Optimization of Enzyme Anode and Cathode with Polyion Complex for the Application to Biofuel Cells

Shinichi KOMABA,a * Toshihiko MITSUHASHI,a and Soshi SHIRAISHIb

aDepartment of Applied Chemistry, Faculty of Science, Tokyo University of Science (Tokyo 162-8601, Japan)
bDepartment of Applied Chemistry, Faculty of Engineering, Gunma University (Kiryu, Gunma 376-8515, Japan)

Received March 14, 2008 : Accepted June 11, 2008

The nanocomposite enzymatic cathode and anode with carbon nanotube were fabricated and optimized for the application to biofuel cells. The nanocomposite electrodes consisted of polyion complex matrix where enzyme, mediator, and carbon nanotube as an electron transport enhancer were immobilized on the glassy carbon electrode. For the fabrication of anode and cathode, glucose oxidase and tetraarylfuvalene and bilirubin oxidase and 2,2’-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium, respectively, were immobilized together in the polymer matrix. Electrochemical observation confirmed the bioelectrocatalytic ability of glucose oxidation and oxygen reduction at the anode and cathode, respectively, due to the enzymatic activity. The biological-fuel cell fabricated by combining the nanocomposite enzyme electrodes demonstrated open circuit voltage of 0.65 V, and its maximum power was 150 μW cm⁻².

Key Words : Biofuel Cell, Glucose Oxidase, Bilirubin Oxidase, Enzyme Electrode, Carbon Nanotube

1 Introduction

A biofuel cell is the electric generation system utilizing the combination of biochemical and electrochemical reactions of biological compounds such as saccharides, vitamins, alcohols, and so on.10 It is able to convert the chemical energy of biofuels into electric energy by bioelectrocatalysis. Since enzyme is usually employed as an electrode catalyst, various substrates are able to be used as biofuels, and it operates under a moderate condition. A biofuel cell has disadvantages; low power output, unstable power generation, and unsatisfactory lifetime compared to those of the conventional fuel cell using hydrogen and oxygen fuels. As is generally accepted, the direct electron transfer between reductant center of enzyme and electrode substrate is difficult due to the long distance for electron hopping, so that the coexistence of additional reductant molecules is required in order to mediate the electron transfer between the reductant center of enzyme and electrode (current collector). For the electrode design and fabrication of biofuel cells, there are the important factors which determine the electric power generation performance of the cell, such as selection of enzyme and mediator, enzymatic activity, immobilization method, and nano-scale structure of electrode, etc.1,10

Mizutani and coworkers found that the polyion complex (PIC) is able to be applied for enzyme immobilized electrodes and has molecular sieving ability.11 In the past ten years, one of us reported the preparation of high sensitivity biosensors by utilizing the molecular sieve variations of the PIC.12-17 The biofuel cell electrodes with the PIC as a matrix for enzyme immobilization were reported recently.18-20 Differently from the application for biosensors, high electric power generation, that is, spontaneous large current at high voltage between anode and cathode is essentially required for biofuel cells, and the selectivity and signal-to-noise ratio of electrochemical response are not essential to biofuel cells. Therefore, the improvement of power generation should be achieved by control of microstructure of enzyme electrode even if the identical enzyme, substrate and redox mediator were employed.

Recently, we fabricated the nanocomposite enzyme electrode and achieved the high current density operation of glucose anode for the biofuel cell by using glucose oxidase (GOD) as a electrode catalyst and tetrathiafulvalene (TTF) as a mediator immobilized in polyion complex, which consisted of poly-L-lysine (PLL) and poly(sodium 4-styrenesulfonate) (PSS). Furthermore, we applied carbon nanotube (CNT) giving the electron conducting network within the polyion complex glucose anode to enhance the power capability20 on the basis of the investigation of CNT by one of us.21 In this study, we reinvestigated the PIC electrode with GOD for the optimization of the anode performance, and the cathode for oxygen reduction was fabricated with the similar electrode configuration to the anode by utilizing bilirubin oxidase (BOD), 2,2’-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) as a mediator, and CNT. The glucose-O₂ biofuel cell equipped with the polyion complex anode and cathode was constructed to evaluate the maximum power.

2 Experimental

GOD (EC 1.1.3.4, from Aspergillus niger, Nagase ChemteX Co.), BOD (EC 1.3.3.5, from Myrothecium sp., Amano Enzyme Co.), TTF, ABTS, PSS (Sigma-Aldrich Co.), multiwalled CNT (ILJIN Nanotech Co., Ltd., 220 m² g⁻¹ of specific surface area21), cetyltrimethylammonium bro-
mide (CTAB), and PLL hydrobromide (Wako Pure Chemical Industries) were used without further purification. Phosphate buffer solutions (PBS, 0.04 mol dm$^{-3}$ pH = 7.0) containing 20 mg cm$^{-3}$ GOD or 30 mg cm$^{-3}$ BOD, a saturated TTF methanol solution, a 0.1 mol dm$^{-3}$ ABTS aqueous solution, a PLL (50 mmol dm$^{-3}$ monomer unit or 30 mg cm$^{-3}$) aqueous solution, a PSS (60 mmol dm$^{-3}$ monomer unit) aqueous solution, and a 1 mg cm$^{-3}$ CNT suspension in 0.1 wt % CTAB aqueous solution or 0.02 mol dm$^{-3}$ ABTS were prepared by sonication to fabricate the enzyme immobilized electrode.

The PIC film incorporating GOD, TTF, and CNT (GOD/TTF/CNT) was prepared on the glassy carbon (GC, geometric area is 7.07 mm$^2$) electrode. First, a total of 8 µl CNT suspension was placed on the glassy carbon electrode followed by drying in vacuum. Then, a total of 15 µl TTF solution, a total of 15 µl GOD solution, and a total of 8 µl CNT suspension were placed and dried for each placement of solutions. Finally, 10 µl PLL (50 mmol dm$^{-3}$ monomer unit) and 6 µl PSS (60 mmol dm$^{-3}$ monomer unit) solutions were successively dropped on the surface, and the electrode was dried in atmosphere to form the insoluble PLL-PSS complex film.$^{20}$ The dropping PLL and PSS solutions and drying were repeated to make the PIC layer thick. Based on these solutions and procedures, the PICs incorporating GOD or GOD and TTF (GOD/TTF) without CNT were prepared by selecting the solutions.

The PIC electrodes incorporating BOD were prepared in the following manner. The PIC film incorporating BOD, ABTS, and CNT (BOD/ABTS-CNT) was prepared on the GC electrode; a total of the 20 µl CNT and ABTS suspension was placed on the glassy carbon electrode with drying in vacuum. Then, 5 µl BOD and 5 µl of 30 mg cm$^{-3}$ PLL solutions were placed and dried in atmosphere. Finally, 10 µl PLL (50 mmol dm$^{-3}$ monomer unit) and 6 µl PSS (60 mmol dm$^{-3}$ monomer unit) solutions were successively dropped and dried to form the complex. The dropping and drying were repeated to make the PIC layer thick. In the same manner, the PICs incorporating BOD or BOD and ABTS (BOD/ABTS) without CNT were prepared by selecting the solutions and compared to the BOD/ABTS-CNT electrode. The BOD electrode was prepared by dropping BOD and PLL solution and dried to form the insoluble BOD-PLL electrostatic binding. The BOD/CTAB-CNT electrode was fabricated by dropping the CTAB-CNT suspension and dried, then BOD and PLL solutions were dropped and dried to form the BOD-PLL electrostatic binding. The BOD/ABTS electrode was fabricated by dropping BOD, ABTS, and PLL solutions and then the electrode was dried to form the insoluble electrostatic binding.

For electrochemical characterization such as cyclic voltammetry and chronoamperometry, the PIC working, platinum counter, and Ag/AgCl reference electrodes were installed in a one-compartment cell. The electrochemical measurements of the enzyme anode were performed in 0.04 mol dm$^{-3}$ PBS at pH = 7.0 in the presence and absence of glucose at 37°C in air. The measurements of the enzyme cathode were performed in the PBS saturated with O$_2$ under 1 atm O$_2$ atmosphere. The biofuel cell which consisted of the GOD/TTF/CNT anode (3 mm in diameter), BOD/ABTS-CNT cathode (3 mm in diameter), and the 0.04 mol dm$^{-3}$ phosphate buffer solution containing 0.1 mol dm$^{-3}$ glucose and saturated with O$_2$ under 1 atm O$_2$ atmosphere was evaluated under fixed constant voltage condition. The electrochemical characterization of biofuel cell was carried out with chronoamperometry mode of potentiostat to obtain a steady-state current.

3 Results and Discussion

3.1 Glucose Anode with GOD

As described recently,$^{20}$ the conductive carbon nanotubes are thoroughly dispersed and provided the conductive network in the nanocomposite to enhance the oxidation current of glucose catalyzed by GOD and mediated by TTF entrapped in the PIC. As previously reported, TTF promotes the electron transfer between the redox center of GOD and GC substrate, resulting in the anodic current.$^{18,19,22}$ Since various (macro)-molecules$^{11-17}$ and nano-sized particles$^{19,20}$ are able to be entrapped and immobilized in the PIC, the PSS-PPL complex was here employed for the formation of the insoluble nanocomposite matrix incorporating the functional molecules and conducting CNT.

We examined three type PIC modified electrodes, GOD, GOD/TTF, and GOD/TTF/CNT, to clarify each function of the immobilized molecules. Figure 1 compares cyclic voltammograms in the presence of glucose. When the voltammograms of GOD and GOD/TTF electrodes are compared in Figs. 1a and 1b, the small current is observed at > 0.1 V by adding TTF. Furthermore, the potential shift toward negative direction and the drastic increase in the oxidation current were confirmed for the PIC film with GOD/TTF/CNT. These characteristics will be advantageous for the application to the anode of biofuel cells. This indicates that the CNT was co-immobilized and dispersed in the whole PIC, resulting in the formation of the nanocomposite of GOD, TTF, and CNT with the PIC. One of us reported the character of multi-walled CNT for the application of electrochemical capacitors.$^{22}$ When we compared car-

![Fig. 1](image_url)

Fig. 1 Cyclic voltammograms of three type GOD electrodes, a) GOD, b) GOD/TTF, and c) GOD/TTF/CNT modified GC electrodes in a 0.1 mol dm$^{-3}$ glucose solution at 10 mV s$^{-1}$. 
bons such as acetylene black, Ketjen black, and CNT as a conducting additive for the PIC anode, the much improved anodic current was observed in the case of CNT because the fibrous shape of CNT, which consists of entangled tubes with 10-20 nm diameter and several hundred nanometer length, would be beneficial to the formation of conducting network in the PIC. We believe that the CNT effectively improved and/or accelerated the oxidation of TTF mediator which is connected to GOD redox in the PIC. Because of the improvement and optimization of the film formation conditions, such as concentrations and volumes of the CNT, TTF, and GOD solutions, the current increased compared to our previous result in.

The glucose permeability in the PIC matrix would be enhanced by selecting the combination of polycation and polyanion. Additionally, the diffusivity of glucose molecules in the PIC and the stability of anode should depend on the PSS-PLL thickness, which increases by increasing the dropped volume of PSS and PLL solutions. We investigated the dependence of glucose oxidative current on the PIC thickness at 0.25 V where the large anodic current flowed in Fig. 1.

As seen in Fig. 2 the current increased when glucose was added into the buffer solution, and the steady-state current density depended on the PIC thickness, which was controlled by changing the number of dropping times of the PSS and PLL solutions. That is, when the number of dropping times increased, the thicker PIC layer was formed on the GC electrode. The thickness of PIC was roughly estimated at 100-300 μm when the dropping was repeated ten times. It is clear that the largest current was observed when the polyanion complex was formed by dropping the PSS and PLL solution once. In case of the PIC formed by dropping the polyanion solutions ten times, the current hardly and slowly changed by the glucose addition. Clearly, the thicker the PIC film led the smaller the anodic current of glucose at the GOD/TTF/CNT electrode, suggesting that it is the diffusion control of glucose within the PIC. The relation between the PIC thickness and current density was plotted in Fig. 2b. The anodic current monotonously decreased by increasing the thickness of PIC. When the oxidation of glucose is catalyzed by GOD, glucose molecules must be diffused in PIC film to reach the redox center of GOD. Since the insoluble PIC film covers the GOD, TTF, and CNT layer as described previously, the thick PIC hinders the diffusion, resulting in the limitation of the supply of glucose molecules to redox center of GOD, therefore, the current depended on the number of dropping times.

The dissolution of the GOD and TTF from the PIC should be one of the main origins of the electrode deterioration. It was expected that the stability of the glucose anode is improved by the thicker PIC formation but the current density was low. To consider the dissolution of GOD and TTF, we compared the current values measured immediately after the electrode fabrication and after five days. As a result, the current was clearly decreased for the thin PIC electrode during five days. In case of thicker PIC, the current was hardly decreased during five days, suggesting that the thick PIC layer suppressed the dissolution, though the absolute current became low compared to that for the thin PIC. In order to achieve both the higher current and higher stability of the GOD/TTF/CNT nanocomposite, the PIC layer was found to be formed by dropping the polyanion solutions four or five times from the above observations.

3.2 Oxygen Cathode with BOD

The application of CNT in the GOD enzyme electrode is able to enhance the electrochemical reaction, because of the conductive CNT network as described recently.

As the electrode system with BOD and ABTS demonstrated as an oxygen cathode in biofuel cells as described, we fabricated and evaluated the BOD modified electrodes based upon the CNT nanocomposite system in order to combine with the glucose cathode to construct glucose-oxygen fuel cells.

Figure 3 compares the cyclic voltammograms in O2 free and O2 saturated PBS solutions of bare glassy carbon, BOD/ABTS, CTAB-CNT, and BOD/CTAB-CNT modified electrodes. The cathodic behavior depended on the modification. In case of the bare GC electrode, the reductive current of O2 was observed at the potential region lower than ~0.1 V. In Fig. 3b, the cathodic current appeared below approximately 0.6 V and the current
became almost constant at the lower potential than ca.
0.4 V for the BOD/ABTS electrode. If the phosphate
solution was deaerated with nitrogen gas, the cathodic
current hardly appeared but the redox couple of ABTS
was observed around 0.5 V. The overpotential of oxygen
reduction was decreased by introducing BOD and ABTS
since the reduction potential was remarkably shifted
toward positive direction from ~0.1 V to 0.6 V. According
to literatures by Tsujimura et al.,9,23,24) the BOD and
ABTS mediator at the electrode catalyzed the reduction of
oxygen, and the BOD and ABTS have the coulombic
interaction with PLL cation as the BOD and ABTS are
negatively charged in the neutral solution. This interaction
would result in the insoluble binding between BOD,
ABTS, and PLL. As the potential shift of oxygen reduc-
tion was confirmed for the modified electrode, the BOD
and ABTS were immobilized on the surface of GC elec-
trode and exhibited the bioelectrocatalysis behavior.

Since the CNT addition is adequate to form the con-
ductive PIC matrix where GOD and TTF were
entrapped as mentioned above, we also applied the CNT
for the BOD cathode for oxygen reduction. The voltam-
mosgrams of the CTAB-CNT and BOD/CTAB-CNT mod-
ified GC electrodes are compared in Figs. 3c and 3d.
When the BOD was added to the CTAB-CNT electrode
to be co-immobilized by using the electrostatic interac-
tion with PLL, we fabricated the BOD/CTAB-CNT elec-
trode. The reduction potential was shifted up to 0.5 V,
which resulted from introducing BOD. In the case of the
CTAB-CNT electrode, the cathodic current also flowed
at the potential lower than ~0.1 V. Comparing the voltam-
mosgrams in Figs. 3b and 3d, the cathodic current
was enhanced by the CNT addition. Tsujimura et al.
described that the direct bioelectrocatalysis of dioxygen
reduction with BOD, of the family of multi-copper
enzymes, occurred as the electrons are transferred from
an current collector to the type 1 Cu site of BOD and
then to the type 2-3 Cu sites where O₂ is reduced to
water.25) Since the mediator free operation for the reduc-
tion was possible in case of the BOD/CTAB-CNT elec-
trode (Fig. 3d), the direct electron transfer might be
applied for this case, though further investigation are
required to prove the exact reaction.

Based on the effects of the BOD/ABTS and
BOD/CTAB-CNT in Fig. 3, we fabricated the ternary
BOD/ABTS-CNT electrode. As drawn in Fig. 4a, the
oxygen reduction clearly starts from 0.6 V. Additionally,
Fig. 4b compares the voltamograms in the O₂ satu-
rated electrolyte. It is obvious that the current density of
BOD/ABTS-CNT was pretty larger than those of the
BOD/ABTS and BOD/CNT electrodes, and the maxi-
mum current was 1.5 mA cm⁻². Because of the fast kinet-
ics of oxygen reduction, the current peak at 0.3-0.4 V
during cathodic scan appears under static condition due
to the O₂ diffusion control dissolved in the PBS solution.

It was found that the CNT can be ultrasonically dis-
persed in water not only with CTAB surfactant but also
with ABTS from our investigation. We compared, there-
fore, the cathode characteristics in the O₂ dissolved solu-
tion for the BOD/ABTS-CNT and BOD/ABTS/CTAB-
CNT which was fabricated by dropping the CTAB-CNT,
ABTS, and BOD solutions. As a result, the BOD/ABTS-
CNT showed the higher current density than the
BOD/ABTS/CTAB-CNT electrode. It is probable that
the nanostructure of the composite and/or the denatura-
tion of BOD by CTAB influenced the resultant activity
of BOD on the electrode. Consequently, the electronically
conductive pathway connecting between ABTS and GC

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**Fig. 3** Cyclic voltammograms of a) bare glassy carbon, b) BOD/ABTS, c) CTAB-CNT, and d) BOD/CTAB-CNT modified electrodes in O₂ free (N₂ saturated) and O₂ saturated PBS solutions at 10 mV s⁻¹.
substrate would be successfully formed within the nanocomposite where the BOD and ABTS were entrapped.

Furthermore, the dependence of the O₂ reductive current on the PIC thickness was investigated as shown in Fig. 5. It was confirmed that the absolute currents on the first day were almost independent of the thickness, on the contrary, the current retention after five days depended on the thickness. The retention was improved with the increase in the thickness of PIC. This result shows that the PIC thickness of 2 or higher is adequate to keep 80% of the initial value after five days. It is believed that the dissolution of the immobilized BOD and ABTS is one of the predominant factors of the electrode deterioration, therefore, the thickness control of the PIC layer was effective to suppress the dissolution not only of the anode but also of the cathode.

3.3 Glucose-O₂ Biofuel Cell with Nanocomposite Electrodes

The glucose anode and oxygen cathode were fabricated on the basis of the polyanion complex possessing the electron conductive network and enzymatic catalysis. Here, we assembled and evaluated the biofuel cell consisted of the GOD/TTF/CNT and BOD/ABTS-CNT with a glucose and O₂ containing electrolyte solution. Figure 6 shows the voltage-current and voltage-power plots of the assembled glucose-O₂ biofuel cell with 0.04 mol dm⁻³ glucose saturated with O₂ (partial pressure of O₂ = 1 atm) at 37 °C. The plots were obtained by keeping and changing the output voltages between the cathode and anode. It was found that the cell exhibited the open circuit voltage of 0.65 V and the maximum power density of 150 μW cm⁻² at 0.35 V. When the cathode and anode were connected directly, the short-circuit current density of 600-700 μA cm⁻² flowed between the cathode and anode.

The relation between the potential and current of the glucose anode and oxygen cathode are shown in Fig. 7. When the constant potential was applied for the electrodes, the current values after three minutes of the potential application were plotted in the figures. The catalytic electrooxidation current of glucose appears at ca. 0 V vs. Ag/AgCl and reached the maximum of ca. 1.3 mA cm⁻² at 0.25 V. The current was almost constant in the potential region between 0.25 and 0.35 V. At higher potentials than 0.35 V, the current suddenly decreases as is seen in Fig. 7a. At the higher potential, the oxidized TTF⁺ molecules underwent the irreversible overoxidation to from TTF²⁺, which is soluble in an aqueous solution and decomposes.²⁶ It is likely that the abrupt decrease in current is due to the irreversible oxidation and/or the leakage of water soluble TTF²⁺ from the polyanion complex matrix. Fig. 7b shows the dependence of current density on potential when the cathode was polarized at the constant potential in the phosphate electrolyte.
containing O₂. The cathodic current of O₂ appears at ca. 0.55 V, and the current was almost constant in the region less than 0.4 V and reaches approximately 700 μA cm⁻².

From Fig. 7, the largest current densities of glucose electrooxidation and O₂ electroreduction were approximately 1300 and −700 μA cm⁻², respectively, in a quiescent solution. The anode and cathode properties in Fig. 7 correspond to the current and power variation in Fig. 6. As the assembled biofuel cell is considered to be the series circuit of the cathode, electrolyte, and anode, the short circuit current should be limited by the oxygen cathode when the anode and cathode in the cell have the same surface area, and the maximum power output was limited by the rate of O₂ electroreduction. It is expected that the specific power and current output density of the biofuel cell will be optimized when the cathode surface area becomes higher.

4 Conclusion

The GOD/TTF/CNT and BOD/ABTS-CNT nanocomposites with the PIC matrix were optimized for the application to the anode and cathode of the glucose-O₂ biofuel cell. The electronic conduction path was developed in the PIC film incorporating enzymes, mediators, and CNT, so that the highly dispersed CNT in the PIC effectively increased the bioelectrocatalytic current. The glucose-O₂ biofuel cell was fabricated by combining these nanocomposite electrodes. The cell operated at a power density of 150 μW cm⁻² at 0.35 V in 0.04 mol dm⁻³ phosphate buffer solution (pH 7.0, 37°C) containing sufficient amounts of glucose and oxygen.

Acknowledgement

The authors thank Ms. M. Takano, Ms. H. Inomata, and Mr. M. Ikeda, Tokyo University of Science, for their helpful assistance for experimental work.

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