

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

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Abstract

Diaphorase catalyzes efficiently the oxidation of NADH to NAD^+ by benzylviologen. This reaction was used to regenerate the NAD^+ required for the biocatalyzed oxidation of methanol to CO_2 using NAD^+ -dependent dehydrogenases. A methanol/dioxygen biofuel cell was assembled using these bioreactions in the anode compartment. The open-circuit voltage of the cell was 0.8 V, and the maximum power output of the cell was 0.67 mW/cm^2 of graphite at 0.49 V. These results reflect the low overpotential at which NADH is oxidized and demonstrate a new approach to lowering voltage losses in biofuel cells due to activation overpotentials. © 1998 Elsevier Science S.A.

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1. Introduction

The efficiency of conversion of chemical energy into electrical energy via combustion is limited by thermodynamics, and is usually analyzed in terms of the Carnot cycle. In theory, fuel cells are thermodynamically more efficient than combustion engines because they are not restricted to Carnot efficiencies. A fuel cell functions by the oxidation of fuel at the anode, simultaneous transfer of electrons and protons to the cathode compartment, and the reduction of a second fuel (typically dioxygen) at the cathode. The power output of a fuel cell is a function of the rate of transfer of electrons through an external circuit (I_{cell}) and the potential difference between the anode and the cathode (V_{cell}).

Presently, the most efficient fuel cells use dihydrogen and dioxygen gas as fuel, transition metal electrocatalysts, and moderate to high operating temperatures. The disadvantages of dihydrogen/dioxygen fuel cells are the cost, engineering and safety associated with the generation, transport, and storage of dihydrogen; and the consequential

need for expensive catalysts, membranes and materials. Methanol is an attractive alternative to dihydrogen as the anodic fuel because it is readily available and easy to transport and store. Moreover, the theoretical $V_{\text{cell max}}$ for a methanol/dioxygen fuel cell (1.19 V) is near that of a dihydrogen/dioxygen fuel cell (1.23 V). Several challenges remain, however, that prevent the use of methanol in fuel cells: development of catalysts that minimize the high overpotentials associated with its oxidation; prevention of incomplete oxidation (e.g., carbon monoxide poisoning of transition metal catalysts); and elimination of fuel 'crossover' between the anode and cathode compartments (common to all liquid fuel cells) [1].

Biocatalysts are an attractive alternative to transition metal catalysts because they oxidize methanol to carbon dioxide at low overpotentials without producing carbon monoxide; they are catalytic at moderate temperatures;³ they are renewable;⁴ and in principle, could be developed for the oxidation of a variety of fuels (e.g., methanol, alkanes, sugars) in the anode compartment and the reduction of dioxygen in the cathode compartment. Additionally, the moderate temperatures for biocatalysis allows for the use of inexpensive fuel cell components.

Fuel cells that use biocatalysts have been described in the literature [2]. These include microbial biofuel cells where the entire organism is used as the catalyst; [3–19]

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Table 1

Operating conditions and performance of CH₃OH/O₂ enzymatic biofuel cells^a

Ref.	Experimental conditions and results ^a
[26]	<i>methanol dehydrogenase</i> oxidizing methanol and formaldehyde using PES ($E^\circ = 0.055$ vs. SCE, pH 7.0) or PMS ($E^\circ = 0.080$ vs. SCE, pH 7.0) as mediator, fuel cell operated at pH 9.5: Pt-gauze anode (50 mesh, 7 cm ²); Pt-gauze cathode (80 mesh, 4.4 cm ²); 0.3 V at open-circuit; 3.7 mA at 10 Ω ; calculated power density = 19 $\mu\text{W}/\text{cm}^2$
[27]	PQQ-linked <i>alcohol dehydrogenase</i> oxidizing methanol using TMPD ($E^\circ = 0.12$ vs. SCE) as mediator, fuel cell operated at pH 10.5: Pt-gauze anode (50 mesh, 7 cm ²); Pt-gauze cathode (80 mesh, 4.4 cm ²); 0.16 V at 133 k Ω ; 20 $\mu\text{A}/\text{cm}^2$ at 10 Ω and 20°C; calculated power density = 28 nW/cm ²
[28]	<i>methanol and formate dehydrogenase</i> oxidizing methanol using PMS or PES as mediator: carbon fabric anode and cathode (40 cm ² each); open-circuit voltage not given; 0.9 mA at 10 Ω and 0.065 V; calculated power density = 1.5 $\mu\text{W}/\text{cm}^2$

^aPower density was calculated from the information given in the reference and is based on the area of the anode. The calculated power density cannot be assumed to be the maximum value without additional current-voltage characterization of the fuel cell.

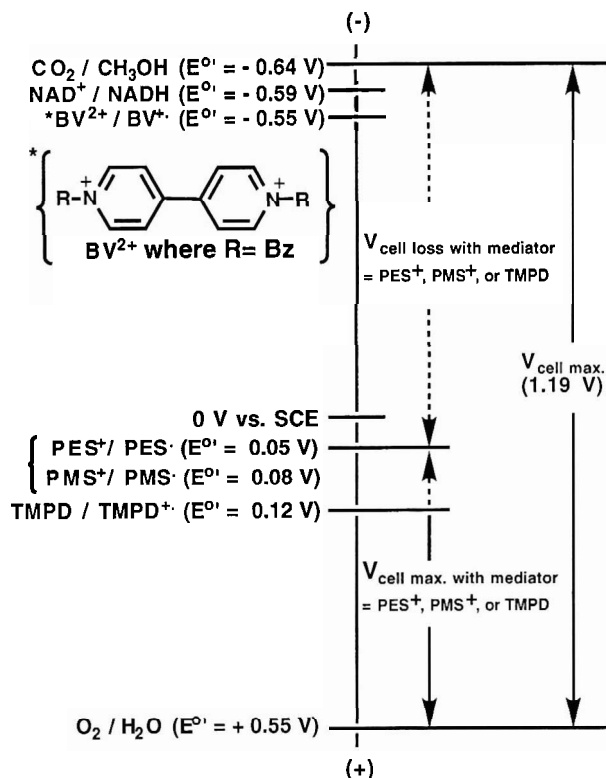
photomicrobial biofuel cells where a photosynthetic organism is used as the catalyst; [20–22] and enzymatic biofuel cells where enzymes, both in solution or immobilized, are used as the catalyst [23–28]. We know of three studies describing enzymatic biofuel cells that used methanol as the anodic fuel and dioxygen as the cathodic fuel (Table 1) [23–25]. The enzymes used in these studies catalyzed the oxidation of methanol without NAD⁺ or other cofactors and were active with redox mediators such as phenazine methosulfate (PMS), phenazine ethosulfate (PES), or *N,N,N',N'*-tetramethyl-4-phenylenediamine (TMPD). Although electrical power was generated by these enzymatic biofuel cells, all studies report low values for I_{cell} and V_{cell} . The poor performance of these fuel cells was caused mainly by the use of redox mediators with high formal potentials to shuttle electrons from the enzyme to the anode (Scheme 1) [29].⁵ Presumably, these redox mediators were chosen because other mediators with lower formal potentials (nearer to E° of CO₂/CH₃OH) were inactive with the enzymes. Consequently, $V_{\text{cell max.}}$ for these fuel cells was limited to < 0.5 V.

To overcome the problem of low cell voltage, we used NAD⁺-dependent enzymes to catalyze the oxidation of methanol to CO₂. The oxidation of one equivalent of methanol to carbon dioxide by these enzymes requires the

³ Catalytic rates of 0.1 to 10 U mg⁻¹ of enzyme at temperatures between 10–100°C are common where 1 U \equiv 1 \times 10⁻⁶ mol substrate oxidized per minute.

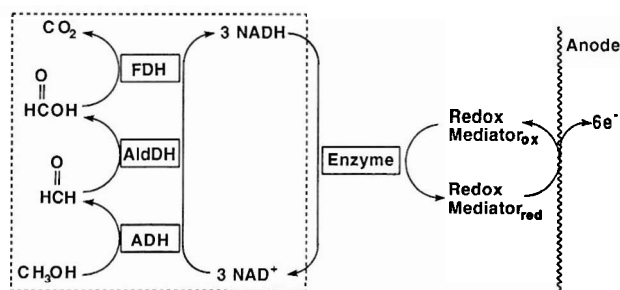
⁴ For example, inexpensive enzymes are routinely produced by fermentation for application in the paper, wine-making and detergent industries.

⁵ An additional problem with the mediators chosen is the slow decomposition of both PES and PMS.



Scheme 1. Formal potentials (vs. SCE at pH 7.5) of CO₂/CH₃OH, NAD⁺/NADH, BV²⁺/BV^{•+} and the redox mediators used in the enzyme catalyzed biofuel cells listed in Table 1. All mediators shown, with the exception of BV²⁺, have formal potentials substantially more oxidizing than the formal potential of CO₂/CH₃OH and consequently, limit $V_{\text{cell max.}}$ to < 0.5 V.

attendant reduction of three equivalents of NAD⁺ to NADH (contents of box in Scheme 2). The NADH cofactor itself, however, is not a useful redox mediator. At platinum or glassy carbon electrodes, NADH is typically oxidized \sim 1 V positive to its formal potential [30–33]. For the purpose of a biofuel cell, the method used to regenerate NAD⁺ must have the following properties: (1) it must be catalytic; (2) it must release reducing equivalents close to E° of NAD⁺/NADH; and (3) it must not



Scheme 2. The oxidation of CH₃OH to CO₂ is catalyzed by NAD⁺-dependent alcohol- (ADH), aldehyde- (AldDH), and formate- (FDH) dehydrogenases (shown within the box). Regeneration of NAD⁺ is accomplished electro-enzymatically with an enzyme coupled to the anode via a redox mediator.

deactivate the cofactor or produce a chemical by-product that will accumulate in the anode compartment of the fuel cell. The reported methods to regenerate NAD^+ do not satisfy these three requirements simultaneously [34–46].

Our approach to the catalytic regeneration of NAD^+ with minimal overpotential was to use in the anode compartment of a fuel cell: (1) a redox mediator that has fast kinetics of heterogeneous electron transfer at or near the formal potential of the NAD^+/NADH couple; and (2) a biocatalyst that lowers the activation barrier for electron transfer between NADH and the redox mediator (Scheme 2). This paper describes how benzylviologen is used to regenerate the NAD^+ cofactor using diaphorase as the catalyst for homogeneous electron transfer. We demonstrate the application of this chemistry in the anode compartment of an enzymatic biofuel cell that uses NAD^+ -dependent dehydrogenases to catalyze the oxidation of methanol to carbon dioxide.

2. Experimental

2.1. Chemicals

Diaphorase ⁶ (E.C.1.6.4.3) (*Clostridium kluyveri*) was purchased from Sigma and UCB; alcohol dehydrogenase (E.C.1.1.1.1), aldehyde dehydrogenase (E.C.1.2.1.5), formate dehydrogenase (E.C.1.2.1.2), nicotinamide adenine dinucleotide, both the oxidized (NAD^+) and reduced form (NADH), and Tris[®] buffer salts were purchased from Sigma. All biological reagents were stored at 0°C prior to use. Benzylviologen (1,1'-dibenzyl-4,4'-bipyridinium dichloride) was purchased from Aldrich and recrystallized twice from ethanol/ H_2O . HPLC grade methanol and proton exchange membrane (Nafion[®] 117, perfluorinated membrane, 0.007 in. thick) were purchased from Aldrich and used as received. Platinum gauze and graphite were purchased from Johnson Matthey.

2.2. Activity of diaphorase

The activity of diaphorase catalyzing the oxidation of NADH using BV^{2+} as the electron acceptor was determined using a modified published procedure [47]. The absorbance of a deoxygenated solution of 0.2 mM NADH, 2.0 mM BV^{2+} , 2×10^{-6} M diaphorase in 0.25 M Tris buffer, pH 7.5, was monitored at 600 nm (BV^{+} , $\epsilon = 10,100 \text{ M}^{-1} \text{ cm}^{-1}$) using a Hitachi U-3210 spectrophotometer. The specific activity of diaphorase ($64 \text{ mU mg}^{-1} \text{ solid}$) was calculated using the slope of an absorbance vs. time plot within the first minute of the assay.

2.3. Stoichiometry of reaction between NADH and BV^{2+}

Measurements were performed on a solution containing 0.25 mM NADH, 5.0 mM BV^{2+} , 6.5×10^{-6} M diaphorase in 0.25 M Tris buffer, pH 7.5. Two separate experiments were conducted. The first experiment determined the concentration of NADH by monitoring the absorbance at 340 nm ($\epsilon = 6300 \text{ M}^{-1} \text{ cm}^{-1}$). Dioxygen was included in this experiment to prevent interference from strong absorption due to BV^{+} . To resolve the absorbance maximum of NADH at 340 nm, difference spectra (spectrum of BV^{2+} subtracted) are shown. The second experiment determined the concentration of BV^{+} formed by monitoring the absorbance at 600 nm ($\epsilon = 10,100 \text{ M}^{-1} \text{ cm}^{-1}$). Dioxygen was rigorously excluded in this experiment.

2.4. Measurement of efficiency of regeneration of NAD^+

An electrolytic cell, comprised of two glass compartments closed to the atmosphere by rubber septa and separated by a proton exchange membrane, was used to measure the efficiency of regenerating NAD^+ electro-enzymatically. The initial composition of the deoxygenated anolyte (49 ml) was 0.7 mM NADH, 10 mM BV^{2+} , and 6.8×10^{-6} M diaphorase in 0.25 M Tris buffer, pH 7.5. At 10 min intervals, a 100 μl aliquot of anolyte was removed from the electrolytic cell using an airtight Hamilton Teflon[®] microsyringe, injected into a sealed quartz cuvette (0.1 cm pathlength, purged with argon) and the absorbance measured at 365 nm (NADH, $\epsilon = 3400 \text{ M}^{-1} \text{ cm}^{-1}$). The charge collected by the anode (graphite felt, $\sim 2.25 \text{ cm}^3$) was monitored with an Electrosynthesis Model 630 coulometer positioned in series between the anode and cathode (Pt-gauze in 0.25 M Tris buffer, pH 7.5, saturated with dioxygen) of the electrolytic cell. The load during cell operation was 11.2 Ω .

2.5. Prototype methanol / dioxygen biofuel cell

A prototype fuel cell was fabricated from glass where the capacity of the anode and cathode compartments were 75 and 50 ml, respectively. The anode and cathode compartments were separated by a Nafion[®] 117, perfluorinated membrane, 0.007 in. thick. The overall dimensions of the fuel cell were 10 cm \times 5 cm \times 5 cm. The anode was a graphite plate with a geometric surface area of 2 cm^2 . The cathode was platinum gauze (3 cm^2 , 52 mesh), electrodeposited with platinum-black.⁷ The anolyte (0.1 M $\text{CH}_3\text{OH} + 5 \text{ U FDH} + 40 \text{ U AldDH} + 11 \text{ U ADH} + 1.0$

⁶ Supplied by Sigma as 5.9 U/mg solid or 6.9 U/mg protein, where 1 U is defined as the amount of enzyme that will oxidize 1×10^{-6} mol of NADH per minute at pH 7.5 at 25°C, with the corresponding reduction of dichlorophenolindophenol.

⁷ The gauze was cleaned with 30% conc. H_2O_2 / 70% conc. H_2SO_4 for 5 min followed by electrodeposition (using a Princeton Applied Research Model 273 potentiostat/galvanostat coupled to a Houston Instruments RE0074 X-Y recorder) of platinum from a solution of 5 mM K_2PtCl_4 dissolved in 1 M HClO_4 . The blackened cathode was rinsed with deionized, distilled water prior to use.

mM NAD^+ + 50 mM BV^{2+} + 1.7 U diaphorase + 1 M LiCl in 0.1 M Tris buffer, pH 7.5) was purged with argon for 20 min prior to operation of the fuel cell and blanketed with argon during operation. The catholyte (1 M LiCl, 0.1 M Tris buffer, pH 7.5) was continuously saturated with O_2 for the duration of the experiment. The potential of the anode and cathode were referenced to a saturated calomel electrode (SCE) located within the anode and cathode compartments, respectively. The fuel cell was operated under external loads by placing a resistor (ranging between 51 Ω and 5 k Ω) in series between the anode and cathode.

3. Results and discussion

3.1. Specific activity of diaphorase using benzylviologen as the acceptor

Our first objective was to identify an enzyme that would catalyze the oxidation of NADH to NAD^+ in the presence of an electroactive acceptor whose formal potential was near that of NAD^+/NADH . Diaphorase is classified as an oxidoreductase that oxidizes NADH or NADPH using a disulfide compound as acceptor [48]. The mechanism of electron transfer is not known; both protein-bound flavin adenine dinucleotide (FAD) and a disulfide–dithiol redox couple, however, appear to be involved in the catalytic reaction [49]. Several electro-active acceptors have been coupled to the diaphorase catalyzed oxidation of NADH including ferricyanide, methylene blue, and 2,6-dichlorophenolindophenol [50–53]. The breadth of acceptable substrates for diaphorase suggests low specificity for the acceptor molecule. These results led us to investigate other electroactive acceptors with formal potentials near that of the NAD^+/NADH couple. At pH 7.5, where both NAD^+ and NADH are chemically stable, E° of NAD^+/NADH is -0.59 V vs. SCE (see Scheme 1). The electro-active 4,4'-bipyridinium salts are known to have formal potentials near this value and are often used as electron donors to reduce NAD^+ to NADH in the presence of diaphorase [54,55]. The formal potential of benzylviologen (BV^{2+}), however, is just positive (-0.55 V vs. SCE) of the NAD^+/NADH couple [56]. Consequently, diaphorase was found to catalyze the oxidation of NADH to NAD^+ by benzylviologen (activity = 64 mU mg^{-1} solid).⁸ In terms of current, this activity corresponds to ~ 0.2 mA per mg of diaphorase.⁹ Other derivatives of 4,4'-bipyridinium were screened as possible acceptors for

diaphorase (*N,N'*-dimethyl-4,4'-bipyridinium; *N,N'*-*p*-benzylsulphonic acid-4,4'-bipyridinium; *N,N'*-(propyl-3-sulphonic acid)-4,4'-bipyridinium). None, however, were active electron acceptors with diaphorase due to their more negative formal potentials than that of the NAD^+/NADH couple (i.e., oxidation of NADH by these derivatives is thermodynamically unfavorable).

3.2. Stoichiometry of the redox reaction between NADH and BV^{2+} catalyzed by diaphorase

The conversion of NADH to NAD^+ is a $2\text{e}^-/1\text{H}^+$ oxidation while the conversion of BV^{2+} to $\text{BV}^{+\cdot}$ is a 1e^- reduction. The stoichiometric ratio of the redox reaction between NADH and BV^{2+} , therefore, should be 1:2 and is confirmed by the results shown in Fig. 1. Fig. 1a shows the change in absorbance of a solution containing NADH, BV^{2+} , and diaphorase in the presence of dioxygen (included to prevent interference from the strong absorbance of $\text{BV}^{+\cdot}$ at 340 nm).¹⁰ Fig. 1b shows the change in absorbance of a solution equivalent to that used in Fig. 1a, except in the absence of dioxygen. From these spectra, we determined that 1.5×10^{-9} mol NADH (340 nm) were oxidized and 3.0×10^{-9} mol BV^{2+} (600 nm) were reduced per minute, confirming that each molecule of NADH oxidized results in the reduction of two molecules of BV^{2+} .

3.3. Efficiency of electro-enzymatic oxidation of NADH

To determine what percentage of reducing species released from the oxidation of NADH were collected at the anode, we compared spectroscopic and coulometric data during operation of an electrolytic cell containing NADH, BV^{2+} and diaphorase in the anode compartment. Spectroscopic data were obtained by measuring the absorbance of an aliquot of anolyte removed at different intervals. Coulometric data were obtained by positioning a coulometer in series between the anode and cathode and measuring the charge that passed through the external circuit of the cell. Fig. 2 shows the results obtained from these measurements. The amount of NADH oxidized was determined from the change in absorbance at 365 nm. From the slope of a line fitted to the absorbance vs. time data, an approximate rate of oxidation of NADH was obtained (1.59×10^{-7} mol min^{-1}), which corresponds to 0.031 C min^{-1} .¹¹ Similarly, from the slope of a line fitted to the coulometric data, an approximate rate at which the anode collected reducing species was obtained (0.033 C min^{-1}). Within experimental error, the absorbance and coulometric data

⁸ The activity of different samples of diaphorase ranged between 7–70 mU mg^{-1} solid depending on the commercial source or production lot. Samples of diaphorase with activities > 50 mU mg^{-1} solid were used for the experimental results given.

⁹ When NADH ($2\text{e}^-/\text{mol}$) is the substrate, a unit of activity is equivalent to 3.22 mA.

¹⁰ The absorption spectrum of NADH does not change in the absence of BV^{2+} , which indicates that diaphorase does not catalyze the oxidation of NADH to NAD^+ by dissolved dioxygen.

¹¹ 0.5 mol NADH (1 mol electrons) is proportional to 96,487 coulombs.

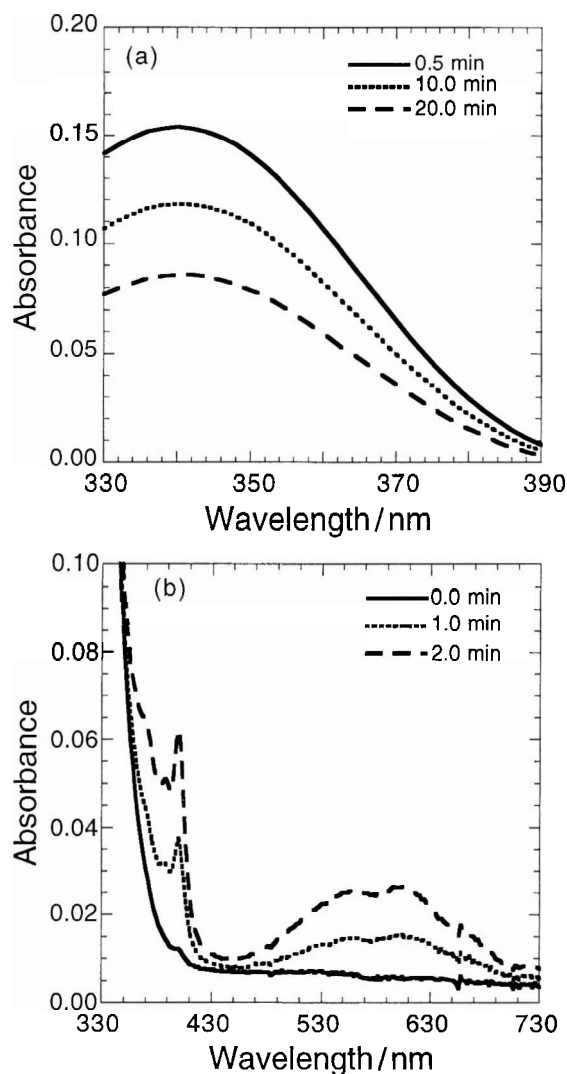


Fig. 1. Absorption spectra used to determine the stoichiometry of the reaction between NADH and BV^{2+} catalyzed by diaphorase. Cuvette (0.1 cm pathlength) contained a solution of 0.25 mM NADH, 5 mM BV^{2+} , and 6.5×10^{-6} M diaphorase buffered with 0.25 M Tris, pH 7.5. (a) difference spectra (absorbance due to BV^{2+} is subtracted) illustrating the oxidation of NADH (dioxxygen present); (b) spectra illustrating the reduction of BV^{2+} to $BV^{+ \cdot}$ (dioxxygen absent).

indicate that the electro-enzymatic oxidation of NADH is highly efficient. We note, however, that the rate of oxidation of NADH or collection of charge at the anode was lower ($\sim 50\%$) than expected based on the amount of diaphorase added to the electrolytic cell. We can only speculate as to the cause of this result, but it is plausible that adsorption of diaphorase onto the surface of Nafion[®] (20 cm² geometric area) prevents the access of substrate or mediator to its active site.¹²

¹² Both in situ impedance and ex situ X-ray photoelectron spectroscopy studies indicate irreversible adsorption of protein on samples of Nafion[®] exposed to protein containing solutions, unpublished results.

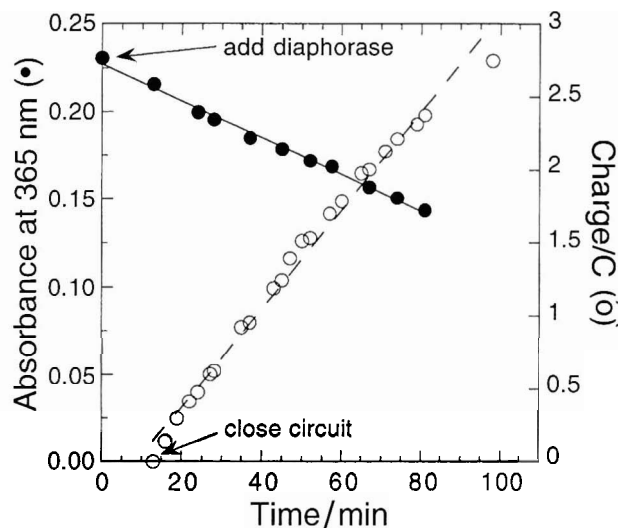
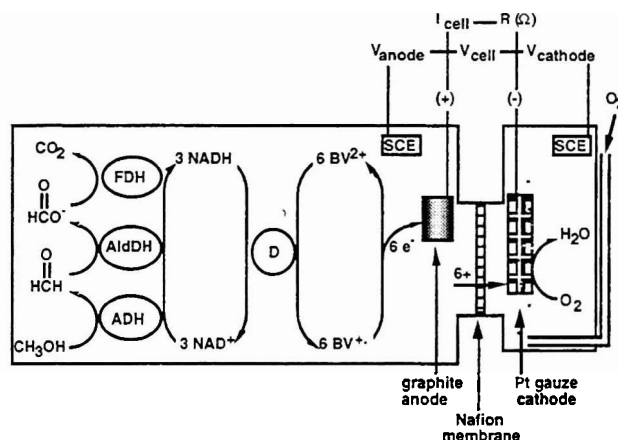


Fig. 2. Measurement of the efficiency of the electro-enzymatic regeneration of NAD⁺. An electrolytic cell was configured with a graphite felt anode immersed in anolyte comprised of NADH, BV^{2+} , and diaphorase in 0.25 M Tris buffer and a Pt-gauze cathode immersed in 0.25 M Tris buffer. Both the anolyte and catholyte were adjusted to pH 7.5 and continuously purged with argon and dioxxygen, respectively. The rate of oxidation of NADH was determined from the slope of the line fitted to the absorbance data. The rate of collection of reducing species at the anode was determined from the slope of the line fitted to the coulometric data.

3.4. Methanol / dioxxygen biofuel cell

An enzymatic biofuel cell was operated using NAD⁺-dependent dehydrogenases to catalyze the oxidation of methanol to carbon dioxide and diaphorase to catalyze the oxidation of NADH to NAD⁺ using benzylviologen as the electron acceptor (Scheme 3). Reduced benzylviologen ($BV^{+ \cdot}$) was oxidized at a graphite anode; dioxxygen was



Scheme 3. Schematic of methanol/dioxxygen biofuel cell. NAD⁺-dependent dehydrogenases oxidize CH_3OH to CO_2 ; diaphorase (D) catalyzes the oxidation of NADH to NAD⁺ using benzylviologen as the electron acceptor; $BV^{+ \cdot}$ is oxidized to BV^{2+} at a graphite anode and thus, releases reducing species for the reduction of dioxxygen at a platinum cathode.

reduced at a platinum-black cathode. Positive ions passed through the Nafion[®] membrane from the anode to cathode compartment to complete the circuit. Potentials of the anode (V_{anode}) and cathode (V_{cathode}) were measured vs. a saturated calomel electrode (SCE).

Fig. 3 shows the current-voltage behavior of the biofuel cell within the first minute of closed circuit at different external loads ($5 \text{ k}\Omega$ to $51 \text{ }\Omega$). The relative amounts of each of the four enzymes were chosen to minimize the concentration of formaldehyde in the anolyte. Under these conditions, the rate of NAD^+ reduction exceeded ($\text{rate}_{\text{max}} = 56 \text{ }\mu\text{mol min}^{-1}$) the rate of NADH oxidation (maximum rate = $1.7 \text{ }\mu\text{mol min}^{-1}$).¹³ The rate determining step in the biofuel cell, therefore, is the oxidation of NADH to NAD^+ and the concomitant reduction of BV^{2+} to BV^{+} . The biofuel cell was operated at room temperature in the absence of external pumps or stirrers and consequently, concentration polarization at the anode was observed as BV^{+} was depleted. A steady current and voltage could be maintained for longer periods of time with stirring. Depletion of BV^{+} (with the consequential drop in current and voltage) eventually occurs with continuous load because the rate of electrochemical oxidation of BV^{+} was faster than its production. The voltage of the biofuel cell (over an 8 h period) repeatedly returned to its initial open-circuit value after removal of load, indicating the continuous biocatalytic production of BV^{+} . Evolution

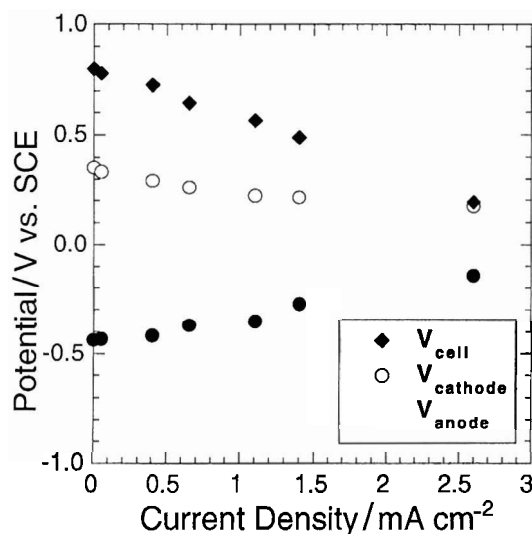


Fig. 3. V_{cell} (\blacklozenge) (the potential difference across the fuel cell), V_{cathode} (\circ) and V_{anode} (\bullet) are plotted against current density (per cm^2 of graphite). V_{cathode} and V_{anode} are vs. SCE, V_{cell} is not IR -corrected.

¹³ Use of a cathode with high surface area (platinum-blackened platinum gauze) insured that the reduction of dioxygen proceeded faster than the rate limiting reaction in the anolyte (i.e., low polarization at the cathode in Fig. 3).

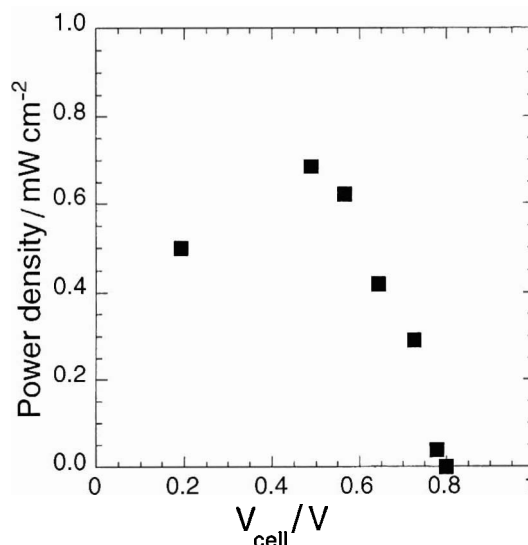


Fig. 4. Power density of the biofuel cell plotted against V_{cell} to illustrate the voltage at which the power density is a maximum (0.68 mW/cm^2 at 0.49 V).

of CO_2 was indicated by precipitation of BaCO_3 when the gas from the anode compartment was vented through a 0.1 M solution of $\text{Ba}(\text{OH})_2$. The rate of CO_2 evolution, however, was not quantified. Fig. 4 shows the power density of the biofuel cell as a function of cell voltage. The maximum power density of the enzymatic biofuel cell was 0.68 mW/cm^2 at 0.49 V .

4. Conclusions

For the first time, an electro-enzymatic method to regenerate NAD^+ from NADH has been developed that operates efficiently near the reduction potential of NAD^+ . In the context of biofuel cells, this method overcomes major impediments suffered by previously reported methods. By applying this method to the regeneration of NAD^+ in a biofuel cell that uses NAD^+ -dependent enzymes as catalysts, we have both circumvented the overpotential for the direct oxidation of methanol to CO_2 and eliminated problems associated with direct electrochemical regeneration of NAD^+ . The result is an enzyme catalyzed methanol/dioxygen biofuel cell with higher $V_{\text{cell max}}$ (0.8 V) and power density (0.68 mW/cm^2 at 0.49 V) than previously reported. The improved performance of the methanol/dioxygen biofuel cell is a consequence of using a mediator with a formal potential near that of $\text{CO}_2/\text{CH}_3\text{OH}$. The kinetic penalty for matching these potentials is enough, however, to warrant further investigation of alternative mediators. Improved fuel cell performance should be observed with mediators that exhibit higher activities with diaphorase and lower sensitivity to

dioxygen.¹⁴ Substantial improvements, however, will require the identification and characterization of enzymes that are both more stable than those currently available and can catalyze more than one oxidative step.

Acknowledgements

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¹⁴ The leakage of dioxygen from the cathode compartment to the anode compartment (fuel crossover) is a concern with any fuel cell that uses Nafion[®] as the separation membrane. In our case, the leakage of dioxygen into the anode compartment would result in the formation of O₂⁻ when dioxygen reacts with BV⁺. The radical anion of dioxygen is known to be detrimental to biocatalysts, and therefore, its formation would destabilize the biofuel cell. Although the ambient temperatures and pressures used in the operation of biofuel cells should limit the amount of dioxygen leakage, the use of mediators less sensitive to dioxygen would eliminate problems associated with the production of O₂⁻.