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Electrosynthesized Bilayer Polymeric Membrane for Effective Elimination of Electroactive Interferents in Amperometric Biosensors

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A lactate amperometric biosensor based on lactate oxidase immobilized in a poly(*o*-phenylenediamine) film grown on a polypyrrole-modified platinum electrode is described. The biosensor, which is fabricated by an all-electrochemical procedure, displays a response time in the low second range and an interferent rejection efficiency never achieved before. Ascorbate, urate, and cysteine, which are the most common endogenous electroactive components, produce a nondetectable bias even at their maximum physiological concentrations in blood. Exogenous paracetamol, which at present is the most difficult to manage electroactive interferent, is also very efficiently excluded; at the maximum observable physiological level of 0.2 mM, a truly negligible lactate bias of only 3 μM (i.e., 0.15% at 2 mM lactate) is produced.

Analytical devices combining the specificity of a biological system with the simplicity and low cost of amperometric transduction play a remarkable role in the field of biosensors.[1] The excellent specificity of the immobilized enzyme (e.g., an *oxidoreductase*) is, however, deteriorated to a certain extent owing to the poor selectivity of the transducer. In a H_2O_2-detecting amperometric biosensor, all those substances electrooxidizable at the working potential represent a potential source of bias. Electrode fouling caused by high molecular weight components (e.g., proteins) normally occurring in biofluids represents a further issue which needs to be addressed.

In conventional amperometric enzyme electrodes,[2] up to three membranes are employed to partially solve problems associated with enzyme immobilization, electroactive interferences, and electrode fouling. However, their use is essentially limited to two-dimensional electrode surfaces, and the resulting biosensors are difficult to miniaturize, must be individually assembled, and usually are not suitable for mass production. Furthermore, while the major contribution of endogenous interferents can be lowered to an often acceptable level by a cellulose acetate membrane, paracetamol causes a dramatically high glucose bias: up to 40% in vitro,[3,4] and up to 200% in vivo.[5]

Sasso et al.[6] have demonstrated the use of electropolymerized 1,2-diaminobenzene film to prevent electroactive interferences and fouling effects at a glucose biosensors based on glucose oxidase immobilized (by cross-linking with glutaraldehyde) at a platinized, reticulated vitreous carbon electrode. Based on a similar approach, Geise et al.[7] have shown that stability and antiinterferent characteristics of the electropolymerized film could be significantly improved by codeposition of 1,3-diaminobenzene and resorcinol.

The use of nonconducting polymer as an enzyme-entrapping, permselective membrane for one-step fabrication of an effective glucose biosensor was first demonstrated in the authors' laboratory.[8] Glucose oxidase has been electrochemically immobilized in an electropolymerized ultrathin film, e.g., poly(*o*-phenylenediamine) (PPD)[8,9] or overoxidized polypyrrole, (PPY_{ox}),[10,11] possessing peculiar permselectivity characteristics.[12] The resulting sensors are characterized by a very low response time, high enzyme activity, satisfactory shelf lifetime, very low faradaic interferences, and fouling in vitro,[9,10,13,14] ex vivo,[11] and in vivo.[15]

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(1) Alvarez-Icaza, M.; Bilitewski, U. *Anal. Chem.* **1993**, *65*, 525A–33A.
(2) Pfeiffer, D.; Setz, K.; Schulmeister, T.; Scheller, F. W.; Luck, H. B.; Pfeiffer, D. *Biosens. Bioelectron.* 1992, *7*, 661–671.
(3) Bindra, D. S.; Zhang, Y.; Wilson, G. S.; Stemberg, R.; Thevenot, D. R.; Moatti, D.; Reach, G. *Anal. Chem.* 1991, *63*, 1692–1696.
(4) Moatti, D.; Velho, G.; Reach, G. *Biosens. Bioelectron.* 1992, *7*, 345–352.
(5) Zhang, Y.; Hu, Y.; Wilson, G.; Moatti-Sirat, D.; Poitout, V.; Reach, G. *Anal. Chem.* 1994, *66*, 1183–1188.
(6) Sasso, S. V.; Pierce, R. J.; Walla, R.; Yacynych, M. *Anal. Chem.* 1990, *62*, 1111–1117.
(7) Geise, R. J.; Adams, J. M.; Barone, N. J.; Yacynych, M. *Biosens. Bioelectron.* 1991, *6*, 151–160.
(8) Malitesta, C.; Palmisano, F.; Torsi, L.; Zambonin, P. G. *Anal. Chem.* 1990, *62*, 2735–2740.
(9) Centonze, D.; Guerrieri, A.; Malitesta, C.; Palmisano, F.; Zambonin, P. G. *Ann. Chim. (Rome)* 1992, *82*, 219–234.
(10) Centonze, D.; Guerrieri, A.; Malitesta, C.; Palmisano, F.; Zambonin, P. G. *Fresenius J. Anal. Chem.* 1992, *342*, 729–733.
(11) Palmisano, F.; Centonze, D.; Guerrieri, A.; Zambonin, P. G. *Biosens. Bioelectron.* 1993, *8*, 393–399.
(12) Centonze, D.; Malitesta, C.; Palmisano, F.; Zambonin, P. G. *Electroanalysis* **1994**, *6*, 423–429.
(13) Centonze, D.; Guerrieri, A.; Palmisano, F.; Torsi, L.; Zambonin, P. G. *Fresenius J. Anal. Chem.* **1994**, *349*, 497–501.
(14) Palmisano, F.; Zambonin, P. G. *Anal. Chem.* 1993, *65*, 2690–2692.
(15) Lowry, J. P.; McAteer, K.; El Atrash, S. S.; Duff, A.; O'Neill, R. D. *Anal. Chem.* 1994, *66*, 1754–1761.

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L-Lactate is a substrate of significant clinical interest[16,17] in monitoring states of shock, respiratory insufficiency, and heart failure; in sports medicine, it is a very useful indicator for assessing the general physical conditions of athletes or racing animals.[18,19] Lactate oxidase (LOD) catalyzes the following reactions,

$$\mathrm{LOD(FAD)} + \text{lactate} \rightarrow \text{pyruvate} + \mathrm{LOD(FADH_2)} \quad (1)$$

$$\mathrm{LOD(FADH_2)} + \mathrm{O_2} \rightarrow \mathrm{LOD(FAD)} + \mathrm{H_2O_2} \quad (2)$$

with the production of hydrogen peroxide, which can be amperometrically detected. Various strategies have been employed to immobilize the enzyme at the electrode surface such as cross-linking with glutaraldehyde,[20-22] physical adsorption,[23] entrapping in a poly(vinyl alcohol) matrix,[24] or using discrete polymer membranes.[25,26] Different sensor operational schemes are also possible in which the natural mediator O_2 is replaced by other mediators such as ferrocene derivatives,[27] methylene green,[28] or conducting organic salts;[29] alternatively, the $FAD/FADH_2$ centers of LOD can be nondiffusionally electrically wired to electrodes through a three-dimensional redox polymer.[30] In this last case, electroactive interferents were chemically removed (by a horseradish peroxidase enzymatic layer catalyzing the oxidation of the interferents by H_2O_2 externally added or generated in situ by a third enzymatic layer of glucose oxidase), giving rise to a very complex biosensor design.

Recently[31] it has been shown that the approach of electrochemical immobilization of enzymes can also be employed to produce a lactate biosensor which could be prepared in situ, i.e., in the detector thin-layer cell of a flow injection analysis (FIA) system, by simple injection of a plug of a solution containing the monomer and the enzyme; lactate determination in diluted serum by FIA has been also achieved. The antiinterferent characteristics of this biosensor were satisfactory; the total bias produced on lactate determination at the 2 mM level by ascorbate, urate, cysteine, and paracetamol, all present at their respective maximum physiological levels, was around 10%, paracetamol and ascorbate contributing about 4 and 3%, respectively. Attempts to improve the interferent rejection by enzyme immobilization in a PPY_{ox} film, which performs significantly better (at least for glucose biosensors) than PPD, failed due to a very low sensor response ascribed (vide infra) to an unfavorable partition and diffusion of lactate into the film. The problem could be circumvented by electrochemical immobilization of lactate oxidase in a PPD film grown on a PPY-modified Pt electrode (see Figure 1). This bilayer polymeric membrane, synthesized by an all-electrochemical procedure, enables the immobilization of an oxidoreductase (e.g., LOD) and the rejection of electroactive interferents with an efficiency never achieved before by any of the existing approaches.

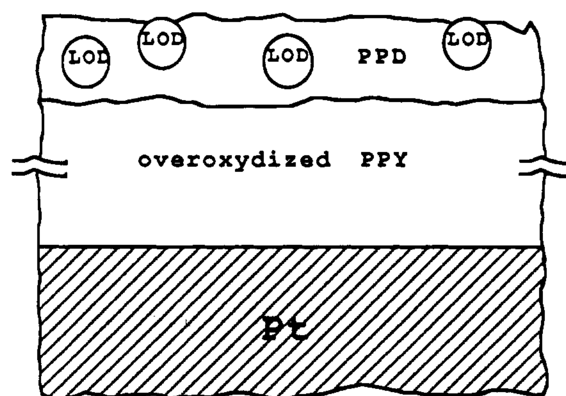

Figure 1. Schematic view of a $Pt/PPY_{ox}/LOD/PPD$ biosensor. The thicknesses of the PPY_{ox} and PPD films are ca. 0.67 μm and 10 nm, respectively.

EXPERIMENTAL SECTION

Chemicals. LOD from pediococcus species (EC1.1.3.2, 35 units/mg), L-(+)-lactic acid (Li salt), ascorbic acid, uric acid, cysteine, and paracetamol were obtained from Sigma Chemical Co. (St. Louis, MO) and used as received. Stock lactate solutions (freshly prepared every 3 days in a phosphate buffer, pH 7.0) were stored at 4 °C when not in use. *o*-Phenylenediamine (*o*-PD) was obtained from Aldrich (Steinheim, Germany) and purified just before use by vacuum sublimation at 90 °C. Pyrrole (Aldrich) was purified by vacuum distillation at 62 °C. All the other chemicals were analytical grade. The carrier stream in FIA was a 0.1 M phosphate buffer, pH 7.0, previously filtered through a 0.45 μm membrane.

Apparatus. All the electrochemical experiments were carried out by a PAR 174A polarographic analyzer (EG&G, Princeton Applied Research) or a PAR 273 potentiostat–galvanostat coupled to a conventional three-electrode system with a Pt wire as counter electrode and a Ag/AgCl as reference. The FIA apparatus consisted in a Gilson Minipuls 3 peristaltic pump, a six-way low-pressure injection valve (Rheodyne Model 5020), a thin-layer flow-through cell, a PAR Model 400 electrochemical detector, and a Y–t strip chart recorder (Kipp & Zoonen Model BD112).

Biosensor Preparation. The base electrode used for biosensor preparation was a Pt disk (3 mm diameter) whose surface was polished with 0.3 μm alumina, washed, and electrochemically pretreated by potential cycling between −0.21 and +1.19 V vs Ag/AgCl in 0.5 M H_2SO_4 until a steady state voltammogram was obtained. A polypyrrole (PPY) film was potentiostatically grown (at +0.7 V vs Ag/AgCl) from a 10 mM KCl supporting electrolyte containing pyrrole at 0.4 M concentration level; the deposition charge employed in polymer growth was typically around 300 mC/cm^2. The Pt/PPy-modified electrode was washed and transferred to another electrochemical cell containing 5 mM *o*-PD and 80–120 units/mL of LOD in a 0.1 M phosphate buffer (pH 7.0)

(16) Hakanson, H.; Kyrolainen, M.; Mattiason, B. *Biosens. Bioelectron.* 1993, *8*, 213–217.
(17) Kyrolainen, M.; Hakason, H.; Ekroth, R.; Mattiason, B. *Anal. Chim. Acta* 1993, *279*, 149–153.
(18) Palleschi, G.; Mascini, M.; Bernardi, L.; Zeppilli, P. *Med. Biol. Eng. Comput.* **1990**, *28*, B25–B28.
(19) Faridnia, M. H.; Palleschi, G.; Lubrano, J. G.; Guilbault, G. G. *Anal. Chim. Acta* 1993, *278*, 35–40.
(20) Dremel, B. A. A.; Li, S. Y.; Schmid, R. D. *Biosens. Bioelectron.* **1992**, *7*, 133–139.
(21) Hu, Y.; Zhang, Y.; Wilson, G. S. *Anal. Chim. Acta* **1993**, *281*, 503–511.
(22) Meyerhoff, C.; Bischof, F.; Mennel, F. J.; Sternberg, F.; Bican, J.; Pfeiffer, E. F. *Biosens. Bioelectron.* 1993, *8*, 409–414.
(23) Palleschi, G.; Turner, A. F. P. *Anal. Chim. Acta* **1990**, *234*, 459–463.
(24) Hajizadeh, K.; Halsall, H. B.; Heineman, R. W. *Talanta* **1991**, *38*, 37–47.
(25) Cunningham, D. D.; Stephens, D. *Anal. Lett.* **1992**, *25*, 1813–1821.
(26) Pfeiffer, D.; Scheller, F. W.; Setz, K. *Anal. Chim. Acta* **1993**, *281*, 489–502.
(27) Loffler, U.; Wiemhofer, H. D.; Gopel, W. *Biosens. Bioelectron.* **1991**, *6*, 343–348.
(28) Kulys, J.; Wang, L.; Maksimoviene, A. *Anal. Chim. Acta* **1993**, *274*, 53–58.
(29) Nguyen, A. L.; Luong, H. T. *Biosens. Bioelectron.* **1993**, *8*, 421–431.
(30) Wang, D. L.; Heller, A. *Anal. Chem.* **1993**, *65*, 1069–1073.
(31) Palmisano, F.; Centonze, D.; Zambonin, P. G. *Biosens. Bioelectron.* **1994**, *9*, 471.

supporting electrolyte. After 10 min of equilibration (during which enzyme adsorption probably occurs), a potential of +0.7 V vs Ag/AgCl is applied for 15 min in order to potentiostatically deposit a PPD film. After preparation, the enzyme electrode was thoroughly washed, fitted to a wall-jet flow-through cell, and potentiostated at +0.7 V overnight in a 0.1 M phosphate buffer (pH 7.0) carrier in order to complete the poly(pyrrole) overoxidation process.

RESULTS AND DISCUSSION

In a conventional three-membrane biosensor, each layer plays a specific role. The inner one serves as the selective membrane for H_2O_2, the second layer provides enzymatic conversion of the substrate, and the third and outermost layer ensures the passage of oxygen and substrate, possibly retarding the last one in order to have an overall diffusion-controlled process. In the approach of electrochemical immobilization of enzymes, the electrosynthesized polymeric membrane performs all of the above functions, which have to be optimized in order to reach the best compromise in terms of the overall performance of the resulting biosensor. Electrosynthesized PPY_{ox} membranes, which give high-performance glucose biosensors, proved useless in the fabrication of lactate biosensors. In effect, attempts were made to immobilize lactate oxidase in a PPY_{ox} film as already done[10] for glucose oxidase; the lactate response of the resulting Pt/LOD/PPY_{ox} sensor was, however, very low. The lack of response could originate from a failure in the enzyme immobilization and/or from lactate rejection by the PPY_{ox} film. To prove the above hypotheses, a Pt/LOD/PPY sensor was polarized at 400 or 500 mV vs Ag/AgCl in a FIA system, and the response to lactate and ascorbate injections was followed at different times. The chosen potentials are far from the optimum value (+0.7 V) for H_2O_2 detection, but it avoids a rapid overoxidation of the PPY film (note, however, that even the pristine film is overoxidized[10] to some extent); the overoxidation of PPY can be followed by the response to ascorbic acid (curve a in Figure 2), which is lower, the higher the degree of overoxidation. As can be seen from Figure 2, at partially overoxidized PPY sensor, a response to lactate is observed even at 400 mV (curve b) which decreases toward negligible values as the time increases. Increasing the applied potential from 400 to 500 mV, the lactate response is again apparent (curve b′) because of the more favorable potential, and the same trend is observed once more. The above findings seem to indicate that the lack of an analytically useful lactate response is to be ascribed to unfavorable substrate partition and diffusion into PPY_{ox} rather than to an inefficient enzyme immobilization. In other words, the PPY_{ox} membrane ensures enzyme entrapping, interferent rejection, and oxygen and H_2O_2 permeation but, contrary to PPD, blocks the access of lactate to the active site of the enzyme. Evidently, another approach had to be found in order to benefit from the excellent antiinterferent characteristics of this membrane, e.g., the use of a bilayer polymeric membrane (e.g., PPY_{ox}/PPD).

A bilayer polymeric membrane in which glucose oxidase is entrapped in a PPD film grown onto a polyaniline (PANI) film has been recently described by Bartlett and Birkin,[32] who have fabricated an enzyme transistor responsive to glucose. The underlying PANI film provides the switching of the device from "off" ($PANI_{ox}$) to "on" ($PANI_{red}$) status through the reaction with the reduced form of an added soluble mediator (tetrathiafulvalenium). In this case, the outermost PPD film provides the

(32) Bartlett, P. N.; Birkin, P. R. *Anal. Chem.* **1994**, *66*, 1552–1559.

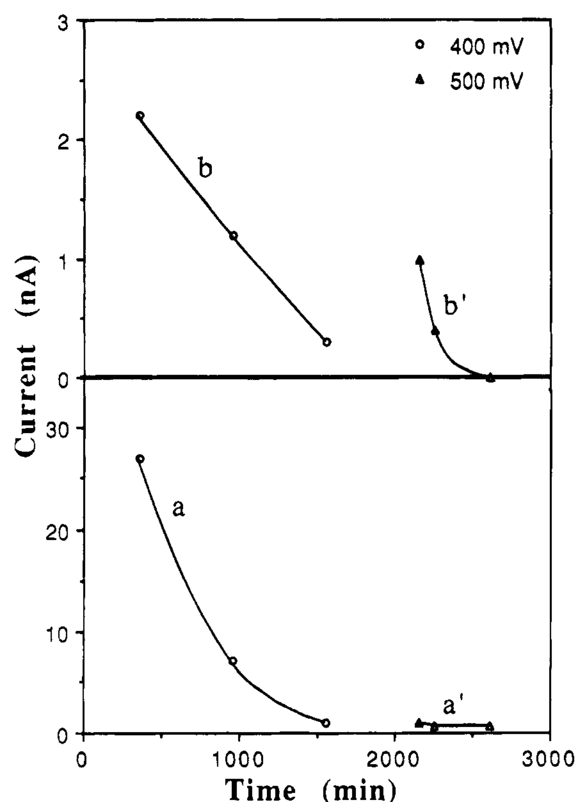

Figure 2. Time dependence of lactate (top) and ascorbate (bottom) FIA responses observed at a Pt/LOD/PPY biosensor during the overoxidation of the polymer at an applied potential of 400 (curves a and b) and 500 mV (curves a′ and b′) vs Ag/AgCl. Flow rate, 0.3 mL/min; injection volume, 110 μL; lactate and ascorbate concentrations, 10 and 0.1 mM, respectively; carrier, 0.1 M phosphate buffer (pH 7.0).

functions of both enzyme entrapment and interferent rejection. The antiinterferent characteristics of the glucose switch are then ultimately determined by the PPD film. As a matter of fact, ascorbate, at its maximum physiological concentration, produced typically a glucose bias of ca. 0.6 mM, the interference arising because of the slow reduction of the PANI film by ascorbate diffusing through the partially blocking PPD film. In the present case we are pursuing the goal of completely eliminating the residual interferents permeating through the PPD film by an additional underlying electropolymerized film. For this purpose, the inner film should be conducting and sufficiently stable in the potential range needed to grow on its top the PPD film entrapping the enzyme. The conductivity of the inner film must then be destroyed (without destroying the enzyme activity) in order to suppress the electrochemistry of common interferents at the inner polymer surface. Finally, the inner polymer in its nonconducting state must possess efficient permselectivity characteristics. An approach based on the electrochemical immobilization of LOD in a self-sealing PPD film grown on a conducting PPY layer, which is then electrochemically overoxidized, proved highly effective, as demonstrated in the following.

The first step in biosensor fabrication was the surface modification of the Pt electrode by potentiostatic growth of a PPY film;[10] the thickness of the film could be controlled through the deposition charge since one can assume[33] that 45 mC/cm² would give a thickness of 0.1 μm. Since the charge typically employed

(33) Fortier, G.; Brassard, E.; Belanger, D. *Biosens. Bioelectron.* **1990**, *5*, 473.

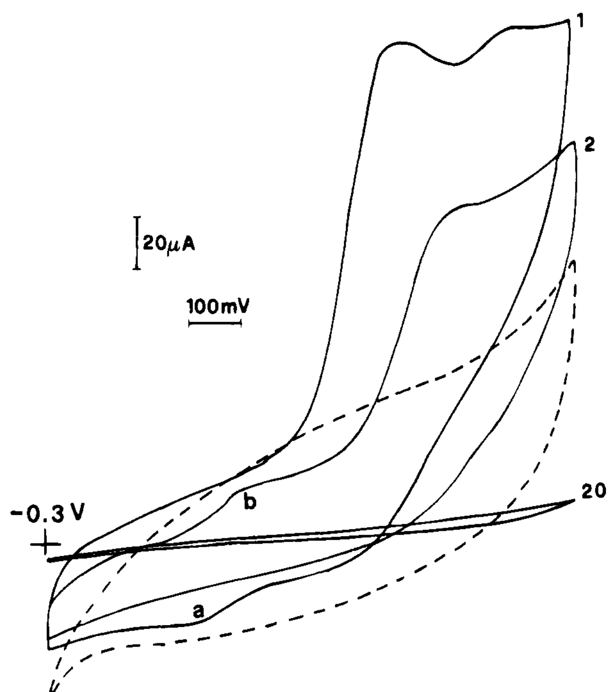

Figure 3. Dashed line: cyclic voltammogram at a Pt/PPY electrode in a 0.1 M phosphate buffer (pH 7) supporting electrolyte. Solid lines: cyclic voltammograms obtained at a Pt/PPY electrode in a supporting electrolyte containing *o*-phenylenediamine at the 5 mM level. Scan rate, 100 mV/s; the scan number is reported on each curve. The potential is scanned between −0.3 and +0.7 V vs Ag/AgCl.

in this work was ca. 300 mC/cm^2, the estimated thickness of the PPY film is around 0.7 μm. Once the Pt/PPY electrode so synthesized was transferred into a 0.1 M phosphate buffer (pH 7.0) supporting electrolyte and the potential cycled between −0.3 and +0.7 V, cyclic voltammograms such as that in Figure 3 (dashed line) were obtained. After addition of lactate oxidase (80–120 units/mL) and *o*-PD (5 mM), cyclic voltammograms 1–20 in Figure 3 were obtained upon repetitive potential scan. The first potential scan (see curve 1 in Figure 3) of *o*-PD on Pt/PPy-modified electrode displays essentially the same features as the one obtainable[8] at a bare Pt electrode; in the reverse and forward scans of cycles 1 and 2, respectively, the peak system *a,b* is observed, which likely originates from the electrochemistry of phenazine units possibly present in the PPD polymer. During further potential cycling (curves not shown for sake of simplicity), the electrochemistry of both PPY and PPD disappears, giving a completely flat cyclic voltammogram (curve 20); the final product is a *bilayer PPY$_{ox}$/PPD nonconducting membrane.* The same result could be achieved by constant potential deposition of PPD film on a Pt/PPY-modified electrode; this was the method normally adopted to prepare (see Experimental Section) the enzyme electrode denoted in the following as Pt/PPY$_{ox}$/LOD/PPD.

Figure 4 shows a FIA calibration curve obtained at a Pt/PPY$_{ox}$/LOD/PPD electrode in a wall-jet flow-through cell; an apparent Michaelis–Menten constant, K_m', of 0.68 ± 0.05 mM and a linear response up to ca. 0.2 mM were obtained, in good agreement with the findings obtained[31] at a Pt/LOD/PPD sensor. This is an indirect indication that the PPD film entrapping LOD grows on the top of the PPY membrane (see Figure 1). The average (n = 5) response time of such a biosensor, measured in a batch addition experiment, is $t_{0.95}$ = 4 ± 1 s.

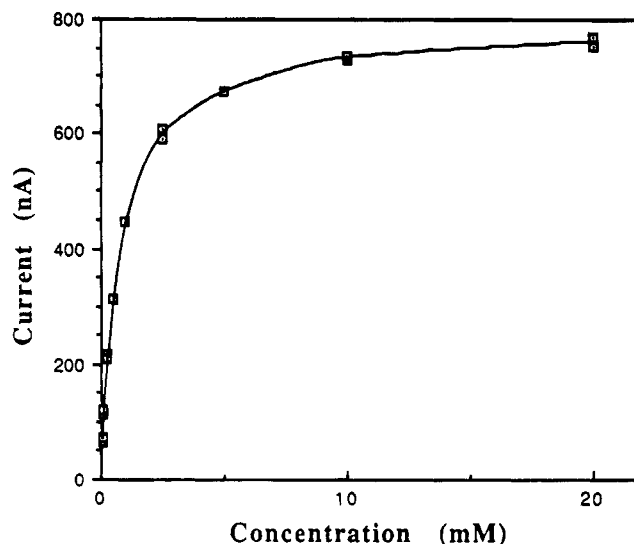

Figure 4. Calibration curve for L-lactate obtained at a Pt/PPY$_{ox}$/LOD/PPD biosensor in a FIA system. Applied potential, 0.7 V vs Ag/AgCl; other conditions as in Figure 2.

Table 1. Lactate Bias Produced by Typical Electroactive Interferents at the Given Concentration in FIA Experiments[a]

interferent	concn (mM)	bias (μM)
ascorbate	0.1	0.3 ± 0.1[b]
cysteine	0.08	0.6 ± 0.1[b]
urate	0.5	not detectable
paracetamol	0.2	3.0 ± 1.1[c]

[a] Data represent average values ±1 SD calculated on three different biosensors. Flow rate, 0.05 mL/min; applied potential, +0.7 V vs Ag/AgCl. The lactate sensitivity is 23.6 ± 4.0 μA cm^{-2} mM^{-1}. Current densities for the various interferents can be easily calculated from the given data. [b] Data extrapolated from measurements at 10 mM concentration level, assuming linearity in the response. [c] Data extrapolated from measurements at 1 mM concentration level, assuming linearity in the response.

The really outstanding feature of the sensor, however, is represented by the interferent rejection characteristics of the PPY$_{ox}$/PPD bilayer membrane. Table 1 gives the bias introduced by the most common electroactive interferents present in biofluids or biotissues, which ultimately determine the usefulness of a biosensor. Ascorbate, urate, cysteine, and paracetamol at their maximum physiological levels in whole blood are not detectable. Consider that the reported ascorbate and cysteine bias could be evaluated only by extrapolation from measurements at 10 mM levels; the same could not be done for uric acid due to its limited solubility. Paracetamol, which at present is the most serious electroactive interferent in H_2O_2-detecting biosensor, is also very efficiently excluded, the maximum interference being only 0.15% at the 2 mM L-lactate level. The overall lactate bias produced by ascorbate, urate, cysteine, and paracetamol, all present at their respective maximum physiological concentrations in blood, is around 0.2%, which represents a ca. 50-fold improvement compared to a Pt/LOD/PPD sensor.[31] Generally speaking, these antiinterferent characteristics are, as far as we know, unrivaled by any existing approaches.

Finally, Figure 5 shows the response vs time plot observed on a typical Pt/PPY$_{ox}$/LOD/PPD biosensor on continuous use in a flow injection system; each point represents the average value obtained on several injections during a working day (see the inset

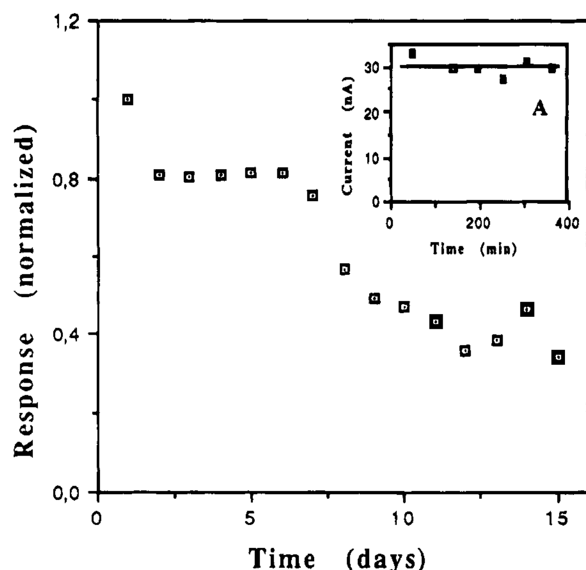

Figure 5. Normalized response vs time for a typical Pt/PPY_{ox}/LOD/PPD biosensor used in a FIA apparatus. Inset: typical lactate response over a ca. 6 h period.

in Figure 5). The biosensor stability appears satisfactory, even if after 5–6 days a periodic recalibration is necessarily required; more importantly, the rejection efficiency of the bilayer polymeric membrane remains unchanged during the lifetime of the immobilized enzyme. A deterioration of the lactate/interferents current ratio consequent to the decrease in the enzyme activity and then in the lactate response (see Figure 5) remains, in practice, not detectable due to the very low interferential bias (i.e., the biosensor remains useful as far as it is responsive to lactate).

In conclusion, this paper demonstrates the possibility of an all-electrochemical fabrication of a really interference-free, fast-response lactate biosensor based on a bilayer polymeric membrane. An apparent drawback of this kind of device could be represented by the reduced linear range due to both the intrinsically low K_m' value of the enzyme and the lack of an outer membrane (normally present in a conventional biosensor design) reducing the substrate diffusion. However, when necessary, this point can be easily overcome, e.g., by using the described sensor in conjunction with microdialysis sampling,[11] providing an "on-line" sample dilution and extension of the linear range.

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