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Strategies for the improvement of an amperometric cholesterol biosensor based on electropolymerization in flow systems: use of charge-transfer mediators and platinization of the electrode

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Abstract

Different configurations based on an amperometric biosensor with cholesterol oxidase entrapped in a polypyrrole film have been developed with a view to improving the analytical properties of this biosensor. The alternatives considered involve the simultaneous entrapment of the enzyme and a charge-transfer mediator as well as previous platinization of the surface of the Pt electrode. Both artificial (a ferrocene derivative) and natural (flavin nucleotides) mediators were studied as constituents of the charge-transfer process between the enzyme and the electrode. The comparative study of these biosensors, which were prepared in situ in a continuous flow system, made it possible to determine the advantages and disadvantages of each configuration when applied to flow-injection determination of cholesterol.

Keywords: Overoxidized polypyrrole films; Charge-transfer mediator; Flavin nucleotides; Platinization; Amperometric cholesterol biosensor; Flow system

1. Introduction

The use of organic conducting polymers for the development of amperometric biosensors has increased notably in recent years. Of special interest are the numerous possibilities electropolymerization provides for the straightforward one-step preparation of biosensors by entrapment of the enzyme within the polymer matrix during the polymer growth stage [1–3]. In particular, the use of polypyrrole (PPy) films combines a series of advantages. As PPy is a conducting film, its growth can be controlled via the charge deposited during the process [4], obtaining a film with size-exclusion properties. Moreover, following its initial formation, the PPy film can be overoxidized to provide a non-conducting polymer with excellent properties for the exclusion of anionic interferences by virtue of the electron density

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generated in its backbone [5], which permits the improvement of the selectivity of the resulting biosensor against the endogenous interferences most frequently present in biological media (e.g. ascorbic acid and uric acid). With this in mind, the preparation of a cholesterol biosensor by entrapment of cholesterol oxidase (COx) in an overoxidized PPy film was successfully carried out [2], in a continuous flow system previously described for glucose biosensors [1]. This configuration provides biosensors with suitable analytical characteristics and results in a significant decrease in the interference caused by uric acid at its normal concentration level in serum. However, complete suppression of the interference caused by ascorbic acid was not achieved.

In order to obtain greater selectivity, other alternative configurations were designed which either make it possible to enhance the exclusion of the polymer layer or decrease the overpotential applied to the amperometric biosensor, at the same time attempting to improve other analytical properties such as biosensor sensitivity and stability.

The first alternative involves the formation of multilayer configurations which combine the permselectivity properties of several polymers in order to achieve greater interference rejection than when they are used individually in monolayer biosensors. With this in mind, different bilayer biosensors have been developed for lactate [6], glucose [7–9] and cholesterol [9] in which the size-exclusion and/or anion exclusion capabilities of overoxidized PPy are enhanced with the permselective properties of poly(*o*-phenylenediamine) due to molecular exclusion and the existence of specific chemical interaction mechanisms [10].

The second alternative proposed, which consists of decreasing the overpotential applied to the amperometric biosensor, is possible by means of charge-transfer mediators or by considering the deposition of metallic layers with a catalytic effect on the detection of the hydrogen peroxide generated during the enzymatic reaction.

The simultaneous entrapment of the enzyme and the charge-transfer mediator during the PPy film growth involves the competitive incorporation of both components as counterions within the polymer. Consequently, an increase in the concentration of one of the two in the polymerization solution results in a decrease in the amount incorporated of the other species [11], making previous control necessary. In this way different oxidase enzymes have been co-immobilized, with ferrocyanide [11], ferrocene derivatives [12] and quinones [13] among others. Simultaneous entrapment of dehydrogenase enzymes and the co-enzyme $NAD(P)^+$ and a suitable mediator for their regeneration has also been carried out [14]. The entrapped mediator should be mobile enough to act as an electron shuttle between the enzyme and the electrode, although excessive diffusion may result in mediator loss from the polymer to the bulk solution, especially if it has a small molecular weight [15]. This has a negative effect on the sensitivity and stability of the biosensor and seriously conditions its application in vivo on account of its potential toxicity.

The study of flavin nucleotides as charge-transfer mediators with redox proteins is another interesting alternative for the development of new electrochemical sensors with natural biocompatible mediator systems for in vivo measurements [16]. The incorporation of flavin mononucleotide (FMN) or flavin adenine dinucleotide (FAD) molecules as a doping anion during the electrosynthesis of PPy has permitted the straightforward preparation of modified electrodes [17]. Pyrrole derivatives with flavin groups have also been synthesized and subsequently electropolymerized [18], giving rise to polymers in which flavin reductase is entrapped for the determination of NADH. Some glucose biosensors have also been developed with co-entrapment of flavin nucleotide and glucose oxidase (GOx) within PPy layers [3,19,20]. In these cases there is no evidence of the existence of nucleotide mediation in the charge-transfer process, although there is discrepancy with regard to the role it plays in the biosensor response. Some authors claim that the activity of the entrapped enzyme is improved [19,20], while others report that its presence in the polymer causes a decrease in the sensitivity of the biosensor [3] due to the incorporation of a smaller amount of enzyme. As a combination of the two

alternatives proposed, the FAD groups in the active site of GOx have been modified with a ferrocene derivative, maintaining the reconstituted enzyme an acceptable level of activity in comparison with the native enzyme and direct communication between electrode and enzyme was shown to exist [21].

On the other hand, platinization of a Pt electrode allows electrocatalysis of the oxidation of the hydrogen peroxide generated in enzymatic reactions involving oxidase enzymes, decreasing the applied overpotential and improving biosensor stability. Two different processes can be used for the platinization of polymer-based amperometric biosensors, including incorporation of Pt particles in the polymer while or after it is obtained [22] or platinization of the electrode surface prior to polymer formation [23,24]. In both cases an increase in biosensor response was detected. There are fewer examples of platinization of the surface of the working electrode prior to synthesis of the polymer on the electrode. The deposited Pt layer in this case, characterized by Scanning Electron Microscopy, has an open porous tridimensional structure formed by a series of Pt aggregates uniformly distributed over the electrode surface [24]. This porous matrix provides a considerable increase in the effective area of the electrode. Consequently, when electrodeposition of the polymer takes place, greater enzyme loading is obtained than with electrodes of the same geometric area. Furthermore, there is an improvement in the efficiency of the oxidation process on the metallised surface of the hydrogen peroxide generated, resulting these two factors in a significant increase in biosensor sensitivity. Platinization of the electrode surface also notably improves the adhesion of the polymer films.

In this paper different alternatives are reported for the improvement of a previously characterized amperometric cholesterol biosensor [2] based on entrapment of the COx enzyme in a PPy layer. For this purpose simultaneous entrapment of the enzyme with an artificial charge-transfer mediator like ferrocenemonocarboxylic acid (FcMC) was studied as well as co-entrapment of COx with FMN or FAD, with a view to studying the effect of these nucleotides on the performance of the resulting biosensor. Alternatively, the surface of the Pt electrode was platinized before electrosynthesis of the PPy enzyme layer and a comparative study of the analytical properties of the different biosensors developed was carried out and in relation to the initial biosensor. All the preparation phases of these biosensors took place in the same flow system in which flow-injection amperometric determination of cholesterol was subsequently carried out and a comparative study was made of the dynamic characteristics of the responses obtained with each biosensor.

2. Experimental

2.1. Apparatus

The preparation of the biosensors and the amperometric determination were carried out using a Coulochem II amperometric detector supplied by ESA (USA) and a BAS thin-layer detection cell (USA) equipped with a plastic block with two Pt disk working electrodes of 3 mm diameter, an auxiliary stainless steel electrode and an Ag/AgCl (3 M NaCl) reference electrode. The two Pt working electrodes can operate in parallel in relation to the carrier flow to make simultaneous independent current measurements of the same carrier solution or in series, which permits detection in the downstream electrode of the species generated or consumed in a reaction that takes place in the upstream electrode.

The volume of the thin-layer detection cell is given by the thickness of the PTFE gaskets intercalated between the auxiliary block and the working electrode. A volume of 175 μl was used for the preparation of the biosensors (platinization and electropolymerization) and 20 μl for the determination of cholesterol. The flow system was completed with a Gilson Minipuls 3 (France) peristaltic pump and a Rheodyne 7125 (USA) injection valve with a loop of 20 μl.

2.2. Reagents

Cholesterol, cholesterol oxidase (COx, E.C. 1.1.3.6. from *Pseudomonas fluorescens*, 4.8 U

mg^{-1} solid), flavin mononucleotide (FMN), flavin adenine dinucleotide (FAD) and ferrocenemonocarboxylic acid (FcMC) were all purchased from Sigma (USA). Pyrrole and hydrogen hexachloroplatinate (IV), used for platinization of the electrodes, were supplied by Aldrich (USA). Cholesterol solutions were prepared in phosphate buffer (pH 7.0; 0.05 M) containing 1% (w/v) Triton X-100 and this solution was also used as a carrier in the flow-injection determination of the substrate. All other reagents used were of analytical grade.

2.3. Biosensor preparation and substrate determination

Electropolymerization of pyrrole and entrapment of the enzyme (and the flavin nucleotides or the artificial charge-transfer mediator) during polymer growth was carried out in the same detection cell of the flow-injection system in which the amperometric determination of the substrate was later performed, in the continuous flow conditions of the polymerization solution previously described [1,2]. For this purpose, a continuous flow of a solution composed of 0.4 M pyrrole monomer and 10 U ml^{-1} COx in a deaerated 0.05 M saline phosphate buffer solution (pH 7.0; 0.05 M + 0.1 M KCl) was circulated in the detection cell at a flow rate of 0.07 ml min^{-1}. A 10 mM concentration of FMN or FAD was added to this solution for the preparation of the biosensors with flavin nucleotides. Nucleotide concentrations of less than 10 mM did not appear to modify the analytical properties of the resulting biosensor with respect to a biosensor without nucleotides.

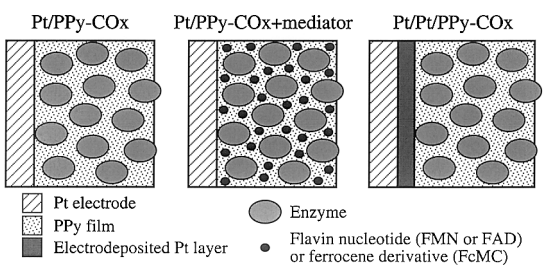

Fig. 1. Scheme of the configurations of the developed biosensors.

However, with concentration values higher than 10 mM a considerable and progressive decrease in sensitivity was observed, so 10 mM was finally selected as the optimum concentration value.

For the preparation of the biosensors using a mediator, $5 \cdot 10^{-4}$ M FcMC were added to the polymerization solution. The electropolymerization of pyrrole was in all cases carried out applying a constant potential of +0.85 V vs Ag/AgCl until a final optimum thickness was reached, controlled via the charge involved during film growth. An optimum thickness of 10 mC cm^{-2} was established for non-platinized biosensors and 15 mC cm^{-2} for platinized biosensors and the electrolysis time needed ranged from 45 to 100 s depending on the type of biosensor.

The platinized biosensors were prepared by electrochemical platinization of the Pt electrode surface before electrosynthesis of the PPy layer. This process was carried out using a continuous flow of a 2 mM deaerated solution of hydrogen hexachloroplatinate (IV) in 0.1 M KCl circulated at a flow rate of 0.08 ml min^{-1} in the detection cell. A potential of −0.25 V vs Ag/AgCl was applied for 10 min and electropolymerization of the PPy film took place after washing the flow system with a 0.1 M KCl solution. A blackish platinization layer was plainly visible on the electrode surface. The amount of Pt deposited on the Pt electrode surface, calculated by integration of the cathodic charge passed during the platinization process, corresponds to a mean value of 106 μg cm^{-2} if it is assumed that a charge of 100 mC cm^{-2} provides a Pt loading of 51 μg cm^{-2} [25].

In all cases the PPy layer was electrochemically overoxidized after preparation of the biosensor by applying a constant potential of +0.7 V vs Ag/AgCl, in the same buffer used as a carrier solution in the determination of cholesterol (phosphate buffer (pH 7.0; 0.05 M) with 1% (w/v) Triton X-100) until the background current levelled off at a constant value lower than 10 nA. The scheme of the resulting biosensors is given in Fig. 1 and the biosensors are subsequently referred to as follows: Pt/PPy-COx for the initial enzymatic biosensor, Pt/PPy-COx + FMN or Pt/PPy-COx + FAD for the FMN biosensor or FAD biosensor, respectively, Pt/PPy-COx + FcMC for

the biosensor with ferrocene and Pt/Pt/PPy-COx for the platinized biosensor.

The amperometric determination of cholesterol was carried out at a potential of +0.7 V vs Ag/AgCl for the biosensors with flavin nucleotides and at +0.5 V for the platinized biosensors. In both cases the detection process was based on the electrochemical oxidation of the hydrogen peroxide generated in the reaction catalyzed by COx. For the biosensors with FcMC, cholesterol determination was carried out at + 0.4 V. This potential corresponded to the electrochemical reoxidation of the mediator molecules entrapped in the PPy film. In all cases, a phosphate buffer solution (pH 7.0; 0.05 M) with 1% (w/v) Triton X-100 was used as a carrier solution at a flow rate of 0.15 ml min^{-1}. When not in use the biosensors were stored in phosphate buffer (pH 7.0; 0.05 M) at 4°C.

3. Results and discussion

The preparation of the Pt/PPy-COx + FcMC and Pt/PPy-COx + flavin nucleotide biosensors was carried out in a continuous flow system, as described for Pt/PPy-COx [2]. This procedure results in lower reagent consumption if compared with conventional batch procedures and offers more effective control of polymer growth and the analytical properties of the resulting biosensor. At pH 7.0 the enzyme, FcMC, FMN and FAD are all present in anionic form, so they can be incorporated in the cationic structure of PPy during the electropolymerization process as counter anions or by physical retention. Consequently, the possible existence of a competitive incorporation of the enzyme molecules and the nucleotide or mediator molecules should be taken into account [11]. This may result in a smaller amount of COx being incorporated in the PPy film, which would have a negative effect on the sensitivity of the resulting biosensor.

With regard to the use of flavin nucleotides in amperometric flavoenzyme biosensors, few papers have been published recently which describe the co-entrapment of flavin nucleotides with GOx in PPy [3,19,20]. There is no agreement about the effect of the entrapped nucleotides on the biosensor response, whether they improve the charge-transfer between the enzyme molecules and the electrode or modify the catalytic activity of the enzyme or its stability. It should be pointed out that COx has a FAD molecule as a prosthetic group, covalently bound to the apoenzyme [26] unlike other enzymes like GOx, in which the FAD group is not covalently attached and can dissociate and be removed from the proteic structure of the enzyme, thus making its reactivation with free FAD molecules possible [26–28].

As described in the Experimental section, platinization of the electrode surface was carried out by electrochemical reduction at a potential of −0.25 V of a solution of $PtCl_6^{2-}$ in KCl in the flow system used for the electrosynthesis of the enzymatic PPy layer. As the solution should not be exposed to the light during the platinization process, all the parts of the flow system through which the solution circulated were suitably protected. Of the two platinization processes commonly used with polymer-modified electrodes, previous platinization of the electrode surface and subsequent electrosynthesis of the PPy film has some advantages over the incorporation of Pt molecules during the formation of the polymer or afterwards, because the layers generated by the latter process have very low porosity, which has an adverse effect on compound diffusion inside the polymer. Moreover, due to the considerable size of the cholesterol molecule, the latter alternative seems less suitable.

As occurred with the Pt/PPy-COx biosensor, the maximum response for Pt/PPy-COx + FcMC and Pt/PPy-COx + flavin nucleotide biosensors was reached for an optimum thickness of 10 mC cm^{-2} and the current recorded decreased for thicknesses higher or lower than this value, as reported previously [2]. It is noteworthy that for the Pt/PPy-COx + FcMC biosensor there is a decrease in the electropolymerization time needed to obtain the optimum thickness (100–120 s for the Pt/PPy-COx biosensor and the biosensors with flavin nucleotides, and 60–75 s for the biosensors with ferrocene) as the presence of the mediator may speed up the electronic transport processes and the whole process of polymer formation and

growth. At the PPy electropolymerization potential, ferrocene oxidation also occurs, but previous assays showed that its contribution to the total charge per unit of time is almost negligible (the charge involved in ferrocene oxidation and capacitative current was estimated about 3.96% of the total charge consumed on PPy electrosynthesis). With the platinized biosensors the optimum thickness value which gave a maximum biosensor response was 15 mC cm^{-2}, but this value was determined in terms of the geometric area of the electrode and is therefore smaller than the real effective area resulting from the platinization of the surface. The increase in the effective area of the electrode after platinization can be verified when a film of non-conducting polymer is electropolymerized onto the surface because polymers of this type have self-limited growth and generate uniform films over the entire electrode surface until growth is blocked, due to a sharp increase in electric resistance. Consequently, the difference between the charge values involved in the electrosynthesis of these non-conducting films on a platinized electrode and an analogous non-platinized electrode reflects the difference in area [23]. The electropolymerization time needed to achieve the optimum thickness ranged from 40–60 s, lower than the electrolysis times required for the preparation of the non-platinized biosensors.

3.1. Measurement conditions: working potential

The operating conditions considered for the three types of biosensor were similar to those established for a Pt/PPy-COx biosensor with regard to injection volume and the detection cell volume (20 μl in all cases), the composition of the carrier solution and the medium in which the substrate solutions were prepared (phosphate buffer (pH 7.0; 0.05 M) with 1% (w/v) Triton X-100) and flow rate (0.15 ml min^{-1}) [2]. Under these conditions, the influence of the applied potential on the determination of the substrate was investigated for each biosensor.

In order to test the possibility of the FMN or FAD molecules acting as charge-transfer mediators (or as electrocatalysts of the enzymatic regeneration) the oxygen present in the carrier solution and the cholesterol solutions injected into the flow system was eliminated by continuous bubbling of nitrogen. No detectable signal was obtained after injection of cholesterol for either the Pt/PPy-COx + FMN or the Pt/PPy-COx + FAD biosensors in the range of potentials between + 0.25 and + 0.8 V vs Ag/AgCl. In order to decide whether the absence of response could be due to a loss of FMN or FAD molecules outside the PPy structure, cholesterol solutions with nucleotide concentrations ranging from 1 to 10 mM were prepared. However, no response was obtained after injection of these substrate solutions in the absence of oxygen in the range of potentials selected, which means that neither FMN nor FAD are capable of producing the regeneration of the enzyme and its electrocatalytic mediation to the Pt electrode.

These studies were carried out with both overoxidized PPy biosensors and biosensors without prior overoxidation of the polymer. In the latter case the polymer retained its conducting properties and could contribute in some way to electron transfer to the Pt surface if the nucleotide molecules were not sufficiently mobile to reach the electrode surface. In this case potential values higher than + 0.6 V were not considered in order to avoid overoxidation of the PPy. No appreciable signal, however, was obtained in any of the situations. It can therefore be concluded that FMN and FAD neither act as charge-transfer mediators (also shown for GOx [3,19,20]) nor improve the enzymatic activity of COx. Consequently, cholesterol determination with Pt/PPy-COx + flavin nucleotide biosensors was carried out at a potential of + 0.7 V in saturated air solutions after overoxidation of PPy.

The optimum working potential of the Pt/PPy-COx + FcMC biosensor was determined in the presence and absence of oxygen in the solutions in order to evaluate the extent of the competition between the two electron acceptors (ferrocene and oxygen) in the reoxidation of the active site of the enzyme. Owing to the need to use surfactants for cholesterol solutions, predominant action of the mediator rather than the oxygen is preferable in the enzyme regeneration. In this way the drawback of oxygen elimination by the passage of the nitrogen stream can be avoided.

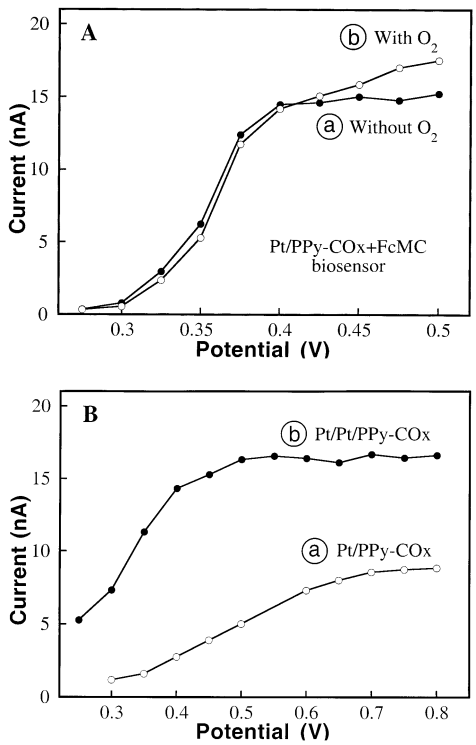

Fig. 2. Hydrodynamic voltammograms obtained with: A) Pt/PPy-COx + FcMC biosensor in the absence (Fig. 2A, curve a) or presence (Fig. 2A, curve b) of oxygen in the carrier solution and in the injected cholesterol solution; and with B) Pt/PPy-COx (Fig. 2B curve a) and Pt/Pt/PPy-COx biosensors (Fig. 2B, curve b). A 0.2 mM solution of cholesterol was used in all cases.

The response of the biosensor with FcMC increased with applied potential values lower than + 0.4 V. With higher potential values the signal recorded remained practically constant in the absence of oxygen in the range considered up to + 0.5 V, as shown in Fig. 2A (curve a), in accordance with the half-wave potential of the mediator. The results were very similar when the dissolved oxygen was not eliminated and only a slight decrease in the current was detected for potential values below + 0.4 V, due to the possible formation of a small amount of hydrogen peroxide not detected in the Pt electrode at these working potentials. Consequently, when the applied potential value is increased, the biosensor response increases (see Fig. 2A (curve b)). Higher current values were obtained with potentials over + 0.45 V when oxygen was present because at these potentials it is possible to detect the electrochemical oxidation of the hydrogen peroxide enzymatically generated due to the regeneration of the active site of a small percentage of COx molecules by the oxygen instead of the charge-transfer mediator entrapped in PPy. The results confirmed that the mediator entrapped in the PPy layer plays an effective part in the reoxidation of the enzyme reduced in the enzymatic reaction with cholesterol.

This study was carried out without previous overoxidation of the PPy film and for this reason potential values higher than + 0.5 V were not considered in order to avoid overoxidation of the polymer. In this way the conducting structure of the polymer would be able to contribute to electron transfer from the mediator molecules to the electrode surface. However, complete prior overoxidation of the PPy film did not significantly affect the potential curve profile with respect to the previously-decribed curve so the conclusion was that the contribution of conducting PPy chains to electron transfer is negligible compared to the predominant role played by the mediator in the charge transfer mechanism. With this biosensor, substrate determination was carried out at a potential of + 0.4 V without removal of the dissolved oxygen, as the amperometric response is independent of the presence of oxygen, thus avoiding the drawbacks of the nitrogen stream bubbling through the solutions containing surfactants. Overoxidation of the PPy film was carried out at + 0.7 V in order to improve selectivity, as described later.

With regard to the Pt/Pt/PPy-COx biosensor, the variation of the response was studied in function of the applied potential to evaluate the electrocatalytic properties of the platinized layer in the electrochemical oxidation of the hydrogen peroxide. The response variation obtained with the non-platinized biosensor (curve a) and a platinized biosensor (curve b) for the same substrate concentration (0.2 mM cholesterol) can be seen in Fig. 2B. The platinized biosensor response increased with the applied potential up to + 0.5 V, after which the signal recorded remained constant. It can be observed that the measured cur-

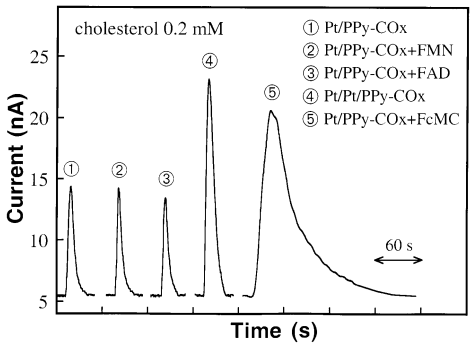

Fig. 3. Amperograms obtained with the biosensors described after injection of 20 μl of a 0.2 mM solution of cholesterol under the optimum measurement conditions for each biosensor. The carrier flow rate was 0.15 ml min^{-1} in all cases.

rent was higher than that generated by the non-platinized biosensor for which a constant maximum response is not obtained until a potential of +0.7 V is applied. This indicates that the presence of the Pt layer deposited on the working electrode has an electrocatalytic effect, allowing the oxidation overpotential of the hydrogen peroxide to decrease and increasing response efficiency in comparison with the non-platinized electrode. The ratio between the response generated for a 10^{-6} M hydrogen peroxide solution in the Pt/Pt/PPy-COx and in Pt/PPy-COx biosensors was 186.47%, considering that the potential applied to the platinized electrode was +0.5 V and that applied to the non-platinized biosensor +0.7 V. As already stated, the platinization layer increases the effective area of the electrode, resulting in the incorporation of a larger amount of COx, which increases the amount of hydrogen peroxide generated. With this biosensor cholesterol determination was carried out at a potential of +0.5 V after overoxidation of the PPy film at +0.7 V.

3.2. Dynamic characteristics of the response

In the optimum operating conditions established, the signals obtained for a 0.2 mM concentration of cholesterol with the biosensors described are given in Fig. 3, where they are compared with the signal corresponding to a Pt/PPy-COx biosensor for the same substrate concentration. Some of the dynamic parameters of the responses generated by the biosensors studied as response time ($t_{95\%}$), signal width ($w_{1/2}$) (calculated at 50% of the maximum height and expressed in units of time) and sample throughput, are given in Table 1.

As can be seen in Fig. 3, there are no significant differences between the profiles of the signals obtained with the biosensors containing entrapped FMN or FAD, which are similar to the response generated by a Pt/PPy-COx biosensor. However, the responses of the biosensors with flavin nucleotides display a smaller peak width which provides greater sample throughput, although the differences are not considerable. With the Pt/PPy-COx + FcMC biosensor it can be observed that the presence of the charge-transfer mediator in the polymer gives rise to a significant increase in the signal in both peak height and area and a notable increase in the peak tail, due to the slow diffusion inside the polymer of the reduced mediator from the active site of the enzyme to the Pt electrode surface for its oxidation. Consequently, sample throughput is very low with this type of biosensor. With a platinized biosensor signal height is higher than that obtained with the Pt/PPy-COx

Table 1
Dynamic parameters of the response of the biosensors described

Type of biosensor	$t_{95\%}$ (s)	$w_{1/2}$ (s)	Sample throughput (samples h^{-1})
Pt/PPy-COx	7.5	9.0	107
Pt/PPy-COx+FMN	7.5	7.7	125
Pt/PPy-COx+FAD	7.4	7.5	127
Pt/PPy-COx+FcMC	28.2	32.9	17.5
Pt/Pt/PPy-COx	6.3	9.7	95

Table 2
Analytical performance of the developed biosensors and their comparison with the Pt/PPy-COx biosensor.

	Pt/PPy-COx	Pt/PPy-COx + FMN	Pt/PPy-COx + FAD	Pt/PPy-COx + FcMC	Pt/Pt/PPy-COx
Reproducibility (RSD (%); $n = 10$)[a]	0.98	1.32	1.23	2.20	2.48
Linear range (mM)	up to 0.3	up to 0.4	up to 0.4	up to 0.4	up to 0.4
Sensitivity (nA mM^{-1})	43.99	42.95	39.85	73.13	77.81
Detection limit[b] (mM)	5.7	12.0	17.3	10.9	14.0
K_M^{app} (mM)	0.59	0.78	0.82	0.79	0.71
I_{max} (nA)	46.51	58.03	50.11	95.17	109.47
α parameter (Hill equation)	0.98	1.02	1.03	1.30	1.06

[a] For 0.2 mM cholesterol solution.
[b] Detection limit expressed as $3s_{y/x}$/sensitivity.

biosensor, although signal width is very similar. A comparison of the dynamic parameters indicates that signal profiles are hardly affected by the platinized layer and sample throughputs are similar.

3.3. Analytical properties

The values determined for a series of analytical parameters evaluated in function of peak height for the different biosensors developed are summarized in Table 2. With regard to reproducibility, there are no significant differences between the coefficient of variation values of the described biosensors, although it should be pointed out that the platinized biosensor displays a high degree of irreproducibility in its first hours of use, with coefficient of variation values exceeding 5% until, after approximately 8 h, it reaches the value given in the table which remains constant during its lifetime. This behaviour may be due to the different processes of initial swelling of the polymer and the reorganization of the COx molecules on the uneven matrix of the platinized layer as this behaviour was not exhibited by biosensors generated on the polished surface of the Pt electrode. It has also been reported that the platinized layer initially exhibits high activity, which decreases in the first hours due to the formation of an oxide layer, obtaining a stable current level [29]. Moreover, the precision of the preparation process was evaluated for each type of biosensor by determining the coefficient of variation from series of $n = 10$ determinations of a 0.2 mM solution of cholesterol carried out with six different biosensors of each of the configurations described, prepared in the same experimental conditions. In all cases the coefficient of variation value was less than 10%, a value considered acceptable taking into account that periods of months sometimes elapsed between the preparation of the biosensors and that different batches of enzyme were used.

The sensitivity of the biosensors with flavin nucleotides is slightly lower than that of the initial Pt/PPy-COx biosensor. This can be explained by the presence of the nucleotide in the polymerization solution with the enzyme, which results in a competitive incorporation of the two species during the PPy film growth process, regardless of whether the entrapment mechanism of both as counterions in the PPy structure is taken into account (both species are present in anionic form in the working pH) or their mere physical retention, which produces a steric hindrance resulting in a smaller enzyme loading in the presence of FMN or FAD, larger if FAD is the nucleotide to co-immobilize on account of its greater molecular size. However, the simultaneous entrapment of FcMC and COx improves sensitivity and the possible loss of response caused by the lesser immobilization of COx in the film (because of its competitive incorporation with FcMC) is compensated for by the increase in response due to the enhanced efficiency in the regeneration process of

the prosthetic FAD groups of the enzyme when the redox charge-transfer mediator acts as an electron acceptor instead of oxygen.

A significant increase in sensitivity was also achieved with the platinized biosensors due to the increase in the effective area of the electrode surface and the electrocatalytic properties provided by the platinized surface for the electrochemical oxidation of hydrogen peroxide. The algebraic transformation of the Michaelis–Menten equation, known as the Hill equation, was selected for the calculation of the apparent Michaelis constant (K_M^{app}) and the maximum current (I_{max}) for the biosensors studied:

$$I = \frac{I_{max}}{1 + (K_M^{app}/[S])^{\alpha}}.$$

This expression allows to obtain greater information about the behaviour of an enzymatic system through parameter α, which is a measure of the degree of deviation of the biosensor response with respect to an ideal enzymatic system governed by Michaelis–Menten enzyme kinetics. Values of $\alpha = 1 \pm 0.1$ can be associated with ideal enzymatic behaviour. The determination of K_M^{app}, I_{max} and α were carried out using a programme of non-linear regression data analysis and the values obtained are given in Table 2 with an r correlation coefficient in all cases higher than 0.9995 for a significance level of $P = 0.05$. All the biosensors display a behaviour in good agreement with Michaelis–Menten kinetics, without inhibitions or limitations caused by substrate transport. The enzymatic reaction rate is the limiting factor of the biosensor response, as indicated by the α values close to the unit, with the exception of the Pt/PPy-COx + FcMC biosensor, whose α value indicates a degree of deviation from the ideal behaviour. In this case the limiting step of the process is the diffusion of the mediator molecules from the active site of the enzyme to the electrode surface through the polymer network. For the flavin nucleotide biosensors, no significant increase in the apparent catalytic activity of the entrapped enzyme was observed which resulted in a marked increase in the I_{max} value, contrary to what had been reported by some authors for other flavoenzymes like GOx [19,20] for which, as previously mentioned, the bonding of the prosthetic group to the apoenzyme or the proteic fraction of the enzyme is not of a covalent nature as it is with COx. This might explain the different behaviour of the two enzymes in the presence of free nucleotide co-entrapped in the polymer film. In view of the results obtained the use of flavin nucleotides does not appear to be an interesting alternative for improving the sensitivity of cholesterol biosensors.

3.4. Selectivity

One of the most important factors to take into account when evaluating the performance of amperometric biosensors is their selectivity against some of the endogenous electroactive species typically present in biological media, like ascorbic acid and uric acid, when these biosensors are applied to the determination of cholesterol in this type of sample. Selectivity was assessed in terms of the film permeation factor (P), defined as the ratio between the signal obtained for the biosensor (I_B) and the signal for a bare electrode (I_{Pt}) arranged in parallel under the same measurement conditions established for each biosensor:

$$\%P = \frac{I_B}{I_{Pt}} 100.$$

Solutions of ascorbic acid and uric acid at their normal upper concentration level in blood serum (5×10^{-5} and 5×10^{-4} M, respectively) were prepared and the $\%P$ values obtained with each biosensor are given in Fig. 4. In all cases the PPy film was submitted to overoxidation prior to the calculation of the P factor. As the optimum working potential of the Pt/PPy-COx + FcMC biosensor is +0.4 V, the determination of P maintaining the PPy layer at its oxidized state was also considered. In this case the PPy film has a polycationic conducting structure and only displays size-exclusion properties depending on the degree of porosity of the polymer, whereas overoxidation of PPy generates a non-conducting structure with high electronic density which gives the film the exclusion capability of anionic species. It can be observed in Fig. 4 that the presence of a flavin nucleotide has little effect on biosensor

selectivity, producing a slight decrease in P which can be attributed to the fact that the resulting film is less permeable. The best results were obtained for the biosensor with FcMC and it is noteworthy that a significant improvement was achieved when the PPy film was overoxidized. However, with the platinized biosensor, high film permeation values were obtained for the interfering species studied and it could be claimed that the platinized layer, which exhibited electrocatalytic properties on the oxidation of hydrogen peroxide, was also catalytically active for any electroactive species. In this case the term film permeation could be misleading as there is no real increase in the permeability of the PPy film but represents, according to its definition, the ratio of the signals between a modified electrode and another identical unmodified electrode under the same measurement conditions.

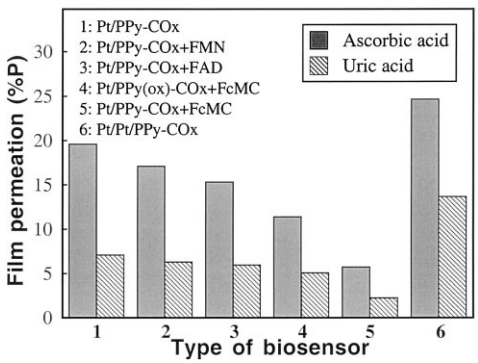

Fig. 4. Selectivity of the different cholesterol biosensor configurations studied: comparison of the film permeation factors (%P) for ascorbic acid (5×10^{-5} M) and uric acid (5×10^{-4} M). The %P is defined in the text. In all cases the PPy film was overoxidized with the exception of the biosensor number 4 (Pt/PPy(ox)-COx + FcMC). In this case, the PPy layer was maintained at its oxidized conducting state.

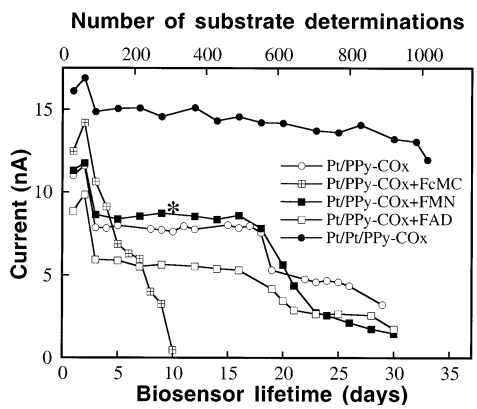

Fig. 5. Evolution of the biosensors response in function of lifetime and the number of substrate determinations carried out. Each point represents the mean value for four series of ten replicate injections of a 0.2 mM cholesterol solution carried out at hourly intervals with three biosensors of each type prepared under the same conditions. The asterisk indicates the mean value obtained on the tenth day with a Pt/PPy-COx + FcMC biosensor using a carrier solution containing 5×10^{-4} M of FcMC.

The application of the biosensors described to the determination of cholesterol in synthetic serum samples by standard addition calibration gave results in accordance with the concentration value of the substrate present in the sample (3.96 mM) for a significance level of $P = 0.05$. Owing to the concentration level of cholesterol, which corresponds to the normal average concentration in blood serum, the sample had to be diluted 50 times in order to bring the substrate concentration within the linear range of determination of the biosensors. In this way the possible interference caused by the electroactive species present in the serum decreased considerably.

3.5. Stability

The most significant differences between the configurations described were related to stability. The variations of the measured current with each of the biosensors for a 0.2 mM cholesterol solution are given in Fig. 5 in function of lifetime and the number of determinations performed. Three analogous biosensors prepared under the same conditions were studied for each configuration. They were all used for 4 h each day and 10 cholesterol determinations were carried out at hourly intervals. The mean value of each day can be seen in Fig. 5. Between successive runs the sensors were stored in PB (pH 7.0; 0.05 M) at 4°C for different periods of time.

It can be observed that all the biosensors display a similar response profile in the first days of use, as reported in previous papers [1,2]. There is an initial increase in the response in the first hours of use due to swelling of the polymer structure

and reorganization of the enzyme molecules in the PPy matrix, followed by a decrease in the response caused by desorption of the enzyme molecules initially adsorbed onto the PPy layer. The latter process did not take place when the PPy layer was subsequently coated with an outer layer of another polymer [8,9].

However, for the biosensor with entrapped FcMC, progressive signal decay with biosensor lifetime was observed and a stable response period with an acceptable duration was not achieved. It was found that this behaviour could be due to a gradual release of mediator molecules from the polymer to the bulk solution. In fact, on the tenth day of life, when the Pt/PPy-COx + FcMC biosensor had a response lower than 1 nA, the determination of cholesterol using, in this case, a carrier solution containing $5 \cdot 10^{-4}$ M of FcMC was carried out and the mean value obtained is shown in Fig. 5 with an asterisk.

The increase in the stable response period of the platinized biosensors is noteworthy — about 30 days after the initial stabilization period during which about 1000 determinations of the substrate can be carried out. This increase in stability may be due to the porous structure of the platinized layer and the possible protection it gives the enzyme molecules against their possible deactivation. In this way it would provide a more favourable environment for their immobilization and enable them to retain their catalytic activity for a longer period of time. In general, it can be stated that the period of stable response for the biosensors described is fundamentally governed by the number of substrate determinations performed and that storage time has little effect on stability. The only exception was the biosensor with FcMC whose stability is mainly conditioned by lifetime due to the gradual loss of immobilized mediator molecules.

4. Conclusion

In view of the studies carried out, it can be concluded that platinization of the electrode surface is of great interest for the development of cholesterol biosensors based on COx enzyme entrapment in PPy films, leading to a significant improvement in the efficiency of the biosensor response (nearly 200% if compared with a similar non-platinized biosensor). Also noteworthy is the increased stability of the biosensor and the high sample throughput (around 100 samples per hour). The dilution of the blood serum samples required to accommodate the normal level of cholesterol within the linear response range of these biosensors permitted the determination of the cholesterol content in these samples satisfactorily using the standard addition calibration. However, the selectivity of these biosensors can be improved by the design of other configurations, which do not significantly modify the above-mentioned advantages. Work in this direction is currently under way in our laboratory to evaluate other alternatives (e.g. multilayer configurations) for the improvement of the selectivity of a Pt/Pt/PPy-COx biosensor.

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