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Immobilization of Enzyme to Platinum Electrode and Its Use as Enzyme Electrode

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ABSTRACT

A glucose electrode was fabricated by immobilizing glucose oxidase covalently onto a platinized platinum electrode. The sensor showed rapid response with response time of 2–4 s, and also the linear response to the glucose concentration, ranging from 2×10^{-3} to 5 m*M*. The sensitivity was found to be correlated with the surface area of a base electrode used.

Index Entries: Glucose sensor; immobilized enzyme; glucose oxidase; galactose oxidase; platinized Pt electrode.

INTRODUCTION

A variety of glucose sensors have been developed for application to biochemical analysis (*1–3*). A conventional glucose electrode is constructed by placing a thin immobilized enzyme membrane around an electrode, so that the composition and thickness of the enzyme-containing membrane play an important role in the response of the enzyme sensor (*4–9*). With growing demand for rapid glucose measurements on one hand, and miniaturization of sensors on the other, active investigations on the improvement of glucose electrodes have been made (*10–12*). Unique method for preparation of glucose electrode that do not use a membrane to entrap the enzyme have been recently demonstrated (*13,14*). The enzyme molecules have been directly attached to the electrode surface by electrochemical incorporation or electrochemical adsorption in these fabrication techniques. A covalent method is one of the most commonly used technique

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for enzyme immobilization, and has also been employed to functionalize electrode surfaces by attaching various electrochemically reactive molecules to the surface (*15*). There have been few attempts, however, to apply this procedure to attach enzyme molecules directly to a metal electrode, such as Pt or Au (*16*). The covalent method can offer a simple construction, and seems to be promising for the improvement of sensor performance and the miniaturization, since it serves to concentrate the enzyme activity in the vicinity of the electrode surface. In the present work, a glucose sensor prepared by immobilizing glucose oxidase (GOD) covalently onto a platinized Pt electrode is described. The sensor is demonstrated to have high performance, and the features arising from its construction and fabrication procedures are discussed.

EXPERIMENTAL METHODS

Materials

Glucose oxidase (type II, from *Aspergillus niger*) was purchased from Sigma. (3-Aminopropyl)triethoxysilane (Tokyo Kasei Kogyo Co.) was distilled under vacuum and stored in a desiccator. Glutaraldehyde (50% aqueous solution, Tokyo Kasei Kogyo Co.) was used as a 2% solution in pH 7.7 (0.1*M*) phosphate buffer. Reagent grade toluene was distilled and dried over sodium. All other reagents were of reagent grade and were used without further purification. Distilled water was used throughout for all electrolytes and buffer solutions.

Platinization and Pretreatment of Electrode

A clean platinum wire of 1 mm in diameter was sealed in a silicone rubber tube, excluding 5 mm in length from an end. The exposed Pt wire was platinized in a ca. 3% solution of hexachloroplatinic acid containing ca. 0.03% lead acetate (*17,18*). The electrodeposition was carried out at a fixed current in the range from 20 to 30 mA for 5 min, with a platinized Pt as a counterelectrode. A platinized Pt electrode thus obtained was washed thoroughly with distilled water and stored in distilled water. As a pretreatment of electrode, a platinized Pt electrode was anodized for 5 min at 1.9 V (vs Ag/AgCl) in 1 *M* H_2SO_4, followed by potential cycling between hydrogen and oxygen discharge until the characteristic wave pattern of clean Pt was observed (*19*). The surface area of the electrode was estimated from the charge associated with hydrogen adsorption (*20*).

Preparation of Enzyme Electrode

The procedure for preparation of enzyme electrode is schematically represented as Eq. (1). The anodic oxidation and alkylamine-silanization

$$\text{Pt]} \xrightarrow{\text{anodization}} \text{Pt]-OH} \xrightarrow{\text{silanization}} \text{Pt]-O)}_x\text{-Si(CH}_2\text{)}_3\text{NH}_2$$

$$\xrightarrow{\text{glutaraldehyde}} \text{Pt]-O)}_x\text{-Si(CH}_2\text{)}_3\text{N=CH-(CH}_2\text{)}_3\text{-CHO}$$

$$\xrightarrow{\text{enzyme}} \text{Pt]-O)}_x\text{-Si(CH}_2\text{)}_3\text{N=CH-(CH}_2\text{)}_3\text{-CH=N-(Enzyme)} \quad (1)$$

are used conventionally for modification of a Pt electrode (*15,21*). Glutaraldehyde is also used extensively to immobilize enzyme molecules onto a carrier substance bearing amino group (*8,22*). The experimental details are as follows:

A platinized Pt electrode was anodized in 1 *M* H_2SO_4 at 1.85 V (vs Ag/AgCl) for a given time. The solution was not stirred during the anodization. The anodized electrode was washed with distilled water and dried under vacuum at room temperature for more than 1 h over P_2O_5. The dried electrode was then allowed to react with a solution of 10% (3-aminopropyl)triethoxysilane in anhydrous toluene under N_2 for 20 h at ambient temperature and was rinsed successively with toluene, acetone, and distilled water. The silanized electrode was treated with a 2% glutaraldehyde solution of pH 7.7 (0.1 *M*) phosphate buffer at 35°C for 1 h. After being washed with distilled water, the electrode was immersed in a ca 0.5 mg/mL GOD solution of pH 7.7 (0.1 *M*) phosphate buffer at 30°C for 2 h. The glucose electrode thus obtained was rinsed with pH 7.0 (0.1 *M*) phosphate buffer and finally stored in the same buffer solution at 4–10°C.

Measurement of Sensor Response

A glucose electrode and an Ag/AgCl reference electrode were placed in a measuring cell (16 mm in inner diameter) filled with 5 mL of pH 7.5 (0.066 *M*) phosphate buffer containing 0.1 *M* KCl. The cell was thermostated at 25°C with water-jacket. The buffer solution was stirred magnetically during a measurement. A potential difference of 0.6 V was applied to the glucose electrode against Ag/AgCl by a potentiostat (Fuso Seisakusho Co., HECS-318B). After a residual current reached a steady state, a sample solution of glucose was injected into the buffer solution with a microsyringe, and the current output was measured. A calibration graph was determined by measuring stationary output currents with successive addition of the sample solution.

RESULTS AND DISCUSSION

Performance of Glucose Electrode

The glucose electrode developed in the present work consists of a layer of immobilized GOD molecules and a platinized Pt electrode. When this

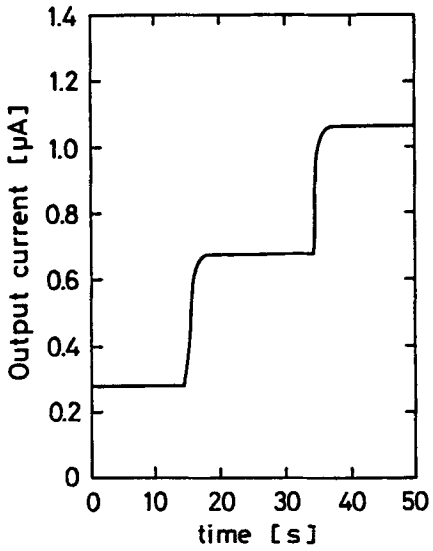

Fig. 1. Response of glucose electrode to a step change in glucose concentration. 10 μL of 0.011 *M* glucose was injected each time.

electrode is placed in glucose solution together with a Ag/AgCl electrode, glucose undergoes an oxidation reaction catalyzed by GOD (Eq. (2)) and the hydrogen peroxide produced is anodically oxidized at the Pt electrode (Eq. (3)). Then, the glucose is measured amperometrically by monitoring the oxidizing current of hydrogen peroxide (*23,24*).

$$\text{glucose} + O_2 + H_2O \xrightarrow{\text{GOD}} \text{gluconic acid} + H_2O_2 \quad (2)$$

$$H_2O \xrightarrow{\text{Pt}} O_2 + 2H^+ + 2e^- \quad (3)$$

A typical response of the sensor with a GOD-immobilized, platinized Pt electrode is shown in Fig. 1. With addition of a glucose solution, the output current increased rapidly and reached another steady state within 2–4 s. This response time is fairly short, as compared with 0.5–3 min for conventional glucose electrodes that are assembled with a membrane and a sensing electrode (*7,23–25*). The response time is almost the same as that observed for the sensor that was fabricated by incorporating GOD molecules into the micropores or growing matrix of platinum black particles deposited on a Pt electrode (*13,26*). The fast response is a striking characteristic of the glucose electrode developed here. Taking its construction into account, there would be no appreciable resistance against the diffusion of reaction species or products. The response time would directly reflect the reaction rate for enzymatic oxidation of glucose, and that for electrochemical oxidation of H_2O_2.

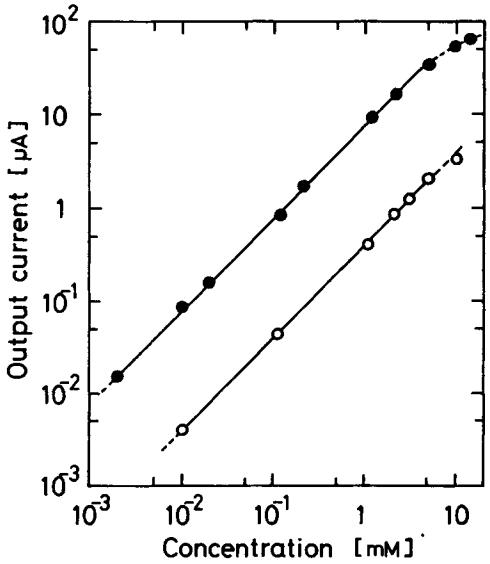

Fig. 2. Calibration graph for glucose determination. ●: platinized electrode-based sensor; ○: wire electrode-based one.

Figure 2 shows a typical current–concentration relationship for glucose. A linear correlation was observed in the 2×10^{-3}–5 m*M* range. The glucose concentration lower than 2×10^{-3} m*M* could not be measured accurately because of the noise produced. A calibration graph is also shown in Fig. 2, which was obtained for a glucose electrode fabricated, using an unplatinized Pt wire as a starting electrode. It is evident that both the sensitivity and the linear response range for the platinized electrode-based sensor are superior to those for the wire electrode-based one. The platinized Pt electrode prepared by electrodeposition of microparticles of platinum onto a Pt electrode is known to have a very large surface area. Actually, the surface area determined by cyclic voltammetric technique was around 200–400 cm^2 for the platinized electrodes, whereas it was 0.3–0.5 cm^2 for original wire electrodes. Thus, the relatively large output current for the platizined electrode-based sensors could be considered to arise from the enlarged surface area. This would be an advantage of the glucose electrode developed here. The output current increased, however, only by one order of magnitude, whereas the surface area increased by about three orders of magnitude. This will be discussed later.

The long-term stability of the glucose electrode was examined by testing the sensor response at least once in 10 d for about 50 d. Measurements were made in the concentration range up to 0.5 m*M* and repeated 5 times/d. The electrode was stored in a pH 7.9 phosphate buffer at 4–10°C when not in use. It can be noted that the sensor shows over 90% of the initial sensitivity for at least 30 d. In Fig. 3, a result is shown for a glucose

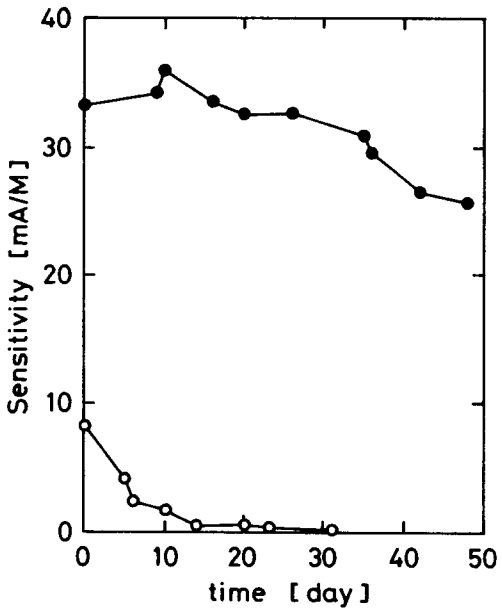

Fig. 3. Long-term stability of glucose electrode. ●: prepared by covalent immobilization; ○: prepared by adsorption.

electrode that has been fabricated by dipping a platinized electrode into a GOD solution without subjecting prescribed treatments, i.e., anodization, aminosilanization, and the reaction with glutaraldehyde. Evidently, the response decreased rather rapidly, and gave about 50% of the initial sensitiveness after 5 d, whereas the sensitivity did not appreciably change after 14 d. This result suggests that most of the GOD molecules are held not strongly by adsorption on the electrode surface. Ikariyama et al. have fabricated a glucose sensor by dipping a platinized Pt electrode into a GOD solution and found the sensitivity to be stable for at least 21 d (*26*). Although the reason for this discrepancy is not clear since the amount of adsorbed GOD and the specific activity of the enzyme employed have not been determined in either work, it is probable that a small amount of GOD molecules would be incorporated almost irreversibly into the micropores of platinum particles. For the sensor described here, most of the GOD molecules are considered to be bound covalently onto the electrode.

Effects of Anodic Oxidation

It should be pointed out for the present glucose sensor that the platinized Pt electrode acts not only as a sensing electrode to hydrogen peroxide oxidation but as a carrier for GOD immobilization. The anodic oxidation to form surface oxide is essential for the immobilization process, whereas such a processing is expected to lower the sensing activity of platinum electrodes. The anodization performed in the course of sensor

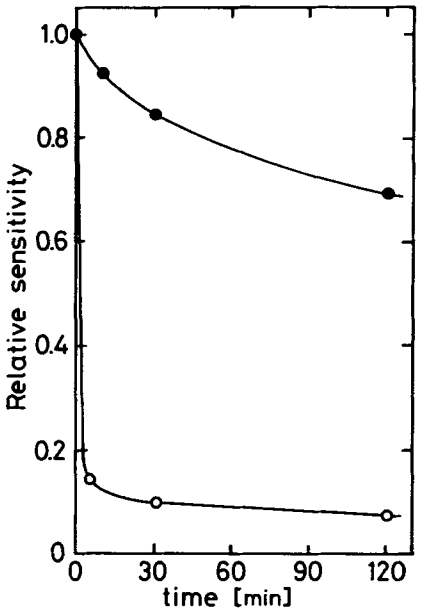

Fig. 4. Variation of sensing activity of Pt electrode to H_2O_2 with anodization in 1 *M* H_2SO_4 at 1.85 V vs Ag/AgCl. ●: platinized electrode; ○: wire electrode.

preparation would exert two mutually competing effects on the performance of resulting sensors. Then, it would be indispensable to examine the influence of anodization processing to characterize the glucose sensor developed here.

At first, the effect on the sensing activity of platinum electrodes to hydrogen peroxide was investigated, using naked electrodes that were not subjected to GOD immobilization. A platinized Pt electrode was anodized in 1 *M* H_2SO_4 at 1.85 V (vs Ag/AgCl) for a given time. The response to H_2O_2 was measured in the same manner as glucose determination, except that a hydrogen peroxide solution was employed instead of a glucose solution. The measurements were made in the range of 10^{-3}–0.04 m*M*, in which the output current was almost the same order of magnitude as that observed for the glucose sensor. The platinized Pt electrode showed a linear response to hydrogen peroxide in this concentration range, regardless of the anodization. A relative sensitivity was determined from the slope of the calibration graph relative to that for the unanodized electrode. Results are depicted in Fig. 4, where the data for a wire electrode are contained for comparison. It can be seen that the relative sensitivity decreases as the anodization is performed for a longer period. The decrease in the response is more drastic for the wire electrode than for the platinized one. These results suggest that the rate of anodization is affected seriously by the surface structure of the electrode. The surface of a platinized electrode is rough and porous, whereas that of a wire electrode is smooth and

Table 1
Sensitivities of Glucose Electrodes

Base electrode	Surface area [cm^2]	Period for anodization[a] [min]	Sensitivity[b] [10^{-2}mA/M]	Sensitivity[b] [10^{-2}mA/cm^2M][c]
Platinized Pt	460	10	860	1.9
	350	30	880	2.5
	250	120	1100	4.4
Wire Pt	0.34	5	45	130
	0.38	30	18	47
	0.48	120	9.1	19

a. Electrolyte: 1 M H_2SO_4, potential: 1.85 V vs. Ag/AgCl.

b. Determined from the slope of calibration graph.

c. Sensitivity per unit area.

nonporous. Anodization of an electrode of complex surface structure would proceed more slowly, compared to an electrode of simple structure.

The sensitiveness of glucose sensors for which the anodization processing has been carried out for various periods are shown in Table 1. The performance of sensors is clearly related to the anodization process. The sensitiveness of the platinized electrode-based sensors seems to rise with the period of anodization applied, whereas the wire electrode-based ones have an opposite tendency. This would be correlated to the difference in base electrodes, and hence, to the difference in the extent of oxide formation. Furthermore, the sensitivities per unit area are appreciably low for the platinized electrode-based sensors, compared with those for the wire electrode-based ones. This implies that the amount of immobilized GOD per unit area for the former is smaller than that for the latter. With a progress of oxide formation, the effect to bring about an increase in the amount of GOD molecules may become predominant when a platinized electrode is employed as a base electrode. In contrast, the effect to result in a lowering of sensing activity to hydrogen peroxide would become more important for the wire electrode-based sensor.

There have been few attempts to employ a platinized Pt electrode as a carrier for enzyme immobilization (*26*). Although this electrode has a disadvantage of lacking a physical strength, its huge surface area would be attractive as a carrier electrode. The electrochemical deposition of microparticles can be achieved with an electrode of any size and shape. The results obtained in this work demonstrate a possibility of further application to other biosensors.

REFERENCES

1. Arnold, M. A. and Solsky, R. L. (1986), *Anal. Chem.* **58,** 84R.
2. Clark, L. C. (1987), *"Biosensors—Fundamentals and Applications,"* Turner, A. P. F., Karube, I., and Wilson, G. S., eds., Oxford University Press, p. 3.
3. Janata, J. and Bezegh, A. (1988), *Anal. Chem.* **60,** 62R.
4. Updike, S. J. and Hicks, G. P. (1967), *Nature* **214,** 986.
5. Williams, D. L., Doig, Jr., A. R., and Korosi, A. (1970), *Anal. Chem.* **42,** 118.
6. Guilbault, G. G. and Lubrano, G. J. (1973), *Anal. Chim. Acta* **64,** 439.
7. Thevenot, D. R., Sternberg, R., Coulet, P. R., Laurent, J., and Gautheron, D. C. (1979), *Anal. Chem.* **51,** 96.
8. Koyama, M., Sato, Y., Aizawa, M., and Suzuki, S. (1980), *Anal. Chim. Acta* **116,** 307.
9. Wingard, Jr., L. B., Cantin, L. A., and Castner, J. F. (1983), *Biochim. Biophys. Acta* **748,** 21.
10. Hanazato, Y., Nakako, M., Maeda, M., and Shiono, S. (1987), *Anal. Chim. Acta* **193,** 87.
11. Kim, J. Y., and Lee, Y. H. (1988), *Biotechnol. Bioeng.* **31,** 755.
12. Umana, M. and Waller, J. (1988), *Anal. Chem.* **58,** 2979.
13. Ikariyama, Y., Yamauchi, S., Yukiashi, T., and Ushioda, H. (1987), *Anal. Lett.* **20,** 1791.
14. Aizawa, M., Chiba, T., and Shinohara, H. (1987), Nippon Kaguka Kaishi, 2210.
15. Murray, R. W. (1980), *Acc. Chem. Res.* **13,** 135.
16. Watanabe, T., Okawa, Y., Tsuzuki, H., Yoshida, S., and Nihei, Y. (1988), *Chem. Lett.* **1988,** 1183.
17. Wroblowa, H., Piersma, B. J., and Bockris, J. O'M. (1963), *J. Electroanal. Chem.* **6,** 401.
18. Green, M., Weber, J., and Drazic, V. (1964), *J. Electrochem. Soc.* **111,** 721.
19. Angerstein-Kozlowska, H., Conway, B. E., and Sharp, B. A. (1973), *J. Electroanal. Chem.* **43,** 9.
20. Barna, G. G., Frank, S. N., and Teherani, T. H. (1982), *J. Electrochem. Soc.* **129,** 746.
21. Lenhard, J. R. and Murray, R. W. (1977), *J. Electroanal. Chem.* **78,** 195.
22. Johanson, G. and Ögren, L. (1976), *Anal. Chim. Acta* **84,** 23.
23. Clark, L. C. and Lyons, C. (1962), *Ann. NY Acad. Sci.* **102,** 29.
24. Guilbault, G. G. and Lubrano, G. L.)1972), *Anal. Chim. Acta* **60,** 254.
25. Williams, D. L., Doig, Jr., A., and Karosi, A. (1970), *Anal. Chem.* **42,** 118.
26. Ikariyama, Y., Yamauchi, S., Yukiashi, T., and Ushioda, H. (1987), *Anal. Lett.* **20,** 1407.