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Glucose-Sensitive Polypyrrole/Poly(Styrenesulfonate) Films Containing Co-immobilized Glucose Oxidase and (Ferrocenylmethyl) Trimethylammonium Bromide

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GLUCOSE-SENSITIVE POLYPYRROLE/ POLY(STYRENESULFONATE) FILMS CONTAINING CO-IMMOBILIZED GLUCOSE OXIDASE AND (FERROCENYLMETHYL) TRIMETHYLAMMONIUM BROMIDE

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ABSTRACT

Glucose oxidase (GOx) and (ferrocenylmethyl) trimethylammonium bromide (FcBr) were co-immobilized into polypyrrole (PP)/poly(styrenesulfonate) (PSS) films during the electropolymerization of pyrrole. PSS was employed as a partial dopant anion for PP (along with Br^-) and as an electrostatic binder for the cationic mediator (FcBr). The electropolymerization of the PP/PSS film was carried out at +0.8 V vs SCE in a 5-cm^3 aqueous solution of glucose oxidase (1 $mg \cdot cm^{-3}$), FcBr (1 mM), PSS (1 mM), and pyrrole (50 mM) at 5°C. These enzyme-mediator derivatized electroactive polymer films (ca. 0.5–2 μm thick) coated on Pt electrodes showed sensitivities to glucose without the use of any mediator in solution, and can be used as amperometric glucose sensors. The responses of the resulting electrodes (0.24 cm^2 Pt plate with

the polymer film) to glucose were measured amperometrically in pH 7, 0.1 M phosphate buffer solution at 5°C, and a constant potential (+0.5 V vs SCE) by using a Pt coil counterelectrode, with a saturated calomel electrode (SCE) as the reference. Co-immobilization of GOx and FcBr in PP/PSS films, responses of the PP/PSS/FcBr/GOx film on Pt electrodes to glucose, and the effect of the temperature on glucose response are discussed.

INTRODUCTION

Glucose oxidase (GOx) is an enzyme of much clinical interest, especially for the development of glucose biosensors. Oxygen is the natural electron acceptor when glucose is oxidized by GOx to form gluconolactone and hydrogen peroxide (H_2O_2). However, electrochemical detection of H_2O_2 can suffer from complications such as the high anodic potential of H_2O_2, diffusion limitations of O_2 (requiring stirring and/or oxygen bubbling), and, most importantly, the deactivation of GOx when H_2O_2 is allowed to accumulate in the reaction medium.

An alternate approach is to use mediators other than oxygen for electron transfer that have low redox potentials, such as ferrocene and its derivatives [1, 2]. The processes that take place in such a system can be shown by the following scheme of coupled reactions [1-3]:

$$\text{GOx(FAD)} + \text{glucose} \rightarrow \text{GOx(FADH}_2\text{)} + \text{gluconolactone} \quad (1)$$

$$\text{GOx(FADH}_2\text{)} + \text{Med}_{(ox)} \rightarrow \text{GOx(FAD)} + \text{Med}_{(red)} + 2\text{H}^+ \quad (2)$$

$$\text{Med}_{(red)} \rightarrow \text{Med}_{(ox)} + ne^- \quad (3)$$

FAD and $FADH_2$ represent the redox center of GOx in its oxidized and reduced forms, respectively. $Med_{(ox)}$ and $Med_{(red)}$ are the oxidized and reduced forms of the electron mediator. The mediators are used to facilitate electron transfer between the enzyme active site and the electrode, and the reaction in Eq. (3) is monitored amperometrically. Ferrocene derivatives are common mediators for this purpose [1], and several polymeric, ferrocene-based mediators have recently been employed [4, 5] in electrochemical glucose biosensors.

A particularly attractive approach for immobilization of GOx involves entrapment of the enzyme in water-stable conducting polymers such as polypyrrole (PP) and poly(*N*-methylpyrrole) [6]. This is easily done during anodic electrosynthesis of PP films. H_2O_2 can be detected amperometrically, but it has been argued that this is possible only after the polymer film has lost its electroactivity and is serving merely the role of an immobilization matrix [7]. Alternatively, it is possible to use a mediator in the analyte [1], or to monitor the reduction current of iodine generated by the reaction of iodide with H_2O_2 [8]. It should be noted that in principle electroactive PP could act as a mediator, although this is not commonly observed, presumably because the PP rigid chains are not in close proximity to the FAD redox center of the enzyme. Recently much research has been done on the co-immobilization of GOx and mediators such as ferrocenecarboxylate [2], hydroquinone sulfonate [3], and ferrocene [5] in polypyrrole films. Here it is anticipated that the mediator will reside near the enzyme redox center and provide a means to shuttle electrons between the redox center and the electrode (and perhaps PP).

The objective of our study was to develop a system in which the enzyme and the mediator were co-immobilized in a conductive polymer film which would be rather stable under ambient conditions and easily prepared electrochemically from aqueous solutions. To meet these conditions, GOx and (ferrocenylmethyl) trimethylammoniumbromide (FcBr) were co-immobilized into PP/poly(styrenesulfonate) [PSS] films deposited on Pt electrodes. PSS was selected to serve several functions. When PSS is the sole electrolyte for pyrrole electropolymerization, a PP/PSS composite is formed at the anode [9] and should electrostatically bind the cationic ferrocene derivative, FcBr. The sulfonates can also function as charge-balancing anions for conductive, oxidized (cationic) PP, along with Br^- from FcBr. In addition, since the surfaces of PP/PSS composites are rich in sulfonates [10, 11], the anionic charge may assist in repelling anionic interfering species from the films. Finally, these PP/PSS films have recently been shown to support cell growth without apparent cytotoxicity, suggesting that they may be useful in implantable sensors and devices [12]. It must be noted that GOx, which has an isoelectric point of 4.2, may also act as a dopant [13]. Different but related approaches were recently disclosed by Yoneyama et al. [3], who employed a sulfonated quinone as a polypyrrole dopant and mediator, and by Schoo and Challa [14], who employed a mediator covalently bound to a polyanion dopant.

EXPERIMENTAL

Reagents

GOx from *Aspergillus niger* (Type VII-S, Sigma), which has an activity of 132 $U \cdot mg^{-1}$, was used. Pyrrole (P) (Aldrich) was passed over alumina before use. PSS (Aldrich) and FcBr (TCI-EP, Imported by American Tokyo Kasei) were used as received. Glucose (Sigma) solutions were prepared in pH 7, 0.1 M phosphate buffer freshly every 2 weeks, left at room temperature for 24 hours prior to use, and thereafter kept in a refrigerator. (In this paper all references to phosphate buffer are for pH 7, 0.1 M phosphate buffer.)

Apparatus

For the electropolymerization step, as well as for amperometric and cyclic voltammetric measurements, a PAR Model 273 potentiostat-galvanostat was used. The electrosynthesis and glucose analysis cell was a one-compartment type employing a Pt plate working electrode (0.24 cm^2), a wound Pt coil counterelectrode, and a saturated calomel reference electrode (SCE). The working electrode was polished with diamond polishing compound and 0.3 μm alumina powder (Buehler Ltd., USA) before use. A chart recorder (Linear) and an X-Y recorder (Hewlett-Packard) were used for amperometric measurements and cyclic voltammetry, respectively. A Zeiss DSM 950 scanning electron microscope was used to take the electron micrographs of the films.

Procedure

In the initial experiments of this study, the polymer films made by anodic electropolymerization at +0.8 V contained P/PSS/GOx but no FcBr. The films thus prepared were soaked in a 10-mM solution of FcBr in phosphate buffer for 6–

12 hours, and they showed sensitivity to glucose. Since the water-soluble mediators can diffuse away from the polymer film, the physically adsorbed mediator on the film was easily leached out within a short period of time. In order to co-immobilize both GOx and FcBr in a PP/PSS film, electropolymerization of pyrrole was carried out at +0.8 V constant potential (vs SCE) from an aqueous solution of pH 6.8 at 5°C, which contained various concentrations of P, PSS, FcBr, and GOx. Twice-distilled water was used for the deposition bath, and N_2 was bubbled into the solution for 5 minutes before the electropolymerization. The charge used for the deposition of PP was read directly from the PAR 273. The electrolysis time was proportional to the deposition charge at the same experimental conditions (e.g., working electrode area, electrolysis solution concentrations). The electrodes were rinsed with water and soaked in phosphate buffer at 5°C for at least 5 hours to eliminate weakly bound enzyme and mediator prior to use in the glucose sensitivity measurements.

The amperometric response to glucose was evaluated in 5 cm^3 phosphate buffer solutions. After 10 minutes of N_2 bubbling into the solution before the addition of glucose, the electrode was potentiostated at +0.5 V vs SCE to decrease the background current to a constant value. Then the 0.5-cm^3 stock glucose solution was added to this buffer solution and the solution was magnetically stirred for 10 seconds under N_2. Current values were recorded in quiescent solutions. The background current was subtracted from the measured current value to obtain the current response. Fresh buffer solutions were used before each glucose addition.

To take the electron micrographs of the films, the electrodes were mounted on aluminum stubs with double-sided carbon tape. Some of them (disk electrodes) were also wrapped with copper tape for better conductivity. Cross-sectional views of the films were taken of the areas that cracked after the film dried by tilting the specimen stage 90°C.

RESULTS AND DISCUSSION

Cyclic Voltammetric Behavior of FcBr

Figure 1 shows cyclic voltammograms of 1 mM FcBr at Pt in deaerated phosphate buffer containing 50 mM glucose in the absence (a) and presence (b) of GOx. In the absence of GOx the oxidation–reduction waves of FcBr appeared near +0.4 V vs SCE. When GOx is added, the oxidation of FcBr, due to Eq. (3), was catalytically enhanced and the reduction peak of FcBr disappeared, demonstrating the effectiveness of FcBr as a mediator. When all the glucose in solution is consumed, the reduction peak can be seen at the cathode.

Co-immobilization of GOx and FcBr in PP/PSS Film

PPS was employed as a partial dopant anion for polypyrrole and electrostatic binder for the mediator during the electropolymerization. Different concentration combinations were tried, and these experiments led us to routinely employ an electropolymerization bath containing 50 mM pyrrole/1 mM PSS/1 mM FcBr/1 $mg \cdot cm^{-3}$ GOx. The resulting composite electrode is shown schematically in Fig. 2. Elemental analysis (Schwarzkopf Microanalytical Laboratory, Woodside, New

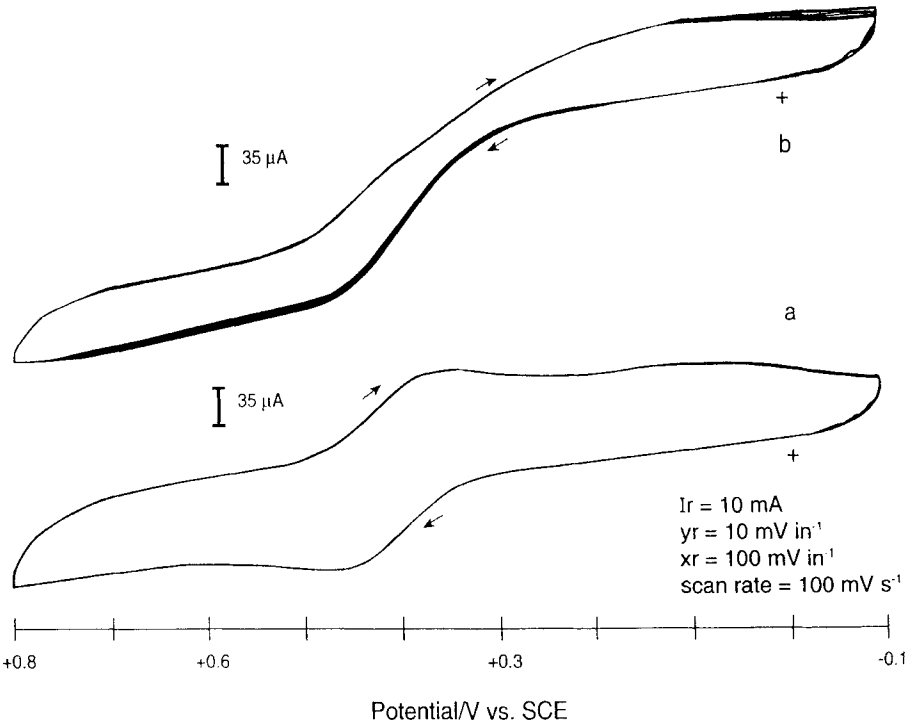

FIG. 1. Cyclic voltammograms of the platinum electrode in pH 7, 0.1 M deaerated phosphate buffer containing 1 mmol/L FcBr and 50 mmol/L glucose, in the absence (a) and presence (b) of GOx.

York) of the films made under these conditions gave the following results: 13.86% N, 0.65% S, 0.14% Fe, and 0.35% Br. From the data we find about 8–9 sulfonates per Fe (ferrocene), and calculate the sulfonate/Br^- anion ratio to be ca. 4–5:1.

The selection of the PSS concentration during electropolymerization is worth discussing briefly. When we used more than 1 mM PSS in the deposition bath, the films grew very quickly (for a 0.24-cm^2 Pt electrode) within a few minutes but nonhomogeneously. Film thicknesses were initially estimated based upon the amount of charge passed during electrosynthesis [15]. Since a film thickness of 0.025 μm requires 10 $mC \cdot cm^{-2}$, a 200-$mC \cdot cm^{-2}$ charge was used in an effort to

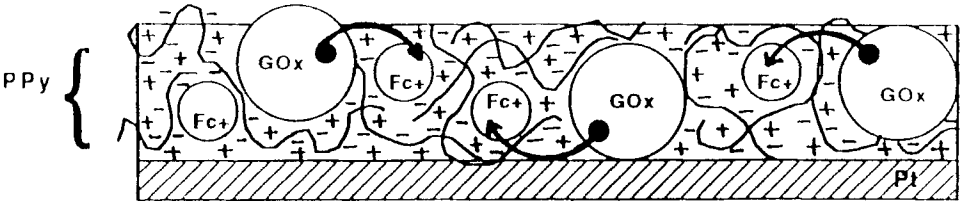

FIG. 2. The anticipated PP/PSS/FcBr/GOx film structure on the Pt electrode.

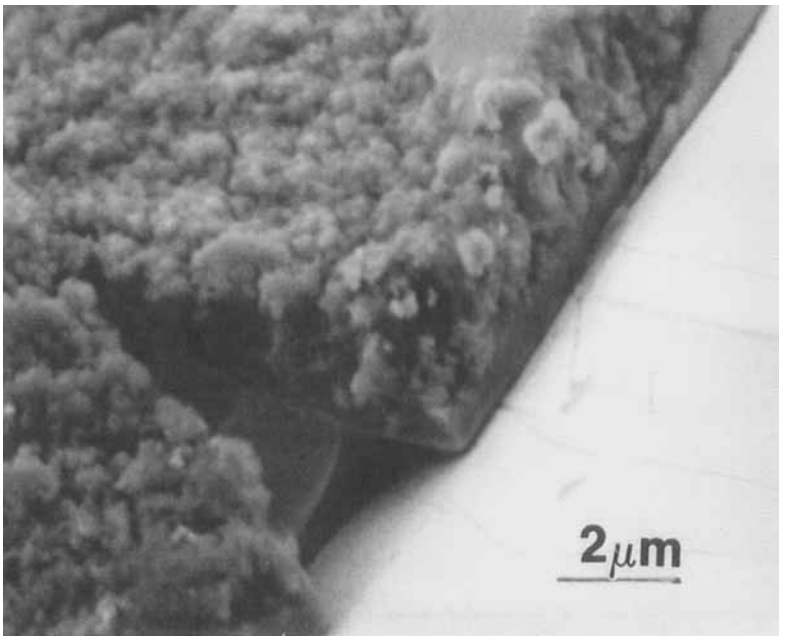

FIG. 3. An SEM photograph of a typical composite film. SEM conditions: 5 kV, 4 mm working distance (charge passed during electrosynthesis: 200 mC·cm $^{-2}$).

grow films 0.5 μm in thickness. The thicknesses of several films were determined experimentally by scanning electron microscopy (SEM) to be ~1.5 μm. We consistently found that the measured thicknesses are 2 to 3 times those predicted based on the amount of charge passed during electrosynthesis. An SEM photograph of a typical film is given in Fig. 3. Note the high porosity of the film, which we found was absent in PP/PSS/FcBr films grown under identical conditions without GOx.

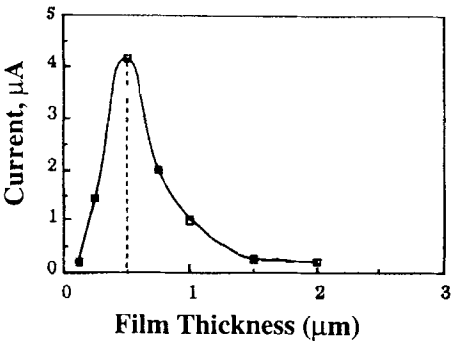

FIG. 4. Dependence of response current on film thickness at 20 mM glucose.

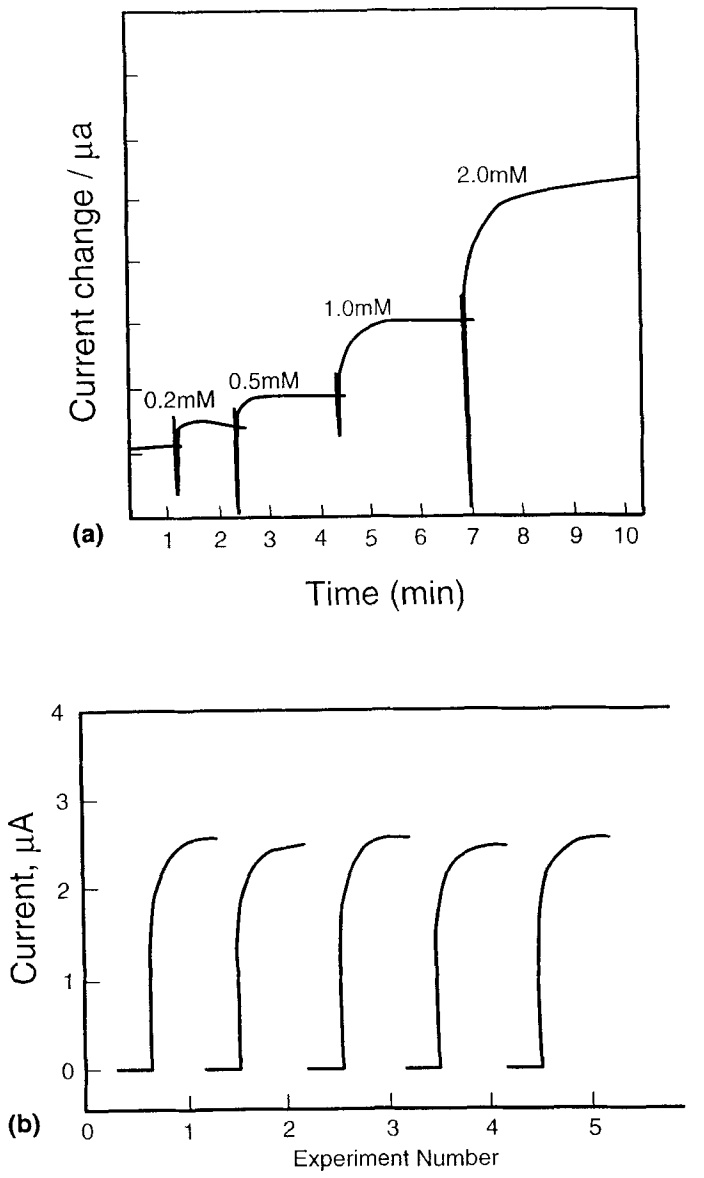

FIG. 5. (a) Raw data of electrode responses to repeated glucose additions (5°C, +0.5 V vs SCE). (b) Reproducibility of responses to 10 mM glucose using the same electrode.

Response of PP/PSS/FcBr/GOx Films on Pt Electrodes to Glucose

The films used in our experiments were prepared by using a 5-cm^3 deposition bath of 50 mM pyrrole, 1 mM PSS, 1 mM FcBr, and 1 $mg \cdot cm^{-3}$ GOx at pH 6.8. The response of these films is the same whether or not the stock glucose solution is

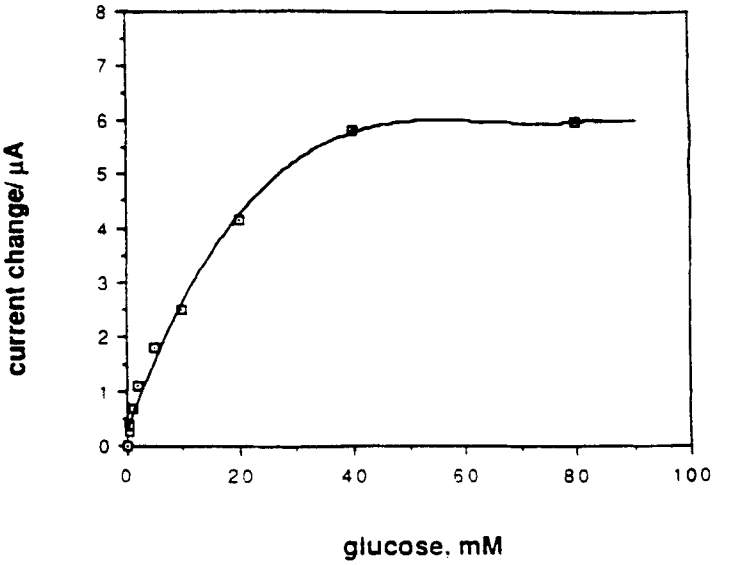

FIG. 6. Dependence of response current on glucose concentration for a 50-mM PP/1 mM PSS/1 mM FcBr/L·mg·cm^{-3} GOx electrode at +0.5 V vs SCE at 5°C (0.34 cm^2 Pt working electrode, 200 mC/cm^2 used to grow film).

deoxygenated before adding to it the phosphate buffer solution, although in all cases the buffer solution was deoxygenated prior to performing the glucose determinations. This is valid as long as the applied potential is less than +0.5 V where H_2O_2 cannot be oxidized. Glucose responses increased and were observed to be proportional to the applied potential between +0.3 and 0.5 V vs SCE. A potential of +0.5 V was used in all experiments unless noted otherwise. Polypyrrole films containing immobilized GOx without any mediator did not show any amperometric sensitivity to glucose in air-saturated solutions at potentials more negative than +0.5 V vs SCE, as reported by others [7].

The glucose sensitivities of the resulting polymer films have been investigated, focusing on the polymer film thickness and amounts of P, PSS, and FcBr incorporated in the electrosynthesis bath. The highest current response was achieved at 200 mC·cm^{-2}. The films were not grown as homogeneously when the amount of charge passed was lower than this value. A plot of current response vs PP film thickness for 20 mM glucose samples is shown in Fig. 4. The reason for the maximum response at a thickness of ca. 0.50 μm likely reflects the competition between the having more enzyme by virtue of having thicker films and the diminishing diffusion rate of glucose in the film. However, slow electron and/or proton diffusion may also be important. Figure 5(a) shows the actual current responses of the electrode (PP film prepared with a 200 mC·cm^{-2} deposition charge in 15 minutes at 5°C, +0.8 V vs SCE) for various glucose concentrations, such as 0.2, 0.5, 1.0, and 2.0 mM by repeated additions of glucose. The reproducibility of an electrode is good as indicated by Fig. 5(b) which shows responses from five successive determinations with the same film where [glucose] = 10 mM. At low glucose concentrations, the electrode response is approximately proportional to glucose concentration (Fig. 6),

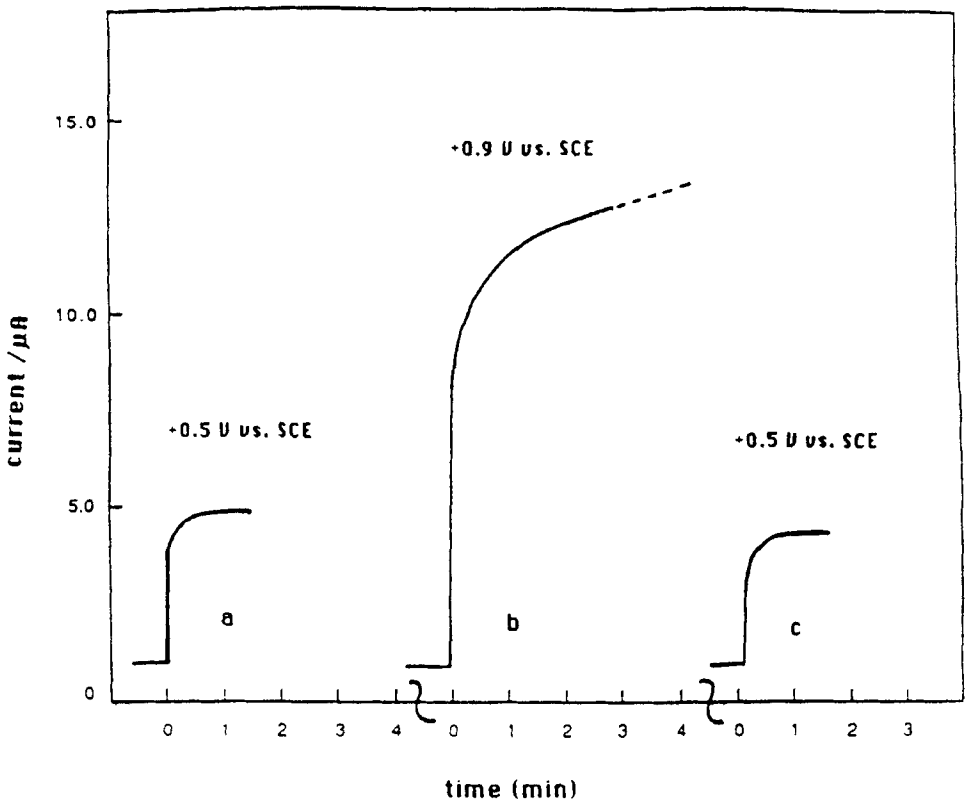

FIG. 7. Glucose response of a composite film potentiostated at (a) +0.5 V, deaerated, before H_2O_2 generation; (b) +0.9 V, with aeration and generation of H_2O_2; and (c) +0.5 V, deaerated, after H_2O_2 generation. [Glucose] = 20 mM. 200 mC/cm^2 used to grow film.

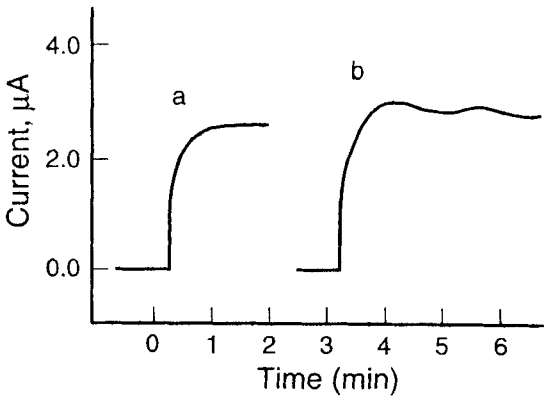

FIG. 8. Glucose responses of a composite film at (a) 5°C and (b) 35°C. [Glucose] = 10 mM. 200 mC/cm^2 used to grow film.

although the plot shows curvature at all concentrations. The greatest nonlinearity begins around a concentration of 40 mM of glucose, suggesting the onset of saturation of the enzyme.

While all of the above experiments were performed under N_2, we note that our electrodes can also be employed in the presence of oxygen to monitor H_2O_2 generated upon glucose oxidation, and then reused at +0.5 V vs SCE, apparently with the ferrocene mediator now functioning. A comparison of the film sensitivities before and after H_2O_2 generation are illustrated in Fig. 7. The first current response for 20 mM of glucose was monitored at +0.5 V as described earlier. A potential of +0.9 V was then applied to the electrode in fresh 4.5 cm^3, O_2-saturated phosphate buffer solution to obtain a constant background current. The response for the same glucose concentration (20 mM), due to H_2O_2 oxidation, was recorded. This response is larger than the previous one but requires much more time to reach a steady-state current. Finally, the film was again potentiostated at +0.5 V in fresh buffer solution, and the same amount of glucose was added. This last current response is slightly smaller (ca. 9% less) than the first one. This may be due to the loss of some GOx activity resulting from H_2O_2 generation, or from the partial loss of conductivity of PP at high potentials. Thus the PP apparently remains electroactive even after H_2O_2 generation. However, cycling of the electrode between +0.8 V and −0.1 V after the first response usually produced more stable and sensitive responses.

The Effect of the Temperature on Response

The glucose sensitivity of the film prepared as described earlier was measured at 5, 25, and 35°C for 10 mM of glucose. Fig. 8 compares glucose responses at 5 and 35°C, respectively. The responses at 25 and 35°C are usually larger than the responses at 5°C, presumably because of higher enzyme activity at the higher temperatures. The responses at 5°C are more stable than the others; responses at 35°C usually require a longer time to reach a steady-state current. Using lower temperatures may allow the GOx to retain its activity for a longer period of time, thereby increasing the lifetime of the electrode. In addition, the lower temperature may retard diffusional loss of GOx and FcBr from the composite film.

CONCLUSIONS

One of the major objectives of this study was to make reproducible and stable polymer films. The yield of working sensor electrodes using our procedures was about 70%, and each showed very close responses for the same glucose concentrations although further improvements of the reproducibility of electrode preparations are expected. We find that at least 35–40 glucose determinations can be performed using the same film. Glucose sensitivity is affected especially by the polymer film thickness [3], which effects the rate of diffusion of glucose in the polymer films and the feasibility of the electron transfer between GOx and FcBr. Therefore, further studies are required for the electrochemical characterization of these polymer films. Future work will be focused on characterization, longevity of the films,

interferences of other species, quantitative analysis of actual biological samples, and statistical evaluation of the reproducibility.

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