Dual gas-diffusion membrane- and mediatorless dihydrogen/air-breathing biofuel cell operating at room temperature

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Abstract

A membraneless direct electron transfer (DET)-type dihydrogen (H₂)/air-breathing biofuel cell without any mediator was constructed wherein bilirubin oxidase from *Myrothecium verrucaria* (BOD) and membrane-bound [NiFe] hydrogenase from *Desulfovibrio vulgaris* Miyazaki F (MBH) were used as biocatalysts for the cathode and the anode, respectively, and Ketjen black-modified water proof carbon paper (KB/WPCC) was used as an electrode material. The KB/WPCC surface was modified with 2-aminobenzoic acid and *p*-phenylenediamine, respectively, to face the positively charged electron-accepting site of BOD and the negatively charged electron-donating site of MBH to the electrode surface. A gas-diffusion system was employed for the electrodes to realize high-speed substrate supply. As result, great improvement in the current density of O₂ reduction with BOD and H₂ reduction with MBH were realized at negatively and positively charged surfaces, respectively. Gas diffusion system also suppressed the oxidative inactivation of MBH at high electrode potentials. Finally, based on the improved bioanode and biocathode, a dual gas-diffusion membrane- and mediatorless H₂/air-breathing biofuel cell was constructed. The maximum power density reached 6.1 mW cm⁻² (at 0.72 V), and the open circuit voltage was 1.12 V using 1 atm of H₂ gas as a fuel at room temperature and under passive and quiescent conditions.

Keywords Biofuel cell, direct electron transfer, bioelectrocatalysis, gas-diffusion

1 Introduction

Enzymatic biofuel cells (EBFCs) are devices for converting chemical energy into electrical energy that utilize enzymes as electrocatalysts for the oxidation of fuels at the anode and the reduction of dioxygen (O_2) or hydrogen peroxide at the cathode [1-4]. In principle, a variety of compounds, such as sugar [5-13], alcohol [14-17], formate [18, 19] and dihydrogen (H_2) [20-31], can be utilized as fuels of EBFCs. Thanks to substrate specificity and high catalytic efficiency under mild conditions (e.g., room temperature, around neutral pH), EBFCs are expected to be one of the next-generation energy conversion systems, comparable to conventional fuel cells, such as polymer electrolyte fuel cell [32]; however, the low power densities of EBFCs hinder technological applications [1-4]. The main factor limiting the performance of EBFCs has been reported to be relatively slow interfacial electron transfer between the enzyme and electrode [1-4].

The first step to construct an EBFC is the immobilization of a redox enzyme onto a solid electrode for bioelectrocatalysis that couples the enzymatic and electrode processes[1-4]; however, for mediatorless direct electron transfer (DET)-type bioelectrocatalysis, the electrochemical communication between the enzyme and electrode is mainly limited due to the long-distance electron transfer from the active center of the enzyme to the electrode surface, and when enzymes are adsorbed in random orientation on electrode surface, the apparent interfacial electron-transfer kinetics decrease [1-4, 33]. In addition, details of the interaction between the enzyme and electrode remain to be elucidated, unlike the well understood interaction between the enzyme and its natural substrate [1-4]. Although some suitable redox mediators may assist electron transfer [34, 35], several drawbacks arise [1-4, 10, 34, 35]: (a) the

toxicity of the mediator, (b) leakage of the mediator, and (c) cell voltage loss to set up the driving force in the electron transfer between the enzyme and mediator. Therefore, multiple studies, such as creating novel electrode materials [36-42], functionalizing electrode surface [9, 43-51], and protein engineering [52-54], to improve DET-type bioelectrocatalysis have been reported.

H₂/O₂ biofuel cells wherein H₂ is oxidized by hydrogenase at the bioanode and O₂ is typically reduced by multi-copper oxidases (MCOs) at the biocathode are known as clean and highly efficient energy conversion devices that have attracted increasing attention for establishing a “hydrogen economy” [20-31].

Bilirubin oxidase from *Myrothecium verrucaria* (BOD, EC 1.3.3.5) is a member of the MCOs, which catalyzes a four-electron reduction of O₂ to water under mild conditions with low overpotentials. The active site of BOD contains four copper atoms, which can be divided into three classes according to their spectroscopic and magnetic properties [56, 57]: type I (T1), type II (T2), and type III (T3) coppers. T2/T3 is responsible for O₂ reduction, and T1 is the electron-accepting site from a donor or electrode [58, 59]. In our previous study, enhanced DET-type bioelectrocatalysis using BOD was accomplished at a negatively charged surface, constructed by modifying 4-aminobenzoic acid on a carbon electrode [49]. The electrostatic and π - π interactions between BOD and the modified electrode surface are responsible for the improvement in DET-type bioelectrocatalysis [49].

Hydrogenase catalyzes the reversible reaction of H₂ oxidation and proton (H⁺) reduction and can be classified according to the metal ion composition of their active sites into [NiFe], [FeFe], and [Fe] hydrogenases [60-65]. Among them, [NiFe] hydrogenases are the most widely investigated [62, 66-71]. Membrane-bound [NiFe]

hydrogenase from *Desulfovibrio Vulgaris* Miyazaki F (MBH) comprises a heterodimer with a total molecular mass of 91 kDa (α : 62.5 kDa and β : 28.8 kDa) [67]. The larger subunit hosts the [NiFe] center while the smaller subunit contains three iron–sulfur (FeS) clusters called the proximal, medial, and distal clusters, relative to the [NiFe] center [67]. The distal FeS cluster has been reported to be responsible for the electronic communication between the enzyme and electrode [67, 71]. From this viewpoint, it appears to be important for MBH to face the distal FeS toward the electrode surface to improve the performance of DET-type bioelectrocatalysis [67]. There are multiple reports focusing on the improvements of the DET-type bioelectrocatalytic performance of several [NiFe] hydrogenases with modified electrodes [72-75], but not for MBH to the best of our knowledge.

Other important issues for H₂/O₂ biofuel cells are the low-saturated concentrations of the gases in solution, the gas supply system, and risk of explosion [29, 31, 76]. Rotating disk electrodes (RDE) or stir bars are usually utilized to minimize the concentration depression near the electrode surface. These methods require external power to drive the RDEs or stir bars. More importantly, it may result in a biofuel cell that is too “fat” to improve the power density (power at a unit of surface area or volume). Recent findings have reported that MCOs [9, 29, 31, 77-79] and several MBHs [76] could efficiently operate in a gas-diffusion system. In addition, a gas-diffusion system is useful for suppressing the oxidative inactivation that occurs at positive electrode potentials and competitively with the catalytic cycle, and is another important issue in hydrogenase applications [76]. On the other hand, to avoid the risk of explosion, the compartmentalization of fuel cells [20, 21, 23, 25, 27, 31] and operation at low concentrations of H₂ and/or O₂ [22, 24, 26, 28-30] are frequently explored. The

former requires sophisticated cell construction and the latter results in low power density.

In this study, we attempt to construct a safe H₂/air-breathing biofuel cell operating at room temperature with a high power density using BOD and MBH on the functionalized mesoporous carbon electrodes. This is the first attempt to fabricate a membrane- and mediatorless H₂/air-breathing biofuel at room temperature.

2 Experimental

2.1 Biocatalysts, materials, and reagents

BOD (EC 1.3.3.5) from *Myrothecium verrucaria* was donated by Amano Enzyme Inc. (Japan) (3.04 unit mg⁻¹) and used without further purification. O₂-sensitive MBH from *Desulfovibrio vulgaris* Miyazaki F was purified according to the literature [80]. Ketjen black EC300J (KB) was kindly donated by Lion Co. (Japan). Waterproof carbon cloth (WPCC; EC-CC1-060T) was purchased from Toyo Co. (Japan), and polytetrafluoroethylene fine power (PTFE, 6-J) was obtained from DuPont Mitsui Fluorochemicals (Japan). 4-Aminobenzoic acid (4-ABA), 4-aminobenzenesulfonic acid (4-ABS), methyl-4-aminobenzoate (MABA), 2-aminobenzoic acid (2-ABA), and *p*-phenylenediamine chloride (*p*-PDA) were purchased from Tokyo Chemical Industry Co. (Japan). All other chemicals used in this study were of analytical grade unless otherwise specified, and all solutions were prepared with distilled water.

2.2 Electrochemical measurement

Cyclic voltammetry and chronoamperometry were performed using an electrochemical analyzer ALS 701 E with a glassy carbon (GC) electrode as a working

electrode, Pt wire as a counter electrode, and an Ag|AgCl|KCl (sat.) electrode as a reference electrode. For a gas-diffusion-type bioelectrode, WPCC and Pt mesh were used as a working electrode and a counter electrode, respectively. All potentials were referenced against the reference electrode.

2.3 Preparation of functionalized biocathodes

The gas-diffusion-type biocathode was prepared according to a previously reported method [31]. In brief, 40 mg of KB powder was mixed with PTFE and homogenized in 3.5-mL 2-propanol for 3 min in ice to prepare a KB slurry ($L = \text{dm}^3$). The slurry (approximately 100 mL) was applied to one side of a WPCC sheet and dried at room temperature to prepare the KB-modified WPCC electrode (KB/WPCC, 0.95 cm^2). The functionalization of the KB/WPCC electrode was conducted by one-step electrochemical oxidation at 0.8 V in a 2-ABA solution (5 mM, 0.1 M KCl) for 40 s [49, 81] ($M = \text{mol dm}^{-3}$) wherein the amino group of 2-ABA was oxidized at the carbon electrode to form a N–C bond to immobilize 2-ABA. The electrode was washed with distilled water and dried at room temperature. The 2-ABA-modified electrode is called 2-ABA/KB/WPCC. A 300- μL BOD solution (20 mg mL^{-1} , pH 7 phosphate buffer) was applied on the 2-ABA/KB/WPCC surface and dried for 2 h under reduced pressure at room temperature. The electrode is called BOD/2-ABA/KB/WPCC. As a control, BOD was adsorbed on pristine KB/WPCC, and the electrode is called BOD/KB/WPCC.

2.4 Adsorption of MBH on functionalized carbon surface

KB-modified GC electrode (KB/GCE) was prepared according to a previously described method [49]. In brief, 3 μL of a KB slurry (PTFE:KB=1:4, w/w) was dropped

on a rotating disk GCE surface (diameter: 3 mm) and dried at room temperature. Amine-functionalized KB/GCE (amine/KB/GCE) was prepared by one-step electrochemical oxidation at 0.8 V in an aniline derivative solution (5 mM, 0.1 M KCl) for 40 s and washed with distilled water [49,81]. Subsequently, a 10- μ L MBH solution (0.02 mM, pH 7.4 Tris-HCl) was applied on the KB/GCE or amine/KB/GCE and dried at room temperature for 2 h. The electrode was washed with a McIlvaine buffer (pH 5) and used for electrochemical measurements.

For the gas-diffusion system, KB/WPCC and *p*-PDA/KB/WPCC were prepared with the methods similar to those described above. A 250- μ L MBH solution (0.02 mM, pH 7.4 Tris-HCl) was applied on KB/WPCC or *p*-PDA/KB/WPCC surface and dried for 2 h under reduced pressure at room temperature.

2.5 DET-type biofuel cell

A DET-type membraneless one-compartment biofuel cell was constructed combining the gas-diffusion biocathode and bioanode, as shown in Fig.1. The projected surface area of the cathode and anode was adjusted to be 0.95 cm² by covering the electrodes with a cell vessel, and the total volume of the electrolysis solution in the vessel was 2.0mL. Titanium mesh was used as a current collector. The cathode and anode of the cell were connected through a variable resistor (Type 2786 Decade Resistance Box, Yokogawa Hokushin Electric, Japan). The cell voltage and potentials of the cathode and anode were measured with an electrometer (HE-106, Hokuto Denko, Japan) at the given values of resistance in the range from 100 k Ω to 10 Ω [5, 6, 9]. The current and power densities were calculated based on the projected surface area of the electrodes. Measurements were performed in 1.5 M citrate buffer (pH 5.0) at room temperature (25

$\pm 2\text{ }^{\circ}\text{C}$).

Fig. 1 Schematic illustration of a gas-diffusion type H_2 /air-breathing biofuel cell.

3 Results and discussion

3.1 Optimization of gas-diffusion biocathode with a negatively charged surface

Fig. 2(A) shows cyclic voltammograms (CVs) of O_2 reduction at a scan rate (ν) of 5 mV s^{-1} with BOD/2-ABA/KB/WPCC (solid line) and BOD/KB/WPCC (dash line) in a quiescent citrate buffer (pH 5, 1.5 M) under an atmosphere of air or argon (dotted line) ($25 \pm 2\text{ }^{\circ}\text{C}$). The O_2 was passively supplied from the air to the electrode. The ratio of PTFE:KB was set to be 1:1 according to a previously described method [31]. The onset potential was approximately 0.6 V at both BOD-adsorbed electrodes. As shown in Fig. 2(A), compared to BOD/KB/WPCC, a much sharper sigmoidal curve was observed at BOD/2-ABA/KB/WPCC. This means kinetic improvement in DET-type bioelectrocatalysis using BOD at the negatively charged 2-ABA-modified KB surface [9, 31, 49]. As shown in Fig. S1, the electron-accepting site (T1) in BOD is located in the positively charged region of the protein surface [82]. Thus, it can be considered that the electrostatic interaction between the positively charged T1 of BOD and the negatively charged electrode surface produced by the carboxy substituent of 2-ABA induces a productive orientation for DET-type bioelectrocatalysis. In addition, the π - π

interaction between BOD and the benzene ring of 2-ABA may assist the productive orientation [49] that improves the kinetic performance in the interfacial electron transfer.

Fig. 2 (A) CVs of BOD/KB/WPCC (broken line) and BOD/2-ABA/KB/WPCC (solid line) at $\nu = 5 \text{ mV s}^{-1}$. (B) CAs of BOD/KB/WPCC (broken line) and BOD/2-ABA/KB/WPCC (solid line) at 0 V. The inset shows the CAs at 0.4 V. Measurements were carried out in 1.5 M citrate buffer (pH 5.0) at room temperature ($25 \pm 2^\circ\text{C}$) under quiescent and air atmospheric conditions. The ratio of PTFE to KB was 1:1. The dotted line in panel A shows the CV of BOD/KB/WPCC under argon atmospheric conditions.

Fig. 2 (B) shows chronoamperograms (CAs) of BOD/KB/WPCC and BOD/2-ABA/KB/WPCC at 0 V. A steady-state catalytic current density was recorded

as $7.0 \pm 0.3 \text{ mA cm}^{-2}$ at BOD/KB/WPCC. At BOD/2-ABA/KB/WPCC, the catalytic current density reached about 30 mA cm^{-2} until about 5 s, but decreased quickly down to $8.0 \pm 0.1 \text{ mA cm}^{-2}$ at 60 s. Since similar behavior was observed in repeated experiments with the same electrode (data not shown), the current decrease after 5 s at BOD/2-ABA/KB/WPCC is not ascribed to a decrease in the enzyme activity but to some change in the bio-three-phase circumstance around the electrode surface during the bioelectrocatalysis at high current densities [31, 77].

In order to check the effect of time on the current density, CAs were measured at 0.4 V. As shown in the inset of Fig. 2 (B), relatively stable steady-state catalytic current was recorded at both of the electrodes and the current density at 20 s was 8.9 ± 0.8 and $5.3 \pm 0.3 \text{ mA cm}^{-2}$ at BOD/2-ABA/KB/WPCC and BOD/KB/WPCC, respectively. The data verify the significance of the 2-ABAmmodification at moderate current densities.

On the other hand, the hydrophobic property of the electrode surface is an important factor for improving the performance of gas-diffusion biocathodes [31, 77]. Although the optimum ratio of PTFE to KB was reported to be 2:3 for copper efflux oxidase [77] and 1:1 for BOD [31] in a gas-diffusion system, we re-optimized the ratio of PTFE to KB for BOD/2-ABA/KB/WPCC.

CVs of the O_2 reduction were recorded with BOD/KB/WPCC and BOD/2-ABA/KB/WPCC at various weight ratios of PTFE to KB in a range from 1:4 to 1:1 (Fig. S2). The ratio of 3:7 was found to be better for BOD/2-ABA/KB/WPCC. The effects of the ratio of PTFE to KB were also examined by chronoamperometry. The result is summarized in Fig. 3(A). At BOD/2-ABA/KB/WPCC, the best ratio was 3:7 and the catalytic current density reached $11.2 \pm 0.7 \text{ mA cm}^{-2}$ at 0.4 V, while the best ratio at BOD/KB/WPCC was 5:5, in accordance with a previous report [31]. We

hypothesize that modification with 2-ABA increased the hydrophobicity of the KB-modified electrode surface. The hydrophobic properties of KB/WPCC and 2-ABA/KB/WPCC surfaces were roughly estimated by measuring the contact angles of the enzyme solution on the electrode surface. As shown in Fig. 3 (B), a larger contact angle was observed on the 2-ABA-modified KB/WPCC surface compared to a pristine KB/WPCC surface. These results support our hypothesis. In this study, we set the weight ratio of PTFE to KB as 3:7 for the gas-diffusion system used in further studies.

Fig. 3 (A) Effects of the ratio of PTFE to KB on the catalytic current densities at BOD/2-ABA/KB/WPCC (filled bar) and BOD/KB/WPCC (shaded bar). The current densities were obtained at 0.4 V after 20 s. Measurements were carried out in 1.5 M citrate buffer (pH 5.0) at room temperature ($25 \pm 2^\circ\text{C}$) under quiescent and air atmospheric conditions. (B) Photographs to show the contact angle of the enzyme solution on KB/WPCC (θ_1) and 2-ABA/KB/WPCC (θ_2). The ratio of PTFE to KB was 1:1 in this experiment.

3.2 DET-type bioelectrocatalysis of MBH at amine-modified electrode

Fig. 4 shows rotating disk CVs of H_2 oxidation (in the potential region from -0.45 to $+0.2$ V) and H^+ reduction (in the potential region from -0.5 to -0.7 V) catalyzed by MBH on p -PDA/KB/GCE, KB/GCE and 4-ABS/KB/GCE. No catalytic current was observed in the absence of MBH. The zero-current potential in the catalytic waves was -0.49 V, which corresponds to the formal potential of the $H_2/2H^+$ redox couple at pH 5.0 due to the reversible and bi-directional catalytic properties of MBH [60].

Fig. 4 CVs at MBH/ p -PDA/KB/GCE (solid line), MBH/KB/GCE (broken line), MBH/4-ABS/KB/GC (dashed-dotted line) and KB/GCE without MBH (dotted line). All measurements were carried out in an H_2 -saturated McIlvaine buffer (pH 5.0, 40 °C) at $\omega = 2000$ rpm and $\nu = 20$ mV s $^{-1}$.

Interestingly, the H^+ -reduction and H_2 -oxidation currents increased with modification using p -PDA on KB/GCE, but decreased with modification using 4-ABS, as compared to unmodified KB/GCE. p -PDA/KB/GCE is expected to produce a positively charged surface, while 4-ABS/KB/GCE produces a negatively charged surface, according to our

previous study[49, 81]. On the other hand, as shown in Fig. S1, the distal FeS cluster (that works as the electron-donating site for the H₂ oxidation and the electron-accepting site for the H⁺ reduction) is located in the negatively charged region of the protein surface. Therefore, the attractive electrostatic interaction between the negatively charged FeS cluster region and the positively charged electrode surface seems to induce MBH to face the distal FeS to the electrode surface. In contrast, at a negatively charged electrode surface produced by modification with 4-ABS, a repulsive electrostatic interaction occurs with the distal FeS cluster region and leads to orientations detrimental for DET-type bioelectrocatalysis of MBH [49].

Further experimental evidence was obtained in favor of the electrostatic interaction between MBH and modified electrode surfaces. The steady-state H₂-oxidation current was measured at −0.3 V with MBH-adsorbed electrodes that were modified with several aniline derivatives. The results are summarized in Fig. S3. Modification with non-charged MABA caused practically no change in the current density compared to pristine KB/GCE. Modification with 4-ABA slightly decreased the current density due to the electrostatic repulsion between the partially deprotonated carboxy substituent of 4-ABA and the distal FeS cluster region.

We also tried to weaken the electrostatic attractive interaction between the positively charged *p*-PDA/KB/GCE and the distal FeS cluster region by increasing the ionic strength (and then by screening the ionic atmosphere). As shown in Fig. S4, the catalytic density decreased to the level of MBH/KB/GCE when MBH was adsorbed on *p*-PDA/KB/GCE in the presence of 1 M KCl. All these results support our hypothesis.

The catalytic turnover of MBH is sufficiently fast and the saturated concentration of H₂ is low. Therefore, the mass transport of H₂ strongly influences the bioelectrocatalytic

density[69]. In order to adequately assess the effect of *p*-PDA modification on the catalytic activity of MBH, rotating disk voltammetry was carried out. The observed density of steady-state limiting catalytic current density (*j*) at a rotating disk electrode can be given by Koutecký–Levich equation as

$$\frac{1}{j} = \frac{1}{j_d} + \frac{1}{j_{\text{cat}}}, \quad (1)$$

$$j_d = 0.62nFD^{2/3}\nu^{-1/6}c\omega^{1/2}, \quad (2)$$

$$j_{\text{cat}} = (n/n_E)Fk_{\text{cat}}\Gamma_e, \quad (3)$$

where j_d and j_{cat} are the diffusion-limiting and catalytic limiting current densities, respectively. Additionally, n is the number of electrons for H₂ (= 2); F is Faraday's constant; D is the diffusion constant of H₂ in a buffer; ν is the kinematic viscosity of the buffer (0.0066 cm² s⁻¹ in 40 °C [83]); c is the bulk concentration of H₂ (0.74 mM in H₂-saturated solution at 40 °C and 1 atm) [84]; k_{cat} is the catalytic rate constant; n_E is the number of electrons of for enzyme in the electron-donating FeS site (=1 in this case); and Γ_e is the surface concentration of effective enzymes oriented conveniently for DET-type bioelectrocatalysis on the electrode. The steady-state limiting current of H₂oxidation was measured at various values of the rotating speed (ω) with MBH/*p*-PDA/KB/GCE and MBH/KB/GCE (Fig. 5S (A)). The Levich plot is shown in Fig. 5. The data were analyzed by Gnuplot[®] 5.0 based on Eq. (1). The refined values of j_{cat} are 20 ± 2 mA cm⁻² at *p*-PDA-KB/GCE and 8.4 ± 0.4 mA cm⁻² at unmodified KB/GCE with a D of $(7 \pm 1) \times 10^{-5}$ cm² s⁻¹. The $k_{\text{cat}}\Gamma_e$ are calculated as $(1.0 \pm 0.1) \times 10^{-7}$ mol cm⁻² s⁻¹ for MBH/*p*-PDA/KB/GCE, which is 2.5 times larger than that of MBH/KB/GCE ($(4.3 \pm 0.2) \times 10^{-8}$ mol cm⁻² s⁻¹). Thus, we conclude that modification with *p*-PDA to produce a positive charge on the KB electrode surface increases Γ_e of

MBH.

Fig.5 Levich plots of ω dependence of the steady-state H_2 oxidation current densities at the MBH/*p*-PDA/KB/GCE (closed circle) and MBH/KB/GCE (open circle). The current density was measured at -0.3 V and 40 °C, and the curves were evaluated by a nonlinear regression analysis with Eq. (1).

Similar analysis was also carried out for H^+ reduction at -0.7 V (Fig. S5(B)); however, H^+ reduction was almost independent of ω . The limited current densities reached -5.8 ± 0.4 mA cm $^{-2}$ at MBH/*p*-PDA/KB/GCE and are about 1.5 times larger than that at MBH/KB/GCE (-3.8 ± 0.3 mA cm $^{-2}$). The activity ratio is close but somewhat smaller than that of H_2 oxidation. At increased current densities of H^+ reduction, the dissociation kinetics of the weak acid (as a buffer component) may affect the overall kinetics (and decrease the current density) [85-87]. In addition, the H^+ -reduction catalytic activity of MBH may be inhibited by H_2 generated near the electrode surface [60, 88-90].

On the other hand, as shown in Fig. 4, the H_2 -oxidation current at the MBH-adsorbed electrodes decrease to zero at potentials over -0.2 V. This is ascribed to oxidative

inactivation (formation of Ni-B) of MBH at high potentials [31, 65]. In this study, a gas-diffusion system was employed to protect from oxidative inactivation according to the method described in our previous papers [31, 76], Fig. 6 shows CVs of MBH/*p*-PDA/KB/WPCC and MBH/KB/WPCC in the gas-diffusion system. Note here that the ratio of PTFE to KB was set as 3:7 according to optimization of the *p*-PDA/KB/WPCC-based gas-diffusion system as described above. Clear H₂-oxidation waves were observed at both MBH-adsorbed electrodes. The current density (at 0.2 V) for H₂ oxidation reached 15.8 mA cm⁻² at MBH/*p*-PDA/KB/WPCC and 5.8 mA cm⁻² at MBH/KB/WPCC. These values are much larger than those at the corresponding GCEs (MBH/*p*-PDA/KB/GCE and MBH/KB/GCE), respectively. In addition, the effects of oxidative inhibition at high potentials were successfully suppressed [76]. These results support the conclusion that H₂ gas is passively supplied at high speeds to the electrodes from the gas phase.

Fig. 6 CVs of MBH/*p*-PDA/KB/WPCC (solid line), MBH/KB/WPCC (broken line) and KB/WPCC (dotted line) at $\nu = 10 \text{ mV s}^{-1}$. Measurements were carried out in 1.5 M citrate buffer (pH 5.0) at room temperature ($25 \pm 2 \text{ }^{\circ}\text{C}$) under quiescent and H₂-atmospheric conditions. The ratio of PTFE to KB was 3:7.

3.3 DET-type H₂/air biofuel cell

Finally, a dual gas-diffusion-type H₂/air-breathing biofuel cell was fabricated by combining BOD/2-ABA/KB/WPCC as a biocathode and MBH/*p*-PDA/KB/WPCC as a bioanode, as shown in Fig. 1. In most H₂/O₂ biofuel cells in the literature, pure O₂ and H₂ gases are separately pumped to the biocathode and bioanode, respectively [20, 21, 23, 25, 27, 31], and the cells are operated at high temperatures [25, 28, 29, 31] to improve their performance; however, such situations risk explosion and are not convenient for practical applications. Therefore, in this study, only H₂ gas was supplied to the bioanode, while the biocathode was open to air at room temperature (25 ± 2 °C). The biofuel cell was operated in a 1.5 M citrate buffer (pH 5.0) under passive and quiescent conditions. The cell voltage, cathode potential, and anode potential were measured at (quasi)-steady state and at given values of resistance. The values were decreased stepwise and the potentials were recorded about 15 s after the change in the resistance.

Fig. 7(A) summarizes the results. The open circuit voltage was 1.12 ± 0.03 V, close to the standard driving force of an ideal H₂/O₂ cell (1.23 V at 25 °C) [20, 31]. The current density increased with a decrease in the voltage, and the maximum current density reached about 11 ± 1 mA cm⁻²; however, the current density decreases suddenly after reaching the maximum. The decrease may be caused by oxidative inactivation of MBH due to fast H₂-consumption near the electrode surface at increased current densities and at positive potentials [31]. The power density is given in Fig. 7 (B) and the maximum power density reached 6.1 ± 0.4 mW cm⁻² at a 0.72 V cell voltage, much higher than that of other H₂ biofuel cells in the literature [20-30].

Fig.7 (A) Polarization curves of the biofuel cell. The cell voltage (closed square) and the cathode (closed circle) and anode potentials (open circle) (vs. $\text{Ag}|\text{AgCl}|\text{KCl}(\text{sat.})$) are plotted as functions of the current density (j). The measurements were performed in 1.5 M citrate buffer (pH 5.0) under quiescent conditions. H_2 gas (1 atm) was supplied to the outside of the bioanode, while the biocathode was opened to air atmosphere (in a passive mode) at room temperature ($25 \pm 2^\circ\text{C}$). (B) The power density (P) as a function of the current density (j) of the biofuel cell.

4 Conclusions

To the best of our knowledge, this is the first report of a membrane- and mediatorless dual gas-diffusion-type H_2 /air-breathing biofuel cell. The orientation of BOD and MBH

was successfully controlled by the electrostatic attractive-interaction between the enzymes and electrodes. Aniline derivatives were utilized to control the surface charge in which the amino group of the aniline derivatives was electrochemically oxidized at the carbon electrodes to form N-C bonds to immobilize the derivatives. For BOD with positively charged electron-accepting sites (T1), 2-ABA was modified on KB-modified electrodes and the deprotonated carboxy group of 2-ABA is responsible for the negative charge on the electrode surface. In contrast, for MBH with negatively charged electron-donating sites (distal FeS), *p*-PDA was modified on KB-modified electrodes and the protonated amino group of *p*-PDA is responsible for the positive charge of the electrode surface at pH 5.0.

A gas-diffusion system was employed using KB/WPCC and the hydrophobicity required for gas permeation through the electrode was controlled by adjusting the ratio of PTFE:KB.

The combination of BOD/2-ABA/KB/WPCC as a biocathode and MBH/*p*-PDA/KB/WPCC as a bioanode realizes a dual gas-diffusion membrane- and mediatorless H₂/air-breathing biofuel cell. The maximum power density reached 6.1 mW cm⁻² at a 0.72 V cell voltage at room temperature (25 ± 2°C) and under quiescent conditions. The performance is comparable to that of a dual gas-diffusion type virtual biofuel cell at 40 °C with pure H₂ and O₂ gases (8.4 mW cm⁻² [31]), and is better than those of real biofuel cells in the literature (0.56 mW cm⁻² at 25 °C with 78% H₂ + 22% air under bubbling[26], 0.72 mW cm⁻² at 45 °C with pure H₂ and air under gas-diffusion [29], 1.5 mW cm⁻² at 60 °C with pure H₂ and O₂ gases and with a membrane as separator [27]). The air-breathing BOD biocathode with improved performance can be utilized for a variety of biofuel cells with fuels other than H₂.

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Figures Captions

Fig. 1 Schematic illustration of a gas-diffusion type H₂/air-breathing biofuel cell.

Fig. 2 (A) CVs of BOD/KB/WPCC (broken line) and BOD/2-ABA/KB/WPCC (solid line) at $\nu = 5 \text{ mV s}^{-1}$. (B) CAs of BOD/KB/WPCC (broken line) and BOD/2-ABA/KB/WPCC (solid line) at 0 V. The inset shows the CAs at 0.4 V. Measurements were carried out in 1.5 M citrate buffer (pH 5.0) at room temperature ($25 \pm 2^\circ\text{C}$) under quiescent and air atmospheric conditions. The ratio of PTFE to KB was 1:1. **The dotted line in panel A shows the CV of BOD/KB/WPCC under argon atmospheric condition.**

Fig. 3 (A) Effects of the ratio of PTFE to KB on the catalytic current densities at BOD/2-ABA/KB/WPCC (filled bar) and BOD/KB/WPCC (shaded bar). The current densities were obtained at 0.4 V after 20 s. Measurements were carried out in 1.5 M citrate buffer (pH 5.0) at room temperature ($25 \pm 2^\circ\text{C}$) under quiescent and air atmospheric conditions. (B) Photographs to show the contact angle of the enzyme solution on KB/WPCC (θ_1) and 2-ABA/KB/WPCC (θ_2). The ratio of PTFE to KB was 1:1 in this experiment.

Fig. 4 CVs at MBH/*p*-PDA/KB/GCE (solid line), MBH/KB/GCE (broken line), MBH/4-ABS/KB/GC (dashed-dotted line) and KB/GCE without MBH (dotted line). All measurements were carried out in an H₂-saturated McIlvaine buffer (pH 5.0, 40 °C) at $\omega = 2000 \text{ rpm}$ and $\nu = 20 \text{ mV s}^{-1}$.

Fig.5 Levich plots of ω dependence of the steady-state H₂ oxidation current densities at the MBH/*p*-PDA/KB/GCE (closed circle) and MBH/KB/GCE (open circle). The current density was measured at -0.3 V and 40°C , and the curves were evaluated by a nonlinear regression analysis with Eq. (1).

Fig. 6 CVs of MBH/*p*-PDA/KB/WPCC (solid line), MBH/KB/WPCC (broken line) and KB/WPCC (dotted line) at $\nu = 10 \text{ mV s}^{-1}$. Measurements were carried out in 1.5 M citrate buffer (pH 5.0) at room temperature ($25 \pm 2^\circ\text{C}$) under quiescent and

H₂-atmospheric conditions. The ratio of PTFE to KB was 3:7.

Fig.7 (A) Polarization curves of the biofuel cell. The cell voltage (closed square) and the cathode (closed circle) and anode potentials (open circle) (vs. Ag|AgCl|KCl (sat.)) are plotted as functions of the current density (j). The measurements were performed in 1.5 M citrate buffer (pH 5.0) under quiescent conditions. H₂ gas (1 atm) was supplied to the outside of the bioanode, while the biocathode was opened to air atmosphere (in a passive mode) at room temperature (25 ± 2 °C). (B) The power density (P) as a function of the current density (j) of the biofuel cell.

Graphical Abstract