NANOMECHANICAL PROPERTIES OF BIOCOMPOSITES USING
ATOMIC FORCE MICROSCOPY - MEASUREMENT AND
MODELING

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by

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ABSTRACT

The goal of this research was to measure, analyze, and simulate the macro- and nano-mechanical properties of the bio-composite structure of plant cell walls using nondestructive evaluation methods. Dimension ICON Atomic Force Microscopy (AFM) and Dynamic Mechanical Analysis (DMA) were utilized as measurement instruments. Onion and celery epidermal peels were used as test samples. High resolution images were taken of microfibril structure of cell walls. This research uncovered, for the first time, an unexpected architecture of intact plant cell wall utilizing extremely low force of the AFM tip in a liquid environment. The newly discovered features include [1] the presence of single fibers, strands of several fibers and large bundles of fibers; [2] the layering of the fiber bundles; [3] the changes in angle of orientation of the fibers between layers, for example, in celery the top layer has an angle of 42 to 50 degrees and the second layer has an angle of 111 to 117 degrees; [4] possible evidence of slight twisting of the bundles. These features are significant because they give insight into the architecture of cell wall structure. The statistical analyses were able to provide a detailed description and understanding of both mechanical properties and experimental data. The results showed that: 1) AFM was determined as an effective method for nondestructive evaluation of materials in nano scale level; 2) an innovative Peak Force Tapping technique in AFM was used to scan the cell walls using extreme low forces (≈200 picoN); 3) the AFM images showed the orientation of the microfibrils in the same cell were consistent, regardless of position of the cell; 4) it was determined that enzyme and buffer solutions had a negative influence on the stiffness and the strength of the cells' epidermis; 5) celery epidermis had comparable behavior to both
viscoelastic and elastoplastic modeling in the linear region; 6) a simulation Model showed the force change as a function of calculated displacement in the same direction for plant cell wall microfibrils, primarily in the first layer; 7) the changes in microfibrillar orientation had an significant role in the change of elastic properties; 8) in modulus of elasticity analysis, onion had the least coefficient of variation (23% or 77% equality), representing the highest consistency during the data collection; 9) the Young’s modulus of onion and celery ranged from 0.2-4.9 MPa and 5-13 MPa, respectively; and 10) the stiffness of onion and celery ranged from 91 to 197 N/m and from 98 to 525 N/m, respectively.
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I dedicate this work to my late father, Heshmatoallah, who passed away while I was far away from him busy with this work. May his soul rest in peace.
Chapter 1

Literature review

1.1 Plant Cell Wall

Plant cells are bound by thin, yet mechanically strong cell walls, containing structural proteins, enzymes, phenolic polymers, and other materials which transform chemical and physical characteristics (Cosgrove, 2006). Cell wall structures consist of a complex mixture of cellulose fiber, polysaccharides, and other polymers that are linked together by both covalent and non-covalent bonds. Cell walls glue the cells together to prevent them from sliding, and determine the mechanical strength of plants. They have many fundamental roles during the growth and development of a plant. This polymeric network (the cell wall) is enlarged by a process of stress relaxation and slipping (creep) of the polysaccharides. As seen in Figure 1.1, the plant cell wall structure consists of three different layers: the middle lamella, the primary cell wall, and plasma membrane. The middle lamella (plural lamellae) is a layer high in pectin, which forms the interface between adjacent plant cells and glues them together (Cosgrove, 2006). The primary cell wall is a thin, flexible and a fiberglass-like structure, with crystalline cellulose microfibrils embedded in a highly hydrated polysaccharide matrix (Cosgrove, 2005). The secondary cell wall also forms in some cells after expansion ceases which is a thick layer preventing collapse of the water-conducting cells during periods of high water tension due to high transpiration (Cosgrove, 2006).
Figure 1.1: Schematic model of plant cell wall that shows three different layers.
(Source: © United States Department of Energy Genome Programs/ genomics.energy.gov)
The Plasma membrane (cell membrane or plasmalemma) is a layer which controls the movement of substances in and out of cells, and contains a wide range of biological molecules, lipids, and primary proteins that are involved in cell adhesion and ion channel conductance (Alberts et al., 2002).

Cellulose, the most common biopolymer in nature, is part of a wide variety of living species including plants. Cellulose is the structural component of plant cell walls that acts as reinforcement material which has a high tensile strength, equivalent to steel (Cosgrove, 2006). Cellulose is a polysaccharide consisting of a linear chain of several hundreds to over ten thousand β (1, 4)-linked β-D-glucan units (Cosgrove, 2005). These parallel glucans form a crystalline microfibril that is chemically stable, insoluble, and relatively resistant to chemical and enzymatic attack (Cosgrove, 2005). These properties make cellulose an excellent candidate to build a strong and complex cell wall.

In addition to all these biological functions, the plant cell wall, specifically cellulose, is very important for human economics. The plant cell wall is used commercially in the form of paper, textile, fibers, charcoal, lumber, and many other wood products (Cosgrove, 2006). Also, structurally modified polysaccharides are often used to make synthetic fibers such as rayon, plastics, films, coatings, adhesives, gels, and many other products. A fundamental understanding of the cellulosic structure of plant cell walls will provide the insight needed to create a new generation of biorenewable nanocomposites with novel properties tailored to diverse applications.
1.2 Atomic Force Microscopy in Plant Cell Wall Microfibrils

The microfibrils in primary cell walls were previously examined by transmission electron microscopy (TEM), but there is little agreement in the literature about their diameters, which are reported to range from 2-3 nm (Roland, et al. 1975, Chanzy et al, 1979) to 13.5 nm (Fujino et al 2000). The major reason for these large differences can be because of the different preparation methods for TEM. Unfortunately this classic method is not appropriate for cell walls, due to the heavy metal staining of polysaccharides. Recently, another method called field emission scanning electron microscopy (FESEM) has been gaining in popularity (Carpita et al.2001, Sugimoto et al., 2002). FESEM combines the typical sample preparation of SEM with the ultrastructural magnification of TEM, but requires dehydration and critical point drying which cause artifacts (Marga et al., 2005).

Probe microscopy provides an alternative to electron microscopy for direct molecular imaging of plant cell wall polysaccharides and cell wall structure (Morris, et al). Specifically, Atomic Force Microscopy (AFM) is a non-destructive technique that presents comparable or even higher resolution (nanometer) images with less sample preparation than other techniques such as electron microscopy. The biological sample can be examined under conditions similar to those in-vivo, so the chance of introducing artifacts from sample preparation decreases significantly.

The first study of plant cells by AFM as a living systems was reported by Butt et al. (Butt et al., 1990). He stuck cut sections of plant leaves, such as Lagerstroemia subcostata, an Indian tree, to stainless steel discs and imaged them under water. High resolution images couldn’t
resolve features less than 200 nm, due to the presence of thick cuticle layers. In more detail, AFM was successfully used to visualize the ultrastructure of hydrated plant cell walls, such as prepared apple (*Malus pumila Mill; Cox orange pippin*), water chestnut (*Eleocharis dulcis L.*), potato (*Solanum tuberosum L.; Bintje*), and carrot (*Daucus carota L.; Amsterdamse bak*) parenchyma (Kirby et al. 1996). Samples were deposited on freshly cleaved mica and imaged in wet conditions. The size and orientation of the microfibrils studied by AFM in extracts of cell wall materials from hairs of corn (*Zea mays*) and radish (*Rhaphanus sativus*) are consistent with the data achieved by electron microscopy (EM) (van der Wel, et al.).

The AFM images only show the cellulose fibers of cell walls, and do not give information for other components, such as pectin or hemicellulose, which is supposed to link the cellulose fibrils (Morris et al., 2009). Microfibril images of both hydrated and partially hydrated celery (*Apium graveolence*) parenchyma cell were examined using AFM (Julian et al., 2000). Hydrated wall microfibril diameters are mostly in the range of 6-25 nm. However, the cellulose microfibrils’ diameters were found to be very dependent on the water content of the sample (Julian et al., 2000). Partially hydrated primary cell walls separated from onion (*Allium cepa L.*) and mouseear cress (*Arabidopsis thaliana Heynh*) were observed using AFM, and the fibrils diameter were found to be about 4.4±0.13 nm for onion, and 5.8±0.17 nm in A. thaliana (Lynette et al., 2003). The AFM images showed the interpenetrating networks (non-covalent bonded and non-soluble polymers) in which the stiffer and the thicker fibers come out brighter than the thinner fibers. Contrast in AFM images derives from a variation in the force between the tip and the sample. To avoid any altering to the structure of the cells, it is best to apply the lowest forces possible.
In addition to taking images of microfibrils, the study of the expansions of plant cell walls is another area of research interest. As discussed in the previous section, the primary wall is a polymeric network of crystalline cellulose microfibrils fixed in a hydrophilic matrix of hemicelluloses and pectins, which make long chains of polymers which form a cohesive network with non-covalent bonding (Cosgrove, 2000). Cellulose microfibrils are effectively inextensible with the tensile modulus greater than \(10^{11} \text{Nm}^{-2}\) (Cosgrove, 2000). A significant group of literature describes this growth as a result of a biochemical/biophysical “loosening” of the wall to permit turgor driven extension of the wall network, and cause the wall to become more plastic or extensible (Tiaz et al., 1984, Carpita et al, 1993, Gibeaut et al., 1993). It is believed that this wall plasticity is maintained by wall loosening enzymes, however more recent research points out that non-enzymatic protein expansin may also cause wall loosening (McQueen-Mason et al., 1992, Cosgrove, 2000). To model the rate of wall expansion the following constitutive equation has been used by other researchers (Veytsman et al., 1998, Lockhart, et al., 1965):

\[
    r = \phi(p - Y),
\]

where \(\phi\) is a yield rate coefficient or extensibility, \(p\) is the turgor, and \(Y\) is the yield threshold.

Plant cells enlarge preferentially in a single direction. Fre-Wyssling (1953) found that the cellulose microfibrils are aligned, on average, perpendicular to the direction of maximum expansion rate. The cellulose co-alignment gives the cell wall a mechanical anisotropy that translates into deformation anisotropy (Marga et al., 2005). The growing cell wall is
characterized as a multi-layer structure of parallel microfibrils tethered, or otherwise secured, by hemicelluloses, usually xyloglucan (Marga et al., 2005). Deep understanding of cell elongation requires a detailed understanding of the movement of cellulose microfibrils. To investigate this, the AFM can be a very useful technique to track the microfibrils with high resolution imaging of cell walls that have been slowly changing under constant tension (Cosgrove, 1989).

1.3 Mathematical Model

Reviews of the structure and composition of primary cell walls have been conducted by Carpita & Gibeau (1993) and McCann & Roberts (1994). The study hypothesized models of primary wall structures at the molecular scale; however the proposed models are conceptual rather than mathematical models. However, the complex structure of polysaccharides can be modeled mathematically with theories for matrix composites or entangled polymers (Bruece et al., 2003). This model allows several levels of structural hierarchy to be connected resulting in a description of cell wall behavior based on its polymeric nature summarized into a constitutive relation for a continuum material. Hettiaratchi et al. (1978) described the pressure-volume relation for pressurized spherical and cylindrical cells. Wu et al. (1985,1988) developed work based on the stress-strain relation for a polymeric material which was previously established in Wu et. al, (1979). In this model, two phases of cell expansion were accounted for; the first happening without the need for stressing the microfibrils and the second as a result of microfibril extension. Chaplin (1993) simplified this model by characterizing the elastic properties of the isotropic cell wall in terms of a general strain energy function. This function
can describe the nonlinear relation between pressure and volume in cell expansion. Veytsman & Cosgrove (1998) modeled plant cell wall extension by using concepts of thermodynamics of polymer mixtures (Veytsman et al., 1998). They showed that macroscopic properties of cell walls are a consequence of the microscopic properties of inter-penetrating networks of cellulose and hemicellulose. The determination of the material characteristics and properties of a cell wall are essential for a mathematical model to be verified. The Young’s modulus of the crystalline domain of microfibrils was measured by applying uniaxial tensile load along the fiber axis and monitoring the lattice deformation along the chain axis using X-ray diffraction (Nishiyama, 2009). The stress on each crystallite is considered to be the same as the stress applied to the macroscopic sample, and the value based on this assumption fall in the range of 115-140 GPa (Sakurada et. al. 1962, Sakurada et. al. 1966, Matsuo et. al., 1990, Nishino et. al., 1995, Ishikawa et. al., 1997).

As discussed previously, the plant cell wall structure consists of three different layers: the middle lamella, the primary cell wall, and plasma membrane. The middle lamella, also called plant cuticle, is a pectin layer which reinforces the cell walls of two adjoining cells. This layer can be assumed to be a waxy hydrophobic material. The plasma membrane could be neglected in our case. The primary cell wall will be simplified to the two groups of material such as cellulose microfibrils and hemicelluloses. Hemicelluloses bind, with pectin, to microfibrils to form a network of cross-linked fibers. In this project, the primary cell wall, in water, is simplified to include only the network of cross-linked fibers and hemicellulose protected by a layer of wax, or cuticle.
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Chapter 2

Intact Primary Cell Wall Imaging using AFM New Ultra-low Force Technique

Abstract

In order to progress the fundamental knowledge regarding the nano-composite structure of plant cell walls to the benefit of biofuel production, nondestructive quantitative techniques are needed. One such nano-quantitative technique is Atomic Force Microscopy (AFM) however, due to the sensitivity of these structures, such as microfibrils, conventional AFM procedures would alter the structure. To overcome this hurdle, a new AFM mode (PeakForce Tapping mode) which allows scanning of sample with extremely low forces is employed. This research uncovered, for the first time, an unexpected architecture of intact plant cell wall utilizing extremely low force of the AFM tip in a liquid environment. The newly discovered features include [1] the presence of single fibers, strands of several fivers and large bundles of fibers; [2] the layering of the fiber bundles; [3] the changes in angle of orientation of the fibers between layers, for example, in celery the top layer has an angle of 42 to 50 degrees and the second layer has an angle of 111 to 117 degrees; [4] possible evidence of slight twisting of the bundles. These features are significant because they give insight into the architecture of cell wall structure.
2.1 Introduction

As the influence of fossil fuel resources approaches an end, the need for economical, sustainable, and environment-friendly alternative fuels becomes essential. Ethanol from lignocellulosic biomass is one source of alternative energy which can substitute directly for its fossil fuel counterpart, namely gasoline. This liquid transportation fuel, from renewable lignocellulosic material, represents a safe source of supply, with limited conflict with land use for food and feed production, and lower fossil fuel inputs (Margeot et al., 2009).

The pretreatment of lignocellulosic materials is one of the major obstacles in the way of commercial scale production of ethanol. Pretreatment processing aims to break down the structure of the lignocellulosic matrix, increasing the accessible surface area through cellulose decrystallization, partial cellulose depolymerization, lignin solubilization, and the modification of the lignin structure (Margeot et al., 2009). Given the complex nature of the pretreatment procedure for a lignocellulosic feedstock in ethanol production, commercial production of ethanol from such sources has been difficult to achieve. Based on these difficulties, there is an important need to understand the polysaccharide nature of cellulose fibrils in plant cell walls which are insoluble, resistant to chemical attack, and cannot be readily fermented.

Plant cells are bound by thin, yet mechanically strong, cell walls containing structural proteins, enzymes, phenolic polymers, and other materials which transform their chemical and physical characteristics (Cosgrove, 2006). Cell wall structures consist of a complex mixture of cellulose fiber, polysaccharides, and other polymers that are linked together by both covalent and non-covalent bonds. Cell walls adhere the cells together to prevent them from sliding and
determine the mechanical strength of plants. They have many fundamental roles during the
growth and development of a plant. This polymeric network (the cell wall) is enlarged by a
process of stress relaxation and slipping (or creep) of the polysaccharides. The plant cell wall
structure consists of three different layers: the middle lamella, the primary cell wall, and plasma
membrane. The middle lamella (plural lamellae) is a layer high in pectin, which forms the
interface between adjacent plant cells and glues them together (Cosgrove, 2006). The primary
cell wall is a thin, flexible, and a fiberglass-like structure, with crystalline cellulose microfibrils
embedded in a highly hydrated polysaccharide matrix (Cosgrove, 2005). The secondary cell wall
also forms in some cells after expansion ceases and comprises a thick layer preventing collapse
of the water-conducting cells during periods of high water tension due to high transpiration
(Cosgrove, 2006). The Plasma membrane (cell membrane or plasmalemma) is a layer which
regulates the movement of substances in and out of cells, and contains a wide range of
biological molecules, lipids, and primary proteins that are involved in cell adhesion and ion
channel conductance (Alberts et al., 2002).

Cellulose, the most common biopolymer in nature, is present in a wide variety of living
species including plants. Cellulose is the structural component of plant cell walls that acts as a
reinforcement material which has a high tensile strength, equivalent to steel (Cosgrove, 2006).
Cellulose is a polysaccharide consisting of a linear chain of β (1, 4)-linked β-D-glucan units from
several hundred to over ten thousand units (Cosgrove, 2005). These parallel glucans form a
crystalline microfibril that is chemically stable, insoluble, and relatively resistant to chemical
and enzymatic attack (Cosgrove, 2005). These properties make cellulose an excellent candidate
to build a strong and complex cell wall.
In addition to the biological functions, the plant cell wall, specifically cellulose, is very important for human economics. The plant cell wall is used commercially in the form of paper, textiles, fibers, charcoal, lumber, and many other wood products (Cosgrove, 2006). Also, structurally modified polysaccharides are often used to make synthetic fibers such as rayon, plastics, films, coatings, adhesives, gels, and many other products. A fundamental understanding of the cellulosic structure of plant cell walls will provide the insight needed to create a new generation of biorenewable, nanocomposites with novel properties tailored to diverse applications.

The main goal of this work was to characterize the nano-biocomposite structure of the plant cell wall through studying the microfibrils. To achieve this goal, Atomic Force Microscopy (AFM) was selected as a unique method which used imaging and measurement techniques to study the microfibrils of cell walls. The following sections present the detailed information of the AFM technique and its importance as a nondestructive evaluation technique in nano-scale studies.

2.2 The Principal of Atomic Force Microscopy (AFM)

The first scanning probe microscope (SPM) was a scanning tunneling microscope (STM) of G. Binnig and H. Rohrer at IBM Zurich Research Laboratory in 1982, for which they received the Nobel prize in 1986. The operation of a STM is based on a tunneling current that starts to flow when a sharp metallic tip approaches a conducting surface at a distance of approximately 10Å (Binning, et al, 1986). The physical principle for this current in vacuum is the tunneling of electrons and it is extremely sensitive to changes in sample height. When the tip, which is
mounted on a piezoelectric tube, moves across the surface, the increasing and decreasing of this current is recorded. The tip allows tiny movements by applying a voltage at its electrodes. By this means, the electronics of the STM system control the tip position in such a way that the tunneling current, and therefore the tip-surface distance, is kept constant, while at the same time scanning a small area of the sample surface. This movement is recorded and can be displayed as an image of the surface topography. Under ideal conditions, individual atoms of a surface can be imaged.

The AFM was invented by Binnig, Quate and Geber in 1986, which is based on a combination of the principles of the scanning tunneling microscope (STM) and the stylus profilometer (Bining et al., 1986). Basically, a sharp tip of silicon or carbon is mounted on a cantilever spring and pulled across the surface of a sample, while a feedback system adjusts the distance between the sample and the probe tip to maintain a constant deflection of the cantilever as it moves over the sample. Typical forces between tip and sample vary from $10^{-11}$ to $10^{-6}$ N. The force necessary to move the cantilever through a minimum distance can be as small as $10^{-18}$ N, so the distance discernable between sample and the cantilever tip can be as small as to $10^4$ Å. Hence, non-destructive imaging is possible with these small forces (Meyer, et al, 1990). The surface contour is determined by monitoring the signals in the feedback loop. The measured cantilever deflection is used by a computer to create a map of surface topography.

As seen in Figure 2.1, the standard AFM consists of five main components; a tip connected to a cantilever, a piezoelectric tube, a position sensitive photo detector, an optical lever system, and a feedback mechanism. The deflection sensor, which is in the scanner-head,
monitors the bending, or deflection, of the cantilever. The scanner can position the cantilever up or down with the purpose of maintaining a constant deflection. This movement of the scanner matches the surface topography and therefore be used to create an image of the surface. The deflection sensor sends deflection signals to the feedback electronics. Hence, the deflection signal is compared to a reference signal, and an error signal is generated, which is used to generate a feedback signal. This feedback signal, which is sent to the piezoelectric scanner, causes the scanner to extend, so raising and lowering the probe to compensate.

Figure 2.1: Basic AFM set up
The cantilever, which is a thin, flexible beam, holds the tip to track its movement over the sample surface and deflects in the same direction in which the topography changes. AFM cantilevers generally have spring constants of about 0.1 N/m. A high flexibility stylus exerts downward forces on the sample, resulting in less distortion and damage while scanning. The cantilevers and tips are typically made from silicon, silicon nitride, or diamond. The reflected laser beam hits a position-sensitive photo-detector (PSPD) consisting of a four-segment photodetector. The differences between the segments of photo-detector of signals indicate the position of the laser spot on the detector and, consequently, the angular deflections of the cantilever. Optical lever detectors are the most common monitoring systems in AFM, and involve a focused laser beam on the end of the cantilever, directly over the tip, reflecting from a mirror to the PSPD. As seen in Figure 2.1, the PSPD has four sections to monitor the movement of the tip and the laser intensity. The photo diode is sensitive to changes in the tip movement on the atomic scale. The difference between the two top segments (1 and 2) and the bottom segments (3 and 4) produces an electrical signal which signifies the vertical motion of the tip. Also, the difference between the left (1 and 3) and the right segments (2 and 4) records lateral or torsional movements of the tip (Morris et al, 1999).

The feedback loop is another main component of an AFM, which is an electrical system to hold the force constant during the movement of the tip across the sample. This control mechanism is important to create an image and the system uses the feedback loop to keep constant the force or cantilever deflection. If the tip has more, or less, interaction force across
the sample, the piezoelectric tube responds by expanding or contracting to maintain the preset constant force between the tip and the sample.

Generally speaking, the tip interacts with the sample surface, while the piezoelectric tube adjusts its movement in all x, y and z direction. The PSPD monitors the cantilever’s movement by detecting the laser position. The resulting map in the x-y direction constructs the topography image of the surface with sub-angstrom resolution.

### 2.2.1 AFM Modes of Operation

There are several modes of operation developed for the AFM that can monitor surface properties of the sample. The primary modes of operation are static and dynamic modes that will be discussed in detail in the following sections. These different imaging modes work by measuring the interaction between the tip and the sample and lead to the topography of the surface.

#### 2.2.1.1 Contact Mode

In the contact mode, also known as repulsive mode, the probe is dragged across the sample with an operation distance of less than a few angstroms. As the scanner gently traces the tip across the sample, the repulsive force causes the cantilever to bend in response to changes in topography. Because the tip is in hard contact with the surface, the stiffness of the lever needs to be less than the effective spring constant holding atoms together (Howland et al, 1996). Most contact mode levers have a spring constant of < 1N/m.
Figure 2.2 shows different force regimes which correspond to interatomic forces. As seen in this figure, the atoms are separated by a large distance on the right side of the curve. As the atoms are brought to less than a few angstroms apart, they attract each other. This attraction increases until they come so close together that their electrons begin to keep away from each other electrostatically. This repulsive electrostatic force opposes the attractive force as the interatomic separation decreases. When the distance between the atoms of the tip and those of the sample reaches a few of angstroms, which is about the length of a chemical bond, the force goes to zero. Therefore, the repulsive van der Waals force balances any force that tries to push the atoms closer together, which, in terms of an AFM, means that when the cantilever pushes the tip against the sample surface, the cantilever bends instead of forcing the tip atoms closer to the sample atoms (Howland et al, 1996). In contact mode AFM, the piezoelectric element and feedback loop control the tip deflection by holding the repulsive force constant. There are also two other methods to detect the cantilever deflection which are: constant-force mode and constant-height mode. In the constant-force mode, the speed of scanning is limited by the response time of the feedback circuitry. However, in the constant-height mode, generating topographic data is based on a fixed scanner height during scanning.

In addition to the van der Waals force explained above, there are two other forces which are important in AFM: (1) the capillary force which results from the thin layer of water present in ambient environment (around $10^{-3}$ N) and (2) the force which the cantilever exerts on the sample. The capillary force begins when water around the tip holds the tip in contact with the sample, applying a strong attractive force which depends on the tip-to-sample separation. During contact mode, this capillary force should be constant. The variable force in the contact
mode is the force exerted by the cantilever, which is as analogous to the force of a compressed spring. The total force that the tip exerts on the surface is the sum of the forces exerted by the cantilever and the capillary force, which should be balanced by the repulsive van der Waals force in the contact mode. The magnitude of this total force is in the range between $10^{-8}$ to $10^{-6}$ N (Howland et al, 1996).

### 2.2.1.2 Non-Contact Mode

To reduce the damage to biological specimens associated with the contact mode, several vibrating techniques are utilized in AFM including non-contact or tapping modes. In non-contact AFM, the system vibrates a stiff cantilever near to its resonant frequency, which is typically from 100 to 400 kHz, with the spacing between the sample (can be seen in the van der Waals curve, Figure 2.2) on the order of tens to hundred of angstroms. The resonance frequency and amplitude of the oscillating probe decreases as the sample surface is approached due to interactions with van der Waals and other long-range forces extending above the surface. These types of forces tend to be quite small (about $10^{-12}$ N) relative to the repulsive forces in the contact mode. This low force is advantageous for the study of soft or elastic samples such as biological cells and does not let the surface become contaminated by contact with the tip. The stiffer cantilevers used to avoid being pulled down to the surface by the attractive forces and weak forces affecting feedback causes the non-contact AFM signal to be small, which can lead to unstable feedback and require slower scan speeds than either contact mode or tapping mode. The resonant frequency of a cantilever changes with the square root of its spring constant. The spring constant varies with the force gradient qualified by the
cantilever. Furthermore, the force gradient used in the derivation of the force distance curve (seen Figure 2.16) varies with tip-to-sample separation (Howland et al, 1996). The feedback system in non-contact AFM keeps the resonant frequency, or vibrational amplitude, of the cantilever and tip-to-sample distance constant to generate the sample topography. In the case of imaging of rigid samples, the result from contact and non-contact mode may be the same. However, the presence of monolayers of water covering the sample surface would cause the images to look completely different. The AFM, operating in contact mode, will go through the water layers to the sample, but an AFM in non-contact mode will image the surface of the liquid layer.

### 2.2.1.3 Intermittent-Contact Mode

Intermittent-Contact, or tapping, mode is another use of dynamic AFM. In the tapping mode, the cantilever vibrates the tip close to its first bending mode resonance frequency, as in non-contact mode. However, the oscillation amplitude of the probe tip is typically much larger than used in the non-contact mode, often in the range of 20 nm to 200 nm. In addition, the tip is permitted to slightly contact, or tap, the sample for a short duration during each oscillation cycle. As the tip moves toward the sample, the tip-sample interactions change the amplitude, resonance frequency, and phase angle of the oscillating cantilever. Tapping mode is a more effective means for imaging in air, particularly for soft samples, as the resolution is similar to the contact mode but with lower forces applied to the samples which mean a less destructive process. There are, however, two disadvantages of the tapping mode compared to the contact mode; (1) slightly slower scan speed and (2) more complex AFM operation. In general, tapping-
mode AFM overcomes some of the limitations of both contact and non-contact AFM and becomes an important technique with improvements to lateral resolution in soft samples.

Figure 2.2: Inter atomic force vs. distance curve.
2.2.1.4 Peak Force Tapping Mode

In Peak Force Tapping® (PFT) mode, the probe and sample are intermittently brought together (similar to Tapping Mode) to contact the surface quickly, which eliminates lateral forces. Both normal forces and lateral forces exerted by the tip can cause damage to the sample and increase the contact area resulting in scan resolution reduction. Unlike Tapping Mode, where the feedback loop keeps the cantilever vibration amplitude constant, Peak Force Tapping® controls the maximum force (Peak Force) on the tip, and protects the tip and sample from damage by decreasing the contact area (Su et al, 2010). The level of force control in PFT® can be in the range of pN, even when scanning in liquid environments. The two biggest challenges of force control in a liquid environment are nonlinear deflection variations and viscous forces when the tip and sample are not in contact. To eliminate this problem, the PFT® mode uses the feedback to maintain a constant peak force for each tap with the force range from pN to μN, depending on the application. This makes the PFT® mode significantly applicable to the imaging and measurement of plant cell walls, which are naturally sensitive to tip movement and associated damage to the structure.

2.2.1.4.1 Peak Force QNM

Peak Force Quantitative Nano-mechanical Mapping (Peak Force QNM)® is a new AFM mode (Su et al, 2010) that uses tapping mode technology to record very fast force response curves at every pixel in the image, and uses the peak tip-sample interaction force as the feedback mechanism. Peak Force QNM® is able to simultaneously obtain quantitative modulus, adhesion, dissipation, and deformation data while imaging topography at high resolution. Also, by maintaining control of direct force to a very low level (pN), the scanning can limit
indentation depths to deliver a non-destructive and high-resolution imaging technique to sensitive samples. Furthermore, material properties can be characterized over a very wide range to address samples in many different research areas (Su, et al, 2010). Figure 2.3 shows a force curve and the parameters that can be obtained from it. The adhesion force represents the minimum force point when the tip starts to pull away from the sample. Energy dissipation is calculated by the hysteresis area between approaching and retracting processes. This dissipation includes the work associated with adhesion and viscous and plastic deformations. When the set point of the peak force is set at, or close to, zero, the energy dissipation is dominated by the work of adhesion. Deformations here represent the total penetration depth, including elastic and plastic deformations (Su, et al, 2010). Figure 2.3 shows the force vs. separation curve instead of the force vs. distance curve. This is because of fitting purposes where the separation is calculated from the piezo position in the Z direction and the cantilever deflection (Su, et al, 2010).
Figure 2.3: AFM Force curve diagram and information that can be obtained from the curve (Su, et al, 2010 with permission from Veeco®).
2.2.2 Force Distance Curve

Force-distance curves have become a fundamental tool for the study of material properties and characterization of different known surface forces since 1989 (Cappella, et al, 1999). Force-distance curves have been used in several measurements such as the determination of; Hamaker constants, surface charge density, elasticity, and degrees of hydrophobicity (Cappella, et al, 1999). The first study of force-distance curves acquired with an AFM focused on the surface forces on LiF and graphite (Meyer, et al, 1988). When acquiring force-distance curves, the piezo element must ramp along the z-axis, which is the axis perpendicular to the surface. There are two principles modes for the acquisition of force-distance curves; static mode and non-contact mode. In the static mode, the sample is displaced along the z-axis in separate steps and the variations in cantilever deflection are collected. In the non-contact mode, the cantilever is vibrated by an additional, external piezoelectric transducer while the sample is approached and the amplitude, or the resonance frequencies, of the cantilever oscillations are collected as a function of tip-sample distance (Cappella, et al, 1999).

In 1991, numerous studies of force-distance curves in liquid were performed in order to measure the adhesion force by Mizes et al. Furthermore, in 1994, other techniques were introduced using functionalized tips (tip covers) with particular molecules that adhere to one another in order to study interaction forces between specific materials by means of force-distance curves (Butt, et al, 2005).

The result of a force-distance determination is a measure of the cantilever deflection, $Z_c$, versus the position of the piezo, $Z_p$, normal to the surface. To acquire a force-distance
curve, $Z_c$ and $Z_p$ must first be converted into force and distance. The force $F$ can be obtained from Hook’s law as in Equation 2.1:

$$ F = k_c Z_c $$  \hspace{1cm} \text{Equation 2.1}

Where $k_c$ is the spring constant of the cantilever. The tip-sample separation, $D$ is calculated by adding the deflection to the position, which is called ‘distance’ (see Figure 2.4)

$$ D = Z_p + Z_c $$  \hspace{1cm} \text{Equation 2.2}

The deflection of the cantilever is measured using optical lever techniques (Meyer, et al, 1990). A beam from a laser diode is focused to the end of the cantilever, which is gold coated on the backside to reflect the beam towards the detector. The position of the reflected beam is monitored by a position-sensitive detector. The cantilever bends while the force is applied and the reflected light beam moves through an angle equal to twice the change in end-slope $d Z_c / d X$ (Butt, et al, 2005), which is given by following equation:

$$ \frac{d Z_c}{d X} = \frac{6 F L^2}{E w t_c^3} $$  \hspace{1cm} \text{Equation 2.3}

For a cantilever with a rectangular cross-section defined by width, $W$, length, $L$, and the thickness, $t_c$; with $E$ as the Young’s modulus of the cantilever material and $F$ is the force applied to the end of the cantilever; the deflection of the cantilever is given by:

$$ Z_c = \frac{4 F L^3}{E w t_c^3} = \frac{2}{3} L \frac{d Z_c}{d X} $$  \hspace{1cm} \text{Equation 2.4}
Both approach and withdrawal force-distance curves can be generally divided into three regions; the contact line, the non-contact region, and the zero line. Zero lines are obtained when the tip is far away from the sample and the cantilever deflection is zero. The zero lines, when working in a liquid, can give information about the viscosity of the specific liquid. When the sample is pushed against the cantilever and the tip is in contact with the sample the corresponding lines in the force-distance curve are called the “contact lines,” which provide the information about stiffness and elastic modulus of the sample. The most interesting regions of the force-distance curve, which are related to this research, are the non-contact regions. These triangular regions contain two main parts; the jump-to-contact and jump-off-contact. The non-contact region is the approach curve which can give information about attractive or repulsive forces before contacting the sample. The maximum value of the attractive force sampled prior to contact is equal to the pull-on force, which is the product of jump-to-contact cantilever deflection and \( k_c \) (Cappella, et al, 1999). The non-contact region in withdrawal curves contains the jump-off-contact and the pull-off force, which is the product of jump-off-contact cantilever deflection and \( k_c \) equal to the adhesion force \( F_{adh} \). Understanding of the relation between the tip and sample surface energies requires evaluating of the deformations and contact area of the sample, which are addressed by several theories.
2.2.2.1 Theories of Contact Region

The first theory regarding the contact region dates back to Hertz, who considered the tip as a smooth elastic sphere and the sample as a rigid, flat surface (Hertz et al, 1881). Based on this theory, adhesion force and surface forces are not taken into account, so the AFM experiment can follow the Hertz’s theory only in the limit of high loads or low surface forces. Hertz’s theory cannot be used to calculate sample deformations by assuming a relatively rigid tip, as in the case of the biological specimens of interest to this study.

When a rigid, spherical tip applied to an elastic surface is considered, Sneddon theory has to be engaged (Cappella et al, 1991). The force, $F$, exerted by the tip on the surface and the surface deformation, $\delta$, are given by:
\[ F = \frac{3}{8} K \left( a^2 + R^2 \right) \ln \left( \frac{R + a}{R - a} \right) - 2aR \]  
Equation 2.5

\[ \delta = \frac{1}{2} a \ln \left( \frac{R + a}{R - a} \right) \]  
Equation 2.6

Where, \( K \), is the reduced Young’s modulus, \( a \) is the contact radius, and \( R \) is the sphere radius.

General speaking, Hertz and Sneddon deformations, which are tip and sample deformations, can be used to calculate the total deformation when the surface forces are insignificant to an AFM measurement. There are three theories which take into account the effect of surface energy on the contact deformation, which are discussed below.

Bradley’s analysis considers two rigid spheres interacting through a Lennard-Jones potential with the total force between the spheres given by Equation 2.7 (Cappella et al, 1999):

\[ F(z) = \frac{8\pi W R}{3} \left[ \frac{1}{4} \left( \frac{z}{z_0} \right)^{-8} - \left( \frac{z}{z_0} \right)^{-3} \right] \]  
Equation 2.7

\[ R = \left( \frac{1}{R_1} + \frac{1}{R_2} \right)^{-1} \]

Where, \( z_0 \) is the equilibrium separation, \( R \) is the reduced radius of the spheres, and \( W \) is the adhesion work at contact.

In the Derjaguin- Muller- Toporov (DMT) theory, the external load, \( F \), and the forces acting between the two bodies outside the contact region are considered (Derjaguin et al, 1975). The DMT theory is applicable for systems which have low adhesion and small tip radii.
\[ F_{\text{adh}} = 2\pi RW \]

Equation 2.8

\[ a = \frac{1}{3} \left( F + 2\pi RW \right) \frac{R}{K} \]

Equation 2.9

\[ a_0 = \frac{1}{3} \sqrt{\frac{2\pi W}{K}} R^2 \]

Equation 2.10

\[ \delta = \frac{a^3}{R} \]

Equation 2.11

Where, \( a_0 \) is the contact radius at zero load, \( \delta \) is the deformation of the spherical tip, and \( K \) is the reduced Young’s modulus.

The Johnson-Kendall-Roberts (JKR) theory, is suitable for highly adhesive systems with low stiffness and large tip radii, ignores long range forces outside the contact area, and considers only short range forces inside the contact region. For the JKR theory, the corresponding equations are:

\[ F_{\text{adh}} = \frac{3}{2} \pi RW \]

Equation 2.12

\[ a = \frac{3}{2} \left[ \frac{R}{K} \left[ F + 3\pi RW + \sqrt{6\pi W F + (3\pi RW)^2} \right] \right] \]

Equation 2.13
In summary, Hertz’s model neglects the adhesion of the sample, while the other two theories consider adhesion outside (DMT) and inside (JKR) the contact area. Thus, Hertz’s theory can be applied only if the adhesion force is much smaller than the maximum load. In the DMT and JKR theories the work of adhesion (W) can be measured from the jump-off-contact, if the tip radius (R) is known (Butt et al, 2005). The JKR theory can be applied if the tip is large and the sample is soft with large adhesion and the DMT theory is applicable in the case of small tips and stiff samples with small adhesion (Table 2. 2).

Another theory, called Maugis’s theory, can describe the transition between DMT and JKR models successfully. Maugis theory is considered the most precise and complete theory applicable to all materials, from large rigid spheres with high surface energies to small compliant bodies with low surface energies. In the Maugis theory, adhesion is considered as a constant, additional stress over an annual region around the contact area. In summary, all theories which have been described are continuum elastic theories and, thus, assume smooth surfaces with no plastic deformation or viscoelastic phenomena (Cappella et al, 1999).
Table 2.2: Relation between the sample deformation $\delta$, the contact radius $a$, and adhesion force $F_{\text{adh}}$ for a spherical tip on a flat solid sample based on Hertz, JKR, and DMT theories.

<table>
<thead>
<tr>
<th>Theory</th>
<th>$a$</th>
<th>$\delta$</th>
<th>$F_{\text{adh}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hertz</td>
<td>$\frac{\sqrt{RF}}{K}$</td>
<td>$\frac{a^2}{R} = \left(\frac{F^2}{RK^2}\right)^{\frac{1}{2}}$</td>
<td>0</td>
</tr>
<tr>
<td>DMT</td>
<td>$\frac{R}{K} (F + 2\piRW)$</td>
<td>$\frac{a^2}{R} = \frac{(F + 2\piRW)^{\frac{2}{3}}}{\sqrt{RK^2}}$</td>
<td>$2\piRW$</td>
</tr>
<tr>
<td>JKR</td>
<td>$\frac{R}{K} \left(F + 3\piRW + \sqrt{6\piRF} + (3\piRW)^{2}\right)$</td>
<td>$\frac{a^2}{R} = \frac{2}{3} \frac{6\piWa}{K}$</td>
<td>$\frac{3\piRW}{2}$</td>
</tr>
</tbody>
</table>

2.2.2.2 The Zero Line

The remaining part of the force-distance curve, which shows no tip deflection, is called the zero line. The zero line corresponds to the section where the tip does not exert any force on the sample and the tip and sample are at a given distance. Though the force cannot be determined in this portion, the zero line has the great advantage that all distances are referenced to the cantilever’s rest position. Accordingly, the forces can only be measured when the deflection of the cantilever, i.e. the difference between the current deflection and the rest position, is known (Cappella et al, 1999). Furthermore, the zero lines exhibit a kind of hysteresis that results in a separation of the approach and withdrawal traces. The hysteresis of zero lines
occurs due to the viscosity of the medium, where the viscous forces pull the cantilever upward when approaching the sample and cause it to twist downward as the sample is withdrawn.

2.2.2.3 Theories of Non-Contact Region

As previously mentioned, the force-distance curve includes two parts; an approach curve and a withdrawal, or retraction, curve. The approach curve, which is also called jump-to-contact, occurs when the gradient of the tip-sample force is larger than the elastic constant of the cantilever. The jump-to-contact may result from a region of attractive forces (Van der Waals or Coulomb) or repulsive forces (Van der Waals force in liquids, double-layer, hydration, and the steric) (Cappella et al, 1999). Thus, this region gives information about attraction forces between the tip and the sample. The maximum value of the attraction force is equal to the jump-to-contact cantilever deflection times the cantilever constant. This part can be predicted by any theory which includes attraction forces, such as JKR or Maugis. Pethica and Sutton (Pethica et al, 1988) have demonstrated the jump-to-contact instability, which is caused by the inherent stiffness of the tip and sample materials. The instability can be predicted by employing Lennard-Jones potentials or using molecular dynamics (MD) simulations. It occurs when, at some small enough separation (\(\approx 1-2\text{Å}\)), the gradient of the surface forces exceed the gradient of the elastic restoring force of the bodies (Cappella et al, 1999). Also, the MD simulation by Landman et al. show the onset of instability when the tip is at a distance of 4.2Å from the sample. When the tip jumps to the surface, the distance decreases from 4.2 to 2.1Å, after which and in addition to the adhesive contact forces between the two surfaces, a partial wetting of the tip bottom by gold substrate atoms induced by adhesion is observed (Cappella et al, 1999).
When the cantilever’s elastic constant is larger than the gradient of tip-sample adhesive forces the jump-off contact occurs during the withdrawal of the sample, or during retraction. The jump-off contact is related to the tip and the sample surface energies through equations that depend on the material’s dimensions, stiffness, and adhesion. The jump-off-contact deflection and the jump-off-contact distance are always greater than the jump-to-contact deflection and the jump-to-contact distance. This happens because; (1) during contact some chemical, or adhesive, bonds may cause non-conservative forces, (2) the sample deforms elastically around the tip, thereby increasing the contact area, and (3) meniscus forces exerted by layers of liquid contaminates act in opposition to the pull-off (Cappella et al, 1999).

In 1995, Agrit et al. measured forces between a gold tip and a gold substrate in vacuum at liquid helium temperature shows that necks are formed during jump-to-contact and jump-off-contact and that those necks elongate during the loading or unloading process. The dependencies of pull-off force and adhesion energy on the loading force have been measured in air and in water (Cappella et al, 1999).

As already discussed, in addition of using AFM as a high resolution imaging tool, results from force-distance curves can be used. This curve provides valuable mechanical property information obtained via recording of binding differences between the AFM tip and the sample. This chapter focused on use of AFM to take images of intact cellulose microfibrils of different plant cell walls. The test samples for AFM imaging were onion and celery. For the first time in AFM technology, PeakForce QNM, a newly developed imaging method, was used to reveal the structure of wall.
2.2 Materials and methods

One of the challenges in extending the AFM technique to the imaging of biological cells was using an intact cell wall, considered as an open structure, for imaging of microfibrils plant cell wall. In order to achieve this goal, the onion and celery epidermal peels’ primary cell walls were used. The benefit of using onion and celery epidermis was that they required simple sample preparations. The onion and celery epidermis were bathed for 1 hour in 1x PBS (Phosphate Buffered Saline) solution with 0.05% Tween 20. The epidermal peels were rinsed in 1x PBS. Due to significant amount of Pectin in celery cell wall, the epidermal tissues required bathing for an additional than four to six hours.

2.2.3 Tip Calibration using Thermal Tuning Techniques

Spring Constant Calibration

During the movement of the cantilever, due to the forces acting on it, the optical lever performs the measurement through reflecting a laser off to the top of cantilever and towards a photo-detector at the cantilever’s free end over certain time period. In the most Contact Mode and Tapping Mode applications, the cantilever deflection is small, which indicates an elastic behavior of the force and cantilever free end position and is defined by Hooke’s law as:

\[ F = -k \times h \]  

Equation 2.16
Where: $F$ is the force on the tip (N),
h is the vertical displacement of the cantilever (m), and
$k$ is the spring constant, which is a property of the cantilever and is the same number in air, water, and vacuum ($N/m$ or in nanoscale work $picoN/pico m$).

Knowing $k$, the measured cantilever deflections can be converted to an inferred tip/sample force, thus, the initial step for an accurate force determination is to find the $k$ of the cantilever.

There are several techniques to measure a cantilever spring constant including: thermal tuning (most preferred method due to less time consuming and accuracy), geometry, comparison, and hydrodynamic model.

**Measuring Thermal Noise**

While the cantilever is away from the sample, the measurement data consists of the time interval of the cantilever deflection signal in contact mode at the thermal equilibrium.

During the sampling, the cantilever is impacted by the Brownian motion of surrounding molecules. The power associated with resonance is obtained from integrating the area under the resonant peak curve. The dynamics of the cantilever can be expressed as an oscillator by the systems’ total energy, which the value for both potential and kinetic energy is defined as:

$$\frac{1}{2} k_B T$$

Where $T$ is the temperature (Kelvin) and

$k_B$ is Boltzmann’s constant (equal to $1.3805 \times 10^{-23}$ joules/Kelvin).
The potential energy can also define as the following:

\[ < \frac{1}{2} m \omega_0^2 z^2 > = \frac{1}{2} k_B T \]

Where \( \omega_0 = \sqrt{\frac{k}{m}} \) is the resonant angular frequency, \( m \) is the effective mass, and \( z \) is the displacement.

The cantilever spring constant is measured by simplifying the temperature and average displacement as:

\[ k = \frac{(k_B T)}{z^2} = \frac{(k_B T)}{P} \]

By integration of the area under the resonance in Power Spectral Density (PSD), and excluding the noise floor and shoulders to either side of the resonance peak, the power can be measured.

When measuring the cantilever spring constant by the thermal method, the accuracy highly depends on the isolation of nonthermal noise and the accuracy of the PSD and its magnitude. The typical nanoscope V controller sample deflection produces a signal every 16.5 seconds, which corresponds to the 64 kHz sampling rate. Accordingly, cantilever resonances with frequencies above 32 KHz can produce distorted to Power Spectral Density.

The thermal tuning method is mostly applicable to soft cantilever, mainly in fluids where cantilever resonance frequencies are significantly lower in air. The cantilevers can generate a smaller signal to analyze due to the magnitude of the PSD is proportional to the mean cantilever displacement.
Calibrating a Cantilever

Measurement of Deflection Sensitivity in Force Mode

Every time the laser beam path changes, the deflection sensitivity must be recalculated due to laser alignment, photodetector adjustment and probe change. The deflection sensitivity calibration is done on the hard and stiff surface.

Below are the procedures for deflection sensitivity measurement:

Engage on the hard surface, like silicon or glass, then switch to ramp mode. Obtain a force diagram, which represent the interaction with the hard surface, then display deflection error vs. Z sensor drag the ramp mode, or force mode, in from the left and/or right edge and displace as far as possible while they are still in the contact region of the force diagram. Figure 2.5 shows the tip deflection error vs. Z sensor on a glass slide in water. The update sensitivity was calculated using software and repeated for five times to calculate the average number for the deflection sensitivity.

The deflection sensitivity is measured based on the assumption of that tip and the test sample do not deform. During the process of measuring the deflection sensitivity, the static force is applied to the end of the cantilever, but during the thermal energy measurement, the cantilever is oscillating at resonance at only one end instead of two. Every time the laser beam path changes, the deflection sensitivity must be recalculated due to change in laser alignment, photodetector adjustment, and probe change. The deflection sensitivity calibration is done on a hard and stiff surface.

Below are the procedures for deflection sensitivity measurement:
Engage the hard surface, like silicon or glass, then switch to ramp mode. Obtain a force diagram which represents the interaction with the hard surface and then display deflection error vs. Z sensor drag during the ramp mode, or force mode, in from the left and/or right edge and displaced as far as possible, while they are still in contact region of the force diagram.

Figure 2.5: Deflection sensitivity determination using force mode in liquid (water).
Measurement of Cantilever Spring Constant by Thermal Tune

The following is the procedures of determining cantilever constant:

1- To choose the least square fit the data click on LORENTZIAN (AIR) or SIMPLE HARMONIC OSCILLATOR (FLUID).

The following equation (Simple Harmonic Oscillator) is used to fit the filtered data

\[ A(\nu) = A_0 + A_{DC} \cdot \frac{\nu_0^2}{\sqrt{(\nu_0^2 - \nu^2)^2 + \frac{\nu_0^2}{Q^2} \nu^2}} \]

Where: \( A(\nu) \) is the amplitude as a function of frequency (\( \nu \)), \( A_0 \) is the base line amplitude, \( A_{DC} \) is the amplitude at DC with zero frequency, \( \nu_0 \) is the center frequency of the resonant peak, and \( Q \) is the quality factor.

Normally, the markers are located where the spectrum rises from the noise floor. The curve fit is not sensitive to the minimal power contributed from these frequencies, which make the precise placement unnecessary. As shown in Figure 2.6, the curve fit along with the acquired data displayed (in red). In order to make sure the data is consistent with the best curve fit at the thermal peak, the marker position was adjusted, then the cantilever temperature and calculated spring constant (K) were entered.

New, sharp ScanAsyst Fluid+ probes were used in this research which had the combination of sharpness of silicon tip with low spring constant and high sensitivity of silicon Nitride
cantilever necessary for this application. This test was conducted for an unprecedented level of high resolution and force control on samples in fluid. The tip height is about 2.5 - 8.0µm, with the radius of about 2-3 nm. The spring constant is between 0.35-1 N/m.

Figure 2.6 : The thermal tune panel
2.3 Results and Discussion

High resolution imaging of microfibrils in liquids, such as water and different chemical buffers, is very difficult with any kind of microscopy, let alone with AFM techniques. However, one important advantage of using AFM is that the sample does not require the preparations or fixations common to other techniques that can cause unrealistic changes in the properties of cellulose microfibrils. Due to the sensitivity of microfibrils to the AFM tip contact during scanning, applying a very low range of force (≈200 picoNewton) is a requirement which cannot be achieved by standard AFMs or any other microscopic techniques. To overcome this difficulty, a new mode (PeakForce Tapping mode) was added to the Dimension ICON AFM which allows for scanning a sample with extremely low forces. In a fluid environment, Peak Force Tapping is significantly more stable and reliable than the traditional tapping mode. This is because there is no need to operate at the cantilever’s resonance frequency, which is notoriously unstable in a fluid.

2.3.1 Onion Microfibril Characterization

As previously explained, the onion epidermis was bathed for 1 hour in 1x PBS (Phosphate Buffered Saline) solution with 0.05% detergent called Tween 20 to remove the proteins and make an accessible structure for AFM tip to scan the plant cell wall’s microfibrils. Before start the experiment, the sample was washed for four to six hours. Based on experience, it was determined that washing onion epidermal peel for a longer period of time did not significantly increase the image resolution. One of the main challenges of taking images is
finding an appropriate position on the sample. The sides of sample were glued to clean, glass sheets and the internal portion remained intact and free.

As already stated, Peak Force Tapping method was used in this study as a quantitative nano-mechanical method using ultra low forces (picoNewton) for tapping the cantilever during the scanning process. This method allowed nondestructive indentation for scanning of sensitive, intact samples in water such as plant cell wall microfibrils. As explained in the tip calibration section, sharp Scan Asyst Fluid + tips were used in this study with a radius of curvature of approximately 2nm. In order to remove the residue from the scanning, the tip was washed after each set of experiments by dipping in a solution of distilled water and 90% ethanol.

As shows in Figure 2.7, the top image shows the optical microscopy of the onion epidermis sample and the position of the AFM tip during scanning. In the bottom image, microfibrils were clearly visible with individual fibrils arranged in parallel with approximate diameter of 5 nm to 13 nm. For the first time in this area of AFM research, high resolution images were captured with a very small force (500 pN), and while intact in water. Figure 2.8 shows a 200 nm image of microfibrils with thickness of 12.7 nm and a radius of 5 nm.
Figure 2.7: 1µm scan of the Onion fibrils, performed with Peak Force Tapping mode in fluid, with Dimension ICON.
Figure 2.8: Individual fibrils are clearly visible and vary in size, but typically measure 5-12nm in diameter. The graph shows the profile corresponding to the oblique line in the image.

Another set of experiments were designed to measure the nano-mechanical properties of individual onion microfibrils. Toward this goal, the quantitative mechanical measurement capability of the AFM was used to measure the relative mechanical modulus, adhesion, deformation, and energy dissipation of the onion microfibrils at the nanometer scale. Using the Peak Force QNM AFM technique, quantitative images of the onion epidermis microfibrils were obtained with the mechanical modulus, adhesion, and deformation providing the contrast for the images. The peak Force QNM AFM technique not only measures the mechanical properties of the plant cell wall interiors, but also provides remarkable textures to the microfibrils which is unseen in the topographical image scans (see Figure 2.9). Unique results for modulus and deformation maps of the primary cell wall revealed a different architecture for cellulose and
matrix polymers including cellulose bundles. The measured diameter for the smallest fibril was approximately 3 nm, which shows that microfibrils bundled together to make cellulose microfibrils of 12 nm. Figure 2.10 illustrates the onion microfibrils modulus distribution with the average modulus of 1.44 MPa.

Figure 2.10 shows post image processing deformation image of onion microfibrils. In the figure, the corresponding three graphs, were given the height distribution for those selected lines. From the results, a very small microfibril with 2.741 nm (blue) diameter, a separation distance of 5.16 nm (green), and large microfibril bundles with approximately 27 nm diameter (red), which represent unique results. The measured distance data shows that the space among microfibrils can be very useful for mechanical modeling of such a biocomposite.
Figure 2.9: Onion microfibrils mechanical map. Different architecture with unseen texture in topographical image was presented.
Figure 2.10: Onion microfibrils modulus distribution. The average modulus was about 1.44MPa
Figure 2.11: Post image processing of onion microfibrils deformation image. Three graphs below corresponded to the three lines show in the image which gives the height distribution for those selected lines. Based on the results, a very small microfibril was about 2.741 nm (blue), the distance between them at some points was about 5.16 nm (green), and one of the big microfibril bundles was about 27 nm.
2.3.2 Celery Microfibril Characterization

The celery epidermis was bathed in 1x PBS (Phosphate Buffered Saline) solution with 0.05% detergent, called Tween 20, to remove the proteins. Due to the significant amount of Pectin, in comparison to the onion cell wall, the sample needed to be bathed in solution for six hours or even longer. Due to the different structure of celery microfibrils, the preparation of a monolayer cell profile in celery epidermis was necessary and required sample preparation that was more complex and time consuming than with the onion samples. As shown in onion images (Figure 2.12), the celery epidermis included both multi layers and a single layer of cell, and cell profile. Therefore, finding a good position on the mono layer cells was a challenge. The sides of celery sample were glued to the clean, glass slides while the internal part remained intact and free.

Similar to onion experiments, Peak Force Tapping method was used with ultra low force (picoNewton) for tapping the cantilever during scanning process. Sharp Scan Asyst Fluid + tips were used in this study with radius of curvature of 2nm. The tip was washed after each set of experiments by dipping in distil water + 90% ethanol to remove the residue from the scanning.

Figure 2.12 and Figure 2.13 show raw 500 nm and 1 µm size images of intact celery microfibrils in water. The right side images are topography, while the left side images are the Peak Force error signal images. Peak Force Error, also called error signal of deflection, is obtained by the subtraction of the set point force from detector signal (actual deflection). In general, the deflection image shows the edges of features in the topography image. In soft
materials, deflection Image was clearer than the Topography Image. If the error signal was too large, which was not in our case, the tip is unable to track the sample accurately.

Post image processing of celery microfibrils topography or height images shows in Figure 2.14. Based on results, the red graph represents small microfibril of 5 nm, the green was distance of 10 nm, and blue was big microfibrils bundle of 21 nm. The results show that celery and onion microfibrils structure had similarity and some differences. The celery plant cell wall microfibrils seemed more stiff and not as flexible and smooth as onion. Although onion cell profile and onion cells had bigger size comparison to celery cells, but their microfibrils were very similar in diameter. It was determined that small difference (nanometer) in measurement could be due to the radius of curvature of the AFM tip. Generally speaking, the results for both samples show remarkable new architecture that is unique in the case of non destructive scanning such a sensitive bio nanocompostire structure. For example, the top layer of microfibrils show an angle of 42 to 50 degrees and the second layer show an angle of 111 to 117 degrees.
Figure 2.12: 500 nm scan of the celery fibrils, performed with Peak Force Tapping mode in fluid, with Dimension ICON. The top layer has an angle of 42 to 50 degrees and the second layer has an angle of 111 to 117 degrees.
Figure 2.13: 1µm scan of the celery fibrils, performed with Peak Force Tapping mode in fluid, with Dimension ICON.
Figure 2.14: Post image processing of celery microfibrils topography or height image. Three graphs below corresponded to the three lines show in the image which gives the height distribution for those selected lines. Based on the results, very small microfibril was about 5 nm (red), the distance between them at some points was about 10 nm (green), and one of the big microfibrils was about 21 nm.
2.4 Conclusion and Future work

Nanocomposite structures of plant cell walls were studied using a Dimension ICON Atomic Force Microscopy (AFM) instrument. Imaging and measurement of microfibrils in onion and celery samples show a different structure of microfibrils in cell walls. The following can be concluded from this study:

1) AFM was an effective and successful method for nondestructive evaluation of materials in nano-scale level

2) Due to the sensitivity of microfibrils, an innovative Peak Force Tapping technique in AFM was used to scan the cell walls using extreme low forces (≈200 picoN)

3) For the first time in AFM research, an unique architecture of intact plant cell was revealed

4) It was determined that the microfibrils diameter in onion and celery samples were very similar in structure

5) Celery cell microfibrils had stiffer structure comparison to onion microfibrils
In our future work we will test competing models involving where the mechanical strength is derived from (i.e. XyG H bonded connections between micro fibrils, coated micro fibrils, or micro fibrils suspended in a matrix of polymers) can be tested by examining samples with altered compositions both through mutant plant species and synthetic cell wall analogues. Comparing cellulose microfibril movements between samples of differing cell wall composition could provide informative correlations to the role of other cell wall components (e.g., hemicelluloses, pectin, etc.) in cell wall elongation. For example, using the cell wall analogs consisting of varying degrees of cellulose, xyloglucan, and pectin could verify literature speculation as to the effects of pectin on mechanical properties by identifying the differences in the resulting structural movements of microfibrils. The relative movement of microfibrils corresponding to a given strain in composites consisting of cellulose/pectin, cellulose/hemicellulose, and cellulose/hemicellulose/pectin can be compared to the motion in pure cellulose composites. This would indicate the relative importance of each component to the emergent mechanical strength. Similarly, natural plant cell wall samples and mutants can be examined.

Also this new AFM technique can be used as a possible alternative approach to the determination of how does the crystallinity vary in natural cellulose microfibers. When comparing values for Young’s modulus from measurements of natural cellulose samples to accepted crystalline cellulose values, lower values are often explained away by the existence of regions of amorphous cellulose. For the QNM AFM technique, these findings amount to a
corroboration of the notion that the Young’s modulus along a nature cellulose fiber will vary based on the type and degree of crystallinity. This translates to contrast in the high resolution quantitative images we obtain, meaning the amorphous regions would show up as dimmer sections of a crystalline microfiber.
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Chapter 3

Nano mechanical behavior of microfibrils plant cell wall during mechanical elongation and enzymatic elongation

Abstract

One of the most important questions regarding the emergent mechanical strength of plant cell walls is: How do the mechanical properties of the individual components within the cell wall matrix compare to one another and evolve during elongation.

In this research, the unique capability of the AFM was used to measure the mechanical properties of onion and celery epidermis on the nanometer scale. The Peak Force Quantitative Nano-mechanical Mapping (Peak Force QNM) technique was utilized to obtain images of the onion microfibrils where quantitative values of the mechanical properties provide the contrast for the image. For the first time, a series of high resolution images of intact onion microfibrils were taken to study the stress relaxation of individual fibrils after applying strain to the epidermis. This study was repeated to investigate the individual fibrils nanoscale movement, or possible reorientation, after mechanical elongation of the onion epidermis. The next step involved taking a sequence of images which were used to investigate the effect of pectin on microfibrils. The literature showed that pectin could inhibit the unfolding of Xyloglucan chains (Abasolo et. al, 2009). This was studied by adding Pectate lyase to both onion and celery epidermis wall to cut the polygalcturonic acid chains.
3.1 Introduction

Extensive research has shown that the bulk properties of plants are comprised from a complex interdependency between the properties of the individual components (i.e. matrix polymers, polysaccharides, and cellulose microfibrils) and the comprised architecture (i.e. the structure and orientation) within a cell wall (Salmen et. al, 2004, Whitney et. al, 1991, Zhou et. al, 2007, Taiz et. al, 1984, McKenna et. al, 2009, Astley et. al, 2003). In order to develop a predictive mechanical model for cell walls, accurate measurements of the mechanical properties of the individual components of a living plant cell wall is the key. However, the published measurements of the Young’s modulus of plant cell walls have mostly relied on interpreting averaged measurements or have obtained the results based on unrealistic conditions. Basically, uniaxial tension tests (~ mm) were used to measure the elastic properties of the materials. For measuring the elastic properties of an individual cellulose microfiber in a synthetic composition of pure bacterial cellulose (Astley et. al 2003, Chanliaud et. al, 1991), this value must be extrapolated. Similarly, Raman spectroscopy has been used to identify crystalline changes in bulk samples of isolated bacterial cellulose. In the Raman spectroscopy method, an estimate for the Young’s modulus of an individual cellulose microfiber is calculated through monitoring the amount of changes in the C-O stretching bond on the cellulose polymer backbone (chanliaud et. al, 2004). One of the disadvantages of this technique is the assumption of complete crystallinity of the cellulose, which is not independently confirmed. Another disadvantage is the unknown effect of the uniformity of orientation of the microfibers under measurement, which would result in bias in the magnitude of the crystallographic changes. Recently, many research studies have attempted to find a three point bending test utilizing an
AFM cantilever to measure force and deflection on a single cellulose microfiber spanning a nano-manufactured gap (Zhao et al., 2005, Geitmann, 2006, Guhados et al., 2005). Aside from the common questions regarding the uniformity and crystallinity of the cellulose microfibril, this research raises a set of issues more specific to the scale of the experiment. For instance, this study used nanometer scale electrostatic forces as a dominant force in mechanical experiments. Such interactions challenge the normal set of assumptions and interpretations of such experiments.

Studying nanoscale phenomena to achieve a quantitative measurement using an AFM, concluded more direct and higher resolution method of measuring the elastic modulus. This method used Force-Distance (F-D) curves of an AFM in tapping mode and advanced theories that correlate the F-D curve to a relative measure of elastic modulus, (Whitne et al., 1999), and further relate to an absolute measure of elastic modulus as demonstrated in hydrated states, (Zhou et al., 2007). The advantage of this technique is that it extends beyond measuring the elastic properties of individual components of the cell wall matrix. These quantitative measurements are continuously made as the AFM probe scans the sample, resulting in enough values for mechanical strength to comprise the contrast data for the individual pixels of the high resolution image. This means that a geometrically (topographically) uniform cellulose microfibril, with variations in crystallinity (amorphous regions) for example, would become evident in a mechanical modulus contrast derived image.

In this research, the quantitative mechanical measurement capability of the AFM was used to measure the relative mechanical properties of both onion and celery epidermis on the nanometer scale. By using the Peak Force QNM AFM technique, quantitative images of the
onion and celery epidermis microfibrils were obtained with the mechanical properties providing the contrast for the image. A series of high resolution images of onion microfibrils were taken to study the stress relaxation of individual fibrils after applying strain to the epidermis for the first time.

This success has been repeated to investigate the individual fibrils’ nanoscale movement, or possible reorientation, after mechanical elongation to the onion epidermis.

In addition, sequences of images were used to investigate the ability of pectin to tether microfibrils or, perhaps, to support them as a filler. Other researchers have suggested that the pectin could inhibit the unfolding of Xyloglucan chains (Abasolo et. al, 2009), which was studied by adding Pectate lyase to both onion, and celery epidermis wall to cut the polygalacturonic acid chains.

3.2 Materials an Methods

One of the challenges for extending the AFM technique was using an intact cell wall which is considered an open structure for the imaging of plant cell wall microfibrils under the AFM tip. In order to achieve this goal, the onion and celery epidermal peels’ primary cell walls were used. The benefit of using onion and celery epidermis was that it required a simple sample preparation. The onion and celery epidermis were bathed for 1 hour in 1x PBS (Phosphate Buffered Saline) solution with 0.05% Tween 20. The epidermal peels were then rinsed in 1x PBS. Due to a significant amount of Pectin in the celery cell wall, the epidermal tissues required bathing for more an additional 4 to 6 hours.
For the enzymatic interaction, Pectate Lyase (Pel10Acm) was used and diluted into 25, 50, and 100 µg/ml with 20mM HEPES buffer, pH 7.0. The enzyme was kept at four degrees Celsius before use, and added about 20 µl enzyme to cover the whole tissue for about 40 min.

3.2.1 Peak Force QNM Technique

Peak Force Quantitative Nano-mechanical Mapping (Peak Force QNM) is a new AFM (Su et al, 2010) technique which uses tapping technology to record very fast force response curves at every pixel in the image while using the peak tip-sample interaction force as the feedback mechanism. Peak Force QNM is able to simultaneously obtain quantitative modulus, adhesion, dissipation, and deformation data while imaging topography at high resolution. Also, by maintaining control of direct force to a very low level (pN), the scanning can limit indentation depths to deliver non-destructive and high-resolution imaging. Furthermore, material properties can be characterized over a wide range to address samples in many different research areas (Su, et al, 2010). As explained in the previous chapter, several mechanical property parameters can be obtained from each force curve. The adhesion force represents the minimum force point when the tip starts to pull away from the sample. Energy dissipation is calculated by the hysteresis area between the approaching and retracting processes. This dissipation includes the work associated with adhesion and viscous or plastic deformations. When the set point of the peak force is set at or close to zero, the energy dissipation is dominated by the work of adhesion. Deformations here represent the total penetration depth, including elastic and plastic deformations (Su, et al, 2010).
3.2.1.1 Mathematical models used in PeakForce QNM

**Elastic Modulus**

To measure the elastic modulus of the system, DMT theory (Maugis 2000) has been used, which is discussed in chapter 2. The relation between adhesion force is as follows:

\[
F - F_{adh} = \frac{4}{3} E^* \sqrt{R(d - d_0)}^3 \tag{3.1}
\]

Where, \(F - F_{adh}\) is the force on the cantilever relative to the adhesion force, \(R\) is the tip radius, and, \(d - d_0\) is the sample deformation, and \(E^*\) is the reduced modulus. The relation between the Young’s Modulus of the sample, \(E_s\), and \(E^*\) is as follows:

\[
E^* = \left[ \frac{1 - \nu_s^2}{E_s} + \frac{1 - \nu_{tip}^2}{E_{tip}} \right]^{-1} \tag{3.2}
\]

Where, \(\nu_s\) and \(\nu_{tip}\) are Poisson’s ratio of the sample and tip respectively (Su, et al. 2010).

**Adhesion**

The next mechanical property that can be acquired by AFM is the adhesion force. The source of adhesion force can be any attractive force between the tip and the sample. In most cases the adhesion force is the combination of electrostatic force \(F_{et}\), van der Waals force \(F_{vdW}\), the capillary, or meniscus, force \(F_{cap}\), and the forces related to chemical bonds or acid-base interactions \(F_{chem}\):

\[
F_{adh} = F_{et} + F_{vdW} + F_{cap} + F_{chem} \tag{3.3}
\]
In some cases, when the tip and the sample are not net charged or when the surfaces are saturated with chemical bonds, the electrostatic or chemical forces can be neglected. But van der Waals force always contributes and, in some cases, is attractive. At ambient conditions, a water neck forms between the AFM tip and substrate by way of capillary condensation and adsorption of thin water films at the surfaces. This attractive interaction depends on the relative humidity and hydrophilicity of the tip and the sample (Butt, et al. 2005). This so-called meniscus, or capillary, force is caused by the pressure difference between the liquid and the surrounding vapor phase which is given by the Young-Laplace equation:

\[ \Delta P = \gamma \left( \frac{1}{R_1} + \frac{1}{R_2} \right) \]  

Equation 3.4

Where \( R_1 \) and \( R_2 \) are the two principal radii of curvature for the water meniscus. So, the capillary force between water on the plate and a sphere, with radius \( R \) and contact angles \( \theta_1 \) and \( \theta_2 \), have been measured as follows (O’Brien et. al, 1973):

\[ F_{\text{cap}} = 2\pi R \gamma (\cos \theta_1 + \cos \theta_2) \]  

Equation 3.5

The area below the zero force reference (the horizontal line in the force curve) and above the withdrawing curve is referred to as “the work of adhesion.”

**Dissipation**

The dissipation energy can be obtained by the product of force and velocity integrated over the period of the vibration (Su, et al 2010):

\[ W = \int F \cdot d\vec{Z} = \int_0^T \dot{F} \cdot \vec{\dot{v}} dt \]  

Equation 3.6
Where \( W \) represents energy dissipated in a cycle of interaction, \( F \) is the interaction force vector, and \( dZ \) is the displacement vector. For pure elastic deformation there is no hysteresis between the repulsive sections of the loading-unloading curve, resulting in very low dissipation. In this case, the work of adhesion becomes the dominant contributor to energy dissipation. Energy dissipation is presented in electron volts as the mechanical energy lost per tapping cycle (Su, et al, 2010).

**Deformation**

The deformation corresponds to total penetration depth of the tip into the sample, including both elastic and plastic deformations. With the knowledge of the tip shape, the deformation can be converted to indentation hardness (Swadene et al, 2002). An accurate description of tip shape can be derived by AFM measurement on a reference sample through morphological dilation (Belikov et al, 2009).

### 3.2.2 AFM Cantilever Calibration

**Spring Constant Calibration**

During the movement of the cantilever, due to the forces acting on it, the optical lever performs the measurement through reflecting a laser off to the top of cantilever and towards a photo-detector at the cantilever’s free end over certain time period. In the most Contact Mode and Tapping Mode applications, the cantilever deflection is small, which indicates a linear behavior of the cantilever free end shown by Hooke’s law as:
\[ F = -k \times h \quad \text{Equation 3.7} \]

Where: \( F \) is the force on the tip (N), \( h \) is the vertical displacement of the cantilever (m), and \( k \) is the spring constant, which is a property of the cantilever and is the same number in air, water, and vacuum (N/m or in nanoscale work picoN/pico m).

Knowing \( k \), the measured cantilever deflections can be converted to an inferred tip/sample force, thus, the initial step for an accurate force determination is to find the \( k \) of the cantilever. There are several techniques to measure a cantilever spring constant including: thermal tuning (most preferred method due to less time consuming and accuracy), geometry, comparison, hydrodynamic model.

**Measuring Thermal Noise**

While the cantilever is away from the sample, the measurement data consists of the time interval of the cantilever deflection signal in contact mode at the thermal equilibrium. During the sampling, the cantilever is impacted by the Brownian motion of surrounding molecules. The power associated with resonance is obtained from integrating the area under the resonant peak curve. The dynamics of the cantilever can be expressed as an oscillator by the systems’ total energy, which the value for both potential and kinetic energy is defined as:

\[ \frac{1}{2} k_B T \quad \text{Equation 3.8} \]

Where \( T \) is the temperature (kelvins) and \( k_B \) is Boltzmann’s constant (equal to \( 1.3805 \times 10^{-23} \) joules/Kelvin).
The potential energy can also be defined as the following:

\[
\langle \frac{1}{2} m \omega_0^2 z^2 \rangle = \frac{1}{2} k_B T
\]

Where, \( \omega_0 = \sqrt{\frac{k}{m}} \) (resonant angular frequency), \( m \) is the effective mass, and \( z \) is the displacement.

The cantilever spring constant, \( k \), is measured by simplifying the temperature and average displacement as:

\[
k = \left( \frac{k_B T}{z^2} \right) = \frac{(k_B T)}{P}
\]

By integration of the area under the resonance in Power Spectral Density (PSD), and excluding the noise floor and shoulders to either side of the resonance peak, the power can be measured.

When measuring the cantilever spring constant by the thermal method, the accuracy highly depends on the isolation of nonthermal noise and the accuracy of the PSD and its magnitude. The typical nanoscope V controller sample deflection produces a signal every 16.5 seconds, which corresponds to the 64 kHz sampling rate. Accordingly, cantilever resonances with frequencies above 32 KHz can produce distorted to Power Spectral Density.

The thermal tuning method is mostly applicable to soft cantilever, mainly in fluids where cantilever resonance frequencies are significantly lower in air. The cantilevers can generate a smaller signal to analyze due to the magnitude of the PSD is proportional to the mean cantilever displacement.
Calibrating a Cantilever

Measurement of Deflection Sensitivity in Force Mode

Every time the laser beam path changes, the deflection sensitivity must be recalculated due to laser alignment, photodetector adjustment and probe change. The deflection sensitivity calibration is done on the hard and stiff surface.

Below are the procedures for deflection sensitivity measurement:

Engage on the hard surface, like silicon or glass, then switch to ramp mode. Obtain a force diagram, which represent the interaction with the hard surface, then display deflection error vs. Z sensor drag the ramp mode, or force mode, in from the left and/or right edge and displace as far as possible while they are still in the contact region of the force diagram. Figure 3.1 shows the tip deflection error vs. Z sensor on a glass slide in water. The update sensitivity was calculated using software and repeated for five times to calculate the average number for the deflection sensitivity.

The deflection sensitivity is measured based on the assumption of that tip and the test sample do not deform. During the process of measuring the deflection sensitivity, the static force is applied to the end of the cantilever, but during the thermal energy measurement, the cantilever is oscillating at resonance at only one end instead of two.

Every time the laser beam path changes, the deflection sensitivity must be recalculated due to change in laser alignment, photodetector adjustment, and probe change. The deflection sensitivity calibration is done on a hard and stiff surface.

Below are the procedures for deflection sensitivity measurement:
Engage the hard surface, like silicon or glass, then switch to ramp mode. Obtain a force diagram which represents the interaction with the hard surface and then display deflection error vs. Z sensor drag during the ramp mode, or force mode, in from the left and/or right edge and displaced as far as possible, while they are still in contact region of the force diagram.

Figure 3.1: Deflection sensitivity determination using force mode in liquid (water).

**Measurement of Cantilever Spring Constant by Thermal Tune**

The following is the procedures of determining cantilever constant:
2- To choose the least square fit the data click on LORENTZIAN (AIR) or SIMPLE HARMONIC OSCILLATOR (FLUID).

The following equation (Simple Harmonic Oscillator) is used to fit the filtered data:

\[
A(\nu) = A_0 + A_{DC} \cdot \frac{\nu_0^2}{\sqrt{(\nu_0^2 - \nu^2)^2 + \frac{\nu_0^2 \nu^2}{Q^2}}}
\]

Where: \( A(\nu) \) is the amplitude as a function of frequency (\( \nu \)), \( A_0 \) is the base line amplitude, \( A_{DC} \) is the amplitude at DC with zero frequency, \( \nu_0 \) is the center frequency of the resonant peak, and \( Q \) is the quality factor.

Normally, the markers are located where the spectrum rises from the noise floor. The curve fit is not sensitive to the minimal power contributed from these frequencies, which make the precise placement unnecessary. The process of the test was explained in this section. The first step was to choose the best fit of the data. As shown in Figure 3.2, the curve fit along with the acquired data displayed (in red). In order to make sure the data is consistent with the best curve fit at the thermal peak, the marker position was adjusted, then the cantilever temperature and calculated spring constant (K) were entered.
New, sharp ScanAsyst Fluid+ probes were used in this research which had the combination of sharpness of silicon tip with low spring constant and high sensitivity of silicon Nitride cantilever necessary for this application. This test was conducted for an unprecedented level of high resolution and force control on samples in fluid. The tip height is about 2.5 - 8.0µm, with the radius of about 2-3 nm. The spring constant is between 0.35-1 N/m.
3.3 Results and discussion

As previously discussed, in order to accurately measure the relative elastic modulus of individual components of a cell wall on the nanoscale, the QNM package of the ICON AFM was used in this work. This calibrated technique accounts for water, allowing for intact cells to be examined. The AFM tip and cantilever were scanned across a section of the sample while recording non-destructive force indentations at each pixel. As a result of the force distance curves, the elastic modulus at each point was calculated. The software package accounted for the contributions of water, isolating the mechanical information of the specimen. The data obtained can be used in form of chromatic images for which the contrast of the image can represent the range of elastic modulus within the image. Under tension, sequential nano-elastography measurements showed the evolution of elastic modulus as stress were distributed throughout the cell wall components.

These experiments provided significant, new information to the field and, for the first time in research analysis, quantified the distribution of stress resulting from tensile loading throughout cell wall components as evidenced by change in elastic compliance. When the tensile load was applied, each component’s elastic modulus should have been altered proportionally to its contribution to load bearing. Such information was revealed how the mechanical interactions between cellulose microfibrils and other cell wall matrix materials attribute strength during loading.
3.3.1 Mechanical Test and Nano level effect on individual Fibrils

One of the main challenges in this research was the suitability of the tensile tester stage in the AFM method. The configuration of the AFM required a very thin (less than 2 inch), horizontal tensile stage allowing for easy, quantitative adjustment of the applied tensile force. As shown Figure 3.3, the size and configuration of the stage was addressed. The remaining efforts were focused on applying a quantitative measure of applied tensile force. That was a technically challenging area based on the inequality in elastic compliance that arises between the need for a stationary (solid) sample holder and the relatively weak (soft) sample.

As showed in Figure 3.3, the tensile test stage included two horizontal plexiglass sheets, one of them was fixed and the other connected to a fine-gauged micrometer. The knob is graduated in µm, where each grade of fine-adjustment knob was about 2.5 µm, and rotation of coarse-adjustment knob was 12.5 µm. The knob provides a maximum separation distance of 5 mm between the plexiglass sheets, which translates to the greatest possible displacement applied to the sample. However, without the micrometer, it was possible to add more separation distance between glasses. The sample was mounted on glass slides and clamped on both sides. The side wall of the epidermal tissue was blocked by regular, adhesive tape to make a small channel for holding water during scanning. As already stated, Peak Force QNM method were used in this study which was represented a quantitative nano-mechanical method using ultra low force (picoNewton) for tapping the cantilever during the scanning process. This method allowed nondestructive indentation for scanning of a sensitive, intact sample in water, such as plant cell wall microfibrils. The first step was to take an image before the applied load,
and then carefully cut the sides of the epidermis with sharp blades to apply load, and used tape to block the sides.

The first set of experiments was conducted in various cells at various positions of the sample. Based on observation, there was drastic change in microfibrils’ orientation before and after stretching. In order to obtain a consistent and reliable result, the sequence of images must be taken in the same cell. At this stage, locating the exact position was almost impossible, even with marking the position, however at least the results can be shown for the same cell, if not the same area. As the previous study (chapter 2) showed, the orientation of the microfibrils in the same cell is consistent, regardless of position in the cell.
For the first time in the area of AFM research, the relaxation phenomena of individual microfibrils were imaged at identical positions to find how each individual component changed their angle, or orientation. Figure 3.4 shows the high resolution images of onion microfibrils at
the same position. The top image shows the onion cell epidermis, including cell profiles, with
the AFM tip on the exact spot of interest, shown as a red cross, which represents the exact
position of scanning. The right side image is the topography, which is the height information of
the sample The left side image shows the Peak Force Error which is called the error signal of
deflection image and represents the subtraction of the set point force from detector signal
(actual deflection). In general, the deflection image shows the edges of features in the
topography image which, in soft materials, subsurface structures were clearer than the
topography Image. If the error signal was too large (which was not in our case) it is interpreted
as the tip not tracking the sample surface very well.

If the tip is calibrated the unit for PeakForce error signal images show variation of
contrast based on force unit. As seen in the top right image, the highest deflection error force is
about 1.3 nN. As explained in tip calibration section, Scan Asyst Fluid + tips were used in this
study. Several force distance curves were gathered at different positions on a smooth, glass
slide in water, and the deflection sensitivity was recorded for each. The calculated average
deflection sensitivity was approximately 17.814nm/v. Based on this parameter, a spring
constant of 0.859 N/m was calculated with AFM software for this specific tip.

For the epidermal tissue with the length of 24 mm and width of 2.5 mm, a displacement
of 6 mm was applied. This displacement was based on a Dynamic Mechanical Analysis result
applying of 2N of force (which will be discussed in following chapter). The average modulus,
average adhesion force, and the average dissipation energy in this image were 1.74 MPa, 1.40
nN, and 1.7 keV, respectively, which was calculated by Peak Force QNM data processing
software. As showed in Figure 3.4, the first set of images corresponded to the time when the
microfibrils have been subjected to a displacement of 6 mm. The second set of images show how each individual component reacts to the applied load after 15 minutes. For example, the microfibril labeled number 1 changed its angle from $72^\circ$ to $101^\circ$. 
Figure 3.4: Topography (left) and Peak error (right) images of onion microfibrils of 24mm Onion Epidermis. A) After 6mm stretching with Tensile Test Stage T1=0. B) 24mm Onion Epidermis after 6mm stretching with Tensile Test Stage T2=15
For the next step, the investigation of the effect of mechanical load on individual microfibrils before and after applying load at the same cell and position was attempted. To achieve this goal, an 18 mm onion epidermis tissue was fixed by clamps and taped on its side walls. Note that the inside tissue was free and intact during scanning process. Figure 3.5 shows high resolution of onion microfibrils image before applying load. The top image shows the onion cell epidermis including cell profiles, AFM tip on the exact spot of interest with a red cross that shows the exact position of the scanning. The bottom right is the topography, and the bottom left image is Peak Force Error or deflection. The Peak force setpoint was selected for this set of experiments which remained constant (1.101 nN) during the experiments. Scan Asyst Fluid and tips were used and calibrated before the experiments. Several force distance curves were taken at different positions on the smooth, glass slide in water and deflection sensitivities were recorded. The calculated average deflection sensitivity was 37.66 nm/v and, based on this parameter, the spring constant of 0.9184 N/m was calculated using AFM software for this specific tip. The average modulus, the average adhesion force, and the average dissipation energy were 1.15 MPa, 500 pN, and 1.2 keV, respectively, which was calculated by Peak Force QNM data processing software.

As shown in Figure 3.6, Figure 3.7, and Figure 3.8, the onion epidermis are displaced for 2mm in the +X direction, which corresponds to a force range of 0.3-0.5 N. As stated before, the Experimental Dynamic Mechanical analysis (DMA) will be discussed in the following chapter.
Figure 3.5: 18mm Onion Epidermis Before stretch, average modulus is 1.15 Mpa, $T_0=0$
Figure 3.6: A) 18mm Onion Epidermis Before stretch Ave Modulus: 1.2 Mpa, T1= 6min. B) 18mm Onion Epidermis After 2mm stretch. average modulus: 1.22 Mpa, T2=11min
Figure 3.7: A) 18mm Onion Epidermis After 2mm stretch. Ave Modulus: 1.2 Mpa, T3 = 18 min.
B) 18mm Onion Epidermis After 2mm stretch. Average modulus: 1.2 Mpa, T4= 25 min
Figure 3.8: A) 18mm Onion Epidermis After 2mm stretch. Ave Modulus: 1.2 Mpa T5=33 min, B) 18mm Onion Epidermis After 2mm stretch. Average modulus: 1.2 Mpa, T6= 40 min
As already explained, all above images were taken in the same cell profile and the same spot. As seen in the first set of images (Figure 3.6), the fibrils after six minutes showed evidence of the applied stress and rearranged, and then returned to the original position with a looser configuration. All of the images together show how individual microfibrils relax and become loose after applied force. The average modulus decreased to 1.2 MPa during stress relaxation. These systematic investigations showed the changes in angles and directions of microfibrils during nondestructive scanning of the intact plant cell wall in water.

3.3.2 Enzymatic treatment and Nano level effect on individual Fibrils

Sequences of images were used to investigate the ability of pectin to tether microfibrils or, perhaps, support them as filler. A study has suggested (Abasolo et al, 2009) that pectin could inhibit the unfolding of Xyloglucan chains. Abasolo et al. studied the role of pectin by adding Pectate lyase to the onion epidermis wall to cut the polygalcturonic acid chains. As higher concentrations of Pectate lyase were reached, images showed the microfibrils eventually collapsing with 3 μL of Pectate Lyase in 300 μL of buffer (Figure 3.9). Unfortunately, quantitative nanomechanical measurements were not made in this study, therefore, the units of images were not calibrated and not converted to force units.
Figure 3.9: From left to right: (A) Microfibrils, before adding pectate lyase to the wall to cut the polygalacturonic acid chains. (B) After treatment—medium (1 μl of the original pectate lyase in 1000 μl of buffer). (C) After treatment—strong (3 μl of the original pectate lyase in 300 μl of buffer): pectate lyase causes microfibrils to collapse completely. Note that some of the horizontal lines, especially in figure (C) are due to vibration noise.
3.4 Conclusion and Future work

The following conclusions were summarized the outcome of this research.

1) For the first time in the area of AFM research, the relaxation phenomena of individual microfibrils were imaged and showed how each individual component changed its angle or orientation as a result of applied stress.

2) The AFM images showed the orientation of the microfibrils in the same cell were consistent, regardless of position of the cell.

3) For an onion epidermal tissue size of 24 mm x 2.5 mm, elastic modulus, adhesion force, and the dissipation energy of microfibrils were measured as 1.74 MPa, 1.40 nN, and 1.7 keV, respectively, using AFM.

4) For an onion epidermal tissue size of 18 mm x 2.5 mm, the average modulus, average adhesion force, and average dissipation energy were calculated as 1.15 MPa, 500 pN, and 1.2 keV, respectively, using AFM.

5) The enzymatic treatment showed the microfibrils eventually collapsing with 3 μL of Pectate Lyase in 300 μL of buffer.
Future Work:

**Using AFM force measurements and functionalized tips on plant cell walls**

For future step functionalized AFM tips could be used to explore the roles of matrix polymers in the primary cell wall. The binding energy between a single molecule of XyG and a cellulose microfibril can be measured. In specific, synthetic composite materials can be used as cell wall analogues to provide baseline comparisons to natural specimens of similar composition (i.e. varying degrees of XyG and Pectin with cellulose microfibrils). Mutant and enzyme treated samples can be used, for instance, to provide accompanying imagery of reorientation of microfibrils in natural plant cell wall samples with normal and deficient amounts of XyG or other matrix polymers. This may provide new insight as to how the mechanical strength of the cell wall can be maintained in XyG deficient samples (perhaps, the orientation of microfibrils begins and/or evolves differently).

Another set of challenges will be extending the above technique to other intact cell wall samples of interest. The anticipated challenge is the preparation of samples to be imaged. For instance, to date the technique has been used on onion primary cell walls, for which a simple sample preparation can reveal the primary cell wall structure. However, other potential samples of interest may require more complicated treatments in order to expose the internal cell wall structure, for example cotton fibers.
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Chapter 4

Nano-Macro mechanical behavior of biocomposites

Abstract

The aim of this research was to measure, analyze, and model the macro- and nano-mechanical properties of biocomposite materials. Onion and celery epidermal tissues were selected as test materials. The macro-mechanical properties of onion and celery epidermis were measured using Dynamic Mechanical Analysis (DMA) system. The nano-mechanical properties of the cells are measured by loading a uniaxial tensile force in the longitudinal direction of onion and celery epidermal peels. Six properties influencing the material’s structural characteristics were studied. These properties included Strain, Stress, Stiffness, Displacement, Static force, and Modulus of elasticity or Young’s modulus. The statistical analysis, namely variation analysis and Gini coefficient, were able to provide a detailed description and understanding of both mechanical properties and experimental data.

The experimental tests were repeated with tissues rinsed in buffer and enzyme solutions. (Pectate Lyase (Pel10Acm) was used to remove Pectin, and diluted into 25, 50 and 100 µg/ml with 20mM HEPES buffer, pH 7.0) A Finite Element Model was used to simulate the mechanical behavior of cells under tension. This study compared the experimental data and model to understand the bio-composite structures of cellulose microfibrils’ movements and reorientation during the elastic deformation. Both elasto plastic and viscoelastic models were used to compare the experimental results. The results showed that: 1) there is a good
agreement between experimental data and the model’s results; 2) the epidermis showed more viscoelastic behavior than elastoplastic under longitudinal tensile test, 3) the Young’s modulus of onion and celery ranged between 0.2-4.9 MPa and 5-13 MPa, respectively; therefore the higher modulus of elasticity of celery can be interpreted of stronger fibril structure in comparison with onion cells; 4) the Young’s modulus of onion and celery in enzyme solution ranged from 0.13-0.47 MPa and 0.27-1.25 MPa, respectively; 5) enzyme and buffer solutions had a significantly negative influence on stiffness and strength of the cells epidermis; 6) in modulus of elasticity analysis, onion had the least coefficient of variation (23% or 77% equality), representing the highest consistency during the data collection; 7) the stiffness of onion and celery ranged from 91-197 N/m and 98-525 N/m, respectively; which shown celery had higher resistance to deformation in compare to onion; 8) the stiffness of onion and celery in enzyme solution ranged from 0.29-4.9 N/m and 1.01-8.4 N/m, respectively; 9) the average coefficient of variation for stiffness analysis is around 23% were shown high consistency of measurement; 10) the entire test for Gini coefficients are between the range of 0.07 (93% equality) and 0.42 (58% equality) which represent the satisfactory data recorded for mechanical property analysis.
4.1 Introduction

Wei et al. (2001) studied the mechanical properties of plant cell tissue using onion (Allium cepa) epidermal cells. They created a unique method of using ball tonometry for measuring cell turgor pressure (Wu et al. 1985). They measured the modulus of elasticity and Poisson’s ratio of the onion cells by applying a tensile force to the onion epidermises while measuring the lateral contraction of epidermises. Single-cell epidermal peels from onion leaves were cut into 18 mm x 33 mm rectangular shape and bathed in pure water or mannitol solution for the experiment. The static load method which was used by Wei et al. is called ball tonometry (Lintilhac et al. 2000).

A glass ball, with diameter of 300 mm, was attached to a loading rod and positioned on the cell. By using the imaging technology, the contact area between the ball and the cell (Wei et al. 1999) were measured. The amount of force applied on the cell wall was 47, 59, and 69 mgF. Due to the uniformity of thickness in the onion tissue, it was assumed that the mechanical properties of multicellular epidermal were identical to the properties of a single cell. Therefore, the relative increase or decrease of epidermal strip thickness was proportional to an individual cell.

The cell was assumed to be a whole unit, and the theory of elasticity (Boussinesq’s method) was used to derive the equation that measured the elastic properties and cell deformation. In order to measure the effect of turgor pressure and cell elasticity on the cell’s ability to axial load, Wei et al. used a small glass ball on a cell and measured the projected contact area between the ball and the cell. The mathematical formula predicts the cell turgor
pressure with standard error of 0.01 MPa. The measured range of Modulus of elasticity and Poisson’s ratio were 3.5-8 MPa and 0.18-0.3, respectively.

Kerstens et al. (2001) also studied the mechanical properties of plant cell walls. They used extensiometry and polarization confocal microscopy techniques. Their studied showed that cell walls can behave like fiber-reinforced composite materials. They used the adaxial side of the onion (*Allium sativum*) bulb scale and from the abaxial side of Kalanchoe (*Kalanchoe blossfeldiana* Poelln) leaf as test materials. Keretens et al. generated a model that separated the cell wall biomechanics at the molecular level.

Micromechanical behavior of onion epidermal tissue, along with the observed micromechanical properties of onion cell and cell structure parameters, were studied by Vanstreels et al. They used a miniature tensile stage mounted under a microscope objective to measure mechanical properties of onion epidermal tissue. The onion tissue was subjected to tensile loading in the longitudinal and transverse directions. They found the cell area had a significantly negative influence on stiffness and strength of the samples. Materials with less elongated cells have a bigger transition zone and a smaller strain than samples with larger cells. The transverse loaded samples showed larger deformation in comparison with the longitudinal samples. The experimental data was then used to model the mechanical properties of plant epidermal tissue (Ng et al. 2000; Wilson et al. 2000; Wei et al. 2001).

The onion epidermal generates a biphasic stress-strain curve under transverse tensile load. The mechanical properties of plant cell walls are significantly different with different scales of the epidermis. For image analysis, Vanstreels et al. used a stereomicroscope equipped
with a CCD camera which monitored cell deformation. Images were processed by image processing software and analyzed using a Matlab program which manually defined the cell walls in each image. The cross-sectional area of each cell was calculated as the width times the thickness of the strip. Young’s modulus was calculated in different sections of the stress-strain curve, which $E_1$ was obtained from the first part of curve and $E_2$ from the second part of curve, after transition zone. The curve contained two noticeable linear portions of different slope divided by a transition section. The cyclic part of the curve showed that after unloading to zero stress, a major plastic deformation remains.

Stamenovic´ and Coughlin created a mathematical model of cellular elasticity based on tensegrity (model of deformability of adherent cells), where the cytoskeleton was defined as a network of interconnected, tension-bearing struts (compression elements). They performed a structural analysis using the tensegrity method (Ingber et al., 1993, Stamenovic´ et al., 1996, Wendling et al., 1999) to discover the basis for cellular deformation. In their model, six compression elements (struts) which were interconnected by 24 tension elements (cables) as frictionless joints. The key assumptions of this research were 1) choice of six-strut model which represents the cell’s steady-state elastic behavior, and 2) choice of the probe diameter as a characteristic length of the mathematical model. The six-strut tensegrity method was used to simulate the steady-state elastic properties of cells. The Young’s modulus ($E$) was calculated through uniaxially stretching the model and calculating the modulus from the elastic portion of the strain-stress curve in an equivalent continuum. Stamenovic´ and Coughlin study showed that the modulus of elasticity is proportional to the pre-existing tension, or compression, to the elements; and inversely proportional to the cable length’s square. The Young’s modulus value
for the cell mechanical probes diameter, ranging from 3 and 5.5 µm, fell within the limits simulated by the model. The experimental results fell outside the range if the probe diameter was less or equal to 2 µm. They concluded that buckling force of microtubules was a key to find the cellular elasticity.

Ng et al. (2000) studied the chemical composition of onion cell walls in relation to its mechanical properties (Ang et al. 1960; Komochi, 1990; Maw et al. 1996). They analyzed the carbohydrate and phenolic composition of cell wall. The cell walls are rich in uronic acid and glucose and have small amounts of the arabinose, galactose, and xylose. The mechanical property results showed that, in reference to strength, the papery scales are higher than fleshy scales, which were in the following order: lower epidermis greater than upper epidermis greater than intermediate parenchyma. The carbohydrate analysis shows that the papery scales and the upper epidermis of the fleshy scales had no galactose in comparison to the intermediate and the lower epidermises. They also showed that the tensile strength of the papery scales was greater than in the fleshy scales, and the upper part of fleshy scales was weaker in the horizontal direction than in the vertical direction. The major difference in mechanical and chemical properties was due to loss of galactose side chains.

The mechanical behavior of an onion epidermis under tension has been studied by Qian et al. They simulated the mechanical deformation of an onion epidermis by combining a finite element model (FEM) with an inverse model (Hamant et al. 2008; Bolduc et al. 2006; Wang et al. 2006; Kha et al. 2010). In finite element model the cell wall was considered to be a homogeneous material (Holzapfel and Weizsacker, 1998; Holzapfel et al. 2000, 2002; Holzapfel,
Since the cell walls are composite materials, the homogenous material model doesn’t have an ability of modeling the anisotropy of cell walls which consist of heterogeneous structures of microfibrils. Fiber-reinforced materials model, which can handle large deformations, would be a suitable model for the simulation of plant cell walls. With Qian et al., the objective was to use the finite element model was to create the numerical solution of a discrete system and create a mathematical formulation by approximating that the true structure as an assembly of simple-shape elements. Although, the cell walls are a composite materials with embedded microfibrils reinforcing the structure, but the model treated the cell walls as a homogenous materials. The main disadvantage of this model was that the homogenous model is not capable of modeling the anisotropy of cell walls induced by heterogeneous structure of microfibrils. The onion epidermis contains a sheet of single cells with circular cross section, with the individual cells a rectangular or an elliptical shape and typically vary in size from 20 to 100 µm in diameter and 50 to 500 µm in length. They used a 10 mm x 4 mm portion of an onion epidermal strip and fitted onto an extensometer device. The onion sample was then immersed in a 10 mM sodium citrate buffer solution for 20 minutes. The tension loading (transverse and longitudinal) of 5-20 g was induced at the ends of the sample. The longitudinal direction is considered to be parallel to the major cellulose microfibril orientation, which is parallel to the axis of the cells, and the transverse direction is perpendicular to the major cellulose microfibril orientation. The finite element model used identical information for geometry and loading. The experimental data for various loads and strains were used for inverse modeling. The model was able to provide detailed
descriptions of both the biomechanics and the experimental data. The simulated results were in
good agreement with the experiment.

4.2 Dynamic Mechanical Analysis

Dynamic mechanical analysis (DMA) can be described as the application of an oscillating
force to a sample while simultaneously analyzing the material response to the applied force. The sample can be subjected to a controlled stress (\( \sigma \)) or a controlled strain(\( \varepsilon \)). For a known stress, the sample is deformed to a certain quantity the amount of which is related to its stiffness. In this study a force motor was used to generate the sinusoidal stress to be applied to the sample, which generates a sinusoidal strain, see Figure 4.1. By measuring both deformation amplitude at the peak of the sinusoidal wave and the lag between the stress and strain response to the sinusoidal wave, quantities like modulus, viscosity, and damping were calculated.

Figure 4.2 illustrates a typical DMA device with grips which hold the sample in place along with the environmental chamber that maintains different temperature conditions. A sample is fixed by the grips and the environmental chamber slides over to enclose the sample. In this study, the static force ramp was applied by a DMA tester which is governed by Hook’s law for a linear elastic material. Hook’s law relates the stress-strain diagram to a spring constant, \( k \), which states that the deformation/strain of a spring is linearly related to the force/stress applied by a constant specific to the spring. Based on Hook’s law, the Young’s modulus can be measured from the slope of stress-strain curve.
Generally, when the modulus is independent of load and loading rate, elastic materials give a linear response. But the majority of materials are not ideal, linear elastic materials. For polymers, which are related to plant cell walls and epidermal tissues, the material’s response to the load is not necessarily linear. The curve presumes to have a specific shape where the linear region is followed by nonlinear region. Usually the concern is stiffness property of the material, which is obtained from the initial slope of stress-strain curves. Also, the yield point can be measured as the point which material will start to deform (see Figure 4.3).

![Figure 4.1: shows how a DMA works (Menard, K., et al., 1999).](image-url)
Figure 4.2: shows a typical DMA instrument with grips to hold sample and environmental chamber to provide different temperature conditions.

Figure 4.3: shows analysis of a stress-strain curve in extension (Menard, K., et al., 1999).
4.3 Results and discussion

Developing a mathematical model is the ultimate goal for scientist wishing to simulate the real world system to understand the response under various experimental conditions. Several engineering mathematical models are built based on purely experimental observations, which treat variables in a physically intuitive manner. The main objective of this technique is to find correlations of the behavior of the dependent and independent variables.

For simulation of natural phenomena, physical, chemical, and biological laws are used to form the basis for the mathematical model. In this situation, the parameters, which are embedded in the mathematical model, have well-defined physical meaning. In order to further the understanding of the behavior of bio-composite materials under tensile force, models were developed and verified to simulate the mechanical behavior of onion and celery epidermal peels.

This section includes a discussion and comparison between experimental results for onion and celery epidermis using Dynamic Mechanical Analysis (DMA) instrument and simulation results with finite element models. Elastoplastic and viscoelastic material models have been used to conclude similar behaviors in reality. Dynamic Mechanical Analysis is a technique which helps to study and characterize the mechanical properties of materials. DMA typically used to study the viscoelestis and viscoplastic behavior of polymers. A sinusoidal stress (MPa) is applied to the sample and the strain is measured, allowing one to measure Young’s modulus as previously described. Due to variation in stress frequency and sample temperature,
one obtains a variation in complex modulus. In this research work, a static force was applied to the sample using DMA instrument in an isothermal situation.

As already stated, both onion and celery epidermis tissues were considered as biocomposite materials and simulated in this study. In order to better understand the mechanical behavior of these materials, onion and celery’s epidermal peels were bathed in enzyme and buffer solutions to alter the composition of the natural biocomposite by selectively removing components, such as pectin. Extensive experiments were conducted on epidermal peels to measure the mechanical properties. It has determined that the enzyme and buffer solutions had a negative influence on the stiffness of cell structure. The following sections will explain in more detail the experimental procedures and results which include statistical analysis on data.

4.3.1 Dynamic Mechanical Analysis Results

Statistical Analysis of Mechanical Properties

The mechanical properties of onion and celery epidermises, and their behavior in buffer and enzyme solutions, were measured by applying a static force using Dynamic Mechanical Analyses (DMA) instrument. The mechanical properties measured were strain (%), stress (MPa), stiffness (N/m), displacement (µm), static force (N), and modulus of elasticity (MPa). In order to quantitatively analyze the results, two statistical analyses were utilized namely; variation analysis and Gini coefficient.
Variation analysis, such as, coefficient of variation (CV %), Max, Min, Mean, and standard deviation (STDEV) were used to assess the variability of the tensile test profile. The usual CV is defined as a ratio between zero and one hundred. For this study, the CV value of zero was interpreted as ideal consistency (i.e., the greatest) among measured properties and 100 as a perfectly random, inconsistent distribution (i.e., the least). Each test combination was repeated six times. It allowed mean values with a coefficient of variation (CV) of less than 20, thus indicating that the data obtained from six replications was sufficiently consistent. Accordingly, the experimental design with six replications per treatment is summarized in Table 4. 9.
<table>
<thead>
<tr>
<th>Samples</th>
<th>Mechanical Properties</th>
<th>Number of Replications</th>
</tr>
</thead>
<tbody>
<tr>
<td>Onion</td>
<td>Stress (MPa)</td>
<td></td>
</tr>
<tr>
<td>Onion in Buffer</td>
<td>Strain (%)</td>
<td></td>
</tr>
<tr>
<td>Onion in Enzyme</td>
<td>Stiffness (N/m)</td>
<td></td>
</tr>
<tr>
<td>Celery</td>
<td>Displacement (µm)</td>
<td></td>
</tr>
<tr>
<td>Celery in Buffer</td>
<td>Static force (N)</td>
<td>Six</td>
</tr>
<tr>
<td>Celery in Enzyme</td>
<td>Modulus of Elasticity (MPa)</td>
<td></td>
</tr>
</tbody>
</table>
The Gini coefficient can measure the inequality of a distribution, which is defined as a ratio between zero (perfect equality) and one (perfect inequality); i.e., the numerator is the area between the Lorenz curve and the uniform (perfect) distribution line and the denominator is the area under the uniform distribution line (Figure 4.4). The Lorenz curve is generated when certain members of the data population contribute more to the total property’s values and certain members less. An estimate of the Gini coefficient can be calculated, where the \( P_i \) are the mechanical properties ranked in order from least to greatest, by Equation 1:

\[
G = \frac{\sum_{i=1}^{n} (2i - n - 1)P_i}{n^2 \overline{P}}
\]

Equation 4.1

where, \( G \) = Gini coefficient, \( 0 \leq G \leq 1 \), \( n \) = total number of data readings, \( P_i \) = mechanical properties \( i \)

\( \overline{P} \) = mean mechanical properties
Figure 4.4: Line of equality (45 degree line) and Lorenz curve for Gini coefficient
1. Variation Analysis

1.1 Strain Analysis

Table 4. 10 shows the variation in the analysis of strain for onion and celery. Based on the results, the highest (max) strain was recorded for celery and onion had the lowest (min) strain. The reason may be a stronger fibril in the epidermises structure of celery in comparison to onion epidermis’s. The coefficient of variation analysis (CV %) in celery and celery in enzyme solution had the highest and the lowest CV, at 75% and 28%, respectively, among all. Small numbers for the CV of celery in enzyme solution can be interpreted as the uniformity of data collected after submerging the samples into enzyme solution. The reason might be due to dissolving the pectin and creating a uniform layer of fibrils in the samples’ epidermises.
Table 4. 10: Statistical Analysis of strain for onion and celery

<table>
<thead>
<tr>
<th>Sample</th>
<th>Max (%)</th>
<th>Min (%)</th>
<th>Mean (%)</th>
<th>STDEV</th>
<th>CV%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Onion</td>
<td>16.41</td>
<td>0.09</td>
<td>6.49</td>
<td>3.28</td>
<td>50.49</td>
</tr>
<tr>
<td>Celery</td>
<td>77.40</td>
<td>0.07</td>
<td>7.20</td>
<td>5.42</td>
<td>75.00</td>
</tr>
<tr>
<td>Onion in buffer</td>
<td>16.67</td>
<td>1.26</td>
<td>8.85</td>
<td>4.33</td>
<td>48.96</td>
</tr>
<tr>
<td>Celery in buffer</td>
<td>69.72</td>
<td>0.00</td>
<td>9.65</td>
<td>7.18</td>
<td>74.00</td>
</tr>
<tr>
<td>Onion in enzyme</td>
<td>18.33</td>
<td>0.97</td>
<td>6.70</td>
<td>4.30</td>
<td>64.15</td>
</tr>
<tr>
<td>Celery in enzyme</td>
<td>41.49</td>
<td>0.66</td>
<td>18.04</td>
<td>5.01</td>
<td>28.00</td>
</tr>
</tbody>
</table>

1.2 Stress Analysis

The stress analysis for onion and celery is shown in Table 4. 11. Celery had shown the highest stress (28.46 MPa) of all, where the onion in enzyme solution had the lowest stress (5.98 MPa). The reason may be due to dissolving textures when the tissues are submerged in the enzyme solution which weakens the epidermis. There are no significant variations of CV% among the samples. The range of CV lies between 56% and 59%, which shows no significant difference among the data collected during the test.
Table 4.11: Statistical analysis of stress for onion and celery

<table>
<thead>
<tr>
<th>Sample</th>
<th>Max (MPa)</th>
<th>Min (MPa)</th>
<th>Mean (MPa)</th>
<th>STDEV</th>
<th>CV%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Onion</td>
<td>17.42</td>
<td>0.12</td>
<td>3.74</td>
<td>2.14</td>
<td>57.09</td>
</tr>
<tr>
<td>Celery</td>
<td>28.46</td>
<td>0.21</td>
<td>4.69</td>
<td>2.66</td>
<td>57.00</td>
</tr>
<tr>
<td>Onion in buffer</td>
<td>5.98</td>
<td>0.26</td>
<td>3.12</td>
<td>1.76</td>
<td>56.34</td>
</tr>
<tr>
<td>Celery in buffer</td>
<td>6.36</td>
<td>0.82</td>
<td>1.94</td>
<td>1.14</td>
<td>59.00</td>
</tr>
<tr>
<td>Onion in enzyme</td>
<td>6.16</td>
<td>0.95</td>
<td>10.48</td>
<td>5.95</td>
<td>56.80</td>
</tr>
<tr>
<td>Celery in enzyme</td>
<td>5.99</td>
<td>0.12</td>
<td>3.10</td>
<td>1.74</td>
<td>56.00</td>
</tr>
</tbody>
</table>

1.3 Stiffness analysis

The statistical data for stiffness measurements are shown in Table 4.12. Celery in enzyme solution and onion are shown as the highest and the lowest stiffness values respectively (841 N/m and 197.9 N/m). This can be interpreted that celery and celery in an enzyme solution were shown to possess the highest resistance to deformation in comparison to other samples. Onion had the lowest coefficient of variation (15%), which represents the most uniform distribution of data collected among the onion samples. Overall, based on CV analysis, stiffness data had highly consistent of data in all samples.
Table 4. 12: Statistical analysis of stiffness for onion and celery

<table>
<thead>
<tr>
<th>Sample</th>
<th>Max (N/m)</th>
<th>Min (N/m)</th>
<th>Mean (N/m)</th>
<th>STDEV</th>
<th>CV%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Onion</td>
<td>197.9</td>
<td>91.27</td>
<td>141.55</td>
<td>22.15</td>
<td>15.65</td>
</tr>
<tr>
<td>Celery</td>
<td>525</td>
<td>98</td>
<td>430</td>
<td>30.15</td>
<td>28.12</td>
</tr>
<tr>
<td>Onion in buffer</td>
<td>249</td>
<td>136.50</td>
<td>217.93</td>
<td>37.40</td>
<td>17.16</td>
</tr>
<tr>
<td>Celery in buffer</td>
<td>418.7</td>
<td>1.21</td>
<td>192.57</td>
<td>62.35</td>
<td>32.00</td>
</tr>
<tr>
<td>Onion in enzyme</td>
<td>493.2</td>
<td>219.60</td>
<td>412.00</td>
<td>80.42</td>
<td>19.52</td>
</tr>
<tr>
<td>Celery in enzyme</td>
<td>841.3</td>
<td>19.92</td>
<td>292.29</td>
<td>83.19</td>
<td>28.00</td>
</tr>
</tbody>
</table>

1.4 Displacement analysis

Table 4. 13 represents the statistical analysis of displacement (µm) for onion and celery samples. The maximum displacement was recorded for onion as 5638 µm (or 0.005638 mm), where the onion in enzyme solution showed the least amount of displacement as 1361 µm (or 0.001361 mm). Due to the stronger structure of celery tissue compared to onion tissue, lesser displacements were recorded under identical stresses. Celery in enzyme solution showed the least coefficient of variation (32%) which represents the least disparity of data points among all, while celery in a buffer solution with CV of 82% represents the highest disparity of the displacement test data.
Table 4.13: Statistical analysis of displacement for onion and celery

<table>
<thead>
<tr>
<th>Sample</th>
<th>Max (µm)</th>
<th>Min (µm)</th>
<th>Mean (µm)</th>
<th>STDEV</th>
<th>CV%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Onion</td>
<td>3,011</td>
<td>16.43</td>
<td>1,189.78</td>
<td>600.73</td>
<td>50</td>
</tr>
<tr>
<td>Celery</td>
<td>5,638</td>
<td>8.89</td>
<td>867.46</td>
<td>652.98</td>
<td>75</td>
</tr>
<tr>
<td>Onion in buffer</td>
<td>1,692</td>
<td>128.20</td>
<td>898.14</td>
<td>439.72</td>
<td>48</td>
</tr>
<tr>
<td>Celery in buffer</td>
<td>4,597</td>
<td>0.01</td>
<td>1,025.50</td>
<td>839.74</td>
<td>82</td>
</tr>
<tr>
<td>Onion in enzyme</td>
<td>1,361</td>
<td>72.09</td>
<td>497.46</td>
<td>319.09</td>
<td>64</td>
</tr>
<tr>
<td>Celery in enzyme</td>
<td>2,576</td>
<td>73.40</td>
<td>1,594.43</td>
<td>516.29</td>
<td>32</td>
</tr>
</tbody>
</table>

1.5 Static force

The statistical data for stiffness is shown on Table 4.14 Celery and onion in enzyme solution are shown as the highest and the lowest stiffness respectively (525 N/m and 4.9 N/m). This can be interpreted as celery was shown to have the highest resistance to deformation as compared to other samples. The low stiffness of samples in buffer and enzyme solutions shows that the resistance to deformation is significantly decreased when samples emerge from the solution. Onion had the lowest coefficient of variation (15.65%) which represents the most uniform distribution of data collected among the onion samples. Overall, based on CV analysis, the stiffness data had a high consistency of data collection in all samples.
Table 4. 14: Statistical analysis of stiffness for onion and celery

<table>
<thead>
<tr>
<th>Sample</th>
<th>Max (N/m)</th>
<th>Min (N/m)</th>
<th>Mean (N/m)</th>
<th>STDEV</th>
<th>CV%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Onion</td>
<td>197.9</td>
<td>91.27</td>
<td>141.55</td>
<td>22.15</td>
<td>15.65</td>
</tr>
<tr>
<td>Celery</td>
<td>525</td>
<td>98</td>
<td>265</td>
<td>74.51</td>
<td>28.12</td>
</tr>
<tr>
<td>Onion in buffer</td>
<td>24.9</td>
<td>1.36</td>
<td>17.4</td>
<td>2.98</td>
<td>17.16</td>
</tr>
<tr>
<td>Celery in buffer</td>
<td>41.8</td>
<td>4.21</td>
<td>12.3</td>
<td>3.9</td>
<td>32.01</td>
</tr>
<tr>
<td>Onion in enzyme</td>
<td>4.9</td>
<td>0.29</td>
<td>2.1</td>
<td>0.4</td>
<td>19.52</td>
</tr>
<tr>
<td>Celery in enzyme</td>
<td>8.4</td>
<td>1.01</td>
<td>4.3</td>
<td>1.2</td>
<td>27.09</td>
</tr>
</tbody>
</table>

1.6 Modulus of Elasticity

The Statistical analysis for modulus of elasticity (E) is shown on Table 4. 15. Based on the data, celery and onion in buffer solutions had the most and the least modulus of elasticity, 13 MPa and 0.28 MPa respectively, among all. Onion had the lowest coefficient of variation representing the lowest variation in data analysis among the six replications of modulus measurements. The average CV% among all samples was around 38%, which indicates an acceptable data variation for computing sample's modulus.

Modulus of elasticity will be drastically decreased when onion and celery tissues are submerge in buffer and enzyme solutions. The reason may be due to the effect of the solutions on the tissues' epidermis, in which the pectin layers of cell are dissolved causing low stability of cell wall and a decrease in the modulus of elasticity.
Table 4.15: Statistical analysis for Modulus of Elasticity

<table>
<thead>
<tr>
<th>Samples</th>
<th>Max (MPa)</th>
<th>Min (MPa)</th>
<th>Mean (MPa)</th>
<th>STDEV</th>
<th>CV%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Onion</td>
<td>4.90</td>
<td>0.02</td>
<td>2.11</td>
<td>0.48</td>
<td>23.02</td>
</tr>
<tr>
<td>Onion in Buffer</td>
<td>0.28</td>
<td>0.07</td>
<td>0.18</td>
<td>0.11</td>
<td>57.81</td>
</tr>
<tr>
<td>Onion in Enzyme</td>
<td>0.47</td>
<td>0.13</td>
<td>0.28</td>
<td>0.13</td>
<td>45.88</td>
</tr>
<tr>
<td>Celery</td>
<td>13.00</td>
<td>5.00</td>
<td>7.92</td>
<td>2.40</td>
<td>30.28</td>
</tr>
<tr>
<td>Celery in Buffer</td>
<td>1.25</td>
<td>0.27</td>
<td>0.94</td>
<td>0.45</td>
<td>48.14</td>
</tr>
<tr>
<td>Celery in Enzyme</td>
<td>0.44</td>
<td>0.27</td>
<td>0.35</td>
<td>0.09</td>
<td>24.11</td>
</tr>
</tbody>
</table>

2. Gini Coefficient

Table 8 represents the Gini analysis for various mechanical properties of onion and celery tissues. The data shows that the stiffness had the lowest coefficient, which represents the highest uniformity (highest equality) of data distributions (recorded) for all sample tissues. Celery in buffer solution for strain and displacement had the highest coefficient, (0.42 and 0.42, respectively), which can be interpreted as the least uniform, or highest inequality, of variables for data recorded in the test. Generally speaking, the entire test for Gini coefficients are in the range between 0.07 (93% equality) and 0.42 (58% equality), which represents the satisfactory data record for mechanical property analysis. The static force resulted in an almost identical Gini coefficient for all of the samples. The Gini factors for modulus of elasticity were shown to have high equality of data for measuring the elasticity. The highest and lowest Gini coefficients were measured for onion and celery in enzyme solutions with 0.4 and 0.11, respectively. There
were not significant differences in Gini coefficient among the data collected, which represents a low sensitivity of Gini to mechanical property determination.

Table 4.16: Gini coefficient analysis for various mechanical properties for onion and celery samples

<table>
<thead>
<tr>
<th>Sample</th>
<th>Strain</th>
<th>Stress</th>
<th>Stiffness</th>
<th>Displacement</th>
<th>Static Force</th>
<th>Modulus of elasticity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Onion</td>
<td>0.27</td>
<td>0.30</td>
<td>0.07</td>
<td>0.27</td>
<td>0.30</td>
<td>0.40</td>
</tr>
<tr>
<td>Celery</td>
<td>0.28</td>
<td>0.32</td>
<td>0.08</td>
<td>0.29</td>
<td>0.32</td>
<td>0.15</td>
</tr>
<tr>
<td>Onion in buffer</td>
<td>0.27</td>
<td>0.31</td>
<td>0.08</td>
<td>0.28</td>
<td>0.32</td>
<td>0.18</td>
</tr>
<tr>
<td>Celery in buffer</td>
<td>0.42</td>
<td>0.33</td>
<td>0.08</td>
<td>0.42</td>
<td>0.33</td>
<td>0.20</td>
</tr>
<tr>
<td>Onion in enzyme</td>
<td>0.34</td>
<td>0.32</td>
<td>0.10</td>
<td>0.34</td>
<td>0.32</td>
<td>0.23</td>
</tr>
<tr>
<td>Celery in enzyme</td>
<td>0.22</td>
<td>0.32</td>
<td>0.17</td>
<td>0.22</td>
<td>0.32</td>
<td>0.11</td>
</tr>
</tbody>
</table>

3. Conclusion of statistical analysis

Various mechanical properties of onion and celery, including strain (%), stress (MPa), stiffness (N/m), displacement (µm), static force (N), and modulus of elasticity (MPa), were studied and analyzed. Two different statistical analyses, namely, variation analysis and the Gini coefficient, were used to evaluate the data collection. The following concluding remarks were obtained from this study:
• Enzyme and buffer solutions significantly influence the mechanical properties of onion and celery.
• Celery tissues show higher strength in comparison to onion tissues.
• In stress analysis, there is not a significant variation in coefficient of variation (CV%) among the samples.
• In strain analysis, celery in enzyme solution has the lowest CV% among all, which represents the highest uniformity of data obtained during testing.
• In stiffness analysis, the average CV is around 23% and shows a high consistency of measurement data for the stiffness analysis.
• In static force data, the CV% numbers were consistent among all samples (56%)
• Celery had the highest and onion had the least average modulus of elasticity (E) with 7.92 MPa and 2.11 MPa, respectively.
• The maximum modulus of elasticity for celery and onion were 13 MPa, and 4.90 MPa, respectively.
• The higher modulus of elasticity in celery can be interpreted as a stronger fibril structure in celery in comparison to onion tissue.
• In modulus of elasticity analysis, onion had the least coefficient of variation (23%, or 77% equality), which represents the highest consistency of the data collection among all.
• Enzyme and buffer solutions significantly decreased the modulus of elasticity of onion and celery samples.
• Stiffness analysis resulted in the lowest Gini coefficient among all, which represents the highest consistency of data collected.
• Overall, there are no significant differences among Gini coefficient among mechanical properties for various samples.
• The Gini coefficient was not sufficiently sensitive to measure and differentiate the consistency of data collected in this study.

### 4.3.2 Mathematical Model

Reviews of the structure and composition of primary cell walls have been conducted by Carpita & Gibeau (1993) and McCann & Roberts (1994). The studies hypothesized models of primary wall structures at the molecular scale; however the proposed models are conceptual rather than mathematical. However, the complex structure of polysaccharides can be modeled mathematically with theories for matrix composites or entangled polymers (Bruce et al., 2003). This model allows several levels of structural hierarchy to be connected, resulting in a description of cell wall behavior based on its polymeric nature summarized into a constitutive relation for a continuum material. Hettiaratchi et. al. (1978) described the pressure-volume relation for pressurized spherical and cylindrical cells. Wu et al. (1985,1988) developed work based on the stress-strain relation for a polymeric materials which was previously established by Wu et. al, (1979). In this model, two phases of cell expansion were accounted for; the first happening without the need of stressing the microfibrils and the second as a result of microfibril extension. Chaplin (1993) simplified this model by characterizing the elastic
properties of the isotropic cell wall in terms of a general strain energy function. This function can describe the nonlinear relation between pressure and volume in cell expansion. Veytsman & Cosgrove (1998) modeled plant cell wall extension by using concepts of thermodynamics of polymer mixtures (Veytsman et al., 1998). They showed that macroscopic properties of cell walls are a consequence of the microscopic properties of inter-penetrating networks of cellulose and hemicellulose. The determination of the material characteristics and properties of a cell wall are essential for a mathematical model to be verified. The Young’s modulus of the crystalline domain of microfibrils was measured by applying uniaxial tensile load along the fiber axis and monitoring the lattice deformation along the chain axis using X-ray diffraction (Nishiyama, 2009). The stress on each crystallite is considered to be the same as the stress applied to the macroscopic sample, and the values, based on this assumption, fall in the range of 115-140 GPa (Sakurada et al. 1962, Sakurada et al. 1966, Matsuo et al., 1990, Nishino et al., 1995, Ishikawa et al., 1997).

As discussed previously, the plant cell wall structure consists of three different layers: the middle lamella, the primary cell wall, and plasma membrane. The middle lamella, also called plant cuticle, is a pectin layer which reinforces the cell walls of two adjoining cells. This layer can be assumed to be a waxy, hydrophobic material. The plasma membrane could be neglected in our case. The primary cell wall will be simplified to the two groups of material cellulose microfibrils and hemicelluloses. Hemicelluloses bind, with pectin, to microfibrils to form a network of cross-linked fibers.
In this work, the primary cell wall, in matrix, is simplified to include only the network of cross-linked fibers and hemicellulose protected by a layer of wax, or cuticle. The goal of this study is to measure the change in mechanical properties of a plant cell wall during stretching and observe the change in displacement of microfibrils under such a load using the Finite Element Method (FEM).

Both nanoscale (Figure 4.5A) and macro-scale (Figure 4.5B) models were designed using SolidWorks©, which is 3D mechanical computer-aided drafting (CAD) program, for subsequent analysis in Comsol©, a commercial multi physics FEM package. Macroscale modeling of the epidermis including cell profiles was implemented to simulate the amount of stress imparted on the nanoscale. The epidermal tissue was fixed at one side and distributed stress was applied on the opposite boundary (in the z direction).

One of obstacles with implementing complex geometries is the conversion of CAD drawings to the FEM program solver’s geometry file; in this case specifically, the treatment of the connections between microfibrils made by hemicelluloses. To overcome this problem, the model was simplified as discussed in detail in the following section.
Figure 4.5.1: A) 3D nano-scale geometry of plant cell wall; B) shows the 3D macro-scale geometry of an epidermis including cell profiles. This macro geometry was designed to estimate the amount of stress applied on individual cell profiles in the nanomodel. The tissue was fixed at one side and distributed stress was applied at the opposite boundary (z direction).
4.3.2.1 Theory: Constitutive Equations using Finite Element Method (FEM)

**Linear Analysis:**

Hooke’s Law states that the stress, $\sigma$, and strain, $\varepsilon$, within a deforming material is related by a constant, $E$, which is the modulus of elasticity.

$$\sigma = E\varepsilon$$  \hspace{1cm} \text{Equation 4.2}

This forms the constitutive relations for a linearly elastic material which can be represented in matrix form as

$$\sigma = D\varepsilon$$  \hspace{1cm} \text{Equation 4.3}

Where, $D$, is the Elasticity Matrix, or, more generally, the Elastic Compliance Matrix, $C$, where

$D^{-1} = C$. For a three-dimensional linear, isotropic material, $C$ is given by

\[
D^{-1} = C = \begin{bmatrix}
\frac{1}{E} & -\frac{\nu}{E} & -\frac{\nu}{E} & 0 & 0 & 0 \\
-\frac{\nu}{E} & \frac{1}{E} & -\frac{\nu}{E} & 0 & 0 & 0 \\
-\frac{\nu}{E} & -\frac{\nu}{E} & \frac{1}{E} & 0 & 0 & 0 \\
0 & 0 & 0 & 1 \frac{G}{E} & 0 & 0 \\
0 & 0 & 0 & 0 & \frac{G}{E} & 0 \\
0 & 0 & 0 & 0 & 0 & \frac{G}{E}
\end{bmatrix}
\]

Where, $\nu$, is the Poisson’s ratio and the shear modulus is given by $G = E / (2(1+\nu))$. 


Linear Viscoelasticity Analysis: Generalized Maxwell Model

The most general form of the linear model for viscoelasticity behavior is the Maxwell model or Maxwell–Weichert model. Maxwell assumed (Lin, 2003) that such materials can undergo viscous flow and also respond elastically. Combining Hooke’s and Newton’s laws and assuming that the strains are additive (both elements feel the same stress). The generalized Maxwell model can be represented as a multiple dashpot-spring combination in parallel. The generalized Maxwell equation is present as (Lin, 2003):

\[ \sigma(t) = \sum \sigma_i(t) \quad \text{Equation 4.5} \]

\[ \frac{d}{dt} \sigma_i(t) + \frac{1}{\tau_i} \sigma_i(t) = -\frac{\eta_i}{\tau_i} \dot{\lambda} \quad \text{Equation 4.6} \]

\[ \tau_i = \frac{\eta_i}{G_i} \quad \text{Equation 4.7} \]

Where, \( \sigma \), is the stress, \( \tau \), is the relaxation time of the viscoelastic system, \( G \), is the modulus of the solid, and \( \lambda \), is the shear strain.

The above equations can be written as:

\[ \sigma(t) = -\int_{-\infty}^{t} \sum_i G_i \exp\left[-\frac{(t-t')}{\tau_i}\right] \dot{\lambda}(t') dt' \quad \text{Equation 4.8} \]

and

\[ \sigma(t) = \int_{-\infty}^{t} \sum_i \frac{G_i}{\tau_i} \exp\left[-\frac{(t-t')}{\tau_i}\right] \lambda(t') dt' \quad \text{Equation 4.9} \]
The arrangement of dashpots-springs provides a phenomenological model. We can then write equations 4, and 5 as:

\[ \sigma(t) = -\int_{-\infty}^{t} G(t - t') \dot{\lambda}(t') dt' \]

Equation 4. 10

Where, \( G(t) \), is referred to as the relaxation modulus (Lin, 2003).

### 4.3.2.2 Finite element Model of Plant Epidermis

In order to validate and compare the experimental analysis, Finite Element Model (FEM) was used to simulate the mechanical behavior (microscale) of plant cell wall for onion and celery epidermal peels using Comsol. Comsol Multiphysics is FEM based simulation software for various engineering applications which interfaces with MATLAB, a numerical computation software. The modeling was operated under the assumption of static, linear stress analysis with two types, visoelastic and elastoplastic, of material models. The inputs for Young’s modulus, which measured from experimental analysis for onion and celery, were 3.16 MPa and 9.54 MPa, respectively. The Poisson’s ratio was assumed to be 0.33 for both onion and celery.

The epidermis size was 1.6 mm x 0.8 mm with the thickness of 10 µm and 50 µm for onion and celery, respectively. The parametric study applied to all four sets of models to simulate the displacement with different forces ranging from 0.02 to 0.8 N in the +x direction, while the other side of epidermis remained fixed. The tensile force is in the same direction as static force applied in DMA experiments.
As seen in Figure 4.6 the coarse mesh size was applied to a 2D geometry of the plant epidermis. Figure 4.7, shows the elastoplastic material models for onion epidermis. As seen in the result, the maximum displacement was 1.341 mm and occurred while 0.12 N force was applied in +x direction.

Figure 4.8 shows the comparison between experimental data and two FEM models. The results indicate a good agreement for onion epidermis DMA experimental results and the viscoelastic model. As presented in the graph 4.5, the static force applied by DMA instrument varied from 0.01583 to 0.3992 N, while the measured displacement varied from 72.09 to 1361 µm, which caused the sample to tear apart from the middle section during the experiment using the DMA sample clamp holder. The force diagram illustrated for that sample showed a linear behavior from an applied force 0.01583 N until 0.2492N; after which the behavior becomes nonlinear up until the fracture point (0.3992 N). As presented in Figure 4.8, linear elastoplastic FEM material model showed a change in force from 0.02 to 0.4 N, while the calculated displacement in +x direction varied from 163 to 3620 µm. These models did not appear to match with experimental results on onion epidermal peels.

The viscoelastic FEM material model had very good agreement with the DMA results for onion epidermal peels. As presented in Figure 4.8, the force varies from 0.02 to 0.4 N, which was similar to elastoplastic model; while the calculated displacement in +x direction changed from 75.3 to 1506 µm. As presented in Figure 4.8, the experimental data has a small shift from the model in the applied force which reached 0.009 N. The reason might be due to the linear configuration of model, while the real world sample acts as only semi-linear or even nonlinear.
Generally speaking, the data confirmed that onion epidermis acts more similar to a visoelastic material than an elastoplastic material.
Figure 4.6: Finite element mesh for epidermis model
Figure 4.7: Elasto-plastic material model result for onion epidermis which maximum displacement was around 1.341 mm occurred at 0.12 N force was applied in +x direction.
Figure 4.8: shows the comparison between experimental data and two FEM models. The results show good agreement between onion epidermis DMA data and viscoelastic model.

As presented in Figure 4.9, experimental data for celery epidermal peels had a more nonlinear behavior in comparison to the onion epidermal tissues. The reason can be found in the different structure of celery which was a relatively non-uniform distribution of fibrils compared with onion. Celery epidermal peels had several types of structures, where some consisted of single cell layers and others included multi cell layers. Based on DMA results, the applied static force varied from 0.0267 N to its fracture point of 0.8767 N. The measured
displacement ranged from 60.04 µm to 2964 µm. The sample exhibited a linear behavior from 0.0267 N to 0.0934N, and then became non linear to the point of fracture occurring at 8767 N.

As illustrated in Figure 4.9, the linear elastoplastic FEM model showed a change in force (N) from 0.02 N to 0.6 N, while the calculated displacement in +x direction varied from 2.76 µm to 1777 µm. These models had good agreement with experimental data in the force range of 0.02 N to 0.05 N, and showed a nonlinear behavior beyond the aforementioned forces.

The viscoelastic FEM model showed both a similar trend to the elastoplastic model and a good agreement with DMA experimental results in the linear regime of data. As shown in Figure 4.9, the force varied from 0.02 N to 0.8 N, while the calculated displacement in +x direction changed from 3.15 µm to 1230 µm. In general, the graph showed that celery epidermis had comparable behavior to both viscoelastic and elastoplastic in linear region.
Figure 4.9: Comparison between experimental data and two FEM models. The results showed good agreement between celery epidermis DMA data and both models.

4.3.2.3 Finite element Model of Plant cell wall

As previously explained, nanocomposite structure models were designed using SolidWorks© for analysis in Comsol© 4.0, a commercial multi physics FEM package. To resolve the connection problem, the model was simplified to ten connections inside microfibril networks protected by a layer of cuticle inside a cubic matrix. The connections are made by randomly oriented hemicelluloses. The diameter of microfibrils and hemicelluloses were 15 nm.
and 1.3 nm, respectively. The length of microfibrils before trimming (in Solid Work) was
120nm. The value for Young’s modulus for microfibrils, hemicelluloses, matrix, and cuticle were
assumed to be 30 GPa, 10 GPa, 2.2 GPa, and 2 GPa respectively. The thickness of the
biocomposite plant cell wall structure was 100 nm.

The model was operated under the assumption of static, linear stress analysis. To start
the simulation, one side of the model, the negative z coordinate, was fixed and the opposite
side, the positive z coordinate, was subjected to small load. Figure 4.10 shows the finite
element model’s mesh using Comsol© multiphysics 4.0a. As illustrated in Figure 4.11, a
maximum displacement of around 17.57 µm was observed when the force (0.01N) was applied
at the boundary. A parametric study was applied to the model to show the force change as a
function of calculated displacement in the same direction for plant cell wall microfibrils,
primarily in the first layer. As showed in Figure 4.12, for nanobiocomposite plant cell wall
models, the force varied from 0.01N to 0.5 N, while the calculated displacements changed from
17.57 µm to 878.6 µm. The model was in good agreement with the linear portion of the
experimental data obtained from onion epidermis using DMA analysis. As illustrated in Figure
4.13, the mathematical model is in good agreement in the range from 0.01 N to 0.12 N of
applied. The experimental data possessed a different trend in comparison to the linear model.
Figure 4.10: Finite element mesh for nano biocomposite structure of plant cell wall, including cuticle, microfibrils, hemicelluloses, and matrix.
Figure 4.11: Plant cell wall model (100 nm thick) when 0.01 nN force is applied in z direction.
Figure 4.12: Comparison between plant cell wall nanocomposite model and onion epidermis experimental data
Figure 4.13: Comparison between plant cell wall nanocomposite model and onion epidermis experimental data
In the second parametric case study, the modulus of the matrix was investigated for different orientations of microfibrils. As seen in Figure 4.14, the maximum modulus occurred when the first and third layers were parallel with the orientation of middle layer deviating from this direction to varying degrees. These changes in plant cell wall microfibrillar orientation show an influential role in the change of modulus.

Figure 4.14: Change in modulus as a function orientation of fibrils.
A simplified nanoscale model was designed with only three microfibrils, with the diameter of 15 nm, and two hemicelluloses, which connected them together with the diameter about 1.3 nm, and modulus of 30 GPa and 10 GPa respectively. Figure 4.15 illustrates the finite element model’s mesh using Comsol© multiphysics 4.0a. Figure 4.16 shows three Microfibrils and two hemicelluloses which connect the fibrils together with the last microfibril connected to the cuticle. The force (1nN) is applied to the upper fibril in the \(-y\) direction, and the maximum resulting displacement in \(y\)-direction is 0.017 \(\mu\)m. This model was operated under the assumption of static, linear stress analysis, and the displacement field is shown by red arrows.

A parametric study was applied to the model to show the force changes as a function of calculated displacements in \(y\)-direction, mainly in the first microfibril. The force varied from 500 pN to 2 nN, and the calculated displacement varied from 0.0084 \(\mu\)m (8 nm) to 0.034 \(\mu\)m (34 nm). As shown in Figure 4.17, the result calculated from the FEM model was compared to deformation results obtained by AFM. The importance of the application of AFM is to measure quantitative data of the response of the AFM tip while pushing on the sample. The disadvantage of the FEM model was that it didn’t include the matrix, while in nature microfibrils are surrounded by a matrix. Although the model and experimental data show totally different trend, this comparison could be valuable in the sense of understanding the influence of matrix component on the mechanical behavior of a plant cell wall.
Figure 4.15: Finite element mesh for a simplified nano structure of plant cell wall, including cuticle, three microfibrils, and two hemicelluloses which connect the microfibrils.
Figure 4.16: Three Microfibrils and two hemicelluloses which connect the fibrils. The last microfibril is connected to cuticle. The force (1nN) is applied to the upper fibril in $-\mathbf{y}$ direction, and the maximum displacement in $y$ direction is 0.017 µm.
Figure 4.17: Force vs. displacement graph for nanofibril and compare the results with deformation data obtained by AFM tip.
4.4 Conclusion and Future Work

The macro- and nano- mechanical properties of bio-composite materials were measured, analyzed, and simulated. Onion and celery epidermal peels were used as test materials. The macro-mechanical properties of onion and celery epidermis were measured using Dynamic Mechanical Analysis (DMA) system. There were two sets of conclusion presented in this section. The first group of finding was proof/modifying of the previous research studied which obtained from literature search, and the second group of result was showed an innovative and unique finding in the field of macro and nano-mechanical properties using bio-composite materials.

Modification and confirmation of existing literature studied were presented below:

1) The Young’s modulus of onion and celery ranged from 0.2-4.9 MPa and 5-13 MPa, respectively. This can be interoperated as celery’s fibril structure being stronger in comparison to onion cells;

2) The Young’s modulus of onion and celery in enzyme solutions ranged from 0.13-0.47 MPa and 0.27-1.25 MPa, respectively;

3) The stiffness of onion and celery ranged from 91 to197 N/m and from 98 to 525 N/m, respectively;

4) the stiffness of onion and celery in enzyme solution ranged from 0.29 to 4.9 N/m and from 1.01 to 8.4 N/m, respectively;
New finding were presented as:

1) Enzyme and buffer solutions had a negative influence on the stiffness and the strength of the cells’ epidermis;

2) In the modulus of elasticity analysis, onion exhibited the least coefficient of variation (23% or 77% equality), which represents the highest consistency of data during the experiments;

3) Celery epidermal peels had a more nonlinear behavior than onion epidermal peels;

4) The average coefficient of variation for the stiffness analysis was 23% showing a high consistency of measurements;

5) For onion epidermis the maximum displacement was 1.341 mm and occurred while 0.12 N force was applied in +x direction.

6) Onion epidermis acts more similar to a viscoelastic material than an elastoplastic material

7) Celery epidermal peels had a more nonlinear behavior in comparison to the onion epidermal tissues due to different structure of celery which was a relatively non-uniform distribution of fibrils compared with onion.

8) Celery epidermis had comparable behavior to both viscoelastic and elastoplastic in linear region

9) Model showed the force change as a function of calculated displacement in the same direction for plant cell wall microfibrils, primarily in the first layer.

10) The changes in plant cell wall microfibrillar orientation had an influential role in the change of elastic modulus.
Reference


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