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Immobilization of cholesterol oxidase in LbL films and detection of cholesterol using ac measurements

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Abstract

The preserved activity of immobilized biomolecules in layer-by-layer (LbL) films can be exploited in various applications, including biosensing. In this study, cholesterol oxidase (COX) layers were alternated with layers of poly(allylamine hydrochloride) (PAH) in LbL films whose morphology was investigated with atomic force microscopy (AFM). The adsorption kinetics of COX layers comprised two regimes, a fast, first-order kinetics process followed by a slow process fitted with a Johnson-Mehl-Avrami (JMA) function, with exponent ~ 2 characteristic of aggregates growing as disks. The concept based on the use of sensor arrays to increase sensitivity, widely employed in electronic tongues, was extended to biosensing with impedance spectroscopy measurements. Using three sensing units, made of LbL films of
PAH/COX and PAH/PVS (polyvinyl sulfonylic acid) and a bare gold interdigitated electrode, we were able to detect cholesterol in aqueous solutions down to the $10^{-6}$ M level. This high sensitivity is attributed to the molecular-recognition interaction between COX and cholesterol, and opens the way for clinical tests to be made with low-cost, fast experimental procedures.

**Keywords:** Layer-by-Layer, Cholesterol Oxidase, Cholesterol, Biosensor, EggPG, Atomic Force Microscopy

1- Introduction

The fabrication of nanostructured films from biomolecules has become an important tool for investigating fundamental adsorption mechanisms and biological activity, in addition to serving for immobilizing materials in different supramolecular architectures for a number of biotechnological applications [1]. In this context, one can benefit from the LbL (layer-by-layer) technique [2] that allows efficient immobilization of enzymes due to the mild conditions under which the film fabrication takes place, in addition to the choice of template materials that may help preserve enzyme activity [3]. Significantly, recent work by Lourenço et al [4] and Silva et al [5] has proven that entrained water is present in LbL films, in spite of the drying procedures, which is important for the preservation of activity of biomolecules. There is now an extensive list of biosensors made with LbL films (for a review see [6]).

Biosensors obtained with immobilized cholesterol oxidase may be advantageous in comparison with standard methods of cholesterol detection, e.g. spectrophotometry [7, 8], gas-liquid chromatography and HPLC [9, 10], owing to the simplicity and low cost involved. This is relevant to the development of reliable methods of cholesterol
detection in blood, a fundamental parameter to identify disorders such as hypercholesterolemia [11, 12]. Furthermore, it is important to control the cholesterol level of foodstuffs for human intake.

Cholesterol is a sterol found in eggs, meats, yellow cheese, and derivatives. Biosensors for cholesterol have been used in biochemical analysis owing to their good selectivity, fast response, low cost, small size and long term stability [8]. Most of the literature on cholesterol biosensors has focused on diagnosing disorders [13-15] and the intake control of foodstuffs [16, 17]. For example, Basu et al [18] developed a cholesterol biosensor based on immobilized cholesterol esterase and cholesterol oxidase to determine the total cholesterol content in foodstuffs, and electrochemical measurements were performed in the cholesterol analysis of food samples.

In this paper we report the immobilization of cholesterol oxidase (COX) in LbL films, with alternating layers of poly(allylamine hydrochloride) (PAH). In addition to studying the adsorption characteristics of the LbL films, including an analysis of the film morphology, we employ an array of sensors to detect cholesterol in chicken eggs at \( \mu \text{M} \) concentrations. Combining the analysis of adsorption experiments with investigation of performance of sensing units is important because film morphology may be a key parameter to determine the suitability of a sensing unit. Indeed, while in some types of devices films with low roughness may be preferred, rough films may be advantageous for sensors as a high surface area may be convenient [19].

2- Experimental Details

2.1- Materials

Cholesterol oxidase (COX) from *Cellulomonas species* (EC 1.1.3.6) (100 units), cholesterol from egg, poly(allylamine hydrochloride) (PAH) and poly(vinylsulfonic
acid) (PVS) were purchased from Sigma-Aldrich. The natural phospholipid L-α-phosphatidyl-DL-glycerol from chicken egg (EggPG) was obtained from Avanti Polar Lipids. All reagents were used without further purification. The structure of some of the materials is shown in Figure 1. Yolk of natural egg chicken was separated from the white part and diluted in Tris-HCl buffer until the formation of a 10^{-3} M cholesterol solution, calculated from the egg nutrition information.

2.2-Enzyme immobilization

Cholesterol oxidase enzyme (COX) was alternately immobilized with PAH onto glass substrates using the LbL technique [2]. The substrates were previously cleaned in HCl/H2O2/H2O (1:1:6) and NH₄OH/H₂O2/H₂O (1:1:5) solutions, both for 10 min at 80°C. Before the PAH/COX film fabrication, 2-bilayers of (PAH/PVS) were deposited onto the solid substrates to reduce substrate effects [20]. All solutions were prepared in 10 mM Tris-HCl buffer pH = 7.5. At this pH COX can be used as a polyanion, as its isoelectric point lies between 4.6 and 5.2 [8]. Both PAH and PVS concentrations were 1 mg/mL, while COX concentration was 0.2 mg/mL. The substrate was immersed in PAH solution for 3 min, being further rinsed with 10 mM Tris-HCl buffer solution to remove loosely adsorbed molecules. Subsequently, it was immersed in the COX solution at 4°C (controlled temperature) for 10 min for enzyme adsorption, being once again washed with Tris-HCl buffer to remove inadequately adsorbed molecules. This procedure was repeated until the desired number of PAH/COX bilayers.

The adsorption time profile of COX multilayers was monitored at each deposition step by measuring the absorbance at 280 nm with a Hitachi U-2001 spectrophotometer, which corresponds to the maximum in absorption. Film morphology was studied by atomic force microscopy (AFM). Figure 2A shows the scheme of the
multilayers by consecutive adsorption of PAH and COX onto a substrate previously modified with two bilayers of PAH/PVS [8].

2.3- Preparation of cholesterol in liposomes

Since cholesterol is insoluble in water and occurs in cell membranes between phospholipids a biomimetic environment was provided using unilamellar vesicles (SUVs) (liposomes). Hence, 1 mM of EggPG (phospholipid) and cholesterol at various concentrations were homogeneously dissolved in chloroform to prepare the SUVs. A thin film was formed by evaporating the solvent with a gentle stream of nitrogen, then the dry lipid film was hydrated overnight in 10 mM Tris-HCl buffer pH = 7.5 resulting in multilamellar vesicles (MLVs). To obtain the SUVs the eggPG/cholesterol mixture was sonicated in ten cycles of 30 seconds, with a delay time of one minute between the sonication period.

2.4- Electrical measurements

Impedance measurements were carried out with a Solartron 1260A impedance/gain phase analyzer in a frequency range from 100 to 1000 Hz. All measurements were performed using three interdigitated (IDE) electrodes: a bare IDE electrode, one coated with seven PAH/PVS bilayers, and another with two PAH/PVS bilayers covered with five PAH/COX bilayers. The experiments were conducted by soaking the electrodes during 20 minutes in liposome/cholesterol solutions containing different molar concentrations of cholesterol (0, 10^-6, 10^-5, 10^-4 and 10^-3 M), as schematically shown in Figure 2B. The same measurements were carried out with yolk egg from chicken in different concentrations of cholesterol (10^{-5}, 10^{-4} and 10^{-3} M). The pH of the solutions was buffered at 7.5 using 10 mM Tris-HCl. The capacitance values...
obtained by modeling the impedance response with an equivalent circuit [21] were treated with Principal Component Analysis (PCA [22], which is a statistical method correlating samples, decreasing the dimensionality of the original data without losing information. Briefly, it makes a linear combination of the data, creating another pair of orthogonal axis into the directions were the higher correlations exist.

2.5- Morphological characterization

Surface morphology was investigated using a Nanoscope IIIa (Digital Instruments) microscope with 512 x 512 pixels per image obtained under ambient conditions in the tapping mode. The average height and average diameter of the aggregates were determined using the software from Digital Instruments.

3- Results and Discussions

3.1- Film growth and adsorption mechanisms for PAH/cholesterol oxidase (COX) LbL films

In order to characterize the COX-containing LbL films, adsorption was carried out onto quartz substrates, which are amenable to optical measurements. As already mentioned, the quartz substrates were initially coated with two PAH/PVS bilayers to minimize substrate effects. The kinetics of adsorption of PAH/COX onto PAH/PVS LbL film is represented in Figure 3, which displays the absorption at 280 nm vs. time. The saturation observed at ca. 200 s indicates that a complete layer is achieved within 3 min. This adsorption kinetics may be modeled with the Johnson-Mehl-Avrami (JMA) equation [23-25], which allows one to infer about growth processes without resorting to molecular-level information [25]. The fitting of the data is shown in the solid line, obtained with the parameters of Table 1. The plateau reached in the absorption is related to the saturation of available sites for COX molecules adsorption from solution, repelled
by those already adsorbed with the same charge. This second process can be explained with a Johnson-Mehl-Avrami (JMA) function (Eq 1) with $n \sim 2$ and characteristic times of 180 s (see Table 1).

$$A = k_1 \left[ 1 - \exp \left( - \frac{t}{\tau_1} \right) \right] + k_2 \left[ 1 - \exp \left( - \left( \frac{t}{\tau_2} \right)^n \right) \right]$$

(1)

where $A$ is the absorbance, $k$ and $n$ are constants and $\tau$ (1 and 2) are the characteristic times [23]. Avrami’s model gives the phenomenological description of adsorption kinetics and has been successfully used to explain the growth of polymer films [24, 25]. The values of $\tau_1$ and $\tau_2$ are typical for adsorption of LbL films. The first process, represented by $\tau_1$ is very fast, usually within a few seconds or less, while the second process has characteristic times of hundreds of seconds. A value of $n \approx 2$ corresponds to a 2D adsorption, without forming new nuclei during the adsorption process. In other words, adsorption takes place with the growth of nuclei already formed in the beginning of the process, which actually corresponds to the first, fast process represented by $\tau_1$.

This is in fact consistent with the morphological analysis presented below.

Figure 4(a) shows an AFM image for a scan window of 10×10 µm$^2$ of a 10-bilayer PAH/COX film. Here, we employ information from surface morphology to analyze growth mechanisms [24]. For instance, from the height profiles in Figure 4b one may infer about the direction of film growth. Figure 5 shows the average diameter of the aggregates increasing linearly with the average height, indicating no preferential direction for the aggregate growth. Note that the average diameter increases much faster than the average height. Therefore, the aggregates grow as discs, consistent with $n=2$ obtained in the kinetics of adsorption (Table 1). The error bars included in the plots of Figure 5 are average values from five scans.
We have also used the dynamic scale theory to obtain roughness exponents. Figure 6 shows the log-log plot of roughness, $W$, against scanning window size, $L$, from AFM images on PAH/COX films and a scanned area of $10 \times 10 \ \mu m^2$. The roughness is typical of LbL films of non-conventional polyelectrolytes. It is possible to apply scaling laws [26] as $W$ saturates at a given $L$, which allows one to investigate the morphology and dynamics of film formation. The parameters involved are the roughness, fractal dimension and the critical exponents, namely, the exponents of roughness ($\alpha$) and growth ($\beta$), and the dynamic exponent ($z$). Scaling laws, therefore, help to describe how film morphology, characterized by rms (root-mean-square) roughness, evolves during film growth, based on two factors: the time formation of the surface and the size of the scanning window [26, 27]. The rms roughness $W$ is taken as a function of the scanning window size, $L$, in the form $W(L) \propto L^\alpha$, where $\alpha$ is independent of the units employed for the lateral dimension $L$. The $\alpha$ should be calculated when the roughness has already reached saturation, as in Figure 6.

The slopes in the $\log W \times \log L$ plots correspond to the roughness exponents ($\alpha$) given in Table 2 along with the fractal dimension (DF=3-$\alpha$). $L_x$, the average diameter, corresponds to the crossover length defining the transition between the two growth regimes, while $\bar{L}$ is the average diameter estimated from the AFM images and calculated with the Nanoscope III software (see Figure 4b). As Table 2 indicates, the dynamic scale analysis provides a length scale that is in good agreement with the average size of domains obtained from AFM.

For length scales larger than the average diameter of the domains, $L > L_x$, the surfaces are characteristic of self-affine fractals that may be described with the Kardar–Parisi–Zhang (KPZ) equation [26, 28]. KPZ is one of the models used to describe stochastic formation at an interface, which can be related to the growth of a film surface.
The $\alpha$ exponent in the KPZ model is close to 0.4 \cite{26, 19, 33-35}. It is possible that this roughness could decrease if additional bilayers were adsorbed, as new molecules could occupy empty spaces \cite{36}. For $L < L_x$ (inside the domains) the measured $\alpha \approx 0.6$ is characteristic of a growing kinetics governed by diffusion along the surface \cite{26, 37}. Indeed, the marked oscillations on top of the domains observed in the height profiles, see Figure 4b, are characteristic of a rough surface, in contrast to other LbL films \cite{36, 38} for which the surface domains are close to a Euclidian surface (DF $\approx$ 2).

3.2- Detection of cholesterol using impedance measurements

Impedance measurements were used to detect cholesterol in eggPG liposome and natural egg yolk using the 3-electrode array with LbL films deposited onto gold interdigitated electrodes (IDEs). The data taken with the 3 electrodes at 1 kHz, which is the frequency most sensitive to changes in film properties \cite{39-41}, were treated with Principal Component Analysis (PCA). The PCA plots in Figures 7 and 8 display the results for the electrical measurements in aqueous solutions with cholesterol in eggPG liposomes or in natural egg yolk, respectively. A clear distinction is made between water and the cholesterol containing solutions at different molar concentrations. In Figure 7 there is a high correlation between the Second Principal Component (PC2) and the cholesterol concentration. The sensor array could also distinguish water from cholesterol egg yolk, as illustrated in Figure 8. In this case a good correlation between PC1 and natural cholesterol concentration was obtained, with a left hand trend to the most diluted samples. It is worth mentioning that the data presented in Figures 7 and 8 show similar results in different sets of independent measurements, taken also during different days of analysis. Such high correlation was attributed to the presence of
specific interactions between COX and cholesterol, increasing the “chemical orthogonality” of the data, as reported in e-tongue analysis of beverages [40].

In PCA plots considering results acquired only with the bare electrodes and PAH/PVS, i.e. without the cholesterol oxidase film, there is no clear distinction between distinct cholesterol concentrations and no correspondence in the data that could be related to the Principal Components (results not shown). Possibly, this is due to the lack of specific interactions due to the absence of COX for cholesterol detection. Nevertheless, in both cases there was a clear distinction between water and liposomes containing cholesterol. In the measurements presented here LbL films with a fixed number of bilayers (seven) were used, and no attempt was made to investigate possible effects of the number of layers on the sensitivity. Previous studies have, however, demonstrated that optimal performance is usually attained with 5 to 10-bilayer films because they are sufficiently to increase sensitivity while providing full coverage of the electrodes, which may not occur for very thin films [42].

4 - Conclusions

Layer-by-layer (LbL) films of cholesterol oxidase (COX) could be produced, in which COX layers were alternated with poly(allylamine hydrochloride), and the COX activity was preserved. This demonstrates the suitability of the film-forming method for biomolecules, which may be largely attributed to the mild conditions under which the films are fabricated. Adsorption of a layer of COX was complete within ca. 180s, with a kinetics that comprises two steps. The nuclei are formed right in the beginning of the adsorption, and then aggregates grow as disks, according to atomic force microscopy data that were analyzed using dynamic scaling theories. LbL films adsorbed onto gold electrodes were used as sensing units, with impedance spectroscopy as the method of
detection. Owing to the specific interaction with COX in the LbL film, cholesterol could be detected in aqueous solutions with a sensitivity reaching $10^{-6}$ M. Because the methodology combining ultrathin, nanostructured films and a.c. electrical measurements can be extended to many other molecular recognition pairs, one may envisage its application in clinical diagnosis.

Acknowledgements

This work was supported by FAPESP, CNPq and CAPES (Brazil).

5- References


São Carlos, 15th July 2008.

Roger Narayan  
Editor Materials Science and Engineering C  
Ms. Ref. No.: MSEC-D-08-00195

Please find attached a revised copy of the manuscript entitled “Immobilization of cholesterol oxidase in LbL films and detection of cholesterol using ac measurements”, which we submitted to the special issue of Materials Science and Engineering C dedicated to the FBPOL 2008 meeting. We have taken onboard all the reviewers’ suggestions and modified the manuscript accordingly.

Yours sincerely

Marli Leite Moraes  
IFSC/USP
São Carlos, 15th July 2008.

Roger Narayan
Editor Materials Science and Engineering C
Ms. Ref. No.: MSEC-D-08-00195

We enclose a revised copy of the manuscript entitled “Immobilization of cholesterol oxidase in LbL films and detection of cholesterol using ac measurements” by M.L. Moraes et al. which we submitted for publication in MSEC. We have taken onboard all the reviewers’ suggestions and modified the manuscript accordingly. A response sheet to the reviewer’s comments is also enclosed.

Yours sincerely

Marli Leite Moraes
IFSC/USP
Response to the reviewers comments

Reviewer 1:


   The reference was replaced in the text.

2. “Page 4 « . to reduce substrate effects [20] » : reference 20 does not provide any information on this question but only refers to another publication.”

   The references were replaced in the text.

3. “Page 4 : the choice to measure the absorbance at 280 nm should be motivated”

   This wavelength is the maximum of the absorbance. This piece of information was added to the revised manuscript.

4. “The reaction of cholesterol into cholestenone mentioned in Figure 2B has to be detailed and commented in the text or references to previous publications have to be given.”

   A reference was added in the text.

5. “The first paragraph on page 6 has to be numbered 2.4 and given a title.”

   The text was corrected.

6. “Page 6 : « .atomic force microscopy with 512*512 pixel images. » has to be replaced by .microscope with 512*512 pixels per image.”

   The text was corrected.

7. “Page 6 : « The saturation observed at ca. 160 s indicates that a complete layer is achieved within 3 min : Figure 3 rather shows that saturation occurs at 200 s, the text should be changed accordingly.”

   The text was corrected.

8. “The error bars included in the plots of Figure 5 are average values from several scans. The number of measurements should be mentioned.”
The number of measurements, 5, was added in the text.

9. “Page 7 and 8: Despite the long introduction to dynamic scale theory, parameters obtained from this analysis listed in Table 2 are not hardly discussed in the text. This analysis has to be clarified and commented.”

The reviewer is absolutely correct. While re-reading the manuscript we noted that the results from the analysis were not commented upon as they should have. We have now added to the manuscript comments on the various parameters used in the dynamic scale analysis.

10. “The PCA analysis has to be explained in the experimental details section. The section dedicated to the detection of cholesterol using impedance measurements is quite convincing. However, it is better if the effect of the number of layers on the sensitivity was not investigated.”

We have added an explanation about PCA in the experimental section. With regard to the possible effects from the number of layers, we have not investigated this specific point in the present study. However, from our previous experiences, we do know that higher sensitivity is obtained with a small number of layers. We have added a comment on this point in the revised manuscript.


The references were corrected.

12. “Figure 2: A and B are missing.”

The caption was corrected.

Reviewer 2:

“This article deals with the production of a new sensor through the preparation of the LbL films using cholesterol oxidase (COX) and poly (allylamine hydrochloride) - (PAH). The authors showed the film growth and the adsorption mechanism for PAH/COX LbL films using absorption measurements and AFM technique. However, the results concerning detection of cholesterol using impedance measurements are not sufficiently clear. In my opinion the Principal Component Analysis (PCA) is a treatment that should be explained. The authors provide details to explain this treatment to the readers. Moreover, there are some problems with the symbols in the figure captions 7 and 8 that must be corrected.”
The piece of the text was added in the new version and the symbols were corrected.
Figure 1: Structures of the materials employed.
Figure 2: Schematic representation of COX immobilization in LbL films (A) and a schematic representation of the enzymatic oxidation of cholesterol in liposomes (B).
**Figure 3:** Absorbance at 280 nm vs. time of adsorption for the COX layer. The solid line represents the fitting with the JMA equation.
Figure 4: (a) AFM image of a 10-bilayer (PAH/COX)$_n$ LbL film adsorbed on a quartz substrate with scanning window of $10 \times 10 \ \mu m^2$. (b) Height profile of the film.
Figure 5: Log-log plot of average diameter of the domains versus height for a 10-bilayer PAH/COX LbL film.
Figure 6: Log-log plot of RMS roughness (W) versus size of the scan window (L).
Figure 7: Principal components analysis (PCA) plots using three electrodes: bare, PAH/PVS and PAH/COX. The capacitance values at 1 kHz for: Milli-Q water (●) and cholesterol into eggC liposome at concentrations of 0 (O), $10^{-6}$ (X), $10^{-5}$ (+), $10^{-4}$ (*) and $10^{-3}$ (□) M.
**Figure 8:** Principal components analysis (PCA) plots using three electrodes: bare, PAH/PVS and PAH/COX. The capacitance values at 1 kHz for: Milli-Q water (●) and egg yolk natural at concentrations of $10^{-5}$ (O), $10^{-4}$ (X) and $10^{-3}$ (+) M.
Table 1: Parameters $n$, $\tau$ and $K$ employed in the JMA equation for fitting the data for kinetics of PAH/COX layers.

<table>
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<th>$K_1$</th>
<th>$\tau_1$</th>
<th>$K_2$</th>
<th>$\tau_2$</th>
<th>$N$</th>
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<td>0.041 ± 0.002</td>
<td>0.8 ± 0.2</td>
<td>0.014 ± 0.003</td>
<td>157 ± 7</td>
<td>1.8 ± 0.1</td>
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</tbody>
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**Table 2** - $L_x$; root mean square roughness (rms); exponents and fractal dimension ($\alpha_1$ and $DF_1$ correspond to the region inside the domains) and $\bar{L}$.

<table>
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<th>$L_x$ (nm)</th>
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<th>$\alpha_2$</th>
<th>$DF_1$</th>
<th>$DF_2$</th>
<th>$\bar{L}$ (nm)</th>
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<td>0.35 ± 0.05</td>
<td>2.39</td>
<td>2.65</td>
<td>410</td>
</tr>
</tbody>
</table>
São Carlos, Brazil, 15th July, 2008.

Materials Science and Engineering C

On behalf of all the authors, I state that the material contained in the present manuscript is original, for it has not been published in any form, and neither has it been submitted to another journal.

Yours sincerely,

Marli L. Moraes
São Carlos, Brazil, 27th may, 2008.

Materials Science and Engineering C

Possible referees:
a) Maria Elena Vela mevela@inifta.unlp.edu.ar with expertise on adsorption processes in nanostructured films
b) Claudio Nicolini - director@nwi.unige.it, expert in sensing and biosensing.

Marli L. Moraes