ENZYMATIC TREATMENT AS A PRE-STEP TO REMOVE CELLULOSE FILMS IN SENSORS

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Abstract

In this work an enzymatic treatment was proposed as a pre-step to remove cellulose film from surface sensors in a cleaning protocol. Quartz crystal gold sensors, coated with cellulose ultrathin films were used in polymer adsorption studies. They had to be cleaned with one of the two treatments with hot acid or ammoniac solutions available for their reuse. Both treatments showed advantages and some disadvantages for cellulose film removal. A mix of cellulase from Aspergillus species, supplied by Sigma, is proposed here as a pre-treatment to improve the cellulose film releasing from the quartz crystal surfaces. Two concentrations of salt solutions are considered in the enzymatic activity, 1 and 10 mM [NaCl], for each fixed conditions of enzyme solution, temperature and pH. It is found that after 80 min, the contact angle for both salt concentrations reaches the equilibrium. The ammoniac solution after the enzymatic treatment showed to be a very practical and safe way to remove the discharged cellulose model films on the gold sensor. This suggested protocol is very reliable and it is a low time demanding step for the researcher. The average contact angle after the integration of the enzymatic and ammoniac treatments was found to be enough to reuse the sensors, between 6.4° and 7.1°.

Keywords: Enzymatic treatment; Chemical treatments; Cleaning protocol, Cellulose model films; Quartz crystal sensors.

Introduction

Cellulose is the most abundant, natural, renewable, and biodegradable polymer synthesized by 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 researches involving cellulosic raw materials and enzymatic process have been frequent in recent studies. In those studies it is usual to apply the model surfaces made by cellulose films to evaluate physical chemical phenomena [1-6].

The model surfaces consist of a small amount of chemically defined compounds which are deposited on a flat substrate [7]. Two examples of cellulose based model films can be mentioned: nanocrystals [1, 4, 8], and amorphous cellulose [2, 9-12].

On the other hand, enzymes in cleaning products currently become popular as they are more biodegradable. 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 compositions.
Cellulose film coated gold sensors for quartz crystal microgravimetry with energy dissipation (QCM-D) [2, 3, 5, 6, 9, 13] in adsorption studies, as well as cellulose film coated in silicon oxide substrate for atomic force microscopy (AFM) and in X-ray photoelectron spectroscopy (XPS) [1, 4, 14] in surface studies, are good examples of model surfaces that have been largely used in recent researches.

Several cellulose film coatings are needed to perform a complete study with quartz crystal microgravimetry, since each trial requires a new flat and uniform film. This can increase largely the cost of the project. Alternatively, to reuse the sensors it is necessary to remove the old cellulose film from their surfaces. In the literature there are two chemical treatments to aim this issue: an acid solution composed by sulfuric acid and hydrogen peroxide adopted by some authors [15, 16], and an ammoniac solution composed by ammonium and hydrogen peroxide as described by QCM-D vendor. The acid treatment uses an extremely strong and corrosive solution and as such has to be handled in a cautious way. The ammoniac solution is not as strong as the acidic one. However, it is a general procedure indicated by the vendor to clean gold sensors and its efficiency in cellulose film removal is low. Frequently the residual fragments of cellulose film over the sensors have to be removed mechanically with a risk of scratching the quartz crystal surface. Also someone has to spend some time to prepare new solutions. Both protocols show the working time demands to finish the process. In this way, these sensors reusing was a challenge for the researchers.

Cellulase activities were studied by Ahola et al. (2008)[17], and Turon et al. (2008)[18] by using cellulose models films. Hydrolysis dynamics of the film at solid-liquid interfaces were clearly verified in real time by quartz crystal microgravimetry instrument. This dynamics proceeds firstly by binding of the protein on to cellulose model film surface with the frequency increasing, and after, by hydrolysis of the cellulose polymer in a reversible process with the frequency decreasing until to reach the equilibrium [17, 18].

Model films from native and amorphous celluloses were used by Ahola et al. (2008) to investigate the dynamics and activities of mixture of cellulase enzymes in real time at different temperatures, starting with 20°C up to 40°C, and several cellulase solution concentrations, starting with 0.01% up to 0.5% (v/v) [17]. They found that the enzymatic degradation of the cellulose nanofibril films was extremely fast for all those 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 [17].
In the work concerning to interfacial phenomena on polymeric organic thin films, Jeong studied the effect of hydrolysis conditions on cellulose film degradation [9]. The effect 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 verified by using quartz crystal microgravimetry with energy dissipation. The best enzyme activity condition was found to be at pH 4.5, with the highest temperature, 38 °C, and the highest enzyme solution concentration, 0.005%. For each variable studied the author fixed the best conditions for the other two.

Based on this efficient enzyme activity to consume cellulosic materials, we are suggesting the enzymatic treatment as a pre-step in the cellulose sensor cleaning procedure. Therefore the main objective of this work is to propose an associated cleaning protocol, enzymatic, followed by a chemical treatment, to remove the cellulose film from recycled sensors in a safe and easier.

**Experimental**

**Materials**

**Solutions**

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, 1 and 10 mM. 0.1N hydrogen chloride was used to adjust the required pH, 4.5. All inorganic chemicals used in this work were from analytical grade.

*Recyclable quartz gold crystal sensors coated with cellulose film*

For these experiments were selected four quartz gold crystal sensors coated with cellulose nanofilms after the polymer adsorption studies. The average contact angles of the 16.2° and 21.5°, for 1 and 10 mM NaCl enzyme solutions, respectively, were found as described in Table 1.

<table>
<thead>
<tr>
<th>Sensor</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>Average</th>
<th>Stand deviations</th>
</tr>
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<tr>
<td>Starting contact angle, °</td>
<td>22</td>
<td>21</td>
<td>20</td>
<td>23</td>
<td>21.5</td>
<td>1.290994</td>
</tr>
<tr>
<td></td>
<td>16</td>
<td>15</td>
<td>16.6</td>
<td>17</td>
<td>16.2</td>
<td>0.869866</td>
</tr>
</tbody>
</table>
**Enzyme solution**

Cellulase enzyme mixture from *Aspergillus* species, supplied by Sigma (Sigma C2605-50 ml, 095K0723), was used in this work for the enzymatic hydrolysis.

**Method**

**Cellulose thin film preparation**

Cellulose nanofilms that were used as surface models for polymer adsorption trails in quartz crystal microgravimetry were prepared as described by Gunnars and co-authors, 2002 [19]. Basically these films were developed by using microcrystalline cellulose dissolved in 50 % wt N-Methylmorpholine-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 poly-vinyl amine (PVAm). The surfaces obtained by spin coating deposition consisted of flat, uniform and ultrathin films. The roughness (RMS) of the dry model films were less than 5 nm as measured by tapping mode AFM (Figure 1), and the average contact angle measured after overnight drying in 43 °C under vacuum, was around 26°.

![Figure 1 – Atomic force microscopy images of the cellulose film, 1x1 µm and 5x5 µm scan](image)

**Chemical treatments**

After using the cellulose coated-gold sensors for adsorption propose, their surfaces can be cleaned to be reused more time. To reuse these sensors it is necessary to remove the used cellulose thin film that is coating the gold surface. There are basically two chemical treatments by using oxidant reagents that are recommended for this purpose:
(a) A hot acid solution called “piranha” solution that is composed by 70% H$_2$SO$_4$, and 30% H$_2$O$_2$, v/v where the recyclable sensors were immersed. As reported by Ayres et al. (2007)[15], and Tran et al. (2006)[16], this solution is extremely reactive and corrosive and as such should be handled with great care. In our case the film releasing was monitored every 10 min and washed with Milli-Q water between the observations. It was common to spend up to 40 min to have a complete releasing (treating four sensors each time).

(b) A hot ammoniac solution that is composed by H$_2$O, 50% NH$_4$, and 25% H$_2$O$_2$, with the ratio of 10:1:2, v/v/v, respectively, also called “piranha” solution that is recommended by the sensors supplier, Q-Sense, to clean new gold quartz crystal sensors was used as well. This solution is less strong and reactive than the first one and as such the time demanding is higher. Some time it was necessary to change the solution three times and to remove some persistent dirt fragments mechanically and very carefully with cotton swab to avoid damages on the sensor surface. It was usual to spend 2 hours for cleaning four sensors each time.

Enzymatic followed by chemical treatment

As we could see, both protocols described above have advantages and disadvantages. Therefore, based on some studies about enzymatic hydrolysis degradation of cellulosic materials and considering that the substrates are covered with cellulose films, their reuse could be facilitated by applying an additional cleaning step before the chemical treatment step (Figure 2). In our studies we considered the ammoniac solution.

The new protocol consists on the immersion of the recyclable gold sensors plus cellulose film in an enzymatic solution (0.01% v/v) under controlled conditions: pH 4.5, at 45°C and 80 min (Figure 2, step 1). Two salt concentration solutions were studied: 1 and 10 mM [NaCl]. After this pretreatment, the sensors were submitted to an ammoniac solution during a short period of time (15 min) as suggested by sensor vendor, Q-Sense (Figure 2, step 4).

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1. AFM images of cellulose film that was developed by Deusamilde Silva and Xiaomeng Liu, NCSU, May 2008.
Based on the literature results [9, 17], the conditions of pH (4.5), temperature (45 °C) and enzyme solution concentration (0.01%) were adopted in the present study. An oven set at 45 °C of temperature was used for the enzymatic activities experiments.

The ultrasonic bath step for 20 min was an important step and it helped to release the fragments of the film when they were easier to be released (Figure 2, step 2).

After the ammoniac treatment, the sensors were washed with milli-Q water and dried with nitrogen gas. The sensors used to prepare new cellulose films, were submitted firstly to UV-ozone treatment for 15 min.

**Contact angle measurement**

The contact angle apparatus with a water drop probe method was used to measure the contact angle. The contact angle was the average of the measurements in both sides.

**Results and Discussion**

The Figure 2 shows the contact angle curves with the time for the proposed treatment in both conditions 1 mM and 10 mM [NaCl]. In this Figure t₁ indicates the time for the proposed treatment, that is, sensor immersed in enzymatic solution for 80 min, and t₂ indicates 15 min for the sensor immersed in ammoniac solution. The symbol (□) in the Figure 2 denotes the application of 1 mM of the salt; and (X) is for 10 mM of the salt.
In order to control the quartz crystals cleaning procedure, our laboratory group usually adopted the contact angle for the cleaned quartz crystal gold sensors between 4° and 6° when applying ammoniac treatment only. Higher contact angle results were found in the present work for both conditions by using enzymatic followed by ammoniac treatment, 6.4° and 7.1°, for 1 mM and 10 mM of salt, respectively. When the sensors are reused several times, the surfaces could become more hydrophobic, mainly due to some damage or scratch.

![Figure 2 – Effect of enzymatic treatment on the contact angle of the cellulose coated-gold sensor surfaces with the time.](image)

The Figure 2 shows that enzymatic treatment only is not possible to clean the sensors completely. For the two salt concentrations and different starting contact angle at least 80 min were necessary to reach the smallest contact angle values with the enzymatic treatment (around 11°). This result was close to the one found by Jeong in his studies about enzyme activity in amorphous cellulose film by using QCM-D for pH 4.5, at 38 °C, and enzyme solution concentration, 0.005% [9]. On the other hand, the time found in this work was higher than the time found in Ahola et al. (2008) work for nanofibril cellulose films that was less than five minutes [17]. These authors used different cellulase solution mixture from the one used in the present work, NS50013 cellulase complex from Novozymes (CelluclastTM).
The proposed cleaning treatment shows to be a bit more hydrophobic, 6.4°-7.1°, than the ammoniac treatment only, 4°-6°. However, this enzymatic pretreatment followed by short time ammoniac treatment was found to be good enough for cleaning and reusing the sensors in the cellulose film preparation.

Conclusions

In this study an enzymatic treatment was proposed as a pre-step to a chemical treatment to remove cellulose thin films from surface sensors. According to the results obtained, the following conclusions can be drawn:

1. The proposed enzymatic pre-treatment was more safe and showed less researcher time demanding when compared with the time required when ammonic solution is using alone;

2. There was no significant difference between the final contact angle results for the two salt solutions studied after the enzymatic hydrolyses have reached the equilibrium, 6.4°± 0.5377 (1 mM), and 7.1°±0.7500 (10 mM);

3. The association of two treatments, enzymatic and ammoniac solution, showed to be a safe and useful protocol to clean cellulose film sensors for reusing proposes. Also, the combined protocol reduces the risk of damaging the sensor surfaces and increases their life circle.

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References


