Surface plasmon resonance imaging of the enzymatic degradation of cellulose microfibrils†

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We present the first study of the interaction of a cellulase enzyme mixture with cellulose microfibrils using surface plasmon resonance (SPR) imaging. The cellulose microfibrils, obtained from the bacterium Acetobacter xylinum, were heterogeneously distributed on a thin layer of thioglucose deposited onto a gold film. SPR images collected as a function of time allowed us to observe the adsorption of the enzymes onto both the cellulose microfibrils and the bare surface, and the subsequent degradation of the cellulose microfibrils in real time. In particular, we were able to characterize the decrease in the thickness and variations in thickness of the cellulose microfibril-coated regions with time, and to define a characteristic time for the removal of cellulose from the surface. These results demonstrate the distinct advantage of the SPR imaging technique for measuring the effectiveness of enzymes on cellulose microfibrils and provide useful metrics of enzyme activity that are of relevance to the cellulosic ethanol industry.

Introduction

The degradation of cellulose by enzymes is a key step in the production of cellulosic ethanol.† Mixtures of enzymes such as endoglucanases, exoglucanases, cellobiohydrolases and β-glucosidases are typically used, mimicking the approach used in nature by various fungi such as Hypocrea jecorina (formerly Trichoderma reesei). ‡-§ Improvements in the efficiency of the enzymatic action are necessary to make the production of cellulosic ethanol more economically viable, however this is challenging because of the physical structure of native cellulose. Native cellulose (cellulose I) consists of linear polymers of β-(1→4) linked D-glucose,‡ and intra- and inter-molecular hydrogen bonding between residues within the polymer chains leads to the formation of fibrils and microfibrils which are the primary structural component in plant cell walls. It is the strongly bonded fibril structures that must be broken down in industrial pretreatment processes to produce cellulosic ethanol,7-11 and so it is of great importance to the cellulosic ethanol industry to study enzymatic digestion using cellulose microfibril samples.

The interaction of enzymes with cellulose is typically studied using biochemical and microbiological techniques,12,13 as well as electron14-16 and fluorescence microscopy.17,18 Recently, there has been considerable effort to develop model cellulose thin films19-21 so that enzymatic degradation studies can be performed using surface-sensitive techniques. A variety of phenomena have been studied in quartz crystal microbalance (QCM) experiments of model cellulose films such as the kinetics of swelling,22,23 and enzymatic adsorption and degradation upon exposure to single enzymes24,25 and enzyme mixtures.26-28 Another important surface-sensitive technique, surface plasmon resonance (SPR), has been used in only a few studies to measure the adsorption of single enzymes to microcrystalline cellulose29 and to study the adsorption of polysaccharides onto model cellulose films.30,31 To allow the use of conventional surface-sensitive techniques such as SPR and QCM, it is necessary to prepare thin coatings of cellulose that have uniform thickness and uniform coverage of the underlying substrate since the analysis of the data relies on the use of uniform layer models. However, the preparation of thin uniform coatings of cellulose requires special treatments, which results in cellulose structure and functionalization that differs from that of native cellulose microfibrils. For example, common solvents cannot be used to dissolve the cellulose. It is possible to dissolve cellulose by using aggressive solvents such as dimethylacetamide/lithium chloride (DMAc/LiCl) and N-methylmorpholine-N-oxide (NMMO), so that model cellulose films can be deposited using spincoating and Langmuir–Blodgett techniques.32 However, the resulting films are quite rough and have a structure (either cellulose II or amorphous cellulose) that is different from the cellulose I structure of native cellulose.
Another approach to creating model cellulose films involves the use of derivatized cellulose, trimethylsilyl cellulose (TMSC), which can also be deposited using Langmuir–Blodgett or spin-coating techniques, and subsequently regenerated in a gaseous wet HCl atmosphere. The regeneration process results in significant changes to the film: a reduction of the film thickness and the production of crystal structures such as cellulose II and amorphous cellulose that differ from the native cellulose I structure.

Other approaches have been used to fabricate model films of cellulose I. Exposure of cellulose microfibrils to sulfuric acid can be used to create nanocrystalline cellulose (NCC), which consists of nanofibrils that are typically several nanometers in diameter and up to 2 μm long. Suspensions of nanocrystalline cellulose can be transferred onto substrates using spincoating. Langmuir–Blodgett and polyelectrolyte deposition techniques, and the resulting films can be quite uniform in thickness (rms roughness of several nanometers). Although NCC has the cellulose I crystal structure, the sulfuric acid treatment produces sulfate groups on the surface that are not present on native cellulose or cellulose that has undergone the steam explosion degradation of thin, non-uniform thickness coatings of cellulose or cellulose that has undergone the steam explosion process that is used in the cellulosic ethanol industry.

The objective of the present work was to study the enzymatic degradation of thin, non-uniform thickness coatings of cellulose microfibrils produced by the bacterium *Acetobacter xylinum*. These cellulose microfibrils have several advantages for the present study: (1) they consist of a native cellulose (cellulose I), with values of the degree of crystallinity (60–90%) and degree of polymerization (2000–8000) that are high; (2) unlike plant-based cellulose, they are not complicated by the presence of hemicellulose or lignin; and (3) we have previously studied the effect of a cellulose enzyme on *A. xylinum* cellulose microfibrils using atomic force microscopy (AFM) which allows us to complement the high resolution imaging data obtained using AFM with the kinetic data obtained in the present study.

Despite the distinct advantages of using the bacterial cellulose microfibrils, their use poses a major challenge for studies of the kinetics of enzymatic degradation. Because the microfibrils do not coat the underlying surface uniformly, these samples cannot be studied accurately using conventional surface plasmon resonance (SPR) that averages over relatively large sample areas (~mm²). The lateral non-uniformity of the cellulose microfibril-coated surfaces requires the use of spatially resolved experimental techniques that can be used to compare the signal from regions of microfibril-coated surface with that from the bare surface. To study the kinetics of enzymatic degradation of cellulose microfibril-coated surfaces, we have adapted one of the surface-sensitive techniques, SPR, to allow imaging of the laterally heterogeneous sample surface.

Surface plasmon resonance (SPR) is a surface-sensitive optical technique that is routinely used to study the kinetics of adsorption of molecules onto surfaces. In the SPR experiment, incident light is coupled into a very thin (~45 nm thick) gold film by using either a high refractive index prism or a surface grating. At the resonance condition, surface-guided collective oscillations of the conduction electrons (surface plasmons) are excited within the gold film, resulting in a decrease of the light reflected from the film. Measurement of the reflected light intensity at angles of incidence near that corresponding to the surface plasmon resonance condition forms the basis for biosensing techniques, such as the analysis of receptor–ligand interactions ranging from small analytes to whole cells. In the conventional SPR experiment, the intensity of the light reflected from the illuminated portion of the sample surface (typically several mm²) is measured, and small changes in the layer adsorbed onto the thin gold film induce significant changes in the reflected light intensity. If the adsorbed layer thickness is very uniform in the experiment, the SPR data can be fit using uniform layer models for the adsorbed layer to achieve sub-nanometer resolution in the determination of changes in the layer thickness.

For adsorbed layers with non-uniform thickness, conventional SPR yields a signal that is an average of many different thicknesses, which leads to a complicated analysis of the data and less resolution in the determination of the film thickness and the kinetics of adsorption/desorption. To allow the study of laterally heterogeneous samples using SPR, SPR imaging (SPRi) techniques have been developed. In these experiments, the photodetector is replaced with a CCD camera, allowing the SPR phenomenon to be studied within laterally heterogeneous samples by tracking small regions of interest (ROIs) within the images in which the coverage of the substrate is rather uniform. SPRi techniques have been implemented using various degrees of sophistication, correcting for different imaging artifacts that are inherent to the experiments.

In the present manuscript, we describe the first use of surface plasmon resonance imaging (SPRi) to study enzymatic degradation of bacterial cellulose microfibrils. This technique allows the concurrent observation of the adsorption of the enzymes onto both the cellulose microfibrils and the bare sample surface, and the subsequent degradation of the cellulose microfibrils in real time. By tracking decreases in the thickness and variations in the thickness of the cellulose microfibril-coated regions with time, we can define a characteristic time constant for the action of the enzymes. These results are significant because they provide a metric of enzyme activity that is likely to be useful to the cellulosic ethanol industry to aid in the development of improvements to enzyme mixtures. The methodology described in this work opens new opportunities for the use of SPR to study laterally heterogeneous samples, and it may find a broad range of new applications in the future.

**Experimental procedures**

**Reagents and solutions**

Solutions of 1-thio-D-glucose (Sigma) in spectroscopic grade methanol (99.96%, Fisher Scientific) were prepared to a concentration of approximately 2 mmol L⁻¹. These solutions were used to form self-assembled monolayers of 1-thio-D-glucose on gold. The electrolyte used for enzyme experiments was a 50 mM sodium citrate buffer (pH 5.4). The buffer was prepared using 0.2286 g citric acid (Fluka) and 1.121 g sodium citrate (Alfa Aesar) in 100 mL of Milli-Q ultrapure water (resistivity of 18.2 MΩ cm). The enzyme mixture used in this work was cellulase from *H. jecorina* (Sigma part number C8546), obtained in lyophilized form. The mixture contained a synergistic complex of enzymes including endoglucanases, exoglucanases, cellobiohydrolases and β-glucosidases. The enzymes were added to the citrate buffer to an approximate concentration of 0.02 mg mL⁻¹.
Cellulose preparation

Cellulose was isolated and purified from cultures of the bacterium Acetobacter xylinum grown for approximately one month in a quiescent solution as previously described. To prepare the cellulose dispersion, a ball milling process (BeadBeater, Biospec Products Inc., USA) was used with 2.5 mm diameter glass beads. Approximately 200 mg dry weight of cellulose was added to the small chamber that was filled to two-thirds capacity with glass beads. Methanol was added carefully to the chamber avoiding air bubble formation. The ball milling process was run for 2 minutes and then allowed to cool for 10 minutes. This procedure was repeated typically 3 times or until a uniform slurry, as observed by eye, was obtained. Ball milling cellulose for only several minutes results in changes that are attributable to a decrease in the degree of crystallinity and/or degree of polymerization of the cellulose.

The beads and cellulose were emptied from the chamber into a small crystallizing dish and the chamber was washed three times with methanol. The cellulose–methanol suspension was removed using a Pasteur pipette as the beads settled to the bottom of the dish. The beads were washed five times with 10–20 mL aliquots of methanol and all of the remaining cellulose was collected. Three 1.5 mL samples of the cellulose dispersion were dried in a small crystallizing dish and the chamber was washed three times with spectroscopic grade methanol and Milli-Q water, creating a self-assembled monolayer of thioglucose on the gold surface. Cellulose microfibrils were deposited onto the thioglucose-coated gold-coated SPR substrates and the thioglucose layer. This allowed the selection of regions of interest (ROIs) in the SPR images for further study.

In addition, to obtain accurate tracking of specific features in the SPR images, algorithms were developed to correct for two imaging artifacts: beam walking, which is due to changes in the refraction of light at the face of the triangular prism as \( \theta_i \) was varied; and image compression or foreshortening, which is due to changes to the illuminated area of the detector as \( \theta_i \) was varied. Both imaging artifacts result in shifts in the position of features in the collected images as \( \theta_i \) was varied. A robust algorithm for calculating the proper offset and scaling for different ROIs in the SPR images was developed to correct for both imaging artifacts. Briefly, the image correction algorithm is implemented as follows: an SPR image, which we call the mask image, is collected at a specific angle of incidence \( \theta_i \) which produces sufficient image contrast between the deposited cellulose microfibrils and the thioglucose layer. This allowed the selection of regions of interest (ROIs) in the SPR images for further study.

An automated angle scanning SPR imaging instrument based on the Kretschmann–Raether geometry was designed and constructed. The instrument is a refinement of a commercially available manual variable angle SPR imaging instrument (SPRImager, GWC Technologies, USA) that was modified to allow automated scanning with higher precision determination of the angles of incidence and reflection, and to correct for imaging artifacts, as described below.

In the SPR imaging experiment, light from a miniature quartz-halogen bulb (3.0 mm length \( \times \) 0.7 mm diameter) was focused onto a pinhole and collimated using a camera lens. The collimated light passed through a polarizer and was incident on the SPR fluid cell assembly at a specified angle of incidence \( \theta_i \). The angle of incidence \( \theta_i \) was determined to within 0.004° by using a precision rotation stage (RT-5, Newmark, USA). The light reflected from the SPR fluid cell assembly was passed through a narrow band-pass filter (FWHM = 1.5 nm, centered at 795–798 nm) and detected using a CCD camera (Watec, 752 \( \times \) 480 pixels). The SPR fluid cell assembly consisted of an equilateral (apex angle of 60°) triangular SF-10 glass prism (Esco Products, USA) coupled to the SF-10 SPR substrate using index matching oil (index of refraction \( n = 1.720 \) at \( \lambda = 589.3 \) nm, Cargille) that is in turn pressed onto a machined Kel-F fluid cell which had a solution volume of approximately 55 \( \mu L \). A second identical precision rotation stage (RT-5, Newmark, USA) was used together with the first precision rotation stage to create the necessary \( \theta-2\theta \) goniometer onto which the SPR fluid cell assembly and detector assembly were mounted. Custom procedures written using V++ (Digital Optics, NZ) were used to automate the \( \theta-2\theta \) goniometer and image acquisition.

At the start of each experiment, a range of angles of incidence \( 45° < \theta_i < 55° \) was scanned as SPR images were collected to determine a value of \( \theta_i \) which produced sufficient image contrast between the deposited cellulose microfibrils and the thioglucose layer. This allowed the selection of regions of interest (ROIs) in the SPR images for further study.

In the present study, two different types of SPR imaging experiments were performed: (1) the collection of SPR images as a function of \( \theta_i \) to obtain reflectivity \( R \) versus \( \theta_i \) curves (which we refer to as SPR curves) for the defined ROIs; and (2) the collection of SPR images at four closely spaced values of \( \theta_i (\theta_i = 50.1°, 50.2°, 50.3° \) and \( 50.4° \)) cyclically as a function of time to obtain the kinetics of enzyme adsorption and cellulose degradation within the defined ROIs. In the SPR imaging experiments, a liquid, either citrate buffer or citrate buffer with enzymes, was pumped into the SPR fluid cell using a 12-roller peristaltic pump (Ismatec) using a...
flow rate of 33.3 μL min⁻¹. All SPR imaging measurements were performed at a temperature of 25.0 ± 0.1 °C.

Results and discussion

SPR images

In Fig. 1A we show a SPR image of the fluid cell chamber that was collected at a fixed angle of incidence $\theta_i = 50.4^\circ$ before the sample was exposed to the enzyme mixture. The large dashed rectangle within the SPR image corresponds to the smaller area that is shown in Fig. 1B. The lighter areas in the SPR images are regions with deposited cellulose microfibrils (cellulose microfibril-coated regions) and the darker areas are the regions in which the thioglucose layer on gold is exposed (uncoated regions), as shown by atomic force microscopy (AFM) images of the coated and uncoated regions (data not shown, similar to data shown previously in ref. 40). The cellulose microfibril-coated regions were stable in citrate buffer before the introduction of the enzyme mixture, as measured using AFM and SPRi. The nonuniform coverage of the surface is immediately apparent in Fig. 1 and this provides a good sample with which to demonstrate the advantages of the new SPRi technique. In Fig. 1, the regions of interest (ROIs) that are used to collect reflectivity versus time data over the course of the experiment are identified by solid rectangles. The solid rectangles labeled 1 to 5 in Fig. 1A indicate ROIs for which a ROI pixel analysis was performed, as described below. Two of the solid rectangle ROIs are highlighted with circles in Fig. 1B and C: the dashed circle highlights an uncoated region, and the solid circle highlights a cellulose microfibril-coated region. The reflectivity $R$ versus $\theta_i$ (SPR) curves for the two highlighted ROIs in Fig. 1B and C are shown in Fig. 2. For this value of $\theta_i = 50.4^\circ$, the reflectivity for the cellulose microfibril-coated regions is higher than for the uncoated regions, corresponding to lighter regions in the SPR images shown in Fig. 1.

The SPR curves shown in Fig. 2 for the cellulose microfibril-coated and uncoated regions differ considerably with respect to the position, depth and width of the minimum in reflectivity. Although the ROI highlighted by a solid circle in Fig. 1B and C appears to be fully covered by adsorbed cellulose microfibrils, the broad, shallow dip in the corresponding SPR curve in Fig. 2 indicates that there is non-uniform or incomplete coverage of cellulose within this ROI. Incomplete cellulose coverage leads to an average reflectivity response for the ROI that is the superposition of different SPR curves, each with its minimum in reflectivity occurring at a different value of $\theta_i$, which leads to a broad and shallow dip in the measured SPR curve.

Enzymatic degradation of cellulose microfibrils

To measure changes that occur in the sample upon the introduction of the enzyme mixture we chose to measure the approximately 130 minutes and subsequent rinse with the citrate buffer, for the same area as shown in (B). For all images, the regions of interest identified by the small solid rectangles are approximately 160 μm × 320 μm (8 × 8 pixels) and are in the same locations for all images. The white arrows in (C) indicate two small areas in which air bubbles developed spontaneously during the course of the experiment and these areas were avoided in the data analysis.
reflectivity in multiple ROIs at four different angles of incidence ($\theta_i = 50.1^\circ$, 50.2$^\circ$, 50.3$^\circ$ and 50.4$^\circ$) cyclically as a function of time. In Fig. 3A we show reflectivity versus time data collected at a fixed angle of incidence of $\theta_i = 50.1^\circ$ for the ROI highlighted in Fig. 1B and C by the solid circle, which initially is an uncoated region. In Fig. 3B we show reflectivity versus time data collected at a fixed angle of incidence of $\theta_i = 50.4^\circ$ for the ROI highlighted in Fig. 1B and C by the solid circle, which initially is a cellulose microfibril-coated region. In Fig. 3, citrate buffer is introduced to the SPR fluid cell at $t = 0$ with a flow rate of 33.3 $\mu$L min$^{-1}$. At $t \approx 370$ s, the citrate buffer solution containing enzymes (concentration $\sim 0.02$ mg mL$^{-1}$) is introduced to the SPR fluid cell. At $t \approx 8000$ s, the flow of the enzyme mixture and data collection were stopped and the fluid cell was flushed with citrate buffer.

In Fig. 3A, a fast initial increase in reflectivity $R$ of approximately 0.1 is observed for the uncoated region at a fixed angle of incidence $\theta_i = 50.1^\circ$. This corresponds to the irreversible adsorption of enzymes to the uncoated region since the reflectivity decreases by only 0.010 (data not shown) after the final rinse with citrate buffer at $t \approx 8000$ s. The change in reflectivity due to the interaction of the enzyme mixture with the cellulose microfibril-coated region is shown in Fig. 3B. An initial increase in reflectivity is observed as the enzymes enter the SPR fluid cell and adsorb to the surface. This initial increase in reflectivity is followed by a large, gradual decrease in reflectivity that we interpret as the enzymatic degradation of the cellulose microfibrils. This interpretation is consistent with our previous direct observation of gradual enzymatic degradation of cellulose microfibrils using AFM. This was shown in the present study by the results of two experiments performed in tandem with a SPR imaging measurement on microfibril-coated SPR slides with comparable surface coverage which showed a time-dependent, gradual decrease in reflectivity with exposure to the enzyme mixture. We note that extensive degradation of cellulose with enzyme action can lead to the eventual detachment of the almost-completely digested microfibrils from the surface, as observed using high-resolution AFM imaging. Other than late-stage detachment of fragments of cellulose microfibrils produced by enzyme action, detachment of microfibrils from the surface was not observed after the introduction of the enzymes, and we interpret the gradual decrease in reflectivity observed in Fig. 3B as enzymatic degradation of the cellulose microfibrils. After

**Fig. 3** (A) Plot of time dependence of average reflectivity $R$ at a fixed angle of incidence of $\theta_i = 50.1^\circ$ of the $8 \times 8$ pixels in the ROI highlighted in Fig. 1B and C by the dashed white circle, which is an uncoated region before the introduction of the enzyme mixture. (B) Plot of time dependence of average reflectivity $R$ at a fixed angle of incidence of $\theta_i = 50.4^\circ$ of the $8 \times 8$ pixels in the ROI highlighted in Fig. 1A and B by the solid white circle, which is a cellulose microfibril-coated region before the introduction of the enzyme mixture. (C) Plot of time dependence of slope of $R$ versus $\theta_i$ calculated using the $R$ versus time data sets collected at four different angles of incidence ($\theta_i = 50.1^\circ$, 50.2$^\circ$, 50.3$^\circ$ and 50.4$^\circ$). The vertical dotted line corresponds to the time for the maximum value of $R$ in (B). The enzyme mixture was introduced at $t \approx 370$ s, as indicated in (B) and (C) by the small vertical arrows.

microfibril-coated SPR slides to the enzyme mixture using a colorimetric carbohydrate assay performed in tandem with a SPR imaging measurement on microfibril-coated SPR slides with comparable surface coverage which showed a time-dependent, gradual decrease in reflectivity with exposure to the enzyme mixture.

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rinsing with citrate buffer at \( t = 8000 \text{ s} \), a further small decrease in reflectivity of 0.015 at a fixed angle of incidence of \( \theta_i = 50.4^\circ \) is observed (data not shown), indicating that an irreversible change to the sample has occurred.

In Fig. 4, we compare the SPR curve that was collected before the sample was exposed to the enzyme mixture with that collected after the sample was exposed to the enzyme mixture. The circular symbols and triangular symbols in Fig. 4 correspond to data obtained from the ROIs highlighted in Fig. 1B and C by the dashed circle and the solid circle, respectively. The solid symbols in Fig. 4 correspond to experimental SPR curves measured for the two ROIs on the SPR substrate after exposure to the enzyme mixture for approximately 130 minutes and a subsequent rinse with citrate buffer. After exposure to the enzyme mixture, the angular position of the SPR minimum of the uncoated region (circles) in Fig. 4 shifts to a larger value of \( \theta_i \) by approximately 0.11°, indicating the irreversible adsorption of enzymes.

The SPR curve obtained for the cellulose microfibril-coated region in Fig. 4 (triangles) becomes narrower and deeper after exposure to the enzyme mixture, with the angular position of the SPR minimum decreasing by approximately 0.19°. The shift of the minimum to smaller values of \( \theta_i \) indicates a reduction of the layer thickness, and the narrowing and deepening of the minimum in the SPR curve indicates that the cellulose layer also becomes more uniform in thickness. It is also worth noting that the final SPR curves (open circles and triangles) are not identical, indicating that the cellulose microfibrils were not completely removed from the thioglucose layer and replaced by adsorbed enzyme molecules. This result is consistent with the visibility of the pattern of the deposited cellulose microfibrils in the final SPR image shown in Fig. 1C.

To quantify the changes in the shape of the SPR curves that occur after introducing the enzyme mixture, it is useful to calculate both the change in the width of the dip in the SPR curves as well as the change in the slope of the low angle side of the minimum in reflectivity as a function of time. We define the half-width of an SPR curve as the angular range between the minimum in reflectivity and the reflectivity value that is halfway between the maximum and minimum reflectivity values on the low angle side of the SPR minimum. The calculated half-widths for the SPR curves shown in Fig. 4 are 0.44° (solid circles), 0.45° (open circles), 0.70° (solid triangles), 0.51° (open triangles). The half-width of the SPR curve for the uncoated region is essentially unchanged (a small increase of only 0.01°) upon adsorption of the enzymes, whereas for the cellulose fibril-coated region the action of the enzymes resulted in a decrease of the half-width of the SPR curve by 0.19°.

It is also useful to compare the average slopes of \( R \) versus \( \theta_i \) on the low angle side of the SPR minimum for the cellulose microfibril-coated region before and after enzyme treatment (solid triangles and open triangles respectively), as indicated in Fig. 4 by the two best-fit straight lines. During the measurement of the action of the enzyme mixture, we chose to measure the reflectivity \( R \) at four different angles of incidence \( (\theta_i = 50.1^\circ, 50.2^\circ, 50.3^\circ \text{ and } 50.4^\circ) \) cyclically as a function of time which allows us to perform a linear fit to each of these angular scan data sets and extract a slope value that characterizes the shape of the SPR curve as a function of time. For each cycle of \( \theta_i \), the four images from which the slope value is extracted are collected within a small time window (≈4 s) and corrected for the beam walking and image compression artifacts associated with changes in \( \theta_i \).

In Fig. 3C we show a plot of slope \( R \) versus time calculated using the reflectivity \( R \) versus \( \theta_i \) data sets collected at the four different angles of incidence for the ROI highlighted in Fig. 1B and C by the solid white circle, which is a cellulose microfibril-coated region. In these plots, the enzyme mixture was introduced at \( t = 370 \text{ s} \) as indicated by the vertical arrows. The vertical dotted line corresponds to the peak in the reflectivity (\( t \approx 800 \text{ s} \)) in Fig. 3B. It can be seen that although an initial increase in \( R \) is observed as the enzymes enter the SPR fluid cell and interact with the cellulose microfibrils (see Fig. 3B), no corresponding change in slope is observed (see Fig. 3C). The lack of change in slope over this time period (\( 370 \text{ s} < t < 800 \text{ s} \)) indicates that there is no change in the shape of the SPR curve, which is consistent with an adsorption of the enzyme to the surface without considerable degradation of the cellulose microfibrils. As \( R \) begins to decrease in Fig. 3B, we observe a corresponding decrease in the magnitude of the negative slope values shown in Fig. 3C, corresponding to a decrease in the width of the SPR curve due to a reduction in the variation of thickness within the ROI. Because the shape of the SPR curve changes as we observe the decrease in \( R \), it is not appropriate to assume that the decrease in \( R \) follows a simple exponential decay. The degradation process observed for the ROI is more complicated than a process that could be characterized by a single time constant. At \( t = 6600 \text{ s} \), we observe that there is no longer any appreciable change in the slope with time, indicating that there is no longer any appreciable change in the shape of the SPR curve and thickness variation within the ROI.
However, we observe in Fig. 3B that $R$ continues to decrease for times $t > 6600$ s which indicates that the overall thickness within the ROI continues to decrease with time.

To obtain a more complete understanding of the effect of the enzyme mixture on the cellulose microfibrils, we performed an analysis of the distribution of reflectivity values for the pixels within individual ROIs in the SPR images. In Fig. 5, we show histograms corresponding to the distribution in reflectivity values measured within the ROIs indicated by the solid rectangles labeled 1 to 5 in Fig. 1A before the introduction of the enzyme mixture (grey) and at the end of the experiment (crosshatched). The ROIs that are labeled 1 to 4 highlight different cellulose microfibril-coated regions, and the ROI that is labeled 5 highlights an uncoated region. In the ROI pixel analysis, the pixels within a given ROI are binned according to their reflectivity values to create reflectivity histograms as shown in Fig. 5. A bin width of 5 was chosen to be sufficiently large to ensure that at least 50 counts were obtained in the maximum of the distribution while being sufficiently small to allow measurement of changes in the width of the distributions with time. It can be seen that the histograms corresponding to the cellulose microfibril-coated regions (ROIs 1 to 4) become narrower and shift to lower values of reflectivity after exposure to enzyme. Table 1 shows the best-fit peak and width values for the histograms labeled 1 to 4 in Fig. 5 obtained by fitting each histogram to a Gaussian distribution.

![Histograms](image)

**Fig. 5** Grey and crosshatched histogram data correspond to ROI pixel analyses performed on SPR images collected at a fixed angle of incidence $\theta_i = 50.4^\circ$ at $t = 3.5$ s and $t = 8003.5$ s, respectively, for the ROIs indicated by the solid rectangles labeled 1 to 5 in Fig. 1A. The bin width for each histogram was chosen to be 0.05. To allow the visibility of both grey and crosshatched bars in each histogram, the width of each bar was reduced to 0.02, the grey bars were shifted in reflectivity by $-0.01$ and the crosshatched bars were shifted in reflectivity by $+0.01$.

<table>
<thead>
<tr>
<th>ROI</th>
<th>Time (s)</th>
<th>Best-fit peak position</th>
<th>Best-fit peak width</th>
<th>$R^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3.5</td>
<td>0.690</td>
<td>0.20</td>
<td>0.991</td>
</tr>
<tr>
<td>2</td>
<td>3.5</td>
<td>0.330</td>
<td>0.087</td>
<td>0.996</td>
</tr>
<tr>
<td>3</td>
<td>3.5</td>
<td>0.290</td>
<td>0.095</td>
<td>0.992</td>
</tr>
<tr>
<td>4</td>
<td>3.5</td>
<td>0.247</td>
<td>0.088</td>
<td>0.998</td>
</tr>
<tr>
<td>5</td>
<td>8003.5</td>
<td>0.329</td>
<td>0.109</td>
<td>0.995</td>
</tr>
</tbody>
</table>

The shift of the histogram peaks to lower values of reflectivity and the decrease in the width of the histograms upon treatment with enzyme supports the interpretation that the enzyme exposure results in a decrease in the cellulose thickness and a decrease in the variation in the cellulose thickness.

Although the choice of $\theta_i = 50.4^\circ$ for the data presented in Fig. 5 gives good contrast in $R$ for the cellulose microfibril-coated regions (ROIs 1 to 4) before and after exposure to the enzyme mixture, it offers little contrast in $R$ for the uncoated region (ROI 5) since these data are collected near the SPR minimum for this ROI. However, the reflectivity histograms observed for the uncoated region provide useful measures of the expected average value of $R$ and spread in $R$ values in the absence of cellulose microfibrils. It can be seen by comparing the histograms for ROI 5 with the crosshatched histograms for ROIs 1 to 4 that there is significant but incomplete removal of cellulose from the surface at the end of the experiment.

The time dependence of the reflectivity within different ROIs can also be used to define a characteristic time $t_{1/2}$ that provides a practical measure of the effectiveness of the enzyme mixture, corresponding to the time for the reflectivity $R$ measured at a fixed angle of incidence $\theta_i$ to decrease to half of the difference between the maximum value of $R$ and the value of $R$ observed at long times. For example, for the plot of $R$ versus time at a fixed angle of incidence $\theta_i$ shown in Fig. 3B, $t_{1/2} \approx 1660$ s. An average value of $t_{1/2} = 1860 \pm 210$ s was obtained by averaging the results measured for the cellulose microfibril-coated ROIs labeled a to d, together with the ROI highlighted by the solid white circle, in Fig. 1B. We see that the characteristic time $t_{1/2}$ can be determined from different cellulose microfibril-coated regions on the sample to within roughly 10%, and so this should be a useful metric for judging the relative effectiveness of different enzyme mixtures on cellulose microfibrils.

**Conclusions**

We have used a custom-built, automated angle scanning surface plasmon resonance imaging system to monitor the interaction of cellulase enzyme mixtures with individual bundles of cellulose microfibrils in liquid. This measurement has been enabled by the development of a novel ROI compensation scheme that allows the accurate tracking of the reflectivity of multiple ROIs both as a function of angle and as a function of time at multiple angles of incidence. These data have allowed us to track decreases in the thickness and thickness variations of the...
cellulose microfibril-coated regions with time upon exposure to enzymes and to calculate a characteristic time that can be used to evaluate the effectiveness of the enzymes. In the past, SPR has been used to investigate time dependent changes in the thickness of laterally homogeneous films. In this study we showed for the first time how to apply this technique to investigate laterally heterogeneous films that are characterized by a distribution of film thicknesses. This is a significant result because it demonstrates that SPR can be used to study industrial samples. We expect that these analysis techniques should be of direct interest to the cellulosic ethanol industry.

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