Imaging and quantitative data acquisition of biological cell walls with Atomic Force Microscopy and Scanning Acoustic Microscopy

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This chapter demonstrates the feasibility of Atomic Force Microscopy (AFM) and High Frequency Scanning Acoustic Microscopy (HF-SAM) as tools to characterize biological tissues. Both the AFM and the SAM have shown to provide imaging (with different resolution) and quantitative elasticity measuring abilities. Plant cell walls with minimal disturbance and under conditions of their native state have been examined with these two kinds of microscopy. After descriptions of both the SAM and AFM, their special features and the typical sample preparation is discussed. The sample preparation is focused here on epidermal peels of onion scales and celery epidermis cells which were sectioned for the AFM to visualize the inner surface (closest to the plasma membrane) of the outer epidermal wall. The nm-wide cellulose microfibrils orientation and multilayer structure were clearly observed. The microfibril orientation and alignment tend to be more organized in older scales compared with younger scales. The onion epidermis cell wall was also used as a test analog to study cell wall elasticity by the AFM nanoindentation and the SAM \( V(z) \) feature. The novelty in this work was to demonstrate the capability of these two techniques to analyze isolated, single layered plant cell walls in their natural state. AFM nanoindentation was also used to probe the effects of Ethylenediaminetetraacetic acid (EDTA), and calcium ion treatment to modify pectin networks in cell walls. The results suggest a significant modulus increase in the calcium ion treatment and a slight decrease in EDTA treatment. To complement the AFM measurements, the HF-SAM was used to obtain the \( V(z) \) signatures of the onion epidermis. These measurements were focused on documenting the effect of pectinase enzyme treatment. The results indicate a significant change in the \( V(z) \) signature curves with time into the enzyme treatment. Thus AFM and HF-SAM open the door to a systematic nondestructive structure and mechanical property study of complex biological cell walls. A unique feature of this approach is that both microscopes allow the biological samples to be examined in their natural fluid (water) environment.

**Keywords:** Atomic Force Microscopy (AFM); High Frequency Scanning Acoustic Microscopy (HF SAM); nanoindentation; \( V(z) \) signature curves; plant cell walls

1. Plant Cell walls

Plant cell walls serve a variety of important biological functions for plants, such as providing mechanical support and determining their size and shape. They have also been an interesting subject for engineering applications. Plant cell walls are essential in various commercial products such as paper, textile, plastic, etc. Most recently, they have gained intensive attention as potential sources for biofuel. Also, the sophisticated composite structure, naturally occurring in cell walls, inspires innovative designs of engineering materials. All these applications require understanding of the composite structure of cell walls and their mechanical properties.

Primary cell walls are formed during the growth and division of plant cells. They provide mechanical support for the cells and can expand to allow cells to grow. Primary plant cell walls are polysaccharide-rich complex structures. The wall contains three main components: cellulose, hemicelluloses and pectin. Cellulose microfibrils are embedded in a highly hydrated polysaccharide matrix which consists of hemicelluloses and pectin.[1-3] Cellulose is comprised of many parallel chains of 1,4-glucan which make cellulose crystalline, strong and indigestible. Cellulose microfibrils serve as reinforcement material of cell wall composite. Cellulose microfibrils are several nm in thickness and have varied length. Hemicellulose is also a polysaccharide, but it is typically made up of chains of xylose interspersed with side chains containing arabinose, galactose, mannose, glucose, acetyl, and other sugar groups, depending on the plant type. [4-6] Hemicelluloses separate microfibrils from each other but may tether them together into a cohesive network. Pectin is a gel phase that embeds the cellulose-hemicelluloses network. Pectin includes relatively simple polysaccharides and becomes soluble with mild treatments such as boiling water or mildly acidic solutions. [7]

2. Atomic Force Microscopy

AFM was invented by Binnig, Quate and Gerber in 1986.[8] AFM can image topography and obtain structural and mechanical information from samples’ surfaces at micrometer to nanometer levels. Most AFM can work in air, liquid or vacuum environment to gather three dimension topographic images of the sample surface without complicated sample preparations. This technique can be applied to different types of materials including semiconductors [9, 10], biological systems [11-13]and polymers [10, 14-16]. Another important usage of AFM is to study surface properties as the force spectroscopy by measuring forces as a function of distance.[17-20] AFM has been proven to be an effective tool for quantitative studies of various surface properties, and has been used in some studies to measure surface adhesion.
forces. AFM has become a powerful and promising tool in the development of micro-systems and nanotechnology.

AFM has been used for more than a decade to examine the plant cell wall structures. However, these studies imaged dehydrated or partially dehydrated cell wall samples where surface structure during drying may greatly change. In our work, fully hydrated epidermis cell walls were examined in peak force tapping mode in liquid environment. The results reflect the properties of cell walls in their natural state.

2.1 AFM Imaging of hydrated cell walls

Onion abaxial epidermis walls and celery epidermis cell walls were prepared for AFM imaging as described by Zhang et al. Freshly peeled cell walls were fixed onto glass slides with edges glued. The epidermal strips were then immersed in a phosphate buffered saline solution (pH 7.4) containing 0.1% Tween-20 for 1 h. After that, the samples were rinsed with distilled water. Epidermal wall strips were probed by AFM (Veeco Dimension ICON from Bruker) in Peak Force QNM fluid mode. Nanoscope (v 8.10b44) was used to control AFM operation and Nanoscope Analysis (v 1.40) was employed for image processing. Figure 1 shows a top view of AFM tip engaged in epidermis cell walls.

![Fig. 1](image1.png) A typical experimental view during imaging and nano-indentation of hydrated onion cell wall with AFM. Magnification 50X.

Figure 2 shows the typical topography images of onion epidermis wall and celery (Apium graveolens L.) parenchyma cell walls. In the topography images, the nm-wide cellulose microfibrils orientation and multilayer structure were clearly observed. In our previous work of imaging epidermis cell wall of different onion scales, the AFM images showed that cellulose microfibrils exposed at the innermost surface of the abaxial epidermis are oriented perpendicular to the bulb axis in the outer scales and more dispersed in the inner scales of onion bulb.

![Fig. 2](image2.png) AFM images of the abaxial epidermal cell wall of a) the onion scale and b) celery epidermis walls. a) 1μm square and b) 500 nm square of the wall surface clearly show cellulose microfibril and matrix detail.

2.2 AFM based nanoindentation

Plant cell walls are complex composites which require delicate instruments and special techniques to study their structures and properties. The analysis of micro- or nano- mechanical properties of plant cell walls has become increasingly important in understanding cell wall structure and cell growth. Because of its micro-scale tip size, indentation technique can investigate the mechanical properties of thin, small and heterogeneous materials. Nanoindentation has been a new application tool on cellulose fibers and plant cell walls. Hardness and Young’s modulus of spruce cell-wall, bamboo cell walls, individual wood fibers and crop stalks cell walls were examined. These experiments were done with add-on force transducers or special indenter testing systems. However, in traditional nanoindentation experiments, samples were dehydrated and many were embedded in epoxy resin. Such sample preparations may cause undesired modifications and influence the mechanical properties of the cell walls.

Recent work has used an improved nanoindentation technique to measure the mechanical properties of single cells or tissue. The indentation depth is comparable to or larger than the cell wall thickness. The indent force is in a range of 1–100 μN. The nano-indentation with an AFM has been recently applied to living plant cells in conditions close to natural. Mechanical properties of suspended grapevine cells cultured in liquid medium, the primary cell wall of shoot apical meristems, rosette leaves, tomato cells and epidermal cells of living roots of Arabidopsis thaliana were measured.
using AFM based nanoindentation.\[36-40\] The indentation depth is much lower (about 100 nm) in most of these studies. However, the experimental samples contain living cells with different turgor pressure which has large influence on measured results. No conclusive results were reached on the cell mechanical properties with the effect of turgor pressure.\[41, 42\] The aim of this mini-review is to focus on the elastic properties of cells walls isolated from onion scales using AFM based nanoindentation and scanning acoustic microscopy in order to assess the feasibility of these methods for further study of plant cell walls. In this review, the focus is further on the freshly peeled single-layer onion abaxial epidermis wall tested in liquid mode. The samples are independent of the turgor pressure influence and reflect only the properties of hydrated cell wall in its natural condition.

With its piconewton force sensitivity and nanometer displacement accuracy, the AFM has been recognized as a promising tool for studying materials properties. AFM with force-distance curve measurement for nanoindentation has emerged as a useful tool measuring the elastic moduli of biological samples. An AFM force-distance (F-D) curve is a plot of tip-to-sample forces versus the extension of the piezoelectric scanner measured with a position-sensitive photo detector. The F-D curve provides information of important mechanical properties such as adhesion, contaminants, viscosity, and local variations in the elastic properties of the surface.

In experiments, F-D curves are quite complex and vary especially with different samples. For the experiments discussed here, the inner side of plant epidermis cell walls and the tip are both negatively charged in water, so there is no adhesion in F-D curves. Figure 3 shows a representative F-D curve obtained on onion epidermis cell wall in liquid.

![Fig. 3](image)

Fig. 3 A representative F-D curve (approach and retract) obtained in the experiment on onion epidermis cell wall in liquid. The y axis is the recorded force in nano-newtons and the x axis is the relative piezo displacement in nm.

The Hertz model is used as the theoretical model for F-D curve analysis. The Hertz model assumes that the sample is isotropic, elastic and occupies infinite half space. It also assumes that the indenter is not deformable and there is no additional interaction between the tip and sample.\[43\] The elastic modulus $E$ can be obtained by a fit to the experimental F-D curve using the Hertz model. The F-D curve obtained by AFM nanoindentation is in fact a force-piezo displacement curve. To understand the force – sample deformation relationship, several specific parameters and equations are introduced.

As shown in Fig. 4, when using the AFM tip to indent the sample, the recorded piezo displacement $D$ is comprised of two parts: the tip cantilever bending $x$ and the sample indentation $\delta$. As shown in Fig. 4, the cantilever is moved towards the sample by a carefully measured distance $D$.

$$D = x + \delta \quad (1)$$

According to Hertz model, the measured force based on a four-sided pyramid can be represented as \[44\]:

$$F = \frac{E}{1-\nu^2} \frac{\tan \alpha}{\sqrt{2}} \delta^2 \quad (2)$$

where $F$ is recorded force, $E$ is sample’s elastic modulus, $\nu$ is sample’s passion ratio, $\alpha$ is geometry angle of the indenter. In our analysis, $\nu$ takes the value 0.3 and $\alpha$ is estimated as 13 according to the parameters given by the manufacturer.

Knowing that $x = F/k$, where $k$ is the cantilever spring constant, from the above two equations, we can easily obtain relationship between recorded force $F$ and recorded piezo displacement $D$:

$$F = \frac{E}{1-\nu^2} \frac{\tan \alpha}{\sqrt{2}} \left( D - \frac{F}{k}\right)^2 \quad (3)$$
By fitting the experimental F-D retrace curves, we can get the sample’s elastic modulus $E$. Figure 5 shows a sample F-D curve fitting result. The experimental F-D retrace curve and fitted curve from the Hertz model are in good agreement.

Fig. 5  A sample of F-D curve fitting. Experiment data in blue dots and fitted data in the red curve from the Hertz model showed good agreement.

2.3 Thickness of hydrated cell walls

For nanoindentation experiments, substrate influence on the measured results is negligible when the sample deformation is less than 10% of sample thickness. The cell wall thickness was measured from scales of three different bulbs to make sure the accuracy of the nanoindentation result. The onions used in this study typically contained twelve scales. The outermost fresh scale will be referred as the 1st scale with the inner scales numbered sequentially through this paper. In the sample preparation, the never-dried cell wall thickness of the abaxial epidermal cells is typically determined with optical microscopy. The onion scale is hand-sectioned tangentially under a dissecting microscope (Olympus). Sections from each of the 4 scales are stained with 0.1 % toluidene blue for 1 min at room temperature on a slide followed by rinsing with deionized water and imaged with an optical microscope (EVOS, AMG). The thickness of different scales is shown in Table 1. The sample deformation is typically less than 150 nm so the substrate influence does not affect the accuracy. The F-D curve generating positions are in the middle of the cells thus avoiding area with vertical adjacent walls.

<table>
<thead>
<tr>
<th>Onion scales</th>
<th>Cell wall thickness ($\mu$m) AVG±SEM (n=3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>11.5±0.7</td>
</tr>
<tr>
<td>5</td>
<td>6.1±0.9</td>
</tr>
<tr>
<td>8</td>
<td>3.4±0.7</td>
</tr>
<tr>
<td>11</td>
<td>2.4±0.7</td>
</tr>
</tbody>
</table>

2.4 Moduli of different onion scales

The scales of onion contain primary cell walls at different growing stage, with inner scales being younger and outer scales being older. Elastic moduli ($E$) of the four scales (11th, 8th, 5th and 2nd scales) in different onions were measured by AFM nanoindentation. 100 curves in 5 random cells in each scale were generated and analyzed. The results are shown in Fig. 6. The error bar shows sample standard deviation. Changes in moduli are statistically significant. Previous research of onion epidermis scales indicate the orientation of cellulose microfibrils variation tendency along scales.[29] Our moduli results indicate the elastic properties increase from inner/younger to outer/older onion scales correspondingly. The plant cell walls become stronger as they rearrange the cellulose microfibrils alignment during growth.

As shown Fig. 6, the bulb-to-bulb moduli variation was larger than the scale-to-scale variation for the same bulb. It is understandable that onion bulbs would have different properties due to different growth environments and status. In our subsequent experiments, the “same” samples were measured avoid original modulus variation.
2.5 Onion epidermis wall with pectin aimed chemical treatment

2.5.1 EDTA effect

In the first chemical treatment, 100 mM EDTA was added onto the cell wall sample taken from onion scale 5. This treatment is known to partially remove the EDTA-soluble pectin from the epidermis walls.[45, 46] Topography images were generated in the same area in water and after adding the EDTA solution. 80 F-D curves were probed in the sample area. Topography images and elastic moduli results are shown in Figure 7.

Topography images became clearer with time after adding in 100 mM EDTA solution. Elastic modulus decreased slightly and remained stable with time after adding EDTA solution. The variation is statistically significant (p=0.04). Elastic modulus decrease with EDTA treatment is probably due to the removal of EDTA-soluble pectin.

2.5.2 Calcium ion effect

In the second chemical treatment, the Calcium ion effect was explored. 100 mM CaCl₂ solution was added onto the cell wall removed from onion scale 5. Topography images were generated in the same area in water and after adding CaCl₂ solution. 120 F-D curves were generated in the same area. Topography images and elastic moduli results are shown in Fig. 8.
Topography images showed apparent changes after adding 100 mM CaCl2 solution. Pectin, especially homogalacturonan (HG) is negatively charged under cell wall physiological pH (around 5.0), Ca2+ serve as a bridge that cross-links the HG chains and increase the regional density of the cell wall surface, which adds rigidity to the cell wall.[47, 48] Due to the formation of gelation structures, elastic modulus increased dramatically after adding CaCl2 solution and the standard deviation increased significantly on the calcium ion treated sample. The topography and elastic modulus remained relatively stable with time.

3. High Frequency Scanning Acoustic Microscopy (HF SAM)

In contrast to Scanning Electron Microscopy (SEM), Optical or Scanning Laser Confocal Microscopy (SLCM), high frequency scanning acoustic microscopy (HF SAM) is capable of imaging not only the surface but also the shallow subsurface regions of materials with high resolution [1-9]. For example, at 500 MHz the resolution in water at Standard Temperature and Pressure is about 1.5 µm. For the focus below the surface of a biological specimen the resolution is approximately 2 micron depending on the acoustical properties of the specimen. The image contrast is based on at least two mechanisms: (1) the contrast resulting from the dissimilar acoustic impedances of the different polymers and (2) the contrast due to absorption of acoustic waves in the material. Conventional acoustic microscopy typically operates in the 20 MHz to 200 MHz range with the use of focused transducers operating in the pulse wave mode. By contrast, HF SAM operates in the 0.4 GHz to 2 GHz range in the long tone burst mode which allows correspondingly much higher resolution [49]. More importantly, the high numerical aperture lens when brought close to the surface produces leaky surface acoustic waves which are sensitive to the presence of changes in structure and materials and mark them in high intensity contrast. This paper provides a description of the HF SAM and a review of the technique’s contrast mechanism and associated simulations involved in data analysis as it is applied to the mechanical characterization of plant cell walls subjected to enzymatic removal of pectin.

3.1 HF SAM Imaging

The HF SAM uses a mechanically scanning, acoustic lens system operating in a pulse-echo reflection mode. The Scanning acoustic microscope provides a non-destructive method for studying the surface/subsurface microstructure of nontransparent solids or biological materials. In scanning acoustic microscopy, a sample is examined by ultrasound waves, and the variation of wave propagation generates the contrast maps which are used to form the resulting image. Mechanical wave propagation and scattering is affected by the structure and elastic properties of the sample.

The working principle of the HF SAM can be described as below: As shown in Fig.9, a transmitter generates an electric signal (usually a tone burst waveform) which travels into a piezoelectric transducer located on the top of a sapphire buffer rod. A circulator limits the signal transmission to one direction: from the transmitter to the transducer or from the transducer to the receiver. The piezoelectric transducer is applied for the electro-acoustic conversion for the transmitter and receiver. The outgoing electric signal is converted to an acoustic plane wave by the transducer operating in transmission mode, and this plane wave is focused into an ultrasonic beam by a spherical or cylindrical lens at the end of the buffer rod. The ultrasonic beam is then transmitted through the fluid buffer, usually de-ionized water, into the...
sample. The wave travels through the sample at the material’s velocity; part of the signal is reflected by the sample and travels back through the lens. The transducer, now in receiving mode, converts the reflected ultrasonic signal into an electrical signal which is collected by the receiver. The returning signal’s amplitude or phase is recorded and modulated on a monitor to show the image of the focal area. Variations in the mechanical properties elicit changes in wave propagation which affect the amplitude and phase of the reflected signal from the sample forming the contrast for features on the grey-scale image. The operating frequencies of HF SAMs are between 400 MHz and 1 GHz with most at 400 MHz. Higher frequency lenses provide more accurate measurement results with a resolution of up to 1 µm at a depth of 10 µm. 600 MHz was found to give optimum results for imaging. For the $V(z)$ measurement the choice of 400 MHz as the operating frequency was based on experience and a practical compromise between resolution and depth of penetration of the waves into the specimen. The parameters of the acoustic lens are shown in below in Fig. 9. The specimen is located in a container filled with coupling medium. The temperature of the coupling medium (i.e., distilled water) is monitored by a thermocouple. The temperature is stabilized at $20.0^\circ C$ (change less than ±0.1°C) in our experiments.

![Fig. 9](image)

**Fig. 9** a) Schematic diagram of the HF SAM and b) Diagram of 400 MHz Acoustic Lens (Olympus, Model: AL4M631). The dimensions in the figure are in cm.

This study focuses on the primary cell wall of onion epidermis as specimens. Typically, the plant epidermis was bathed in 1x PBS (Phosphate Buffered Saline) solution with 0.05% Tween 20 for 1 hour. The edges of the sample were glued to a clean glass slide while the internal part remained intact and free. During experiments, water was used as the buffer liquid so the samples were kept hydrated. Acoustic images were obtained before $V(z)$ measurement. In Fig. 10 are displayed typical HF SAM images of onion epidermal walls at a frequency of $f = 600$ MHz and at two different scales. The white lines in (a) are identified as cuticle structures located between adjacent cell walls making up the risers in the “shoe-box-like” structure.

![Fig. 10](image)

**Fig. 10** Typical acoustic images of onion epidermal walls at a frequency of 600 MHz. The white lines are identified as cuticle structures located between adjacent cell walls making up the risers in the “shoe-box-like” structure. In (a) the scan width is 1 mm, whereas in (b) the scan width is 0.5 mm.

### 3.2 $V(z)$ measurement with HF SAM of Plant Cell wall

In addition to contrast variations provided by elastic properties of materials, another important usage of high frequency HF SAM is measuring the velocity of surface acoustic waves. Atalar, Quate, and Wickramasinghe established that the amplitude of the output voltage $V$ as a function of lens-to-sample distance $z$ "has a characteristic response that is dependent upon the elastic properties of the reflecting surface". [50] Later Weglein reported the periodicity of dips in the
The periodicity of the $V(z)$ curve was related to surface wave propagation. Parmon and Bertoni proposed a simple formula for determining the SAW velocity from $V(z)$ curves measurement using a ray model.[52] Wave velocity in the sample derived from the $V(z)$ curve can be calculated according to Atalar’s model.[53] Following well-known procedures to obtain, measure, and interpret $V(z)$ curves, the longitudinal wave velocities were obtained by implementing a computer parameter-fitting technique. The algorithm of the $V(z)$ curve simulation is described in the following steps. First, initialize parameters of acoustic lens, specimen (thinly sectioned biological tissue), and soda-lime substrate. Second, calculate parameters of acoustic field at the back focal plane, pupil function of the lens, and reflectance function. Third, calculate and draw the $V(z)$ curve. The velocity of the longitudinal wave velocity of water was set as 1,487 m/s. Based on the previous research study, the speed of sound in most plant cells is quite constant and close to that of water, with ultrasound velocities are in the range of about 1600 m/s. Therefore the longitudinal velocity of the tissue (thickness: 8$\mu$m) was set to a range from 1540 m/s to 1650 m/s. Moreover, the plant cell wall density is set within a range of 1.3 – 1.6 (10$^3$ kg m$^{-3}$). A Soda-lime glass slide was chosen as the substrate, its velocities of longitudinal and shear waves are 6,000 m/s and 3200 m/s respectively. The summarized physical parameters of biological tissue for computer simulation are shown in Table 2.

<table>
<thead>
<tr>
<th>Lens</th>
<th>AL4M350</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liquid</td>
<td>De-ionized Water</td>
</tr>
<tr>
<td>Temperature of Water</td>
<td>20ºC</td>
</tr>
<tr>
<td>Substrate</td>
<td>soda-lime glass</td>
</tr>
<tr>
<td>Radius of Lens</td>
<td>577.52 $\mu$m</td>
</tr>
<tr>
<td>Wave Length in Water</td>
<td>3.725 $\mu$m</td>
</tr>
<tr>
<td>Longitudinal Velocity of Tissue</td>
<td>1540 – 1650 m/s</td>
</tr>
<tr>
<td>Density of Tissue</td>
<td>1.4 g / cm$^3$</td>
</tr>
<tr>
<td>Thickness of Tissue</td>
<td>5.00 – 8.00 $\mu$m</td>
</tr>
</tbody>
</table>

Changing the values of the longitudinal wave velocity and the thickness, the simulations were continued until the periodicities in the interference pattern were nearly coincident with the experimental values. Figure 11 shows a typical experimental $V(z)$ curve generated on an onion epidermal wall, as well as the matching curve from simulation. The vertical axis is the intensity of the received signal. The horizontal axis is the defocusing distance of the lens. $z=0$ represents the position of the lens when it is focused on the top surface of the specimen. The curves are obtained as the lens is moved deeper into the interior of the specimen. The longitudinal wave velocity in epidermal wall was calculated as 1628 m/s. Ten curves were measured in different positions along the sample surface. The wave velocity resulting from simulation was found to be 1626 ±3 m/s.

The sample material longitudinal velocity $V_L$ is related to the elastic modulus with the relationships below:

$$V_L = \sqrt{\frac{K}{\rho}}$$  \hspace{1cm} (4)

where $V_L$ is the longitudinal wave velocity in the sample, $K$ is the sample material bulk modulus which is related to the Young’s modulus $E$ by:

$$K = \frac{(1+\nu)(1-2\nu)}{1-\nu}E$$  \hspace{1cm} (5)
where $\nu$ is the material Poisson ratio. Then the elastic modulus $E$ can be presented as

$$E = \frac{1-\nu}{(1+\nu)(1-2\nu)} \rho V_s^2$$  \hspace{1cm} (6)

According to Eq. (6), the elastic modulus $E$ could be calculated as

$$E = 3.3 \text{ GPa}$$

using a standard assumed value for Poisson’s ratio and density, respectively, of $\nu=0.3$ and $\rho=1.4 \text{ g/cm}^3$.

Those values are in approximate agreement with the modulus values in the literature. HF SAM measures elastic properties at the macroscopic level. The macroscopic properties incorporate the mechanical composition of a sample over a distance proportionally large in comparison with the dimension of cellulose microfibrils and bundles. The $V(z)$ measurement is therefore, potentially more sensitive to visco-elasticity of the plant cell wall as a composite as compared to for example AFM techniques. AFM techniques do have the ability to measure the viscoelastic response of a single microfiber embedded in the plant cell wall matrix, however, the loss of the leaky surface acoustic wave resulting from a $V(z)$ measurement over millimeters of the surface of a sample is significantly more apparent.

3.3 Enzyme treatment

The $V(z)$ curve was also used to study the influence to the mechanical properties of the plant cell wall by using pectinase to remove the biopolymer pectin, which is one of the important structural components of a plant cell wall. Ten to twenty units of Pectinase were mixed with 1mL of NaOAc buffer and 100 to 150 microliters of the mixed liquid was applied to the prepared onion abaxial epidermis sample during the experiment. The $V(z)$ measurements were taken before the enzyme treatment, directly after the enzyme was applied, and at elapsed increments of 10, 30, 60, and 180 minutes after the enzyme was applied. Figure 12 shows the $V(z)$ curves of onion skin under the enzyme treatment with time. The gradual decrease of the amplitude at $z=0$ is obvious as a function of time, which means that less acoustic energy is reflected back to the transducer from the sample surface. Under the action of the enzyme, the biopolymer pectin was moved away from the cell wall, which changed the cell wall structurally. More acoustic energy was absorbed by the cell wall loosened by the removal of the supporting pectin. To illustrate the peak amplitude change more clearly, Fig. 13 shows the relative peak value of the $V(z)$ curve at $z=0$ of as a function of time under enzyme treatment.

![Fig. 12](image)

**Fig. 12** $V(z)$ curve of onion skin under enzyme treatment. Notice the gradual decrease of the surface echo $V(z=0)$ in amplitude as a function of time.

The control experiment in water was undertaken to compare with the enzyme treatment experiment. All the steps and measurements were the same except instead of using enzyme, water was applied. Figure 13 shows the measured $V(z)$ curves peak value at $z=0$ of onion epidermis scale in water. It shows that the peak amplitude at $z=0$ of different times remain at a static value with small amount of variation in water. This means the structure and mechanics of the cell wall remained unchanged during the experiment.
4. Discussion and Conclusions

In summary, this mini-review presents an educational description of two methods in advanced microscopy. The two methods stand out from other microscopies in that they are used with water-immersed samples. The two methods are acoustic microscopy for microscale resolution and atomic force microscopy for nanometer resolution. The two methods are not restricted to but are demonstrated for biological samples, in particular plant cell walls, which are currently of particular interest. The interest arise in the desire to understand the composition and properties of plant cell walls which are the fundamental building blocks for all natural plants ubiquitous around the globe and are therefore of great importance to mankind and civilization.

In the feasibility demonstration of atomic force microscopy (AFM) measurements on single layered, fully hydrated onion epidermis cell walls are used as test analogs. In the tests the AFM based nano-indentation method is described to observe changes in the elastic properties of onion epidermis cell walls. In particular, the effects on the cell wall modulus by different chemical treatments on pectins in the cell wall structure are shown. The results demonstrate that this approach can effectively track variations in cell wall properties and thus show the feasibility of AFM based nanoindentation as a tool to characterize - nondestructively – intact fully hydrated plant cell walls.

In the observations the AFM is shown to provide both imaging and quantitative elasticity measuring capabilities. The influence on cell wall elasticity when pectin is chemically modified was examined. Pectin has long been suspected of playing an important role in cell wall mechanics. Two chemical methods were used to modify the pectin network: Ca\(^{2+}\) to cross-link the pectin network and EDTA to partially remove pectin. Ca\(^{2+}\) treatment caused the formation of cross-linked pectin gelation structures, which can be clearly observed in the topography images. Correspondingly, the elastic moduli were found to increase dramatically. On the other hand, the topography images after the EDTA treatment became clearer as a result of partial pectin removal. Correspondingly, the moduli decreased, indicating the weakening of pectin structure. This verifies that the pectin network makes significant contribution to cell wall property. Based on these findings the conclusion can be drawn that the AFM based nanoindentation method is a viable technique and thus opens the door to a systematic nondestructive examination and evaluation of complex cell wall structures in the future for education and research.

The objective of this mini-review is also to demonstrate the feasibility of High Frequency Scanning Acoustic Microscopy (HF SAM) as a bio-technological tool to characterize biological tissues, in particular primary plant cell wall tissues. Currently, there is interest in the structure of cellulose micro-fibrils in primary cell walls from celery collenchymas.[26, 54] A combination of x-ray and neutron scattering methods with vibration and nuclear magnetic resonance spectroscopy were found useful in the elucidation of many details on the micro-fibril structure. These observations were all obtained with a dry primary wall structure. In contrast, the approach using the HF SAM is shown to provide both imaging capability and quantitative measurements of stiffness in the natural wet state of plant cell walls.

The plant cell wall is used as a test analog to demonstrate the influence on stiffness when one important structural component is removed from a complex polymer structure. In particular, this work tested whether the biopolymer pectin has a strong effect on the mechanical properties of primary plant cell walls. Onion epidermis were used as examples because they are thought to contain about 42% of pectin. The mechanism for the effect on the elastic wave propagation is thought to be the viscous absorption by the plant cell wall. The bio-technical approach is to use HF SAM to document the effect of pectinase enzyme treatment to remove pectin from onion primary cell wall. The results indicate a significant change in the $V(z)$ signature with time after enzymatic pectin removal. Thus the HF SAM method opens the door to a systematic study of complex polymer structures.
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