# In Situ Monitoring of Cellulase Activity by Microgravimetry with a Quartz Crystal Microbalance

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*Received: July 27, 2009; Revised Manuscript Received: September 19, 2009*

Quartz crystal microgravimetry (QCM) was used to investigate the interactions between cellulase enzymes and model cellulose substrates. The substrates consisted of thin films of cellulose that were spin-coated onto polyvinylamine (PVAm) precoated quartz crystal sensors carrying conductive gold surfaces. In QCM the quartz crystals are piezoelectrically driven and the frequency and dissipation shifts allow monitoring of substrate hydrolysis at various temperatures and enzyme concentrations in situ and in real time. The changes in frequency of cellulose-coated quartz resonators during their incubation in cellulase solutions were related to contributions from the liquid phase properties, the adsorptions of cellulase enzymes, and the hydrolysis of the substrate. Cellulase adsorption was found to be nonspecific and irreversible on gold-, PVAm-, and cellulose-coated quartz crystal sensors. The contribution to frequency shifts due to the bulk fluid properties of the cellulase solutions (at concentrations lower than 0.5 mg/mL) was minimal compared to the frequency shifts produced by cellulase binding. The maximum frequency decreases were fitted to a Langmuir model. The adsorption constant and the maximum adsorption were estimated by the fitting parameters of this model. The hydrolysis process was modeled by using a dose−response model that was then used to estimate the maximum hydrolysis rate, to compare the relative effects of temperature on adsorption and hydrolysis rate, and to obtain the apparent activation energy of cellulose hydrolysis. The hydrolysis rate increased with incubation temperature while apparent adsorption decreased. The apparent activation energy for the hydrolysis of the cellulose films employed was calculated to be 37 kJ/mol.

## 1.. Introduction

The broad application of the piezoelectric quartz crystal technique was developed long after Marie Curie discovered this property of crystals. The quartz crystal microbalance (QCM) has become a useful technique to study interfacial phenomena in different fields of science. Since Sauerbrey reported that the mass sensitivity of a quartz crystal could be used to measure the thickness of vacuum-deposited metals, significant progress has been made in understanding other interaction mechanisms between acoustic devices and contacting media.[1] The various reported investigations discussed theoretical models,[2,3] thin film deposition,[4,5] applications in electrochemical reactions,[6,7] and biological/biochemical developments.[8] In the case of cellulose research, cellulose hydration,[9] enzyme adsorption,[10] and enzyme kinetics (cellulase binding and substrate hydrolysis rates)[11−13] have been also reported.

According to the Sauerbrey equation,[14] when a crystal is loaded with a uniform rigid film that has a much smaller mass than that of the quartz resonator, the change in resonance frequency can be related to the effective mass on the sensor by a simple linear relation:

$$\Delta f = -\frac{2nf^2}{v_q \rho_q}\Delta m = -\frac{nf}{\rho_q t_q}\Delta m = -\frac{n\Delta m}{C} \qquad (1)$$

where $\rho_q$ and $v_q$ are the specific density and the shear wave velocity in quartz, respectively; $t_q$ is the thickness of the quartz crystal, $f$ is the resonant frequency, $n$ is the overtone number, and $\Delta m$ is the mass change per unit area. The minus sign indicates that a decrease of mass will result in an increased frequency. The constant in eq 1 is 17.7 ng $Hz^{-1}$ $cm^{-2}$ for a 5 MHz AT-cut quartz crystal sensor.

For soft films eq 1 is not exactly obeyed due to viscoelastic dissipation. In such cases dissipation data can be used to characterize the film viscoelasticity more comprehensively and accurately. This dissipation factor, which measures the energy dissipated during dampening of oscillations as the vibration amplitude decays exponentially, can be expressed as

$$D = \frac{E_{\text{dissipated}}}{2\pi E_{\text{stored}}} \qquad (2)$$

For soft films the linear relationship with frequency as described in eq 1 is not obeyed and quantification of changes in film mass requires the use of viscoelastic parameters.

In addition to the mass effect of the film, the physical properties of the liquid or gas filling the QCM chamber have an effect on the measured resonant frequency,[15−17] the change of which is proportional to the square root of the product of liquid density and liquid viscosity.[18] By measuring the changes

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10.1021/jp907155v CCC: $40.75 
Published on Web 10/14/2009

in QCM frequency and dissipation, one can monitor the dynamics of hydrolysis of a cellulose film deposited on the quartz crystal sensors.

The deposition of cellulose films has been successfully practiced for various purposes.[19−21] Both spin-coated and Langmuir−Blodgett[21−23] films of cellulose have been investigated in terms of their degradation behavior when exposed to cellulase enzymes. Several research efforts opened the way to study the interactions between cellulose films and fungal cellulase systems using QCM-D. The investigated parameters included temperature and pH,[10] the nature of the substrate,[13] and enzyme systems (pure isozymes versus cellulase mixtures).[9,12] One significant contribution among these is the study by Turon et al.,[12] who modeled the dynamics of events as a cellulose film was hydrolyzed by a fungal cellulase solution in a QCM chamber. In this work the frequency−time profile was fitted to a two-part function to describe an initial drop in resonance frequency followed by an increase in frequency that occurs in a typical incubation experiment. However, in this and other reports the complicating contributions from changes in viscosity and density of the fluid were assumed to play a negligible role compared to changes brought about by variations in substrate mass. On the other hand, our recent work[24] associated with methods to measure enzyme activity based on changes in viscosity and/or density of the medium relied on such contributions to obtain the enzyme activity from the shift of frequency in a sensor in contact with the incubating solution only. This work showed that contributions from solution viscosity and density can play a measurable role in the net frequency that can be measured. Nevertheless, the question still remains as to what extent the fluid properties affect the frequency signal while enzyme adsorption and substrate degradation occur simultaneously.

Other approaches such as those used in refs 24 and 27 are based on measurements of changes in solution viscosity or density, which relaxes the demand of using a thin film of cellulose on the quartz crystal sensors. In these cases other limitations exist such as the inability to obtain real-time data and the need to separate the solid substrate from the enzyme (protein) and sugar fractions. Therefore, if results from the two main QCM techniques are to be compared, understanding to what extent the properties of cellulase solution affect the measured frequency shift becomes pertinent.

Thus, in this work the effects of solution viscosity and density on the frequency shift relevant to protocols that rely on thin films of cellulose[12,13] were investigated. A pseudo-Langmuir model was fitted to the maximum QCM frequency decreases at different cellulase loadings. Additionally, a dose−response model was applied to describe the dynamics of the hydrolysis process. The maximum hydrolysis rate was obtained by analyzing the time-course response of the QCM frequency. The effect of temperature was also determined by using this model. From these results the activation energy of cellulose hydrolysis was obtained by using the maximum rate constant from the dose−response model in combination with the Arrhenius equation applied to experiments at various temperatures.

## 2. Experimental Section

**2.1. Materials.** Microcrystalline cellulose (MCC, AvicelPH-101) was purchased from Fluka. *N*-Methylmorpholine *N*-oxide (NMMO) and dimethyl sulfoxide (DMSO) were supplied by Aldrich. Polyvinylacrylamine (PVAm) was supplied by BASF. DyAdic EXP Cellulase, with a protein content 520 ± 28.7 mg/g (BSA equivalent) and FPA activity of 417 ± 53.0 U based on per gram of crude mixture, was supplied by DyAdic (Jupiter, FL). The protein content was determined by following the Lowry procedure,[25] and the FPA activity was determined by IUPAC method.[26] This cellulase was a yellow powder and was dissolved in buffer solution before use. Another commercial cellulase (Celluclast) was purchased from Sigma Aldrich. This cellulase was produced from *Trichoderma reesei*. The Modified Lowry Assay Kit was supplied by Pierce (Rockford, IL). Glacial acetic acid and sodium acetate were used in the preparation of buffer solutions (pH 4.8, ionic strength 100 mM). Crystal sensors coated with silicon dioxide (QSX303) were purchased from Q-Sense (Baltimore, MD). A Milli-Q Gradient system was the source of all the water (resistivity >18 MΩ) used in all experiments. Both Q-Sense D-300 and Q-Sense E-4 were from Q-Sense.

**2.2. Preparation of Cellulose Film on Sensor Disks.** The quartz crystal sensor disks were cleaned by an ultraviolet ozone treatment (UVO) for 10 min followed by soaking in a sodium dodecyl sulfate solution for 30 min. An immersion for 10 min in polyvinylamine (PVAm, 100 ppm) solutions was used to precoat the sensors. After rinsing with water, the excess solution was removed by drying with a gentle jet of filtered air, and then the crystal sensors were dried in an oven at 40 °C for 20 min. Microcrystalline cellulose (MCC) was dissolved in NMMO at a concentration of 2% at 115 °C. Care was taken that the temperature did not exceed 130 °C. A small amount of DMSO was carefully added to improve the dispersion, viscosity, and solubility. Additional DMSO was added so that the final concentration of cellulose in the solution was 0.5%. The cellulose solution was then spin-coated on the PVAm-coated resonator using a spin-coater (Laurell Technologies Model WS-400A-6NPP) at 5000 rpm for 40 s.[21] The resulting cellulose-coated resonators were heated at 80 °C for 2 h, followed by washing with Milli-Q water for 1 h. The water was replaced with fresh water and the disks were soaked for another 3 h.

**2.3. Density and Viscosity of Cellulase Solution.** A 250 mL volumetric flask was used to measure the densities of cellulase solutions. The kinematic viscosities of the solutions were determined from the transit time of liquid through the two meniscuses in a Cannon-Fenske capillary viscometer (size 50, viscometer constant = 0.004 $mm^2/s^2$). Dynamic viscosities were calculated using the following equation:

$$\eta = \nu\rho \tag{3}$$

where $\eta$ is the dynamic viscosity in N s/$m^2$, $\nu$ is the kinematic viscosity in $m^2/s$, and $\rho$ is the density in kg/$m^3$. The calculation of the sensor's frequency change due to variations in density and viscosity of the surrounding medium followed the procedure introduced elsewhere.[24]

**2.4. In Situ Monitoring of Cellulase Activity with QCM-D.** The QCM instrument used was a Q-Sense D300 from Q-Sense (Q-Sense AB, Gothenburg, Sweden). The instrument can monitor both the frequency and dissipation changes of the quartz resonator. During the measurement, the crystal sensor coated with a cellulose film was mounted in the QCM-D chamber, which is built with a temperature control system to provide a rapid exchange of the liquid in contact with one side of the sensor without any disturbance. Cellulose-coated resonators were conditioned with buffer in the QCM chamber and were hydrated until no appreciable frequency shifts were observed. After the stabilization, a cellulase solution was introduced and QCM was operated in a batch mode. All liquid samples were degassed and preheated to a temperature close to the desired temperature for the QCM chamber before they were

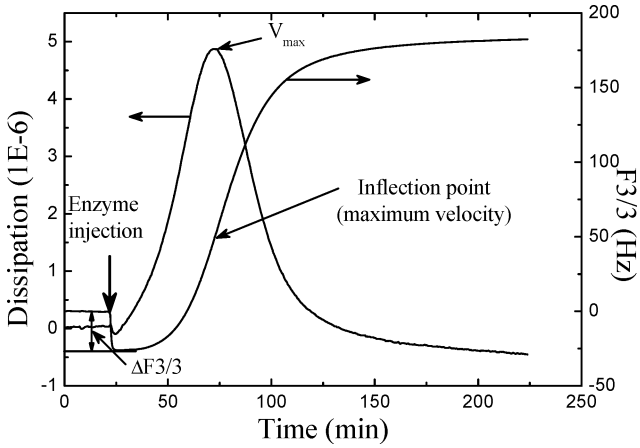


**Figure 1.** QCM-D frequency and dissipation change response in cellulose film hydrolysis by DyAdic EXP cellulase. Cellulose film was made from AvicelPH101 microcrystalline cellulose. Cellulase concentration: 0.15 mg/mL in pH 4.8 buffer having an ionic strength of 100 mM. The QCM-D was operated in a batch mode and the records shown here were based on the third overtone. The maximum velocity ($V_{max}$) is believed to occur when the dissipation curve is at or near its peak value, which corresponds to the inflection point in the frequency curve.

injected into the system. A syringe pump (Cole-Parmer 74900 series) was employed to inject the solutions into the QCM-D chamber at a flow rate of 0.1 mL/min. A 0.5 mL volume of cellulase solution was injected in each run. Experiments were terminated when a plateau in each respective run was observed. The Q-Sense D300 recorded frequency and dissipation responses at 5, 15, 25, and 35 MHz, which corresponds to the overtones $n = 1, 3, 5,$ and 7, respectively. In this report, the third overtone for frequency and dissipation were used for analysis.

**2.5. Measurement of Adsorption Reversibility.** A Q-Sense E4 (Q-Sense, Baltimore, MD) was used to check the specificity and reversibility of adsorption. A gold, a PVAm precoated, and two cellulose-coated quartz crystal sensors were used in the four cells of the E4. After hydration, as described in section 2.4, cellulase solution (DyAdic EXP cellulase, 0.25 mg/mL concentration) was introduced to the E4 chambers. This was followed by a continuous rinsing process for 60−90 min to check the binding affinity between cellulase enzymes and the substrates. Rinsing by injection of enzyme-free buffer solution was started at the point when the resonant frequency reached a minimum in the respective $\Delta f$−time profiles. The injection speeds for both the addition of cellulase solution and the rinsing buffer were controlled at 0.1 mL/min and no pronounced temporal and shear stresses were generated to disrupt the natural desorption due to the concentration gradient of cellullase. All four QCM chambers shared the same cellulase solution and rinsing buffer.

## 3. Results and Discussion

**3.1. Monitoring Cellulose Film Hydrolysis.** All cellulose films were hydrated in the QCM-D chamber until no significant frequency and dissipation changes were observed. Figure 1 shows a typical QCM-D response for cellulose film hydrolysis by cellulase solutions. The first frequency shift corresponds to the injection of cellulase solution into the QCM-D chamber thereby replacing the buffer solution. After a decrease of frequency for about 5 min, the frequency typically started to increase rapidly for about 2 h and then leveled off. The dissipation curve showed an increase until a maximum point was reached. After the maximum point, the dissipation started to decrease and finally leveled off as the frequency change attained a plateau value.

Many researchers have ascribed the initial decrease in frequency primarily to contributions from cellulase binding,[10−13] with the assumption that the frequency change due to the change of liquid properties would be minimal. As reported by Stockbridge[18] and Kanazawa,[15,17] the physical properties of the liquid medium can affect the change in resonance frequency. Further developments have confirmed these effects from liquid properties in the absence of cellulose films on quartz crystal sensors.[24,27] Based on these previous investigations, the frequency shift in response to the cellulase injection can be expected to be the result from contributions from both cellulase adsorption (binding) and changes in liquid properties (density and viscosity). There are three different ways in which the liquid properties can be changed: introduction of cellulase solution, adsorption of cellulase to the substrate, and release of hydrolysis products from the cellulose film. In this work, we have attempted to build on previous work by investigating the significance of liquid properties during in situ measurement of cellulose hydrolysis.[12,13,24,27] To this end, experiments were conducted to measure the cellulase interaction with gold surface, PVAm surface, and cellulose thin film surface, by which the adsorption reversibility and specificity may be observed (see Figure 2).

The dissipation curve indicates changes in the viscoelasticity of the cellulose film bound to the quartz crystal sensor used in the QCM-D. It can be expected that the increase of dissipation energy is caused by the adsorption and penetration of cellulase enzyme onto and into the cellulase film. As illustrated in Figure 1, appreciable cellulase film degradation occurred after the frequency drop, which was followed by a large increase of frequency. It can be hypothesized that several different physical and/or chemical phenomena occurred at this stage. These include the cellulase adsorption on the cellulose film, the penetration of cellulase into the different regions of the cellulose film, and the release of soluble products during cellulose hydrolysis. Cellulase enzyme did not have a complete interaction until the substrate films became highly viscous, which is indicated by the peak point of the dissipation curve in Figure 1. At this time, the hydrolysis of the cellulose film reached its maximum rate, as can be interpreted from the inflection point in the frequency curve in Figure 1, where the frequency curve has its maximum slope. After this time (about 75 min), the hydrolysis of the cellulose film began to slow down, which can be observed from the decrease of the slop of the frequency curve in Figure 1. This rate decrease was caused by the depletion of available substrates as the hydrolysis continued.

It should be pointed out that in these circumstances QCM-D may be less valuable than expected in exploring the Michaelis−Menten kinetics, which are broadly used to describe the relationship between the initial velocity and the soluble substrate concentration in biological reactions.[28,29] There are various limitations that may prevent this application. The first difficulty is in describing the soluble substrate concentration in the QCM-D setting. Films produced using the spin-coating technique can have different thicknesses, porosities, and surface roughnesses as has been reported by various researchers.[19,30−32] The modeling of the relationship between surface roughness and the amount of cellulose substrate deposited on the quartz crystal sensor is one practical issue that should be addressed in order to study Michaelis−Menten kinetics. A further difficulty is that the maxium hydrolysis rate in the QCM setting (Figure 1) is not at the beginning of the enzymatic reaction, compared to the hydrolysis rates obtained for the Michaelis−Menten relation

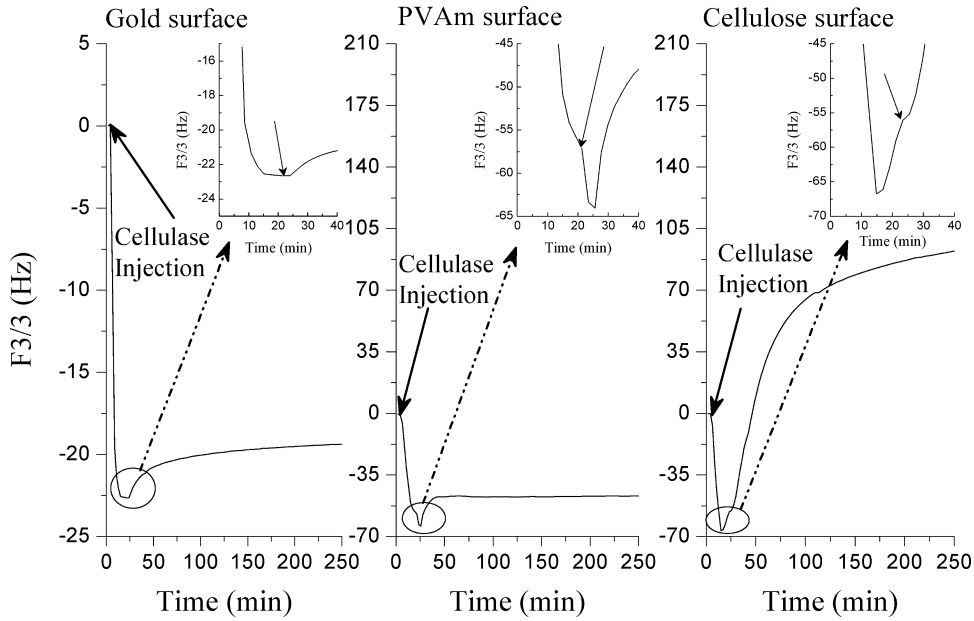


**Figure 2.** Rinsing effect on the adsorption of cellulase enzymes on gold-coated, PVAm-coated, and cellulose-coated quartz crystal sensors. Rinsing was started after the frequency shifts passed their maximum frequency decreases, as indicated by the arrows in the insets. The left, middle, and right graphs correspond to the situations in which gold-coated (gold surface), PVAm-precoated (PVAm surface), and cellulose-coated (cellulose surface) quartz crystal sensors were used, respectively. The experiments were conducted at 25 °C with DyAdic EXP cellulase (0.25 mg/mL) in the Q-Sense E4. Both enzyme injection rate and buffer rinsing rates were controlled at 0.1 mL/min.

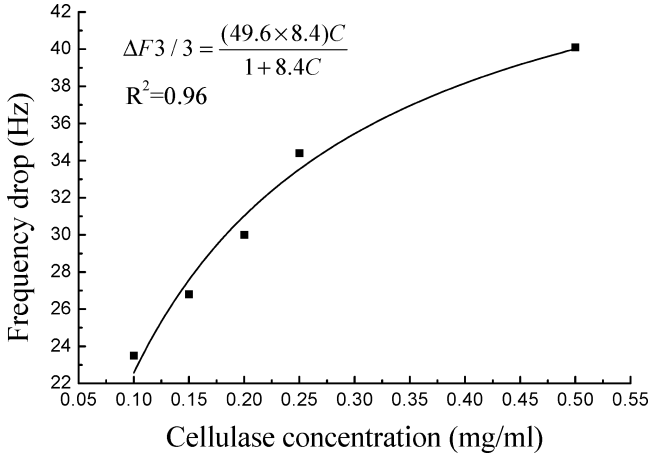


**Figure 3.** QCM-D frequency drop as a function of cellulase concentration at 25 °C. A Langmuir equation was employed to fit the frequency ($\Delta F_3/3$) and cellulase concentration ($C$). The cellulase was a DyAdic EXP Cellulase. Cellulose films were used at each cellulase charge. The fitted equation was $\Delta F_3/3 = [(49.6)(8.4)C]/(1 + 8.4C)$ with a correlation coefficient of 0.96. The maximum possible frequency drop is 49.6 Hz with a quasi-equilibrium constant of 8.4 mL/mg.

using soluble substrates. Furthermore, as pointed out by Hu et al.,[24] the morphology of the native cellulose is altered in preparation of cellulose film; amorphous cellulose may form using the DMAc/LiCl procedure,[33,34] whereas the NMMO/DMSO solutions produce cellulose II.[19]

**3.2. Cellulase Adsorption and Liquid Properties of Cellulase Solutions.** In an effort to decouple the contribution of hydrolysis on the frequency signal, experiments on enzyme adsorption and desorption were conducted with noncellulosic substrates and then compared to observations made on thin films of cellulose. The substrates consisted of gold and gold coated with PVAm, which was the polymer used to bind cellulose to the gold-coated sensor (see section 2.5). After hydration of the cellulose films, cellulase solutions were injected in each of the four channels of the QCM by using a peristaltic pump at 0.1 mL/min (Figure 2). Rinsing with buffer solution was initiated at about 23 min, which was after the frequency drop passed its minimum value for gold and cellulose surfaces, as indicated by the arrows in the insets in Figure 2. It was observed that the gold and cellulose surfaces reached their minimum resonance frequencies at about 15 min, −22.5 Hz for the gold surface and −66.7 Hz for the cellulose surface. However, the minimum resonance frequency of −64.5 Hz for the PVAm surface was not reached until about 25 min (insets in Figure 2), which was about 2 min after rinsing was started. After rinsing began, the resonance frequencies for the gold and PVAm surfaces started to rapidly increase and then leveled off with the continuation of rinsing. Resonance frequencies reached a plateau at ca. −18.9 and −47.5 Hz for gold and PVAm surfaces, respectively. Since the resonance frequency shifts due to the change in the physical properties of enzyme solutions were minimal (see the following), these two figures show that 83% (18.9/22.5 = 83%) and 74% (47.5/64.5 = 74%) of the initial adsorbed enzymes remained on the gold and PVAm surfaces after rinsing, assuming that cellulase adsorption is proportional to the resonance frequency drop in both cases. It may be hypothesized that the cellulase adsorption on gold and PVAm surfaces was caused by electrostatic interactions brought about by the zwitterionic properties of cellulase proteins. It can also be speculated that the higher adsorption on PVAm surfaces was caused by the larger cationic charge density of this substrate.

Cellulase adsorption on the cellulose film showed a different trend, in which no appreciable desorption with the rinsing could be observed. Arguably, it can be proposed that this effect is due to the confounding hydrolysis events that occurred on the cellulose film. It is therefore not clear how much of the cellulase adsorbed on the cellulose film was removed, if any. However, it is expected that a large amount was still adsorbed on the film, which produced the subsequent hydrolysis of the cellulose film, as indicated by the rapid increase of resonance frequency (Figure 2). It can be expected that the adsorption mechanism for cellulase adsorption on the cellulose surface is different from

**TABLE 1: Density, Viscosity, and Calculated Frequency Change Using the Stockbridge Equation[a,b]**

| | buffer | | | 0.5 mg/mL cellulase soln | | | |
|---|---|---|---|---|---|---|---|
| temp (°C) | $\rho$ (kg/m$^3$) | $\eta$ ($10^{-3}$ N s m$^{-2}$) | $\Delta f_1$ (Hz) | $\rho$ (kg/m$^3$) | $\eta$ ($10^{-3}$ N s m$^{-2}$) | $\Delta f_2$ (Hz) | $\Delta f^*$ (Hz) |
| 25 | 999.562 | 0.991 | −434.52 | 999.867 | 0.992 | −434.89 | −0.37 |
| 32 | 996.880 | 0.820 | −394.73 | 997.400 | 0.823 | −395.55 | −0.82 |
| 40 | 993.938 | 0.671 | −356.54 | 994.950 | 0.684 | −360.16 | −3.62 |

[a] Cellulase concentration was 0.5 mg/mL. [b] $\rho$ = density; $\eta$ = dynamic viscosity; $\Delta f_1$ and $\Delta f_2$ = frequency change from Stockbridge equation; $\Delta f^* = \Delta f_2 - \Delta f_1$.

those for cellulase adsorption on gold and PVAm surfaces. In the first case, the cellulase binding domain may play an essential role as reported elsewhere.[35] Additionally, the high amount of cellulase adsorption on the cellulose film (minimum frequency was −66.8 Hz), compared to that for gold and PVAm surfaces, could also be partially due to the higher surface area (higher roughness) of the spin-coated cellulose film. Finally, the interaction of the cellulase enzyme with gold and PVAm surfaces showed that the binding was irreversible.

The contribution of the physical properties of cellulase solutions to the frequency shifts were investigated by using the Stockbridge equation[18] in which experimentally measured liquid densities and viscosities were used. The densities and viscosities were determined at three different temperatures (25, 32, and 40 °C) and are shown in Table 1. It can be seen that both density and dynamic viscosity decreased for the buffer and the 0.5 mg/mL cellulase solutions as the temperature increased. Also, the viscosities and densities of the cellulase solutions were greater than those for the buffer solutions in which the cellulase solutions were prepared. $\Delta f_1$ and $\Delta f_2$ show the frequency decreases caused by the buffer and cellulase solutions with respect to air. The calculation is illustrated elsewhere.[24] $\Delta f^*$ was calculated by subtracting $\Delta f_1$ from $\Delta f_2$, which indicates the frequency shift due to change in the physical properties of enzyme solutions with respect to those of the buffer solution in which they were prepared. Table 1 shows that the physical properties of cellulase solutions caused a greater frequency drop at higher temperatures, but these changes were much lower than those observed during the experiments described before for cellulase adsorption (Figure 2). The actual contribution of cellulase solution to frequency shift is smaller than the calculated values in Table 1 because cellulase adsorption reduces the cellulase concentration and thus brings the liquid properties back closer to the buffer solutions in which the cellulase solutions were prepared. This confirms the assumption that the effect of solution properties on frequency drops are minimal, as stated in the works of Turon et al.[12] and Ahola et al.[13]

**3.3. Effect of Cellulase Concentration.** The normalized frequency drop ($\Delta F_3/3$) similar to that indicated in Figure 1 was obtained for five different cellulase concentrations at 25 °C. Figure 3 shows the relationship of maximum frequency drop to cellulase concentrations measured on cellulose-coated quartz crystal sensors. A Langmuir model was used to fit the frequency drop to the corresponding concentrations. The generated model has a maximum frequency drop of 49.6 Hz and quasi-equilibrium constant of 8.4 mL/mg, which can be obtained from the fit equation $\Delta F_3/3 = [(49.6)(8.4)C]/(1 + 8.4C)$, as also presented in Figure 3; the correlation coefficient is 0.96, which indicates a fairly good fit. As pointed out elsewhere,[13] the two parameters, $A_{max}$ and $K_p$, in a Langmuir isotherm, indicate the maximum adsorption and adsorption at certain conditions. The maximum frequency drop in this report is directly related to the cellulase adsorption (section 3.2). A rough estimation using the Sauerbrey equation (eq 1) gives a maximum adsorption of 0.3 $\mu$g. Much research has determined the maximum cellulase adsorption on cellulose substrates,[36−38] but it would be difficult to compare these results with others' due to the different techniques employed for parameter determination and because of the differences in substrate morphology, such as thickness, porosity, roughness, and crystallinity.

According to the cellulase−substrate complex hypothesis, the adsorption constant can indicate how much cellulase enzyme adsorbs on the substrate at the equilibrium state.[16,38] The adsorption constant shows that only about 0.8% of the cellulase adsorbed on the substrate upon adsorption equilibrium. This result is in agreement with the maximum adsorption as reported above. A comparison of these results with those found in the literature is difficult due to the different substrate morphologies and techniques employed. This is probably the first work that can be used to determine the maximum adsorption and equilibrium constant in a QCM setting. It should be noted that the continuous slow addition of cellulase solution may have affected the equilibrium process due to the continuous change of concentration until the end of addition of cellulase solutions. The enzymatic hydrolysis becomes more complicated to interpret in this setting. However, the results did support that cellulase adsorption was an appreciable process in cellulose hydrolysis as reported previously.[35]

**3.4. Hydrolysis Kinetics.** A dose−response model was adopted to investigate the hydrolysis kinetics. Equation 4 shows a typical dose−response equation.

$$F_3/3 = A_1 + \frac{A_2 - A_1}{1 + 10^{P(\log t_0 - t)}} \quad (4)$$

where $F_3/3$ is the dependent variable indicating the frequency change during cellulose film hydrolysis and $t$ is the time. $A_1$ is the bottom asymptote, $A_2$ is the top asymptote, $P$ is the hill slope, and log $t_0$ is a value corresponding to the time required to reach $(A_1 + A_2)/2$. Figure 4 shows a sigmoidal dose−response fitting of frequency response to time. The different parameters for different cellulase concentrations are listed in Table 3. Each fitting has an adjusted correlation coefficient greater than 0.99, which indicates that the dose−response model can model the frequency−time course data in the QCM fairly well.

The bottom asymptote $A_1$ can be interpreted as the maximum frequency drop caused by the adsorption of cellulases, which can be a net effect that includes the frequency shift due to the liquid properties, including viscosity and density. We expect that in most cases the contribution from adsorption is larger than that from the liquid property changes. This adsorption depletes the cellulase content in the solutions and therefore reduces the change of liquid density and viscosity. In this investigation, the maximum frequency drops, as that indicated in Figure 3, have the same trend as the ones shown in Table 2 for $A_1$.

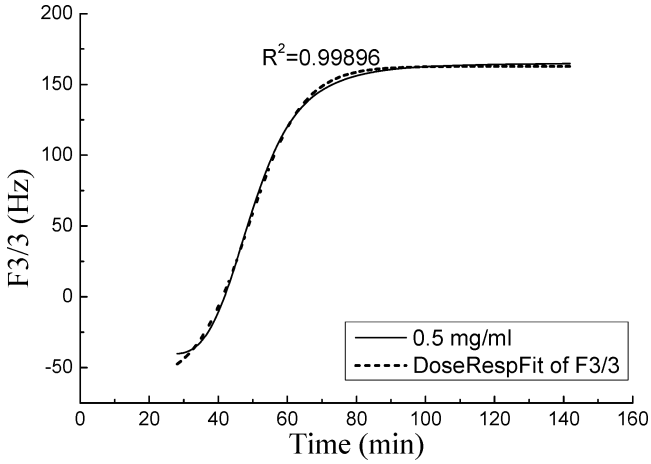


**Figure 4.** Dose−response fit of $F_3/3$ to time in cellulose film hydrolysis by 0.5 mg/mL DyAdic EXP cellulase solution at 25 °C. The fitting was generated using Origin and has an adjusted $R^2 = 0.999\,73$. The fitting parameters are $A_1 = -61.9$ Hz, $A_2 = 162.7$ Hz, log $t_0 = 48.8$ min, and hill slope $P = 0.56$ Hz$^{-1}$, respectively.

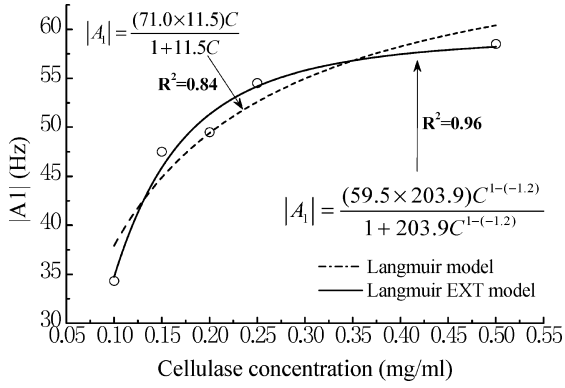


**Figure 5.** Plot of $|A_1|$ as a function of cellulase DyAdic EXP concentrations. The Langmuir model and Langmuir extension model were used to fit these data respectively. A Langmuir model produces a fit equation $|A_1| = [(71.0)(11.5)C]/(1 + 11.5C)$ with a correlation coefficient of 0.84, whereas a Langmuir extension model generates a fit equation $|A_1| = [(59.5)(203.9)C^{1-(-1.2)}]/(1 + 203.9C^{1-(-1.2)})$ with a correlation coefficient of 0.96.

**TABLE 2: Dose−Response Parameter Summary for Cellulose Film Hydrolysis by DyAdic EXP Cellulase Solutions at 25 °C**

| | cellulase concentration (mg/mL) | | | | |
|---|---|---|---|---|---|
| parameters | 0.10 | 0.15 | 0.20 | 0.25 | 0.50 |
| $A_1$ (Hz) | −30.0 | −47.5 | −53.6 | −54.9 | −61.9 |
| $A_2$ (Hz) | 166.9 | 159.0 | 213.9 | 139.7 | 162.7 |
| log $t_0$ (min) | 77.2 | 93.9 | 66.5 | 146.5 | 48.8 |
| $P$ (Hz$^{-1}$) | 0.040 | 0.013 | 0.046 | 0.0077 | 0.056 |
| adjusted $R^2$ | 0.999 73 | 0.999 98 | 0.999 27 | 0.999 84 | 0.998 96 |

The top asymptote $A_2$ can be considered to indicate the maximum hydrolyzable substrate mass on the quartz crystal sensors, which may be related to the film thickness. Different $A_2$ values indicate that the cellulose films produced in spin coating varies in film thickness and hydrolyzable masses.

Figure 5 shows the fit of $|A_1|$ as a function of cellulase concentration. The two Langmuir fits (Figures 3 and 5) show that QCM-D data can be fitted to the Langmuir isotherm. A Langmuir extension model gives a better fit in Figure 5, but the parameter interpretation would be different. A higher power of the concentration effect and greater adsorption constant (203.9 mL$^{2.2}$/mg$^{2.2}$) is observed in the Langmuir extension model fitting.

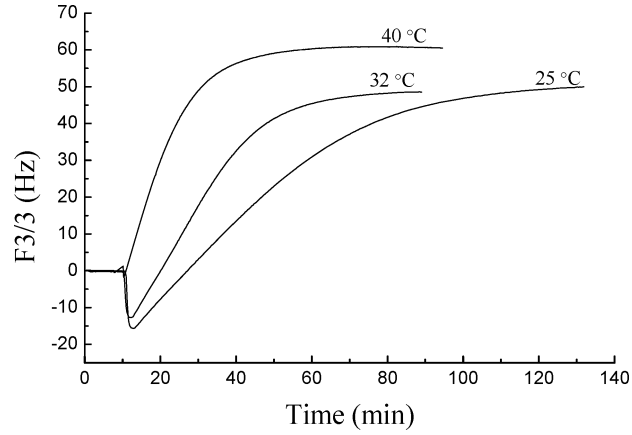


**Figure 6.** QCM-D frequency response for cellulose film hydrolysis at three different temperatures. Cellulase solutions used were from Celluclast from *T. reesei*; the concentration for cellulase solution was 500 ppm based on volume ratio from the as-received solution.

This implies that a faster saturation is achieved with an increased cellulase charge. A plausible hypothesis is that fewer binding sites can be found on the cellulose films than are expected from the Langmuir monolayer assumption. Because of the complex heterogeneous surface and the use of a mixture of various isozymes, some of which have binding domains, the good fit to the Langmuir model cannot be taken as evidence for compliance with the assumptions in the Langmuir model (monolayer adsorption, homogeneous surface energy contributions, and no lateral interactions).

The log $t_0$ varies with the running time and the data selected for model fitting. Extended experimental time increases the value for log $t_0$, especially as the frequency curve (Figure 1) gradually levels off. It is perhaps not practical to expect physical interpretation of these log $t_0$ values. $P$ is the hill slope in the dose−response model, which may used to estimate the maximum hydrolysis rate in cellulose film hydrolysis. Lynd et al. have reported that the fastest hydrolysis occurred at the beginning of cellulose hydrolysis.[39] However, their work was focused on extensive hydrolysis over a longer period of time. The QCM technique allows investigation of a very short time hydrolysis rate; it has been seen that there is an increase of hydrolysis rate in the first minutes of the reaction.[12,13] Turon et al. has explained that this is a complex process which includes the swelling of the cellulose film, the penetration of cellulase into the film substructure, and the increased accessibility of the cellulose surface to enzyme attack. The cellulase−cellulose interaction would be maximized only at the time when the accessible area reached its maximum value, which corresponds to its maximum hydrolysis rate.[12]

**3.5. Temperature Effect.** The temperature effect was investigated using a cellulase enzyme from *T. reesei* (Celluclast). Figure 6 shows the QCM-D frequency response at three different temperatures. It is apparent from the curve slopes that higher temperature produced faster hydrolysis rates. Table 3 shows the dose−response model parameters for frequency response curves obtained by fitting the dose−response model for Figure 6 (fitting figure not shown). It can be observed that higher temperature corresponds to higher $P$ values.

As indicated in Figure 6, the initial frequency drop became smaller as the temperature increased. However, the fitted $A_1$ shows an opposite trend, with higher temperature showing greater maximum frequency drop. This may also indicate that adsorption is not the only factor that affects the frequency shift at the initial stage when cellulase was injected, although it may

**TABLE 3: Dose−Response Parameters for Cellulose Film Hydrolysis by *T. reesei* Cellulase at 25, 32, and 40°C in QCM-D Chamber[a]**

| | temperature (°C) | | |
|---|---|---|---|
| parameters | 25 | 32 | 40 |
| $A_1$ (Hz) | −27.1 | −33.2 | −81.9 |
| $A_2$ (Hz) | 49.8 | 48.7 | 60.8 |
| log $t_0$ (min) | 38.8 | 24.5 | 8.3 |
| $P$ ($min^{-1}$) | 0.0232 | 0.0397 | 0.0478 |
| adjusted $R^2$ | 0.993 37 | 0.999 96 | 0.999 87 |

[a] Determined from frequency response curves by using the dose−response model.

be the leading factor. It appears that $A_1$ may not be a good indicator of initial adsorption at different temperatures. $A_2$ can be used to estimate the substrate mass on the quartz crystal sensors under the premise that the whole cellulose film was completely degraded, which is indicated by the leveling off of the frequency response curve from the QCM. This was in general agreement with the observations we obtained during the spin-coating process. Variation in cellulose film mass can be reflected in this parameter. For example, the thinner films came from less concentrated solutions, which produced a lower $A_2$ value. Lower amounts of cellulose solutions applied (1 drop vs 2 drops) can also cause differences in mass deposited on the quartz crystal resonator. A comparison of the hill slope $P$ values shows an increasing maximum hydrolysis rate as temperature rose. This temperature effect is in agreement with various reports in the literature.[13,40−42]

**3.6. Activation Energy of Cellulose Hydrolysis by Celluclast.** The $P$ values in Table 3 were treated as the maximum hydrolysis rate constant. By assuming first-order kinetics with respect to cellulase concentration, the apparent activation energy was calculated to be 37 kJ/mol for Celluclast cellulase to hydrolyze the cellulose film in this research. This was calculated by using the simple Arrhenius equation:

$$k = A \exp\left(-\frac{E_a}{RT}\right) \quad (5)$$

where $k$ is the rate constant, $A$ is a preexponential factor, and $E_a$ is the activation energy. The linear plot of ln $k$ against $1/T$ rendered a correlation coefficient of 0.83, which indicates a relatively poor fit (figure not shown). Due to the complexity of components in the cellulase mixtures, this is not a true activation energy but an overall or apparent activation energy. The value is lower than that obtained by He et al., who reported that the activation energy was 52 kJ/mol for enzymatic hydrolysis of CMC.[27] This difference may result from the different enzymes, substrates, and reaction systems used. Banka et al. reported that the activation energy of a low molecular weight fibril-forming protein purified from *T. reesei* was 4.18 kcal/mol (17.5 kJ/mol) in a filter paper disruption process.[43] The higher activation energy in this research was more related to hydrolysis reaction, in which covalent bonds are broken, while in the work by Banka et al. the disruption was interpreted to be physical and no reducing sugars were produced.[43]

## 4. Conclusion

The quartz crystal microbalance technique with dissipation monitoring was used to investigate the kinetic behavior of cellulose hydrolysis by cellulase enzymes. The kinetic data were fitted to a dose−response model. The model parameters were used to obtain kinetic information such as the maximum hydrolysis rates and substrate information such as the maximum hydrolyzable film mass on the crystal resonators. The maximum frequency drops at the initial stage in cellulase solution injection were fitted to a Langmuir model with their respective cellulase charges. Parameters in this model can be used to estimate the interfacial adsorption and saturation of adsorption. The contribution of liquid properties, i.e., density and viscosity of cellulase solutions, to frequency shift was also studied. While decoupling these effects from each is a difficult task, the evidence clearly indicated that the solution properties had a minimal effect in determining the maximum frequency drop after cellulase injection (at least at the employed cellulase concentrations which were lower than 0.5 mg/mL). By using the hydrolysis parameters from the dose−response model, the activation energy of enzymatic hydrolysis by Celluclast was estimated to be 37 kJ/mol. The dose−response model can be employed to accurately model cellulose film hydrolysis kinetics by cellulase enzymes as indicated by results from two different cellulase systems, the Langmuir data, and the activation energy calculations.

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JP907155V