Glucose Sensing Based on Interdigitated Array Microelectrode

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A micro glucose sensor consisting of an interdigitated array gold microelectrode was developed. The interdigitated array structure, which has 10 μm band width and 10 μm band gap, was fabricated in a small region ($2.5 \times 5 \text{ mm}^2$) on a quartz substrate. Glucose oxidase was chemically fixed onto the electrode surface through self-assembled monolayer of 11-mercaptopundecanoic acid; ferroceneacetic acid was used as electron mediator. Electrochemical properties of the glucose oxidase-immobilized microelectrode were investigated by cyclic voltammogram measurements. Results confirmed that the reductive ferroceneacetic acid generated at counter electrode diffuses through a narrow band gap (10 μm) and can reach the working electrode surface.

(Received February 2, 2001; Accepted May 2, 2001)

Introduction

In recent years, a miniaturized enzyme sensor has been developed for small-volume sample analysis.¹ Small-volume sample analysis leads to alleviation of burden for patients, faster and cheaper diagnosis, and a reduction of reagent waste.² Some types of electrochemical sensors have been developed and investigated for selective and sensitive enzyme detection.³⁻⁶ A microelectrode with interdigitated array (IDA) structure is one of the effective approaches for miniaturization of the electrochemical sensors because of its inherent features, such as large currents, high sensitivity, and rapid rise to a steady state.⁷⁻⁹ In particular, the IDA structure enables detector device or/and sample volume to be miniaturized while sustaining its sensitivity and selectivity, so this type of microelectrode have been applied to fabrication of microdetectors.^{10,11}

Until now, enzyme sensors consisting of IDA microelectrode have been developed as detectors of glucose, urea, lipase, and so on.¹²⁻¹⁸ In these sensors, enzyme oxidase was immobilized by using an immobilization matrix, such as conducting polymers, inorganic gels, organic-inorganic composite film, and organic film. As Gooding pointed out, however, these immobilization techniques have some problems.¹⁹ When an enzyme is immobilized in such a three-dimensional reaction matrix placed over a planar electrode, the response of the sensor depends on the thickness of the reaction matrix and the density of enzyme in the matrix. Moreover, enzymes physically entrapped in the matrix dissolve into the sample solution. These problems lead to decrease in reproducibility and accuracy. An immobilization method using self-assembled monolayers was suggested to overcome the problems mentioned above.¹⁹ The molecular sizes of alkanethiols are generally smaller than the oxidase, so that the dimension of immobilization matrix can be

neglected, that is, the oxidase layer can be regarded as a two-dimensional reaction layer. Alkanethiols can easily adsorb on a metal (particularly gold) surface with high density and can chemically bond to enzyme oxidase through cross-linkers.²⁰ We consider that this immobilization method using self-assembled monolayers is suitable for enzyme sensors consisting of IDA microelectrode to improve sensitivity and reproducibility of electrochemical response. Furthermore, it is expected that selective modification of the oxidase at each arrayed microelectrode can be achieved. Alkanethiols adsorbed onto metal electrodes can be easily desorbed by applying negative potential.²¹ Following desorption of alkanethiols, the oxidase is also removed from the electrode surface. From the point of development of enzyme sensor with IDA microelectrode, this selective immobilization has some possible advantages. There is a possibility to construct arrayed microelectrodes with different enzyme oxidase by repeating the immobilization and desorption. This is difficult to do by using an immobilization matrix such as a conducting polymer and some films.

In this work, we firstly applied the self-assembly method to immobilize enzyme oxidase on an IDA microelectrode surface. A gold film IDA microelectrode (electrode width/gap is 10 μm) was fabricated by photolithographic technique and its surface was modified by glucose oxidase (GOD) through self-assembled monolayer of 11-mercaptopundecanoic acid (MUA). Electrochemical response for glucose was measured by using ferroceneacetic acid (FA), which is the most popular electron mediator for electrochemical detection of enzyme reaction.²² In addition, by controlling the modification of GOD on microelectrode, we could study the diffusion of the mediator between each IDA microelectrode by cyclic voltammogram measurements.

Experimental

Materials

Quartz substrates were from Shinetsu; photoresist (HPR-1183) and developer (MIF) used in microelectrode fabrication were from Fuji Film Olin; MUA was from Aldrich; and *N*-hydroxysuccinimide (NHS) was from Wako Pure Chemicals. Glucose Oxidase (GOD) was from Sigma. 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (WSC) and Tris buffer solution were from Dojindo. Other reagents, prepared from Kanto Chemicals, were of analytical reagent grade and were used as received. Milli-Q water was used for all electrochemical experiments and for fabrication of microelectrodes.

Microelectrode fabrication

The microelectrode was fabricated on a quartz substrate ($23 \times 23 \times 1 \text{ mm}^3$). Before film deposition, each quartz substrate was cleaned by following procedures: wet etched in 5% HF for 20 s, and then sonicated in acetone, methanol, Milli-Q water for 5 min, respectively. By use of a magnetron sputtering system (ULVAC: Model MPS-4000) the substrates were coated with a 30 nm layer of chromium, followed by a 350 nm layer of gold. The thickness of the metal and resist layer was measured by DEKTAK profilometer (SLOAN).

Each IDA microelectrode was fabricated by standard photolithography technique.²³ A thin layer (3–4 μm) of photoresist was spin-coated on the gold surface at 400 rpm/5 s and 3000 rpm/15 s. After pre-bake at 110°C for 4 min, the pattern of microelectrode was transferred by contact exposure to mid-UV light through a positive photomask, and immediately post-baked at 90°C for 8 min. After development, the coated substrate was etched by an iodine etch (0.5 g I_2 , 2 g NH_4I in 10 ml H_2O and 15 ml ethanol) and a ferricyanide etch (2 g $\text{K}_3\text{Fe}(\text{CN})_6$ and 1 g NaOH in 16 ml H_2O).²⁴ The etching times were 1.5 min and 3 min, respectively. Finally, the photoresist was removed by acetone. The layout of each microelectrode consisted of a 3 mm long filament, with an electrode gap and width of 10 μm . A schematic diagram of a fabricated microelectrode is shown in Fig. 1.

An insulated Si_3N_4 film was deposited on the electrode substrate to separate the working area and the connection tracks. A standard lift-off technique was employed to make the pattern of the Si_3N_4 film.²⁵ The photoresist was coated on the substrate and the pattern was transferred by the contact exposure of mid-UV light, as mentioned above. A 500 nm layer of Si_3N_4 was deposited by sputtering. Finally the Si_3N_4 layer on the photoresist was removed in acetone solution.

The film Ag/AgCl reference electrode was formed on the quartz substrate. The gold film fabricated on the substrate was electroplated in Silverex JS-2 (Tanaka Noble Metal) at 60°C by application of +1.6 V potential for 30 s, and then by subsequent electroplating in 3 M (= mol dm^{-3}) NaCl solution at room temperature with –1.2 V for 15 s.

GOD immobilization on gold surface

The fabricated microelectrode surface was cleaned by voltammetrical cycles in 1 M H_2SO_4 solution from –0.3 V to +1.2 V vs. Ag/AgCl until a stable cyclic voltammogram was obtained.²⁶ The self-assembled monolayer of MUA was formed onto the cleaned gold surface by immersion in 5 mM MUA ethanol solution for 12 h. After sonication in ethanol solution, MUA was activated by immersion in 5 mM WSC phosphate buffer (pH 7.0) and then in 5 mM NHS phosphate buffer (pH

Fig. 1 Schematic diagram of the microelectrode and scanning electron microscopy image of part of IDA structure. The 3 mm long IDA contains 25 bands on each side.

7.0) for 30 min. At last, GOD was immobilized in 20 mg/ml GOD phosphate buffer (pH 7.0) for 1 h. After GOD immobilization, the microelectrode was washed by 0.1 M Tris buffer (pH 7.0) and stored in the buffer solution at 4°C until use.

Electrochemical measurements

The electrochemical measurements were performed with an electrochemical analyzer (ALS: Model CHI832) at room temperature. The electrolyte used in all experiments was 0.5 mM FA in 50 mM Tris buffer (pH 7.0). In this electrolyte condition, the potential of the film Ag/AgCl reference electrode on the substrate was shifted by 70 mV as compared to a commercial Ag/AgCl reference electrode in 3 M NaCl (BAS). The measurements were performed by dropping of 2–3 μl sample solutions onto the working area.

Results and Discussion

Electrochemical response of microelectrode

Figure 2 shows cyclic voltammograms of the microelectrodes with MUA monolayer and GOD/MUA monolayer in 0.5 mM FA buffer solution. Since a reversible cyclic voltammogram was observed for both monolayer systems, FA exhibits electrical communication with the modified electrodes. The response current went to a steady state or quasi-steady state and was not peak-shaped as would be expected from an ordinary band electrode.⁷ In the absence of GOD at the electrode surface, no significant change in cyclic voltammograms was

Fig. 2 Cyclic voltammograms of FA in buffer solution at (a) MUA modified microelectrode and (b) GOD immobilized microelectrode. Concentration of glucose is 0 mM (dashed line) and 20 mM (solid line). Scan rate 50 mV/s.

Fig. 3 Electrochemical response of the GOD immobilized microelectrode with (1) no enzyme, (2) 20 mM glucose, (3) 20 mM lactic acid, (4) 20 mM urea. Scan rate 50 mV/s.

observed on adding glucose (Fig. 2(a)). On the other hand, a remarkable enhancement of oxidized current was observed in the presence of GOD (Fig. 2(b)). These results confirm that the enzyme reaction takes place at the electrode surface and FA can work as an electron mediator with the enzyme reaction.

Figure 3 shows the cyclic voltammograms of the GOD immobilized microelectrodes in 20 mM urea solution, 20 mM L-

Fig. 4 Cross-talking behavior of the microelectrode. Current response is normalized to that of just before adding glucose. Potential is 500 mV.

lactic acid, 20 mM glucose and buffer solution, respectively. Comparing the cyclic voltammograms between +100 mV and +400 mV, we see a small difference with L-lactic acid, urea and buffer solution. On the other hand, above +500 mV of applied potential, the effects of L-lactic acid and urea become limited, and an obvious response can be observed only for glucose. It seems that the applied potential set above 500 mV should be used for selective glucose sensing. Figure 4 shows the cross-talking behavior caused by adding the glucose solution and substance solution at a potential of +500 mV. When 10 μ l of 40 mM glucose solution was added into 10 μ l of the buffer solution, the response current was immediately increasing and reached a steady state within a few tens of seconds. The current response was reduced by about 3% by adding the substance solution, which contains 20 mM glucose, lactic acid and urea. The concentration of glucose was constant within this experimental condition. Thus, interfering effects with lactic acid and urea are small and the microelectrode can be used for selective glucose detection.

The sensor response was not changed during a measurement, and was retained for one week while stored in Tris buffer solution at 4°C. It seems that the GOD once fixed at electrode surface is chemically stable until the activity of GOD decreases. This is an advantage compared to methods of physically entrapment in immobilization matrixes. On the other hand, the response of each microelectrode was not constant; the difference was about 30%. This difference is considered to be caused by the surface amount of GOD. For the use of sensor device, one must develop some procedures to keep GOD density constant.

Effect of oxygen on the microelectrode

In these experiments, the concentration of FA used in all measurements is comparable to that of oxygen solved in air-saturated solution (*ca.* 10^{-4} M). Hence, it is expected that the current response would be inhibited by competition of electron transfer between FA and oxygen molecules.²⁷ Figure 5 shows the cyclic voltammograms in air-saturated and nitrogen saturated solution. The current response with/without glucose seems almost the same and no significant change can be observed. As shown in Fig. 6, the amperometric response in air-saturated solution is linearly related to glucose concentration until 15 mM without base-line shift, suggesting that the FA acts as a non-threshold type electron acceptor in our electrode

Fig. 5 Cyclic voltammograms of FA in (1, 2) air-saturated and (3, 4) nitrogen-saturated buffer solution at GOD immobilized microelectrode. Concentration of glucose is 0 mM (dashed line) and 20 mM (solid line). Scan rate 50 mV/s.

Fig. 6 Calibration curve of glucose with the microelectrode.

system.^{27,28} Therefore, it can be considered that the free FA molecules are close to the GOD and mediate the electron between GOD and electrode surface with high efficiency, as predicted by Dicks *et al.*²⁷

Diffusion effects of microelectrode

In our GOD attachment procedure, all of the IDA microelectrode surface must be modified with GOD. This means that GOD is fixed on both working and counter electrode surfaces. It can be considered that the reduction of FA takes place not only on the working electrode but also on the counter electrode by enzyme reaction. The reductive species generated at the counter electrode may diffuse to the working electrode through a narrow band gap. This diffusion causes an increment of reductive FA in the vicinity of the working electrode, and induces an enhancement of the electrochemical response. In order to confirm such considerations, we performed the following experiments.

The GOD immobilized on one side of IDA microelectrode was removed by reductive desorption of MUA; cycle +100 to -1200 mV until the reduction peak of the MUA disappeared.²¹ Thus, we get IDA microelectrodes with/without GOD as illustrated in Fig. 7. In order to diffuse the desorbed MUA and

Fig. 7 Electrochemical setup used for studying diffusion effects of the microelectrode.

GOD into the bulk solution easily, the desorption process was carried out using outer Pt counter electrode and Ag/AgCl (3 M NaCl) reference electrode by immersing the working area of the microelectrode into a buffer solution in the sample vial. This sample assembly, except for the outer Pt electrode, was employed for the measurements reported in this section in order to prevent heterogeneous diffusion by using small droplets on the microelectrode.

We measured cyclic voltammograms in the following ways: (1) GOD modified electrode (GOD electrode) as a working electrode and GOD desorbed electrode (non-GOD electrode) as a counter electrode, (2) GOD electrode as a counter electrode and non-GOD electrode as a working electrode. The sample assembly is illustrated in Fig. 7. As shown in Fig. 8(a), increasing of response current with adding glucose at a potential above +200 mV was observed in the former electrode connection. This is a reasonable result because the reductive FA is generated just on the detection electrode. On the other hand, the same behavior was observed even though GOD was not located at the detection electrode, Fig. 8(b). In the latter electrode connection, GOD exists at counter electrode and enzyme reaction does not take place at detection electrode surface. This result indicates that the reductive FA generated at counter electrode surface by enzyme reaction diffuses toward the working electrode and is also oxidized at the working electrode, as illustrated in Fig. 9.

According to such experiments, we can say that both electrodes with GOD layer act as the generator electrode and the working electrode is used as collector electrode. This means that the reductive FA is collected in the vicinity of the working electrode and the concentration becomes higher than that around the isolated working electrode. It is expected that the concentration of FA around the working electrode leads to large current response in the case of GOD immobilization on both electrodes. Contrary to our expectation, however, the current response, shown in Fig. 3 (curves 1 and 2), seems slightly smaller compared to GOD removal condition, Fig. 8(a).

In order to explain this weak response, we considered that some electron-insulating took place due to the large organic molecules which covered the surface of the electrode. It is well known that an organic layer on an electrode surface prevents the electron transfer between electroactive species and electrode surface and leads to the reduction of current response in cyclic voltammograms.^{29,30} Such electron-insulating can be observed

Fig. 8 Diffusion of reductive FA generated by enzyme reaction at the counter electrode. Electrochemical configurations are (a) GOD electrode as working electrode, (b) non-GOD electrode as working electrode. Concentration of glucose is 0 mM (dashed line) and 20 mM (solid line). Scan rate 50 mV/s.

even in the case of small alkanethiol derivatives, which possess four or eight alkyl chains.²⁹ Moreover, a large biochemical molecule such as a protein near the electrode surface can reduce the effective rates of the electron transfer reactions.^{31,32} In this case, the surface of microelectrode is covered with large enzyme molecules through MUA. Therefore, it is possible to consider that the oxidation of FA and its counter reaction at working and counter electrodes are prevented by GOD and/or MUA monolayer.

Conclusion

We fabricated microelectrodes with IDA structure. GOD was chemically attached onto the electrode surface through self-assembled monolayer of MUA. The electrochemical response of this microelectrode has selectivity for glucose without interference from urea and lactic acid. By controlling the GOD modification, we confirmed that the microelectrode with GOD layer acts as generator electrode of reductive FA resulting from enzyme reaction. The reductive FA is collected at the vicinity of working electrode by diffusion through a narrow band gap (10 μm). Thus, the GOD modification at both working and counter electrodes is useful to increase the concentration of electron mediator at working electrode. There is a possibility that the electrochemical response of the microelectrode will be enhanced by improving the collection efficiency of the mediator

Fig. 9 Schematic diagram of the generation and diffusion of reductive FA.

by IDA structure with narrower band gap.

Acknowledgements

This present work was supported in part by a NEDO (New Energy Industrial Technology Development Organization) regional consortium project "Development of Ultrasensitive Micro-Integrated Analysis Systems", a Grant for the Collaboration between University and Society (No. 11793004) from the Ministry of Education, Culture, Sports, Science and Technology, Japan, and by the Satellite Venture Business Laboratory of the University of Tokushima.

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