



## Spectroscopy and atomic force microscopy of biomass

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### ARTICLE INFO

#### Keywords:

Atomic force microscopy Spectroscopy

Plant cells

Biomass

Nanomechanics

### ABSTRACT

Scanning probe microscopy has emerged as a powerful approach to a broader understanding of the molecular architecture of cell walls, which may shed light on the challenge of efficient cellulosic ethanol production. We have obtained preliminary images of both *Populus* and switchgrass samples using atomic force microscopy (AFM). The results show distinctive features that are shared by switchgrass and *Populus*. These features may be attributable to the lignocellulosic cell wall composition, as the collected images exhibit the characteristic macromolecular globule structures attributable to the lignocellulosic systems. Using both AFM and a single case of mode synthesizing atomic force microscopy (MSAFM) to characterize *Populus*, we obtained images that clearly show the cell wall structure. The results are of importance in providing a better understanding of the characteristic features of both mature cells as well as developing plant cells. In addition, we present spectroscopic investigation of the same samples.

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### 1. Introduction

One of the current global challenges is the development of new sources of energy. In the quest for new sustainable energy sources, which would be renewable and economically competitive and could provide a substantial percentage of the resources in the near decades, cellulosic ethanol seems to take the lead. Recent interest in lignocellulosic biomass towards providing a new source of energy, which would offer a renewable, clean and carbon free reliable alternative to existing technologies (oil, fossil fuels for example), has prompted extensive research efforts to overcome existing challenges [1]. The goal is to overcome the world challenges posed or influenced by the shortcomings of energy supply. A significant amount of work targeting the various properties of biological tissues at different system-levels (molecular, cellular or as a whole) has been reported [2–5].

Due to the diversity of biological samples that can be studied by the various modalities of the atomic force microscope (AFM) [2,6], specific information on biomass characterization appears scattered throughout the literature. The global initiatives for acquiring alternative energy resources [7] have intensified research efforts to understand the morphological and chemical characteristics of the biomass [8]. Therefore, in this article we begin with a short review of the relevant biomaterial and AFM techniques that are of current importance and practice.

The recalcitrance of lignocellulosic material is a major constraint on the advancement of efficiency to create biofuel from lignocellulosic materials. The plant features contributing to natural recalcitrance of biomass to breakdown into simple sugars are designed to protect the plant from various abiotic and biotic stressors at various levels. Such features are found in the epidermal (bark) tissue, vascular bundles, primary cell walls (PCW), secondary cell walls (SCW), and genes with associated proteins. For example, cellulose is the most abundant biopolymer; however production of cellulosic ethanol is currently not straightforward. It is known that structural heterogeneity and complexity of plant cell walls, insolubility of substrates and certain wall constituents (such as hemicellulose, phenolic and acetylated compounds) inhibit wall deconstruction and fermentation during the treatment process [9]. Much of the specifics, however, are unknown due to limitations of existing characterization approaches.

Although its potential is now acknowledged, a better understanding of biomass morphology, structure, as well as chemical composition, at the micro- or nano-scale is necessary in order to reach an optimization of the production processes. Optical microscopy combined with chemical staining is commonly used to study samples extracted from plants [10–12]. Advances in scanning electron microscopy (SEM) provided higher resolution information of plant samples [12]. Currently, SEM and TEM are used to characterize lignocellulosic material at the nanoscale. While optical micrographs depict the variety of cell sizes present in the samples, SEM and transmission electron microscopy (TEM) images reveal the microfibrils and successive layers within the cell walls [12–17]. Recent advances in environmental SEM

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electron microscopy have been very important in analyzing lignocellulosic materials [13]. Indeed the conditions of operation of this type of techniques have long caused major drawbacks to their application in biology since they require the samples to be conductive, and the electron beam often damages the samples. Another major technique used to obtain atomic resolution images of biomass samples is AFM [18,19]. In their study of cellulose structure, Baker et al. [20] reported near-atomic resolution of the structure of cellulose corresponding to the model of the  $\alpha$  crystal phase. The strength of AFM-based techniques consists of its capacity to image the samples in their natural environment.

A greater understanding of the plant cell wall architecture is necessary to improve lignocellulosic feedstock properties. The molecular architecture of the plant cell wall is still a largely underexplored area. The lack of a complete comprehensive model for plant cell walls at the molecular level is a major drawback. Plant cell walls are three-dimensional nanocomposites. They constitute a dynamic mixture of multifunctional components, assembled into an intricate network of mixed sugars, such as cellulose and hemicellulose, of lignin, and other less abundant compounds, such as pectins, proteoglycans, etc. Variations in the environment of the plants (temperature, humidity, stress, etc.) have a great influence on the composition of the cells. The present gap in understanding the structure of such systems results from the significant lack of tools to access the information at the nanoscale level by nondestructive means (Fig. 1). The composition of lignin and its interactions with cellulose need to be further characterized.

In this report, we present results obtained using a variety of lignocellulosic materials (namely *Populus* and switchgrass) to establish how AFM and MSAFM [21] should be part of the characterization tools that will contribute to the development of a

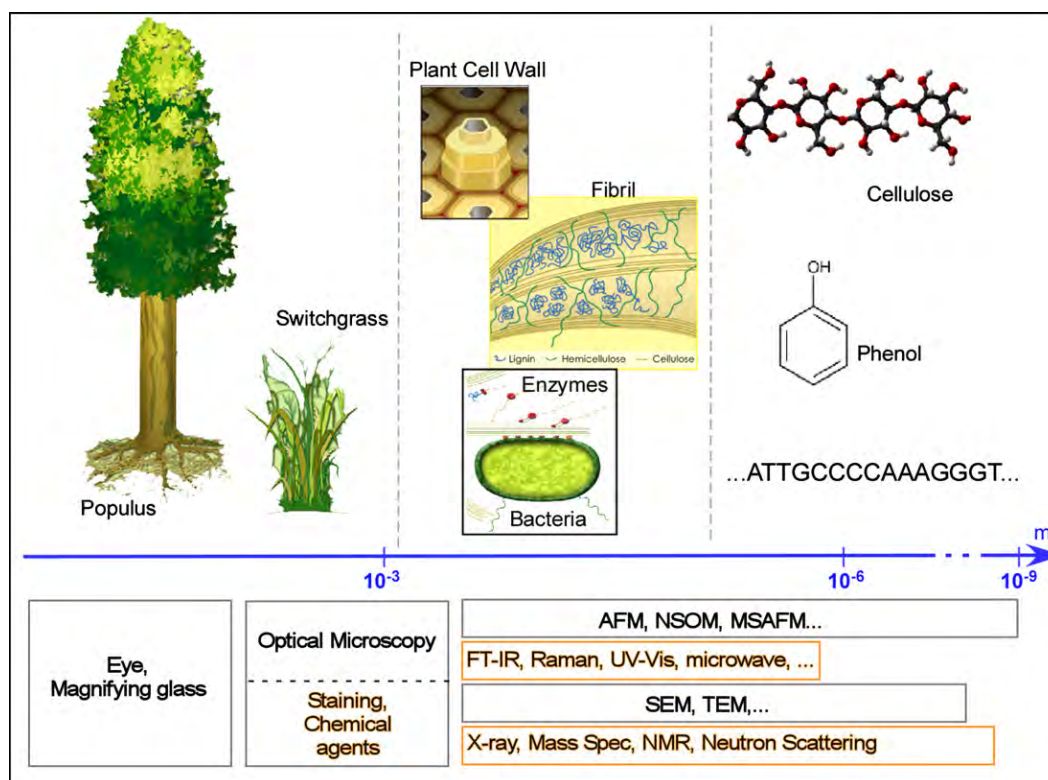
complete model for the plant cell wall and a detailed understanding of the various processes involved in the deconstruction of the plant cell wall.

Here, using AFM and a particular mode of MSAFM, we present results of topography and subsurface imaging for various preparations of both *Populus* and switchgrass samples in order to evaluate a new approach to characterize these samples at the nanoscale. The number of AFM-related methods used as a tool to map the composition of materials at the nanoscale has been increasing in the past few years [6,22–25]. In the case of MSAFM, both the probe and the sample are driven using multi-harmonic forcing to create a multitude of functioning modes. A number of techniques inspired by early work on ultrasonic microscopy [26,27] and the introduction of the concept of ultrasonic force microscopy by Quate in 1984 [28] have been implemented in AFM such as those described by Kolosov et al. [29] in 1993, followed by various heterodyning schemes [25,30]. The particular MSAFM mode used in this work was chosen to allow comparison to these previous schemes.

## 2. Experimental procedures

In order to carry out the subsurface measurements using MSAFM, an AFM was modified as described recently [21]. A Multimode AFM (Veeco) with a Nanoscope III controller was used to acquire the results presented here.

In all measurements, the AFM probes predominantly used were selected to be better suitable for imaging rough samples in contact mode. We found that stiffer cantilevers were more appropriate for this study. Experimentally, the dynamic properties of the microcantilever are determined by measuring the



**Fig. 1.** Imaging and spectroscopy techniques used for characterization of lignocellulosic materials of various dimensions. The techniques listed in the first row are considered as non-destructive techniques, whereas the ones in the second are invasive or require a specific sample preparation. Atomic force microscopy (AFM), mode synthesizing atomic force microscopy (MSAFM) and Fourier transform infrared spectroscopy (FTIR) are non-invasive techniques of important to access micro- and nanoscale mechanical and chemical properties of plant cell walls.

instantaneous position of a segment of the cantilever using the reflection of a laser from the illuminated segment and a position sensitive detector (PSD). From the spectrum of the random motion of the cantilever, or by explicit frequency sweeping of the voltage that drives the piezoelectric bimorph on which the cantilever rests, the resonant frequencies can readily be measured. The first resonant frequency of the stiffer cantilever ( $k=42$  N/m), corresponding to a flexural mode of the cantilever beam, was in average on the order of 300 kHz. Hanley et al. [18] have reported use of stiff cantilevers in previous work on cellulose from *Micrasterias denticulate*. In our measurements, spectral shifts did not exhibit a qualitatively strong dependence on the values of the driving frequencies and amplitudes for the sample and the cantilever except for the very large driving amplitudes, where a maximum shift of up to 2 kHz was measured.

Different lock-in amplifiers (SR844 with a range of 25 kHz–200 MHz and SR530 covering the range 0–100 kHz) were used to acquire the data. Due to the bandwidth limitation of the PSD, around 1 MHz frequency roll-off, an appropriate frequency window has to be considered so that the signal measured by the lock-in will adequately contain information on the difference frequency  $\omega_{\text{ref}} = |f_s - f_c|$  [22].

Shavings of *Populus* were obtained and mounted on a sample-holder using epoxy. Similarly, shavings of Alamo varietal switchgrass were Wiley milled to a 80–20 mesh and mounted on a sample-holder using epoxy. Stem and petiole (leaf stalk) samples, taken from greenhouse growing *Populus* plants were cryosectioned (Leica Cryostat) to 10–50  $\mu\text{m}$  and fixed on sample holder without additional staining with 0.05% (w/v) Toluidine Blue O (TBO). Stem samples, stored in a dry environment between two microscope glass slides to prevent distortion, were then immobilized on a substrate using an adhesive film typically used in SEM. Petiole sections were prepared on glass cover slides, which were then glued on the sample holder's piezoelectric bimorph prior to imaging.

Infrared (IR) spectroscopy was carried out using a Fourier transform infrared spectrometer (Spectrum GX, Perkin Elmer). The spectrometer was used in transmission mode using a mid-IR source (10,000–30  $\text{cm}^{-1}$ ), an optimized KBr Beam splitter (7000–370  $\text{cm}^{-1}$ ) and a mid-IR detector. The spectrometer's parameters were set to achieve a resolution of 4  $\text{cm}^{-1}$ . The background of the measurements was air, and each measurement was obtained by averaging four scans.

### 3. Results and discussion

We focus our discussions on two particular samples, *Populus* and switchgrass. Noting that *Populus*, a woody plant belonging to *Salicaceae* family and its woody biomass are important in the bioenergy production, we describe our findings as follows. In order to determine the kind of information which can be extracted using atomic force microscopy on lignocellulosic samples, measurements were carried out for coarsely ground chips (of few millimeters in size) of wood and switchgrass (Figs. 2 and 3) and cross sections of petiole or leaf stalk (Fig. 4) and *Populus* stem tissue (Figs. 5 and 6).

Fig. 2 is an example of *Populus* shavings images obtained by tapping mode AFM. The cell wall boundary (CW) and middle lamella (ML), as well as globular features of the lignocellulosic structure. While these images show topographical details, differentiation between primary and secondary cell walls was not possible. Fig. 3 shows the surface morphology of switchgrass shavings as obtained by AFM. With the coarsely ground chips of wood and switchgrass samples, the shavings were too rough to accurately resolve the cell wall structure. The unevenness of the

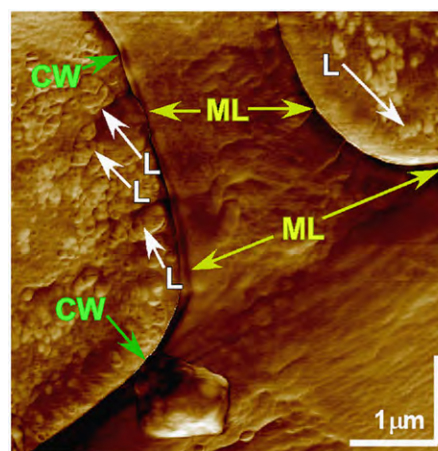


Fig. 2. AFM image of *Populus* cells observed on shavings sample. Phase data (obtained by Tapping mode) is presented. The cell wall and two partial cells are clearly visible. ML refers to the middle lamella, CW to the cell wall boundary, and L to the lignocellulosic cell wall structures. As these images show topographical details, differentiation between primary and secondary cell walls was not possible.

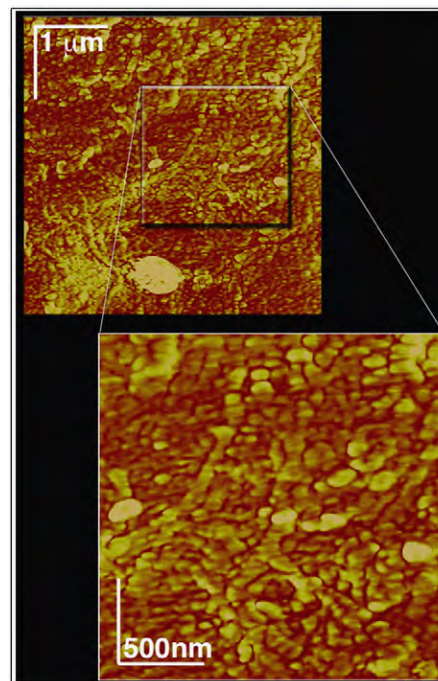
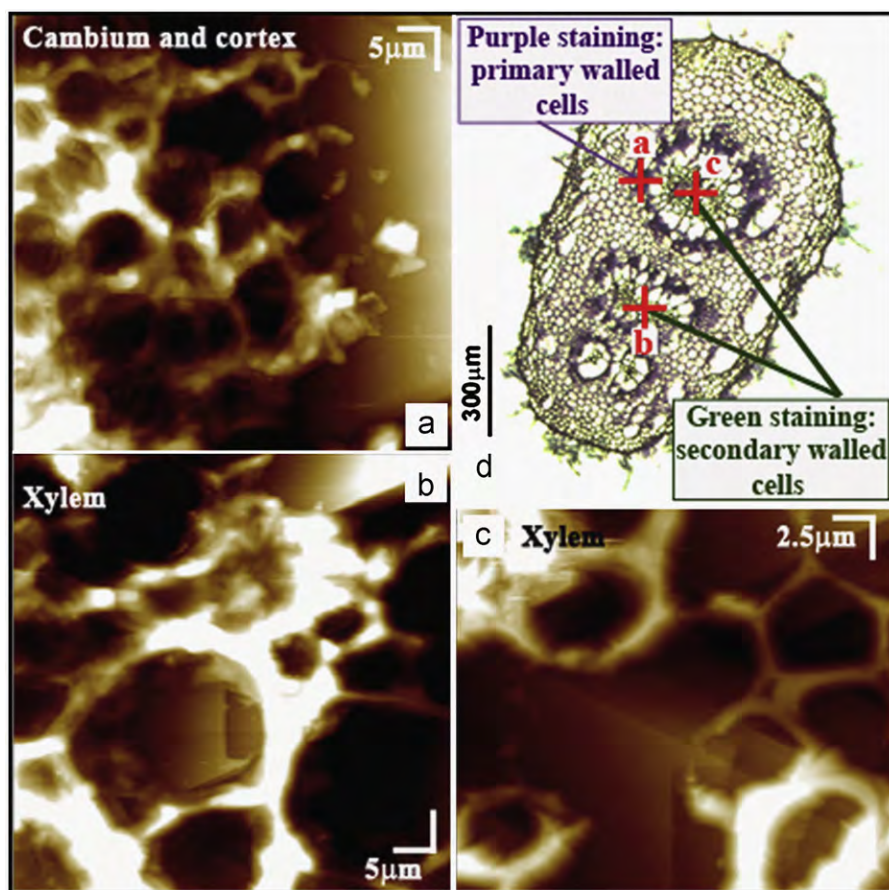


Fig. 3. AFM images of switchgrass shavings. Phase data (obtained by Tapping mode) is presented. The macromolecular globules structures attributable to lignin are visible. With the coarsely ground shavings of *Populus* wood and switchgrass, the samples were too rough to accurately picture the cell wall structure. The unevenness of the samples made it challenging to image a large area.

samples made it challenging to image a large area. Although the acquired image does not clearly reveal the cell wall structure, the close accordance of these globular structures with those reported in previous work by Micic et al. [31] who synthetically created lignin on a cellulose substrate leads us to argue that one may be seeing the lignocellulosic structure in the stem sample. These results are important in that these images are from native samples, giving us a perspective on the actual lignocellulosic structure in *Populus* and switchgrass.

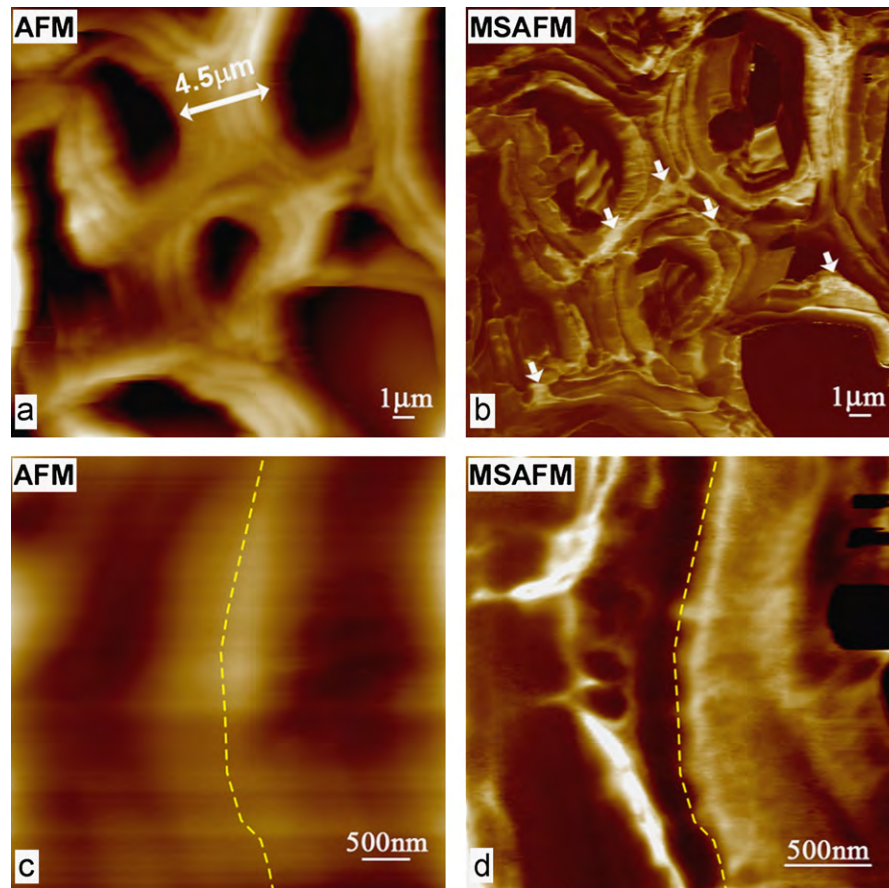
The primary motivation of the present work is to develop new characterization tools to gain a greater understanding of the plant



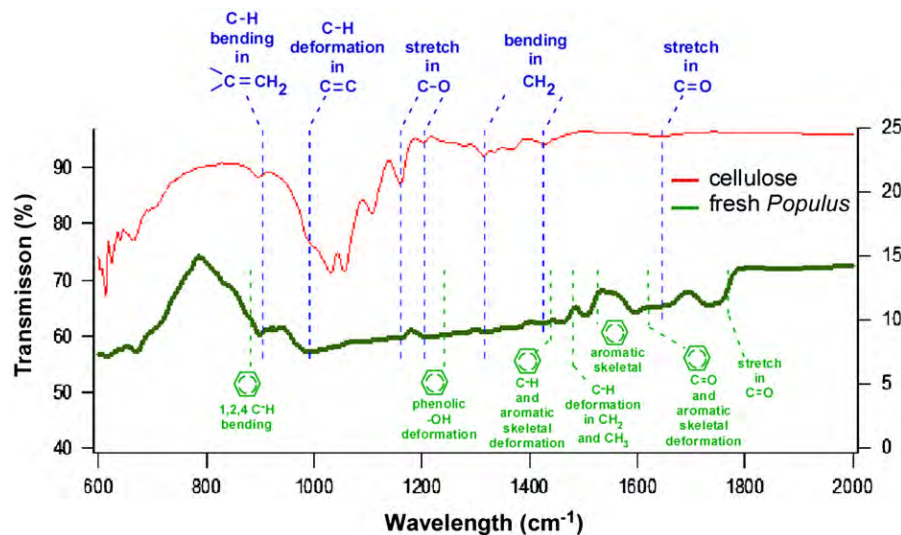
**Fig. 4.** (top right) (a–c) AFM imaging representative of the primary (cambium and cortex) (a) and secondary (xylem) (b, c) cell walls. Diameters of the cells and thickness of the walls are indicated on the picture obtained by contact mode imaging and will be used as part of the structural parameters available to determine the nature of the cell wall (primary or secondary for example). (d) Optical image ( $5\times$  magnification) of *Populus* petiole 10- $\mu\text{m}$ -thick cross section stained with a Toluidine Blue O stain that stains primary walled cells purple and secondary walled cells green.

cell wall structure, organization and chemistry. As can be observed in Figs. 2 and 3, the samples prepared from coarsely ground *Populus* or switchgrass do not exhibit the kind of structures needed to understand the physical and structural organization of the systems. In order to access the properties of the cell walls we focused on cross sections of the plant material (here *Populus* only will be presented, see Experimental procedures section). An optical image of the petiole sample is presented in Fig. 4 (d), and the corresponding AFM images in Fig. 4(a–c). The purple and green regions in the Toluidine Blue O stained *Populus* petiole section represent primary and secondary walled cells, respectively. A stiff probe ( $k=42\text{ N/m}$ ) was used for these images in order to avoid the damage on softer probes caused by the voids of the samples. These hollow regions enclosed by cell walls represent dead intracellular cleared region or vacuoles or intercellular spaces. The hollow dead xylem vessel cells that form conductive tissue in plants, present a pathway for water transport. We further investigated the structure of the cell wall using MSAFM, which makes it possible to access the physical information of materials (surface and subsurface) using mechanical excitation of the probe and the sample [21]. The results from a sample extracted from fresh *Populus* are presented in Fig. 5, where the images of the left column were obtained with regular contact mode AFM, and the images of the right column were obtained using MSAFM. The MSAFM images reveal new details on the structure of the plant cell wall that the topography could not resolve. In an effort to correlate the organization of the different layers of the cell wall that were resolved in Fig. 5, we carried out

FTIR spectroscopy on the fresh *Populus* sample and compared the results with the spectrum of pure cellulose. Based on previous work by Wadsworth and Kataoka [32,33], we labeled the spectrum associated with our sample of fresh *Populus* (Fig. 6) and indicated the peaks relative to the carbohydrates in blue and the peaks relative to aromatic compounds (lignin) in green. The complexity seen in the AFM and MSAFM images (Fig. 5) is reflected in the IR spectrum of the *Populus* sample. Several peaks indicative of aromatic groups are revealed by the spectroscopy measurement, verifying the presence of lignin in tissue sample. From the IR spectra of fresh *Populus* and cellulose, as well as the previous measurements described in the literature [32,33], some peaks of the cellulose and the lignin will be more relevant than others in the differentiation of carbohydrate or aromatic content of the wall layer. The vibration of the aromatic skeletal at  $1595\text{ cm}^{-1}$  could for example be used to detect the presence of lignin in a sample. This peak is important since no major vibration associated with the cellulose could be found in the surrounding range. Vibrations of C=O or C–H, although expected to take place at different wavelengths due to the difference in linkage between cellulose and lignin, are located in similar regions. For this reason, it will be interesting to use them as support for the identification of the chemical composition, but they will not be sufficient. The major peaks used to determine the carbohydrate content (cellulose) are extracted from the red curve in Fig. 6 (pure cellulose). By direct comparison to the spectrum obtained from a fresh *Populus* sample, it is obvious that cellulose mixed in a lignocellulosic type of material will exhibit very different features,



**Fig. 5.** AFM (left) and MSAFM (right) images of a cross section of *Populus* wood. MSAFM images exhibit different information from regular topography. The two complementary techniques should be used in order to determine the structural and mechanical properties of the lignocellulosic samples. The arrows indicate the brighter areas of the cell walls, corresponding to the Middle Lamella (ML), which exhibit different mechanical properties revealed by MSAFM.



**Fig. 6.** Infrared spectroscopy spectra of the *Populus* cross section sample compared to cellulose. The fresh *Populus* sample (no treatment) exhibits features that can readily be associated with the peaks of cellulose corresponding to the carbohydrates bonds. The spectra have been labeled at the wavelength corresponding to the vibrations of the aromatic group (green) and carbohydrates (blue), which have been reported in the literature (for interpretation of the references to color in this figure legend, the reader is referred to the web version of this article).

but the C-H bending and deformation in various groups (alkene and C=O) remain, and could be used for chemical mapping. For example the relative IR absorbance of particular bands ( $a_{1429 \text{ cm}^{-1}}/a_{893 \text{ cm}^{-1}}$  and  $a_{1429 \text{ cm}^{-1}}/a_{2900 \text{ cm}^{-1}}$ ) can be used to determine the crystallinity ratio of cellulose in a material [31,32].

**Fig. 5(b)** and **(d)** also depict materials of different mechanical properties within the cell wall. In light of the data presented here and recent work by Ding et al. [17], it is clear that the chemical and physical/structural properties of the plant cell wall at the nanoscale are closely related. In addition, the cell corner and

middle lamella composition appear to be very different from the primary and secondary cell wall [17]. In fact, Fig. 5(d) accentuates the difference in the structure of the different layers: on the right hand side of the yellow dashed line, corresponding to the secondary cell wall, fibrous material is clearly observable whereas on the left hand side of the line the composition is not as dense and regions of different density are observed.

#### 4. Conclusion

The quest to develop sustainable biomass-based biofuels has given rise to new questions related to overcoming the barrier of biomass recalcitrance, challenging the technological state of characterization tools appropriate for lignocellulosic materials. In particular, tools to characterize feedstock materials in order to simultaneously obtain physical, structural and spectroscopic information at different length scales would be of great interest to expand the current understanding of the plant cell wall and biomass materials.

The level of heterogeneity of the chemico-structural wall characteristics that determine conversion efficiency, the link between the physical features and the composition at the molecular level, the influence of various parameters (temperature, pressure, humidity, pH, etc.) on the spatiotemporal changes in the cell wall, as well as how pretreatments impact plant cell walls to facilitate the deconstruction of cellulose, are major biological questions that remain unexplained today. A more detailed understanding of the complexity of plant cell walls and the specific structural and chemical features that contribute to recalcitrance will require the development of novel analytical and imaging techniques with greater detection and resolution capabilities.

Here standard AFM was performed on various forms of *Populus* and switchgrass samples. Although native coarsely ground shavings of wood and switchgrass present few characteristics that could be used for an extended characterization of the plant cell wall structure at high resolution, they give us a perspective on the actual lignocellulosic structure in *Populus* and switchgrass. Strengthened by their versatility, AFM-based techniques will be of tremendous importance in acquiring a systematic understanding of the plant cell wall architecture, crucial in the development of biomass-based alternative sources of energy. Recently, MSAFM revealed a great potential in the study of complex biological and synthetic materials. We presented results from a single mode of MSAFM so as to better evaluate its capabilities on plant tissues. The results showed variations observed in mechanical properties of the different layers of the cell walls, as well as the differences of composition within the tissue sample.

The results suggest that cross-linking the information from various technologies is needed to overcome the challenges related to the use of biomass for energy production. Although the local (nanoscale) chemical mapping of the sample is not available among the variety of tools for the study of lignocellulosic material at the nanoscale, the value of simultaneous structural and chemical information of such samples is obvious. The presented results help evaluating the applicability of a new approach to characterizing the complexity of plant cell wall properties at the nanoscale so that the full potential of MSAFM may be exploited to further explore biomass.

#### Acknowledgments

This research was sponsored by the ORNL BioEnergy Science Center (BESC). The BioEnergy Science Center is a US Department

of Energy Bioenergy Research Center supported by the Office of Biological and Environmental Research in the DOE Office of Science. We are indebted to Jung Seokwon and Art Ragauskas at the Georgia Institute of Technology for providing the *Populus* wood samples. We also thank Martin Keller at ORNL for his support. ORNL is managed by UT-Battelle, LLC, for the US Department of Energy under contract DE-AC05-00OR22725.

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