ABSTRACT

ERNEST-SAUNDERS, RACHEL ALYSSA. Surface Modifications of Microfibrillated and Bacterial Cellulose with Acetylation and Biosynthesized Hygroscopic Polyamide to Study Bonding and Material Property Augmentation. (Under the direction of Dr. Joel J. Pawlak).

Cellulose has traditionally been studied for its use in paper products. However, recent research has focused on both microfibrillated cellulose (MFC) and on cellulose synthesized by bacterial species (BC). These areas offer the possibility for advances in biomedical and packaging applications that traditional paper-making cellulosic fibers could not accomplish.

To study the bonding characteristics of MFC in thin films, surface acetylation of the MFC was carried out at 100 °C with acetic anhydride and acetic acid. The acetylated MFC was then formed into thin films via a vacuum-dewatering method followed by restrain-drying. MFC can form more inter-fibril hydrogen bonds than traditional paper-making fibers due to their increased surface area, which allows MFC films to carry a higher applied load. Even when the MFC is acetylated, it has enough remaining hydrogen bonds to maintain load carrying capacity. However, the amorphous regions of the fibrils are acetylated first and combined with the reduction of hydrogen bonds, the films have a more uniform structure that can only absorb energy as well as traditional paper fibers.

To further examine how modification of fibrils changed the material properties of resultant films, a hydrophilic polyamide called poly(gamma-glutamic acid) (γ-PGA) was combined with MFC films and bacterial cellulose pellicles. This polymer has a high molecular weight and is highly hygroscopic due to its pendant carboxyl groups.
In this work, aqueous \(\gamma\)-PGA was applied to MFC films. After air-drying, the films were disintegrated and re-formed. The addition of \(\gamma\)-PGA allowed for a 96% recovery of tensile strength as compared to only 75% recovery when no \(\gamma\)-PGA was applied. A second set of films were heat treated after \(\gamma\)-PGA entrainment at 150 °C for 30 minutes. Thermal analysis showed a possible interaction between the polymers, but spectral analysis was not conclusive. Material properties, though, revealed that heat treatment alone improved the tensile strength and hydrophilicity of the MFC films. Entraining \(\gamma\)-PGA further altered the material properties, including an increase in hydrophilicity, as well as water absorption and vapor transmission rate. Heat treatment to films entrained with \(\gamma\)-PGA, though, reduced the latter water interaction properties.

The \(\gamma\)-PGA used in the blending study was commercially-fermented, but \(\gamma\)-PGA was also fermented in-house. Bacterial isolates from soybeans were identified as *Bacilli*. When grown on complex media solid-substrate plates, the bacteria produced \(\gamma\)-PGA. The solid-substrate method was chosen so that less acetone was necessary to precipitate the polymer.

To further study the formation of strong, hygroscopic materials, carbodiimide chemistry was used with BC and \(\gamma\)-PGA. The BC was carboxymethylated, and ethylenediamine was the cross-linker for the amidization. Carbodiimide chemistry is performed in an aqueous media, and the reaction was successful in that di-amine was attached to the carboxymethylated cellulose surface. However, the second stage of the cross-linking did not seem to result in the \(\gamma\)-PGA linked to the cross-linker. The molecular weight, charge, and conformation of the \(\gamma\)-PGA are suspected as the cause for the steric hindrance and the lack of amidization.
Surface Modifications of Microfibrillated and Bacterial Cellulose with Acetylation and Biosynthesized Hygroscopic Polyamide to Study Bonding and Material Property Augmentation

by
Rachel Alyssa Ernest-Saunders

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APPROVED BY:

Dr. Joel J. Pawlak
Chair of Advisory Committee

Dr. Amy M. Grunden
Member of Advisory Committee

Dr. Orlando J. Rojas
Member of Advisory Committee

Dr. Richard A. Venditti
Member of Advisory Committee

Dr. Lucian A. Lucia
Member of Advisory Committee
I dedicate this work to two men who have helped me be the successful woman I am today. As per my dad’s joking instruction to “always thank your wonderful father”, I do love and appreciate him for always expecting the best of me and teaching me to be responsible and dedicated and to always act with integrity. The other man, I was not to marry until after the conferment of this degree – as per another of my dad’s instructions of “no dating until you earn a graduate degree”. But I couldn’t wait, and married him already! So, to my husband, Christopher, who has been my backbone for many difficult challenges and from whom I learn something new each day. I hope that he will always be my wingman.
Rachel Ernest-Saunders received her Bachelor of Science in Chemical Engineering and in Paper Science and Engineering from North Carolina State University in 2004.

After graduation, she joined Teach for America and taught secondary mathematics in under-privileged and under-resourced communities. With the conclusion of her commitment with Teach for America, she moved into teaching other teachers how to teach with the NCSU NCTeach program.

In 2008, she was granted the USDA/CSREES Higher Education Food and Agricultural Sciences National Needs Fellowship. She, thus, began her current work in the Department of Forest Biomaterials under the guidance of Dr. Joel J. Pawlak.
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1. Introduction
1.1 RESEARCH OBJECTIVES and MOTIVATION

Creating renewable and sustainable alternatives to petrochemical-derived materials is important for building an economy that is sustainable. In this research, various forms of cellulose are modified, blended, and reacted to create materials that can be used in a variety of applications and to better understand how cellulosic materials develop their strength. Chapters Two, Three, and Five focus on different aspects of the cellulose research, while Chapter Four describes a method for producing another biologically-derived polymer, poly (gamma-glutamic acid) (γ-PGA)

Chapter Two uses acetylation of cellulose microfibrils to systematically degrade the strength of films made of microfibrillated cellulose (MFC). The objective of this chapter is to understand how MFC films create such high strength and to understand the structural aspects governing the strength development. This work is critical to develop basic scientific understanding of the material science behind cellulose fiber/fibril materials.

Chapter Three describes blends of MFC with γ-PGA to enhance the strength of the MFC films and improve the hydrophilicity of the films. The objective of this chapter is to understand how a polyamide with a high carboxylic acid content affects the material properties of MFC films. This chapter establishes a means for adding a hydrophilic polymer to MFC and increasing the water uptake, while also improving the strength.
Chapter Four examines a technique for cultivating $\gamma$-PGA on solid media. This technique improves the ease of harvesting and isolation of the polymer. This chapter establishes a means for growing bacteria that produces large amounts of extracellular polymer on solid media. This polymer can then be purified and used for subsequent experimentation.

Chapter Five targets the creation of a cross-linked cellulose and $\gamma$-PGA material. The objective was to establish a means for creating a strong highly hydrophilic material from bacterial cellulose. This material would have direct application in biomedical applications.

The remainder of this chapter establishes the necessary background to understand this work as a whole. An introduction to cellulose chemistry, cellulose derivatization and fibrillation, bacterial cellulose, and poly (gamma-glutamic acid) is given in this first chapter.

1.2 CELLULOSE CHEMISTRY

1.2.1 Origin

Cellulose is a biosynthesized polymer that is considered the most abundant polymer on Earth. It is estimated that at least 100 billion tons per year are produced [1]. Although both Eukarya and Bacteria species can generate cellulose (Table 1.1), the most common and profuse source of cellulose is from higher order plants, such as trees or grasses. In these organisms, the cellulose polymer is associated with other polymers, most notably lignin and hemicelluloses (see Section 1.2.4: Associated Polymers in Wood).
Table 1.1: Selected cellulose-producing species of *Eukarya* and *Bacteria*.

<table>
<thead>
<tr>
<th>SPECIES</th>
<th>CELLULOSE FUNCTION</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Eukarya</strong></td>
<td></td>
</tr>
<tr>
<td><em>Valonia ventricosa</em> (algae)</td>
<td>cell membrane</td>
</tr>
<tr>
<td><em>Halicystis</em> (algae)</td>
<td>cell membrane</td>
</tr>
<tr>
<td><em>Tunicata</em> (animal)</td>
<td>mantle</td>
</tr>
<tr>
<td>Cotton</td>
<td>structural component</td>
</tr>
<tr>
<td>Grass</td>
<td>structural component</td>
</tr>
<tr>
<td><strong>Softwood Trees</strong></td>
<td></td>
</tr>
<tr>
<td><em>Pinus</em> (pine)</td>
<td>structural component (42% ± 2%)</td>
</tr>
<tr>
<td><em>Picea</em> (spruce)</td>
<td></td>
</tr>
<tr>
<td><em>Abies</em> (fir)</td>
<td></td>
</tr>
<tr>
<td><strong>Hardwood Trees</strong></td>
<td></td>
</tr>
<tr>
<td><em>Quercus</em> (oak)</td>
<td>structural component (45% ± 2%)</td>
</tr>
<tr>
<td><em>Fagus</em> (beech)</td>
<td></td>
</tr>
<tr>
<td><em>Eucalyptus</em> (eucalyptus)</td>
<td></td>
</tr>
<tr>
<td><strong>Bacteria</strong></td>
<td></td>
</tr>
<tr>
<td><em>Acetobacter xylinus</em></td>
<td>biofilm</td>
</tr>
<tr>
<td><em>Agrobacterium mefaciens</em></td>
<td>virulence factor</td>
</tr>
</tbody>
</table>

Cellulose has been recognized by humans as an important raw material for many civilized activities. From combustion for energy to building materials to the formation of paper, cellulose has become an integral aspect of human life. In the past century, research has revealed more advanced uses for cellulose where the macro-structure and even the atomic structure can be utilized for sophisticated materials, second generation fuels, or even foundation chemicals.
1.2.2 Molecular Structure

Cellulose was first identified by Anselme Payen in 1838 when he treated different woody material with nitric acid and always obtained the same carbohydrate of glucose (empirical ratio of $C_6H_{10}O_5$). He called the carbohydrate, cellulose. Later researchers confirmed the actual structure and monomeric unit of the polymer [2].

The cellulose polymer at the molecular level is a carbohydrate composed of glucopyranose. Long chains of glucopyranose are bound by glycosidic beta-1-4 linkages, thus the repeating unit of cellulose is called celllobiose (Figure 1.1). Cellulose chains contain a reducing end and a non-reducing end group where the reducing end group has the potential of forming a reactive aldehyde. The degree of polymerization (DP) of cellulose depends on the origin. Wood pulp DP can range from 300 – 1700 units whereas cotton and bacterial cellulose reportedly have DP values of 800 – 10,000 units [3]. Though, to date, the exact degree of polymerization of a cellulosic sample cannot be accurately determined. To purify cellulose, the polymer must be separated from residual molecules and these techniques can cause chain scissoring which interferes with measuring the degree of polymerization.

![Figure 1.1](image_url)

**Figure 1.1**: Molecular structure of cellulose showing beta-1-4 glycosidic linkage forming celllobiose repeating unit as well as the reducing and non-reducing end groups.
Another familiar glycosidic-bound anhydroglucose structure is starch; however, starch has *alpha*-1-4 glycosidic bonds (Figure 1.2). Starch synthase enzymes polymerize units of adenosine-diphosphate-glucose (ADP-glucose) [4] as shown in Figure 1.3a. For cellulose, though, rosette and linear terminal complexes (TC’s) [5] located in the plasma membrane contain cellulose synthase enzymes (from CesA genes) which polymerize units of uridine-diphosphate-glucose (UDP-glucose) (Figure 1.3b) with *beta*-1-4 glycosidic bonds [6]. The *alpha* linkage in starch creates an open and flexible structure, whereas the *beta* linkage in cellulose confers a straight, rigid structure to the cellulose chain. This structure and its subsequent bonding properties are responsible for the macro-scale properties of cellulose.

**Figure 1.2:** Molecular structure of starch showing *alpha*-1-4 glycosidic linkage.

**Figure 1.3:** Polymerized units for (a) starch (adenosine diphosphate-glucose [ADP-glucose]) and (b) cellulose (uridine diphosphate-glucose [UDP-glucose]).
Analysis of the cellulosic macromolecular structure has revealed four different allomorphs of crystalline cellulose, called cellulose I, II, III, and IV. Some are naturally generated, while others are created artificially through chemical and/or physical processing. Cellulose I is referred to as native cellulose and is produced most abundantly by plants (thus it is also the most abundant form). Its cellulose strands are arranged in a parallel fashion. It has two subgroups called cellulose Iα (single chain per unit cell) and cellulose Iβ (two chains per unit cell, second chain staggered). These phases are found in varying proportions in different organisms – cellulose from bacteria and algae contains predominately Iα while higher-order plants and animals generate structures of cellulose Iβ [7, 8]. Through annealing above 260 °C in an autoclave, cellulose Iα can be irreversibly converted to Iβ [9]. When cellulose is regenerated from solution or treated with strong (17 – 20 %) sodium hydroxide (“mercerization”), the macromolecule takes on a different structure called cellulose II. “Regenerated cellulose” is most commonly formed via this method. This allomorph has cellulose strands arranged in an anti-parallel fashion and has a lower crystallinity than native cellulose. The formation of cellulose II is irreversible which indicates that cellulose II is more thermodynamically stable than cellulose I [10, 11]. If either cellulose I or cellulose II is treated with liquid ammonia (under extreme conditions: either high heat and pressure or extremely low temperatures), then cellulose III is formed [12]. Cellulose III has the same parallel strand arrangement as cellulose I but it is not nearly as crystalline [13]. Cellulose III can be transformed into the final allomorph, cellulose IV, with the application of select amides, such as dimethylformamide [12].
Along with the allomorphs of the cellulose strands, the larger macro-structure can take on a combination of forms via intra- and inter-molecular hydrogen bonding (Figure 1.4). Since each hydroxyl of the cellobiose unit is located in the equatorial position, the configuration of all the hydroxyl groups along the cellulose chain help to build complex hydrogen-bond bound structures. Intra-molecular hydrogen bonding builds rigidity in a single cellulose chain, whereas inter-molecular hydrogen bonding builds sheet-like or block structures from multiple cellulose chains [5]. These structures can be considered as either crystalline or amorphous (Figure 1.5). The crystallinity of the cellulose structure is source dependent, for example: algae >> animal > cotton > wood [14]. The crystalline regions (with inter-molecular hydrogen bonds along the entire chain of a cellulose strand) give cellulosic macromolecules rigidity and a high elastic modulus [15]. However, the amorphous regions are integral to the fundamental properties of a cellulosic structure. Amorphous regions can more readily hydrogen bond with water (due to fewer inter-molecular bonds) in comparison to crystalline regions. These regions swell the cellulosic macromolecule, allow dispersion of stress (resulting in higher tensile loading), and increase flexibility of the supra-structure [16]. The macro-structure is comprised of micro-fibrils that contain both of these crystalline and amorphous regions. For higher-order plants, electron microscopy has verified that micro-fibrils are then arranged into fibrils, followed by fibers that form the cell walls of plants (Figure 1.6 and 1.7) – see Section 1.2: Microfibrillated Cellulose.
Figure 1.4: Hydrogen bonding within cellulose structure, (a) *intra*-molecular bonding and (b) *inter*-molecular bonding between cellulose chains.

Figure 1.5: Possible configurations of amorphous and crystalline regions of cellulosic micro-fibrils in higher-order plants.
Figure 1.6: Cellulose organization in higher-order plants from glucose to cell walls. [17]

Figure 1.7: Cellulosic plant structures, (a) plant fiber and (b) microfibrils liberated from fiber (taken from Nakagaito and Yano, 2005 [18]). Scale bar is 10 µm.
1.2.3 Cellulose Derivatives

Although cellulose itself has many uses, there exists much interest in developing derivatives that add beneficial properties to the cellulosic structure or chemistry. These derivatives are formed by the substitution of some or all of the cellulosic hydroxyl groups with a more desired functional group. On cellulose’s anhydrous unit of glucose, there are three available hydroxyl groups (C2, C3, and C6 positions). Their substitution is reported as degree of substitution (DS) where a DS of three represents complete substitution. DS is usually calculated based upon weight (e.g. ASTM D1439-03 and ASTM D871-96), so there is not a clear indication of where the substitution has taken place just that a substitution has occurred. Alterations to cellulose can be in the form of complete dissolution of the cellulose structure in tandem with modification (homogeneous) or, more commonly, modification of the intact structure (heterogeneous). Figure 1.8 shows some of the most commonly researched or industrially important cellulose derivatives.

![Cellulose Derivatives Diagram](image)

**Figure 1.8:** Some common cellulose derivatives in their fully substituted forms, (a) cellulose acetate, (b) carboxymethyl cellulose, and (c) silylation of cellulose.
**Cellulose Acetate**

Cellulose acetate is generally used in photography film, separation membranes, and cigarette filters. Acetylation through esterification is the replacement of the cellulosic hydroxyl groups with an acetyl group (Figure 1.8a). Most acetylation is accomplished heterogeneously; thus, the substitution is somewhat random along the cellulose backbone. Therefore, industrially, cellulose acetate is often produced to complete substitution (triacetate) and then de-acetylated to the desired DS that will create a material with the preferred properties. The acetylated cellulose with the desired DS can be dissolved in an appropriate solvent (Table 1.2) and then extruded to form fibers or materials. In many research programs, though, complete substitution is not desired. Rather, only the surface hydroxyls are substituted which leaves the macroscopic structure of the cellulosic fiber/fibril more intact. In these schemes, the cellulose acetate is not dissolved in a solvent; it is merely cleaned of un-reacted reagents and formed into desired material structures.

<table>
<thead>
<tr>
<th>Table 1.2: Solvents for cellulose acetates at various levels of substitution. [2]</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>DS</strong></td>
</tr>
<tr>
<td>1.8 – 1.9</td>
</tr>
<tr>
<td>2.2 – 2.6</td>
</tr>
<tr>
<td>2.8 – 2.9</td>
</tr>
<tr>
<td>2.9 – 3.0</td>
</tr>
</tbody>
</table>

Many methods are used to transform cellulose into cellulose acetate (Table 1.3) using acetic anhydride (Figure 1.9). Each requires some form of catalyst since the hydroxyl group is not
a very good chemical leaving group. It has been reported that in heterogeneous reactions, substitution at the surface of fibers/fibrils occurs first, and that the C6 hydroxyl is preferentially acetylated (followed by the C2 and then the C3 positions). As heterogeneous acetylation proceeds, the crystallinity of the macrostructure of acetylated regions decreases but the acetylated cellulose material remains with the ultrastructure. However, for homogenous reactions (e.g. ionic liquids or cellulose solvents), the acetylated regions of the fiber/fibril peel away from the fiber/fibril – thus, exposing new surfaces to acetylate [19]. With increased acetylation, the cellulose becomes more hydrophobic and less able to create hydrogen bonds [20]. However, these changes to the chemistry of cellulose can increase compatibility with non-polar matrices such as acrylic resin [21, 22].

Figure 1.9: Reaction of cellulose hydroxyl with acetic anhydride to produce ester.
Table 1.3: Methods for transforming cellulose into cellulose acetate.

<table>
<thead>
<tr>
<th>HETEROGENEOUS REACTIONS</th>
<th>Reagents</th>
<th>Temp</th>
<th>Catalyst</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>glacial acetic acid</td>
<td>60 °C</td>
<td>sulfuric acid</td>
<td>Sassi and Chanzy (1995 [19])</td>
</tr>
<tr>
<td></td>
<td>acetic anhydride</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>sulfuric acid</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>glacial acetic acid</td>
<td>25 °C</td>
<td>perchloric acid</td>
<td>Ifuku et al. (2007 [21])</td>
</tr>
<tr>
<td></td>
<td>toluene</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>acetic anhydride</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>perchloric acid</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>acetic anhydride</td>
<td>100 °C</td>
<td>temperature</td>
<td>Jonoobi et al. (2009 [24])</td>
</tr>
<tr>
<td></td>
<td>pyridine</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>acetic anhydride</td>
<td>120 °C</td>
<td>temperature</td>
<td>Nogi et al. (2006 [22])</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>acetic anhydride</td>
<td>100 °C</td>
<td>temperature</td>
<td>unpublished results (Ernest-Saunders et al.)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HOMOGENEOUS REACTIONS</td>
<td>Reagents</td>
<td>Temp</td>
<td>Solvent</td>
<td>Reference</td>
</tr>
<tr>
<td>--------------------------</td>
<td>----------</td>
<td>------</td>
<td>---------</td>
<td>------------</td>
</tr>
<tr>
<td></td>
<td>acetic anhydride</td>
<td>20 °C</td>
<td>ionic liquid</td>
<td>Wu et al. (2004 [25])</td>
</tr>
<tr>
<td></td>
<td></td>
<td>80 °C</td>
<td></td>
<td>Schlufter et al. (2006 [26])</td>
</tr>
<tr>
<td></td>
<td></td>
<td>100 °C</td>
<td></td>
<td>Cao et al. (2007 [27])</td>
</tr>
<tr>
<td></td>
<td>acetic anhydride</td>
<td>60 °C</td>
<td>DMSO / tetrabutylammonium fluoride trihydrate</td>
<td>Ass et al. (2004 [28])</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>acetic anhydride</td>
<td>60 °C</td>
<td>DMAC / LiCl</td>
<td>El-Seoud et al. (2000 [29])</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>acetic anhydride</td>
<td>25 °C</td>
<td>DMAC / LiCl with sulfuric acid catalyst</td>
<td>McCormick and Callais (1987 [30])</td>
</tr>
</tbody>
</table>
**Carboxymethyl Cellulose**

Carboxymethyl cellulose (CMC) (Figure 1.8b) is commonly used in many commercial products. Among the many applications of CMC, this derivative can be used as a food thickener, an emulsion stabilizer, or an enhancer of detergents.

Carboxymethylation can be achieved with a heterogeneous alkali-catalyzed reaction with chloroacetic acid (Figure 1.10) at elevated temperatures (~65 °C) with increasing time to give increasing substitution levels. Niemelä and Sjöström [31] found that for CMC-generating reactions, substitution of cellulose hydroxyls began with C6 and C2 being roughly equivalent, followed by C3 substitution. Sjöström [32] has even proposed that the C2 hydroxyl (the most acidic of the hydroxyl groups) has the highest reactivity with chloroacetic acid and is therefore the most substituted.

![Figure 1.10: Reaction of monochloroacetic acid with hydroxyl of cellulose to yield carboxymethyl cellulose.](image)

Since cellulose is not soluble in the suspending reagents used in carboxymethylation, the reaction is considered to be heterogeneous. However, carboxymethylated cellulose is more soluble in water than unmodified cellulose. The derivative has high hygroscopicity due to
the added carboxylic acid. Also the pendant carboxylic acid groups confer greater chemical reactivity for further chemistry.

**Silylated Cellulose**

At a bench scale, silyl groups have been added to cellulose (Figure 1.8c) to create substrates with varying properties. Depending on the R-group of the silyl functional group, these modifications can allow cellulose to better disperse in non-polar solvents or can make the cellulose more reactive for adsorption of charged polymers. Silylation has been accomplished by various methods in organic solvents (Table 1.4) but most involve the use of a silyl chloride in a strongly basic solvent in order to deprotonate cellulose’s hydroxyls.

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Solvent</th>
<th>Temp</th>
<th>Time</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cellulose</td>
<td></td>
<td></td>
<td></td>
<td>Goussé et al. (&lt;2002 [33], 2004 [34])</td>
</tr>
<tr>
<td>spruce sulfite microfibrils</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Silyl Group</td>
<td>toluene</td>
<td>25 °C</td>
<td>&lt; 16 hr</td>
<td>Andresen et al. (2006 [35], 2007 [36])</td>
</tr>
<tr>
<td>isopropyl dimethylchlorosilane (IPDMSiCl)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>chlorodimethylisopropylsilane</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(3-chloropropyl)trimethoxysilane</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cellulose</td>
<td>DMF</td>
<td>−15 °C</td>
<td>1 hr</td>
<td>Klemm and Stein (1995 [37])</td>
</tr>
<tr>
<td>Avicel (PH 101)</td>
<td></td>
<td>60 °C</td>
<td>6 hr</td>
<td></td>
</tr>
<tr>
<td>Silyl Group</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>thexyldimethylchlorosilane</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cellulose</td>
<td>DMAc/LiCl</td>
<td>80 °C</td>
<td>not given</td>
<td>Klemm and Stein (1995 [37])</td>
</tr>
<tr>
<td>Avicel (PH 101)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spruce sulfite pulp</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cotton linters</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Silyl Group</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>hexamethyldisilazane</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
1.2.4 Associated Polymers in Plants

Although cellulose is the predominant macromolecule in plants, there are two other major groups of macromolecules that are typically associated with cellulose. These two macromolecules are hemicellulose and lignin (Table 1.5).

<table>
<thead>
<tr>
<th></th>
<th>Cellulose</th>
<th>Hemicellulose</th>
<th>Lignin</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Softwood</strong></td>
<td>42 ± 2 %</td>
<td>27 ± 2 %</td>
<td>28 ± 3 %</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(galactoglucomannans ~20 % of wood)</td>
<td></td>
</tr>
<tr>
<td><strong>Hardwood</strong></td>
<td>45 ± 2 %</td>
<td>30 ± 5 %</td>
<td>20 ± 4 %</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(glucuronoxylans ~15-30 % of wood)</td>
<td></td>
</tr>
</tbody>
</table>

**Hemicellulose**

Hemicellulose is similar to cellulose in that it is comprised of a carbohydrate backbone; however, several distinct differences exist (Table 1.6). Since hemicelluloses have a more random and/or branched structure (Table 1.7), it is much more amorphous and thus more responsive to water and other chemical attack than cellulose. Also unlike cellulose, hemicellulose can more easily be extracted using alkali treatments, but the hemicellulose product will be nearly completely deacetylated [38]. Some hemicellulose structures incorporate 5-carbon sugars (like xylose and arabinose) – a feature that offers up new opportunities for those researchers attempting to utilize the entirety of the wood structure.
Table 1.6: Comparison of cellulose and hemicellulose. [2, 38]

<table>
<thead>
<tr>
<th>Cellulose</th>
<th>Hemicellulose</th>
</tr>
</thead>
<tbody>
<tr>
<td>always linear</td>
<td>often branched</td>
</tr>
<tr>
<td>contains residues only of glucose</td>
<td>contains residues of glucose, xylose, arabinose, mannose, galactose, among others</td>
</tr>
<tr>
<td>contains only 6-carbon sugars (glucose)</td>
<td>contains both 5- and 6-carbon sugars (xylose and arabinose)</td>
</tr>
<tr>
<td>utilizes β-1-4 glycosidic bonding</td>
<td>utilizes both α and β bonding</td>
</tr>
</tbody>
</table>

Table 1.7: Typical hemicellulose fractions in wood and their typical structures. [2, 38]

<table>
<thead>
<tr>
<th>Hemicellulose Type</th>
<th>Wood Type</th>
<th>Amount (% of wood)</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Units</td>
</tr>
<tr>
<td>Galactoglucomannan</td>
<td>Softwood</td>
<td>5 – 8 %</td>
<td>β-D-Manp</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>β-D-Glc</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>α-D-Galp</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Acetyl</td>
</tr>
<tr>
<td>(Galacto)glucomannan</td>
<td>Softwood</td>
<td>10 – 15 %</td>
<td>β-D-Manp</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>β-D-Glc</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>α-D-Galp</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Acetyl</td>
</tr>
<tr>
<td>Arabinogluconuroxylan</td>
<td>Softwood</td>
<td>7 – 10 %</td>
<td>β-D-Xylp</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>4-O-Me-α-D-Glc</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>α-L-Araf</td>
</tr>
<tr>
<td>Glucuronoxylan</td>
<td>Hardwood</td>
<td>15 – 30 %</td>
<td>β-D-Xylp</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>4-O-Me-α-D-Glc</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Acetyl</td>
</tr>
<tr>
<td>Glucomannan</td>
<td>Hardwood</td>
<td>2 – 5 %</td>
<td>β-D-Manp</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>β-D-Glc</td>
</tr>
</tbody>
</table>
The presence of hemicellulose in the processing and end-products of cellulosic materials is generally recognized as positive. Many hemicelluloses have pendant carboxylic acid or acetyl groups which can confer a reactive nature to hemicellulose-containing composites. Several studies [39, 40] have found that materials made from cellulose with higher fractions of hemicellulose have a higher Young’s modulus (GPa) and tensile strength (MPa). Cellulosic fibers after hemicellulose extraction create a weaker recycled paper [41]. Likewise, Iwamoto et al. [39] found that higher levels of hemicellulose allowed better fibrillation of both once-dried and never-dried holocellulose into microfibrils than those pulps that had been alkali-treated. Their research also found that lower hemicellulose levels resulted in poorer thermal stability of resultant materials.

**Lignin**

Lignin is a network polymer that is often described as a natural glue for the fibrous structure of plants. In wood, the middle lamella contains the highest concentration of lignin though the greatest amount of lignin is found in a fiber’s S2 layer (due predominantly to the thickness of this layer) [38]. Lignin’s main three monomer components (monolignols) are coniferyl alcohol / guaiacyl (G), syringyl alcohol / sinapyl (S), and p-coumaryl alcohol / hydroxyphenyl (H) (Figure 1.11). Enzymes dehydrogenate these phenolic monolignols to form radicals; subsequent reactions with other lignin intermediates create a network polymer that is seemingly a random structure [38, 42]. The proportion of the monolignol units varies with species, but the aspect given most attention by researchers is the S/G ratio. Lignin with
a high $S/G$ ratio is more reactive and, therefore, more easily separated from the cellulosic components of wood [43].

![Monolignols](image)

**Figure 1.11:** Monolignols (basic monomers) of lignin; (a) hydroxyphenyl / $p$-coumaryl alcohol, (b) guaiacyl / coniferyl alcohol, and (c) sinapyl / syringyl alcohol.

Many methods are used to remove lignin and liberate cellulose fibers from its supra-structure, although the two most dominant are sulfite pulping and sulfate kraft pulping. These methods rely predominantly upon making the lignin more soluble in the reaction medium by hydrolyzing ether bonds and creating free phenolic hydroxyl groups [38]. Sulfite pulping generates a more hydrophilic lignin product due to sulfonation (addition of sulfonic acid groups) than sulfate kraft pulping [38]. These differences can affect the purified lignin product’s reactivity and property conferment to composites with cellulose.

### 1.2.5 Isolation of Cellulose from Wood

Although wood in itself can be used for energy or as a building material, civilization has found more advanced ways to utilize its unique chemistry and actual structure – especially that of cellulose. In order to access cellulose and its fiber structure, a process called pulping
was developed. Pulping de-lignifies the wood and allows the fibers to separate so that they can be rearranged into desirable forms (e.g. paper).

Mechanical grinding can be used to liberate fibers from the supra-structure of wood, though modern mechanical process use some form of chemical and/or temperature to fully liberate the fibers. Mechanical methods leave the lignin in the pulp, so the material properties of the product are quite different than material made from de-lignified fibers. In sunlight, lignin oxidizes and turns the fibrous material yellow. It also makes the material more brittle by inhibiting the formation of hydrogen bonds between adjacent fibers.

The earliest commercial form of chemical pulping is called the soda process, which has since morphed into the Kraft process. In its earliest form – developed by Watt and Burgess – caustic sodium hydroxide was cooked with wood chips at elevated pressures and temperatures. Then, several years later, several independent researchers found that using sodium sulfate to make-up for soda losses created a strong fiber – thus, naming the process kraft (German for strength). The kraft process is also called the sulfate process. The $\text{HS}^-$ ion from sodium sulfate is a stronger base and a stronger nucleophile than the $\text{OH}^-$ ion from soda, so using sulfate has proven to be much more efficient and effective at cleave ether linkages found in the dendritic lignin polymer. In the alkaline pulping process, lignin is broken down into smaller molecular weight fragments that can be more easily be dissolved and removed from the fibrous structure.
The kraft pulping process is perhaps the most widely used de-lignification process, but another process based on sulfonation of carbon cations uses acids with sulfite ions. Though the first iteration of sulfite pulping used calcium sulfite, the modern process uses sodium, magnesium, and ammonia bases to improve chemical recovery due to their increased solubility. In sulfite pulping, the predominant action is creating a more soluble lignin product by adding hydrophilic sulfonic acid groups to the lignin structure. There is relatively low ether cleavage because ethers are stable under acidic conditions; however, lignin-carbohydrate bonds can be broken to liberate the cellulose from the lignin. Sulfite pulping has the drawback that it can hydrolyze carbohydrates, so the fiber product is not as strong as alkaline pulped fiber.

1.2.6 Industrial and Commercial Uses for Cellulose

Cellulose in its native form is bound into wood. Of course, traditionally, wood can be burned for energy production or it can be maintained in its supra-structure to use in building or crafts. However, the cellulose structure and/or its chemistry can be used for many more advanced applications.

Paper is the most ubiquitous use for cellulose fibers. Material for writing upon was first developed by Egyptians from papyrus about 3000 BCE, but this paper was made from whole biomass – the fibers were never liberated from their supra-structure. However, Chinese inventors in 105 CE, liberated fibers by cooking an inner bark material in water, followed by
softening the fibers by beating and then forming the fibers into paper. Modern-day paper-making still retains many of the same features of ancient paper-making, including: separation of fibers (pulping), softening the fibers (refining), formation of a sheet and drainage of water (wet-end), and finally pressing (press-section) and drying (dry-end) the last of the water from the sheet. To accomplish their work, modern day papermakers use machines (Figure 1.12) to quickly create many varieties of paper products.

![Figure 1.12: The basics of a modern-day paper machine, shown in the form of a Fourdrinier type machine with a flat table for forming the wet sheet.](image)

Besides paper, there are many more advanced uses for cellulose. Although wood has been used as an energy source for millennia, there are other ways to use the energy stored in the wood structure. Since the backbone of cellulose is glucose, it can be used as a feedstock for fermentation into fuel ethanol. Improving the efficiency of this process is still being researched heavily. Another fuel source could be torrified wood where the energy density of the structure is increased by treating the wood with temperatures of 200 – 300 °C in the absence of oxygen [45]. Other researchers have suggested that through various chemical
modifications and reactions, the cellulosic glucose could be used not for fuel but as foundation chemicals (benzene, toluene, xylene) for chemical processing [46]. These alternative uses for cellulose would replace currently petrochemical-derived materials.

1.3 MICROFIBRILLATED CELLULOSE (MFC)

1.3.1 Production

Cellulose microfibrils are typically liberated from the cellulosic fiber via mechanical methods – sometimes with chemical pre-treatments – and result in a fibril structure quite similar to the parent fiber. On the other hand, if chemicals or enzymes are used as the primary liberator of the smaller components of a whole fiber, the process is often detrimental to the amorphous portion of the fiber, thus creating a highly crystalline structure [47]. These chemically liberated components also generally have a lower aspect ratio and higher stiffness than those liberated via mechanical means.

When Herrick et al. [48] and Turbak et al. [49] first liberated microfibrils they used a high-pressure homogenizer (Figure 1.13). This method is still in wide use today; however, a refining step has been generally added as a “pretreatment” step to cause external fibrillation to the primary and S1 layers and subsequent exposure of the S2 layer [50]. Dilute slurries of refined fibers (consistency of ~2 %) are passed through the homogenizer where a large pressure drop causes intense shear and impact to the fiber structure. Microfluidizers work according to this same principle but utilize an “interaction chamber” with fixed
“microchannels” to cause the intense pressure drop [51]. One pass through these homogenizing machines is not enough to produce microfibrillated cellulose. Depending on the applied pressure, 15 – 30 passes create an adequately microfibrillated structure [52].

Figure 1.13: High-pressure homogenizer showing internal mechanisms. (APV Gaulin, Inc.)

Another commonly used method to liberate microfibrils is with a grinder (Figure 1.14). A refining step can be used prior to grinding as with homogenization. Dilute slurries of cellulosic fibers (consistency of ~2 %) are passed between two specially modified grooved disks – one static, the other revolving at variable high rates. The extent of fibrillation is dependent upon the distance between the disks, the morphology of the disk channels, and the number of passes through the grinder. Like with a homogenizer, many passes are necessary (anywhere from 3 – 10 passes) are required to generate adequate liberation of fibrillated cellulosic structures though refining pre-treatment is not necessary [53].
Prior to mechanical microfibril liberation, researchers have investigated various pretreatment techniques. The most common is refining, similar to the refining used in traditional paper-making, whether it be with a beater or a disk refiner. These refining techniques cause disruption of the primary and S1 layers of the fiber and initial fibrillation of the thick S2 layer. In addition to refining, more advanced techniques are used as pretreatments. Oxidation using 2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPO) radicals applies carboxylic acid groups to the surface of fibers. It is suggested that the negative charge conferred to the surfaces of the cellulose microfibrils cause repulsion and easier liberation [54]. In fact, liberation of microfibrils after fiber oxidation with TEMPO is performed in a blender rather than a homogenizer or grinder. Enzymatic pretreatment may also be used to degrade the outer layers of the fiber [55]. In particular, endoglucanases are used to create general disorder in the cellulosic structure; exoglucanases and beta-glucosidases are avoided due to their more extensive degradation of the cellulose chains.
1.3.2 Structure

An often lauded aspect of microfibrillated cellulose is that it generally maintains the amorphous and crystalline ratios of its source whole fiber. Unlike microcrystalline cellulose, cellulose whiskers, or cellulose nanocrystals, microfibrillated cellulose is predominantly produced mechanically and therefore does not lose its amorphous region. For cellulose model surface or composite materials, a component that is too crystalline does not accurately represent cellulose in its native state or necessarily create beneficial material properties.

Since the sources of microfibrillated cellulose vary, as well as the production techniques, there is not a genuine consensus about what constitutes microfibrillated cellulose. However, most microfibrillated cellulose produced with homogenizers or grinding has widths of less than 100 nm and various lengths, usually several micrometers [56]. Even within one research program, though, the dimensions of the fibrils produced can have a very wide distribution [53]. However, even with a wide distribution of dimensions, the aspect ratio of microfibrillated cellulose is still measured at approximately three orders of magnitude larger than its source fiber [57]. This markedly high aspect ratio leads to many of the beneficial material properties of microfibrillated cellulose.

As a source fiber is fibrillated into its component microfibrils, the specific surface area of the resultant microfibrillated cellulose becomes larger than its source fiber. Several methods have been attempted to measure the surface area of microfibrillated cellulose, including Brunauer–Emmett–Teller (BET) N₂ physisorption, Congo Red, and visual estimations based
on image analysis. Due to the differences in source fiber, processing methods, and measuring techniques, the specific surface areas are markedly diverse. For example, Sehaqui et al. [58] report a specific surface area of 45 m²/g for softwood microfibrils. However, using Congo Red, Spence et al. [57] claim to measure specific surface areas from 68 m²/g for bleached hardwood fibers to 195 m²/g for bleached softwood fibers. However, even at the lowest specific surface area measurement, these surface areas are still an order of magnitude larger than the source fiber’s specific surface area [57]. The increase in surface area results in more exposure of surface hydroxyl groups which can allow for a more interactive surface.

1.3.3 Properties of Microfibrillated Films

Thin films can be easily generated from microfibrillated cellulose slurries via either a casting or vacuum filtration method followed by air or oven drying. Most researchers have also found that some type of restraint during drying is a necessary requirement to produce flat, testable films. Each source fiber and method, though, produces films exhibiting varying levels property improvement over the whole source fiber. Microscopic viewing and X-ray scattering [59] can verify that the fibrils have a random orientation in these films.

The dimensional and specific surface area changes caused by complete fibrillation cause a notable increase in mechanical properties over materials formed from whole fibers (even refined fibers). Table 1.8 lists values for the elastic modulus, tensile index, and strain at break for several groups researching microfibrillated cellulose.
Table 1.8: Mechanical properties of microfibrillated cellulose films.

<table>
<thead>
<tr>
<th>Source Fiber</th>
<th>Processing Method</th>
<th>Film Method</th>
<th>Elastic Modulus (GPa)</th>
<th>Tensile Index* (N m g⁻¹)</th>
<th>Strain at Break (%)</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>softwood</td>
<td>TEMPO</td>
<td>casting</td>
<td>6.9</td>
<td>160</td>
<td>7.6</td>
<td>[54]</td>
</tr>
<tr>
<td>hardwood</td>
<td>TEMPO</td>
<td>casting</td>
<td>6.2</td>
<td>153</td>
<td>7.0</td>
<td>[54]</td>
</tr>
<tr>
<td>softwood</td>
<td>homogenizer</td>
<td>casting</td>
<td>15.7 – 17.5</td>
<td>129 – 146</td>
<td>5.3 – 8.6</td>
<td>[60]</td>
</tr>
</tbody>
</table>

Since cellulose microfibrils have widths of less than one micron (the wavelength of visible light), fibrils may not be capable of scattering light. Therefore, well-formed films of microfibrillated cellulose can be highly translucent or even transparent. Several researchers [57, 61-63] have demonstrated that increased fibrillation (smaller particle size) resulted in decreased opacity. In using microfibrillated cellulose produced via TEMPO-oxidization, Fukuzumi et al. [54] found that films formed from softwood fibrils (C6 substituted glucomannan) were more transparent (90% vs. 78%) than hardwood fibrils (no C6 to substitute in xylan) presumably because the softwood fibrils were more dispersible in the casting solvent.

Another benefit of microfibrillated cellulose films is their outstanding barrier properties which could be useful if these films are used as packaging materials. Several research studies have looked at microfibrillated cellulose as a coating, rather than itself being the base material. Fukuzumi et al. [54] lowered the oxygen permeability (0% RH) of 25 µm thick poly-lactic acid films from 746 mL m⁻² d⁻¹ to only 1 mL m⁻² d⁻¹ by using a 0.4 µm thick
coating of TEMPO-oxidized microfibrillated cellulose. Aulin et al. [63] performed several barrier confirmation tests with both microfibrillated cellulose coating and casted films – their promising coating results are shown in Table 1.9. In this study, oxygen permeability of very low grammage films was highly dependent upon grammage (Figure 1.15). Somewhat dissimilar, Syverud and Stenius [60] found that air permeability of microfibrillated films in the 17 – 35 g/m² range had no real dependence on grammage (average air permeability of 11 nm Pa⁻¹ s⁻¹). These same researchers found that adding a 2 g/m² layer of microfibrillated cellulose to softwood paper (dynamic sheet former) could cut the air permeability in half.

Table 1.9: Barrier properties of unbleached Kraft paper coated with microfibrillated cellulose prepared from carboxymethylated fibers. [63]

<table>
<thead>
<tr>
<th>MFC Coating of 1.3 g m⁻²</th>
<th>Unbleached Kraft</th>
<th>Kraft with Coating</th>
</tr>
</thead>
<tbody>
<tr>
<td>Air Permeability (nm Pa⁻¹ s⁻¹)</td>
<td>69,000</td>
<td>0.3</td>
</tr>
<tr>
<td>Castor Oil (seconds)</td>
<td>1</td>
<td>1800</td>
</tr>
<tr>
<td>Turpentine Oil (seconds)</td>
<td>1</td>
<td>200</td>
</tr>
</tbody>
</table>
1.4  BACTERIAL CELLULOSE

1.4.1  Production

Although the most abundant form of cellulose is that produced by plants, bacteria also naturally produce the polymer. The most commonly researched bacterial species that synthesizes cellulose is *Acetobacter xylinum*; however, there are many others that can produce cellulose, including species from the genera *Agrobacterium*, *Pseudomonas*, *Rhizobium*, and *Sarcina* [64]. Bacterial cellulose is chemically identical to plant-derived cellulose, with many differences being mainly macro-structural. Both consist of polymerized glucose with $\beta$-1-4 glycosidic bonds that form into larger macromolecular fibril structures. For bacterial cellulose, these fibrils have dimensions of 3 nm x 130 nm [65], though there can be slight changes to structure and size based on cultivation conditions (Figure 1.16) [66]. A significant distinction of bacterial cellulose, in comparison to plant cellulose, is that bacterial cellulose is free of all hemicellulose and lignin.

![Figure 1.15: Oxygen permeability of microfibrillated cellulose films. [63]](image)
Figure 1.16: Bacterial cellulose fibril structures from cultivation with various carbon sources, (a) mannitol, (b) fructose, (c) galactose, (d) glucose, (e) glycerol, (f) sucrose. (taken from Mikkelsen et al., 2009 [66]). Scale bar is 100 nm.

For cultivation of \textit{Acetobacter xylinum}, most researchers use a modified Hestrin and Schramm [67] media. This media (\%, w/v) generally consists of glucose (2.0), peptone (0.5), yeast extract (0.5), anhydrous disodium phosphate (0.27), and monohydrate citric acid (0.115) with an adjusted pH of 5 – 6. Several researchers [65, 68] have also found that the addition of 1 \% ethanol or the replacement of glucose with sucrose in the media can increase cellulose production by up to six-fold. The optimum cultivation temperature for cellulose production is 28 – 30 °C under aerobic conditions.

The culture can either be agitated or static for bacterial cellulose production, with static cultures resulting in fewer \( Cel^{-} \) mutants and, thus, much higher cellulose yields [65]. Agitated cultures do produce cellulose, but the structure is round pellets instead of flat pellicles [69]. Under static cultivation conditions, the bacteria produce a dense, layered pellicle from individual cellulosic fibrils (Figure 1.17) at the air/media interface. It is
hypothesized that the pellicle helps to maintain the cells near the oxygen source since the fresh cellulose is produced at the air/pellicle interface, not the pellicle/media interface [70]. The rate of cellulose production can vary in accordance to several factors, including surface to volume ratio (Figure 1.18 [71]), glucose concentration (Figure 1.19 [71]), and carbon/nitrogen source (Table 1.10 [66]). Production of cellulose is not necessarily linear with cultivation time or nutrient source concentration – some conditions result in a constant production (after an initial lag) while others cause a tremendous spurt near the end of cultivation time [66].

Figure 1.17: SEM images of bacterial cellulose pellicles showing fibril network and layered internal structure, (a) surface of pellicle (taken from Schumann et al., 2009 [72]) and (b) cross-section of pellicle (taken from Bodin et al., 2006 [73]). Scale bar is 10 µm.
Figure 1.18: Cellulose production increases as surface to volume ratio increases (taken from Masaoka et al., 1993 [71]).

Figure 1.19: Bacterial cellulose yield can decrease if initial glucose concentration is above optimum (recreated from Masaoka et al., 1993 [71]).
Table 1.10: Bacterial cellulose yield of *G. xylinus* strain ATCC 53524 with various carbon sources at 30 °C with initial pH 5. (taken from Mikkelsen et al., 2009 [66]).

<table>
<thead>
<tr>
<th>Carbon Source</th>
<th>Cellulose Yield 48 h (g/L)</th>
<th>Cellulose Yield 96 h (g/L)</th>
<th>cFinal pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>1.89</td>
<td>3.10</td>
<td>5.33</td>
</tr>
<tr>
<td>Mannitol</td>
<td>2.04</td>
<td>3.37</td>
<td>4.91</td>
</tr>
<tr>
<td>Glycerol</td>
<td>0.82</td>
<td>3.75</td>
<td>4.81</td>
</tr>
<tr>
<td>Fructose</td>
<td>1.79</td>
<td>2.81</td>
<td>5.09</td>
</tr>
<tr>
<td>Sucrose</td>
<td>0.34</td>
<td>3.83</td>
<td>5.23</td>
</tr>
<tr>
<td>Galactose</td>
<td>0.10</td>
<td>0.09</td>
<td>5.52</td>
</tr>
</tbody>
</table>

1.4.2 Physical Properties

Like plant-derived cellulose, bacterial cellulose offers noted properties that may be beneficial for many applications. For bacterial cellulose, microfibrils do not form into individual fibers; rather, fibrils are inter-twined with each other into a dense pellicle. Thus, the fibrils cannot be easily separated from the greater structure, so physical properties are usually measured for the pellicle under different cultivation, purification, and drying conditions.

Yamanaka et al. [74] determined several mechanical properties of a restrained, air-dried bacterial cellulose pellicle sheet, including the Young’s modulus (17 GPa) and the tensile strength (256 MPa). In their work, the only altered property was tensile strength; it decreased with increasing drying pressure. They also reported that cultivation conditions did not affect the physical properties of the dried bacterial cellulose sheets. Air-dried sheets are less than 10 µm thick.
A harvested bacterial cellulose pellicle is tactilely similar to a jellyfish mantle. Seifert and co-workers [75] report that bacterial cellulose pellicle structures are approximately 87.2 % water. However, after drying, these structures cannot be fully re-swollen. For example, after freeze-drying, these structures will only contain about 73.3 % water, and after air-drying, the structure cannot be re-swollen at all. Autoclaving and alkali treatments have a rearranging or compacting effect on the structure of the pellicle, but they do not cause a change in allomorph structure [76].

1.4.3 Biocompatibility

Much research has shown that bacterial cellulose nanofibers are not cytotoxic or genotoxic through conducting Organisation for Economic Co-operation and Development (OECD) guideline protocols, including reversion and forward mutation assays, comet assays, cytogenetics assays, UDS assays, and pyrogenicity studies. Schmitt and coworkers [77] found no eye or dermal irritation in rabbits exposed to bacterial cellulose, and they also determined that the acute oral LD$_{50}$ is greater than 2000 mg per kg body weight. In addition because of the similarity of size, shape, and persistence of bacterial cellulose nanofibers to asbestos fiber, bacterial cellulose nanofiber toxicity is of concern. Yet, when bacterial cellulose was used as an in vitro matrix for fibroblasts and Chinese hamster ovary (CHO) cells, Moreira et al. [78] reported a slight reduction in cell proliferation but no change in cell morphology. Subcutaneous implantation of bacterial cellulose fibers into rats for up to 12 weeks showed no micro- or macroscopic indications of inflammation and no fibrotic capsules or giant cells. In fact, Helenius and coworkers observed that host tissue (fibroblasts) was
integrated with the bacterial cellulose [79]. Another study by Mendes et al. [80] found that only in the first 30 days of a 90 day trial was there a mild inflammation after subcutaneous implantation into mice. Also, in this study, mesenchymal stem cells attached to the bacterial cellulose exhibited normal morphology and that 95% of the attached cells were alive. Finally, Jonas and Farah [64] describe several animal and human case studies for near-commercial grade bacterial cellulose products that demonstrated success.

1.4.4 Biomedical Material

*In vitro* studies with bacterial cellulose material abound. Andersson et al. [81] successfully cultivated human chondrocyte cells on a highly porous bacterial cellulose scaffold. The depth of cell infiltration reached up to 100 µm (one-third of the scaffold thickness), and the number of cells increased four-fold over the course of two weeks. In another study, within the lumen of bacterial cellulose tubes for possible vein replacement, seeded endothelial cells grew a confluent layer within seven days [73].

With the success of *in vitro* studies, several research groups have demonstrated *in vivo* biocompatibility and functionality of bacterial cellulose materials. Working in conjunction, Schumann et al. [72] and Wippermann et al. [82] demonstrated that bacterial cellulose (BASYC®) tubes could replace small-diameter blood vessels in rat and pig specimens. Esguerra and co-workers [83] were also successful in showing initial progress in utilizing bacterial cellulose as a vascular scaffold in hamsters; although their work also reported that polyglycolic acid materials exhibited more rapid vascularization than bacterial cellulose.
1.5 POLY (GAMMA-GLUTAMIC ACID) (γ-PGA)

1.5.1 Origin

Poly (gamma-glutamic acid) or γ-PGA is an extracellular polymer excreted by several species of the bacterial genus *Bacillus*. Ivánovics and Brückner first identified γ-PGA in the capsule of the gram-positive *Bacillus anthracis* in 1937 [84]. It is considered a virulence factor for *Bacillus anthracis* as it is anti-phagocytic [85, 86], and thereby protects the cells from attack by a host’s immune system. However, most *Bacillus* species that excrete γ-PGA are non-pathogenic. The most frequently researched species of γ-PGA excreting *Bacillus* are *Bacillus licheniformis* and *Bacillus subtilis*. In addition, a common non-scientific interaction humans have with γ-PGA is in the naturally fermented soybean food called *natto* (Japan) or *chungkookjang* (Korea) (Figure 1.20).

![Mucilage of fermented soybeans containing γ-PGA.](image)

**Figure 1.20:** Mucilage of fermented soybeans containing γ-PGA.
1.5.2 Molecular Structure

Poly (gamma-glutamic acid) is a homopolymer of the amino acid glutamic acid. It is classified as a polyamide along with other naturally-occurring polyamides poly-lysine and poly-aspartic acid (Figure 1.21) [87].

![Chemical structures of three naturally occurring polyamides](image)

*Figure 1.21: Chemical structures of three naturally occurring polyamides, (a) poly(gamma-glutamic acid), (b) poly(lysine), and (c) poly(asparatic acid). [87]*

Amino acids are most commonly utilized by living cells to build protein biomacromolecules. In the primary structure of proteins, amino acids are polymerized with peptide bonds using the alpha-carbon and the alpha-amine. However, in the primary structure of γ-PGA, glutamic acid is polymerized with amide bonds using the gamma-carbon and alpha-amine [88] (Figure 1.22) – hence its name referencing the gamma linkage.
Polymerized glutamic acid can be produced synthetically or via bacterial fermentation. Artificially synthesizing poly(glutamic acid) is quite laborious [89], but it can be bought commercially. These polymers are a low-molecular weight (2 – 100 kDa) and are comprised of either L-glutamic acid or D-glutamic acid (no mixed isomer composition). These products also cost well over $1000 per gram (Sigma-Aldrich), which is well out of range for most commercial applications. Fermented polymerized glutamic acid, though, is significantly less expensive, and the polymer has different characteristics than the artificially synthesized version. The glutamic acid in the naturally-occurring polymer is a mixture of the L- and D-glutamic acid stereo-isomers (Figure 1.23) – the concentration of each isomer depends on the cultivation conditions and the strain of Bacillus producing the polymer. The D-glutamic acid
isomer is usually predominant because the turn-over rate for the polymerizing enzyme (PgsBCA) is about four-fold higher with D-isomer versus the L-isomer [90, 91]. The degree of polymerization (DP) of γ-PGA can be up to 700 units with molecular weights ranging from 10 – 3000 kDa [92].

Figure 1.23: Stereo-isomers of glutamic acid, (a) L-glutamic acid and (b) D-glutamic acid.

With its pendant carboxylic acid, the γ-PGA polymer can exist in either a free-acid form, a salt-form, or in a mixed ionization state. The polymer is generally in its ionized form after it is excreted from the bacterial cell. Its pKa is dependent on the alpha-carboxylic acid and is considered to be 2.3. The conformation of γ-PGA in solution depends on the ionization degree of the polymer. Generally, in its free-acid form, it is helical [93, 94]. Circular dichroism techniques have described the conformations for γ-PGA as highly dependent on pH, and in an aqueous solution of low ionic strength, the polymer exhibits a highly extended chain conformation [89].
1.5.3 Properties

Even though γ-PGA is a virulence factor for *Bacillus anthracis*, the polymer and its monomer units in themselves are non-toxic [95, 96]. It is also biodegradable [87] and edible (common East Asian food). Both the free-acid and salt-forms of γ-PGA are water-soluble. The carboxylic acid found on every unit of the polymer confers a hygroscopic nature. Precipitation of γ-PGA from aqueous solutions can be accomplished using common protein precipitation chemicals, such as alcohols or acetone.

Several means to degrade γ-PGA have been described. As an amide-bonded polymer, γ-PGA can be hydrolyzed with strong acids. Oppermann et al. found that at least 12 different bacteria species isolated from soil, fresh water, and sewage could degrade γ-PGA [87]. Ultra-sonic irradiation (20,000 Hz for 2 hours) fragments high molecular weight γ-PGA into average molecular weights that are 25% of their original size [97]. Temperatures higher than 60 °C in the presence of free-radicals from radiation treatments can also hydrolyze the polymer. When no free-radicals are present, γ-PGA was found to not hydrolyze significantly until exposure to sustained temperatures above 80 °C [98]. However, even 15 minutes exposure to temperatures of 100 °C or 120 °C resulted in number-average molecular weights that were 80% or 30%, respectively, of the original weights. Portilla-Arias et al. [99] completed a thermo-degradation study on free-acid form γ-PGA that supported degradation of γ-PGA to volatile pyroglutamic acid between 250 – 300 °C by end-of-chain unzipping (Figure 1.24). This same study corroborated their results by showing that number-average molecular weight was inversely proportional to increasing temperature.
1.5.4 Synthesis

Unlike proteins which are polymerized by a ribosome, γ-PGA is polymerized using an enzymatic complex called pgsBCA [100] (Accession No. AB016245). A simplified synthesis route is illustrated in Figure 1.25 [96, 101, 102].

**Figure 1.24:** End-of-chain unzipping of γ-PGA to pyroglutamic acid at 250 °C. [99]

**Figure 1.25:** Microbial synthesis route for γ-PGA involving enzymatic complex pgsBCA (taken from Buescher and Margaritis, 2007). [96, 101, 102]
1.4.5 Fermentation

Determining the essential components and methods of producing γ-PGA was initially focused on understanding the pathogenic *Bacillus anthracis*. However, with γ-PGA recognized as a polymer with desirable properties, scientific inquiry shifted toward optimizing the cultivation.

Species of *Bacillus* are aerobic chemo-organo-heterotrophs, so cultivation studies have primarily focused on selecting carbon sources to push γ-PGA production rather than cell growth or unwanted chemical/polymer production. Table 1.11 lists several selected *Bacillus* strains cultivated with various carbon and nitrogen sources. Cultivation temperature of γ-PGA is between 30 and 37 °C.
Table 1.11: Selected production results of several γ-PGA producing *Bacillus* strains.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Main Nutrients (g/L)</th>
<th>Culture Time (hrs)</th>
<th>γ-PGA (g/L)</th>
<th>Molecular Wgt (Da)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>B. licheniformis</em> ATC 9945a</td>
<td>citric acid (12) glycerol (80) glutamic acid (20)</td>
<td>96</td>
<td>20</td>
<td>$9.8 \times 10^5$</td>
<td>[103]</td>
</tr>
<tr>
<td><em>B. subtilis</em> IFO3335</td>
<td>citric acid (20) glutamic acid (30) NH$_4$SO$_4$ (20)</td>
<td>80</td>
<td>20</td>
<td>$2.0 \times 10^6$</td>
<td>[104]</td>
</tr>
<tr>
<td><em>B. subtilis</em> TAM-4</td>
<td>fructose (75) NH$_4$Cl (18)</td>
<td>96</td>
<td>22</td>
<td>$1.6 \times 10^6$</td>
<td>Ito</td>
</tr>
<tr>
<td><em>B. subtilis</em> F-2-01</td>
<td>glucose (1) glutamic acid (70)</td>
<td>96</td>
<td>48</td>
<td>$1.0 \times 10^6$</td>
<td>[105]</td>
</tr>
<tr>
<td><em>B. subtilis</em> ZJU-7</td>
<td>glucose (60) tryptone (60) glutamic acid (80)</td>
<td>24</td>
<td>54</td>
<td>$1.2 \times 10^6$</td>
<td>[106]</td>
</tr>
<tr>
<td><em>B. subtilis</em> NRRL B-2612</td>
<td>wheat gluten (200) K$_2$HPO$_4$ (10)</td>
<td>30</td>
<td>10</td>
<td>$2.0 \times 10^4$</td>
<td>[107]</td>
</tr>
</tbody>
</table>

Most strains of *Bacillus* do not naturally synthesize glutamic acid, so an exogenous source of glutamic acid must be supplied. Only a few strains have been identified as glutamic acid synthesizers, including *Bacillus subtilis* TAM-4 and *Bacillus licheniformis* A35 [92]. Some researchers have circumvented this obstacle by transforming glutamic acid producing bacteria with a plasmid containing the *pgsBCA* gene or co-cultivating a non-glutamic acid producing *Bacillus* with a glutamic acid producing bacteria [96].
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2. Properties of Surface Acetylated Microfibrillated Cellulose Relative to Intra- and Inter-Fibril Bonding
2.1 ABSTRACT

Microfibrillated cellulose (MFC) created via the grinding method was heterogeneously acetylated to different substitution levels using acetic anhydride and heat rather than strong acid catalysis. The acetylated MFC was formed into thin films and characterized by infrared spectroscopy and mechanical testing. Spectral and chemical analysis confirmed controlled acetylation with increased reaction time. Due to microfibrils forming more inter-fibril bonds than whole fibers, it is generally accepted that there are more hydrogen bonds to carry the applied load. However, with increased acetylation, the initial number of possible hydrogen bonds was decreased which led to a lower level of internal stresses and a more uniform material structure. At 28% substitution, the tensile index decreased to 50% of the initial tensile, whereas the rupture energy was nearly completely eliminated. These acetylated fibrils, thus, created a structure that resembled the energy absorption behavior of whole fiber sheets but with the maintenance of load-bearing capacity of unmodified fibrils.

2.2 INTRODUCTION

Cellulosic paper-making fibers are renowned for their substantial strength properties, with the added benefits of abundance, renewability, and biodegradability. Studies have also shown that more extensively processed cellulosic fibers (Figure 2.1), such as microfibrillated cellulose (MFC), have particularly advantageous properties. Microfibrils are mechanically liberated from the fiber through homogenization (Figure 2.2) or grinding (Figure 2.3).
Figure 2.1: Cellulosic plant structures, (a) plant fiber and (b) microfibrils liberated from fiber (taken from Nakagaito and Yano, 2005 [1]). Scale bar is 10 µm.

Figure 2.2: High-pressure homogenizer showing internal mechanisms. (APV Gaulin, Inc.)

Figure 2.3: Grinder unit and bottom plate of a typical grinder to generate microfibrillated cellulose from cellulose fiber. (Masuko Sangyo Co.,Ltd.)
The resultant microfibril has typical dimensions of less than 100 nm with varied lengths (usually in the range of several micrometers) [2], which means an aspect ratio of three times that of a papermaking fiber [3]. This aspect ratio can be beneficial for mechanical properties of resultant materials. For papermakers, it might be somewhat analogous to paper made from softwood fiber having higher strength properties than paper from hardwood fibers. MFC produced through homogenization also has about a 10-fold increase in specific surface area over non-processed fiber [3], which corresponds to exponential increases in exposed surface hydroxyl groups. By mechanically processing the fibers into microfibrils, both the amorphous and crystalline regions of the cellulose structure are maintained [4], – whereas with chemical breakdown of fiber structure, much of the amorphous region is lost [5]. Both regions are important for the unique properties of cellulosic fibers and fibrils. Research has shown that MFC can be the sole constituent for innovative materials. When the microfibrils are formed into thin films or used as a coating, they exhibit markedly high barrier [6-8] and strength properties (Table 2.1).

<table>
<thead>
<tr>
<th>Source Fiber</th>
<th>Processing Method</th>
<th>Film Method</th>
<th>Elastic Modulus (GPa)</th>
<th>Tensile Index* (N m g⁻¹)</th>
<th>Strain at Break (%)</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>softwood</td>
<td>TEMPO</td>
<td>casting</td>
<td>6.9</td>
<td>160</td>
<td>7.6</td>
<td>[6]</td>
</tr>
<tr>
<td>hardwood</td>
<td>TEMPO</td>
<td>casting</td>
<td>6.2</td>
<td>153</td>
<td>7.0</td>
<td>[6]</td>
</tr>
<tr>
<td>softwood</td>
<td>homogenizer</td>
<td>casting</td>
<td>15.7 – 17.5</td>
<td>129 – 146</td>
<td>5.3 – 8.6</td>
<td>[8]</td>
</tr>
</tbody>
</table>
The first reports of MFC by Herrick et al. and Turbak et al. demonstrated their use as additives, binders, or emulsifiers [9, 10]. In the past decade, studies are now revealing that MFC can be used as the reinforcing agent or the matrix for advanced materials [1, 6, 11-17]. The hydrophilic nature of cellulose, though, is often problematic for interface compatibility in composites with non-polar matrices [18-20]. Chemical modification of the cellulosic fibril surface can help overcome this obstacle. Cellulose derivatives, such as cellulose acetate or carboxymethyl cellulose, can be produced in a manner that destroys the beneficial internal structure of the cellulosic microfibril [21]. Therefore, research into reactions that modify only the cellulose surface [22, 23] has been performed to maintain the native cellulosic structure. These derivatives of MFC include carboxymethylation [24], silylation [15, 16, 25, 26], and charged surfaces [17]. In addition, there has been progress in the modification of cellulose surfaces by acetylating micro-crystals to create a more matrix-compatible reinforcing agent [23].

Heterogeneous acetylation causes a decrease in paper sheet properties [27, 28] (Figure 2.4 and Figure 2.5) at substitution levels of approximately 25 – 30 %. Nissan and Higgins used this data to conclude that only 33 % of the hydroxyls in cellulose were responsible for building sheet strength. However, experiments have shown the commercial potential for acetylated cellulose fibers. Herdle and Griggs [29] reported on the increased hydrophobicity of cellulose fibers which caused a decrease in water absorption and water/oil permeability.
Figure 2.4: Elastic modulus and rupture energy of acetylated whole fiber sheets.
(taken from Nissan and Higgins, 1959 [27])

Figure 2.5: (a) Tensile index and (b) elastic modulus of acetylated whole fiber sheets.
(re-constructed from Aiken, 1943 [28])
On a practical level, the acetylation of microfibrils – rather than whole fiber [27] – offer an opportunity in future research to maintain a percentage of sheet strength while benefiting from the hydrophobicity of the acetylated fibril. However, this work primarily focuses on examining the response of MFC to various degrees of acetylation, so that one can better understand the mechanism by which the MFC films generate strength.

2.3 MATERIALS and METHODS

2.3.1 Materials

All chemicals were Certified ACS grade, purchased from FisherScientific. Acetic acid and acetic anhydride were used as received. Hydrochloric acid and sodium hydroxide solutions were made from purchased 1 N solutions, and ethanol was diluted to a 70 % solution with deionized water. The starting material for the microfibrillated cellulose was bleached softwood kraft pulpsheets. Deionized water was used in all experiments.

A Büchner funnel apparatus complete with a 1 L vacuum flask and 11 inch funnel was used. Filters included Whatman 540 and 41. To generate microfibrillated cellulose, a Masuko SuperMassColloider and Hamek PFI Mill were employed. Other equipment included a Wiley Mill fitted with a 20-mesh screen (aperture size of 0.89 mm), silicon oil bath controlled by a temperature probe, stirring motor with propeller, and a 10 L water bath. Instruments included a Thermo-Nicolet Nexus 670 FTIR ESP spectrophotometer equipped with an ATR accessory (germanium crystal) and an Instron 4411 with a 500 N load cell.
2.3.2 Methods

2.3.2.1 Production of Microfibrillated Cellulose (MFC)

To create MFC, disintegration followed by grinding and PFI milling was performed. Bleached kraft hardwood pulpsheets were hand torn into approximately 2 inch squares (avoiding the edges of the pulpsheet) and soaked overnight in a cold room at 2 % solids in order to swell the fibers and allow for better fibril processing. The swelled pulpsheet pieces were disintegrated in a hydropulper at 2 % consistency. The resulting pulp was then passed nine times through a 10 inch Masuko SuperMassColloider at 2 % solids. Using a Büchner funnel apparatus, the pulp was vacuum-dewatered over a Whatman 540 filter paper to about 6 % solids and further refined with a PFI mill for 9000 revolutions. The resulting microfibrillated cellulose (MFC) was stored at 4 °C at 6 % solids until use.

2.3.2.2 Acetylation of Fibrils

MFC (2.5 g for each batch) was soaked in 100 mL of glacial acetic acid for two days in stoppered flasks. Using a Büchner funnel apparatus, the MFC was rinsed three times with fresh glacial acetic acid to remove the water associated with the fibers. The MFC was at about 20 % solids after the final rinsing and filtering. Each batch of filtered MFC was placed in a condenser-equipped round bottom flask followed in sequence with 75 ml glacial acetic acid and 175 ml acetic anhydride. Each batch was vigorously agitated with a propeller-type mixer to maintain homogeneous heating and chemical mixing.
The reaction (Figure 2.6) was performed at 100 °C by immersing the round bottom flask in a silicon oil bath; feedback on the temperature was accomplished by equipping the flask with an immersed temperature probe. After the 15 minute heating to 100 °C, the batch mixtures reacted for 0.5, 6, and 12 hours. The samples were removed from the flasks into a Büchner funnel apparatus with Whatman 540 filters to vacuum-remove any remaining reagents. Once the samples appeared free of reagents, the samples were rinsed three times with water in the Büchner funnel apparatus to remove all un-reacted reagent and to achieve a pH between 6 and 7 (checked by litmus paper). All samples were then soaked overnight in water and stored at 4 °C. The next day, the samples were all rinsed for a final time in the Büchner funnel apparatus with water and then stored at 4 °C at about 1 % consistency.

2.3.2.3 Determination of Degree of Substitution

Samples from each reaction time were dewatered to about 20 % solids, frozen at −80 °C, and then freeze-dried overnight. The samples were then heated in an oven at 105 °C for 1 hour to remove remaining water. The samples were then Wiley milled using a 20 mesh screen followed by an additional hour in a 105 °C oven to ensure proper sample weight for titration.
In a glass Erlenmeyer flask, 100 mg of sample was suspended in 40 mL of 75 % (v/v) ethanol. The flask, loosely stoppered, was heated to 50 – 60 °C in a water bath for 30 min for better swelling of the material. Then, 40 mL of 0.5 N NaOH solution was added to the sample flask (loosely stoppered), and the mixture was heated to 50 – 60 °C in a water bath for 15 min. The flask was stoppered tightly and allowed to stand at room temperature for 72 hours. Blank flasks were also prepared according to the same procedure, with the exception that no sample was added to the blank flasks.

The excess alkali (the volume which was not used to saponify the acetyl groups) was then titrated with 0.5 N HCl using phenolphthalein as an indicator. At the endpoint, 1 mL excess 0.5 N HCl solution was added, and any remaining NaOH was allowed to diffuse from the fibers overnight. Any excess HCl was then titrated with 0.5 N NaOH solution using phenolphthalein as an indicator. The mixture was vigorously shaken to ensure all excess HCl was consumed, which was indicated by a persistent faint pink endpoint.

Percentage of acetylation is determined on the whole fibril fraction, not just for the surfaces. Also, the calculation is on a mass basis and can be defined as the ratio of acetyl group mass to the mass of the entire sample (Equation 1).

\[
\% Acetyl = \frac{\text{mass of acetyl ester}}{\text{mass of sample}} \times 100
\]  
(1)
It is important in calculating the level of substitution that the molar mass of the actual substitute group be used. For acetylation, the substitute group is the acetyl ester (containing 2 carbon atoms, 3 hydrogen atoms, and 1 oxygen atom), so its molar mass is equivalent to 43.05 grams. Thus, the mass of the acetyl ester can be found using Equation 1.1 below,

\[
\text{mass of acetyl ester} = \frac{(D - C) \text{ mL HCl}}{1 \text{ L HCl}} \times \frac{N_a \text{ eq. HCl}}{10^3 \text{ mL HCl}} \times \frac{1 \text{ L HCl}}{1 \text{ eq. HCl}} \times \frac{1 \text{ eq. acetyl ester}}{1 \text{ eq. acetyl ester}} \times \frac{43.05 \text{ g acetyl ester}}{1 \text{ eq. acetyl ester}}
\]

where,

\[
C = \text{HCl solution required for titration of sample, mL}
\]
\[
D = \text{HCl solution required for titration of blank, mL}
\]
\[
N_a = \text{normality of HCl solution}
\]

simplified,

\[
\text{mass of acetyl ester} = [(D - C) \cdot N_a \cdot 10^{-3} \cdot 43.05] \text{ grams}
\]

substituting the mass of acetyl ester calculation above (Eqn 1.2) into Equation 1, which generates Equation 1.3

\[
\%\text{Acetyl} = \frac{(D - C) \cdot N_a \cdot 10^{-3} \cdot 43.05}{\text{mass of sample}} \times 100
\]
Thus, the final calculation for percentage of acetylation can be found using Equation 1.4

\[
\%Acetyl = \frac{(D - C) \cdot N_a \cdot 4.305}{mass\ of\ sample} \quad (1.4)
\]

The degree of substitution indicates the number of hydroxyl groups substituted (thus number of acetyl esters) per anhydrous glucose unit. It can be derived from the percentage of acetylation value found using Equation 1 above. The actual calculation is shown by Equation 1.8 below.

\[
\%Acetyl = \frac{mass\ of\ acetyl\ ester}{mass\ of\ sample} \times 100 \quad (1)
\]

\[
\%Acetyl = \frac{43 \cdot DS}{162 + 42 \cdot DS} \times 100 \quad (1.5)
\]

where,

- mass of acetyl ester = 43 grams
- number of acetyl esters per anhydrous glucose = DS
- mass of anhydrous glucose unit = 162 grams
- additional mass from esterification with acetate = 42 grams
rearranging,

\[ DS = \frac{162 \cdot (\text{\%Acetyl}/100)}{43 - 42 \cdot (\text{\%Acetyl}/100)} \]  

(1.6)

simplifying,

\[ DS = \frac{162 \cdot \text{\%Acetyl}}{4300 - 42 \cdot \text{\%Acetyl}} \]  

(1.7)

\[ DS = \frac{3.86 \cdot \text{\%Acetyl}}{102.4 - \text{\%Acetyl}} \]  

(1.8)

2.3.2.4 Film-Making

Films were prepared by vacuum-dewatering followed by restrain-drying (Figure 2.7). The acetylated MFC slurry (diluted to 0.2 % solids) was vigorously mixed to ensure complete fibril dispersion. A Büchner funnel with two 11 cm-diameter Whatman 41 filters was set-up by wetting the filters and vacuum-sealing them to the mouth of the funnel. Vacuum was removed, and the acetylated MFC slurry was poured into the funnel – care was taken to not overload one section of the funnel with fibrils. The vacuum was quickly reapplied. Also, the funnel was adjusted to level with a one-foot level.
Once the films were at about 20% solids, vacuum was removed and the films plus filters were removed from the Büchner funnel. The films were carefully peeled from the filter papers and pressed flat onto a stainless steel plate. Using rings, the films were restrained dried overnight at room temperature. Thicknesses of the resultant films were 30 – 70 µm.

2.3.2.5 Fourier Transform Infrared Spectroscopy – Attenuated Total Reflectance

Thin film sample spectra were recorded using the OMNI-Sampler single-reflection ATR accessory of a Thermo-Nicolet Nexus 670 FTIR ESP spectrophotometer. The spectra were an accumulation of 128 scans, with a resolution of 4 cm\(^{-1}\) from 800-4000 cm\(^{-1}\). Absorbance spectra were analyzed using Omnic software. The absorbance of the CH band at 2890 cm\(^{-1}\) was measured and all peaks were normalized to this value. The CH band should remain constant through the reaction process and thus serves as an internal standard. The absorbencies of three major bands associated with acetylation were measured: C=O band (1745 cm\(^{-1}\)), C–CH\(_3\) band (1375 cm\(^{-1}\)), and C–O (1240 cm\(^{-1}\)).
2.3.2.6  Mechanical Property Testing

Rectangular sample strips (5 x 30 mm) were cut from the center sections of the prepared films. An Instron 4411 was equipped with a 500 N load cell and operated with a 2 mm/min extension rate and 10 mm gap width. The resulting stress-strain data (Figure 2.8) was then analyzed to determine several mechanical properties, including tensile index, elastic modulus, and the rupture energy (Table 2.2).

Table 2.2: Mechanical properties determined from Instron tensile testing

<table>
<thead>
<tr>
<th>Mechanical Property</th>
<th>Calculation Basis*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tensile Index</td>
<td>max load per unit width normalized to the basis weight</td>
</tr>
<tr>
<td>Elastic Modulus</td>
<td>slope of the initial linear region of the load-elongation curve</td>
</tr>
<tr>
<td>Rupture Energy</td>
<td>integration under the entire load-elongation curve</td>
</tr>
</tbody>
</table>

* All calculations performed using MatLab script created by Christopher S. Saunders

Figure 2.8: Representation tensile loading curves for each reaction time.
2.3.2.7 Contact Angle

Samples with 1 cm edges were cut from films. Each sample was secured to a glass slide with double-sided tape. A SEO Phoenix Dynamic Contact Angle instrument with Image XP software captured the droplet images. The contact angle from each image was measured using the NIH software ImageJ.

2.4 RESULTS and DISCUSSION

2.4.1 Determination of Degree of Substitution

The degree of substitution (DS) of acetylated MFC as determined by titration is plotted in Figure 2.9 against reaction time (hours at 100 °C). After 12 hours, the highest DS obtained was 0.77 DS. This level of substitution indicated that approximately 26 % of the available hydroxyl groups in the whole fibril fraction were substituted with an acetyl group. The discrepancy between the two 12 hour samples is most likely caused by slow initial heat-up.

![Figure 2.9](image)

**Figure 2.9:** (a) Degree of substitution (DS) and (b) % OH substituted of acetylated MFC.
2.4.2 Film-Making

Thin films were flat and translucent and had uniform appearance. Images of the films after varying reactions times are shown in Figure 2.10. It is noted that little change in the appearance between the films can be seen, which can be attributed to the relatively high level of compacting of the fibrils and elimination of air voids within the matrix even at the highest levels of acetylation. The material properties for the films are listed in Table 2.3. All films were strong enough to handle.

![Figure 2.10: Translucent thin films formed from fibrils acetylated for 0, 0.5, 6, and 12 hr.](image)

<table>
<thead>
<tr>
<th>Reaction</th>
<th>Thickness (µm)</th>
<th>Grammage (g / m²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0hr – Rxn 1</td>
<td>32.43 (0.20)</td>
<td>37.59 (0.20)</td>
</tr>
<tr>
<td>0hr – Rxn 2</td>
<td>32.86 (1.41)</td>
<td>37.73 (0.93)</td>
</tr>
<tr>
<td>0.5hr – Rxn 1</td>
<td>30.71 (3.03)</td>
<td>34.48 (4.08)</td>
</tr>
<tr>
<td>0.5hr – Rxn 2</td>
<td>43.43 (3.03)</td>
<td>49.98 (3.83)</td>
</tr>
<tr>
<td>6hr – Rxn 1</td>
<td>45.86 (1.41)</td>
<td>42.80 (3.36)</td>
</tr>
<tr>
<td>6hr – Rxn 2</td>
<td>67.71 (7.07)</td>
<td>46.13 (4.63)</td>
</tr>
<tr>
<td>12hr – Rxn 1</td>
<td>70.17 (0.71)</td>
<td>55.58</td>
</tr>
<tr>
<td>12hr – Rxn 2</td>
<td>42.92 (2.47)</td>
<td>35.68 (1.13)</td>
</tr>
</tbody>
</table>
For the more highly acetylated MFC films, the densities decreased (Figure 2.11), although the materials remained relatively translucent through the range of reaction times. Kubelka and Munk [30] noted that light is scattered from interfaces within a material. Thus, the lack of opacity is closely associated with the lack of scattering surfaces (i.e. no large voids in the structure). One contributing explanation for the decrease in film density and the maintenance of translucence, then, is that for a heterogeneous reaction, the density of a cellulose fiber decreases linearly with increased acetylation [31]. Density changes in the MFC itself would not be large enough to scatter visible light (wavelength of visible light is 400 – 800 nm; much larger than any “pores” developing on the MFC).

Figure 2.11: Densities of acetylated MFC films for each level of substitution.
Assuming that the MFC has the same density as cellulose (1550 kg/m³), we can determine the air void content within the material (Equation 2):

\[
(1 - v) \left( 1550 \frac{kg}{m^3} \right) + (v) \left( 1.23 \frac{kg}{m^3} \right) = 1200 \frac{kg}{m^3}
\]

where \(v\) represents the air void content, 1550 kg/m³ is the density of cellulose (or non-acetylated MFC), 1.23 kg/m³ is the density of air, and 1200 kg/m³ is the density of the non-acetylated MFC film. Thus, an air void content of 0.23 can be calculated for the non-acetylated MFC film.

Assuming that the air void content (0.23) remains constant, the density for each acetylated MFC can be calculated. These densities are plotted in Figure 2.12.

![Figure 2.12](image.png)

**Figure 2.12:** The calculated densities of the acetylated MFC via Eqn 2, assuming constant air void fraction. Density of cellulose acetate is marked with dashed line.
The calculated acetylated MFC film densities fall well below the literature value for cellulose acetate (1300 kg/m³) at hydroxyl substitution levels above 15%. This observation indicates that the structure of the material must be changing, which could result from a number of causes. First, the increased hydrophobic nature of the cellulose acetate could reduce the surface tension effects on the consolidation of the MFC structure during drying. Second, the MFC could become less flexible making it more difficult for these materials to conform to one and other. Third, the acetylation reaction could be disrupting hydrogen bonds with the fibril structure itself, thereby releasing natural internal stresses within the material. The release of these stresses could cause the expansion of the material which lowers its overall density. The phenomenon in the third explanation was also observed by Lee and Pawlak (unpublished results) during successive drying and humidification cycles. They attributed the changes in dimension and volume in part to the release of internal stresses within the fibrils. The acetylation may have a similar effect in that it disrupts intra-fibril hydrogen bonding resulting in a lower density of MFC films.

2.4.3 Fourier Transform Infrared Spectroscopy − Attenuated Total Reflectance

Figure 2.13 shows the spectra obtained from FTIR-ATR analysis of acetylated MFC. The peaks of interest are C=O (1745 cm⁻¹), C−CH₃ (1375 cm⁻¹), and C−O (1240 cm⁻¹). These peaks can be used to characterize the amount of acetylation owing to the fact that they are only present when the cellulose is acetylated. The absorbance spectra are normalized to the CH peak, which is unaffected by acetylation and, thus, can be used as a direct indication of the amount of cellulose examined during the FTIR-ATR analysis. The spectral
characterization of the degree of substitution showed a direct relationship to the degree of substitution characterized by titration (Figure 2.14). This correlation is an indication that FTIR is representing changes in the chemistry of the material as expected.

**Figure 2.13:** FTIR-ATR spectra of MFC acetylated to various levels by increasing the reaction time at 100 °C.
FTIR-ATR can also be used to better understand the structure of the materials. The Lateral Order Index (LOI) (absorbance 1428 cm\(^{-1}\)/absorbance 898 cm\(^{-1}\)) is one measurement of the amount of ordering with the structure. A number of researchers [32-35] have reported on the LOI for cellulose of various forms and crystallinity. As the LOI increases, the degree of ordering (crystallinity) increases. Figure 2.15 shows the LOI for the acetylated MFC over the levels of substitution. The LOI is relatively constant over this range of reactions times with the mean being slightly higher for the samples substituted at levels higher than 15%. Order within the material may be increasing slightly during these later stages of acetylation. It is widely accepted that acetylation proceeds from the surface hydroxyls and into the amorphous regions of the structure. This process would increase the overall crystallinity of the structure. Also, if the acetylation is disrupting the inter-fibril hydrogen bonding network and relieving stress on the crystal structure, then the removal of the stress may allow for the
crystals within the fibrils to increase in order slightly. This hypothesis is consistent with what is observed in humidity cycling where stress is relieved on the fibril structure, and it returns to a lower thermodynamic state.

![Figure 2.15: The Lateral Order Index (LOI) for acetylated MFC. (error bars show one standard deviation)](image)

**2.4.4 Mechanical Property Testing**

The mechanical properties of these materials can provide significant insight into how micro-fibrillated cellulose films develop their strength characteristics. MFC films are noted for their toughness, high modulus, and overall tensile strength. These characteristics can be attributed to the large amount of inter-fibril bonding that takes place in the films. The small size and relatively high specific surface area of the fibrils allows for these materials to generate very high tensile strength when compared to traditional paper sheets. Table 2.4 contains a listing of the tensile index (tensile strength/basis weight), elastic modulus, and
rupture energy for the acetylated MFC films. As expected, the materials lose a large fraction of their tensile index and modulus with increased acetylation. This observation is attributed to the fact that acetylation disrupts the hydrogen bonds between fibrils, which reduces the strength. In fact, the rupture modulus approaches zero at the highest level of acetylation.

Table 2.4: Mechanical properties of thin films created with acetylated MFC.

<table>
<thead>
<tr>
<th>Reaction</th>
<th>Tensile Index (Nm/g)</th>
<th>Elastic Modulus (MPa)</th>
<th>Rupture Energy (MJ/m³)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0hr – Rxn 1</td>
<td>76.67 (2.90)</td>
<td>3202.06 (236.35)</td>
<td>8.32 (0.09)</td>
</tr>
<tr>
<td>0hr – Rxn 2</td>
<td></td>
<td>3210.84 (348.02)</td>
<td>7.31 (0.65)</td>
</tr>
<tr>
<td>0.5hr – Rxn 1</td>
<td>84.51 (2.25)</td>
<td>3127.66 (231.23)</td>
<td>9.33 (0.09)</td>
</tr>
<tr>
<td>0.5hr – Rxn 2</td>
<td></td>
<td>3248.33 (186.17)</td>
<td>9.50 (0.27)</td>
</tr>
<tr>
<td>6hr – Rxn 1</td>
<td>52.65 (3.47)</td>
<td>2232.15 (223.92)</td>
<td>1.14 (0.01)</td>
</tr>
<tr>
<td>6hr – Rxn 2</td>
<td></td>
<td>1676.99 (194.25)</td>
<td>0.88 (0.19)</td>
</tr>
<tr>
<td>12hr – Rxn 1</td>
<td>44.41 (2.00)</td>
<td>1673.23 (114.95)</td>
<td>0.53</td>
</tr>
<tr>
<td>12hr – Rxn 2</td>
<td></td>
<td>1808.43 (122.60)</td>
<td>0.73 (0.09)</td>
</tr>
</tbody>
</table>

It is worth pointing out that at a hydroxyl substitution level of 28% (12 hours of reaction), the rupture energy is almost zero (but not completely eliminated). A 28% substitution level is far below the value of all the hydroxyl groups being substituted. This observation is a direct indication of the importance of the surface hydroxyl groups on the strength of the material. Nissan et al. [27] as well as Aiken [28] demonstrated a similar phenomenon with paper sheets, although they lost the strength of the paper sheet (and all measureable rupture
energy) at a noticeably lower degree of substitution (Figures 2.4 and 2.5). This difference can be attributed to the fact that MFC has a higher specific surface area when compared to whole wood fibers. It has also been hypothesized, though, that since MFC have such a pronounced aspect ratio (in comparison to whole fibers), there may be an element of tangling which could enhance the integrity of the resultant films.

In the comparison of whole fiber versus MFC sheets, the MFC material will undoubtedly contain more inter-constituent bonds per volume due to the 10-fold increase in specific surface area. The un-acetylated MFC has a lower number of intra-constituent bonds, but according to the Page equation, the fiber strength is less dominant than fiber-fiber bonds in developing strength. In both systems, though, the bonding network can be considered non-uniform. As loading is placed on the system, localized stresses will be relieved causing both bond breakage and unrecovered elastic energy losses. It must be kept in mind that the number of bonds participating in localized stresses for an MFC sheet is many more than that involved with a paper sheet – thus causing an additive effect for load carrying capacity. As the localized stresses are relieved, the system will continue to carry load (and exhibit strain) until all other bonds are broken (increasing the energy absorbed by the system). Thus, with MFC sheets, there will be an increased load over paper sheets as well as an increase in absorbed energy. An increased load measurement from the increase in number of bonds in the system results in an increase in tensile strength. Also, as the number of bonds broken increases, the amount of energy absorbed (rupture energy) and lost to the system increases.
Figure 2.16: Preservation of mechanical properties of acetylated MFC as a function of hydroxyl groups substituted (a) tensile index, (b) energy absorption (toughness).
However, as the MFC film is acetylated, the number of inter-constituent bonds decreases as well as the number of intra-constituent bonds. During acetylation, the most vulnerable hydroxyl groups are substituted first, i.e. those on the surface and through the amorphous region of the fibril. These structural changes result in the release of internal fibril stresses even prior to material formation. Thus, this un-recovered energy is not even measured in the tensile testing regime. In measuring increasingly acetylated MFC films, the tensile strength is largely maintained, while the rupture energy is virtually eliminated. Yet, in acetylated whole fiber sheets, both tensile strength and rupture energy are eliminated at the same levels of substitution. This is expected because with acetylation, the density changes which indicates a loss in the number of inter-constituent bonds, so the load-carrying capacity will consequentially decrease. However, with MFC sheets, there are so many more bonds in the entire system initially that even a high bond-number reduction may not be as detrimental to developing strength as the same reduction in acetylated whole fiber sheets. The heterogeneous acetylation reaction has not been fully characterized, but it is understood that hydroxyl substitution will occur on the surface and throughout the amorphous region which means the low-energy crystalline region will remain. Plus with the loss of internal stresses, the intra-fibril bonds will also dampen to a lower energy state. Crystalline regions carry loads much more uniformly, which dictates that, upon loading, the acetylated MFC sheet (which has a higher crystalline load-bearing fraction) may not be able to absorb as much energy as the un-acetylated MFC sheets. Combining the affect of structural changes along with the reduction in the number of breakable bonds outside the fracture zone of the material, the rupture energy of the MFC sheets should follow the same trend as whole fiber sheets.
2.4.5 Contact Angle

Preliminary results for further research in applications of acetylated fibrils were collected by measuring the contact angle of the films. As shown in Figure 2.17, an increase in hydrophobicity occurred with an increase in substitution. The hydrophobicity plateaus which could further indicate that the acetylation is proceeding to the interior of the fibril and no longer affecting the surface hydroxyls but affecting the interior fibril structure.

![Figure 2.17: Contact angles of acetylated MFC films.](image)

2.5 CONCLUSIONS

Microfibrillated cellulose (MFC) was generated via the grinding method. Heterogeneous acetylation with heat rather than using a strong acid was accomplished using acetic
anhydride. The acetylated MFC was formed into thin films and characterized. Fibrils are capable of forming more fibril-to-fibril bonds than traditional fibers, so they can carry a much higher applied load. The acetylation retards the ability of fibrils to form bonds during the sheet formation process which would lead to lower internal stresses and a more uniform, crystalline structure. The plateau in the contact angle above 15 % hydroxyl substitution shows that acetylation proceeded to the interior of the fibrils. The densities of the films substituted at above 15 % were used to show that fibril density was decreasing with acetylation and was, in fact, lower than the density of cellulose acetate. Since the acetylation attacks the amorphous regions first, the crystallinity of the fibrils may have also increased slightly as indicated by the LOI analysis. This change leads to even more uniform distribution of stress. Both of these effects could explain how the acetylated fibril structure could not absorb as much energy as the unmodified fibril structure and even resembled the behavior of a paper sheet. The tensile strength was, nevertheless, maintained because even if the acetylation restricted hydrogen bonding, the level of initial hydrogen bonding was much augmented in comparison to traditional whole fibers.

2.6 REFERENCES


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3. Blends of Microfibrillated Cellulose with Poly (gamma-Glutamic Acid)
3.1 ABSTRACT

Biosynthesized poly(γ-glutamic acid) (γ-PGA) has desirable water-interaction properties. Alone, cellulose fibers can be used to produce biodegradable, high-strength materials; however, by mixing an interactive polymer with cellulosic fiber derivatives, materials with augmented properties can be generated. Through mechanical refining, microfibrillated cellulose (MFC) was liberated from chemically pulped wood fibers. After forming the MFC into thin films of 80 g/m², increasing levels of γ-PGA was entrained in the films. The films were heat treated at 150 °C to alter mechanical and water-interaction properties. Also, non-heat-treated films were recycled, and the presence of γ-PGA aided in the recovery of material properties for the recycled-fibril films. The presence of γ-PGA also increased tensile strength, water absorption and transmission, as well as contact angle. However, heat treatment only increased the tensile strength of the MFC matrix and decreased the water absorption and transmission of films with entrained γ-PGA. These effects are attributed to the ability of γ-PGA to form hydrogen bonds but its susceptibility to scission upon exposure to heat. These cellulose-polyamide films show promise as a step in the creation of strong and sustainable biomaterials.

3.2 INTRODUCTION

Changes in energy and raw material sourcing, as well as global policy, will demand creative solutions for material production. The current use of petro-chemical based plastics is not generally considered sustainable. However, a replacement that incorporates a matrix derived
from renewable sources and uses green processing techniques would be a more sustainable product. An innovative composite material could be favored, if it has improved or new properties and addresses the concerns of society and is environmentally friendly and economical. Utilizing renewable naturally-synthesized raw materials, such as cellulose, starch, chitin, biological enzymes and polymers, is an area many researchers are exploring in their pursuit of sustainable materials. This current work involves two bio-polymers, cellulose and poly (gamma-glutamic acid) (γ-PGA).

Cellulose (Figure 3.1) is considered the most abundant polymer on Earth [1]. It is a linear chain of \( \beta \)-1-4 glucopyranose that can be synthesized by bacteria, animals, and plants and is commonly used as a structural component of fibers and plant cell walls [2, 3].

![Figure 3.1](image)

**Figure 3.1:** Cellulose structure showing \( \beta \)-1-4 glycosidic bond of cellobiose repeating unit.

As a biodegradable and renewable polymer, many researchers are looking into physical incorporation of cellulose into novel materials [4-6] or its chemical conversion into fuels and fine chemicals [7]. Cellulosic plant fibers have been used for centuries to make paper, and current research is advancing in regards to the liberation of fibrils and crystalline segments.
from the fiber through both physical and/or chemical techniques. The physical technique results in fibrils, called microfibrillated cellulose (MFC) \([8, 9]\) (Figure 3.2b), which are mechanically liberated from the fiber so that the amorphous and crystalline regions are maintained along with an increased aspect ratio and surface area. The inter-fibril bonding of MFC when formed into thin films confers augmented material properties for the cellulosic material \([6, 10-18]\), particularly high strength and toughness.

![Figure 3.2: Cellulosic plant structures, (a) plant fiber and (b) microfibrils liberated from fiber (taken from Nakagaito and Yano, 2005 [10]). Scale bar is 10 µm.](image)

It has been observed, though, that once these MFC are dried, they do not recover their original properties. As a result, the MFC must be produced on-site or be shipped in swollen form, both options present logistic and economic difficulties. In a related area, researchers have shown that fibrillating fibers that have first been oxidized reduces the level of required mechanical processing to create fibrillated cellulose \([15, 19]\). Thus, it was conjectured that blending MFC with a polymer exhibiting carboxylic functionality may have a similar affect on MFC recovery after drying.
The γ-PGA polymer (Figure 3.3) is water-soluble, edible, non-toxic, and biodegradable [20]. It is used in varied applications, from hydrogel formation to a food additive [21]. Structurally, γ-PGA is a homopolymer formed by amide linkages between the α-amino group and the γ-carboxylic acid of the functional R-group of the amino acid, glutamic acid. Its molecular weight can vary between 10 – 10,000 kDa [20] with a reported degree of polymerization of 700 – 7000 units [22].

Several different species of bacteria naturally synthesize γ-PGA, most commonly members of the Bacillus genus. Although γ-PGA was first discovered in the capsule of Bacillus anthracis in 1937 [23], the most frequently – and safely – researched species today are
*Bacillus licheniformis* and *Bacillus subtilis*. Researchers have found that at least twelve Bacilli species studied produce enzymes that can degrade γ-PGA [24]. Research also reports that ultrasonic irradiation (20,000 Hz for 2 hours) fragments high molecular weight γ-PGA [25], and temperatures above 80 °C can hydrolyze the polymer [26].

The goal of this work was to create a material with improved strength and water-interaction properties that could with further research be suitable for biomedical applications. A covalent linkage between the two polymers could aid with the overall goal of this work. Literature has reported that temperatures in the 150 °C range had been successful at creating ester bonds between carboxyl-bearing polymers with a cellulosic matrix [27]. Thus, a research scheme was developed to determine if the application and heat treatment of MFC films entrained with γ-PGA would have beneficial affects on the fibril films.

### 3.3 MATERIALS AND METHODS

#### 3.3.1 Materials

Hardwood pulp sheets were the starting material for microfibrillated cellulose (MFC). The sodium form of powdered poly (gamma-glutamic acid) (γ-PGA) with molecular weight of 2250 kDa was kindly supplied by NattoBiosciences (Montreal, Canada). De-ionized water was used in all the experiments.
3.3.2 Methods

3.3.2.1 Preparation of Microfibrillated Cellulose (MFC)

Hardwood pulp sheets were prepared for beating as described in TAPPI Standard T200 sp-96 in a Voith Valley Beater (Appleton, WI). The sheets were torn into approximately two-inch squares (avoiding cut edges) and soaked overnight at 4 °C at 2 % consistency. The swelled pulp sheet pieces were disintegrated in a hyrapulper at 2 % consistency. The disintegrated pulp was refined into MFC after a total of 3.5 hours at a consistency and loading to maintain pulp flow. The MFC (Figure 3.4) was stored in a cold room at 2 % solids until use.

![Image of microscopic images of the progression of cellulose fiber to MFC.](image)

**Figure 3.4:** Microscopic images of the progression of cellulose fiber to MFC.

3.3.2.2 Preparation of γ-PGA Solutions

All solutions of γ-PGA were prepared by vigorously stirring powdered γ-PGA in water until the solution was clear (Figure 3.5). Prepared solutions were 0.0, 0.5, and 1.0 % w/w for the heat treatment trials. For the recyclability of fibrils investigation, the solutions were 0, 1, 4,
6, and 10 % w/w. Solutions were stored at 4 °C until use and allowed to equilibrate to room temperature on the day of use.

**Figure 3.5:** (a) Freeze-dried γ-PGA as received; (b) γ-PGA suspended in water at 1 % w/w.

The viscosities of the solutions were measured (Figure 3.6) using a Brookfield viscometer equipped with an LV1 spindle at 20 rpm for 30 minutes at room temperature. For comparison, the viscosity of olive oil is approximately 80 cP.

**Figure 3.6:** Measured viscosities of five γ-PGA solutions.
3.3.2.3 Preparation of γ-PGA Entrained MFC Films

Films were prepared by vacuum-dewatering followed by restrain-drying (Figure 3.7). To prepare films, the MFC (diluted to 0.2% solids) was vigorously mixed to ensure complete fibril dispersion. A Büchner funnel with two 18.5 cm-diameter Fisherbrand P8 filters was set-up by wetting the filters and vacuum-sealing them to the mouth of the funnel. Vacuum was removed, and the MFC slurry was poured into the funnel – care was taken to not overload one section of the funnel with fibrils. The vacuum was quickly reapplied. A bubble level was used to ensure that film formed evenly across the width of the funnel.

![Diagram](image)

**Figure 3.7:** MFC film-forming method using vacuum-dewatering and restrained drying.

Once the films were at about 20% solids, vacuum was removed. The filter and film were removed from the Büchner funnel, weighed, and then immersed in a 1 cm deep γ-PGA solution bath for 30 minutes. After removal from the bath, the filter and film were weighed a
second time, then placed back into the Büchner funnel for 6 minutes of vacuum-dewatering, followed by a final weighing. The filter and film were removed from the funnel and then separated; the filter alone was then weighed. The film was laid on a metal plate, restrained in paper handsheet rings, and dried overnight at 25 °C and 50 % RH. After removal from the restraining rings, the films were stored at 25 °C and 50 % RH.

For the recyclability investigation, the film was not immersed in a γ-PGA bath. Rather, the γ-PGA solution was spread evenly over the 20 % solids film and then restrained-dried overnight at 25 °C and 50 % RH.

### 3.3.2.4 Heat Treatment of γ-PGA Entrained MFC Films

For heat treatment, each air-dried film was cut into 2 cm x 1 cm sample sections (from the centers of the films), laid in a single layer in glass Petri dishes, and placed in a 150 °C [27, 28] oven for 30 minutes. The heat-treated samples were then stored at 25 °C and 50 % RH until further testing.

### 3.3.2.5 Recovery of MFC after Drying

For the recyclability investigation, the dry films were immersed in water overnight followed by disintegration with a Waring blender for 2.5 minutes. The MFC was then reformed into thin films following the vacuum-dewatering procedure previously described.
3.3.2.6 Fourier Transform Infrared Spectroscopy – Attenuated Total Reflectance

Thin film sample spectra were recorded using the OMNI-Sampler single-reflection ATR accessory of a Thermo-Nicolet Nexus 670 FTIR ESP spectrophotometer. The spectra were an accumulation of 128 scans, with a resolution of 4 cm\(^{-1}\) from 800-4000 cm\(^{-1}\). Absorbance spectra were analyzed using Omnic software. The absorbance of the CH band at 2890 cm\(^{-1}\) was measured and all peaks were normalized to this value. The CH band should remain constant through the reaction process and thus serves as an internal standard. The absorbencies of three major bands associated with γ-PGA (Figure 3.8) and ester formation were measured: C=O band (1745 cm\(^{-1}\)) and C−O (1240 cm\(^{-1}\)).

![Absorbance spectrum of γ-PGA](image)

**Figure 3.8:** FTIR-ATR spectra of commercial γ-PGA.

3.3.2.7 Thermal Gravimetric Analysis (TGA)

The maximum degradation rate temperature was measured using a TGA Q500 (Thermal Analysis Instruments, New Castle, DE). Samples (14 mg) from heat treated films were heated at a rate of 5 °C/min from 30 °C to 500 °C. The Universal Analysis software provided by the TGA manufacturer was used to determine the maximum degradation rate temperature.
3.3.2.8 Tensile Index

Rectangular sample strips (5 x 30 mm) were cut from the heat-treated sample sections. An Instron 4411 equipped with a 500 N load cell and operated with a 2 mm/min extension rate and 10 mm gap width was used to test the sample strips in tensile mode.

3.3.2.9 Contact Angle

Triangular samples with 1 cm edges were cut from the original air-dried films and heat-treated films. Each sample was secured to a glass slide with double-sided tape. A SEO Phoenix Dynamic Contact Angle instrument with Image XP software captured the droplet images. The contact angle from each image was measured using the NIH software ImageJ.

3.3.2.10 Water Absorption Rate

The water absorption apparatus (Figure 3.9) was prepared with a water-filled reservoir connected with a tube to a second reservoir filled with Oasis foam, upon which a sample was placed. The water-filled reservoir was positioned on a balance that would record its weight loss as the sample absorbed the water through the Oasis foam. Plastic Petri dishes were placed on top of the water-filled reservoir to reduce the evaporation rate. A 5.5 cm diameter Whatman 540 filter paper was allowed to equilibrate on the Oasis foam-filled reservoir for 3 minutes prior to sample placement. Then, a 3 cm diameter sample was placed on the filter paper, and the weight loss in the water-filled reservoir was recorded over time [29] by
computer recording. The weight loss in the water-filled reservoir was assumed to correspond to weight gain by the sample.

**Figure 3.9:** Water absorption rate schematic where $H$ is the height of the water in the water reservoir and foam structure. (Taken from Hayden, 2008 [29]).

### 3.3.2.11 Water Vapor Transmission Rate

The open-end of a water-filled reservoir was covered with a sample and secured with a screw ring (Figure 3.10). A sample area of 12.6 cm$^2$ was left exposed and used in the calculation of the water transmission rate. The sealed reservoir was positioned on a balance, and the weight loss over time was recorded by computer recording.

**Figure 3.10:** Water vapor transmission rate schematic.
3.4 RESULTS and DISCUSSION

3.4.1 Recovery of MFC Films after γ-PGA Entrainment

The reformed MFC films were tested for recovery of load carrying capacity (Figure 3.11). If no γ-PGA is added to the MFC prior to air-drying, the recovery is only 75% of the strength exhibited by “virgin” MFC. However, with the addition of a γ-PGA, the strength recovery can be as much as 96% of the “virgin” MFC films. The γ-PGA increased the water absorption capacity of the films (see Section 3.4.4); in fact, the γ-PGA films when soaked in water for less than an hour had no coherence. Thus, it could be concluded that the γ-PGA allowed water to better permeate the fibril films even after drying.

![Figure 3.11: Recovery of MFC film tensile strength by the addition of γ-PGA solution prior to drying of MFC films.](image-url)
3.4.2 Spectral and Thermal Analysis

FTIR-ATR spectra were acquired for three different sets of films, initial and heat treated. The baseline film (Figure 3.12a) had no entrained γ-PGA (lack of 1600 cm\(^{-1}\) peak). The films immersed in the 0.5 % and 1.0 % γ-PGA bath were also examined (Figures 3.12a and 3.12b). The spectra between the three film sets were remarkably similar, save for the slightest increase in the 1.0 % γ-PGA bath spectra. This increase is emphasized by the spectral subtraction shown in Figure 3.13. The moieties associated with γ-PGA (Figure 3.8) are located near 1600 cm\(^{-1}\) and 1400 cm\(^{-1}\). Although there was a relatively low uptake of the γ-PGA in the MFC films, material property measurements show a clear change for the films that were soaked in baths of γ-PGA. Thus, it should be assumed that some amount of γ-PGA is present in the MFC films. The heat-treated films from each set of films were also similar possibly indicating that little to no ester formation occurred.
Figure 3.12: FTIR-ATR spectra of films from water baths of increasing levels of $\gamma$-PGA and a heat treatment of 150 °C for 30 minutes.
Reports indicate that γ-PGA may form esters with carbohydrates [27, 28, 30]. Cellulose esters are known to have lower decomposition temperatures than unmodified cellulose [31], so a thermal analysis of MFC films entrained with increasing levels of γ-PGA could reveal whether cross-linking between the two polymers occurred. With increasing γ-PGA bath concentration, the maximum degradation rate temperature (Figure 3.14, inset) does decrease slightly. It is difficult to definitively attribute the degradation temperature change to ester formation (or other chemical changes) without further confirmation from spectral analysis. Furthermore, it is known that ionic complexes involving poly (γ-glutamic acid) have also shown lower decomposition temperatures [32]. Thus, simple complexing behavior could also account for the differences in thermal degradation temperature.
Figure 3.14: Rate of weight loss from films with temperature ramping. Inset shows maximum degradation rate temperature for γ-PGA entrained films.
3.4.3 Tensile Index

Tensile index (Figure 3.15) increased even with no $\gamma$-PGA entrainment after heat treatment. This change could indicate that remaining water in the films was driven off, thus forming more fibril-to-fibril hydrogen bonding to develop more strength.

![Figure 3.15: Tensile index of MFC films from water baths of increasing levels of $\gamma$-PGA and a heat treatment of 150 °C for 30 minutes.](image)

The addition of $\gamma$-PGA further increased the tensile index of the MFC films, even with no heat treatment. Since the films were formed before the entrainment of $\gamma$-PGA and they were air-dried before heat treatment, the $\gamma$-PGA should not have affected the hydrogen bonding within the film (the main strength-building complex for these fibrous materials). Thus, it could be conjectured that the carboxylic-acid groups of the $\gamma$-PGA formed hydrogen bonds with both itself and its fibril matrix to augment the strength of the MFC films [33]. The further increase in strength with the higher application level of $\gamma$-PGA could also support this
explanation as more γ-PGA was able to form complexes. Since the increase in strength from heat treatment for all γ-PGA application levels is on par with the strength increase from the 0.0 % γ-PGA bathed film, it can be assumed that the 10% strength increase after heat treatment for γ-PGA entrained films is due to the MFC matrix strength increase from the heat treatment to the MFC fibrils themselves and not an additive effect from the γ-PGA.

### 3.4.4 Water Interaction Properties

As illustrated in Figure 3.16, the addition of γ-PGA to the films increases hydrophilicity. The γ-PGA polymer is a highly hydrophilic polymer because each of its monomeric units contains a carboxylic acid group.

![Figure 3.16](image.png)

**Figure 3.16**: Contact angle of films from water baths of increasing levels of γ-PGA and a heat treatment of 150 °C for 30 minutes.
Simply adding the polymer to the already hydrophilic cellulose further increased the hydrophilicity of the MFC film. Applying more polymer to the film did not further increase the contact angle, so it can be assumed that application of a 0.5% bath was enough to create coverage over the surface of the films. Heat treatment at 150 °C can cause γ-PGA scission which may have resulted in a small increase in hydrophilic carboxylic groups exposed by the breakage of the amide bonds.

Water absorption (Figure 3.17) of the cellulosic MFC alone was not affected by the heat treatment. Drying causes the porous structure of fibers to collapse which reduces the ability of dry fibers to absorb water. Further drying did not cause any further collapse.

![Water Absorption Graph](image)

**Figure 3.17:** Water absorption of films from water baths of increasing levels of γ-PGA and a heat treatment of 150 °C for 30 minutes.
The addition of γ-PGA polymer, as anticipated, created an over 150% increase in water absorption. This increase is undoubtedly generated by the carboxylic acid functionality of the monomers of γ-PGA. It is interesting to note the contrast between the contact angle and the water absorption. While the heat treatment affected the contact angle for all three γ-PGA level films, the heat treatment only seemed to affect the water absorption for the films with entrained γ-PGA. This implies that the changes which took place during heat treatment mainly affected the surface characteristics of the film and not the bulk of the film.

Even though heat treatment caused an increase in strength and contact angle, it had the opposite affect for water absorption for films entrained with γ-PGA. The γ-PGA polymer scissions and oxidation may occur at the temperature used for heat treatment, and a reduced polymer DP could explain the depressed water absorption levels. Water absorption is dependent on polymer matrix formation, and with the decrease in γ-PGA polymerization, this matrix may not have been as well-formed leading to the decrease in water absorption.

Similar to the water absorption rates, further drying of the MFC films with the heat treatment did not cause a change in the water vapor transmission properties of the bare MFC films (Figure 3.18). Also, as expected, the entrainment of γ-PGA in the films did allow water to transpire more efficiently through the films.
However, the γ-PGA entrained films had a depressed transmission rate when heat treated. Since the water vapor must first be absorbed into the channels of the film, the decreased water absorption and vapor transmission rates with heat treatment seem to be retarded by the same phenomenon. Increased densification of the films was an initial hypothesis, but all of the films experienced a density decrease with heat treatment (decreases of 3.5%, 3.9%, and 4.9% with each increasing γ-PGA bath). An observation is that when γ-PGA solution is dried alone, it forms a solid material similar to a brittle plastic. A possible explanation could be that heat treatment caused a continuous layer of γ-PGA to form over the film which retarded the transmission of water.
3.5 CONCLUSIONS

Microfibrillated cellulose (MFC) and poly(gamma-glutamic acid) (γ-PGA) were blended together and then subjected to heat treatment at 150 °C for 30 minutes. Spectral and thermal analysis indicated that there is an interaction between the cellulose and the γ-PGA. Although low level of γ-PGA was detected by FTIR-ATR, a more robust analysis technique may reveal more accurate levels. Thermal analysis showed a shift in the maximum degradation rate temperature which could be attributed to either a chemical bonding between γ-PGA and cellulose or to complexing between γ-PGA and cellulose.

Exposure to 150 °C alone increases the strength of the MFC films by 10 %. The strength of the film further improved by 10 % with addition of γ-PGA, but no synergistic effect between heat treatment and γ-PGA was observed. Heat treatment decreased the contact angle making the films more water loving as did the addition of γ-PGA (a highly hydrophilic polymer). Moreover, the addition of γ-PGA to an MFC matrix shows increased material and water interaction properties. Increased water absorption (+150 %) and hydrophilicity as well as increased water vapor transmission rate (+15 %) are effects of cellulosic fibers entrained with γ-PGA. These improved water interaction properties are possible causes for the 96 % recovery of fibril film strength after recycling. Heat treatment causes an additive effect for films entrained with γ-PGA in terms of tensile strength and hydrophilicity. However, heat treatment of the γ-PGA polymer with MFC presumably breaks the polymer and also may form barriers which may be the cause of impeding the water absorption and transmission rates, respectively, of the MFC films.
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4. Method for Production of Poly (gamma-Glutamic Acid) on Solid Substrate Media
4.1 INTRODUCTION

Poly (gamma-glutamic acid) or γ-PGA is an extracellular polymer excreted by several species of the bacterial genus Bacillus. Ivánovics and Brückner first identified γ-PGA in the capsule of the gram-positive Bacillus anthracis in 1937 [1]. It is considered a virulence factor for Bacillus anthracis as it is anti-phagocytic [2, 3], and thereby protects the cells from attack by a host’s immune system. However, most Bacillus species that excrete γ-PGA are non-pathogenic. The most frequently researched species of γ-PGA excreting Bacillus are Bacillus licheniformis and Bacillus subtilis. In addition, a common non-scientific interaction humans have with γ-PGA is in the naturally fermented soybean food called natto (Japan) or chungkookjang (Korea) (Figure 4.1).

![Figure 4.1: Mucilage of fermented soybeans containing γ-PGA.](image)

Poly (gamma-glutamic acid) is a homopolymer of the amino acid glutamic acid. Amino acids are most commonly utilized by living cells to build protein biomacromolecules. In the primary structure of proteins, amino acids are polymerized with peptide bonds using the
alpha-amine and the alpha-carbon. However, in the primary structure of γ-PGA, glutamic acid is polymerized with amide bonds using the alpha-amine and the gamma-carbon [4] (Figure 4.2) – hence its name referencing the gamma linkage.

![Figure 4.2](image)

**Figure 4.2**: Structure of (a) generalized form of an amino acid, (b) glutamic acid, (c) glutamic acid (rotated), and (d) free-acid form of γ-PGA.

Polymerized glutamic acid can be produced synthetically or via bacterial fermentation. Artificially synthesizing poly(glutamic acid) is quite laborious [5], but it can be bought commercially. These polymers are a low-molecular weight (2 – 100 kDa) and are comprised of either L-glutamic acid or D-glutamic acid (no mixed isomer composition). These products also cost well over $1000 per gram (Sigma-Aldrich), which is well out of range for most commercial applications. Fermented polymerized glutamic acid, though, is significantly less
The degree of polymerization (DP) of γ-PGA can be up to 700 units with molecular weights ranging from 10 – 3000 kDa [6].

Even though γ-PGA is a virulence factor for *Bacillus anthracis*, the polymer and its monomer units in themselves are non-toxic [7, 8]. It is also biodegradable [9] and edible (common East Asian food). Both the free-acid and salt-forms of γ-PGA are water-soluble. The carboxylic acid found on every unit of the polymer confers a hygroscopic nature. Precipitation of γ-PGA from aqueous solutions can be accomplished using common protein precipitation chemicals, such as alcohols or acetone.

Determining the essential components and methods of producing γ-PGA was initially focused on understanding the pathogenic *Bacillus anthracis*. However, with γ-PGA recognized as a polymer with desirable properties, scientific inquiry shifted toward optimizing the cultivation.

Species of *Bacillus* are aerobic chemo-organo-heterotrophs, so cultivation studies have primarily focused on selecting carbon sources to push γ-PGA production rather than cell growth or unwanted chemical/polymer production. Table 4.1 lists several selected *Bacillus* strains cultivated with various carbon and nitrogen sources. Cultivation temperature of γ-PGA is between 30 and 37 °C.
Table 4.1: Selected production results of several γ-PGA producing *Bacillus* species.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Main Nutrients (g/L)</th>
<th>Culture Time (hrs)</th>
<th>γ-PGA (g/L)</th>
<th>Molecular Wgt (Da)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>B. licheniformis</em> ATC 9945a</td>
<td>citric acid (12) glycerol (80) glutamic acid (20)</td>
<td>96</td>
<td>20</td>
<td>9.8 x 10⁵</td>
<td>[10]</td>
</tr>
<tr>
<td><em>B. subtilis</em> IFO3335</td>
<td>citric acid (20) glutamic acid (30) NH₄SO₄ (20)</td>
<td>80</td>
<td>20</td>
<td>2.0 x 10⁶</td>
<td>[11]</td>
</tr>
<tr>
<td><em>B. subtilis</em> TAM-4</td>
<td>fructose (75) NH₄Cl (18)</td>
<td>96</td>
<td>22</td>
<td>1.6 x 10⁶</td>
<td>Ito</td>
</tr>
<tr>
<td><em>B. subtilis</em> F-2-01</td>
<td>glucose (1) glutamic acid (70)</td>
<td>96</td>
<td>48</td>
<td>1.0 x 10⁶</td>
<td>[12]</td>
</tr>
<tr>
<td><em>B. subtilis</em> ZJU-7</td>
<td>glucose (60) tryptone (60) glutamic acid (80)</td>
<td>24</td>
<td>54</td>
<td>1.2 x 10⁶</td>
<td>[13]</td>
</tr>
<tr>
<td><em>B. subtilis</em> NRRL B-2612</td>
<td>wheat gluten (200) K₂HPO₄ (10)</td>
<td>30</td>
<td>10</td>
<td>2.0 x 10⁴</td>
<td>[14]</td>
</tr>
</tbody>
</table>

Most strains of *Bacillus* do not naturally synthesize glutamic acid, so an exogenous source of glutamic acid must be supplied. Only a few strains have been identified as glutamic acid synthesizers, including *Bacillus subtilis* TAM-4 and *Bacillus licheniformis* A35 [6]. Some researchers have circumvented this obstacle by transforming glutamic acid producing bacteria with a plasmid containing the pgsBCA gene or co-cultivating a non-glutamic acid producing *Bacillus* with a glutamic acid producing bacteria [8].
4.2 MATERIALS and METHODS

4.2.1 Materials

All media components (glucose, peptone, yeast extract, and agar) as well as chemicals (acetone) were purchased from Thomas Scientific and used as received. Water used in all experiments was de-ionized water.

Sterile polystyrene Petri dishes (9 cm) were used as growth plates in an isotemp incubator. Centrifuge tubes (15 mL, 50 mL, and 250 mL) purchased from Fisher-Scientific were used for polyamide purification; they were used multiple times with detergent and water washing between each use. A Beckman J-21C centrifuge equipped with a JA-14 rotor was used for polyamide purification in afore mentioned centrifuge tubes.

4.2.2 Methods

4.2.2.1 Media Preparation

A complex media substrate (YPD) was used for all isolation and growth media.

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount (g/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>20</td>
</tr>
<tr>
<td>Peptone</td>
<td>20</td>
</tr>
<tr>
<td>Yeast Extract</td>
<td>10</td>
</tr>
<tr>
<td>Agar</td>
<td>16</td>
</tr>
</tbody>
</table>

Table 4.2: Media make-up for isolation and growth of bacteria.
Media batches of 500 mL (Table 4.1) were prepared in 1 liter Erlenmeyer flasks, mixed with a magnetic stir-bar for 10 minutes, and then sterilized in an autoclave (25 min at 121 °C). Media was cooled to 60 °C for 45 minutes in a water bath and then poured quickly into Petri dishes. About 20 plates were made per 500 mL – each plate had media about 1 cm thick. Closed Petri dish lids were used during the solidification stage on the bench top overnight.

4.2.2.2 Bacteria Isolation and Growth

Natto, a traditional Japanese food product made from fermented soybeans (Figure 4.1), was purchased from a local Asian supermarket and stored at −20 °C until use. Samples of the mucilage was taken from the natto and suspended in water and heated at 80 °C for 10 minutes to kill vegetative cells – leaving only the Bacillus spores. The heated suspension was streaked onto solid YPD media plates. The plates were incubated at 35 °C for 48 hours. The most mucous colonies were sampled and streaked onto fresh solid YPD media plates until pure colonies were obtained. Highly mucous colonies (Figure 4.3) were grown for up to four days on solid YPD media plates at 35 °C and then harvested. New plates were cultivated from a single seed plate stored at 4 °C.

Figure 4.3: Highly mucous colonies isolated from natto.
4.2.2.3 Microscopic Identification of Bacteria

Samples from the isolated bacteria were observed using a light microscope (Olympus BH-2). Glass slides were prepared by swirling a water drop with a loop touched to a colony. A cover slip was placed onto the slide, and the slide was quickly passed twice over a Bunsen burner flame. The slides were observed under both low (10x) and high (50x) magnification.

4.2.2.4 Purification of Polymer

Highly mucous plates were grown at 35 °C for four days. To collect the polymer, entire colonies, especially the bulbous structures, were scraped from the plates into centrifuge tubes, and one part water was added. The tubes were shook by hand until the suspension was well distributed in the tube. The cells were spun down at 12,000 rpm for 30 minutes at 4 °C. The supernatant was poured into fresh centrifuge tubes and spun down to eliminate all cell debris within the supernatant (Figure 4.4). Four parts acetone at −20 °C was added to the supernatant to precipitate the polyamide (Figure 4.5). The precipitate which formed a compact ball of polymer was removed from the acetone:water and air-dried overnight.

Figure 4.4: (a) Pelleted cells with polyamide polymer in the (b) supernatant.
4.2.2.5 Characterization of Polymer via FTIR-ATR

Samples of the dried precipitate were dissolved in water. Drops of the solution were placed on glass slides and allowed to dry overnight. The resultant thin films were transparent and somewhat brittle. Sample spectra of the thin films were recorded using the OMNI-Sampler single-reflection ATR accessory of a Thermo-Nicolet Nexus 670 FTIR ESP spectrophotometer. The spectra were an accumulation of 128 scans, with a resolution of 4 cm\(^{-1}\) from 800-4000 cm\(^{-1}\). Absorbance spectra were analyzed using Omnic software.

4.3 RESULTS and OBSERVATIONS

4.3.1 Bacteria Isolation and Growth

A bacterial isolate from the *natto* was easily obtained in two isolation stages. The YPD plates streaked from the seed plate were cultivated for four days to produce highly-mucous colonies (Figure 4.6). The highly hydrophilic γ-PGA polymer produced and excreted from
the bacteria absorbed the water in the plates so that after day four, the plates were dehydrated and did not produce further extracellular polymer. It was important to not over-streak the plates initially because colonies tended to not become as mucous, and the plates dehydrated much too quickly.

![Image of bacteria growth on YPD plates at 35°C over four days]

**Figure 4.6:** Isolated bacteria colony grown on YPD plates at 35 °C over four days.

### 4.3.2 Microscopic Identification of Bacteria

Using a light microscope, under low power (100x total magnification), many clusters and streaks could be seen. At higher power (500x total magnification), the expected cell morphology for *Bacillus* was observed (Figure 4.7), including double- and multiple-chained rod-shaped cells with rounded edges and approximately a 3:1 aspect ratio. The cells had a very bright outer edging that surrounded a speckly cytoplasm containing a very bright round spot (spore structure).
4.3.3 Purification of the Polymer

After centrifugation, the supernatant was highly viscous and transparent (Figure 4.4b). With the addition of four parts acetone, a precipitate formed that was a mass of extremely sticky and elastic whitish material (Figure 4.5b) that dried to a light orange color (Figure 4.5c) on the bench top overnight. When water was added to the dried material, it would return to being a highly viscous liquid.

4.3.4 Characterization of Polymer via FTIR-ATR

Spectral analysis with FTIR-ATR (Figure 4.8) revealed characteristic peaks associated with the salt-form of poly (gamma-glutamic acid). The strong peaks at 1650 cm$^{-1}$ and 1400 cm$^{-1}$ indicate the presence of the salt form of a carboxylic acid (-COO$^-$). At 1650 cm$^{-1}$ (Amide II
region), open-chain secondary amides can be detected, and a portion of the broad band below 800 cm$^{-1}$ indicates N–H bonding, as well. The intense band at 1050 cm$^{-1}$ has yet to be identified. The additional peaks in the laboratory-fermented γ-PGA could be impurities such as cellular proteins from dead or lysed cells during the purification process. These impurities could possibly be removed with dialysis since γ-PGA is a high molecular weight polymer and any impurities most likely have a much lower molecular weight in comparison to γ-PGA.

**Figure 4.8**: FTIR-ATR spectra of sodium-form γ-PGA, commercially-fermented γ-PGA (---) and laboratory-fermented crude γ-PGA (—).
4.4 CONCLUSIONS

Production of γ-PGA from an isolate identified as a member of Bacilli can be accomplished on solid complex media. Solid substrate was preferred in this method to reduce the amount of acetone necessary for polymer precipitation. Since a four to one ratio of acetone to water is necessary, precipitating polymer from 500 mL of fermentation liquid is quite arduous and expensive (especially if the acetone is not recovered). However, a probable limiting factor in high production levels of the γ-PGA polymer is water for biosynthesis and growth – in that as the polymer is excreted, it begins to absorb the water from the plates. Growing fewer colonies per plate helps reduce this obstacle.

4.5 REFERENCES


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5. Trial of Cross-linking Reactions Involving Poly (gamma-Glutamic Acid) with Bacterial Cellulose
5.1 INTRODUCTION

Surface modification of cellulose to create unique functionalities is a common research objective [1-5]. Cellulose has a hydrophilic nature due to its hydroxyl moieties (Figure 5.1), but creating an even more water loving material from cellulose is often desired. The polyamide poly(gamma-glutamic acid) (γ-PGA) is a polymer that if attached to the cellulose surface could impart a highly hygroscopic function. It would be desired to bond the γ-PGA to the cellulose through a direct ester linkage between the cellulosic hydroxyl and the pendant carboxyl group of the γ-PGA (Figure 5.2).

![Figure 5.1: Cellulose structure showing β-1-4 glycosidic bond of cellobiose repeating unit.](image1)

![Figure 5.2: Structure of γ-PGA polymer showing pendant carboxyl group.](image2)
Unfortunately, esterification reactions of this type require hazardous chemicals for catalysis and solvent and a water-free environment [6-9]. More benign methods are available, though the route involves modifying the cellulosic surface and using a cross-linker rather than directly grafting the \(\gamma\)-PGA to the cellulose surface. In this work, the cellulosic surface was prepared for cross-linking by conferring a carboxylic functionality to the C6 position and then performing an amidization reaction with a di-amine (ethylenediamine) as the cross-linker between the cellulose and the \(\gamma\)-PGA (Figure 5.3).

![Diagram of \(\gamma\)-PGA cross-linked to cellulosic surface]

**Figure 5.3:** Desired final product of \(\gamma\)-PGA cross-linked to cellulosic surface.

Carboxymethylation of cellulose can be achieved with a heterogeneous alkali-catalyzed reaction with chloroacetic acid (Figure 5.4) at elevated temperatures (~65 °C) with increasing...
time to give increasing substitution levels. Niemelä and Sjöström [10] found that for CMC-generating reactions, substitution of cellulose hydroxyls began with C6 and C2 being roughly equivalent, followed by C3 substitution. Sjöström [11] has even proposed that the C2 hydroxyl (the most acidic of the hydroxyl groups) has the highest reactivity with chloroacetic acid and is therefore the most substituted.

Figure 5.4: Reaction of monochloroacetic acid with hydroxyl of cellulose to yield carboxymethyl cellulose.

To perform an amidization reaction, carbodiimide chemistry [2, 3, 12-19] can be used in an aqueous environment, which is beneficial when using cellulose. Replacing water with a non-polar solvent in cellulose is difficult and can result in unwanted structural changes. The complete scheme of reactions to achieve cross-linking is shown in Figure 5.5.
Figure 5.5: Crosslinking of carboxymethyl cellulose and γ-PGA using amidization of the cross-linker ethylenediamine.

5.2 MATERIALS and METHODS

5.2.1 Materials

Cultures of Acetobacter xylinum were supplied by Ann G. Matthysse (UNC-CH). All media components for bacterial growth media (glucose, peptone, yeast extract, sodium hydrogen phosphate hepta-hydrate, and citric acid hydrate) were purchased from Thomas Scientific and used as received. Chemicals (chloroacetic acid, methanol, sodium hydroxide, and hydrochloric acid) used in the carboxymethylation reaction and its characterization were purchased from Fisher Scientific. The sodium hydroxide and hydrochloric acid solutions were diluted from 1 N stock solutions of each.
Poly (gamma-glutamic acid) or γ-PGA was grown and purified in the lab as described in Technical Report I. For the cross-linking reaction between the carboxymethyl bacterial cellulose and the γ-PGA, the chemicals (2-(N-morpholino)ethanesulfonic acid, N-hydroxysuccinimide, 1-ethyl-3-[3 dimethylaminopropyl] carbodiimide hydrochloride, and ethylenediamine) were purchased from Sigma-Aldrich and used as received. The MES buffer used as a suspending medium for the reaction was made at a 50 mM concentration.

5.2.2 Methods

5.2.2.1 Bacterial Cellulose Generation

Cultures of Acetobacter xylinum were seeded into 500 mL of Schramm-Hestrin media (Table 5.1) in 1 L sealed Erlenmeyer flasks. The cultures were incubated at 30 °C without agitation for four days after which the pellicles were harvested. To purify the pellicles of media components, proteins, and cells, they were rinsed under running water and then immersed in 0.1 M NaOH for 2 hours with agitation. They were then autoclaved to kill vegetative cells. The pellicles were rinsed again in water and the purification procedure was repeated. The pellicles were stored in water at 4 °C until use.

<table>
<thead>
<tr>
<th>Table 5.1: Schramm and Hestrin media</th>
</tr>
</thead>
<tbody>
<tr>
<td>Component</td>
</tr>
<tr>
<td>Glucose</td>
</tr>
<tr>
<td>Peptone</td>
</tr>
<tr>
<td>Yeast Extract</td>
</tr>
<tr>
<td>Na₂HPO₄ • 7H₂O</td>
</tr>
<tr>
<td>Citric acid • H₂O</td>
</tr>
</tbody>
</table>
5.2.2.2 Fourier Transform Infrared Spectroscopy-Attenuated Total Reflectance

Sample spectra of the thin films were recorded using the OMNI-Sampler single-reflection ATR accessory of a Thermo-Nicolet Nexus 670 FTIR ESP spectrophotometer. The spectra were an accumulation of 128 scans, with a resolution of 4 cm$^{-1}$ from 800-4000 cm$^{-1}$. Absorbance spectra were analyzed using Omnic software.

5.2.2.3 Carboxymethylation of Bacterial Cellulose

5.2.2.3.1 Reaction

Pellicles of bacterial cellulose (1 gram, oven-dry weight) were suspended in 200 mL of methanol/water (85/15 v/v) and soaked for 20 minutes. The pellicle was removed from the suspension and heated to 60 – 70 °C with an oil bath in a one liter 3-mouth reaction jar equipped with a reflux condenser, temperature control probe, and stirrer.

For the reaction, the solids content was 2.5%. Thus, the volume of methanol/water required to bring the wet pellicle to this consistency was used to dissolve the other reaction reagents. Into 20 mL of below room temperature methanol/water, chloroacetic acid (7.6 g) was dissolved using a magnetic stir bar. Into 20 mL of above room temperature methanol/water, sodium hydroxide (6.5 g) was dissolved using a magnetic stir bar. The two volumes were mixed and kept at 60 – 70 °C and then added to the heated wet bacterial cellulose pellicle. An additional 20 mL of methanol/water was added to bring the reaction mixture to the correct consistency. The reaction (Figure 5.4) was performed for 3 hours.
The carboxymethyl bacterial cellulose pellicle was removed from the reaction mixture and placed in a Büchner funnel apparatus with a Whatman 541 filter. The pellicle was rinsed repeatedly with 500 mL of water, but the pellicle was never allowed to dewater on the filter. Two 100 mL aliquots of 0.1 N HCl were washed over the pellicle to form the free-acid form of carboxymethylated cellulose. The excess hydrochloric acid was washed away with an additional 200 mL of water.

### 5.2.2.3.2 Degree of Substitution

The carboxyl content of the carboxymethyl bacterial cellulose was determined by acid-base titration. Lyophilized carboxymethyl bacterial cellulose was Wiley milled through a 40 mesh screen and then placed in a 90 °C oven for one hour to ensure proper weight measurement for titration. A sample (50 mg oven-dried weight) was suspended into 0.01 M HCl (15 mL) with stirring overnight to fully suspend the sample. The sample was then titrated with 0.01 M NaOH solution, and the pH was measured and recorded by hand with each addition of base.

The volume of NaOH bound by the inflection points in the curve of pH versus NaOH volume (cf Figure 5.6) corresponds to the carboxyl content (weak acid region). The degree of substitution (DS) is given by the following equation:

\[
DS = \frac{162 \times (V_2 - V_1) \times N_b}{w - [58 \times (V_2 - V_1) \times N_b]}
\]
Where \((V_2 - V_1)\) is the NaOH (in liters) used to neutralize the carboxylic acid groups, \(N_b\) is the normality of the NaOH solution (in mol/L), \(w\) is the weight of sample titrated (in grams), 162 corresponds to the molecular weight of the anhydroglucose unit of cellulose, and 58 corresponds to the net increase in the weight of the anhydrogluclose unit for each hydroxyl substituted by a carboxymethyl group.

### 5.2.2.4 Cross-linking Reaction with Carboxymethyl Bacterial Cellulose

Carboxymethyl bacterial cellulose (10 mg oven-dried weight) in swelled pellicle form was immersed in 2-(N-morpholino)ethanesulfonic acid (MES) buffer (50 mM, 5 mL) in a glass beaker secured in a room temperature shaker cabinet. For the reaction, 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (EDC•HCl) (50 mg) and \(N\)-hydroxysuccinimide (NHS) (50 mg) were added to the MES buffer with the carboxymethyl bacterial cellulose. The creation of the amine-reactive NHS-ester occurred over two hours. At this point, ethylenediamine (10 μL) was added to the reaction mixture and allowed to react for two hours. During this time, a reaction of \(\gamma\)-PGA (20 mg) in MES buffer (5 mL) with EDC•HCl (100 mg) and NHS (100 mg) was allowed to form an amine-reactive NHS-ester. When the reactions were complete (two hours), the bacterial cellulose grafted with ethylenediamine was drip-dried and added to the \(\gamma\)-PGA reaction flask. This reaction was allowed to proceed for two hours. After this reaction, the sample was rinsed under running water for two minutes.
5.3 RESULTS and OBSERVATIONS

5.3.1 Carboxymethyl Bacterial Cellulose – Degree of Substitution

The degree of substitution (DS) was found by determining the weak acid region of the carboxymethyl bacterial cellulose. Figure 5.6 shows the measured pH at each volume of NaOH added to an initially free-acid form of the oxidized cellulose.

![Titration of free-acid carboxymethyl bacterial cellulose to determine DS.](image)

Figure 5.6: Titration of free-acid carboxymethyl bacterial cellulose to determine DS.

From three titrations of the carboxymethyl bacterial cellulose, the weak acid region was found to be bound by the volume on average between 14.9 mL and 18.2 mL which corresponded to an average DS of 0.109. This DS was expected from the amount of chemicals and length of time used in the carboxymethylation reaction.
5.3.2 Carboxymethyl Bacterial Cellulose – FTIR-ATR

The carboxymethylation of bacterial cellulose was confirmed by FTIR-ATR spectral analysis (Figure 5.7). The appearances of the peaks for the salt-form of a carboxyl group are clearly shown at 1600 cm$^{-1}$ (asymmetrical stretch) and 1420 cm$^{-1}$ (symmetrical stretch) in the blue curve (reacted bacterial cellulose). The peak at 1150 cm$^{-1}$ is a moiety of –CO and –CC stretching and –CCH bending [20] – all which are stronger in the carboxymethyl bacterial cellulose than in the unmodified bacterial cellulose.

![FTIR-ATR Spectra](image)

**Figure 5.7**: FTIR-ATR of unmodified bacterial cellulose (---) and carboxymethyl bacterial cellulose (—).
5.3.3 Cross-linking Reaction with Carboxymethyl Bacterial Cellulose

As shown in the FTIR-ATR spectra in Figure 5.8, the reaction between the carboxymethyl bacterial cellulose and ethylenediamine proceeded as expected. The disappearance of the peak at 1580 cm\(^{-1}\) (corresponding to the COO\(^{-}\) stretch of a carboxylic salt) \([20]\) indicates that it was substituted. In the red curve, the peak at 1600 cm\(^{-1}\) was replaced by a 1660 cm\(^{-1}\) peak – an amide carbonyl stretch. Also, two peaks around 1570 cm\(^{-1}\) and 1220 cm\(^{-1}\) develop which correspond to N–H in-plane stretching and C–N bond stretching, respectively. The appearance of these three peaks helps to corroborate the grafting of ethylenediamine to the carboxymethyl bacterial cellulose \([21]\).

![Figure 5.8: FTIR-ATR spectra of amidization reaction stages.](image-url)
A spectra for the activated-ester of γ-PGA is not available because γ-PGA is soluble in water; thus, any washing would wash away the γ-PGA. If the research is continued, then dialysis may be a method to remove the un-reacted reagents; however, dialysis is long process that would compound upon the actual reaction time.

The reaction between the amine-functionalized bacterial cellulose (red curve) and γ-PGA apparently did not proceed as expected. The green curve in Figure 5.8 demonstrates that the amine functional group still existed and no γ-PGA moieties appeared (Figure 5.9). FTIR-ATR may not be a sensitive enough analysis tool for this detection, though. In future studies, it might be useful to use either elemental analysis or XPS to detect a change in nitrogen levels after the final reaction and washing stage.

![FTIR-ATR spectra of sodium-form γ-PGA, commercially-fermented γ-PGA (---) and laboratory-fermented crude γ-PGA (—).](image)

**Figure 5.9:** FTIR-ATR spectra of sodium-form γ-PGA, commercially-fermented γ-PGA (---) and laboratory-fermented crude γ-PGA (—).
5.4 CONCLUSIONS

Production of bacterial cellulose is accomplished by fermenting *Acetobacter xylinum* in Schramm and Hestrin media. The cellulose surface, though, must be modified to efficiently react with the γ-PGA polymer to create new functionality. Carboxymethylation of the cellulose to a substitution level of just over 0.1 was accomplished to meet this goal. A cross-linker of ethylenediamine between the carboxyl groups of the γ-PGA and the newly carboxymethylated cellulose was chosen so that carbodiimide chemistry could be used in an aqueous reaction. Although attachment of the ethylenediamine was successful, the γ-PGA did not appear to bond to the pendant amine of the cross-linker. One reason could be the length of the cross-linker in comparison to the morphology of the cellulosic surface. A second possibility is that there was a great amount of steric hindrance from the high molecular weight of the γ-PGA. Other researchers have been successful with carbodiimide chemistry, and further investigations with this system using lower molecular weight γ-PGA, free-acid form γ-PGA, longer cross-linkers, and even different buffers to modify the charge of the amines should also be successful. Also, it is possible that more robust analysis techniques could show the presence of γ-PGA bound to the cellulosic surface.

5.5 REFERENCES


5. Andresen, Martin; Stenstad, Per; Møretrø, Trond; Langsrud, Solveig; Syverud, Kristin; Johansson, Leena-Sisko; and Stenius, Per (2007): Nonleaching antimicrobial films prepared from surface-modified microfibrillated cellulose. *Biomacromolecules*, 8(7), 2149-2155.


6. General Conclusions and Suggestions for Future Work
6.1 GENERAL CONCLUSIONS

Traditional uses of cellulose have benefited society, and new developments with highly-refined cellulosic fibers (generally called microfibrillated cellulose (MFC)) are gaining research interest. In this work, surface modification was investigated to examine the bonding structure of these MFC fibrils as well as to create materials with new functionalities.

Surface acetylation of MFC was accomplished with a heterogeneous reaction with acid anhydride and acetic acid with only heat as the catalyst. In comparison to whole fibers, MFC have a higher surface area and are, thus, capable of forming more fibril-to-fibril bonds. This increase in bonds enables the structure to carry a much higher applied load. Even with acetylation, the tensile strength of this heavily bonded structure was largely maintained. However, the acetylation of the amorphous region and the decrease in number of bonds means a more uniform structure which, in turn, creates a material that absorbs energy similar to a traditional paper fiber.

The identification of poly (gamma-glutamic acid) (γ-PGA) offered an opportunity to study hydrophilic modifications to the MFC films. Adding a hygroscopic polymer to the MFC surface could make the material useful in biomedical applications, such as bandages or tissue scaffolds. A commercial source for the polymer was found, but in-house laboratory fermentation was also explored. The most common method for the production of the polymer is via liquid fermentation; however, this work attempted solid-substrate fermentation to reduce the amount of precipitating reagent required. The polymer production
from a bacterial isolate was successful and the laboratory-fermented polymer was used in further experimentation with MFC films.

In one experiment of combining γ-PGA with MFC, the γ-PGA was applied to MFC films followed by heat treatment at 150 °C for 30 minutes. Previous reports had suggested that esters should form between the carboxyl of the γ-PGA and the hydroxyl of the cellulose, yet conclusive spectral evidence could not be found for this reaction. It was assumed that some kind of complexing had occurring, though, due to the changes observed during thermal analysis. However, simply entraining the films with γ-PGA caused a positive effect in tensile strength, hydrophilicity, and water absorption, as well as an increased water vapor transmission rate. The heat treatment improved the tensile strength and hydrophilicity of the bare MFC films, but reduced the water absorption and vapor transmission rates of films entrained with γ-PGA.

To determine if a more involved chemistry could yield the desired binding of γ-PGA with cellulose, a carbodiimide trial was initiated to create amide bonds using a di-amine cross-linker. To achieve this goal, the cellulose surface was carboxymethylated so that amidization could be accomplished in an aqueous media. The carboxymethylation of the cellulose and the attachment of the di-amine were successful; however, the linking of the γ-PGA polymer did not yield desired results. The conformation of the high molecular weight γ-PGA as well as the length of the cross-linker most likely led to steric hindrances.
6.2 SUGGESTIONS for FUTURE WORK

Surface modification of cellulose as the main material matrix is a large focus of current research. Controlled acetylation of MFC with heat catalysis was accomplished in this work, but optimizing this heterogeneous reaction is necessary. Another investigation would be into uses for these acetylated fibrils, perhaps in a layered structure with non-modified fibrils or as a component in whole fiber sheets. Acetylated fibrils have the potential of delivering a moderately strong material that has desirable chemical characteristics, most notably hydrophobicity, that traditional paper fibers cannot deliver.

In addition to creating hydrophobic films, an advance in highly hydrophilic films could be useful in creating bandages or tissue scaffolds. Heat treatment could cause esterification under the right conditions, but even complexing of polymers, such as cellulose and γ-PGA, could help in augmenting the material properties of MFC films for use in biomedical applications. Further research into the chemistries associated with heat-catalyzed esterification, particularly the charge, molecular weight, and conformation of the γ-PGA would be necessary. The research trials with carbodiimide chemistry and the use of a cross-linker to achieve these goals for amidization rather than esterification were begun, but developing these concepts further could provide positive results. Most importantly, the characteristics of the γ-PGA must be controlled with appropriate molecular weight, functional group charges, and choice of buffers for each stage of the reaction.