Langmuir

ARTICLE

pubs.acs.org/Langmuir

# Quantitative Assessment of the Enzymatic Degradation of Amorphous Cellulose by Using a Quartz Crystal Microbalance with Dissipation Monitoring

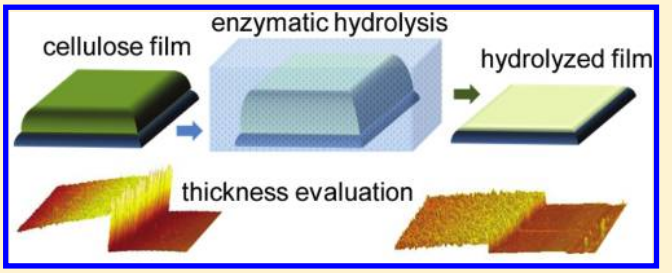
Miro Suchy,[†] Markus B. Linder,[‡] Tekla Tammelin,[‡] J. M. Campbell,[†] Tapani Vuorinen,[†] and Eero Kontturi*[,†]

[†]Department of Forest Products Technology, School of Chemical Technology, Aalto University, P.O. Box 16300, FIN-00076 Aalto, Finland

[‡]VTT Technical Research Centre of Finland, P.O. Box 1000, FI-02044 VTT, Finland

Supporting Information

**ABSTRACT:** The systematic evaluation of the degradation of an amorphous cellulose film by a monocomponent endoglucanase (EG I) by using a quartz crystal microbalance with dissipation monitoring (QCM-D) identified several important aspects relevant to the study the kinetics of cellulose degradation by enzymes. It was demonstrated that, to properly evaluate the mechanism of action, steady state conditions in the experimental set up need to be reached. Rinsing or diluting the enzyme, as well as concentration of the enzyme, can have a pronounced effect on the hydrolysis. Quantification of the actual hydrolysis was carried out by measuring the film thickness reduction by atomic force microscopy after the enzymatic treatment. The values correlated well with the frequency data obtained by QCM-D measurement for corresponding films. This demonstrated that the evaluation of hydrolysis by QCM-D can be done quantitatively. Tuning of the initial thickness of films enabled variation of the volume of substrate available for hydrolysis which was then utilized in establishing a correlation between substrate volume and hydrolytic activity of EG I as measured by QCM-D. It was shown that, although the amount of substrate affects the absolute rate of hydrolysis, the relative rate of hydrolysis does not depend on the initial amount of substrate in steady state system. With this experimental setup it was also possible to demonstrate the impact of concentration on crowding of enzyme and subsequent hydrolysis efficiency. This effort also shows the action of EG I on a fully amorphous substrate as observed by QCM-D. The enzyme was shown to work uniformly within the whole volume of swollen film, however being unable to fully degrade the amorphous film.




## 1. INTRODUCTION

Cellulose, the main structural component of higher plants, is seen as a major potential source of glucose for the subsequent production of biofuels, bioethanol in particular. However, the recalcitrance of cellulose to hydrolytic degradation[1,2] has been a major impediment in such technology and the facilitation of degradation has recently been subject to intense research efforts. Enzymatic hydrolysis with cellulase enzymes is currently regarded as the most viable option for industrial degradation of cellulose. Fundamental research on enzymatic hydrolysis of cellulose is therefore instrumental for its efficient exploitation. In this context, physical features of the cellulosic substrates affecting hydrolysis have been identified,[3,4] including crystallinity, pore size, and chemical heterogeneity. Similarly, enzyme related features influencing the efficiency of hydrolysis have been investigated.[4−7] Despite all these efforts, the mechanisms of enzymatic cellulose hydrolysis are still not fully understood.

Since typical biomass is chemically heterogeneous, fundamental research on enzymatic hydrolysis is often carried out with pure cellulose substrates of different origin and characteristics. However, as the morphology of the substrate also plays a significant role in the reactivity of cellulose, model films have proved very useful in fundamental cellulose research. They essentially reduce the morphology to the level of a smooth ultrathin film and enable the use of various surface analytical techniques which are not applicable for bulk samples. In this contribution, an amorphous cellulose model film with well-defined characteristics has been applied in fundamental, quantitative studies of cellulose degradation by various cellulase enzymes. In particular, we have studied two of the major cellulases from *Trichoderma reesei*, namely EG I and CBH I. Both enzymes have a modular structure typical for cellulases. In this arrangement a catalytic domain (CD) is linked to a smaller cellulose-binding domain (CBD). This structure apparently helps to anchor the CD to the cellulose and thereby increase its catalytic efficiency. Functional studies on the two domain structure of cellulases show that binding is critical for catalysis to occur but binding and catalysis are not necessary concurrent events.[8]

**Received:** January 26, 2011
**Revised:** May 25, 2011
**Published:** June 23, 2011

The quartz crystal microbalance with dissipation monitoring (QCM-D) is a recently developed methodology for in situ studies of phenomena at the solid−liquid interface. In essence, it is a very sensitive balance, where frequency changes are indicative of, and proportional to, the changes of mass deposited on the crystal. Simultaneously, dissipation monitoring provides useful information on viscoelastic properties of the film. The technique is well established in studying adsorption of, for example, polyelectrolytes or proteins on a variety of model substrates.[9−13] Recent advances in the preparation of cellulose ultrathin model films have allowed utilization of QCM-D to obtain valuable information on the behavior of cellulose, such as swelling and the adsorption of polyelectrolytes. The exceptional sensitivity of QCM-D makes it suitable for studies on the enzymatic degradation of cellulose that consist of adsorption (binding) and degradation (hydrolysis) stages. Previous investigations have included evaluations of the treatment conditions (enzyme concentration, temperature and pH)[14−17] and model film characteristics (cellulose II, nanofirillar cellulose and cellulose nanocrystals)[18] on the rate of hydrolysis. The technique has also been used to demonstrate the difference in mode of action for two individual cellulases and their synthetic mixtures.[19] The common features of these efforts were utilization of semicrystalline cellulose films and/or usage of a commercial cellulase mixture. Only indirect quantification of the degradation was attainable in QCM-D by using the Sauerbrey equation that correlates the change in frequency to the change of mass on the crystal.[20] Although visual AFM analysis of surfaces before and after hydrolysis demonstrated removal of deposited cellulose,[18] the method could not reliably quantify the extent of hydrolysis. A modified method (measurement of density and viscosity of hydrolysate) of utilizing QCM-D to quantify hydrolysis has been described,[21] however a quantification of cellulose degradation by analyzing the films after hydrolysis and correlation with the data obtained by QCM-D testing has not been achieved.

In this contribution, we attempted to investigate several issues that have not been properly addressed by previously cited accounts. The model substrate used in this investigation was a film of amorphous cellulose prepared by spin coating from trimethylsilyl cellulose (TMSC) and subsequently regenerated by acid hydrolysis. The films were extensively characterized, particularly their crystallinity and swelling behavior.[22] The use of an amorphous substrate circumvents the ambiguous interpretation resulting from semicrystalline cellulose where the contributions of crystalline and disordered cellulose are often difficult, if not impossible, to distinguish. Furthermore, this amorphous film has been shown to exhibit uniform swelling in water,[22] enabling uniform binding of the enzyme throughout the whole film. Then, as opposed to a cellulase mixture, the action of individual monocomponent cellulases was evaluated, with a focus on endoglucanase (EG I) that acts on amorphous part of the cellulose structure.[5,23,24] Investigation of the action of EG is slowly gaining momentum. The monocomponent EG is finding its application, for instance, in increasing solubility[25] and reactivity[26,27] of dissolving pulps, or in modification of delignified wood pulp fibers prior to preparation of nanofibrillated cellulose.[28] Another objective of this effort was to assess the impact of the amount of substrate on the rate of hydrolysis measured by QCM-D technique. Finally, we attempted a direct quantification of the mass reduction by hydrolysis of the film and correlate the data with the data obtained in QCM-D measurements. Contrary to the bulk monitoring of enzymatic hydrolysis of cellulose, this approach does not employ the characterization of the hydrolysate by, e.g., released sugar analysis. Instead, we are applying QCM-D as an in situ tool for following the hydrolysis and thickness measurements as an ex situ tool for validating the quantification. In other words, changes in the residual cellulose substrate are followed instead of monitoring of the hydrolyzed sugars from solution.

## 2. EXPERIMENTAL SECTION

**Materials.** Trimethylsilyl cellulose (TMSC, degree of substitution 2.2; $M_w$ = 231 320 g mol$^{-1}$, $M_n$ = 82 660 g mol$^{-1}$, $M_w/M_n$ = 2.80) was synthesized from cellulose powder from Spruce (Fluka) as described previously.[29] Toluene was p.a. grade from Aldrich. The HCl solution was prepared from a VWR stock solution (11.76 M as determined by titration with NaOH). Milli-Q (Millipore Corporation) purified water was used throughout the experiments. Substrates used for model film preparation were silica QCM-D crystals (Q-sense AB, Västra Frölunda, Sweden) and smooth silicon wafers (Okmetic, Espoo, Finland). Acetate buffer solution (50 mM, pH 4.5) for enzyme dilution, film swelling, and rinsing was prepared using p.a. grade anhydrous sodium acetate (Fluka) and glacial acetic acid (p.a. Merck).

**Enzymes.** The enzymes used were EG I and CBH I from *Trichoderma reesei*. EG I was purified from culture filtrates lacking CBH I and CBH II, as described by Suurnäkki et al.[30] Briefly, culture supernatant was desalted on a Sephadex G-25 coarse column (GE Life Science), equilibrated with 6 mM sodium phosphate buffer, pH 7.0, and then loaded on a DEAE Sepharose FF (GE Life Science). EG I was eluted using a NaCl gradient and then loaded on a Phenyl Sepharose FF (GE Life Science) column equilibrated with 25 mM sodium phosphate at pH 6.0 with 0.4 M $(NH_4)_2SO_4$. EG I was eluted by a decreasing gradient of 20 mM pH 6.0. EG I containing fractions were further concentrated by ultrafiltration with 25 mM sodium acetate, pH 4.5, and applied to a DEAE Sepharose FF column, and eluted by a NaCl gradient. Finally the protein was desalted and concentrated by ultrafiltration.

CBH I was purified from culture filtrate devoid of EG I and EG II as described by Rahkamo et al.[31] Briefly, bentonite treated and concentrated culture was desalted on Sephadex G-25 (GE Life Science) using 10 mM sodium phosphate buffer, pH 7.0 and applied on DEAE Sepharose FF (GE Life Science). Elution was with a NaCl gradient. The CBH I fraction was then affinity purified using p-aminobentzyl 1-thio-B-D-cellobioside linked Sepharose (GE Life Science) column. The CBH I pool was then applied on Phenyl Sepharose FF equilibrated with 20 mM sodium acetate pH 5.0 containing 0.4 M $(NH_4)_2SO_4$. Elution was performed with a decreasing gradient of $(NH_4)_2SO_4$ 20 mM sodium acetate. The protein was finally desalted and concentrated by ultrafiltration.

Commercial cellulase mixture, NS50013 cellulase complex from Novozymes (Celluclast) was used for complementary evaluation.

**Model Films Preparation.** Ultrathin model films of cellulose of different thickness were prepared by spin coating from TMSC solutions of different concentrations. TMSC was dissolved in toluene to obtain solutions in concentration range from 1 to 20 g dm$^{-3}$ (1, 2.5, 5, 7.5, 10, 12.5, 15, 17.5, and 20 g dm$^{-3}$). Silicon wafers were cut to ca. 1 × 1 cm$^2$ squares and cleaned in an UV/ozonator (Bioforce Nanosciences, Ames, IA) for 15 min. The cleaned wafers were rinsed twice with toluene (4000 rpm for 15 s) right before spin coating with the TMSC solutions. Spin coating was carried out at 4000 rpm (acceleration of 2200 rpm s$^{-1}$) for ca. 45 s. The TMCS films for QCM-D studies were prepared in a similar manner using silica QCM-D crystals (Q-Sense AB, Gothenburg, Sweden) as substrates. The spin coater used was a WS-400B-6NPP/LITE (Laurell Technologies Corporation, North Wales, PA). The spin coated TMSC films were then converted to cellulose by exposure to the vapor of 2 M HCl for 2 min.[29]


dx.doi.org/10.1021/la2014418 |*Langmuir* 2011, 27, 8819–8828

**Quartz Crystal Microbalance with Dissipation Monitoring (QCM-D).** Enzyme interactions including enzyme adsorption and enzymatic hydrolysis with cellulose model films were studied using a Q-Sense E4 instrument (Q-Sense AB, Gothenburg, Sweden). The QCM-D technique enables in situ studies of mass change at solid/liquid interface due to adsorption, desorption[20] and swelling.[32] The piezoelectric quartz crystal oscillates at a resonant frequency $f_0$ which is lowered or increased when mass changes are sensed on the surface of the crystal. If the layer on the crystal surface is evenly distributed, rigidly attached, fully elastic and small compared to the mass of the crystal, the shift in the resonant frequency is related to the mass change by the Sauerbrey equation,[33]

$$\Delta m = -\frac{C\Delta f}{n} \quad (1)$$

where $\Delta m$ is the adsorbed or desorbed mass per unit surface, $\Delta f = f - f_0$ is the frequency shift, $n$ is the overtone number ($n = 1, 3, 5, 7, ...$), and $C$ is a constant that describes the sensitivity of the device to changes in mass. For the crystals used, $C \approx 0.177$ mg m$^{-2}$ Hz$^{-1}$.[34]

The resonant frequency of the crystal depends on the total oscillating mass, including water coupled to the oscillation. By measuring several frequencies and the dissipation, it becomes possible to determine whether the film on the sensor surface is rigid or water-rich and mobile (soft) which is not possible by looking only at the frequency response. If the film on the sensor surface is not fully elastic, frictional losses occur that lead to a damping of the oscillation with a decay rate of amplitude that depends on the viscoelastic properties of the material. With the QCM-D instrument the change in the dissipation factor, $\Delta D = D - D_0$, can be measured. $D$ is defined by

$$D = \frac{E_{diss}}{2\pi E_{stor}} \quad (2)$$

where $E_{diss}$ is the total dissipated energy during one oscillation cycle and $E_{stor}$ is the total energy stored in the oscillation.

The spin-coated and acid hydrolyzed crystals were left to swell in buffer solution overnight. The crystals were then rinsed with water and thoroughly dried with nitrogen before being mounted in the measurement chamber. Prior to the measurement, the cells (40 $\mu$L volume above sensor) were filled with buffer solution to allow the films fully swell, as indicated by stabilized frequency and dissipation values. The measurement commenced by running buffer solution for ca. 5 min (0.1 mL min$^{-1}$) to attain the measurement baseline, and then the buffer solution was replaced by enzyme. The enzyme was pumped for a total of 60 min, and then the eluent was switched back to buffer solution for another 60 min (both at the same 0.1 mL min$^{-1}$ flow rate). The pump was then turned off, and the measurement continued for an additional 2 h. Frequency and dissipation changes were recorded as a function of time and the total time of measurement was 4 h. All measurements were recorded at the 5 MHz fundamental resonance frequency and its overtones 15, 25, 35, 55, and 75 MHz. The third overtone (15 MHz) was used in the evaluation of the data. The temperature inside the measurement chamber was maintained at 25 °C.

**Hydrolysis of Films on Silicon Wafers.** The films deposited on silicon wafers were treated similarly as those on the quartz crystals. The cellulase treatment followed the same protocol as the QCM-D measurement, using the same pump at the same flow rate. The outlets of the tubes were placed directly above the films, so that eluent could continually flow on the film surface. To further simulate the continuous flow as in the QCM-D cell, the excess of the eluent was periodically (5 min intervals) suctioned from the film by micropipet.

**Film Thickness Analysis by AFM.** Cellulose model film (on silicon wafers) thickness was determined using a Nanoscope IIIa Multimode scanning probe microscope (Digital Instruments, Inc., Santa Barbara, CA). The images were scanned in tapping mode with J-scanner and silicon cantilevers (NSC15/AIBS from Ultrasharp $\mu$masch, Tallinn, Estonia). The radius of curvature for the tip according to the manufacturer was less than 10 nm and typical resonance frequency of the cantilever was 325 kHz. Relatively soft tapping (50−70% of the free oscillating amplitude) was applied in the imaging. The films on silicon wafers, freshly prepared and the films after enzymatic hydrolysis were dried in oven at 80 °C for 15 min. Then the films were scratched using a sharp needle and the scratched area was scanned by the AFM (10 $\mu$m × 10 $\mu$m). The height difference between the revealed substrate and the intact areas of the film determined the film thickness. A minimum of three scans were imaged for each film sample and at least three points in the image were measured. All quantitative data were extracted from the height images. No image processing except flattening was performed. Detailed description of the thickness measurements complete with exemplary scratched AFM images are available in the Supporting Information (Figure S1).

**X-ray Photoelectron Spectroscopy (XPS).** A Kratos Analytical AXIS 165 electron spectrometer with a monochromatic A1$_{K\alpha}$ X-ray source was used to analyze the elemental and chemical compositions of the sample surfaces in a manner reported previously.[35] All spectra were collected at an electron takeoff angle of 90° from sample areas less than 1 mm in diameter. Survey spectra were recorded at 1 eV intervals at a pass energy of 80 eV. Higher resolution regional spectra were recorded at 0.1 eV intervals at a pass energy of 20 eV. The spectra were recorded at three different spots on each sample and the analysis vacuum was monitored during the experiments with an in situ reference sample. No X-ray induced degradation was detected during the experiments.

## 3. RESULTS AND DISCUSSION

**3.1. Cellulose Degradation by Endoglucanase: Impact of Concentration.** QCM-D is a useful technique for providing information on the mechanism and kinetics of cellulose degradation by cellulases. In previous studies, however, the semicrystalline nature of the films used, coupled with the complexity of the used enzyme (cellulase mixture), did not allow proper assessment of cellulase action, providing mostly qualitative information on the hydrolysis.[14−19] The commercial cellulose mixtures are not suited for fundamental studies by QCM-D. The complete composition of these enzymes is often not available, and the enzyme usually contains other components (e.g., preservatives) that may have an influence on the measurement, particularly when using highly sensitive techniques such as QCM-D. In this investigation, we selected an experimental system consisting of well-defined and noncomplex components that would allow a clear analysis of the enzyme action. More specifically, the substrate used was a homogeneous film of amorphous cellulose, whose characteristics and swelling behavior have recently been described.[22] The cellulases used in this investigation were purified monocomponent cellulases. The more detailed and systematic study was carried out using endoglucanase (EG I), which is known to act on amorphous cellulose.[5] In addition, we decided to use markedly lower concentrations of the enzyme as that used in the previous studies, which would allow finer distinction in the action of cellulase. QCM-D data of the action of EG I on amorphous cellulose and the impact of the enzyme concentration is shown in Figure 1. It should be noted that hydrolysis occurs under a continuous flow of enzyme. Only at the position marked "rinse" was the liquid changed to plain buffer. This leads to conditions where the load of enzyme is constant during hydrolysis. This approach is different from typical hydrolysis setups where an initial amount of enzyme is introduced in

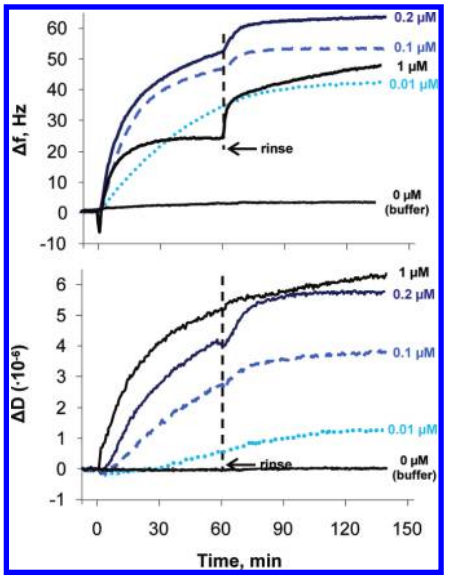



**Figure 1.** Dependence of hydrolytic activity on enzyme concentration. Change in frequency (top) and dissipation (bottom) measured by QCM-D for films of amorphous cellulose (18 nm thick) exposed to EG I of different concentrations. ($f_0$ = 5 MHz, $n$ = 3, $f_3/n$).

the system which leads to changing ratios of free and bound enzyme as hydrolysis proceeds and cellulose is disintegrated.

The action of EG I on amorphous films leads to rapid degradation of the film (Figure 1). This is demonstrated by the changes in frequency which were not observed when the film was exposed to pure buffer solution. The hydrolysis commences almost immediately after contact of EG I with the substrate, indicated by a rapid increase in frequency. The rate of degradation starts to slow after ca. 30 min. Interestingly, after continuous exposure to the enzyme (60 min), the introduction of a buffer to the system (rinse stage) resulted in a rapid short burst of mass loss from the film, in particular at higher concentrations. It appears that rinsing frees the activity of the enzyme, as replacement of enzyme solution with pure buffer reduces crowding of the enzyme on cellulose surface. In addition, it is possible that the hydrolyzed cellulose is more readily dissolved in pure buffer solution. Possible removal of cellulose fragments released from the film by the action of enzyme should not be enhanced by the exchange of enzyme to pure buffer in the cell, considering the properties (pH and ionic strength) of EG I solution in buffer and pure buffer are very similar, and the flow was constant during both hydrolysis and rinsing stages. When compared to the previously described investigation of EG I hydrolysis of semicrystalline cellulose model films studied with QCM-D,[19] EG I exhibits markedly different action here. In that investigation, the interaction of the EG I with a partially crystalline film and at higher enzyme loads resulted in adsorption of EG I on the substrate and subsequent swelling of the film. However, no detachment of the cellulose from the film was detected over extensive periods of exposure of the film to the enzyme.

The enzyme concentration was shown to be a factor in the hydrolysis as monitored by QCM-D. The comparison in Figure 1 showed that very low concentrations (0.01 $\mu$M) and particularly high (1 $\mu$M) concentrations have a different impact on the hydrolysis. At low concentration, a slower rate and a lower extent of hydrolysis were observed. The concentration in the middle range showed an increase in both the rate and the extent of hydrolysis. The high concentration, however, behaved differently. At this concentration a transient drop in frequency within ca. 2 min from the initial contact was noticeable, indicating rapid adsorption of the enzyme that was affected to a lesser extent by the concurrent hydrolytic action of EG I. The adsorption at 0.2 $\mu$M enzyme dosage or less resulted in a drop in frequency of less than 2 Hz, whereas the high enzyme concentration (1 $\mu$M) measured a drop in frequency of ca. 7 Hz. The adsorption was rapidly surpassed by the hydrolytic action denoted by the rapid increase in frequency. At the initial stage, the rate of hydrolysis was among the fastest from the concentrations evaluated, when considering the initial adsorption. Shortly afterward, however, the hydrolysis starts to rapidly level off and appears to stop within 30 min of exposure. Introduction of the buffer to the system resulted in a burst of hydrolytic activity of the EG I. The frequency jumps almost 20 Hz within a minute after beginning the rinsing stage. This frequency increase is likely enhanced by the desorption of EG I. At this concentration, both adsorption and desorption after rinsing were more pronounced when compared to measurements at lower EG I concentrations. Then the rate of hydrolysis starts to level off again, and gradually continues until the end of the measurement. Overall, the extent of cellulose film degradation is lower than that observed for lower concentrations, even that of the 100 times weaker enzyme concentration. This indicates that once the film is saturated with the enzyme, overcrowding starts to impact the hydrolysis probably because the dense binding of the enzyme molecules hinder each other from accessing the cellulose with their catalytic domains. The impact of overcrowding on the efficiency and kinetics evaluation of cellulose hydrolysis by cellobiohydrolase (CBH) has recently been reported.[6,36,37] Although no data is available for EG I and an amorphous model film, it appears that overcrowding is also associated with the endoglucanase hydrolysis of cellulose. Already at 1 $\mu$M concentration it appears that the excess of the enzyme significantly reduces the hydrolysis, and even leads to its apparent stopping of the hydrolysis. However, dissipation data in Figure 1 indicate that, despite no change in frequency after 30 min of exposure, the action of EG I leads to change in rheological properties of the film. Positive changes in dissipation indicate the formation of viscoelastic, gel-like material. Thus, the rigid and fully elastic cellulose film became soft and mobile due to the partial degradation and simultaneously increased water uptake. The available surface for EG I binding appears to be fully saturated at that point. Therefore this increase in dissipation is most likely due to continuous loosening of the swollen film and while no significant amount of cellulose is released as indicated by the lack of frequency change, the hydrolytic activity of the enzyme continues. Previous findings on the action of monocomponent EG I on a semicrystalline cellulose model film investigated by QCM-D showed a similar, continuous increase of dissipation while a relatively stable frequency was maintained.[19]

The data also suggest that hydrolysis at lower concentrations was affected by overcrowding, but to a much lesser extent. The overcrowding is likely related to the burst of activity at the beginning of the rinse stage, where removal of the excess of enzyme frees further enzyme activity. As mentioned above, the exchange of enzyme solution to buffer should not have an impact on the conditions in the system since the properties of pure buffer and enzyme solution are comparable. Interestingly, at the lowest concentration (0.01 $\mu$M) the rinsing stage had no effect on the rate of hydrolysis, indicated by the smooth progression in

**Table 1. XPS Data for the Amorphous Films Exposed to the EG I and CBH I Treatment for 60 min and Subsequent Rinsing[a]**

| | atomic concentrations | | | |
|---|---|---|---|---|
| sample | O 1s | C 1s | Si 2p | N 1s |
| EG | 40.4% | 57.6% | 1.7% | 0.3% |
| CBH | 38.1% | 57.8% | 0.1% | 4.1% |

[a] The initial thickness of the films was 60 nm. The XPS spectra are available in the Supporting Information (Figure S7).

frequency change. This could indicate that the enzyme concentration is low enough for the enzyme to work efficiently without the impact of overcrowding. Thus it appears that the optimal concentration for the maximal hydrolytic efficiency would be somewhere between 0.2 and 1 $\mu$M. For the purpose of this investigation, however, 0.2 $\mu$M was selected as the enzyme concentration for further experiments. Because rinsing, i.e., dilution of the EG concentration, clearly alters the kinetics of hydrolysis, the results also demonstrate that for a proper evaluation of hydrolysis it is important to maintain the same enzyme load to obtain a system with steady state conditions.

The impact of rinsing was further investigated in detail. For a constant flow of 0.2 $\mu$M EG I solution, the time of the exposure of the film to the enzyme was varied from 5 to 90 min followed by rinsing for 90 min (Supporting Information, Figure S2). The time of exposure proportionally affects the overall extent of the degradation and led to an initial increase in the rate of hydrolysis. It appears that the enzyme is gradually washed away, and the hydrolysis eventually stops within 20 min after the rinsing commences. This time lag corresponds to the off-rate for EG I to desorb from the amorphous cellulose and shows the reversibility of the protein−substrate interaction. The XPS analysis of the QCM-D crystals after endoglucanase exposure and subsequent rinsing confirmed the absence of protein within the film as indicated by the absence of surface nitrogen (protein marker) as shown in Table 1. The data measured for CBH I treated film are included in the table and are discussed below.

In summary, both concentration (Figure 1) and time of exposure (Figure S2 in Supporting Information) were shown to have a pronounced effect on the hydrolysis of the cellulose films by EG I. Too high a concentration of the enzyme can lead to self-inhibition of the enzyme activity. This also shows that the mode of enzyme action is affected by the experimental conditions. The hydrolysis of the model films has previously been investigated using various experimental protocols. Some explored a batch mode, where enzyme is added for a short time, in a pulse-like fashion, followed by rinsing, thus letting only the initially adsorbed enzyme degrade the cellulose in the film.[19] Others, similarly to this investigation, used continuous mode with a constant enzyme flow.[17] The constant flow of the enzyme ensures that the concentration of the enzyme is constant throughout the hydrolysis, therefore the ratio between free and bound enzyme is also constant. In addition, the homogeneous film maintains its characteristics during the hydrolysis, thus morphology of the film and the type of interaction do not change during the hydrolysis. In this investigation we demonstrated that, for a proper quantitative investigation of enzymatic action, a steady state system should be achieved by maintaining the same enzyme load. The rinsing sequence that leads to dilution of the enzyme can alter the rate of the degradation, making a proper kinetic evaluation of the process difficult.

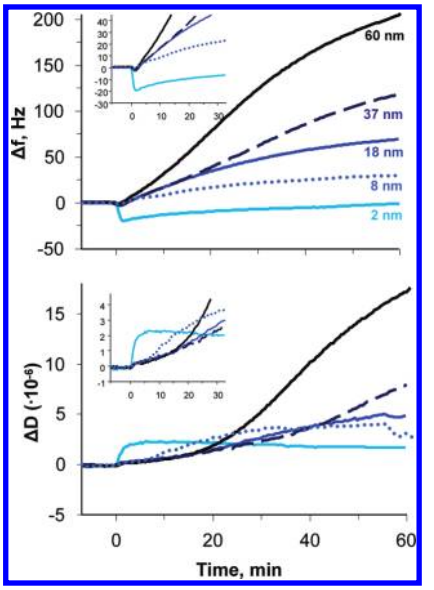



**Figure 2.** Frequency and dissipation change development during hydrolysis of the films of different thickness. Time 0 indicates the start of enzyme (EG I) addition ($f_0$ = 5 MHz, $n$ = 3, $f_3/n$).

Many models have been proposed to describe the kinetics of cellulose degradation by cellulases and the majority of them are based on the Michaelis−Menten kinetics.[4,5] Despite controlled and simplified conditions, the enzyme load applied and dilution (rinsing) of the enzyme indicate additional factors that may have an impact of the rate of hydrolysis and subsequently on kinetic evaluation of the process. This demonstrates that the kinetic evaluation based on Michaelis−Menten equation should not be applied for this system in particular.

**3.2. Impact of Film Thickness on Rate of Hydrolysis.** The next step in evaluating the action of EG I on amorphous cellulose film was to assess the impact of the amount of substrate available for hydrolysis on the rate of hydrolysis as detected by QCM-D. In our recent characterization, we quantitatively demonstrated that the regenerated cellulose model films prepared from spin coated TMSC swell extensively in water and that this swelling is homogeneous throughout the whole film.[22] During the swelling, the film doubles its volume while remaining stable over a long period of time.[38] Spin coating, on the other hand, allows for the preparation of homogeneous amorphous films of different thickness. The thickness of these homogeneous films can be controlled by the concentration of the TMSC solution used in spin coating. The correlation between the concentrations of the solutions of TMSC and resulting thickness of the spin coated regenerated cellulose films in a dry state is shown in the Supporting Information (Figure S3 and Table S1). Because of the homogeneous swelling, we are able to control the amount of cellulose that is susceptible to enzymatic attack by casting films of different thickness values. Previous fundamental studies of the enzymatic degradation of cellulose model films[14−19,39] have not addressed the issue of substrate available for hydrolysis, and as such, a direct comparison with previous work is not possible. The ability to control the film thickness, or volume of available substrate, however, can be valuable for obtaining additional information pertinent to the kinetic evaluation of cellulose degradation by cellulases.

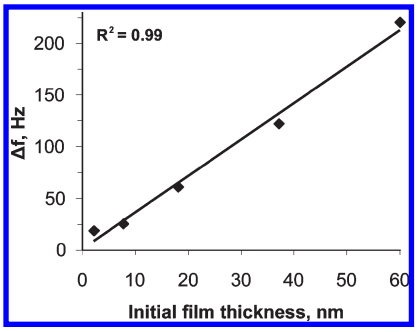



**Figure 3.** Correlation between initial film thickness and the change of frequency as measured by QCM-D after 60 min EG I treatment. Positive frequency change indicates mass reduction on the sensor surface.

The films swollen in buffer solution were exposed to the solution of EG I (0.2 $\mu$M) in the QCM-D chamber for a period of 60 min. Preliminary investigation (see previous section) showed that this time is sufficient for achieving the bulk of hydrolysis of cellulose in the films. The development of frequency and dissipation during the hydrolysis of the films with different thickness (2, 8, 18, 37, and 60 nm) is shown in Figure 2. The measurements of films of additional thicknesses (i.e., 14, 32, and 48 nm) are included in the Supporting Information (Figure S4).

Except for the thinnest film (2 nm), all other films of different thicknesses showed a rapid degradation almost immediately following contact with the enzyme. The initial adsorption is rapidly surpassed by the concurrent hydrolytic action. The minimum frequency is reached for films of all thickness (except for the thinnest film) within ca. 2 min from the initial contact, followed by a rapid increase in frequency indicating the removal of the cellulose from the film. Development of the dissipation values showed a similar overall trend (Figure 2). The initial response appeared to be delayed proportionally to increasing thickness, but in later stages the rate and extent of dissipation change increases proportionally with film thickness. The thinner films (2 and 8 nm) were readily saturated with the EG I faster and were also more sensitive to the viscoelastic change due to adsorbed enzyme, thus the faster initial response (increase in dissipation). The adsorption of the EG I did not affect the thicker films (18, 37, and 60 nm) to the same extent, but later the rate of dissipation change of the films increased proportionally with the film thickness. The thinnest film (2 nm) exhibited an anomaly in both frequency and dissipation development (Figure 2) compared to the thicker films. A clearly visible and more pronounced adsorption stage and an instant increase in dissipation differ from the trends observed for the thicker films. The reason for this difference has not yet been identified, although it is possible that the cellulose in close contact with the silica substrate form an arrangement that cannot be disrupted by the EG I treatment. Nonspecific binding of the EG I onto possibly exposed base substrate (silica) can be excluded, as indicated by a very small change in frequency values measured for blank silica crystals exposed to EG I (Supporting Information Figure S5). In the case of the thinnest film, however, the effect of water uptake is minimal, and it is possible that its behavior reflects the true expected interaction between EG and cellulose. In other words, both stages can be distinguished: rapid adsorption and hydrolysis. At the hydrolysis stage, a detectable decrease in dissipation occurs, which is indicative of detachment of cellulose. However, slight swelling is also visible in this curve, seen as the different rates of change in frequency and dissipation curves.

The comparison in Figure 2 clearly demonstrates the impact of the film thickness on the rate of hydrolysis. The larger amount of available cellulose substrate (film thickness) showed faster rates of hydrolysis, represented by the slope of frequency change. Despite different behavior of the 2 nm film (adsorption), the hydrolytic part of the EG I action correlates well with the rest of the films. A correlation between the initial film thickness (mass of cellulose) and the absolute increase in frequency change (due to mass reduction) after 60 min of EG I hydrolysis is shown in Figure 3.

The frequency change correlates well with the initial film thickness, indicating that a greater amount of available cellulose on the model surface results in a proportionally greater frequency change after 60 min of EG I exposure. This shows that the swollen amorphous cellulose film creates a freely diffusible system. We previously demonstrated that upon swelling the thickness of the film increases to reach almost twice that of the original dry film, and that despite this marked swelling the film retained its substantial supramolecular cellulose network.[22] This allows the EG I to readily diffuse into the whole volume of the swollen film of amorphous cellulose. Indeed, it appears that EG I readily penetrates into the whole volume of the cellulose in the film and that the cellulose is released from the whole volume.

In general, this finding should be considered when a direct comparison of different cellulose model films, particularly their rate of hydrolysis, is evaluated with QCM-D. Although the correlation is well-defined due to the homogeneity of the cellulose in these films, it is reasonable to assume that the amount of available substrate for hydrolysis should be comparable to obtain proper data on the kinetics of hydrolysis.

**3.3. Quantification of the Extent of Hydrolysis.** The measured change in frequency can be under some conditions converted to actual mass change of the substrate deposited on the crystal.[20] Previous investigations have utilized various models to express the kinetics of the hydrolysis.[16,17,19] In this investigation, we attempted a direct quantification of the data obtained by QCM-D measurement. Parallel to the experiments carried out by QCM-D, experiments using identical model films deposited on silicon wafers were conducted. This was necessary because the quartz crystal sensors used in the actual QCM-D measurement could not be used in thickness profiling by AFM. The silicon layer on the top of the blank QCM-D crystal is deposited on gold and is affected by scratching with the sharp needle. As such, the thickness of the films on QCM-D crystals could not be analyzed by this technique. Identically prepared films deposited on silicon wafers were exposed to the flow of the enzyme. We tried to closely simulate the conditions in the QCM-D measurements by using the same pump, tubing, and experimental conditions (flow). The enzyme, at the same rate as that in the QCM-D measurement, was introduced on top of the film, and the excess of the liquid (enzyme) was periodically removed from the top of the films by suctioning with a micropipet. To measure mass reduction, the thickness of the film was measured before and after the EG I treatment. The mass reduction was then determined from the difference between the initial thickness and that after the enzymatic treatment. This thickness difference can be considered a viable indicator of the mass reduction, based on the homogeneous characteristics of the films used in this investigation.[22] We emphasize that the residual thickness was analyzed from dry films after the EG hydrolysis, thereby omitting the possible artifacts arising from nonuniformities that may result in the swollen film during the hydrolysis. The correlation

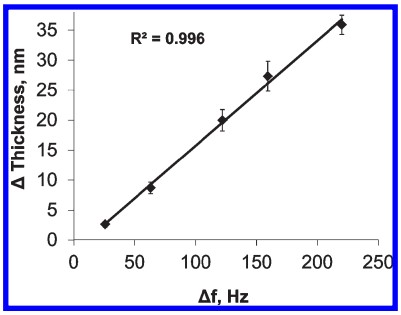



**Figure 4.** Correlation between measured QCM-D decrease and decrease of film thickness after 60 min EG I treatment.

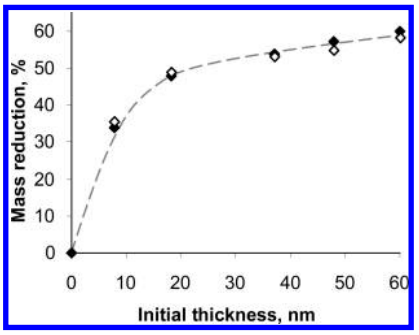



**Figure 5.** Correlation between relative decreases of the film thickness after 60 min EG I treatment and initial film thickness. The solid points are measured AFM data, and white points are calculated form the QCM-D frequency change (Figure 2) and the correlation of Figure 4.

between the change of frequency and the decrease in film thickness after treatment is shown in Figure 4.

The films of 8, 18, 37, 49, and 60 nm thicknesses were included in the evaluation for thickness change. The tabulated measured data are included in the Supporting Information (Table S2). The data for the thinnest film (2 nm) is not included because the experiments showed that it was too thin for reliable measurements. The AFM images of films after hydrolysis did not reveal visible changes in morphology (Supporting Information Figure S6). In addition, variation of the thickness measurements taken from different spots of the film were within an acceptable range, confirming that the degradation is even over the whole film surface. Both QCM-D frequency change and the thickness reduction measured by the AFM correlated very well with each other (Figure 4). This shows that the QCM-D frequency change reliably measures the mass loss of the cellulose model films for these films and conditions. These measured mass reduction data were used to determine the overall extent of hydrolysis for the film of different thicknesses. The relative mass reduction by EG I treatment (mass decrease of initial mass) plotted against the initial thickness of the corresponding films is shown in Figure 5. The figure also contains data on mass reduction calculated from the frequency change measured by QCM-D using the correlation shown in Figure 4.

Although an inadequate amount of data points is available in the lower film thickness region, it appears that the relative amount of cellulose that is degraded by the action of EG I is similar for all films thicker than ca. 20 nm. This indicates that the cellulose layer in close contact with the base substrate possesses different characteristics that prevent its efficient hydrolysis and subsequent detachment from the crystal. This is probably also

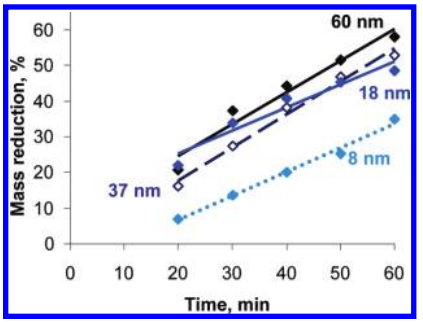



**Figure 6.** Correlation between relative degradation of the film and hydrolysis time is linear and does not depend on initial film thickness for films over 8 nm initial thickness. This shows that the cellulose film allows free diffusion of EG I and hydrolysis is uniform except close to the base of the cellulose film.

the reason why the thinnest film exhibited markedly different adsorption and dissipation patterns when compared to the thicker films. In general, the films with thicknesses above 20 nm appear to yield a similar relative extent of degradation of ca. 50 to 60% range. The lower amount of available substrate may be affecting the quantitative evaluation of the hydrolysis of cellulose model films by QCM-D.

The thickness measurements and quantification of the extent of hydrolysis also clearly demonstrates that the complete hydrolysis of the film by EG I after 60 min is not achieved. Although it has been shown that the individual, pure monocomponent cellulases do not fully degrade the insoluble cellulose substrate (e.g., bacterial, acid hydrolyzed and phosphoric acid swollen cellulose),[40−42] it was interesting to notice that even under these optimal conditions, the extent of degradation did not exceed 60%. Although mostly identified for cellobiohydrolases and cellulase mixtures, the main factors responsible for inhibition of hydrolytic action include enzyme deactivation, inhibition by cellobiose, jamming or overcrowding, and substrate related limitations.[3,6,43,44] Our experimental setup allows very good control of these factors, since fresh enzyme was constantly available, low concentration should prevent enzyme overcrowding, constant flow continuously removed dissolved or detached cellulose, and the homogeneous swollen amorphous cellulose does not present any limitations in substrate accessibility. Although reduction of the mass, represented by the film thickness reduction, demonstrated the hydrolysis of the films, this information does not elucidate the causes of this limited hydrolysis. Detailed investigation of structural changes in the hydrolyzed films was not within the scope of this initial investigation and will be evaluated in future investigations.

Next, we attempted to assess the rate of hydrolysis for films of different thicknesses. Using the correlation of frequency and mass reduction (Figure 4) and the measured frequency development (Figure 1), the time dependent rate of hydrolysis was compared. The comparison of the rate of hydrolysis for films of different thicknesses (8, 18, 37, and 60 nm) is shown in Figure 6.

The linear relationship demonstrated in Figure 6 implies that the contribution of the enzyme binding is over after the first 10 min, that is, a steady state is rapidly reached between free and bound enzymes. Substantial additional binding during the cellulose degradation would render the relative rate of degradation different, i.e., the mass reduction would not be linear against time. Moreover, the slopes of the correlation were similar for films of


dx.doi.org/10.1021/la2014418 |*Langmuir* 2011, 27, 8819–8828

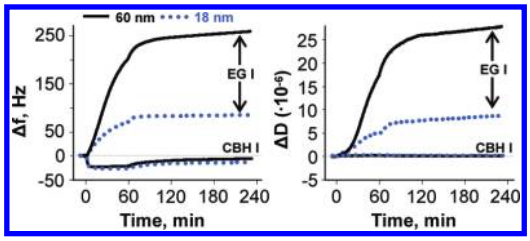



**Figure 7.** QCM-D evaluation of EG I and CBH I action on amorphous cellulose model films with different thickness. Change in frequency (left) and dissipation (right). Enzyme (0.2 $\mu$M) introduced at $t$ = 0, rinsing after 60 min of treatment. ($f_0$ = 5 MHz, $n$ = 3, $f_3/n$).

all measured thicknesses. The kinetics of hydrolysis for the 18 nm film appears to be closer to the thicker films in the initial stage, but with time (of hydrolysis) it inclines toward the kinetics of the 8 nm film. This comparison indicates that the relative rate of hydrolysis for EG I hydrolysis of amorphous cellulose in the film is independent of film thickness (Figure 6), although the absolute rate is thickness-dependent (Figure 2). This shows that hydrolysis occurs throughout the cellulose film (except close to the base) and EG I diffuses throughout the film layer.

**3.4. Comparison of EG I and CBH I Action.** For a broader understanding of cellulose−substrate interactions, we compare the action of monocomponent cellobiohydrolase (CBH I) to EG I using 18 and 60 nm thick films. This comparison was carried out to obtain the initial information on action of CBH I on amorphous cellulose film and to compare it to the action of EG I. The comparison is shown in Figure 7.

The differences in action of CBH I and EG I were observed on two levels: extent of hydrolysis and the impact of film thickness. The films exposed to CBH I exhibited a rapid drop in frequency (ca. 25 Hz), that reached a minimum within ca. 10 min and remained constant over the period of enzyme exposure (60 min). This indicates that no or a limited decrease of mass occurred during that time. In addition, the film thickness did not appear to have an impact on the extent of frequency change. In contrast, the hydrolytic action of EG I, as shown previously, commenced almost immediately after the contact with the substrate, indicated by an instant rapid increase in frequency. Contrary to the action of CBH I, the impact of the film thickness is clearly evident; the change in frequency after 60 min of exposure markedly increases with the film thickness. Replacement of the enzyme with buffer solution (at 60 min) resulted in a gradual increase in frequency observed for CBH I treated film, while EG I treated sample exhibited a rapid increase in frequency followed by gradual leveling off. It appears that the CBH I initially tightly adsorbs on the surface of the film, forming a thin, rigid layer of adsorbed enzyme, as indicated by no change in measured dissipation (Figure 7). The fact that the thickness of the film (initial volume of cellulose available) does not affect the extent of the adsorption, nor the subsequent hydrolysis, would suggest that the adsorption of CBH I takes place on surface only. The gradual increase of the frequency after switching to buffer solution is a result of either desorption of the enzyme or limited hydrolysis of cellulose. It is possible that both, desorption and hydrolysis, take place simultaneously. The XPS analysis of the hydrolyzed films (Table 1) indicated the presence of protein in the film. Based on the nitrogen content, the amount of CBH I is approximately ten times higher that that of EG I. This would support the assumption that limited hydrolysis indeed takes place, whereas the CBH I irreversibly adsorbs on the cellulose and proceeds with limited hydrolysis. The bound CBH I is not removed even by extensive washing after the hydrolysis, which also supports the finding of a strongly bound rigid layer of CBH I on cellulose detected by QCM-D. Irreversible adsorption of CBH I on a crystalline substrate and its impact on the hydrolysis rate has been demonstrated.[37] It was reported that, despite preference for crystalline cellulose, this irreversible adsorption takes place also on amorphous cellulose.[45] Comparison of the films of different thickness (initial thickness of 8 and 60 nm) showed that the amount of protein is similar for the film of both thicknesses, supporting the assumption that the binding takes place on surface of the films (Supporting Information, Figure S7 and Table S3). In contrast, the protein was not detected in the films (18 and 60 nm thick) treated with EG I under identical conditions, indicating a much quicker desorption rate for EG I.

The previously reported comparison of the action of monocomponent CBH and EG cellulases in the degradation of semicrystalline cellulose films showed different trends.[19] The exposure of the film to EG resulted in a rapid decrease in frequency and continuous increase of dissipation, attributed to increased water uptake of the film due to film swelling. However, no mass reduction was observed during the extended period of measurement. In contrast, the action of CBH resulted in rapid hydrolysis, indicated by an increase in frequency. In this investigation, the opposite trend was observed for both enzymes. A significant degradation of an amorphous cellulose film was observed in the EG I treatment of the film, while only marginal hydrolysis was observed for CBH I. This agrees with the preference of EG I toward the amorphous parts of cellulose.[5,23,24] Dissipation development also showed extensive continuous swelling of the film. It appears that the cellulose released from the volume was replaced by water, resulting in a softer film. The swelling had a markedly greater impact on the dissipation measurement than the detachment of cellulose, which would be indicated by decrease in the dissipation values. The limited activity of CBH I is also different to that measured using semicrystalline films. Although CBH I preferentially acts on the crystalline part of cellulose, partial activity on amorphous cellulose has been reported.[40,44] In fact, experiments with bulk amorphous cellulose and CBH I indicate activity which is in comparable quantities with the present results.[44] It appears that the action of CBH I on swollen amorphous cellulose films is very limited.

The action of both EG I and CBH I on amorphous cellulose differs from the action of cellulase mixture as described in previous QCM-D evaluations.[18] In those studies, a rapid removal of cellulose was observed, and the enzyme was able to degrade and eventually remove the whole cellulose film. The dissipation development showed an increase as the film becomes more diffuse, reaching a dissipation maximum (at the maximum hydrolysis rate) when the thickness of the film has decreased enough and it starts to become more compact, and finally the dissipation reduces to zero when the whole film is removed. Similar trend was observed for this film of amorphous cellulose when exposed to the same commercial cellulose mixture at comparable concentration, as shown in the Supporting Information (Figure S8). Therefore, the different dissipation profile measured for this system and EG I is not due to film characteristics. We showed that even the fully amorphous cellulose film cannot be completely degraded by EG I alone. That could explain why the dissipation value does not reach maximum with

subsequent rapid drop in value that would indicate the complete removal of the film.

With this comparison we intended to demonstrate the difference in action of CBH I and EG I in hydrolysis of amorphous substrates. Further investigation and more detailed study of the hydrolysis by CBH I are necessary to properly evaluate the mechanism of action of CBH I on the substrate.

## 4. CONCLUSIONS

The systematic evaluation of the degradation of amorphous cellulose film by a monocomponent endoglucanase identified several important aspects relevant to the study of the kinetics of cellulose degradation. We have shown that QCM-D together with specifically prepared surfaces offer a tool to study such aspects of cellulose hydrolysis for which otherwise tools are not available.

Quantification of the actual hydrolysis was carried out by measuring the film thickness reduction by AFM after the enzymatic treatment. The values correlated well with the frequency data obtained by QCM-D measurement for corresponding films, which shows that the QCM-D frequency change reliably measures the mass loss of the hydrolyzed cellulose model films for these films and conditions, and thus that the evaluation of the hydrolysis by QCM-D can be done quantitatively.

Explicitly, it was demonstrated that to properly evaluate the mechanism of action, steady state conditions in the experimental set up need to be reached. Rinsing, or diluting the enzyme, as well as concentration of the enzyme have a pronounced effect on the hydrolysis. Furthermore, substrate (cellulose) volume does not have an impact on relative rate of hydrolysis. The absolute rate of hydrolysis, as observed by the rate of frequency response measured by QCM-D, varies according to the thickness (volume) of the film. However, when expressed as a relative mass reduction, it was shown that the relative rate of film hydrolysis is not affected by the amount of the substrate available. This was considered to support the presumption that the ratio of free to bound enzyme stays constant during cellulose degradation, i.e., that the system meets steady state conditions.

This experimental setup allowed the study of the impact of enzyme concentration on the efficiency of hydrolysis. The data clearly shows that high loads of enzyme easily lead to overcrowding and results in markedly slower rate of degradation. Also, with this experimental setup, the conditions at which crowding occurs can be controlled.

A specific finding with respect to amorphous cellulose and EG was that EG I alone was not able to hydrolyze the whole film of amorphous cellulose, reaching a maximum degradation of around 60% for films thicker than 20 nm. Moreover, EG I works uniformly within the whole volume of cellulose film. The model film of amorphous cellulose, upon swelling, forms a freely diffusible substrate.

The model film of fully amorphous cellulose used in this investigation does not entirely correspond to native cellulose. Native cellulose is morphologically complex and experiments with several model systems are required to put down the pieces of information for that would form a bigger picture leading to resolving the nature of enzymatic hydrolysis of native cellulose. This effort using a well-defined model substrate represents such a piece.

## ■ ASSOCIATED CONTENT

**Ⓢ Supporting Information.** Detailed description of film thickness measurement by AFM (S1), a description of the effect of enzyme exposure time on hydrolysis of amorphous films measured as frequency change with QCMD (S2), correlation between concentration of TMSC solution used in spin coating and thickness of the regenerated films (S3) with the corresponding tabulated data, Table (S1), the frequency change measured for EG I hydrolysis of films with different thickness for the entire set of films (S4), EG I interaction (frequency change) with a blank silica QCMD sensor (S5); table of relative mass reduction calculated from film thickness measured before and after hydrolysis, Table (S2), AFM images of dried films before and after hydrolysis (S6), XP Spectra of cellulose model films after exposure to EG I and CBH I and subsequent rinsing (S7), with the corresponding XP Atomic concentrations Table (S3), QCM-D evaluation of hydrolysis of amorphous film by commercial cellulase mixture (S8). This material is available free of charge via the Internet at http://pubs.acs.org.

## ■ AUTHOR INFORMATION

**Corresponding Author**

*Telephone: +358 9 470 24250. Fax: +358 9 470 24259. E-mail: eero.kontturi@aalto.fi.

## ■ ACKNOWLEDGMENT

M.S. thanks Multidisciplinary Institute of Digitalisation and Energy (MIDE, http://mide.tkk.fi) for financial support. E.K. acknowledges the Academy of Finland (Project No. 129068) for financial support. Matti Siika-aho is thanked for the EG I and CBH I enzymes. Dr. L.-S. Johansson is thanked for assisting with the XPS analysis.

## ■ REFERENCES

(1) Himmel, M. E. *Science* **2007**, *315*, 804–807.

(2) Gross, A. S.; Chu, J.-W. *J. Phys. Chem. B* **2010**, *114*, 13333–13341.

(3) Mansfield, S. D.; Mooney, C.; Saddler, J. N. *Biotechnol. Prog.* **1999**, *15*, 804–816.

(4) Zhang, Y.-H. P.; Lynd, L. R. *Biotechnol. Bioeng.* **2004**, *88*, 797–824.

(5) Lynd, L. R.; Weimer, P. J.; van Zyl, W. H.; Pretorius, I. S. *Microbiol. Mol. Biol. Rev.* **2002**, *66*, 506–577.

(6) Xu, F.; Ding, H. *Appl. Catal., A* **2007**, *317*, 70–81.

(7) Bansal, P.; Hall, M.; Realff, M. J.; Lee, J. H.; Bommarius, A. S. *Biotechnol. Adv.* **2009**, *27*, 833–848.

(8) Palonen, H.; Tenkanen, M.; Linder, M. *Appl. Environ. Microbiol.* **1999**, *65*, 5229–5233.

(9) Tammelin, T.; Merta, J.; Johansson, L.-S.; Stenius, P. *Langmuir* **2004**, *20*, 10900–10909.

(10) Kontturi, K. S.; Tammelin, T.; Johansson, L.-S.; Stenius, P. *Langmuir* **2008**, *24*, 4743–4749.

(11) Halthur, T. J.; Björklund, A.; Elofsson, U. M. *Langmuir* **2006**, *22*, 2227–2234.

(12) Halthur, T. J.; Claesson, P. M.; Elofsson, U. M. *Langmuir* **2006**, *22*, 11065–11071.

(13) Malmström, J.; Agheli, H.; Kingshott, P.; Sutherland, D. S. *Langmuir* **2007**, *23*, 9760–9768.

(14) Jeong, C.; Maciel, A. M.; Pawlak, J. J.; Heitmann, J. A.; Argyropoulos, D. S.; Rojas, O. J. Proceeding of the 13th International Symposium on Wood, *Fibre and Pulping Chemistry*, Auckland, New Zealand, May 16−19, 2005; Vol. 2: 495-502.

(15) Rojas, O. J.; Jeong, C.; Turon, X.; Argyropoulos, D. S. *ACS Symp. Series* **2007**, *954* (Materials, Chemicals, and Energy from Forest Biomass), 478−494.

(16) Hu, G.; Heitmann, J. A., Jr.; Rojas, O. J. *J. Phys. Chem. B* **2009**, *113*, 14761–14768.
(17) Turon, X.; Rojas, O. J.; Deinhammer, R. S. *Langmuir* **2008**, *24*, 3880–3887.
(18) Ahola, S.; Turon, X.; Osterberg, M.; Laine, J.; Rojas, O. J. *Langmuir* **2008**, *24*, 11592–11599.
(19) Josefsson, P.; Henriksson, G.; Wagberg, L. *Biomacromolecules* **2008**, *9*, 249–254.
(20) Rodahl, M.; Höök, F.; Krozer, A.; Brzezinski, P.; Kasemo, B. *Rev. Sci. Instrum.* **1995**, *66*, 3924–3930.
(21) Hu, G.; Heitmann, J. A., Jr; Rojas, O. J. *Anal. Chem.* **2009**, *81*, 1872–1880.
(22) Kontturi, E.; Suchy, M.; Penttilä, P.; Jean, B.; Pirkkalainen, K.; Torkkeli, M.; Serimaa, R. *Biomacromolecules* **2011**, *12*, 770–777.
(23) Bailey, M. J.; Siika-Aho, M.; Valkeajärvi, A.; Penttilä, M. E. *Biotechnol. Appl. Biochem.* **1993**, *17*, 65–76.
(24) Kleman-Leyer, K. M.; Siika-Aho, M.; Teeri, T. T.; Kirk, T. K. *Appl. Environ. Microbiol.* **1996**, *62*, 2883–2887.
(25) Rahkamo, L.; Siika-aho, M.; Viikari, L.; Leppanen, T.; Buchert, J. *Holzforschung* **1998**, *52*, 630–634.
(26) Henriksson, G; Christiernin, M.; Agnemo, R. *J. Ind. Microbiol. Biotechnol.* **2005**, *32*, 211–214.
(27) Engström, A.-C.; Ek, M.; Henriksson, G. *Biomacromolecules* **2006**, *7*, 2027–2031.
(28) Pääkkö, M.; Ankerfors, M.; Kosonen, H.; Nykänen, A.; Ahola, S.; Österberg, M.; Ruokolainen, J.; Laine, J.; Larsson, P. T.; Ikkala, O.; Lindström, T. *Biomacromolecules* **2007**, *8*, 1934–1941.
(29) Kontturi, E.; Thüne, P. C.; Niemantsverdriet, J. W. *Langmuir* **2003**, *19*, 5735–5741.
(30) Suurnäkki, A.; Tenkanen, M.; Siika-Aho, M.; Niku-Paavola, M.-L.; Viikari, L.; Buchert, J. *Cellulose* **2000**, *7*, 189–209.
(31) Rahkamo, L.; Siika-aho, M.; Vehvilainen, M.; Dolk, M.; Viikari, L.; Nousiainen, P.; Buchert, J. *Cellulose* **1996**, *3*, 153–163.
(32) Tammelin, T.; Saarinen, T.; Österberg, M.; Laine, J. *Cellulose* **2006**, *13*, 519–535.
(33) Höök, F.; Rodahl, M.; Brzezinski, P.; Kasemo, B. *Langmuir* **1998**, *14*, 729–734.
(34) Edvardsson, M.; Rodahl, M.; Kasemo, B.; Höök, F. *Anal. Chem.* **2005**, *77*, 4918–4926.
(35) Johansson, L.-S.; Campbell, J. M. *Surf. Interface Anal.* **2004**, *36*, 1018–1022.
(36) Igarashi, K.; Wada, M.; Hori, R.; Samejima, M. *FEBS J.* **2006**, *273*, 2869–2878.
(37) Ma, A.; Hu, Q.; Qu, Y.; Bai, Z.; Liu, W.; Zhuang, G. *Enzyme Microb. Technol.* **2008**, *42*, 543–547.
(38) Nyfors, L.; Suchy, M.; Laine, J.; Kontturi, E. *Biomacromolecules* **2009**, *10*, 1276–1281.
(39) Eriksson, J.; Malmsten, M.; Tiberg, F.; Callisen, T. H.; Damhus, T.; Johansen, K. S. *J. Colloid Interface Sci.* **2005**, *284*, 99–106.
(40) Nidetzky, B.; Hayn, M.; Macarron, R.; Steiner, W. *Biotechnol. Lett.* **1993**, *15*, 71–76.
(41) Väljamäe, P.; Sild, V.; Nutt, A.; Pettersson, G.; Johansson, G. *Eur. J. Biochem.* **1999**, *266*, 327–334.
(42) Rabinovich, M. L.; Melnick, M. S.; Bolobova, A. V. *Biochemistry (Moscow)* **2002**, *67*, 850–871.
(43) Eriksson, T.; Karlsson, J.; Tjerneld, F. *Appl. Biochem. Biotechnol.* **2002**, *101*, 41–60.
(44) Gruno, M.; Väljamäe, P.; Pettersson, G.; Johansson, G. *Biotechnol. Bioeng.* **2004**, *86*, 503–511.
(45) Hall, M.; Bansal, P.; Lee, J. H.; Realff, M. J.; Bommarius, A. S. *FEBS J.* **2010**, *277*, 1571–1582.