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ORIGINAL PAPER

Immobilization of uricase in layer-by-layer films used in amperometric biosensors for uric acid

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Abstract The layer-by-layer technique was exploited to immobilize the enzyme uricase onto indium tin oxide substrates coated with a layer of Prussian Blue. Uricase layers were alternated with either poly(ethylene imine) or poly(diallyldimethylammoniumchloride), and the resulting films were used as amperometric biosensors for uric acid. Biosensors with optimum performance had a limit of detection of 0.15 μA μmol l^{-1} cm^{-2} with a linear response between 0.1 and 0.6 μM of uric acid, which is sufficient for use in clinical tests. Bioactivity was preserved for weeks, and there was negligible influence from interferents, as detection was carried out at 0.0 V vs saturated calomel electrode.

Keywords Layer-by-layer · Amperometric biosensor · Uricase · Prussian Blue

Introduction

The determination of uric acid dissolved in human fluids is required in diagnosing of disorders such as gout, hyperuricemia, and Fanconi syndrome, which are caused by failures in the purine catabolism [1]. Uric acid sensors have been developed since 1970s, with uricase, referred to here as UOx, employed as a molecular recognition element [2] and based on the decrease in oxygen level or increase of CO_2 level. The equation of the enzymatic reaction catalyzed by uricase is:

$$\text{Uric acid} + O_2 + H_2O \xrightarrow{\text{uricase}} \text{allantoin} + H_2O_2 + CO_2 \quad (1)$$

An alternative strategy is the amperometric method for oxidase enzymes. For uric acid, use can be made of the current produced by anodic oxidation of H_2O_2 in the following reaction:

$$H_2O_2 \rightarrow O_2 + 2H^+ + 2e^- \quad (2)$$

One problem with this approach though is that uric acid itself is oxidized at electrodes such as platinum (Pt), gold (Au), and carbon at potentials that are sufficient to oxidize H_2O_2 [2–5]. Several methods have been employed to overcome this difficulty. Kuwabata et al. [6] immobilized uricase together with cystamine, 2-aminoethane-thiolate, and glutaraldehyde on Au and used $\left[Fe(CN)_6^{3-}\right]$ as electron-transfer mediator so that H_2O_2 could be detected at low potentials. Miland et al. [5] mixed uricase and horseradish peroxidase (HRP) into carbon paste and covered the electrode surface with poly(*o*-aminophenol). Then, H_2O_2 could be detected at 0.05 V without an electron-transfer mediator. Luo et al. [7] immobilized uricase with glutaraldehyde as cross-linking agent onto the surface of iridium-modified carbon electrodes using the casting method. H_2O_2 detection was carried at low potential of 0.25 V to eliminate effects of the interference from the

This paper is dedicated to the memory of Francisco C. Nart.

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electroactive biological species, such as ascorbic acid (electrode sensitivity at 16.60 μA mM^{-1}). Hoshi et al. [8] prepared amperometric sensors using a membrane polyelectrolyte multilayer method. They deposited UOx and poly(allylamine) (PAA) alternately on the Pt electrode modified with permselective films consisting of two bilayers of alternated PAA and poly(vinyl sulfate) (PVS) deposited with the layer-by-layer (LbL) method [9]. H_2O_2 could pass through freely, but uric acid could not permeate. Shaolin [10] and Uchiyama and Sakamoto [11] immobilized uricase on a carbon or Pt electrode coated with a conducting polymer film. Reports have also been made of detection of uric acid in the presence of interferents with modified electrodes that did not contain uricase [12, 13]. For example, Kalimuthu et al. [12] detected uric acid in the presence of ascorbic acid using cyclic voltammetry and a self-assembled sub-monolayer of hetero-aromatic dithiol, 2,5-dimercapto-1,3,4-thiadiazole (DMcT), on a Au electrode. Safavi et al. [13] reported the simultaneous determination of dopamine, ascorbic acid, and uric acid using differential pulse voltammetry in a carbon ionic liquid electrode. Although detection of uric acid can be made in the presence of some interferents, selectivity is still poor due to the absence of an enzyme that would ensure specific interactions.

The advantages of the LbL method for immobilizing biomolecules are now well known, stemming basically from the possible minimization of protein denaturing, as mild conditions are used to form the films [14, 15]. In this paper, we prepared and characterized amperometric biosensors for uric acid using LbL films from UOx alternated with poly(ethylene imine) (PEI) or poly(diallyldimethylammoniumchloride) (PDAC). The LbL films were adsorbed onto ITO electrodes previously modified with a Prussian Blue (PB) layer, which has been used as electrochemical mediators for catalysis of H_2O_2 reduction in oxidase enzyme-based biosensors [16]. The detection of H_2O_2 could be carried out at 0.0 V, thus avoiding oxidation of uric acid. The proposed architectures for the biosensor are ITO/PB/$(PEI/UOx)_{(n)}$ and ITO/PB/$(PDAC/UOx)_{(n)}$.

Experimental section

Reagents and solutions

UOx from *Bacillus fastidiosus* (15 U/mg), uric acid (MW= 168.11), and the polyelectrolytes PEI and PDAC were purchased from Sigma-Aldrich. The chemical structures of the polymeric materials are shown in Fig. 1. All other reagents were of analytical grade and used without further purification. UOx was dissolved in sodium borate buffer 0.1 mol/l (pH 9) at a concentration of 2.5 mg/ml and then diluted to 0.2 mg/ml in a phosphate buffered saline solution (PBS), pH 7.4. PBS was prepared with dibasic sodium phosphate (6 mmol/l) and sodium chloride (150 mmol/l), with the pH adjusted to 7.4 with monobasic potassium phosphate (150 mmol/l). A stock uric acid solution was prepared in NaOH 1 mol/l at a concentration of 0.01 mol/l and then diluted to 0.001 mol/l in PBS, with pH adjusted to about 7.4 with HCl 1 mol/l. Both PEI and PDAC were dissolved in PBS at a concentration of 1 mg/ml.

Layer-by-layer films

PDAC/UOx and PEI/UOx LbL films were assembled onto quartz slides for ultraviolet-visible (UV-vis) and fluorescence spectroscopy measurements and onto ITO-coated glass (indium–tin oxide, one-side coated on glass by Delta Technologies), previously modified with a PB layer [17] for electrochemistry measurements. These pairs of materials were suitable for LbL film formation, as the isoelectric point of UOx is 5.4, and therefore, the enzyme is negatively charged at pH 7.4, while PDAC and PEI are positively charged. The procedure for preparing LbL films was as follows: The first layer of polymer was prepared by immersing the substrate in the PEI or PDAC solution containing 1 mg/ml in PBS for 5 min at room temperature, T=22 °C. For the second layer, the substrate coated with the polymer layer was immersed into the 0.2 mg/ml UOx solution for 10 min at T=10 °C, as this temperature did not affect enzyme activity. Each immersion was followed by

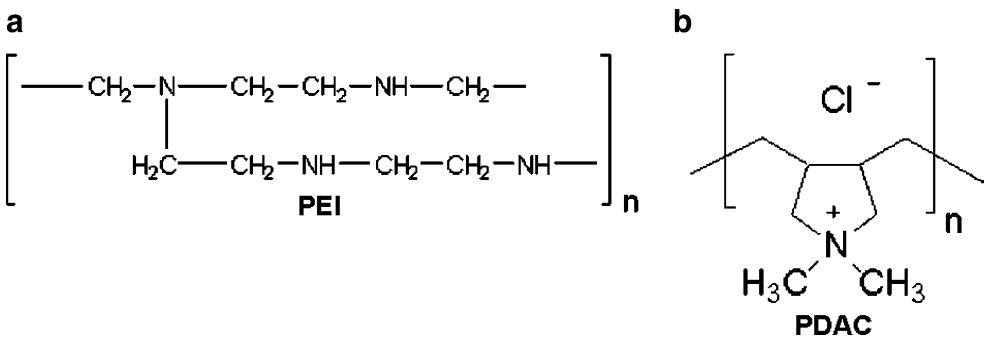


Fig. 1 Chemical structures of polyelectrolytes used

washing with PBS to remove the excess of non-adsorbed molecules. This procedure was repeated until the desired number of PDAC/UOx or PEI/UOx bilayers were deposited. The multilayer growth was monitored with UV-vis spectroscopy (Hitachi U-2001) and fluorescence spectroscopy using a Shimadzu RF-5301 PC Spectrofluorimeter, with excitation at 280 nm. A neat quartz substrate was used as reference.

Electrochemical measurements

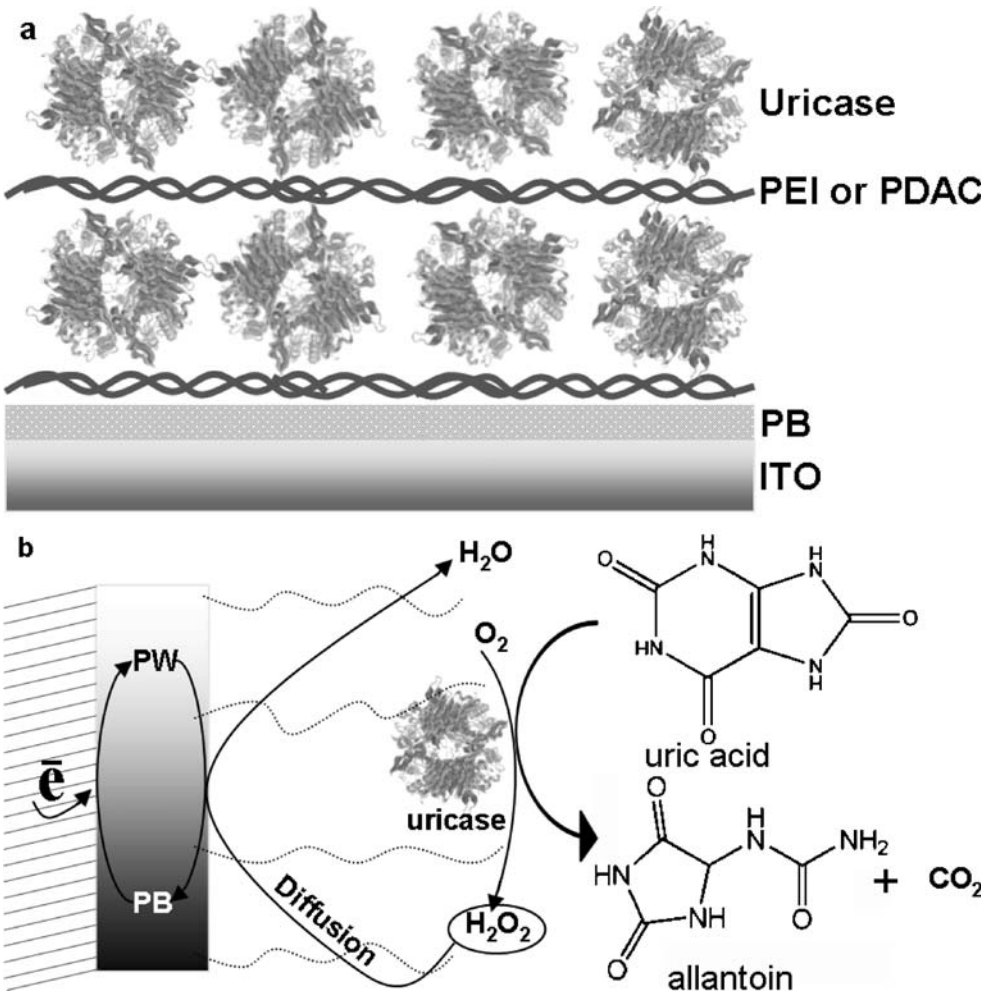
Amperometric measurements were performed at 0.0 V [vs satured calomel electrode (SCE)] in a conventional three-electrode electrochemical cell (10 ml) containing PBS (pH= 7.4) as electrolyte, at room temperature, using an Autolab PGSTAT 30. The reference electrode was $Hg/Hg_2Cl_2/KCl_{(sat.)}$ (SCE); a 1.5 cm^2 Pt foil was used as auxiliary electrode, and the working electrode was the LbL film on ITO modified with a PB layer as follows: ITO/PB/$(PEI/UOx)_n$ or ITO/PB/$(PDAC/UOx/)_n$. The PB film was potentiostatically deposited onto ITO at a potential of +0.40 V (vs Ag/AgCl electrode) during 60 s from aqueous 2×10^{-3} mol/l $K_3[Fe(CN)_6]+2\times10^{-3}$ mol/l $FeCl_3$ solutions in 0.1 mol/l KCl+0.01 mol/l HCl. After deposition, the modified electrodes were rinsed with Milli-Q water and immersed into a solution containing 0.1 mol/l KCl+0.01 mol/l HCl, where the electrode potential was cycled between 0.0 and 1.0 V vs Ag/AgCl electrode) at a scan rate of 0.05 V/s, until a stable voltammetric response was obtained [16, 18].

The potential of each biosensor was kept at the operating value 0.0 V allowing the background current to decay to a steady-state value before the amperometric experiments for each addition of uric acid (100 μl, 0.001 mol/l) under stirring. Usually, 1 min elapsed before the measurements were taken, to get a stable signal. The effect from interferents and the stability of the biosensor were also studied.

Results and discussion

Electrode preparation

Scheme 1 shows the idealized structure of an LbL film containing the enzyme immobilized onto ITO, previously modified with PB (a). The mechanisms for detection of uric



Scheme 1 Schematic architecture of the PEI or PDAC/uricase multilayers onto ITO modified with a PB layer (**a**). Mechanisms involved in detection of uric acid with generation of H_2O_2 (**b**)

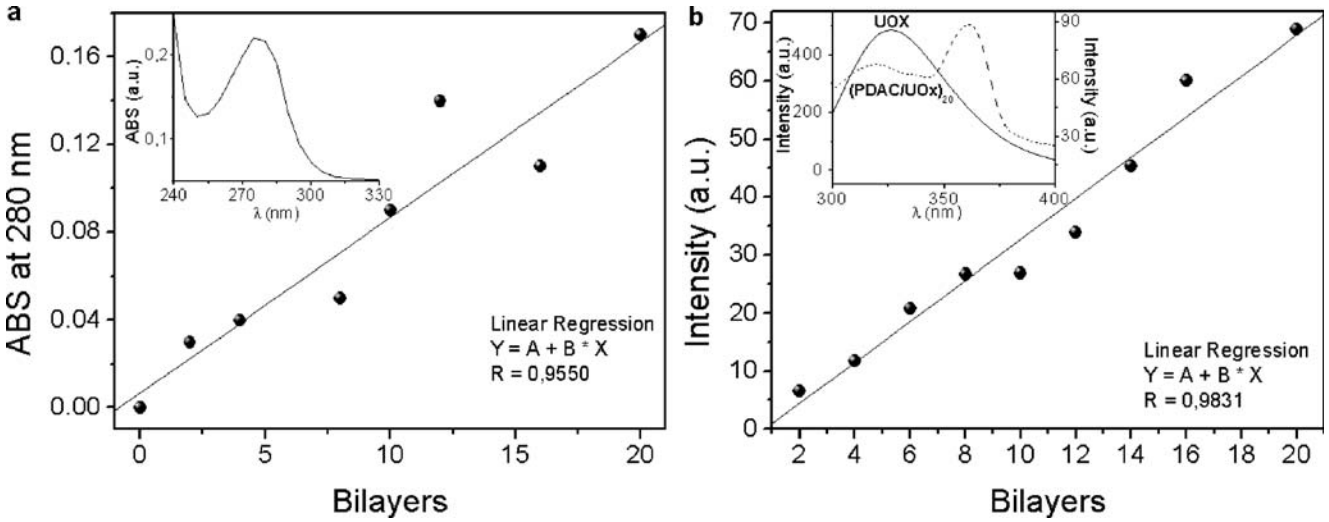


Fig. 2 **a** Increase in the absorption at 280 nm for a PDAC/UOx film as a function of the number of deposited bilayers. Absorption spectra of UOx in solution (*inset*). **b** Increase in the emission intensity at 320 nm for a PDAC/UOx film as a function of the number of deposited bilayers. Fluorescence spectra (*inset*) of a 20-bilayer PDAC/UOx film (*broken line*) and UOx in solution (*solid line*). Excitation wavelength at 280 nm and a quartz substrate used as reference

acid with generation of H_2O_2 are depicted in Scheme 1b. PB films can be reduced to the colorless form, referred to as Prussian White (PW) [16], according to Eq. 3:

$$KFe^{III}Fe^{II}(CN)_6 + e^- + K^+ \rightarrow K_2Fe^{II}Fe^{II}(CN)_6 \qquad (3)$$

where Fe^{III} and Fe^{II} are the oxidation states of Fe atoms in the PB structure. In subsidiary experiments, we observed that bare ITO and ITO modified with PB used in amperometric measurements failed to detect uric acid.

Film growth

The growth of LbL films was monitored with UV-vis absorption and fluorescence spectroscopies, whose results appear in Figs. 2 and 3 for PDAC and PEI polyelectrolytes, respectively. For both films, the almost linear increase at the maximum absorption (280 nm) with the number of bilayers indicates that the same amount of material was probably adsorbed in each deposition step. The differences in the amount of material adsorbed may be associated with the

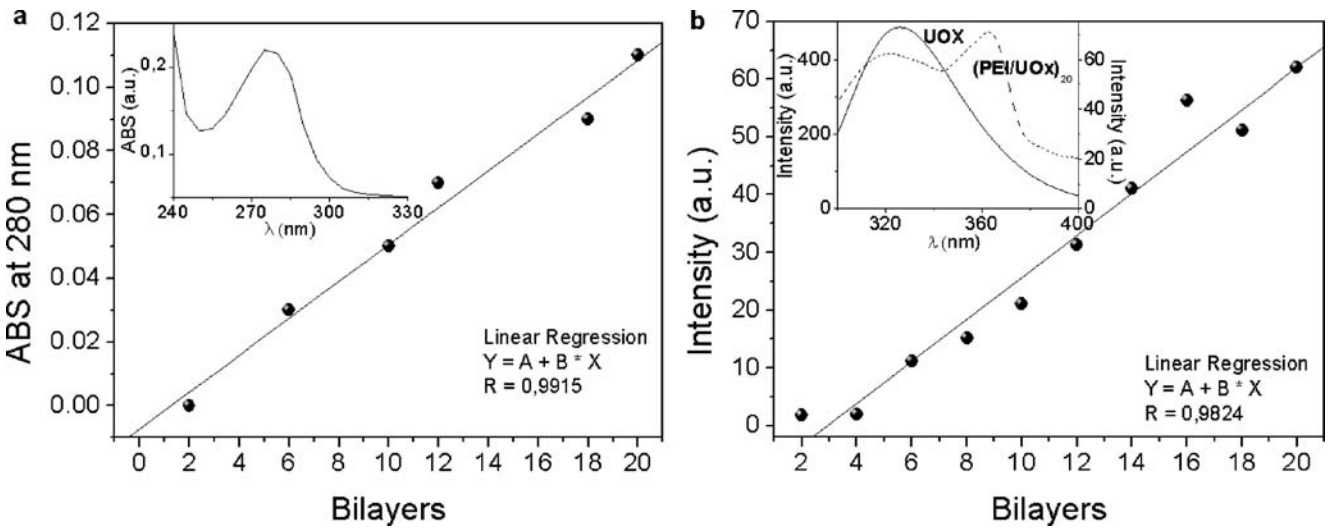


Fig. 3 **a** Increase in the absorption at 280 nm for a PEI/UOx film as a function of the number of deposited bilayers. Absorption spectrum of UOx in solution (*inset*). **b** Increase in the emission intensity at 320 nm for a PEI/UOx film as a function of the number of deposited bilayers. Fluorescence spectra (*inset*) of a 20-bilayer PEI/UOx film (*broken line*) and UOx in solution (*solid line*). Excitation wavelength at 280 nm and quartz substrate used as reference

distinct structures of the polyelectrolytes used in conjunction with UOx to build the films. Film growth was also confirmed by plotting the fluorescence intensity at 320 nm, attributed to phenylalanine and tryptophan chromophores in the enzyme, vs the number of bilayers of UOx/PDAC and UOx/PEI in Figs. 2b and 3b, respectively. The insets show the fluorescence spectra of UOx in solution and immobilized in LbL films.

Amperometric measurements

The amperometric response is shown in Fig. 4 for a biosensor built with 10 bilayers (i.e., ITO/PB/(PDAC/UOx)$_{10}$ and operating at 0.0 V (vs SCE) in a PBS buffer solution at pH 7.4. The biofunctionality of UOx and the feasibility of the method for biosensing are demonstrated by the increase in reduction current upon addition of successive aliquots of uric acid. Each addition corresponds to an increase of 100 μl of 1×10^{-3} mol l^{-1} uric acid in 10 ml (PBS) of supporting electrolyte. A similar response was obtained for the biosensor using PEI as polyelectrolyte, and the data were therefore omitted. It should be noted in Fig. 4 that the sensor responds fast to addition of uric acid—with a response time estimated to be about 2 and 6 s for biosensors containing PDAC and PEI, respectively. The difference between the two biosensors may be associated with wrapping of the molecules in the LbL film and with distinct conformations of the enzyme, as it is known that the scaffolding material affects enzyme conformation (see, for instance, comparison of glucose oxidase in polyaniline [16] and in chitosan [19]). In addition, the slower response for the PEI/UOx biosensor could be related to the differences in adsorption indicated in Figs. 2 and 3. In fact, the most likely reason for the differences is the conformation adopted by the two polyelectrolytes both in solution and in the films. For PEI is expected to form a globular structure, similarly to poly (allylamine hydrochloride), while PDAC should adopt a helical structure as in collagen.

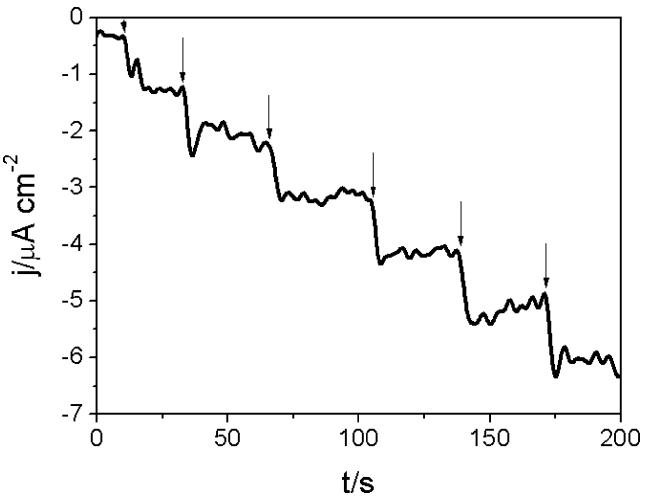


Fig. 4 Amperometric response obtained at 0.0V in a buffer solution at pH 7.4 with a biosensor ITO/PB/(PDAC/UOx)$_{10}$ 10-bilayer LbL film. Each current step corresponds to an increase of 1×10^{-3}mol l^{-1} in uric acid

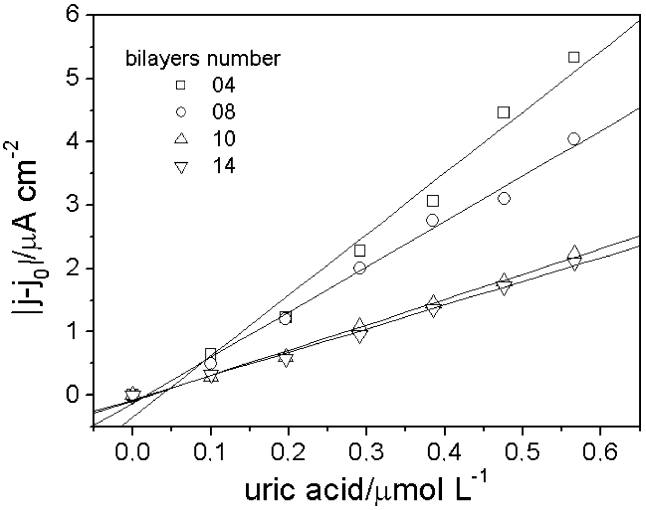


Fig. 5 Analytical curves showing the linear range for UOx/PEI LbL films with 4 (*square*), 8 (*circle*), 10 (*triangle*), and 14 (*inverted triangle*) bilayers

Figure 5 shows the analytical curves for ITO/PB/(PEI/UOx)$_n$ biosensors, with n varying from 4 to 14. The sensitivity decreased with the number of bilayers, probably because thicker films hindered H_2O_2 diffusion when uric acid was injected. The limit of detection was about 0.15 μA μmol l^{-1} cm^{-2} (three times the noise) for a 10-bilayer LbL film. Similar results were obtained using the PDAC polyelectrolyte with a slightly higher detection limit of 0.36 μA μmol l^{-1} cm^{-2}. The main difference between the performance for the distinct polyelectrolytes was on the stability, as will be discussed later.

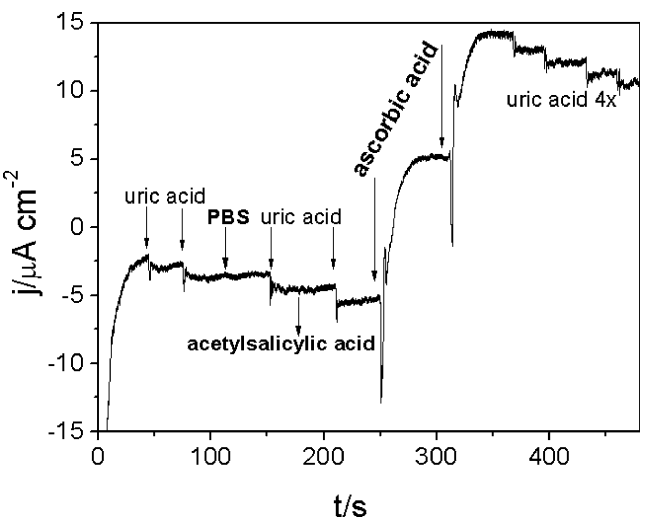


Fig. 6 Effect of the addition of PBS, acetylsalicylic acid and ascorbic acid

Our results can be compared to those of Hoshi et al. [8] who produced biosensors for uric acid in which UOx was immobilized on a Pt electrode and a PAA/PVS LbL film functioned as selective for H_2O_2. The biosensors reported here are advantageous because detection was performed at 0.0 V vs the reference electrode (as ITO was modified with a PB layer), in contrast to +0.6 V employed by Hoshi et al. [8]. In any case, both of these biosensors are simpler than others reported in the literature, in which H_2O_2 detection is carried out with HRP or mediators [3–5]. In fact, using the LbL method to produce biosensors has been proven excellent [20] not only because enzymes have their activity preserved for long times due to the mild film-forming conditions but also because a smaller amount of enzymes is required in comparison with cross-linking methods [21].

The sensitivity achieved in the biosensors made with PDAC/UOx and PEI/UOx LbL films, with a linear response between 0.1 and 0.6 μM of uric acid, is sufficient for these biosensors to be used in detecting uric acid in serum. Indeed, reported uric acid concentrations in serum were 0.305±0.066 (SD) mM in 2,283 men and 0.239± 0.056 (SD) mM in 2,844 women [1].

Effects from interferents

As already mentioned, effects from interferents represent a major difficulty for the suitability of a biosensor. With the architecture used for the biosensors studied here, comprising a PB layer below the LbL films, detection was performed at 0.0 V vs SCE, which helped prevent interferent effects. This is demonstrated in the results depicted in Fig. 6, from experiments where the unbiased electrodes (0.0 V vs SCE) were first subjected to 0.1 mmol l^{-1} uric acid solution until the current reached a steady-state value and then injections were made of 1 mmol l^{-1} of each of the interferents, indicated by arrows in the figure. It is readily seen that the interferents caused no change in current, with the exception of ascorbic acid, which made the current to increase rather than decrease as it occurs for uric acid. Therefore, although ascorbic acid gives a signal, it can still be distinguished from uric acid.

Biosensor stability

The stability of the biosensors was investigated during 30 days. The sensors were stored in PBS buffer (pH 7.4) at 10 °C when not in use. For the ITO/PB/$(PEI/UOx)_n$ system, the response was unaltered even 10 days after the biosensor was built. After the 11th day, the current gradually decreased to 30–40% the original value at the 30th day. For the biosensor ITO/PB/$(PDAC/UOx)_n$, the current decreased gradually from the first day, reaching 70% of the original value at the 30th day. In addition to a possible denaturing of the immobilized UOx, the decrease in activity may be explained by the phosphate from PBS—in which solution the sensing device was stored—diffusing through the film and destroying the PB structure. Then, only the ferrocyanide acts as mediator, which decreases the biosensor performance because it cannot electrocatalyze the decomposition of hydrogen peroxide at 0 V. Note that in spite of the change in response, the sensor can still be used, as the response degradation does stabilize after the 30th day. All measurements were made in duplicate and the stability tests confirmed the film reproducibility.

Conclusions

It has been demonstrated that uricase (UOx) may be efficiently immobilized with two types of polyelectrolyte in LbL films, in which the enzyme preserves its activity for several weeks. The molecular architecture of the films played an important role, particularly because an initial PB layer deposited before the polyelectrolyte/UOx bilayers allowed the films to be used in sensing uric acid, with hydrogen peroxide being detected at 0 V vs SCE. Furthermore, the biocatalytic activity depended on the structure of the polyelectrolyte. More robust electrodes were obtained with PDAC alternated with UOx, while those containing PEI/UOx were more stable with the time. Significantly, the biosensors' sensitivity is sufficient for applications in clinical tests to detect uric acid, with negligible effects from interferents.

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