Microbiosensing Device for Real Time Determination

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A microbiosensing device fabricated by assembling an enzyme-embodied microelectrode with a counter electrode and a reference electrode was applied to the real time determination of trace droplet of glucose sample. The microdevice demonstrated unique properties such as real time response, independence of sample volume, unnecessity of sample mixing by the rendezvous of microfabricated biosensing device with pulse voltammetry. A linear relationship between sensor output and glucose concentration was obtained in the concentration range from 10^{-3} to 10^{-1} mol L^{-1}. The sensing device responded to every glucose sample of different concentration in 2 ms, and the coefficient of variation for 10 mM glucose determination was within 5%.

Microfabrication is one of the common trends in science and technology of our age, since microfabricated sensors will play indispensable roles for the construction of very small sophisticated instrument as well as the microinstrumentation of analytical tools.[1] Being small has many advantages in electrochemical analysis of trace substances of biological importance with biosensors. In the early step of biosensor research many enzyme-immobilized membranes have been attached to Clark-type oxygen electrodes.[2-6] In recent years further application of enzyme electrodes has been extended by the microfabrication of electrode-based biosensors, since multifuctional biosensors can be developed by taking advantages of silicon technology.[7-9] Recent photolithographic techniques for deposition and patterning of thin noble metal films (50—300 nm) have been developed for the purposes of fabrication of planar integrated circuits.[10] These techniques are also promising for the preparation of microtransducer for miniaturized biosensors. However, new favorable techniques for immobilizing enzyme onto microtransducers are strongly required for the fabrication of microenzyme electrodes, which sizes are in the μm range, because the conventional enzyme immobilization techniques are not necessarily compatible with miniaturized transducers as long as enzymes are employed in polymer matrix-supported forms.

In previous papers we have reported fundamental properties of microenzyme electrodes with diameters in the range from 10 to 200 μm prepared by incorporating enzyme molecules onto a platinized surface of microplatinum electrode.[11,12] It was the platinization technique that enabled the microfabrication of the biosensor. It is a well-known fact that platinized platinum, porous microparticles of platinum, possesses very large surface area and high catalytic activities for electrolytic processes. By employing a microelectrode as a base electrode for platinization and simultaneous enzyme immobilization, a microenzyme sensor can be easily fabricated.[13] Enzyme molecules are stably retained in the porous platinum particles.[14] In other words, the platinum particles play the dual roles of transducer of enzyme electrode and matrix for enzyme immobilization.

The fabricated microbiosensors for glucose demonstrates high performances such as low detection limit of 5×10^{-7} mol L^{-1}, wide dynamic range from 5×10^{-7} to 2×10^{-2} mol L^{-1}, rabid response of 3 s, satisfactory coefficient of variation [2%], and time stability of over one month.[14,15]

Here we described a unique microbiosensing device for the real time determination of droplet glucose sample. As the operation mode of the microdevice is a voltammetry driven by the application of pulsed potential, stationary trace glucose sample can be determined in 2 ms.

Experimental

A microplatinum wire having a diameter from 10 to 200 μm, a platinum wire (200 μm diameter), and a silver wire (500 μm diameter) were sealed in a Teflon casing (2.5-mm inner diameter) with polyester resin, ground, and polished to leave exposed a circular surface. Electrodes were polished with Imperial™ wrapping films of alumina powders of different particle sizes. At the final stage of polishing 0.1 μm Al_2O_3 was used as the finish and only the microplatinum electrode for enzyme immobilization was cycled between −0.25 and +1.28 V vs. a silver-silver chloride electrode (reference electrode) in 0.5 M H_2SO_4 (1 M=1 mol dm^{-3}) prior to platinization with a conventional three electrode system. The silver electrode was anodized in 1 M NaCl solution for the preparation of a silver-silver chloride electrode. The microplatinum electrode was platinized in a mixed solution of hexachloroplatinate and lead acetate by following the procedure described previously.[14] After the platinization high purity glucose oxidase (EC 1.1.3.4) (from A. *niger*, 120 units/mg) was embodied with the porous surface of microplatinum electrode, following which crosslinking of the enzyme molecules with glutaraldehyde was performed.[14] Figure 1 shows a schematic illustration of

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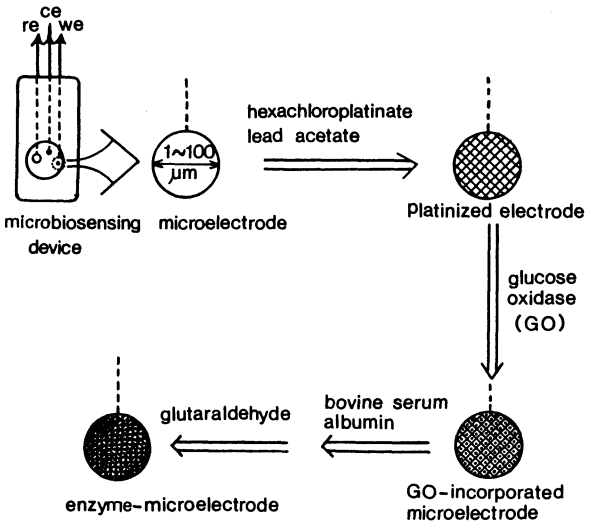

Fig. 1. Schematic illustration of electrochemical fabrication technique.
After the preparation of microplatinum electrode in a Teflon casing, platinization is performed by electrochemical reduction of hexachloroplatinate in the presence of lead acetate, following which enzyme incorporation is carried out. The electrode is immersed in an albumin solution and then treated with glutaraldehyde.

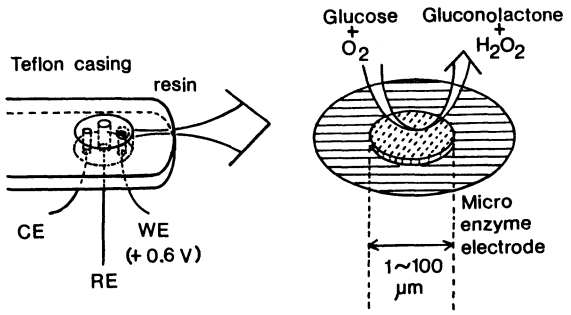

Fig. 2. Schematic drawing of microbiosensing device.
Glucose oxidase immobilized in the porous surface of platinized microelectrode oxidizes glucose in a droplet sample, and the generated hydrogen peroxide is electrochemically oxidized to give anodic sensor signal.

electrochemical microfabrication techniques. The measuring principle of the microbiosensing device thus prepared is schematically shown in Fig. 2.

A stock buffer solution of pH 6.8 was prepared from phosphate salts (50 mM) and NaCl (50 mM) as working electrolyte.

Experimental assays of droplet glucose sample were carried out with a potentiostat in a three-electrode configuation with the Ag/AgCl reference electrode of the microbiosensing device. The potentiostat was operated in on-off potential application mode. A potential of +0.6 V vs. Ag/AgCl was applied to the enzyme-embodied working electrode. The resulting amperometric signals were recorded by using an x-y-t recorder or a memory scope (MOS static RAM=4X12 bitX4096 word). The response time of the x-y-t recorder was 200 ms. In the later case each recorded datum was stored in a floppy disk, and then recorded on an x-y-t recorder. A schematic diagram of the microbiosensing principle is shown in Fig. 3. Glucose determination was performed by addition of aliquot of glucose stock solution. Potential was applied at 10 s aftter the addition of a droplet glucose sample.

Results and Discussion

Typical Responses of Microbiosensing Device. The effectiveness of the microbiosensing device, pulse voltammetry combination was evaluated in glucose sample by loading aliquot of standard glucose sample (10 mM). Every glucose sample (10 μL) was dropped on the three-electrode biosensing device, and a potential of +0.6 V was applied at 10 s after sample loading. Figure 4 shows typical data recorded on the x-y-t recorder. Also shown is the response curve of a blank

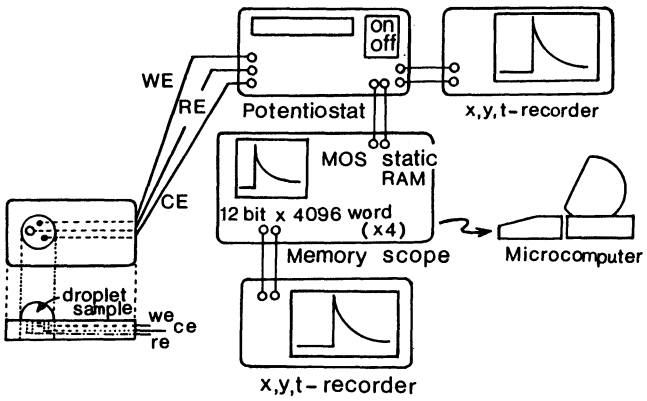

Fig. 3. Schematic diagram of the sensing principle of microbiosensing device.
After the addition of a droplet glucose sample on the three-electrode biosensing device, a potential of +0.6 V is applied to the microdevice and the resulting transient response is recorded on an x-y-t recorder or on a memory scope.

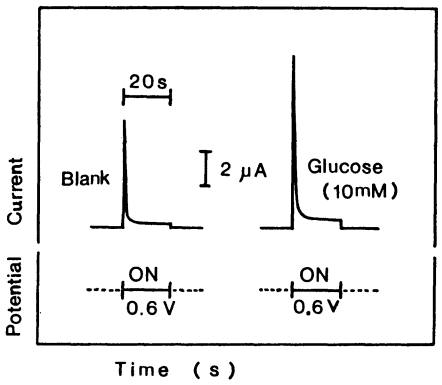

Fig. 4. Typical responses of microbiosensing device.
The responses of the biosensing device to a blank sample (the phosphate-buffered NaCl solution) and a 10 mM glucose sample (in the same buffered solution) were recorded on an x-y-t recorder whose response time is 200 ms.

sample (the phosphate-buffered NaCl solution). After the initial sharp peak, gradually decaying curve was observed. As the response time of the x-y-t recorder is 200 ms, the initial peak seems to the sums of capacitive and faradaic currents. The difference between the peak height of glucose sample and that of blank sample was plotted to the concentration of glucose sample, whose result gave a linear relationship between the peak height current and the glucose concentration in the range from 1 mM to 20 mM against a double logarithmic expression. The calibration curve was a straight line with slope equaled to ca. one-third. Since the slope of the curve seemed worth remarking, we applied a platinized microplatinum electrode to the pulse voltammetric determination of hydrogen peroxide, whose result gave a linear relation against a double logarithmic expression, with a slope of +1, between the initial peak height and the H_2O_2 concentration in the range of 1—100 mM hydrogen peroxide. These results explain the distinction between the enzyme-embodied and the platinized (non-enzyme) electrodes.

The above results make it apparent that in order to measure faradaic current a sufficient recording instrument of fast electrodic process will significantly separate faradaic current from capacitive one.

A sufficiently equipped memory scope was employed to investigate the transient response of the microdevice in further detail. In chronoamperometry current as a function of time is not the response of the microbiosensing devise described in this report but that of the recording system, as long as the x-y-t recorder whose response time is 200 ms is taken. Figure 5 shows a detailed response of the microbiosensing device to 10 mM droplet glucose sample (10 μL). In addition to a blank sample, we employed a fructose sample of the same volume and concentration to know the specificity of the microdevice. All three samples demonstrated similar responses in the rapid decay of initial peak, however, considerable difference among three samples was observed after the decay of charging current. The potential application initiated an instantaneous current as a result of the oxidation of hydrogen peroxide. The resulting current decreases as the electrodic reaction proceeds. In the faradaic process, therefore, the charge passed across the electrode interface is related to the amount of material that has been converted, and the generated current is related to the instantaneous rate at which the oxidation of hydrogen peroxide produced by the immobilized glucose oxidase occurs. In general monitored response to the potential excitation signal for a solution containing supporting electrolyte only is the background response (capacitive current) which should decay rapidly when the electrode has been charged to the applied potential. However, the response to a blank sample shows the faradaic current even after the decay of capacitive current. It should be noted that when platinum electrode is used as a transducer electrode, faradaic current is observed due to the formation of surface oxide of platinum.[16,17] After the anodic polarization the faradaic current for the surface oxide formation decreases significantly.[18] With respect to the response curve of the sensing device in Fig. 5, the difference in the transient response between a glucose sample and a blank sample can be a reasonable sensor signal of the pulse voltammetric biosensing device. As the behavior of the device to a fructose sample was almost similar to that to a blank sample, the microdevice was regarded to have specificity to glucose.

Relation between Transient Response and Glucose Concentration. As the greater difference in the tran-

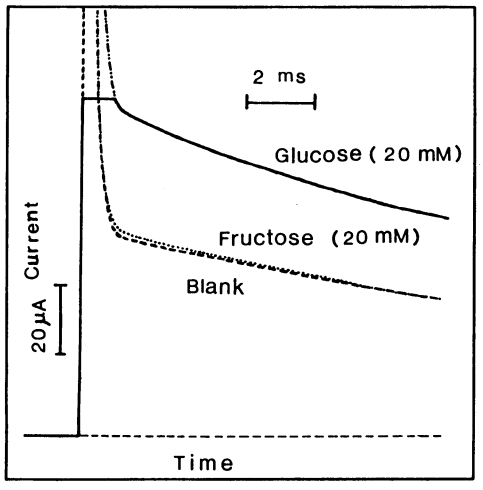

Fig. 5. Transient responses of microbiosensing device.
The responses of the microdevice were obtained by applying a potential of 0.6 V after a glucose sample (20 mM), a fructose sample and a blank sample were separately loaded on the device surface.

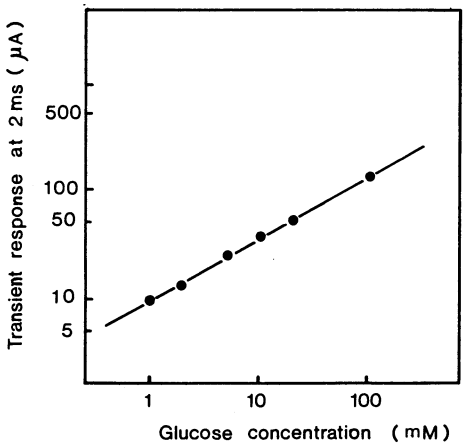

Fig. 6. Calibration curve for real time determination of glucose.
The difference in transient response at 2 ms between glucose sample and blank sample is plotted to the glucose concentration.

sient current between a glucose sample and a fructose sample was observed at 2 ms after the application of +0.6 V to the three-electrode device, the relation between the difference in sensor output and glucose concentration was investigated. Figure 6 shows a linear calibration curve for real time determination of glucose in the range from 1 mM to 100 mM. We have also here a linear relation with a slope of one-third. So far we can not find a resonable explanation for the slope of the calibration curve. In the case of glucose determination in a batch system[14] and a flow system[15] where samples are in hydrodynamic state, we have obtained a linear calibration with a slope of +1. In the determination with the present pulse voltammetric device, droplet samples are in hydrostatic conditions where the flux of hydrogen peroxide to the microelectrode is diffusion control. It is noteworthy that the enzyme reaction is also diffusion-controlled process in the stationary solution. A microplatinized electrode was applied for the pulse-voltammetric determination of hydrogen peroxide in a similar manner, and the resulting calibration curve showed a linear relationship with a slope of +1 in the concentration range from 1 mM to 100 mM hydrogen peroxide, as shown in Fig. 7. A complicated mass transfer process in the enzyme-embodied electrode is reflected on this experimental fact. With this in mind one can easily understand that the transient response seemed to reflect the non-steady state of the enzyme reaction as well as the electrodic reaction. The reasonable explanation for this intriguing phenomenon still remains to be studied in further detail.

Relation between Sensor Output and Sample Volume. The responses of the microbiosensing device to 10 mM glucose sample of different sample volume were obtained to know the relation between sensor output and sample volume. Almost identical responses were observed in this series of measurements where sample volume was varied from 1 to 20 μL. When the sample volume was less than 1 μL, the three-electrode microbiosensing device was not covered with the sample droplet. On the other band, the sample droplet more than 20 μL spilt from the device surface. Table 1 shows the relation between sensor output and sample volume. It should be noted that the sensing principle is independent of sample volume, which is one of the advantages for the microinstrumentation in clinical situations, because the pulse voltammetric device does not require any attached stirring machines.

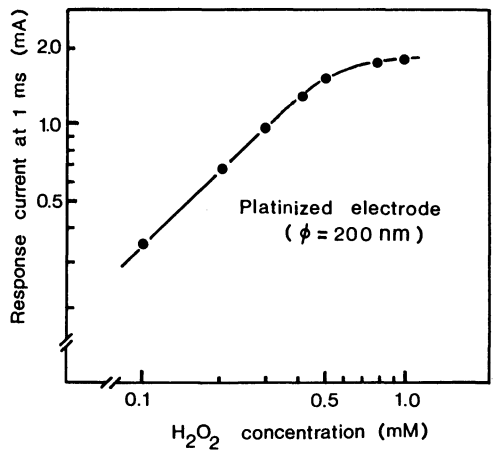

Fig. 7. Relation between transient response and hydrogen peroxide concentration.
The difference in transient responce at 1 ms between hydrogen peroxide sample and blank sample is plotted to the H_2O_2 concentration. The electrode employed here was a platinized microelectrode.

Table 1. Relation between Sensor Output and Sample Volume

Volume (μL)	1	2	5	10	15	20
Sensor output (μA)	29	30	31	31	30	30

The sensor output taken here is the difference in transient response at 1 ms between droplet glucose sample and droplet blank sample.

As the conventional voltammetry such as pulse voltammetry is independent of solution volume being tested, the results in Table 1 are consistent with typical data obtained by pulse voltammetric techniques. It is also worth noting that almost all the measurements with chemical sensors are also independent of sample volume. To our knowledge, however, this is the first experimental demonstration that biosensors are applicable to hydrostatic samples without paying attention to the volume of the sample being tested. Although the present work is in the early stage of experimentation, we examined the accuracy of the pulse voltammetric biosensing device by using 10 mM glucose sample (10 μL). The resulting coefficient of variation was calculated to be +4.4% (N=10).

Conclusion

The microbiosensing device demonstrated several performance characteristics such as real time determination, unnecessity of stirring equipment, and unnecessity of sampling tool. These advantages will allow several design criteria for microinstrumentation of clinical analyzers. The performance characteristics of the platinized platinum microelectrode-based biosensors in batch system, flow system, and pulse voltammetric measurements are summarized in the table.

By combining three microelectrodes into a single sensing unit, we have succeeded in fabricating a miniaturized biosensing device. Therefore, very small amount of sample can be applied to the microdevice, even if the droplet sample volume is a few μL. The microsensing device described here has

a couple of advantages over the dry chemistry-based clinical test papers. Microinstrumentation can be easily performed, because the device does not require additional equipments for the sampling of exact sample and for the stirring of sample solution. Furthermore, the microbiosensing device can be repeatedly used because of the stable, intact immobilization of enzyme molecules in the porous platinized electrode matrix. In addition to these advantages, another most important performance characteristic is that every sample can be determined instantaneously by taking advantage of pulse voltammetric technique. Finally, while we have focused on the fabrication of microbiosensing device which can be operated in the real time determination of analytes, the principle described here will also be a useful means of the microinstrumentation of portable-type biosensors for the daily surveillance of diabetic patients, especially of diabetic blind patients.

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