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An interference-free biosensor based on glucose oxidase electrochemically immobilized in a non-conducting poly(pyrrole) film for continuous subcutaneous monitoring of glucose through microdialysis sampling

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Abstract: A glucose biosensor, based on glucose oxidase immobilized in a non-conducting (overoxidised) polypyrrole film, is described which proved practically immune from faradaic interference arising from endogeneous (ascorbate, urate, cysteine) and exogeneous (acetaminophen) electroactive interferents. The bias introduced in the measurement of 5 mM glucose by the given interferents at their maximum physiological levels never exceeded 2% which is, by far, the lowest value ever reported. The biosensor has been used for continuous subcutaneous monitoring of glucose in a rabbit implanted with a microdialysis probe. The potential and limits of this approach are discussed.

Keywords: glucose sensor, microdialysis, *in vivo* monitoring, immobilized enzymes, non-conducting polymers.

INTRODUCTION

In the course of the past two decades intense efforts have been directed towards the development of biosensors. One of the main goals pursued by most researchers in the field is the realization of a biosensor capable of continuous blood glucose monitoring as an aid for the treatment of diabetes (Reach & Wilson, 1992). The aim of such a glucose sensor is to provide an accurate measurement of glucose concentration *in vivo* to be used as the basis for an alarm system detecting abnormal changes in glycaemia or as part of a closed loop insulin delivery system (Rebrin *et al.*, 1989) regulated by the sensor output.

The two sites where glucose concentration is

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identical to the plasma one, under both stationary and dynamic conditions, are the peritoneal cavity and the subcutaneous tissue, the last being currently (Reach & Wilson, 1992) the most realistic site for glucose sensing.

Over the past few years, a great deal of work has been devoted to the development of needle type sensors to be implanted in the subcutaneous tissue (Ege, 1989; Claremont *et al.*, 1986; Velho *et al.*, 1989*a*,*b*; Johnson *et al.*, 1992). In addition to size and shape requirements, the sensor must have certain characteristics: linear range up to 15–20 mM, response time less than 5 min and sufficient sensitivity to produce an acceptable signal-to-noise ratio. Several systems that meet these requirements have been described in the past few years; particularly effective are those designs (Bindra *et al.*, 1991) based on glucose oxidase (GOx) immobilized at a Pt transducer (where H_2O_2 is amperometrically detected) protected by a permselective membrane (e.g. cellulose acetate). An outer protective membrane (e.g. polyurethane) is also used to prevent enzyme leakage, improve the biocompatibility of the sensor and extend the linear range of its response. A major drawback, however, remains which is represented by residual faradaic interferences caused by endogeneous (e.g. ascorbate) and/or exogeneous (e.g. acetaminophen) electroactive compounds. It has been recently demonstrated that while ascorbate interference could be efficiently removed, an unacceptable bias was introduced by acetaminophen (Moatti *et al.*, 1992) both *in vitro* and *in vivo*.

In the past few years, our group has demonstrated (Malitesta *et al.*, 1990; Centonze *et al.*, 1992*a*,*b*) that amperometric glucose biosensors, based on GOx immobilized in electrochemically synthesized non-conducting polymeric films, possess distinctive features such as: one-step all-chemical construction, fast response time (1–5 s), efficient rejection of electroactive interferents and reduced fouling effects. The application of our sensors to flow injection glucose determination in undiluted serum samples has also been described (Centonze *et al.*, 1992*a*,*b*).

Although our technology possesses all the necessary requirements for the construction of a miniaturized sensor for a "direct" subcutaneous implant, an "indirect" approach involving a microdialysis probe was exploited. Microdialysis (Lunte *et al.*, 1991; Menacherry *et al.*, 1992; Moscone *et al.*, 1992; Moscone & Mascini, 1992; Mascini *et al.*, 1992) is an *in vivo* sampling technique for continuous monitoring of chemical processes occurring in a living organism whose driving force is passive diffusion. No macroscopic matter is transferred to or from the sampling site (e.g. the subcutaneous tissue) and only well-tested biocompatible materials are employed to avoid (or greatly reduce) adverse tissue reactions, such as inflammation and clotting.

The usefulness of a biosensor based on GOx electrochemically immobilized in overoxidised poly(pyrrole) coupled to microdialysis sampling for *in vivo* continuous monitoring of glucose will be demonstrated in this paper.

EXPERIMENTAL

Chemicals

Glucose oxidase (EC 1.1.3.4 from *Aspergillus niger*, type VIIS) was obtained from Sigma. Glucose stock solutions were prepared from $\beta-$D(+)-glucose (Sigma) and allowed to mutarotate overnight; more diluted solutions were prepared just before use by dilution with a phosphate or Dulbecco buffer (pH 7·4).

Pyrrole (Aldrich) was purified by vacuum distillation at 62°C. Acetaminophen, cysteine, ascorbic and uric acid (Sigma) were used without further purification. All the other chemicals were analytical grade.

Apparatus

Cyclic voltammetry was performed by a PAR 174A (EG&G) polarographic analyser coupled to a Philips PM8121 X-Y recorder. Electrosynthesis of the polymeric film immobilizing GOx was performed under potentiostatic control and the charge involved in the process (used to estimate the film thickness) was measured by the digital coulometer of a PAR 273 potentiostat-galvanostat. A PAR 400 electrochemical detector coupled to a Kipp & Zoonen BD112 Y-t recorder was used to monitor the response of the glucose biosensor. A home-made electrochemical flow-cell (wall-jet configuration) was used; all potentials were referred to an Ag/AgCl, 3 M Cl- reference electrode. The working electrode (home-made) was a 3 mm diameter Pt rod embedded in a PTFE body.

A Gilson minipuls 3 peristaltic pump was used in flow experiments.

Spectra/por hollow fibres (regenerated cellulose i.d. 150 μm, wall thickness 9 μm, MWCO 9000 Dalton) obtained from Spectrum Medical Industries Inc. (Los Angeles, CA, USA) were used for *in vivo* microdialysis sampling.

Biosensor preparation

Poly(pyrrole) enzyme electrodes (Pt/GOx/PPy) were prepared by electrochemical polymerization at +0·7 V vs Ag/AgCl from a 10 mM KCl solution containing 0·4 M pyrrole and 250 U/ml of GOx. The deposition charge was typically 300 mC/cm^2. Pt/GOx/PPy electrodes were overoxidised at +0·7 V vs Ag/AgCl overnight in a phosphate buffer. Detection potential was 0·7 V vs. Ag/AgCl. The response time (0 to 95%) evaluated on five different sensors was 4 ± 1 s. When not in use the sensor was stored in a phosphate buffer at 4°C.

***In vivo* measurements**

The set-up for *in vivo* measurements is shown in Fig. 1. The microdialysis fibre was subcutaneously inserted for a length of about 2 cm in a ca. 3 kg awake rabbit as previously described (Mascini & Moscone, 1992; Moscone & Mascini, 1993). Connection of the fibre to the peristaltic pump and to the flow cell was realized through nylon tubes. A glucose load of about 2 g (ca. 30 mL of a 7% w/v glucose solution in physiological buffer) was manually infused through the ear vein in a period of about 8 min.

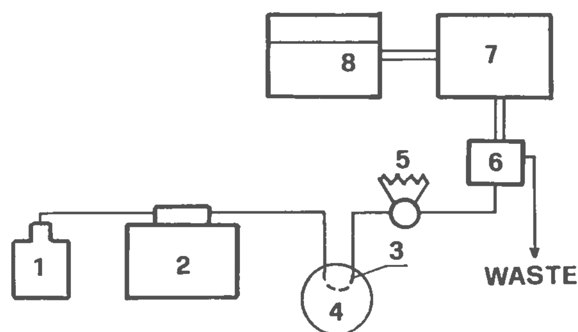

Fig. 1. Schematic diagram of the experimental set-up. 1: perfusion medium reservoir; 2: peristaltic pump; 3: microdialysis probe; 4: sampling site (glucose standard solutions or subcutaneous tissue of an awake rabbit); 5: six-port injection valve; 6: three-electrode amperometric wall-jet cell fitted with a glucose biosensor (working electrode), an Ag/AgCl reference electrode and a stainless steel block (counter electrode); 7: potentiostat; 8: recorder.

RESULTS AND DISCUSSION

***In vitro* assessement of interferences**

The *in vitro* response of the sensor to the most common electroactive interferents was evaluated in a continuous flow experiment pumping, through the flow cell assembled with a Pt/GOx/PPY sensor, a phosphate buffer containing 5 mM glucose and/or a known concentration of the interferent.

Figure 2 (curves *b*, *c*) shows the response to a 5 mM glucose solution and to 5 mM glucose

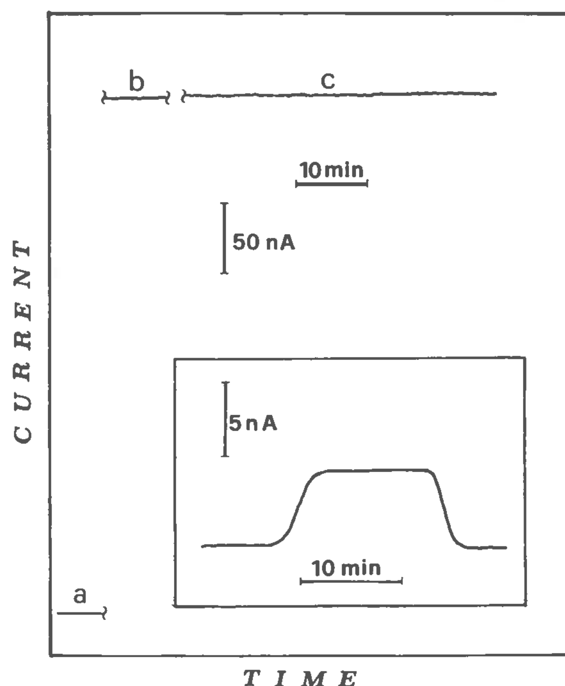

Fig. 2. Current-time responses, obtained under continuous flow conditions, relevant to a buffer solution (curve a), a buffered 5 mM glucose solution (curve b) and a buffered 5 mM glucose solution additioned with ascorbic acid at 0·5 mM level (curve c). The insert in the same figure shows the response of the glucose sensor to a loop injection of a buffered 0·5 mM ascorbic acid solution. Flow rate: 100 μl/min. Experimental set-up: as in Fig. 1 (microdialysis probe by-passed).

plus 0·5 mM AA, respectively at a flow rate of 100 μL/min. As can be seen the sensor displayed a remarkable glucose sensitivity (75 nA/mM or 10 nA/mM mm^2) appearing at the same time practically immune to Faradaic interference arising from ascorbic acid; the insert in the same figure shows the response to a ca. 2 mL plug injection of 0·5 mM AA: a plateau current of 5 nA is obtained compared to 375 nA observed for 5 mM glucose. The current observed at a given concentration of the interferent could be easily converted in terms of a concentration bias introduced in the measurement of a 5 mM glucose concentration. Table 1 summarizes the results obtained for the most common electroactive interferents. As can be seen, the sensor possesses excellent, unrivalled rejection characteristics. Ascorbate at 500 μM level, an unusually high concentration observable in brain fluids, produced a glucose bias of 0·07 mM only. At the maximum ascorbate concentration (i.e. 100 μM) observed in other biofluids (e.g. serum) the glucose bias would be approximately 14 μM which, as far as we know, is indeed the lowest value reported until now. Even more interesting is the capability of the sensor of completely rejecting acetaminophen which, currently, is the most difficult inteferent to manage. Consider, for instance, that in a conventional three-membrane glucose sensor (Moatti *et al.*, 1992), acetaminophen at 200 μM produces a bias of approximately 7 mM glucose both *in vitro* and *in vivo*.

The storage stability of the sensor was not less than three weeks. The sensor response under continuous operation (a 5 mM glucose solution pumped at 0·1 mL/min) was followed for one week. The response remained practically unchanged for three days and then started to drift slowly (in the worst situation the drift was typically 0·4%/hour or less).

TABLE 1 Bias introduced in the measurement of a 5 mM glucose concentration by different interferents at the given concentration level.

Interferent	*Interferent conc. (μM)*	*glucose bias (mM)*
Cysteine	100	0·05
Uric acid	400	0·1
Ascorbic acid	500	0·07
Acetaminophen	200	ND*

*ND = not detected (i.e. current less than 3 times the base line noise originated by the peristaltic pump pulsation).

Microdialysis sampling

Microdialysis is a dynamic sampling method (a perfusion medium is continuously pumped through the probe) based on analyte diffusion across a semipermeable membrane in the presence of a concentration gradient. So the concentration in the dialysate medium is not in true equilibrium with the external (sample) concentration. The concentration ratio at the two sides of the microdialysis probe is dependent on a number of factors, the most important being (for a given probe) perfusion rate, temperature, analyte species and nature of the external medium. For a given set of experimental conditions, the ratio between the two concentration values is a measure of the probe recovery.

At a perfusion rate of 30 μL/min (the same used for *in vivo* experiments) the glucose sensitivity of the sensor in a typical experiment (see Fig. 3A and Fig. 4A) was found to be 85·6 nA/mM with a linear response up to ca. 2 mM (the linearity range was of course dependent on the flow rate). Ratioing the slope obtained in a calibration experiment through the microdialysis probe (see Fig. 3B and Fig. 4B) and the slope given above, a dilution factor of ca. 1:20 (i.e. a recovery of

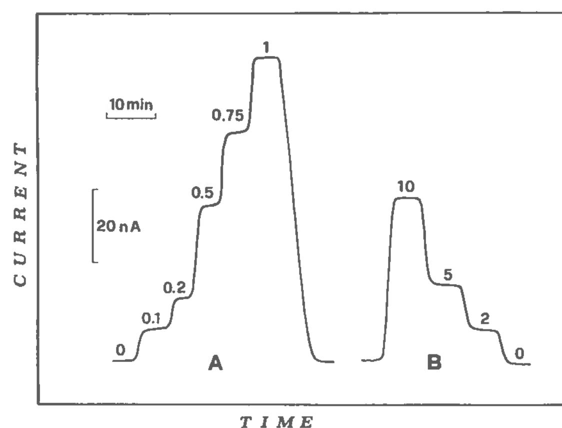

Fig. 3. Current-time response obtained at different glucose concentrations without (experiment A) and with the microdialysis probe (experiment B). Numbers on each current plateau indicate the glucose concentration (mM). Flow rate: 30 μL/min. Experimental set-up as in Fig. 1.

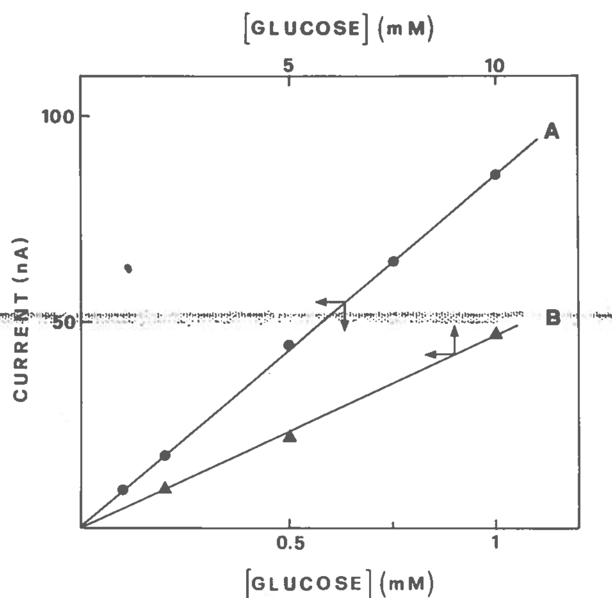

Fig. 4. Calibration curves for the glucose biosensor without (curve A) and with (curve B) microdialysis fibre. Experimental conditions as in Fig. 3. Curve A is described by the following regression equation: $y=(0{\cdot}8 \pm 0{\cdot}3) + (85{\cdot}6 \pm 0{\cdot}9)\,x;\ r = 0{\cdot}9998$.

about 5%) was found under the given experimental conditions. Higher recovery could be, of course, obtained at a lower perfusion rate; consider however that quite fast perfusion rates are necessary to monitor transient concentration changes in the external medium as in the case of the glucose load experiment described in the following section.

In vivo experiment

Figure 5 shows an example of the sensor output in a continuous glucose monitoring experiment in a rabbit implanted with a subcutaneous microdialysis probe. The segment *a* represents the response to the perfusion medium (baseline at [Glucose]=0); after connecting the microdialysis probe, the biosensor response jumped to segment *b* which gives (in nearly real-time, *vide infra*) the glucose concentration in the perfusion medium which is proportional (through the "*in vivo* recovery" of the probe) to the subcutaneous glucose concentration. Segment *c* in the figure shows the response to a glucose load infused in a 8 min period. At the end of the experiment described in Fig. 4, the probe was by-passed and a new sensor calibration was performed; a

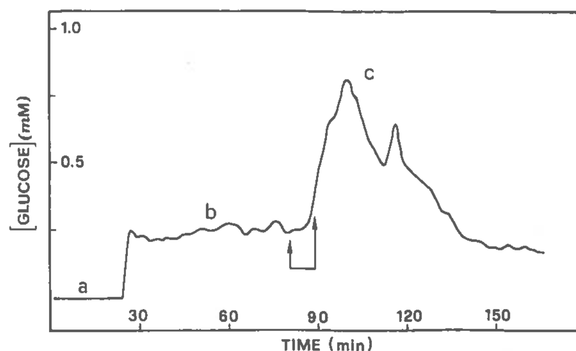

Fig. 5. Glucose concentration (in the subcutaneous dialysate) vs. time dependence in the course of an in vivo *experiment. Experimental set-up as in Fig. 1. Perfusion medium flow rate: 30 μL/min. Curve a: perfusion medium only (microdialysis probe by-passed); curve b: glucose level in the subcutaneous dialysate; curve c: glucose level variation following a 2 g glucose load. Arrows indicate the infusion period (8 min duration).*

sensitivity not significantly different (according to the proper t-test at 95% confidence level) from the one before the *in vivo* experiment was obtained, demonstrating that no fouling or loss of sensitivity occurred.

Because of a finite, not negligible, dead volume (connecting tube) between sampling and sensing sites a lag-time exists, which in the present experimental conditions, is in the order of ca. 2 min. Dispersion in the tube connecting the probe and the amperometric cell can also "smooth" concentration changes at the sampling site; on the contrary no significant smoothing could be introduced by our sensor (response time in the low second range). Consider that dispersion, lag-time, recovery and linear range are dependent on the perfusion rate which has to be optimized in order to have the best compromise in terms of all the cited requirements. The shape of the response curve in Fig. 5 (in particular the continuous oscillation of the sensor output) suggests however that the described experimental set-up works in a very satisfactory way.

Subcutaneous glucose concentration could be easily calculated from the dialysate concentration provided the *in vivo* recovery of the probe is known (which is not the case here). It must be pointed out that *in vitro* recovery could be used as a rough estimator of the *in vivo* recovery provided permeation through the membrane is the limiting factor in determining analyte recovery;

under this assumption the average subcutaneous glucose concentration, extrapolated from segment *b* in Fig. 5, resulted around 5 mM which is a quite plausible value (Johnson *et al.*, 1992). Such an approach, however, must be adopted with a certain caution also because of "active processes" in certain biological tissues (e.g. brain tissue) influencing the mass transport to the probe. Other approaches enabling the *in vivo* calibration of the probe have been proposed (Menacherry *et al.*, 1992) such as the extrapolation to zero flow rate and the method of no net flux point. Alternatively a one point external calibration method (Moscone & Mascini, 1993) could be adopted.

CONCLUSION

In conclusion, the present work reached the goal of demonstrating, for the first time, that the electrochemical immobilization of GOx in non-conducting polymeric films is mature enough even for *in vivo* applications, where some peculiarities of the resulting sensors (e.g. high immunity to electroactive interferents, high sensitivity, etc) could be fruitfully exploited. The described approach appears worthy of more experimental work (e.g. *in vivo* assessment of interferences, *in vivo* calibration etc.) for a definite evaluation of its potentialities.

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