ABSTRACT

LEE, JUNG MYOUNG. An Atomic Force Microscopy Study of the Local Hygro-expansion Behavior of Cellulose Microfibrils. (Under the direction of Dr. Joel J. Pawlak and Dr. John A. Heitmann.)

Structure-property relationships of cellulose-based materials including paper, micro- and nano-fiber composites are often strongly influenced by environmental variables. The interaction of polymeric and crystalline structure in cellulose bio-based materials is of high technological importance. Therefore, understanding the underlying mechanism of environmental/material interactions is crucial for engineering products from bio-based materials. This study was undertaken in an effort to develop a technique for the assessment of dimensional stability of cellulose microfibrils as a function of different relative humidity. Analysis of atomic force microscopy images showed that the local dimensional properties of cellulose microfibrils are highly responsive to variable relative humidity, and their hygro-expansive behavior depends on the relative humidity history, and their method of preparation. The results obtained suggested that dimensional and hygro-expansive behaviors of cellulose microfibrils are related to their ultra-structural arrangements and their origin, either directly or indirectly. These findings, hopefully, will prompt an open discussion regarding the dynamic interactions between cellulose and water molecules at a nano-scale.
AN ATOMIC FORCE MICROSCOPY STUDY
OF THE LOCAL HYGRO-EXPANSION BEHAVIOR
OF CELLULOSE MICROFIBRILS

by

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To the memories of my father who belongs to heaven three months ago…,

To my mother and my wife,

With my love.
BIOGRAPHY

Jung Myoung Lee received a Bachelor of Science degree and a Master of Science degree in Wood Science and Technology in 1996 and 1998, respectively, from Kyungpook National University, Daegu, South Korea. He continued his Ph.D. work at his alma mater until 2001 and then came to join the graduate program in the Department of Wood and Paper Science at North Carolina State University in 2002 under the guidance of Dr. Joel J. Pawlak and Dr. John A. Heitmann.
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<tr>
<td>$A_0$</td>
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<tr>
<td>AFM</td>
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<td>$A_{sp}$</td>
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<td>CAFs</td>
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<td>NS</td>
<td>fully bleached northern U.S. softwood pulp</td>
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CHAPTER ONE

INTRODUCTION
A. Background

The interaction of the polymeric and crystalline structure of the fiber cell wall with surrounding environments is of high technological importance. For example, the functional properties of bio-based materials such as paper, micro- and nano-fiber composites often change significantly when they are exposed to environmental factors [1, 2]. The interaction underlies the functional properties of the final product from cellulosic fibers and controls the mechanical, dimensional, electrical, thermal, and optical properties, either directly or indirectly. Therefore, understanding the underlying mechanisms of these interactions is crucial for further developing efficient and economical processes for producing final from bio-based raw materials.

Paper is a common final product from cellulosic materials (approx. 300,000,000 tons/year). When paper is dried it shrinks and upon rewetting it swells. The ability to resist shrinkage and/or swelling is thus called \textit{dimensional stability}. The development of an effective means to enhance dimensional stability of paper has been a goal of papermakers for many years, since dimensional stability is a factor of primary importance in various types of board and paper [3]. Hence an understanding of the fundamental parameters affecting dimensional properties, called \textit{hygro-expansion}, of paper is essential in order to develop final product with better dimensional stability. Dimensional properties of paper have been extensively studied from both fundamental and practical viewpoints. Dimensional stability of paper is directly connected with the effect of moisture content on expansion and contraction of the sheet.
To improve the dimensional properties, paper mills have used mechanical means to control the ratio of the machine direction (MD) and cross direction (CD) orientation in paper web shrinkage. Edge shrinkage in the CD can be controlled to a limited extent by mechanical means, but the fibers themselves are the underlying cause for the shrinkage of the sheet [4-6].

Paper is a consolidation of fibers, which consist of partially crystalline and non-crystalline cellulose microfibrils arranged at a certain angle in a lamellar structure making up the cell wall. These fibers are typically harvested from wood, where the microfibrils are surrounded by a discontinuous amorphous matrix composed of lignin and hemicellulose. The fibers are typically liberated from the wood by a mechanical or chemical pulping process. The chemical pulping process primarily removes the lignin and hemicellulose, leaving a fiber consisting mostly of cellulose. These physicochemical characteristics of the fiber are significantly influenced by the morphology of the wood source and by the particular pulping, bleaching and papermaking processes the fiber is subjected to during papermaking.

Pulp fibers are hydro-plastic and hygro-expansive in nature, which make them highly responsive to the environment [5]. Therefore, individual fibers will also expand and contract with changes of moisture content, although the movement of the fibers in relation to one another will govern the net changes in the paper. Ultimately, the outer and inner physicochemical properties of the papermaking fiber play a crucial role in final paper properties.

The root of the hygro-expansion properties of the cellulosic materials is the swelling capability of the polymeric and crystalline structure of the cellulose microfibrils. Understanding the hygro-expansive properties of cellulose microfibrils is, therefore, an
essential part of understanding the hygro-expansive properties of the fiber, the paper, as well as micro- and nano-composites. Thus, the dimensional properties of the microfibril and its dynamic interaction with moisture are very important aspects of the fundamentals of dimensional stability.

In the following section, a review of the literature pertaining to high resolution microscopy and its principle and applications will be given. In addition, reviews of physicochemical properties of cellulose microfibrils at the molecular, supra-molecular, and morphological aspects will be presented. Finally, the utilization of cellulase hydrolysis in the pulp and paper science will be discussed. The following sections are intended to provide a way to evaluate dimensional changes of individual cellulose microfibrils and assist in a better understanding of the dimensional changes of individual cellulose microfibrils from variable sources.

B. High resolution microscopy techniques

A number of microscopy techniques have been used to digitize dimensional profile information of materials. These techniques include scanning electron microscopy (SEM), scanning tunneling microscopy (STM), optical interferometry, microfocusing, and atomic force microscopy (AFM) [7]. The majority of these techniques are non-contacting, but many require extensive sample preparation. AFM uses a mechanical stylus to image the surface, but requires little sample preparation [8].
In addition, the techniques mentioned above have specific vertical and horizontal measurement ranges for which they are suitable. Figure 1.1 shows the working range of 3-D topography measurement techniques based on amplitude-wavelength space (AW space) [10].

In the figure, the two axes represent the resolution and the ranges of the techniques in both vertical and horizontal directions. Each block in the figure indicates the working area of the technique. The large working area of the stylus instruments illustrates its wide applicability. Interferometric systems have high resolution but a greater operational range than the scanning techniques such as STM and AFM systems. Compared to conventional electron microscopes, e.g. transmission electron microscope (TEM) and SEM, vertical resolution at the sub-nm and nm level is only attained through the use of STM and AFM.
This level of vertical resolution is of interest in this study. Thus, the advantages and disadvantages of AFM and STM will be discussed.

The STM, invented in 1982, provided unique opportunities for obtaining three-dimensional images of surfaces with atomic resolution [11, 12]. STM requires a voltage to be applied to the surface and the scanning tip to create a tunneling current. This creates one of the main limitations of the STM, which is that it only works for conducting or semiconducting surfaces. The magnitude of this tunneling current is an exponential function of the tip-sample distance, and it is this strong distance dependence which is utilized for topographic imaging. The limitation of application to conducting materials was overcome by Binning, Quate and Geber, who developed the AFM with a novel force sensing technique which could be used regardless of material composition [13]. Compared to STM and SEM, the advantage of AFM as an imaging tool is that samples can be imaged under ambient conditions without severe loss in resolution and that no surface coating is needed. However, perhaps the greatest advantage is that AFM can be used to measure more than surface topography. At the same time as a topography image is being recorded, information about local properties such as surface chemistry, stiffness, friction, and magnetic properties can be obtained [14].

1. Fundamental principles of AFM

The basic working principle of AFM is the measurement of forces between the sample surface and a sharp micro-fabricated tip (probe) at the end of a cantilever. Figure 1.2 shows a general description of AFM configuration.
As the probe is “rastered” over the surface, the electronics maintains a condition of constant force between surface and probe by adjusting the height (z dimension) of the probe support. The movement of the tip over the surface is controlled by the piezoelectric scanner, which can move in the x, y, and z directions in response to applied voltages. Movement of the piezo probe system in the x and y directions scans the sample. A feedback circuit controls the voltage applied to the z piezo so that the bending of the probe cantilever is held constant as the tip is scanned across the surface. As the tip moves over the surface, the cantilever bends back and forth in the z direction. A laser beam is directed onto the back of the cantilever, and as the cantilever bends, the movement of the reflected beam is detected by a position-sensitive photodiode. A feedback circuit integrates this signal and applies a feedback voltage to the z piezo to exactly balance the cantilever bending. The piezoelectric
scanner and photodiode are linked via a computer, employing various feedback options to display the deflection as a function of position at the sample surface, as shown in Figure 1.2.

When the AFM is configured for imaging in the contact mode, the repulsive force between the sample surface and tip is kept constant by the feedback loop, and the piezoelectric scanner adjusts the separation distance. Hence, the topographic information corresponding to a height image in contact mode is deduced by measuring the cantilever deflection during scanning. However, the tip can exert considerable force on the sample surface, thereby causing sample deformation. Thus, the height images may not represent the true topographic information of the sample surface.

Figure 1.3 Schematic description of the tip-sample interaction (modified from [17]).
(a) The cantilever is away from the surface and oscillates freely with an amplitude of $A_0$. 
(b) The tip just touches the surface at the lowest point of its oscillation. $A_0$ is equivalent to the real amplitude of the cantilever $A_{sp}$, since there is no indentation into the surface. $A_{sp}$ also is equal to the tip-sample distance and the setpoint distance, $d_{sp}$.
(c) The sample is brought into contact with the tip, and $A_{sp}$ becomes smaller than $A_0$, because of damping of the tip induced by indentation into the sample. $A_{sp}$ is equal to the tip-sample distance, $d_{sp}$, plus the indentation depth, $Z_{ind}$. 

$d_{sp}/A_0 = r_{sp} = 1$

$d_{sp}/A_0 = r_{sp} < 1$
In order to overcome possible sample deformation, tapping mode AFM was introduced [15]. In this method the cantilever where the tip is mounted is vertically oscillated above the sample surface near its resonance frequency as shown in Figure 1.3a. The free amplitude $A_0$ is the oscillation amplitude of the cantilever when there is no interaction with the surface of the sample. The amplitude oscillation of the cantilever is monitored by the laser photodetector, and it is the signal which is used in the Z feedback control circuitry.

When the probe is lowered down to a surface, the amplitude oscillation of the cantilever is dampened due to the tip-sample interaction. The feedback control loop of the system then maintains this new amplitude constant as the oscillating tip scans the surface. The feedback control loop of tapping mode AFM is controlled by the set-point amplitude ratio $\gamma_{sp} = A_{sp}/A_0$, where $A_0$ is the amplitude of the free oscillation and $A_{sp}$ is the set-point amplitude such that during scanning the observed amplitude of oscillation is maintained at $A_{sp}$ by adjusting the vertical position of the sample. In other words, setpoint control is adjusted to determine how much damping the Z feedback circuit seeks to maintain. In this mode, the cantilever periodically contacts the surface. This approach eliminates the frictional forces that can be a problem in contact mode. Simultaneous detection of the phase shift between the driving oscillator and the detected oscillation signal during intermittent contact is used to create a phase image. The phase differences between the driven and the resulting oscillation can also give information about adhesion and viscoelastic surface properties [8]. However, the features in height and phase images of materials with different chemical components should be carefully assigned, since topographical information from the images depends on changes in the set-point amplitude ratio [16].
For imaging the true topography of a sample surface by tapping mode AFM, the tapping force, which is defined as the degree to which the energy of the tip will be dissipated into the sample surface during scanning, should be considered [17]. When the setpoint ratio ($\gamma_{sp}$) equals the ratio of setpoint amplitude ($A_{sp}$) and its free oscillation ($A_0$), it has been called “soft tapping” which does not apply tapping force onto the sample surface, so that there is negligible indentation of the tip on the surface, as illustrated in Figure 1.3b. However, when the setpoint ratio is less than the unity, called “hard tapping”, a significant indentation will be introduced into the resulting image, as shown in Figure 1.3c. The indentation depth depends on the energy of the tip coming into contact with the surface. In order to reduce the force applied to the sample, leading to a plastic deformation of the sample surface, the setpoint ratio should be close to unity and its free oscillation should be as low as possible. It was found that changing setpoint ratio [18] or changing free oscillation while keeping the setpoint ratio constant [19] results in significant differences in the resulting images.

The effects of setpoint ratio and free oscillation are not fully investigated in the AFM imaging technique. Thus, the tip-sample interaction based on the tapping force applied to the sample surface during imaging should be systematically investigated in order to get “true” topographical images from a sample surface.

2. Tip geometry and step height

To get a true sample image with AFM, a sharper tip and slower scan rate would be needed to remove the artifacts from the convolution effect [8, 20]. A model illustrating the
tip geometry effect has been developed [21, 22]. The artificial enlargement of width and height in real samples depends on the AFM tip radius and cone angle as shown in Figure 1.4.

![Figure 1.4 Artifacts caused by AFM tip and cone angle. E is artificial enlargement, R is tip radius, W is the real sample width, H is the real sample height, and A is cone angle [22].](image)

Several researchers have investigated the AFM as a metrology instrument, which can be used to measure the width and height of a sample in terms of two-point algorithm and histogram techniques [23]. In addition, algorithms for the calculation of profiling information on the model materials were developed and prototype programs using BASIC and MATLAB software were developed [24, 25].

### 3. AFM applications in characterization research

Atomic force microscopy (AFM) is now a widely available microscopy technique, offering the ability to image single individual macromolecules adsorbed on a substrate for visualization on its conformational properties and morphological changes of synthetic [26]
and natural polymers materials [27, 28] at the nanometer scale in many different native environments.

Although AFM techniques have been applied to investigate the surface characterization of wood, fiber, pulp and paper [29-31], the application of AFM for elucidating characterization of the structure-property relationships of cellulosic materials is still at an early stage [32]. AFM application to study the dynamic changes of macromolecules in situ and in real-time, however, is still a challenge due to several limiting factors, such as lateral resolution, tip-and-sample interaction, and variable scanning parameters [33].

Nevertheless, AFM imaging at a nano-meter scale, where a variable environment induced changes of the macromolecular structure and its morphology, can be used for the characterization of behaviors of macromolecular dynamics [33]. In addition, it has become increasingly evident that this technique can provide answers in quantitative terms to several questions about the structure and functional properties of cellulosic materials [34-36].

C. Ultrastructure in cell wall

1. Wood polymers

Wood is the principal source of lignocellulosic fibers. The cell wall of a softwood fiber is considered as a gel built up mainly of three polymers, cellulose, hemicelluloses, and lignin [37]. Cellulose is a high-molecular weight linear homopolymer composed of repeating
β-D-(1-4)-glucopyranose units in a variety of arrangements [38]. The anhydroglucose unit $\text{C}_6\text{H}_{10}\text{O}_5$ is in the form of a six-sided ring consisting of five carbon atoms and one oxygen atom. The basic chemical structure of cellulose is presented in Figure 1.5. The degree of polymerization (DP) of wood cellulose is around 10,000 [39]. The hydroxyl groups of cellulose form intra- and inter-molecular hydrogen bonds. The intra- and inter- molecular hydrogen bonds hinder the free rotation of the rings on their linking glucosidic bonds resulting in the stiffening of the chain. Without destroying the intermolecular bonds, the hydrogen bonds also contribute to the insolubility of the cellulose chains in solvents. However, cellulose is a hydrophilic polymer with mainly three hydroxyl groups per glucosidic unit available for water adsorption.

![Figure 1.5 Chemical structure of cellulose](image)

Hemicelluloses are polysaccharides generally classified according to the main sugar residue in the backbone, e.g. xylans, mannans, and galactans. Hemicellulose in hardwoods is mainly xylan (15-30 %), whereas softwood hemicelluloses consist of galactoglucomannans (15-20 %) and xylan (7-10 %). The hydroxyl groups and carboxylic acid groups in the
hemicelluloses result in hydrophilic behavior. The average degree of polymerization of hemicelluloses in softwood and hardwood is approximately 100 and 200, respectively [39].

Lignin is a cross-linked aromatic polymer based on phenylpropane units; p-coumaryl alcohol, coniferyl alcohol, and sinaphyl alcohol. The proportions of these monomers are dependent on the plant species. Softwood lignin is built up mainly of coniferyl alcohol, whereas hardwood lignin is a copolymer of sinapyl and coniferyl alcohol units. The lignin network is less hydrophilic than the polysaccharides in wood.

2. Organization in the cell wall

Wood fiber is composed of a heterogeneous mixture of lignin, hemicellulose, and cellulose arranged in a complex fibrillar structure. The average length of fiber in typical softwood is 3 mm and the width is 25 to 30 microns. When the fiber is collapsed, the thickness is 5-8 microns [40]. The wood cells are composed of different layers, which differ from one another with respect to their structure and chemical composition.

Figure 1.6 illustrates hierarchical structure of wood cells which are made of three main layers; the middle lamella, the primary wall, and the secondary wall. There are three sub-layers in the secondary wall, a thin outer layer (S₁), a thick middle layer (S₂), and a thin inner layer (S₃) towards the lumen. The microfibril angle (MFA) in the S₂ layer, defined as the angular deviation of the microfibrils in the S₂ layer relative to the longitudinal cell axis, plays a crucial role in the mechanical properties of wood fiber since the S₂ layer forms the main fraction (ca. 75 %) of the fiber wall volume [41].
3. Physicochemical properties of cellulose microfibrils

It has been shown in the literature [42-44] that the cellulose chains are biosynthesized and self-assembled into bundles of molecules, called elementary fibrils, microfibrils, and protofibrils which are the smallest morphological structure of the fiber. Various organization models of plant cell wall have been proposed and well reviewed [45].

Figure 1.7 shows a proposed lamellar model for ultra-structural arrangement of the secondary cell wall in spruce wood and its corresponding chemical pulp fiber [36]. Cellulose, hemicellulose, and lignin are differently distributed in the cell wall and they are closely associated with each other. The hemicellulose glucomannan in softwood is closely associated with the cellulose microfibril with a diameter of approximately 3 nm [46], while
xylan seems to appear in combination with the lignin [47]. The cellulose microfibrils are aggregated with a portion of glucomannan, forming a larger structural unit called a cellulose fibril aggregate. The cellulose fibril aggregate is approximately 15 nm in diameter.

Figure 1.7 Schematic illustration of a cross-section of S2 layer in spruce wood (left) and its corresponding chemical pulp (right) [36].

Away from the main controversy, the existence of two families of native cellulose was confirmed by the application of solid state NMR ($^{13}$C CP/MAS) to a range of cellulose samples of different origins [48-50]. From a detailed analysis of the carbon atom couplings observed in the solid state NMR spectrum, Vanderhart and Atalla [51] established that native cellulose was a composite of two distinct crystalline forms called I$_\alpha$ and I$_\beta$ lattices. Figure 1.8 [52] shows two different crystal forms based on results from Nishiyama et al. [53, 54]. The chains in cellulose I$_\alpha$ are crystallographically identical but alternating glucose units in each chain are slightly different in conformation. Cellulose I$_\beta$ consists of alternating distinct sheets of two distinct chain arrangements. The latter, predominant in cotton and ramie, is
characterized by two polysaccharide chains in a parallel arrangement resulting in a monoclinic unit cell. The lattice $I_\alpha$ is generally present in all algae and bacteria, and has a triclinic unit cell structure. The $I_\alpha$ may be converted into the $I_\beta$ form by annealing the cellulose chain in the solid state [55].

![Figure 1.8](image)  
**Figure 1.8** Symmetry and directions of hydrogen bonding in cellulose [52]. (a) Cellulose $I_\alpha$, (b) Cellulose $I_\beta$.

Various theories regarding the supra-molecular arrangement of the wood polymer chains in cellulose microfibrils have been proposed [56]. **Figure 1.9** shows some of the proposed structures in the cellulose microfibrils [57]. Two principal models for the cellulose microfibril structure have been proposed, continuous and discontinuous structures. The discontinuous structure was originally derived from the fringed micellar hypothesis [58]. According to this hypothesis, cellulose chain molecules are tied laterally together and formed parallel crystallites. Cellulose chains are transversely linked from one crystallite to another by regions, which have no regular arrangement. This region is considered to be amorphous.
Thus, the amorphous and crystalline regions separately exist in a semicrystalline polymer. A continuous microfibril structure called the fringed fibrillar model was proposed by Hearle [59]. In this model, the cellulose microfibril consists of crystalline fibrils of indefinite length helically wound in a matrix of amorphous cellulose.

![Figure 1.9 Schematic descriptions of several theories of component structures in fibers [57].](image)

Although the exact arrangement of cellulose microfibrils is still subject of controversy, many studies based on various physical and chemical methods [60-62] have indicated that the microfibrils are not completely crystalline, but instead contain two distinctly different regions; the crystalline area, composed of highly ordered cellulose molecules, and the amorphous or paracrystalline region, which is less highly ordered cellulose molecules. The ratio between the mass of ordered regions and the total mass of the cellulose, is called the degree of crystallinity, and typical dimensions are dependent on their
origin [63]. Crystallinity of cellulose controls not only its physical properties, but also its accessibility to chemical modification, swelling, and adsorption behaviors.

Figure 1.10 Developing a two-phase structure in cell wall with processing variables [69].

This structure influences the physical and chemical behavior of the wood fiber. Consequently, altering this structure by pulping or papermaking processes will change the physicochemical properties of the fiber and end products produced from the fiber (e.g. paper). Wood fiber can be separated either by mechanical pulping and chemical pulping or a
combination of both types of pulping (semi-chemical). Figure 1.7 shows a schematic description of pulp fiber after chemical pulping. During chemical pulping, the lignin-hemicellulose matrix is gradually removed, leading to formation of pores in the cell wall structure [36, 37, 64] and an increase in crystallinity of cellulose microfibrils [65], as well as an enlargement of cellulose microfibrils due to low residual hemicellulose content [66, 67]. Thus, the properties of a wood fiber are interconnected with its chemical composition and macromolecular arrangement of each chemical component.

Stockman [68] also proposed that the paracrystalline regions are converted to amorphous regions by chemical or mechanical treatment of the fibers as shown in Figure 1.10. He also proposed that longitudinal contraction of microfibrils is from an entropy-elastic deformation of amorphous regions of microfibrils. With the anisotropy of the thermal fluctuations of the atoms in the cellulose molecules, the atoms vibrate more strongly in the perpendicular than in the longitudinal direction of the chain, resulting in a kinetic tensile force. The built-in tensile stresses from cell growth stresses in wood and supra-structural changes of elementary fibrils during fiber processing including pulping, beating, and mercerization cause elementary fibrils to contract longitudinally [68-70]. Clair et al. [71] demonstrated the maturation stresses cause the contraction of cellulose microfibrils/crystallites in a tension wood by synchrotron x-ray diffraction experiment. He proposed possible shrinkage models of cellulose microfibrils as shown in Figure 1.11. In addition, Nakano et al. [72] showed that the contraction of wood samples depend on the concentration of alkali and heating temperature during mercerization. They also concluded that the longitudinal contraction of the sample is due to entropy elasticity, which is caused by the transformation of microfibrils in wood.
Figure 1.11 Possible shrinkage model of cellulose microfibril [71]. $L$ represents the length of wood and $d$ the fiber repeat distance, so that the macroscopic strain is $(L_1 - L_0)/L_0$ and the lattice strain is $(d_1 - d_0)/d_0$. The dashed lines represent H-bondings in lateral direction (a) Lattice strain corresponding to the macroscopic strain; (b) Microfibril buckling; (c) Reorganization of the metastable crystal.

The removal of hemicellulose and lignin from wood fibers during the pulping and bleaching processes also leads to changes in the physical and chemical properties. The ionic groups at the surface of the fiber, mainly carboxylic acid groups attached to the xylan and phenolic hydroxyl groups in the lignin, make fiber properties especially sensitive to the environment. After dissolutions of the lignin and hemicellulose located between lamellae, gaps remain between the lamellae. These gaps between the lamellae were called
“macropores” and were compared to a smaller class of intralamellar pores, the “micropores” [73]. The native fiber has a 0.02 cm$^3$/g void volume in the fiber wall, but the void volume will increase to around 0.6 cm$^3$/g with kraft pulping at a yield of 47 % [73]. These changes in structure of the fiber wall have a great influence on the ability of the fibers to consolidate with each other during drying of the paper.

In addition to gaps in the fiber wall, pulp fibers have inherent defects influencing reactivity toward swelling or hydrolytic agents. The defects described as dislocations, slip planes, microcompressions, and nodes [74] exist in the internal structure of wood and pulp fibers. The deformations come from natural growth, wind stress on the tree, or they may be artificially induced by mechanical treatments in pulp and paper processing. Due to the less ordered or exposed cellulose in dislocations, these deformations are more readily attacked by chemical, mechanical, and enzymatic treatments. Although it makes fibers more flexible, it can affect paper properties including strength, degree of swelling, and rate of dissolution.

Natural fibers can be processed in different ways to yield reinforcing elements having different mechanical properties. The elastic modulus of natural fibers from wood is about 10 GPa, and the stiffness of fiber from chemical pulping processes is only in the range of 20-40 GPa, since lignin and hemicellulose have elastic moduli of 2 GPa and 7 GPa respectively [75]. The elastic modulus of the fibers up to 150 GPa can be achieved by hydrolysis, followed by mechanical disintegration into microfibrils. Theoretical calculation of the elastic modulus in crystallites parallel to the chain direction in an elementary cellulose I fibril is 128 to 167.5 GPa [76]. This remarkable mechanical performance of wood fiber is due to its complex architecture at all hierarchical levels, as well as to specific molecular mechanistic phenomena in the cell wall.
4. The relation between a single fiber and paper

The manufacturing and converting of paper and paperboard, in common with other cellulose-based composite materials, can be affected by dimensional behavior of paper and paperboard toward its surrounding environments [2]. Dimensional stability of paper and board, in turn, is important for many reasons. For example, the dimensions of printing and xerographic paper were significantly changed when moistened or dried [77]. Tensile loading occurs during the transport of a web in papermaking machines, and printing presses can stretch paper permanently. In offset printing, paper can expand from moisture pickup or be stretched when peeled off the printing blanket. Paper stored on pallets may develop wavy edges in high humidity and tight edges in low humidity, both of which can cause poor feeding in printing processes.

Dimensional stability of paper is indirectly and/or directly connected with the effect of moisture content on expansion (swelling) and contraction (shrinkage) of the fibers of which the paper is composed. The changes of individual fibers in dimensions, basically, come from their physicochemical response to water [78]. The amount of moisture take-up is governed by the degree of crystallinity of a fiber [79]. As the proportion of amorphous cellulose increases, so also does the accessibility of the cellulose to water. The degree of swelling which takes place and the limit of swelling depend on the degree of crystallinity of the fiber and the strength of the bonds holding the crystalline regions together. Therefore, the swelling of fiber by water is a question of fundamental importance in dealing with the manufacture of paper, and the relationship between the extent of swelling of fiber and the properties of the paper made from these fibers is of great interest from the fundamental point of view and may be of great significance in practical papermaking [4,5,80].
The mechanical behavior of paper is, in common with other cellulose-based composite materials, time and humidity-dependent [40]. The question is to what extent phenomena such as relaxation, creep, and hygro-expansion should be taken into account in an analysis of the behavior of paper during its end-use as well as its manufacture.

Cellulose microfibril material has been exploited for many decades and could be important for the coming decades as a source of biopolymer, due to its ubiquity and industrial potential. Although considerable research has focused on the elucidation of the structure-property relationships of cellulose microfibrils, the industrial application of cellulose constantly generates questions of a scientific nature for researchers.

**D. Enzyme technology**

It has been clearly established that cellulose and hemicellulose can be converted to soluble sugars by enzyme hydrolysis actions. These enzymes are called cellulases and hemicellulases. Simultaneous synergistic actions of several different enzymes are required to the complete hydrolysis of the heterogeneous cellulose as shown in Figure 1.12. Based on their biophysical action on cellulose, cellulases can be classified into endoglucanases (EGs), cellobiohydrases (CBHs), and β-glucosidases [81]. EGs preferentially hydrolyze the internal β-(1-4) linkages of amorphous cellulose in a random manner. CBHs hydrolyze crystalline cellulose by cleavage of cellobiose from the chain ends with synergic action with EGs. The cellobiose released from EGs and CBHs actions is finally converted into glucose by action of β-glucosidases. Thus, the structure-function relationships between the multiple cellulase
components and its heterogeneous substrate enhance our knowledge of biodegradation of cellulose as well as facilitate the usage of cellulose as a source of monomeric chemicals [82].

![Diagram of cellulase action](image1)

**Figure 1.12** A model demonstrating the action mode of two different types of cellulase.

Various cellulase/hemicellulase preparations are now commercially available. These enzymes have been widely applied in fundamental aspects and in many industrial applications in the field of food, textile, and pulp and paper industries [83]. However, the performance of cellulases is considered to be rather low compared to cost. Thus, more powerful cellulases are being developed in order to increase their conversion rate of cellulose into glucose per unit cost. The conversion rate, called cellulase activity, is an indicator of the efficiency of cellulases. The determination of cellulase activities is a complicated process because hydrolysis of the water-insoluble cellulose does not necessarily have a linear relationship with enzyme dosage or with reaction time. Sometime over-dosage and over-
reaction time have a significant detrimental impact on the final products. For such reasons, enzymatic activities should be carefully determined.

Interactions of cellulases and cellulose not only facilitate physical contact between enzyme and substrate, but also play an important role in the efficiency of the enzymatic hydrolysis of crystalline cellulose [84, 85]. Therefore, an understanding of enzyme interactions with substrates is of great importance in processing wood and cellulosic fibers. The rate of enzymatic hydrolysis and its yield are dependent on the adsorption of enzyme onto the substrate surface, although the activity of the cellulase seems to be dependent on the physicochemical properties of the substrate [86, 87], the multi-components of the cellulases [88], and physical reaction parameters such as mass transfer [89] and temperature [87]. Various kinetic models have been used to explain the cellulose hydrolysis rate, which is based on the amount of adsorbed enzyme on the cellulose surface [90], the structural characteristics of the substrate including pore size distribution, crystallinity index, specific surface [91,92], and cellulase-cellulose adsorption [93-95].

All of the kinetic models developed so far assume that the initial rate of hydrolysis is proportional to the amount of enzyme-substrate complex formed by adsorption of cellulase. However, the proportional hydrolysis rate rapidly decreases with reaction time. Several studies proposed the reasons; thermal instability of the cellulases [96], inactivation of the adsorbed cellulase due to the diffusion into the cellulose fibrils [97], strong inhibition by by-products such as cellobiose and glucose [98], transformation of the cellulose into a less digestible form [99], and the heterogeneous structure of the substrate itself [100]. Overall, it is difficult to completely understand the enzymatic degradation of cellulose from a
theoretical approach or to even develop a kinetic model for pure cellulose due to the complex enzymatic actions of multi-component cellulases.

Monitoring reductions in viscosity of CMC solutions is considered to be the most accurate method for detecting the EGs action, which hydrolyzes internal bonds within in a polymer [101, 102]. However, the viscometric measurement of soluble cellulose derivatives using the manual viscometers for EGs activity is not commonly used, due to its laborious and discontinuous nature. For this reason, the colorimetric measurement of reducing sugars has been routinely used for measurement of EGs activity.

Various types of automated viscometers are now used to measure the rheological change in polymer solution. Viscosity is a fundamental rheological parameter in polymer solution, characterizing fluid resistance to flow. The viscosity of a polymer solution is related to the polymer concentration, the extent of polymer-solvent interaction, and the polymer structure such as size, shape, molecular flexibility, and configuration of the molecules. It is functionally related to the molecular weight under appropriate experimental conditions [103]. However, more fundamental studies of the rheological change in cellulase-polymer solutions should be investigated in order to monitor degradation patterns of polymers by enzyme hydrolysis.

Like all proteins, enzymes are basically made up of amino acids linked by peptide bonds between the carboxyl groups of one amino acid and the amino group of the next amino acid. The hydrophobic and hydrophilic nature of amino acids often makes the surface of enzymes an amphipathic interfacial structure, i.e. proteins tend to adsorb at interfaces as a surface-active polymer [104]. Moreover, CMC is a semiflexible anionic polymer [105]. The adsorption of enzymes (protein) on the CMC leads to a change of the functional properties
such as mechanical, physical and/or chemical properties of the interface. Mixtures of proteins (enzymes) and polymers in aqueous dispersion are often accompanied by either segregative or associative phase separation [106]. Thus, physico-chemical parameters of proteins (enzymes) and substrates such as pH, ionic strength, charge density, concentration and its ratio should be considered. Moreover, processing variables such as temperature, shear rate and time also strongly influence the rheological behavior of the complexes. For example, changes in functional properties due to soluble complexes between a globular protein (BSA) and a polysaccharide (CMC) were reported [107, 108]. The protein was adsorbed onto the CMC coil segments in the dilute regime or entrapped in the cross-links of the entangled network in the semi-dilute region. After thermal treatment of the soluble complexes, a considerable change in the visco-elastic properties of the network was also observed [109].

The mechanism of adsorption to cellulose is still unknown, but the presence of aromatic amino acids such as tyrosin and tryptophan in CBDs suggests that these amino acids have critical roles in binding [110]. The binding abilities of different CBDs vary, but they seem to allow the celllobiohydrases to have a processive hydrolysis and the endoglucanases to have mobilities to access new sites on the substrate surface [111]. However, the role of the different structural domains in cellulase action is not clear and therefore their effects on substrates needs to be examined further. 
E. References


CHAPTER TWO

RESEARCH OBJECTIVE
As described in the Chapter One, the interaction between cellulose microfibrils and the surrounding environment is critical for functional properties of final products from bio-based materials. Despite its obvious significance, there is still not a thorough understanding of how a cellulose microfibril interacts with water molecules and of its concurrent dimensional changes. This may be due to the complicated behaviors of the heterogeneous fiber materials. In addition, the experimental techniques were cumbersome and difficult to interpret at the nano-scale. Only a limited number of studies have yet been made on dimensional changes of cellulosic material with water molecules.

Therefore, the main emphasis in this study has to establish working routines for the AFM as well as to develop an image analysis technique. Based on the developed novel technique, dynamic interaction of the concurrent dimensional changes occurring in individual cellulose microfibril with variable relative humidity will be clarified.

The interaction between water molecules and cellulose microfibrils is certainly a field where this new technique offers notable advantages. The measurements of the hygro-expansive properties of cellulose microfibrils will be applicable in understanding the hygro-expansive properties of paper, the accelerated creep properties of paper, and how cellulose fibrils may perform for nano- and micro-composite applications. These findings will have significant implications in paper dimensional stability and the engineering of cellulose micro- and nano-fiber composites.
CHAPTER THREE

TECHNIQUE FOR THE MEASUREMENT OF DIMENSIONAL CHANGES OF NATURAL MICROFIBRIL MATERIALS UNDER VARIABLE HUMIDITY ENVIRONMENTS

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A. Summary

An algorithm was developed to analyze the dimensions of line scan data of step-shaped discontinuities acquired with an atomic force microscope. The effect of a number of AFM parameters on the quantitative imaging of step features is discussed. Quantitative imaging using AFM was shown to be very reproducible as five successive scans of a standard step height grating produced less than 3 % variation in measured parameters. A cellulose microfibril, called cellulose aggregate fibrils (CAFs), with dimensions of ~ 50,000 × 2,000 × 300 nm derived from papermaking fibers was scanned under cyclic relative humidity conditions with the relative humidity starting at 50 % then rising to 80 %, followed by a decrease in the relative humidity to 28 %. Changes in the width of the CAFs were weakly correlated with changes in the relative humidity, while changes in the height and area of the CAFs were positively correlated with the relative humidity. The length of the CAFs was negatively correlated with the given relative humidity cycle. These findings have significant implications in paper dimensional stability and the engineering of cellulose micro and nano-fiber composites.

B. Introduction

Paper is a consolidation of randomly deposited fibers, which typically originate from wood. These fibers may be liberated from the tree by a number of processes (i.e. chemical,
mechanical, or semi-chemical pulping). Chemical pulping produces fibers consisting of partially crystalline cellulose microfibrils discontinuously surrounded by a matrix of lignin and hemicellulose.

The specific pulping and bleaching processes used to produce the fibers can affect the final properties of the fibers produced. The pulping and bleaching processes used are often optimized to increase pulp yield on wood, or to reach a limited number of quality control parameters (ex. Kappa number, conductivity, and brightness). During this optimization, little regard is given to the final performance of the wood fiber in the papermaking operation. However, manipulation of the pulping process can lead to differences in the deposition of the discontinuous matrix of amorphous hemicellulose and lignin leading to differences in papermaking performance. Ultimately, the distribution of the cellulose, hemicellulose, and lignin throughout the fiber plays a crucial role in the final paper properties. Dimensions of cellulosic papermaking fiber are found to be related to relative humidity [1]. Consequently, the response of papermaking fiber to environmental variables is related to the dimensional stability of the paper. Dimensional instability causes end-use problems and limits the wider application of paper and paperboard. Thus, good dimensional stability of paper and paperboard is important for the satisfactory performance of paper and paperboard [2].

When paper dries it shrinks, and upon exposure to moisture it swells. The ability to resist swelling is called dimensional stability. The dimensional changes that take place during exposure to moisture are characterized by the hygro-expansive properties of the sheet. Paper is an anisotropic material and its properties may be different in the three principal directions. These differences arise from the manufacturing process and the anisotropic nature of the fibers that constitute paper. Differences in the hygro-expansive properties lead
to macroscopic deformation of the paper such as curl, cockle (waviness), and twisting of the paper (i.e. multi-axis curl).

The hygro-expansive properties of paper have been extensively studied from both fundamental and applied viewpoints [3-6]. To control the hygro-expansive properties, paper mills have used mechanical means to control the anisotropy of paper web shrinkage [7]. Shrinkage of paper near the edge of the paper machine can be controlled to a limited extent with mechanical means, but the fibers themselves are the underlying cause for the shrinkage of the sheet [8, 9]. Therefore, research on dimensional stability of the fiber should be carried out in order to improve the dimensional stability of the sheet.

Understanding the hygro-expansive properties of microfibrils is an essential part of understanding the hygro-expansive properties of the fiber and thus the paper sheet. However, only limited research exists on relationships between the hygro-expansive behavior of individual fibers at the microfibril scale and the paper sheet [10]. This might be due to the complicated behaviors of non-homogeneous fiber materials (i.e. crystalline and non-crystalline cellulose, hemicelluloses, and residual lignin) or to the fact that techniques for detecting dimensional changes at the nanometer scale in variable humidity conditions were not previously readily available.

Atomic force microscopy (AFM) is a widely available microscopy technique. This technique offers the ability to examine materials at the nanometer scale in many different native environments. It is recognized that nano- and micro-scale materials properties measured in the laboratory affect the performance of materials in end-use applications. It is important that reliable and statistically significant measurements of nano- and micro-scale properties can be made. This necessitates robust data processing techniques that have high
fidelity for the material property being measured. In the current situation, it is of interest to
determine the measured length, width, and height changes of a cellulose aggregate fibrils
(CAFs) under varying humidity conditions. This problem is similar to determining the step
height of a calibration grating, which has been previously studied.

Although many researchers have addressed the metrological aspects of AFM imaging
of materials, uncertainty of instrumentation parameters has been shown to lead to
inconsistency in the accurate measurement of dimensions [11]. The step height of a structure
is generally defined as the vertical distance between the top of a plateau discontinuity (step)
and the bottom of the discontinuity (baseline) [12]. The step height can be measured from an
AFM image using histogram or manual point-to-point measurement methods [13]. The
histogram method does not provide a unique step height, as there are no written standards for
correcting the baseline of the observed line profile. The manual single-point method, which
is also commonly used, is not satisfactory for actual samples due to the low number of points
that are sampled and the possible bias in the selection of the points. In addition to these
methods, AFM instrument manufacturers may offer measurement capabilities in their
standard software packages; however, the measurement software still may require an
extensive time-consuming image processing routine. Automated algorithms for determining
the apparent dimension of nanoscale materials are not readily available. In this study, a step
height characterization technique is described and the effects of AFM imaging parameters on
the measured dimensions of step height standards are described.

This work focuses on developing a well-characterized method for the metrological
imaging (measured length, width, height, and cross-sectional area) of cellulose aggregate
fibrils under varying relative humidity conditions. The study and the resulting method take
account of the actual surface topographical characteristics, AFM instrument parameters, and the effects of probe/surface convolutions. Measurements of the hygro-expansive properties of cellulose aggregate fibrils are applicable in understanding the hygro-expansive properties of paper, the accelerated creep properties of paper, and how cellulose fibrils may perform in nano- and micro-composite applications.

C. Experimental

1. Experimental set-up

The experimental apparatus was placed in a controlled temperature and humidity environment (23 °C and 50 % relative humidity). Images were acquired with a Q-scope™ 250 (Quesant Instrument Corporation, USA) using a NCS tip (MikroMasch, USA) with 230 μm long cantilever with a spring constant of approximately 3.5 N/m in intermittent contact mode. An acoustic/vibration isolation chamber (Quesant Instrument Corporation, USA) served as the test chamber for the experiments. The atmosphere above two saturated salts solutions, potassium acetate and potassium sulfate, were combined with an air pump, as shown in Figure 3.1, to generate various humidity conditions in the test chamber. Samples were subjected to different humidity cycles from ca. 28 % to ca. 80 % relative humidity (RH).
Figure 3.1 Diagram of the equipment used to control the humidity within the AFM test chamber. Each of saturated potassium acetate solution and saturated potassium sulfate gives ca. 28 % and 80 % RHs, respectively, in the AFM chamber for 1 hour after pumping. This setup allowed for the imaging of samples over a wide range of relative humidity from ca. 28 % to ca. 80 %.

Two different humidity cycles were followed: 1) Forced Change (FC) - The air pump supplying humid air to the test chamber was turned on to bring the relative humidity to the desired level as rapidly as possible. The pump was left on for two hours during which time images were continuously acquired with the AFM. Air flow was then switched to a different combination of saturates salt solutions and the humidity in the test chamber was brought to the new humidity level during which time images were continuously acquired. 2) Free Decay (FD) - The air pump was used to raise the humidity in the test chamber as rapidly as possible. During two hours of pumping, the sample was imaged and then the pump was shut
down. The door to the test chamber remained closed and the humidity in the test chamber was allowed to slowly return (decay) to ambient conditions (50 % RH) over at least a 24 hour period. During this period of free decay, the sample was imaged. Once ambient conditions were reached and 24 hours had passed, the air pump was turned on bringing the test chamber to the next relative humidity. The humidity within the test chamber was measured with an electronic hygrometer (OMEGA® RH411 Relative humidity meter, OMEGA Co.).

Each image typically took ~10 minutes to complete during which time the relative humidity changed. The humidity was recorded at the start and completion of each scan. The average of these two values was then recorded as the relative humidity value for the corresponding image. The range of these two values was typically less than 5 % relative humidity.

2. Reference grating

To calibrate the height of samples, standard step height gratings were used. The reference gratings (TGZ series, MikroMasch, USA) consist of a silicon chip with one dimensional (1D) arrays of rectangular SiO2 steps coated by Si3N4 to prevent the silicon from oxidizing. The gratings had nominal step heights of 20 nm (TGZ 01), 102 nm (TGZ 02), and 513 nm (TGZ 03) with a nominal pitch of 3.0 μm.

3. Preparation of cellulose aggregate fibrils (CAFs)

Cellulose aggregate fibrils were prepared from a NIST bleached softwood kraft pulp [14]. The NIST softwood pulp was beaten in a valley beater (Tappi Method T200 om-89)
and fractionated in a Bauer-McNett classifier (Tappi Method T233 cm-82) to remove small particle fragments of cell wall and primary cell wall fractions released from the fiber. The secondary cell wall fiber fraction retained on Tyler screen No. 12 was beaten in a PFI mill (Tappi Method T248 cm-85) for 75,000 revolutions to increase the degree of fibrillation of the pulps. The pulps were then screened with a Britt dynamic drainage jar (Tappi Method T261 cm-90) at 0.5 % consistency. The filtrates, called cellulose aggregate fibrils (CAF), were thickened ca. 2 % consistency with a 0.45 micron membrane filter and were refrigerated during storage. These cellulose aggregate fibrils are presumed to be composed of cellulose fibrils mixed with lignin and hemicellulose in varying amounts depending on yield of the original pulp and pulping process.

In order to obtain images of CAFs using AFM, a glass slide sample of the CAFs was prepared by dropping 10 μl of diluted CAFs suspension (less than 0.0002 %) onto 20 different spots on a cleaned glass slide. The glass was first cleaned by immersing the slide in a mixture of absolute ethanol and 1 N HCl (1:1, v/v) overnight followed by ultrasonic cleaning in deionized water three times. The glass slide with the CAFs sample attached was conditioned at 50 % RH and 23ºC for more than one week.

4. Image processing procedures

AFM images were obtained at a variety of magnifications under the conditions described in the experimental set-up. Since the surfaces scanned by the AFM are not generally parallel to the scanning plane, the resulting images have unwanted tilt and/or bow [15]. The plane distortion should be corrected by a plane flattening algorithm before
carrying out further analysis. In order to remove the tilt, scanned images were processed with a tilt-removal algorithm in the AFM software (Quesant Instrument Corporation, Version 4.00.3). The parabolic tilt-removal algorithm fits a paraboloid section (i.e. a surface described by parabolic functions) to the image contours and then subtracts this from the image data [16]. The main purpose of the tilt-removal processing is to facilitate further image processing. Raw data was exported from the instrument manufacturer’s software in ASCII format consisting of X, Y, and Z values for the corresponding image. The Z values, which correspond to the piezoelectric tube voltage, must be converted into height values using a Z scaling factor provided in the ASCII file header. Once this is performed the image can be recreated using a variety of image processing and data processing software packages.

5. Processing algorithm to detect edges and calculate the step width, height, and cross-sectional area

Figure 3.2 shows the line profile of original (2A) and baseline adjusted images (2B) of 102 nm step height standard grating. The slope of the baseline in the original image (2A) may be corrected by a least square fitting method. However, this may lead to errors in the step height measurement derived from cosine errors during the slope correction of the X-Z plane and Y-Z plane. In addition, after slope correction, the corrected left and right data points were set to zero making it difficult to automate further image processing. An essential first step to develop an automated image processing algorithm was to remove the slope in such a manner that the starting positions of left and right sidewall of a feature in an AFM scan line can be easily detected by the computational algorithms.
Figure 3.2 AFM images and associated line scans of TGZ 02 grating. (A) Original image (top) and a line profile (bottom); (B) The same image after parabolic baseline correction (top) and a line profile (bottom); (C) Line profiles of original and leveled images from A and B; (D) Geometry definition in a line profile. The arrow in (A) and (B) indicates a line segment out of 400 line segment at each image.
In order to remove the slope of the baseline in the original image, the parabolic tilt-removal algorithm in the ScanAtomic software was used. The baseline of the corrected image had a parabolic-type profile where features on the far left had a negative slope associated with them and the features on the far right had a positive slope associated with them. Features near the center of the image had little slope associated with them due to the parabolic nature of the baseline, the bottom of the sidewall of the center feature (step) on the left and right were lowest values in the line scan and can be readily detected, cf. Figure 3.2C and 3.2D. Note that the image is not translated or dilated in the X-Y plane.

The line profile acquired by AFM is a combination of both the actual surface topography and the probe shape used during scanning. The probe shape and radius has a large influence on the perceived shape of the grating as shown in Figure 3.2D. The AFM line scan profile shows a convolution between the tip geometry and the real feature shape, so that a highly accurate measurement of width is hard to realize. However, one can estimate the artificial enlargement of the real width. Knowing that the standard grating has a well defined sharp edge and defined height (102 nm in height, \( h \)) and knowing the characteristics of the probe (30° cone angle, \( \alpha \)), a geometrical convolution of the two can be easily determined to estimate the real width of the feature [17].

A generally accepted definition of the line width has not yet been developed. In the current situation, the perpendicular distance from the lowest point on the left sidewall (a) and the right sidewall (b) after parabolic tilt removal as shown in Figure 3.2D was used as the line width. The area underneath the line scan of the feature was determined by calculating the area underneath the entire feature and then subtracting out the area of the baseline between (a and b) as follows:
\begin{align*}
\text{Area} &= \int_{a}^{b} f(x) \cdot dx - \int_{a}^{b} F(x) \cdot dx \\
\text{[Eq. 1]}
\end{align*}

The average height of the feature was calculated by dividing the area by the line width. This algorithm was termed rapid feature analysis (RFA) algorithm and allowed for a significant reduction in the amount of time required to characterize images compared to manual point-to-point methods. Changes in the measured length of the CAFs were determined by identifying random defects in the CAFs and monitoring the change in position of these defects in the resulting image. The point-to-point distance between these defects was measured. This indicates a measurement of a segment length, which may or may not be indicative of how the dimensions of the entire CAFs change with relative humidity. For all measurements the average value of multiple measurements is displayed. For the width, cross sectional area, and height, 400 data points are used unless noted.

D. Results and discussion

1. Comparison of two analysis techniques

Figure 3.2B shows an example of an AFM image after tilt removal. The dimensions of the gratings were calculated from the line profiles of these images. Figure 3.3 shows the average area, width and height of the standard grating determined by three different algorithms: a manual point-to-point measurement, rapid feature analysis using 100 line scans.
(RFA-100), and rapid feature analysis using 400 line scans (RFA-400). For this analysis, images of the standard grating were taken over a 10 µm (X) × 10 µm (Y) area and the tilt from the image was removed as described above. The center step was then cropped from the image to form a new image 4 µm (X) × 10 µm (Y) in size. An upper portion of the 4 µm x 10 µm image, corresponding to 4 µm (X) × 2.5 µm (Y) of the cropped area, was then analyzed by the three algorithms described above. As shown in Figure 3.3, the average heights of 90 and 96 nm were quite close to the nominal height value (102 nm). The point-to-point analysis showed little difference in dimensions compared to the computational measurement in the same area and entire image. The result showed that the dimensions of 1D-grating standards were well characterized based on the algorithms.

![Figure 3.3](image)

*Figure 3.3* Comparison between the manual point-to-point and computational algorithm for the analysis of images of the TGZ 02 standard grating (102 nm in nominal height).
2. Optimization of scanning parameter

The accuracy and precision of quantitative measurements made with an AFM can be affected by instrumentation error (noise) and inattention to details of instrument settings. Noise is unavoidable, however, in this study, careful attention was given to noting and ensuring consistent instrument settings were used.

Atomic force microscopy is in reality a profilometric technique. The AFM records the height profile of the surface of a sample from which three-dimensional information can be extract. However, AFM imaging techniques are often designed to visualize rather than extract quantitative surface topographical information. The RFA algorithm developed in this paper was designed to take advantage of the topographical information provided by the AFM and enhance the contrast of the specific topographical information. Using this algorithm, the effect of altering microscope imaging parameters can be systematically investigated. The fidelity of measurements extracted from an AFM image is most likely affected by instrument settings and noise associated with the measurement. There are several basic instrument parameters that introduce uncertainties into feature size measurements in addition to noise (stochastic and stationary) and image pre-processing procedures. A selection of these factors (image pre-processing procedure, set point ratio, and scan rate) was studied and the impact on the measured feature size was determined. This was accomplished by systematically altering the parameters of interest, while imaging standard step height gratings.
Figure 3.4 Line profiles of standard gratings with different nominal step heights after leveling (A) TGZ 01 with 20 μm × 20 μm scanned area; (B) TGZ 01 with 4 μm × 4 μm; (C) TGZ 02 with 10 μm × 10 μm; (D) TGZ 03 with 10 μm × 10 μm. The dotted line indicates the original line profile and the solid line indicates the leveled line profile at each image.
3. Effect of image pre-processing procedure

AFM images typically have an unwanted baseline tilt that must be removed to quantify feature dimensions [18]. There are several plane flattening algorithms available with the software provided with most AFM instruments. In the current situation, the tilt in the AFM images was removed using a parabolic tilt-removal algorithm. One benefit of using this algorithm, for adjusting the baseline, is that the resulting line scans have readily identifiable edges at the left and right sidewalls of step like features as shown in Figure 3.2D. In order to examine the artifacts introduced by the tilt algorithm, three different standard gratings (nominal heights 20, 102, and 513 nm) were scanned with different scan sizes. Figure 3.4 shows a line scan of each standard grating after baseline correction. The baseline correction for the 20 µm × 20 µm image results in a baseline that is concave down. This curvature in the baseline is undesirable for further processing. The small scan size (4 µm × 4 µm) yields an image that has the features “compressed” in the vertical direction. However, the line scan profile in the 10 µm × 10 µm scan area showed a concave upward curvature, which enables the RFA algorithm to readily detect the sidewall edges. The results from the 10 µm × 10 µm image indicate that the feature of interest should be positioned near the center of the scanned area and that a scanned area should be around 10 µm × 10 µm to apply the parabolic baseline correction effectively.

Figure 3.5 shows the measured dimensions of each grating with different scan area. The average measured heights were 23.8 nm ± 2 nm (TGZ 01, 4 µm × 4 µm), 101.4 nm ± 17 nm (TGZ 02, 4 µm × 4 µm), 98.1 nm ± 8 nm (TGZ 02, 10 µm × 10 µm), and 357.3 nm ± 15
nm (TGZ 03, 10 μm × 10 μm). Those values were close to the nominal heights provided by the manufacturer (20 nm (TGZ01) and 102 nm (TGZ01)), except the TGZ03 (513 nm). The TGZ03 grating was improperly manufactured and the normally square step had a shoulder on one of the edges, cf. Figure 3.4D. This shoulder on the grating resulted in significantly lower average height than the nominal height of 513 nm. This defect also serves to demonstrate the ability of the algorithm to characterize imperfect steps, cf. Figure 3.4D.

![Figure 3.5](image)

**Figure 3.5** Effect of different scan area sizes on the measured height of step gratings (TGZ 01, TGZ 02, and TGZ 03). The insert re-plots the result of TGZ 01 with 4 μm × 4 μm scan size.
4. Effect of scan rate

In order to evaluate the effect of scan rate on the measured dimensions of the 102 nm standard grating (TGZ 02), scan rates were varied from 0.25 to 2.0 Hz over a 10 µm x 10 µm image.

Figure 3.6 Effect of scan rate on the measured dimensions of the 102 nm standard grating (TGZ 02) with 10 µm x 10 µm scan area.

Figure 3.6 illustrates the effect of increasing scan rate on the measured width, height, and area of the step feature. It is clear from this figure that scan rate has a significant impact on the measured values. In particular, it is observed that for this system an increase in width occurs with higher scan rates. The average width for each scan rate was observed to be 1.09
µm ± 0.02 µm, 1.91 µm ± 0.02 µm, 1.95 µm ± 0.02 µm and 2.05 µm ± 0.02 µm from 0.25 to 2.0 Hz. This indicated that the measured width of the step feature depends on the scan rates. The measured height and area were observed to decrease as the scan rate increased, cf. Figure 3.6. The average height for 1.0 Hz was observed to be 98.1 nm ± 8 nm, which was the closest value for the nominal height of 102 nm. The observed trends in the measured values were expected, due to the fact that AFM imaging is a physical interaction and faster scan rates will result in the probe not following the topography as closely as at slower scan rates. This results in an apparent widening of features and an apparent decrease in the feature height at higher scan rates.

5. Effect of setpoint ratio

In the current study, wave mode was used to image the standard gratings. In this mode, the probe is intermittently brought into contact with the surface of the sample at a frequency of 70-72 kHz. The amplitude of this oscillation may be altered by altering the drive voltage. Once the probe is brought into contact with the surface the amplitude of the cantilever oscillation becomes less when compared to the free oscillation; this is termed dampening. The amount of dampening dictates how firmly the probe strikes the surface at a given drive oscillation amplitude. By adjusting the setpoint (A_{setpoint}) the dampening can be adjusted. The setpoint ratio (setpoint to drive amplitude) is an indirect measure of the dampening. Lower setpoint ratio indicates more dampening. The effect of the setpoint ratio with constant drive amplitude on the measured height, width, and area of the 102 nm (TGZ 02) standard grating is shown in Figure 3.7. The same drive amplitude (0.187 V) was used
with three different setpoints (0.15, 0.375, and 0.5 V) to create the three setpoint ratios. A decrease in the setpoint ratio, corresponding to “hard tapping”, resulted in a substantial decrease in the measured dimensions compared to higher setpoint ratios, corresponding to “soft tapping”.

**Figure 3.7** Dimensions of the 102 nm standard grating (TGZ 02) with a fixed drive amplitude ($A_0$) of 0.187 V and three different setpoints ($A_{setpoint}$); (left) 0.15 V, (middle) 0.375 V, (right) 0.5 V.

Alternatively, the drive amplitude may be changed, while the dampening remains constant, to adjust the setpoint ratio. **Figure 3.8** shows the dimensions of the 102 nm standard grating (TGZ 02) scanned with different drive amplitudes (0.14 V, 0.187 V and 0.3
V) and a fixed setpoint (50 % damping). Compared to 0.14 V and 0.3 V drive amplitudes, the height measurement with the amplitude of 0.187 V was closest to the nominal step height.

<table>
<thead>
<tr>
<th>Setpoint ratio ((A_{\text{setpoint}} / A_0))</th>
<th>Area (nm² X 1,000)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.5</td>
<td>200</td>
</tr>
<tr>
<td>1.6</td>
<td>210</td>
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<tr>
<td>1.7</td>
<td>220</td>
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<td>1.8</td>
<td>230</td>
</tr>
<tr>
<td>1.9</td>
<td>240</td>
</tr>
<tr>
<td>2.0</td>
<td>250</td>
</tr>
<tr>
<td>2.1</td>
<td>260</td>
</tr>
<tr>
<td>2.2</td>
<td>270</td>
</tr>
</tbody>
</table>

Figure 3.8 Dimensions of the 102 nm standard grating (TGZ 02) with three different drive amplitudes; (left) 0.14 V, (middle) 0.187 V, (right) 0.3 V and 50 % dampening.

6. Repeatability of measurements

In order to investigate the repeatability of quantitative measurements with an AFM, a selected set of imaging parameters were chosen and repeated measurements of the same CAFs were made. The imaging parameters were as follows: 10 μm × 10 μm scan size, 1 Hz scan rate, fixed drive amplitude of 0.187 V, and fixed setpoint (50 % damping). The dimensions of the CAFs were then measured at 50 % RH in the AFM test chamber. The
probe was repeatedly disengaged and reengaged with the sample surface. Upon each engagement, an image of the surface was obtained and analyzed. The variations over five engagements were 12 nm² in area (2 %), 10 nm in height (3 %), and 5 nm in width (0.2 %) as shown in Figure 3.9.

![Figure 3.9 AFM image of a CAFs (top) and dimensional profiles (bottom) of each fresh engagement of the tip and sample with constant scanning parameter in 50 % RH and 23 ºC. The rectangular area in the AFM image was analyzed by the RFA algorithm.](image-url)
Figure 3.10 shows the dimensional percent changes of 102 nm step grating as a function of relative humidity from 28 % to 80 % RH with both free decay (FD) and forced change (FC) humidity cycles. Our experiments have shown that whether the RH in the AFM chamber is successively and/or discontinuously increased and decreased, the dimensions can be reliably and repeatedly measured to within a 5 % variation. A slight positive correlation is found for the width of the grating step. This may be attributed to moisture accumulating in the corners of the step, differences in the interaction of the tip with the surface, and/or changes in the performance of the AFM as the humidity increases.

Figure 3.10 Profile changes in width and height of 102 nm standard grating (TGZ 02) as a function of relative humidity.
7. Dimensional changes of CAFs at variable humidity

Figure 3.11 shows the dimensional changes of the CAFs under variable humidity conditions. The width, area, height and length of the CAFs were observed. The data in Figure 3.11 combines together all data for both free decay (FD) and forced change (FC) humidity cycles. It is worth noting that the not all dimensions change significantly during relative humidity changes. Notably the width of the CAFs does not change significantly over the relative humidity observed. This lack of significant change in the width of the CAFs may be attributed to the CAFs being attached to the glass slide, which restricts the movement of the CAFs. The area and height change in a similar manner. Both of these measurements are found to increase with increases in relative humidity, such changes are expected due to the hygroscopic nature of cellulose.

Somewhat unexpectedly, the length of the CAFs was found to shrink as the relative humidity increased. A relatively good linear correlation was found between the relative humidity and the shrinkage of the CAFs. This change may be due to simple conservation of volume of the sample or arrangement of the cellulose molecules within the CAFs. Expansion in the height of the sample results in an increase in the volume unless there is a decrease in another dimension. However, one would expect that increasing the moisture content would typically result in swelling in all dimensions. Further work is needed to understand this phenomenon in detail as it has significant implications for paper dimensional stability and the use of cellulose micro- and nano-fibers in composite materials.
Figure 3.11 Four plots showing the change in dimensions of a CAFs from a bleached softwood pulp. The $R^2$ values for the regressed lines are shown on each plot. It is worth noting that the length of the CAFs is found to reduce as humidity increases.
E. Conclusions

The AFM scan rate and dampening was found to significantly affect the measured dimensions of step discontinuities. The tip shape produced a geometric convolution of the step and the tip and was taken to introduce a constant error in the images. The reproducibility of quantitative AFM imaging was also examined by repeatedly measuring the same sample using constant conditions. Results showed that variations in the height, width, and area were less than 3 % at the ambient condition of 50 % RH and 23 °C as well as 5 % in variable relative humidity conditions.

Cellulose aggregate fibrils (CAFs) was also scanned under varying relative humidity conditions. Changes in the width of the CAFs were not correlated with changes in the relative humidity, while changes in the height and area of the CAFs were positively correlated with the relative humidity. The stability in the width of the fiber may be attributed to the fact that the fiber is attached to a glass slide and that the width is stabilized in that direction. The length of the CAFs was found to be negatively correlated with the relative humidity, such that as the relative humidity increases the CAFs becomes shorter for current relative humidity cycle. This could be attributed to the simple conservation of volume of the CAFs as it undergoes a strain, or to the specific arrangement of cellulose chains within the CAFs. Further work is needed to understand this unexpected phenomenon. This finding has significant implications in paper dimensional stability and the application of cellulose micro and nano-fiber composites.
F. References


[16]. *Operator’s Manual* Provided the manufactures and *Personal Communication*.


CHAPTER FOUR

AFM OBSERVATIONS LONGITUDINAL CONTRACTION AND ITS CONCURRENT DIMENSIONAL CHANGES OF CELLULOSE AGGREGATE FIBRILS DURING DESORPTION AND ADSORPTION

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A. Summary

Atomic force microscopy (AFM) studies on dimensional changes of cellulose aggregate fibrils (CAF) exposed to two distinct relative humidities of saturated salt solutions, corresponding to 80 % and 23 % for 24 hours and then suddenly subjected to 50 % RH and 23 °C show that the CAFs respond to the surrounding environments in a nonspecific fashion. The moisture uptake and loss of the sheet from CAFs under each saturated salt condition were less than 3 % (w/w). Hysteresis behavior of fibril sheets made by water evaporation casting was observed during desorption and adsorption stages. AFM images of the same area (10 μm × 10 μm) in the individual straight CAFs (approx. 100 μm × 3 μm × 300 nm) were taken as a function of elapse time during both desorption and adsorption stages. The length of the area of interest was first measured and then its concurrent dimensions in terms of cross-sectional area, width, and height were analyzed. The measured length of the CAFs was observed to have shrunk just after being removed from each RH and progressively relaxed to its original length as a function of elapsed time during both desorption and adsorption stages. However, the relaxation rate under the adsorption stage was faster than that of the desorption stage, and even its length was more extended at the same time scale. Possible explanations were discussed with reference to the sample preparation method, entropy elasticity, volume conservation, and free volume theory. Based on the measured length as a function of elapsed time, their concurrent dimensional changes were also evaluated. The concurrent dimensional results were not conclusive, since plastic deformations were introduced during sorption.
stages. However, the changes of the measured length of the CAFs might be from nature of cellulosic material.

**B. Introduction**

Bio-based materials such as wood have long been used as a raw material for building construction as well as pulp and paper production. Today, there is a growing interest to transform bio-based materials into building blocks for new functional materials such as composites [1-6], electronic display [7] and electro-active papers [8]. These applications of bio-based material are due to their excellent physical and mechanical performances, and their availability. However, the material properties of the cellulose-based materials including paper, micro- and nano-fiber composites are often strongly influenced by environmental factors such as relative humidity (moisture content) and temperature [9, 10]. The interaction of polymeric and crystalline structure in the bio-based materials with surrounding environments is thus of high technological importance. The interaction underlies the functional properties of the final product from bio-based materials. The interactions also control the mechanical, dimensional, electrical, thermal, and optical properties of the final products, either directly or indirectly. Therefore, understanding the underlying mechanisms of these interactions is crucial for further developing the efficient and economical process of producing products from bio-based raw materials.

Paper is a network of natural polymer fibers, which typically originate from wood. The walls of wood fibers are constructed of a matrix of hemicellulose and lignin, reinforced
by microfibrils of partly crystallized cellulose chains arranged at different angles to the fiber axis [11, 12]. Wood fibers are currently used in a wide variety of consumer products. Currently, the single biggest use of wood fibers is in paper products (approx. 300,000,000 tons/year). To make paper, the fibers are separated from wood using a variety of pulping processes (ex. mechanical pulping and chemical pulping including sulfite pulping, kraft pulping, semichemical pulping). Depending on the pulping process and subsequent bleaching sequence, various amounts of amorphous hemicelluloses and lignin remain between the partially crystalline cellulose microfibrils. To increase the fiber conformability, a refining process is used that removes the constraining primary wall of the wood fiber and partly fibrillates the fibers. Thus, several structural imperfections are induced into the remaining fiber structure. These microscopic defects and natural imperfections of the fiber often lead to some macroscopic effects: curl, twist, kinks, and dislocations [13]. In a drying process, the fibers lose water and shrink primarily in their transverse direction [14]. The shrinkage of the fibers causes contraction of the paper sheet and creates various deformations in the fibers, such as dislocations and compression zones at the fiber crossings. This results in internal stresses in the resulting paper [15]. These internal stresses are believed to be in part responsible for the time dependent properties toward external stimuli such as loads, moisture and temperature. Paper is thus considered as a visco-elastic, plastic, hygroscopic material [16].

Paper swells and shrinks, depending on its environments. The ability to resist shrinkage and/or swelling is called dimensional stability. Hence an understanding of the fundamental parameters affecting dimensional properties, called hygro-expansion of paper is essential in order to develop final products with better dimensional stability. Factors that
have been found to be most important in paper dimensional stability are the degree of beating, drying shrinkage of the pulp, sheet anisotropy, and drying restraint imposed upon the paper [17]. Pulp fibers are hygro-expansive in nature, which makes them highly responsive to the environment [18]. Therefore, individual fibers also expand and contract with changes of moisture content, although the movement of the fibers in relation to one another will govern the net changes in the paper. Ultimately, the root of the hygro-expansion properties is the swelling capability of the wood polymers in the fiber. Hygro-expansion is, therefore, dependent on the fiber composition as well as the distribution of these components in the fiber and fibrous network.

Cellulose chains are aggregated as a form of cellulose microfibril with a network structure during biosynthesis. The macromolecular arrangement of cellulose chains is stabilized via intermolecular hydrogen bonds, dipole interactions, and van der Waals interactions [19, 20]. It is considered that cellulose microfibrils are composed of both ordered, called crystalline, and disordered, called amorphous, phases. The mechanical properties of cellulose microfibril material strongly depend on the ratio between crystalline and amorphous phases. The crystalline domain is responsible for the load-bearing capacity. The properties of the amorphous domains are not extensively characterized [9, 10]. But it is considered that the amorphous cellulose is strongly related to visco-elastic properties of the cellulose microfibrils [21, 22]. Cellulosic fibers expand approximately 1 % in the longitudinal direction and 20 % in the lateral direction in the range of 0 to 100 % RH conditions [13]. The moisture acts as a plasticizer, which weakens the hydrogen bonding between cellulose microfibrils and another matrix between cellulose microfibrils (e.g. hemicellulose and residual lignin) [9, 10]. The adsorbed moisture strongly influences the
time dependent characteristics of the material. The alteration of the hydrogen bonding of the amorphous components of the fiber wall, such as non-crystallized cellulose, hemicellulose, and lignin, greatly influences the visco-elastic properties of paper. If the external conditions suddenly change, there is a delay in the absorption of moisture and thus the change in the physical and mechanical properties of paper. Thus, the dimensional properties of the microfibril and its dynamic interaction with moisture are very important to understand the fundamentals of both dimensional stability and time dependent behaviors.

Despite its obvious significance, there is still not a thorough understanding of how a microfibril interacts with water molecules and its concurrent dimensional changes. This may be due to the complicated behaviors of the heterogeneous fiber materials. In addition, the experimental techniques have been cumbersome and difficult to interpret at a nano-scale. The measurements of the hygro-expansive properties of cellulose microfibrils, if possible, will be applicable in understanding the hygro-expansive properties of paper, the accelerated creep properties of paper, and how cellulose fibrils may perform in nano- and micro-composite applications. These findings will have significant implications in paper dimensional stability and the engineering of cellulose micro and nano-fiber composites.

Atomic force microscopy (AFM) technique provides the ability to image single individual macromolecules adsorbed on a substrate for visualization on its conformational properties and morphological changes of synthetic [23] and natural polymers [24, 25]. AFM application to study the dynamic changes of macromolecules in situ and in real-time, however, is still a challenge due to several limiting factors, such as the lateral resolution, tip-and-sample interaction, and variable scanning parameters. In relation to this, a technique was
developed to measure the dimensional changes of cellulose aggregate fibrils (CAFs) from natural fibers [26].

The main focus of this research was to study one aspect of CAFs behavior and its concurrent dimensional changes toward surrounding environments in order to better understanding the relation of CAFs and the interaction of water molecules with the fiber wall.

C. Experimental

1. Preparation of cellulose aggregate fibrils (CAFs)

Cellulose aggregate fibrils were prepared from a NIST bleached softwood kraft pulp. The NIST pulps were beaten in a valley beater (Tappi Method T200 om-89, Figure 4.1a) and fractionated in a Bauer-McNett classifier (Tappi Method T233 cm-82) to obtain a fines and primary cell wall-free fiber fraction. The fiber fraction retained on Tyler screen No. 12 was beaten in a PFI mill (Tappi Method T248 cm-85, Figure 4.1b) for 75,000 revolutions to increase the degree of fibrillation of the pulps. The pulps were then screened with a Britt dynamic drainage jar (Tappi Method T261 cm-90) at 0.5 % consistency. The filtrates, called cellulose aggregate fibrils (CAFs, Figure 4.1c), were thickened ca. 2 % consistency with a 0.45 micron membrane filter and were refrigerated during storage.

In order to obtain images of CAFs using an atomic force microscope (AFM), a glass slide sample of the CAFs was prepared by dropping 10 μl of diluted CAFs suspension (less than 0.0002 %) onto 20 different spots on a cleaned glass slide. The glass was cleaned by
immersing the slide in a mixture of absolute ethanol and 1 N HCl (1:1, v/v) overnight, followed by ultrasonic cleaning in deionized water three times. The glass slide with the CAFs sample attached was conditioned at 50 % RH and 23 °C for more than one week.

Figure 4.1 Optical microscopy images of each preparation step (a to c) and CAFs sheet (d). (a) Valley beater, (b) PFI milling filtercake, (c) PFI milling filtrate (CAFs), (d) CAFs sheet.
2. Apparatus and measurement

The experimental apparatus was set up in a controlled environment (23 °C and 50 % RH). Images were acquired with a Q-scope™ 250 (Quesant Instrument Corporation, USA) using a NCS tip (MikroMasch, USA) with 230 μm long cantilever with a spring constant of approximately 3.5 N/m in intermittent contact mode provide by the manufacturer. An acoustic/vibration isolation chamber (Quesant Instrument Corporation, USA) served as the test chamber for the experiments.

Saturated solutions of different salts (ammonium chloride and potassium acetate) were used to achieve constant relative humidities, 80 % and 23 % respectively, over the samples for 24 hours. The saturated salts solutions were employed to create humid atmospheres in a sealed container. Samples were allowed to equilibrate with the atmosphere inside the desiccator for 24 hours. Thereafter, the sample were quickly transferred to the AFM test chamber at 50 % RH and 23 °C and imaged as a function of elapsed time. Each image typical took approximately 10 minutes to complete. The elapsed time formed removal from the saturated salt solution atmosphere was recorded at the start and completion of each scan. The average of these two values was then recorded as the elapsed time for the corresponding image.

3. Preparation of CAFs sheet

Sheets of CAFs were prepared for use in determining the moisture content of the sheets at different humidities. In this procedure, 50 ml of ca. 0.2 % CAFs suspension was cast onto a polystyrene disposable Petri dish (size 150 × 15 mm, VWR). The CAFs
suspension Petri dish was allowed to evaporate the water until a sheet (apparent density of ca. 590 kg/m³), called CAFs sheet, had been obtained as shown in Figure 4.1d. The sheets were dried at 50 % RH and 23 °C and the sheets were allowed to dry until they maintained a constant mass.

4. Moisture change

In order to estimate moisture content of the CAFs samples being imaged by the AFM as a function of elapsed time, CAFs sheets were conditioned for 24 hours in a sealed container with an atmosphere created by a saturated salt solutions. One of two salts were used, either potassium chloride or ammonium chloride, to achieve a relative humidity of 80 % and 23 % respectively. After conditioning the samples were removed from the seal container and weighed. The sample was then subjected to a similar humidity cycle as the CAFs slide samples. The moisture change was calculated as follows,

\[
\text{Moisture change (\%)} = \frac{W - W_0}{W_0} \times 100
\]

where \( W \) and \( W_0 \) are the conditioned and oven dry weights measured by TGA of the sample, respectively.

The oven-dried weight of the CAFs sheet was determined using thermogravimetric analysis (TGA Q500, TA instrument, USA) at a heating rate of 10 °C/min. over a heating temperature range from room temperature to 110 °C. Based on these data on the oven dry
weight of the conditioned CAFs sheet, the moisture regain at the observation image as a function of elapsed time was estimated.

5. Dimensional measurement

An individual straight CAFs attached to a glass slide was chosen for observation (e.g. rectangular area in Figure 4.6a) and a series of images were then taken with the AFM during the adsorption and desorption of moisture. Each image was 10 µm × 10 µm in size and had a 400 line vertical and horizontal resolution (1 line segment corresponds to 0.025 µm in the image). For each image two distinct defects (Y₁ and Y₂) were identified. At least 290 line segments out of 400 line segments in each CAFs sample were selected. The difference in length between the defects was used to characterize the changes in the measured length of CAFs segment. The change in length was measured by manual point-to-point method and this change in length was taken to be representative of the change in measured length of the entire CAFs. The mean CAFs cross sectional area, height, and width was measured 5 times using a rapid feature analysis (RFA) developed by the authors and described in an earlier work [26]. The changes of each dimensional measurement were calculated as follows,

\[ \text{Changes in dimensions (\%)} = \frac{D - D_0}{D_0} \times 100 \]
where $D$ and $D_0$ are the dimensions at each elapsed time and starting dimensions of each sample, respectively. The experimental variation was measured by repeated imaging of a reference grating (TGZ 02, MikroMasch, USA) which is insensitive to moisture.

**D. Results and discussion**

**1. Moisture content in the CAFs sheet as a function of time elapsed**

In order to measure the sorption isotherm of the CAFs sheet, its moisture changes were monitored until a stable weight was achieved, as shown in Figure 4.2. The moisture uptake of the CAFs sheet subjected to the 80 % RH salt solution for 24 hours was 2.86 %. The loss of moisture kept over the 23 % RH salt solution for 24 hours was 2.3 %. After being taken out of each atmosphere, the moisture content of the CAFs sheets progressively decreased during the desorption stage and increased during the adsorption stage. It took 60 to 70 minutes to reach a plateau, whether the sheet was in the adsorption or desorption stage. As the desorption proceeds, the CAFs sheet did not reach its original equilibrium moisture content after 300 minutes, whereas during the adsorption stage the CAFs sheet reached its original equilibrium moisture content at the same time scale. These results indicated that hysteresis was present and that the behavior of moisture transport at the each stage was different.
Figure 4.2 Sorption isotherms of CAFs sheet from kraft pulps as a function of time elapsed. The dotted-line in the figure shows moisture content (7.5 %) of the sheet at ambient condition of 50 % RH and 23 °C measured by TGA.

2. Experimental variations

Figure 4.3 shows AFM images from two discrete trials of the reference grating under the ambient condition of 50 % RH and 23 °C for 24 hours. The dimensional changes of the grating were evaluated based on two distinguished defects (Y₁ and Y₂ in Figure 4.3a and
The variations over 24 hours were less than ±1%, ±3%, ±5%, and ±5% in measured length, width, and area and height, respectively, as shown in Figure 4.4. This fact indicates that the AFM measurements are not seriously affected by interactions between the tip and the sample under this experimental setup.

Figure 4.3 Tapping mode (TM)-AFM images of two different trials ((a) to (c) and (d) to (f)) of the reference grating (TGZ 02, 10 µm × 10 µm scan area) as a function of time elapsed under the ambient condition of 50% RH and 23°C for 24 hours. First trial (a) to (c), (a) 0 minute elapsed, (b) 30 minutes, (c) 1400 minutes; Second trial (d) to (f), (d) 0 minute, (e) 38 minutes, (f) 1320 minutes elapsed.
Figure 4.4 Concurrent dimensional variations of two discrete trials with the reference grating (TGZ 02) under 50 % RH and 23 °C as a function of elapsed time.
3. Dimensional behaviors at desorption stage

Three different CAFs slide samples were prepared and their dimensional changes were evaluated as a function of time with different surrounding environments.

Figures 4.5 to 4.7 show AFM images successively taken of an area (10 µm × 10 µm) of each CAFs slide sample (e.g. Figure 4.6a) as a function of time at the desorption stage. Before taking images from the sample glass slide, the sample slide was conditioned at the ambient condition of 50 % RH and 23 °C for one week.

A straight CAFs out of several hundred CAFs in a glass slide was chosen by optical microscopy (BH2-UMA, Olympus Co.). The CAFs chosen was manually placed on the AFM stage to make vertical alignment with the AFM cantilever to prevent processing errors from digital rotation. With a couple of scans of the CAFs, a 10 µm × 10 µm area having two distinct defects (Y₁ and Y₂ in Figures 4.5a, 4.6b, and 4.7a) was selected. Before the glass slide sample was transferred to the saturated ammonium chloride solution corresponding to 80 % RH, the area selected was scanned (Figures 4.5a, 4.6b, 4.7a) at the ambient condition of 50 % RH and 23 °C. After 24 hours the sample slide was quickly transferred from the 80 % RH to 50 % RH, 23 °C, and then the same area in the CAFs was manually aligned as soon as possible. It took less than 30 minutes to get a first scan of the same area (Figures 4.5b, 4.6c, and 4.7b) and then intermittently the images were taken as a function of elapsed time over 24 hours.
Compared to the initial starting image (4.5a, 4.6b, and 4.7a) of the three CAFs samples, called Sample A, B, and C in Figures 4.5 to 4.7, the AFM images obtained after removing the sample from high humidity have changed as a function of elapsed time. Figures 4.5b to 4.5d show blurred images of the CAFs up to 70 minutes. The blurriness diminished with time (Figures 4.5e to 4.5h). After scanning the Sample A for 300 minutes, it was stored under 50 % RH, 23 °C for 5 additional months. After 5 months, the same area in the Sample A was scanned as shown in Figure 4.5i. Plastic deformations were observed in the resulting image (Figure 4.5i). In Samples B (Figure 4.6) and C (Figure 4.7), their morphological changes were also observed as a function of elapsed time. The width of Sample C (Figures 4.7b to 4.7i) was remarkably changed compared to the initial image (Figure 4.7a).

The most noticeable difference between initial images and later images of the three samples was in the measured length of the CAFs. All of the samples shrunk when they were exposed to the ambient conditions of 50 % RH and 23 °C after the high humidity condition. It was difficult to scan the exact same area of the CAFs in every scan, but each of the images taken has two identifying defects (Y1 and Y2) which allow measurement of the segment length of the CAFs as a function of time. Based on the resulting images, changes in the segment length of the CAFs were measured by manual point-to-point method. It was clearly shown that the measured length of the CAFs significantly changed as shown in Figure 4.11.
Figure 4.5 Tapping mode (TM)-AFM images of an area 10 µm × 10 µm of the CAFs (Sample A) as a function of elapsed time under desorption stage. (a) before the sample subjected to the 80 % RH, (b) 20 minutes elapsed after exposure to the ambient condition of 50 % RH and 23 °C, (c) 40 minutes , (d) 70 minutes, (e) 150 minutes, (f) 170 minutes, (g) 250 minutes, (h) 270 minutes, (i) 5 months elapsed (arrows indicate plastic deformations).
Figure 4.6 Tapping mode-AFM images of 80 µm × 80 µm area (a) and its an area of 10 µm × 10 µm (b to i) of the CAFs (Sample B) as a function of elapsed time under desorption stage. (b) before the sample subjected to the 80 % RH, (c) 26 minutes elapsed after exposure to the ambient condition of 50 % RH and 23 °C, (d) 70 minutes, (e) 170 minutes, (f) 315 minutes, (g) 630 minutes, (h) 750 minutes, (i) 1450 minutes elapsed.
Figure 4.7 Tapping mode (TM)-AFM images of an area 10 μm × 10 μm of the CAFs (Sample C) as a function of elapsed time under desorption stage. (a) before the sample subjected to the 80 % RH, (b) 35 minutes elapsed after exposure to the ambient condition of 50 % RH and 23 °C, (c) 55 minutes, (d) 80 minutes, (e) 180 minutes, (f) 310 minutes, (g) 580 minutes, (h) 720 minutes, (i) 1400 minutes elapsed.
Figure 4.8 TM-AFM images of the same area 10 μm × 10 μm of the CAFs (Sample A) after 5 months under adsorption stage. (a) before the sample subjected to the 23 % RH, (b) 20 minutes elapsed after exposure to the ambient condition of 50 % RH and 23 °C, (c) 40 minutes, (d) 60 minutes, (e) 140 minutes, (f) 160 minutes, (g) 240 minutes, (h) 580 minutes, (i) 600 minutes elapsed.
Figure 4.9 TM-AFM images of the same area 10 µm × 10 µm of the CAFs (Sample B) under adsorption stage. (a) before the sample subjected to the 23 % RH, (b) 30 minutes elapsed after exposure to the ambient condition of 50 % RH and 23 °C, (c) 50 minutes, (d) 65 minutes, (e) 180 minutes, (f) 260 minutes, (g) 350 minutes, (h) 740 minutes, (i) 1340 minutes elapsed.
Figure 4.10 TM-AFM images of the same area 10 µm × 10 µm of the CAFs (Sample C) under adsorption stage. (a) before the sample subjected to the 23 % RH, (b) 20 minutes elapsed after exposure to the ambient condition of 50 % RH and 23 °C, (c) 70 minutes, (d) 140 minutes, (e) 160 minutes, (f) 320 minutes, (g) 400 minutes, (h) 450 minutes, (i) 1430 minutes elapsed.
Figure 4.11 Concurrent dimensional changes of CAFs exposed to 80 % RH for 24 hours, followed by returning to ambient condition of 50 % RH and 23 °C, and exposed to 23 % RH for 24 hours, followed by returning to ambient condition of 50 % RH and 23 °C.
Compared with the initial images (0 minute), the measured length of the samples exposed to the ambient condition of 50 % RH and 23 °C for 30 minutes decreased by 10 % in Sample A and B, 6 % in Sample C. In the case of Sample A, as the exposure time to the ambient condition of 50 % RH and 23 °C increased, the strain in the length gradually increased up to 4.6 % over the experimental time scale (300 minutes). After 5 months it also showed a tendency to relax toward its original length. Although it is difficult to directly compare the measured length of the CAFs in Figures 4.5a and 4.5i due to the swelling of the defects (Y₁ and Y₂), the length increased by 1 % after 5 months. This indicated that the original length of the CAFs can be recovered through a long relaxation process. The measured length of the Sample B and C was recovered in the 24 hours time scale. It could be expected, however, that when the sample was just taking out of the high humidity chamber, the strain in length was larger than that of the first scanned sample, although the experimental setup made it only possible to scan the sample after 20 minutes elapsed.

Figure 4.11 shows concurrent dimensional changes of the CAFs as function of time. The cross-sectional area and height of Sample A decreased up to 70 minutes by 8 % and 13 %, respectively. The width of the CAFs increased by 6 % up to 70 minutes and gradually approached to a volume equilibrium state. Although the moisture content (Figure 4.2) and width reach an equilibrium state after 70 minutes (Figure 4.11), the cross-sectional area, height and measured length still gradually increased. After 5 months, the cross-sectional area, height, and width of the Sample A were increased by 9 %, 7 %, and 2.5 %, respectively, compared to the initial dimensions. In the case of Sample B, the dimensions decreased, but the dimensions recovered to their original state as time elapsed. The
measured length of the *Sample C*, which had a 14 % decreases in width, gradually relaxed back to its original value. However, the width, area, and height of *Sample C* had not recovered within 24 hours. This result showed that the dimensions of the CAFs subjected to a high RH and then suddenly exposed to 50 % RH and 23°C were responsive toward surrounding environments in a non-specific fashion. Their dimensional changes vary from sample to sample.

**4. Dimensional behaviors at adsorption stage**

*Sample A*, which was kept in the 50 % RH and 23 °C for 5 months, and *Samples B* and *C*, allowed to equilibrate 1 day under the same condition, were used to examine the effect of moisture adsorption. The samples were evaluated in an adsorption experiment where the CAFs was exposed to 23 % RH for 24 hours and suddenly exposed to the ambient condition of 50 % RH and 23 °C. **Figures 4.8** to **4.10** show AFM images successively taken of the same area (10 µm × 10 µm) of the CAFs before being transferred to 23 % RH condition (**4.8a**, **4.9a**, and **4.10a**) followed by exposure to the 50 % RH as a function of time. Compared with the images of the desorption experiment, the morphology of the samples was more dramatically changed, as shown in **Figures 4.8** to **4.10**. A swollen state of the external microfibrils was observable in *Sample A*. In *Samples B* and *C*, several deformations were apparent, and slip deformations were observable (**Figures 4.9b** and **4.10h**). These deformations did not disappear as a function of time. This result suggested that the moisture absorbed (2.3 %) during the adsorption stage significantly influences the morphology of the samples. This may be attributed to plastic deformations caused by swelling stress, as
proposed by Barkas [27]. The water adsorbed on the cellulose chains may act as a lubricant, which may facilitates the slippage of weekly bonded micelles, leading to irreversible plastic deformations.

It is difficult to directly compare the changes in dimensions of the CAFs at desorption and adsorption stages due to the changes of the morphology. In order to evaluate the changes in dimensions of the samples under the adsorption stage, the dimension of the samples was calculated by the last couple of images of each sample at the desorption stage, i.e., 5 month elapsed images in the Sample A and 24 hours elapsed images in the Samples B and C. Figure 4.11 shows the measured length of the CAFs when the sample was suddenly exposed to the ambient condition of 50 % RH and 23 °C from 23 % RH and 23 °C. The lengths of the samples decreased by 3, 9, and 5 % in the Sample A, B, and C, respectively, and progressively increased by up to 9 %. Compared to the desorption stage, the relaxation rate was faster at the same time scale. Figure 4.11 also shows the changes in cross-sectional area, width, and height. The overall dimensions increased as a function of time.

5. Possible explanations of the dimensional behaviors of the CAFs

The magnitude of changes in the measured length of the CAFs under the experimental conditions was larger than our previous results with different experimental conditions [26]. However, we consistently observed the length changes of CAFs by simply exposing them to different relative humidities. Several possible phenomenons at the molecular level may contribute to the observed CAFs behavior. It might be possible that the manner of drying the CAFs sample affects the configuration of the microfibril when it was
deposited on the glass slide in this work. For example, Hanley et al. [28] speculated that observations of chiral helical twist of microfibrils from algae might be from an artifact of the sample preparation method. As the algae microfibril was drying on a substrate, the surface tension forces draw the microfibril to the surface of the substrate, giving rise to shorter and twisted segments in the microfibril. The twisting of the CAFs was not observed because the cross-sectional dimension of the CAFs was at least 100 times larger in width and 50 times bigger in height than that of the algae microfibril (30 nm wide and 5 nm high).

Another possibility, related to the drying method is that “poor” adhesion of the CAFs to the substrate may result in a part or the entire CAFs debonding from the substrate. When debonding occurs in the sample, stress is relaxed locally and transferred to another adjacent layer in the substrate. In this case, the substrate does not constraint the contraction and/or elongation of the CAFs. It should be noted, however, that whether the CAFs is partly or wholly attached to the surface layer of the glass, the measured length of the CAFs is shorter and relaxing to the original length. This observation suggests that a built-in stress of the CAFs causes the contraction of the CAFs. The changes in the length of cellulose microfibrils have been a subject of significant discussion. Stöckmann [29-31] proposed that longitudinal contraction of microfibrils is from an entropy-elastic deformation of amorphous regions of microfibrils. With the anisotropy of the thermal fluctuations of the atoms in the cellulose molecules, the atoms vibrate more strongly perpendicularly than longitudinally to the chain direction, resulting in a kinetic tensile force. The built-in tensile stress results from cell growth stresses in wood and supra-structural changes of elementary fibrils during fiber processing, including pulping, beating, and mercerization, causing the fibrils to contract longitudinally. By synchrotron X-ray diffraction experiments Clair et al. [32] demonstrated
that the maturation stresses cause the contraction of cellulose microfibrils/crystallites in tension wood. In addition, Nakano et al. [33] showed that the contraction of wood samples depend on the concentration of alkali and heating temperature during mercerization. They also concluded that the longitudinal contraction of the sample is due to entropy elasticity, which is caused by the molecular movements in the microfibrils in wood.

Given these considerations, changes in supra-structure of the microfibril are likely to be the source of the longitudinal contraction of the CAFs. However, the changes in the length of the CAFs as a function of elapsed time can not be adequately explained by the entropy elasticity hypothesis. Padanyi [34] has suggested that adsorption and desorption of water vapors involved a physical change at the molecular level of amorphous or partially amorphous materials. In order to explain time-dependent changes related to temperature, humidity, or applied stress, the author introduced a concept of molecular free volume, called physical aging. This is defined as relaxation by molecular movement into existing free volume. Physical aging is a process, occurring below the glass transition temperature, \( T_g \), where the macromolecules gradually change their packing in order to approach the equilibrium free volume state. He argued that moisture existing in the sample has a strong effect on lowering the \( T_g \) based on Salmén and Back’s result [35]. He postulated that both desorption and adsorption stages generate the free volume, resulting in a thermodynamic non-equilibrium state of the material. The effects of physical aging continue until the material reaches its volume equilibrium.

Thus, dimensional behaviors of CAFs exposed to dramatic changes in relative humidities might be related to specific arrangements of the cellulose chains and/or the volume conservation of the CAFs. In addition, the behaviors of CAFs are complex and
dependent on local areas. When *Sample A* was subjected to a sudden changes in the RH from 50 % to 80 %, the CAFs adsorbed moisture and continued to swell for 24 hours. The adsorbed water molecules might be responsible for the swelling in the transverse direction, since the original widths were at least 10 times bigger than the heights based on the geometrical consideration of the CAFs. As the width increases, the measured length of the CAFs shrinks. Consequently, its area and height would be expected to decrease. Under the quenching process from 80 % to 50 % RH, the free volume in the *Sample A* generated by the quenching may result in a transient state in the CAFs. Thus, the width, area, and height did not follow the behavior of moisture content. In other words, when the moisture was progressively desorbed from the CAFs, the width was supposed to be decreased and consequently its corresponding cross-sectional area and height should be increased. However, when the moisture reaches an equilibrium state at 70 minutes, the changes in the width increased by 6 % but the area and height decreased by 8 % and 11 %, respectively. After 70 minutes, the cross-sectional area and height progressively increased as a function of time. Over long periods, the dimensions reach a new equilibrium state. This result indicated that there might be structural rearrangements within the CAFs during the transient condition. The dramatic changes of relative humidity also results in more densely packed configurations of a molecular structure, based on the observations of *Samples B* and *C*. All dimensions of *Samples B* and *C* dramatically shrunk just after placing it in 50 % from 80 % RH. Depending on the packing, the CAFs will respond to the surrounding environments. In the case of *Sample B*, all dimensions were gradually increased and relaxed to their initial values as a function of time. However, the more condensed structure in the case of *Sample C* did not respond to the surrounding environment under the desorption stage, except in terms of
the measured length. However, the denser structure exposed to adsorption stage adsorbs a small amount of moisture, leading to a dramatic change in the configuration. For example, *Samples B* and *C* had irreversible plastic deformations as shown in *Figures 4.9* and *4.10*. The dimensions of the *Sample B* slowly increased, but *Sample C* progressively increased.

The changes of moisture during sorption stages results in the CAFs reaching a thermodynamically unstable state. Consequently, the CAFs are in an intermolecularly unfavorable state, leading to equilibrium state whenever the intermolecular forces are weakened. During sorption stages, measured lengths of the CAFs contracted and gradually relaxed to their original lengths. The built-in tensile force might be responsible for the longitudinal contraction of the CAFs, if there are no plastic deformations. If plastic deformations existed, the length is considerably extended, as shown in *Figure 4.11*. Thus, specific re-arrangements of cellulose microfibrils into the free volume influence the dimensional behaviors of CAFs during the desorption stage. However, moisture adsorbed during adsorption stages might be a controlling factor of the behavior of CAFs. In this present study, the long-term dimensional behavior of CAFs has been evaluated. However, future work is necessary to clarify the underlying mechanism of the changes in measured length and its concurrent dimensions with a short-term interaction between CAFs and water molecules. In addition, one might use ozone radiation drying in order to make more hydrophilic surface layer in the glass slide, so that debonding from the substrate would not occur. Using different geometrical CAFs might be a great experimental setup in order to explain the volume conservation of the CAFs.
E. Conclusions

Atomic force microscopy was used for the measurement of dimensional changes of cellulose microfibrils, called cellulose aggregate fibrils (CAFs), exposed to two distinct relative humidities (80 % and 23 %) for 24 hours and then suddenly exposed to the 50 % RH and 23 °C. The moisture change of the CAFs sheet when subject to 80 % and 23 % RH was less than ± 3 % (w/w). The sorption isotherms reached an equilibrium state when the sheet was subjected to ambient condition of 50 % RH and 23 °C for 70 minutes. In such an experiment, hysteresis behavior was observed during desorption and adsorption stages. This indicated that the sheet from CAFs has the same tendency to respond to its surrounding environments. AFM images were taken before the sample was subjected to the 80 % RH and 23 % RH chambers and after it suddenly was exposed to the ambient condition of 50 % RH and 23 °C. AFM images taken during both desorption and adsorption stages of the CAFs were evaluated for segment length of the CAFs as well as the cross-sectional area, width, and height. The measured length of the CAFs was observed to decrease just after being taken out of each humidity condition and progressively relaxed to its original length as a function of elapsed time. However, the relaxation rate under the adsorption stage was faster than that of desorption stage, and even its length was more extended at the same time scale. This result clearly showed that the adsorption and desorption of moisture could change the supra-structure of microfibrils at the molecular level. This could be attributed to unfavorable thermodynamic conditions relating to the free volume in the CAFs under adsorption and
desorption stages. The concurrent dimensional results in terms of cross-sectional area, width, and height were not conclusive, since several plastic deformations were introduced.

F. References


CHAPTER FIVE

DIMENSIONAL AND HYGRO-EXPANSIVE BEHAVIORS OF CELLULOSE AGGREGATE FIBRILS FROM KRAFT PULP-BASED FIBERS

Parts of the content were presented in 231st ACS National meeting, Atlanta, Georgia USA, March 26-30, 2006.
A. Summary

Cellulose aggregate fibrils (CAFs) with dimensions of 100,000 × 3,000 × 300 nm from unbleached kraft pulp, oxygen delignified kraft pulp, and fully bleached kraft pulp were liberated by a series of high shear and fractionation operations. The CAFs were used as a cellulose microfibril model material to evaluate their dimensional and hygro-expansive behaviors with variable relative humidities (RHs) in this study. The AFM images of CAFs from different sources of kraft fibers were dynamically obtained during a RH cycle from 50 % RH to 78 % RH and then to 21 % RH while being kept at 23 °C. The resulting images were analyzed to determine dimensional changes in terms of measured length, its concurrent cross-sectional area, width, and height. There were no significant differences in dimensional changes of CAFs from different sources. The mean value of changes in length and width was in the range of 2.3 to 3.2 % and 1.9 to 3.3 %, respectively. The changes in area and height were in the range of 14.5 to 18.2 % and 12.4 to 17.3 %, respectively. The measured length of CAFs showed a negative correlation, whereas cross-sectional area, width, and height showed positive correlations at the given RHs. In the out-of-plane direction such as area and height, the hygro-expansivity was one order of magnitude larger than the in-plane hygro-expansivity, including measured length and width.
B. Introduction

Cellulose is biosynthesized as a form of microfibrils in an extended chain conformation and fibrillar morphology. Wood fiber is one of the most available feedstocks for producing cellulose microfibrils. The cell wall of wood is constructed from a matrix of hemicellulose and lignin reinforced by cellulose microfibrils of partly crystallized cellulose arranged at different angles to the fiber axis [1, 2]. Concerning its mechanical properties, the ratio of crystalline and amorphous moieties affects the mechanical properties of the cellulose microfibril. The elastic modulus of the crystalline cellulose microfibrils, which are the reinforcing constituent of the wood cell wall, has been reported as 150 GPa [3], whereas the amorphous cellulose showed visco-elastic properties [4, 5]. For this reason it is of interest to explore the possibilities for new materials based on crystalline cellulose microfibrils in polymer matrices [6].

Although the natural fibers have remarkable stiffness, it is difficult to extract the cellulose microfibrils from surrounding hemicellulose-lignin matrix in the cell wall without degrading the reinforcing potential. In order to take full advantage of the mechanical properties of microfibrils, efforts have been extensively carried out to characterize the aspect ratio in terms of the source material, its surface properties, and the microfibril’s ability to interact with polymer matrices [6, 7]. For example, the size and surface properties of microfibrils depends on the isolation procedure and cellulose sources. Compared to hydrochloric acid hydrolysis, sulfuric acid hydrolysis introduced acidic sulfate ester groups
onto the microfibril surface, creating an electrostatic repulsion layer between the microfibrils in suspension, which plays a big role in their interaction with a polymer matrix and with each other. Since cellulose microfibrils have a high density of hydroxyl groups on the surface, agglomeration is a problem, particularly when combined with non-polar thermoplastic polymers.

It is becoming better known that the organization of ultra-structural elements and the distribution of chemical constituents of the material, and their interfacial adhesion influence the behaviors of these natural materials very significantly [6, 7]. The hierarchical structure and surface properties of cellulose microfibrils can be significantly changed by pulping and bleaching process. During kraft pulping, which is the most common commercial chemical delignification method, and oxygen delignification processes, which has more selectivity, the lignin in the middle lamella and parts of hemicelluloses are dissolved, resulting in the formation of pores in the cell wall [8 -10], an increase in crystallinity of cellulose microfibrils [11], and an enlargement of cellulose microfibrils due to low residual hemicellulose content [12, 13]. Most of the residual lignin in the resulting pulp was covalently linked to the hemicelluloses [14]. Compared to kraft cooked pulps, fiber charge of the oxygen-delignified kraft pulp can be varied as the final pulp kappa number, which is a measure of residual lignin in the pulp, and different structural properties for residual lignin and hemicellulose can be changed [15]. Furthermore, the residual lignin can be removed by a bleaching process. Therefore, these delignification and bleaching processes can modify the hierarchical structure, mechanical and physical properties of the cell wall, transforming the anatomical characteristics of plants into structural materials with entirely different compositions. Thus, single individual microfibrils assembled with a large number of monomer units with various
functional groups arranged in a special sequence may provide a base material for creating a high functional material and alternative to inorganic-based composites [6]. However, the cellulose microfibrils showed high moisture adsorption, resulting swelling and decreases in mechanical properties [16, 17]. There are only a few investigations of the dimensional properties of cellulose microfibrils from pulp fibers and, to the best of our knowledge; the dimensional properties at the level of the cellulose microfibrils are not fully explored.

Atomic force microscopy (AFM) is a useful tool for the characterization of materials at a nano-scale. AFM has been applied to image the conformational behaviors of single macromolecules [18-21] as well as evaluate functional properties of materials [22-24]. Although AFM application to image dynamic changes of macromolecules is still a challenge due to several limiting factors such as lateral resolution, tip-and-sample interaction, and variable scanning parameters, the AFM images where a variable environmentally-induced changes of the macromolecular structure and its morphology can be used for the characterization on behaviors of macromolecular dynamics [25]. However, robust image analysis procedures that have high fidelity for the resulting images are necessary. The authors developed an automated algorithm for determining the apparent dimensions of nano-scale materials from AFM images [26]. The technique was applied to measure local dimensional properties of CAFs, providing new insights of its local hygro-expansion behavior toward surrounding environments [27]. However, the knowledge of dynamic interactions between CAFs and surrounding environments is still limited. In order to broaden the spectrum of the dynamic interactions between CAFs with relative humidity, three different cellulose aggregate fibrils (CAFs) from unbleached, oxygen delignified, and fully bleached kraft pulps were liberated and used as a cellulose microfibril model material.
The aim of this work is to measure the dimensional properties of CAFs from the different kraft-pulp sources in relation to variable relative humidities using the developed image analysis technique. Based on their dimensional behavior as a function of RHs, their hygro-expansion behavior at the given RHs is to be evaluated.

C. Experimental section

1. Pulp samples

Unbleached softwood kraft (SL50) pulp, its extended oxygen delignified softwood kraft (SLO16) pulp, and fully bleached northern U.S. softwood (NS, RM 8495) were used to prepare cellulose aggregate fibrils (CAFs). The SL50 and SLO16 pulps were kindly provided by Dr. Hasan Jameel and Dr. Ved Naithani (NCSU). The SL50 with a kappa number of 50 was prepared by cooking in an M&K digester at 15 % active alkali charge with 1650 H-factors. After kraft pulping, the pulp was disintegrated, screened, washed thoroughly with water, and stored at 4 °C. The resultant pulp was bleached with two stages of a typical oxygen delignification [15] and had a kappa number of 16.0. The northern softwood bleached kraft pulp was supplied from National institute of standards and technology (NIST) and their characteristics have been reported elsewhere [28].
2. Preparation of CAFs

CAFs were prepared in the laboratory from the three kraft pulps by a series of high shear refining and fractionation according to Lee et al. [26]. Each kraft pulp was passed through a valley beater for 80 minutes, followed by a fiber length based Bauer-McNett classifier to remove a fines and primary cell wall-free fiber fraction on Tyler screen No. 12. The fiber fraction was beaten again in a PFI mill for 75,000 revolutions. The fibrillated pulps were then screened with a Britt dynamic jar at 0.5 % consistency. The filtrates, called cellulose aggregate fibrils (CAFs), were filtered through a 0.45 micron membrane filter (47mm, Gelman Sciences, USA) to thicken ca. 2 % consistency and were refrigerated during storage. The liberated CAFs had dimensions of ~100,000 (L) × 3,000 (W) × 300 (H) nm. In order to obtain images of CAFs by AFM, original cellulose suspensions were diluted to about 0.0002 % concentration by weight. 10 µl of the diluted suspension was placed on a cleaned glass slide (Superfrost®, Fisher scientific) in at least 20 different spots. The glass slides were first cleaned by immersing the glass slide in a mixture of absolute ethanol and 1 N HCl (1:1, v/v) overnight, followed by three stages of ultrasonic cleaning in deionized water. The glass slide where the CAFs attached was carefully placed in an ambient condition of 50 % RH and 23 °C for more than one week.

3. Experimental set-up

The experimental apparatus was set up in ambient conditions of 50 % RH and 23 °C. Images were acquired with a Q-scope™ 250 (Quesant Instrument Corporation, USA) using a NCS tip (MikroMasch, USA) with 230 µm long cantilever with a spring constant of
approximately 3.5 N/m (provided by the manufacturer) in intermittent contact mode. An acoustic/vibration isolation chamber (Quesant Instrument Corporation, USA) served as the test chamber for the experiments. The atmosphere above two saturated salts solutions, potassium acetate (20 % RH) and potassium sulfate (80 % RH), were combined with an air pump to generate various humidity conditions in the test chamber from 78 % to 21 %. The humidity in the test chamber was varied in two hour increasements. For example, the 80 % RH atmosphere was pumped into the test chamber for two hours. After two hours, a low humidity (20 %) atmosphere was pumped into the test chamber for two hours. The CAFs sample was imaged. The humidity within the test chamber was measured with an electronic hygrometer (OMEGA® RH 411 relative humidity meter, OMEGA Co.). Each image typically took ~ 10 minutes to complete, during which time the relative humidity changed. The humidity was recorded at the start and completion of each scan. The average of these two values was then recorded as the relative humidity value for the corresponding images.

4. Dimensional measurement by image processing

An individual straight CAFs of each sample was chosen for observation on the slide CAFs sample and a series of images were then taken with the AFM with variable humidity conditions described in the experimental set-up session. Each image was 10 µm × 10 µm in size and had a 400 line vertical and horizontal resolution (1 line segment corresponds to 0.025 µm in the image). For each image two distinct defects (Y₁ and Y₂) were identified. The difference in measured length between the defects was used to characterize the changes in the measured length of CAFs segment. The change in length was measured by manual point-to-point method and this change in length was taken to be representative of the change
in measured length of the entire CAFs. The mean CAFs cross sectional area, height, and width was measured 5 times using a rapid feature analysis (RFA) developed by the authors and described in an earlier work [26]. The changes of each dimensional measurement were calculated as follows.

\[
\text{Changes in dimensions(\%)} = \frac{D - D_0}{D_0} \times 100
\]

where \( D \) and \( D_0 \) are real-time dimensions and starting dimensions of each sample at a given RH, respectively.

D. Results and discussion

1. Cellulose aggregates fibrils (CAFs) as a cellulose model material

Figure 5.1 shows photomicrographs of an intact (parent) fiber from unbleached softwood kraft pulp (SL50), and CAFs isolated from their parent kraft pulps such as SL50, SL016 (oxygen bleached kraft pulp), and NS (fully bleached kraft pulp) by a series of high shear refining and fractionation. The image of the parent fiber showed a typical morphology of kraft fibers, i.e., no external fibrillations and dislocation areas (notches) could be observed.
Figure 5.1 Photomicrographs of intact SL50 fiber (a) and isolated CAFs (b to d) from different parent kraft fiber sources: (b) SL50, (c) SLO16, (d) NS. The solid arrow indicates dislocations in the fiber and the empty arrow indicates a small aspect ratio of CAFs.
Although the thickness of the intact fiber could not be directly measured by high-resolution optical microscopy (BJ2-UMA, Olympus Co.), its thickness is considered a half of the width of the parent fiber which is approximately 25 microns, due to collapse of the cell wall after drying [29]. A refining process increases conformability of fibers by removal of the constraining primary layer of wood fiber, and fibrillates the fibers. Thus, several structural imperfections such as twist, kinks, and dislocations are induced in the remaining fiber structure [30]. Images obtained for each isolated CAFs show some defects including kinks and twists. In addition, the CAFs were externally fibrillated and well individualized by a series of refining steps. The isolated CAFs shows two distinct classes of CAFs; one is approximately 10 microns in length and less than 1 micron in width, and another with at least 10 times longer and 5 times wider. The former has small aspect ratio (length-to-width ratio), which might be from externally fibrillated fragments of the longer CAFs. The longer CAFs are a ribbon-shape with dimensions ~100,000 (L) × 3,000 (W) × 300 (H) nm. The isolated longer CAFs represents a typical cell wall structure from kraft fibers and thus was used as a cellulose microfibril model material in this study.

2. Initial dimensions of CAFs

In order to evaluate dimensional changes of the CAFs with variable humidity conditions, a long and straight CAFs was carefully selected out of several hundred CAFs in a slide sample. With the optical microscopy, an area of 10 µm × 10 µm in the CAFs selected was marked and placed on the AFM stage. The marked area was manually aligned with the AFM tip to prevent an error from electronic rotations. Before RHs changed, a couple of
AFM images at ambient conditions of 50 % RH and 23 °C were obtained. Figures 5.2 to 5.4 show the initial image (a, d, and g) of each sample. Two distinct defects (Y_1 and Y_2) at each initial image were first identified. The length between these defects was measured by a manual point-to-point method. The difference detected was termed measured length. Based on the length, its initial dimensions in terms of area, width, and height were calculated by Rapid Feature Analysis (RFA). Various initial dimensions of CAFs were selected and were subjected to variable RHs as shown in Table 5.1.

### Table 5.1 Initial dimensions of each CAFs and its experimental conditions.

<table>
<thead>
<tr>
<th>Samples</th>
<th>Measured length (µm)</th>
<th>Cross-sectional area (nm² × 1,000)</th>
<th>Width (µm)</th>
<th>Height (nm)</th>
<th>Initial</th>
<th>Max.</th>
<th>Min.</th>
</tr>
</thead>
<tbody>
<tr>
<td>SL50</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>9.060 ± 0.014</td>
<td>685.7 ± 6.5</td>
<td>2.904 ± 0.002</td>
<td>236.4 ± 2.3</td>
<td>48</td>
<td>78</td>
<td>22</td>
</tr>
<tr>
<td>B</td>
<td>9.301 ± 0.044</td>
<td>440.1 ± 2.5</td>
<td>2.863 ± 0.009</td>
<td>153.7 ± 8.3</td>
<td>45</td>
<td>76</td>
<td>27</td>
</tr>
<tr>
<td>C</td>
<td>9.223 ± 0.020</td>
<td>974.9 ± 3.0</td>
<td>3.756 ± 0.004</td>
<td>259.8 ± 1.0</td>
<td>38</td>
<td>70</td>
<td>27</td>
</tr>
<tr>
<td>SLO16</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>7.219 ± 0.010</td>
<td>463.2 ± 0.3</td>
<td>2.482 ± 0.002</td>
<td>187.0 ± 8.1</td>
<td>48</td>
<td>74</td>
<td>22</td>
</tr>
<tr>
<td>B</td>
<td>8.668 ± 0.014</td>
<td>378.6 ± 2.7</td>
<td>2.440 ± 0.008</td>
<td>155.7 ± 1.1</td>
<td>40</td>
<td>76</td>
<td>27</td>
</tr>
<tr>
<td>C</td>
<td>8.833 ± 0.020</td>
<td>426.6 ± 2.9</td>
<td>2.768 ± 0.009</td>
<td>154.1 ± 1.3</td>
<td>39</td>
<td>70</td>
<td>27</td>
</tr>
<tr>
<td>NS</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>6.767 ± 0.014</td>
<td>683.6 ± 5.6</td>
<td>2.097 ± 0.001</td>
<td>327.5 ± 2.6</td>
<td>49</td>
<td>75</td>
<td>37</td>
</tr>
<tr>
<td>B</td>
<td>7.729 ± 0.035</td>
<td>327.7 ± 4.9</td>
<td>2.314 ± 0.004</td>
<td>143.0 ± 2.2</td>
<td>43</td>
<td>76</td>
<td>27</td>
</tr>
<tr>
<td>C</td>
<td>8.935 ± 0.005</td>
<td>425.8 ± 3.5</td>
<td>2.556 ± 0.004</td>
<td>167.1 ± 1.3</td>
<td>41</td>
<td>70</td>
<td>27</td>
</tr>
</tbody>
</table>
3. Morphology of CAFs as function of RH

Figures 5.2 to 5.4 show some of AFM images obtained from different CAFs samples as a function of relative humidity, especially start, maximum, and minimum RH, as shown in Table 5.1. Compared to our previous study where CAFs was subjected to a high humidity condition (80 % RH) for 24 hours, followed by quenching the CAFs down at an ambient condition of 50 % RH and 23 °C or subjected to an low humidity (23 % RH) for 24 hours, followed by exposure to the ambient condition of 50 % RH and 23 °C, several plastic deformations on the CAFs samples were observed [26]. Under our experimental conditions in this study, no significant morphological change on the CAFs samples was observed compared to the initial image of each samples as a function of relative humidity as shown in Figures 5.2 and 5.3. However, some deformations in the NS-B and NS-C samples from the fully bleached softwood pulp were observed at top area in Figures 5.4e and 5.4f and the left side in Figures 5.4h and 5.4i. In order to remove an experimental error by these deformations, the deformed area was manually excluded when the measured length was being selected.
Figure 5.2 AFM images of unbleached kraft pulp (SL50) as a function of RHs. (a-c) SL50-A, (d-f) SL50-B, and (g-i) SL50-C.
Figure 5.3 AFM images of oxygen delignified kraft pulp (SLO16) as a function of RHs. (a-c) SLO16-A, (d-f) SLO16-B, and (g-i) SLO16-C.
Figure 5.4 AFM images of fully bleached kraft pulp (NS) as a function of RHs. (a-c) NS-A, (d-f) NS-B, and (g-i) NS-C.
4. Dimensional changes of CAFs as function of RHs

Figures 5.5a to 5.5c show the changes in measured length of each CAFs sample as a function of RH. Their mean percent changes (top), and maximum and minimum values (bottom) were presented in Figure 5.5d. Although there was no significant morphology change of the CAFs, it should be noted that the measured length of each sample was changed as a function of relative humidity. When each CAFs was subjected to high humidity condition up to 78 % RH, the percent changes in the measured length compared to their initial length varied from sample to sample. All of samples except SLO16-A and NS-A expanded as the RHs increased. The SLO16-A and NS-A shrunk by up to 2 % at the given RHs. However, the percent changes of each sample followed by exposure to lower humidity down to 21 % RH increased in the range of 0.4 to 5.0 %, compared to their initial measured length. With different initial RHs, no significant percent change of length was observed. The mean value of three discrete trials of SL50 sample was a little smaller than those of SLO16 and NS, as shown in Figure 5.5d. However, the variations were more significant in the CAFs from NS fibers.

Figures 5.6a to 5.6c show the changes in the apparent width of each CAFs sample at given measured lengths as shown in Figure 5.5. When CAFs samples were exposed to high humidity, the width of all CAFs samples except SLO16-A sample tend to expand. Compared to their initial width, the SL50 and SLO16 samples were expanded less than 2 %, but the NS samples showed more significant expansion, up to 4.5 %. The expanded widths were gradually and/or progressively shrunk as the CAFs were exposed to the lower humidity.
Most of samples were shrunk, but the widths were still larger than their initial values. However, some samples (SL50-A, SLO16-A, NS-A) were more profoundly shrunk, leading to smaller width compared to their initial width. The mean values based on percent changes in width of NS and SLO16 were bigger than those for the SL50.

The changes in the cross-sectional area and height of each sample with variable humidities are shown in Figures 5.7 and 5.8. The cross-sectional area and height of all of samples were responsive to surrounding environments, i.e. the cross-sectional areas and height were swollen in high humidity and shrunk in low humidity. The cross-sectional area of each CAFs sample evolved as a function of RHs in the range of 8 to 20 %. The height is swollen up to 17 % compared to their initial height. When the swollen area and height of samples were exposed to low humidity, they were shrunk by up to 10 % compared to their initial area and height. The variations based on discrete trials showed that there is no significant difference between different CAFs samples as shown in Figures 5.7d and 5.8d.

It has been known that cellulosic fibers expand approximately 1 % in the longitudinal direction of the fibrils and approximately 20 % in the lateral direction over the range of RH from 0 % to 100 % at room temperature [31]. However, mean changes in measured length of the CAFs used in this study at a given RH from ambient condition (50 % RH) to 78 % RH and followed by to 21 % RH were in the range of 2.3 to 3.2 % and mean changes in width were 1.9 to 3.3 %. The changes in area and height were in the range of 14.5 to 18.2 % and 12.4 to 17.3 %, respectively.
Figure 5.5 Changes in measured length of each CAFs sample from different kraft-pulp sources (a to c) and their minimum, maximum, and mean values (d). (a) SL50, (b) SLO16, and (c) NS.
Figure 5.6 Changes in width of each CAFs sample from different kraft-pulp sources (a to c) and their minimum, maximum, and mean values (d). (a) SL50, (b) SLO16, and (c) NS.
Figure 5.7 Changes in cross-sectional area of each CAFs sample from different kraft-pulp sources (a to c) and their minimum, maximum, and mean values (d). (a) SL50, (b) SLO16, and (c) NS.
Figure 5.8 Changes in height of each CAFs sample from different kraft-pulp sources (a to c) and their minimum, maximum, and mean values (d). (a) SL50, (b) SLO16, and (c) NS.
5. Relationship between behaviors of each dimension as a function of RHs

The changes in measured length of CAFs samples from NS fiber have been reported earlier [27], where CAFs were subjected to two distinct RHs (80 % and 23 %) for 24 hours. When the CAFs samples were taken out of the distinct RHs where they were subjected to 80 % or 23 % RHs to ambient condition of 50 % RH and 23 °C, their length shrunk by up to 10 % and gradually relaxed to their original length as a function of elapsed time [27]. In the present work, a different surrounding condition was used, where CAFs were exposed to high humidity followed by low humidity. Although the changes of measured length of the CAFs were also observed, the behavior was rather different, compared to the previous results, i.e. when CAFs were exposed to a high humidity condition, the measured length of CAFs expanded or shrunk depending on the local area of the sample. However, after they were exposed to a low humidity, all of their lengths expanded compared to the initial length. This result suggested that the measured length increased or decreased depending on RH history.

Stöckmann proposed that a built-in tensile stress from growth stress, morphological changes during variable processing stages such as pulping, beating, and mercerization is a main driving force to change the longitudinal changes in the microfibril [32-34]. The changes of microfibril in length by growth stress [35] and mercerization [36] have been reported. The CAFs used in the work has a ribbon shape where the width is at least 10 times wider than the height. When it adsorbs moisture, the width increased as a function of RH. However, the changes in width did not correspond to the changes in measured length of each CAFs, i.e. as
the width is increasing the measured length should be decreased but the length is increasing. Thus it is reasonable that molecular re-arrangement of elementary fibril, induced by variable RHs might be a contribution to the changes in measured length of CAFs in this work. In addition to expansion in measured length and width, the cross-sectional area and height also increased as the RH is increasing. Those behaviors where expansion occurs all of directions in terms of length, width, area, height suggest that the CAFs samples are auxetic responses under the high humidity condition, i.e., in-plane and out-of-plane directions of CAFs show a negative Poisson ratio. Auxetic behaviors of cellulose microfibrils from kraft-pulp [37] and paper [38] have been reported. When the RH is decreasing down to 21 %, the expanded dimensions decrease except for the measured length. Thus, the behavior of measured length as a function of RHs is not dependent on the other dimensions. The mean value of percent changes in the measured length and width from three discrete trials shows that there is a little difference between each CAFs sample. The magnitude of changes in length and width of CAFs samples from oxygen bleached (SLO16) and fully bleached kraft (NS) pulps is bigger than that of CAFs sample from unbleached kraft pulp (SN50). The result suggests that the periodicity of longitudinal phase, called string of beads [32], of the elementary microfibrils from fully bleached kraft pulp (NS) and extended delignified microfibril (SLO16) have higher frequency in unit dimension than that of unbleached microfibrils (SL50).
6. Hygro-expansion behavior of each CAFs sample

Although the hygro-expansivity of mechanical and chemical pulps did not show a substantial difference [39], three different CAFs from different pulping and bleaching processing were used to evaluate their hygro-expansion behaviors. Based on the Figures 5.5 to 5.8, the slope of hygro-expansion behaviors of each dimensions at the given RH was calculated by a linear regression analysis. The slope shown in each figure is plotted in Figure 5.9. The slopes of measured length and width in NS CAFs samples were a little steeper than those of SL50 and SLO16 samples. However, the slope of the regressed line for cross-sectional area and height did not show a substantial difference between the three samples. These results suggested that the hygro-expansivity in in-plane direction is influenced by the proportional content of cellulose, since the removal of the hemicellulose and lignin in samples by the pulping and bleaching processing increases its effective crystallinity. In addition, the chemical differences in the cell wall did not result a substantial differences in the hygro-expansion behavior in the out-of-plane direction. The ultrastructure altered by the pulping and bleaching processes may be involved their behavior.

The average hygro-expansivity of three discrete trials corresponding measured length, width, area, and height was -0.025, 0.022, 0.21, and 0.18, respectively. In the out-of-plane direction such as area and height, the hygro-expansivity was one order of magnitude larger than the in-plane hygro-expansivity, including measured length and width.
<table>
<thead>
<tr>
<th></th>
<th>SL50</th>
<th>SLO16</th>
<th>NS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Length</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Width</td>
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<td></td>
</tr>
<tr>
<td>Area</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Height</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Figure 5.9** Hygro-expansion behavior of each CAFs as a function of RHs. (a) measured length, (b) width, (c) cross-sectional area, and (d) height.
**E. Conclusions**

AFM images of CAFs from different fiber sources were dynamically obtained over the range of RH from 78 % to 21 % at 23 °C and analyzed for length, cross-sectional area, width, and height. There was no significant difference between CAFs sources. The mean value of changes in length and width was in the range of 2.3 to 3.2 % and 1.9 to 3.3 %, respectively. The changes in area and height were in the range of 14.5 to 18.2 % and 12.4 to 17.3 %, respectively. The measured length of CAFs showed a negative correlation when it exposed to high humidity condition up to 78 % and followed by low humidity up to 21 %, whereas cross-sectional area, width, and height showed positive correlations with this humidity cycle. Overall, the variability in chemical composition did not appear to have a significant effect on the dimensional stability of the CAFs.

**F. References**


CHAPTER SIX

HYGRO-EXPANSIVE PROPERTIES OF ENZYME-DEGRADED CELLULOSE AGGREGATE FIBRILS

Parts of the content were presented in 2006 Progress in Paper Physics Seminar, Oxford, Ohio USA, October 1-5, 2006.
A. Summary

A study of the degradation effects of enzyme treatment on the hygro-expansive properties of CAFs with dimensions of ~100,000 × 3,000 × 300 nm from fully bleached kraft fiber was performed. CAFs were incubated with cellulase for up to 32 hours. The insoluble CAFs fragments after enzymatic hydrolysis were then subjected to variable relative humidity. Each sample was imaged by an atomic force microscope (AFM) in tapping mode. The images were analyzed to determine the dimensional changes and hygro-expansive properties of the insoluble CAFs. Enzymatic hydrolysis continuously depolymerized the CAFs over 32 hr, ultimately causing 20 % of the CAFs to become soluble. Compared to initial dimensions of the reference CAFs with no enzymatic treatment, the dimensions of the enzyme treated CAFs were more responsive to high humidity and exhibited an increased frequency of plastic deformations.

B. Introduction

Native cellulose is the most abundant renewable biopolymer, which is biosynthesized as a form of cellulose microfibril with diversity of macromolecular arrangement through intra- and inter-molecular hydrogen bonds [1, 2]. Depending on their origin, various lateral sizes and shapes of cellulose microfibrils were produced and are well-documented [3]. The cellulose microfibril from lignocellulosic fibers has been considered as an alternative source
to inorganic filler due to their potential reinforcing properties and wide availability [4]. Consequently, the demand to increase the sustainability and biodegradability of materials has been a driving force to engineer the cellulose microfibril from biological sources into advanced materials such as composites [5-9].

However, one challenge in utilizing microfibrils in composites is understanding the effect of the separation processes used to obtain the microfibrils on the microfibril properties. For example, hydrochloric acid hydrolysis leads to lower aspect ratio (length-to-width ratio) of microfibrils and dispersion problems. In order to overcome these aggregations, sulfuric acid hydrolysis [10] and TEMPO-mediated oxidation [11] may be applied to create more stable aqueous microfibril suspensions. This is done by the introduction of sulfate ester groups and carboxylic groups on the surface of isolated microfibrils. In addition, several methods have been reported to isolate microfibrils with high aspect ratio from the cellulosic fibers including a combination of high-pressure and mechanical treatment [12], high shear refining, and cryocrushing [13]. Another pathway for production of microfibrils from cellulosic fibers is by hydrolytic enzyme degradation with cellulases.

Knowledge of the structure-property relationships of isolated microfibrils from lignocellulosic fibers in a matrix network is still limited. In addition, physical and mechanical properties of the network often change significantly in response to environmental factors [4, 14, 15]. The dimensional behaviors of cellulose microfibrils are an important factor when one would like to engineer composite materials. These properties have not been studied in detail. Thus, dimensional changes of cellulose microfibrils to environmental factors are an important parameter for the quantitative assessment of composites, paper, and natural fiber.
An algorithm based on atomic force microscopy (AFM) imaging and computational analysis techniques was introduced to determine the apparent dimensions of nano-scale materials [16]. The technique has been applied to measure local dimensional properties of CAFs, providing new insights of its local hygro-expansion behavior toward surrounding environments [17, 18]. In this paper, the hygro-expansive properties of CAFs degraded by an enzymatic treatment were evaluated.

C. Experimental section

1. Preparation of cellulose aggregate fibrils (CAFs)

CAFs were prepared in the laboratory from a fully bleached northern softwood pulp (NIST, RM 8495) by a series of high shear refining and fractionation according to Lee et al. [16]. The pulp was passed through a valley beater for 80 minutes, followed by a fiber length based Bauer-McNett classifier to remove a fines and primary cell wall-free fiber fraction on Tyler screen No. 12. The fiber fraction was beaten again in a PFI mill for 75,000 revolutions. The fibrillated pulps were then screened with a Britt dynamic jar at 0.5 % consistency. The filtrates, called cellulose aggregate fibrils (CAFs), were filtered through a 0.45 micron membrane filter (47mm, Gelman Sciences, USA) to thicken ca. 2 % consistency and were refrigerated during storage. These CAFs with dimensions of ~100,000 (L) × 3,000 (W) × 300 (H) nm were liberated.
2. Enzymatic treatment of the CAFs

The enzyme used is produced from *Trichoderma reesei* (Fluka, 22173) and its cellulolytic activity provided by the manufacturer was 6 U/mg. In order to obtain a homogeneous distribution of the enzyme, the enzyme (0.1g) was first added to distilled water (100 g, pH 7.0, and 48 °C) before 1 ml of the enzyme solution was added to the CAFs (0.1 g/g dry solid, 48 °C), to a final CAFs concentration of 1 % (g/g). Two separated series of enzyme treatments were performed with various incubation times. The incubation times were 0 (reference), 1, 2, 4, 8, 16, and 32 hours. The enzymatic incubation was carried out in a gyratory water bath (250 rpm, New Brunswick scientific, USA) at 48 °C. After incubation, the samples were filtered on a 0.2 µm membrane filter (47 mm, Gelman Sciences, USA) and washed extensively with deionized water to remove the residual enzymes and soluble sugars.

3. Preparation of sample glass slide

In order to obtain images of CAFs by AFM, cellulose suspensions of each enzymatic treatment were diluted to about 0.0002 % concentration by weight. A 10 µl aliquot of the diluted suspensions was placed on a cleaned glass slide (Superfrost®, Fisher scientific) in at least 20 different spots. The glass slide was first cleaned by immersing the glass slide in a mixture of absolute ethanol and 1 N HCl (1:1, v/v) overnight, followed by ultrasonic cleaning in deionized water for three times. The glass slide with the CAFs attached was carefully placed in an ambient room for more than one week to dry.
4. Experimental set-up

The experimental apparatus was set up in an ambient condition of 50 % RH and 23 °C. Images were acquired with a Q-scope™ 250 atomic force microscopy (Quesant Instrument Corporation, USA) using a NCS tip (MikroMasch, USA) with 230 μm long cantilever with a spring constant of approximately 3.5 N/m (provided by manufacturer) in intermittent contact mode.

An acoustic/vibration isolation chamber (Quesant Instrument Corporation, USA) served as the test chamber for the experiments. The atmosphere above two saturated salts solutions, potassium acetate (20 % RH) and potassium sulfate (80% RH), were combined with an air pump to generate various humidity conditions in the test chamber from 80 % to 26 %. The humidity in the test chamber was varied in two hour increments. For example, the 80 % RH atmosphere was pumped into the test chamber for two hours. After two hours, a low humidity (20 %) atmosphere was pumped into the test chamber for two hours. The CAFs sample was imaged. The humidity within the test chamber was measured with an electronic hygrometer (OMEGA® RH 411 relative humidity meter, OMEGA Co.). Each image typically took ~ 10 minutes to complete, during which time the relative humidity changed. The humidity was recorded at the start and completion of each scan. The average of these two values was then recorded as the relative humidity value for the corresponding images.
5. Dimensional measurement by image processing

An individual straight CAFs of each sample was chosen for observation on the slide. CAFs sample and a series of images were then taken with the AFM with variable humidity conditions described in the experimental set-up. Each image was 10 µm × 10 µm in size and had a 400 line vertical and horizontal resolution (1 line segment corresponds to 0.025 µm in the image). For each image two distinct defects (Y₁ and Y₂) were identified. The difference in length between the defects was used to characterize the changes in the measured length of CAFs segment. The change in length was measured by manual point-to-point method and this change in length was taken to be representative of the change in measured length of the entire CAFs. The mean CAFs cross sectional area, height, and width were measured 5 times using a rapid feature analysis (RFA) developed by the authors and described in an earlier work [16]. The changes of each dimensional measurement were calculated as follows;

\[
\text{Changes in dimensions (\%)} = \frac{D - D_0}{D_0} \times 100
\]

where \( D \) and \( D_0 \) are real-time dimensions and starting dimensions of each sample at a given RH, respectively.
D. Results and discussion

1. Mass reductions and the morphology of CAFs

In the enzymatic hydrolysis experiments of CAFs from fully bleached kraft pulp, two separated fractions at each incubation time were obtained after extensive washing with deionized water.

![Graph showing mass reductions of enzyme treated CAFs as a function of incubation times: 0, 1, 2, 4, 8, 16, and 32 hrs. The enzyme dosage was 0.01g/g dry weight CAFs.](image)

**Figure 6.1** Mass reductions of enzyme treated CAFs as a function of incubation times: 0, 1, 2, 4, 8, 16, and 32 hrs. The enzyme dosage was 0.01g/g dry weight CAFs.

**Figure 6.1** shows the mass reduction of the CAFs by enzymatic hydrolysis. There is a linear relationship between the mass reduction and the logarithm of the incubation time. Enzymatic degradation occurred rapidly in the early stage of the incubation time and progressively depolymerized the CAFs up to 20 % (g/g) after 32 hours.
Figure 6.2 Optical micrographs of the isolated CAFs after enzymatic treatment as a function of incubation time: (a) reference (0 hr), (b) 1 hr, (c) 2 hrs, (d) 4 hrs, (e) 8 hrs, (f) 16 hrs, (g) 32 hrs.
Figure 6.2 shows optical micrographs of the isolated CAFs as a function of incubation time. In the pictures, the CAFs are well separated. Kinks were observed on CAFs in the samples, probably due to damage from the mechanical treatment and enzymatic action. The initial CAFs showed two classes of dimensions of CAFs, one with a longitudinal dimension of over 100 µm and the other with a few micrometers. When the initial CAFs is treated with the enzyme, the lengths were reduced. After 32 hours of incubation time, their measured lengths were progressively reduced and the two dimensions were more distinct. This result suggested that the mass reduction as shown in Figure 6.1 corresponds to the hydrolysis of the amorphous zones and to the removal of CAFs with low aspect ratio (fines).

2. Initial dimensions of isolated CAFs

Among seven variable incubation times, four different incubation times (0, 1, 8, and 32 hrs.) were selected to be evaluated for dimensional stability under variable RHs. After each incubation time, a sample was prepared on a glass slide described above. The CAFs with at least 100 µm in measured length were chosen for the measurement of their dimensional stability properties. The selected CAFs were carefully placed onto the AFM stage. Before changing the RHs, a number of images were obtained at 50 % RH and 23 °C to be used as a baseline for the initial dimensions of the CAFs. Based on the two distinct naturally occurring defects (e.g. Y₁ and Y₂ in Figures 6.3 to 6.12), the length of the segment of the CAFs was determined. In addition, its corresponding cross-sectional area, width, and height were calculated by the RFA analysis [16]. Table 6.1 summarized the initial dimensions of CAFs at each incubation time and their RH experimental schedule.
Table 6.1 Initial dimensions of each CAFs and its experimental conditions.

<table>
<thead>
<tr>
<th>Enzyme treatment (hr.)</th>
<th>Measured length (µm)</th>
<th>Area (nm² ×1,000)</th>
<th>Width (µm)</th>
<th>Height (nm)</th>
<th>Initial Max.</th>
<th>Min.</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>8.094 ± 0.027</td>
<td>772.3 ± 19.1</td>
<td>2.208 ± 0.004</td>
<td>350.5 ± 8.9</td>
<td>41</td>
<td>75</td>
</tr>
<tr>
<td>A</td>
<td>6.665 ± 0.010</td>
<td>254.4 ± 10.8</td>
<td>1.645 ± 0.005</td>
<td>156.8 ± 6.3</td>
<td>45</td>
<td>75</td>
</tr>
<tr>
<td>B</td>
<td>8.863 ± 0.022</td>
<td>331.4 ± 2.30</td>
<td>1.858 ± 0.010</td>
<td>181.5 ± 0.5</td>
<td>51</td>
<td>76</td>
</tr>
<tr>
<td>C</td>
<td>9.274 ± 0.010</td>
<td>215.2 ± 10.3</td>
<td>1.957 ± 0.005</td>
<td>110.2 ± 5.0</td>
<td>49</td>
<td>80</td>
</tr>
<tr>
<td>A</td>
<td>7.525 ± 0.026</td>
<td>784.5 ± 29.4</td>
<td>2.769 ± 0.004</td>
<td>284.0 ± 11</td>
<td>45</td>
<td>76</td>
</tr>
<tr>
<td>8</td>
<td>4.015 ± 0.029</td>
<td>256.8 ± 4.90</td>
<td>1.371 ± 0.007</td>
<td>187.5 ± 3.6</td>
<td>51</td>
<td>76</td>
</tr>
<tr>
<td>B</td>
<td>4.231 ± 0.031</td>
<td>244.7 ± 1.10</td>
<td>1.539 ± 0.013</td>
<td>160.5 ± 1.7</td>
<td>50</td>
<td>82</td>
</tr>
<tr>
<td>C</td>
<td>8.573 ± 0.017</td>
<td>921.5 ± 21.8</td>
<td>3.379 ± 0.006</td>
<td>274.3 ± 5.4</td>
<td>42</td>
<td>75</td>
</tr>
<tr>
<td>32</td>
<td>8.552 ± 0.103</td>
<td>225.1 ± 5.00</td>
<td>1.595 ± 0.019</td>
<td>141.6 ± 4.7</td>
<td>53</td>
<td>81</td>
</tr>
<tr>
<td>B</td>
<td>8.192 ± 0.017</td>
<td>417.0 ± 12.1</td>
<td>2.041 ± 0.015</td>
<td>204.8 ± 4.3</td>
<td>48</td>
<td>77</td>
</tr>
</tbody>
</table>

3. Morphology of isolated CAFs as function of RHs

Isolated CAFs were selected to be subjected to variable RH. Figure 6.3 showed AFM images in 10 µm × 10 µm scan size of the reference CAFs (0 hour) from the fully bleached kraft pulp as a function of relative humidity from 41 % to 75 % RH during adsorption and followed by 26 % RH during desorption stage. During the sorption stages, significant morphological changes were not observed. Figures 6.4 to 6.12 showed AFM
images of the isolated CAFs with different enzymatic incubation times during sorption stages, i.e., 1 hour in Figures 6.4 to 6.6, 8 hours in Figures 6.7 to 6.9, and 32 hours in Figures 6.10 to 6.12. In the images, significant morphological changes and various plastic deformations were observed as a function of RHs. After scanning a number of images of the isolated CAFs by AFM at ambient condition, the humidity in test chamber was slowly increased by turning on the air pumping. In the meantime, the same scan area was imaged as a function of RH. When the RH in the test chamber was reaching to around 60 %, morphological changes were often observed, regardless of the CAFs treatment time. In some samples which are not shown in this paper, we could not get images just after turning on the pump due to a significant morphological change. After significant morphological changes during the adsorption stage, the sample was successively imaged during the desorption stage. When the RH decreases, the morphological features observed during adsorption stages diminished, but the resulting images had significant plastic deformations (e.g. Figure 6.8). Most of plastic deformations were observed during adsorption stage but sometime we could observe the plastic deformation during the desorption stage, as shown in Figure 6.6.
Figure 6.3 AFM images of reference CAFs (0 hour) during adsorption (a to e) and desorption (f to i) stages.
Figure 6.4 AFM images of enzymatic treatment for 1 hour (1hr-A sample) during adsorption (a to e) and desorption (f to i) stages.
Figure 6.5 AFM images of enzymatic treatment for 1 hour (1hr-B sample) during adsorption (a to d) and desorption (e to i) stages.
Figure 6.6 AFM images of enzymatic treatment for 1 hour (1hr-C sample) during adsorption (a to d) and desorption (e to i) stages.
Figure 6.7 AFM images of enzymatic treatment for 8 hours (8hrs-A sample) during adsorption (a to e) and desorption (f to i) stages.
Figure 6.8 AFM images of enzymatic treatment for 8 hours (8hrs-B sample) during adsorption (a to d) and desorption (e to i) stages.
Figure 6.9 AFM images of enzymatic treatment for 8 hours (8hrs-C sample) during adsorption (a to d) and desorption (e to i) stages.
Figure 6.10 AFM images of enzymatic treatment for 32 hours (32hrs-A sample) during adsorption (a to d) and desorption (e to i) stages.
Figure 6.11 AFM images of enzymatic treatment for 32 hours (32hrs-B sample) during adsorption (a to e) and desorption (f to i) stages.
Figure 6.12 AFM images of enzymatic treatment for 32 hours (32hrs-C sample) during adsorption (a to d) and desorption (e to i) stages.
4. Dimensional changes of isolated CAFs as function of RHs

Figures 6.13 to 6.16 showed the dimensional changes in terms of length, width, area, and height of the isolated CAFs with each incubation time. Their mean percent value (top) and maximum and minimum values (bottom) were presented in Figure 6.17. In the reference CAFs with no enzyme treatment, the dimensional changes were 2.3 % in measured length, 2.4 % in width, 13.4 % in cross-sectional area, and 11.9 % in height. All of dimension was positive correlations except the measured length, which showed a negative correlation at the given experimental RHs schedule. The magnitude of the dimensional changes of the reference CAFs is a good agreement with our previous result [18].

Figure 6.13 showed the percent changes in measured length of the CAFs from each incubation time. The measured length of each CAFs was expanded and/or shrunk as the RH was reached up to their maximum level. However, the percent changes of each sample followed by exposure to lower humidity up to 26 % RH were expanded compared to their initial measured length. At the given RHs, negative correlation was observed in all samples. The mean value of the treated CAFs from each incubation time was 4.4 %, 5.8 %, and 4.0 % in 1 hour, 8 hours, and 32 hours, respectively as shown in Figure 6.17a. The magnitude of changes in measured length of the treated is in the range of our previous results although the treated undergoes significant morphological changes. This result suggested that the observation of a negative correlation in the measured length of CAFs under a cyclic humidity condition where RH was increased up to 80 % and followed by 23 % is from CAFs itself.
Figure 6.13 Changes in measured length of CAFs at each incubation time. (a) 0 hour, (b) 1 hour, (c) 8 hours, (d) 32 hours.
Figure 6.14 Changes in width of CAFs at each incubation time. (a) 0 hour, (b) 1 hour, (c) 8 hours, (d) 32 hours.
Figure 6.15 Changes in cross-sectional area of CAFs at each incubation time. (a) 0 hour, (b) 1 hour, (c) 8 hours, (d) 32 hours.
Figure 6.16 Changes in height of CAFs at each incubation time. (a) 0 hour, (b) 1 hour, (c) 8 hours, (d) 32 hours.
Figure 6.17 Mean values of each dimensional change (top) and maximum and minimum values (bottom) during cyclic humidity changes of CAFs of each incubation time. (a) measured length, (b) width, (c) area, (d) height.
Figure 6.14 showed the percentage changes in width of CAFs at the given measured lengths as shown in Figure 6.13. Compared to the percentage changes in width of the reference, the treated CAFs showed significant changes in width, i.e. 12 %, 55 %, and 16 % in 1 hour, 8 hours, and 32 hours, respectively as shown in Figure 6.17b. The morphological changes and plastic deformations observed give rise to the considerable changes in width. These morphological features also affect the changes in the cross-sectional area and height of samples. The magnitude of changes in area and height is more significant than that of width, as shown in Figures 6.15 and 6.16. The mean value of percent change in the cross-sectional area of the treated is 59 %, 118 %, and 43 % in 1 hour, 8 hours, and 32 hours, respectively. The mean percentage change in height of the treated CAFs is at least three times bigger than the untreated as shown in Figure 6.17d.

It is impossible to directly compare the dimensional changes of the CAFs from each incubation time due to the significant morphological features observed. However, compared to the untreated CAFs, the dimensions of the treated CAFs of each incubation time significantly increased, except the measured length. This result suggested that the isolated CAFs treated with enzymatic treatment is highly responsive to the surrounding environment even with a small change of moisture content (ca. 6 %). With mild enzymatic treatment, the supra-structure of the CAFs could be changed, leading to more open arrangements in the outer layer of the surface as well as the whole area in the CAFs [19].
E. Conclusions

Cellulose aggregate fibrils (CAFs) were incubated with cellulase for up to 32 hours. A significant amount of cellulose became soluble during this time (ca. 20 %). The remaining insoluble CAFs fragments were subjected to variable relative humidity. The treated CAFs were more responsive to the variable humidity and exhibited greater changes in dimensions compared to untreated control samples. They were also observed to be more prone to plastic deformation during the humidity cycle.

F. References


CHAPTER SEVEN

RHEOLOGY OF CARBOXYMETHYL CELLULOSE SOLUTIONS TREATED WITH CELLULASES

Parts of the content were submitted for publication in *BioResources*
A. Summary

The effect of cellulase treatments on the rheology of carboxymethyl cellulose (CMC) solutions was studied using a rotational viscometer. The rheological behaviors of CMC solutions of different molecular mass and degrees of substitution were studied as a function of time after various treatments. These solutions were subjected to active and heat-denatured cellulase, a cationic polyelectrolyte (C-PAM), as well as different shear rates. A complex protein-polymer interaction was observed, leading to a potential error source in the measurement of enzymatic activity by changes in the intrinsic viscosity. The interaction was termed a polymeric effect and defined as a reduction in viscosity of the substrate solution without significant formation of reducing sugars from enzymatic hydrolysis. The cause of the reduction in viscosity appears to be related to the interaction between the enzymes as an amphipathic particle and the soluble CMC. Thus, the polymeric effect may cause a considerable experimental error in the measurement of enzymatic activity by a viscometric method.

B. Introduction

Cellulases are produced by a variety of bacteria and fungi. These enzymes have been used in a number of industrial applications in the fields of food science, textiles, and pulp and paper [1]. These enzymes act on cellulosic substrates both soluble and insoluble. Native
cellulose has a complex ultrastructure with both crystalline and amorphous regions. The simultaneous synergistic action of several different isozymes of cellulase is required to complete the hydrolysis of solid cellulose. Based on their activity on cellulose, cellulases can be classified as endoglucanases (EGs), cellobiohydrases (CBHs), and β-glucosidases [2].

EGs preferentially hydrolyze the β-(1-4) linkages of amorphous cellulose in a random manner. CBHs hydrolyze crystalline cellulose by cleaving the β-(1-4) linkage of cellulose to produce cellobiose and may work synergistically with EG to depolymerize the cellulose. The cellobiose released from EG and CBH actions is finally converted into glucose by action of β-glucosidase. Thus, the structure-function relationship between the cellulase isozymes and the cellulosic substrate enhances our knowledge of cellulose biodegradation [3].

The conversion rate of cellulose into glucose is called cellulase activity. It is an indication of the efficiency of the cellulase hydrolyzing reaction. The determination of cellulase activity is a complicated process, because the hydrolysis of insoluble cellulose may not be linear with enzyme dosage and/or reaction time. Thus, the dosage of enzyme and the point in the reaction at which the activity is measured is critical. For such reasons, enzymatic activity should be carefully determined. Several standard substrates are available for the determination of cellulase activity in terms of the overall and EG activity [4]. Filter papers have been used as a standard substrate to measure the total cellulase activity. The filter paper activity, termed as FPase, expressed the summations of the simultaneous synergistic action of EGs, CBHs, and β-glucosidase in a cellulase preparation. Soluble cellulose derivatives, such as carboxymethyl cellulose (CMC) and hydroxyethyl cellulose (HEC), may be used as substrates for determining EG activity. These substrates are rather specific to EG activity since CBHs are not generally able to degrade substituted cellulose [5]. The hydrolysis rate of
EG was conventionally determined by reduction of viscosity or by increase of reducing sugar end groups of the cellulose derivatives [6, 7]. Recently, based on adsorption behaviors of free and bound enzymes on microcrystalline cellulose (MCC), a new measurement of EG activity has been introduced [8]. Among these methods, monitoring the reduction in CMC solution viscosity is considered to be the most accurate method to detect EG activity [9, 10]. However, the viscometric method for measuring activity is not commonly used due to its laborious and discontinuous nature. For this reason, the colorimetric measurement of reducing sugars has been routinely used for measurement of EG activity.

Various types of automated viscometers are available to measure the rheological changes of a polymer solution. Viscosity is a fundamental rheological parameter that characterizes the resistance of the fluid to flow. The viscosity of a polymer solution is related to the polymer concentration, the extent of polymer-solvent interaction, and the polymer structure such as molecular weight, shape, molecular flexibility, and molecular conformation. Under appropriate experimental conditions, it is functionally related to the molecular weight [11]. However, a more complete understanding of the rheological changes in a cellulase-polymer solution is required to accurately monitor cellulose hydrolysis by viscosity measurements.

Like all proteins, enzymes are basically made up of amino acids linked by peptide bonds between the carboxyl groups of one amino acid and the amino group of the next amino acid. The hydrophobic and hydrophilic nature of amino acids often makes the surface of enzymes an amphipathic interfacial structure, i.e. proteins tend to adsorb at interfaces as a surface-active polymer [12]. Moreover, CMC is a semiflexible anionic polymer [13]. The adsorption of enzymes (protein) on the CMC leads to a change of the polymer behavior. Of
particular interest is the effect of enzyme absorption on the rheology of a cellulase-CMC solution. Mixtures of proteins (enzymes) and polymers in aqueous dispersion are often accompanied by either segregative or associative phase separation [14]. Thus, physical and chemical parameters of the proteins (enzymes) and substrate and solution characteristics such as solution pH, ionic strength, concentration and protein/polymer ratio should be considered. Moreover, processing variables such as temperature, shear rate and time also strongly influence the rheological behavior of the complexes. For example, changes in functional properties due to soluble complexes between a globular protein (BSA) and a polysaccharide (CMC) have been reported [15, 16]. The protein was adsorbed onto the CMC segments in the dilute regime or entrapped in the polymer network in the semi-dilute region. After thermal treatment of the soluble complexes, a considerable change in the visco-elastic properties of the network was observed [17].

When a cellulase is added to a soluble cellulose derivative (e.g. CMC), one might expect that the rheological behavior of the mixture will change considerably as a function of reaction time. The change in the viscosity may be due to the cellulase ad- and de-sorbing or entrapping the substrate. The configuration and conformation of the substrate molecules will then be changed. These interactions between cellulases and substrates may be expected to result in considerable changes in the solution viscosity. Thus, significant changes in the solution viscosity may result without any enzymatic hydrolysis taking place. It should also be noted that reductions of enzymatic activity and its binding ability are expected when enzymes are continuously subjected to shear or exposed to an air-liquid interface during mixing and agitation processes [18, 19].
The study reported herein investigates the rheological behavior of CMC solutions during enzyme hydrolysis and the influence of enzymes on CMC solutions without hydrolysis. The ability of enzymes to reduce CMC solution viscosity without hydrolysis was investigated in two manners. First a heat-denatured enzyme was applied to the CMC solution. Second, a high degree of substitution CMC was used. This CMC could not be degraded by the enzymes. In addition, the effects of shear rate and contact time were evaluated during the cellulase-polymer interaction.

C. Experimental

1. Materials

A commercial enzyme preparation supplied by Dyadic international (Fibrezyme L, Florida, USA) was used in this study. The cellulase preparation was fractionated by using a 10kD cut-off filter (Amicon, USA) and then used as an active cellulase preparation. In order to make a heat-denatured cellulase preparation, a 10 g l⁻¹ solution of the active cellulase preparation was transferred to a glass tube and covered. The tube was then placed in a boiling water bath for 2 hours with vigorous agitation. After the boiling, the suspension was homogenized for 15 minutes with an ultrasonic homogenizer (Omni Ruptor 250, Omni International Inc, USA) in order to make a well dispersed heat-denatured cellulase suspension. The active and heat-denatured cellulase suspensions were refrigerated during storage.
Five commercial carboxymethyl celluloses (CMCs) in the sodium salt form of different average degrees of polymerization (DP) and degrees of substitution (DS) were obtained from Aqualon Hercules Inc. (Wilmington, Delaware, USA): 7L (0.7 DS and 400 DP), 7M (0.7 DS and 1,100 DP), 7H (0.7 DS and 3,200 DP), 9M8 (0.9 and 800 DP), and 12M8 (1.2 DS and 800 DP). The letters L, M, and H correspond to low, medium, and high molecular mass. According to the manufacturer, the typical molecular mass values corresponding to these categories are 90,000, 250,000, and 700,000 Daltons. A cationic polyacrylamide (C-PAM) (Percol® 175, Ciba Specialty Chemicals) was also used. Its molecular mass is approximately 5,000,000 Daltons.

2. Methods

a) Preparation of CMC solutions

An appropriate amount of the CMCs was slowly added into the sodium acetate buffer (50mM, pH 4.7) to make 1.0 % and 1.4 % concentrations of CMC suspensions under vigorous agitation. After completing the addition, the suspensions were transferred to a blender and vigorously mixed for one minute at room temperature. The blended suspension was stirred for one hour, while heating to 80 °C. In order to make homogenous CMC solution, the suspension was then filtered through a sintered glass (coarse) funnel into a vacuum filtering flask. The suspension was then agitated for one hour at room temperature under the vacuum to remove entrapped air bubbles. The CMC solutions were stored at 4 °C and used within one week. After one week, fresh CMC solutions were again prepared.
b) Enzymatic hydrolysis and its viscosity measurement

Enzymatic hydrolysis of CMC solutions was performed using a Brookfield rotational viscometer with a UL adaptor (Brookfield DV-II+2, USA). 20 ml of each CMC suspension was preheated at 50 °C in a water bath before being transferred to the UL adaptor, which was controlled to 50 °C. One ml of each cellulase preparation was added to the CMC suspension to start the enzymatic reaction. The apparent viscosity of CMCs was collected every one minute with an automated program (Rheocalc®, Brookfield instrument, USA). The viscometer used a rotational speed of 10 rpm except during the shear rate dependence experiments. During this set of experiments the shear rates were varied in the range of 1 to 32 rpm.

c) Reducing sugar measurement

The reaction products of enzymatic hydrolysis were transferred into a test tube as quickly as possible to determine the concentration of reducing sugar end-groups in the sample. The amounts of reducing sugar end groups formed by cellulase action were determined by the dinitrosalicylic acid (DNS) method [20] using glucose to generate a standard curve.

D. Results and discussion

1. Polymeric and enzymatic effects of cellulase
Figure 7.1 shows the change of the apparent viscosity as a function of time with the addition of active and heat-denatured enzymes. It is clear from this figure that both the active and the heat-denatured enzyme reduce the viscosity of the CMC solution as a function of contact time. This seems to imply that the heat-denatured enzyme preparation retains a portion of its activity, although the preparation was brought to boiling for 2 hours. In order to examine whether the change in viscosity of the solution with the heat-denatured enzyme is from its residual activity, the production of reducing end groups in the hydrolysates from the active and heat-denatured cellulases were analyzed by the DNS method. The concentration of reducing sugar (glucose equivalent) liberated from the CMC can be used as a measure of hydrolytic efficiency of the active and heat-denatured cellulase preparations.

The active cellulase depolymerized the substrate as a function of reaction time, leading to increase formations of the reducing sugar, whereas the heat-denatured preparation showed no significant change in the reducing sugar. It appears likely that the thermal denaturation of cellulases [21, 22] and proteins [23, 24] caused partial unfolding of the proteins due to the disruption of the hydrogen bonds responsible for the three-dimensional structure. This may expose highly reactive groups buried inside the protein structure to the solution. This may lead to increased sites available for the CMC to interact with the denatured enzyme causing conformational changes of the substrate [23]. Based on this observation, the results in Figure 7.1 imply that the heat-denatured enzyme alters the conformation of the CMC and thus changes the viscosity of the solution. The change in the viscosity due to the amphipathic nature of the enzyme has been the termed the polymeric effect in this study.
Figure 7.1 Changes in apparent viscosities (50 °C and 10 rpm) and reducing sugars of 7 M CMC (1.0 % conc.) treated with active and its heat-denatured cellulase as a function of time.

2. Effect of cationic polymer

A decrease in the viscosity of a CMC solution was also observed when a cationic polymer was added to the solution, cf. Figure 7.2.

On this figure, one can see a decrease in the CMC solution viscosity over time with cationic polymer and heat-denatured enzyme addition. A similar effect is noted. After 90 minutes of elapsed time, the solutions were left in the viscometer for 500 minutes without
shearing, and then the viscosities of the solutions were again measured. The viscosity with heat-denatured enzyme decreased slightly (3 cP) whereas the viscosity with the cationic polymer increased over 500 cP. This indicates that the cationic polyelectrolyte (C-PAM) interacts with the anionic polymer (CMC) as a function of time, forming a complex, and thus leading to an increase in the viscosity. This may be attributed to the high degree of polymerization of the C-PAM.

Figure 7.2 Changes in apparent viscosity of 7 M CMC (1.4 % conc.) treated with the heat-denatured enzyme or C-PAM (0.1 % conc.) at 50 °C and 10 rpm as a function of time. The inserted plot represents the apparent viscosity of the CMC after 500 minute elapsed.
3. Effect of CMCs with different DS

The polymeric effect, which reduces the CMC viscosity without the creation of reducing end groups, can also be observed using CMC with different degrees of substitution.

Figure 7.3 Changes of apparent viscosities and reducing sugars of 7 M, 9 M, and 12 M CMCs (1.4 % conc.) treated with the active cellulase.

Figure 7.3 shows the viscosity of CMC with different degrees of substitution as a function of enzyme contact time. Also shown in this figure is the concentration of reducing
end groups as a function of time. It is known that the susceptibility of CMC to enzyme hydrolysis is dependent on degree of substitution (DS) of the CMC. When the DS of CMC reaches an average of one substitution per glucose unit, steric factors strongly retard the enzyme activity [25]. Thus the International Union of Pure and Applied Chemistry (IUPAC) recommended CMC having a DS of 0.7 as a substrate for measuring cellulase activity [4]. The inhibition of enzyme activity at high DS is shown again here by the lack of increase in reducing end group concentration with increased reaction time for the 1.2 DS CMC. However, it is noted that the viscosities of CMC decreased in a similar manner for all degrees of substitution regardless of the change in the reducing end groups. This again indicates that a “polymeric effect” plays an important role in determining the viscosity of the CMC solution independent of hydrolytic activity.

4. Changes of intrinsic viscosity

When measuring the enzymatic activity via changes in solution viscosity, the polymeric effect can result in significant errors in the measured activity as shown in Figures 7.1-7.3. To evaluate the impact of the polymeric effect on the activity measurement, one needs to observe the intrinsic viscosity as a function of time, as the intrinsic viscosity is related to the molecular weight of the polymer. The intrinsic viscosity of a polymer solution is determined by measuring apparent viscosity at a series of different solute concentrations (0.5, 1.0, 2.0, and 3.0 % with the 7 M CMC). The intrinsic viscosity was evaluated from the extrapolation to zero concentration of \( \ln \eta_0/C \) against concentration C, using the Kraemer equation [26]:

\[
\eta_0/C = K \times \ln \eta_0/C
\]
\[
\frac{\ln \eta_{rel}}{C} = [\eta] + \kappa'' [\eta]^2 C \quad \text{Eq. [1]}
\]

where \([\eta]\) is intrinsic viscosity, \(\eta_{rel}\) is relative viscosity of the polymer solution compared with the solvent, \(C\) is the concentration of the polymer solution (in g/dl), and \(\kappa''\) is Kraemer’s constant.

**Figure 7.4** Intrinsic viscosity of CMC with active and heat-denatured cellulase at 50 °C and 10 rpm.
Figure 7.4 shows the profiles of intrinsic viscosity of different concentrations of 7M CMC treated with active and heat-denatured cellulases for 2 hours at 50 °C and a shear rate of 10 rpm. One observes a significant decrease in the intrinsic viscosity for both the heat-denatured and active enzymes. Thus, significant errors could occur in measuring the enzymatic activity by solution viscosity, if the polymeric effect is strong or varies from sample to sample.

To accommodate the polymeric effect, the solution viscosity can be adjusted. Assuming that the viscosity of a solution is related to friction between molecules within the solution and that the different components contributing to the solution viscosity are linear, then one can separate the decrease in viscosity of the active enzyme into two components. The first component is related to the decrease in the molecular weight of the CMC. The second component is related to the polymeric effect. The change in viscosity can be expressed as,

\[
\left( \frac{d\eta}{dt} \right) = \left( \frac{d\eta}{dt} \right)_{MW} + \left( \frac{d\eta}{dt} \right)_{polymer}
\]

\[\text{Eq. [2]}\]

where the subscript \(MW\) indicates the viscosity changes associated with changes in the molecular weight, and the subscript \(polymer\) indicates viscosity changes associated with polymeric effects. Equation 2 is not applicable when a large amount of enzymatic degradation has taken place, as the magnitude of the polymeric effect is influenced by the molecular weight, cf. Figure 7.5.
Figure 7.5 Apparent viscosity of different molecular weights of CMC (1 % conc.) treated with active cellulase (top) and heat-denatured cellulase (bottom) at 50 °C and 10 rpm as a function of time.
Figure 7.6 Adjusted viscosities of the different molecular weights of CMC (1 % conc.) as a function of time.

For relatively short degradation times, the change in viscosity as measured with the heat-denatured enzyme may be subtracted from the viscosity of the active enzyme solution to provide an estimate of the polymeric effect. For purposes of this estimation it is necessary to assume that the heat-denatured enzymes retain a molecular conformation that is not too different from that of the native enzymes. Using this modified viscosity data, a measurement of the change in viscosity attributed strictly to the catalytic activity can be made. Figure 7.6 shows the change in the viscosity adjusted for the polymeric effect compared to the
unadjusted viscosity. As one can see in the figure, the polymeric effect is significant and should be compensated for in rheological measurements of enzyme activity. However, one also can see that this adjustment for the polymeric effect is nonsensical for large contact times, as the adjusted viscosity become negative. Thus, such a correction method should only be used for short contact times.

Figure 7.7 Effect of shear rates in the range of 1 to 32 rpm of 7 H CMC (1 % conc.) treated with active cellulase as a function of reaction time.
5. Effect of Shear rate on viscosity

Figure 7.7 shows the effect of shear rate on the measured viscosity of 1 % 7 H CMC at 50 ºC treated with a fixed enzyme dosage. A complex pattern of viscosity change is detected. A possible explanation for this complex pattern is the interaction of enzyme mixing and the effect of shear rate on the disruption of cellulase binding. At low shear rates, the enzymes must diffuse throughout the CMC solution with little assistance from mixing. This results in a slower drop in the viscosity of the CMC solution with time. As the shear rate increases, the mixing assistance becomes better at distributing the cellulase throughout the solution. This results in a more rapid decrease in the solution viscosity. However, at higher shear rates the viscosity does not decrease as fast as at intermediate shear rates. This may be attributed to the shear rate disrupting the cellulase/CMC binding and thus reducing the cellulase efficiency [18, 19]. Thus, differences in shear rate can affect the measured viscosity change and the perceived cellulase activity.

E. Conclusions

The rheological behavior of anionic cellulose derivatives during the course of enzyme hydrolysis has been investigated. The effects of the active and heat-denatured cellulases, and a cationic polyelectrolyte (C-PAM) have been examined. Cellulose derivatives with different degrees of polymerization and substitution were examined. A polymeric effect, defined as a reduction in viscosity of the CMCs without significant formation of reducing sugars released
from the degradation of the CMCs by enzymatic hydrolysis, has been observed in this study.
The polymeric effect may be attributed to the interactions of the enzyme with the polymer in
solution. This effect was observed with heat-denatured enzyme, active enzyme and high DS
CMC (preventing enzymatic hydrolysis), and with the addition of C-PAM. The intrinsic
viscosity, which may be related to the molecular weight of a dissolved material, is reduced
by the polymeric effect. This may cause a significant error in the measurement of enzymatic
activity with viscometric methods. Assuming a linear relationship between the changes in the
solution viscosity attributed to enzyme hydrolysis and the polymeric effect, a means for
correcting for the polymeric effect was proposed. This allows for making a correction for the
polymeric effect during the onset of the hydrolysis reaction. Finally, the effect of shear rate
on the viscosity change caused by enzymatic hydrolysis was examined. A complex behavior
of viscosity change was found with shear rate. The results were interpreted to be a
combination of improved mixing with higher shear rates and disruption in binding by higher
shear rates.

F. References


CHAPTER EIGHT

FUTURE ASPECTS
I regretted that more practical measurements with different surrounding environments were not carried out in this dissertation, but it is my hope that this dissertation will help someone standing on the shoulders of giants to see the dynamic interactions between visco-elastic, dimensional, and hygro-expansive properties of nano-, micro-, and macro-cellulosic fibers and water molecules and/or chemicals (bio-chemicals) in the future. As my work came to its end, it became more and more clear that there is still much work to be done in order to unravel the underlying mechanism of water molecules-structural properties. To this end the following suggestions are made for continuation of the current work in order to elucidate the relation between these interactions and help us better understanding of the mechanisms behind each.

A. Multiple humidity schedule systems with different sequences

In the dissertation, I have studied mainly one humidity schedule system where the relative humidity in the AFM chamber was first increased up to 82 % and followed by a decrease down to 21 %. I then tried to correlate the response of CAFs from various sources to the dynamic interaction with water molecules. However, when CAFs were exposed to the opposite humidity sequence, where the humidity in the chamber was first lowered to 21 %, followed by an increase to high humidity condition, the dimensional changes of the CAFs
were significantly different, compared with present humidity schedule used in the dissertation.

Figures 8.1 and 8.2 show the dimensional changes of CAFs from fully bleached softwood and hardwood kraft pulp with a different sequence humidity schedule system. The measured length of the CAFs from both of softwood and hardwood was expanded when they were first exposed to low humidity and then the length was progressively expanded when the RH was approaching to 50 % RH and 23 °C. After being exposed to high humidity, the length slowly decreased and then slowly increased again when exposed to the ambient condition of 50 % RH and 23 °C. At this point, the CAFs from the hardwood pulp had a plastic deformation as shown in Figure 8.2. However, compared to the initial length of both samples, the final lengths of the samples expanded in the humidity cycles in a helix-like fashion if they did not show plastic deformation. The magnitude of their concurrent dimensional changes in terms of area, height, and width also showed a significant difference compared to the CAFs used in Chapter Five in the dissertation. Very large dimensional changes were observed. It would be of interest to correlate the interaction of different humidity cycles with CAFs behaviors, to better understand the relationship between these two different ways of measuring dynamic interaction.
Figure 8.1 Dimensional changes of CAFs from fully bleached softwood kraft pulp exposed to lower humidity and followed by high humidity.
Figure 8.2 Dimensional changes of CAFs from fully bleached hardwood kraft pulp exposed to lower humidity and followed by high humidity.
B. Cyclic pre-conditions with CAFs

The CAFs used in this dissertation were dried only under 50 % RH and 23 °C. The observed dimensional behaviors of the CAFs in the dissertation were an expression of the built-in stress in the CAFs, since the cellulosic fibers from bio-based sources experience growth stresses and effects of processing conditions. It would be interesting to study the dimensional behavior of CAFs if the built-in stresses in the CAFs were released by different cyclic sequences of pre-conditions.

C. Nanocrystals from acid hydrolysis and enzymatic hydrolysis

In Chapter Six, the dimensional changes of CAFs treated with cellulase were studied, but the CAFs selected were morphologically and dimensionally similar to the initial CAFs in order to evaluate only the effect of enzymatic treatment on the CAFs. However, enzymatic hydrolysis might be an alternative way to produce a nanocrystal with defect- and amorphous region-free character from bio-based materials, compared to acid hydrolysis. With selective degradations of amorphous cellulose in the CAFs through combinations of enzymatic and acid hydrolysis, the dimensional effect on its crystallinity of the local microfibrils will be of interest.
Although dimensional changes of CAFs from different pulping processes have been characterized in Chapter Five, there was no significant difference observed. However, the behavior of the amorphous cellulose is assumed to be responsible for the viscoelastic properties of cellulosic material. This would allow a comprehensive study of the relationship between amorphous and native crystalline phases.

**D. Cast microfibril film as a model cellulose surface**

The CAFs from various cellulosic pulps have been used as a starting material to evaluate their dimensional properties. When a cellulose film is cast by water evaporation on a polystyrene Petri dish according to Edgar and Gray [1], it shows a flat cellulose surface. Thus the cast film from the CAFs might be a great substrate for making a cellulose model film to examine cellulose interactions in the field of cellulose-cellulose, cellulose-polyelectrolyte, and cellulose-cellulase interaction. In Chapter Seven, we observed a polymeric interaction of carboxymethyl cellulose (CMC) with the heat-denatured cellulase, and hydrodynamic shear disrupted the interaction of the cellulase toward the surface of CMC. It would be of interest to know how the heat-denatured cellulase will interact with the “real” cellulose surface from cellulosic fibers, not a modified cellulose surface from CMC. Quartz crystal microbalance with dissipation (QCM-D) could easily be used to monitor this process as a change in the visco-elastic properties of the substrate. In addition, it would be of interest to investigate the interaction of cellulose binding domains (CBDs) from cellulases and heat-
denatured cellulase and/or proteins having similar surface composition with the CBDs with the cellulose surface.

Hopefully, such measurements will be done in the future and if so, I believe that the results presented here will help to better understand these properties.

E. References

CHAPTER NINE

APPENDIX
A. Appendix 1

MATLAB codes: Rapid feature analysis

function [cutdata,width,height,area,length1] = cafdim(filename,zres,xyres,contrast)

asciidata = dlmread(filename);

zd = (asciidata(:,3).*zres);

raw_data1 = reshape (zd,400,400);

raw_data1 = raw_data1';

raw_data1 = double(raw_data1);

[r,c] = size(raw_data1);

slopeimage = raw_data1(5:r,1:c);

catmat = zeros(4,c);

slopeimage = cat(1,catmat,slopeimage);

slopeimage = raw_data1(1:r-contrast,1:c)-raw_data1(contrast+1:r,1:c);

imagemin = min(min(slopeimage))

imagemax = max(max(slopeimage))

slopeimage = slopeimage-imagemin;

%slopeimage = (slopeimage./(imagemax-imagemin)).*254;

imshow((slopeimage))

colormap(prism)

[ulr,ulc]=GETUPPERLEFT;
ulr = int32(ulr)+contrast-1
ulc = int32(ulc)
[lrr,lrc]=GETUPPERLEFT;
lrr = (int32(lrr))+contrast-1
lrc = int32(lrc)
cutdata = raw_data(1:ulr:lrr,1:ulc:lrc);
raw_data = cutdata;
res = xyres;
calculationsa;
width = width;
height=height;
area = area;
length1 = res*(double(lrc-ulc));
imshow(cutdata)
colormap(gray)
area = mean(area)
width= mean(width)
height = mean(height)
B. Appendix 2

MATLAB codes: GETUPPERLEFT

function [ulr,ulc] = GETUPPERLEFT;

This function returns the mouse pointer location for the upper left hand corner of the area to
be turned into a text file. When the upper left corner button is selected on the textfilem gui,
the pointer becomes a crosshair. The location of the crosshair on the image is recorded when
the right mouse button is pushed.

ulr =[1];
ulc = [1];
but = [1];

edithandle = findobj(gcf,'Tag','Fig1');
    set(edithandle,'Pointer','crosshair');

while but == 1
    [ulc,ulr,but] = ginput(1);
end

set(edithandle,'Pointer','arrow');
edithandle = findobj(gcf,'Tag','ULR');
set(edithandle,'String',ulr);
edithandle = findobj(gcf,'Tag','ULC');
set(edithandle,'String',ulc);
C. Appendix 3

MATLAB codes: GETLOWERRIGHT

function [lrr,lrc] = GETLOWERRIGHT;

This function returns the value of the the lower right hand corner of the area being selected. Once the lower right corner button is actived on the text filem gui, the mouse pointer becomes a cross hair. The location is then selected by pressing the right mouse button.

lrr = [1];
lrc = [1];
but = [1];
edithandle = findobj(gcf,'Tag','Fig1');
    set(edithandle,'Pointer','crosshair');
    check = 1;
    while but == 1
        [lrc,lrr,but] = ginput(1);
    end
    set(edithandle,'Pointer','arrow');
edithandle = findobj(gcf,'Tag','LRR');
    set(edithandle,'String',lrr);
edithandle = findobj(gcf,'Tag','LRC');
    set(edithandle,'String',lrc);
D. Appendix 4

MATLAB codes: Calculations

---

[r,c] = size(raw_data); % determining the size of the matrix

just_fibril = zeros(r,c);

% % % % % % % % % % % % % % % % % % % % % % % % %

for n=1:c
    maximum(1,n) = max(raw_data(:,n));
end

for m=1:c
    for n=1:r
        if (raw_data(n,m)== maximum(1,m))
            loc_max(1,m) = n;
        end;
    end
end

for m=1:c
    [first_min(1,m),location_first_min(1,m)] = min(raw_data(1:(loc_max(1,m)),m));
    [second_min(1,m),location_second_min(1,m)] = min(raw_data(loc_max(1,m):r,m));
    location_second_min(1,m)=location_second_min(1,m)+loc_max(1,m)-1;
end
for m=1:c
    for n=1:r
        if (raw_data(n,m)== first_min(1,m))
            location_first_min(1,m) = n
        end;
    end
end

for m=1:c
    for n=1:r
        if (raw_data(n,m)== second_min(1,m))
            location_second_min(1,m) = n
        end;
    end
end

for m=1:c
    if ((raw_data(location_first_min(1,m),m)>= (raw_data(location_second_min(1,m),m))))
        correction = 0.5*(raw_data(location_first_min(1,m),m)-
                         raw_data(location_second_min(1,m),m))*location_second_min(1,m)*res...
                            + ((raw_data(location_second_min(1,m),m)))*(location_first_min(1,m)-
                            location_second_min(1,m))*res;
    else
        correction = 0.5*(raw_data(location_second_min(1,m),m)-
                         raw_data(location_first_min(1,m),m))*res...
                            + ((raw_data(location_first_min(1,m),m)))*(location_second_min(1,m)-
                            location_first_min(1,m))*res;
    end
end
(raw_data(location_first_min(1,m),m))*(location_first_min(1,m)-location_second_min(1,m))*res... + ((raw_data(location_first_min(1,m),m))*(location_first_min(1,m)-location_second_min(1,m))*res);
end;
area(1,m)=((sum(raw_data(location_first_min(1,m):location_second_min(1,m),m))*res)-correction);
width(1,m)=-(location_first_min(1,m)-location_second_min(1,m))*res;
end
x=1:r;
for m=1:c
    if ((location_first_min(1,m))<= (location_second_min(1,m)))
        just_fibrils(location_first_min(1,m):location_second_min(1,m),m) = raw_data(location_first_min(1,m):location_second_min(1,m),m);
    else
        just_fibrils(location_second_min(1,m):location_first_min(1,m),m) = raw_data(location_second_min(1,m):location_first_min(1,m),m);
    end;
plot(x,raw_data(:,m),location_first_min(1,m),raw_data(location_first_min(1,m),m),'or',location_second_min(1,m),raw_data(location_second_min(1,m),m),'og')
    pause(0.01)
end
height = area(:,:)./width(:,:);