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Amperometric cholesterol biosensors based on the electropolymerization of pyrrole and the electrocatalytic effect of Prussian-Blue layers helped with self-assembled monolayers

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Abstract

Three cholesterol biosensor configurations based on the formation of a layer of Prussian-Blue (PB) on a Pt electrode for the electrocatalytic detection of the H_2O_2 generated during the enzymatic reaction of cholesterol with cholesterol oxidase (ChOx) were constructed. The enzyme was entrapped within a polypyrrole (PPy) layer electropolymerized onto the PB film. The influence of the formation of self-assembled monolayers (SAMs) on the Pt surface on the adherence and stability of the PB layer and the formation of an outer layer of nafion (Nf) as a means of improving selectivity were both studied. A comparative study was made of the analytical properties of the biosensors corresponding to the three configurations named: Pt/PB/PPy-ChOx, Pt/SAM/PB/PPy-ChOx and Pt/SAM/PB/PPy-ChOx/Nf. The sensitivity (from 600 to 8500 nA mM^{-1} cm^{-2}) and selectivity of the developed biosensors permitted the determination of the cholesterol content in reference and synthetic serum samples. The detection limit for the Pt/SAM/PB/PPy-ChOx/Nf biosensor was 8 μM. Formation of the SAM on the electrode surface and covering with a Nf film considerably improved the stability and lifetime of the biosensor based on the catalytic effect of the PB layer (as the PB layer was retained longer on the electrode), and the Nf layer protects the enzyme from the external flowing solutions. Lifetime is up to 25 days of use. The formation of the SAM also has an effect on the charge transfer and the formation of the PB layer.

Keywords: Amperometric cholesterol biosensor; Self-assembled monolayers; Prussian-Blue; Electropolymerization; Polypyrrole

1. Introduction

Cholesterol has aroused considerable interest in recent years on account of its being a very important parameter in clinical diagnosis. The clinical analysis of cholesterol in serum samples is important in the diagnosis and prevention of a large number of clinical disorders such as hypertension, cerebral thrombosis, heart attack and artheriosclerosis [1]. However, few biosensors have been developed for cholesterol determination compared with those reported for glucose determination [2,3].

Oxido-reductase enzyme electrodes are a large group of biosensors, accounting for over 90% of the existing amperometric enzyme-based biosensors. The side product of the flavin-oxidase enzymes reactions is usually hydrogen peroxide, formed by the enzyme-catalyzed oxidation of the analyte by dissolved molecular oxygen [4]. Immobilization of the cholesterol oxidase enzyme (ChOx) is one of the most selective methods for determining its substrate, although the signals obtained are usually very small (a few nanoamperes), basically due to the small number of immobilized enzymatic units [5].

Prussian-Blue (PB) is a three-dimensional polymeric network of a ferric hexacyanoferrate(II) complex, and forms electroactive layers after its electrochemical deposition on the electrode surface which are very efficient transducers for hydrogen peroxide [6]. PB is stable, highly active and selective towards hydrogen peroxide reduction in the presence of molecular oxygen, which allows sensing with oxido-reductase enzyme biosensors at reductive potentials of around 0 V. (versus Ag/AgCl) and a great improvement in the sensitivity and selectivity [7].

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The amperometric detection of hydrogen peroxide is normally carried out anodically (e.g. oxidation at +700 mV with a Pt working electrode), but is strongly influenced by many easily oxidizable interferents present in real samples. For this reason, sensitive and selective detection of H_2O_2 with PB using a cathodic biosensing scheme has aroused considerable interest [6,8].

PB was first used in 1994 in amperometric biosensors owing to its attractive electrocatalytic characteristics [9]. Numerous PB-based electrochemical biosensors have since been developed, mainly with glucose oxidase and other oxidase enzymes, for the determination of ethanol, glutamate, D-alanine and choline [10]. To our knowledge, PB has never been used with ChOx for the construction of a cholesterol biosensor.

But one of the main drawbacks of the PB layer is the progressive loss of its catalytic effect due to its rapid desorption from the electrode surface (with oxidizing potentials in particular) and the biosensor lifetimes with PB reported in the literature are usually short [7,11,12], in some cases a matter of hours [13,14]. Moreover, Prussian-White (PW, the reduced redox state of PB at 0.0 V) is thermodynamically unstable on electrode surfaces, and hydroxyl ions (produced in the hydrogen peroxide reduction in neutral media) may solubilize the inorganic polycrystal [6].

In order to overcome this limitation, an attempt was made in this paper for the first time to retain the PB film on the electrode surface with the aid of a self-assembled monolayer. Alkanethiols spontaneously absorb from solutions onto metallic electrode surfaces to form self-assembled monolayers (SAMs), consisting of a single layer of molecules on a substrate with a high degree of orientation, molecular order and packing [15]. These close-packed monolayers may partially inhibit the electron-transfer reactions, but also serve as a basis for anchoring proteins and redox molecules at fixed and controllable distances from the electrode [16,17].

However, the catalytic effect of the PB layer also leads to an increase in the signal due to interference from electroactive species. An improvement on selectivity can be achieved with an additional layer of polypyrrole (PPy), tipically formed by electrochemical oxidation of pyrrole (Py) monomer onto the electrode surface [18]. The electropolymerized PPy layer, in which the enzyme is immobilized, produces a significant exclusion of these interfering species, although in some cases (especially with small thicknesses fabricated in this way to obtain rapid biosensor responses) the exclusion is incomplete and selectivity needs to be improved, for example, by electropolymerising a second excluding layer of o-phenylenediamine or poly(naphthalene) onto the PPy inner conducting layer [19,20].

Another way of improving selectivity uses a nafion (Nf) layer formed by deposition of a small volume of Nf solution in ethanol and its subsequent evaporation. Nf is a commercially available perfluorinated sulphonate ionomer widely used in biosensor making as a protective coating material against electrode fouling and as a support for enzyme immobilisation [9,21]. A Nf layer efficiently excludes molecular and anionic species by charge and/or size from reaching the electrode surface.

In this paper, electrodeposition of the PB layer in amperometric conditions onto a SAM, followed by electropolymerization of Py and entrapment of the enzyme is achieved. Higher sensitivity was observed due to the electrocatalytic reduction of the enzymatically generated H_2O_2. The influence of the SAM on the PB layer was also studied.

Three configurations of enzymatic amperometric biosensors were designed for cholesterol determination, and the analytical performances of all the biosensors were compared. The three biosensors were based on the electrocatalytic properties of the PB layer and the electropolymerization of Py for entrapment of ChOx.

2. Experimental

2.1. Reagents and solutions

Cholesterol oxidase (ChOx, EC 1.1.3.6, C-7149) with a 4.2 units mg^{-1} concentration, from *Pseudonomas fluorescens*, cholesterol (C-8667) and Triton X-100 were all purchased from Sigma (St. Louis, USA). A stock 0.01 M cholesterol solution was prepared in phosphate buffer (0.05 M., pH = 7) containing 10% (w/w) of Triton X-100 in a thermostated bath at 65 °C. This solution was stored at 4 °C in the dark and was stable for at least 10–15 days (until turbidity was observed). More dilute working solutions of cholesterol were prepared by dilution of the stock solution using a 0.05 M phosphate buffer solution containing 1% (w/w) of Triton X-100. This solution was used as the carrier solution in the FIA. Py and Nf were products of Aldrich (Milwaukee, USA). Py was distilled in vacuo and stored in an inert nitrogen atmosphere. 1-Hexadecanethiol and 1-propanethiol were from Fluka (Milwaukee, USA). Potassium ferricyanide and ferric chloride were supplied by Merck (Darmstadt, Germany). All the other reagents used were reagent-grade. High purity water was obtained from a Milli-Q system (Millipore, Bedford, USA). Synthetic cholesterol serum samples were prepared with the concentrations of interferents indicated in the text. *Accutrol*™ Chemistry control (A-2034, Sigma Diagnostics), a control human serum sample with a certified cholesterol concentration of 3.96 mM, was also used.

2.2. Instrumentation

The electroanalytical experiments for the preparation of the biosensors were performed with a potentiostat modular electrochemical system Autolab (Eco Chemie, Utrecht, The Netherlands), equipped with PGSTAT12 and ECD modules, and driven by GPES software (GPES, V.4.9). A conditioning-cell (ACE-Cell, MF-1021), and a thin-layer cell (model LC-4), with a dual-Pt working electrode block

(3 mm diameter), an Ag/AgCl (3 M NaCl) reference electrode and a Pt auxiliary electrode, were from Bioanalytical Systems (BAS, West Lafayette, USA). All the potentials cited in this paper refer to Ag/AgCl reference electrode. The volume of this cell was controlled by the thickness of the PTFE gaskets employed (20 μL). The amperometric detector used was a Coulochem II (ESA, Bedford, USA). Other components of the system included a Minipuls 3 peristaltic pump (Gilson, Villiers le Bel, France) and an injection valve (model 7125, Rheodyne, USA) equipped with a 100 μL sample loop.

2.3. Preparation of the cholesterol biosensor

Carefully cleaning of the Pt electrode surface prior to fabrication of the biosensor was necessary. Organic contaminants were removed with a strong "piranha" oxidant solution for 2 min (1:3 mixture of 30% hydrogen peroxide and concentrated sulfuric acid at ca. 100 °C) (*caution*: this mixture reacts violently with organic material and may explode when stored in closed containers!) and then washed with bi-distilled water. The electrode was then polished with 1 μm diamond slurry on a nylon surface, polished with 0.05 μm alumina slurry and washed again with bi-distilled water.

The working electrode block was then immersed in an ultrasound bath for 5 min to completely remove adsorbed slurry particles and cleaned with a soft wet ethanol cloth. All these steps are important to prevent holes from appearing in the SAM due to accumulated redox reaction products.

The Pt electrode surface was first covered with a SAM film from a solution of 1 mM in 1-propanethiol in water for 2 h, rinsed with distilled water several times to remove any physically adsorbed molecules and dried in a N_2 stream.

PB was amperometrically deposited from a solution containing 2 mM in $FeCl_3$, 2 mM in $K_3Fe(CN)_6$, 0.1 M in KCl and 0.1 M in HCl. As soon as the Pt/SAM electrode was formed it was immersed in this solution and a working potential of +400 mV applied for 30 s. The PB layer was then activated in a 0.1 M KCl solution with cyclic voltammetry scans ranging from −0.05 to +0.35 V (50 mV s^{-1}, 25 scans).

An electropolymerized layer of PPy was formed on the PB from a 0.4 M Py monomer solution containing 10 units mL^{-1} of cholesterol oxidase enzyme. The ionic strength of the solution was adjusted with a 0.1 M KCl and 0.05 M. phosphate buffer solution. A constant potential of +0.750 V versus (Ag/AgCl 3 M) reference electrode was then applied until the total current generated corresponded to a 10 mC cm^{-2} film growth (PPy thickness of about 20–25 nm).

An outer protective layer consisting of a Nf film on the PPy-enzyme layer was formed by dropping 10 μL solution containing 0.5% (v/v) of Nf (90:10) ethanolic solution over the Pt/SAM/PB/PPy-ChOx surface, which was left to dry (about 15 min). The biosensor was then stored at 4 °C in 0.05 M phosphate buffer solution (pH = 7.05) for 24 h before use.

The biosensor electrode must be conditioned before cholesterol is analysed in a Flow-Injection Analysis manifold (FIA). A −0.05 V potential was applied to the working electrode for this purpose until the anodic background current decreased to under 30 nA.

2.4. Cholesterol determination in an artificial serum sample

The amperometric determination of cholesterol was performed in a FIA system at −50 mV, a potential corresponding to the electrochemical reduction of the hydrogen peroxide. A phosphate buffer solution (pH = 7; 0.05 M) with 1% (w/w) Triton X-100 was used as a carrier at a flow rate of 0.20 mL min^{-1}. The sample volume injected was 100 μL, and the detection-cell volume was 20 μL. The standard addition method was used with injection of the same volume of the sample solution (increments of 0.05 mM in cholesterol) and additional volumes of cholesterol at a known concentration were added in triplicate. The current obtained was found to be limited by the kinetics of the enzymatic reaction and not by diffusion of the substrate towards the enzymatic layer or hydrogen peroxide to the PB layer. The biosensors were stored in a phosphate buffer solution (pH = 7.0; 0.05 M) at 4 °C when not in use.

3. Results and discussion

As can be seen in Fig. 1, three different configurations were studied in order to compare the performance of the three cholesterol biosensors, for which the following nomenclature was used: Pt/PB/PPy-ChOx for the basic enzymatic biosensor with the PB layer, Pt/SAM/PB/PPy-ChOx, the same but with a SAM layer on the Pt surface and Pt/SAM/PB/PPy-ChOx/Nf, the same as the second configuration but with an additional outer Nf layer on the electropolymerized PPy layer. The results and discussion for the three are below.

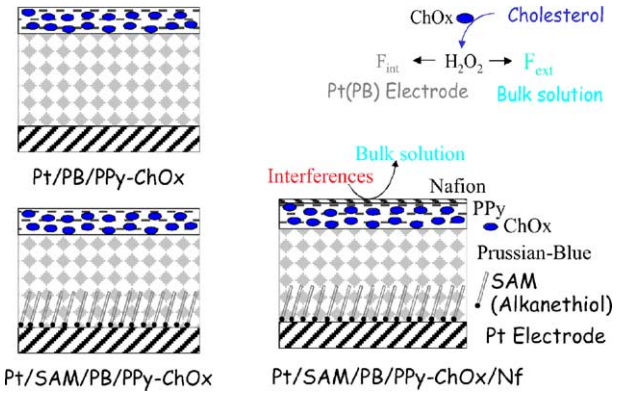

Fig. 1. Scheme of the three configurations studied for the cholesterol amperometric biosensors.

3.1. Pt/PB/PPy-ChOx biosensor configuration

The first biosensor tested was a Pt/PB/PPy-ChOx configuration with the enzyme entrapped within the outer layer of the conducting PPy polymer which made diffusion of the cholesterol across the porosity and thickness of the polymer easy and provided an ideal environment for the enzymatic reaction.

PB-based sensors were tested first to detect the hydrogen peroxide in both anodically (+0.6 V) and cathodically (0 and −0.05 V) [18]. Currents dependent on the cholesterol concentration were obtained in all cases, and they all were kinetically limited by the enzyme reaction. As reported in the bibliography, although PB is electrocatalytic for the reduction of both oxygen and hydrogen, the dissolved oxygen in the measured solutions has no effect on biosensor response (that is oxygen does not compete with reduction of the hydrogen peroxide) at the above-mentioned cathodic potentials [6,18].

Electrolysis times of 15, 30 and 60 s at a constant potential of +0.4 V were tested in the PB formation in order to determine the influence of the thickness. A slight increase in signal was observed in the resulting biosensor when 30 s was used instead of 15 s. The cyclic voltammograms in a 0.1 M KCl solution recorded once the PB layer had formed can be seen in Fig. 2 for each of the three electrolysis times tested. Electroformation of PB at a constant potential for 60 s did not improve the total charge contained in the anodic and cathodic currents of the cyclic voltammograms of PB, and the distribution of the PB layer (deep blue) on the Pt surface was not homogeneous whereas it was much more uniform when an electrolysis time of 30 s was used. Even less adherence of the PB layer were observed in this last case. The preparation of PB by cyclic scans with potentials ranging from −50 to +400 mV and scan rates of 40 mV s^{-1} was also tested with no noticeable improvement, and so the amperometric procedure was selected on account of its simplicity and the optimum results obtained. With an electrolysis time of 30 s the total amount of deposited PB layer onto the bare electrode was about 2–3 nmol cm^{-2}, according to the faradic measured current, if it is assumed a transfer of 4 electrons per unit cell.

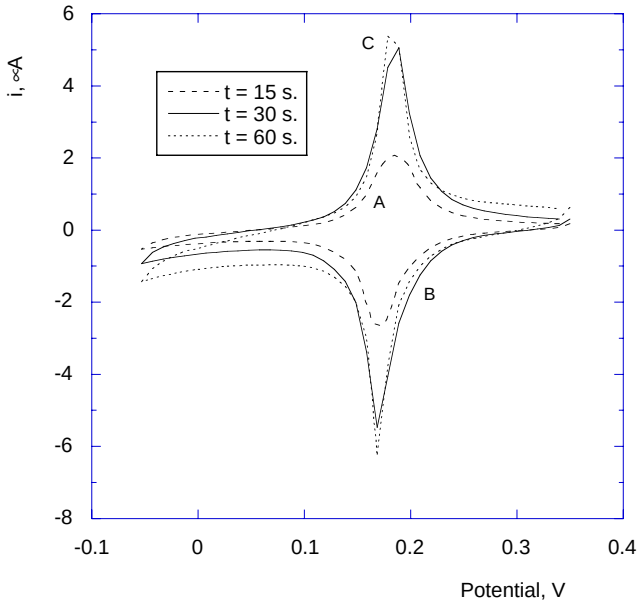

Fig. 2. Cyclic voltammograms of the PB layer recorded immediately after their amperometric formation ($E = +400$ mV) by using electrolysis times (t) of: (A) 15 s; (B) 30 s; (C) 60 s.

Electropolymerization of Py occurs at positive potentials (above 0.7 V), whereas electrodeposition of PB occurs at cathodic potentials. In theory it should be possible to control both processes independently, and by cycling the potential both films can be simultaneously deposited from an initial solution containing all the required monomers. So PPy and PB can both be simultaneously deposited in cyclic voltammetric conditions [22]. This procedure involves the preparation of a solution containing the Py monomer, the entrapped enzyme and the acid solution of the PB precursors. This alternative method for simultaneous formation of the corresponding composite was tested, but because of the acidity needed to form PB (ferric ions are easily hydrolized, and the hydroxyl ions cannot be substituted in their coordination sphere in course of PB crystallization), it is incompatible with ChOx stability for the correct formation of the PB layer, then the results obtained for the resulting cholesterol biosensors were poor.

As reported by other authors [23], electrochemical activation pretreatment of the PB layer prior to formation of the PPy is necessary for improving reproducibility. The pretreatment was carried out by cycling the potential between −0.05 and +0.35 V. at a scan rate of 50 mV s^{-1} for 25 full cycles, in a 0.1 M KCl electrolyte at pH = 7.

The electropolymerization of PPy is easily achieved over the cubic lattice of PB, which acts as a three-dimensional catalyst, and so a short electropolymerization time (around 30 s for a PPy thickness of about 20–25 nm) was required. In the short time needed for the formation of PPy in these conditions the amount of PB that can be oxidized to Berlin Green (also named “Prussian-Yellow”, the oxidized state of PB lacking the electrocatalytical properties of PB and more soluble which leads to electrode loss) is very small. The formation of Berlin Green takes place at potentials higher than +600 mV, whereas PPy formation occurs at approximately +750 mV. By comparing the peaks of the cyclic voltammograms of the PB film before and after the electropolymerization of the Py step, the quantity of PB oxidized to Berlin Green can be evaluated in less than 0.5% percentage.

Electropolymerization should be started immediately after mixing the Py monomer with the enzyme solution, on account of the poor stability of the monomer and because a long delay could cause enzyme denaturation. This solution is deoxygenated and using a glass vessel especially designed for the working electrode PEEK (polyether–ether–ketone) support block (dimensions of 25 mm × 25 mm × 9 mm) the

Table 1
Analytical performance of the Pt/PB/PPy-ChOx cholesterol biosensor with a PB electrocatalytic layer

Potential of the electrode (mV)	−50	0	+600
I_{max} (nA)	−120	−75	40
Linearity (mM)[a]	0.025–0.300	0.025–0.350	0.025–0.350
K_M^{app} (mM)[b]	0.08	0.14	0.28
Lifetime (days) – (no. of injections)	9 (335)	10 (375)	10 (375)
Sensitivity (nA mM^{-1} cm^{-2})	3253	1768	441
Reproducibility (% CV)[c]	3.5	2.3	2.5
Detection limit (mM)[d]	0.008	0.009	0.01
Relative error (%)[e]	1.2	−0.8	1.1
Relative error (%)[f]	1.3	−0.8	1.2

Results with three different amperometrically applied potentials are compared: at −50, 0 mV (cathodic) and at +600 mV (anodic potential). The currents correspond to the reduction or oxidation of the H_2O_2 enzymatically generated.

[a] For $r > 0.9990$.

[b] K_M^{app}: Michaelis apparent constant.

[c] For a 0.20 mM cholesterol solution.

[d] Expressed as $3s_{y/x}$/sensitivity.

[e] Free cholesterol content: 3.98 mM (synthetic serum sample).

[f] Total cholesterol content reported: 3.96 mM ("*Accutrol*™").

volume needed was only 1.15 mL, which is important if the high cost of ChOx is taken into account. The solubility of cholesterol oxidase was good when mixed with the Py monomer solution. Another alternative method tested for constructing PPy onto the PB layer tested was a cyclic scan ranging from 0 to +800 mV at a rate of +50 mV s^{-1}. No improvement in the characteristics of the resulting biosensor were observed with this method and reproducibility was even worse if compared with similar thicknesses (total charge in the electropolymerization step of Py of 10 mC cm^{-2}) [25].

It was observed that the biosensor should be allowed to stand for at least 24 h after preparation to allow natural conditioning of the enzyme inside the PPy structure (usually the first day the electrode give small signals). The analytical characteristics of the Pt/PB/PPy-ChOx biosensor are given in Table 1.

The influence of the potential for the determination of cholesterol was also studied. Results are shown in Fig. 3 with a Pt/PB/PPy-ChOx biosensor, where it can be seen that reductive potentials are more sensitive than oxidative for hydrogen peroxide detection with the PB layer. Similar behaviour was observed with the other biosensor configurations. Cholesterol concentrations ranging from 0.025 to 1.0 mM (0.05 M phosphate buffer at pH = 7.00 and 1% (w/w)) Triton X-100) were injected sequentially into the FIA system, and the currents obtained with applied potentials of −50, 0 and +600 mV are shown in this figure. A cathodic potential of −50 mV is preferably selected for the determination of the cholesterol in real samples (reduction of the hydrogen peroxide) on account of a greater sensitivity.

The Pt/PB/PPy-ChOx biosensor had a short lifetime (not more than 9–10 days) due to the loss of PB which was reduced to a more soluble form, owing to the application of the −50 mV potential which converts PB into Prussian-White (more soluble in solution) [6]. The PB loss can be easily monitored using cyclic voltammetry in a KCl aqueous solution by a decrease of the current peaks of PB (not shown).

3.2. Pt/SAM/PB/PPy-ChOx biosensor configuration

For the second configuration studied (Pt/SAM/PB/PPy-ChOx) a SAM was formed on the electrode prior to the formation of the PB layer. The main purpose was to determine whether the SAM layer permitted the formation of the PB layer and improved adherence of the PB to the Pt electrode surface.

Good electrical contact with the electrode surface through the SAM layer is necessary for the electrochemical formation of the PB layer and so a priori the use of long-chain SAMs is not advisable. On the other hand, different chain lengths of the mercaptane could change adherence of the PB

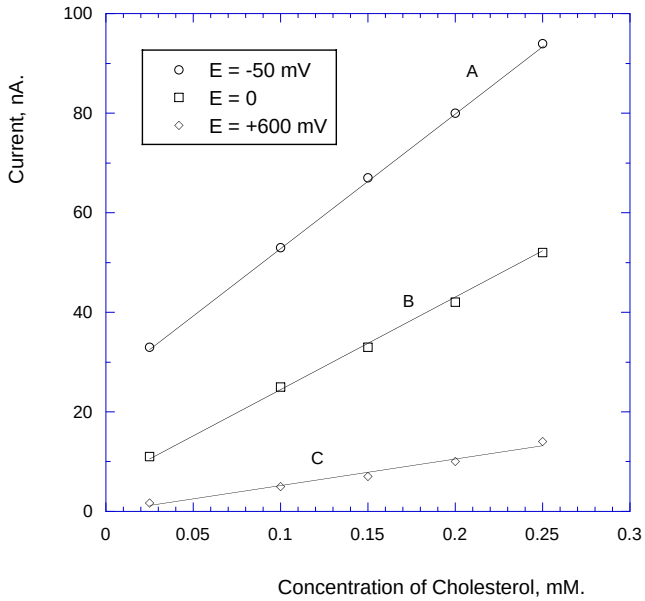

Fig. 3. Influence of the potential on the measured current of cholesterol solutions, with a Pt/SAM/PB/PPy-ChOx: (A) $E = -50$ mV; (B) $E = 0$ mV; (C) $E = +600$ mV. All currents are shown in absolute (positive) values.

layer. Good electronic transfering can easily be evaluated by measuring the total current involved during the formation stage of PB, and adherence can be related to the time in which the measured current is due to the catalytic effect of the PB layer.

When the mercaptane solution first comes into contact with the electrode a disordered monolayer is deposited in a few seconds, but this monolayer is slowly transformed over a period of hours to days into a much more oriented and densely-packed monolayer. This is due to lateral hydrophobic interactions and Van der Waals interactions between the carbon chains to yield a close-packed array of molecules, which produces a monolayer that is oriented 30–35° from the normal surface with an orderly formation of channels [15].

Permeability of the SAM can be evaluated using a marker solution (5 mM $Fe(CN)_6^{3-}$ in KCl 0.1 M) which is detected by cyclic voltammetry. The currents given by the marker solution depends on the permeability of different kinds of SAM preparation. Some results are shown in Fig. 4.

C_3 mercaptanes with high concentrations (e.g. 1-propanethiol 10 mM) and short formation times on the Pt electrode (t_{SAM} = 15 min) gave a disordered arrangement of the monolayer and small voltammetric currents of the marker. On the other hand, low concentrations of C_3 (e.g. 1-propanethiol 1 mM) and longer times (t_{SAM} about 2 h) produced bigger voltammetric currents. Intermediate values of concentration and/or t_{SAM} displayed a gradual intermediate behaviour. In any case, the arrangement should be as ordered as possible but not to hinder to a great extent the charge transfer of the PB precursors with the electrode.

A short alkyl chain will, a priori, produce greater adherence of the PB layer which may be formed in a "mushroom" shape across the spacing of the alkyl chains. SAM layers with shorter chain lengths have also a shorter tunnel distance to the electrode surface, resulting a priori in better charge transfer between electroactive molecules to the electrode surface. Mercaptanes with C_3 and C_{16} chains at a low concentration (1–10 mM) were assayed during the chemisorption time needed for a SAM which permitted charge transfer and proper formation of the PB.

With C_{16} the PB layer is hardly formed because the charge transfer between the diffusional species of Fe^{3+} and $Fe(CN)_6^{3-}$ and the electrode surface is hindered and so C_3 was preferred (permeability through the SAM layer can be drawn from the Fig. 4). The formation of the PB layer was also carried out more easily (shorter times or lower potentials to obtain similar thicknesses of the PB) with the C_3 alkanethiol.

Using a 1 mM solution of 1-propanethiol (C_3) mercaptane, the SAM was developed in 2 h as the best alternative, because of the best stability and sensitivity obtained in the resulting Pt/SAM/PB/PPy-ChOx cholesterol biosensor. The PB layer deposited onto the SAM electrode ranged from 3 to 5 nmol cm^{-2} with an electrolysis time of 30 s. The PPy layer containing the enzyme was then superimposed in the aforementioned conditions in a similar process from that of the biosensor previously prepared without a SAM layer.

The cyclic voltammograms of the PB layer when the biosensor was prepared and after 12 days of use can be seen in Fig. 5, for the two configurations Pt/PB/PPy-ChOx and Pt/SAM/PB/PPy-ChOx. Usually, 40 injections of concentra-

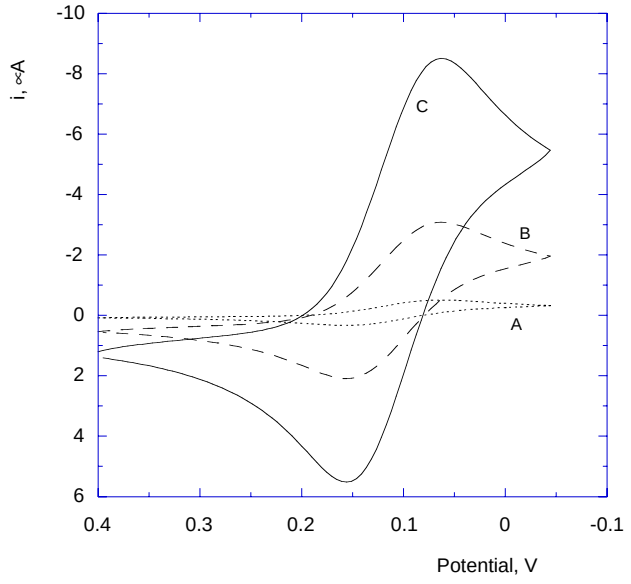

Fig. 4. Cyclic voltammograms of a 5 mM hexacyanoferrate(III) solution on a Pt electrode surface after coating the surface with SAMs: (A) 1 mM of 1-hexadecanethiol, t_{SAM} = 10 min; (b) 5 mM of 1-propanethiol, t_{SAM} = 1 h; (C) 1 mM of 1-propanethiol, t_{SAM} = 2 h.

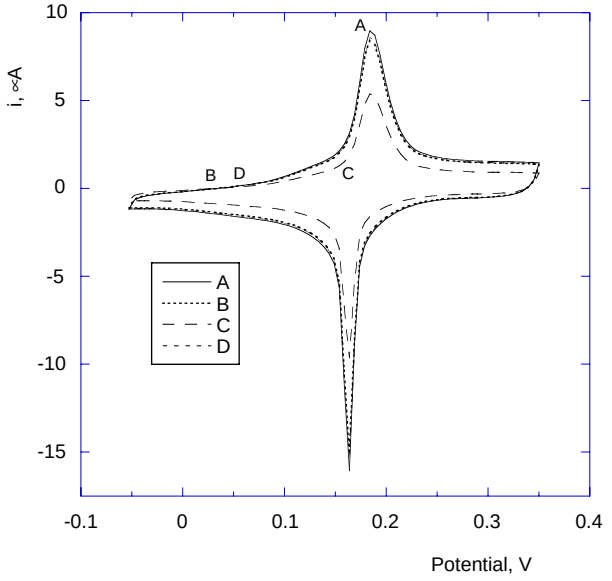

Fig. 5. Cyclic voltammograms of the PB layer in a KCl 0.1 M solution for: (A) a recently prepared Pt/PB/PPy-ChOx biosensor, without having it been used; (B) a recently prepared Pt/SAM/PB/PPy-ChOx biosensor; (C) a Pt/PB/PPy-ChOx biosensor, after 12 days of use (cholesterol determinations, see text); (D) a Pt/SAM/PB/PPy-ChOx biosensor, after 12 days of use. Biosensors were prepared as indicated in the experimental section, and by using 1-propanethiol as the mercaptane.

Table 2
Analytical performance of the Pt/SAM/PB/PPy-ChOx cholesterol biosensor with a SAM film and a PB electrocatalytic layer

Potential of the electrode (mV)	−50	0	+600
I_{max} (nA)	−130	−85	47
Linearity (mM)[a]	0.025–0.300	0.025–0.350	0.025–0.350
K_M^{app} (mM)[b]	0.09	0.13	0.29
Lifetime (days) – (no. of injections)	14 (520)	16 (590)	16 (585)
Sensitivity (nA mM^{-1} cm^{-2})	5714	2857	714
Reproducibility (% CV)[c]	3.5	2.3	2.5
Detection limit (mM)[d]	0.008	0.009	0.01
Relative error (%)[e]	1.4	−0.9	1.3
Relative error (%)[f]	1.6	−0.9	1.2

Results with three different amperometrically applied potentials are compared: at −50, 0 mV (cathodic) and at +600 mV (anodic potential). The currents correspond to the reduction or oxidation of the H_2O_2 enzymatically generated.

[a] For $r > 0.9990$.
[b] K_M^{app}: Michaelis apparent constant.
[c] For a 0.20 mM cholesterol solution.
[d] Expressed as $3s_{y/x}$/sensitivity.
[e] Free cholesterol content: 3.98 mM (synthetic serum sample).
[f] Total cholesterol content reported: 3.96 mM ("*Accutrol*^{TM}").

tions ranging from 0.05 to 0.4 mM of cholesterol were performed each day and the biosensors were used for 4 h daily. With the second configuration the typical cyclic voltammogram reached higher total currents going from PB to PW after using these time, compared with the Pt/PB/PPy-ChOx configuration. Then, the PB layer adhered longer and with electrical contact on the electrode when helped with the SAM layer.

The analytical characteristics of the Pt/SAM/PB/PPy-ChOx biosensor are summarized in Table 2. An improvement in the stability and the biosensor lifetime of the biosensor with the SAM layer was observed with respect to the biosensor without this SAM film (Pt/PB/PPy-ChOx configuration). Sensitivity (around 5700 nA mM^{-1} cm^{-2} at −50 mV.) is also increased due to better anchorage of the PB layer and a better formation of the PB helped with the SAM layer. The lifetime was over 2 weeks, and a decrease in the sensitivity was observed after about 17–20 days of use.

3.3. Pt/SAM/PB/PPy-ChOx/Nf biosensor configuration

The PPy layer in which the entrapped enzyme is immobilised gives the biosensor a degree of selectivity owing to its permselective properties. However, at times PPy was not sufficiently selective to exclude the interference of electroactive species completely and a second polymer layer was frequently used as an additional barrier which easily prevented the diffusion of these species towards the electrode surface [19,20].

Another option is polymer casting onto the electrode, the most commonly used polymers being cellulose acetate or Nf [21]. This solid-state polyelectrolyte has an special ability for reducing the interference of anionic species, like ascorbate and urate. The main advantage of a protective Nf layer compared with a second polymerisation on the PPy is that, apart from simplifying the fabrication, the biosensor does not need to be subjected to oxidising electrical potentials which involve small losses due to oxidation of PB to Berlin Green.

Different Nf thicknesses were tested controlling the volume and concentration of the solution applied directly onto the PPy layer by means of a micropipette. The concentrations studied were 1% (w/v) and 0.5% (w/v) in a EtOH/H_2O (1/5) (v/v) solution, injecting 10 μL in both cases and allowing it to dry at room temperature for at least 15 min. In the first case, cholesterol diffusion across the Nf is prevented owing to the low currents obtained in the cholesterol determination (around 0.05 nA). Sensitivity increased considerably in the second case and so this amount of Nf was used.

The determination of cholesterol was carried out with this biosensor in the previously described conditions and the results are gathered in Table 3. Pt/SAM/PB/PPy-ChOx/Nf had the best analytical performance for cholesterol determination, as can be appreciated from the results gathered in Table 3 for the optimum potential of −50 mV.

Table 3
Analytical performance of the Pt/SAM/PB/PPy-ChOx/Nf cholesterol biosensor. The currents correspond to the reduction of the H_2O_2 generated by the cholesterol

Potential of the electrode (mV)	−50
I_{max} (nA)	−210
Linearity (mM)[a]	0.050–0.300
K_M^{app} (mM)[b]	0.09
Lifetime (days) – (no. of injections)	25 (730)
Sensitivity (nA mM^{-1} cm^{-2})	8572
Reproducibility (% CV)[c]	3.8
Detection limit (mM)[d]	0.012
Relative error (%)[e]	−3.05
Relative error (%)[f]	−5.05

[a] For $r > 0.9990$.
[b] K_M^{app}: Michaelis apparent constant.
[c] For a 0.20 mM cholesterol solution.
[d] Expressed as $3s_{y/x}$/sensitivity.
[e] Free cholesterol content: 3.98 mM (synthetic serum sample).
[f] Total cholesterol content reported: 3.96 mM ("*Accutrol*^{TM}").

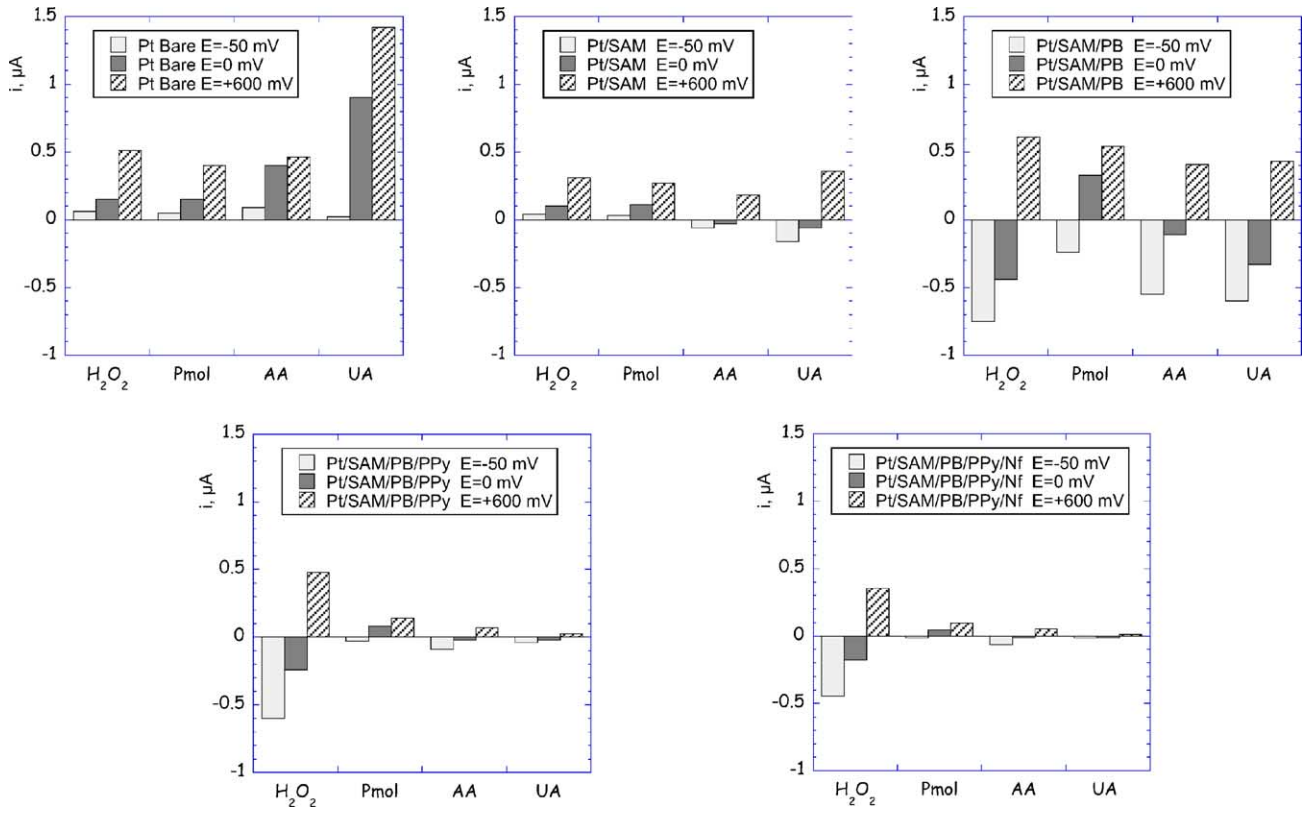

Fig. 6. Currents obtained for hydrogen peroxide (H_2O_2) and for the electroactive interferents acetominophenone (Pmol), ascorbic acid (AA) and uric acid (UA), at potentials of −50 mV, 0 and +600 mV. Plots correspond to the different electrode configuration layers, as described in text.

Improvements in lifetime and selectivity were observed with respect to the other two configurations. The sensitivity of the biosensor was about 8500 nA mM^{-1} cm^{-2} at an applied potential of −50 mV and the biosensor lifetime was around 25 days (750 measurements). Moreover, biosensor selectivity was also better, as explained bellow. An ideal behaviour of the enzymatic systems according to Michaelis–Menten kinetics were observed in all designed biosensors, and the values of K_M are given in the corresponding tables. Values of K_M are very similar with the three configurations at the same working potential.

The Nf layer shields the enzyme from the external solution flow, generates a much smaller and more stable background current, and protects the inner conducting layer of the flow fluctuations of the FIA system. Biosensor lifetime was also extended due to protection of the enzyme. A greater sensitivity was also observed possibly caused by a partial adsorption of the cholesterol onto the Nf layer [24]. Moreover, the sensitivity can be controlled by regulating the thickness of the Nf deposited.

The constructed electrode must also be stored for 24 h at 4 °C in 0.05 M phosphate buffer solution (pH = 7.05) before measurement to accommodate the enzyme within the PPy structure. The best results were obtained when the biosensor was stored at 4 °C in 0.05 M phosphate buffer solution at pH = 7.05. A dry biosensor storage was also tried (at 4 °C), but the biosensor lifetime decreased.

3.4. Selectivity of biosensors

The biosensor selectivity is enhanced by the presence of the successive permselective layers. However, the PB layer may also serve electrocatalytically towards the aforementioned species, facilitating their oxidation or reduction according to the potential applied. To provide information about the electroactivity of these molecules, the currents at potentials of −50, 0 and +600 mV on a bare Pt electrode, Pt/SAM, Pt/SAM/PB, Pt/SAM/PB/PPy and a Pt/SAM/PB/PPy/Nf sensors were measured in order to study the electrocatalysis produced by the PB layer and the effect of the polymeric layers used in the biosensor construction. The results can be seen in Fig. 6.

Great care should be taken with the cleaning and polishing of the Pt surface in order to obtain the most reproducible results possible in the direct electrolysis of the molecules studied and the formation of the SAM and the PB film.

The SAM produces a reductive current at potentials of 0 and −50 mV for UA and AA, while the PB layer generates a greater cathodic current at the same potentials for UA and AA, and acetaminophenone also is reduced at −50 mV. However, the most important conclusion that can be drawn is that the PPy layer and, in particular, the joint use of PPy and Nf cause a significant suppression of the current caused by these interferents.

It should be also pointed out that this degree of exclusion is both inwards and outwards, producing the Nf layer a preconcentration effect towards the interior by decreasing the outward diffusion of the H_2O_2 generated inside the PPy layer in which the enzyme is entrapped [19].

3.5. Cholesterol determination in reference serum samples

A synthetic cholesterol serum sample containing ascorbic acid (AA), uric acid (UA), acetaminophenone (Pmol), cysteine (Cys), glucose (Gl) and bovine serum albumin (BSA) was fabricated to test the reliability of the biosensors. The concentration of the sample components were: 50 mM AA, 0.50 mM UA, 0.01 mM Pmol, 3.98 mM cholesterol, 5 mM Gl and 0.8 g/100 mL of BSA.

The determination was carried out by the standard addition method (3 additions were made of 50 μL for additions of 0.05 mM cholesterol). Concentrations of cholesterol substrate ranging from 0.05 mM to 0.50 mM were determined after dilutions of the samples. All diluted cholesterol standard solutions were prepared daily, from a stock solution of 10 mM of cholesterol.

The biosensors proved to be fairly sensitive for typical cholesterol concentrations in serum samples, and selectivity was also satisfactory with exclusion of the typical interferences. Resulting relative errors are summarized for each biosensor in Tables 1 to 3, these values corresponding to the mean value of 4 independent determinations.

The biosensors were also used for determining the total cholesterol concentration of a certified reference sample of "*Accutrol*™", a lyophilized preparation containing enzymes and analytes in a human serum medium. The concentration in this sample was 3.96 mM as total cholesterol (e.g. 30% of the total cholesterol in free form and 70% in sterified form). A pretreating of the sample with the enzyme cholesterol esterase (*Sigma* reference C-9281) was performed to hydrolyse cholesterol esters in order to determine all cholesterol in its free form. All concentrations measured by the biosensors were consistents with the certified value (results are also shown in Tables 1 to 3).

4. Conclusions

The formation of a PB layer on the Pt surface permits the electrocatalytic detection of the enzymatically generated hydrogen peroxide in a cholesterol biosensor with greater sensitivity. However, the PB on the electrode surface is easily lost and the lifetime of biosensors based on PB is usually short. The formation of SAMs with short chains (C_3 mercaptanes) on the Pt electrode surface and subsequent formation of a PB layer improved PB retention, thereby increasing the biosensor lifetime considerably.

A comparative study was made of three configurations of a cholesterol biosensor based on the formation of a PB layer with entrapment of the ChOx enzyme in PPy: Pt/PB/PPy-ChOx, Pt/SAM/PB/PPy-ChOx and Pt/SAM/PB/PPy-ChOx/Nf. The Pt/SAM/PB/PPy-ChOx/Nf biosensor has the best combination of sensitivity and selectivity of the three configurations studied, and the outer Nf layer improved stability and biosensor lifetime due to the protection of the enzyme entrapped within PPy. The biosensor lifetime for the determination of cholesterol was up to 25 days, an important advance taking into account the poor stability of the ChOx enzyme and the lifetimes of biosensors developed to date [5,24].

The sensitivity achieved (from about 600 to 8500 nA $mM^{-1}\,cm^{-2}$) permits the determination of much lower cholesterol concentrations than those typically found in serum samples and so dilution of this type of sample, with the consequent dilution of all its components, allows the biosensor to be used without any interference of electroactive molecules usually present. Application of a reductive potential of −50 mV proved better sensitivity than with e.g. 0 and +600 mV (reductive or oxidising potentials, respectively), because of the PB layer.

The reliability of the biosensors was verified with synthetic and reference serum samples. The future of this biosensor configuration is related to the versatility the SAM confers. Mercaptanes with different alkyl chain lengths or different functional groups at their ends might be tested with the consequent modifications of sensitivity, stability and the PB characteristics. These configurations could also be slightly modified to adapt their analytical properties to the determination of cholesterol in other kinds of sample.

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