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Electrosynthesized non-conducting polymers as permselective membranes in amperometric enzyme electrodes: a glucose biosensor based on a co-crosslinked glucose oxidase/overoxidized polypyrrole bilayer

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Abstract: A glucose amperometric biosensor based on glucose oxidase immobilized on an overoxidized polypyrrole (PPy_{ox}) platinum modified electrode, by glutaraldehyde co-crosslinking with bovine serum albumine, is described. The advantages of covalent immobilization techniques (e.g. high loading and long-term stability of the enzyme) are coupled with the excellent interferent rejection of electrosynthesized non-conducting polymers. The sensor showed an apparent Michaelis–Menten constant of 16 ± 0·8 mM, a maximum current density of 490 $\mu A/cm^2$ and a shelf lifetime of at least 3 months. Ascorbate, urate, cysteine and acetaminophen at their maximum physiological concentrations produced a glucose bias in the low micromolar range. Flow-injection response was linear up to 20 mM glucose with typical sensitivity of 84·0 ± 1·5 nA/mM.

The sensor was tested for glucose determination of untreated serum samples from both normal and diabetic subjects; results of amperometric assay compared well with those obtained by a standard enzymatic-colorimetric method. ©1998 Published by Elsevier Science Limited

Keywords: enzyme immobilization, biosensor, overoxidized polypyrrole, enzyme co-crosslinking, glucose, flow-injection analysis, serum

INTRODUCTION

An effective method for the fabrication of amperometric enzyme electrodes consists of enzyme entrapment in electrosynthesized polymers (see Bartlett & Cooper, 1993, for a review). This approach usually involves the electrochemical oxidation of an appropriate monomer from a supporting electrolyte containing the enzyme, to form a polymer membrane on the electrode surface. One-step/all-chemical construction, control of the

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spatial distribution of the enzyme and of the layer thickness, miniaturization, and the possibility of building up multilayer and/or multienzyme structures are the main advantages of this method. However, conducting polymer-based membranes lack permselectivity towards anionic endogenous electroactive interferents (e.g. ascorbate, urate) which can be directly oxidized at the polymer surface.

In this respect, significant improvements have been achieved by using non-conducting polymers with built-in permselectivity, such as poly-*o*-phenylenediamine (PPD) (Malitesta *et al.*, 1990; Centonze *et al.*, 1992a; Palmisano *et al.*, 1994), polyphenol (Bartlett *et al.*, 1992) and overoxidized polypyrrole (PPy_{ox}) (Centonze *et al.*, 1992b; Palmisano *et al.*, 1993). These polymers prevent interfering species from reaching the electrode surface, greatly improving the selectivity of the sensor. By such an approach, glucose (Malitesta *et al.*, 1990; Centonze *et al.*, (1992a, b); Palmisano *et al.*, 1993) and lactate (Palmisano *et al.*, 1994) fast-response and interference-free amperometric biosensors have been fabricated and successfully employed in the analysis of biological fluids or even for subcutaneous continuous monitoring (Palmisano *et al.*, 1993) using a microdialysis fibre as sampling system. Furthermore, rejection of electroactive interferents with an efficiency never achieved before has been reported for a lactate amperometric biosensor obtained by enzyme immobilization in an electrosynthesized bilayer non-conducting polymeric membrane (Palmisano *et al.*, 1995).

Unfortunately, the electrochemical immobilization of enzyme possesses some drawbacks. Some substrates and/or enzymes seem, in fact, incompatible with certain polymeric matrices or with the chemical environment generated at the electrode surface during the electropolymerization step (Bartlett & Cooper, 1993; Palmisano *et al.*, 1995; Pantano & Kuhr, 1995). Furthermore, electrochemical immobilization seems successful only with enzymes exhibiting a significant, non-denaturing adsorption on the electrode surface (Bartlett & Whitaker, 1987b; De Benedetto *et al.*, 1994). Even in the case of the thicker layers obtained by conducting polymers, incorporation of the enzyme into the growing film requires electrostatic affinity between the polymer and the enzyme so that, for example, positively charged proteins cannot be efficiently entrapped in polypyrrole films (Bartlett & Cooper, 1993). Finally, the ageing of the enzyme-entrapping polymer (i.e. the modification of its diffusion/partition characteristics) as well as the low amount (typically a monolayer) of the immobilized enzyme reduce significantly the long-term stability of these biosensors.

A way to overcome the above problems could be represented by a 'hybrid' biosensor design in which the advantages of electrosynthesized non-conducting polymers are coupled with those of classical enzyme immobilization methods. Indeed, some examples of such an approach have been already reported. Sasso *et al.* (1990), for example, described a glucose sensor based on glucose oxidase (GOD) immobilized onto a platinized, reticulated vitreous carbon electrode by crosslinking with glutaraldehyde (GLU), followed by electropolymerization of 1,2-diaminobenzene film providing interferents rejection. However, this sensor displayed satisfactory anti-interferent characteristic up to about 1 week so that frequent repolymerization was necessary (Geise *et al.*, 1991). In order to avoid this, different films prepared from diaminobenzene and dihydroxybenzene isomers have been studied (Geise *et al.*, 1991); codeposition of 1,3-diaminobenzene and 1,3-dihydroxybenzene permitted the development of a glucose biosensor whose lifetime was limited by the stability of the immobilized enzyme instead of the polymer film. Schuhmann (1991) and Schalkhammer *et al.* (1991) attempted to fabricate glucose biosensors in which the enzyme was covalently immobilized on properly functionalized polypyrrole layers. However, owing to the low enzyme loading, the long-term stability of these sensors was limited by the enzyme stability, even in the case where the immobilized enzyme was crosslinked (Schuhmann, 1991). More important, owing to the poor permselective properties of the polypyrrole layers, interference from electroactive compounds could be suppressed only by differential measurements on a four-electrode assembly (Schuhmann, 1991; Schalkhammer *et al.*, 1991). Finally, a further membrane was required to extend the linear range of the sensor and to avoid unwanted adsorption on the top of the enzyme film.

In this paper, a glucose amperometric biosensor based on GOD immobilized on a PPy_{ox} platinum modified electrode by GLU co-crosslinking (Guerrieri *et al.*, 1995) with bovine serum albumin (BSA) is described. The co-crosslinking procedure permitted high loading and long-term stab-

ility of the immobilized enzyme while retaining the permselective properties of the underlying PPy_{ox} layer. A glucose biosensor with high sensitivity and wide linear range, completely free from interference and fouling problems, with response times in the low seconds range, was fabricated. The sensor was tested for real matrices analysis by glucose determination in untreated serum samples from both normal and diabetic subjects; results by amperometric assay compared well with those obtained by a standard enzymatic-colorimetric method.

EXPERIMENTAL

Materials

β-D-glucose, ascorbic acid, uric acid, acetaminophen (4-acetamidophenol or paracetamol), L-cysteine, glucose oxidase (EC 1.1.3.4., type VIIS, from *Aspergillus niger*, 180 U/mg solid), bovine albumine (fraction V) and glutaraldehyde (grade II, 25% aqueous solution) were purchased from Sigma (Sigma Chemical Co., St Louis, MO, USA) and used without further purification. Pyrrole (Aldrich, Steinheim, Germany) was purified just before use by vacuum distillation at 62°C. All the other chemicals were of analytical reagent grade. Stock solutions were prepared in tridistilled water or buffer and stored in the dark at 4°C. Dilute solutions were prepared just before use. Glucose solutions were allowed to mutarotate at room temperature overnight before use. A Glucose Trinder (Sigma) reagent kit was used for the enzymatic-colorimetric determination of glucose in serum samples.

Apparatus

Batch electrochemical experiments were carried out by a PAR 174A polarographic analyser (EG&G Princeton Applied Research, Princeton, NJ, USA). The electrochemical cell was a conventional three-electrode system with a Pt rod as auxiliary electrode and an Ag/AgCl, KCl saturated reference electrode. Rotating disk electrode experiments were performed by an EDI-Controvit system (Tacussel, Villeurbane, France) equipped with a Pt disk electrode (3 mm diameter) embedded in a PTFE body. Unless stated otherwise, the rotation rate was 1000 r.p.m. In some experiments, a Pt working electrode constructed by embedding a platinum disk (polycrystalline 99·95%, 4·0 mm diameter, Goodfellow, Cambridge, UK) in a glass body was used. In these latter cases, solutions were stirred by an EG&G Mod. 305 magnetic stirrer. Signals were recorded by a Linseis LY 16100-II X-Y-t recorder (Linseis, GmbH, Germany).

Flow-injection experiments were performed by using a Gilson (Gilson Medical Electronics, Villiers-Le-Bel, France) Minipuls 3 peristaltic pump and a two/six-way low pressure injection valve (Rheodyne mod. 5020, Cotati, CA, USA) equipped with a 100 microlitres sample loop. The injected samples were dispersed by a PTFE tubing (0·5 mm i.d., 130 cm length). An EG&G mod. 400 electrochemical detector including a thin-layer electrochemical cell with a Pt disk (3 mm diameter) working electrode and an Ag/AgCl, 3 M NaCl reference electrode was used. Two thin-layer flow cell gaskets (Bioanalytical Systems, West Lafayette, IN, USA) of 0·005 inch thickness were used. Flow-injection signals were recorded by a Kipp&Zoonen (Delft BV, Holland) mod. BD 112 Yt recorder.

An EG&G PAR 273 potentiostat-galvanostat was used for the controlled electrochemical deposition of polypyrrole film. Spectrophotometric measurements were performed on a Perkin-Elmer (Norwalk, CT, USA) 555 spectrophotometer.

Biosensor preparation

Before each electrode modification, the Pt working electrode was cleaned by hot nitric acid followed by an alumina (0·05 μm particles) polishing procedure, extensive washing and sonication in tridistilled water.

Polypyrrole films (PPy) were electrochemically grown at + 0·7 V versus Ag/AgCl in a 0·1 M KCl supporting electrolyte containing 0·4 M pyrrole. The deposition charge was typically 300 mC/cm^2 giving an estimated (Fortier *et al.*, 1990) film thickness of about 0·67 μm. The Pt/PPy modified electrodes were overoxidized at + 0·7 V versus Ag/AgCl in a phosphate buffer (I = 0·1 M, pH 6·8) for at least 6 h until a steady-state background current was obtained. Overoxidized Pt/PPy electrodes (Pt/PPy_{ox}) were then washed and air-dried at room temperature.

A typical glucose biosensor was prepared as follows. Three hundred microlitres of a phosphate buffer (I = 0·1 M, pH 6·8) solution containing 8 mg BSA and 0·1 mg GOD were carefully mixed

with 30 microlitres of 2·5% glutaraldehyde solution (25% glutaraldehyde solution diluted 1:10 with phosphate buffer). One to two microlitres of the resulting solution were pipetted onto the Pt/PPy_{ox} working electrode surface (avoiding air bubble formation), carefully spread out to cover the electrode surface completely and air-dried at room temperature; after a few minutes, the enzyme electrode was ready to use. To remove any weakly bound and/or adsorbed enzyme and to permit swelling of the enzyme layer, the electrode was preliminarily soaked in the background electrolyte under stirring for a few minutes. When not in use, the enzyme electrode was stored in a phosphate buffer (I = 0·1 M, pH 6·8) at 4°C in the dark.

Electrochemical conditions

A detection potential of + 0·7 V versus Ag/AgCl was used in all the electrochemical experiments. Solutions were left air-saturated and the temperature was ambient. Unless stated otherwise, all the electrochemical experiments were performed in a phosphate buffer (I = 0·1 M); a flow rate of 1 ml/min was used in flow-injection experiments.

RESULTS AND DISCUSSION

Biosensor design

Co-crosslinking of GOD with BSA by GLU was preferred to the simple crosslinking procedure (e.g. Sasso *et al.*, 1990; Geise *et al.*, 1991; Schuhmann, 1991), mainly because (Kennedy & White, (1985a, b)) it allows a higher degree of intermolecular bonding, a lower extent of enzyme crowding and enzyme deactivation and results in an immobilized enzyme layer showing high enzyme stability and good mechanical properties (*vide infra*).

Two alternative approaches were explored for biosensor preparation. In the first one, a thin enzyme layer was cast directly onto the bare Pt surface followed by the electrosynthesis of the anti-interferant, anti-fouling overoxidized polypyrrole (PPy_{ox}) layer. Biosensors prepared in this way, however, showed low glucose responses and high response times (typically greater than 1 min). Microscopic inspections showed a relatively thick PPy_{ox} layer spread out in a non-uniform way inside the proteinaceous membrane. Attempts to reduce the thickness of the PPy layer by using lower deposition charges were unsuccessful because only a partial electrode coverage was obtained. Probably, PPy grew mainly inside some preferential channels of the proteinaceous membrane so that in such a case an uncontrolled, three-dimensional growth instead of a two-dimensional one was obtained.

The second approach, which was successful, consisted of the preliminary electrode position/overoxidation of the PPy_{ox} layer onto the bare Pt electrode followed by the casting of the GOD solution. In this way, it was possible to obtain a thin, two-dimensional PPy_{ox} layer assuring high glucose response and low response times. Obviously, the volume V_c of the enzyme solution cast onto the electrode surface (i.e. the thickness of the enzyme layer) influenced the overall response time of the sensor (Guerrieri *et al.*, 1995). The use of a V_c of 1–2 μl assured a typical response time ($t_{95\%}$) of 1·2 ± 0·4 s (n = 5) which was quite satisfactory for biosensor application in flow analysis.

GOD co-crosslinking with BSA by GLU and the relevant electrodic modification procedure permitted one to obtain a thin enzyme layer strongly adherent to the PPy_{ox} surface and (mechanically) very stable in stirred or flowing solutions.

Enzyme immobilization

In agreement with previous findings (Guerrieri *et al.*, 1995), pH values in the range 6–9 were optimal for biosensor preparation by enzyme co-crosslinking. Moreover, a GLU concentration around 0·2% ensured the best compromise between the amounts of enzyme immobilized and deactivated by crosslinking, giving rise, at the same time, to an enzymatic layer with satisfactory diffusional characteristics.

GOD loading was optimized by testing several sensors prepared from solutions with enzyme concentrations ranging from 0·2 to 20 mg protein/ml (i.e. 36–3600 U/ml); the underlying assumption was that the concentration of active enzyme in the immobilized layer was proportional to its concentration in the casting solution. Glucose sensitivity (i.e. the slope of the glucose calibration curve in the linear region, see Fig. 1) and I_{max} (i.e. the maximum steady-state current) increased on increasing the enzyme loading; in particular, biosensors prepared with the higher volumes V_c

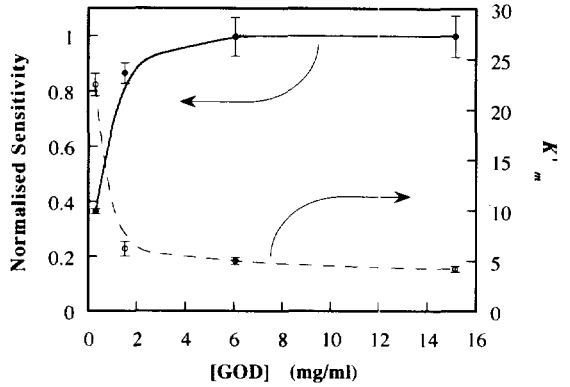

Fig. 1. Normalized sensitivities to (•) glucose and (○) apparent Michaelis constants K_m' as a function of GOD concentration in the enzyme solution used for sensor preparation. [BSA]: 48 mg/ml; [GLU]: 0·2% v/v. Supporting electrolyte: phosphate buffer pH 6·8, I = 0·1 M. Error bars indicate standard deviations calculated on five different sensors.

of enzyme solution cast onto the electrode surface (i.e. with the higher enzyme layer tickness) showed the higher glucose responses. Such behaviours are expected for amperometric biosensors in which the rate of enzyme catalysis is comparable or slower than the diffusion of enzyme substrates inside the immobilized layer (Mell & Maloy, 1975; Bartlett & Whitaker, (1987a, b)). However, at the higher enzyme loadings, sensitivity (see Fig. 1) and I_{max} levelled off and the relevant Eadie–Hofstee plots (see Fig. 2) deviated from the expected linearity suggesting a change in the rate determining step, i.e. substrate diffusion limitations. On increasing the enzyme

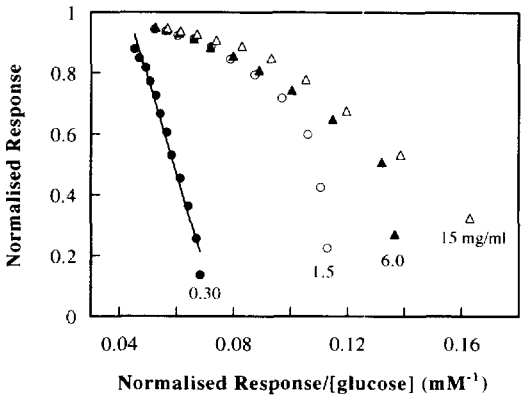

Fig. 2. Normalized Eadie–Hofstee plots for biosensors prepared with GOD concentrations of (•) 0·30, (○) 1·5, (▲) 6·0 and (Δ) 15 mg/ml. Experimental conditions as in Fig. 1.

loading, a significant decrease of the apparent Michaelis constant K_m' was also evident (see Fig. 2). Reported values for the Michaelis constant of GOD lie around 20 mM (Wilson & Turner, 1992), whereas the apparent Michaelis constant K_m' decreased down to 5 mM at the highest GOD loading. Similar findings have already been observed for a choline and acetylcholine amperometric sensor based on a Pt electrode modified with a co-crosslinked bienzymatic system (Guerrieri *et al.*, 1995). The GOD catalytic mechanism is known to be of the 'ping-pong' type (Weibel & Bright, 1971) so that the apparent Michaelis constant $K_m'^{G}$ for glucose (G) when the enzyme is free in solution becomes (Cornish-Bowden, 1976):

$$K_m'^{G} = K_m^G[O_2]/(K_m^{O_2} + [O_2]) \quad (1)$$

where K_m^G and $K_m^{O_2}$ are the true K_m for G and O_2, respectively, when the other substrate is at saturating concentrations. The decrease of K_m' outlined in Fig. 2 seems to be explained by this two-substrate enzyme model. Increasing the GOD loading, the O_2 supply (required for enzyme reactivation) becomes increasingly important so as to become the rate-determining step at the higher enzyme loadings. In this situation, i.e. when $[O_2] \leq K_m^{O_2}$, $K_m'^{G}$ is lowered by a factor depending on the $[O_2]/K_m^{O_2}$ ratio, thus explaining the above experimental findings. Owing to this, low GOD loadings (i.e. about 0·3 mg/ml equivalent to 0·3 μg or 0·054 units for each biosensor) were used for further studies since it represented a good compromise between sensitivity and K_m' optimization. In spite of the low enzyme amount required for each electrode and the permselective behaviour of the Pt/PPy_{ox} electrode (reducing the hydrogen peroxide response too), the biosensor showed a good glucose sensitivity as well as good operational and long-term stabilities (*vide infra*).

Figure 3 shows the sensitivities and the relevant I_{max} of a series of glucose biosensors prepared with enzyme solutions at several BSA concentrations up to 60 mg protein/ml. As can be seen, there is an optimal BSA concentration maximizing both glucose sensitivity and I_{max}. Since K_m' (see Fig. 3) and sensor response time did not change appreciably, a thickening of the enzyme layer and/or a decrease in layer diffusitivity (both involving an increase of enzyme catalysis with respect to diffusion of enzyme substrates (Mell & Maloy, 1975; Bartlett & Whitaker, (1987a, b))) can reasonably be excluded. The

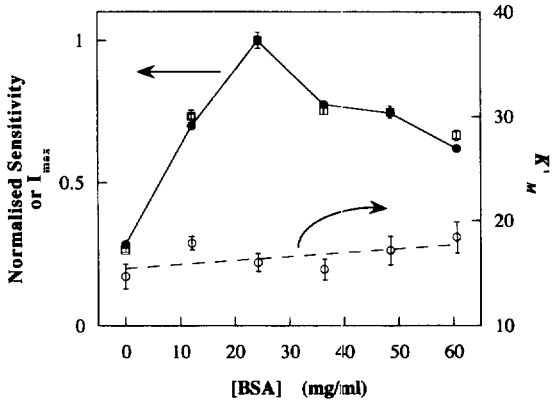

Fig. 3. Normalized (●) glucose sensitivities, (□) I_{max} and (○) apparent Michaelis constants K_m' as a function of BSA concentration; [GOD]: 0·3 mg/ml; [GLU]: 0·2% v/v. Supporting electrolyte: phosphate buffer pH 6·8, I = 0·1 M. Error bars indicate standard deviations calculated on five different sensors.

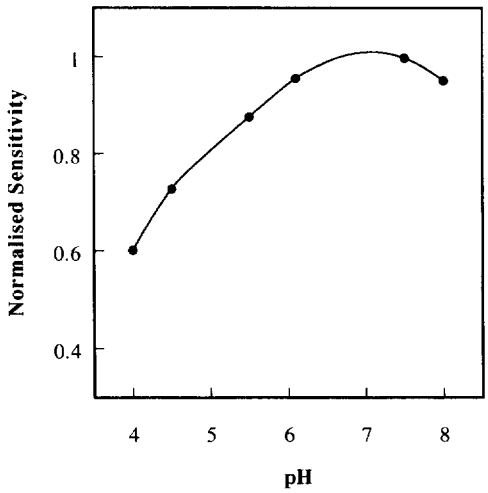

Fig. 4. Normalized glucose sensitivities of a typical biosensor as a function of pH. Supporting electrolyte: acetate/phosphate/borate buffer I = 0·1 M.

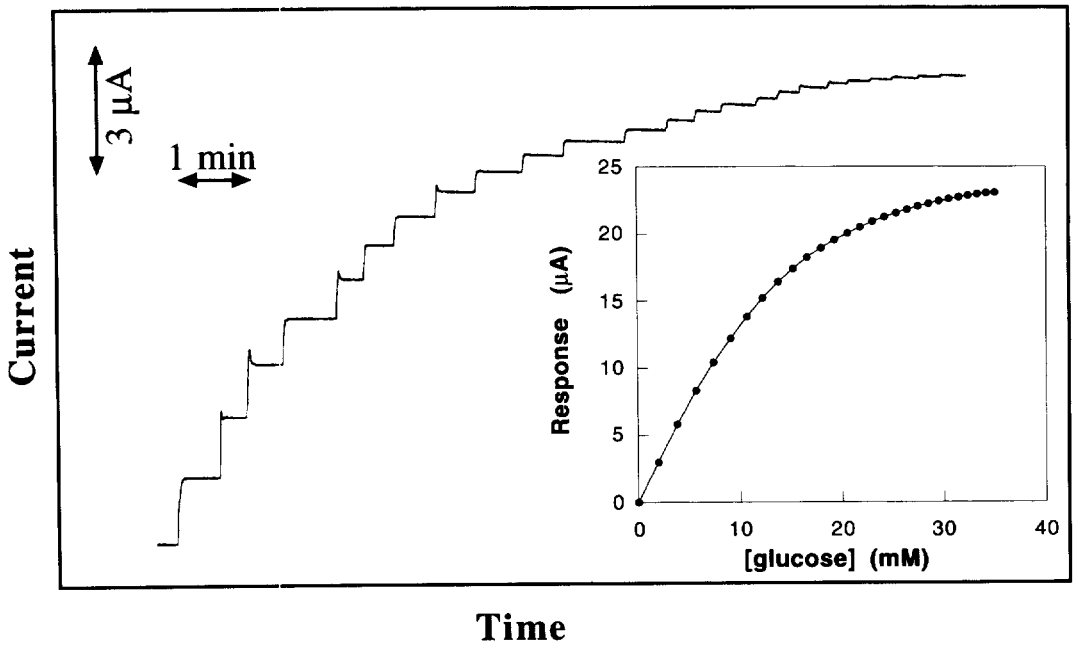

Fig. 5. Current–time responses of a typical biosensor for successive addition of a glucose standard solution to an air-saturated, stirred buffer solution. Inset: relevant calibration curve. Supporting electrolyte: phosphate buffer pH 6·8, I = 0·1 M.

behaviours depicted in Fig. 3 are apparently in contrast to those already reported elsewhere (Guerrieri *et al.*, 1995). Probably the behaviour observed in the present case derived (Kennedy & White, 1985a) from the interplay between retainment of enzyme activity and incoming crowding of proteinaceous layer for which enzyme molecules are out of contact with the substrate. As a consequence, a BSA concentration of about 25 mg/ml was used for biosensor preparation.

Biosensor behaviour

Figure 4 shows the glucose sensitivities of a typical biosensor as a function of pH. As can be seen, the characteristic bell-shaped curve presents a maximum located at pH of about 7 significantly higher than that (pH 5·6) of the free enzyme (Wilson & Turner, 1992). Shifting of the pH of maximal enzyme activity (usually observed after enzyme immobilization) was, in the present case, in the desired direction since biosensor sensitivity was maximized at pH values near to that of biological fluids where glucose measurement is usually performed.

Current–time responses of a typical biosensor obtained for successive additions of a stock glucose solution in an air-saturated, stirred buffer solution are shown in Fig. 5. The response time was very low (*vide ante*) and probably mainly determined by the mixing time. Calibration curves (inset of Fig. 5) were linear up to 12 mM. The relevant Eadie–Hofstee plots (not shown) were linear, as expected for an enzyme catalysis-controlled amperometric biosensor (Mell & Maloy, 1975; Bartlett & Whitaker, (1987a, b)). Linear fitting of data gave a K_m' of 16 ± 0·8 mM and an I_{max} value of 34·7 ± 0·7 μA, corresponding to a maximum current density of 490 μA/cm^2.

Owing to the very low response time, the biosensor was ideally suited for flow-injection analysis applications. The electrodeposition of the anti-interferant, anti-fouling PPy_{ox} layer and the enzyme immobilization procedure developed permitted the fabrication of the glucose biosensor on the platinum working electrode of a conventional thin-layer electrochemical cell. In this respect, no tailored cell geometries and/or unusual flow cell are required, as has sometimes been reported elsewhere.

Typical flow-injection peaks relevant to standard glucose injections are depicted in Fig. 6. Optimization of both biosensor (*vide ante*) and some hydrodynamic parameters (i.e. sample dispersion) permitted linear glucose responses (typical sensitivity of 84·0 ± 1·5 nA/mM) up to about 20 mM (see inset in Fig. 6), which seems more than adequate for the glucose analysis in blood samples from diabetic subjects.

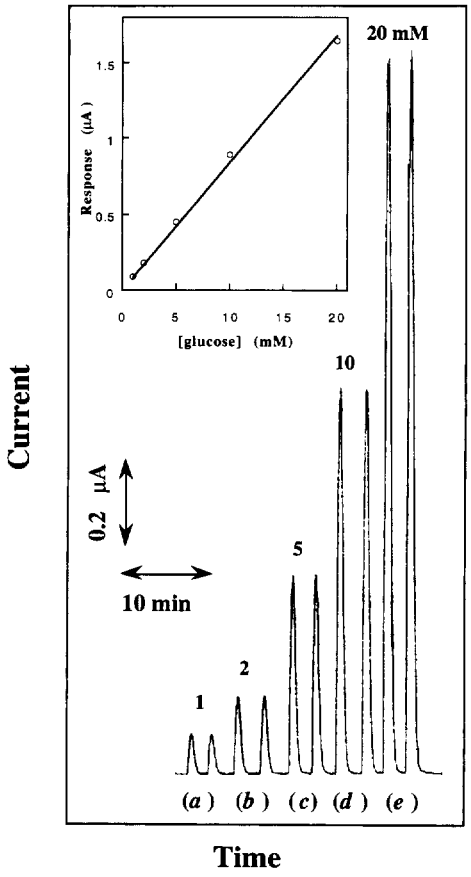

Fig. 6. Typical flow-injection peaks for duplicate injections of (a) 1, (b) 2, (c) 5, (d) 10 and (e) 20 mM glucose standard solutions. Flow cell: thin-layer configuration. Carrier: phosphate buffer pH 6·8, I = 0·1 M; flow rate: 1 ml/min; injection volume: 110 μl. Inset: relevant calibration curve.

Operational and long-term stabilities of the biosensor

The operational stability of the glucose biosensor was tested by continuously monitoring the steady-state response to 5 mM glucose at room temperature. With the exclusion of low frequency slight fluctuations due to the use of a non-thermostatted system, the glucose response did not change appreciably during a 24 h period of continuous use.

The long-term stability was investigated by discontinuously monitoring, for several days, the glucose response of some biosensors stored in a pH 6·5 phosphate buffer at 4°C in the dark. In this respect, no particular effort was made to avoid bacterial growth in the storage buffer. No appreciable loss in glucose sensitivity was observed up to about 3 months.

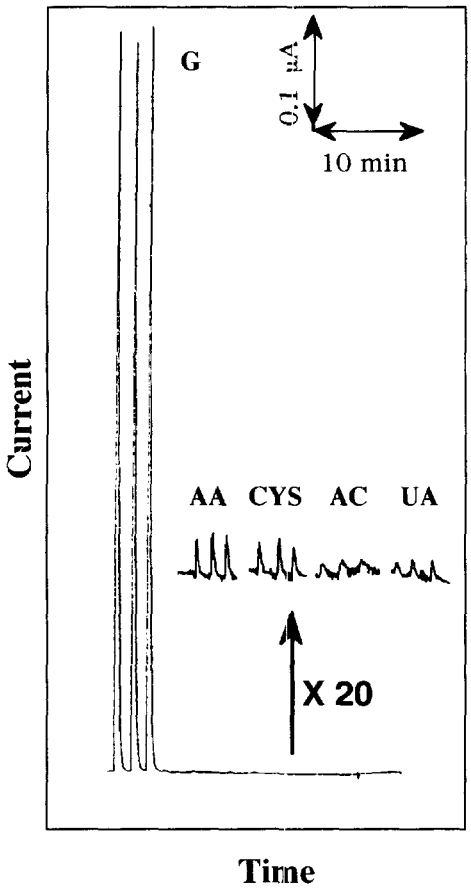

Fig. 7. Flow-injection peaks for triplicate injections of 2 mM glucose (G), 0·1 mM ascorbic acid (AA), 0·08 mM cysteine (CYS), 0·2 mM acetaminophen (AC) and 0·5 mM uric acid (UA). Experimental conditions as in Fig. 6.

Anti-Interferential and anti-fouling properties of the biosensor.

Figure 7 compares the flow-injection responses generated by injection of a 2 mM glucose solution with those relevant to some common interferents at concentrations near the upper limits of their respective physiological concentration ranges. Table 1 reports the relevant bias introduced in the measurement of glucose at the given concentration levels. Finally, Fig. 8 shows the flow-injection peaks relevant to repetitive injection of untreated serum samples and glucose standard. The glucose signal remained practically unchanged between the set of the serum injection runs showing negligible fouling.

Determination of glucose in untreated serum.

Serum samples from both normal and diabetic subjects, with a glucose content in the range 5–16 mM (corresponding to 90–290 mg/dl), were

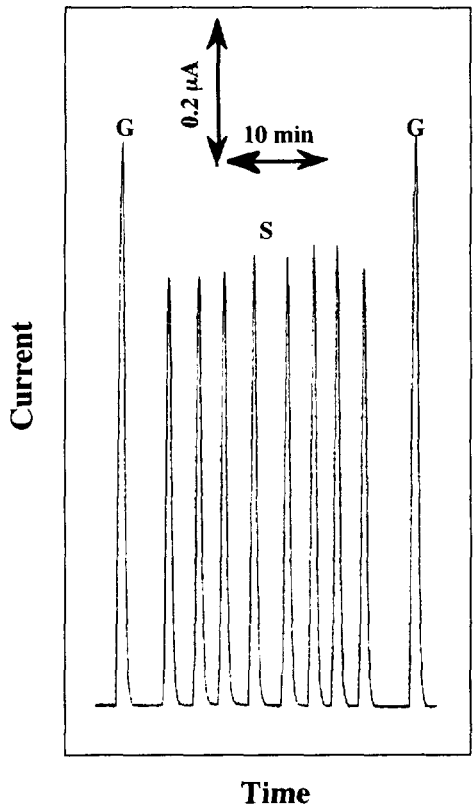

Fig. 8. Flow-injection peaks for replicate injections of untreated serum (S) and 8 mM glucose standard solution (G). Experimental conditions as in Fig. 6.

TABLE 1 Bias introduced in FIA glucose measurement by common electroactive interferents at the given concentration

	Ascorbic acid	*Cysteine*	*Acetaminophen*	*Uric acid*
Interferent concentration (μM)	100	80	200	500
Glucose bias (mM)	5×10^{-3}	4×10^{-3}	$1{\cdot}3 \times 10^{-3}$	$1{\cdot}4 \times 10^{-3}$

analysed by the flow-injection analysis amperometric assay (see Fig. 9) and by a routine enzymatic-colorimetric method. According to a paired-*t*-test (95% confidence level), no significant difference between the results of the two methods has been observed.

CONCLUSIONS

This paper demonstrates how a classical immobilization method (enzyme co-crosslinking) combined with an advanced membrane production technology (electrosynthesis of polymer films with built-in permselectivity) leads to biosensing devices with significantly improved performances. A glucose biosensor showing long-term stability (> 3 months), fast response time (1·2 s), wide linear range (up to 20 mM) and excellent interferent rejection characteristics (glucose bias in the low micromolar range) can easily be fabricated.

A further development would be to employ enzyme casting by ink-jet printing technology on disposable electrode substrates electrochemically covered with polymeric membranes. Both technologies are amenable to mass production. Work is in progress in this direction.

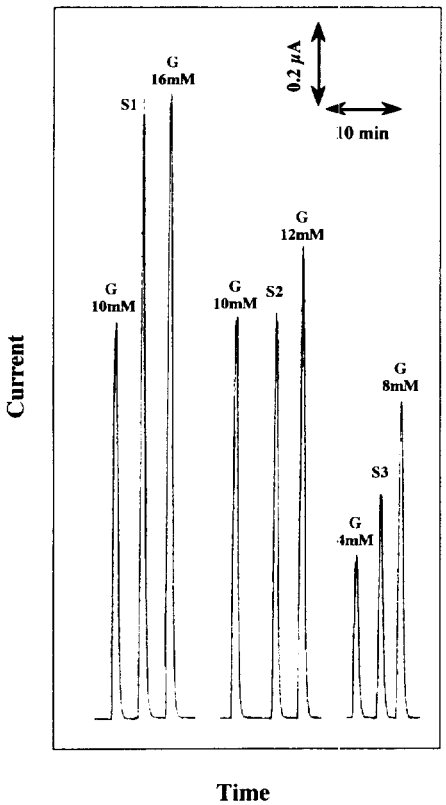

Fig. 9. Flow-injection peaks relevant to glucose standard (G) and untreated serum (S) injections. S1 and S2: serum samples from diabetic subjects; S3: serum from normal subject. Serum concentration found by the amperometric assay: S1 = 15·9 mM; S2 = 10·0 mM; S3 = 5·3 mM.

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