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Comparison of Direct and Mediated Electron Transfer in Electrodes with Novel Fungal Flavin Adenine Dinucleotide Glucose Dehydrogenase

Kazuya ISHIDA,*1 Kouhei ORIHARA,*1,*2 Hitoshi MUGURUMA,*1,*2† Hisanori IWASA,*2 Atsunori HIRATSUKA,*2 Katsumi TSUJI,*3 and Takahide KISHIMOTO*4

*1 *Graduate School of Engineering and Science, Shibaura Institute of Technology, 3-7-5 Toyosu, Koto, Tokyo 135–8548, Japan*
*2 *Nanomaterials Research Institute, National Institute of Advanced Industrial Science and Technology (AIST), Tsukuba Central 5-41, 1-1-1 Higashi, Tsukuba, Ibaraki 305–8565, Japan*
*3 *Research Center, TOYOBO CO., LTD., 2-1-1 Katata, Otsu, Shiga 520–0292, Japan*
*4 *Tsuruga Institute of Biotechnology, TOYOBO CO., LTD., 10-24 Toyo-cho, Tsuruga, Fukui 914–8550, Japan*

Direct and mediated electron transfer (DET and MET) in enzyme electrodes with a novel flavin adenine dinucleotide-dependent glucose dehydrogenase (FAD-GDH) from fungi are compared for the first time. DET is achieved by placing a single-walled carbon nanotube (CNT) between GDH and a flat gold electrode where the CNT is close to FAD within the distance for DET. MET is induced by using a free electron transfer mediator, potassium hexacyanoferrate, and shuttles electrons from FAD to the gold electrode. Cyclic voltammetry shows that the onset potential for glucose response current in DET is smaller than in MET, and that the distinct redox current peak pairs in MET are observed whereas no peaks are found in DET. The chronoamperometry with respect to a glucose biosensor shows that (i) the response in DET is more rapid than in MET; (ii) the current at more than +0.45V in DET is larger than the current at the current-peak potential in MET; (iii) a DET electrode covers the glucose concentration range for clinical requirements and is not susceptible to interfering agents at +0.45 V; and (iv) a DET electrode with the novel fungal FAD-GDH does not affect sensing accuracy in the presence of up to 5 mM xylose, while it often shows a similar response level to glucose with other conventionally used fungus-derived FAD-GDHs. It is concluded that our DET system overcomes the disadvantage of MET.

Keywords Flavin adenine dinucleotide-dependent glucose dehydrogenase, mediated electron transfer, direct electron transfer, single-walled carbon nanotube, biosensor

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Introduction

Oxygen-insensitive flavin adenine dinucleotide-dependent glucose dehydrogenase (FAD-GDH) has been given much attention recently and there has been an increasing numbers of related reports on biosensors[1-15] and biofuel cells.[13,14,16-18] Recently, the structure of FAD-GDH from *Aspergillus flavus* was unveiled and it was found that an FAD cofactor is buried deeply (~1.4 nm) below the protein surface.[19] Therefore, the main issue with FAD-GDH is the construction of an electron transfer configuration as in the case of FAD-glucose oxidase (GOD).[20] There are two approaches: mediated electron transfer (MET) and direct electron transfer (DET). MET is the utilization of electron transfer mediators such as potassium hexacyanoferrate ($K_3[Fe(CN)_6]$),[5] phenazine methosulfate,[3,7] osmium complexes,[8-10] phenothiazin,[13] and naphthoquiobne.[15] Small and mobile mediators ferry electrons from FAD, which originates from an enzyme-catalytic reaction, to the electron collector (Fig. 1(A)). However, some practical limitations exist; the instability of the oxidized form of a mediator coexisting with an enzyme in an electrode is naturally reduced during long-term storage, which causes a reduction in the sensitivity. Leaching of the free mediators also reduces the sensitivity, and even worse, it causes poisoning problems. In the case of mediator immobilization, the process is complicated, including the preparation of an accommodating polymer matrix and requiring fine-tuning between a hydrophobic mediator and a hydrophilic enzyme, and is not avoidable.

DET is a so-called mediatorless approach (Fig. 1(B)) and overcomes the disadvantages of MET partly because the electron at FAD does not come *via* a mediator having the specific redox potential but is sent directly to the electrode. Sode and coworkers reported DET of FAD-GDH from *Burkholderia cepacia* where a cytochrome *c* subunit in this enzyme can configure DET from FAD to heme domein in the cytochrome *c*.[11,12] However, the utilization of this system is limited. Our groups have reported DET between FAD-GDH and a single-walled carbon nanotube (CNT).[1,2] A nanometer-scale CNT can be close to a cofactor FAD of GDH deeply embedded in a structurally rigid glycoprotein shell within the distance for DET.

† To whom correspondence should be addressed.
E-mail: muguruma@shibaura-it.ac.jp

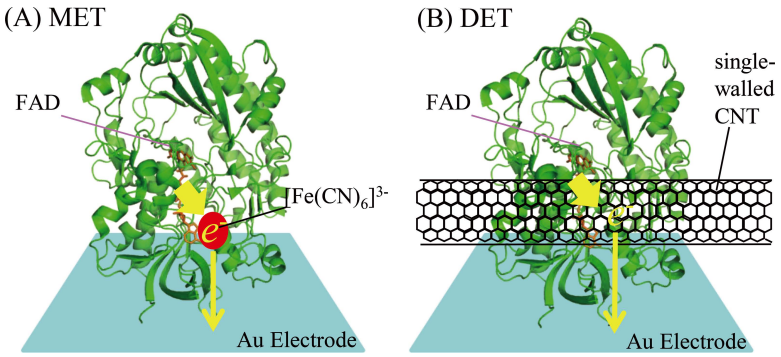

Fig. 1 (A) MET and (B) DET model between FAD-GDH and Au electrode. The diameter of the single-walled CNT is 1.2 nm. The three-dimensional structure of FAD-GDH was drawn with PyMOL (Schrödinger, LLC. The PyMOL Molecular Graphics System, Ver. 1.7. https://www.pymol.org/citing) using the PDB file 4YNT of A. flavus FAD-GDH.[19]

On the other hand, DET in another flavoenzyme, FAD-GOD, has been mainly reported by Willner's group,[21-24] where wiring or plugging with reconstruction of apo-GOD was conducted to achieve distances close to that required for DET. Another feasible approach was the combination of protein engineering and binding nanoparticles.[25,26] However, the reported DET procedures in FAD-GOD still involve some problems: the elimination of oxygen or non-DET active GOD is required to achieve maximum performance (a large turnover rate); the discussion for the achievement of DET is controversial.[27-30] In practical use, FAD-GOD has substantial drawbacks in that its enzyme-catalytic reaction depends strongly on the concentration of dissolved oxygen and the generated hydrogen peroxide is harmful in biocathode enzymes in biofuel cells.[17]

We adopted the combination of oxygen-insensitive FAD-GDH and a single-walled CNT for DET because it is a simple procedure and can be applied to any kind of FAD-GDH.[1,2] We adopted $K_3[Fe(CN)_6]$ as an electron transfer mediator for MET because it is low cost, highly water-soluble, highly stable, and widely used in commercial blood sugar biosensors.[31] The advantage of this system is that the oxygen-insensitive GDH can distinguish the effects of DET and MET because it can be controlled with and without CNT and $K_3[Fe(CN)_6]$. Herein, a comparative study between DET and MET for biosensor and biofuel cell applications is conducted for the first time. A similar study has been reported on cellobiose dehydrogenase. However, DET in cellobiose dehydrogenase is heme domein, which exists inherently in the enzyme system,[32,33] and so it cannot be extended.

The other issue of this report is substrate specificity of the novel fungal FAD-GDH, which has much less catalytic activity to xylose (monosaccharide) compared with the conventionally used FAD-GDH.[34,35] Therefore, we demonstrate that our DET electrode avoids an overlapped bias of the current toward glucose due to xylose.

Experimental

Reagents

D-Glucose, L-ascorbic acid, uric acid, acetaminophen, xylose, potassium dihydrogenphosphate, disodium hydrogenphosphate, and sodium cholate were purchased from Kanto Chemical Co., Inc. (Tokyo, Japan). Single-walled CNT (1.2 – 1.7 nm diameter, 0.1 – 4 µm long, SuperPureTubes) was purchased from Nanointegris (Boisbrin, Canada). $K_3[Fe(CN)_6]$ and carboxymethylcellulose (CMC) was purchased from Aldrich (St. Louis, USA). The novel FAD-GDH was produced by the species of a eukaryote fungus and was purified with our procedure.[34,35]

Electrode preparation

The electrode was formed on sputtered Au on plastic (PET) film. The area of the opening for the working electrode was 9 mm^2 (3 × 3 mm). For the DET electrode, a CNT dispersed solution was prepared with 0.15% (w/v) CNT and 2.0% (w/v) sodium cholate in water. Next, 3 – 5 µL aliquots of the CNT solutions were dropped onto the Au surface and dried. Then, 3 – 10 µL aliquots of enzyme (100 U) in phosphate buffer (40 mM, pH 7.4) were dropped onto the surface. The actual composition of supporting salt is the mixture of potassium dihydrogenphosphate and disodium hydrogenphosphate. Finally, 5 µL aliquots of 1.0% CMC solution were dropped onto the surface. The CMC layer plays a role as an enzyme immobilized matrix, which prevents the enzyme from leaching. For the MET electrode, the same procedure as for DET without CNT was conducted and 100 mM $K_3[Fe(CN)_6]$ was added in solution.

Measurement

Electrochemical measurements were performed using an electrochemical analyzer (701A, ALS Instruments, West Lafayette, USA) with a three-electrode configuration. The reference (Ag/AgCl, RE-1C) and counter (platinum wire) electrodes were purchased from BAS Co., Ltd. (Tokyo, Japan). Electrochemical measurements were conducted in a 5-mL vessel at ambient temperature (20°C) using a phosphate buffer (40 mM, pH 7.4) as the supporting electrolyte. Stock glucose solutions of 250 mM were successively added to prepare samples with designated concentrations. For the sake of brevity, "CMC" will be omitted from the electrodes' abbreviated names. The DET electrode will be denoted as GDH/CNT/Au and the MET electrode will be denoted as $K_3[Fe(CN)_6]$/GDH/Au where $K_3[Fe(CN)_6]$ is not immobilized.

Results and Discussion

Comparison of DET and MET for biosensor

Cyclic voltammetry (CV) is a useful tool for fundamental evaluation of the electrode. Cyclic voltammograms ranging

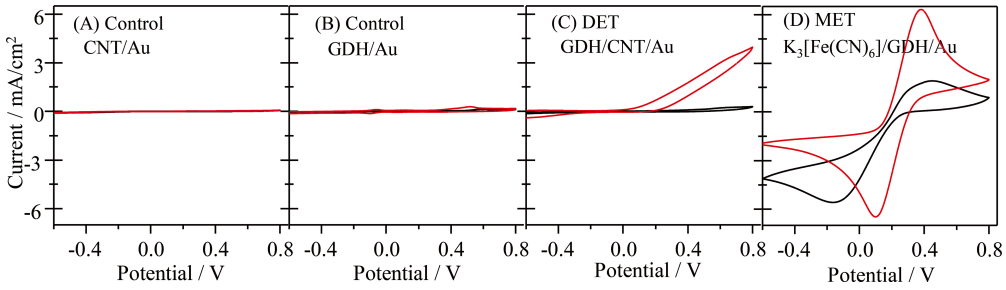

Fig. 2 CV profiles of (A) CNT/Au (control), (B) GDH/Au (control), (C) GDH/CNT/Au (DET), and (D) $K_3[Fe(CN)_6]$/GDH/Au electrodes (MET). Concentration of $K_3[Fe(CN)_6]$ in MET is 100 mM. Sweep rate: 50 mV s^{-1}. Glucose concentrations: 0 (black) and 48 (red) mM. pH 7.4, 40 mM, phosphate buffer solution.

from –0.80 to +0.80 V (*vs*. Ag/AgCl) of the fabricated control, DET, and MET electrodes are shown in Fig. 2. In the control electrodes (CNT/Au, Fig. 2(A) and GDH/Au, Fig. 2(B)), no glucose-concentration-dependent current (GCDC) is observed. DET occurred in the combination of CNT and GDH (Fig. 2(C)). MET occurred in the combination of $K_3[Fe(CN)_6]$ and GDH (Fig. 2(D)). This electrode structure involving FAD-GDH can separate DET and MET without oxygen elimination, unlike FAD-GOD, and therefore is suitable for a comparative study of DET and MET.

In a DET electrode (Fig. 2(C)), the GCDC due to the increase of the polarized potential increases steeply and the oxidative current starts at around +0.1 V (onset potential). The GCDC at +0.4 – 0.8 V is not due to hydrogen peroxide because oxygen-insensitive FAD-GDH does not produce hydrogen peroxide. The origin of GCDC due to enzymatic reaction is represented as follows:

$$\beta\text{-D-Glucose} + \text{GDH(FAD)} \longrightarrow \text{D-glucono-1,5-lactone} + \text{GDH(FADH}_2\text{)}, \quad (1)$$

In the case of DET, the electron at FAD is directly transported to CNT as follows:

$$\text{GDH(FADH}_2\text{)} \longrightarrow \text{GDH(FAD)} + 2H^+ + 2e^-. \quad (2)$$

Therefore, the observation of a large GCDC at +0.4 – 0.8 V is evidence of DET between FAD-GDH and CNT. The reason why GCDC did not appear at –0.20 V (redox potential of FAD)[36] but at +0.4 – 0.8 V is due to the overpotential (η); CNT did not attach to FAD directly but CNT is close to the electron tunneling distance. As a consequence, at least η = +0.30 V of overpotential is required for DET.[1] The CV profile of a novel fungal FAD-GDH/CNT electrode is similar to that with the conventionally used FAD-GDH counterpart, suggesting that the novel fungal FAD-GDH has a similar structure to the conventionally used FAD-GDH, and so the DET model in Fig. 1(B) is valid.

In the MET electrode (Fig. 2(D)), the large GCDC is also observed. Unlike the case of DET, the distinct redox peak pairs are observed, which is a specific characteristic for MET. MET between FAD-GDH and an electron transfer mediator after the enzymatic reaction of Eq. (1) is represented by:

$$\text{GDH(FADH}_2\text{)} + 2[Fe(CN)_6]^{3-} \longrightarrow \text{GDH(FAD)} + 2[Fe(CN)_6]^{4-} + 2H^+, \quad (3)$$

$$[Fe(CN)_6]^{4-} \longrightarrow [Fe(CN)_6]^{3-} + e^-. \quad (4)$$

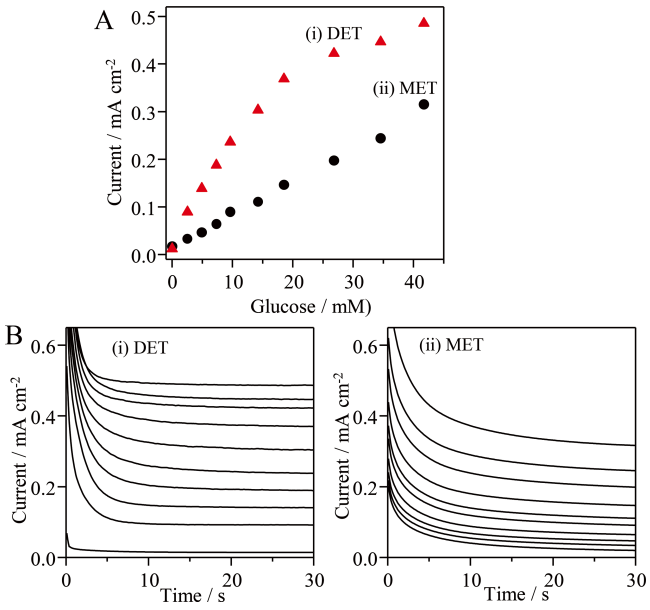

Fig. 3 (A) Glucose concentration *vs*. current of CA after 30 s for (i) DET and (ii) MET electrodes. (B) CA profiles of (i) DET and (ii) MET electrodes. Glucose concentrations of 0, 2.5, 4.9, 7.3, 9.6, 14, 19, 26, 34, 41, and 48 mM. The polarized potential was (i) +0.45 V and (ii) +0.35 V *vs*. Ag/AgCl with an electrolyte of pH 7.4 40 mM phosphate buffer solution. The temperature was set at 20°C.

The redox pair peaks (+0.37/+0.11 V) correspond to the redox reaction of $[Fe(CN)_6]^{3-}/[Fe(CN)_6]^{4-}$. The control electrodes (GDH/Au) show that no GCDC is observed, indicating that MET without an electron transfer mediator does not occur and the FAD cofactor in novel fungal FAD-GDH is also buried deeply below the protein surface as in conventionally used FAD-GDH.

Chronoamperometry (CA) is adopted for comparison of sensing performance between DET and MET because this method is used in most commercial blood sugar sensing devices. One of the important parameters is the value of a polarized potential. Figure 3A shows the measured current with CA of DET and MET due to glucose addition. In both electrodes, the quantitative current depending on the glucose concentration at a physiological level is observed. In DET, the current increases while polarized potential increases, which is also consistent with the CV trace in Fig. 2(C). Therefore, the higher the potential, the higher the sensitivity. However, the higher

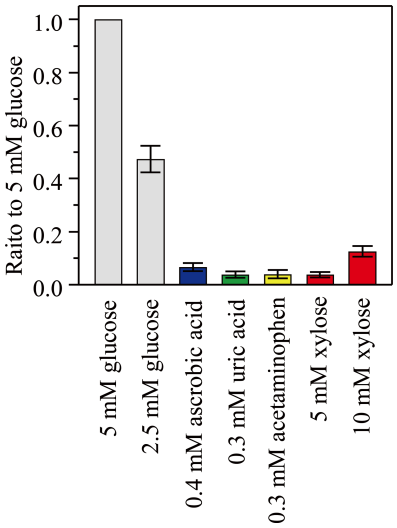

Fig. 4 Selectivity of various interfering agents in a DET electrode. The polarized potential was +0.45 V.

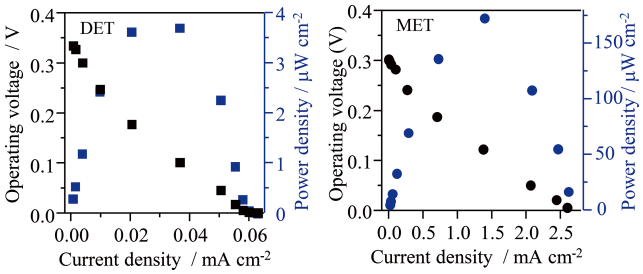

Fig. 5 Polarization curve (black dots) and output power (blue dots) of a biofuel cell with a DET and MET anode of GDH at 20°C. Platinum wire was used as the cathode. Glucose concentration is 19 mM. pH 7.4, 40 mM, phosphate buffer solution.

potential is affected by interfering agents such as ascorbic acid, uric acid, and acetaminophen, which are electrochemically active materials (easily oxidized). In a word, the sensitivity and the selectivity are trade-offs. In this case, we adopted +0.45 V in DET because it is the minimum potential that surpasses the current-peak potential (+0.35 V) in MET (discussed in the following).

Figure 3B shows the time course of CA for the DET and MET electrodes at +0.45 and +0.35 V, respectively. The potential was applied after glucose addition and the enzymatic reaction rapidly reached equilibrium. Then, the response current obeys the Cottrell equation where it is proportional to the glucose concentration. The typical profiles are an initial decay current until a steady plateau is reached. To be noted is that the time to reach the steady plateau in the DET electrode is shorter than that in MET. This is probably because the current response in the DET system was faster than that in the MET system due to the mass transfer of the mediator. The rapid response time is one of the advantages of the DET electrode.

The polarized potential at DET (+0.45 V *vs*. Ag/AgCl) sensing may be disadvantageous mainly because of the coexistence of interfering agents such as ascorbic acid, uric acid, and acetaminophen, which are electrochemically active materials (easily oxidized around at +0.2 V) in the physiological range. The larger the potential, the easier the interfering agents are oxidized at an electrode. As a consequence, the measurement current contains an incorrect positive bias. However, Fig. 4 and Fig. S1 (Supporting Information) show that the present DET biosensor is not affected by glucose detection where the current due to ascorbic acid, uric acid, or acetaminophen is less than 5% compared to that due to glucose at a physiological level. This is attributed to the large glucose response current with a GDH/CNT/Au electrode. It is concluded that DET constructed using a single-walled CNT exhibited suitable biosensor performance similar to its commercially used MET counterpart. Furthermore, this system overcomes the disadvantage of an MET system, which suffers from a reduction sensitivity in the case of long-term storage.

Substrate specificity of novel fungal FAD-GDH

Oxygen-insensitive GDH has been increasingly used in commercial biosensors for self-monitoring of blood glucose.[31] One problem with FAD-GDH is that it catalyzes xylose (monosaccharide) to almost the same level as glucose. Patients may receive the xylose as part of a clinical test. Diabetes patients may give themselves too high a dose of insulin due to appraisal of a blood sample containing glucose and xylose, resulting in hypoglycemia. The novel fungal FAD-GDH shows a lower response to xylose than the conventionally used FAD-GDH from *Aspergillus species* (Figs. S2 and S3, Supporting Information). The novel fungal FAD-GDH also shows a lower response to maltose (Fig. S2). In Fig. 4, the GDH/CNT/Au electrode shows a less than 5% response for 5 mM xylose compared to 5 mM glucose. This is a similar result to the electrode for the MET system with *Aspergillus niger* FAD-GDH, which was recombinantly produced by *Escherichia coli* and exhibited a small response to xylose.[3] Therefore, this is the first report of glucose selectivity toward xylose in a DET system. However, *A. niger* FAD-GDH is a recombinant product expressed by the prokaryote bacteria *E. coli.*, which has no ability to be post-translationally modified. Thus, it is mainly expressed as inactive inclusion bodies, which are difficult to fold correctly. Since our novel FAD-GDH is from a eukaryote fungus and is glycosylated, an active and soluble-form FAD-GDH can be efficiently produced. Glycosylation by fungus stabilized FAD-GDH against heat and pH was demonstrated previously.[2,34,35] Therefore, in addition to being able to be produced efficiently, our novel glycosylated FAD-GDH is stable in harsh conditions.

Comparison of DET and MET for biofuel cells

Here, the performance of the GDH bioanodes was tested for biofuel cell application. A platinum electrode was used as a cathode to eliminate limitations from the cathode. Figure 5 shows the polarization curve and power output of a biofuel cell constructed from DET and MET electrodes as an anode. This shows that both DET and MET electrodes can be applied to an anode of biofuel cells. The output voltage of a biofuel cell is determined by the redox potential difference between an anode and a cathode. In this case, this value depends on only the anode because of eliminating limitations from the cathode. The fact that the current density of the MET anode is larger than that of the DET electrode also corresponds to the fact that the current at +0.3 – 0.4 V in the MET biosensor is larger than that in DET (Figs. 2(C) and 2(D)). The maximum output power of MET (170 μW/cm^2) is superior to that with a conventionally used FAD-GDH anode.[14,17,18]

Conclusions

DET and MET in the enzyme electrodes with oxygen-insensitive novel FAD-GDH from fungi are compared for the first time. DET is achieved by placing a single-walled CNT between GDH and a flat gold electrode where the CNT is close to FAD within the distance for DET. MET is induced by a free electron transfer mediator, potassium hexacyanoferrate, shuttling electrons from FAD to the electron collector. Electrochemical measurements showed that (i) the response in DET is more rapid than that in MET; (ii) the current at above +0.45 V in DET is larger than the current at the current-peak potential in MET; (iii) a DET electrode covers the glucose concentration range for clinical requirements and is not susceptible to interfering agents at +0.45 V; and (iv) a DET electrode with the novel fungal FAD-GDH does not affect sensing accuracy in the presence of up to 5 mM xylose. Therefore, a DET electrode shows suitable biosensor performance similar to its commercially used MET counterpart. Furthermore, the system overcomes the disadvantages of the MET system. Both DET and MET electrodes can be applied to the anode of biofuel cells. The presented work will be a significant contribution toward development of high-end electrochemical biosensors and biofuel cells.

Supporting Information

CA of DET electrodes for various materials, CV of DET electrode with novel fungal FAD-GDH and conventionally used FAD-GDH, and glucose or xylose concentration *vs.* current of CV in a DET electrode. This material is available free of charge on the Web at http://www.jsac.or.jp/analsci/.

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