Influence of polymer preadsorption on the performance of an electrochemical serum biosensor

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Abstract

We compare the performance of platinum and glassy carbon electrodes with and without polymer pretreatment, in detecting the presence of $\text{H}_2\text{O}_2$ after passage of blood serum through an enzymatic minireactor. The simple pretreatment is performed by physisorption from a dilute aqueous solution of the polymer, leading to a diffuse layer of polymer segments which protects the electrode from large entities such as proteins, while allowing the small electroactive species to reach the electrode surface. Three polymers, all with a high polyoxyethylene content, were tested and the results were evaluated. The signal on starting sequences of serum injections is less high in the pretreated electrode than in the untreated electrode. However, the loss of signal on increasing the number of injections is lower and leads to a more stable response and finally, after 20 injections, to a higher signal than at untreated electrodes.

INTRODUCTION

One of the most important problems arising in in-vivo electrochemical biosensors is the adsorption of biological species on their surfaces when implanted. The points where the implanted electrodes cause tissue damage are rapidly regenerated and covered by conjunctive tissue or even by antibodies from electrode rejection. The formation and growth of conjunctive tissue is influenced by the form and nature of the electrode material, i.e. its biocompatibility. Therefore it is
important to develop biomaterials for in-vivo sensor applications since neither the conjunctive tissue nor the antibody layer on the electrode are conducting, and a large decrease in electrode response after implantation is observed. The development of chemically modified electrodes better suited for biosensors and the use of mediators are being studied in several research laboratories [1,2].

In this paper we report results obtained using electrodes modified by the physical adsorption of synthetic polymers to create a haemocompatible surface. They were specifically designed to avoid protein adsorption and cell adhesion, and have been developed recently with the aim of pretreating hollow-fibre modules used in haemodialysis [3,4].

These physically modified electrodes were studied using a rotating-disc electrode (RDE) and a flow-through electrochemical wall-jet cell with a mini-reactor attached to the inlet [5] containing glucose oxidase (GOx) immobilized on a nylon membrane. The mechanism of reaction of GOx with glucose is of the double-displacement (ping-pong) type [6]. The active sites of the enzyme are the flavin (FAD) molecules and the reaction can be summarized as follows. In the membrane

\[
glucose + FAD \rightarrow \text{gluconolactone} + FADH_2
\]
\[
FADH_2 + O_2 \rightarrow FAD + H_2O_2
\]

At the wall-jet electrode

\[
H_2O_2 \rightarrow O_2 + 2H^+ + 2e^-
\]

We assume enzyme kinetics following the Michaelis–Menten theory. The amount of $H_2O_2$ oxidized at the wall-jet electrode, which is held at a potential corresponding to transport-limited oxidation, is proportional to the rate of the enzyme reaction (first-order kinetics). Therefore measurements of current vs. glucose concentration will furnish data on the GOx + glucose reaction mechanism.

EXPERIMENTAL

Chemicals and solutions

The enzyme glucose oxidase used was from *Aspergillus niger* (type X, E.C.1.1.3.4, activity 125,000 $\mu$g$^{-1}$ with O$_2$-saturated reaction) obtained from the Sigma Chemical Company (Poole, UK) and was immobilized on Nylon-66 material.

All other chemicals were analytical grade. Solutions were made up with triple-distilled water. The oxygen used to saturate the solution was from Ar Liquido (Air Liquide). Experiments were carried out in 0.10 M acetate buffer at pH 5.1.

The glucose standard solution was prepared by dissolving $\alpha$-D-glucose in acetate buffer, and before being used this solution was always left for 24 h at room temperature to allow equilibration of the isomers.
Blood serum samples were taken from patients without any specification of their type of illness; we obtained information about the values of glucose in their blood and they follow the accepted values of 3.34–5.55 mM for normal individuals in the fasting state. The serum samples were mixed, homogenized, diluted with acetate buffer (1:4) and kept frozen. They were defrosted and homogenized again before use.

All experiments were performed at room temperature (T = 19–22°C).

**Immobilization of GOx on nylon**

The enzyme (GOx) was covalently bound to discs of nylon material. Several immobilization procedures have been tried previously [7]. In this work the immobilization sequence nylon–G–BSA–G–GOx was used, where G denotes glutaraldehyde and BSA denotes bovine serum albumin denatured by heat.

The immobilization procedure was as follows. The nylon membrane was hydrolysed under reflux in 3 M HCl for 10 min and then activated with glutaraldehyde (12.5%) for 3 h at 40°C in phosphate buffer (0.10 M, pH 8.0). The same buffer was used for coupling the BSA for 4 h at 40°C. To denature the BSA coupled to the membranes, they were refluxed for 10 min and then dried under vacuum for 4 h at 40°C. The membranes were again activated with glutaraldehyde and finally the enzyme was immobilized: 1.5–1.8 mg cm\(^{-3}\) of GOx in phosphate buffer (0.10 M, pH 6.0) for 24 h at 20°C. In each phase of immobilization the membranes were thoroughly rinsed with deionized water in a porous Buchner filter. These membranes were obtained with good immobilization yields, a good enzyme retention, maximum enzyme activity and a minimum \(K_M\), i.e. maximum enzyme affinity, determined electrochemically.

**Preparation of electrodes and characteristics of the polymers**

Glassy carbon or platinum electrodes were polished with alumina (0.3 µm particle size) and cleaned with triple-distilled water. The polymeric films were formed by physical adsorption of the polymer onto the cleaned surface of the working electrode.

A solution of ca. 1.0 mg cm\(^{-3}\) of polymer in acetate buffer was prepared. The electrode was carefully immersed in 2 cm\(^3\) of a solution of acetate buffer to which 4 cm\(^3\) of the solution of the polymer was added; the concentration of the final solution was ca. 0.67 mg cm\(^{-3}\). The electrode was immersed in this solution for 15 min with constant stirring. Afterwards, the electrode was cleaned with triple-distilled water and immersed in the buffer solution at room temperature until use. Films were prepared daily.

Radical polymerization of trimethylaminoethyl chloride methacrylate (MQ), methoxy-poly(ethyleneglycol) methacrylate (MG\(_2\)), and acrylonitrile (AN) was performed in water at 30°C for 3.25 h with the couple KClO\(_3\) + NaHSO\(_3\) as initiator:
Different proportions of the monomers led to the polymers described in Table 1. Monomer MQ contains quaternary ammonium for the purpose of ionic binding with the sulphonate groups of the haemodialysis membrane. Monomer MG₃ contains a chain of poly(ethylene oxide). Monomers MQ and AN were chosen so as to anchor the polymer to the membrane, while monomer MG₃ was chosen to make a haemocompatible water-rich layer. This terpolymer is a comb-like polymer with branches of poly(ethylene oxide) which is known to be a good candidate for haemocompatibilization.

**Other apparatus and procedures**

RDE experiments were performed using a platinum disc (r_D = 3.48 mm) or a glassy carbon disc (r_D = 3.50 mm). The reference was a saturated calomel electrode (SCE) and the auxiliary electrode was a platinum foil.

**Table 1**

<table>
<thead>
<tr>
<th>Polymer</th>
<th>Monomers</th>
<th>MQ</th>
<th>MG₃</th>
<th>AN</th>
</tr>
</thead>
<tbody>
<tr>
<td>A5</td>
<td></td>
<td>0.52</td>
<td>0.46</td>
<td>0.02</td>
</tr>
<tr>
<td>A3</td>
<td></td>
<td>0.00</td>
<td>0.71</td>
<td>0.29</td>
</tr>
<tr>
<td>C5</td>
<td></td>
<td>0.02</td>
<td>0.85</td>
<td>0.13</td>
</tr>
</tbody>
</table>
Electrode potentials were controlled and currents measured using a potentiostat developed in our laboratory. For platinum working electrodes the potential applied to ensure mass-transport-limited oxidation of $\text{H}_2\text{O}_2$ was $+0.65$ V/SCE and for glassy carbon it was $+1.10$ V/SCE. Currents were registered on an HP 7015B x-y-t recorder.

The working electrode of the electrochemical wall-jet detector was either a platinum disc ($r_D = 1.48$ mm) or a glassy carbon disc ($r_D = 2.49$ mm). The reference electrode was $\text{Ag/AgCl} (0.1 \text{ M KCl})$ and the platinum tube outlet of the cell functioned as the auxiliary electrode.

Flow injection analysis was performed according to the scheme in Fig. 1. The injector valve was a Valco (GCW-HC) titanium valve with a 15 $\mu$l loop, and the solution was pumped with a Pharmacia P3 three-channel variable-flow-rate peristaltic pump. The flow rate was in the range $0.50-0.65$ cm$^3$ min$^{-1}$.

RESULTS AND DISCUSSION

Evaluation of the different polymers

The rotating-disc electrodes were coated with polymers using the adsorption procedure described above. In order to evaluate their electrochemical performance the modified electrodes were tested in a solution containing 2 mM potassium ferrocyanide in 0.10 M acetate buffer (pH 5.1).

The adsorption of the polymer creates a diffuse layer of polymer segments near the interface, described in terms of loops and tails, while segments directly attached to the electrode surface are called trains. The thickness of the trains layer is of the order of magnitude of a segment size, a few ångströms. As we observed a slower rate of the ferrocyanide reaction, two qualitative arguments can be given for such an effect. First, the trains layer constitutes a non-conducting barrier covering the greater part of the accessible surface of the electrode. However, the disorder in
this layer in addition to the dynamic exchange between loops and trains allows small electroactive species to reach the electrode surface. Second, this diffuse layer can be characterized by a hydrodynamic thickness [8]. Even when convection occurs near the surface of the covered electrode, the fluid velocity within this diffuse layer is almost zero. Therefore the final transport of the electroactive species to the actual surface of the electrode is purely diffusive.

In Fig. 2 we present the voltammograms obtained with the polymer designated A5. They were much better than those of the other films as confirmed in Fig. 3 by the cyclic voltammograms of all three polymers. The A5 polymer was used on the electrodes for the rest of the experiments. These were with the flowthrough
Fig. 4. Effect of flow rate on the kinetics of the enzyme GOx immobilized on the membrane used in the biosensor; O $I$; • $K'_{M}$.

electrochemical wall-jet cell and the minireactor containing a nylon membrane with GOx covalently immobilized on it which was attached to the cell inlet.

**Effect of flow rate on membrane performance**

We studied the kinetic behaviour of GOx immobilized on nylon membranes in order to optimize the flow conditions. The membranes were placed in the minire-

![Graph showing the effect of flow rate on membrane performance](image)

**Fig. 5. Flow injection analysis of glucose samples in 0.1 M acetate buffer. The working electrode is platinum covered with a film of AS.**
actor, and Fig. 4 shows the effect of different solution flow rates on the characteristics of the enzyme, evaluated electrochemically. We observe that the enzymatic yield decreases with increasing flow rate since there is an increase in the apparent value of $K_M$. The current observed has a maximum for a flow rate $V_f = 0.50 \text{ cm}^3 \text{ min}^{-1} (\approx 0.00835 \text{ cm}^3 \text{ s}^{-1})$. Therefore, on choosing this flow rate, we are compromising between several factors affecting the performance of the biosensor.

**Electrochemical flowthrough biosensor**

The flow injection system was calibrated with glucose standards in a region that included the concentration of glucose in normal individuals (Fig. 5).

Blood serum samples were used to test the flow-through glucose minireactor with wall-jet electrochemical detection using real sample conditions. The injection of blood serum directly into the flow system causes blockage of the injection loop and a decrease in the observed signal with the number of injections performed.

To prevent blockage of the injection loop the serum was diluted (1:4) and standard samples of glucose were injected twice between the serum samples. By using this procedure we were able to calibrate the system continuously as well as avoid blockage problems.

![Graph](image1)

![Graph](image2)

*Fig. 6. Plots of flow injection of glucose standard samples (1) 8.34 mM; (2) 4.55 mM; (3) 2.83 mM; (4) 1.92 mM; (5) 0.98 mM) in 0.1 M acetate buffer, followed by injections of serum using a glassy carbon working electrode: (a) uncoated; (b) coated with A5 polymer film.*
We performed flow injection analysis (FIA) experiments with glassy carbon (Fig. 6) and platinum (Fig. 7) working electrodes with and without a covering of a film of A5; Figs. 8 and 9 show the respective calibration curves with glucose. The experiments consisted in injecting a standard amount of glucose between the injections of serum samples (up to 25), comparing the stability of the signals and repeating for a different concentration of glucose in order to construct the calibration curve.

Using uncoated glassy carbon and platinum electrodes (Figs. 6(a) and 7(a)) we found that the signal decreased, first very rapidly and then more slowly, even after 25 injections. Figures 8(a) and 9(a) show the respective calibration curves with glucose. Cleaning and polishing the electrode surface led to precisely the same type of behaviour on repeating the series of injections. These observations suggest that the blood serum does not affect the activity of GOx in the membrane.

We prepared the platinum and glassy carbon electrodes with the surface covered with a film of polymer A5 and repeated these experiments. The results are shown in Figs. 6(b) and 7(b). The signal was not as high to start with but a smaller decrease in signal size with increasing number of injections was observed. After the fourth injection the signal became constant, and after 20 injections the signal

![Graph](attachment:image.png)

**Fig. 7.** Plots of flow injection of glucose standard samples ((1) 8.34 mM; (2) 4.55 mM; (3) 2.83 mM; (4) 1.92 mM; (5) 0.98 mM) in 0.1 M acetate buffer, followed by injections of serum, using a platinum working electrode: (a) uncoated; (b) coated with A5 polymer film.
was higher than that without the film. The respective calibration curves are shown in Figs. 8(b) and 9(b).

When the same type of experiment was repeated without using membrane with immobilized GOx in the minireactor and injecting H₂O₂ instead of glucose between serum injections, the same decrease in current as before was observed. Therefore we deduce that the decrease in response is due to partial blocking of the electrode surface through adsorption of components of the blood serum sample, i.e. fats (triglycerides, esterified cholesterol etc.), forming a film which is porous to H₂O₂. Further blocking does not occur, probably for reasons of physical size. It seems that the film of polymer A5 prevents fouling of the electrodes by the serum constituents and leads to a longer life for the electrode. Better reproducibility is attained.

Nevertheless platinum electrodes gave higher currents compared with glassy carbon electrodes and a less positive potential for the oxidation of H₂O₂ could be employed.

CONCLUSIONS

The application of physically modified electrodes through the adsorption of synthetic polymers that have been developed with the aim of creating a haemo-compatible surface seems promising for future application in in-vivo biosensors. Pretreatment of the electrode with biocompatible polymers prevents poisoning of
the electrode by the biological constituents, and the rejection and deposition of connective tissue onto the electrode surface. The polymer covering is permeable to small molecules that are electroactive and will react at the electrode.

Flow injection results showed that these physically modified electrodes can be used for flow injection analysis, and that they can be automated and used for continuous determination of glucose in blood serum samples or, in the stationary mode, for in-vivo measurements. The pretreatment of microelectrodes for experiments in vivo should give confirmation of this assumption and is under investigation.

ACKNOWLEDGEMENT

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REFERENCES