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Enzymatic digestion of partially and fully regenerated cellulose model films from trimethylsilyl cellulose

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ABSTRACT

Partially and fully regenerated cellulose model films from trimethylsilyl cellulose (TMSC) were prepared by a time dependent regeneration approach. These thin films were characterized with contact angle measurements and attenuated total reflectance infrared spectroscopy (ATR-IR). In order to get further insights into the completeness of the regeneration we studied the interaction of cellulase enzymes from *Trichoderma viride* with the cellulose films using a quartz crystal microbalance with dissipation (QCM-D). To support the results from the QCM-D experiments capillary zone electrophoresis (CZE) and atomic force microscopy (AFM) were applied. The changes in mass and energy dissipation due to the interaction of the enzymes with the substrates were correlated with the surface wettability and elemental composition of the regenerated films. The highest interaction activity between the films and the enzyme, as well as the highest cellulose degradation, was observed on fully regenerated cellulose films, but some degradation also occurred on pure TMSC films. The enzymatic degradation rate correlated well with the rate of regeneration. It was demonstrated that CZE can be used to support QCM-D data via the detection of enzyme hydrolysis products in the eluates of the QCM-D cells. Glucose release peaked at the same time as the maximum mass loss was detected via QCM-D. It was shown that a combination of QCM-D and CZE together with enzymatic digestion is a reliable method to determine the conversion rate of TMSC to cellulose. In addition QCM-D and AFM revealed that cellulase is irreversibly bound to hydrophobic TMSC surfaces, while pure cellulose is digested almost completely in the course of hydrolysis.

1. Introduction

The surface properties of cellulose can be studied using well defined model substrates in the form of nanometric thin films. Commonly used methods for the preparation of these films are spin coating or Langmuir–Blodgett film forming of cellulose suspensions or derivatives (Ahola, Salmi, Johansson, Laine, & Österberg, 2008; Ahola, Turon, Österberg, Laine, & Rojas, 2008; Geffroy, Labeau, Wong, Cabane, & Cohen Stuart, 2000; Gunnars, Wågberg, & Cohen Stuart, 2002; Holmberg et al., 1997; Kontturi & Lankinen, 2010; Schaub, Wenz, Wegner, Stein, & Klemm, 1993; Wegner, Buchholz, Stemme, & Ödberg, 1996). As cellulose derivative, trimethylsilyl cellulose (TMSC) is often used for substrate coatings or surface interaction studies (Findenig et al., 2012; Spirk et al., 2010). These thin TMSC films can be partially or completely regenerated back to pure cellulose by hydrochloric acid vapours which cleave the Si—O bonds under formation of trimethylsilyl chloride and further hydrolysis to trimethylsilanol (Spirk, Nieger, Belaj, & Pietschnig, 2009). In this case films with different wettabilities and silicon contents can be obtained by adjusting the regeneration parameters during the desilylation reaction (Mohan et al., 2011). The desilylation can conventionally be monitored using well established techniques such as attenuated total reflectance infrared spectroscopy (ATR-IR), X-ray photoelectron spectroscopy (XPS) (Kontturi, Thüne, & Niemantsverdriet, 2003a) or Raman spectroscopy (Woods, Petkov, & Bain, 2011). Besides the investigations of surface properties such as wettabilities or elemental compositions, model films were also used to study the enzymatic digestion of cellulose or cellulose derivatives from different origins (Jausovec, Angelescu, Voncina, Nylander, & Lindman, 2008; Wang, Wang, & Ragauskas, 2010). In particular, the quartz crystal microbalance with dissipation monitoring (QCM-D) as a nanogram sensitive balance (Rodahl, Höök, Krozer, Brzezinski, & Kasemo, 1995) in combination with well defined model films of cellulose, was used to study the adsorption properties and digestion rates of cellulase enzymes systematically (Cheng et al., 2011; Eriksson et al., 2005; Esaki et al., 2009; Suchy et al., 2011). A significant contribution to this was the study of Ahola et al., who investigated cellulase

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interaction with different spin coated model films including regenerated cellulose from TMSC (Ahola, Salmi, et al., 2008; Ahola, Turon, et al., 2008). Overall, the changes in frequency and dissipation were interpreted in terms of swelling, cellulase binding capacity, digestion rates, enzyme system, temperature, solution pH, and ionic strength (Fält, Wågberg, & Vesterlind, 2003; Hu, Heitmann, & Rojas, 2009a; Hu, Heitmann, & Rojas, 2009b; Josefsson, Henriksson, & Wågberg, 2008; Turon, Rojas, & Deinharmmer, 2008). These studies are important contributions to the basic understanding of the enzymatic digestion of cellulosic materials and for the utilization of renewable resources in general (Wilson, 2009). Alternatively enzymatic digestion could be used to study the surface or bulk properties of thin films of cellulose and its derivatives. A combination of digestion with cellulases and the QCM-D method could provide further insights into the regeneration process of the aforementioned TMSC and show whether a film is completely digestible or not. Such information is crucial if one aims to understand the regeneration process of TMSC and the properties of the derived cellulose thin films in general. In order to verify and support the enzymatic digestion experiments from QCM-D, analysis of the hydrolysis products can be performed (Hu et al., 2009a, 2009b; Johnston, Shoemaker, Smith, & Whitaker, 1998). For this analysis capillary zone electrophoresis (CZE) with photometric detection can be used to determine the concentration of monosaccharides in the enzyme hydrolysates (Dahlman, Jacobs, Liljenberg, & Olsson, 2000; Doliška et al., 2009). The combination of the enzymatic digestion with QCM-D and CZE in this study should provide a better understanding of the desilylation reaction and the structure of cellulose thin films that were regenerated from TMSC. It should further provide information about the digestibility of pure and partially desilylated TMSC films which is interesting in terms of the biodegradability of hydrophobic cellulose derivatives (Puls, Wilson, & Hölter, 2011). The aim of this work was therefore to prepare partially and fully regenerated cellulose films from trimethylsilyl cellulose (TMSC) using a time dependent regeneration approach. The desilylation is monitored by contact angle and ATR-IR measurements. The films are further subjected to enzyme solutions in order to study the adsorption properties and hydrolytic efficiency of cellulases from *Trichoderma viride*. These enzymatic digestions should provide details about the completeness of the desilylation reaction. As a complementary method CZE is used to analyse the glucose content in the enzyme hydrolysates. Morphological AFM investigations are further applied to elucidate the influence of enzyme adsorption and digestion on the film surfaces. This study should offer information about the digestibility of hydrophilic and hydrophobic cellulose films, and show that cellulase incubation in combination with QCM-D and CZE can be used to monitor the time dependent regeneration of TMSC to cellulose.

2. Materials and methods

2.1. Materials

Trimethylsilyl cellulose (TMSC), with a degree of substitution (DS) of 2.55 (M_w: 175.4 kDa) (kindly supplied by the Centre of Excellence for Polysaccharide Research, University of Jena) was used as the starting material for the cellulose model films preparation (Köhler, Liebert, & Heinze, 2008). Cellulase from *T. viride*, a yellow crude powder (3–10 units mg^{-1} solid), sodium acetate (p.a.), glacial acetic acid (98.5%) toluene (99.9%), 4-aminobenzoic acid ethyl ester (ABEE), sodium dodecyl sulphate, acetonitrile and sodium cyanoborohydride were purchased from Sigma–Aldrich (Germany) and used without purification. D (+) glucose, D (+) xylose, and sodium tetraborate decahydrate were purchased from Merck (Germany). QCM-D sensor crystals (QSX-301) were purchased from LOT-Oriel (Germany). A Milli-Q water system was the source of pure water (resistivity = 18.2 M Ω cm at 25 °C) used in all solutions and measurements. All enzyme solutions were prepared by dissolving 1 mg ml^{-1} cellulase in a 100 mM sodium acetate buffer at pH 4.8.

2.2. Model film preparation

QCM-D Au-crystals were used as substrates that were spin coated with TMSC films. The crystals were first soaked in a mixture of H_2O/H_2O_2 (30 wt.%)/NH_4OH (5:1:1; v/v/v) for 10 min at 70 °C, then immersed in a "piranha" solution containing H_2O_2 (30 wt.%)/H_2SO_4 (1:3; v/v) for 40 s, and then rinsed with water and finally blow dried with nitrogen. Silicon wafers supplied by Silchem (Germany), were cut into 1 cm × 1 cm pieces, cleaned with the "piranha" solution for 60 min, rinsed and stored in water for 15 min, rinsed with water again and blow dried with nitrogen. 50 μl of TMSC (1 wt.% solution; dissolved in toluene) was deposited on a static QCM-D Au-crystal, which was then rotated for 60 s at a spinning speed of 4000 rpm and an acceleration of 2500 rpm s^{-1}. This follows a modified procedure published by Kontturi, Thüne, and Niemantsverdriet (2003b) which can be found elsewhere. As was published recently and verified by optical thickness measurement, spin coating resulted in TMSC layers with a thickness of 68 nm whereas fully regenerated cellulose resulted in layers that were 25 nm thick (Mohan et al., 2011). Two methods were used to prepare the time dependent regeneration of cellulose films with gaseous HCl. In method I, the spin coated crystals were placed in 20 ml polystyrene petri-dishes containing 2 ml of 10 wt.% HCl. These dishes were covered with their caps, and the films were exposed to HCl vapours for different times (0, 1, 2, 4, 6, 10, and 12 min). The details of this procedure are published elsewhere (Mohan et al., 2011). In method II, the regeneration was performed in a higher volume of HCl vapours. 200 ml of 10 wt.% HCl were stabilized for 1 h in a 300 ml vacuum desiccator. The spin coated TMSC Au-crystals were kept in 20 ml polystyrene petri-dishes and were regenerated for either 1, 2, or 4 min by placing them into the desiccator. For contact angle measurements, all surfaces were prepared in the same way but on silicon wafers measuring 1 cm × 1 cm.

2.3. Contact angle measurements

Static contact angles (SCA) of water were measured using the Dataphysics contact angle measurement system OCA15+ (Dataphysics, Germany) with the sessile drop method and a drop volume of 3 μl. All measurements were carried out at room temperature on pure TMSC and regenerated surfaces. Determination of the SCA was based on the analysis of the drop shape and was performed with the software provided by the manufacturer (software version SCA 20.2.0). All the measurements were performed on at least two independent surfaces per regeneration time with a minimum of three drops per surface.

2.4. Infrared spectroscopy

Attenuated total reflectance infrared spectroscopy (ATR-IR) of partially and fully regenerated cellulose films was performed using a PerkinElmer Spectrum GX Series-73565 FTIR-spectrometer at a scan range of 4000–650 cm^{-1}. 32 scans were performed for each sample with a resolution of 4 cm^{-1}. QCM-D Au-crystals (QSX-301, LOT-Oriel, Germany) were used as base substrates for the ATR-IR measurements.

2.5. Enzyme interaction studies with a quartz crystal microbalance

A QCM-D instrument (model E4) from Q-Sense, Gothenburg, Sweden was used. The instrument simultaneously measures

changes in the resonance frequency (Δf) and energy dissipation (ΔD) when the mass of an oscillating piezoelectric crystal changes upon adsorption on the crystal surface (Fält et al., 2003; Hu et al., 2009a, 2009b; Josefsson et al., 2008; Turon et al., 2008). A decrease in resonance frequency reflects a mass increase, whereas higher dissipation values are caused by a higher damping of the layer on the crystal surface. This damping can be attributed to a less rigid surface. A detailed description of the QCM-D method can be found elsewhere (Marx, 2003; Rodahl et al., 1997). For the data analysis in this study, the changes in the third overtone's frequency and dissipation (Δf_3, ΔD_3) were determined. The spin coated and regenerated films on Au-crystals were assembled in the QCM-D chamber. In each run, water (flow rate = 0.2 ml min^{-1}) was pumped into the chamber for 10 min to ensure the stability of the baseline frequency. The temperature was kept at 37 °C for the duration of the measurement. The film surfaces were then rinsed with a 100 mM sodium acetate buffer solution (pH 4.8, flow rate = 0.2 ml min^{-1}) until there was no observable change in the oscillation frequency. After that the buffer flow rate was reduced to 0.1 ml min^{-1} and the data acquisition system was reset to zero. Following this, the cellulase enzyme solution was introduced into each chamber at a flow rate of 0.1 ml min^{-1}. All experiments were conducted in continuous flow mode, in which the film surfaces were constantly exposed to fresh enzyme solution. The spin coated films were allowed to interact with the cellulase solution until no pronounced changes in f_3 and D_3 were observed. The films were then rinsed again with the buffer solution (flow rate = 0.1 ml min^{-1}) until the measurement was stopped.

2.6. Capillary zone electrophoresis

CZE experiments were carried out using an Agilent CE3D Instrument G-1600 (Agilent, USA) equipped with a diode array detector. CZE is a reliable method for the quantification of monosaccharides in solution (Santos, Duarte, & Esteves, 2007). It can be used to determine the concentration of glucose within a short analysis time and with reliable reproducibility. The quantitative results obtained from CZE monomer analysis are comparable with those obtained from standard analysis methods such as ion-exchange chromatography (Doliška et al., 2009). Therefore CZE was used as a method to determine the amount of glucose in the eluates of the QCM-D cells. Untreated fused silica capillaries of 40 cm effective length and 48 cm total length with a 50 μm internal diameter were used. The running background electrolyte (BGE) for CZE was an aqueous solution of 0.1 M sodium tetraborate with 30% (v/v) acetonitrile as a modifier. Prior to experiments the capillary was rinsed first with 1 M NaOH for 20 min, after that with Milli-Q H_2O for 10 min and finally with BGE for 20 min. Before each run the capillary was preconditioned with 1 M NaOH for 2 min, with H_2O for 1 min and with BGE for 2 min. BGE, NaOH and H_2O were filtrated through PVDF (polyvinylidenfluoride) syringe filters (0.2 μm) before use. The samples were hydrodynamically injected at 50 mbar for 5 s, followed by a plug of BGE at 50 mbar for 2 s. A voltage of 25 kV was applied, the temperature was constant at 20 °C and UV absorbance was detected at 306 nm. By the use of an extended light path (bulb factor 3), the detection limit was decreased to 2×10^{-3} mg ml^{-1} with the same injection procedure (Doliška et al., 2009). For derivatization of the enzyme hydrolysates, a stock solution was prepared by dissolving ABEE (100 mg ml^{-1}) and acetic acid (100 mg ml^{-1}) in MeOH. The solution used for derivatization was prepared by adding 10 mg of $NaBH_3CN$ to 1 ml stock solution. 240 μl of this solution was added to 200 μl of the hydrolysis product and placed into a thermoblock (LAbEna) for 1 h at 80 °C. Then 200 μl of 0.1 M tetraborate buffer (pH 10.5) was added and the solutions were shaken vigorously, which caused precipitation of the excess ABEE. The samples were filtrated through 0.45 μm syringe filters (PVDF) prior to CZE analysis. A linear calibration was established with peak area versus concentration of the monosaccharide derivatives ranging from 50 to 150 mg l^{-1}. The samples of enzyme hydrolysates were collected at the outlet of the QCM-D flow module after the initial enzyme adsorption (5 min) and at various incubation times (15, 25, 35, 65, 95, and 125 min). The amount of glucose in the hydrolysate was normalized with respect to the QCM-D quartz crystal surface area (1.5394 cm^2) and expressed in ng cm^{-2} film area.

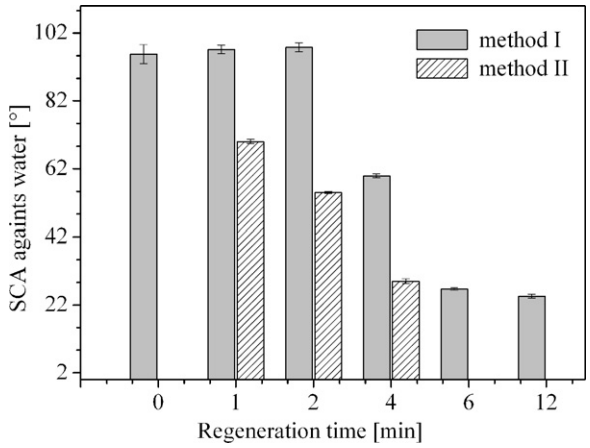

Fig. 1. Static contact angles (SCA) of water on partially and fully regenerated cellulose films. Two different methods of regeneration are shown. 2 ml of HCl (method I) and 200 ml of HCl (method II) were used to generate HCl vapours.

2.7. Atomic force microscopy (AFM)

The QCM-D crystals that were coated with partially and fully regenerated films were investigated by AFM before and after the digestion experiments in the QCM-D cells. The topographical features of the surfaces (before and after enzyme treatment) were characterized by AFM in tapping mode with an Agilent 5500 AFM multimode scanning probe microscope (Digital Instruments, Santa Barbara, CA, USA). The images were scanned using silicon cantilevers (ATEC-NC-20, Nanosensors, Germany) with a resonance frequency of 210–490 kHz and a force constant of 12–110 N m^{-1}. All measurements were performed at room temperature under ambient air. The samples were measured without any pre-treatment. For the determination of the root mean square roughness, an image size of 5 μm × 5 μm was taken.

3. Results and discussion

3.1. Surface properties of regenerated films

When method I was used for regeneration (a low volume of HCl) a water contact angle of 25 ± 0.6° and full regeneration from TMSC to cellulose were reached after 10–12 min exposure (Mohan et al., 2011). Using method II (a higher volume of HCl vapour), a low water contact angle (29 ± 0.7°) and a fully regenerated cellulose film were already obtained after 4 min of regeneration (see Fig. 1). Fig. 2 shows the ATR-IR spectra of the TMSC film and the regenerated cellulose films that were obtained with both methods. These spectra show that there is a good correlation between contact angle values and the amount of silicon that can be found in the films. In agreement with earlier studies, the spectra in Fig. 2 show that the peaks due to Si—C rocking at 757, 848, and 1252 cm^{-1} decrease concomitantly with the emergence of OH vibrations at 3000–3700 cm^{-1}. It is evident that method II cleaved more TMS groups, introduced more OH groups and improved wetting by water in shorter reaction times than method I. Nevertheless both methods allow the controlled

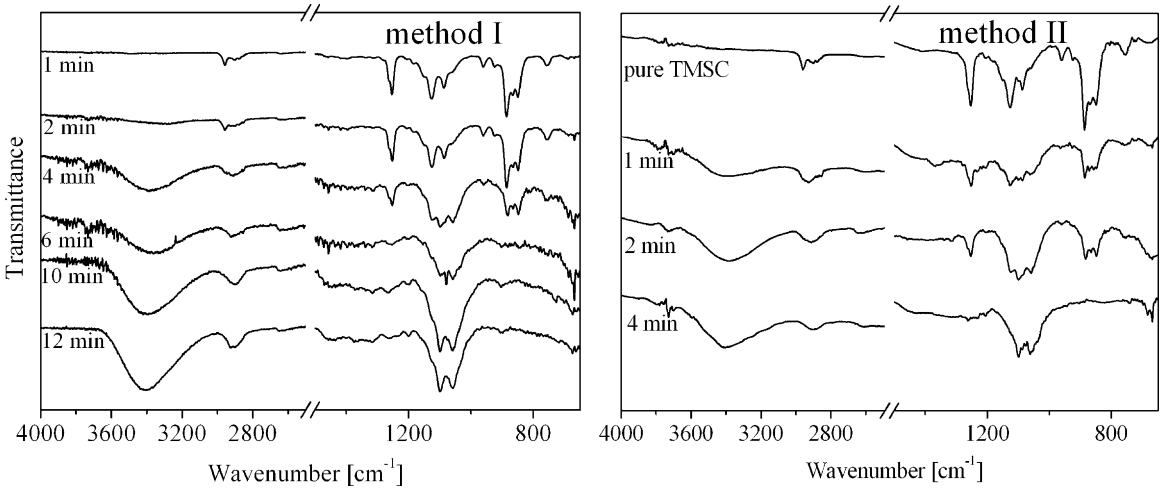

Fig. 2. ATR-IR spectra of spin coated TMSC films after different regeneration times. Left method I, right method II.

manufacturing of partially and fully regenerated cellulose model films from TMSC and can be used to study the interaction ability of cellulases on these surfaces. With the two different methods of regeneration we could show that the wettability of the films correlates well with their composition as revealed by ATR-IR measurements. Nevertheless in the following QCM-D studies, only films derived from method I were subjected to cellulase incubation. This method offered the possibility to gradually regenerate TMSC to cellulose and to produce a greater variety of films with different elemental compositions and wettabilities which can be subjected to enzyme interaction studies.

3.2. QCM-D studies

3.2.1. Enzyme interaction with hydrophobic TMSC films

The pure TMSC and films regenerated for shorter times (1 and 2 min using method I) were subjected to cellulase incubation in the QCM-D cell as described above. The results of this incubation are shown in Fig. 3 (left: Δf_3, right: ΔD_3). For all films cellulase was introduced 5 min after the stabilization of the baseline frequency. A constant frequency signal before the introduction of the enzyme solution also indicates that the films are stable under the chemical conditions (acetate buffer, pH 4.8) and are not hydrolyzed in pure buffer. This is also in agreement with other studies, where degradation only started after the application of the enzyme solutions (Ahola, Salmi, et al., 2008; Ahola, Turon, et al., 2008). As shown in the following sections, also partially and fully regenerated films are not degraded by pure buffer without the addition of enzyme.

After application of the enzyme solution the frequency immediately started to decrease until a steady state was reached. No increase in frequency was noted even after extensive rinsing with the buffer solution. The measured final changes in frequencies were −31 Hz (pure TMSC), −30 Hz (1 min), and −29 Hz (2 min). There was a slower decrease in the time dependent adsorbed mass, and a strong increase in dissipation when the regeneration time was increased. The results indicate that although there was no significant change in the contact angles of water (see Fig. 1), some changes did take place when the enzyme was adsorbed. A likely explanation is that some material was already hydrolyzed and released when the cellulase bound to the surface. The increase in dissipation could then be explained by a combination of adsorbed cellulase and its coupled water molecules with a simultaneous release of hydrolysis products from the cellulase activity (Hu et al., 2009a, 2009b). For the hydrophobic films, the mass loss during a probable hydrolysis is low compared to the amount of enzyme and water that are adsorbed on the model films. Therefore the possible onset of the enzymatic digestion of non regenerated films could not be detected through the observation of frequency changes in the QCM-D experiment.

Nevertheless the dissipation values show that there are differences in the characteristics of the films. The results from the QCM-D measurements for pure TMSC and shorter regeneration times show no frequency increase after rinsing the surface with the buffer solution, which confirms irreversible enzyme adsorption and a stable film. The binding of cellulase itself was probably due to non polar interactions since the surface energy of pure TMSC films is dominated by dispersive forces (Mohan et al., 2011). It seems that as long as the surface remained hydrophobic, hydrolysis was very slow, most probably due to steric hindrance caused by the association of the hydrophobic part of the enzyme with the TMS-groups of the TMSC film. Therefore even though from dissipation values the films seem different because of the onset of TMS-cleavage by HCl vapours, the main parts of films are not digestible by cellulase since it consists mainly of highly substituted TMSC.

3.2.2. Enzyme interaction with partially and fully regenerated films

Compared to hydrophobic TMSC films, the QCM-D frequency curves for films regenerated for 4–12 min using method I showed different behaviour (Fig. 4). A steep decrease in frequency was detected after incubation with cellulase. Before rapid hydrolysis, the largest negative frequency shifts, of about −25 Hz, were similar for all regenerated films. This frequency decrease can be attributed to the initial binding of cellulase to the surface as well as to the extended incorporation of water into more hydrophilic films (Ahola, Salmi, et al., 2008; Ahola, Turon, et al., 2008; Hu et al., 2009a, 2009b; Turon et al., 2008). Interestingly, the absolute values in frequency change during cellulase binding are very similar to that on pure TMSC. In this case it has to be considered that enzyme adsorption and cellulose hydrolysis are overlapping, and a higher rate of enzyme adsorption can be covered by a higher mass loss during hydrolysis. This mass loss can be observed after the initial binding of cellulase, when the frequency increased and levelled off after a certain time. The plateau of maximum frequency change was reached at approximately 200 and 248 Hz for 6 and 10 min regenerated films (Fig. 4; left). The film regenerated for 12 min showed the largest hydrolytic activity, and no frequency plateau was achieved within the experiment's duration (100 min). The hydrolytic activity on the 6, 10, and 12 min films, which are all hydrophilic (SCA = 24–26°), was markedly higher compared to the film regenerated for 4 min. The likely explanation is the partially hydrophobic structure of the

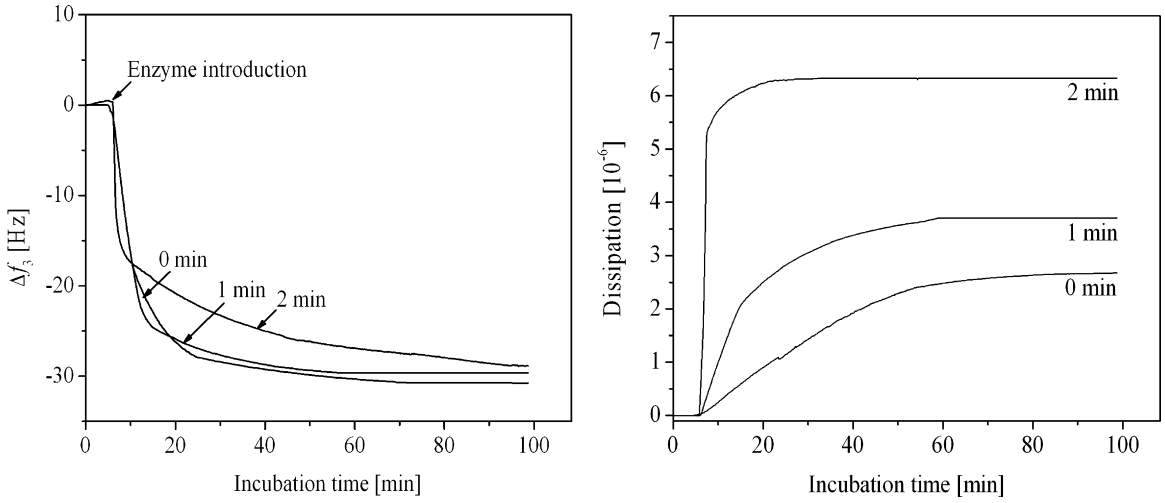

Fig. 3. QCM-D change in frequency and dissipation during cellulase incubation of pure TMSC (0 min) and films obtained from shorter regeneration times (1 and 2 min, method I).

4 min regenerated film, which makes them resistant to complete enzymatic hydrolysis. In conclusion, the more hydrophilic the film is, the stronger is the interaction with cellulase, and the larger the digestion rates are. This is a result of a completely regenerated cellulose film. Even though the contact angles of water levelled off after 6 min of regeneration, the combination of QCM-D and enzymatic digestion showed that the films regenerated for 6 min are not completely digestible by cellulases and, thus, are most likely not fully regenerated.

The changes observed in the energy dissipation for films regenerated for 4–12 min are shown in Fig. 4 (right). Initially, a sharp increase in dissipation was detected after the enzyme solution was applied. Similarly to pure TMSC this increase can be attributed to the coupled effect of adsorption and penetration by the enzyme and water into the accessible region of the cellulose surfaces. After longer incubation times dissipation decreased below the initial values and reached a plateau. Maximum dissipation occurred around the time when the hydrolysis rate started to slow down. These results clearly show that maximum dissipation increased markedly with increasing regeneration time. These higher dissipation changes indicate the loosening of a larger number of digestible cellulose chains. A reasonable explanation for the initial rapid increase in dissipation up to the maximum is that the cellulase binds to the surface of the regenerated cellulose and thus enabled extensive swelling, which resulted in a higher substrate concentration for the cellulase to act on (Hu et al., 2009a, 2009b; Turon et al., 2008). The decrease in dissipation energy after a peak can be attributed to the degradation of cellulose chains and desorption of the enzyme, which leaves behind a thin and rigid layer of indigestible higher substituted cellulose. The decrease in energy dissipation was less pronounced with shorter regeneration times indicating the presence of higher amounts of indigestible material.

3.2.3. Notes on the enzymatic activity

The commercial cellulase (*T. viride*) mixture contains six endoglucanases (endo I, II, etc.) and three exoglucanases (exo I, II, III). The exoglucanases act from either the reducing or the non-reducing end of the cellulose chain and produce a shortened chain and cellobiose (Beldman, Searle-Van Leeuwen, Rombouts, & Voragen, 1985). Endoglucanases cleave the internal β-1,4-glycosidic bonds of the cellulose chain randomly along its length and produce free chain ends that are acted upon by exoglucanases. The commercial cellulase also contains β-1,4-glycosidase, which hydrolyzes cellobiose units to glucose monomers.

The cellulase binding and subsequent enzymatic hydrolysis were evident from the measured changes in frequency and dissipation during the enzyme-substrate interaction. The frequency profiles (Fig. 4) showed several distinct features. Active binding of the cellulase enzyme occurred immediately after the enzyme's introduction. When sufficient enzyme had adsorbed and

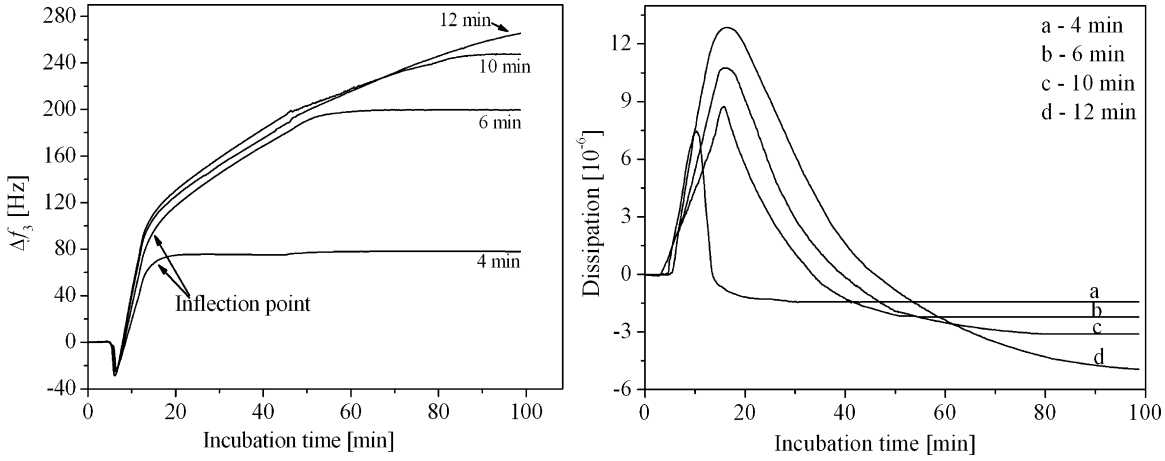

Fig. 4. QCM-D change in frequency and dissipation of longer RT (4, 6, 10, and 12 min, method I) incubated with 1 g l^{-1} cellulase.

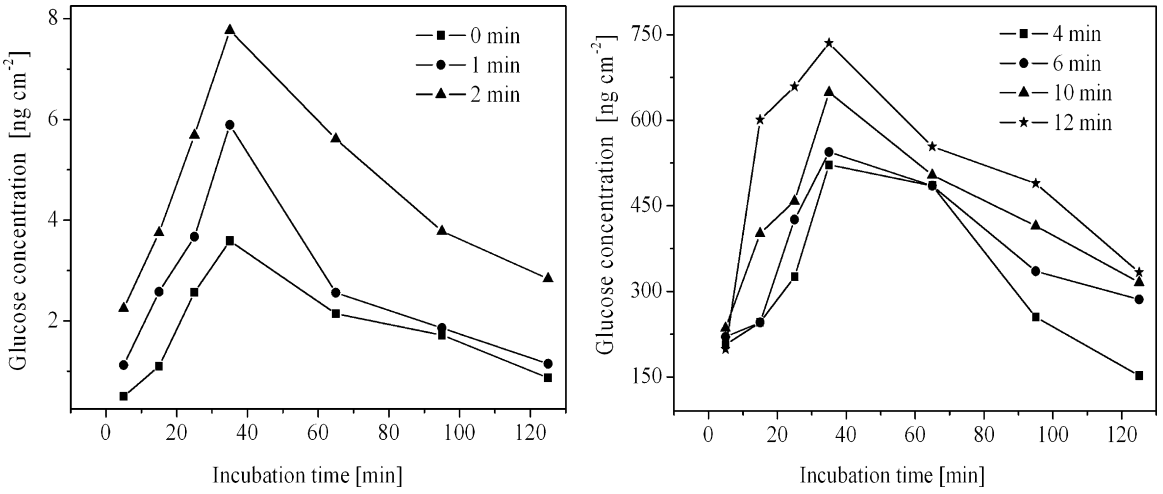

Fig. 5. Calculated amounts of glucose in the enzyme hydrolysates of films that were regenerated for different times (0–12 min) using method I.

penetrated the film, the hydrolysis of cellulose became rapid, which resulted in a change in the direction of the frequency shift due to the combined effect of enzyme binding and hydrolysis (Ahola, Salmi, et al., 2008; Ahola, Turon, et al., 2008; Hu et al., 2009a, 2009b; Turon et al., 2008). In this degradation state the enzyme activity was high and brought most of the topographical changes as confirmed by AFM (see Section 3.4). The continuous degradation of the film released oligosaccharides and monosaccharides to the bulk solution, causing the observed frequency increase. Eventually the rate of release of reaction products decreased as indicated by the decrease in the slope of the frequency curve starting from the inflection point (Fig. 4). At this stage the film was either completely digested, or the cellulose film that remained could not be hydrolyzed any further. The main reason for this rate-retarding effect is still not completely understood. It has been assumed to be related alternatively to the cellulase's thermal instability during the course of the reaction, the inactivation of the adsorbed cellulase due to the diffusion into the different parts of the cellulose (Caminal, Lopez-Santin, & Sola, 1985; Gonzalez, Caminal, De Mas, & Lopez-Santin, 1989) or to a decrease in the degree of saturation of adsorption sites on the film surfaces, which decreases the generation of chain ends by endoglucanases (Converse & Optekar, 1993).

3.3. Glucose analysis of hydrolysis products

The CZE analysis showed the absence of glucose in all samples collected from the pure buffer incubation, which confirms the stability of cellulose and TMSC films at a pH of 4.8. The determination of glucose in the eluates of the enzymatic hydrolysis indicated that β-glucosidase in the enzyme mixture was able to degrade cellobiose units into glucose. Other authors applied ion chromatography and found glucose as well as cellobiose after hydrolysis of cellulose with cellulases (Hu et al., 2009a, 2009b). They also found that the amount of glucose correlated well with the released QCM-D mass after enzymatic digestion. In our case no cellobiose could be detected within the measurement time of CZE. We therefore did not include the analysis of cellobiose in the eluates. The glucose content resulting from cellulase activity as a function of cellulase incubation time is shown in Fig. 5. In this case the cellulose films were prepared with method I as described in Section 2.2. The highest glucose concentrations were obtained after approximately 35 min of incubation. These glucose contents decreased continuously at longer incubation times, supporting the QCM-D data where a maximum mass release over time was observed after approximately 20 min of incubation. The deviation in time can be easily explained by the fact that there is a time lag between the QCM-D measurement and the sample collection.

Interestingly the cellulases were also able to release glucose from pure TMSC and the films regenerated for the shorter times, although, as discussed above, there were no frequency changes in the QCM-D experiments that could be related to film digestion. This indicates that hydrolysis had already stopped at a low concentration of glucose and cellulase was not able to digest the films anymore. Nevertheless CZE could show the differences in the films regenerated for 0, 1 and 2 min, since the glucose concentration in the eluates is higher for longer regeneration times. This also supports the observed differences in dissipation after enzyme adsorptions that are described in Section 3.2.1. The films that were regenerated for longer times (4–12 min) show higher amounts of glucose in the eluates. As expected, the overall amount of glucose increased as the regeneration time increased. These findings correlate well with the QCM-D data. The overall mass release of glucose after 125 min from films regenerated for 12 min was calculated to be 3.6 $\mu g\,cm^{-2}$. This value is in the range of the theoretical mass of the initial pure cellulose film (2.9 $\mu g\,cm^{-2}$) which was calculated from the density of cellulose (1.51 $g\,cm^{-3}$) (Kontturi & Lankinen, 2010) with a thickness of 25 nm and a radius of 0.5 cm.

In summary, it could be shown that CZE is a sensitive and rapid method of elucidating the production of glucose from the

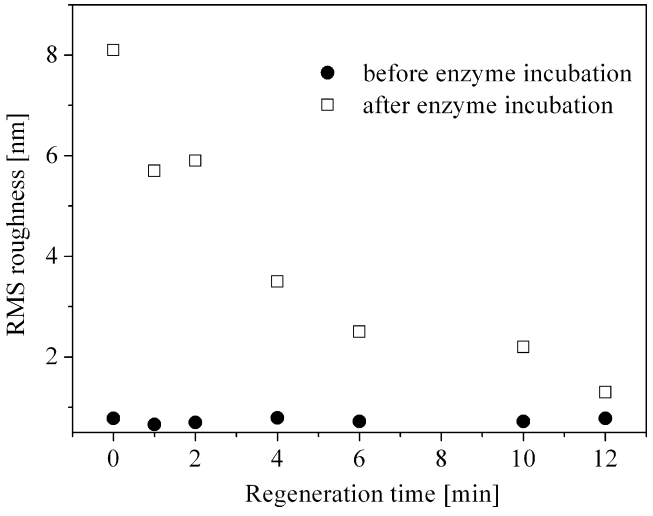

Fig. 6. AFM root mean square roughness of partially and fully regenerated films on QCM-D crystals before and after enzyme incubation.

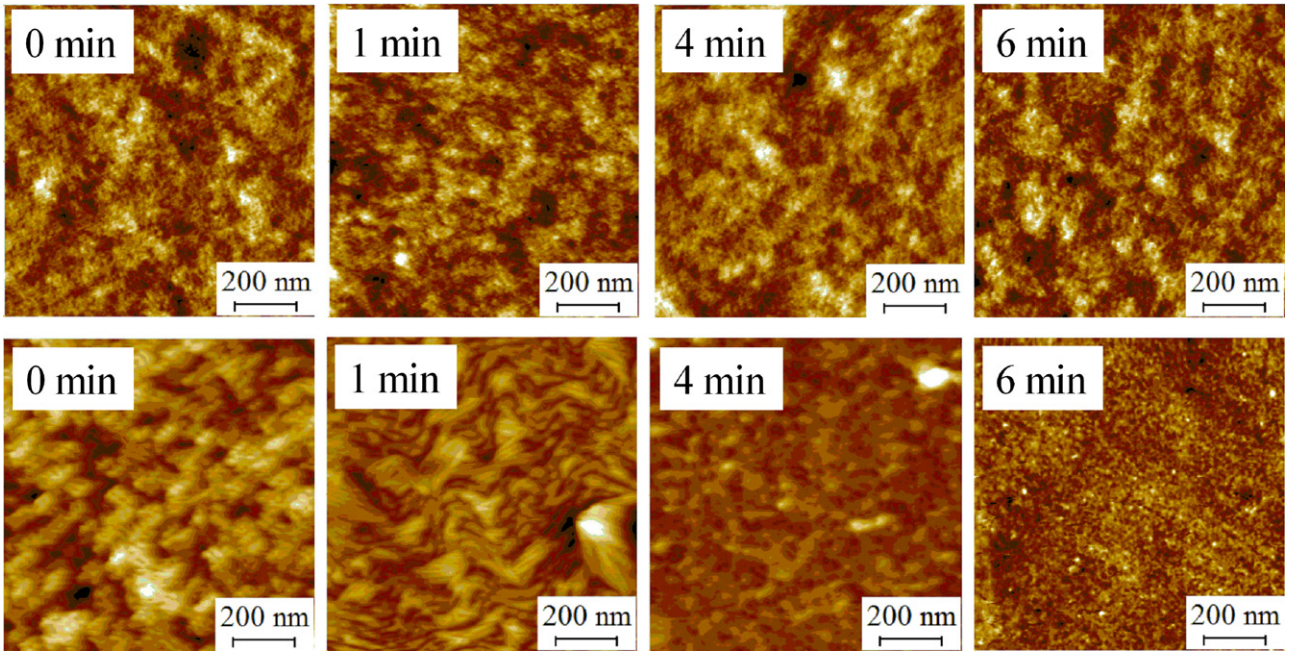

Fig. 7. AFM topography images of cellulose films obtained from different regeneration times. Upper row: films before enzyme incubation; lower row: films after enzyme incubation in the QCM-D.

hydrolysis products of pure TMSC or partially and fully regenerated cellulose. In particular, when the QCM-D changes in frequency do not show a release of mass, CZE can be used to detect released products and to explain changes in dissipation. Contact angle data and ATR-IR measurements of films obtained from 10 to 12 min of regeneration do not show quantitative differences but CZE and QCM-D revealed differences in the released mass and the amount of glucose. The combination of QCM-D and CZE supported the data from surface analytical methods but additionally allowed to verify whether the whole cellulose film is completely digestible or not. Since this digestibility is an indirect measure for the completeness of regeneration, CZE and QCM-D can be regarded as reliable tools to measure the regeneration of TMSC to cellulose.

3.4. Atomic force microscopy (AFM)

The changes in surface topography of the spin coated films before and after enzyme interaction were investigated by AFM (Fig. 7). Pure TMSC and other regenerated films were smooth and their manufacturing was reproducible (Mohan et al., 2011). The surface roughness of freshly regenerated films did not differ significantly. After cellulase treatment the roughness of all surfaces changed markedly as shown in Fig. 6. The roughness decreased as more and more regenerated cellulose was subjected to the enzymatic digestion. In comparison to TMSC, pure cellulose was completely digested and removed from the crystal, which led to a roughness close to a clean QCM-D gold crystal (rms roughness of a 25 μm^2 AFM image: 1.6 nm). The high roughness of pure TMSC films after enzyme incubation also indicates that cellulases are irreversibly bound to the surface and cannot be removed by washing with buffer. For films that were regenerated for 1 min, a certain pattern can be detected, which might be caused by fibril formation of the substrate, similar to dried cellulose model films (Mohan et al., 2011).

4. Conclusion

The interaction of cellulase enzymes from *T. viride* with hydrophobic trimethylsilyl cellulose (TMSC) and partially and fully regenerated cellulose was studied using QCM-D and capillary zone electrophoresis. The course of regeneration from TMSC to cellulose is strongly influenced by the applied volume of gaseous hydrochloric acid. Higher volumes of HCl reduce the time needed to produce pure cellulose. Lower volumes can be used to create partially and fully regenerated films. Cellulases bind irreversibly to hydrophobic TMSC films and are, to a minor extent, able to release glucose from these films. As expected the more the films are regenerated by exposure to HCl vapours, the more cellulose chains become available for enzymatic digestion, which results in higher mass-releases from the crystal's surface. QCM-D in combination with capillary zone electrophoresis is a reliable method of investigating the completeness of the regeneration of cellulose from TMSC. Even if analytical tools such as contact angle measurements and ATR-IR do not show differences in the film properties, the enzyme treatment elucidates differences in the digestibility. Furthermore capillary zone electrophoresis can be used to detect, support and quantify the release of monomers from the films even if frequency changes from QCM-D are covered by adsorbed masses. Capillary zone electrophoresis is even useful for the quantitative determination of released monomers of glucose.

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