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Catalytic Current Based on Direct Electron Transfer Reactions of Enzymes Immobilized onto Carbon Nanotubes

M. Tominaga, S. Kaneko, S. Nomura, S. Sakamoto, H. Yamaguchi, T. Nishimura, and I. Taniguchi

Graduate School of Science and Technology, Kumamoto University, Kumamoto 860-8555, Japan

Multi-walled and single-walled carbon nanotubes were synthesized on platinum plate (MWCNTs/Pt) and gold wire (SWCNTs/Au) electrodes, respectively, using a chemical vapor deposition (CVD) method. These carbon nanotube-modified electrodes were immersed into solutions of glucose oxidase (GOX) and D-fructose dehydrogenase (FDH) to immobilize these enzymes onto the electrode surfaces. After GOX was immobilized onto the MWCNT/Pt electrode, the well-defined catalytic oxidation current was increased from ca. -0.45 V (vs. Ag/AgCl/saturated KCl), which was close to the redox potential of flavin adenine dinucleotide as a prosthetic group of GOX under physiological pH values. Furthermore, catalytic oxidation currents of fructose based on direct heterogeneous electron transfer reactions between FDH and the SWCNT/Pt electrode were observed.

Introduction

The increasing interest in direct electron transfer reaction type bioelectrocatalysis is driven by its important applications as biosensors, biofuel cells and bioreactors. However, in general, the electron transfer rate of an enzyme on the electrode surface is generally slow, or cannot be detected due to the fact that the redox center of the enzyme is buried deep within the protein shell (1-4). Since the discovery of carbon nanotubes (CNTs) in 1991, they have been one of the most actively studied materials because of their unique properties in fundamental studies as atomically ordered materials, and as promising materials for nano-technological applications (5-9). It is thought that CNTs can communicate with the enzyme redox centers as molecular wires because of their small diameters and conductivities. In fact, direct electron transfer reactions between enzymes and CNTs have been previously reported (10-20). In this study, we observed for the first time that the direct electron transfer reaction of glucose oxidase (GOX) immobilized onto CNTs modified electrode at the redox potential of flavin adenine dinucleotide as a prosthetic group of GOX under physiological pH values. Furthermore, the effects of the surface conditions of the CNTs on direct electron transfer reactions with fructose dehydrogenase were investigated.
Experimental section

Materials

Glucose oxidase (GOX, EC 1.1.3.4, from *Aspergillus niger*, 12 units mg\(^{-1}\), Tokyo Chemical Ind. Co., Japan) and D-fructose dehydrogenase (FDH, EC 1.1.99.11, from *Gluconobacter* sp., Toyobo Co., Japan) were used as received.

Instrumentation

The electrochemical measurements were carried out in a three-electrode cell. The cyclic voltammetric measurements were performed in phosphate buffer (pH 7) and phosphate (pH 5) solutions using 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 solution was deaerated with high purity argon, and a positive pressure of argon was kept over the solution during all electrochemical experiments.

The Raman spectra were observed using a 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.

X-ray photoelectron spectroscopic (XPS) measurements were performed using a Thermo VG Scientific Sigma Probe HA6000II. This instrument uses a focused monochromatic Al K\(\alpha\) X-ray (1486.68 eV) source for excitation. The binding energies were calculated on the basis of a binding energy of C(1s) (284.5 eV).

A UV-ozone treatment system (Model OC-2503, Eye Graphics Co., Japan) was used in this study. The UV light had main emissions at 185 and 254 nm. The density at 230 ~ 280 nm (sensitivity peak: ca. 255 nm) was evaluated as 11 mW cm\(^{-2}\) by an ultraviolet ray integration luminance meter (UVPF-A1, Eye Graphics Co., Japan). The concentration of ozone was ~ 100 ppm.

Carbon nanotube (CNT) synthesis and characterization

The CNTs were synthesized onto a platinum plate by a chemical vapor deposition (CVD) method using iron nanoparticles derived from ferritin molecules (19-21). To prepare the iron nanoparticles as a catalyst for the CNTs, a 2 \(\mu\)mol dm\(^{-3}\) ferritin solution was cast 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 CNTs were also synthesized onto a gold wire using Co-Mo alloy nanoparticles as the catalyst. The CVD growth of the CNTs was synthesized in a quartz tube (inner diameter: 35 mm, length: 840 mm) equipped with temperature and gas flow controls. The prepared catalyst nanoparticles on the electrodes were first reduced in H\(_2\) for 10 min at 850 °C, and then a mixture of ethanol (99.8 %) and H\(_2\) gas was introduced into the system at a flow rate of 100 ml min\(^{-1}\) H\(_2\) at 800 °C for 10 min.

The synthesized CNTs were characterized by Raman spectroscopy, SEM and TEM. Two Raman shift peaks corresponding to the G- \((ca. 1593\text{ cm}^{-1})\) and D-bands \((ca. 1350\text{ cm}^{-1})\) were observed on the platinum plate and gold wire electrode surfaces after the synthesis of the CNTs, which indicated that CNTs were present on the electrode surface. The ratios of the G-band and D-band (G/D) were evaluated to be \(ca. 5\) and \(ca. 40\) for the
platinum and gold electrode surface, respectively, when a laser at 532 nm was used for excitation. From the results of SEM and TEM images (Figures 1 and 2), the synthesized CNTs were evaluated to be 5–10 nm in diameter on the platinum electrode surface, which could be classified as multi-walled carbon nanotubes (MWCNTs). For the CNTs on the gold wire electrode, the diameter was evaluated to be 1.3 (± 0.2) nm, and they were classified as mainly single-walled CNTs (SWCNTs) from the results of the TEM images and the radial breathing mode (RBM) of the Raman spectroscopic measurements (22, 23).

**Figure 1.** SEM image of CNTs grown on a gold wire.

**Figure 2.** TEM images of CNTs grown on a platinum plate (a) and a gold wire (b).

**Immobilization of enzymes**

The MWCNT/Pt electrode was immersed into a phosphate buffer (pH 7, μ = 0.1) containing 560 units ml⁻¹ GOX for 12 hours to immobilize the GOX onto its surface, and then rinsed with buffer solution to remove any free GOX. In the case of FDH, the SWCNT/Au 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 showed the N(1s) peak at 400.5 eV for both GOX and FDH immobilized onto MWCNT/Pt and SWCNT/Au electrodes, respectively. The N(1s) peak was not observed before this modification. Furthermore, C(1s) peaks at ca. 289 and 287 eV corresponding to the C-N and C-O functional groups, respectively, were observed after the enzyme modification (24). These results clearly indicated that GOX and FDH were immobilized onto the MWCNT/Pt and SWCNT/Au electrode surfaces.

**Results and Discussion**

**Catalytic oxidation current based on the direct electron transfer reactions of GOX immobilized onto MWCNT/Pt electrodes**

Figure 3a shows typical cyclic voltammograms at GOX immobilized onto the MWCNTs/Pt electrode in the presence of glucose. A well-defined catalytic oxidation current was observed from ca. -0.45 V (vs. Ag/AgCl/saturated KCl). Such a catalytic oxidation current was not observed at the MWCNT/Pt or Pt electrodes. Furthermore, substrate selectivity was observed: no catalytic oxidation current was observed in the presence of mannose or galactose. The potential by which the catalytic current increased was close to the redox potential of flavin adenine dinucleotide (FAD) as a prosthetic group of GOX under physiological pH values. Thus, the catalytic oxidation current would be due to the direct electron transfer reaction of GOX and the MWCNTs/Pt electrode, as shown in the following equation (Eq. 1):

\[
\text{GOX (FAD)} + \text{glucose} \rightarrow \text{GOX (FADH}_2) + \text{gluconolactone} \\
\text{GOX (FADH}_2) \rightarrow \text{GOX (FAD)} + 2e^- + 2H^+ 
\]

[1]

where GOX(FAD) is GOX with the oxidized form of FAD and GOX(FADH\(_2\)) is GOX with the reduced form of FAD.

![Figure 3](image-url)

**Figure 3.** Cyclic voltammograms at GOX immobilized onto MWCNT/Pt electrodes in phosphate buffer (pH 7) in the presence of 5 mmol dm\(^{-3}\) glucose at 30 (a), 140 (b) and 270 (c) hours after GOX immobilization when the modified electrodes were stored in a
phosphate buffer solution at 10 °C. Potential sweep rate: 5 mV s⁻¹. Electrode area: 0.25 cm².

The direct electron transfer reactions of GOX at CNT-modified electrodes have been reported in previous papers (11,13). However, a catalytic glucose oxidation current increasing to near the redox potential of FAD, like in this study, has not been reported previously. Figures 3b and c show the cyclic voltammograms at GOX immobilized MWCNT/Pt electrode in the presence of glucose at 30, 140 and 170 hours after the GOX modification when the modified electrode was kept in a phosphate buffer solution at 10 °C. Figure 4 shows a plot of the time course of the catalytic glucose oxidation current observed in the voltammograms. The oxidation current gradually decreased, and eventually no catalytic oxidation current was observed at ca. 1 week after the GOX modification.

![Figure 4](image)

**Figure 4.** Time course of the catalytic peak currents of glucose oxidation obtained from the cyclic voltammograms at the GOX immobilized onto MWCNT/Pt electrodes in phosphate buffer (pH 7) in the presence of 5 mmol dm⁻³ glucose, when the modified electrodes were stored in a phosphate buffer solution at 10 °C. Potential sweep rate: 5 mV s⁻¹. Electrode area: 0.25 cm².

Catalytic oxidation current based on the direct electron transfer reactions of FDH immobilized onto UV-ozone-treated SWCNT/Au electrodes

To induce surface structural defects onto the CNTs (25, 26), UV-ozone treatment was performed for 1 and 5 min. The XPS results of the UV-ozone treated SWCNT/Au electrode in the C(1s) region showed peaks corresponding to C-C (ca. 285 eV), and other oxidized carbon species such as C-O, C=O and O-C=O (ca. 286-290 eV) were observed (27). The peak area ratios of the oxidized carbon species to the total carbon species were evaluated to be ca. 10, 20-30 and ca. 60 % for UV-ozone exposure for 0, 1 and 5 min, respectively. These results indicated that surface structural defects of the CNTs were caused by the UV-ozone treatment. The Raman spectroscopic results also supported this hypothesis. The G/D ratio decreased with the processing time of the UV-ozone treatment: G/D = 40, 10 and 5 for 0, 1 and 5 min, respectively.

Figure 5 show typical cyclic voltammograms at FDH immobilized and unimmobilized onto SWCNT/Au electrodes. Catalytic oxidation currents were observed from ca. -0.1 V in a phosphate solution (pH 5) in the presence of 0.1 mol dm⁻³ fructose. On the other hand, no catalytic oxidation current was observed at the FDH
unimmobilized SWCNT/Au electrode. These results indicate that the observed catalytic oxidation current was due to direct electron transfer reactions based on the FDH immobilized onto the SWCNT/Au electrode.

Figure 5. Cyclic voltammograms at UV-ozone treated (0 (a), 1 (b) and 5 (c) min) SWCNT/Au electrodes modified with FDH in phosphate solution (pH 5) in the presence (solid line) and absence (broken line) of 0.1 mol dm$^{-3}$ fructose. Potential sweep rate: 5 mV s$^{-1}$. Electrode area: 0.25 cm$^2$.

Although a catalytic oxidation current was observed at both UV-ozone-treated and -untreated SWCNT/Au electrodes, the highest oxidation current was observed at the SWCNT/Au electrode treated for 1 min. To explain this observation, we evaluated the surface excess of FDH on the SWCNT/Au electrode by fluorescent measurements. The surface excess was evaluated to be $4.5 \pm 1.0 \times 10^{-11}$ mol cm$^{-2}$ (apparent surface area), which did not depend on the UV-ozone treatment time. These results indicate that the difference in the catalytic current values was not due to the surface excess of FDH. It is well known that the molecular orientation of an enzyme on the electrode surface is one of the most important requirements for fast direct electron transfer reactions, because the redox center of an enzyme is buried deep within the protein shell (4). The proper orientation of FDH on an electrode surface is also important for its successful direct electron transfer reactions (28). Based on the fact that the catalytic oxidation current based on the direct electron transfer reaction of FDH increased from $ca. -0.1$ V, the direct electron transfer reaction of FDH at the electrode surface occurred at the heme c-containing subunit (29), as shown in Figure 6, which was similar to previous results (28).
The overall fructose oxidation process catalyzed by FDH can be described as follows: fructose is oxidized at the flavin site, and the intermolecular electron transfer takes place from the flavin site to the heme c site, and the direct electron transfer reaction at the SWCNT surface occurs at the heme c site. From the observation of the catalytic oxidation from -0.1 V together with the fact that the surface excess of FDH did not depend on the UV-ozone treatment time, the difference in the observed catalytic current values at the UV-ozone treated SWCNT/Au electrodes must be due to differences in the orientation of FDH on the SWCNT surface. In other words, these results indicated that the concentration of oxidized carbon species on the SWCNTs played an important role for the direct electron transfer reaction of FDH at the electrode surface.

Conclusions

MWCNTs of 5-10 nm and SWCNTs of ca. 1.3 nm in diameter were synthesized on a platinum plate and gold wire electrodes, respectively using the CVD method. Glucose oxidase (GOX) and D-fructose dehydrogenase (FDH) were immobilized onto these CNTs modified electrode surfaces, and this was confirmed by their XPS spectra. On the GOX immobilized MWCNTs/Pt electrode, a well-defined catalytic glucose oxidation current was increased from ca. -0.45 V. We concluded that the observed catalytic oxidation current was based on direct heterogeneous electron transfer reactions between GOX and the MWCNTs/Pt electrode. Furthermore, direct heterogeneous electron transfer reactions of FDH at the SWCNT/Au electrode were also observed. The concentration of oxidized carbon species on the SWCNTs played an important role for the direct electron transfer reaction of FDH at the electrode surface.

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