DOI: 10.1002/masy.200900081

Enzymatic Treatment as a Pre-Step to Remove Cellulose Films from Sensors

Deusanilde J. Silva,[1] Orlando J. Rojas,[2,3] Martin A. Hubbe,[2] Song W. Park[1]*

Summary: In this work an enzymatic treatment is proposed as a preparative, cleaning protocol to remove cellulose films from resonators and sensors. Quartz crystal and surface plasmon gold sensors, coated with ultrathin films of cellulose are used in studies of molecular (for example, polymer and surfactant) adsorption. The sensors are usually recycled after removal of the film, with limited success, after one of two treatments, either hot acid or ammoniac solutions. In the proposed, improved protocol a mixture of cellulases from *Aspergillus* species, are used as a pre-treatment to facilitate the release of the cellulose film from the surfaces of the sensors. Two concentrations of NaCl solutions were considered in the enzymatic treatment, 1 and 10 mM, at given enzyme solution concentration, temperature and pH. It was found that after 80 min, the water contact angle after treatment with both salt concentration conditions reached a plateau. The average water contact angle after integration of the enzymatic and ammoniac treatments was found to be low enough, between 6.4 and 7.1 deg to allow reuse the sensors. It is concluded that the use of the ammoniac cleaning solution after the enzymatic treatment is a very convenient, safe and less time consuming way to remove the cellulose films from the sensors to be recycled.

Keywords: cellulases; cellulose films; cleaning protocol; enzymes; sensors

Introduction

Cellulose is the most abundant, natural, renewable, and biodegradable polymer, and it is naturally synthesized by an enzymatic process. This polymer has been considered as an important raw material for many applications, e.g., in the production of second generation alcohol. The bio-processing research involving cellulosic raw materials and enzymatic processes have been cited extensively in recent studies. It is common practice to use cellulose thin films as surfaces to evaluate physical-chemical phenomena. [1–10]

Typical model surfaces consist of a small amount of chemically defined compounds that are deposited on a flat substrate.[11] Two examples of cellulose-based model films can be mentioned: nanocrystals,[2,5,9,12] and amorphous cellulose. The latter has been studied in terms of its development [13–15] and use.[3,16–19]

On the other hand, enzymes have become popular in cleaning products because they are biodegradable and facilitate selective reactions. In some applications, e.g., dishes and cotton clothes, it is usual to find several cleaning detergents with some amount of lipase and cellulase enzymes in their formulations.

Cellulose-coated gold sensors have been used in adsorption studies by using quartz crystal microgravimetry (QCM) [1,3,4,6–9,20–22] and surface Plasmon Resonance.[23] Also, cellulose-coated silica substrates have been developed for studies involving atomic force microscopy (AFM) and X-ray photoelectron spectroscopy (XPS).[2,5,12,24]

[1] Sao Paulo University, Campus Sao Paulo, Sao Paulo, SP, Brazil
E-mail: deusanilde@gmail.com
[2] North Carolina State University, Raleigh-NC, United States of America
[3] Aalto University of Technology, Espoo, Finland

Regeneration of cellulose-coated sensors are needed in order to perform multiple QCM and SPR experiments since each trial requires fresh, pristine films. This can greatly increase the cost of any project. Therefore, in order to reuse the sensors it is necessary to remove the used cellulose films from the substrate. In the literature there are two chemical treatments to achieve this aim: an acid solution composed of sulfuric acid and hydrogen peroxide has been adopted by some authors [25,26] and an ammoniac solution composed of ammonium and hydrogen peroxide has been described by a QCM-D manufacturer. The acid treatment uses an extremely strong and corrosive solution, and as such has to be handled with caution. The ammoniac solution is not as strong as the acidic one. Although it is a general procedure recommended by the manufacturer to clean gold sensors, its efficiency in the removal of cellulose film is very limited. Frequently, residual fragments of cellulose film over the sensors have to be removed mechanically, with a risk of scratching the surface of the sensor. Also time needs to be spent to prepare new solutions. In summary, both chemical protocols are very time-demanding yet the re-use of cleaned sensors remains a challenge.

Cellulase activities have been studied by using cellulose models films.[1,9] Hydrolysis dynamics of the film at solid-liquid interfaces were clearly verified in real time by quartz crystal microgravimetry. This dynamics proceeds with binding of the protein onto cellulose film, resulting in a decrease in frequency and also with, as hydrolysis of cellulose accompanied by an increase in frequency until it reaching a plateau value, once all of the cellulose has been removed.

In work concerning interfacial phenomena on polymeric organic thin films, Jeong studied the effect of hydrolysis conditions on cellulose film degradation.[20] The effects of pH (4.5, 7, and 10), temperature (28, 33, and 38 °C) and enzyme concentration (0.00056, 0.00167, and 0.005%) on the enzymatic activity were evaluated by using quartz crystal microgravimetry. The best enzyme activity condition was found to be at pH 4.5, 38 °C, and with the highest enzyme solution concentration, 0.005%.

Model films from native and amorphous celluloses were used by Ahola et al. (2008) to investigate the dynamics and activities of mixtures of cellulase enzymes in real time at different temperatures, starting at 20 and up to 40 °C, and several cellulase solution concentrations, in the range of 0.01% up to 0.5% (v/v).[1] It was found that the enzymatic degradation of cellulose nanofibril films was very fast throughout the temperature range. As the authors expected, faster rates of cellulose degradation were found at higher temperatures. The effect of enzyme solution concentration on the kinetics and degradation of cellulose nanofibril films was studied at 40 °C, pH 5.0, and 0.1 mM of ionic strength. The authors found that higher enzyme concentration did not dramatically increase the already fast degradation rate.[1]

Based on its efficient activity to hydrolyze cellulosic films, the use of an enzymatic treatment is suggested here as a preparative step to remove cellulose from sensors. Therefore the main objective of this work is to propose a method for the reuse of sensors in a safe, convenient, and reproducible manner. To this end the evaluation of the proposed enzymatic protocol followed by a chemical treatment, is carried out and the main results presented.

Experimental Part

Materials

A milli-Q unit was used as source of ultra pure water in all experiments. Sodium chloride was used as a supporting salt to adjust the ionic strength of the buffer solutions to either 1 or 10 mM. 0.1N hydrogen chloride was used to adjust the required pH to 4.5. All inorganic chemicals used in this work were of analytical grade. A cellulase enzyme mixture from *Aspergillus* species, supplied by Sigma (Sigma C2605-50 ml, 095K0723), was used in this work for

Table 1.
Water contact angle of gold sensors coated with cellulose nanofilms.

	Initial contact angle, °				Average	Standard deviation
1 mM [NaCl]	22	21	20	23	**22**	1.3
10 mM [NaCl]	16	15	16.6	17	**16**	0.9

the enzymatic hydrolysis. For these experiments gold sensors coated with cellulose nanofilms that had been used for polymer adsorption studies [18] were selected. Average water contact angles of 16 and 22 deg were observed for 1 and 10 mM NaCl enzyme solutions on these surfaces, as described in Table 1.

Methods

Cellulose thin Film Preparation

Cellulose nanofilms that were used for polymer adsorption trials in quartz crystal microgravimetry were prepared as described by Gunnars and co-authors.[14] Basically, these films were produced by using microcrystalline cellulose dissolved in 50% wt N-methylmopholine-oxide (NMMO) at 115 °C followed by dilution with dimethyl sulfoxide (DMSO) to adjust the viscosity. Gold-coated quartz sensors were used as base substrate for the cellulose thin films. A thin layer of cellulose solution was spin-coated at 50 °C onto gold quartz crystals with a pre-adsorbed layer of polyvinyl amine (PVAm). The surfaces obtained by spin coating deposition consisted of flat, uniform and ultrathin films. The root mean square roughness of the dry model films were less than 5 nm as measured by tapping mode Atomic Force Microscopy (Figure 1), and the average contact angle measured after overnight drying in 43 °C under vacuum, was around 26 deg.

Chemical Treatments

A hot ammoniac solution composed of H_2O, 50% NH_4, and 25% H_2O_2, at a ratio of 10:1:2, v/v/v, respectively, was used to clean the sensors, as recommended by the supplier (Q-Sense, Sweden). When applied to remove cellulose films, this solution demanded a long treatment time for complete film removal. Furthermore, exchanging the solution several times (three times, typically) was required to remove the more recalcitrant cellulose fragments. Mechanical removal by using a cotton swab was required in some cases and therefore typical cleaning times of ca. 2 hours were required.

Enzymatic Hydrolysis Followed by Chemical Treatment

Based on studies the enzymatic hydrolysis of cellulosic materials coating QCM and SPR sensors, it is proposed that reuse or regeneration of the sensors could be facilitated by applying an additional cleaning step before the chemical (ammoniac solution) treatment (Figure 2). The proposed protocol consists of the immersion of the used gold sensors coated with residual

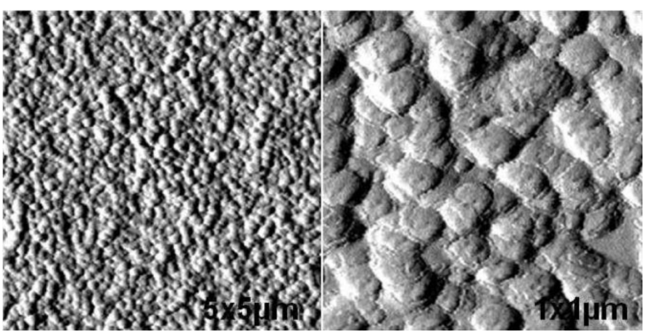

Figure 1.
Atomic force microscopy images of the cellulose film with a scan size of 5 × 5 μm (left) and 1 × 1 μm (right).

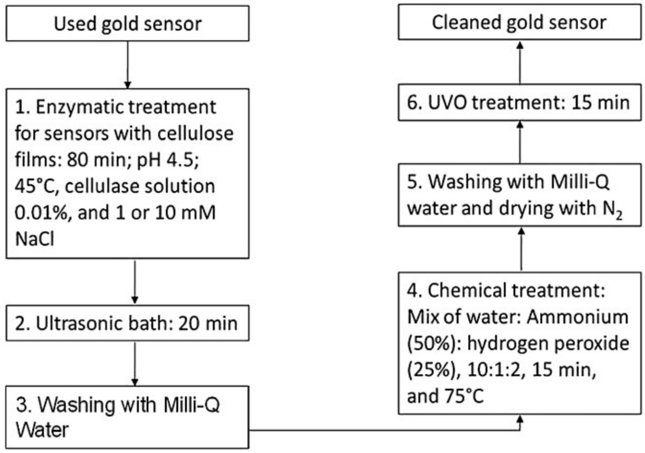

Figure 2.
Block diagram of the proposed protocol of combined enzymatic and oxidative treatment for sensor cleaning and reuse.

cellulose in an enzymatic solution (0.01% v/v) under controlled conditions of pH (4.5), temperature (45 °C) and time (80 min) (see Figure 2, step 1). To this end, 1 and 10 mM NaCl aqueous solutions were used. After this pretreatment, the sensors were submitted to an ultrasonic bath and washed (Figure 2, steps 2 and 3), followed by an immersion an ammoniac solution during a short period of time (15 min), as suggested by the sensor manufacturer, Q-Sense (Figure 2, step 4).

The conditions used were selected from earlier reports [1,20] which indicated optimal conditions of pH (4.5), temperature (45 °C) and enzyme solution concentration (0.01%)

The ultrasonic bath treatment for 20 min was important, since it helped to release the fragments of the cellulose film (Figure 2, step 2). After the ammoniac treatment, the sensors were rinsed with milli-Q water and dried with nitrogen gas. The cleaned sensors, now ready for deposition of a new cellulose films were first submitted to UV-ozone treatment for 15 min (Figure 2, steps 5, and 6, respectively).

Contact Angle Measurement

The water contact angles (WCA) on the different surfaces were measured with a Phoenix 300 system (SEO Corporation, Korea) by computer-controlled application of a water droplet (4 μl volume) from a syringe. The images of the sessile drop were analyzed with respect to their width and height to yield the contact angle and drop volume by using the "Image J" software (National Institutes of Health, USA). The contact angle was the average of measurements on both sides.

Results and Discussion

Figure 3 shows the water contact angle for surfaces after the proposed treatment for both salt conditions, 1 mM and 10 mM [NaCl] as a function of time. In this Figure t_1 indicates the time for the proposed treatment, that is, sensor immersed in enzymatic solution for 80 min, and t_2 indicates 15 min during which the sensor was immersed in ammoniac solution. The symbol (□) in Figure 3 denotes the application of 1 mM of the salt; and (x) represents the 10 mM NaCl aqueous solution.

When sensors are reused several times, the surfaces could become more hydrophobic, mainly due to damage, scratching or residual contaminants. In order to assess the quality of the sensor after the respective

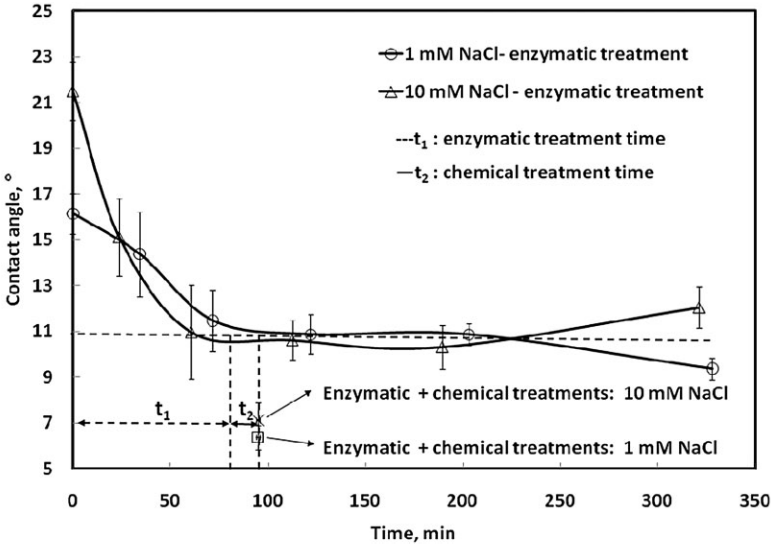

Figure 3.
Effect of enzymatic treatment on the contact angle of the cellulose coated-gold sensor surfaces with the time.

cleaning procedure, the criterion of a contact angle for the cleaned quartz crystal gold sensors between 4 and 6 deg (after applying ammoniac treatment only) is usually used. Higher contact angles were found in the present work after using enzymatic treatment followed by ammoniac treatment, 6 and 7 deg for 1 mM and 10 mM salt concentration, respectively.

Figure 3 shows that by using only the enzymatic treatment it was not possible to clean the sensors completely. For the two NaCl concentrations and different initial contact angles a minimum of 80 min were necessary to reach the targeted low contact angles after enzymatic treatment (around 11 deg). This result was close to that found by Jeong concerning enzyme activity in regenerated cellulose films by using QCM at pH 4.5, 38 °C, and 0.005% enzyme solution concentration.[20] On the other hand, the time found in this work was higher than that found by Ahola et al. (2008) for the hydrolysis of nanofibril cellulose, which was less than five minutes.[1] However, it is noted that these authors used a different cellulase solution mixture from that used in the present work, NS50013 cellulase complex from Novozymes (CelluclastTM).

The proposed cleaning treatment resulted in sensor surfaces with a slightly higher water contact angle compared to that after ammoniac treatment only, 6–7 deg vs 4–6 deg, respectively. However, this enzymatic pretreatment followed by a brief ammoniac treatment was found to be good enough for removing the cellulose films and to clean the sensors for recycling (repeated preparation of cellulose model films).

Conclusion

In this study an enzymatic hydrolysis was proposed as a pre-treatment to be followed by a chemical cleaning to remove cellulose thin films from the surface of sensors for QCM and SPR work. According to the results obtained, the following conclusions can be drawn:

(a) The proposed enzymatic pre-treatment is safe and requires less time than the conventional treatment using only the ammoniac solution;

(b) There was no significant difference between the final contact angles for two aqueous NaCl solutions employed after the enzymatic hydrolyses had reached the equilibrium, 6, and 7 deg for 1 and 10 mM NaCl, respectively;

(c) The sequence of two treatments, enzymatic followed by ammoniac treatment, was found to be effective to clean cellulose film sensors, allowing for their reuse. Also, the combined protocol reduces the risk of damaging the sensor surfaces and increases their expected useful life.

Acknowledgements: The financial support of CAPES, Brazilian Ministry of Education agency for graduate studies and the major scholarships, is acknowledged. The authors also would like to express their thanks to Xiaomeng Liu for her assistance in AFM imaging.

[1] S. Ahola, X. Turon, M. Österberg, J. Laine, O. J. Rojas, *Langmuir* **2008**, *24*, 11592.
[2] C. D. Edgar, D. G. Gray, *Cellulose* **2003**, *10*, 299.
[3] L.-E. Enarsson, L. Wågberg, *Biomacromolecules* **2009**, *10*, 134.
[4] S. Fält, L. Wågberg, E.-L. Vesterlind, *Langmuir* **2003**, *19*, 7895.
[5] Y. Habibi, L. Foulon, V. Aguié-Béghin, M. Molinari, R. Douillard, *Journal of Colloid and Interface Science* **2007**, *316*, 388.
[6] K. S. Kontturi, T. Tammelin, L.-S. Johansson, P. Stenius, *Langmuir* **2008**, *24*, 4743.
[7] J. Song, T. Yamagushi, D. J. Silva, M. A. Hubbe, O. J. Rojas, *J. Phys. Chem. B* **2010**, *114*, 719.
[8] T. Tammelin, T. Saarinen, M. Österberg, J. Laine, *Cellulose* **2006**, *13*, 519.
[9] X. Turon, S. Ahola, M. Österberg, O. J. Rojas, "Enzymatic hydrolysis of cellulose thin films with different crystallinities and morphologies studied by QCM-D and AFM techniques", in *235th National Meeting & Exposition*, New Orleans, LA, **2008**.
[10] X. Turon, O. J. Rojas, R. S. Deinhammer, *Langmuir* **2008**, *24*, 3880.
[11] E. Kontturi, T. Tammelin, M. Österberg, *Chem. Soc. Rev.* **2006**, *35*, 1287.
[12] J. Lefebvre, D. G. Gray, *Cellulose* **2005**, 12.
[13] S. Fält, L. Wågberg, E.-L. Vesterlind, P. T. Larsson, *Cellulose* **2004**, *11*, 151.
[14] S. Gunnars, L. Wågberg, M. A. Cohen Stuart, *Cellulose* **2002**, *9*, 239.
[15] S. Gunnars, L. Wågberg, M. A. Cohen Stuart, *Cellulose* **2003**, *10*, 185.
[16] D. J. Silva, M. A. Hubbe, S. W. Park, O. J. Rojas, "Adsorption and viscoelastic properties of polyampholytes monitored by QCM-D", in *V Congreso Iberoamericano de Investigacion en Celulosa y Papel*, Guadalajara, JL, Mexico **2008**.
[17] D. J. Silva, O. J. Rojas, M. A. Hubbe, S. W. Park, "Dynamics of Polyampholyte Adsorption on Cellulose Nanofilms", in *VII Annual Meeting of the Brazilian Materials Research Society (SBPMat)*, Guarujá, SP, Brazil **2008**.
[18] D. J. Silva, O. J. Rojas, S. W. Park, M. A. Hubbe, "Evaluation of Adsorbed Polyampholyte Layers by Using Quartz Crystal Microbalance ", in *10th International Symposium on Process Systems Engineering - PSE2009*, Salvador, BA **2009**.
[19] D. J. Silva, O. J. Rojas, M. A. Hubbe, S. W. Park, "Adsorption behavior of dry-strength polyampholytes as a function of solution pH", in *42nd Pulp and Paper International Congress & Exposition*, Sao Paulo, SP, Brazil **2009**.
[20] C. Jeong, "Interfacial Phenomena in Polymeric Thin Films", in *Wood and Paper Science*, North Carolina State University, Raleigh **2006**, MSc, p. 70.
[21] S. Ahola, M. Österberg, J. Laine, *Cellulose* **2008**, *15*, 303.
[22] G. Hu, J. A. Heitmann, O. J. Rojas, *Journal of Physical Chemistry B* **2009**, *113*, 14761.
[23] J. Song, J. Liang, X. Liu, W. E. Krause, J. P. Hinestroza, O. J. Rojas, *Thin Solid Films* **2009**, *517*, 4348.
[24] S. Yokota, T. Kitaoka, H. Wariishi, *Applied Surface Science* **2007**, *253*, 4208.
[25] N. Ayres, C. D. Cyrus, W. J. Brittain, *Langmuir* **2007**, *23*, 3744.
[26] Y. Tran, P. Perrin, S. Deroo, F. Lafuma, *Langmuir* **2006**, *22*, 7543.

