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D-fructose detection based on the direct heterogeneous electron transfer reaction of fructose dehydrogenase adsorbed onto multi-walled carbon nanotubes synthesized on platinum electrode

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Abstract

Multi-walled carbon nanotubes (MWCNTs) were synthesized on platinum plate electrodes by the chemical vapor deposition (CVD) method. From the results of X-ray photoelectron spectroscopy and voltammetric investigation, the iron nanoparticles used as a catalyst for the MWCNT synthesis were enclosed with MWCNTs. The MWCNTs synthesized on the Pt plate (MWCNTs/Pt) electrode were immediately immersed into solutions of D-fructose dehydrogenase (FDH) to immobilize the enzyme onto the MWCNTs/Pt electrode surfaces. After the FDH was immobilized onto the MWCNTs/Pt electrode, a well-defined catalytic oxidation current based on FDH was observed from ca. -0.15 V (vs. Ag/AgCl/sat’d KCl), which was close to the redox potential of heme c as a prosthetic group of FDH. From an analysis of a plot of the catalytic current vs. substrate, the calibration range for the fructose concentration was up to ca. 40 mmol dm\(^{-3}\), and the apparent Michaelis-Menten constant was evaluated to be 11 (± 1) mmol dm\(^{-3}\).

Keywords

Fructose, Fructose dehydrogenase, Electron transfer reaction, Catalytic current, Carbon nanotube
Introduction

The increasing interest in direct electron transfer reaction type bioelectrocatalysis is driven by its important applications as biosensors, biofuel cells and bioreactors. The first direct electron transfer reactions of proteins were performed by the Hill (Eddowes and Hill, 1977), Kuwana (Yeh and Kuwana, 1977) and Niki (Niki et al., 1979) groups, in which the reversible cyclic voltammetric responses of cytochrome c and cytochrome c₃ were reported. Recently, direct heterogeneous electron transfer reactions between enzymes and electrodes have also been reported. Direct electron transfer reactions on electrodes were achieved for heme-containing enzymes such as cytochrome oxidases (Cullison et al., 1994; Burgess et al., 1998), cellobiose dehydrogenase (Gorton et al., 1999; Lindgren et al., 2001; Stoica et al, 2005 and 2006; Harreither 2007), theophylline oxidase (Christenson et al., 2004), alcohol dehydrogenase (Gorton et al., 1999; Ikeda et al., 1993; Ramanavicius et al., 1999; Razumine et al., 2001), sulfite oxidase (Ferapontova et al., 2003) and peroxidase (Gorton et al., 1999; Ferapontova et al., 2001; Andreu et al., 2007), as well as for multicopper enzymes such as laccase (Gorton et al., 1999; Shleev and Tkac et al., 2005; Gupta et al., 2004; Pita et al., 2006), ascorbate oxidase (Gorton et al., 1999; Shleev Tkac et al., 2005), bilirubin oxidase (Shleev and Tkac et al., 2005; Tsujimura et al. 2004; Tsujimura et al., 2005; Shleev et al., 2004; Shleev and Wilkolazka et al., 2005; Zheng et al., 2006; Tominaga et al. 2006), ceruloplasmin (Shleev and Tkac et al., 2005) and Cu efflux oxidase (Miura et al., 2007). It was also reported that the direct electron transfer reactions of glucose oxidase at highly-oriented pyrolytic graphite and at carbon nanotubes (Patolsky et al., 2004; Gong et al., 2005; Wang et al 2005; Gooding, 2005; Ivnitski et al., 2006; Banks et al., 2006; Gooding et al., 2003; Zhang et al., 2007; Liu et al., 2007; Tominaga et al., in press), and superoxide dismutase (Gorton et al., 1999; Tian et
al., 2004) were achieved. In addition, various functionalized electrodes were developed for protein electrochemistry such as self-assembled monolayer-modified electrodes (Taniguchi et al., 1982; Nuzzo and Allara, 1983; Troughton et al., 1988; Porter et al., 1987; Chidsey, 1991; Tarlov Bowden, 1991; Feng et al., 1997; Kepley et al., 1992; Terrettaz et al., 1996; Sigal et al., 1998; Tominaga et al., 2007) and lipid film-modified electrodes (Rusling, 1998; Bianco and Haladjian, 1997; Ferri et al. 1998; Tominaga et al., 1999) have been developed. Although such huge efforts have been paid to achieve a direct electron transfer reaction on an electrode, success in this area remains still limited, because the redox center of the enzyme is buried deeply within the protein shell (Armstrong and Wilson, 2000; Barton et al., 2004; Armstrong and Hill, 1998; Ikeda, 2003). Therefore, shortening the electron transfer distance between the active center of the enzyme and the electrode is one of the most important key requirements. Carbon nanotubes (CNTs) could communicate with redox centers buried deep within protein shells as molecular wires, because of their small diameters and conductivities (Wiles and Abrahamson, 1978; Iijima, 1991; Ajayan, 1999; Li et al., 2001; Tominaga et al., 2004). CNTs are described as a graphite sheet rolled up into a nanoscale-tube (single-walled CNTs), and with additional graphene tubes around the core of single-walled CNT (multi-walled CNTs). These CNTs have diameters in the range between fractions of nanometers and tens of nanometers and lengths up to several centimeters with both their ends normally capped by fullerene-like structures.

In the present study, the direct electron transfer reactions of D-fructose dehydrogenase (FDH) immobilized onto CNTs were investigated. FDH is a membrane-bound enzyme with a molecular weight of ca. 140 kDa containing flavin and heme c as prosthetic groups, and catalyzes the oxidation of D-fructose to 2-keto-D-fructose (Ameyama et al., 1981). This enzyme shows high
substrate specificity for D-fructose, which is expected to be utilized in the field of food analysis and for clinical use (Matsumoto et al., 1986; Prado and Sampietro, 1994; Parades et al., 1997; Bassi et al., 1998; Nakashima et al., 1985). The direct electron transfer reactions of FDH on electrode surfaces were reported in previous studies (Ferapontova and Gorton, 2005; Kamitaka et al., 2007; Tominaga et al., 2007). However, investigations on the direct electron transfer reaction on CNTs have not been reported. In this study, using multi-walled carbon nanotubes (MWCNTs) synthesized onto Pt electrodes, we demonstrated the direct electron transfer reaction of FDH, and the apparent Michaelis-Menten constant was determined. The obtained results are useful in applications to prepare the third-generation biosensors and other future bioelectrochemical devices.

2. Experimental

2.1. Materials

Horse spleen ferritin (Type I, Sigma) was purified by size exclusion chromatography (Tominaga et al., 2004). The number of iron atoms per ferritin molecule used in this study was evaluated to be approximately 3 x 10^3 atoms by inductively coupled plasma-atomic emission and atomic absorption spectroscopy. D-fructose dehydrogenase (FDH, EC 1.1.99.11, from *Gluconobacter* sp., Toyobo Co., Japan) was used as received. D-Fructose of analytical grade was used as the substrate for FDH. Water was purified with a Millipore Milli-Q water system. All other reagents used in this study were of analytical grade.

2.2. Instrumentation
The Raman spectra were observed using JASCO Raman spectrometer NRS-3100. Transmission electron microscopy (TEM) characterization was performed with a JEOL-2000FX electron microscope at an acceleration voltage of 200 kV. CNTs synthesized on a platinum plate were transferred onto a carbon-coated copper grid sample holder. X-ray photoelectron spectroscopic (XPS) measurements were then performed using a Thermo VG Scientific, Sigma Probe HA6000II. The instrument uses a focused monochromatic Al Kα X-ray (1486.68 eV) source for excitation, a spherical section analyzer, and a 6-element multichannel detection system. The binding energies were calculated on the basis of the binding energy of C(1s) (284.5 eV).

The electrochemical measurements were carried out in a three-electrode cell. Cyclic voltammetric measurements were performed in a phosphate buffer solution (µ = 0.1, pH 7.0) using a Toho Giken 2020 potentiostat with a function generator or an electrochemical analyzer (Bioanalytical Systems, BAS 100B/W). An Ag/AgCl (saturated KCl) electrode and a platinum electrode were used as the reference and counter electrodes, respectively. All potentials are reported with respect to the Ag/AgCl (saturated KCl) electrode. Prior to the cyclic voltammetric measurements, the buffer solution was deaerated with high purity argon, and a positive pressure of nitrogen was kept over the solution during all electrochemical experiments.

2.3. Carbon nanotubes synthesis on Pt plate electrode surface

The CNTs were synthesized onto a platinum plate by the chemical vapor deposition (CVD) method using iron nanoparticles derived from ferritin molecules (Tominaga et al., 2004). To prepare the iron nanoparticles as a catalyst for the CNTs, a 2 mmol dm⁻³ ferritin solution was casted onto the platinum plate electrode, and then the modified electrode was heated at 400 °C for 60 min to
eliminate the protein moiety of ferritin. The CVD growth of the carbon nanotubes was synthesized in a quartz tube (diameter: 40 mm, volume: 500 ml) equipped with temperature and gas flow controls. The prepared iron nanoparticles adsorbed onto the platinum plate were first reduced in H$_2$ for 30 min at 900 °C, and then a mixture of methane (99.999%) and H$_2$ gas was introduced into the system at a flow rate of 200 ml min$^{-1}$ methane and 200 ml min$^{-1}$ H$_2$ at 900 °C for 30 min. The synthesized CNTs were characterized by Raman spectroscopy and TEM. Two Raman shift peaks corresponding to the G- (ca. 1580 cm$^{-1}$) and D-bands (ca. 1350 cm$^{-1}$) were observed on the platinum electrode surface after the synthesis of the CNTs, which indicated that CNTs were present on the electrode surface. Fig. 1 shows a TEM image of the CNTs synthesized on the Pt plate. The diameters of individual CNTs were evaluated to be ca. 5 to 10 nm, which could be classified as multi-walled carbon nanotubes (MWCNTs). Immediately thereafter, the MWCNTs on the Pt plate (MWCNTs/Pt) electrode were immobilized into an enzyme solution to protect against surface contamination of the MWCNTs/Pt electrode in air.

2.4. Immobilization of FDH onto MWCNTs/Pt electrodes

A MWCNTs/Pt electrode was immersed into a phosphate solution (pH 5, µ = 0.1) containing 1200 units ml$^{-1}$ FDH for 1 min, and then rinsed with phosphate solution. After the immobilization procedure, XPS measurements were carried out. Fig. 2A shows the N(1s) peak region. A N(1s) peak at ca. 400.5 eV was observed after the MWCNTs/Pt electrodes were immersed into the FDH solution for 1 min, which was not observed before the immersion. Furthermore, C(1s) peaks at ca. 287 and 289 eV corresponding to C-N and C-O functional groups, respectively, were observed after the enzyme modification (Moulder et al., 1995), as shown in Fig. 2B. These results clearly
indicated that FDH was immobilized onto the MWCNTs/Pt electrode surface. The polished Pt plate electrode was also modified with FDH by the same procedure of MWCNTs/Pt electrode.

3. Results and Discussion

3.1. Characterization of MWCNTs/Pt electrode surfaces

Iron and its oxidized species were present on the MWCNTs/Pt electrode as an electrocatalyst for CNT synthesis. Such nanometallic impurities influence the electrochemical responses, because electrochemical techniques are highly sensitive to surface conditions (Banks et al., 2006). Thus, we should investigate the effect of the iron nanoparticles on the electrochemical response. First, XPS measurements were carried out. Fig. 3A shows the Fe(2s) peak region for MWCNTs/Pt electrode surfaces. Before the MWCNTs synthesis, XPS peaks around 712 and 725 eV corresponding to Fe(2p) were observed (Moulder et al., 1995). However, these Fe(2p) peaks could not be detected after the MWCNT synthesis. To clarify that the iron nanoparticles were present on the surface, the MWCNTs on the electrode were eliminated by heat-treatment at 900 °C for 60 min. This elimination was confirmed by Raman spectroscopy, because the characterised G- and D-band peaks corresponding to the MWCNTs were not detected after the heat-treatment. After this process, peaks corresponding to Fe(2p) were observed again. These facts indicated that iron nanoparticles were present on the electrode surface, but the nanoparticle surface was enclosed with MWCNTs. The cyclic voltammetric investigation also supports such a hypothesis. A peak based on iron impurity species could not be detected on the MWCNTs/Pt electrode in a phosphate buffer (pH 7) solution, indicating that the iron nanoparticle surfaces were electrochemically inactive, because the nanoparticle surface was enclosed with MWCNTs. Fig. 3B shows the Pt(4f) peak region for the
MWCNTs/Pt electrode surfaces. The peaks at 71.3 and 74.6 eV corresponding to Pt(4f) were observed, even after the MWCNT synthesis. These results indicated that part of the Pt electrode surface was exposed. In other words, the surface density of the MWCNTs was not sufficient to cover the entire surface.

3.2. Voltammetric studies on FDH immobilized onto MWCNTs/Pt electrodes

Fig. 4 shows typical cyclic voltammograms at Pt, MWCNTs/Pt, FDH immobilized Pt, and FDH immobilized MWCNTs/Pt electrodes in the presence and absence of fructose. No catalytic current was observed at the Pt, MWCNTs/Pt and FDH immobilized Pt electrodes in the potential region of -0.2 to 0.6 V in the presence of fructose. In contrast, a well-defined catalytic oxidation current was observed from ca. -0.15 V at the FDH immobilized MWCNTs/Pt electrode, as shown in Fig. 4d, which was close to the redox potential of heme c as a prosthetic group of FDH. In our previous report, direct heterogeneous electron transfer reactions of FDH adsorbed onto a HOPG electrode were also observed from about -0.15 V (Kamitaka et al., 2007; Tominaga et al., 2007), although the catalytic current density was ca. fifty times smaller than that in the case of MWCNT/Pt. Thus, in the case of FDH immobilized onto a MWCNTs/Pt electrode, the electron transfer reactions would pass between the MWCNTs/Pt electrode and FDH, which was similar to the case of FDH adsorbed onto HOPG. In other words, fructose was oxidized at the flavin site, and the intramolecular electron transfer took place from the flavin site to the heme site (Tominaga et al., 2007). Thus, the direct electron transfer reaction of FDH at the electrode surface occurred at the heme c-containing subunit, as shown in the following equation (1):

\[
\text{FDH (FAD-oxidized heme c)} + \text{D-fructose} \rightarrow \text{FDH (FADH}_{2}\text{-oxidized heme c)} + \text{2-keto-D-fructose}
\]

\[
\text{FDH (FADH}_{2}\text{-oxidized heme c)} \rightarrow \text{FDH (FAD-reduced heme c)}
\]

\[
\text{FDH (FAD-reduced heme c)} \rightarrow \text{FDH (FAD-reduced heme c)} + e^- + H^+
\]

\[
\text{FDH (FAD-reduced heme c)} \rightarrow 9 \text{FDH (FAD- oxidized heme c)} + e^- + H^+
\]
where FAD, FADH and FADH$_2$ are oxidized, semiquinone and the reduced form of flavin adenine dinucleotide in FDH, respectively. Oxidized- and reduced-heme c are the oxidized and reduced forms of heme c in FDH, respectively.

Fig. 5 shows the dependence of the catalytic oxidation current observed at the FDH immobilized MWCNTs/Pt electrode on the fructose concentration. The inset shows a linear plot of the catalytic peak currents versus the fructose concentration. The calibration range for the fructose concentration was up to ca. 40 mmol dm$^{-3}$, and the detection limit was ca. 5 mmol dm$^{-3}$. No significant change in the catalytic oxidation current was observed while keeping the electrode in the buffer solution at ca. 20 °C for at least 3 days. At high fructose concentrations, a platform response was observed, indicating that this was a characteristic Michaelis-Menten kinetic mechanism. The apparent Michaelis-Menten constant was determined by an analysis of the Lineweaver-Burke equation (Kamin and Wilson, 1980), as shown in the following equation (2):

$$\frac{1}{I} = \frac{1}{I_{\text{max}}} + \frac{K_m c}{I_{\text{max}}}$$

(2)

where $K_m$ is the apparent Michaelis-Menten constant for D-fructose including a factor representing the mass transfer of the substrate, $c$ is the bulk concentration of D-fructose, $I$ is the...
obtained current after the addition of the substrate, and $I_{\text{max}}$ is the maximum current. The $K_m$ value was determined to be 11 (± 1) mmol dm$^{-3}$, which is almost the same as that obtained in the case of an enzymatic reaction in bulk solution (pH 4.5), 10 mmol dm$^{-3}$ (Ameyama et al., 1981). This suggests that in this study, the concentration depression of the substrate is negligible. This result would come from the low surface concentration of FDH immobilized onto the electrode surface. There are two possibilities for reason. First, it would be due to the low surface density of MWCNTs, as described in the above section. Second, it would be due to that FDH immobilization onto the MWCNTs would be inhibited in comparison with other carbon materials.

4. Conclusions

MWCNTs of 5-10 nm diameter were synthesized on a platinum plate electrode by the CVD method using iron nanoparticles as a catalyst. From the results of XPS measurements, iron nanoparticles were enclosed with the MWCNTs, and did not influence the electrochemical response. FDH immobilized onto the MWCNTs/Pt electrode surfaces showed a heterogeneous direct electron transfer reaction. From the dependence of the catalytic oxidation current at the FDH immobilized MWCNTs/Pt electrode on the fructose concentration, the calibration range for the fructose concentration was up to $ca.$ 40 mmol dm$^{-3}$. The apparent Michaelis-Menten constant was determined to be 11 (± 1) mmol dm$^{-3}$.

Acknowledgements
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References


**Figure Captions**

Fig. 1. TEM image for MWCNTs grown on a Pt electrode surface.

Fig. 2. XPS spectra in the N(1s) (A) and C(1s) (B) regions for FDH immobilized MWCNTs/Pt (FDH/MWCNTs/Pt) (a), MWCNTs/Pt (b) and Pt (c) electrode surfaces.

Fig. 3. XPS spectra in the Fe(2p) and Pt(4f) regions for the iron nanoparticle modified Pt electrode surface before (a) and after (b) MWCNTs synthesis, and the surface of the MWCNTs/Pt electrode after heat-treatment at 900 °C for 60 min (c).

Fig. 4. Typical cyclic voltammograms at bare Pt (a), MWCNTs/Pt (b), FDH immobilized Pt (c) and FDH immobilized MWCNTs/Pt (d) electrodes in a phosphate solution (pH 5) in the presence (solid
lines) and absence (broken lines) of 10 mmol dm$^{-3}$ fructose. Potential sweep rate: 5 mV s$^{-1}$. Electrode area: 0.25 cm$^2$.

Fig. 5. Catalytic peak currents as a function of the fructose concentration. The peak currents were observed at a FDH immobilized MWCNTs/Pt electrode in a phosphate solution (pH 5) at a potential sweep rate of 5 mV s$^{-1}$. Electrode area: 0.25 cm$^2$. 
Figure 2

A  N(1s)

a)  

b)  
c)  

Binding energy / eV

390 395 400 405 410

B  C(1s)

a)  

b)  
c)  

Binding energy / eV

275 280 285 290 295

C-N  
C-O  

1000 cps

Fig. 2
Figure 3

(A) Fe(2p)

- a)
- b)
- c)

(B) Pt(4f)

- a)
- b)
- c)

Binding energy / eV

Fig. 3
Figure 4

a) 

\[ \uparrow 5 \, \mu A \]

E/V vs. Ag/AgCl

b) 

\[ \uparrow 5 \, \mu A \]

E/V vs. Ag/AgCl

c) 

\[ \uparrow 5 \, \mu A \]

E/V vs. Ag/AgCl

d) 

\[ \uparrow 5 \, \mu A \]

E/V vs. Ag/AgCl

Fig. 4