., 2011; Mao et al., 2015; Tan et al., 2015; Zhao et al., 2017a). According to a study by Tan (Tan et al., 2015), the dissociated [Bmim]⁺ cation and [HSO₄]⁻ anion could diffuse into cellulose chain and formed the electron donor and acceptor complex with oxygen and hydrogen atoms, resulting in the breakage of cellulose hydrogen bonding network and the separation of cellulose chains. Furthermore, carbon atoms and oxygen atoms of β-1,4-glycosidic bonds can also be attached by the dissociated ions, resulting in the cleavage of cellulose chains. The amorphous regions of cellulose are more accessible to [Bmim]⁺ and [HSO₄]⁻, leading to the selective hydrolysis and removal of the amorphous regions, resulting in the crystalline regions, known as CNCs. High yields of CNCs were obtained from different biomass (e.g., 57.7 ± 3.0% for bleached softwood kraft pulp, 57.0 ± 2.0% for bleached hardwood kraft pulp, and 75.6 ± 3.0% for microcrystalline cellulose) with favorable properties (e.g., high aspect ratios (35 - 45), low sulfur content (0.02 - 0.2%)) by optimizing the production of CNCs using [Bmim]HSO₄ (Mao et al., 2015). Deep eutectic solvents (DESs), as introduced previously, exhibit similar properties with ILs and can be used as alternatives. CNCs, with width of 9 - 17 nm, length of 310 - 410 nm, and crystallinity indexes of 66.0 - 71.0%, were successfully obtained from chlorine chloride/oxalic acid dihydrate-treated wood fiber (Sirviö et al., 2016). Two energy-intensive mechanical pretreatments (i.e., homogenization and microfluidization) were applied after chlorine chloride/oxalic acid dihydrate pretreatment. The combination of DES pretreatment and
high-energy mechanical pretreatments could be the reason for CNCs extraction in this study.

Subcritical water pretreatment is another green method to prepare CNCs with high crystallinity index (79.0%) (Novo et al., 2015). It is mainly due to the oxidation ability of $\text{H}_3\text{O}^+$. Under sub- or supercritical condition, higher concentrations of ionized species ($\text{H}_2\text{O}^+$) can be obtained, contributing to the effectiveness of the hydrolysis of cellulose.

Liquefaction was also reported to be an efficient method for preparing CNCs from different types of biomass. CNCs with high yield (55.6% - 75.1%) was successfully extracted from different cellulose materials (i.e., cotton linters, spruce wood, eucalyptus wood, and Chinese silver grass) by one-step liquefaction reaction with glycerol using 3% methane sulphonic acid as a catalyst (Kunaver et al., 2016). During the one-step reaction, lignin, hemicellulose, and the amorphous regions of cellulose were hydrolyzed or oxidized, dissolving in the reaction mixture, leaving the solid crystalline cellulose, known as CNCs.

Figure 2.8. Typical procedures for producing CNCs with distinct surface chemical properties.
2.4. Surface modification of CNCs

Different preparation methods of CNCs lead to different surface properties. The active sites on the CNCs surface (hydroxyl groups, carboxylic groups, etc.) provide a unique platform for modification to target on specific purposes, which facilitate more advanced applications of CNCs.

2.4.1. Non-covalent surface modification of CNCs

Non-covalent surface modifications of CNCs are usually achieved through the electrostatic attraction, hydrogen bonding, or van der Walls forces between the modifier and CNCs. Typical modifiers include surfactants and oppositely charged polymers. The Beycostat NA (BNA) surfactant, which is a phosphoric ester of polyoxyethylene (9) nonylphenyl ether, was used to modify CNCs (Heux et al., 2000). The resultant CNCs dispersed very well and showed good compatibility in non-polar solvents. Further work showed that the improved dispersibility and compatibility of CNCs were mainly achieved by coating CNCs surface with a BNA layer of 15 Angstroms (Bonini et al., 2002). Other types of surfactants, such as non-ionic surfactants (Bondeson & Oksman, 2007; Kim et al., 2009; Rojas et al., 2009) are used to modify CNCs to improve the dispersion properties in the hydrophobic polymer matrix. By mimicking the natural lignocellulosic copolymer, xyloglucan oligosaccharide-poly(ethylene glycol)-polystyrene triblock copolymer was used to modified the surface of CNCs by simply mixing and stirring at room temperature for 4 h (Zhou et al., 2009). The resultant CNCs showed good dispersion stability in toluene. By adsorption of cationic quaternary ammonium salts, the anionic oxidized CNCs showed a significant increase in hydrophobicity and was able to form good dispersion in non-polar systems (Salajková et al., 2012).
With distinct surface properties, CNCs can be utilized as a promising template for synthesizing various types of antibacterial agents by electrostatic attraction with the reactants or the catalysts for the reaction. The synthesis of silver nanoparticles (AgNPs) are extensively investigated by utilizing the negatively charged CNCs. CNCs are usually used as the reducing agent, structure-directing agent, or stabilizer in the synthesis process (Lin et al., 2012b). The synthesized AgNPs using CNCs exhibited higher antibacterial activity for both Gram-negative and Gram-positive bacteria than the commercial AgNPs (Wang et al., 2016). Another antibacterial agent, polyrhodanine were successfully coated on the surface of negative charged CNCs by polymerization of monomer rhodamine using Fe$^{3+}$ as the initiator and oxidant (Tang et al., 2015).

2.4.2. Covalent surface modification of CNCs

Covalent surface modification of CNCs mainly focus on hydroxyl groups on the surface of CNCs. There are three main methods used to modify CNCs covalently. The common covalent modifications of CNCs were shown in Figure 2.9.

1) By different types of chemical reactions (e.g., oxidation, esterification, etherification, silylation, urethanization and amidation), the surface hydroxyl groups of CNCs are substituted with other functional groups.

2) By electrostatic attachment of pre-synthesized polymer chains, which carry reactive end groups, on the surface of CNCs, the polymer is synthesized and grafted onto CNCs, known as “grafted onto” strategy (Lin et al., 2012b).

3) By using surface hydroxyl groups as initiating sites, the polymer can be synthesized in situ on the surface of CNCs directly by ring opening polymerization (ROP) (Goffin et al., 2011; Labet & Thielemans, 2011), atom
transfer radical polymerization (ATRP) (Majoinen et al., 2011; Morandi et al., 2009), single-electron transfer living radical polymerization (SET-LP) (Zoppe et al., 2010), or other radical polymerization techniques, known as “grafted from” strategy.

Several antibacterial agents were found to be able to be reacted with the active hydroxyl groups on the surface of CNCs, resulting in the formation of antibacterial CNCs. By chloroacetylation and subsequently reacting with tertiary amines (Bespalova et al., 2017) or ring opening reaction (Liu et al., 2017b), a series of quaternary ammonium with different alkyl groups were successfully grafted onto the surface of CNCs, gaining the antibacterial activity. Rosin, a kind of essential oil, was grafted onto the surface of CNCs using a sustainable and green process (de Castro et al., 2016). Bespalova proposed that the covalently modified CNCs with antibacterial agents would gain antibacterial activity as well as improving their dispersion stability in the polymer system, which further expands their application (Bespalova et al., 2017).
Figure 2.9. Common covalent modifications of cellulose nanocrystals (CNCs) (Juntao, 2016; Lin et al., 2012b) [PEG: poly(ethylene glycol); PEO: poly(ethylene oxide); PLA: poly(lactic acid); PAA: poly(acrylic acid); PNiPAAm: poly(N-isopropylacrylamide); and PDMAEMA: poly(N,N-dimethylaminoethyl methacrylate)].

2.5. Application of nanocellulose in food packaging

Active packaging refers to packaging systems having active functions in addition to the inert containment and protection of the products. Active packaging has been extensively investigated and utilized in food packaging to reduce food deterioration.
Nanocellulose with its unique intrinsic properties (e.g., high specific surface area and high tensile strength), non-toxicity, and renewability gains much interest in the application of food packaging. Surface modification can endow new properties with nanocellulose, such as antibacterial activity. Such surface-modified nanocellulose have the potential to serve as both antibacterial activity and reinforcement agents applied in active food packaging.

2.5.1. Antibacterial food packaging

The Centers for Disease Control and Prevention (CDC) indicates that foodborne pathogens lead to 48 million people getting sick each year in the United States, of which 128000 people are hospitalized and 3000 die finally (Hosseinnejad & Jafari, 2016; Sundaram et al., 2016a). Additionally, the foodborne pathogens can ruin the food, causing the increase of food waste. Safe packaging, especially antimicrobial food packaging has been proved to be one of the most important and effective ways to ensure food safety from food manufactures to customers.

There are mainly two types of antibacterial food packaging (Imran et al., 2010): leaching antibacterial food packaging and non-leaching antibacterial food packaging. Table 2.4 compares the key features of these two packaging systems. For the former one, antibacterial agents (AAs) are usually physically incorporated in packaging systems. Bioactive agents such as silver nanoparticles (Yan et al., 2016) and essential oil (Chen & Liu, 2016), can slowly release toward the environment and food products to keep the quality of food. These kinds of bioactive agents are usually small with low molecular weight so that they can diffuse into the targeted microorganisms (Wei et al., 2016) to kill them. Non-leaching antibacterial food packaging is also called active contact food packaging. Antibacterial agents are usually chemically grafted in the major packaging
polymer. Bioactive agents are usually antibacterial cationic polymers, such as antibacterial peptides (Goddard & Hotchkiss, 2007; Steven, 2004) and quaternary ammonium salts (Anthierens et al., 2012; Bespalova et al., 2017; Liu et al., 2017b). They are large with high molecular weight and interact with negatively charged microbial membranes through electrostatic interaction, resulting in the damage of the membrane and the leakage of cytoplasm (Wei et al., 2016).

The direct incorporation of bioactive agents is still the most popular method to prepare the antibacterial food packaging. However, many disadvantages are coupled with this kind of food packaging. They may cause the environmental pollution and influence organoleptic food properties after releasing out of the packaging system. In addition, the antibacterial activity of the systems is only temporary since the release rate of the bioactive agents will decrease with the time and eventually exhaust. What’s worse, the decreasing biocide concentration will provide the condition for developing bacteria resistance, which may cause more environmental and health problems. Chemically binding the antibacterial agents in the food packaging can potentially solve those problems as the biocide could not migrate out of the packaging system while maintaining the antibacterial activity (Nigmatullin et al., 2009). Due to the above advantages, considerable attention has been paid on the development of contact active food packaging systems. By quaternizing cellulose, the final products showed notable antibacterial activity against Gram-positive bacteria Staphylococcus aureus and bacteriostatic activity against Gram-negative bacteria Escherichia coli (Hu & Wang, 2016; Saini et al., 2017). The detailed comparison between leaching and non-leaching antibacterial food packaging is shown in Table 2.4.
Table 2.4. Comparison between leaching and non-leaching antibacterial food packaging.

<table>
<thead>
<tr>
<th>Type of antibacterial food packaging</th>
<th>Antibacterial agents’ incorporation methods</th>
<th>Properties of AAs</th>
<th>Mechanism of antibacterial activity</th>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leaching</td>
<td>Physically incorporation</td>
<td>Small molecular weight</td>
<td>Penetrating the microorganisms to act on the targeted sites, like DNA or respiratory enzymes</td>
<td>Strong antibacterial activity in the initial stage</td>
<td>Short-term antibacterial protection; inducing bacteria resistance; causing the environmental pollution and influencing organoleptic food properties;</td>
</tr>
<tr>
<td>Non-leaching</td>
<td>Chemically grafting</td>
<td>Large molecular weight</td>
<td>Interacting with the negative charged microbial membranes through electrostatic interaction, resulting in the damage of the membrane and the leakage of the cytoplasm</td>
<td>Long-term antibacterial activity</td>
<td>The relatively complex synthesis and production process</td>
</tr>
</tbody>
</table>

2.5.2. Application of CNCs in food packaging

CNCs have been extensively studied as the reinforcement agent in the packaging materials due to their biodegradability and non-toxicity (Kovacs et al., 2010), as shown in Table 2.5. The pristine CNCs (with hydroxyl groups or carboxylic groups on the surface), with their high hydrophilicity, are usually directly incorporated into the hydrophilic systems such as wheat gluten (El-Wakil et al., 2015), natural rubber (Neto et al., 2016), and polyvinyl alcohol (PVA) (George et al., 2011) to improve their mechanical properties. With the direct addition of 4 wt% CNCs into the PVA film, the tensile strength has increased from 62.5 MPa to 128 MPa (George et al., 2011). However, in the hydrophobic systems, CNCs are commonly surface modified to avoid agglomeration and improve the biocompatibility (Fortunati et al., 2012b; Tian et al., 2017), resulting in good dispersion.
As shown in Table 2.5, additional antibacterial agents (e.g., essential oil, AgNPs, and nisin) are usually added to food packaging to make it antimicrobially active. Nanocellulose can be modified to have antimicrobial activity. Antimicrobially active nanocellulose can not only act as a reinforcement agent to improve mechanical properties of food packaging, but also an antibacterial agent. It was found that with the addition of 5% CNCs-DMOEPAC (CNCs modified with N, N, N-dimethyl-N-octadecyl-N-(1,2-epoxypropyl) ammonium chloride), the tensile strength of chitosan film was increased along with increased antibacterial activity (Liu et al., 2017b).
Table 2.5: Application of nanocellulose in food packaging.

<table>
<thead>
<tr>
<th>Materials</th>
<th>Antibacterial agents</th>
<th>Antibacterial assay method</th>
<th>Antibacterial activity</th>
<th>Water vapor permeability g mm/m2 day KPa</th>
<th>Young modulus (MPa)</th>
<th>Tensile strength (MPa)</th>
<th>Thermal degradation temperature (°C)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chitosan/CNF</td>
<td>Chitosan</td>
<td>Agar diffusion</td>
<td>No bacteria growth on the contact area</td>
<td>4.6</td>
<td>65</td>
<td>115.88</td>
<td>(Dehnad et al., 2014)</td>
<td></td>
</tr>
<tr>
<td>Paper/CNF/AgNP</td>
<td>AgNP</td>
<td>ASTM E2 149* (Bacteria reduction**)</td>
<td>Staphylococcus aureus 100% Escherichia coli 100%</td>
<td>12.91</td>
<td>1424.03</td>
<td>55.53</td>
<td>132.5</td>
<td>(Amini et al., 2016)</td>
</tr>
<tr>
<td>Starch/CNF/chitosan</td>
<td>Chitosan</td>
<td>Agar diffusion (Inhibition zone)</td>
<td>Bacillus subtilis 3.2 cm E. coli 3.1 cm</td>
<td>2.3 (8 days)</td>
<td>63.59</td>
<td>4.3</td>
<td>(Salehudin et al., 2014)</td>
<td></td>
</tr>
<tr>
<td>Starch/CNF/chitosan</td>
<td>Chitosan</td>
<td>Food model (Log increase**)</td>
<td></td>
<td>3.8</td>
<td>150</td>
<td>13</td>
<td>(Yu et al., 2017)</td>
<td></td>
</tr>
<tr>
<td>Chitosan/CNF</td>
<td>CNF</td>
<td>Agar diffusion (Inhibition zone)</td>
<td>B. subtilis 38 ± 1 mm E. coli 33 ± 1 mm S. aureus 50 mm Enterococcus faecalis 35 mm</td>
<td>3.8</td>
<td>100</td>
<td>100</td>
<td>(Sundaram et al., 2016b)</td>
<td></td>
</tr>
<tr>
<td>PLACNF/essential oil/propolis</td>
<td>Essential oil and propolis</td>
<td>JIS Z 2801* (Bacterial viability**)</td>
<td></td>
<td>3.8</td>
<td>100</td>
<td>100</td>
<td>(Sundaram et al., 2016b)</td>
<td></td>
</tr>
<tr>
<td>Chitosan/CNF/SNP</td>
<td>CNF</td>
<td>Agar diffusion (Inhibition zone)</td>
<td>S. aureus 47% E. coli 50%</td>
<td>3000 ± 200</td>
<td>48.0 ± 3</td>
<td>149.7 ± 0.7</td>
<td>(Fortunati et al., 2012a)</td>
<td></td>
</tr>
<tr>
<td>PLA/CNCs/AgNP</td>
<td>CNF</td>
<td>JIS Z 2801* (Bacterial viability**)</td>
<td></td>
<td>2.23</td>
<td>2971</td>
<td>99</td>
<td>280</td>
<td>(Khan et al., 2012)</td>
</tr>
<tr>
<td>Gelatin/CNCs</td>
<td>Essential oil</td>
<td>Food model test (Bacterial viability**)</td>
<td>Listeria monocytogenes 12.5%</td>
<td>1.6</td>
<td>2350.4 ± 65</td>
<td>44.8</td>
<td>348.7 ± 2.4</td>
<td>(George, 2012)</td>
</tr>
<tr>
<td>PLA/CNCs/essential oil</td>
<td>Essential oil</td>
<td>Food model test (Bacterial viability**)</td>
<td></td>
<td>2.33</td>
<td>693</td>
<td>99</td>
<td>348.7 ± 2.4</td>
<td>(Salmieri et al., 2014b)</td>
</tr>
<tr>
<td>PLA/CNCs/Nisin</td>
<td>Nisin</td>
<td>Agar diffusion (Inhibition zone)</td>
<td></td>
<td>1000 IU/ml for 18 mm</td>
<td>1574.0 ± 206.7</td>
<td>35.9 ± 2.7</td>
<td>(Salmieri et al., 2014a)</td>
<td></td>
</tr>
<tr>
<td>Wheat gluten/CNCs/TiO2NP</td>
<td>TiO2NP</td>
<td>JIS Z 2801* (Bacterial reduction**)</td>
<td></td>
<td>0.011</td>
<td>34.7</td>
<td>2.16</td>
<td>18</td>
<td>(El-Wakil et al., 2015)</td>
</tr>
<tr>
<td>PHBV/CNCs/AgNP</td>
<td>CNCs-AgNP</td>
<td>Agar diffusion (Inhibition zone)</td>
<td></td>
<td>0.12</td>
<td>170</td>
<td>30.5</td>
<td>264.7 ± 2.6</td>
<td>(Yu et al., 2014)</td>
</tr>
<tr>
<td>PVA/CNCs/AgNP</td>
<td>AgNP</td>
<td>JIS Z 2801* (Bacterial viability**)</td>
<td></td>
<td>3700</td>
<td>108</td>
<td>52.06</td>
<td>(Fortunati et al., 2013)</td>
<td></td>
</tr>
<tr>
<td>Chitosan/CNCs</td>
<td>Chitosan</td>
<td>JIS Z 2801* (Log reduction**)</td>
<td>S. aureus 6.01 E. coli 6.34</td>
<td>3700</td>
<td>108</td>
<td>52.06</td>
<td>(de Mesquit et al., 2012)</td>
<td></td>
</tr>
<tr>
<td>Chitosan/Quaternized CNCs</td>
<td>Chitosan/Quaternized CNCs</td>
<td>JIS Z 2801* (Log reduction**)</td>
<td>S. aureus 6.01 E. coli 6.34</td>
<td>3700</td>
<td>108</td>
<td>52.06</td>
<td>(Liu et al., 2017b)</td>
<td></td>
</tr>
</tbody>
</table>

* ASTM E2 149: The packaging materials are co-cultured with bacteria while shaking;
** JIS Z 2801: A drop of bacteria suspension is placed on the film and allowed to incubate for a certain time;
** Bacterial reduction = (viable count in control tube – viable count in experimental tube)/viable count in control tube × 100%;
** Log increase (log₁₀ CFU/g) = log₁₀ (the number of viable cells during storage CFU/g) – log₁₀ (the initial number of viable cells CFU/g);
** Bacterial viability = viable count in experimental tube/viable count in control tube × 100%.
Chapter 3 Materials and Methods

3.1. Feedstock and chemicals

Switchgrass was harvested from South Farm at University of Missouri in Columbia, Missouri, USA. Switchgrass was air-dried and ground through 2 mm screen using Wiley Laboratory Mill, Model 4. All the chemicals were of analytical grade and purchased from Fisher Scientific.

3.2. Pulp preparation via different fractionation methods

3.2.1. Acid-chlorite pretreatment

Acid-chlorite pretreatment was used to fractionate switchgrass for pulp preparation. The schematic diagram is shown in Figure 3.1. First, extractives (e.g., wax, pectin, and pigments) were removed from raw switchgrass following the method described by Oun and Rhim (Oun & Rhim, 2016). Specially, 10 g dried switchgrass was mixed with 150 ml toluene/ethanol (1:2 v/v) solvent at room temperature for 20 h, and then washed with ethanol several times until the filtrate became colorless to remove extractives. Secondly, the resultant extractive-free switchgrass was mixed with 330 ml 1.4% (w/v) sodium chlorite solution (pH was adjusted to 4 by 0.5 M HCl) and then pretreated at 70 ºC for 5 h for lignin removal. The solids were recovered via vacuum filtration and then washed with deionized (DI) water several times until the filtrate became neutral. Subsequently, the solids (around 7.78 g) were further treated using 200 ml 5% potassium hydroxide solution at room temperature for 24 h followed by 90 ºC for 2 h. The highly delignified solids were finally recovered by vacuum filtration, washed with DI water until the pH reached neutral.
The pulp obtained (BSW) was dried at 105 ºC for 4 h and stored in an air tight container at room temperature prior to use.

![Diagram of pulp pretreatment process]

Figure 3.1. Acid-chlorite pretreatment for pulp preparation from switchgrass.

### 3.2.2. Organosolv pretreatment

Organosolv pulp was prepared following the method reported in literature with slight modification (Katahira et al., 2014). The major steps are outlined in Figure 3.2. Briefly, 25 g switchgrass (dry base) was mixed with 250 ml mixed solvent comprised of methyl isobutyl ketone (MIBK), ethanol, and water (1:4:4, w/w/w) in a Parr reactor (Parr Instrument Company, Moline, Illinois, USA). Sulfuric acid of 0.025 M (based on the total solvent) was used as the catalyst. The reactor was sealed and heated to 160 ºC and then held for 70 min. After the reaction, the reactor was cooled down to room temperature and the reaction mixture was filtered for solid recovery. The recovered solids were then washed
with the same solvent (500 ml) followed by washing with DI water until the filtrate became colorless and neutral. The solids were dried at 105 ºC for 4 h and stored at an air tight container for further use. For post-treatment, the dried solids were mixed with 0.8 g NaOH/g fiber and 1 g H₂O₂/g fiber with a solid loading of 1:20 (w/v) at 50 ºC for 90 min followed by washing with DI water until the pH reached neutral (Li et al., 2016). The resultant pulp (BOSW) was oven-dried and stored in an air tight container at room temperature for further use.

Figure 3.2. Organosolv pretreatment for pulp preparation from switchgrass.

3.2.3. Deep eutectic solvent (DES) pretreatment

3.2.3.1. DES preparation

Two types of deep eutectic solvents, namely choline chloride: formic acid (ChCl: FA) and choline chloride: lactic acid (ChCl: LA) were synthesized by mixing chlorine chloride and formic acid (or lactic acid) with the molar ratio of 1:2 at 60 ºC and 300 rpm until a
clear, homogenous liquid was formed. The synthesized ChCl: FA and ChCl: LA were stored in a desiccator at room temperature for further use. The DESs synthesis process is shown in Figure 3.3.

![Chemical Structures](image)

**Figure 3.3. Process for ChCl: FA and Ch: LA synthesis.**

### 3.2.3.2. DES pretreatment

The procedure of DES pretreatment is outlined in Figure 3.4. Briefly, 10 g switchgrass was pretreated with 90 g ChCl: FA for 15 min at 130 °C and 200 rpm and with 90 g ChCl: LA for 30 min at 130 °C and 200 rpm, respectively. After the reaction, the pretreated mixture was cooled down to room temperature, and then filtered for solid recovery. The recovered solids were then washed with the solvent mixture of acetone and water (1:1 v/v) by vacuum filtration several times using 1.6-μm glass fiber filter paper until the filtrate
became transparent. Subsequently, the solid was post-treated with an alkaline peroxide solution containing 0.8 g NaOH/g fiber and 1 g H₂O₂/g fiber (severe alkaline peroxide (AHP) treatment) and, 0.08 g NaOH/g fiber and 0.1 g H₂O₂/g fiber (mild AHP treatment) with a solid loading of 1:20 (w/v) at 50 °C for 90 min. After post-treatment, the solids were washed with DI water until the pH reached 7 (Li et al., 2016). The resultant pulps were denoted as BFA-S, BFA-M, BLA-S, and BLA-M, respectively. The pulps were oven-dried and stored in an air tight container at room temperature for further use.

Figure 3.4. DES pretreatment for pulp preparation from switchgrass.

3.3. CNCs extraction from switchgrass-based pulps via sulfuric acid hydrolysis

CNCs were extracted from the pulps prepared above via sulfuric acid hydrolysis (Figure 3.5). The pulps were first blended using a laboratory scale blender (Green Mix, model DA700-G) to get the fine power for CNCs extraction. The pulp power (1.5 g) was added to 10 ml preheated 64 wt% sulfuric acid. The hydrolysis was conducted at 45 °C for 30 min (excluding heating-up time) with continuous stirring at 300 rpm. Thereafter, the
reaction was stopped by adding 10 folds cold DI water followed by dialysis with DI water until the pH did not change. The suspension was then sonicated at 200 W for 15 min in an ice water bath using a bench-top ultrasonic processor (Branson Sonifier 450). After sonication, the suspension was filtered through 1.6-μm glass filter paper to remove the large particles. The filtrate was collected and centrifuged at 2603 ×g for 10 min. The CNCs-containing supernatant was then collected. The CNC yield was calculated based on the total dry mass of pulp used for CNC extraction.

Figure 3.5. CNCs extraction from the pulp via acid hydrolysis.

3.4. TEMPO oxidation of pulp and microcrystalline cellulose for preparing CNCs with carboxylic groups (CNCs-COOH)

The TEMPO oxidation of the pulps and microcrystalline cellulose was conducted to prepare CNCs with carboxylic groups following the method modified from prior study (Li et al., 2015). Briefly, 1 g cellulose was suspended in 100 mL DI water with 0.1 mmol TEMPO and 1 mmol NaBr dissolved inside. The TEMPO oxidation was initiated by the dropwise addition of NaClO solution (10 mmol). The reaction was carried out at room temperature with stirring at 300 rpm and pH 10 (adjusted by dropwise addition of 0.4 M
NaOH) until all NaClO (10 mmol) was consumed and no NaOH consumption was observed. Subsequently, 10 ml ethanol was added to stop the reaction. The reaction mixture was centrifuged at 19632 ×g for 10 min. The solids were collected and washed with DI water twice via centrifugation (19632 ×g for 10 min) to remove the remaining chemicals. The solid was re-suspended and then dialyzed against DI water until the pH did not change. The suspension was collected and further sonicated followed by centrifuging and filtering, which were the same as described for CNCs preparation via acid hydrolysis (Section 3.4).

3.5. Surface modification of CNCs-COOH with polyethyleneimine (PEI)

CNCs extracted via TEMPO oxidation were modified by grafting PEI onto their carboxylic groups following the method reported in prior study (Liu et al., 2017a). In brief, 0.3% CNCs was mixed with PEI (4 g), N-hydroxysuccinimide (NHS) (0.24 g), and 1-ethyl-(3-dimethylaminopropyl aminopropyl) carbodiimide hydrochloride (EDC) (0.45 g). The pH of the mixture was adjusted to 7.0 using 2 M HCl solution. The reaction was conducted at room temperature for 24 h with stirring at 300 rpm. Thereafter, the resultant mixture was centrifuged at 19632 ×g for 15 min. The solids were collected and washed with DI water for 2-3 times via centrifugation (19632 ×g for 15 min). The final products, denoted as CNCs-PEI, was re-suspended in DI water and then stored at 4 ºC for further use.

3.6. Synthesis of PVA/CNCs-PEI film

The PVA/CNCs-PEI nanocomposite film was synthesized following the method described below. In brief, 1.5 polyvinyl alcohol was fully dissolved in 30 mL DI water by heating the mixture at 121 ºC for 15 min. After cooling the mixture to 90 ºC, CNC-PEI was added at varying contents (1 - 9 wt% on a basis of dry weight of PVA) with continuous
stirring at 300 rpm. After full dispersion, DI water was added to bring the total volume of the mixture to 50 mL. The PVA/CNCs-PEI nanocomposite film was prepared by casting 15 ml mixture into a Petri dish (9 cm in diameter) followed by drying at 45 °C for 12 h. The film without the addition of CNCs-PEI was also made as a control. All the dried films were stored at 30 °C with humid air before further characterization. The detailed process for synthesizing PVA/CNCs-PEI nanocomposite film is shown in Figure 3.6.

Figure 3.6. Solution casting for preparing the PVA/CNCs-PEI films.

3.7. Antimicrobial assay of CNC-PEI and PVA/CNCs-PEI film

Gram-negative bacteria *Escherichia coli* and Gram-positive bacteria *Bacillus megaterium* were kindly provided by the Food Microbiology Laboratory in University of Missouri, Columbia. The strains were maintained on Luria-Bertain (LB) agar plates (1% tryptone, 0.5% yeast extract, 1% NaCl, and 1.5% agar) at 4 °C. The inoculum for antibacterial assay was prepared by growing a colony in 5 mL LB medium at 37 °C for *E. coli* and 30 °C for *B. megaterium* until the cell densities reached approximately 10^9 CFU/mL at 12 h of cultivation. DI water and CNCs-COOH (without surface modification) were used as controls.
The antibacterial activity CNCs-PEI was evaluated using agar diffusion zone test and bacterial killing kinetics test. For agar diffusion zone test, 0.1 mL inoculum was evenly seeded onto the entire surface of LB agar plates. CNCs-PEI suspension was added with a varying amount (12.5, 25, 50, 75, 100 µg CNCs-PEI) to the pre-inoculated plates. The CNCs-PEI suspension was added to the agar plates in two ways: (1) fixed volume (5 µL with different concentrations (i.e., 2.5 mg/mL and 5 mg/mL) with 5 µL DI water and 5 µL unmodified CNCs (CNCs-COOH, 5 mg/mL) as the controls; and (2) fixed concentration (5 mg/mL) with different volume (i.e., 5 µL, 10 µL, 15 µL, and 20 µL). After 24 h cultivation, the formation of inhibition zone was determined by visual inspection. For bacterial survival rate test, 0.1 ml inoculum was added to 5 mL LB broth containing CNCs-PEI at varying concentrations (i.e., 0 mg/mL, 0.01 mg/mL, 0.1 mg/mL, 0.5 mg/mL, 1 mg/mL, 2 mg/mL, and 5 mg/mL). After 6 hours of liquid cultivation at 500 rpm for both strains at proper temperatures, the numbers of viable bacteria were determined by colony forming units (CFU). Bacterial survival (%) was calculated following the equation: Bacterial survival (%) = CFU in sample broth/CFU in LB broth ×100%.

3.8. Analytical methods and characterization techniques

3.8.1. Compositional analysis of switchgrass and pulps

The cellulose, hemicellulose and lignin contents of raw switchgrass and pulps were determined using two-stage acid hydrolysis according to NREL Laboratory Analytical Procedure (Sluiter et al., 2008). Briefly, the biomass sample was first hydrolyzed by 72% sulfuric acid at 30 ºC for 1 h followed by dilute acid (4% sulfuric acid) hydrolysis at 121 ºC for 1 h. After acid hydrolysis, the solid was collected for the determination of acid-insoluble lignin by a gravimetric method. The filtrate was analyzed for the determination
of acid-soluble lignin and monomeric sugars. Acid-soluble lignin was measured at 320 nm by UV-Vis spectrophotometer. The concentrations of monomeric sugars were measured by high performance liquid chromatograp (HPLC) (Agilent 1100 Series) equipped with reflective index detector (RID). Bio-Rad Aminex HPX-87P (300 × 7.8 mm) column was used for the monomeric sugar analysis and its temperature was kept at 80 °C. The mobile phase was HPLC grade water, eluting at a flow rate of 0.6 ml/min. The cellulose and hemicellulose were calculated from monomeric sugars using a conversion factor of 0.9 and 0.88, respectively. The solid recovery was calculated as a percentage of solid recovered after pretreatment. The removal of individual cell wall components was reported as a percentage of the corresponding component removed upon pretreatment.

3.8.2. Average particle size and zeta potentials of CNCs

The average particle size and zeta potential /surface charge of the CNCs samples were determined by Laser Doppler Velocimetry (LDV) using a Zetasizer Nano ZS (Malvern Instrument, Malvern, UK). Freshly prepared CNCs samples (1.2 ml, 0.05 - 0.5 wt% CNC) were used for the measurement. The specific parameters for measurement were as follows: dispersant water, material refractive index of 1.47, dispersion refractive index of 1.33, viscosity of 0.8872 CP, temperature of 25 ºC. General calculation model for irregular particles was also used by the instruments (Zhou et al., 2012). The average particles and zeta potential were reported as the range of three measurements.

3.8.3. Atomic force microscopy (AFM)

AFM imaging was performed on a Multimodal AFM (MFP-3D BIO™, Asylum Research, Santa Barbara, CA) in tapping model (Espino-Pérez et al., 2014). The imaging
was conducted following the method described by Jiang and Hsieh (Jiang & Hsieh, 2013). The sample for the imaging was prepared as follows: CNCs suspension was sonicated at 200 W for 5 min and then adjusted to a CNCs concentration of 0.01 wt%; One drop (about 10 µL) of the above CNCs suspension was deposited on a mica substrate and oven-dried at 50 °C for 2 h. The dried mica substrate was scanned by AFM for the imaging.

3.8.4. Fourier transform infrared (FTIR) spectroscopy

The surface functional groups of raw switchgrass, pulps, and CNC samples were determined using FTIR. FTIR spectra were recorded using a Nicolet 4700 FT-IR spectrophotometer (Thermo Electron Corporation, Maltham, MA) with an attenuated total reflection (ATR) probe. All samples were scanned from 4000 to 400 cm\(^{-1}\) in a resolution of 4 cm\(^{-1}\).

3.8.5. Characterization of mechanical properties of PVA/CNCs-PEI film

The mechanical properties of the synthesized PVA film and PVA/ CNCs-PEI nanocomposite films were characterized using a TA-XT2i texture analyzer (Texture Technologies Corp., Scarsdale, NY, USA). Texture analyzer (TA) probe (TA version N° 07.144) was first calibrated with a return distance of 25 mm and a contact force of 30 N. Then the pre-cut film (20 mm × 70 mm) was fixed by a clamp grip at initial distance of 50 mm and pulled apart with the speed of the upper grip at 5 mm/min. A force (N) versus distance (mm) curves were recorded by the software. Young’s modules (N/m\(^2\) or Pa) was determined by measuring the slope of the axial stress-strain curve in the elastic region. The thickness of the film samples was measured by a caliper (sensitivity of 0.01 mm) prior to
the stress-strain tests. The stress and strain (%) were calculated following Equation (1) and Equation (2), respectively.

\[
\text{Stress (Pa)} = \frac{F}{A} \quad (1)
\]

\[
\text{Strain (\%)} = \frac{\Delta H}{H_0} \times 100 \quad (2)
\]

where \(F\) is the force (N) applied to the film sample, \(A\) is the cross-sectional area (thickness \(\times\) width, m\(^2\)), \(\Delta H\) is the length changed, and \(H_0\) is the initial length of the film samples between grips.

3.8.6. Measurement of color of PVA/CNCs-PEI nanocomposite films

The color of the PVA/CNCs-PEI nanocomposite films was measured by a Chroma Meter CR-410 (Konica Minolta Sensing, Inc., Japan) equipped with a pulsed xenon lamp. Three parameters (\(L^*, a^*,\) and \(b^*\)) of color were reported by \(L^*\) value with a range of 0 to 100 indicates the color from black to white, \(a^*\) value with a range of -100 to 100 indicates color from green (-) to red (+), and \(b^*\) value with a range of -128 to 127 indicates color from blue (-) to yellow (+). The colorimeter was calibrated with the standard white plate before the PVA/CNC-PEI film test. The total color (\(\Delta E\)) of the film samples was calculated following Equation (3) (Yu et al., 2017):

\[
\Delta E = [(L^* - L_0^*)^2 + (a^* - a_0^*)^2 + (b^* - b_0^*)^2]^{1/2} \quad (3)
\]

where \(L_0^*, a_0^*,\) and \(b_0^*\) are the color parameters of the white plate and \(L^*, a^*,\) and \(b^*\) are the color parameters of the film samples.
3.9. Statistical analysis

Analysis of Variance (ANOVA) and Waller-Duncan K-ratio t tests were performed using SAS GLM procedure. The significant difference among treatments was determined at a level of significance ($\alpha = 0.05$). The data with different letters (e.g., a, b and c) were significantly different ($p < 0.05$) while the data with the same letter were not significantly different ($p > 0.05$).
Chapter 4 Results and Discussion

4.1. Pulp fractionated from switchgrass

4.1.1. Compositional change

Pulp was prepared from switchgrass via three different pretreatment methods (i.e., sodium chlorite, organosolv, and DES pretreatment). Acidified sodium chlorite, as a traditional bleaching agent, was highly effective for fractionating biomass, particularly removing lignin. As a result, 97.79% lignin and 59.58% xylan were removed while cellulose loss upon this pretreatment was minor (6.62%) (Figure 4.1). Such effective delignification generated bleached pretreated solid (also called pulp) that showed a bright white color (Figure 4.2). Only 1.12% lignin was left in pulp, but cellulose was highly enriched, reaching 77.53% of dry pulp (Table 4.1). The pretreatment also reduced xylan content in switchgrass down to 11.47% due to partial removal of hemicellulose.

Organosolv pretreatment is another method used to solubilize lignin and hemicellulose from biomass. It uses organic solvents, which can be easily recovered via distillation. It is an environmentally benign alternative to other popular pulping methods (e.g., kraft pulping and sulfite pulping). Acid is often added to catalyze organosolv fractionation. In this study, organosolv pretreatment was conducted using a mixture of MIBK, ethanol, and water (1:4:4, w/w/w) with the addition of 0.025 M H₂SO₄ as the catalyst. Additional bleaching using an agent of 0.8 g/g NaOH and 1 g/g H₂O₂ effectively whitened organosolv pulp that was originally grayish (Figure 4.2.). Organosolv pretreatment with additional bleaching is highly effective for delignification and xylan removal. Lignin and xylan removal both reached 90%, but cellulose was slightly affected (Figure 4.1). Subsequently, cellulose was enriched to 86% along with small amounts of xylan and lignin in the pretreated solid (Table
4.1). Compared to the above acid-chlorite pretreatment, organosolv with additional bleaching post-treatment removed 10% more xylan but 5% less lignin.

DES pretreatment is an emerging pretreatment method based on designer solvents. Both DESs (i.e., ChCl: FA and ChCl: LA) were able to effectively solubilize xylan and lignin. ChCl: FA pretreatment caused more hemicellulose removal while ChCl: LA removed more lignin. About 80% lignin and 82% xylan were removed by ChCl: FA while 79% lignin and 80% xylan were removed by ChCl: LA. The resultant pulp from two DESs had similar cellulose content (73%) and lignin content (8.2%) while ChCl: FA pulp contained a lower xylan content (5.95%) than ChCl: LA pulp. Additional bleaching, regardless of bleaching agent loading, was not only beneficial for further delignification but also whitening DES pulp. High loading of bleaching agent (i.e., 0.8 g/g NaOH and 1 g/g H$_2$O$_2$) solubilized slightly more lignin and xylan than low loading of bleaching agent (0.08 g/g NaOH and 0.1 g/g H$_2$O$_2$). Bleaching for post-treatment led to cellulose-rich DES pulp (about 84%). Xylan and lignin contents were reduced markedly. ChCl: FA pulp contained more lignin and less xylan than ChCl: LA pulp since these two DESs differed slightly in fractionation capability.

Pulp obtained via the above three pretreatment methods had adequately high cellulose content for further nanocellulose manufacture. All effectively removed lignin, but varied in the degree of delignification. Acid-chlorite was particularly effective for delignification, directly generating bleached pulp. The results were in agreement with the pulps obtained from garlic straw and kenaf fiber that the lignin content after acid-chlorite pretreatment was lower than 2% (Kallel et al., 2016; Kargarzadeh et al., 2012). Organosolv pretreatment was known as an environmentally friendly method to fractionation cellulose from
lignocellulosic biomass. The lignin and xylan content in the organosolv treated switchgrass-based pulp were higher than organosolv treated Aspen-based pulp (Bozell et al., 2011). The reason might be due to different feedstocks which differ in the recalcitrance. DES pretreatment, as an emerging pretreatment, was reported to remove lignin by the cleavage of ether bond in lignin, which leads to the dissociation of lignin from lignocellulose complex (Alvarez-Vasco et al., 2016). The DES pulp obtained in our study contained less lignin and xylan content than that from corn stover (Xu et al., 2016). This was largely due to the short reaction time and the washing solvent (a mixture of acetone and water) used in our study, which may prevent the lignin from re-deposition (Procentese et al., 2015).

Post-treatment via bleaching led to comparable bleached pulp for both organosolv and DES pretreatment (Li et al., 2016). However, DES pretreatment had many advantages over acid-chlorite. It was faster and less tedious, and required less energy input. This method also imposed less stress to environment due to limited waste generation. DES pretreatment may also have a comparable economy to organosolv pretreatment provided that most of DESs are recyclable.
Figure 4.1. Degradation of switchgrass upon pretreatment.

Table 4.1. Chemical compositions of switchgrass and the fractionated pulps.

<table>
<thead>
<tr>
<th>Samples</th>
<th>Glucan (%)</th>
<th>Xylan (%)</th>
<th>Lignin (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SW</td>
<td>33.56°e</td>
<td>18.5°a</td>
<td>20.47 ± 0.5°a</td>
</tr>
<tr>
<td>BSW</td>
<td>77.53 ± 0.5°c</td>
<td>11.47 ± 0.05°b</td>
<td>1.12 ± 0.25°e</td>
</tr>
<tr>
<td>BOSW</td>
<td>86.83 ± 2.0°a</td>
<td>4.18 ± 0.32°a</td>
<td>4.13 ± 0.01°c</td>
</tr>
<tr>
<td>LA</td>
<td>73.48 ± 0.2°d</td>
<td>8.63 ± 0.51°d</td>
<td>8.29 ± 0.16°b</td>
</tr>
<tr>
<td>BLA-M</td>
<td>82.13 ± 0.4°b</td>
<td>9.52 ± 0.12°c</td>
<td>4.72 ± 0.16°b</td>
</tr>
<tr>
<td>BLA-S</td>
<td>83.21 ± 0.2°b</td>
<td>8.14 ± 0.10°d</td>
<td>3.53 ± 0.23°f</td>
</tr>
<tr>
<td>FA</td>
<td>73.18 ± 0.1°d</td>
<td>6.34 ± 0.27°c</td>
<td>8.45 ± 0.44°b</td>
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<tr>
<td>BFA-M</td>
<td>82.15 ± 1.0°b</td>
<td>5.94 ± 0.11°c</td>
<td>6.50 ± 0.48°c</td>
</tr>
<tr>
<td>BFA-S</td>
<td>84.53 ± 0.9°b</td>
<td>5.03 ± 0.04°d</td>
<td>5.33 ± 0.08°d</td>
</tr>
</tbody>
</table>

§ Values with different letters within a column were significantly different (p < 0.05).

§ SW: switchgrass; BSW: pulp resulting from acid-chlorite pretreatment; BOSW: pulp resulting from organosolv pretreatment followed by severe alkaline peroxide post-treatment; LA and FA: pulp resulting from ChCl: LA and ChCl: FA pretreatment, respectively; BLA-M and BFA-M: pulp resulting from ChCl: LA and ChCl: FA pretreatment, respectively, followed by mild alkaline peroxide post-treatment; BLA-S and BFA-S: pulp resulting from ChCl: LA and ChCl: FA pretreatment, respectively, followed by severe alkaline peroxide post-treatment.
Figure 4.1.2. Pulp obtained from switchgrass via different pretreatment methods.

4.1.2. Surface functional groups of pulp

The functional groups of switchgrass before and after different pretreatment was characterized by FTIR spectroscopy. As shown in Figure 4.3, the peak at 1510 cm\(^{-1}\) corresponding to the aromatic skeletal vibrations from lignin (Mirmohamadsadeghi et al., 2016), were almost disappeared in all the pulp samples, indicating significant delignification upon pretreatment. The peak at 1250 cm\(^{-1}\) attributing to C-O stretching in hemicellulose and lignin (Zhang & Wu, 2015), also disappeared in pulp, further confirming lignin and xylan solublization after pretreatment. The peaks at 898 cm\(^{-1}\) and 1105 cm\(^{-1}\) were attributed to C-O-C vibration at β-(1-4)-glycosidic linkages between the glucose units and the peak at 1158 cm\(^{-1}\) corresponds to C-O-C stretching at β-(1-4)-
glycosidic bonds (Mirmohamadsadeghi et al., 2016). These peaks had increased intensity for the pretreated solids, suggesting enriched cellulose after pretreatment (Kumar et al., 2014). The intensity of these peaks also increased compared with ChCl: FA pulp (FA) and bleached ChCl: FA pulp (BFA-S), indicating further enriched cellulose after additional bleaching. The FTIR results agreed with the compositional analysis results as presented in Table 4.1.

Figure 4.3. FTIR spectra of switchgrass and pulp.

4.2. CNCs extraction from pulps via acid hydrolysis

CNCs were extracted from different pulps via acid hydrolysis. The yields of CNCs across the pulps ranged from 30 - 36% (Figure 4.5). As shown in Figure 4.5, the highest yield was observed with ChCl: FA pulp which was mildly post-treated. Figure 4.4 showed both slurry and freeze dried CNCs samples. Acid-chlorite pulp and post-treated organosolv pulp yielded white CNCs. Post-treated DES pulp at high severity (0.8 g/g NaOH and 1 g/g
H₂O₂) led to the formation of white CNCs via acid hydrolysis. However, less severely post-treated (0.08 g/g NaOH and 0.1 g/g H₂O₂) DES pulp yielded brownish CNCs, largely due to relatively high lignin content. All the original pulp except acid-chlorite pulp gave dark slurry after acid hydrolysis (pictures not shown), indicating that post-pretreatment via bleaching is necessary to obtain white CNCs. Further study is needed to characterize the potential CNCs products from non-bleaching pulps. Across all the pulps, lignin appeared to be a determining factor for the color of CNCs. The more lignin was contained in the substrates, the less whitish CNCs were obtained.

Figure 4.4. CNCs samples: slurry (top) and lyophilized power (bottom).
Figure 4.5. Yield of CNCs after acid hydrolysis of different pulps.

The average particle size and zeta potential of CNCs are shown in Table 4.2. Acid-chlorite pulp yielded CNCs with the smallest average particle size (about 64 nm) while CNCs obtained from mildly post-treated ChCl: LA pulp had the largest average particle size (218 - 222 nm) (Table 4.2). In addition, CNCs from acid-chlorite pulp had relatively uniform size distribution compared with other pulps (Figure 4.6). The difference in particle size was largely due to the varying crystallinity of pulps (Beck-Candanedo et al., 2005; de Souza Lima & Borsali, 2002). Specifically, the pulp with low crystallinity will lead to short nanocrystals (Fengel & Wegener, 1983).

The zeta potential of CNCs were negative due to the formation of anionic sulfate ester groups (-OSO$_3^-$) on the surface of CNCs samples upon acid hydrolysis (Lin et al., 2012b). The surface charge of CNCs from BOSW was lower than those from other pulps. Different fractionation methods endowed the pulps and subsequent CNCs with different surface
functional groups, which would lead to varying surface charges of CNCs (Eley & Thielemans, 2014).

Table 4.2. Average particle size and zeta potential of CNCs.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Average Particle size (nm)</th>
<th>Zeta potential (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BSW</td>
<td>63.55 - 64.11</td>
<td>-23 - -24.1</td>
</tr>
<tr>
<td>BOSW</td>
<td>118.8 - 119.5</td>
<td>-26.1 - -26.7</td>
</tr>
<tr>
<td>BLA-S</td>
<td>183.4 - 187.6</td>
<td>-22.5 - -23.7</td>
</tr>
<tr>
<td>BLA-M</td>
<td>217.5 - 222.2</td>
<td>-21.1 - -21.9</td>
</tr>
<tr>
<td>BFA-S</td>
<td>181 - 185.7</td>
<td>-21.1 - -22.3</td>
</tr>
<tr>
<td>BFA-M</td>
<td>179.2 - 180.7</td>
<td>-20.3 - -21.4</td>
</tr>
</tbody>
</table>

Figure 4.6. Size distribution of CNCs obtained from different pulps.
The dimensions of CNCs were determined by AFM. BLA-S was selected as the representative sample for AFM imaging. As shown in Figure 4.7, all the particles were rod-shaped or needle-shaped. The length (< 200 nm) and width (< 40 nm) of typical particles are shown in Figure 4.7. CNCs aggregates are shown in the AFM image. Since CNCs were re-dispersed via ultrasound prior to coating, aggregation could largely result from the coating step which involved water evaporation.

Figure 4.7. AFM image of CNCs from BLA-S.
CNCs prepared from raw switchgrass in our study are compared with CNCs prepared from other raw biomass (Table 4.2). Our study showed comparable CNCs yields and CNCs length. The difference in yield and CNCs length might be due to the varying crystallinity of pulp after fractionation and the different acid hydrolysis conditions. The pulps fractionated from different feedstocks (i.e., switchgrass, corn stover and Miscanthus) via the same DES pretreatment were reported to yield pulps with varying crystallinities (Chen & Wan). Different reaction conditions (acid concentration, hydrolysis time and acid fiber ratio) will also cause different characteristics of CNCs. Specifically, longer hydrolysis time, more concentrated acid or higher acid/fiber ratio usually lead to shorter CNCs (Azizi Samir, Alloin, & Dufresne, 2005; X. M. Dong, Revol, & Gray, 1998). However, if the reaction time is too long or the acid/fiber ratio is too high, the acid would completely digest the cellulose fiber into its component sugar molecules. On the contrary, short reaction time or low acid/fiber ratio may lead to large unhydrolyzed cellulose chain (Beck-Candanedo et al., 2005; Siqueira et al., 2010).

As shown from the Table 4.2, acid-chlorite pretreatment was highly popular for pulp preparation (Fengel & Wegener, 1983). Our study demonstrated that DES pretreatment with alkaline peroxide post-treatment was also effective for pulp preparation and CNCs extraction.
Table 4.3. CNCs preparation from raw biomass.

<table>
<thead>
<tr>
<th>Lignocellulosic biomass</th>
<th>Pulp making methods</th>
<th>Post-treatment</th>
<th>CNCs preparation methods</th>
<th>Conditions</th>
<th>Solid loading (%)</th>
<th>CNCs yield* (%)</th>
<th>CNCs length (nm)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Corn cob residue</td>
<td>Acid-chlorite</td>
<td>Acid hydrolysis</td>
<td>64 wt% H$_2$SO$_4$, 45 °C for 1h</td>
<td>5</td>
<td>34.5</td>
<td>198 ± 51</td>
<td>(Liu et al., 2016)</td>
<td></td>
</tr>
<tr>
<td>Garlic straw</td>
<td>Acid-chlorite</td>
<td>Acid hydrolysis</td>
<td>64 wt% H$_2$SO$_4$, 45 °C for 45 min</td>
<td>5</td>
<td>19.6</td>
<td>400 - 700</td>
<td>(Kallel et al., 2016)</td>
<td></td>
</tr>
<tr>
<td><em>Posidonia oceanica</em></td>
<td>Acid-chlorite</td>
<td>Acid hydrolysis</td>
<td>6.5 mol/L H$_2$SO$_4$, 55 °C for 40 min</td>
<td>5</td>
<td>27.9</td>
<td>276 - 676.2</td>
<td>(Bettaieb et al., 2015)</td>
<td></td>
</tr>
<tr>
<td>Posidonia oceanica</td>
<td>Acid-chlorite</td>
<td>Acid hydrolysis</td>
<td>65 wt% H$_2$SO$_4$, 45 °C for 90 min</td>
<td>5</td>
<td>30</td>
<td>148.7 ± 43</td>
<td>(Kargarzadeh et al., 2012)</td>
<td></td>
</tr>
<tr>
<td>Kenaf fibers</td>
<td>Acid-chlorite</td>
<td>Acid hydrolysis</td>
<td>65 wt% H$_2$SO$_4$, 45 °C for 90 min</td>
<td>5</td>
<td>27.9</td>
<td>122.7 ± 39.4</td>
<td>(Neto et al., 2013)</td>
<td></td>
</tr>
<tr>
<td>Soy hull</td>
<td>Acid chlorite</td>
<td>Acid hydrolysis</td>
<td>65 wt% H$_2$SO$_4$, 45 °C for 30 min</td>
<td>10</td>
<td>17.2</td>
<td>200 - 400</td>
<td>(Dong et al., 2016)</td>
<td></td>
</tr>
<tr>
<td>Soft wood</td>
<td>Acid chlorite</td>
<td>Acid hydrolysis</td>
<td>65 wt% H$_2$SO$_4$, 45 °C for 30 min</td>
<td>15</td>
<td>33.78</td>
<td>63.55 - 64.11</td>
<td>This study</td>
<td></td>
</tr>
<tr>
<td>Wheat straw</td>
<td>Acid chlorite</td>
<td>Enzymatic hydrolysis</td>
<td>2g fiber/mL cellulase; 50 °C for 120 h</td>
<td>3</td>
<td>22.57</td>
<td>200 - 400</td>
<td>This study</td>
<td></td>
</tr>
<tr>
<td>Switchgrass</td>
<td>Acid chlorite</td>
<td>Acid hydrolysis</td>
<td>64 wt% H$_2$SO$_4$, 45 °C for 30 min</td>
<td>15</td>
<td>33.78</td>
<td>63.55 - 64.11</td>
<td>This study</td>
<td></td>
</tr>
<tr>
<td>Switchgrass</td>
<td>Organosolvent</td>
<td>S-AP* Acid hydrolysis</td>
<td>64 wt% H$_2$SO$_4$, 45 °C for 30 min</td>
<td>15</td>
<td>32.87</td>
<td>118.8 - 119.5</td>
<td>This study</td>
<td></td>
</tr>
<tr>
<td>Switchgrass</td>
<td>ChCl: LA</td>
<td>S-AP Acid hydrolysis</td>
<td>64 wt% H$_2$SO$_4$, 45 °C for 30 min</td>
<td>15</td>
<td>30.96</td>
<td>183.4 - 187.6</td>
<td>This study</td>
<td></td>
</tr>
<tr>
<td>Switchgrass</td>
<td>ChCl: LA</td>
<td>M-AP* Acid hydrolysis</td>
<td>64 wt% H$_2$SO$_4$, 45 °C for 30 min</td>
<td>15</td>
<td>32.31</td>
<td>217.5 - 222.2</td>
<td>This study</td>
<td></td>
</tr>
<tr>
<td>Switchgrass</td>
<td>ChCl: FA</td>
<td>S-AP Acid hydrolysis</td>
<td>64 wt% H$_2$SO$_4$, 45 °C for 30 min</td>
<td>15</td>
<td>30.52</td>
<td>181 - 185.7</td>
<td>This study</td>
<td></td>
</tr>
<tr>
<td>Switchgrass</td>
<td>ChCl: FA</td>
<td>M-AP Acid hydrolysis</td>
<td>64 wt% H$_2$SO$_4$, 45 °C for 30 min</td>
<td>15</td>
<td>35.27</td>
<td>179.2 - 180.7</td>
<td>This study</td>
<td></td>
</tr>
</tbody>
</table>

* Yield (%) = Dry weight of CNCs obtained/Dry weight of pulp loaded × 100%;
* S-AP: Severe alkaline peroxide post-treatment (0.8 g/g NaOH and 1 g/g H$_2$O$_2$);
* M-AP: Mild alkaline peroxide post-treatment (0.08 g/g NaOH and 0.1 g/g H$_2$O$_2$).
4.3. Cationic CNCs obtained via surface modification with PEI

Cationic CNCs was prepared via surface modification with PEI. CNCs prepared from microcrystalline cellulose via TEMPO oxidation were used for surface modification. Differing from acid hydrolysis, TEMPO oxidation endowed CNCs with carboxylic groups on their surface which would be more favorable for further modification. As shown in Table 4.3, the yield of CNCs-COOH was 33.91% and their average particle size ranged from 173.5 to 194.6 nm and zeta potential ranged from -25.9 to -26.5 mV. After being modified with PEI, the average particle size of CNCs had increased to 251 - 254 nm. The particle size distribution became broader after surface modification (Figure 4.8). The surface charge of modified CNCs became positive, reaching 47.8 - 48.8 mV, confirming the successful grafting of PEI on the surface of CNCs. Figure 4.9 shows both slurry and freeze dried CNCs-COOH and CNCs-PEI samples.

Table 4.4. Yield, average particle size and zeta potential of CNCs obtained from TEMPO oxidation (CNCs-COOH) and CNCs surface modification (CNCs-PEI).

<table>
<thead>
<tr>
<th>Samples</th>
<th>Yield (%)</th>
<th>Average Particle size (nm)</th>
<th>Zeta potential (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CNCs-COOH</td>
<td>33.91 ± 0.15</td>
<td>173.5 - 194.6</td>
<td>-25.9 - -26.5</td>
</tr>
<tr>
<td>CNCs-PEI</td>
<td>-</td>
<td>251.3 - 253.6</td>
<td>47.8 - 48.8</td>
</tr>
</tbody>
</table>
Figure 4.8. Size distribution of CNCs-COOH and CNCs-PEI.

Figure 4.9. CNCs-COOH and CNCs-PEI samples: slurry (top) and lyophilized power (bottom) (CNCs-COOH suspension is more dilute than CNCs-PEI suspension).
The surface functional groups of CNCs-COOH and CNCs-PEI were analyzed by FTIR and the spectra are shown in Figure 4.10. The characteristic peaks at 1620 cm\(^{-1}\) and 3360 cm\(^{-1}\) were assigned to be carbonyl groups (C=O) in carboxylic acid (-COOH) and O-H stretching, respectively (Liu et al., 2017a). The band at 3300 cm\(^{-1}\) is typical for N-H stretching of primary amines (Jin et al., 2015). The increased intensity of band around 3360 cm\(^{-1}\) could be due to the superposition of O-H stretching and N-H stretching, suggesting the amino groups present in CNCs-PEI. The new peaks at 1380 cm\(^{-1}\) and 1510 cm\(^{-1}\) (Du et al., 2015) shown in modified CNCs corresponded to the C-N stretching, further confirming that PEI were successfully grafted on the surface of CNCs upon the reaction between carboxyl groups (-COOH) on the surface of CNCs and amine groups (-NH\(_2\)) on the surface of PEI.

![FTIR spectra of CNCs-COOH and CNCs-PEI.](image)

Figure 4.10. FTIR spectra of CNCs-COOH and CNCs-PEI.

### 4.4. Antibacterial activity test of surfaced modified CNCs (CNCs-PEI)

Cationic PEI is reported be antibacterially active and has been used for many applications (Koplin et al., 2008). It is expected that CNCs modified with PEI would be endowed with antibacterial activity. In this study, antibacterial activity of CNCs-PEI was
evaluated against Gram-positive bacteria *Bacillus megaterium* and Gram-negative bacteria *Escherichia coli* using agar diffusion zone test and bacterial killing kinetics test.

### 4.4.1. Agar diffusion zone test

CNCs-PEI with different concentrations were directly added to the pre-inoculated agar plate. Deionized (DI) water and unmodified CNCs (CNCs-COOH) were used as controls. CNCs-PEI showed inhibition zone towards Gram-positive *B. megaterium* while DI water and unmodified CNCs (CNCs-COOH) had no inhibition zone. High concentration at 5 mg/mL had clearer inhibition zone than low concentration at 2.5 mg/mL (Figure 4.11a). When the concentration of CNCs-PEI remained same (5 mg/mL), the bigger volume was added, the larger inhibition zone was obtained (Figure 4.11b). In contrast, for *E. coli*, CNCs-PEI appeared to have little inhibition effect. These results were in agreement with antimicrobial activity of PEI that also showed strong inhibition toward Gram-positive bacteria, but was less active toward Gram-negative bacteria (Briones et al., 2014).
Figure 4.1. Agar diffusion zone test against Gram-positive bacteria *B. megaterium* (a and b) and Gram-negative bacteria *E. coli* (c and d).

### 4.4.2 Bacterial killing kinetics test

The antibacterial activity of CNCs-PEI was further evaluated by following bacterial killing kinetics test. The test was conducted in a liquid culture and the results are shown in Figure 4.12, Figure 4.13, and Figure 4.14. CNCs-PEI had little inhibitory effect on *B. megaterium* when its concentration was lower than 0.5 mg/mL. With an increase in the CNCs-PEI concentration, the bacterial survival decreased. No *B. megaterium* survived when CNCs-PEI concentration reached 2 mg/mL or above. The inhibitory effect of CNCs-PEI on *E. coli* was also concentration-dependent, but did not become significantly active until CNCs-PEI concentration reached 2 mg/mL or above. In agreement with agar
diffusion zone test, liquid culture test also showed that CNCs-PEI was more effective to
gram-positive bacteria than gram-negative ones. Our study was in agreement with prior
study that cationized cellulose nanofibrils showed more obvious inhibitory effect toward
Gram-positive bacteria than Gram-negative bacteria (Saini et al., 2016).

PEI is polycationic owing to the presence of primary, secondary and tertiary amino
groups (Khalil et al., 2008). It endowed CNCs with polycationic properties once it was
grafted on the surface of CNCs. The positively charged CNCs-PEI could interact with the
negatively charged membrane of bacteria, inducing the damage of bacterial cell wall and
subsequently the death of microorganism (Saini et al., 2016; Tang et al., 2015).

The different antibacterial inhibitory effect of CNCs-PEI toward Gram-negative
bacteria *E. coli* and Gram-positive bacteria *B. megaterium* might be due to the difference
of the cell wall structures and surface charge change when subjected to cationic CNCs-PEI.
As shown in Figure 4.15, irrespective of peptidoglycan layer, Gram-negative bacteria
contains outer membrane and periplasmic space, which could serve as an effective
protective barrier, helping maintain the integrity of the cell.

Surface charge neutralization was found to lead to alter membrane permeability (Alves
et al., 2010), resulting in the decrease of cell viability. Gram-negative bacteria were found
to have more negative charge than Gram-positive bacteria (Halder et al., 2015) due to the
additional layer of negative charged lipopolysaccharides and phospholipids. Specifically,
the surface charges of *E. coli* and *B. megaterium* were reported to be around -20 mV and –
13 mV, respectively, in 0.1 M sodium phosphate buffer solution (pH 6.8) (OD$_{660}$ = 2)
(Hanpanich et al., 2017). In addition, the high density of anionic groups in the outer
membrane in Gram-negative bacteria help to maintain their surface charge. When
subjected to identical concentration of cationic agents, the decrease of zeta potential was more in Gram-positive bacteria than that in Gram-negative bacteria (Halder et al., 2015). Thus, higher concentration of cationic agents, specifically higher concentration of CNCs-PEI in our study, was needed to neutralize the surface charge of Gram-negative bacteria *E. coli* than the Gram-positive bacteria *B. megaterium*. In other words, higher concentration of CNCs-PEI was needed to alter the membrane permeability of *E. coli*. It resulted in less decrease of *E. coli* viability compared to *B. megaterium* when the same concentration of CNCs-PEI was used for both strains. Thus, CNCs-PEI showed higher inhibitory effect toward *B. megaterium*.

![Image](image_url)

**Figure 4.12.** Antibacterial assessments of CNCs-COOH and CNCs-PEI suspensions toward *B. megaterium* and *E. coli*.
Figure 4.13. *B. megaterium* colonies grown on LB plates after incubation with CNCs-COOH and CNCs-PEI.

Figure 4.14. *E. coli* colonies grown on LB plates after incubation with CNCs-COOH and CNCs-PEI.
4.5. PVA/CNCs-PEI films

4.5.1. Characteristics of the synthesized films

The PVA film incorporating CNCs-PEI was synthesized and characterized for mechanical strength. The typical stress-strain curves and Young’s modulus for PVA/CNCs-PEI films are shown in Figure 4.16. Young’s modulus increased with the increasing contents of CNCs-PEI up to 5 wt%. The highest Young’s modulus was 209.24 MPa, which was more than two folds that of PVA film only, proving the reinforcing effect of CNCs-PEI. The increased stiffness could be attributed to hydrogen bonding and the electrostatic interaction between the CNCs-PEI and PVA. However, PVA with CNCs-PEI content above 5% had a lower Young’s modulus that that with 5% CNCs-PEI. The aggregation of CNCs could happen with higher loading in PVA matrix, resulting in the non-homogeneous dispersion in the polymer matrix and lower mechanical strength of the film compared to PVA film with optimal CNCs loading (Cho & Park, 2011). The results

Figure 4.15. Cell wall structures of Gram-negative bacteria and Gram-positive bacteria (Brown et al., 2015).
were in agreement with previous studies that PVA or chitosan could obtain the highest elastic modulus with the addition of 5 wt% of CNCs (Cho & Park, 2011; Liu et al., 2017b).

Figure 4.16. (a) Typical stress-strain curves obtained from tensile tests for neat PVA & PVA/CNCs-PEI films; (b) Young’s modulus for neat PVA & PVA/CNCs-PEI film.
4.5.2. Color of the synthesized films

Color of the packaging is important regarding product appearance and consumer acceptance (Peng & Li, 2014). The effect of CNCs-PEI on the color of the synthesized nanocomposite films are shown in Figure 4.17 and Table 4.5.

As shown in Figure 4.17, the actual color of the film looks almost same by visual inspection. However, the color showed significantly different by Chroma Meter (Table 4.5). With the increases in contents of CNCs-PEI, $L^*$ value did not change, while $a^*$ value showed a significant decreasing trend and $b^*$ values showed a significant increasing trend. The difference of $a^*$ and $b^*$ values for individual film samples resulted in significant increase of $\Delta E$ value. The reducing $a^*$ values and the increasing $b^*$, $\Delta E$ values indicated that the film samples were greener and yellower when more CNCs-PEI added to PVA film. The CNCs-PEI powder was yellowish (Figure 4.9). The color difference between the nanocomposite films might be attributed to the natural color of CNCs-PEI as used in the film synthesis.

![Figure 4.17. PVA film containing 0% (a), 1% (b), 3% (c), 5% (d), 7% (e) and 9% (f) CNCs-PEI.](image)
Table 4.5. Effects of CNCs-PEI contents on the color of PVA/CNCs-PEI films.

<table>
<thead>
<tr>
<th>CNCs-PEI content (%)</th>
<th>$L^*$</th>
<th>$a^*$</th>
<th>$b^*$</th>
<th>$\Delta E$</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>97.83 ± 0.13$^a$</td>
<td>-0.04 ± 0.02$^a$</td>
<td>2.28 ± 0.06$^e$</td>
<td>0.38 ± 0.06$^e$</td>
</tr>
<tr>
<td>1</td>
<td>97.90 ± 0.03$^a$</td>
<td>-0.43 ± 0.08$^b$</td>
<td>3.24 ± 0.24$^d$</td>
<td>1.4 ± 0.25$^d$</td>
</tr>
<tr>
<td>3</td>
<td>97.82 ± 0.13$^a$</td>
<td>-0.74 ± 0.06$^c$</td>
<td>4.23 ± 0.11$^c$</td>
<td>2.43 ± 0.13$^c$</td>
</tr>
<tr>
<td>5</td>
<td>98.01 ± 0.28$^a$</td>
<td>-0.88 ± 0.03$^{cd}$</td>
<td>4.50 ± 0.12$^{bc}$</td>
<td>2.75 ± 0.14$^{bc}$</td>
</tr>
<tr>
<td>7</td>
<td>98.06 ± 0.21$^a$</td>
<td>-1.10 ± 0.15$^d$</td>
<td>5.21 ± 0.37$^b$</td>
<td>3.49 ± 0.39$^b$</td>
</tr>
<tr>
<td>9</td>
<td>98.12 ± 0.37$^a$</td>
<td>-1.42 ± 0.21$^e$</td>
<td>6.05 ± 0.61$^a$</td>
<td>4.39 ± 0.63$^a$</td>
</tr>
</tbody>
</table>

$^$ Each value was expressed as mean ± SD (n = 3).

$^$ Values with different letters within a column were significantly different ($p < 0.05$).

$^$ $L^*$ value (0 - 100) indicates the color from black to white; $a^*$ value (-100 - 100) indicates color from green (-) to red (+); $b^*$ value (-128 - 127) indicates color from blue (-) to yellow (+).
Chapter 5 Conclusions and Suggestions for Future Research

5.1. Conclusions

This study aimed at developing switchgrass-based CNCs with antimicrobial activity for their application in active packaging. Switchgrass was first fractionated into cellulose-enriched pulp via different pretreatment/pulping methods (i.e., acid-chlorite pretreatment, organosolv pretreatment, and DES pretreatment). CNCs were then extracted via acid hydrolysis from the prepared pulps and characterized for their properties. CNCs extracted via TEMPO oxidation were cationized by grafting PEI onto the surface and evaluated for their antimicrobial activity. The PVA/CNCs-PEI nanocomposite films were synthesized in order to evaluate the applicability of CNCs-PEI in improving mechanical properties of packaging materials. Below are the major conclusions drawn from this study:

1) Switchgrass was effectively fractionated into pulp by the above mentioned three methods. Acid-chlorite pretreatment was particularly effective for delignification, yielding white pulp. Acid-chlorite pulp contained a significantly low lignin content (1.12%) while organosolv pulp and DES pulps contained 3.53 - 8.45% lignin. DES pretreatment was shown to be an effective pulping method, especially for hemicellulose removal (> 79.55%). As a result, DES pulps contained less than half of xylan compared to raw switchgrass. Post-treatment using a mixture of NaOH and H₂O₂ bleached organosolv pulp and DES pulps, which made these pulps more suitable for CNCs extraction. The FTIR results were in agreement with the results obtained from the compositional analysis, confirming lignin and hemicellulose removal as well as cellulose enrichment.
2) The resultant pulps yielded 30.52 - 35.82% CNCs (based on the dry mass of pulp loaded) via sulfuric acid hydrolysis with the highest yield observed with mildly post-treated ChCl: FA pulp. The average particle size ranged from 63.55 to 222.20 nm and relatively uniform distribution of particle sizes was observed with CNCs from acid-chlorite pulp. The surface charges of all the extracted CNCs ranged from -20.30 to -26.70 mV. AFM image showed that the prepared CNCs had typical dimensions reported in literature.

3) Surface modification with PEI successfully cationized CNCs. In this case, CNCs with carboxylic groups were extracted from microcrystalline cellulose via TEMPO oxidation. CNCs, with a yield of 33.91%, an average particle size of 173.50 - 194.60 nm, and surface charge of -25.90 - -26.50 mV were obtained. PEI was grafted onto the surface of CNCs with carboxylic groups, which was confirmed by FTIR spectra, resulting in cationized CNCs (CNCs-PEI) with an average particle size of 251.30 - 253.60 nm and surface charge of 47.80 - 48.80 mV.

4) Antimicrobial assays showed that CNCs-PEI were successfully endowed with antimicrobial activity via cationization. CNCs-PEI were more active toward Gram-positive bacteria *B. megaterium* than Gram-negative bacteria *E. coli*. Bacterial killing kinetics test demonstrated that 2 mg/mL CNCs-PEI could kill *B. megaterium*, but a doubled concentration was required for killing *E. coli*.

5) Antibacterial CNCs-PEI were evaluated for their application in packaging materials. PVA/CNCs-PEI nanocomposite films were synthesized by incorporating CNCs-PEI at 1 - 9% into the films. The Young’s modulus of the film was increased from 99.20 MPa to 209.24 MPa with the addition of 5 wt% CNCs-PEI. However, with
higher loading of CNCs-PEI, the Young’s modulus tended to decrease likely due to the aggregation of CNCs-PEI. The color analysis of the synthesized film demonstrated that with higher concentrations of CNCs-PEI incorporated into the films, the film samples tended to be greener and yellower.

In short, this study demonstrated that switchgrass was effectively fractionated into pulp, which was suitable for CNCs extraction. Cationized CNCs (CNCs-PEI), thanks to their antimicrobial activity, have great potential to be used as a multifunctional nanomaterial for advanced applications, such as active packaging.

5.2. Suggestions for future research

There are several aspects to be studied in the future research. One is the optimization of acid hydrolysis to improve CNCs yields. More characterization of CNCs (e.g., X-ray crystallography and Fourier-transform infrared spectroscopy) and more efficient extraction methods for CNCs extraction could also be explored. Another one is to explore CNCs-like products extracted from organosolv pulp and DES pulp without post-treatment. Other cationization methods will be exploited to make surface active to a broad spectrum of pathogens. Possible aggregation of CNCs at high loading in the film matrix is another issue to be addressed. Nano-emulsification techniques may be used to improve the dispersion stability of CNCs and their contact with the polymers (Lefebvre et al., 2017). Cytotoxicity of CNCs-PEI and antimicrobial activities of the PVA/CNCs-PEI film would also be evaluated using proper methods. More applications of CNCs-PEI can be explored such as 3D antibacterial gel and their multifunctionality for enhancing the scaffold properties used in biomedical engineering.
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