A New Amperometric Glucose Sensor Based on Bilayer Film Coating of Redox-Active Clay Film and Glucose Oxidase Enzyme Film

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A new amperometric glucose sensor based on bilayer film coating has been fabricated and its sensor characteristics under a steady-state condition have been examined. The electrode substrate was coated with two kinds of polymer films in a bilayer state, i.e., first with the redox-active montmorillonite clay film into which $[Ru(NH_3)_6]^{3+}$ complexes are incorporated electrostatically and they function as an electron shuttle which delivers electrons to redox-active sites in the clay film, and then with the enzyme film consisting of bovine serum albumin and glucose oxidase (GOx). The glucose concentration could be monitored by measuring the current for H_2O_2 reduction electrocatalyzed by the inner clay film where H_2O_2 was produced by the glucose–GOx enzyme reaction in the outer enzyme film. The mechanism of the overall electrode/enzyme reaction for this amperometric glucose sensor is represented as follows:

$$\beta\text{-D-glucose} + O_2 \xrightarrow{\text{glucose oxidase}} \text{D-glucono-}\delta\text{-lactone} + H_2O_2 \quad (1)$$

$$\text{D-glucono-}\delta\text{-lactone} + H_2O \longrightarrow \text{D-gluconic acid} \quad (2)$$

$$[Ru(NH_3)_6]^{2+} + H_2O_2 \xrightarrow{\text{montmorillonite, } H^+} [Ru(NH_3)_6]^{3+} + H_2O \quad (3)$$

$$[Ru(NH_3)_6]^{3+} + e \longrightarrow [Ru(NH_3)_6]^{2+} \quad (4)$$

where reactions 1,2,3, and 4 correspond to the glucose–glucose oxidase enzyme reaction, the hydrolysis reaction of D-glucono-δ-lactone, the electrocatalytic reduction of H_2O_2 by the montmorillonite clay containing $[Ru(NH_3)_6]^{2+}$ and the electrode reaction of the $[Ru(NH_3)_6]^{2+/3+}$ couple, respectively. The reproducible relationship between glucose concentration and sensor output was obtained. The sensitivity and the dynamic range were found to depend on the amount of GOx confined in the enzyme film and its thickness.

Recently, we have demonstrated the usefulness of the immobilized enzyme chemically modified electrodes (IECMEs) based on bilayer film coating for amperometric determination of glucose.[1] The idea presented therein is as follows. The electrode is coated with two kinds of polymeric films in a bilayer state, i.e., the construction of the entire electrode system is expressed by

electrode substrae/film(I)/film(II).

The inner film(I) functions as a "catalyst" which electrocatalyzes the redox reaction of the species (e.g., O_2 or H_2O_2) of interest which are associated with the enzyme reaction, while the outer film(II) is the enzyme film where the enzyme reaction of interest occurs. In a previous work,[1] we have employed the cobalt tetrakis-(*o*-aminophenyl)porphyrin polymer film and the glucose oxidase (GOx) enzyme film as film(I) and film(II), respectively. In this glucose sensor system, the O_2 depletion resulting from the glucose–GOx enzyme reaction is monitored by measuring the current for O_2 reduction electrocatalyzed by the cobalt porphyrin polymer film.

In the present paper, a new amperometric glucose sensor based on such a bilayer film coating will be described: film(I) is the redox-active montmorillonite clay film into which $[Ru(NH_3)_6]^{3+}$ complexes are incorporated electrostatically and they function as an "electron shuttle" which delivers electrons to the redox-active sites, while film(II) is the GOx enzyme film. Recently, Oyama and Anson[2] have reported that the $[Ru(NH_3)_6]^{3+}$-containing montmorillonite clay coating on electrodes effectively electrocatalyzes the reduction of H_2O_2 to H_2O. They have suspected that the iron cations, which commonly replace some of the aluminum ions in octahedral sites within the structure of montmorillonite clay, might be responsible for the catalytic activity exhibited by coated electrodes.[2,3] According to these facts and the principle of the glucose–GOx enzyme reaction,[4] the mechanism of the overall eletrode/enzyme reaction for our new amperometric glucose sensor may be depicted as in Fig. 1. The feature of the present sensor is that the overall process of the reaction shown in Fig. 1 can be monitored by measuring the current produced by the electrocatalytic reduction (to H_2O) of the hydrogen peroxide formed by the glucose–glucose oxidase

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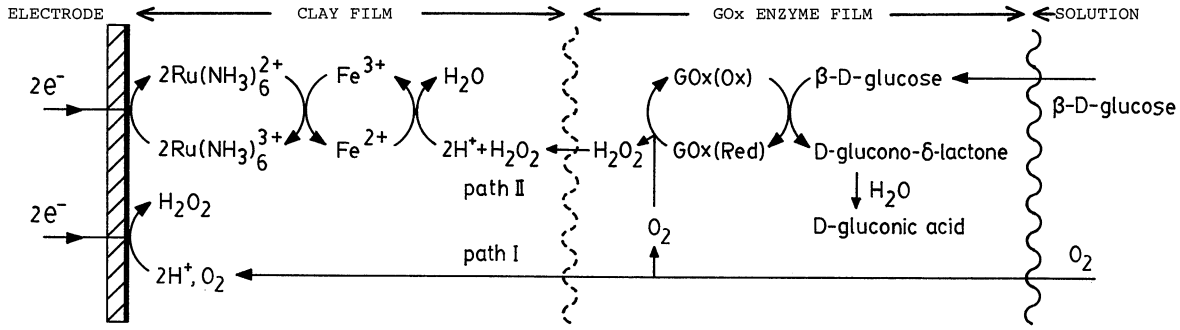

Fig. 1. A schematic depiction of the overall electrode/enzyme reaction at the IECME based on bilayer film coating of the $[Ru(NH_3)_6]^{3+}$-containing montmorillonite clay film and the glucose oxidase enzyme film. GOx(Ox) and GOx(Red) represent the oxidized and reduced forms of glucose oxidase, respectively.

reaction. In the common so-called glucose sensors, the hydrogen peroxide generated is detected amperometrically at a platinum electrode.[4,5] The oxidation of hydrogen peroxide is irreversible and requires a high potential (typically 0.6—0.8 V vs. Ag/AgCl). At such a high potential, many organic compounds (e.g., ascorbic acid, uric acid, and amino acids), which are common components of biological fluids, are readily oxidizable.[6–9] This is a severe problem in developing glucose sensors based on electrochemical oxidation of hydrogen peroxide. Several attempts have been made to overcome this problem, e.g., the coating of the enzyme electrode surface by a membrane with a decreased permeability to organic compounds and the decreasing the electrode potential for the current measurement using an electron-transfer mediator.[4,5] On the other hand, for our new glucose sensor based on electrochemical reduction of hydrogen peroxide, such a problem does not need to be considered. The concept of the bilayer film coating mentioned above would be straightforwardly applicable to the enzyme sensors other than glucose sensor.

Experimental

Reagents. The sodium montmorillonite clay is the same as that employed previously.[2,10] $[Ru(NH_3)_6]Cl_3$ was obtained from Strem Chem. Co. and was recrystallized from water. Glucose oxidase (type II, from *Aspergillus niger*, abbreviated as GOx) was used as supplied by Sigma Chemical Co. β-D-Glucose (anhydrous, for biochemistry) was obtained from Merck Co.. Bovine serum albumin (fraction V, abbreviated as BSA) powder was obtained from Kodak Co. Glutaraldehyde was aqueous 50% solution (Kanto Chemical Co.). Hydrogen peroxide (30 wt%) of reagent grade was obtained from Kanto. Basal-plane pyrolytic graphite (BPG) disks used as an electrode substrate was obtained from Union Carbide Corp. The side of the disk electrode (diameter: ca. 0.1 cm) was sealed with heat-shrinkable polyolefin tubing. Solutions were made up with distilled deionized water. All other chemicals were of reagent grade and were used as received.

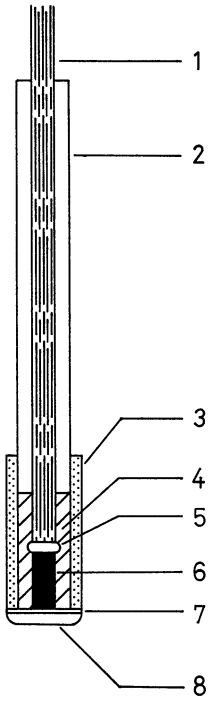

Fig. 2. A schematic construction of the IECME based on bilayer film coating. 1, lead wire; 2, insulater tube; 3, heat shrinkable tube; 4, epoxy resin; 5, silver paste; 6, BPG electrode substrate (diameter: ca. 1 mm); 7, clay film (thickness ca. 1 μm); 8, enzyme film (thickness ca. 0.1—0.6 mm).

Preparation of IECMEs Based on Bilayer Film Coating. Figure 2 shows the schematic construction of the IECMEs employed in this study. The BPG rod electrode substrates were directly coated with two kinds of polymeric films in a bilayer fashion. The inner layer was the montmorillonite clay film (thickness: ca. 1 μm). The clay coating was carried out by pipetting aliquots of the colloidal suspension solution of clay (0.5 wt%), which was well suspended by magnetic stirrer for more than 2 days before use, on the freshly cleaved electrode surface and then by evaporating the solvent in air. The coating of the clay suspension of 2.3 μl resulted in the film of ca. 1 μm thick. After that, the clay

film-coated BPG electrode thus prepared was soaked in 0.1 M (1 M=1mol dm^{-3}) phosphate buffer solution (pH 7.0) containing 0.2 mM $[Ru(NH_3)_6]^{3+}$ and the electrode potential was cycled between −0.6 and 0.2 V vs. a sodium chloride saturated calomel electrode (SSCE) at 50 mV s^{-1}. The peak current corresponding to the redox reaction of $[Ru(NH_3)_6]^{3+}$ complex incorporated into the clay coating on the electrode increased gradually and the almost steady-state peak current was obtained after the scanning time of about 1 h. In this case, the concentration of the $[Ru(NH_3)_6]^{3+}$ complex confined in the clay coating was about 0.1 M and the formal redox potential of the $[Ru(NH_3)_6]^{3+/2+}$ redox couple was −0.22 V vs. SSCE.

The $[Ru(NH_3)_6]^{3+}$ -containing montmorillonite clay film-coated BPG electrode so prepared was further coated with the GOx enzyme film consisting of a matrix of BSA and GOx that were held together by cross-linking with glutaraldehyde according to a published procedure.[11-14] 1 μl of enzyme matrix solution consisting of 4.0 mg ml^{-1} GOx and 15 wt% BSA in 50 mM phosphate buffer (pH 7.0) and 0.6 μl of 25 wt% glutaraldehyde solution were mixed on the previously prepared $[Ru(NH_3)_6]^{3+}$ -containing montmorillonite film-coated BPG electrode with a microsyringe and were allowed to cross-link in air for 10 min and then 0.1 M phosphate buffer solution containing 0.05 mM $[Ru(NH_3)_6]^{3+}$ for about 20 min. After the cross-linking reaction was completed, the electrode was washed by immersion in 10 wt% glycine solution to remove any glutaraldehyde excess from the elecrode surface. The thickness of the outer GOx enzyme film was typically 0.3 mm. When not in use, the electrode was stored in 0.1 M phosphate buffer solution (pH 7.0) containing 0.05 mM $[Ru(NH_3)_6]^{3+}$ in a refrigerator.

Apparatus. A standard three-electrode electrochemical cell was used for all the electrochemical experiments. The electrode assembly consisted of an immobilized enzyme chemically modified BPG electrode (area: 7.8×10^{-3} cm^2) as the working electrode, an SSCE as the reference electrode, and a spiral platinum electrode as the counter electrode. For cyclic voltammetry, a polarization unit (Toho-giken Co., PS-02) was employed, together with an XY-recorder (Graphtec Co., WX 4421). The current-time curves were measured with a potentiostat (Toho-giken Co., PS-12) and a servocorder (Graphtec Co., SR 6342). The flow rates of air and O_2 gas were controlled using a mass flow controller (Kojima Flow Instrument Corp., PSK-6MF). All the measurements were performed at room temperature (25±1 °C) in an earthed Faraday cage.

Results and Discussion

Sensor Characteristics under N_2 Atmosphere. Prior to the examination of the sensor characteristics of the IECMEs in the presence of glucose under an O_2 (or air) atmosphere, we examined their electrochemical behaviors under an N_2 atmosphere. The catalytic H_2O_2 reduction by $[Ru(NH_3)_6]^{3+}$-containing montmorillonite clay film-coated electrode in 0.2 M CF_3COONa solution (pH 2.9) has been recently reported by Oyama and Anson.[2] Figure 3 shows the typical cyclic voltammograms for the IECME based on bilayer film coating in a deaerated phosphate buffer solution (0.1 M, pH 7.0) in the absence and the presence of H_2O_2. The

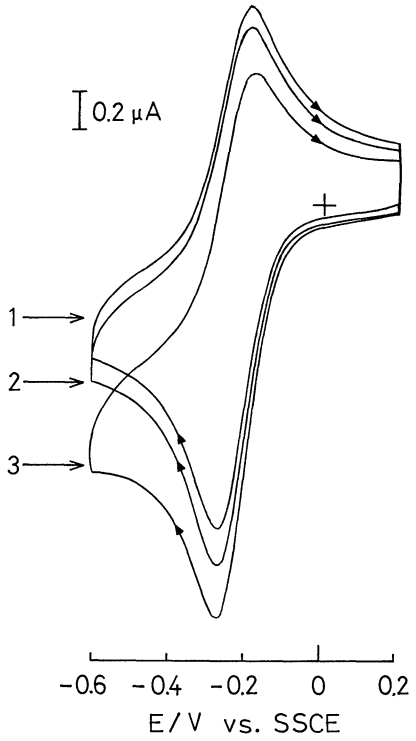

Fig. 3. Cyclic voltammograms for the IECME in phosphate buffer solutions (0.1 M, pH 7.0) containing 0.016 mM $[Ru(NH_3)_6]^{3+}$ and H_2O_2 under N_2 atmosphere. Concentration of H_2O_2: (1) 0, (2) 0.1, and (3) 5.0 mM. Amount of GOx in the enzyme film: 0.085 mg cm^{-2} (thickness ca. 0.3 mm). Concentration of $[Ru(NH_3)_6]^{3+}$ in the clay film: 0.1 M (thickness ca. 1 μm). Electrode area: $7.8\times10^{-3}cm^2$. Flow rate of N_2 gas: 0.1 l min^{-1}. Potential scan rate: 200 mV s^{-1}.

reversible redox response observed around −0.22 V in the absence of H_2O_2 can be assigned to the redox reaction of the $[Ru(NH_3)_6]^{3+/2+}$ couple confined in the clay film, since no redox response was observed at the IECME without $[Ru(NH_3)_6]^{3+}$ in the clay film in 0.1 M phosphate buffer solution containing no $[Ru(NH_3)_6]^{3+}$. In the presence of 0.1 mM H_2O_2, the reduction current at ca. −0.27 V is large compared with that in the absence of H_2O_2, and at higher concentration (5.0 mM) of H_2O_2 the reduction current becomes larger. On the other hand, the corresponding oxidation current at ca. −0.21 V decreases with increasing the H_2O_2 concentration. This fact demonstrates the electrocatalytic reduction of H_2O_2 by the IECME based on bilayer film coating. Note that $[Ru(NH_3)_6]^{2+}$ is not an efficient catalyst for the reduction of H_2O_2 at uncoated BPG electrode.[2] Thus, the observed catalytic activity may be responsible for iron cations which commonly replace some of the aluminum ions in octahedral sites within the structure of montmorillonite clays, as suggested by Oyama and Anson,[2] and $[Ru(NH_3)_6]^{3+/2+}$ complexes are thought to function as an electron shuttle which delivers electrons into the redox-active sites of the iron

cations.[3] The same results as those shown in Fig. 3 were also obtained in the presence of glucose, being in agreement with the expectations based on the principle of the sensor system under consideration.

Figure 4A shows the correlation between the "catalytic current" and the concentration of H_2O_2 under an N_2 atmosphere. Here, the term "catalytic current" represents the difference in the cathodic peak currents observed at ca. −0.27 V in the absence and the presence of H_2O_2, which are estimated from the same cyclic voltammograms as those shown in Fig. 3. A similar correlation was also obtained between the steady-state reduction current and H_2O_2 concentration (Fig. 4B) where the steady-state current was obtained by holding the electrode potential at −0.27 V under the condition of constant flow (0.1 l min^{-1}) of N_2 gas (Fig. 5). From Figs. 4 and 5, it is apparent that the IECME responds to H_2O_2 in the examined range of H_2O_2 concentration 5×10^{-6}—10^{-2} M.

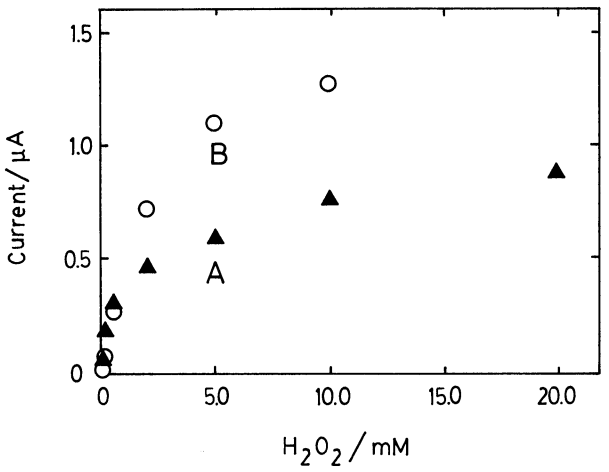

Fig. 4. Correlations between the current and the H_2O_2 concentration. The current represents the difference in the currents obtained in the presence and the absence of H_2O_2; (A) in the cathodic peak currents of the same cyclic voltammograms as those shown in Fig. 3; (B) in the steady-state reduction currents (estimated from the steady-state current response shown in Fig. 5.).

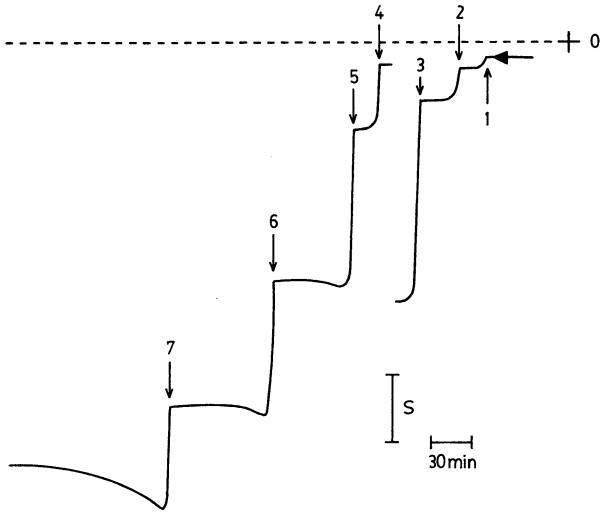

Fig. 5. Typical steady-state current response of the IECME based on bilayer film coating to change in H_2O_2 concentration. The current was measured by holding the electrode potential at −0.27 V vs. SSCE under the condition of N_2 bubbling (0.1 l min^{-1}). Steady-state currents 1, 2, 3, 4, 5, 6, and 7 correspond to H_2O_2 solutions of 0.005, 0.02, 0.1, 0.5, 2, 5, and 10 mM, respectively. The arrows indicate the injection points of H_2O_2 solution. Sensitivity (S): (1,2,3) 20 and (4,5,6,7) 200 nA. Other experimental conditions are the same as those in Fig. 3.

Sensor Characteristics under O_2 (or air) Atmosphere. Figure 6 shows the cyclic voltammetric responses of the IECME in the absence and the presence of glucose under the condition of air bubbling (0.1 l min^{-1}). The solution is composed of 0.1 M phosphate buffer (pH 7.0), 0.016 mM $[Ru(NH_3)_6]^{3+}$, and 5.5 mM glucose. The cathodic peak current at ca. −0.27 V in the presence of glucose is

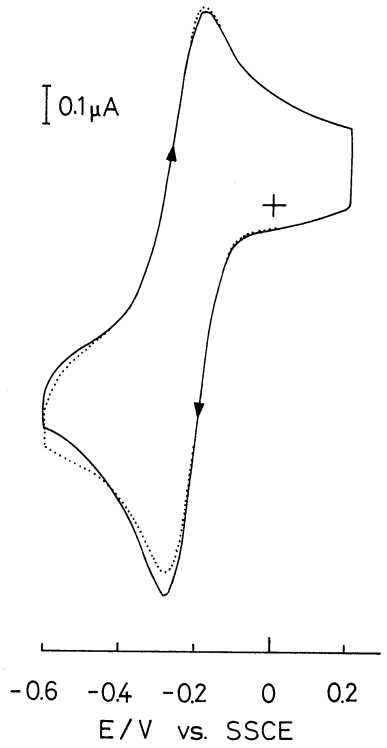

Fig. 6. Cyclic voltammetric responses of the IECME in phosphate buffer solutions (0.1 M, pH 7.0) containing 0.016 mM $[Ru(NH_3)_6]^{3+}$ and glucose under the condition of air bubbling. Concentration of glucose: (----) 0; (—) 5.5 mM. Amount of GOx in the enzyme film: 0.087 mg cm^{-2} (thickness ca. 0.3 mm). Concentration of $[Ru(NH_3)_6]^{3+}$ in the clay film: 0.1 M (thickness ca. 1 μm). Electrode area: 7.8×10^{-3} cm^2. Flow rate of air: 0.1 l min^{-1}. Potential scan rate: 200 mV s^{-1}.

larger than that in the absence of it, while the corresponding anodic peak current at ca. −0.21 V is, though slightly, smaller in the former than in the latter. This increased reduction current reflects the electrocatalytic reduction of H_2O_2, produced by the glucose-GOx enzyme reaction in the outer enzyme film, by the inner clay film. Thus, the IECME used in this study responds amperometrically to changes in glucose concentration and allows us to detect glucose.

It is of particular interest to note that the reduction current in the potential region of −0.6 to ca. −0.4 V in the presence of 5.5 mM glucose is smaller than that in the absence of glucose. This fact may be reasonably explained as follows. In the presence of glucose, O_2 molecules, which penetrate into the bilayer film, are in part consumed in the GOx-glucose enzyme reaction within the outer enzyme film and thus the flux of O_2 molecules reaching the electrode surface is small compared with that in the absence of glucose. In other words, in the absence of glucose, O_2 molecules, which diffuse through the clay film and reach the electrode surface via path I (shown in Fig. 1), are directly reduced at the electrode surface. On the other hand, in the presence of glucose, O_2 molecules diffusing from the solution into the bilayer film partly take part in the enzyme reaction and are reduced to H_2O_2 and subsequently the further reduction of H_2O_2 to H_2O is electrocatalyzed by the inner clay film (path II). In this case, O_2 molecules, which are not required for the GOx-glucose enzyme reaction, diffuse to the electrode surface via path I and are reduced there.

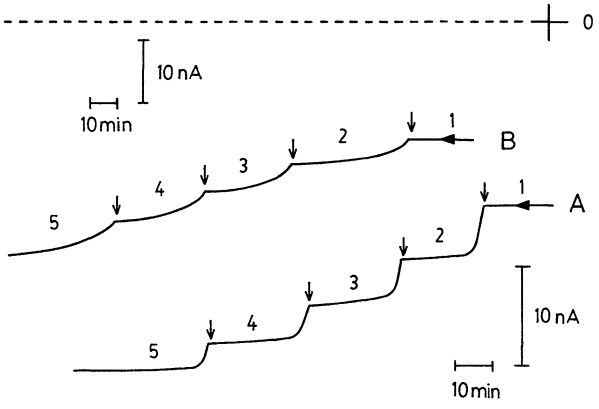

Fig. 7. Typical steady-state current response of the IECME based on bilayer film coating as a function of glucose concentration. The current was measured by holding the electrode potential at −0.18 V vs. SSCE under the condition of O_2 bubbling (flow rate: 0.1 l min^{-1}). The amount of GOx in the enzyme films: (A) 0.085 mg cm^{-2} (thickness ca. 0.3 mm); (B) 0.17 mg cm^{-2} (ca. 0.6 mm). Steady-state currents 1, 2, 3, 4, and 5 correspond to glucose solutions of 0, 0.44, 0.89, 1.3, and 1.8 mM, respectively. The arrows indicate the injection points of glucose solution. Other experimental conditions are the same as those in Fig. 6.

Figure 7 shows typical steady-state current response of the IECMEs based on bilayer film coating to change in glucose concentration. In this case, the current was measured by holding the electrode potential at −0.18 V vs. SSCE under the condition of O_2 bubbling. The current for the direct reduction of O_2 at uncoated BPG electrode was found to be negligible at −0.18 V; The reduction of O_2 at a bare BPG electrode in a phosphate buffer solution (pH 7.0) commences at about −0.4 V vs. SSCE at a scan rate of 200 mV s^{-1} and the reduction peak is observed at ca. −0.8 V.[1] Thus, the steady-state reduction current observed in the absence of glucose is ascribable to the reduction of O_2 and/or $[Ru(NH_3)_6]^{3+}$ in solution which is mediated by the $[Ru(NH_3)_6]^{2+}$ confined in the clay film.[15,18] The reduction current started to increase at ca. 30—60 s after injection of glucose solution, and a 95% increase in current response occurred over the course of ca. 6—9 and 20—25 min at the IECMEs of Γ_{GOx}=0.085 and 0.17 mg cm^{-2}, respectively (Γ_{GOx}: the amount of GOx confined in the enzyme film). This suggests that enzymatic production of H_2O_2 determines the extent of the amperometric signal. The different response times are considered to be due to the different film thickness (ca. 0.3 and 0.6 mm). That is to say, the response time at a steady-state current has been predicted to be less than 1.5 l^2/D for typical amperometric enzyme electrodes,[16,17] where l is the average film thickness and D is the substrate diffusion coefficient. Thus, if this idea is applicable to the present sensors, one can roughly estimate the diffusion coefficient of glucose within the enzyme film, i.e., the D value was estimated to be (2—5)×10^{-6} cm^2 s^{-1}, and was almost comparable to the reported diffusion coefficient for glucose within a polyacrylamide gel where GOx was immobilized.[16] The D value obtained is only slightly smaller than that in aqueous solution;[19] this suggests that the diffusion of glucose to the enzymatic active sites in the enzyme film is not seriously impaired by the enzyme matrix. As can be expected from the principle of this sensor, the reduction current was found to increase with increasing the glucose when other experimental conditions were held constant. In addition, it was found that ascorbic acid, uric acid, urea, and glycine do not influence the determination of glucose concentration at the same concentrations as those in serum solution.

The steady-state current responses for various concentrations of glucose were employed to construct response curves for glucose. The typical results are shown in Fig. 8, where the current was measured by holding the electrode potential at −0.18 V vs. SSCE in phosphate buffer solutions (0.1 M, pH 7.0) containing various concentrations of glucose under the condition of O_2 (or air) bubbling. The ordinate represents the difference in the currents obtained in the presence and the absence of glucose. Figure 8 also involves the

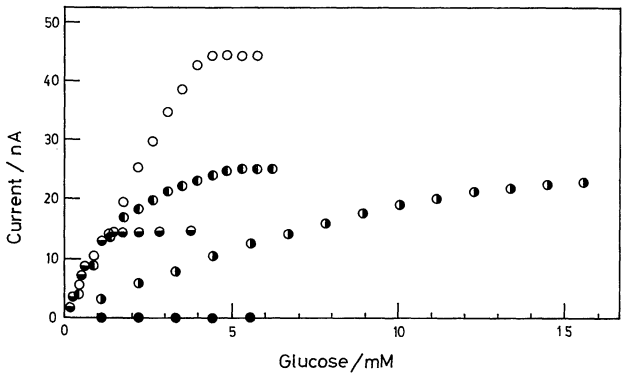

Fig. 8. Calibration curves of experimental steady-state current vs. glucose concentration for the IECMEs based on bilayer film coating. The amount of GOx confined in enzyme films: (◑) 0.021, (◒,◐) 0.085, and (○) 0.17 mg cm^{-2}. The thickness of the outer enzyme films: (◑,◒,◐) ca. 0.3 and (○) ca. 0.6 mm. The points indicated by solid circle (●) represent the blank current responses obtained at (i) the IECME without GOx in the enzyme film, (ii) the IECME without $[Ru(NH_3)_6]^{3+}$ in the clay film (In this case, the sample solutions also contain no $[Ru(NH_3)_6]^{3+}$), and (iii) the electrode coated with the GOx enzyme film alone (Γ_{GOx}: 0.085 mg cm^{-2}). The data were obtained under the condition of O_2 bubbling (0.1 l min^{-1}), except for those represented by symbol ◒ which were obtained under the condition of air bubbling (0.1 l min^{-1}). The current was measured by holding the electrode potentials at −0.18 V vs. SSCE. Other experimental conditions are the same as those in Fig. 6.

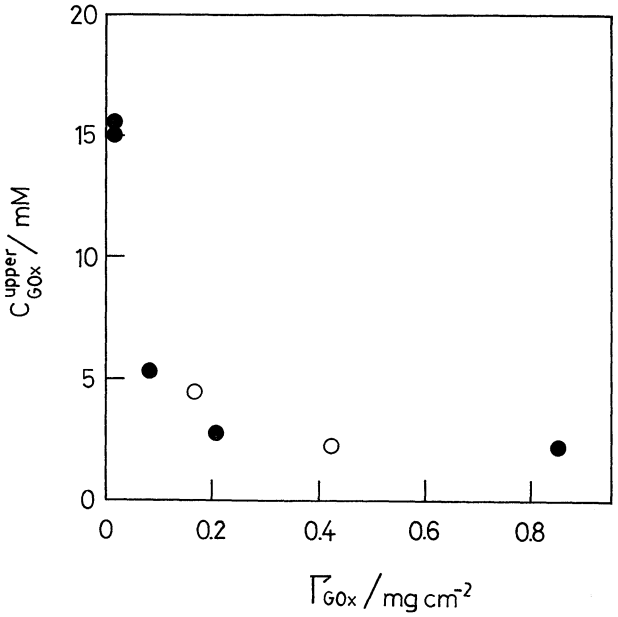

Fig. 9. Correlation between the upper limit of the dynamic range (C_{GOx}^{upper}) and the amount of GOx confined in the enzyme films (Γ_{GOx}). Thickness of the enzyme films: (●) 0.3; (○) 0.6 mm. The current was measured by holding the electrode potential at −0.18 V vs. SSCE. Other experimental conditions are the same as those in Fig. 6.

blank current responses obtained at (i) the IECME without GOx in the enzyme film, (ii) the IECME without $[Ru(NH_3)_6]^{3+}$ in the clay film and (iii) the electrode coated with the GOx enzyme film alone (i.e., without the inner clay film). For these blank experiments, the current was, as expected from the reaction scheme in Fig. 1, zero irrespective of the glucose concentration. On the other hand, the current obtained at the IECMEs increased gradually with increasing the glucose concentration, depending on the amount of GOx confined in the enzyme film and its thickness, and at high concentrations the steady-state current approached a limiting value. At a given concentration of glucose, the higher is the GOx loading, the larger is the current. In other words, the sensitivity, which can be estimated as the initial slopes of the current vs. glucose concentration curves shown in Fig. 8, is higher for the electrode with higher loading of GOx. For example, the slope of the almost linear portion of the calibration curve for the electrode of Γ_{GOx}=0.17 mg cm^{-2} is about four times that for the electrode of Γ_{GOx}=0.021 mg cm^{-2}. On the contrary, the dynamic range where the observed current significantly changes with glucose concentration became narrow with increasing Γ_{GOx}, e.g., the dynamic range is ca. 1—5 and 1—15 mM for the IECMEs with GOx of 0.17 and 0.021 mg cm^{-2}, respectively. More detailed response characteristics are shown in Fig. 9. The observed Γ_{GOx} dependence of the dynamic range remains to be elucidated.

Under the condition of air bubbling the current reaches its saturated value in lower concentrations of glucose, compared with the case of O_2 bubbling. The saturated currents obtained at a given concentration of glucose and under the condition of a constant O_2 bubbling increase with the GOx loading. These facts may suggest that under the experimental conditions employed the enzymatic reaction within the outer enzyme film is current limiting.

Further, we should demonstrate that the present IECMEs based on bilayer film coating do possess a dynamic range wide enough to apply them to assay of whole blood or serum samples. A dynamic range of ca. 0.5—15 mM is usually required for a glucose sensor for a usual clinical use.[11,20-27] Among our sensors, the sensor with Γ_{GOx}=0.021 mg cm^{-2} appears to be suitable for this purpose. In addition, the stable response was obtained for a period of more than 2 months.

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