Micro-Enzyme Electrode as a High-Performance Detector of Flow Injection Analysis

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(Received January 20, 1989)

The advantages of miniaturized enzyme electrode have been applied to flow injection analysis for the development of a rapid and selective method for the continuous determination of glucose. By assembling a micro-enzyme electrode having a diameter of 10 μm for an amperometric flow-through detector, at least 600 glucose samples can be determined in one hour. And micro-enzyme electrodes are satisfactorily stable for over 4 weeks even after 1000 glucose injections.

Miniaturization is one of the common trends in science and technology of our age. As far as chemistry is concerned, miniaturization is closely related to the microinstrurmentation of analytical tools. As Haugen et al. pointed out in a recent journal,[1] microfabricated sensors will play indispensable roles for the construction of very small sophisticated instruments.

A great number of approaches for microfabrication of chemical sensors are currently under way, particularly in the areas of field effect transistor (FET)-based chemical sensors, metal oxide gas sensors, and biosensors. Since Janata et al. reported the first micro-enzyme electrodes based on FET,[2] a number of groups have been taking microfabrication techniques such as semiconductor technology and photolithography for fabricating micro-enzyme electrodes. Despite enormous efforts of many groups the FET-based micro-enzyme electrodes of practical use have not been realized yet, because this potentiometric method lacks fast response, high sensitivity, and wide dynamic range.

Over several years, microelectrodes having diameters less than 20 μm have been introduced as the in vivo chemical sensors of neurotransmitter.[3-5] Because of their nonselective voltammetric principle, microelectrodes of high selectivity have been earnestly desired to be developed.

Obviously selective membrane layers such as enzyme membranes exhibit for the target substances affinities by taking advantages of specificity and selectivity to enzymes' substrates.

In our previous papers we have successfuly fabricated amperometric micro-enzyme electrodes having diameters from 10 μm to 500 μm by the embodiment of enzyme molecules with platinized microelectrode.[6,7] We have taken two approaches for the construction of micro-enzyme electrodes. One of them is to incorporate enzyme molecules to an platinized microelectrode,[8] and the other is to make enzyme molecules adsorb electrochemically on the surface of an electrochemically growing platinized electrode matrix.[9]

Here we describe an effectiveness of the smallness of enzyme (glucose oxidase, GO) electrodes whose diameters from 10 μm 500 μm by applying them to amperometric detectors of flow injection analysis (FIA) of glucose. In addition to the fundamental characteristics of micro-enzyme electrodes as high-performance detectors for FIA, the authors would like to mention the importance of anodic polarization of the platinized electrodes for the improvement of the selectivity of the enzyme electrodes. The benefits of miniaturization of enzyme-electrodes are also discussed in this paper with respect to the microinstrumentation of FIA.

Experimental

Reagents and Materials. Hexachloroplatinic acid and lead(II) acetate were the products of Wako Chem. Co. (Osaka). Glucose was obtained from Nakarai Chem. Co. (Kyoto), and glucose oxidase (EC 1.1.3.4, 110 u/mg) was the product of Toyobo (Osaka). Bovine serum albumin (Grade V) and glutaraldehyde (50% water solution) were purchased from Tokyo Kasei (Tokyo). All the other reagents were of analytical grade; their solutions were prepared with glass-distilled water.

Platinum wires having diameters from 10 μm to 500 μm were manufactured by Sanwa Metal Co. (Tokyo). Imperial™ wrapping films of alumina powders of different particle sizes from 0.1 μm to 30 μm were obtained from Sumitomo 3M Co. (Tokyo).

Apparatus. The flow-through cell used for a transducer electrode in the FIA system is shown in Fig. 1. The working electrode (WE) was a platinized platinum whose diameter is from 10 μm to 500 μm, the reference electrode (RE) was an Ag/AgCl electrode, and the auxiliary electrode (AE) was a stainless steel upper cell for a thin-layer transducer cell for LCEC (BAS, West Lafayette, IN). Every microelectrode was fixed in an acrylic block bottom cell with a polyester resin. The reference electrode was positioned downstream from the biosensing cell. Potential application and the generated current observation were carried out by a Potentiostat/Galvanostat (Model

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2090 with Low Noise of TOHO Technical Research, (Tokyo). The output current was recorded with an X-t recorder (Model R-302V of RIKADENKI, Tokyo). A double plumger pump (Model NF-EX-2 of NIHON SEIMITSU KAGAKU Co., Tokyo) was employed with a pulse damper (NIHON SEIMITSU KAGAKU Co.). A 5 μL sample loop was used for all experiments. The length of the connective tube (inner diameter=200 μm) from the injection valve to the detector was 50 cm. The flow rate of the pump was controlled at 0.8 mL min^{-1}. The electrode potential of micro-enzyme electrode was 0.6 V, and all the electrode potentials are reported vs. an Ag/AgCl reference electrode. All the experiments were carried out at room temperature (23 °C).

Preparation of Micro-Enzyme Electrode in an Electrode-Assembled Transducer Cell. A microplatinum electrode (diameter=10 μm—500 μm) in an acrylic block bottom cell for the thin-layer transducer cell concerned was platinized with a conventional three-electrode system using a platinum wire electrode (diameter=1 mm, l=10 mm) and an Ag/AgCl reference electrode. Electrolytic reduction of hexachloroplatinate (33 mg mL^{-1}) was carried out in the presence of lead acetate (0.6 mg mL^{-1}) at a potential of −0.08 V for 5 min. After the reductive deposition of platinum particles, the platinized electrode was thoroughly rinsed in 0.1M phosphate buffer (pH 6.8). Incorporation of GO molecules in the platinized electrode was carried out by immersing the electrode in an enzyme solution for 10 min before assembling the three electrode detector cell. The solution contained 50 mg of GO in one mL of 0.1 M phosphate buffer (pH 6.8). The electrode was then kept in a bovine serum albumin (20 mg) in 1 mL of 0.1 M phosphate buffer of pH 6.8 for 10 min. The albumin-incorporated platinized microelectrode was treated with a 2% glutaraldehyde solution buffered with 0.1 M phosphate of pH 6.8 for 10 min for the preparation of thin-layer albumin film over the porous electrode surface, following which thorough washing of the electrode was performed overnight. A gasket having a thickness of 100 μm was employed between the upper stainless steel cell and the enzyme electrode-embedded acrylic bottom cell as shown in Fig. 1. Also is shown a schematic diagram of instrumentation.

Anodic polarization of the micro-enzyme electrode was performed before enzyme incorporation by applying +1.2 V to the electrode for 10 min in 0.1 M phosphate buffer (pH 6.8).

Results

Responses of Enzyme-Electrode to the Repeated Injection of Glucose Sample. A glucose sample (10 mM, 5 μl) was repeatedly injected to the transducer assembly. Figure 2 illustrates the typical responses of the detector system. Every response reached a peak within 2 or 3 s and decayed to the baseline within a few seconds. Each peak was highly reproducible, and gave ca. 200 nA maximum peak to 10 mM glucose sample. The micro-enzyme electrode having a diameter of 100 μm demonstrated responses neither to blank samples (0.1 M phosphate buffer of pH 6.80 nor to fructose and galactose samples after the anodic polarization of the platinized transducer electrode at 1.2 V for 10 min. Without anodic polarization the platinized electrodes as well as the enzyme-embodied platinized electrodes demonstrated responses not only to glucose but to galactose, fructose, and maltose. Before anodic polarization, the relative responses of the enzyme electrode were 7.7%, 6.2%, and 3.8% for galactose, fructose, and maltose, respectively, when the responses were normalized to that of glucose. After the anodic treat-

§ 1 M=1 mol dm^{-3}.

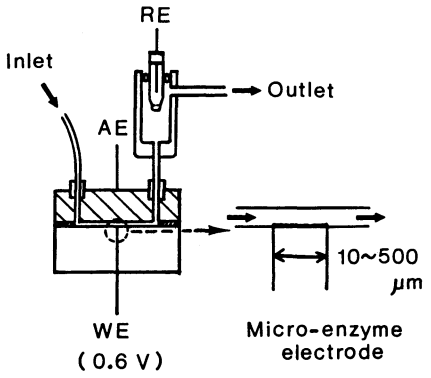

Fig. 1. Schematic diagram of instrumentation for FIA with micro-enzyme electrode.
The upper stainless steel cell is employed as an auxiliary electrode. The micro-enzyme electrode (WE) is embedded with a polyester resin in an acrylic bottom cell. The potential of the enzyme electrode is controlled at 0.6 V vs. Ag/AgCl reference electrode.

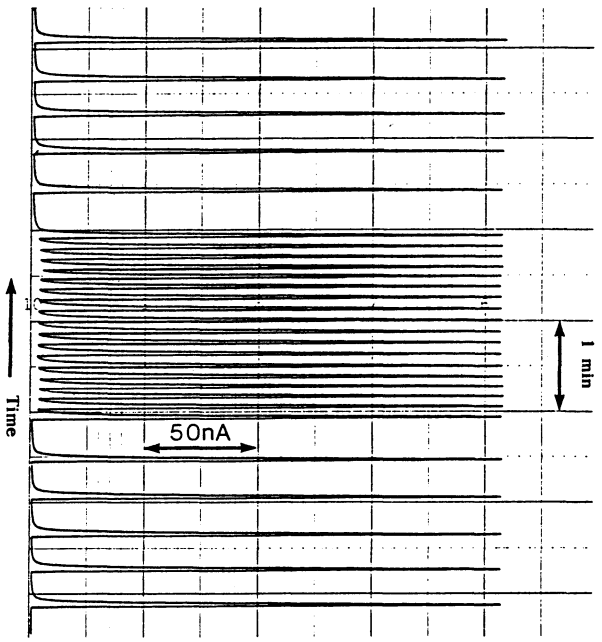

Fig. 2. Typical responses of micro-enzyme sensor system.
A micro-enzyme electrode having a diameter of 100 μm was used at a flow rate of 0.8 mL min^{-1}. Glucose (10 mM) samples were repeatedly injected. The flow-through detector showed no responses to fructose and galactose after anodic polarization.

ment the platinized enzyme electrodes showed no responses to these carbohydrates, and the enzyme electrodes demonstrated selective response to glucose. On the other hand, the anodically polarized platinized electrodes showed no responses to any carbohydrates. However, gradual polarization was observed at 0.6 V when the enzyme electrode was kept at the potential for a few hours. After the gradual oxide formation on the electrode surface at the potential, the enzyme electrode demonstrated selective response to glucose. Since a double plumger pump with a pulse damper was employed, the baseline slightly showed typical behavior of peristaltic wave.

The Relation between the Glucose Concentration and the Peak Current. A series of glucose samples whose concentrations varied from 200 μM to 20 mM were injected to the thin-layer transducer cell consisted of a 100 μm micro-enzyme electrode. Figure 3 shows the responses for the repeated injections. All the responses showed sharp peaks, reaching the peak heights in 2 or 3 s, and returned to the steady state current (residual current) in 7 or 8 s. Every glucose sample was injected 3 times, and all of the resulting three peak heights were almost same. The micro-enzyme electrode demonstrated little response to the injection of blank samples (0.1 M phosphate mobile buffer of pH 6.2). Figure 4 shows a linear relationship between the glucose concentration injected and the peak height in the glucose concentration range from 50 μM to 20 mM. As was already reported,[6,7] the micro-enzyme electrode showed high sensitivity (lowest detection limit : 0.5 μM), and wide dynamic range (0.5 μM—20 mM) in a batch system measurement, however, in this flow type measurement the detection limit still remained to be 50 μM range because of the sample dilution in the tube from the injection value to the transducer cell and of the poor signal to noise ratio due to a peristaltic wave caused by the double plunger pump. The improvement of the sensitivity seems to be easily attained by the employment of a nonperistaltic pump, by which mass transfer process from the mobile phase to the electrode is kept constant.

Figure 5 shows the relationship between the flow rate of the system and the peak height for a glucose sample of 10 mM. In the hydrodynamic voltammetry the transducer output is a function of the thickness of diffusion layer. As far as the electrochemistry between an electrode and an analyte is diffusion-controlled process, a greater response is anticipated at

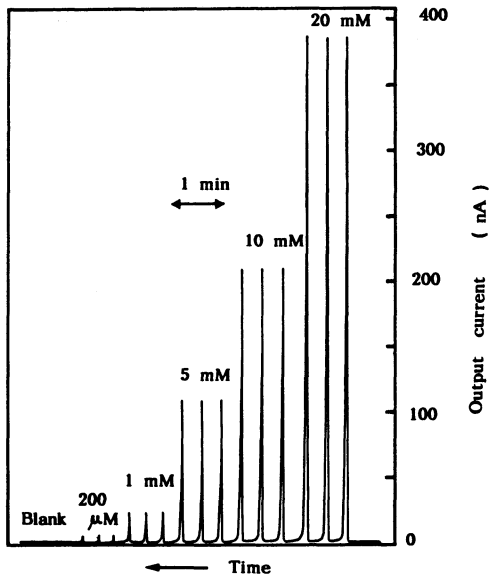

Fig. 3. Responses of micro-enzyme detector system to a series of glucose samples.
A series of glucose samples were injected to a detector system composed of a 100 μm micro-enzyme electrode at a flow rate of 0.8 mL min^{-1}.

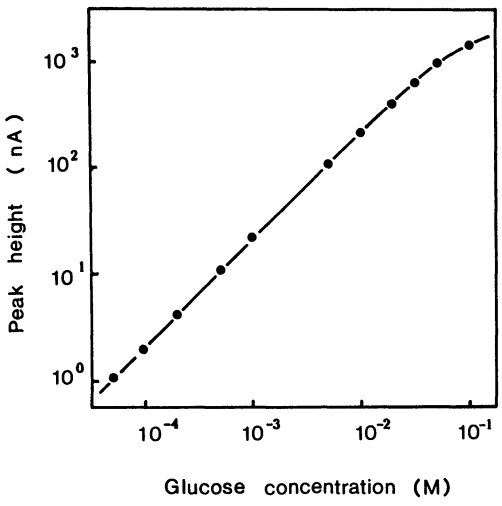

Fig. 4. Relationship between glucose concentration and peak height.

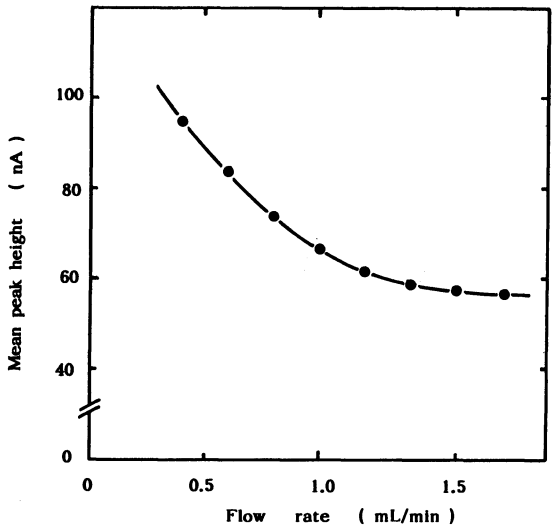

Fig. 5. Relationship between flow rate and peak height.
Micro-emyme electrode having a diameter of 50 μm and glucose sample of 10 mM was employed.

a higher flow rate.[11,12] In the case of highly porous transducer bioelectrode such as the platinized enzyme electrode being concerned, however, there are mainly three processes for the generation of sensor output: mass transfer process, enzyme reaction, and electrochemical reaction (charge transfer process). The mass transfer process in a porous electrode can be divided into two processes, i.e., mass transfer from mobile bulk solution to the apparent surface of electrode by convective flow and that within the crevasses of platinized microparticles. Both the enzyme reaction of glucose oxidase and the electrochemical oxidation of the produced hydrogen peroxide seem to be high enough to generate sensor output, since apparent K_m value (ca. 50 mM) for the enzyme electrode was much greater than the analyte concentration (10 mM), and the applied potential (0.6 V) was higher than the oxidation potential (ca. 0.45 V) of hydrogen peroxide, respectively. Therefore, it would be the mass transfer within the porous particles mainly by diffusion that determines the relation in Fig. 5.

Relation of the Micro-Enzyme Electrode Diameter to the Processing Speed. A variety of enzyme electrodes whose diameters were from 10 μm to 3 mm were prepared to study the relationship between the size of enzyme-microelectrodes and the measuring performance of the detecting system. An enzyme electrode having a diameter of 3000 μm was here employed as a reference to know the effect of miniaturization of enzyme electrode. The flow rate for this experimentation was 0.8 mL min^{-1}. Figure 6 shows the relationship of enzyme electrode diameter and the time required for the measurement of every glucose sample (10 mM). The solid line shows the time when each sample peak returns completely to the baseline, and the dotted one shows the time in which every sample peak has no interaction with each other in the continuous injections of glucose sample. One can easily understand that the smaller the enzyme electrode is employed, the faster measurement is carried out. Figure 6 shows one of the most beneficial characteristics of the microfabricated enzyme electrodes, because smaller electrodes are in shorter contact time with the injected samples.

Table 1 lists the relation of sensor diameter to coefficient of variation. Also listed are the mean sensor response of the respective electrode and the number of injection. Although the mean sensor response was related to the size of micro-enzyme electrode, the enzyme-electrode size was not reflected on the coefficient of variation as long as the enzyme-electrode diameter was in this range. On the other hand, in the case of a macro-enzyme electrode having a diameter of 3000 μm, the coefficient of variation was 1.1% for N=10.

Table 1. Coefficients of Variations of a Series of Micro-Enzyme Sensor Systems

Diameter	Mean sensor response	CV	N
μm	nA	%	
10	13.0	0.61	20
50	75.5	0.59	20
100	205	0.73	20
200	260	0.40	20
500	610	0.61	20

Precision and Reproducibility of the Micro-Enzyme Electrode for FIA of Glucose. A micro-enzyme electrode having a diameter of 100 μm was assembled in a transducer assembly for the repeated injections of 10 mM glucose. The flow rate for this time stability checking in FIA of glucose was 0.8 mL min^{-1}. The micro-enzyme electrode embedded in an acrylic bottom cell was kept in a refrigerator (4 °C) when the electrode was not used. One hundred glucose samples were injected every three days in order to evaluate precision and reproducibility. On the first and fourth days, however, fifty glucose samples were repeatedly injected. Table 2 lists the accuracy of the micro-enzyme electrode. The precision and reproducibility are here expressed as coefficient of variation (CV,%) and relative activity (%) of the micro-enzyme electrode. It is noted that the microfabricated enzyme electrodes demonstrated satisfactory precision in the FIA of glucose.

In another series of experimentations six hundred samples were injected to the flow-through enzyme electrode having a diameter of 100 μm at room temperature, and the coefficient of variation was then calculated. The mean response to 10 mM glucose sample was 252 nA at a flow rate of 0.8 mL min^{-1}. The CV was 1.9 (%) for the six hundred sample injections. One can easily notice the discrepancy that the CV for N=600 is larger than that for N=100 as listed in Table 2. Although all the experimentations were carried out at a room temperature of 23±2 °C, subtle change of the room temperature in one hour seemed to have affected the output of the flow-through enzyme electrodes. We are convinced that the CV for a great number of sample injections can be improved to be

Table 2. Precision and Reproducibility of Micro-Enzyme Sensor in FIA

Time	Relative activity	CV	N
day	%	%	
0	100	1.00	50
4	99	0.73	50
7	100	1.30	100
9	103	0.62	100
12	105	0.51	100
15	100	0.74	100
18	100	1.30	100
21	95	1.10	100
24	99	1.30	100
27	101	0.83	100
30	100	0.75	100

much smaller if the system is strictly thermostated. In the one hundred injections in ca. 10 min the subtle influence of the temperature change seemed to be negligible.

Discussion

The rapid responses of the enzyme (GO)-incorporated platinized microelectrodes can be explained by the properties of the porous platinum surface where sufficient amount of the enzyme molecules (GO) can be embodied with the highly porous platinum, and electrolytic oxidation of hydrogen peroxide proceeds smoothly on the highly catalytic surface of platinized electrode. The asymmetric shape of the response of every electrode can also be interpreted by the porous structure where a part of glucose sample can be retained within the micropores for a relatively long time, although the sample solution was throughly washed away by the mobile buffer.

The surface conditions of the platinized electrodes, both bare platinized electrodes and enzyme (GO)-embodied electrodes, play very important roles in the selective detection of glucose. The improvement of the enzyme electrode selectivity may be elucidated by the surface oxide formation at an anodic potential of ca. 1.2 V. The surface oxide formation of platinum has been uniquely utilized by Johnson et al.[13-15] for the amperometric detection of electroinactive species. In the present study, the hydrogen peroxide to be detected at the electrode seems to promote the surface oxide formation, because the enzyme-incorporated microelectrodes gradually became irresponsive to other sacccharides than glucose after several glucose injections even without anodic polarization at a potential of 0.6 V for 10 min.

One of the benefits of hydrodynamic voltammetry is the impressive detection limit,[16] however, in the FIA of glucose described here we have not succeeded in the sensitive determination of the analyte, which may have been caused mainly by the low signal-to-noise ratio due to the peristaltic wave, as long as a double plumger pump is adopted. Current and future developments are certain to improve detection limits.

A couple of literatures concerning thin-layer electrochemical cells have recently confirmed that the electrode output current depends on the solution velocity to the one-third power. The major reason why the micro-enzyme transducer cell does not obey this theoretical and experimental evidence would be the porous electrode matrix structure where diffusion-controlled as well as convection-controlled mass transfers contribute the output current. From the relationship between the output peak current and the flow rate, it may be stated that there are a few complicated processes until the peak current is generated. In the flow-through electrode like the enzyme electrode being considered, the diffusion process within the crevasses of porous matrix seems to be a rate-determining process, because the output current decreased when the flow rate increased.

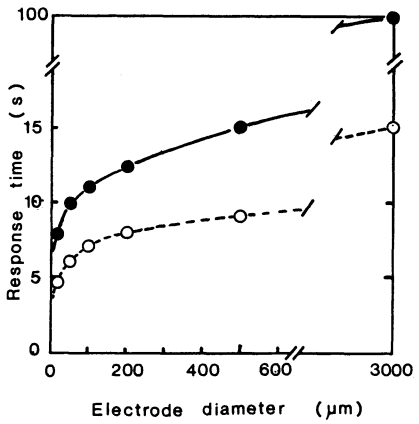

Fig. 6. Relation of micro-enzyme electrode diameter to response time.
The response times in single injection and repeated injections are represented by a solid and dotted lines, respectively.

The most remarkable benefit of miniaturization is the high processing speed of the microfabricated channel electrodes. The evidence is clear in Fig. 6. Another advantage may be the development of microinstrumentation by combining the microsensors with a micropump fabricated by silicon technology.[17]

The satisfactory precision and reproducibility of the FIA electrodes reflect the intact immobilization of enzyme (GO) molecules. Although the GO-entrapped electrode was treated in a glutaraldehyde solution for the preparation of a thin film of albumin, the enzyme molecules within the electrode matrix seemed to be kept as intact as possible.

Coating with anion membrane such as Nafion™ can be a promising approach to make the amperometric enzyme electrode irresponsive to concomitant oxidizable substances such as uricate and ascorbate,[18] however, usage of two electrodes, an enzyme-deactivated electrode and an enzyme-embodied electrode is a general strategy in practical situations, because by measuring the difference between two electrodes the FIA analyzer for glucose would be unaffected by those undesired ingredients. This approach is now under progress.

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