CHAPTER 3

Advances in the Measurement and Characterization of Biomass Structure and Processing

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3.1 Introduction

The recalcitrance of lignocellulosic plant biomass makes the cost of biofuel higher than that of fossil fuel. Plant cell wall has a highly complex macromolecular structure mainly consisting of crystalline cellulose that is embedded in an amorphous matrix of cross-linked lignin and hemicelluloses. To break down the lignin–hemicellulose barrier, thermochemical and/or biological pretreatment is usually applied. Therefore, to reduce the cost of the lignocellulose-to-ethanol process, we need to control the cost of feedstock, thermochemical pretreatment, enzymes, and fermentation. In contrast to cornstarch for human food, non-food plant biomass feedstock includes forestry waste and grasses, as well as agricultural residues (corn stover). Deconstructing the plant cell wall alters the cell-wall structure making it more permeable to enzymes, and therefore understanding the ultra-structure of the plant cell wall and how it changes during the pretreatment process, provides insight into fundamental

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mechanisms that contribute to the native recalcitrance of cell walls. In this chapter, we review the various microscopy and spectroscopy tools used to characterize plant-cell-wall structure, including optical microscopy, atomic force microscopy, and electron microscopy. We start with the principle of the instruments followed by their application in biomass conversion. The instruments can also be combined with one other to make them more powerful and able to characterize more aspects of biomass.

3.2 Atomic Force Microscopy

Atomic Force Microscopy (AFM) is a type of scanning probe microscopy with unprecedented atomic-level resolution. It can image a sample's surface providing a three-dimensional (3D) profile by measuring the force between the tip and sample, which follows Hooke's Law. The sharp tip with radius of a few nm is supported on the end of a flexible cantilever. The tip is used to scan across the sample surface by 'gently' touching it, and thus providing sample morphology information. AFM images not only represent the topography of the sample surface at very high magnification, but also measure the attractive and repulsive forces between the scanning probe tip and the sample surface, thus providing both height and phase images. For biological applications, AFM has the capability of operating in an aqueous environment and enables the observation of dynamic molecular events in real time and under real physiological conditions without the need for complex sample preparation procedures (*e.g.*, staining, freezing, chemical extraction, dehydration or embedding), which is essential for conserving native biomass structure. To probe plant tissues, "tapping mode" (or "intermittent contact mode") is usually used to prevent damage to these soft materials. Unlike contact mode, the probe does not contact the sample surface, but oscillates up and down, near its resonance frequency, above the adsorbed fluid layer on the surface during scanning.

In biomass conversion, AFM provides not only plant-cell-wall architecture (primary plant cell wall, secondary plant cell wall, and fibrils) but can also trace the interaction dynamics of biocatalysts and substrates. Several different cellulose and plant-cell-wall microfibrils have been characterized, such as *Valonia* (primarily I_α crystals ~ 20 nm in diameter), tunicate (primarily in the I_β form), bacterial microcrystalline cellulose (ribbon-like cellulose microfibrils), maize parenchyma cell wall (cellulose in the form of small microfibrils 3–5 nm in diameter), Avicel (microfibrils shown as small crystals or bundles ranging from 100 to 200 nm in length, and 5 to 10 nm in diameter) and phosphoric acid treated cellulose (amorphous).[1–3] The detailed structure of the primary cell wall of maize parenchyma and the cellulose synthesis process have also been studied by AFM: well-shaped pits, 2 μm wide and 150–200 nm deep, are found in the cell wall; parallel-microfibrils are embedded in the wall matrices forming cell wall lamella; and macrofibrils were found to exist only in the uppermost layer of the native primary cell wall and appeared to be bundles of elementary fibrils.[4]

Real-time AFM images were acquired to study *T. reesei* cellobiohydrolase I (CBH I) hydrolyze crystalline cellulose (*Valonia*) over the course of 11 hours. CBH I is bound to, and moves on the cellulose hydrophobic faces. The authors observed cellulose structural changes over time: a decrease in width; an increase in surface roughness; and a change in the shape of the fiber ends.[5] High-speed AFM (1–4 frames per second) enabled the study of the sliding movement of *Tr*Cel7A on crystalline cellulose (*Cladophora* sp.), and an average velocity of 3.5 nm s^{-1} was measured.[6]

3.3 Fluorescence Microscopy

Fluorescence microscopy is a type of optical microscopy. The sample is illuminated with light of a short wavelength (high energy) which causes fluorescence. The light emitted by fluorescence is of a longer wavelength (lower energy) than the illumination, and is then detected through a microscope objective and an image is recorded using a CCD camera. The basic system components include a light source (lamp or laser), filter sets (excitation, dichroic, emission), and a detection device (camera). The filter sets are chosen to match the excitation and emission spectra of the labeled fluorophore. The sample can be fluoresced in its natural form (primary fluorescence or auto-fluorescence) or labeled with fluorophores (dyes, fluorescence proteins, quantum dots *etc.*). In plant biology, various types of fluorescence microscopy like epifluorescence microscopy, confocal microscopy, and total internal reflection fluorescence microscopy (TIRF) are used widely.

Auto-fluorescence is an intrinsic optical property of biological materials, where the materials fluoresce in a similar manner to the fluorophore dyes or markers used to label biological objects of interest. The intrinsic auto-fluorescence of biomass is mainly from lignin and phenolic compounds. Lignin is a complex compound with strong auto-fluorescence in the visible as well as far-infrared regions.[7] The broad emission spectrum of lignin contains contributions from its three monolignols: *p*-hydroxyphenyl (H), guaiacyl (G), and syringal (S). Auto-fluorescence offers a direct, nearly native method to visualize the distribution of lignin in the cell wall without the need of labor-intensive and time-consuming chemical and immunological labeling. Thus, auto-fluorescence imaging provides insight into lignin location and its relative concentration (intensity). Greater lignin accumulation in the middle lamella and cell corners has been observed, and lignin-rich sclerenchyma cells are near the epidermis.[8] In addition, dynamic tracking of lignin solubilization during an ionic liquid pretreatment of switchgrass has been reported.[8] The ionic liquid, EmimAc (1-*n*-ethyl-3-methylimidazolium acetate), is an effective biomass pretreatment method to dissolve lignocellulose and later help hydrolyze the resulting liquor into sugars. After 2 h of EmimAc ionic liquid treatment, the organized switchgrass plant-cell-wall structure has been completely broken down. A rapid swelling of the secondary plant cell walls within 10 min at 120 °C, disrupting the inter- and intra-molecular hydrogen bonding between

cellulose fibrils and lignin was observed. The swelling was followed by complete dissolution of the biomass over 3 h.[8]

In addition to the presence of auto-fluorescence, labeling cell-type-specific cell-wall polymers can elucidate the sophisticated polymer configurations. Due to the complex polymers located in the plant cell wall, understanding the spatial distribution of cell-wall components is challenging. The molecular probes known as "carbohydrate-binding modules" (CBMs) and monoclonal antibodies can be used to recognize cell-wall macromolecules.

3.3.1 Carbohydrate-Binding Modules

CBMs are non-catalytic proteins found in many glycoside hydrolases. They serve as attachment devices for glycoside hydrolases and their target substrates and therefore are usually used as polysaccharide-specific probes. CBMs are classified into three types according to the functions. Type A CBMs, or "surface-binding" CBMs, comprise a planar hydrophobic surface, which binds specifically to insoluble crystalline cellulose. Type B CBMs, or "glycan-chain-binding" CBMs, bind to single-chain polysaccharides. Type C CBMs, or "small-sugar-binding" CBMs specifically bind to the ends of polysaccharides or oligosaccharides.[9] Researchers have identified CBMs that specifically bind to crystalline cellulose,[10–14] xylan,[15] and amorphous cellulose.[9,16]

To make CBMs visible under fluorescence microscopy, fluorescent tags need to be incorporated into them. Using fluorescent quantum dots (QDs) and fluorescent proteins (FPs) to label CBMs has been reported.[13,14,17] NAC/histidine-capped CdSe/ZnS QDs have been bound to a CBM (*Ac*CBM2 or *Ct*CBM3) fusion protein with dual hexahistidine-tags at its N- and C- termini. The CBM–His probes bound to crystalline cellulose, *Valonia*, were examined by TEM and fluorescence microscopy.[13,14] Three different types CBM (*Ct*CBM3, *Ct*CBM6, *Sl*CBM20) each exhibiting different carbohydrate specificities were each fused with either green fluorescent protein (GFP) or red fluorescent protein (RFP) and employed for dual-labeling fluorescence microscopy studies of primary cell walls and various carbohydrate target molecules. *Ct*CBM3 and *Sl*CBM20 were specifically bound to crystalline cellulose and starch, respectively. *Ct*CBM6 had a strong interaction with xylan and xylodextrins and a weak interaction with cellodextrins, non-crystalline cellulose, and xyloglucan.[17] The fluorescent CBM probes were used for characterizing both native complex carbohydrates and pretreated biomass.

3.3.2 Monoclonal Antibodies

Immunofluorescence monoclonal antibodies (Abs) against polysaccharides have been applied to investigate polymer distribution in plants by confocal microscopy.[18–20] Two rat monoclonal antibodies, LM10 (rat immunoglobulin class IgG2c) and LM11 (class IgM), bind to transverse sections of *Silene* and tobacco stems, flax hypocotyls, and wheat grain. A study showed that LM10 is specific to non- or low-substituted xylans, whereas LM11 binds to wheat

arabinoxylan and unsubstituted xylans indicating the presence of both epitopes in the secondary cell walls of xylem, but differences in occurrence in other cell types.[19] In another study, the rat monoclonal Abs JIM7, JIM5, LM5 and LM6 and the mouse monoclonal Ab 2F4 were used to map pectic motif distribution in sugarbeet root cell walls. Control sections were treated in parallel but without the primary Abs. All cell walls remained unlabeled when the primary Abs were omitted from control sections. The results showed that pectic polysaccharides are spatially regulated in sugarbeet root cell walls and the spatial patterns vary between cell types implying that structural variants of pectic polymers are involved in the modulation of cell-wall properties.[18] In addition to fluorescent dyes, immunolabeling techniques with Abs can also be applied for conjugation with gold (immunogold labeling) for TEM characterization.[18,20]

3.4 Fourier Transform Infrared Microspectroscopy

Infrared spectroscopy is a technology that is used to detect molecular vibrational energy (or frequency) which lies in the infrared (IR) region. Infrared light is passed through molecules and the intensity of the transmitted light is measured at each frequency. When molecules absorb IR radiation, transitions occur from a ground vibrational state to an excited vibrational state. In order for a molecule to be IR active there must be a change in dipole moment as a result of the vibration that occurs when IR radiation is absorbed, and hence asymmetric vibrations cause the most intense IR absorption. Fourier transform is a data-processing technique that turns raw data (interferogram) into the desired result (the sample's spectrum): light output as a function of IR wavelength (or equivalently, wavenumber). FTIR spectra of pure compounds are generally so unique that they are like a molecular 'fingerprint'. The most useful fingerprint region lies from 4000 to 400 cm^{-1}. FTIR spectroscopy can be upgraded to FTIR microspectroscopy when equipped with a microscope. This enables the visualization of the sample, the choice of a specific region for analysis, and two-dimensional (2D) data acquisition by raster scanning the sample simultaneously. IR spectra are acquired at each pixel of 1D or 2D maps, and chemical maps can thus be derived. FTIR microspectroscopy can be used to directly characterize polysaccharide and aromatic molecules in the cell wall with minimal sample preparation.[21,22] It can also map the chemical distribution in plant cells and thus reveal intrinsic heterogeneities in a single cell wall or in many different cell-wall types within a tissue.[15,21] FTIR microspectroscopy can be more powerful when integrated with the attenuated total reflectance (ATR) technique, synchrotron sources, a focal plane array (FPA) IR detector, or chemometric analysis.

3.4.1 Attenuated Total Reflectance Fourier Transform Infrared Microspectroscopy

ATR uses the property of total internal reflection resulting in an evanescent wave. An IR beam is passed through an ATR crystal with a high refractive

index, such that at a certain angle the light undergoes multiple internal reflections in contact with the sample. This reflection creates the evanescent wave which extends into the sample. The penetration depth into the sample is typically between 0.5 and 2 μm, and therefore, there must be direct contact between the sample and the ATR crystal surface. The ATR crystals must have refractive index values greater than that of the sample, typically, between 2.38 and 4.01 at 2000 cm^{-1}. Crystals made of zinc selenide (ZnSe), germanium (Ge), and diamond are often used in ATR-FTIR to study plant materials due to faster sampling, improved reproducibility and reliability, and less impedance by water absorption.

In biomass-conversion studies, ATR-FTIR has been applied to study switchgrass pretreated by an ionic liquid and dilute acid.[23] The ratio of two peaks 1510 : 900 cm^{-1} representing the lignin-to-cellulose ratio was compared between the ionic liquid and dilute acid pretreatment methods. The ratio decreased from 0.49 (untreated switchgrass) to 0.13 (ionic liquid pretreatment), but increased to 0.68 for dilute acid pretreatment indicating that ionic liquid pretreatment is a promising alternative to dilute acid pretreatment for switchgrass.

ATR-FTIR has also been used to measure crystallinity changes in cellulose I polymers.[24] After normalization the obtained spectra of the absorbance of the O – H in-plane deformation band at 1336 cm^{-1}, the lateral order index (LOI, α 1429/893) and hydrogen bond intensity (HBI, α 3336/1336) can be used to interpret qualitative changes in cellulose crystallinity. Kljun *et al.*[24] used ATR-FTIR and X-ray diffraction (XRD) to observe crystallinity changes in cotton fibers after sodium hydroxide (NaOH) treatment, and compared the results with CBM probes. They found close agreement with changes in crystallinity observed with ATR-FTIR techniques compared to CBM probes when NaOH concentrations greater than 2.0 mol dm^{-3} were used.

3.4.2 Synchrotron-Radiation-Based Fourier Transform Infrared Microspectroscopy

Synchrotron-radiation-based FTIR (SR-FTIR) microspectroscopy uses synchrotron emission as its IR source, which is 100–1000 times brighter than a standard globar (conventional thermal IR source). Due to the greater brightness of synchrotron IR light, it enables the beam size to be reduced below 10 μm without a significant loss of photons, and thus allows a very small area to be explored providing higher accuracy and precision. The technique allows faster data collection, reaches the diffraction limit, and provides a very good signal-to-noise (S/N) ratio with high ultra-spatial resolution.[25–27] The main drawbacks are the requirement for a bright synchrotron beam and special instrumentation, and the difficulties in sample preparation for some feed materials. Using the SR-FTIR technique, the intensities and distributions of the biological components (such as lignin, proteins, lipids, structural and non-structural carbohydrates and their ratios) in the microstructure of plant tissue within cellular dimensions could be imaged.[28–33]

3.5 Raman Spectroscopy

Raman spectroscopy is based on inelastic scattering (Raman scattering) of a photon that interacts with molecular vibrations, resulting in an energy shift (Raman shift) of the photon. Raman shift provides information about vibrational, rotational and other low-frequency transitions in molecules. To be Raman active, the polarizability of the molecule must change with the vibrational motion. Thus, Raman spectroscopy complements IR spectroscopy. A Raman microspectroscopy system typically consists of an excitation source (laser), a spectrophotometer, a CCD detector, and a confocal microscope. It is widely used because it offers label-free chemical contrast with high resolution and chemical specificity,[34–37] and can be used in solid, liquid, and gas samples. Several potential bioenergy crop candidates have been characterized by Raman microspectroscopy including black spruce wood,[34] poplar wood,[36] flax stem,[38] *Arabidopsis thaliana* and *Poplus trichocarpa* stem wood,[37] and corn stover and *Eucalyptus globulus*.[39] Raman signals are normally weak, and needs long acquisition time (0.1–1 s per pixel). To overcome these drawbacks, many different ways of sample preparation, sample illumination and scattered light detection have been invented.

3.5.1 Coherent Anti-Stokes Raman Scattering Microscopy

Coherent anti-Stokes Raman scattering (CARS) microscopy produces a signal in which the emitted waves are coherent with one another. As a result, a CARS signal is orders of magnitude greater than that from spontaneous Raman scattering. Unlike Raman spectroscopy using a single continuous wave (CW) laser, CARS generally requires two pulsed laser sources to focus on the sample, a pump beam with frequency ω_p, and a Stokes beam with frequency ω_S. Two laser beams are temporally and spatially overlapped to generate anti-Stokes signals at frequency $\omega_{aS} = 2\omega_p - \omega_S$ such that the frequency difference $(\omega_p - \omega_S)$ is tuned to match a particular Raman active molecular vibration frequency.[40,41] CARS microscopy provides a contrast mechanism based on molecular vibrations, which are intrinsic to the samples, as well as high spatiotemporal resolution, and is free of background noise from one-photon-excited fluorescence.[42] It also offers much greater sensitivity and faster acquisition times than traditional Raman spectroscopy.[43]

CARS imaging of lignin distribution in the cell wall has been demonstrated in the lignocellulosic biomass alfalfa[44] and corn stover.[45] The $1600\,cm^{-1}$ Raman mode was used to represent the lignin signal in wild-type (WT) and two lignin-down-regulated alfalfa lines: shikimate hydroxycinnamoyl transferase (HCT) and coumaroyl shikimate 3-hydroxylase (C3H) for CARS imaging. A reduction of the CARS signal in the cell corners and the compound middle lamella (the middle lamella + primary walls) areas was observed.[44]

3.5.2 Stimulated Raman Scattering Microscopy

In stimulated Raman scattering (SRS) microscopy, the pump beam with frequency ω_p and the Stokes beam with frequency ω_S are incident on the

sample. If the frequency difference ($\omega_p - \omega_S$) matches a molecular vibration, stimulated excitation of vibration transitions occurs. The intensity of the pump beam is annihilated (stimulated Raman loss: SRL) and the Stokes beam is created (stimulated Raman gain: SRG). SRS cannot occur when the frequency difference ($\omega_p - \omega_S$) does not match any vibrational resonance that absorbs the difference in energy from the beams. Thus, SRS does not have a non-resonant background signal. The major advantages of SRS are identical spectral response to spontaneous Raman scattering but with orders of magnitude larger signal which is linearly dependent on the analyte concentration, fast image acquisition (50 μs per pixel) and a non-resonant background.[46] SRS thus is an ideal technique for investigating biomass-conversion processes since it has the capability of real-time monitoring, it is label-free and non-invasive, and has high spatial resolution.

The real-time image delignification process by acid chlorite in corn stover has been demonstrated.[47] The Raman peaks at 1600 cm^{-1} (aromatic stretching of lignin) and 1100 cm^{-1} (C–C and C–O stretching) were used to represent lignin and cellulose respectively for SRS imaging. After applying acid chlorite (0.1 M HCl (aq) and 10% $NaClO_2$) for 53 min, the lignin signal reduced significantly (more than 8×) whereas the cellulose signals remained at the same level.[47] This real-time SRS imaging technique demonstrates the possibility of studying enzymatic breakdown cellulose processes or the *in vivo* imaging of lignification and cellulose biosynthesis in plant cell wall development.

3.6 Transmission Electron Microscopy

In transmission electron microscopy (TEM) a high-energy electron beam emitted by an electron gun is transmitted through a very thin sample in a vacuum to image and determine the internal structure of materials. The electrons are focused with electromagnetic lenses and the image is observed on a fluorescent screen, or recorded on photographic film or by digital camera. Because the wavelength of electrons is much smaller than that of light, the resolution for TEM images is a 1000× better than those obtained with a light microscope. Thus, TEM can reveal the finest details of internal structure at atomic resolution. In order to allow electrons to pass through the sample, the sample needs to be ultra-thin (ideally 100–300 nm), and hence requires sophisticated preparation techniques, such as chemical fixation, shadowing or staining with high-contrast compounds, resin embedding, or microtoming. However, these preparation procedures are usually considered to alter the native sample structure. Compositional analysis of a material can be obtained when TEM is equipped with energy-dispersive spectroscopy (EDS). Images obtained from TEM are 2D projections of a 3D volume of the material under study, but for structural biology, cryo- or cold-stage transmission electron microscopy (cryo-TEM) can resolve of multi-subunit proteins and clusters and tomography provides reconstruction of complex images in a 3D profile, and in their native state.

A recent study visualized corn stover cell-wall pretreated with ammonia fiber expansion (AFEX) by 3D electron tomography.[48] Nanoporous tunnel-like networks that enhance enzyme accessibility are embedded in cellulosic microfibrils. The estimated exposed pore surface area was between 0.005 and 0.05 nm^2 per nm^3 which resulted in AFEX-pretreated cell wall enhancing the enzymatic hydrolysis yield by 4–5 fold over untreated cell walls.

3.7 Scanning Electron Microscopy

In scanning electron microscopy (SEM) electrons are focused into a small beam by a series of electromagnetic lenses in the SEM column under vacuum, and scan the sample in a raster scan pattern. When the electrons hit the sample, secondary electrons are produced by inelastic scattering and backscattered electrons are produced by elastic scattering. These electrons are collected by a secondary detector or a backscatter detector, converted to a voltage, and amplified. A 2D distribution of the voltage signal is displayed on the monitor, which is a representation of the topography of the sample. SEM has a larger depth of field than light microscopy, which allows a large amount of the sample to be in focus at one time, and thus can produce the characteristic 3D appearance useful for understanding the surface structure of a sample. SEM also produces images of high resolution (less than 1 nm), which means that closely spaced features can be examined at a high magnification. Sample preparation is easier than for TEM since most SEMs only require the sample to be electrically conductive. Similar to TEM, qualitative and quantitative chemical analysis information can be obtained by using EDS.

SEM has been widely used to study the structure of biomass which usually coated with a thin layer of electron-dense material, such as carbon or atomized gold. A cross-section of a native maize stem tissue showed vascular bundles and pith tissues, as well as diverse cell sizes, shapes, and cell walls.[1] After thermochemical pretreatment of corn stover stalks, lignin coalescence and a range of lignin droplets are formed in the xylem and sclerenchyma cells.[49] A recent SEM study showed significant structural differences between corn leaves, stalk pith, and stalk rind before and after liquid hot water pretreatment and correlated the changes to sugar yields from enzymatic hydrolysis of the corn fractions.[50]

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