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Real-Time Detection of the Morphological Change in Cellulose by A Nanomechanical Sensor

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Abstract

Up to now, experimental limitations have prevented researchers from achieving the molecular-level understanding for the initial steps of the enzymatic hydrolysis of cellulose, where cellulase breaks down the crystal structure on the surface region of cellulose and exposes cellulose chains for the subsequent hydrolysis by cellulase. Because one of these non-hydrolytic enzymatic steps could be the rate-limiting step for the entire enzymatic hydrolysis of crystalline cellulose by cellulase, being able to analyze and understand these steps is instrumental in uncovering novel leads for improving the efficiency of cellulase. In this communication, we report an innovative application of the microcantilever technique for a real-time assessment of the morphological change of cellulose induced by a treatment of sodium chloride. This sensitive nanomechanical approach to define changes in surface structure of cellulose has the potential to permit a real time assessment of the effect of the non-hydrolytic activities of cellulase on cellulose and thereby to provide a comprehensive understanding of the initial steps of the enzymatic hydrolysis of cellulose.

Keywords

cellulose; structural change; microcantilever; cellulase; nanomechanical; surface detection

One of the most critical aspects of biofuel production is conversion of cellulosic biomass to fermentable sugars via enzymatic hydrolysis of cellulose (Wilson 2009). The enzymatic hydrolysis of cellulose requires the enzyme cellulase first to break down the crystal structure on the surface region of cellulose, then to bind to the partially liberated single glucan chains of cellulose, and finally to hydrolytically cleave these liberated glucan chains. The non-hydrolytic breakdown of the crystal structure on the surface region of cellulose has been speculated to be the rate-limiting step for the entire enzymatic hydrolysis of crystalline cellulose (Wilson 2009).

Such disruption of the cellulose surface is expected to be localized in the outer layer, and to occur intermittently on very small areas of cellulose. Most surface detection methods, such as Fourier transform infrared spectroscopy (Fengel et al. 1995), Raman spectroscopy (Eronen et al. 2009; Schenzel et al. 2005), and x-ray photoelectron spectroscopy (Fardim et al. 2005) are unable to monitor such changes because they are focused on global changes of cellulose that occur over relatively large surface areas. Although atomic force microscopic (AFM) imaging has the capability and sensitivity to assess the surface structure of cellulose

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(Eronen et al. 2009), it is limited to analysis at discrete time points. Both scanning electron microscope and transmission electron microscopy have the same limitations.

In recent years, both quartz crystal microbalance with dissipation (QCM-D) and ellipsometry have been also used to examine the property of cellulose in response to salts (Ahola et al. 2008; Freudenberg et al. 2007; Tammelin 2006) and enzymes (Josefsson et al. 2008; Turon et al. 2008). Measurements by these two techniques are mainly based on changes in total mass and/or thickness of the thin film of cellulose and the results are not very informative about changes in the structural property of the surface region of cellulose. Technical limitations such as these have contributed at various extents to our limited knowledge of a detailed molecular mechanism of non-hydrolytic breakdown of the crystal structure on the surface region of cellulose. Such knowledge is essential to the success of improving catalytic efficiency of cellulase in biomass conversion.

Many of the aforementioned limitations in detecting minute changes in surface structure of cellulose in real time can be overcome with the use of the microcantilever technique. This technique uses microcantilevers (Datskos 2005) that are widely used as force transducers in AFMs. Microcantilevers are usually made of silicon or silicon nitride and are available in a variety of shapes and stiffnesses. Their typical dimensions are 200 μm long, 1 μm thick, and 20 μm wide. Such small size makes for high sensitivity, fast response, and the ability to investigate small areas (microenvironments). In the static mode of microcantilever detection, microcantilevers can be bent by stresses that occur on or within its surface (Figure 1a) (Datskos 2005), such as those caused by dimensional changes in the surface coatings. Stoney's equation relates the amount of microcantilever bending to the differential change in surface stress (Figure 1b). The deformation of a coated microcantilever is in the range of 10^{-6} to 10^{-12} m and can be measured by a laser beam reflecting from the tip of the microcantilever (Figure 1a) in the AFM.

The application of such technique allows the detection of the surface stress induced by conformational (structural) change of surface-bound molecules, including proteins, DNA, and polymers (Godin et al. 2004; Karnati et al. 2007; Moulin et al. 1999; Shu et al. 2005). For example, Zhou and coworkers (Zhou et al. 2006) used the microcantilever technique to probe the structural change of polyelectrolyte brushes (20 nm thickness) immobilized on a microcantilever in response to a variety of environmental changes, such as pH and salt concentrations. These reports have established that the microcantilever technique has the specificity and sensitivity to detect real-time structural changes in a thin layer of surface-bound macromolecules.

In the present study, we apply the microcantilever technique to monitor changes in structure of the surface region of a cellulose coating on a microcantilever in response to a concentration gradient of sodium chloride. The results of our study demonstrate the capability of such nanomechanical approach in real-time detection of the minute change in surface structure of cellulose. This nanomechanical approach has the potential to reveal the non-hydrolytic breakdown of the crystal structure on the surface region of cellulose by cellulase and to eventually achieve a comprehensive understanding of cellulase actions on cellulose.

It is generally agreed that the natural substrate is often too complicated to be useful for detailed characterizations of cellulases (Kontturi et al. 2006). Various cellulose model surfaces have therefore been developed in the past decade (Kontturi et al. 2006) and utilized extensively in a variety of studies including investigation of interaction between cellulose and cellulase. We have developed a protocol to coat the surface of microcantilever with a model film of cellulose II having a thickness between 10 to 20 nm. The reason we decided

to use cellulose II was that this model film has been used most often and there is much technical information available about it. We also took advantage of the fact that the surface of cellulose II is relatively easier to prepare and characterize. All these made the cellulose II film as an ideal model surface for the current study. We are also in the process of exploring a film of native cellulose, which can be prepared from a nanocrystal suspension (Edgar and Gray 2003; Habibi et al. 2007) or from a nanofibril dispersion (Ahola et al. 2008). For the future study, we intend to use the nanofibril film, which is expected to preserve most of the structural properties of wood fibrils and to offer the best option for the surface of cellulose.

Attachment of a thin layer of cellulose to the surface of a microcantilever requires a thin anchoring layer, such as polyvinyl amine (PVAM), to improve the adhesion between the substrate and the cellulose (Figure 2a). The anchoring layer was prepared by the introduction of PVAM directly onto the silicon surface of a microcantilever beam. In our case, both the front side and the back side of the microcantilever were coated with PVAM. The cellulose was then deposited on the top of the anchoring layer on the front side of the microcantilever (Figure 2a) in the form of microcrystalline cellulose dissolved in a mixture of N-methylmorpholine-N-oxide (NMMO) and dimethyl sulfoxide (DMSO) with a desired viscosity (Falt et al. 2003). The characterization of each layer by means of AFM imaging shows the modification in the surface topography after each coating step (Figure 2 b–d). AFM imaging was performed on at least three different areas of the same specimen.

The microcantilever coated with cellulose II was treated with a successive exposure of different water/salt environments, and the bending of the microcantilever was monitored by the deflection of the microcantilever at its apex. To compensate for bulk effects of the buffer and the salt, we measured the differential bending, termed simply “bending” hereafter. The bending is the difference in deflection of the microcantilever with the cellulose coating and without the cellulose coating.

The result of bending of the microcantilever is presented in Figure 3a. In the experiment, the coated microcantilever was allowed to equilibrate in water for 2 hours prior to the first injection to achieve a stable baseline. Such a short time to achieve the equilibrium can be attributed to the unique size of microcantilever and the thin layer of cellulose coating, which allow a fast response to change of the solution environment. This has also benefit all the following studies involving changes of the salt concentrations. The measurement was initiated with the injection of water and a constant bending was observed during the first 45 minutes. After injection of a solution of 0.1M NaCl, the level of bending remained virtually constant. During the next two stages, higher concentrations of NaCl (0.5 M and 1 M) were applied. All salt solutions were prepared in water. A continuous rise in bending was detected at a pace of roughly 1 nm/min and a cumulative bending of more than 100 nm was seen at the end of the experiment.

We attribute the observed bending of the microcantilever to the change in the surface structure of the cellulose coating resulting from change in repulsive interactions among cellulose molecules. When a cellulose model film is exposed to an electrolyte solution, the cellulose undergoes a change in internal charge density, the magnitude of which depends on the concentration of the electrolyte (Ahola et al. 2008; Freudenberg et al. 2007; Tammelin 2006). The change in charge density in the cellulose film will change the intermolecular repulsion among cellulose molecules and will cause the surface structure of the cellulose to alter. When the cellulose is coated on one surface of the microcantilever (Figure 2a), such changes in the surface structure of the cellulose coating can exert a differential mechanical stress between the opposite surfaces of the microcantilever, leading to bending of the microcantilever.

Unlike QCM-D and ellipsometry, the bending of the microcantilever does not respond to minor changes in mass or thickness of the surface coating (Datskos 2005). The microcantilever technique specifically measures the mechanical stress induced by the structural changes in cellulose (Datskos 2005). Thus, such measurement is expected to be more informative about the non-hydrolytic disruption of the surface structure of cellulose by cellulase than those of QCM-D and ellipsometry.

The level of change in the microcantilever bending is indicative of the extent of change in the surface structure of the cellulose coating. As the increasing salt concentration results in a continuous change in the repulsive interactions among cellulose molecules in the cellulose coating, a more significant alteration of the surface structure of the cellulose coating as well as a larger rise in the microcantilever bending would be expected. This was verified when we characterized the surface of the microcantilever by means of AFM imaging to evaluate changes in surface morphology of cellulose after each salt treatment. We found no visible change in surface morphology (2.2% increase in mean roughness) after 0.1 M NaCl treatment, which is consistent with very little change in bending (Figure 3c). When the surface was further treated with 1 M NaCl, a significant change in morphology (43% increase in mean roughness) was observed (Figure 3d), which is correlated well with the large rise in bending. This AFM imaging study further confirms the link between the bending of microcantilever and the change in surface structure of cellulose.

Overall, microcantilever bending correlates well with the change in molecular structure of the surface region of the cellulose film. This validates that the microcantilever technique is highly sensitive and specific in detecting real-time changes in surface structure of cellulose. In our further work, we will apply the microcantilever technique to the study of the effect of cellulase on the surface structure of cellulose.

Materials and methods

Materials

Microcrystalline cellulose, DMSO, and N-methylmorpholine oxide (NMMO) were purchased from Sigma-Aldrich (St. Louis, MO). Polyvinylamine (PVAM) was purchased from BASF (Florham Park, New Jersey). The microcantilevers ($200\text{ }\mu\text{m} \times 25\text{ }\mu\text{m} \times 2\text{ }\mu\text{m}$, 0.1N/m) were home-made.

Preparation of cellulose II model surface

The cellulose II surface was prepared on the front side of a microcantilever that was made of SiO_2 . The surface of the microcantilever ($200\text{ }\mu\text{m} \times 25\text{ }\mu\text{m} \times 2\text{ }\mu\text{m}$, 0.1N/m) was first treated with UV ozone for 20 min. It was then immersed in 0.22% polyvinylamine (PVAM) for 60 min followed by rinsing with water. Both the front side and the back side of the microcantilever were coated with PVAM. A suspension of 0.5 mg of microcrystalline cellulose powder in 25 ml of 50% N-methylmorpholine oxide (NMMO) was heated while stirring until a transparent brown solution was obtained. While still warm, DMSO was added to afford the cellulose solution with a final concentration of 1%. Such solution was evenly applied onto the surface on the front side of the PVAM-coated microcantilever. The microcantilever was allowed to sit for about one hour, and then a drop of water was added to precipitate the cellulose. The cellulose film was soaked in water for four hours and the water was replaced every 30 minutes. The resulting cellulose film was then incubated in the oven at $80\text{ }^\circ\text{C}$ for one hour.

Microcantilever experiment

All the experiments were performed using a modified commercial scanning probe microscope (Nanoscope III from Digital Instruments/Veeco, Santa Barbara, CA). The cellulose-coated microcantilever was mounted with the coating facing down in a liquid cell with a volume of 50 μl . All the solutions were introduced through injection in a batch mode, where each solution was injected all at once. The cellulose was usually allowed to equilibrate with water for at least 2 hours prior to any addition. Each assay was run against a reference assay where the microcantilever was coated with only PVAM to allow the subtraction of the background signal and the bulk solvent effect.

A desktop PC, running programs written in LabView (National Instruments, Austin, TX), was used to record the cantilever deflection signal from the AFM via a data acquisition board with a maximum data acquisition rate of 300 kHz. During a measurement, 100,000 data points were taken every 30 seconds at a rate of 100 kHz. The bending of the cantilever was obtained by simply averaging the data points.

AFM imaging

To characterize the coverage, morphology, roughness, and thickness of model films, AFM imaging was performed using a Nanoscope IIIa multimode scanning probe microscope from Digital Instruments Inc., Santa Barbara, CA. The samples were scanned in contact mode in air using silicon nitride cantilevers (MLCT) manufactured by Veeco, Camarillo, CA, with a nominal spring constant of 0.05 N/m. The size of the images was $5\text{ }\mu\text{m} \times 5\text{ }\mu\text{m}$, and images were scanned on at least three different areas of the sample. The images of both height and deflection modes were captured. The surface morphology was analyzed using image processing software.

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Fig. 1.

The deflection of a microcantilever in an AFM. a. Deformation in the range of 10^{-6} to 10^{-12} m of a coated microcantilever can be precisely measured by the reflection of a laser beam from the tip of the microcantilever. b. The deformation of the microcantilever ΔZ follows the Stoney equation (Datskos 2005). R is the radius of the curvature of the microcantilever, ν is Poisson's ratio for the substrate, E is Young's modulus for the substrate, l is the length of the microcantilever, t is the thickness of the microcantilever, and $\Delta\sigma$ is the differential surface stress. The AFM instrument is modified so that it can be used to detect and record the deflection in the microcantilever in real-time.

Fig. 2.

Surface coating on the microcantilever. a. The coating scheme. Attachment of a thin layer of cellulose to the surface of the microcantilever required a thin anchoring layer, such as polyvinyl amine (PVAM), to improve its adhesion to the microcantilever. The anchoring layer was prepared by introducing PVAM directly onto the silicon surface of a microcantilever beam. Microcrystalline cellulose was dissolved in a mixture of N-methylmorpholine-N-oxide (NMMO) and dimethyl sulfoxide (DMSO) and was deposited on the top of the anchoring layer on the front side of the microcantilever. b–d. AFM images of surface coatings ($5\ \mu\text{m} \times 5\ \mu\text{m}$). b. The surface without any coating. c. The surface coated with PVAM. d. The surface coated with cellulose. The characterization of each layer by means of AFM imaging shows the modification in the surface topography after each coating step (Figure 2 b–d). e. The picture of the microcantilever used in the experiment.

Fig. 3.

The morphological and structural changes of cellulose monitored by means of the microcantilever technique. a. The bending of the microcantilever increased with the increasing NaCl concentration. The bulk effects of the buffer were compensated by measurement of differential bending, which is the difference in deflection between the microcantilever with the cellulose coating and without the cellulose coating. b. AFM image of the surface of the cellulose coating on the microcantilever. Mean roughness: 1.80 nm with a Z range of 25 nm. c. AFM image of the cellulose surface (as shown in b) after being treated with 0.1 M NaCl. Mean roughness: 1.84 nm with a Z range of 25 nm. d. The cellulose surface (as shown in c) after being treated with 1.0 M NaCl. Mean roughness: 2.59 nm with a Z range of 25 nm.