Usefulness and Performance Comparison of Complex Enzyme-type Biofuel Cell Using Electrode Modified with Two DET-type Enzymes by Covalent Bonding

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Abstract. The demand for enzymatic biofuel cells (EBFCs) as power sources or auxiliary power sources for small devices is expected to increase in the near future. EBFCs have advanced properties and do not require a separator, depending on the substrate specificity of the enzyme. Two direct electron transfer (DET)-type enzymes were used to modify anodes (length 5 mm, width 4 mm) by a chemical modification method using a condensation agent. The DET-type enzymes used in this study were pyrroloquinoline quinone-dependent glucose dehydrogenase (PQQ-GDH) with glucose as a reaction substrate and fructose dehydrogenase (FDH) using fructose as a reaction substrate. Carboxyl groups were attached to multi-walled carbon nanotubes (MWCNTs) that act as catalyst carriers, activated to other functional groups, and reacted with the amino groups of the enzyme by the condensation agent to form covalent bonds. As a result, the upper limit of the concentration, considered to be the reaction limit, was raised as compared with that of EBFC modified with only one kind of enzyme for each electrode prepared by the same process. The output voltage was 0.155 V, and the maximum power density was 80.08 μW/cm². Also the deterioration characteristics were confirmed with the passage of time for EBFC-Z; the maximum power density was almost unchanged for three months, and output reduction due to the passage of time was not observed.

1. Introduction

An enzymatic biofuel cell (EBFC) is a fuel cell that performs an oxidation-reduction reaction using an enzyme as a catalyst, and converts the obtained chemical energy into electrical energy to generate electricity [1, 2]. Since the enzyme is derived from living organisms, it is possible to generate electricity with ordinary temperature, normal pressure, and neutral fuel. This fuel cell can be manufactured with a separator-free structure due to the substrate specificity of the enzyme. There are several reports on EBFCs [3-8], but none on modifying the anode with multiple DET-type oxidoreductases. By modifying an anode with multiple enzymes, it is possible to increase the number of substrates that can react with it, and thus, produce a battery that can handle various fuels. Herein, this battery is referred to as a complex enzyme-type biofuel cell (CEBFC). In our study, we tested and evaluated CEBFCs to demonstrate the usefulness of combining direct electron transfer (DET)-type enzymes such as pyrroloquinoline quinone-dependent glucose dehydrogenase (PQQ-GDH; from microorganisms) and fructose dehydrogenase (FDH; from gluconobacter sp.).

Physical adsorption, which is one of the conventional carrier bonding methods [9], has been widely used for immobilizing enzymes. This is simpler than other methods, and the possibility of enzyme deactivation is also relatively low. However, since the binding force between the carrier and the enzyme is small and it is not possible to adjust the orientation of the enzyme, the possibility of peeling of the enzyme from the carrier and a reduction in output are disadvantages in this method. With this method, it is difficult to solve the low power and
low lifetime cited as the characteristics of EBFCs [10]. Therefore, in order to make these improvements, we adopted covalent bonding, which is one of the chemical bonding methods [11].

In order to show the usefulness of CEBFC, three types of battery patterns were prepared: EBFC–X (anode: PQQ-GDH and cathode: Laccase (Lac)), EBFC–Y (anode: FDH and cathode: Lac), and EBFC–Z (anode: PQQ-GDH, FDH and cathode: Lac). In EBFC–X and Y, we used glucose and fructose as the fuel, respectively, and a glucose:fructose ratio of 1:1 was used as EBFC–Z’s fuel.

The power densities, along with the outputs of each modification method and concentration in the above three patterns were evaluated.

2. Experimental

2.1. Power generation principle

For understanding the principle, EBFC–X rather than CEBFC is cited as an example. Fig 1 shows the principle of power generation in this cell. The electrode is immersed in the glucose fuel solution. At the anode, glucose is oxidized to gluconolactone. During this reaction, protons (hydrogen ions) are released into the fuel, and electrons flow in the circuit. At the cathode, oxygen dissolved in the fuel is reduced, and water is generated by the reaction of reduced oxygen with the released hydrogen ions and electrons in the circuit. Due to this electron transfer/redox reaction cycle, a current flows and electromotive force is obtained.

![Proposed power generation principle for EBFC–X](image)

2.2. Design of CEBFC

The structure of the fabricated CEBFC is shown in Fig 2. Polyimide (PI) was selected as the battery material, platinum (Pt) as the metal substrate for flowing current, and MWCNTs as the catalyst carriers of the enzyme. Also, carbon black ink (CBI) was adopted as the adhesive between Pt and MWCNTs, because peeling occurs easily when the MWCNTs dispersion is directly applied to a metal substrate.

![Design of CEBFC](image)
2.3. Fabrication process of carbon electrodes

The fabrication of the carbon electrode is illustrated in Fig 3. First, in order to pattern a Pt electrode on a substrate of PI, sputtering was carried out by attaching a mask in which an OHP sheet was patterned with holes. Second, CBI was coated on the electrode and fired at 150 °C for 6 min. Since the MWCNT dispersion is aqueous, it is not suitable for hydrophobic CBI. Subsequently, UV/O₃ was used to irradiate the electrode as a hydrophilic treatment for 30 min. Immediately thereafter, the MWCNT dispersion liquid was dropped onto the electrode and slowly baked at 100°C for 15 to 20 minutes until the water evaporated to prepare a carbon electrode.

![Fabrication of carbon electrodes: (a) Pt mask sputtering on PI substrate, (b) CBI application to Pt electrode, (c) UV / O₃ irradiation of electrode, and (d) application of MWCNT](image)

2.4. Fabrication of enzymatic electrodes

The chemical reaction between the enzyme and the catalyst carrier is shown in Fig 4. 1-Cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-p-toluenesulfonate (CMC) was adopted as a condensation agent. However, CMC cannot directly bond with carboxyl groups, and it may cause racemization. Therefore, N-hydroxysuccinimide (NHS), which activates the carboxyl group, was introduced. With this reagent, the carboxyl group becomes an NHS active ester, and a condensation reaction can occur with the amino group present on the surface of the enzyme.

Furthermore, we consider that the orientation of the enzyme can be aligned by chemical modification. Therefore, the enzyme does not exfoliate, most of it can be modified, the orientation of the enzyme is more uniform, and the electron transfer is performed smoothly, the output becomes larger than that of the physical adsorption method. First, the surface of the electrode was modified by UV/O₃ irradiation for 1 h to apply a carboxyl group to the carbon electrode, which acts as a hydrophilic treatment. Second, in order to activate the carboxyl group to effect a condensation reaction with the amino group, the device was immersed in 2 ml of an aqueous solution mixed with 100 mg of CMC and 20 mg of NHS in a McIlvain buffer at pH 5.0 for 30 min. Thereafter, the device was washed and dried; 4 μl of an aqueous enzyme solution (50 mg/ml) was added dropwise to each electrode and allowed to stand undisturbed at room temperature (23 to 26 °C) for 30 min or more to effect a condensation reaction. Finally, the residual moisture on the surface was dried to prepare the EBFC. Furthermore, an aqueous enzyme solution for EBFC-Z was complexed in advance at a PQQ-GDH:FDH ratio of 1:1 to prepare an aqueous solution and dropped onto the electrode.

![Chemical reaction of enzyme and catalyst carrier: (a) Carboxyl group attached to the carbon electrode surface, (b) NHS active ester, and (c) enzyme electrode after condensation reaction](image)
2.5. Electrochemical characterization

Variable resistors were connected to the anode and the cathode, the electrodes were immersed in the fuel contained in the beaker and the voltage was measured while varying the resistance from 0 to 2 MΩ. The battery performance was evaluated based on the power density calculated from those numerical values. Fuel concentration was increased in steps of 100 mM from 0 to 1000 mM and the concentration limit was determined. Furthermore, in order to demonstrate the usefulness of the CEBFC, the difference between the outputs of the physical adsorption method and the covalent bonding method adopted in the study was measured. These conditions were adopted for the EBFC-X, Y, and Z prototypes, and output measurements were carried out. (All of fuel temperature is 37 °C because of optimal temperature for these enzymes)

3. Results and discussion

The output graphs based on the difference in the enzyme immobilization method for each pattern are shown in Fig 5. A large difference was observed in the output value of all the patterns, and it was concluded that the chemical modification method was appropriate. The output graphs from varying concentration in each pattern are shown in Fig 6 (a-c). In EBFC-X, the output value was highest when the concentration was 500 mM, with an output voltage of 0.103 V and maximum power density of 35.36 μW/cm². Similarly, in EBFC-Y, the output value was highest when the concentration was 500 mM, with an output voltage of 0.083 V, and maximum power density of 22.96 μW/cm². Furthermore, in EBFC-Z, the output value was highest when the concentration was 800 mM, with an output voltage of 0.155 V, and maximum power density of 80.08 μW/cm².

When only one enzyme was used to modify the anode, the peak output values were at roughly the same concentrations. This suggests that there is an upper limit of the concentration of fuel that can react with each enzyme dropping amount, that is, there is a saturation limit of the reaction. If this modified multiple types of enzymes to the anode, several problems could occur. Those were the upper limit of the concentration of fuel does not change, or multiple kinds of enzymes are reacted in the same electrode, the speed of electrons becomes rate limiting, and the output becomes the same as rate limiting enzyme side. The experimental results, as shown in Fig. 10, illustrate that the peak output was obtained when the concentration was 800 mM, and the output was much higher than those of the other patterns. This is an indication that the speed of the electrons is not rate limiting and that they effect the redox reaction without interfering with each other. This result shows that various enzymes can be combined into the electrode by taking advantage of the substrate specificity of the enzyme.

Also the deterioration characteristics were confirmed with the passage of time for EBFC-Z; the maximum power density was almost unchanged for three months, and output reduction due to the passage of time was not observed (Fig 6 (d)). It was preserved by being immersed in a buffer solution at around 4 °C. The reason why the maximum output density did not change so much is considered to be that the enzyme electrode was not dried but stored at a low temperature and the enzyme on the electrode surface was not inactivated. Therefore, differences in the established environmental conditions such as the optimum pH and optimum temperature of each enzyme do not significantly affect the CEBFC output.

![Fig 5. Relation between power density and output voltage of (a) EBFC-X, (b) EBFC-Y, and (c) EBFC-Z due to difference in modification method](image-url)
Fig 6. Relation between power density and concentration in (a) EBFC-X, (b) EBFC-Y, (c) EBFC-Z, and (d) Relation between power density and elapsed time in EBFC-Z

4. Conclusion
In this study, a CEBFC using an anode modified with two enzymes was prepared and its performance was compared with that of an EBFC modified using only one enzyme. Using the chemical modification method resulted in a large difference in output compared with that of the physical adsorption method, demonstrating the advantage of this CEBFC fabrication process. Under these conditions, the output was the highest in EBFC-Z with 800 mM fuel concentration, with an output voltage of 0.155 V and maximum power density of 80.08 μW/cm². EBFC-Z's the maximum power density was almost unchanged for three months, and output reduction due to the passage of time was not observed. Therefore, we continue to experiment and investigate the degradation characteristics. To the best of our knowledge, there are no reports on EBFCs with multiple DET-type enzymes immobilized on MWCNT by chemical modification; hence, this work shows promise for the design of the world's first CEBFC.

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