Title

Multi-enzyme anode composed of FAD-dependent and NAD-dependent enzymes with a single ruthenium polymer mediator for biofuel cells

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Abstract

A multi-enzyme electrode composed of FAD-dependent and NAD-dependent enzymes was fabricated using a poly-ruthenium complex (PAHA-Ru), which has two 1, 10-phenanthroline-5, 6-dione molecules as ligands. PAHA-Ru was used to immobilize FAD-dependent glucose dehydrogenase (FAD-GDH) onto an electrode and to examine PAHA-Ru containing the quinone moieties as an electron mediator. In cyclic voltammetry measurements of the FAD-GDH modified electrode in the presence of D-glucose, a catalytic current was obtained, which indicated electron transfer from FAD-GDH to PAHA-Ru. Our previous study has reported that PAHA-Ru with the quinone ligands also works as a mediator for NADH oxidation on an NAD-dependent alcohol dehydrogenase (NAD-ADH) modified electrode. Hence, FAD-GDH and NAD-ADH were co-immobilized with PAHA-Ru to make a multi-enzyme electrode. Using this multi-enzyme electrode as an anode, catalytic currents were observed in D-glucose solution, ethanol solution, and a mixed D-glucose and ethanol solution. The catalytic current in the mixed solution was greater than the currents obtained in the single substrate solutions, indicating bioelectrocatalysis reactions by the two enzymes and the single mediator in the mixed solution. Thus, we demonstrated that PAHA-Ru modified electrode enables selection of enzymes and their substrates from a wider range for

enzymatic biofuel cells.

Keywords

redox polymer mediator; 1,10-phenanthroline-5,6-dione; flavin adenine dinucleotide dependent-glucose dehydrogenase; nicotinamide adenine dinucleotide-dependent alcohol dehydrogenase; multi-enzyme electrode; entrapping immobilization method

1. Introduction

Biofuel cells that possess enzymes on their anode and cathode can gain electrons through anodic reaction of safe and familiar donors, such as saccharides and alcohols, and can pass electrons to acceptors such as molecular oxygen through cathodic reactions to generate electricity [1][2].

Electrodes modified with more than one type of enzyme, multi-enzyme electrodes, can oxidize substrates such as methanol [3], ethanol [4], lactic acid [5], glucose [6, 7], and glycerol [8] in a multi-step manner. Multi-step oxidation with biofuel-cell-anodes improves the amount of energy derived per unit mass substrate, reducing the amount of fuel consumed and possibly increasing the current density [6]. Furthermore,

multi-enzyme anodes allow us to generate electricity from two or more types of fuel in a single mixed solution.

To procure electrical energy from each enzymatic reaction different in substrates on a multi-enzyme electrode, electrons should be transferred efficiently from each enzyme to the electrode. The more enzymes are loaded on the electrode, the greater the electrical current from each enzymatic reaction will be expected. However, electron transfer can be inhibited by the protein mass itself because peptide chains are electrically non-conductive. Thus, it is desirable to use fewer types of redox enzymes that can oxidize the substrate in a multi-step pathway [6-9]. Using electron mediators facilitates the electron transfer from enzyme active sites to an electrode around non-conductive peptide chains. For multi-enzyme anodes, it is desired to apply redox polymer mediators that can be co-immobilized with enzymes. Simple cell structures are achieved by entrapping enzymes with a single polymer mediator on an electrode.

FAD-dependent enzymes and NAD-dependent enzymes are related to many biological reactions and are frequently used as catalysts in bioelectronic devices [10]. They are also expected to be useful in multi-enzyme electrodes. A multi-enzyme electrode

composed of enzymes with different coenzymes allows us to select enzymes and fuels from a wider range. A multi-enzyme anode composed of FAD- and NAD-dependent enzymes was fabricated by Minteer's group [4]. Most of the enzymes employed on the electrode were from the enzymes in the citric acid cycle, so the anode was able to completely oxidize ethanol to carbon dioxide. In their case, the anode used poly(methylene green), an electropolymerized film, for the mediator and tetrabutylammonium bromide modified Nafion® for the entrapping polymer. In this research, we entrapped FAD- and NAD-dependent enzymes with a redox polymer mediator itself on an electrode to create a simple cell structure.

FAD-dependent glucose dehydrogenase (FAD-GDH) is different from widely used FAD-dependent glucose oxidase (FAD-GOx) in one respect; GDH does not deliver electrons to oxygen molecules. Therefore, FAD-GDH is a good electrode catalyst in blood glucose sensors and biofuel cells because their measured value and power output, respectively, do not fluctuate with dissolved oxygen. Moreover, FAD-GDH utilization in bioelectronics devices does not require cofactor addition because GDH couples with FAD within itself. This property permits the fabrication of bioelectronic devices with simple structures [11]. Electronic devices with FAD-GDH appeared around 2005. FAD-GDH

was employed in a biosensor with high substrate selectivity that was not affected by oxygen [12]. Using FAD-GDH from *Burkholderia cepacia* as an anode catalyst, a biofuel cell was constructed [13]. Because FAD-GDH includes cytochrome *c* as the electron transfer subunit, the enzyme performed direct electron transfer with an electrode. However, most groups have used polymer electron mediators to achieve efficient electron transfer from FAD-GDHs to electrodes [11, 14]. Recently, enormously high catalytic current densities were observed employing an FAD-GDH modified electrode with magnesium oxide-templated mesoporous carbon particles and a redox hydrogel [15].

Because FAD-GDH is expected to be useful as an anode catalyst in multi-enzyme electrodes, it is important to find a redox polymer mediator for the enzyme that can be co-immobilized on an electrode. Quinone derivatives, such as benzoquinone and pyrroloquinoline quinone, are often reported as mediators for FAD-GOx [16-18]. We have already reported that a poly-ruthenium complex, $\text{Ru}(\text{PQ})_2(\text{NH}_2\text{-phen})](\text{NH}_3)_2$ (PAHA-Ru), with quinone derivative ligands worked as a mediator for NADH oxidation, and we have previously constructed an NAD-dependent alcohol dehydrogenase (NAD-ADH) modified electrode for ethanol oxidation [19]. Because electron transfer occurred through

1,10-phenanthroline-5,6-diones, the quinone moieties, PAHA-Ru is expected to function as a new mediator between FAD-GDH and the electrodes. Moreover, if the ruthenium polymer works as a redox polymer mediator between the enzyme and electrodes, a multi-enzyme electrode composed of FAD-GDH and NAD-dependent enzymes will be achieved.

In this study, FAD-GDH was co-immobilized with PAHA-Ru onto an electrode to examine PAHA-Ru as a mediator. Furthermore, FAD-GDH and NAD-ADH were co-immobilized with PAHA-Ru to fabricate a multi-enzyme electrode on which a single redox polymer mediates the electrochemical reactions of an FAD-dependent enzyme and an NAD-dependent enzyme.

2. Experimental

2.1. Materials

Glucose dehydrogenase dependent on FAD was gifted from Ikedatohka Industries (Hiroshima, Japan). Alcohol dehydrogenase from yeast was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Poly (diallyldimethylammonium chloride) aqueous solution and NAD⁺ were purchased from Sigma-Aldrich Japan G.K. (Tokyo,

Japan).

2.2. Electrode preparation

Glassy carbon electrodes (3 mm in diameter) were polished with alumina (particle size 3 μm : 3 M Japan Limited, Tokyo, Japan) and chromium oxide (particle size 0.5 μm : 3 M Japan Limited, Tokyo, Japan). This step was followed by ultrasonic cleaning and rinsing with deionized water.

PAHA-Ru was synthesized as previously reported [19]. PAHA-Ru phosphate buffer solution (1 wt%), FAD-GDH aqueous solution (0.137 mM), and poly(diallyldimethylammonium chloride) (PDDA) aqueous solution (1 wt%) were mixed at a ratio of 1:1.25:1, and the mixture (3.25 μL) was dropped onto a pretreated glassy carbon electrode. After 30 minutes of drying at room temperature, the FAD-GDH modified electrode was obtained.

PAHA-Ru phosphate buffer solution (1 wt%), FAD-GDH aqueous solution (0.137 mM), NAD-ADH aqueous solution (0.271 mM) and poly(diallyldimethylammonium chloride) (PDDA) aqueous solution (1 wt%) were mixed at a ratio of 1:1.25:1:1, and the mixture

(4.25 μ L) was dropped onto another pretreated glassy carbon electrode. After 30 minutes of drying at room temperature, the FAD-GDH/NAD-ADH modified multi-enzyme electrode was obtained.

2.3. Electrochemical measurements

All electrochemical measurements were conducted using an ALS electrochemical analyzer 600 (BAS Inc., Tokyo, Japan). A conventional three-electrode cell was used, in which a platinum wire served as the counter electrode and Ag/AgCl (3 M NaCl) served as the reference electrode. All redox potentials in this report are relative to the Ag/AgCl (3 M NaCl) electrode. The cyclic voltammetry (CV) measurements of the FAD-GDH modified electrode were conducted in 100 mM potassium phosphate buffer solution (pH = 7.0) containing 100 mM D-glucose. For the CV measurements of the FAD-GDH/NAD-ADH modified multi-enzyme electrode, three solutions of 100 mM potassium phosphate buffer (pH = 7.0) containing 10 mM NAD⁺ were prepared. One solution contained 100 mM D-glucose as a substrate. Another solution contained 100 mM ethanol. The last solution contained both 100 mM D-glucose and 100 mM ethanol. For all measurements, the scan rate and range were set to 10 mVs⁻¹ and -0.30 V to 0.35 V.

3. Results and discussion

3.1. CV measurements of FAD-GDH modified electrode

A redox couple with a half-wave potential ($E_{1/2}$) of approximately -10 mV was observed when a CV measurement of the FAD-GDH modified electrodes was conducted in potassium phosphate buffer without D-glucose (Fig. 1 a). The previous report and its following experiments showed that redox reactions of PAHA-Ru modified electrodes without enzyme substrates were assigned to 1,10-phenanthroline-5,6-dione, the quinone moieties of PAHA-Ru, considering the redox potential and its pH dependency [19]. In D-glucose solution, an oxidative catalytic current of $36 \mu\text{Acm}^{-2}$ (at 0.35 V) was obtained, which started from approximately - 0.1 V (Fig. 1 b). This result demonstrated that PAHA-Ru containing quinone derivatives could mediate the electron transfer from FAD-GDH to the electrode. The potential from which the catalytic current started, is one of the lowest values among reports on FAD-GDH modified electrodes with redox polymer mediators, as far as we know [11, 14, 20]. It is expected that an open circuit voltage of a biofuel cell with FAD-GDH would increase when using PAHA-Ru.

3.2. CV measurements of FAD-GDH/NAD-ADH modified multi-enzyme electrode

In the CV measurements of the FAD-GDH/NAD-ADH modified multi-enzyme electrode

in both ethanol solution and D-glucose solution, catalytic currents were obtained (Fig. 2 b, c). The current densities at 0.35 V were $10.4 \pm 2.1 \mu\text{Acm}^{-2}$ in the ethanol solution and $23.1 \pm 5.7 \mu\text{Acm}^{-2}$ in the D-glucose solution. In the case of the mixed solution of ethanol and D-glucose, we also observed catalytic currents of $39 \pm 9.0 \mu\text{Acm}^{-2}$ at 0.35 V. Because the current density of the combined solution was greater than the current densities obtained in the single substrate solutions, it was demonstrated that a bioelectrocatalysis reaction of the two enzymes and the single mediator proceeded on the electrode in the mixed solution (Scheme 1). The current density in the mixed solution was almost the same as the sum of those in each substrate solution. Hence, the electron transfer from each enzyme to the mediator proceeded without competition between enzymes with different coenzymes when excess mediator moieties existed.

Conclusions

Using an FAD-GDH electrode modified with PAHA-Ru, a catalytic current was observed in D-glucose solution. This result showed the possibility that PAHA-Ru could act as a redox polymer mediator between FAD-dependent enzymes and electrodes. The measurements of the FAD-GDH/NAD-ADH multi-enzyme electrodes demonstrated that the sum of the catalytic current densities in D-glucose solution and ethanol solution was almost equal to the

current density in the mixed solution. FAD-dependent and NAD-dependent enzymes are important in a large number of biochemical processes. Hence, fabrication of a multi-enzyme electrode composed of FAD-dependent and NAD-dependent enzymes allows us to select enzymes and substrates from a wider range for enzymatic biofuel cells, which can generate electricity by multi-step oxidation of substrates or produce electric power from mixed fuel solutions.

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References

- [1] R.A. Bullen, T.C. Arnote, J.B. Lakeman, F.C. Walsh, Biofuel cells and their development, *Biosens. Bioelectron.* 21 (2006) 2015-2045.
- [2] J.A. Cracknell, K.A. Vincent, F.A. Armstrong, Enzymes as Working or Inspirational Electrocatalysts for Fuel Cells and Electrolysis, *Chem. Rev.* 108 (2008) 2439-2461.

- [3] G.T.R. Palmore, H. Bertschy, S.H. Bergens, G.M. Whitesides, A methanol/dioxygen biofuel cell that uses NAD⁺-dependent dehydrogenases as catalysts: application of an electro-enzymatic method to regenerate nicotinamide adenine dinucleotide at low overpotentials, *J. Electroanal. Chem.* 443 (1998) 155-161.
- [4] D.S. Lazic, S.D. Minteer, Citric acid cycle biomimic on a carbon electrode, *Biosens. Bioelectron.* 24 (2008) 939-944.
- [5] S. Tsujimura, J. Fukuda, O. Shirai, K. Kano, H. Sakai, Y. Tokita, T. Hatazawa, Micro-coulometric study of bioelectrochemical reaction coupled with TCA cycle, *Biosens. Bioelectron.* 34 (2012) 244-248.
- [6] M. Shao, M.N. Zafar, C. Sygmund, D.A. Guschin, R. Ludwig, C.K. Peterbauer, W. Schuhmann, L. Gorton, Mutual enhancement of the current density and the coulombic efficiency for a bioanode by entrapping bi-enzymes with Os-complex modified electrodeposition paints, *Biosens. Bioelectron.* 40 (2013) 308-314.
- [7] S. Xu, S.D. Minteer, Enzymatic Biofuel Cell for Oxidation of Glucose to CO₂, *ACS Catal.* 2 (2012) 91-94.
- [8] R.L. Arechederra, S.D. Minteer, Complete Oxidation of Glycerol in an Enzymatic Biofuel Cell, *Fuel Cells* 09 (2009) 63-69.
- [9] K. Takeda, H. Matsumura, T. Ishida, M. Samejima, K. Igarashi, N. Nakamura, H.

Ohno, The two-step electrochemical oxidation of alcohols using a novel recombinant PQQ alcohol dehydrogenase as a catalyst for a bioanode, *Bioelectrochemistry*, 94 (2013) 75-78.

[10] P.N. Bartlett (Ed.), *Bioelectrochemistry: Fundamentals, Experimental Techniques and Applications*, Wiley, New Jersey, 2008.

[11] M.N. Zafar, X. Wang, C. Sygmond, R. Ludwig, D. Leech, L. Gorton, Electron-Transfer Studies with a New Flavin Adenine Dinucleotide Dependent Glucose Dehydrogenase and Osmium Polymers of Different Redox Potentials, *Anal. Chem.* 84 (2012) 334-341.

[12] S. Tsujimura, S. Kojima, K. Kano, T. Ikeda, M. Sato, H. Sanada, H. Omura, Novel FAD-Dependent Glucose Dehydrogenase for a Dioxygen-Insensitive Glucose Biosensor, *Biosci. Biotechnol. Biochem.* 70 (2006) 654-659.

[13] N. Kakehi, T. Yamazaki, W. Tsugawa, K. Sode, A novel wireless glucose sensor employing direct electron transfer principle based enzyme fuel cell, *Biosens. Bioelectron.* 22 (2007) 2250-2255.

[14] O. Yehezkeli, R.T. Vered, S. Raichlin, I. Willner, Nano-engineered Flavin-Dependent Glucose Dehydrogenase/Gold Nanoparticle-Modified Electrodes for Glucose Sensing and Biofuel Cell Applications, *ACS Nano* 5 (2011) 2385-2391.

- [15] S. Tsujimura, K. Murata, W. Akatsuka, Exceptionally High Glucose Current on a Hierarchically Structured Porous Carbon Electrode with “Wired” Flavin Adenine Dinucleotide-Dependent Glucose Dehydrogenase, *J. Am. Chem. Soc.* 136 (2014) 14432-14437.
- [16] D.L. Williams, A.R. Doig Jr., A. Korosi, Electrochemical-enzymatic analysis of blood glucose and lactate, *Anal. Chem.*, 42 (1970) 118-121.
- [17] T.W. Hui, K.Y. Wong, K.K. Shiu, Kinetics of o-benzoquinone mediated oxidation of glucose by glucose oxidase at edge plane pyrolytic graphite electrode, *Electroanalysis* 8 (1996) 597-601.
- [18] I. Willner, V.H. Shabtai, R. Blonder, E. Katz, G. Tao, A. F. Bückmann, A. Heller, Electrical Wiring of Glucose Oxidase by Reconstitution of FAD-Modified Monolayers Assembled onto Au-Electrodes, *J. Am. Chem. Soc.* 118 (1996) 10321-10322.
- [19] Y. Motoyama, N. Nakamura, H. Ohno, Preparation of an Electrode That Immobilizes NAD-Dependent Alcohol Dehydrogenase by Using a Redox Polymer Containing 1,10-Phenanthroline-5,6-dione, *Electroanalysis* 20 (2008) 923-926.
- [20] S. Tsujimura, S. Kojima, K. Kano, T. Ikeda, M. Sato, H. Sanada, H. Omura, Novel FAD-dependent glucose dehydrogenase for a dioxygen-insensitive glucose biosensor, *Biosci. Biotechnol. Biochem.* 70 (2006) 654-659.

[21] Z. Liu, M. Cardosi, J. Rodgers, G. Lillie, L. Simpson, Synthesis and study of copolymer of vinylferrocene, acrylamide and 2-(diethylamino)ethyl methacrylate as a polymeric mediator for electrochemical biosensors, *React. Funct. Polym.* 70 (2010) 715-725.

Figure legends

Fig. 1 Cyclic voltammograms of the FAD-GDH modified electrode at a scan rate of 10 mVs⁻¹ measured in 100 mM phosphate buffer (a) and 100 mM D-glucose / 100 mM phosphate buffer solution (b).

Fig. 2 Cyclic voltammograms of the FAD-GDH/NAD-ADH modified electrode at a scan rate of 10 mVs⁻¹ measured in 10 mM NAD⁺ / 100 mM phosphate buffer solution (a), containing 100 mM ethanol (b), 100 mM D-glucose (c), and 100 mM D-glucose and 100 mM ethanol (d).

Scheme 1. Schematic description of the electron transfer. Electrons from the D-glucose oxidation by FAD-GDH transfer to an electrode via PAHA-Ru. Electrons from the

ethanol oxidation by NAD-ADH are brought by NADH-diffusion to PAHA-Ru and then to an electrode.

d. Figure 1

d. Figure 2