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Poly 3,4-ethylenedioxythiophene as an entrapment support for amperometric enzyme sensor

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Abstract

A conducting polymer of poly 3,4-ethylenedioxythiophene (PEDT) was used as a matrix for entrapment of enzymes onto a platinum electrode surface in order to construct amperometric biosensors. Glucose oxidase (GOD) was used as an example, and it was entrapped in the polymer during the electrochemical polymerization. Glucose in oxygenated solutions was tested by amperometric measurements at +650 mV (vs. SCE) in a batch system. The influence of several experimental parameters in the electropolymerization process was explored to optimize the analytical performance.

The detection limit and sensitivity for this biosensor were 4×10^{-5} M and $15.2 \text{ mA M}^{-1}\text{cm}^{-2}$, respectively. A linear range of response was found from 0.2 to 8 mM of glucose. The response time was 2–5 s. The stability of the electropolymerized films was evaluated in operational conditions. The glucose probe, stored in buffer at 4 °C when not in use, showed a residual activity of 40% after about 1 month. Glucose in synthetic serum was determined under flow injection conditions using an amperometric flow cell.

Keywords:

Electropolymerization; Conducting polymer; Glucose enzyme electrodes; FIA

I. Introduction

Amperometric enzyme electrodes have generally been constructed by direct immobilization of oxidoreductases on electrode surfaces. Platinum, gold, graphite or glassy carbon electrodes have been used. They often have produced unstable responses attributed to a poor charge transfer between the electrode and the enzymes. This is due to the fact that biocatalytic sites are usually buried in the inner part of the enzymatic protein [1]. Therefore, natural or artificial redox mediators are added in the soluble [2] or immobilized [3] form to shuttle the electrons between the various redox chemicals involved in the oxidoreductase process [4]. Conducting polymers have also been used in amperometric enzyme electrodes with the intention to couple the electron transfer reaction between enzyme and electrode via the ramified conducting network of the polymer [2, 5 and 6]. They could also have appropriate functional groups to which allowed enzymes can be attached. Finally, many conducting polymers can be deposited electrochemically [7]. In most polymers such as polypyrrole [8, 9, 10, 11, 12, 13, 14 and 15], polyaniline [16, 17 and 18] and polythiophene [19 and 20], redox charges are delocalised over some polymer groups that could facilitate the oxidation–reduction processes. Among the promising family of polythiophenes, poly 3,4-ethylenedioxythiophene (PEDT) shows remarkable stability, provides homogeneous films [21] and can be synthesized electrochemically even in aqueous medium [22]. Oligonucleotides have then been

incorporated into PEDT films by electrochemical method [23]. Therefore, we recently used PEDT to covalently attach glucose oxidase (GOD) onto a glassy carbon electrode to obtain an enzyme electrode for glucose sensing [24]. In this paper, this polymer is directly electrogenerated on a platinum electrode to produce thin films for a single-step entrapment of the enzyme. The amperometric biosensor obtained from this technique is designed to be integrated in a flow injection system to determine glucose in real samples.

Amperometric flow injection analysis (FIA) associated with immobilized enzyme sensor has become recognized as a selective, simple, accurate and rapid technique for the determination of a variety of biologically important compounds [25]. In such a system, solid electrode-based detectors are commonly used for the detection of electroactive products generated enzymatically. This system offers advantages of highly reproducible timing and high sample throughput and eliminates the need to attain steady states [26]. As the PEDT biosensor has never been tested in FIA conditions, the PEDT biosensor described in this paper is therefore integrated in a FIA equipment to determine the performance of this coupling.

In order to construct the amperometric enzyme sensor, glucose oxidase is used as an example of a redox protein. The enzyme catalyses, in the presence of molecular oxygen, the oxidation of β -D-glucose into gluconic acid and hydrogen peroxide. The conversion of β -D-glucose to gluconic acid involves the transfer of two protons and two electrons from the substrate to the flavin moiety of the enzyme [27]. The electron transfer from the redox cofactor to the sensing electrode might also be facilitated by the presence of a polymeric conducting material.

II. Experimental

II.1. Chemicals

3,4-Ethylenedioxythiophene monomer (EDT) was kindly provided by Bayer (France). Polyethylene glycol (PEG) of 15,000 MW was purchased from Aldrich. -(+)-Glucose and glucose oxidase (EC 1.1.3.4.) type VII from *Aspergillus niger* were purchased from Sigma (France) and used as received. Accutrol™ normal chemistry control serum was purchased from Sigma. All other chemicals were of analytical grade.

Glucose solutions were allowed to mutarotate overnight at room temperature before use. Phosphate buffer was prepared using dihydrogen phosphate dihydrate (Prolabo, France). pH was adjusted with sodium hydroxide solution. All solutions were prepared using milliQ® pure water (Millipore, France).

II.2. Apparatus

Voltammetric and amperometric measurements were performed using a Tacussel PGP 201 potentiostat. The potentiostat output was recorded and processed by means of a Volta Master 1 software. The electrochemical cell was a three-electrode cell where the PEDT/GOD-modified platinum disk electrode acted as the working electrode and a platinum wire as the counter electrode. All potentials were measured vs. SCE. The working electrodes were constructed using a BAS® platinum electrode with a disk-shaped active surface with a diameter of 1.6 mm for batch system and a diameter of 3 mm for flow injection system. A miniaturized flow cell from BAS® was used for FIA measurements (Figure 1). One side of the flow cell contains the inlet and outlet cannulas, the auxiliary electrode and the reference electrode. The other side holds the working electrode. The reference electrode is an electrode without porous junction. It consists of silver metal (Ag) coated with a layer of silver chloride salt (AgCl). The auxiliary electrode is an adjacent 2-mm stainless-steel disk. The inlet cannula is connected to the source of the flow stream, and the outlet cannula is directed to a waste container. The flow system volume using a 51- μ m-thick Teflon gasket is 6.9 μ l.

All the lines were made from a 0.41-mm internal diameter Teflon tubing. A sample line was used to fill the sample loop on a Rheodyne (USA) sample injection valve that was used to inject samples into a buffer stream. An Ismatec peristaltic pump (Switzerland) was used in conjunction with an injection valve.

II.3. Immobilization procedure

Bare electrodes used for preparing the biosensors consisted of Pt disks. The working electrode surface was polished before use with alumina (polishing alumina fluid provided by BAS®, England) of 0.05 μm particle size. After careful rinsing with distilled water, the electrodes were pretreated by potential cycling in 0.5 M H_2SO_4 from -1500 to $+1500$ mV (vs. SCE) at a scan rate of 2 V/s until no changes were observed in the cyclic voltammograms.

A film of enzymatic PEDT was deposited on the Pt electrode by electropolymerizing 3,4-ethylenedioxythiophene (EDT) through cyclic voltammetry. A 10^{-2} M solution of EDT was obtained by dissolving EDT in a 0.02-M, pH 6.2 phosphate buffer solution containing 10^{-3} M PEG and 1000 IU glucose oxidase ml^{-1} . Electrochemical growth of PEDT was carried out using potential cycling between $+200$ and $+1500$ mV at a scan rate of 0.1 V/s for a total of 15 cycles. The same immobilization procedures were used to construct glucose enzyme sensor for batch and flow injection systems.

II.4. Batch measurements

For the batch amperometric measurement of glucose, the PEDT/GOD biosensor was dipped in a 20-ml beaker containing 10 ml of potassium phosphate buffer solution. The electrochemical cell was completed with a SCE reference electrode and a platinum wire counter electrode. The solution was continuously stirred in the open air with a magnetic stirrer to maintain a constant oxygen concentration in the solution for the enzymatic reaction to operate properly. The temperature was controlled using a thermostat (Lauda-Thermostat, Germany). After the cell was assembled, a potential of $+650$ mV was applied to the working electrode to detect hydrogen peroxide. When a baseline was reached, glucose was spiked. The current generated on the biosensor was measured when a steady state was reached. Unless otherwise stated, all experiments were carried out at 25 °C, with an applied potential of $+650$ mV vs. SCE, in a sodium phosphate buffer (0.01 M, pH 7.0) containing 0.1 M KCl.

II.5. Flow injection measurements

The configuration of the FIA manifold has already been described [28]. A peristaltic pump was used to transport the carrier liquid and inject the sample and an injection valve with a loop to receive sample and carrier liquid alternately. The determination of glucose was obtained with a biosensor placed in a miniaturized flow cell. An potential of $+650$ mV vs. Ag/AgCl was applied to the amperometric enzyme electrode by means of a potentiostat connected to a recorder. In this method, time, temperature and dispersion were all reproducible conditions. The peristaltic pump operated with a $75 \mu\text{l min}^{-1}$ flow rate of a carrier stream of potassium phosphate buffer (0.01 M, pH 7.0) containing 0.1 M KCl. The volume of injection loop was $50 \mu\text{l}$. The response curve appears in the form of peaks whose heights depend on the substrate concentration. The concentration of the analyte in a sample can thus be determined from a calibration curve setup with known substrate standards in duplicate measurements.

III. Results and discussion

III.1. Optimization of the enzyme immobilization

Current–potential curves at steady state for glucose are shown in Figure 2. The current response increases as soon as the potential is over $+200$ mV and a plateau is reached at $+500$ mV. Therefore, $+650$ mV was chosen as the working potential for glucose determination. It corresponds to the oxidation of H_2O_2 produced by the enzyme–substrate reaction.

To control the electroentrapment of the enzyme, electropolymerization of this solution was carried out using a number of voltammetric cycles: The first cycles were applied to induce the polymerization process and the following cycles to achieve the overall coating of the electrode. Fifteen cycles were found sufficient to ensure an effective enzyme immobilization, too many cycles would have deleterious effects on mechanical properties of the polymer layer [23]. The thickness of the polymer film can be controlled by the charge consumed in the polymerization

of the monomer. The enzyme concentration has little effect on the membrane thickness because the matrix is mainly represented by PEDT [2].

The influence of enzyme loading in the polymer on the sensor response to glucose was examined (Figure 3). Responses of the PEDT/GOD biosensor to 5 mM glucose increase when the enzyme concentration in the electropolymerization solution increases from 50 to 800 IU ml⁻¹ and reach a plateau at 800 IU ml⁻¹ corresponding to the enzyme saturation in the solution. However, a 1000 IU ml⁻¹ concentration, which is the saturation limit, was adopted for enzyme immobilization in case of enzyme activity loss during electropolymerization. A specific activity of immobilized GOD is estimated to be 0.4 IU/ml from spectrophotometric determination of glucose oxidase activity described elsewhere.

III.2. Response of the biosensor in a batch system

Figure 4 shows steady-state amperometric responses of the PEDT/GOD biosensor to the addition of aliquots of the stock glucose solution into an air-saturated phosphate buffer solution in a batch system. Oxygen concentration should be kept constant because it is a cosubstrate of the enzymatic reaction. These responses are used to construct a calibration curve for glucose. Although a time lag is necessary for the sample solution to be homogeneous after substrate addition, a rapid response of the PEDT/GOD biosensor is observed for each glucose concentration: 2–5 s is estimated to reach a stable plateau.

The enzyme electrode calibration curve using a PEDT film is presented in Figure 5. The data are obtained from measurements repeated three times for each concentration, and the mean value is reported in this figure. The linear range of the biosensor response to glucose concentration from 0.2 up to 8 mM would allow to determine human blood glucose, which lies within the narrow limits of 3.5–5.0 mM. The detection limit of the enzyme electrode is 40 μM, which corresponds to a signal-to-noise ratio of 3. The sensitivity corresponding to the linear range is about 15.2 mA M⁻¹ cm⁻². This sensitivity gradually decreases at higher glucose concentrations.

III.3. Effects of pH

The effect of pH of the electropolymerization solution on the amperometric response of the PEDT/GOD electrode was studied for pHs ranging from 5 to 8 (Figure 6). Optimum response to 5 mM of glucose was observed for an electropolymerization at pH 6.2. This pH value was previously adopted for polymerization of the same polymer [21]. An optimized polymerization pH should allow an efficient entrapment of the enzyme while preventing loss of enzyme activity under polymerization conditions. PEG was added to the EDT solution to improve the enzyme activity by increasing the hydrophobicity of the polymer.

The enzyme sensor response also depends on the working pH of the sampling solution. The effect of pH on the behaviour of the enzyme electrode was studied with 0.01 M phosphate buffer solution containing 5 mM glucose. The pH of the solution ranges from 5 to 8. The response of biosensor as a function of working pH is shown in Figure 6. A maximal response is obtained for pH 7.0, which is different from the optimal electropolymerization pH and higher than the optimal pH of glucose oxidase dissolved in a solution which has been reported as 5.6 [29]. In fact, the kinetics of immobilized enzyme is different from the enzyme kinetics in a homogeneous solution. On the biosensor where the diffusion–reaction process should be taken into account, the oxidation of glucose by immobilized glucose oxidase in the active layer produces gluconic acid, which decreases the local value of pH. The pH value in the enzyme layer bound to the electrode then is lower than the pH of the bulk solution. In order to have the enzyme optimal pH in the PEDT film containing GOD, a higher pH is necessary in the sample solution.

III.4. Stability

Thermal stability of the enzyme electrode was investigated up to 50 °C. The response of the PEDT/GOD electrode to glucose increases with temperature from 15 to 30 °C, then a decrease in enzyme activity is observed (Figure 7). A 25 °C temperature is adopted in our experiments in order to prevent any risk of enzyme denaturation.

The main objective of enzyme immobilization on a transducer for analytical purposes is to stabilise the enzyme for the biosensor to be used repeatedly over a long period. The long-term stability of a biosensor is then tested everyday with the PEDT/GOD electrode. The biosensor was stored between measurements in a phosphate buffer solution at 4 °C. This testing procedure is carried out with a 5-mM glucose solution over 4 weeks. The enzyme electrode retained about 40% of its initial activity after 25 days (Figure 8).

III.5. Flow injection system for the determination of glucose in synthetic serum sample

The batch system previously described is quite suitable to study the effects of pH and temperature on the response of the biosensor as well as its long-term stability. In such a system, the various parameters can easily be adjusted and controlled in order to allow normal working conditions of the biosensor to be optimized.

In biochemical analysis, the need of high sample throughput requires continuous measurements and limitation of manual handling. FIA associated with a biosensor provides a fast, reliable and continuous method to determine glucose without any additional reagent.

Repeatability of FIA measurements is conducted with injections of glucose in duplicate at various concentrations (Figure 9). A mean RSD value of 3.7% is obtained from those responses.

Figure 10 shows typical calibration graph for glucose. A linear relationship between the signal and the concentration is observed up to 10 mM for glucose. The sensitivity calculated from the linear range is about $7.8 \text{ mA M}^{-1} \text{ cm}^{-2}$.

The detection limit with this FIA biosensor is $5 \mu\text{M}$ for a glucose concentration corresponding to a signal-to-noise ratio of 3. This good detection limit is due to a very stable baseline: It is eight times lower than that obtained from the batch system.

Monitoring of glucose in blood is important for diagnosis and surveillance of diabetes, the assays should also be conducted with glucose in serum samples to assess the practical applicability of this method. In this work, the control human serum used consists of an assayed lyophilized preparation containing the analytes commonly observed in man following physiological or pharmacological intakes. Some electroactive species except ascorbic acid are present in this control serum at their usual concentrations: acetaminophen ($86\text{--}139 \mu\text{M}$), salicylate ($0.5\text{--}0.8 \text{ mM}$) and uric acid ($321\text{--}393 \mu\text{M}$).

Table 1 shows the results obtained from this FIA biosensor in comparison with others techniques used to determine glucose in human control serum. Assigned means are of replicate assays performed in reference laboratories using the indicated procedure. The concentrations obtained with this FIA biosensor are similar to those given by other procedures.

IV. Conclusion

In this study, the possibility of using conducting polymer as a support for single-step immobilization of glucose oxidase has been investigated. The use of PEDT as an entrapment support of glucose oxidase in the development of a glucose biosensor is motivated by the conductivity and stability properties of PEDT [21, 30, 31 and 32]. We have shown that electrochemical polymerization of EDT results in an efficient technique to immobilize glucose oxidase onto a platinum electrode surface. The batch system provides an easy method to optimize the various operating parameters of the biosensor. Its rapid response time and stability are compatible with its application in flow analysis systems. The determination of human blood glucose is possible due to its wide dynamic range.

In spite of a large number of species present in control human serum, the results given by the FIA/biosensor are in agreement with those obtained from other methods currently in use.

This immobilization technique, which can be extended to other oxidoreductases, is quite suitable for the design and construction of microbiosensors.

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Figures

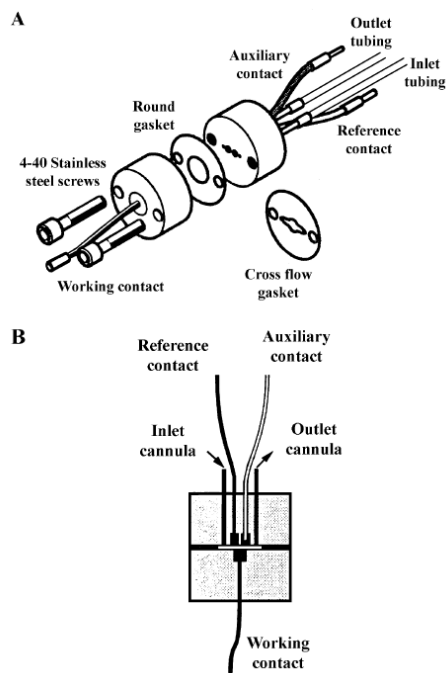


Figure 1: Configuration (A) and side view (B) of a miniaturized amperometric flow cell.

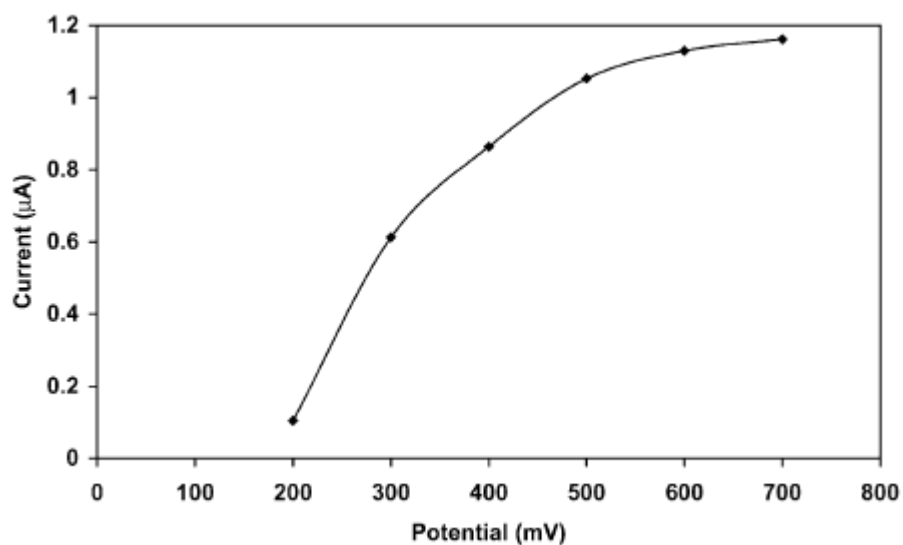


Figure 2: Hydrodynamic voltammogram of a glucose solution using a PEDT/GOD electrode. Conditions: 5 mM glucose, 0.01 M, pH 7.0 sodium phosphate buffer and 0.1 M KCl, 25 °C.

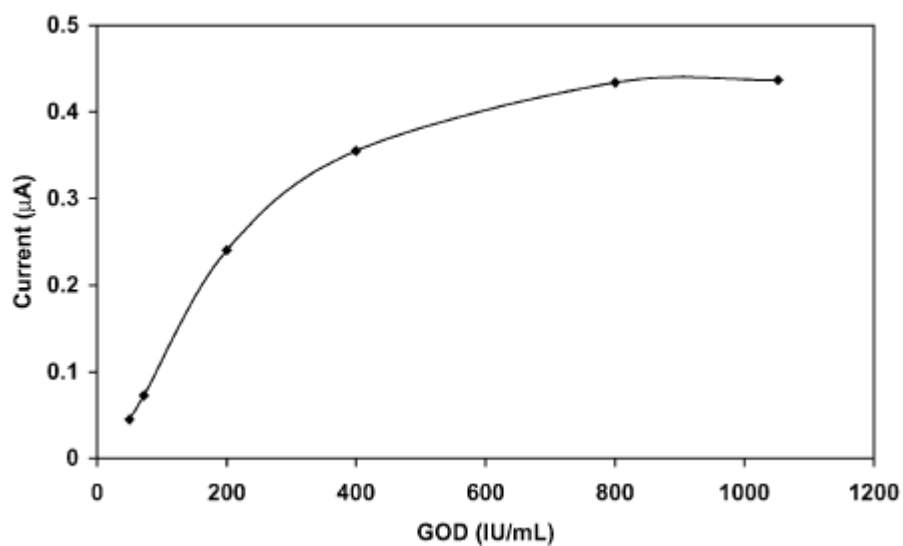


Figure 3: Effect of enzyme concentration on the PEDT/GOD electrode response to glucose. Conditions: 5 mM glucose, 0.01 M, pH 7.0 sodium phosphate buffer and 0.1 M KCl, 25 °C, +650 mV vs. SCE.

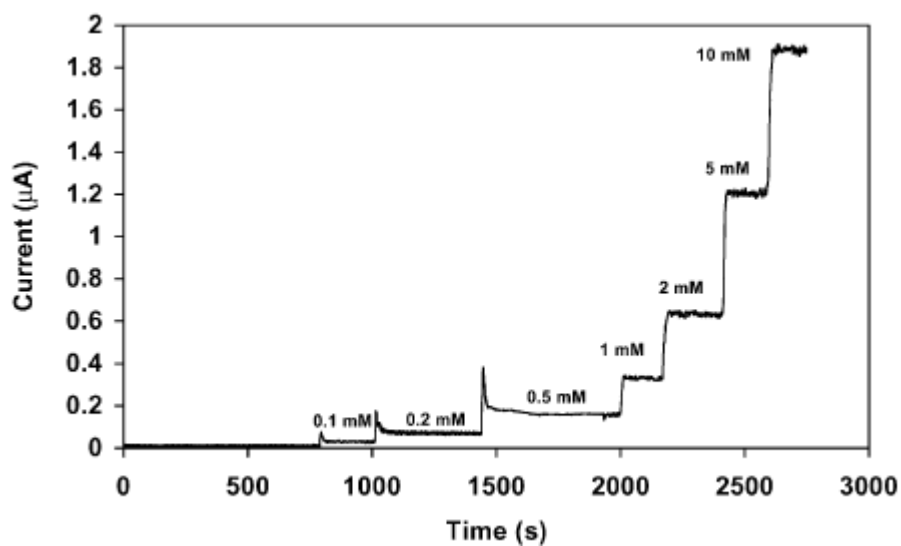


Figure 4: Typical responses of a PEDT/GOD electrode to glucose in an air-saturated buffer. Conditions: 0.01 M, pH 7.0 sodium phosphate buffer and 0.1 M KCl, 25 °C, +650 mV vs. SCE.

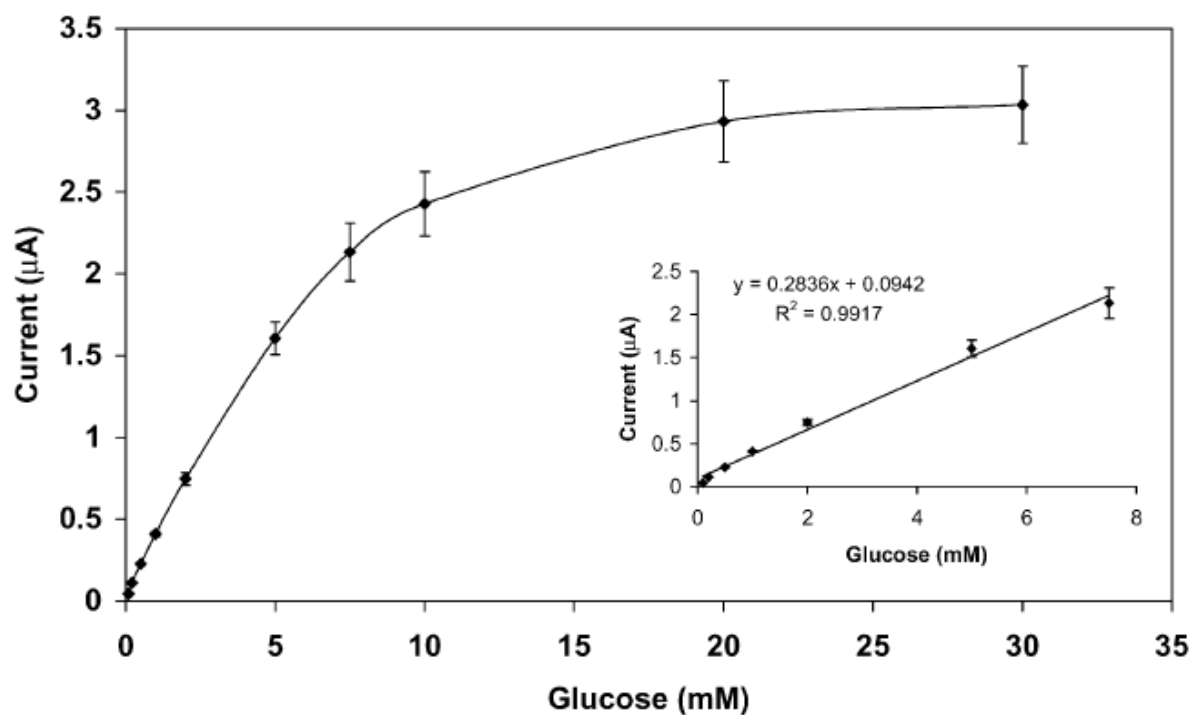


Figure 5: Calibration curve of a Pt/PEDT/GOD electrode sensitive to glucose. Conditions: 0.01 M, pH 7.0 sodium phosphate buffer and 0.1 M KCl, 25 °C, +650 mV vs. SCE.

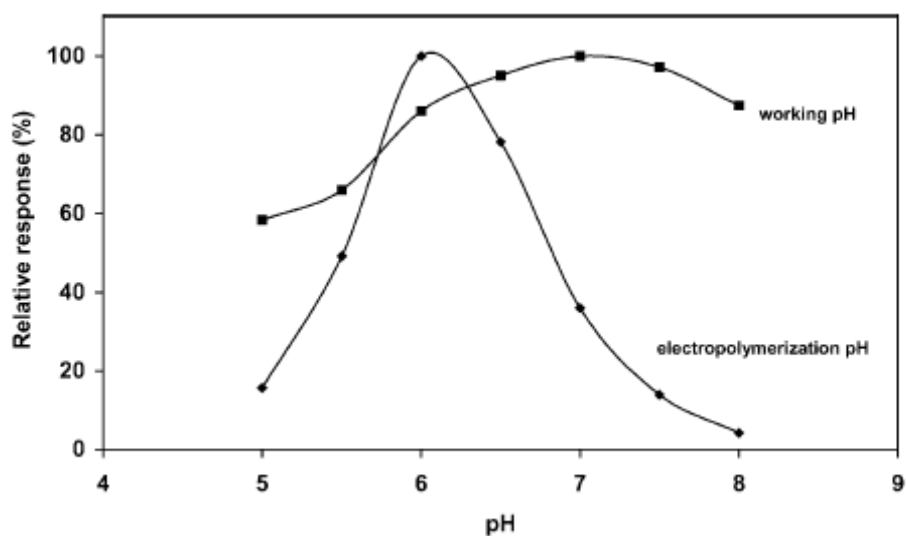


Figure 6: Effect of electropolymerization and working pHs on the PEDT/GOD electrode response to glucose. Conditions: 5 mM glucose, 0.01 M sodium phosphate buffer and 0.1 M KCl, 25 °C, +650 mV vs. SCE.

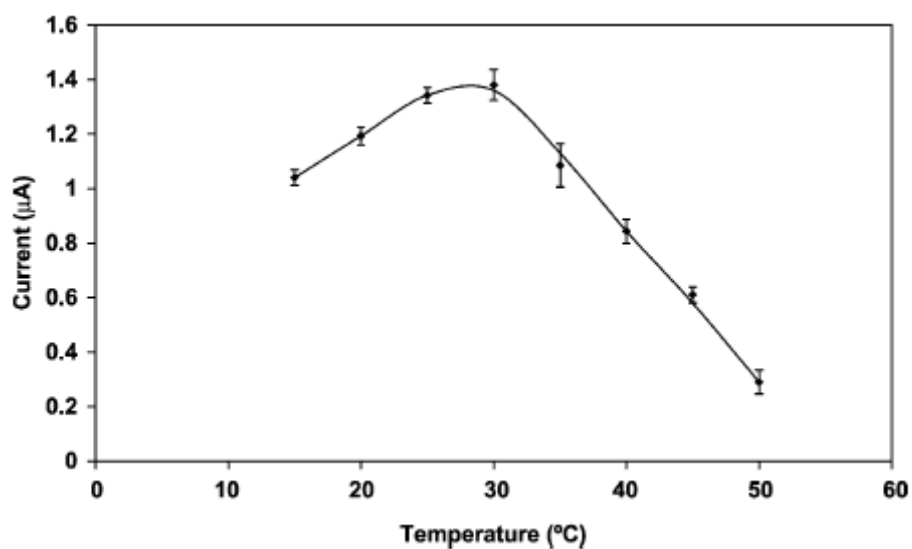


Figure 7: Effect of temperature on the PEDT/GOD electrode response to glucose. Conditions: 5 mM glucose, 0.01 M, pH 7.0 sodium phosphate buffer and 0.1 M KCl, +650 mV vs. SCE.

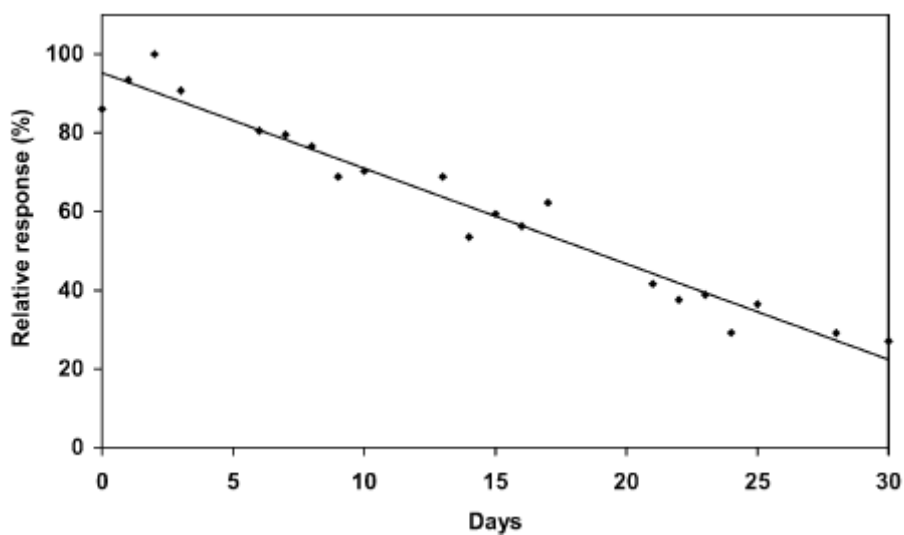


Figure 8: Long term stability of a PEDT/GOD electrode. Conditions: 5 mM glucose, 0.01 M, pH 7.0 sodium phosphate buffer and 0.1 M KCl, 25 °C, +650 mV vs. SCE.

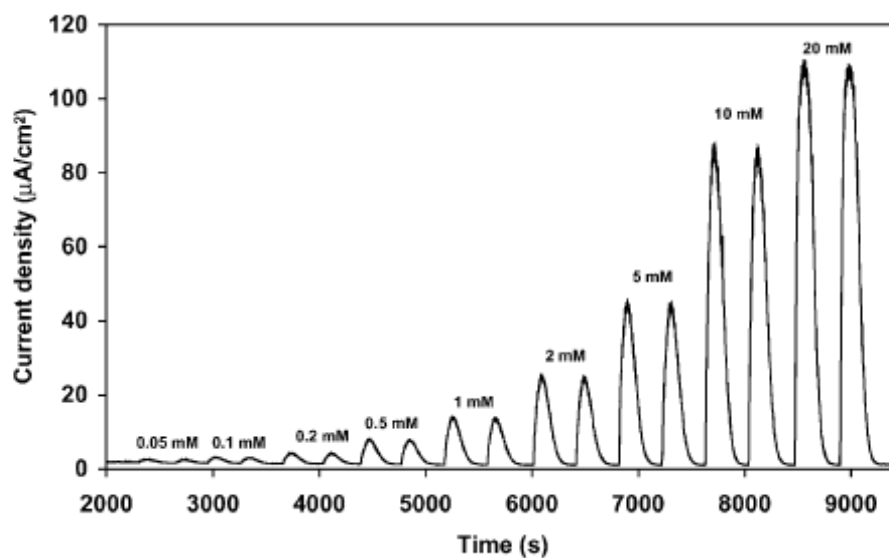


Figure 9: Flow injection analysis of the biosensor to various injections of glucose samples in duplicate. Conditions: 0.01 M, pH 7.0 sodium phosphate buffer and 0.1 M KCl, 25 °C, +650 mV vs. Ag/AgCl, 75 $\mu\text{l min}^{-1}$ flow rate.

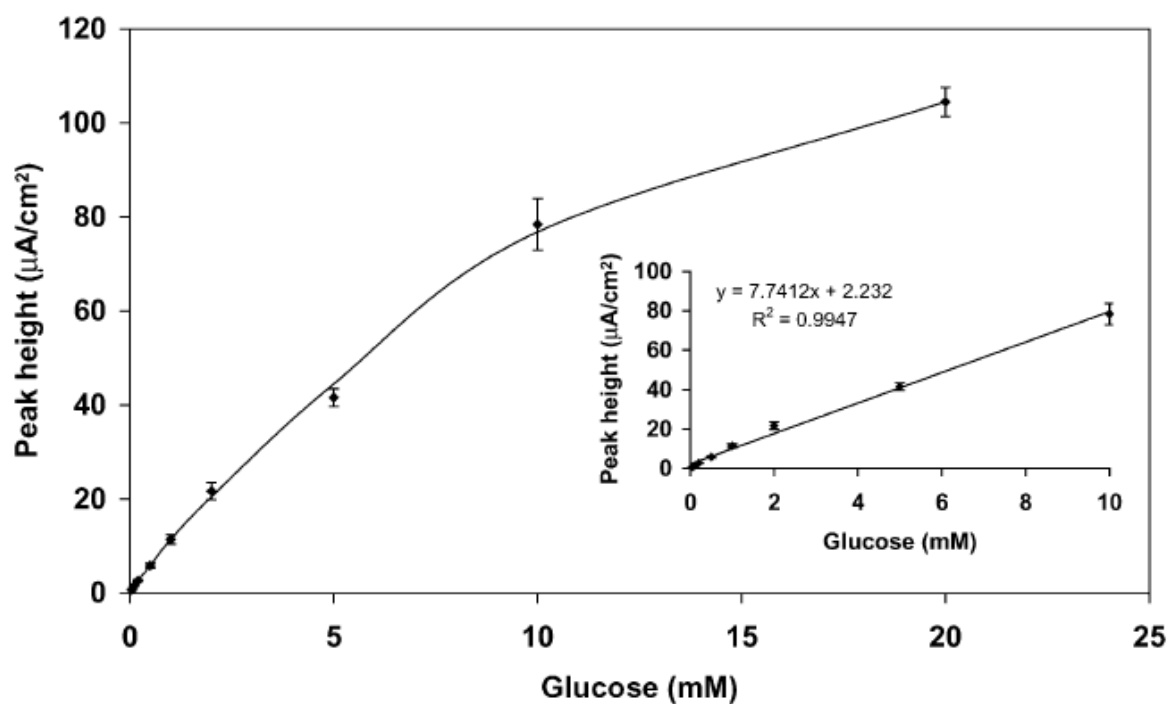


Figure 10: Calibration plot in FIA determination of glucose with the FIA/biosensor. Conditions: 0.01 M, pH 7.0 sodium phosphate buffer and 0.1 M KCl, 25 °C, +650 mV vs. Ag/AgCl.

Tables

Table 1: Comparison of the results obtained with this FIA/biosensor to those obtained with standards glucose kits, in serum glucose analysis

Method	SIU ^a unit	SIU Mean	SIU Range
Abbott Spectrum/VP series (hexokinase)	mmol/l	4.9	4.4-5.4
Bayer-Opera/RA series (hexokinase/340 nm)	mmol/l	5.0	4.5-5.5
Beckman-CX/LX/Synchron series (hexokinase)	mmol/l	4.8	4.3-5.3
BioChem-ATAC (hexokinase/340 nm)	mmol/l	5.3	4.7-5.9
Ortho-Vitros (oxidase)	mmol/l	4.8	4.3-5.3
Pointe Scientific (oxidase)	mmol/l	5.0	4.5-5.5
Roche-Cobas Mira series (hexokinase/340 nm)	mmol/l	4.6	4.2-5.0
Sigma (enzymatic, Trinder)	mmol/l	4.7	3.7-5.7
Present FIA biosensor	mmol/l	4.1	3.9-4.3

^a System of International Units.