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Article *in* Bioscience Biotechnology and Biochemistry · April 2006

DOI: 10.1271/bbb.70.654 · Source: PubMed

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Biosci. Biotechnol. Biochem., **70** (3), 654–659, 2006

Novel FAD-Dependent Glucose Dehydrogenase for a Dioxygen-Insensitive Glucose Biosensor

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Received September 6, 2005; Accepted November 11, 2005

A novel FAD-dependent glucose dehydrogenase (FAD-GDH) was found and its enzymatic property for glucose sensing was characterized. FAD-GDH oxidized glucose in the presence of some artificial electron acceptors, except for O_2, and exhibited thermostability, high substrate specificity and a large Michaelis constant for glucose. FAD-GDH was applied to an amperometric glucose sensor with $Fe(CN)_6^{3-}$ as a soluble mediator. The use of a relatively high concentration of $Fe(CN)_6^{3-}$ resulted in a good linearity between the current response and the glucose concentration, taking into account a large Michaelis constant for $Fe(CN)_6^{3-}$. The glucose sensor was completely insensitive to O_2 and responded linearly to glucose up to 30 mM. Compared to glucose, the response to other saccharides was negligible. The sensor can be stored at room temperature in a desiccator for at least one month without any change in the response or activity.

Key words: glucose sensor; glucose dehydrogenase; FAD; amperometric sensor

A blood glucose monitoring system that enables an individual to measure his/her own blood glucose level is essential to managing diabetes. Numerous amperometric glucose sensors have been constructed and commercialized during the last 2–3 decades as a result of huge efforts toward developing and improving the performance of such systems.[1–4] Most of these sensors utilize glucose oxidoreductase as a catalyst and an appropriate redox mediator to assist the electron transfer from enzyme to electrode in order to achieve the enzymatic electrochemical oxidation of glucose. Commercially available glucose sensors still have problems needing to be solved, some problems arising from the inherent characteristics of the enzymes used for the sensors.

Among the glucose oxidoreductases, glucose oxidase (GOD) has been widely utilized as a mediated amperometric sensor because of its high thermostability and high glucose selectivity.[5] However, glucose measurement is often interfered with dioxygen (O_2), since the mediator competes with O_2 for reduced GOD. The Michaelis constant of GOD for O_2 is as large as 0.2 mM, resulting in the sensor being susceptible to O_2-concentration-dependent drift; an increase in the O_2 concentration leads to a decrease in the signal and underestimation of the glucose concentration.[6]

In construct, glucose dehydrogenases, which do not utilize O_2 as an electron acceptor, are promising candidates for the electrocatalyst in an O_2-insensitive glucose sensor. Two types of dehydrogenase have been utilized for glucose sensors.

One is quinoprotein glucose dehydrogenase, possessing pyrroloquinoline quinone as the cofactor (PQQ-GDH), which can use a variety of electron acceptors as a redox mediator except for O_2 in acceptable electron transfer kinetics.[4] There are two kinds of PQQ-GDHs; one is intracellular and soluble, while the other is tightly bound to the outer surface of the cytoplasmic membrane. Membrane-bound quinoprotein glucose dehydrogenase (mPQQ-GDH) exhibits high glucose selectivity, but requires suitable detergents for solubilization and purification.[7] It would therefore seem to be difficult to apply mPQQ-GDH to glucose sensors on a commercial basis.

In contrast, water-soluble pyrroloquinoline quinone glucose dehydrogenase (sPQQ-GDH) has low substrate specificity; it can oxidize a variety of monosaccharides and disaccharides (mannose, maltose, lactose *etc.*).[8] A glucose sensor based on sPQQ-GDH may overestimate the glucose concentration of a blood sample containing such saccharides. In particular, the marked response to maltose is serious, because the infusion solution for diabetes contains a high concentration of maltose.[9] Moreover, sPQQ-GDH lacks thermal stability, although some attempts have been made to improve the substrate specificity and thermal stability of sPQQ-GDH by protein engineering.[10]

The other type of dehydrogenase is nicotinamide

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adenine dinucleotide (NAD)-dependent glucose dehydrogenase (NAD-GDH) which exhibits higher substrate specificity and stability than sPQQ-GDH does. One drawback of this enzyme is that a detection system based on NAD-GDH would be more complicated than that of a GOD- or sPQQ-GDH-based glucose sensor, because such catalysts as NADH dehydrogenases (EC: 1.6.99.- :diaphorase) or organic compounds must be incorporated into the system to oxidize NADH to NAD^+ electrochemically.[11)]

A novel thermostable heme-containing FAD-dependent glucose dehydrogenase has been isolated from the moderately thermophilic bacterium, *Burkholderia cepacia*.[12)] The gene encoding this glucose dehydrogenase has recently been clarified and expressed in *Escherichia coli*; however, the catalytic activity was not very high at room temperature.[13)] another FAD-dependent glucose dehydrogenase was discovered from *Aspergillus oryzae* in the 1960s,[14,15)] although there have been no subsequent reports on this enzyme, as far as we aware.

We have recently discovered a novel FAD-dependent glucose dehydrogenase from *Aspergillus terreus* (FAD-GDH).[16)] FAD-GDH showed thermostability and high substrate specificity, and did not require additional cofactors or activators to exhibit dye-mediated glucose dehydrogenase activity. FAD-GDH would therefore, be much better than other glucose oxidoreductases found to date to construct an O_2-insensitive amperometric glucose sensor. In this paper, we partially characterize FAD-GDH in respect ofits sensor application, and report the construction of a prototype FAD-GDH-immobilized amperometric glucose sensor. Hexacyano ferrate (III) ($Fe(CN)_6^{3-}$) is used in this sensor as a mediator, because it is stable, inexpensive, insensitive to O_2, and has already been utilized in the commercial amperometric glucose biosensors.[3)]

Materials and Methods

Reagents. FAD-GDH from A. terreus with an activity of 1000–1500 units/mg was prepared according to the literature[16)] and dissolved in a 50 mM phosphate buffer (pH 7.5). The concentration of FAD-GDH was determined spectrophotometrically, using the molar extinction coefficient of free FAD of $11.3 \times 10^3\ M^{-1}\ cm^{-1}$ at 465 nm.[17)] Potassium hexacyano ferrate (III), D(+)-glucose, and glucose oxidase (GOD) from Aspergillus niger were purchased from Wako Pure Chemical Industries (Japan) and used without further purification. A glucose stock solution was made with a phosphate buffer (100 mM at pH 7) and stored overnight to achieve mutarotative equilibrium. The concentration of GOD was determined by using a molar extinction coefficient of $13.1 \times 10^3\ M^{-1}\ cm^{-1}$ at 450 nm.[18)]

Preparation of a FAD-GDH-modified electrode. A 5-µl aliquot of the FAD-GDH solution (32 pmol) was dropped onto the surface of a glassy carbon electrode of 3 mm in diameter. The solvent was allowed to evaporate in a desiccator at room temperature, and then the surface was covered with a dialysis membrane with a thickness of 20 µm in the dry state.

Electrochemical measurement with the enzyme-modified electrode. Electrochemical measurements were carried out with a three-electrode system, using a BAS100B voltammetric analyzer (BAS), in which a platinum wire and Ag|AgCl|KCl (sat.) electrode were respectively used as the counter and reference electrodes. Measurements were performed in a laboratory-made water jacket-equipped electrolysis cell (3 ml) at 40 °C. The solution was stirred with a magnetic stirrer at 1000 rpm in chronoamperometry. The concentration of dissolved O_2 was continuously monitored with a Clark-type O_2 electrode (Opto Science, Kyoto, Japan).

Results and Discussion

Spectroscopy of FAD-GDH

The purified FAD-GDH solution was yellow in color and exhibited two characteristic peaks at 385 and 465 nm (curve A, Fig. 1). When glucose was added to the enzyme solution, these peaks immediately disappeared (curve B). These spectral properties are characteristics of flavoproteins. The spectrum revealed the absence of heme in FAD-GDH, and was distinct from FAD-containing glucose dehydrogenase from *B. cepacia*.[12)]

Reactivity to dissolved O_2

Dehydrogenases often exhibit some degree of oxidase activity. In order to check the oxidase activity of FAD-GDH, the O_2 concentration change was monitored with a Clark-type O_2 electrode during the enzyme reaction in a glucose solution (100 mM) at pH 7.0. As shown by

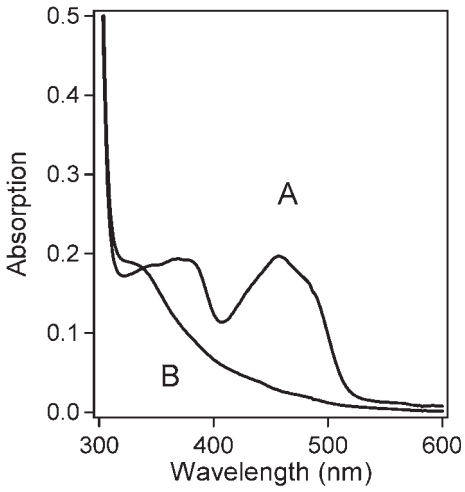

Fig. 1. Absorption Spectra of Purified FAD-GDH.

FAD-GDH (18 µM) was dissolved in a phosphate buffer solution at pH 7.5. (A) Native enzyme (oxidized form), (B) reduced enzyme after adding 50 mM glucose.

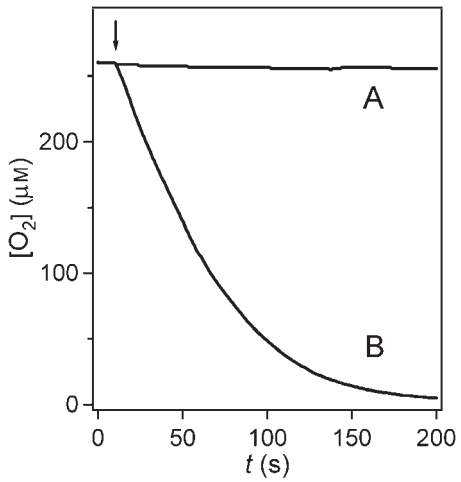

Fig. 2. Time-Course Characteristics of the O_2 Consumption during the (A) FAD-GDH and (B) GOD Reactions.

Each enzyme was added to an air-saturated buffer solution (pH 7.0) containing 100 mM glucose at the point indicated by the arrow at a final concentration of 20 nM. The O_2 concentration was measured with a Clark-type O_2 electrode, and the signal was converted to the O_2 concentration.

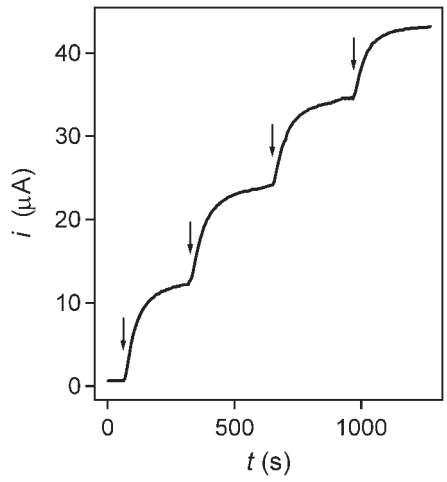

Fig. 3. Time-Course Characteristics of the Current Response (i) against Glucose at the FAD-GDH-Modified Electrode in an Air-Saturated Buffer Solution (pH 7.0, 40 °C) in the Presence of 100 mM $Fe(CN)_6^{3-}$.

At the pointes indicated by the arrows, a glucose stock solution was successively added at final concentrations of 10, 20, 30, and 40 mM. The current was measured at 0.5 V while stirring.

curve B in Fig. 2, when GOD was added to the air-saturated glucose solution, a rapid decrease in the O_2 concentration was observed; two-electron reduction of O_2 occurred concomitant with enzymatic glucose oxidation to gluconolactone. In contrast, no change was apparent in the O_2 concentration in the presence of FAD-GDH (curve A). Similar results were obtained in the pH range from 4 to 10 (data not shown). These finding clearly indicate that FAD-GDH completely excluded O_2 as an electron acceptor.

Amperometric measurements by the enzyme-modified electrode

We first tried to measure the catalytic current without a mediator aiming at a 3rd generation biosensor. A catalytic current, however, was not generated in a glucose solution in the absence of a mediator, indicating that it was difficult for FAD-GDH to transfer electrons directly from embedded reduced FAD to the electrodes as in the case of most redox enzymes. We therefore used $Fe(CN)_6^{3-/4-}$ as a mediator, and measured the steady-state catalytic current at a fixed potential of 0.5 V while stirring. Figure 3 shows the typical time dependence of the steady-state catalytic current on the successive addition of glucose to a buffer solution (pH 7) containing 100 mM of $Fe(CN)_6^{3-}$. Part of $Fe(CN)_6^{3-}$ transferred convectively to the enzyme-trapped layer was reduced by the FAD-GDH reaction to $Fe(CN)_6^{4-}$, which was re-oxidized at the electrode surface to generate the steady-state current. The current reached its limiting value within about 200 s, this relatively slow response being due to the permeability of glucose (and $Fe(CN)_6^{3-}$). The steady-state catalytic limiting current increased with successive glucose injection. It is noteworthy that a relatively high concentration of $Fe(CN)_6^{3-}$ was essential to observe a detectable steady-state current making it difficult to observe the catalytic current in cyclic voltammetry in quiet solution. This suggests a large value of the Michaelis constant for $Fe(CN)_6^{3-}$.

The current response was not inhibited by EDTA (1–10 mM) as a typical inhibitor of PQQ-GDH, and was independent of Mg^{2+} or Ca^{2+} that are essential for the activation of PQQ-GDH. The electrode allowed glucose detection to a level as low as 10 μM ($S/N \geq 3$). The relative standard deviation of the current response obtained for a 5–30 mM glucose concentration was 1% or better in run-to-run measurements ($n = 4$) and within 5% in electrode-to-electrode measurements ($n = 5$). The current response time and magnitude were stable for at least one month when the FAD-GDH-modified electrode was stored in a desiccator at room temperature.

$Fe(CN)_6^{3-}$ concentration dependence of the catalytic current

The enzymatic reaction would proceed under Ping Pong Bi Bi-mechanism assuming analogy with other flavin-dependent oxidoreductases. The steady-state current (i_s) of the enzyme-modified electrode while stirring can be approximately expressed as a function of the total concentration of the mediator ([M]*) and substrate ([S]*) in the bulk solution:[19]

$$i_s = \frac{(n_s/n_m)FAk_{cat.app}[E]_0}{1 + K_{S.app}/[S]^* + K_{M.app}/[M]^*} \quad (1)$$

where n_s, n_m, F, A, and $[E]_0$ respectively express the number of electrons of the substrate, the number of electrons of the mediator, the Faraday constant, the electrode surface area, and the surface concentration of FAD-GDH in the immobilized layer. $k_{cat.app}$ is the apparent catalytic constant of the immobilized enzyme,

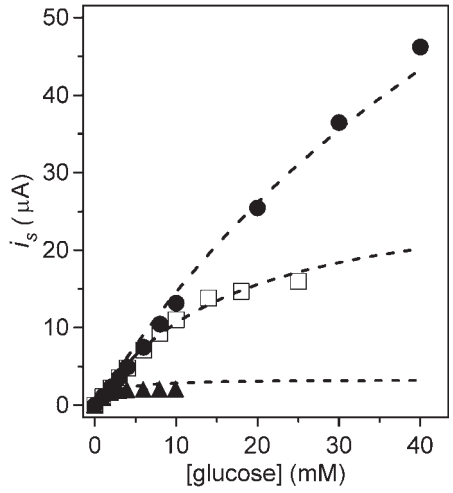

Fig. 4. Dependence of the Steady-State Catalytic Current (i_s) at the FAD-GDH-Modified Electrode on the Glucose Concentration.

The $Fe(CN)_6^{3-}$ concentration was (▲) 1 mM, (□) 10 mM, and (●) 100 mM, the other conditions being identical to those given in Fig. 3. Dashed lines represent the regression curves based on Eq. 1 with the parameters given in the text.

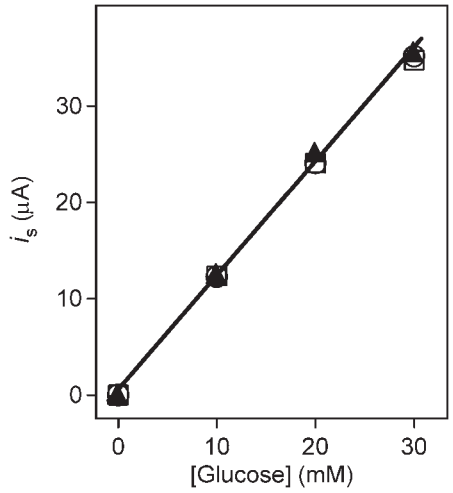

Fig. 5. Effect of Dissolved Dioxygen.

Calibration curves of glucose measured with the FAD-GDH-modified electrodes under (○) air-saturated, (▲) O_2-saturated, and (□) Ar-saturated conditions. Other conditions are identical with those given in Fig. 3.

and $K_{S.app}$ and $K_{M.app}$ are apparent Michaelis constants for the substrate and mediator, respectively. Parameters $k_{cat.app}$, $K_{S.app}$ and $K_{M.app}$ involve the effect of the concentration depression in the immobilized layer and the permeability through the membrane.[20)]

Figure 4 shows the dependence of i_s on the glucose concentration at various concentrations of $Fe(CN)_6^{3-}$. The i_s value increases with the glucose concentration in the low concentration range, and reaches the saturated value at higher concentration. The saturated value is elevated with increasing $Fe(CN)_6^{3-}$ concentration. These data suggest that the $K_{M.app}$ value of FAD-GDH for $Fe(CN)_6^{3-}$ was relatively large. The data in Fig. 4 would be fairly represented by Eq. 1 with $k_{cat.app}$ of 230 s^{-1}, $K_{M.app}$ of 65 mM, and $K_{S.app}$ of 140 mM as shown by the dashed lines. At low $Fe(CN)_6^{3-}$ concentration, the $K_{M.app}/[M]^*$ term in Eq. 1 is not negligible, and i_s reaches the saturated value at a low glucose concentration. When 100 mM of $Fe(CN)_6^{3-}$ was used, the linear range between i_s and the glucose concentration extended up to 30 mM. When $[M]^* > K_{M.app}$ and $[S]^* < K_{S.app}$, the current-response becomes linear against the substrate concentration, and i_s can be approximately expressed by the following equation:

$$i_s = \frac{(n_s/n_m)FAk_{cat.app}[E]_0[S]^*}{K_{S.app}} \quad (2)$$

This was the case at 100 mM of $Fe(CN)_6^{3-}$ and 0–30 mM of glucose, although the dashed line depicted in Fig. 4 is based on Eq. 1. Considering the requirement that 30 mM is the upper limit of detection required for a commercial blood glucose sensor,[3)] the FAD-GDH-modified electrode can satisfactorily meet this requirement.

The $K_{S.app}$ value is much larger than the Michaelis constants of FAD-GDH for glucose obtained in a bulk solution (49.5 mM with phenazine methosulfate as an electron acceptor[16)]). The low permeability of glucose through the dialysis membrane (compared with the bioelectrochemical reaction rate) may have been responsible for the large $K_{S.app}$ value. The permeability of $Fe(CN)_6^{3-}$ through the dialysis membrane might also have been responsible in part for the large $K_{M.app}$ value. However, as compared to the $K_{M.app}$ value of 0.5 mM for 2,3-dimethoxy-5-methyl-1,4-benzoquinone (Okumura, N., Tsujimura, S., and Kano, K., unpublished data), there seems to be some barrier in the access of $Fe(CN)_6^{3-}$ to the active center of FAD-GDH such as electrostatic repulsion or structural hindrance. Further details are under investigation.

Effect of O_2 on the current response

Figure 5 shows the calibration curves obtained with the FAD-GDH-modified electrode in the presence of 100 mM $Fe(CN)_6^{3-}$ in air-saturated, O_2-saturated and Ar-saturated buffer solutions. The values measured under the three conditions are almost identical. FAD-GDH can also utilize hexaammineruthenium (III), a variety of quinones and naphthoquinones, and such redox dyes as thionine in place of $Fe(CN)_6^{3-}$ as an electron acceptor (data not shown). However, the FAD-GDH-modified electrode utilizing these chemicals was affected by dissolved O_2, because the reduced forms of these chemicals are susceptible to autoxidation by O_2.

Substrate specificity-response to other saccharides

The current response to other saccharides was measured with the FAD-GDH-modified electrode in the presence of 100 mM $Fe(CN)_6^{3-}$ at pH 7. A negligibly small response was observed against maltose, mannose, galactose, and lactose. The slopes of the linear relationship between the steady-state current and the

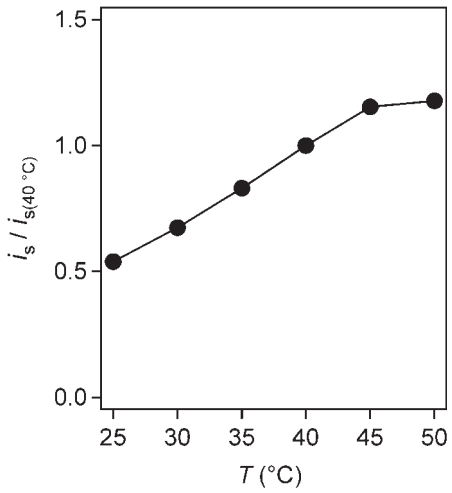

Fig. 6. Temperature Dependence of the Steady-State Catalytic Current at the FAD-GDH-Modified Electrode at 5 mM of Glucose.

The conditions other than the temperature are identical to those given in Fig. 3. The current (i_s) is expressed as a relative value against that at 40 °C ($i_{s(40\,°C)}$).

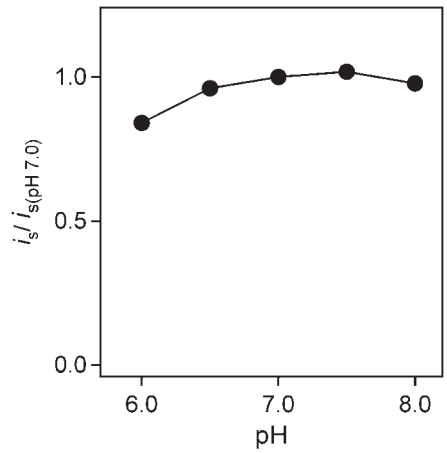

Fig. 7. pH Dependence of the Steady-State Catalytic Current (i_s) at the FAD-GDH-Modified Electrode at 5 mM of Glucose.

A phosphate buffer of 50 mM was used for the test solution, the other conditions being identical to those given in Fig. 3. The current (i_s) are expressed as a relative value against that at pH 7.0 ($i_{s(pH\ 7.0)}$).

saccharide concentration were 2.4% (maltose), 1.7% (mannose), 0.4% (galactose), and 0.4% (lactose) against the slope for glucose with a relative standard deviation of 12%, 10%, 15% and 8% (n = 5), respectively. Acceptably high selectivity was confirmed against glucose, and the relative values for the slope were similar to the relative activity measured in a solution containing 333 mM saccharides (1.4%, 2.8%, 1.2% and less than 0.1% for maltose, mannose, galactose, and lactose, respectively).[16)]

Effects of temperature and pH on the current response

Figure 6 shows the temperature dependence of the catalytic current for the FAD-GDH-modified electrode. The current response was measured in 5 mM of the glucose solution in the temperature range of 25–55 °C. The steady-state current increased linearly up to 45 °C and reached the maximum value at 50 °C. However, at temperatures above 55 °C, the current decreased with time during measurement, and a steady-state current response could not be obtained. The temperature profile of the FAD-GDH modified electrode was very similar to that of the FAD-GDH activity in a bulk solution.[16)] The pH dependence of the current is illustrated in Fig. 7. The FAD-GDH-modified electrode shows acceptable stability in a broad pH range of 6.0–8.0. This property is somewhat different from the pH-activity profile of FAD-GDH dissolved in a buffer solution.[16)] The difference might be due to the fact that the steady-state current is governed in part by the permeability of the substrate. In addition, a difference in the electron acceptor seems to affect the pH-activity profile.

In conclusion, we have demonstrated that novel FAD-GDH could utilize $Fe(CN)_6^{3-}$ as a mediator and be suitable for use in an O_2-insensitive glucose sensor. A high concentrations of $Fe(CN)_6^{3-}$ ($\geq$ 100 mM) was required to achieve a wide range of linear response up to 30 mM of glucose because of the large $K_{M.app}$ value for $Fe(CN)_6^{3-}$. The sensor constructed in this work showed long-term stability at least for one month in the dry state, and worked adequately over a wide range of pH and temperature. Its selectivity against glucose was sufficient for practical use. Since the Michaelis constant of FAD-GDH for glucose in solution is rather high (49.5 mM[16)]), the outer membrane used in this study would not be essential in an FAD-GDH sensor, especially in the disposable type.

Acknowledgments

This work was supported in part by grant-aid for scientific research from the Ministry of Education, Culture, Sports, Science and Technology of Japan (MECSSTJ), and by COE for Microbial Process Development Pioneering Future Production Systems (COE program of MECSSTJ).

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