High Performance Micro-Enzyme Sensor Using Platinized Microelectrode

Yoshihito IKARIYAMA,*,† Shigeru YAMAUCHI, Masuo AIZAWA,†† Tomoaki YUKIASHI,††† and Hiroo USHIODA†††
Research Institute of National Rehabilitation Center, Namiki 4-1, Tokorozawa, Saitama 359
††Department of Bioengineering, Tokyo Institute of Technology, O-okayama 2-12-1, Meguro-ku, Tokyo 152
†††Central Research Laboratory, Sumitomo Cemment Co., Ltd., Toyotomi 5-8-5, Funabashi, Chiba 274
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A unique micro-enzyme sensor for the determination of glucose has been prepared by incorporating glucose oxidase into the micropores of a platinized microelectrode. The electrode diameter is in the range from 10 μm to 200 μm. The detection limit is 5×10^{-7} mol L^{-1} for glucose, and an excellent linearity is obtained in the concentration range from 5×10^{-7} to 10^{-2} mol L^{-1} in the case of micro-enzyme electrode whose diameter is larger than 50 μm. In the case of micro-enzyme electrode whose diameter is 10 μm, the detection limit for glucose is 10^{-5} mol L^{-1}, and the linearity is observed in the concentration range from 10^{-5} to 5×10^{-2} mol L^{-1}. The sensor responds to every glucose sample of different concentration in 3 s or less, and the coefficients of variation for glucose (1 mM) determination were within 2%. The sensor output was stable over 4 weeks. This unique microfabrication approach to micro-enzyme electrodes enables the immobilization of intact enzyme molecules by a simple procedure.

Since the introduction of the enzyme electrode by Clark and Updike,[1,2] a wide variety of biosensors have been developed. In the early stage of biosensor research many enzyme-immobilized membranes have been attached to a Clark-type oxygen electrode.[3-5] In addition, antibodies,[6-9] organelles,[10,11] plant tissues,[12] animal tissues,[13] and microorganisms[14,15] have been immobilized for the preparation of a wide variety of Clark-type oxygen electrode-based biosensors.

The further practical application of enzyme electrodes has been extended by the microfabrication of electrode-based biosensors, since multifunctional biosensors can be developed by taking advantage of silicon technology.[16-18] Disposable biosensors can also be constructed.

In almost all areas of chemical measurement the philosophy "the smaller, the better" seems to be prevailing. Well-known examples are capillary columns for gas and liquid chromatography, and microelectrodes for in vivo neurochemical sensors.[19,20] These microinstruments have been gradually replacing the conventional ones because of their increased resolution, sensitivity, and saving in running cost. Miniaturization of biosensors is particularly important for the realization of not only high performance autoanalyzing systems with compactness in clinical laboratory but sensing devices for artificial organs.

Microelectrodes the size of which are less than 10 μm radius have been investigated and employed to obtain neurochemical information.[19,20] Small size also has many advantages in electrochemical analysis particularly with biosensors by virtue of specific reaction of an enzyme, and specific binding between an antigen and its corresponding antibody. Microfabrication of enzyme sensors has been mainly pursued by employing field effect transistors as potentiometric transducers.[16-18] This semiconductor-based approach is promising, since disposable enzyme electrodes are prepared by covering the gate of a field effect transistor with an enzyme-immobilized membrane by employing the conventional immobilization techniques. However, as long as the conventional immobilization techniques of enzymes are employed, enzyme electrodes with sizes in the μm range are beyond realization. By the conventional techniques the authors mean the polymer matrix-supported enzyme membranes. In our previous rapid communication,[21] we have reported fundamental properties of an enzyme electrode (diameter=200 μm) prepared by incorporating enzyme molecules on a platinized surface of platinum electrode.

Here we describe the details and further information on micro-enzyme sensors (diameter=10—200 μm) based on platinized microplatinum electrode, especially with the additional information of our world-smallest enzyme electrode which can be applied as an in vivo micro-enzyme sensor for glucose sensitive neurons. The relationship between the porosity of platinized microelectrode and the sensor output as well as long term stability are also described. Enzyme molecules are intactly inmobilized in the micropores of a transducer electrode. It is the platinization technique that enables the microfabrication of the biosensor. It is a well-known fact that platinized platinum, porous microparticles of platinum, possesses very huge surface and high catalytic activities for electrolytic processes. By employing a microelectrode as a base electrode for platinization and enzyme immobilization, a micro-enzyme sensor can be easily fabricated. Therefore, the size of the micro-enzyme sensor is directly related to that of the base electrode. Enzyme molecules are stably retained in the porous platinum particles with active surface. In other words, the platinum particles play the dual roles of transducing material and matrix for enzyme immobilization.

† Present address: Department of Bioengineering, Tokyo Institute of Technology, O-okayama 2-12-1, Meguro-ku, Tokyo 152.

Experimental

Reagents and Materials. Hexachloroplatinic acid and lead acetate were purchased from Wako Chem. Co. (Osaka). Glucose was the product of Nakarai Chem. Co. (Kyoto), and glucose oxidase (110 u mg^{-1}) was purchased from Toyobo Co. (Osaka). Bovine serum albumin and glutaraldehyde were obtained from Tokyo Kasei (Tokyo). All the chemicals were analytical reagent grade, and all solutions were prepared with glass-distilled water.

Platinum wires (diameter=10—200 μm) were the products of Sanwa Metal Co. (Tokyo). Imperial™ wrapping films of alumina powders of different particle sizes were obtained from Sumitomo 3M Co. (Tokyo).

Preparation of Glucose Oxidase-Immobilized Platinized Microelectrode. Platinum wire whose diameter is in the range from 50 μm to 200 μm was sealed by heating a platinum wire-inserted small soda glass tubing and then polished with the polishing films to prepare a flat surface. In the case of 10 μm platinum electrode, a 10 μm platinum wire was placed in a glass capillary tubing and then sealed at a mild heating temperature. The glass tubing was pulled gently by creeping for several minutes. This was performed by a glass microelectrode puller for patch-clamp pipet (type PP-83) of Narishige Scientific Instrument Laboratory (Tokyo). After sufficient encapsulation of the platinum wire in the capillary tubing, the wire-encapsulating tubing was cut into two microelectrodes. The tip of every platinum electrode was polished with a film of 30 μm alumina powder and then with a film of 9 μm alumina powder. At the last stage, the tip was polished with alumina powder of 1 μm diameter with the exception of the 10 μm microplatinum electrodes, which are polished carefully with 0.1 μm alumina powder. After the smoothing of the surface of the platinum microelectrode, platinization was carried out by reducing hexachloroplatinate (33 mg mL^{-1}) in the presence of lead acetate (0.6 mg mL^{-1}). The electrode potential is referred to a Ag/AgCl reference electrode in the following description. A platinum wire (d=200 μm, l=5 mm) was employed as an auxiliary electrode. The electrolytic reduction of the platinum complex was performed either potentiostatically or galvanostatically. In the potentiostatic preparation, the potential was varied from −0.15 to −0.20 V, and the deposition time was 10 min. On the other hand, in the case of galvanostatic preparation, the deposition current was changed in the range from −0.5 to −60 μA, and the deposition time was 5 min. After the reductive deposition of platinum particles, the platinized electrode was thoroughly washed in 0.1 M phosphate buffer (pH 6.8; 1 M=1 mol dm^{-3}). Enzyme incorporation was then carried out by immersing the electrode in a phosphate-buffered solution (pH 6.8) containing 50 mg glucose oxidase for 10 min. The enzyme-incorporated platinized microelectrode was rinsed thoroughly in 0.1 M phosphate buffer (pH 6.8) for over 12 hours. The electrode, if necessary, was then immersed in a bovine serum albumin solution (20 mg in 1 mL of 0.1 M phosphate buffer of pH 6.8) for 10 min. The albumin-incorporated platinized surface was then immersed in a glutaraldehyde solution (2% in 0.1 M phosphate buffer) for 10 min to form a thin albumin film over the platinized surface. The electrochemical microfabrication technique is schematically shown in Fig. 1.

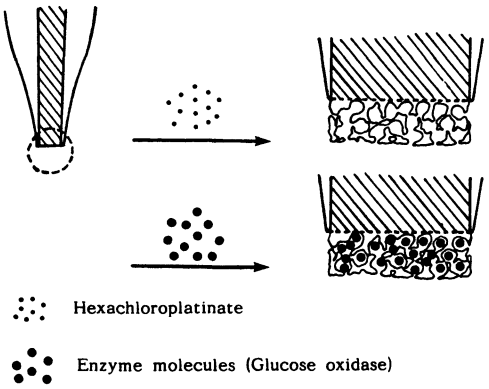

Fig. 1. Schematic illustration of the fabrication technique for the preparation of micro-enzyme sensor. After the encapsulation of micro-platinum wire, platinization is performed by electrochemical reduction of hexachloroplatinate, which is followed by the incorporation of enzyme molecules. The electrode was immersed in an albumin solution and then treated with glutaraldehyde.

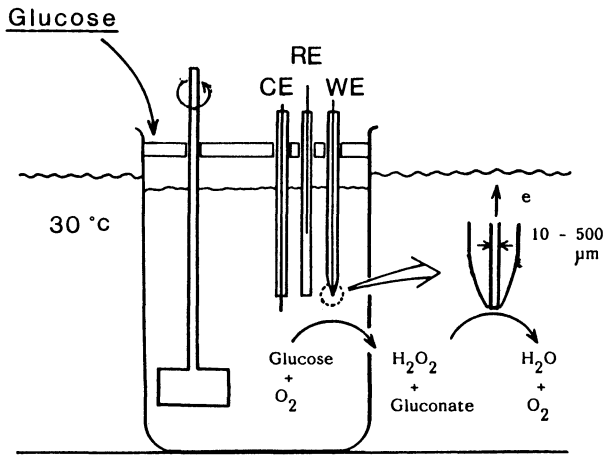

Fig. 2. Measuring apparatus for glucose determination with a micro-enzyme electrode. Glucose oxidase generates hydrogen peroxide from glucose, and the peroxide is oxidized by the transducer electrode of which potential is kept at 0.6 V vs. Ag/AgCl reference electrode. The anodic current is the sensor output.

Observation of Enzyme-Immobilized Microelectrode. A typical micro-enzyme electrode was observed by a SZH optical microscope of Olympus Co. (Tokyo). The surface of the platinized electrode was observed by a Hitachi S-450 scanning microscopy before and after enzyme incorporation. Each electrode was coated with gold by a Eiko IB-3 ion coater after the lyophylization of the electrodes. All the electrodes were kept at −60 °C overnight and then lyophylized with a freeze dryer (Model DC-55A) of Yamato Scientific Co., Ltd. (Tokyo). The gold coating (5 min) was repeated three times to keep the coating at moderate temperature.

Glucose Determination by Micro-Enzyme Electrode. Glucose was determined with a three electrode system by applying 0.6 V to the working micro-enzyme electrode. At the applied potential the hydrogen peroxide generated by glucose oxidase is oxidized to give anodic current. Figure 2

shows the measuring apparatus for glucose determination with micro-enzyme electrodes. A series of glucose samples were injected into the measuring buffer solution (0.1 M phosphate of pH 6.8) under the stirring with a motor-driven screw mixer. The mixing with a motor-driven screw was required to obtain a good signal to noise ratio especially in the case of an enzyme electrode of 10 μm diameter. The glucose determination was performed at 30 °C.

Results

Evaluation of Platinized Platinum Surface. After platinization the microelectrode was cleaned by sweeping the electrode potential repeatedly in 0.5 M sulfuric acid for the estimation of electrode surface area. A oxygen and hydrogen adsorption/desorption curve for a platinized electrode (diameter=10 μm) was investigated and compared with the curve for a flat electrode of the same diameter. From the two voltammograms the surface area of a platinized electrode is estimated to be about 5000 times that of a plain electrode. The surface area of a platinum electrode is easily controllable by changing the deposition time. The platinization is also controlled by choosing the applied potential in the case of potentiostatic preparation and by choosing the reducing current in the case of galvanostatic prepartation.

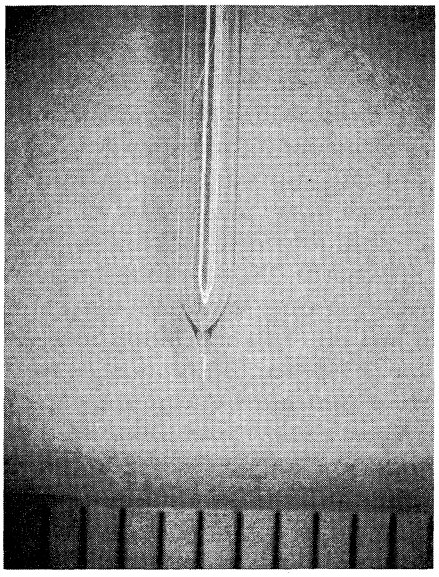

Fig. 3. A typical micro-enzyme electrode (diameter=10 μm) encapsulated in a soda glass tubing. The graduation lines of the scale is 1 mm.

Observation of Enzyme-Immobilized Microelectrode. A typical micro-enzyme electrode (10 μm) was observed by the microscope. Figure 3 shows that a 10 μm platinum wire is encapsulated in a capillary tubing. The surfaces of three different electrodes were observed by the scanning electron microscope. Figure 4 shows the surface of a platinized electrode before enzyme incorporation. Microparticles of platinum were seen on the surface. A photograph of a glucose oxidase-incorporated platinized electrode is shown in Fig. 4(B). Despite considerable enzyme activity in the porous electrode surface, no membrane-like structure was observed on the platinized surface. The last photograph, Fig.4(C), shows the surface of the enzyme-incorporated platinized electrode treated in the albumin solution and in the glutaraldehyde solution. A very thin protein layer was observed over the electrode surface after the crosslinking of albumin molecules by the bifunctional aldehyde.

The thickness of the albumin film was estimated to be a few hundred A from the cross section of the broken protein film during lyophylization.

Determination of Glucose by the Micro-Enzyme Electrode. The micro-enzyme electrode (diameter: 50 μm) was immersed in 0.1 M phosphate buffer (pH 6.8). After the residual current became a constant value, each glucose sample was injected. The output current increased promptly after the injection of a glucose

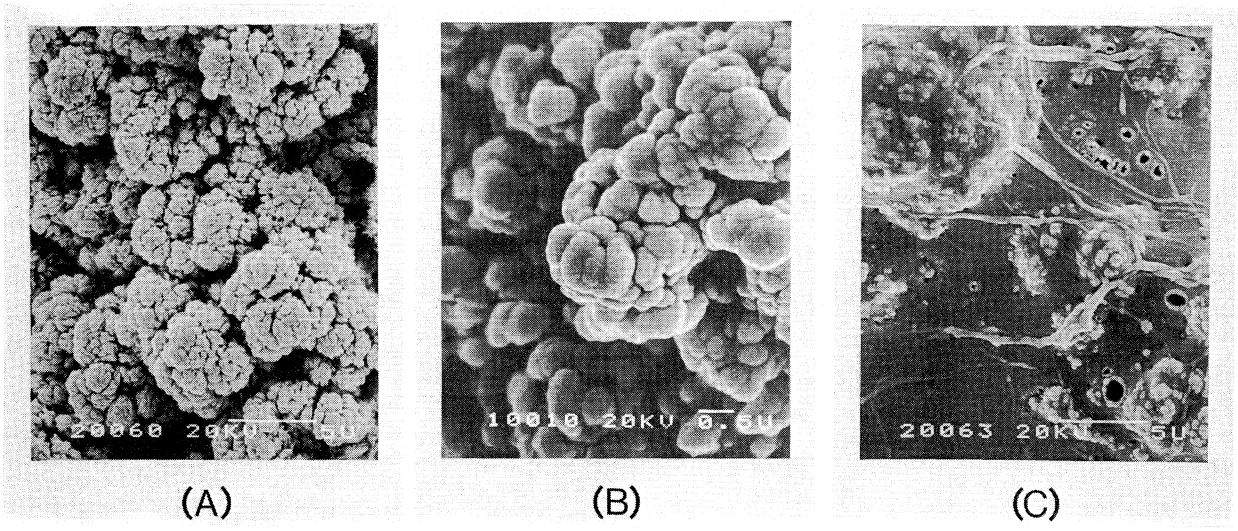

Fig. 4. SEM photographs of a platinized microelectrode (A), an enzyme-incorporated platinized microelectrode (B), and an enzyme-incorporated platinized microelectrode treated with albumin and glutaraldehyde (C).

sample as illustrated in Fig. 5. The output of a micro-enzyme electrode reached a steady state current in 3 s or less. The increase in the oxidizing current was caused by the oxidation of the hydrogen peroxide generated by immobilized glucose oxidase molecules. The response in Fig. 5 was measured repeatedly by changing the glucose concentration.

The change in sensor output was plotted versus glucose concentration to give a calibration curve for glucose as shown in Fig. 6. The lowest and highest detectable glucose concentrations were 0.5 μM and 10 mM,

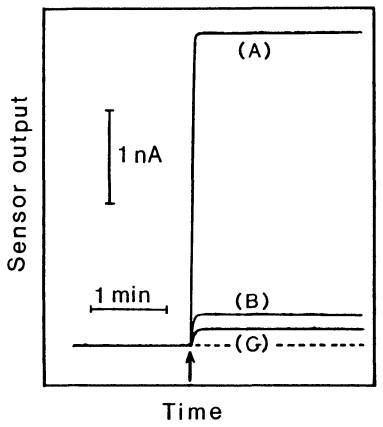

Fig. 5. Typical responses of a micro-enzyme electrode (diameter=50 μm). Glucose concentrations were 100 μM (A), 10 μM (B), and 5 μM (C).

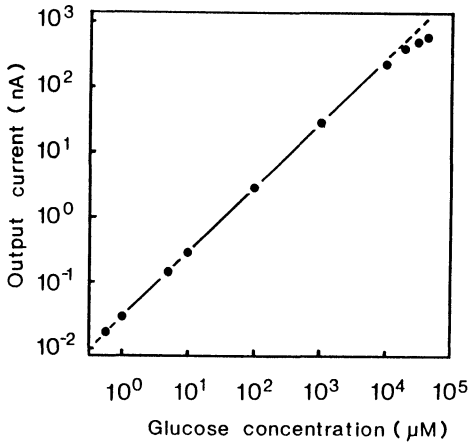

Fig. 6. Calibration curve for glucose with a micro-enzyme electrode (diameter=50 μm).

respectively. When the microenzyme electrode diameter is larger than 50 μm, the calibration curve and dynamic range of a given micro-enzyme electrode were not influenced by the size of a base platinum micro-electrode.

However, the 10 μm enzyme electrode showed a narrower dynamic range between 5 μM and 10 mM, although good linearity and rapid-response was observed within this range. Since the determination of 5 μM glucose sample caused a sensor output of only a few pA, the authors believe that a more sensitive recorder could make the 10 μm enzyme electrode more sensitive because of the high signal to noise ratio of the 10 μm enzyme electrode.

Three different enzyme electrodes (diameter: 10, 50, 200, 500 μm) prepared under different conditions were studied for the comparison of sensor characteristics such as response to 1 mM glucose and the Michelis-Menten constant with respect to the porosity of the platinized electrode and the electrode diameter. The calibration curves obtained by four enzyme electrodes allow the calculation of apparent Michaelis-Menten constants. These values are listed in Table 1. All the enzyme electrodes were prepared by following the galvanostatic conditions described in the experimental section. The charge, i.e., the product of reducing current and reducing time, needed for the platinization is also listed in Table 1. The relation between the peak current for hydrogen adsorption wave and the response to 1 mM glucose was compared for every enzyme electrode. One can understand that the electrode response to 1 mM glucose is related to the porosity of the platinized electrode where the enzyme molecules are immobilized. The relation was more clearly investigated by using three platinum electrodes of the same diameter (200 μm). Figure 7 shows the relation between the sensor response to 1 mM glucose and the total charge required for platinization.

The coefficients of variation for glucose analysis are also shown in Table 1. Here micro-enzyme electrodes prepared by using four different diameter electrodes were employed to evaluate the precision of the micro-enzyme sensors. The sensor response to 1 mM glucose was repeated studied. A micro-enzyme electrode prepared by greater reducing current for platinization showed greater response to 1 mM glucose. However,

Table 1. Relation between the Fabrication Conditions and the Characteristics of Micro-Enzyme Electrode

Fabrication condition		Characteristics of micro-enzyme electrode			
Electrode diameter	Peak height of H_2 adsorption wave/μA	Mean response to 1 mM glucose/nA	Detectable range	K_m	CV(N)
μm			M	mM	%
10	7	5.0	10^{-5}—5×10^{-2}	60	2.5(10)
50	20	29.2	5×10^{-7}— 10^{-2}	45	1.5(20)
200	65	166	5×10^{-7}— 10^{-2}	35	0.8(20)
500	105	278	5×10^{-7}— 10^{-2}	30	0.6(20)

The deposition currents for the platinization were −0.5 μA, −3 μA, −15 μA, and −60 μA for 10 μm, 50 μm, 200 μm, and 500 μm platinum electrode, respectively. And the deposition time was 5 min in every electrode.

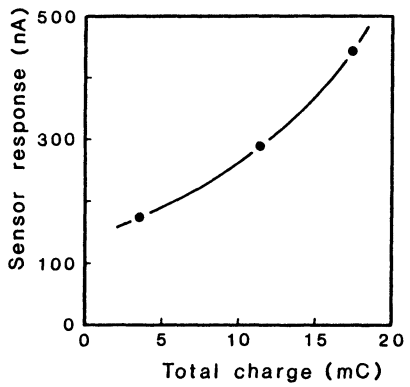

Fig. 7. The relation between the sensor response to 1 mM glucose and total charge required for the platinization. The diameter of the enzyme sensors is 200 μm, and the total current is shown in Coulomb.

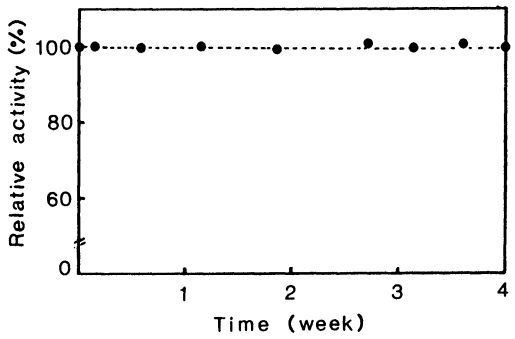

Fig. 8. Time stability of a typical micro-enzyme electrode (diameter=50 μm). The response of the micro-enzyme electrode (diameter=50 μm) was studied by the two injections of 1 mM glucose.

the microelectrodes which have greater output showed greater residual current. The mean response in Table 1 corresponds to the mean current increase when the same glucose sample (1 mM) was repeatedly determined, and the number of repeated analysis is shown in parenthesis.

Time Stability of Micro-Enzyme Electrode. The stability of the micro-enzyme electrode (diameter: 50 μm) was studied by measuring the sensor response to 1 mM glucose. The measuring conditions were the same as described in the above description. The enzyme electrode was kept in the refrigerator when not in use. The storage buffer was 0.1 M phosphate of pH 6.8. The sensor output on the first day was normalized to 100%.

Figure 8 is the time stability of one of the typical micro-enzyme electrode that was mainly stood in a refrigerator. On the 28th day the sensor output still demonstrated nearly 100% response, of which detailed reason is now being investigated. The time stability for an enzyme electrode that was repeatedly used was also studied. All the electrodes tested showed almost same response to the same glucose samples after six hundred injections.

The time-stability of the micro-enzyme electrode was improved by the crosslinking of the incorporated enzyme molecules by glutaraldehyde. The stability of the albumin membrane-coated enzyme electrode was almost the same as that of the glutaraldehyde-treated one. That fact suggests that the enzyme molecules after crosslinking are effectively retained on the microparticles because of the formation of conglomerate enzyme within the porous transducer matrix. On the other hand, without the glutaraldehyde treatment, the enzyme-incorporated electrode lost its activity gradually. The enzyme electrodes without glutaraldehyde treatment retained their activity of approximately 80% on the 28th day when they were kept in a refrigerator when not in use.

Discussion

The microfabrication technique described here is a unique approach by which method micro-enzyme electrodes are easily prepared in a simplified manner. The miniaturized enzyme electrode can be applied to the fabrication of a micro-biosensing device by which stationary trace glucose can be determined in 2 ms.[22] By micro-enzyme electrode the authors do not necessarily mean that the micro-enzyme electrode behaves as a conventional microvoltammetric electrode where the steady-state current is observed when the electrolysis rate is approximately equal to the rate of diffusion of molecules to the electrode surface.[20] Although the size of the electrode is in the range of a microelectrode (diameter=10 μm), the platinized electrode smaller than 10 μm in diameter is shown to behave as a macroelectrode as is shown in Fig. 2. The high sensitivity of the present enzyme electrodes can be explained by the behaviors as macroelectrodes. The smallest size of the enzyme electrode the authors have ever tried to fabricate is 10 μm, because of the difficulty in the preparation of the platinum wire. However, smaller enzyme electrode can be fabricated by either using a carbon fiber electrode or an electropolished platinum microelectrode on which surfaces platinization can also be easily carried out.

The main reason for the remarkable improvement of the long term stability by the bifunctional aldehyde treatment seems to have been caused by the crosslinking of the enzyme molecules, by which the polymerized enzyme can be retained stably in the micropores of the transducer electrode.

One of the advantages of the present fabrication technique is that the sensor response is easily controllable by selecting the current for platinization. Another advantage is the efficient transduction of biochemical signal to electric signal, since the porous surface provides efficient mass transfer from bulk solution to the electrode itself and the hydrogen peroxide generated within the electrode is efficiently oxidized to give sensor response, since the enzyme is embodied

with the porous matrix electrode. These can be the reasons of the fast response and the high sensitivity of the enzyme electrode.

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