Separator-less Amino Acid-powered Biofuel Cell Through an Artificial Multi-enzyme Cascade Reaction

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Biotechnology letters

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Title: Separator-less amino acid-powered biofuel cell through an artificial multi-enzyme cascade reaction

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19 **Abstract**

20 *Objective*

21 This study was aimed at constructing a highly stable one-compartment enzymatic biofuel cell (EFC)
22 without a separator through a multi-enzyme cascade reaction pathway.

23 *Results*

24 A separator-less EFC composed of a multi-enzyme cascade anode containing four dehydrogenases
25 from a thermophile and a cathode devised using a multi-copper oxidase mutant with enhanced enzyme
26 activity from a hyperthermophile was developed. To fabricate an EFC without a separator, redox
27 mediators utilized in the enzymatic cascade reaction were also immobilized on the anode. In the
28 presence of the fuel 100 mM L-proline, the separator-less EFC with four thermophilic dehydrogenase-
29 modified anode achieved a maximum power density of 11.3 $\mu\text{W}/\text{cm}^2$ at 37°C, which was 1.6-fold
30 higher than that of a similar EFC fabricated with a one enzyme-modified anode. The separator-less
31 EFC composed of a multi-enzyme modified anode maintained approximately 57% of the load current
32 at 0.3 V measured on the first day of EFC fabrication, even after 4 days.

33 *Conclusion*

34 Efficient L-proline electric generation utilizing a separator-less EFC composed of a multi-enzyme
35 modified anode through a multi-step fuel oxidation reaction and a highly stable multi-copper oxidase
36 mutant-modified cathode was successfully achieved over a long period. The long-term stability of the
37 separator-less EFC can facilitate its application as an efficient power source for implantable medical
38 devices requiring continuous operation.

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40 **Keywords:** Separator-less enzymatic biofuel cell, thermophilic enzyme, enzyme cascade, polymer-
41 bound redox mediator

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Introduction

Enzymatic biofuel cell (EFC) is an electrochemical device that uses oxidoreductase as an electrocatalyst and converts the chemical energy of its substrate into electric energy. EFCs have high potential for application in implantable medical devices that can generate electricity without added toxicity concerns, unlike traditional batteries such as lithium batteries (Cosnier et al. 2014). However, the practical applications of EFCs are still limited because of their low battery life due to enzyme instability. To address this issue, we have developed EFCs using oxidoreductases from thermophiles as a specific electrode element. Enzymes from thermophiles are known to be considerably more stable at room temperature and across a wide range of pH than their mesophilic counterparts (Ohshima and Soda 1989; Adams 1993; Li et al. 2005). We have already succeeded in constructing a novel high-efficiency bioanode for the multi-step oxidation of L-proline by an artificial electro-metabolic pathway using thermophilic enzymes (Fig. 1) (Satomura et al. 2019). The novel electrode performing multi-step oxidation using L-proline as a fuel comprised four kinds of thermophilic dehydrogenases: dye-linked L-proline dehydrogenase from *Aeropyrum pernix* (ApLPDH), NAD-dependent Δ^1 -pyrroline-5-carboxylic acid dehydrogenase from *Thermus thermophilus* (ThP5CDH), NAD-dependent glutamate dehydrogenase from *Pyrobaculum islandicum* (PiGDH), and dye-linked NADH dehydrogenase from *Geobacillus kaustophilus* (GkNADHDH). The electrode modified with these 4 enzymes exhibited an approximately 4.2-fold higher current response than the electrode modified with ApLPDH alone.

Moreover, the current response of the multi-enzyme modified electrode remained at more than 50% of its initial current response even after 15 days at room temperature. In addition to the development of a highly efficient anode, we have successfully constructed a highly stable multi-copper oxidase mutant from the hyperthermophilic archaeon, *P. aerophilum*, with enhanced electrocatalytic activity (eMcoP) as an element for cathode (Satomura et al. 2021). In this study, we aimed to construct a highly efficient EFC with the multi-enzyme modified electrode as the anode and the eMcoP-modified electrode as the cathode.

In several cases of EFCs, redox mediators are required to shuttle electrons between the enzymes and electrodes. In addition, the anode used in this study requires redox mediators to perform a multi-enzyme cascade reaction on the electrode. Free redox mediators are known to cause serious crossover reactions with electrodes in the EFC (Katz et al. 1999; Tujimura et al., 2001; Tujimura et al. 2002; Kamitaka et al. 2007). Redox mediators required for the anodic reaction react at the cathode, leading to a decrease in the power of the EFC. In general, to avoid the crossover reactions, separators such as Nafion membranes are incorporated into the EFC; however, these modifications increase the structural complexity of the EFC.

Therefore, we investigated the conditions for immobilizing the redox mediators to the anode and developed a simplified one-compartment EFC without a separator. The use of immobilized redox mediators together with the multi-enzyme cascade based electrodes was aimed at building simple and

efficient EFCs for biomedical application. [INSERT Fig.1]

Materials and methods

Materials

L-Proline and Nafion perfluorinated resin solution were obtained from Sigma-Aldrich (St. Louis, MO, USA). NAD⁺ was purchased from Oriental Yeast (Tokyo, Japan). Carbon paper (CP, TGP-H-120) was purchased from Toray (Tokyo, Japan). 1-Aminopyrene (Py-NH₂), multi-walled carbon nanotubes (MWCNT, 10-20 nm in diameter and 5-15 mm in length), 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC), *N*-hydroxysuccinimide (NHS), and aminoferrocene were purchased from Tokyo Chemical Industry (Tokyo, Japan). Sodium alginate was obtained from Nacalai Tesque (Kyoto, Japan). *N*-methylpyrrolidone (NMP) was purchased from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan). All other chemicals were of reagent grade.

Preparation of bioanode.

The enzymes composing the artificial enzyme cascade for the bioanode were prepared as described previously (Satomura et al. 2019). Polymer-bound NAD⁺ (Alg-NAD) with alginic acid was prepared as previously described (Nakamura et al. 1996). To prepare the polymer-bound ferrocene (Alg-Fc), 20

103 mL of 50 mM potassium phosphate buffer (PKB, pH7.2) containing 10 mg sodium alginate, 2.5 mM
104 EDC and 2.5 mM NHS was prepared, and the solution was stirred for 1h at 30°C. Then, 1.25 mM
105 aminoferrocene was added and the resulting solution was stirred for 6 h at 30°C. The reaction mixture
106 was dialyzed against 50 mM PKB (pH 7.2) for 6 h and then dialyzed against ethanol and water for 24
107 h and 20 h, respectively. Alg-Fc was recovered after lyophilization and stored at -20°C in the dark.
108 To prepare the enzyme-modified electrode to oxidize L-proline, a 200 μ L of 4.0 mg CNT /mL in NMP
109 solvent was added on a carbon paper (5×5 mm) and dried at 200°C to prepare the CP/CNT electrode.
110 The resulting electrode was dipped in 5.0 mM solution of Py-NH₂ in NMP for 1 h at 30°C.
111 Subsequently, the electrode was rinsed with NMP and water to prepare the CP/MWCNT/Py-NH₂
112 electrode. An aliquot of 200 μ L from a solution containing 0.5 mg Alg-NAD/mL, 0.5 mg Alg-Fc/mL,
113 100 mM EDC, 100 mM NHS, and 50 mM PKB (pH 7.2) was dropped onto the CP/CNT/Py-NH₂
114 electrode. The electrode was incubated for 6 h at 30°C and then rinsed with 50 mM PKB (pH 7.2) to
115 prepare the CP/CNT/Py-NH(-Alg-Fc/-Alg-NAD) electrode. The enzyme solution containing
116 ApLPDH, ThP5CDH, PiGDH and GkNADHHDH (the activity of all enzymes except GkNADHHDH
117 was adjusted to 0.01 μ mol/min and that of GkNADHHDH was adjusted to 0.05 μ mol/min) was dropped
118 onto the CP/CNT/Py-NH₂(-Alg-Fc/-Alg-NAD) electrode. After drying, Nafion solution (20 μ L of
119 0.5%, v/v) was dropped onto the electrode and dried overnight at 4°C.
120

Preparation of biocathode

The eMcoP for the biocathode was prepared as described elsewhere (Satomura et al. 2021); after soaking the CP/CNT electrode in 20 % (v/v) ethanol, 20 μ L eMcoP (0.01 μ mol/min) was dropped onto the electrode. The electrode was dried at room temperature and then cross-linked in 25% glutaraldehyde vapor for 30 min. After washing the electrode with 10 mM Tris-HCl (pH 7.2), the cathode was stored at 4°C in 100 mM PKB (pH 7.0) until use.

Electrochemical measurements

PKB (200 mM, pH 7.2) was used as the electrolyte for all electrochemical measurements. Cyclic voltammetry (CV) was performed at 37°C using a three-electrode cell on an ALS electrochemical analyzer (Model 1205 B, BAS Inc., Tokyo, Japan). The enzyme-modified electrode, platinum wire and Ag/AgCl electrode were used as the working, counter and reference electrodes, respectively. The CV measurements were recorded at 10 mV/s.

Linear sweep voltammetry (LSV) was performed from -0.45 to 0 V with a scanning rate of 5 mV/s at 37°C using an ALS electrochemical analyzer (Model 1205 B).

Evaluation of the long-term stability of EFC was performed using the Bio Battery Tester Model 1703 (Keisokuki Center, Osaka, Japan). The voltage of the biofuel cell was adjusted to 0.3 V, and the temporal changes in the value of the load current of the EFC were measured.

Results and discussion

Electrochemical evaluation of polymer-bound redox mediator-immobilized electrode

In our previous study, the efficient oxidation of L-proline to 2-oxogutarate by multi-enzyme cascade reactions was successfully achieved on our designed electrode (Fig. 1) (Satomura et al. 2019). The multi-enzyme cascade reaction was achieved using four thermophilic dehydrogenases (ApLPDH, ThP5CDH, PisGDH, and GKNADHDH) in the presence of two kinds of free redox mediators (NAD^+ and ferrocene). In this study, to construct a separator-less EFC, we immobilized both NAD^+ and ferrocene on the electrode. First, NAD^+ or aminoferrocene was covalently modified at the carboxylic group of alginic acid using EDC and NHS to generate Alg-NAD and Alg-Fc, respectively (Fig. 1). Next, Py-NH₂ was immobilized on the CP/CNT electrode. Our previous study revealed that pyrene derivatives containing Py-NH₂ specifically interact with CNTs by π - π stacking (Sakamoto et al. 2019). After the immobilization of Py-NH₂ on the CP/CNT electrode, the amino group in Py-NH₂ and the non-modified carboxylic groups in Alg-NAD and Alg-Fc were conjugated using NHS and EDC to generate a CP/CNT/Py-NH₂(-Alg-Fc/-Alg-NAD) electrode. To investigate the function of the immobilized redox mediators on the electrode, the CV of the CP/CNT/Py-NH₂(-Alg-Fc/-Alg-NAD) electrode modified with four thermophilic enzymes was measured without redox mediators in the electrolyte. As a result, an electrocatalytic oxidation current of approximately 200 μA was observed for ferrocene vs. Ag/AgCl, which was further markedly increased by the addition of 100 mM L-proline

(Fig. 2).

This indicates that polymer-bound ferrocene and NAD^+ can function as electron mediators for the deep oxidation of L-proline through a multi-enzyme cascade pathway. [INSERT Fig. 2]

Evaluation of EFC through the multi-step enzymatic L-proline oxidation pathway

The separator-less EFCs were constructed with an eMcoP-modified cathode and three different thermophilic dehydrogenase-modified anodes (ApLPDH, ApLPDH/P5CDH/GkNADHHDH or ApLPDH/P5CDH/PisGDH/GkNADHHDH) as the electrodes (Fig. 1), and their electrochemical characteristics were evaluated. The maximum power densities of one-compartment EFCs without separator in the presence of 100 mM L-proline using ApLPDH, ApLPDH/P5CDH/GkNADHHDH and ApLPDH/P5CDH/PisGDH/GkNADHHDH immobilized anodes were $6.82 \mu\text{W}/\text{cm}^2$ at 0.20V, $8.49 \mu\text{W}/\text{cm}^2$ at 0.21V, and $11.2 \mu\text{W}/\text{cm}^2$ at 0.22V, respectively (Fig. 3). These results clearly show that the power density of the EFC increases with an increase in the number of thermophilic dehydrogenases, and the multi-enzyme cascade reaction occurs efficiently on the electron mediator immobilized anode. [INSERT Fig. 3] In addition, they indicate that the separator-less EFC composed of anode modified with four dehydrogenases and an eMcoP-modified cathode can generate the highest power output among all EFCs tested in this study. Several separator-less EFCs have been reported so far (Tsuji-mura et al. 2002; Kamikita et al. 2007; Coman et al. 2008; Bellino and GJ. Soler-Illia 2014), but all these

involved only single-step oxidation of the fuel. To our knowledge, this is the first successful example of a separator-less EFC through a multi-enzymatic cascade reaction.

The long-term stability of the EFC composed of four thermophilic dehydrogenase-immobilized anode and eMcoP cathode was evaluated at 37°C by monitoring the relative load current at 0.3V. The EFC retained 62% of the initial load current after continuous operation for 2 days, and 57% of the initial load current even after 4 days (Fig. 4). **[INSERT Fig. 4]**

Separator-less EFCs can be miniaturized owing to their structural simplicity. Therefore, a high-powered separator-less EFC with long life shows high potential for application as a power source for implantable medical devices such as implanted drug-delivering pumps for the management of chronic pain, cerebral palsy, and diabetes (Barton et al. 2004). Further studies are currently underway to realize the practical biomedical application of such a power source.

Conclusions

A novel separator-less EFC was fabricated through a multi-enzyme cascade pathway using thermophilic enzymes. The maximum power density of the EFC composed of the four dehydrogenase-immobilized anode was 1.6-fold higher than that of the EFC composed of the anode immobilized with ApLPDH alone. Furthermore, the separator-less EFC retained more than 50% of its initial load current

at 0.3V even after continuous operation for 4 days at 37 °C. The high-performance separator-less EFC with a long life exhibits a high potential utility as power source for implantable bio-devices.

Declarations

Funding

This work was supported by the research grants from the Research and Education Program for Life Science, University of Fukui.

Conflict of Interest

The authors declare that they have no competing interests.

Data and material availability

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Kousaku Horinaga, and Takenori Satomura. The first draft of the

211 manuscript was written by Takenori Satomura and all authors commented on previous versions of the
212 manuscript. All authors read and approved the final manuscript.

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246 **Figure legends**

Fig. 1 Schematic illustration of separator-less biofuel cell operated through a multi-enzyme cascade reaction. ApLPDH, dye-linked L-proline dehydrogenase from *Aeropyrum pernix*; TthP5CDH, NAD-dependent Δ^1 -pyrroline-5-carboxylate dehydrogenase from *Thermus thermophilus*; PiGDH, NAD-dependent L-glutamate dehydrogenase from *Pyrobaculum islandicum*; GkNADHDH, dye-linked NADH dehydrogenase from *Geobacillus kaustophilus*; eMcoP, multi-copper oxidase mutant from *P. aerophilum*; P5C, Δ^1 -pyrroline-5-carboxylate; Alg-Fc, polymer-bound ferrocene; Alg-NAD, polymer-bound NAD⁺; Pyr-NH₂, 1-aminopyrene

Fig. 2 Cyclic voltammetry curves of ApLPDH/TthP5CDH/PisGDH/GkNADHDH-modified CP/CNT/Py-NH₂(-Alg-Fc/-Alg-NAD) electrode at 37°C in 200 mM potassium phosphate buffer (pH 7.0) in the presence (black line) and absence (gray line) of 100 mM L-proline at 10 mV/s

Fig. 3 Power density curves of L-proline enzymatic biofuel cell (EFC) through enzyme cascade reaction.

L-Proline EFC was constructed using the eMcoP-immobilized electrode as a cathode, and Ap-LPDH (dotted line), Ap-LPDH/TthP5CDH/GkNADHDH (gray line) or ApLPDH/TthP5CDH/PisGDH/GkNADHDH (black line) -modified electrode as the anode

265 **Fig. 4** Long-term stability of L-proline EFC with eMcoP-immobilized cathode and
266 ApLPDH/ThP5CDH/PisGDH/GkNADHDH-immobilized anode. The relative load current was
267 defined as the percentage of the load current relative to the initial load current measured on the first
268 day of EFC fabrication

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270

Figures

Fig. 1 Schematic illustration of separate-less biofuel cell operated through a multi-enzyme cascade reaction: ApLPDH, dye-linked L-proline dehydrogenase from *Aeropyrum pernix*; ThP5CDH, NAD⁺-dependent 4'-pyrimidine-5-carboxylate dehydrogenase from *Thermus thermophilus*; PGDH, NAD⁺-dependent L-glutamate dehydrogenase from *Pyrobaculum islandicum*; GKNADHHDH, dye-linked NADH dehydrogenase from *Geobacillus kaustophilus*; eMcoP, multi-copper oxidase mutant from *P. aerophilus*; P5C, 4'-pyrimidine-5-carboxylate; Alg-Fc, polymer-bound ferrocene; Alg-NAD, polymer-bound NAD⁺; Pyr-NH₂, 1-aminopyrene

Figure 1

Please See image above for figure legend.

Fig. 2 Cyclic voltammetry curves of ApLPDH/ThP5CDH/PisGDH/GkNADHDH-modified CP/CNT/Py-NH₂(-Alg-Fe/-Alg-NAD) electrode at 37°C in 200 mM potassium phosphate buffer (pH 7.0) in the presence (black line) and absence (gray line) of 100 mM L-proline at 10 mV/s

Figure 2

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Figure 3

Please See image above for figure legend.

Fig. 4 Long-term stability of L-proline EFC with eMcoP-immobilized cathode and ApLPDH/ThP5CDH/PisGDH/GkNADHDH-immobilized anode. The relative load current was defined as the percentage of the load current relative to the initial load current measured on the first day of EFC fabrication

Figure 4

Please See image above for figure legend.