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POLYMER-COMPLEX MEDIATED ENZYME ELECTRODES FOR AMPEROMETRIC DETERMINATION OF GLUCOSE

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ABSTRACT

The immobilized enzyme chemically modified solid-state electrodes based on bilayer-film coating for amperometric determination of glucose have been fabricated and their sensor characteristics have been examined. The electrode substrate was coated with two kinds of polymeric films in a bilayer state, that is, System I: first with the cobalt tetrakis(*o*-aminophenyl)porphyrin polymer (poly-CoTAPP) film and then with an enzyme film consisting of bovine serum albumin and glucose oxidase (GOx), and System II: first with the $Ru(NH_3)_6^{3+}$-containing montmorillonite clay film and then with the GOx enzyme film. The glucose concentration could be monitored by measuring the currents corresponding to the O_2 reduction and the H_2O_2 reduction which are electrocatalyzed by the poly-CoTAPP film (System I) and the clay film (System II), respectively. The reproducible relationship between glucose concentration and sensor output was obtained for both systems with a dynamic range of ~1–100 m*M* (for System I as an electrochemical detector for a flow injection analysis) and ~0.4–4 m*M* (for System II). In addition, the sensors showed long-term stability (more than 1 and 2 months in System I and System II, respectively) and relatively rapid response (response times of System I and System II are ~5–10 and 40–60 s, respectively).

INTRODUCTION

One of the most important applications of chemically modified electrodes is their use as an electrochemical (amperometric and potentiometric) sensor [1]. In particular, immobilized enzyme chemically modified electrodes (IECMEs), which combine the specificity and selectivity of an enzyme for its natural substrate with the advantages of electrochemical detection, have become a research area of great interest [2–10] because of their simplicity of operation, superior amperometric response characteristics, capability of miniaturization, etc. Recently, we [11] reported on the electrode characteristics of the IECME based on bilayer-film coating for the amperometric determination of glucose. In this case the electrode substrate was coated with two kinds of polymeric films in a bilayer state, that is, first with the cobalt tetrakis(*o*-aminophenyl)porphyrin polymer (poly-CoTAPP) film and then with the glucose oxidase (GOx) enzyme film (this electrode system is abbreviated as System I). The inner poly-CoTAPP film electrocatalyzes the reduction of O_2 [12] which still remains without participating in the glucose–GOx enzyme reaction within the outer GOx film, and the concentration of glucose in solution can be correlated to the current of O_2 reduction [11].

In the present paper the sensor characteristics of two kinds of IECMEs based on bilayer-film coating for amperometric determination of glucose will be described. One is System I as an electrochemical detector for flow injection analysis (FIA). Another is the IECME based on a bilayer coating of montmorillonite clay film (inner layer) and GOx film (outer layer) (abbreviated as System II). In this case the clay film, into which $Ru(NH_3)_6^{2+/3+}$ complexes are incorporated electrostatically and function as an "electron shuttle," can be expected to electrocatalyze the reduction of H_2O_2 resulting from the glucose–GOx enzyme reaction [13]. Both these electrode systems are regarded as solid-state sensors.

EXPERIMENTAL

Reagents

Glucose oxidase (Type II, from *Aspergillus niger*, abbreviated GOx) was used as supplied by Sigma Chemical Co.. Tetrakis(*o*-aminophenyl)-porphyrin (TAPP) was synthesized and metalated with cobalt in refluxing pyridine under an N_2 atmosphere, as described previously [11]. Bovine serum albumin (Fraction V, abbreviated BSA) powder was obtained from

Kodak Co. Glutaraldehyde was an aqueous 50% solution (Kanto Chemical Co.). β-D-Glucose (anhydrous, for biochemistry) was obtained from Merck Co. The montmorillonite clay was the same as that employed previously [13]. $Ru(NH_3)_6Cl_3$ was obtained from Strem Chemical Co. Basal-plane pyrolytic graphite (BPG), used as an electrode substrate, was obtained from Union Carbide Corp. All other chemicals were of reagent grade.

Apparatus

A BAS-200 electrochemical analyzer (Bioanalytical Systems Inc.) was used in the flow injection experiments. The flow-cell used for electrochemical detection was of thin-layer design (LC-17A, BAS). The Ag/AgCl reference electrode was positioned downstream of the working electrode, and the auxiliary electrode was positioned directly across the thin-layer channel from the working electrode. The working electrode was glassy carbon (area: 7.07×10^{-2} cm^2) and the auxiliary electrode was stainless steel. The flow rate was varied in the 0.5–2.0 mL/min range.

A standard three-electrode electrochemical cell was used for measuring the steady-state current response of the sensors. The electrode assembly consisted of an immobilized enzyme chemically modified BPG electrode (area: 7.8×10^{-3} cm^2) as the working electrode, a sodium chloride saturated calomel electrode (SSCE) as the reference electrode, and a spiral platinum electrode as the auxiliary electrode. All the experiments were performed at room temperature (25 ± 1°C).

Preparation of IECMEs

In the case of System I the glassy carbon electrode substrate was coated with two kinds of polymeric films in a bilayer state, that is, first with poly-CoTAPP film (thickness: ~1 μm), prepared by electrooxidative polymerization of the monomer (CoTAPP) [11, 12] and then with the enzyme film (~0.1–0.6 mm) consisting of BSA and GOx that were held together by crosslinking with glutaraldehyde.

In System II the BPG electrode substrate was coated with the $Ru(NH_3)_6^{3+}$-containing montmorillonite clay film (~1 μm) and then with the GOx enzyme film (~0.1–0.6 mm). The incorporation of $Ru(NH_3)_6^{3+}$ into the montmorillonite clay film on the BPG electrode, which is based on its cation-exchange property [13], was conducted by soaking the clay film-coated electrode in 0.1 *M* phosphate buffer solution containing 0.2

m*M* $Ru(NH_3)_6^{3+}$. The clay film-coated BPG electrode thus prepared was further coated with the GOx film as described previously [11]. When not in use, the electrode was stored in pH 7.0 phosphate buffer solution containing 0.05 m*M* $Ru(NH_3)_6^{3+}$ at 4°C.

RESULTS AND DISCUSSION

System I as an Electrochemical Detector for FIA

A schematic depiction of the overall electrode/enzyme reaction for System I is shown in Fig. 1(A). In principle, as seen from this figure, the glucose concentration can be monitored by measuring 1) the O_2 consumption, 2) the formation of H_2O_2, or 3) the change in pH due to the formation of D-gluconic acid. However, the last procedure may not be employed in blood samples as well as in buffered solutions, since the pH change resulting from the enzyme reaction is virtually canceled under these conditions. As demonstrated previously [11, 12], it is important to note that in this system the poly-CoTAPP film effectively electrocatalyzes the O_2 reduction. Figure 2 shows characteristic flow-injection current–time profiles for 20, 30, and 40 m*M* glucose at the bilayer-coated electrode of System I. Note that the peak current obtained in the flow injection experiments represents the difference in the currents obtained in the absence and in the presence of glucose. It is apparent that a highly reproducible response is obtained, that is, no difference in peak size at a given concentration is observed. In addition, it can be seen that the electrode exhibits rapid increase and decrease of the current. The response times to reach 90% of the maximum signal are less than 5–10 s. The relatively fast response indicates rapid replenishment of the solution from the surface, as desired for dynamic flow systems.

The flow-rate dependence of the current response at a constant concentration of glucose was examined by recording the peak currents at different flow rates. A typical example of such experiments is shown in Fig. 3. The current response was found to decrease with increasing flow rate, in contrast with the case of a bare thin-layer detector where the response increases with increasing flow rate. For example, the peak current decreased from 0.59 μA at 0.5 mL/min to 0.29 μA at 2.0 mL/min. This fact indicates that restricted diffusion of glucose and/or O_2 to the enzyme can be controlled by the time of exposure of the sample to the electrode, i.e., by varying the flow rate. A similar flow-rate dependence

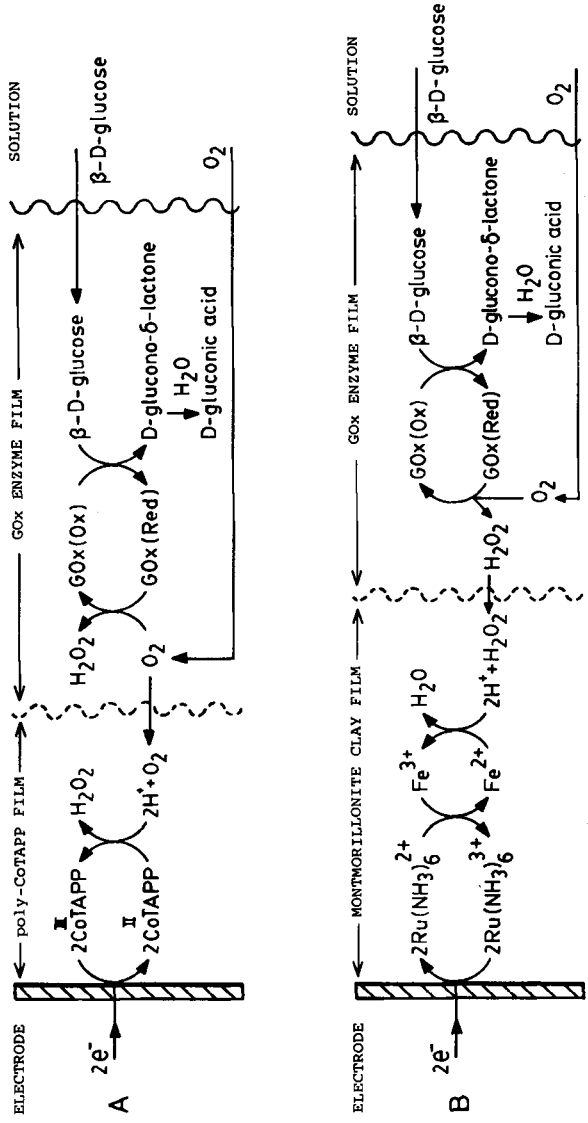

FIG. 1. A schematic depiction of the overall electrode/enzyme reactions at (A) GOx enzyme film/poly-CoTAPP film-coated electrode (System I) and (B) GOx enzyme film/montmorillonite clay film-coated electrode (System II). GOx (Ox) and GOx (Red) represent the oxidized and reduced forms of glucose oxidase, respectively.

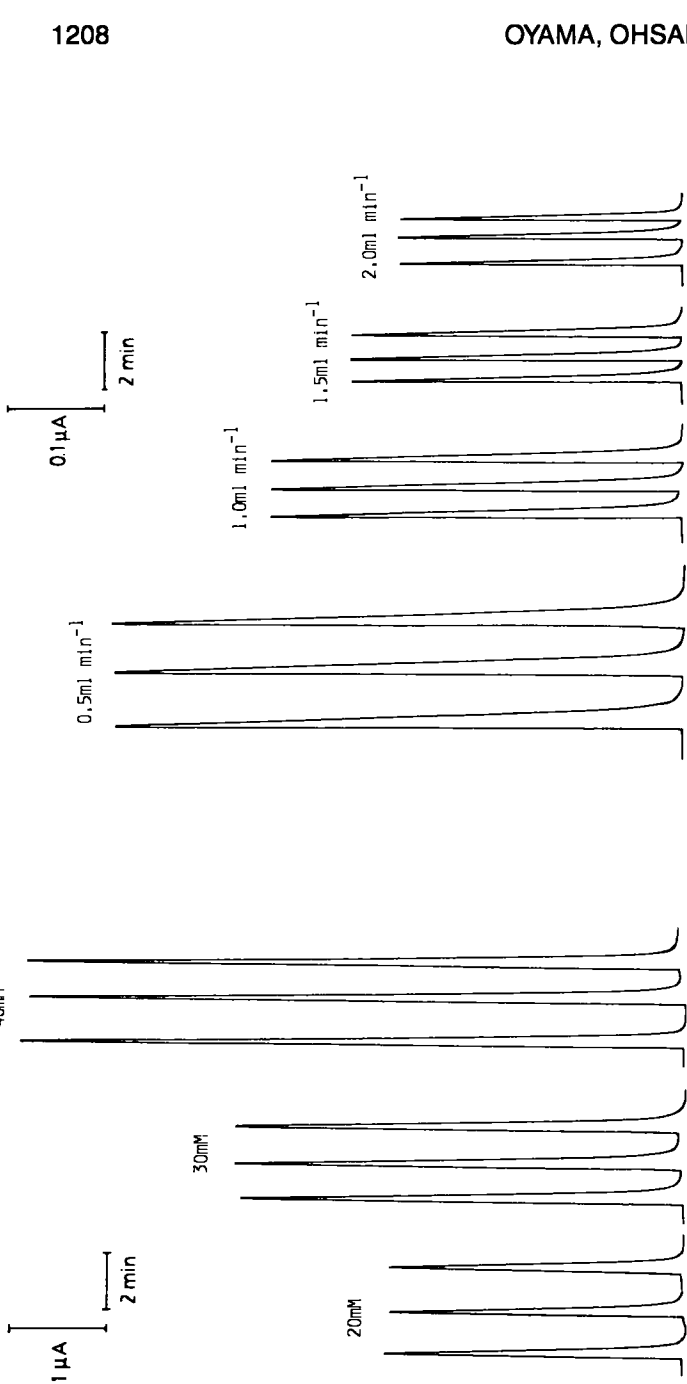

FIG. 2. Typical flow-injection current–time profiles for 20, 30, and 40 m*M* glucose at System I. Flow rate: 1.0 mL/min. Injected volume of glucose solution: 10 μL. Applied potential: −0.35 V vs Ag/AgCl. Electrolyte and carrier: air-saturated phosphate buffer solution (0.05 *M*, pH 7.0). The amount of GOx in the enzyme film: 0.14 mg/cm^2.

FIG. 3. Flow-rate dependence of the current response. Injected solution: 30 m*M* glucose (10 μL). Other experimental conditions are the same as in Fig. 2.

has been also reported for the poly(4-vinylpyridine) (PVP)-coated glassy carbon flow detector [14]. In this case, permselective positively charged PVP coatings offer significant selectivity improvements based on charge exclusion. In other words, they permit effective discrimination against cationic interferants. Such a flow-rate dependence is unique to the film-coated electrode system, and the related kinetics remain to be elucidated.

The hydrodynamic voltammograms (Fig. 4) for glucose obtained at the bilayer-coated electrode (●) and the electrode coated with the GOx enzyme film alone (○) under flow injection conditions demonstrate that the poly-CoTAPP film electrocatalyzes the O_2 reduction, as previously reported [11, 12]. For example, the ratios of the current obtained at the bilayer-coated electrode to that at the GOx film-coated electrode de-

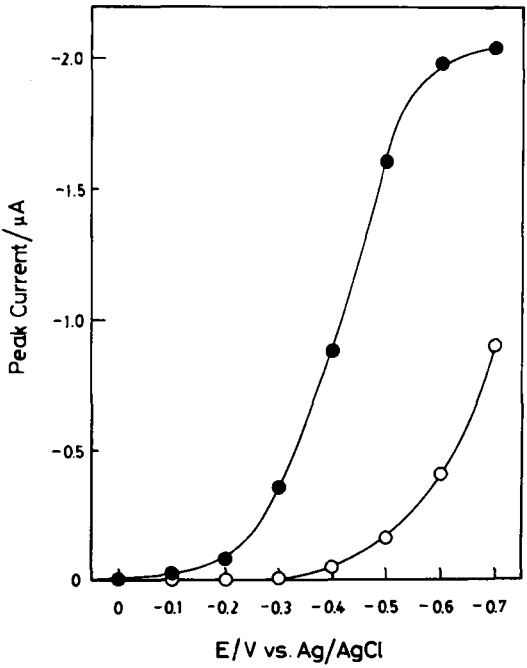

FIG. 4. Hydrodynamic voltammograms for 10 μL injections of 30 m*M* glucose. (●) At GOx film/poly-CoTAPP film-coated electrode (System I). (○) At GOx film-coated electrode. In both cases the amount of GOx in the enzyme films is 0.14 mg/cm^2. Other experimental conditions are the same as in Fig. 2.

creased with increasing electrode potential in the negative direction, i.e., they are ~36, 17.6, 10, and 4.8 at −0.3, −0.4, −0.5, and −0.6 V vs Ag/AgCl, respectively. On the other hand, the magnitude of the reduction current increased with a negatively increasing electrode potential. Thus, a potential of −0.5 to −0.3 V was normally chosen.

Figure 5 illustrates typical calibration curves obtained at the bilayer film-coated electrodes with different loadings of GOx. The peak current decreased gradually with increasing glucose concentration, depending upon the amount of GOx confined in the enzyme films. For an electrode with a higher loading of GOx, the dynamic range where the observed current significantly changes with glucose concentration is more narrow. For example, the dynamic range of the electrode with GOx of 0.71 mg/cm² was ~1–50 m*M*, while for the electrode with GOx of 0.071 mg/cm² it was ~1–100 m*M*. On the other hand, the sensitivity increased with the loading of GOx, that is, the initial slopes of the current vs glucose concentration curves were −6, −17, and −28 nA/m*M* for the electrodes with

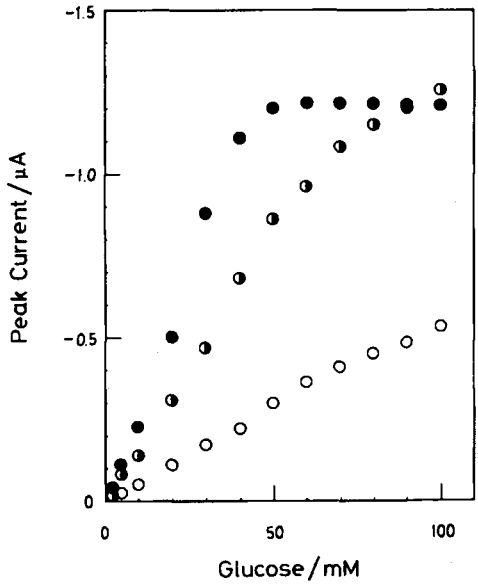

FIG. 5. Typical calibration curves for glucose in System I. The amount of GOx in the enzyme film: (●) 0.71, (◑) 0.14, and (○) 0.071 mg/cm². Other experimental conditions are the same as in Fig. 2.

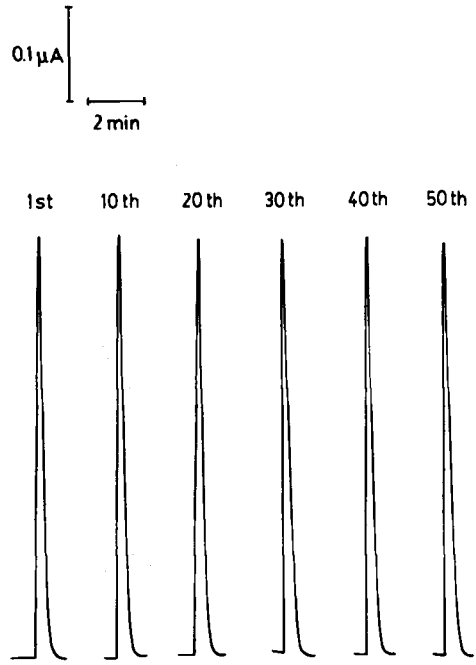

FIG. 6. Flow injection response obtained in System I for successive injections of 30 m*M* glucose over a period of an hour. Other experimental conditions are the same as in Fig. 2.

GOx of ~0.071, 0.14, and 0.71 mg/cm^2, respectively, in the enzyme films. As can be readily seen from Fig. 5, the present IECMEs based on bilayer-film coating possess a dynamic range wide enough to apply them to assays of whole blood serum samples (typically ~0.5–15 m*M*) [15–17].

Figure 6 shows the flow injection response obtained for 50 injections of a 30-m*M* glucose solution over a period of an hour. After the 50th injection, the peak response was still more than 97% of its initial value, and the relative standard deviation of the series of injections was less than 7%. Figure 7 shows the long-term sensor characteristics of the bilayer film-coated electrode with GOx of 0.14 mg/cm^2. Both the blank current obtained under the condition of the absence of glucose and the slope of the linear calibration curves, which represents the sensitivity of the electrode, decreased gradually with time. These decreases in the blank cur-

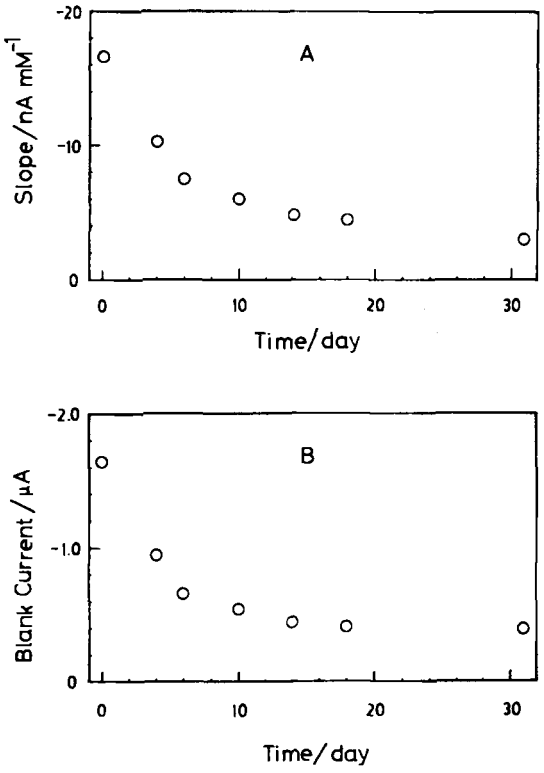

FIG. 7. Long-term sensor characteristics of System I. (A) Plot of slope of the linear calibration curves vs time. (B) Plot of blank current in the absence of glucose vs time. Experimental conditions are the same as in Fig. 2.

rent and the slope may be due to the loss of film integrity. However, the dynamic range remained almost constant (1–60 m*M*) over more than 1 month.

System II

The mechanism of the overall electrode/enzyme reaction in System II is shown in Fig. 1(B). In contrast with System I, the glucose concentration can be monitored by measuring the formation of H_2O_2. Recently, Oyama and Anson [13] reported that the $Ru(NH_3)_6^{3+}$-containing mont-

morillonite clay coating electrocatalyzes the reduction of H_2O_2 to H_2O. The $Ru(NH_3)_6^{2+/3+}$ complexes incorporated into the clay coating are thought to function as an "electron shuttle" which delivers electrons to the redox active sites [13]. The iron cations which commonly replace some of the aluminum ions in octahedral sites within the structure of montmorillonite clay (the clay we employed contains 2.7% iron) [13] are considered to be responsible for the catalytic activity since it was shown by Taube and coworkers [18] that traces of iron greatly enhance the rate of reaction between $Ru(NH_3)_6^{2+}$ and H_2O_2 in homogeneous solution, and $Ru(NH_3)_6^{2+}$ is not an efficient catalyst for the reduction of H_2O_2 at an uncoated graphite electrode [13].

Figure 8 shows a typical steady-state current response of the bilayer-film-coated GOx electrode (System II) to change the glucose concentration in phosphate buffer solutions (0.1 *M*, pH 7.0) containing 0.016 m*M* $Ru(NH_3)_6^{3+}$. In this case, O_2 gas was bubbled at a flow rate of 0.1 L/min, and the current was measured by holding the electrode potential at -0.18 V vs SSCE. After injecting the glucose solution, the electrode showed a

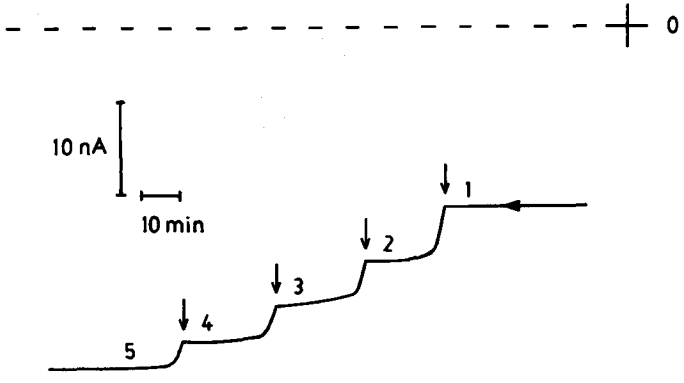

FIG. 8. Typical steady-state current response of System II to changes in glucose concentration. The current was measured by holding the electrode potential at -0.18 V vs SSCE under the condition of O_2 bubbling (0.1 L/min). The amount of GOx in the enzyme film (thickness 0.3 mm): 0.085 mg/cm^2. Steady-state currents 1, 2, 3, 4, and 5 correspond to glucose solutions of 0, 0.44, 0.88, 1.33, and 1.77 m*M*, respectively. The arrows indicate the injection points of glucose solution. Electrolyte solution: phosphate buffer solution (0.1 *M*, pH 7.0) containing 0.016 m*M* $Ru(NH_3)_6^{3+}$. BPG electrode area: 7.8×10^{-3} cm^2.

relatively rapid response time, reaching 95% of the steady-state current in ~40–60 s. As expected from the principle of this sensor system (shown in Fig. 1B), the reduction current was found to increase with increasing concentration of glucose when other experimental conditions were held constant. A typical calibration curve of reduction current vs glucose concentration is shown in Fig. 9, together with the blank current response obtained with the bilayer-film-coated electrode without GOx in the outer enzyme film. The blank current is actually zero, as expected, irrespective of glucose concentration. The electrode responded to a change in glucose concentration of ~0.4 to 4 m*M*. The dynamic range changes with the GOx loading and the enzyme film thickness [19]. The stable response was obtained for a period of more than 2 months.

It is concluded that IECMEs based on a bilayer-film coating with two kinds of polymeric films, i.e., the GOx enzyme film/poly-CoTAPP film electrode system and the GOx enzyme film/montmorillonite clay film

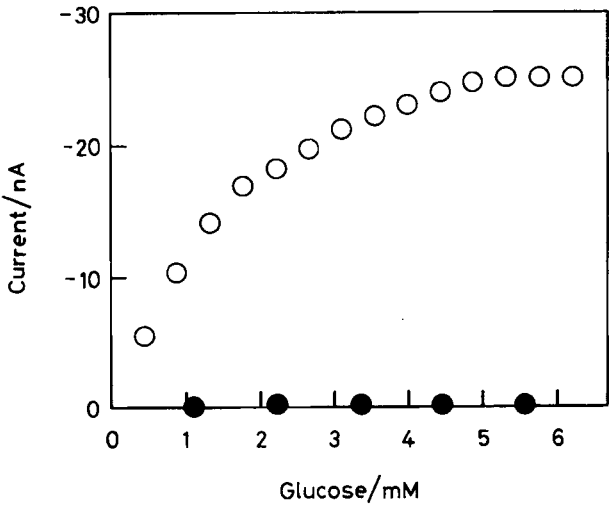

FIG. 9. Calibration curves of experimental steady-state current vs glucose concentration for the GOx film/montmorillonite clay film-coated BPG electrode (System II) with and without GOx. The amount of GOx confined in the enzyme films was (●) 0 and (○) 0.085 mg/cm^2. Other experimental conditions are the same as in Fig. 8.

electrode system, are promising as a new solid-state type of glucose sensor.

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