Enzymatic Kinetics of Cellulose Hydrolysis: A QCM-D Study

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The interactions between films of cellulose and cellulase enzymes were monitored using a quartz crystal microbalance (QCM). Real-time measurements of the coupled contributions of enzyme binding and hydrolytic reactions were fitted to a kinetic model that described closely significant cellulase activities. The proposed model combines simple Boltzmann sigmoidal and 1 - exp expressions. The obtained kinetics parameters were proven to be useful to discriminate the effects of incubation variables and also to perform enzyme screening. Furthermore, it is proposed that the energy dissipation of a film subject to enzymatic hydrolysis brings to light its structural changes. Overall, it is demonstrated that the variations registered in QCM frequency and dissipation of the film are indicative of mass and morphological transformations due to enzyme activities; these include binding phenomena, progressive degradation of the cellulose film, existence of residual, recalcitrant cellulose fragments, and the occurrence of other less apparent changes throughout the course of incubation.

1. Introduction

Unraveling the mechanisms that influence the interactions between biological substrates and enzymes is essential to engineer biomolecular processes. During the last few decades, considerable research efforts have been directed to understand the mechanisms of enzyme functions. At present, the chemical makeup of most relevant enzymes is fairly well-known, and a large number of studies has focused on the molecular and three-dimensional structure of cellulolytic enzymes.1-4 Efforts are underway to develop enzyme binding domains and to promote specificity that necessitates data concerning enzyme dynamics during complex formation at the interface.1,5 The importance of cellulose hydrolysis in the context of conversion of photosynthesized biomass into fuels or chemicals is well-recognized and documented. Plant biomass makes available a vast amount of mass and energy to be exploited. Cellulose as the most abundant carbohydrate in the biosphere is obviously of foremost relevance,6 and its enzymatic hydrolysis is at the center stage of bioconversion efforts. For these reasons, it is essential to understand ensuing enzyme—substrate interactions and the effect of external conditions in the rates of binding and degradation. A complete enzymatic hydrolysis of the cellulose molecule to glucose is accomplished with the complementary activity of different enzymes, called cellulases. Cellulases are divided into three different types or subcategories: endo-1,4-β-glucanases (EG) that target cellulose chains in random locations away from the chain ends; exo-glucanases or -cellobiohydrolases (CBH) that degrade cellulose by splitting off molecules from both ends of the chain, producing the cellobiose dimer; and finally, the cellobiose units produced during EG and CBH attack that are hydrolyzed to glucose by cellobiose β-glucosidase (BGL). While each component in the cellulase systems plays an individual role, it is important to recognize that the overall effect observed in cellulose degradation is a combined, synergistic interaction between the various cellulase components. Current research addresses such issues as the optimum composition of enzyme mixtures and the best conditions for promoting synergisms with different types of cellulase activities.7,8 It is apparent that the involved mechanisms are extremely complex since the enzyme effect is also coupled to contributions from the substrate (i.e., its intrinsic properties such as the degree of polymerization, crystallinity, or accessible surface area), all of which play leading roles in the outcome of enzymatic degradation.

The physicochemical or environmental conditions prevailing during enzyme action need to be taken into account to gain a better understanding of any experimental observations. Specifically, substrate composition, crystallinity, and recalcitrance9-11 have a distinctive influence on the mechanisms and dynamics of cellulose binding and hydrolysis.1,12

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In this investigation, we built up from our early efforts involving the use of a piezoelectric sensing technique the quartz crystal microbalance (QCM), to monitor in situ and in real-time the binding and catalytic activity of cellulases on model cellulose substrates. Our aim ultimately was to gain insightful information relevant to complex systems by probing the phenomena involved in simpler, better-controlled conditions of enzymatic degradation.

The morphology and chemical composition of the substrate (which in the case of the actual cell wall includes cellulose, hemicelluloses, lignin, etc.) adds to the degree of complexity. Therefore, we concentrated our efforts on smooth, model cellulose systems. It is expected that by using such substrates, we gain valuable information to foster research efforts in this area, especially in activities related to enzyme screening.

To ensure experimental reproducibility and meaningful results, the development of robust cellulose films is of paramount importance. Several techniques have been proposed to create such substrates, the most significant of which involves spin-coating, self-assembly, and Langmuir–Blodgett deposition techniques.

We report experimental results obtained by using the QCM technique in conjunction with atomic force microscopy (AFM) and X-ray photoelectron spectroscopy (XPS) and propose a simple model to quantify the enzyme binding and hydrolysis rate of cellulose thin films. Our quantification of enzyme dynamics then was used to interpret the main features exhibited by the cellulose films during the action of the multicomponent enzymes.

2. Experimental Procedures

2.1. Cellulose Thin Films. Thin films of cellulose were used as model substrates. The process for preparing cellulose surfaces typically started with QCM resonators consisting of quartz crystals (Q-sense) sandwiched between two conductive gold layers and a top surface and the cellulose thin film.8

Spin-coating of the cellulose solution on the sensor was then performed to create a uniform, thin film on top of the PVAm layer. A number of solvents are available to prepare cellulose solutions for spin-coating,15 including N-methylmorpholine-N-oxide (NMNO).17 In our case, cellulose solutions were prepared by dissolving microcrystalline cellulose (Avicel) in 50 wt % water/NMMO at 115 °C. Dimethyl sulfoxide (DMSO) was added to adjust the concentration and viscosity of the polymer (0.05%) in the mixture. A reduced viscosity allows the manufacture of thinner films, provided that DMSO is added judiciously to avoid suspension instability.8 The cellulose solution was then spin-coated on the sensors using a piezoelectric resonator (quartz) undergoing electric polarization effect (i.e., deformation of a resonator by applying an electric field) within a frequency range of 20 kHz. Finally, a Rudolph single-wavelength UV light oxidizes any spurious adsorbed organic matter that could remain on the surface of the sensor and also activates silanol groups required in later coating steps. Cleaned QCM-D sensors were then immersed in a diluted polyvinyl amide (PVAm) (BASF) solution before spin-coating. PVAm thus adsorbed from the solution on the sensor and was used as an anchoring layer between the sensor surface and the cellulose thin film.

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2.2. Cellulose Characterization. Ellipsometry, XPS, and AFM were used to characterize the cellulose films. XPS was performed on bare silica, PVAm-coated, and cellulose-coated sensors to quantify the respective chemical composition. This was accomplished by using a RIBER LAS-3000 XPS system with Mg Kα (1253.6 eV) as the X-ray source. The thin cellulose films were characterized in terms of material distribution, surface roughness, and topography using a Q-Scope 250 AFM model (Quesant Inst. Corp.), prior to and after enzyme treatment. The scans were performed in dried conditions using a nitrogen pulse and a nitrogen stream. Finally, a Rudolph single-wavelength ellipsometer was used to determine the thickness of the films on the surface of the sensors (a refractive index of cellulose of 1.56 was assumed).

2.3. Enzymes. The enzyme mixture used in this work was the NS5013 cellulase complex from Novozymes (Celluclast), which is available as an aqueous solution (≥700 U/g). This commercial enzyme mixture from Trichoderma reesei fungus contains endo- 

Among the enzymes used in this work was the NS5013 cellulase complex from Novozymes (Celluclast), which is available as an aqueous solution (≥700 U/g). This commercial enzyme mixture from Trichoderma reesei fungus contains endoglucanases, exo-glucanases, cellobiohydrolases, and β-glucosidases and was designed for the efficient saccharification of lignocellulosic materials18–21 with maximum activity in mild acidic conditions (pH of ca. 5) and temperatures between 50 and 60 °C (a decrease in cellulase activity was detected for experimental temperatures higher than 50 °C). A purified cellulase enzyme known to be inactive at room temperature was also used in one of the experiments to illustrate binding phenomena. We note that the rate and extent of hydrolysis as described in this paper depend heavily on the composition of the enzyme blend. The selected systems were used to illustrate fittings with our kinetic model. However, it was equally suitable in the case of other enzymes tested (pure and commercial cellulases).

Sodium acetate, sodium hydroxide, sodium chloride, and acetic acid (all from Fisher Scientific) were used for the pH 5 buffer solutions. The ionic strength used in all experiments was kept at 0.1 M, and all solutions were prepared with water from a Milli-Q gradient system (resistivity >18 MΩ).

2.4. Enzyme–Cellulose Surface Interactions Studied by QCM. A QCM with dissipation monitoring, QCM-D (Q-sense D-300), was used to study enzyme binding and activity on the cellulose thin films deposited on the sensors (that were coated with a 50 nm silica or gold layer). The temperature in our experiments was controlled within ±0.02 °C of the respective set point via a Peltier element that was built in the QCM apparatus.

A piezoelectric resonator (quartz) undergoes electric polarization due to applied mechanical stresses (piezoelectricity). The converse effect (i.e., deformation of a resonator by applying an electric field)
is the principle of the QCM technique: when an ac voltage is applied over the electrodes, the crystal is made to oscillate. Resonance is obtained when the thickness of the plate is an odd integer, \( n \), of half wavelengths of the induced wave (\( n \) is an integer since the applied potential over the electrodes is always in the anti-phase). QCM-D measures simultaneously changes in resonance frequency, \( \Delta f \), and energy dissipation, \( \Delta D \). Here, dissipation refers to the frictional and viscoelastic energy losses due to changes in the sensing surface. The frequency is measured intermittently, while the oscillator or sensor is driven to resonate, and \( D \) is measured during periods when the driving field is disconnected. More specifically, energy dissipation is quantified from the dampening of the oscillating signal as its driving field is disconnected. The shift in the resonator, \( \Delta f \), is measured during periods when the thickness of the plate is an odd integer, \( n \), of half wavelengths of the induced wave (\( n \) is an integer since the applied potential over the electrodes is always in the anti-phase). QCM-D measures simultaneously changes in resonance frequency, \( \Delta f \), and energy dissipation, \( \Delta D \). Here, dissipation refers to the frictional and viscoelastic energy losses due to changes in the sensing surface. The frequency is measured intermittently, while the oscillator or sensor is driven to resonate, and \( D \) is measured during periods when the driving field is disconnected. More specifically, energy dissipation is quantified from the dampening of the oscillating signal as its driving field is disconnected.

The dissipation factor is proportional to the power dissipation in the oscillatory system and can give valuable information concerning the rigidity of the film. For example, a soft (viscoelastic) film will not fully couple to the oscillation of the crystal as obtained with the dissipation value \( D \) according to

\[
D = \frac{E_{\text{dissipated}}}{2\pi E_{\text{stored}}} \tag{2}
\]

Here, \( E_{\text{dissipated}} \) and \( E_{\text{stored}} \) are the values of energy dissipation and stored energy, respectively, during one oscillation in the oscillating system. As a result, the measured changes in \( D \) are due to contributions from, for example, slip and viscous losses. For QCM measurements in liquids, the major contribution to \( D \) comes from frictional (viscous) losses within the liquid contacting the crystal. According to Stockbridge, the shift in the dissipation factor in a liquid environment is

\[
\Delta D = \frac{1}{\rho_l \eta_l} \sqrt{\frac{\rho_l \eta_l}{2\pi f}} \tag{3}
\]

where \( \eta \) and \( \rho \) are the viscosity and density of the fluid, respectively, and \( t \) and \( r \) are the thickness and the density of the quartz plate. When the adsorbed film slips on the electrode, frictional energy is created that increases the dissipation. Furthermore, if the film is

\[\text{viscous, energy is also dissipated due to the oscillatory motion induced in the film (internal friction in the film).}\]

A rigid adsorbed layer gives no change in dissipation, while a loose layer gives a dissipation increase.

### 2.4.2. Batch and Continuous QCM Modes.

In a typical experiment, degassed buffer solution (no enzyme present) was injected into the QCM flow (sensor) module assembled with a cellulose-coated QCM resonator. The coated sensor remained in contact with the background electrolyte solution for several hours to allow the cellulose film to fully swell and reach equilibrium with the medium (to produce the base QCM signal or baseline). Following this, the enzyme solution was continuously introduced into the cell with a syringe pump at a flow rate of 0.2 mL/min. The majority of the experiments reported here were conducted in batch conditions. In this mode of operation, the enzyme solution was introduced in the cell while ensuring that the buffer solution present initially was fully replaced (a minimum exchange of six cell volume equivalents were carried out in each run).

Some of our reported experiments were conducted in an open or continuous mode. Here, the enzyme solution was continuously injected at a constant flow rate (the surface was continually exposed to a fresh enzyme mixture). By comparing the two flow modes, it was possible to determine any effect produced by the hydrolysis byproducts as they remained in the sensor module or continuously renewed from the buffer. In the type of experiments reported here, no significant differences were observed.

In each experiment, the enzyme solution was injected only after ensuring that the drift of the third overtone frequency (\( \Delta f_3 \)) for the cellulose-coated sensor in the background electrolyte solution was lower than 2 Hz h\(^{-1}\). This ensures a full stabilization of the film in the buffer solution (fully swelled film and stress-free system). Enzyme incubation was terminated when no appreciable changes in frequency were observed (\( \Delta f_3 \) lower than 0.2 Hz min\(^{-1}\)).

### 3. Results and Discussion

#### 3.1. Substrate Characterization

Results from ellipsometry measurements on the PVAm/cellulose-coated substrates indicated a layer thickness of the PVAm and cellulose films of ca. 1 and 12 nm, respectively. AFM height images obtained after an incision on the cellulose thin film with a scalpel gave similar thicknesses. Furthermore, line profiles showed a uniform and homogeneous cellulose surface with a roughness (2 \( \mu \)m x 2 \( \mu \)m) of 2.6–2.8 nm before enzyme treatment. The sampling depth in XPS is comparable to the film thickness. However, the effective thickness in the valley areas may be smaller than the nominal thickness (due to the local roughness), and therefore, the underlying material can contribute to the XPS signal: Emission of photoelectrons from the metal substrate or intermediate PVAm layers was present in the spectra collected.

The carbon emission of the XPS spectrum showed a larger contribution of the bonded carbons in the cellulose film than the Si, Au, and N peaks from inner layers (substrate and PVAm anchoring layer). Low-resolution XPS survey spectra showed an O/C atom % ratio of 0.85 with measured standard deviations of less than 1% for the surface concentrations of carbon and oxygen. The ratio of relative area % obtained from high-resolution C1s spectra for C bonding two oxygens (O–C–O or C3) to that bonding one oxygen (O–C or C2) was 0.22, similar to the theoretical value for pure cellulose (0.2). The differences (experimental with respect to the theoretical) for these ratios are explained by the signal contribution from carbon atoms from the underlying PVAm. Note that a small C1 carbon peak (carbon bonded to other carbons or hydrogen) was detected (less than 9% of total carbon area). This peak is mainly due to the adsorption of airborne contaminants during sample preparation and handling.

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The nature of the surface, enzyme concentration, incubation. In this very short phase, only enzyme binding is detected. In this step, the film is accessible to the enzyme due to high crystallinity or due to steric barriers imposed by the enzymes themselves.

Replacing the enzyme solution by buffer solution (rinsing with enzyme-free solution) did not produce any noticeable change in the frequency response. This indicates that there is no further change in the adsorbed mass. Furthermore, any possible effect of variations of density and viscosity in the bulk solution was confirmed to be negligible.

In binding stage I, the variation in frequency is related to enzyme binding but in the next stage, ii, the catalytic activity of cellulase is initiated. In a case such as the one illustrated in Figure 1, equilibrium in enzyme binding was not reached, and degradation occurred concurrently. Therefore, the experimental frequency values that were observed during this stage resulted from the combined effect of enzyme binding (increased effective mass or decreased frequency) and enzymatic hydrolysis (onset of degradation of fragments of cellulose chains in the film).

Continued degradation of the film released oligosaccharide and monosaccharide units to the bulk solution, causing a frequency increase. As enzyme binding reached equilibrium, the variation in successive frequency values was unequivocally related to enzymatic hydrolysis (stage iii). At some point, the maximum rate of degradation was reached (linear phase) until the film began to become depleted, and the degradation rate slowed down (reduction of the frequency slope during stage iv).

As seen in the energy dissipation profiles illustrated in Figures 1b and 2, no changes occurred after injection of enzyme-free buffer solution (0 min). This fact reveals the existence of an initial thin, relatively rigid film of cellulose. When the enzyme solution was injected (15 min in Figures 1 and 2), a very rapid increase in energy dissipation was observed. This effect is due to changes in the viscoelastic properties of the adjacent bulk
solution and also, most importantly, due to the effect of enzyme binding on the cellulose substrate. Both the adsorbed protein layer and its coupled water contribute to this distinctive increase of energy dissipation.

Once cellulose degradation took place, the energy dissipation continued to increase, indicating changes in film morphology. Cellulose chains in the film were exposed to the enzyme solution, and thus, hydration was favored (the coupled water further increased the energy dissipation). At this stage of the process, some differences in the values of \( D \) could be observed for the various overtones (\( D_1, D_2, \) and \( D_7 \); see Figure 2). These differences are typical of a frequency-dependent dissipative film that in our case is subject to structural changes, likely at the film–bulk solution and film–substrate interfaces. At a certain point, the dissipation values reached a maximum. Interestingly, this maximum occurred at a certain time during degradation phase iii. This event is hypothesized to be a consequence of the initiation of a slower enzymatic degradation as the film becomes depleted (the available surface concentration of cellulose was reduced). This behavior, observed as a peak in the \( D \) profile, is viewed as the competition of two effects that take place at the same time.

AFM phase mode imaging of the film was performed before and after enzyme incubation (see Figure 3). Typical globular structures were observed in the spin-coated cellulose film (see 2 \( \mu \)m \( \times \) 2 \( \mu \)m AFM image in Figure 3a). Most of the features visible in the AFM image of the untreated cellulose film disappeared after enzymatic degradation (Figure 3b). It is worth noting that the roughness and average height measured in the topographic image of the cellulose film were reduced considerably after enzyme treatment (from a rms roughness of 2.6–2.8 nm before treatment to 1–1.2 nm after enzymatic treatment). Despite the long incubation times (longer than those used in the experiments shown in Figures 1 and 2), it is evident that residual cellulose still remained. Therefore, as was explained previously, the \( \Delta f \) and \( \Delta D \) plateau values observed at the end of the QCM experiments could indicate either a complete depletion of the film or the inability to degrade recalcitrant, residual fragments of cellulose. As is the case with cellulose fibers that contain both amorphous and crystalline regions, the thin films used here also can have crystalline regions that are more difficult to degrade. All in all, these regions are expected to significantly affect the rate and extent of enzyme hydrolysis, as indicated by the plateau values in QCM frequency.

The rate of enzymatic hydrolysis and its yield are dependent on the adsorption of enzymes on the surface of the substrate. The accessibility of cellulose to cellulase enzymes is controlled by the physicochemical properties of the substrate, the multiplicity of the cellulase complexes, and reaction parameters including those associated with pH, dosage levels, mass transport, and temperature. All these effects can be modeled explicitly by using an empirical approach, as is presented in the next sections.

### 3.3. QCM Frequency Modeling

Various kinetic models have been developed to describe the hydrolysis rate by cellulases.\(^{15,16}\) These models are based on parameters such as the amount of adsorbed enzyme, the structural characteristics of the substrate (including pore size distribution, crystallinity index, and specific surface area), and cellulase–cellulose adsorption rates. The quantification of the hydrolysis rate demands knowledge of the exact concentration of both cellulose and enzymes. Typical models include the Michaelis–Menten equation\(^{16–18}\) and other models that assume quasi-steady-state conditions during hydrolysis.\(^{16}\) The data presented in this investigation deal with cellulose surfaces. In this case, the concentration of the substrate is not known since cellulose is not in the dispersed state but it is rather a solid thin film in contact with the enzyme solution. From these considerations, classical, deterministic kinetic models cannot be applied to our experimental results. Thus, the need for suitable models to describe enzymatic degradation of (cellulose) thin films is warranted.

An empirical model was used to fit and interpret the changes in QCM frequency during cellulose hydrolysis by the action of cellulase enzymes. We began this discussion by focusing our attention on the experimental \( \Delta f \) data obtained by the piezoelectric resonator, for example, as presented in Figure 1. The frequency values corresponding to film swelling and signal stabilization were taken before the baseline (starting at 0 min), and the modeling was applied to the time range after enzyme binding was detected (upon enzyme injection). As introduced before, the main challenge for any quantification is the fact that two events overlap, namely, binding and hydrolysis occur as soon as enzymatic activity is initiated. The model presented here involves two different equations, one to fit data for binding and the other to describe cellulose degradation. Thus, binding and degradation models are presented next, and then their combination to fit the effective (observed) values of frequency is introduced.

#### 3.3.1. Binding Model

It is expected a priori that enzyme binding to the cellulose film be detected as a progressive decrease in the frequency signal, until a plateau is reached (as is the case for typical molecular adsorption). This assumption is supported by experiments that showed that inactive purified cellulases (used at low temperatures) adsorbed effectively onto the substrate but that no hydrolytic activity was detected (Figure 4). In the case illustrated in Figure 4, the frequency decreased as a consequence of the added mass (due to enzyme binding) until an equilibrium state at the interface was reached (equal enzyme adsorption and desorption rates).
It is assumed that the enzymes bind to the substrate, forming an enzyme–substrate complex (ES), and that this enzyme–substrate complex then is irreversibly decomposed to form the final product P. Finally, the reducing sugars inhibit the enzyme in a reversible and competitive manner, by forming the complex EP.

On the basis of these assumptions, enzymatic cellulose hydrolysis can be characterized by the following steps: E + S $\rightarrow$ ES, ES $\rightarrow$ E + P, and E + P $\rightarrow$ EP. For the present study, the parameters obtained by our empirical models are expressed as single kinetic constants. The objective is thus to provide a simple model that allows a quantitative determination of the key kinetic parameters to determine the effect of the different variables on the hydrolysis rates (i.e., enzyme dosage, type of enzyme, temperature, pH, ionic strength, etc.).

Qualitative evaluation of the dynamics of hydrolysis can be found in the literature, as well as simplifications to describe enzymatic kinetics, including the initial rate of degradation or linearization. However, in most cases, aqueous suspensions with known concentrations of both cellulose and enzymes are used. In the present cases, the interaction involves free enzymes and cellulose film substrates.

For enzymatic degradation of the film, an increase in frequency is detected as the cellulose chains are hydrolyzed. The maximum rate of degradation should be reached after a certain time. The initial degradation rate is lower because a certain time is needed to reach the maximum synergistic effect between endo- and exo-
glucanases. The initial activity detected corresponds to endo-
glucanase binding to the surface of the film and cleaving randomly the cellulose chains. Once the endo-
glucanases are bound to the surface, their activities have no measurable effect in frequency. This is because there is no significant amount of mass released from the cellulose film. However, endo-
glucanases are effective in opening new reducing and non-reducing ends in the cellulose chain, allowing exo-
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lulojose units. This effect of exo-
glucanases is unambiguously probed by the piezoelectric sensor, which signals the highest rate of hydrolysis after a certain time after initiation of the enzyme activity. As the amount of available cellulose is reduced (and also as the morphological characteristics of the film prevent degradation), the hydrolytic rate is slowed down. It is hypothesized that non-crystalline and easily accessible cellulose is hydrolyzed first (producing a rapid increase in frequency), but once these fractions are removed, the enzyme action is slowed down when exposed to the more crystalline domains. Unfortunately, the measurement of crystallinity of ultrathin films of cellulose is very challenging, and confirmation of this hypothesis is not possible at this time.

As for the mathematical modeling of the enzymatic degradation, several empirical models were tested. However, the experimental results were best fitted by a Boltzmann–sigmoidal equation (eq 5)

$$\Delta f = A + \frac{B - A}{1 + e^{(t_0 + t_{1/2})}}$$

where $\Delta f$ is the change in QCM frequency (Hz) as monitored as a function of time $t$ (min), and $B$ is the maximum frequency value in hertz that corresponds to the plateau region (stage iii). The frequency maximum is reached when all the hydrolyzable

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Table 1. Binding Parameters (Eq 4) Calculated from Frequency Data for Cellulose Thin Films Incubated with Cellulases

<table>
<thead>
<tr>
<th>temp (°C)</th>
<th>$M_{\text{max}}$ (Hz)</th>
<th>$\tau$ (min$^{-1}$)</th>
<th>$R^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>25</td>
<td>40.2</td>
<td>0.60</td>
<td>0.991</td>
</tr>
<tr>
<td>35</td>
<td>41.7</td>
<td>0.38</td>
<td>0.985</td>
</tr>
</tbody>
</table>

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It is assumed that the enzymes bind to the substrate, forming an enzyme–substrate complex (ES), and that this enzyme–substrate complex then is irreversibly decomposed to form the final product P. Finally, the reducing sugars inhibit the enzyme in a reversible and competitive manner, by forming the complex EP.

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substrate has been complexed (ES) and transformed into products (P). At V50, the conversion from the complex form (ES) to final products (P) is maximized; the reaction equilibrium is strongly displaced to the right (formation of E and P). C is the inverse of the hydrolysis rate in reciprocal minutes. The value of parameter A corresponds to the minimum frequency in the sigmoidal curve, related to the time period for the transition between binding and hydrolysis. Note that in eq 5, the term V50 is closely related to the half-maximum speed (P).

The Boltzmann–sigmoidal equation fitted quite well with the experimental degradation signal, as illustrated in Figure 6. The parameters fitted to the experimental values previously reported (see also the binding model) are summarized in Table 2. As can be expected for the effect of temperature, the hydrolysis rate B is increased as the temperature is closer to the optimum (around 50 °C). The parameter C (i.e., the reciprocal of the hydrolysis rate) decreases significantly. Also, V50, which indicates the inflection point where the maximum degradation rate is achieved, is clearly reduced from 37.8 to 9.6 min. In all cases, significant differences in the values of the model parameters are observed for the different incubation temperatures.

Finally, binding and hydrolysis models were combined to produce an excellent fit with the measured QCM frequency, as observed in Figure 7. Here, eq 6, representing a function by parts (based on eqs 4 and 5), was used. The value of I represents the time for the transition between the two stages of the dynamic process, namely, binding and degradation

$$\Delta f = M_{\max}(1 - e^{-\omega t}), \ t < I$$

$$\Delta f = A + \frac{B - A}{1 + e^{(V_{50} - B/C)}}, \ t > I$$

3.4. Energy Dissipation and Degradation of Cellulose Films.

As was discussed before, the energy dissipation of the film during the incubation process was consistent with a rigid film of cellulose; this is especially noticeable at the beginning of the experiments. At some point, the dissipation begins to increase as the enzyme binds to the substrate, and the initial phases of degradation are initiated (see also Figures 1 and 2). At a later time, the dissipation reaches a maximum value, which is related to a soft, viscoelastic film. Finally, the dissipation decreases as the hydrolysis activity slows down. The maximum in energy dissipation of the film occurs in all experiments when both binding and degradation are present (active enzyme).

We performed surface plasmon resonance (SPR) experiments similar to those reported here with QCM and observed that the changes in the refractive index of the film followed the same trends as the QCM frequency profiles. However, the dissipation behaviors were distinctively monitored via QCM. We further propose that this energy dissipation provides a fingerprint of the structural events that occur during binding and hydrolysis of films of cellulose exposed to cellulase enzymes. Figure 8 shows frequency–dissipation profiles (the so-called ΔD–Δf plot), where a maximum in dissipation can be clearly observed at ca. 80 Hz. The ΔD–Δf plot helps to elucidate the changes of the film/layer adsorbed on the surface.31,32

The question then arises as to whether a link exists between the maximum energy dissipation and the frequency variation (amount of cellulose degraded). Is this maximum related to the

Table 2. Hydrolytic Parameters (Eq 5) Calculated from Frequency Data for Cellulose Thin Films Incubated with Cellulases

<table>
<thead>
<tr>
<th>temp (°C)</th>
<th>B</th>
<th>V50</th>
<th>C</th>
<th>R²</th>
</tr>
</thead>
<tbody>
<tr>
<td>25</td>
<td>187</td>
<td>37.8</td>
<td>13.9</td>
<td>0.9993</td>
</tr>
<tr>
<td>35</td>
<td>414</td>
<td>9.6</td>
<td>4.1</td>
<td>0.9998</td>
</tr>
</tbody>
</table>

* 0.5 v/v % concentration and two different temperatures.
parameters determined by the models presented before? And more importantly, does it relate to the changes in the film during incubation?

The maximum in energy dissipation reveals changes in the structure of the cellulose film: As the enzyme binds to the film, it becomes softer and thicker. Coupled (hydration) water increases, and the cellulose structure becomes more open. As enzymatic hydrolysis takes place, some chains of the polysaccharide are cleaved randomly by endo-glucanases. This initial enzymatic attack opens some new cellulose chains, carrying both reducing and non-reducing end groups. The surface becomes more prone to further hydration, thus promoting a swollen and softer film that dissipates more energy. A sketch of this phenomenon is presented in Figure 9, which emphasizes the superficial hydration before and after the initial attack by endo-glucanases.

The synergistic effect of the different types of cellulases produces a progressive degradation of the cellulose film and opens the cellulose chain network. This leads to the formation of cavities that further expose hydrolyzable cellulose molecules (less crystalline regions). As time progresses, the film becomes more hydrated, losing the packed structure (Figure 9). This effect reaches a maximum at a certain point, depending on the enzymes and experimental conditions. After this maximum, the dissipation begins to decrease. This reduction is related to a severe change in the film structure. The film is degraded by enzymatic attack, and the surface turns into cellulose clusters. These clusters, which are evident in AFM topographic images (Figure 3b), contain the less hydrolyzable residual cellulose chains.

The experimental results presented here and also those from tests with other enzyme mixtures as well as with purified enzymes indicate that the $V_{50}$ parameter is closely related to the maximum in dissipation. At around the $V_{50}$ time, the highest rate of degradation is reached; thereafter, the degradation rate is progressively slowed down. In the case presented in Figure 9, this occurs at approximately a few minutes after $V_{50}$. We argue that the $V_{50}$ value, obtained from frequency data, signals the onset of the maximum dissipation (maximum viscoelasticity of the film).

We observed that as the incubation temperature is increased, the difference between $V_{50}$ and the maximum dissipation tends to become smaller. This is believed to be a consequence of a smaller energy barrier for the changes (that were described previously) to take place. In conclusion, the hydrolysis rate decreases as the film becomes more degraded and begins to lose its original structure. The remaining film becomes more difficult to degrade due to the higher crystallinity of the residual cellulose and/or the non-homogeneity of the surface that promotes increased non-specific binding.

**Conclusion**

QCM-D with cellulose biosensors was used to investigate the dynamics and activities of cellulase enzymes by monitoring the changes in frequency and energy dissipation during incubation. These changes were explained by events such as enzyme binding and hydrolytic activity. A simple model that accounts for the binding and degradation stages was proposed. Binding was described by a $1 - \exp$ expression, while cellulose hydrolysis was described by a Boltzmann–sigmoidal model. It is proposed that QCM energy dissipation correlates with the changes in substrate morphology. It was observed that residual, recalcitrant cellulose fragments remained on the sensor even for the longest incubation times. This is probably due to the higher crystallinity in these fragments, which prevents hydrolytic reactions, or due to non-specific adsorption of enzymes on exposed subsurface areas. The combination of the piezoelectric sensing technique and AFM to measure enzymatic degradation offers a great potential to screen enzymes and enzyme mixtures as well as to study the effect of reaction conditions.

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