Self–Powered Sugar Indicator Using CNT–Enzyme Ensemble Film

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Abstract. We report the stepwise modification of Os-complex mediator (polyvinylimidazole-[Os(bipyridine)2Cl] (PVI-[Os(bpy)2Cl]) and glucose oxidase (GOD) within the inner nanospace of a carbon nanotube forest (CNTF) film. Owing to the controlled alignment of enzyme/mediator/electrode in the ensemble, the prepared film electrode has both a high-efficiency (turnover rate of ca. 650 s\(^{-1}\)) and a large net oxidation current (ca. 15 mA cm\(^{-2}\)). The previous GOD electrodes developed by monolayer-based and polymer-based approaches have either of the performances (efficiency or net activity). In addition, the present GOD electrode is a flexible film that could be used by winding on needle devices.

1. Introduction
Controlling the electrical contact of redox enzymes with electrodes is a critical issue for enzymatic biodevices such as biofuel cells and biosensors.\(^{(1-4)}\) The mutual positioning between enzyme molecules, mediator molecules (not always necessary), and electrode surface determines the efficiency, reproducibility, and stability of the bioelectrocatalysis systems. A conventional engineering for accelerating the electron transfer to the redox enzymes is the inclusive immobilization with mediator polymer matrices, in which the successive electron exchange between the neighboring mediator groups connects the enzyme redox center and the electrode surface. For example, the Os-complex-pendant polymers are successful mediating matrices for glucose oxidase (GOD) to provide glucose oxidation current at mA cm\(^{-2}\) level.\(^{(1-3)}\) However, because of random mutual positioning in the 3D composite, many enzyme molecules are often isolated from the molecular network for continuous bioelectrocatalysis. On the other hand, direct immobilization of enzyme monolayer on the electrode surface has improved the efficiency of enzyme utilization. A striking example is the reconstituting apo-GOD on a relay-FAD monolayer linked to electrode surfaces.\(^{(1,3)}\) Since all the enzyme units are oriented in an optimal position with respect to the electrode surface, a high electron-transfer turnover rate comparable to that for bulk GOD reaction (\(~\)700 s\(^{-1}\) at 25 °C) has been achieved. However, the drawback of such 2D monolayer engineering is the lower bioelectrocatalytic performance due to the limited amount of immobilized enzymes.

We present herein an enzyme/mediator/electrode ordered ensemble that shows both “high turnover rate” and “large catalytic current”. In order to satisfy both of these requirements, the larger amount of enzymes than monolayer should be immobilized with keeping effective contact with electrodes. We
realize such ideal condition by taking advantage of a film of well-aligned carbon nanotube forest (CNTF) \(^{(5)}\) consisting of single-walled CNTs arrayed with a pitch of 16 nm. The CNTF was synthesized by water-assisted chemical vapor deposition on a line-patterned Al\(_2\)O\(_3\) / Fe catalyst on silicon wafers (see Experimental for details). \(^{(5)}\) As shown in Figure 1a, the synthesized CNTF film (1.5 mm × 1 mm) was pulled from the substrate and pinched by inverse operating tweezers (electrical lead), to produce an exposed electrode geometric area of ca. 2 mm\(^2\) (sum of both faces of a 1mm × 1 mm sheet). The thickness of the CNTF films (4μm, 12μm or 20μm) was determined by the width of the line-patterns of Al\(_2\)O\(_3\) / Fe catalyst. Recently, we reported that the intraspace of the CNTF is useful for immobilization of fructose dehydrogenase and laccase, which are the direct electron transfer (DET)-type enzymes. \(^{(4)}\) Although there are a few recent reports that also GOD is capable of direct communication with electrodes, repeated attempts to prepare a workable GOD/CNTF ensemble electrode without any mediators have failed. Then we have developed the stepwise process to construct molecular architecture with polyvinylimidazole-\[Os(bipyridine)\_2Cl\] (PVI-\[Os(bpy)\_2Cl\], MW: 15000) and GOD (EC:1.1.3.4, MW: 186 kDa), as illustrated in Figure 1b and 1c. The PVI-\[Os(bpy)\_2Cl\] was synthesized according to a literature method, with a molar ratio of imidazole group to \[Os(bpy)\_2Cl\] of 5.

![Figure 1. Schematic illustration of the stepwise process for constructing bioelectrocatalytic composite inside a CNTF film.](image)

### 2. Results and discussion

#### 2.1. Adsorption of PVI-\[Os(bpy)\_2Cl\] inside CNTF films.

The CNTF film was first treated with 0.1 % Triton X-100 to be hydrophilic, and then soaked in a stirred phosphate buffer solution (PBS, pH7.0) containing 1 mg ml\(^{-1}\) PVI-\[Os(bpy)\_2Cl\] at 4 °C. As shown in Figure 2a, the cyclic voltammogram (CV) of the treated CNTF showed a symmetric shape typical for adsorbed redox species. In fact, the amplitude of peak currents were proportional to the scan rates (Figure 2b). The amount of PVI-\[Os(bpy)\_2Cl\] adsorbed within the CNTF films were estimated by integrating the CV currents and is plotted in Figure 2c against the soaking time in the 1 mg ml\(^{-1}\) PVI-\[Os(bpy)\_2Cl\] solution. The amount of PVI-\[Os(bpy)\_2Cl\] in a CNTF film increased with the soaking time and reached a maximum after 2 hours. Importantly, these values are proportional to the CNTF film thickness (7.2 × 10\(^{-10}\) mol for a 12 μm thick film and 12.8 × 10\(^{-10}\) mol for a 20 μm thick film), indicating that the PVI-\[Os(bpy)\_2Cl\] molecules can entirely and uniformly adsorbed inside the CNTF films, as illustrated in Figure 1b. A part of the free imidazole groups of the mediator polymer would adsorb on CNT surfaces via π-π interaction. The adsorption density of PVI-\[Os(bpy)\_2Cl\] calculated using the effective inner surface area of the CNTF films (8.2 cm\(^2\) for 20μm thick film) \(^{(5)}\) was 1.6 ± 0.1 × 10\(^{-10}\) mol cm\(^{-2}\), which is comparable with the value for a PVI- \[Os(bpy)\_2Cl\] film adsorbed on a flat Au surface (3.2 × 10\(^{-10}\)mol cm\(^{-2}\)).
2.2. Electrocatalytic activity of GOD/PVI-[Os(bpy)2Cl]CNTF ensemble films.

Subsequent loading of the enzyme GOD was conducted by immersing the PVI-[Os(bpy)2Cl]-adsorbed CNTF films in a stirred PBS (pH 7.0) solution containing 3 mg ml\(^{-1}\) GOD for 1 hour. Figure 3a shows the CVs of GOD/PVI-[Os(bpy)2Cl]/CNTF ensemble films at 10 mV s\(^{-1}\) in a stirred 200 mM D-glucose PBS solution. The catalytic current for glucose oxidation increased in response to the thickness of CNTF films (3.7 mA cm\(^{-2}\) for 4 μm thickness and 14.7 mA cm\(^{-2}\) for 20 μm thickness), indicating that also GOD can entirely penetrate inside the PVI-[Os(bpy)2Cl]-modified CNTF films. For example, the content of GOD incorporated in a 20μm-thick film was measured as ca. 0.86 μg by a C-6667 Protein Quantitation Kit, the value being a little below the case when GOD molecules (6.7 × 6.7 × 21 nm\(^3\)) align to form lines in the interspace of CNTs (1.17μg). The current density was enhanced to as high as 26.7 mA cm\(^{-2}\) by turning up the buffer temperature to 37.5 °C. Importantly, more than 90 % of the electrode activity could be maintained even after 6 days storage in an air-saturated PBS solution (Figure 3b), proving the stability of bioelectrocatalytic architecture with the composite of PVI-[Os(bpy)2Cl] polymer and GOD. The anionic GOD molecules could be stabilized by cationic Os complex of the mediator polymer anchored on the CNT surface via π-π interaction.

The electron turnover rate for the 20 μm-thick film was calculated from the current value at 25 °C (0.29 mA), the Faraday constant (96500 C mol\(^{-1}\)), the molecular weight of GOD (186000 g mol\(^{-1}\)), and the content of GOD molecules in a piece of the ensemble film (ca. 0.86 μg). The derived turnover rate was ca. 650 s\(^{-1}\), being comparable with that of GOD in bulk solution containing an electron acceptor of O\(_2\) (700 s\(^{-1}\)) or ferrocene (600 s\(^{-1}\)) at 25 °C. These results indicate that most of ca. 3 × 10\(^{12}\) GOD units within the film could efficiently work to the full, presumably owing to the molecularly ordered alignment of enzyme/mediator/electrode in the ensemble. Such a high efficiency of the present GOD electrode resulted in a resistance to oxygen inhibition, as shown in Figure 3c. The catalytic performance was almost identical in N\(_2\)-saturated, air-saturated and even O\(_2\)-saturated solutions. In general, glucose oxidation with GOD-modified electrodes is often disturbed by dissolved O\(_2\), which is troublesome for glucose sensing. However, the ordered Os(bpy)\(_2\) groups in the present ensemble electrode could effectively accept the electron from GOD in preference to O\(_2\), resulted in excellent O\(_2\) resistance.

2.3. Application as a windable anode of biofuel cells.

The present free-standing, bioelectrocatalytic film could be used for miniature biofuel cell devices. We demonstrate here the application of the film to a self-powered sugar indicator designed for inserting into a fruit. For indicating the glucose concentration, the net performance of the biofuel cell system should be controlled by the glucose anode. Because the oxygen in fruits is limited to a lower concentration than glucose, we employ a gas-diffusion biocathode for utilizing the abundant oxygen in air outside of the fruits (see Experimental for details). Figure 4a shows the biofuel cell performance measured using 200 mM glucose PBS solution with a couple consisting of a GOD/PVI-[Os(bpy)2Cl]...
CNTF film anode (20μm thickness) and a cathode made from bilirubin oxidase (BOD)-modified carbon fabric (1cm × 1cm). The open-circuit voltage of the cell was 0.5 V in agreement with the difference between the potentials at which glucose oxidation and oxygen reduction start to occur in cyclic voltammetry. The maximum output current (0.27 mA) indicates that the system is limited by the anode even in 200 mM glucose, a concentration that markedly higher than that found in raw fruits (a few tens of mM). As shown in Figure 4b, a piece of GOD/PVI-[Os(bby)2Cl]/CNTF film was wound on one lead of a light-emitting-diode (LED) device, whose blinking interval is inversely proportional to the power of the biofuel cell. The other lead was connected to the BOD-based gas-diffusion cathode. The blinking interval of the LED upon inserting the device to a grape was coincident with that for the extracted juice (Figure 4c), proving that this device could serve as a sugar indicator by simply being inserted into a grape. This principle of the self-powered sensor could be applied to more important blood sugar monitoring applications. We are planning to develop a GOD/PVI-[Os(bby)2Cl]/CNTF-based device structure suitable for low-invasive insertion into a blood vessel through skins.

3. Conclusion

The larger amount of enzyme units than monolayer were successfully immobilized with keeping the effective electrical contact with the electrode (CNTs). In particular, we have succeeded in forming the entirely uniform bioelectrocatalytic architecture with PVI-[Os(bpy)2Cl] and GOD inside a CNTF film. The voltammograms of the PVI-[Os(bpy)2Cl]-modified CNTF indicated the uniform adsorption of PVI-[Os(bpy)2Cl] on the CNT surface via π–π interaction with the density of ca. 1.6 ± 0.1 × 1010 mol cm−2. The subsequent modification of GOD seemed to form at the interspaces of PVI-[Os(bpy)2Cl]-modified CNTs, as judged from the protein quantitation assay. Owing to such ordered positional relationship between GOD, PVI-[Os(bpy)2Cl] and CNT, the composite film showed both “high activity” for glucose oxidation (ca. 15 mA cm−2) and “high turnover rate” (ca. 650 s−1), indicating almost every enzyme molecules within the film could work to the full.

4. Experimental section

CNTF preparation: CNTF was synthesized in a 1-in. tube furnace by water-assisted chemical vapor deposition at 750 °C with a C2H4 carbon source and an Al2O3 (10 nm)/Fe (1.0 nm) thin-film catalyst grown on silicon wafers. (5) We used He with H2 as the carrier gas (total flow 1000 standard cubic
centimeters per minute (sccm) at 1 atm with a controlled amount of water vapor with ethylene (100 sccm) for 10 min. Quantitative Analysis of the Entrapped Enzymes: The quantitative analysis of GOD was conducted as explained in our previous paper. The enzyme-incorporated CNTF film was first washed and immersed in 20 mM sodium phosphate buffer (pH 9.3) containing 0.1 M sodium borate and 1% sodium cholate and dispersed with an ultrasonic homogenizer for 15 min. The GOD in the dispersion was then analyzed using a C-6667 Protein Quantitation Kit (Molecular Probes), using 5 mM (3-(4-carboxybenzoyl)-quinoline-2-carboxaldehyde) (ATTO-TAG CBQCA) and 20 mM KCN to label the enzyme with CBQCA. After 1.5 h of incubation, the fluorescent intensity was measured by a luminescent image analyzer system (Fuji Photo Film, LAS-3000 mini), and the amount of enzyme was determined by referencing a calibration curve.

Preparation of gas-diffusion carbon fabric (CF) cathodes: The preparation of the cathode basically followed the procedures used for our previous work. A 40 μl aliquot of a 10 mg ml⁻¹ multiwalled CNT solution was put on a CF strip and dried in air, followed by thoroughly washing out the surfactant by soaking in an ethanol solution for more than 1 h with stirring. The surface of the CNT-modified CF electrode was further modified with a 0.1 ml solution of 5 mg ml⁻¹ bilirubin oxidase (BOD, EC 1.3.3.5, 2.5 U/mg, from Myrothecium) in vacuum oven (0.09 MPa, 35 °C). The strip was additionally coated with the CNT solution to make the surface hydrophobic.

Electrochemical Measurements: The GOD/PVI-[Os(bpy)₂Cl]/CNTF ensemble films, anchored at the edge with SUS316L fine tweezers, was analyzed by a three-electrode system (BSA, 730C electrochemical analyzer) in stirred solutions using a Ag/AgCl reference and a platinum counter electrode. The gas-diffusion cathode (BOD-modified CF strip) was put on an air-saturated solution so as to contact the solution by the BOD-modified face during the cyclic voltammetry. The performance of a biofuel cell constructed from an GOD-based CNTF anode and an BOD-based CF cathode was evaluated on the basis of the cell voltage upon changing the external resistance between 1 kΩ and 2 MΩ at the time step of 60 s. Unless otherwise indicated, the electrochemical measurements were carried out at room temperature (25 °C).

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