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Bioelectrocatalysts

Engineered Oxidoreductase System for Utilization of Fumarate Reductase in Chemical Synthesis, Detection, and Fuel Cells

Doo Hyun Park, C. Vieille, and J. G. Zeikus***

Department of Biochemistry and Microbiology, Michigan State University, East Lansing, MI 48824-1319, E-mail: zeikus@msu.edu

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Abstract

Fumarate reductase was used as a model oxidoreductase to demonstrate continuous electrical cofactor reduction–oxidation during the bioelectrochemical synthesis and detection of chemicals. The enzyme preparation was immobilized onto a graphite felt electrode that was modified with carboxymethylcellulose (CMC). Nicotinamide adenine dinucleotide (NAD), neutral red, and fumarate reductase (which contained menaquinone) were covalently linked by peptide bonds to the CMC. The electron mediator neutral red allowed NAD and menaquinone to be recycled electrically during enzymatic chemical synthesis. Succinate detection by the bioelectrocatalyst was linear from 5 μ*M* to 10 m*M* succinate. Fumarate synthesis using this bioelectrode was dependent on succinate utilization and resulted in proportional production of electricity and fumarate. Succinate synthesis using this bioelectrocatalyst was dependent on current and fumarate concentration. This bioelectrocatalyst system may enhance the utility of menaquinone- and/or pyridine nucleotide-linked oxidoreductases in diverse enzymatic fuel cells and sensors. It may also enhance the utility of oxidoreductase-based chemical synthesis systems because it eliminates the problem of cofactor recycling.

Index Entries: Bioelectrochemistry; fumarate reductase; biosensor; cofactor recycling.

Introduction

One major limitation to the utilization of oxidoreductases in chemical synthesis *(1,2)*, in chemical detection (i.e., biosensors *[1,3,4]*), or in biofuel

*Author to whom all correspondence and reprint requests should be addressed.
**Present address: Department of Biological Engineering, SeoKyeong University, 16-1 Jungneung-dong, Sungbuk-gu, Seoul 136-704 Korea.

cells *(5,6)* is the lack of a simple regeneration or recycling system for the electron-transferring cofactors (e.g., nicotinamide adenine dinucleotide [NAD], quinones, flavine adenine dinucleotide [FAD], etc.). Many different kinds of approach have been used to electrically recycle NAD in enzyme-immobilized electrodes used as biosensors and in biofuel cells *(4–15)*. These approaches include the use of different electron mediators such as conducting polymers [e.g., polypyrroles, osmium complexes, ferrocene gels, polyaniline, and poly(*O*) amino-phenol], and redox dyes (e.g., Meldola blue, methylene green, Nile blue, toluidine blue O, thionine, azure C, and *N*-methyl-phenazinium) to recycle NAD *(16–26)*.

We discovered *(27)* that neutral red (NR) would undergo reversible chemical oxidoreduction with NAD and that we could continuously recycle NAD electrochemically with NR. By using soluble NR in microbial electrochemical reactors, we also showed that (1) microbes could grow on electricity as the sole electron donor, (2) diverse microbes could overproduce a variety of reduced biochemicals during fermentations or biotransformations, and (3) microbes could generate electricity during organic matter digestion *(28–30)*. NR appears to function as a chemical mimic of menaquinone, as it both transfers electrons and translocates protons *(27)*. Recently, we enhanced the electron-transfer rate in and out of microbes by immobilizing NR onto the electrode *(30)*.

We are developing an industrial succinic acid fermentation process *(31)* and we have characterized the biochemical route of succinate production in *Actinobacillus succinogenes (32)*. A present, a detector is not available for on-line measurement of succinic acid. We previously showed that *A. succinogenes*'s fumarate reductase contained menaquinone and that this enzyme could function with either NAD or NR as the electron acceptor *(27)*. We also showed that *A. succinogenes*'s fumarate reductase was electrochemically reduced or oxidized by NR in vivo *(27,28)*. Our interest in the present work was to study the electrochemical oxidoreduction of fumarate reductase by NR in vitro using an immobilized enzyme electrode system suitable for use as a succinate biosensor, biofuel cell, and biocatalyst for fumarate production. Here, we have developed a generic enzyme immobilization protocol to link NAD^+, NR, and an oxidoreductase (i.e., fumarate reductase) in juxtaposition on a graphite electrode. We call this kind of system for recycling enzyme cofactors (i.e., NAD and/or menaquinone) on an electrode a *bioelectrocatalyst*. This bioelectrocatalyst was suitable for continuous measurement of succinate levels present in fermentation broths.

Materials and Methods

Growth of A. succinogenes

Actinobacillus succinogenes 130Z *(33)* was grown in the following medium per liter: 10 g glucose, 5 g yeast extract, 15.5 g anhydrous potassium phosphate dibasic, 8.5 g sodium phosphate monobasic, and 10 g sodium bicarbonate. The pH was adjusted to 7.4 before autoclaving. Cells were

grown anaerobically under a N_2–CO_2 (80:20) atmosphere at 37°C in a rubber-bunged, 4-L bottle for 16 h.

Fumarate Reductase Preparation

Cell extracts were prepared at 4°C under a strict anaerobic N_2 atmosphere. A 16-h *A. succinogenes* culture was harvested by centrifugation (5000*g*, 30 min) at 4°C, and cells were washed three times with 1500 mL of 100 m*M* Tris-HCl buffer (pH 7.2) containing 1 m*M* dithiothreitol (DTT). The cells were then resuspended in 500 mL of 100 m*M* Tris-HCl buffer (pH 7.2) containing 1 m*M* DTT and 0.05 mg DNAse. The bacterial cells were disrupted by passing them twice through a French pressure cell at 20,000 lb/in^2. The cell debris was removed by centrifuging three times at 40,000*g* for 30 min. The purified membranes were obtained from cell extracts by ultracentrifugation at 100,000*g* for 120 min. The clear, brown precipitate was washed twice with 100 m*M* Tris-HCl buffer (pH 7.2) containing 1 m*M* DTT and resuspended by homogenization in the same buffer. The suspended membrane fraction was used as the enzyme source for the fumarate reductase. Fumarate reductase activity in the membrane protein fraction was 9.5 µmol/mg protein/min.

Electrode Composition and Preparation

The Fe(III)–graphite electrode was made from a mixture of 60% (w/w) fine graphite powder (particle size below 600 mesh), 36% (w/w) inorganic binder (Kaolin, with particle size below 400 mesh), 3.0% (w/w) ferric sulfate, and 1.0% (w/w) nickel chloride. Two parts of this mixture plus one part distilled water were mixed into a paste. This paste was shaped into a square plate (20 cm × 20 cm × 1 cm) that was compacted by applying pressure at 1.0 kg/cm^2. The plate was then dried in air for 48–72 h at room temperature and baked in a kiln at 1100°C for 12 h under anaerobic conditions.

Carboxymethylcellulose (CMC), NR, NAD^+, and fumarate reductase were immobilized on the graphite felt electrode (*see* Figs. 1 and 2) as follows:

1. Graphite felt electrode modified with NR
   a. The graphite felt electrode was cleaned by soaking in methanol for 12 h and then in distilled water for 12 h.
   b. The electrode was dried at 120°C for 1 h.
   c. It was converted to the carboxy form by heating at 200°C under air for 24–48 h.
   d. The electrode was soaked in a 2-mg/mL dicyclohexylcarbodiimide solution in chloroform at 4°C for 6 h and then dried in air.
   e. The electrode was soaked in a 100-µ*M* NR solution in chloroform at 4°C for 12 h.
   f. Finally, the electrode was washed three times by soaking in methanol at 4°C for 3 h each time (until the unbound NR was completely removed), before it was dried at 40°C for 5 h.

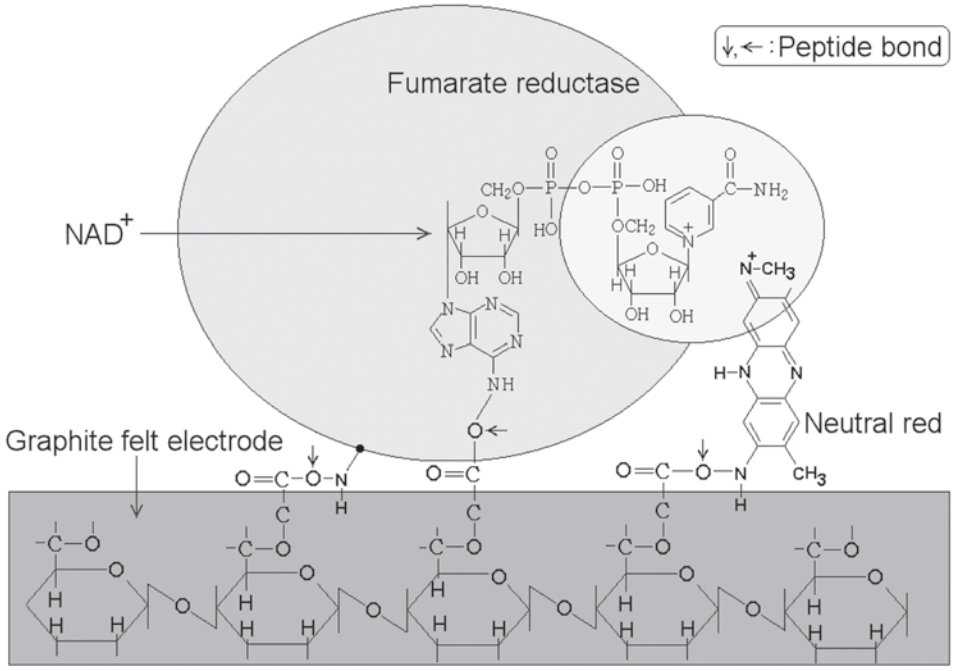

Fig. 1. Diagram of the graphite felt electrode modified with the CMC–NR–NAD^+–fumarate reductase complex. The complex was a thin film that coated the graphite felt electrode. The electrons can be reversibly transferred from the graphite felt to fumarate or from fumarate to the graphite felt through the coupling oxidoreductions of NR and NAD.

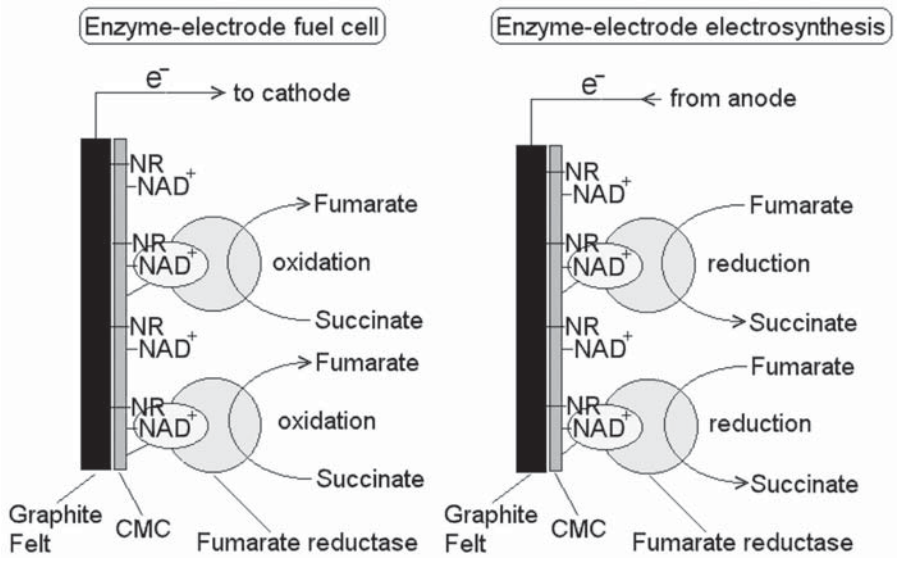

Fig. 2. Mechanisms for succinate oxidation to fumarate coupled to electricity production *(left)* and for fumarate reduction to succinate coupled to electricity consumption *(right)* by the CMC–NR–NAD^+–fumarate reductase complex immobilized on the graphite felt electrode.

2. Graphite felt electrode modified with NR and CMC
   a. The graphite felt electrode was cleaned by soaking in methanol for 12 h and then in distilled water for 12 h.
   b. It was then soaked in a 0.07% CMC (Sigma, C5678, low viscosity, average molecular weight [MW] 700,000) solution in distilled water and dried at 60°C for 6 h.
   c. Neutral red was immobilized on the CMC-linked electrode by following steps 4, 5, and 6 of the previous procedure.
3. NR–CMC graphite felt electrode modified with NAD^+ and fumarate reductase
   a. The NR–CMC–graphite felt electrode was treated in 100 m*M* of 1-cyclohexyl-3-(2-morpholinoethyl) carbodiimide metho-*p*-toluenesulfonate at 4°C for 6–12 h.
   b. The electrode was then soaked in a 1-m*M* NAD^+ solution in 100 m*M* Tris-HCl (pH 7.0) (buffer A) at 4°C for 12 h and then washed in buffer A.
   c. The electrode was soaked in an *A. succinogenes* fumarate reductase membrane fraction (adjusted to 1.635 mg/mL protein in buffer A) at 4°C for 12 h.
   d. The graphite felt electrode linked to NR, CMC, NAD^+, and fumarate reductase was stored in buffer A at 4°C until use.

Bioelectrochemical Reactor Systems

A two-compartment glass electrochemical system was used either as a biofuel cell or for chemical synthesis (*see* Fig. 3B). In the biofuel cell mode (i.e., succinate oxidation to fumarate), the anode was the graphite felt electrode modified with CMC–NR–NAD^+–fumarate reductase ($1.75 \times 4.0 \times 0.6$ cm, 0.47 m^2/g, 0.141 m^2 surface area); the cathode was the Fe(III)–graphite electrode (0.0014 m^2 surface area) (*see* Fig. 3B, *right*). With a redox potential (E_0) of + 0.78 V versus a normal hydrogen electrode (NHE), Fe(III) acted as an electron acceptor and the cathodic process in the biofuel mode was the conversion of Fe(III) to Fe(II). Fe(II) was then reoxidized to Fe(III) by reaction with the oxygen dissolved in the catholyte.

In the biocatalyst mode (i.e., fumarate reduction to succinate), the anode was the Fe(III)–graphite electrode and the cathode was the CMC–NR–NAD^+–fumarate reductase-modified graphite felt electrode (*see* Fig. 3B, left). The anode and cathode compartment volumes were 15 mL each. The two glass compartments were separated by a porcelain micropore, ion-selective membrane ($3 \times 5 \times 0.3$ cm).

A one-compartment rubber-stopper system was used as the biosensor system (*see* Fig. 3A). In this system, the anode was a cylinder (1.0 cm in diameter; 0.6 cm in height; 0.05 g; 0.47 m^2/g; 0.0235 cm^2 surface area) of graphite felt modified with CMC–NR–NAD^+–fumarate reductase. The cathode was Fe(III)–graphite with a surface area of 0.785 cm^2. The inside diameter and height of the electrochemical system were 1 cm and 0.6 cm, respectively. The thickness of the porcelain membrane and anode were

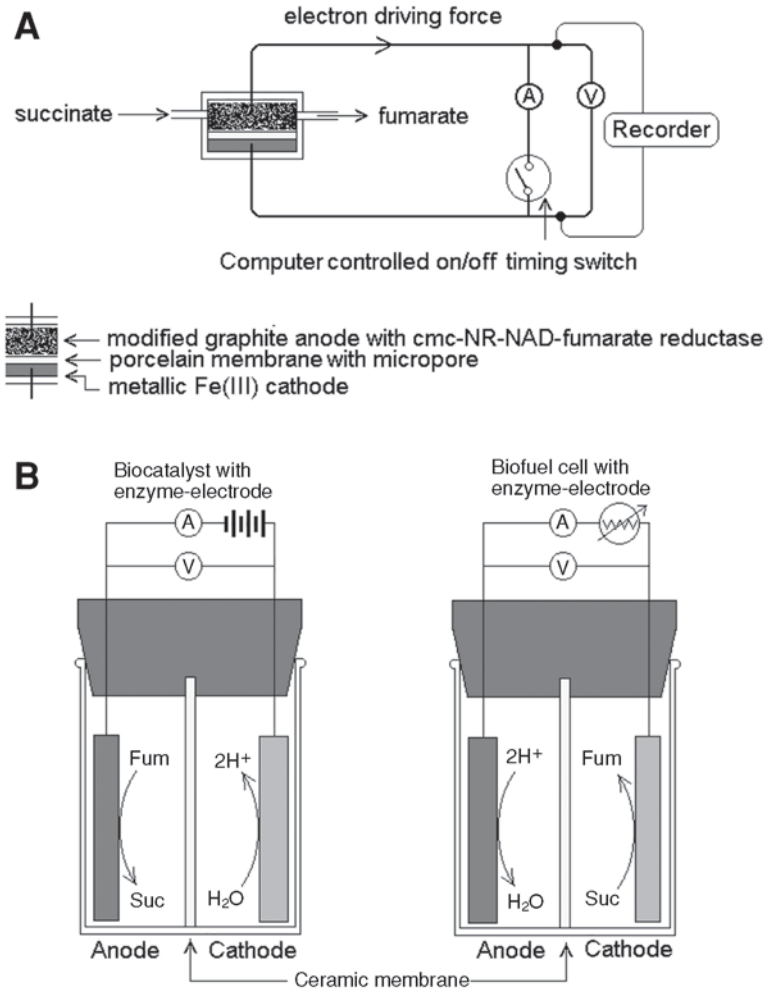

Fig. 3. Diagrams of the two-compartment biocatalyst **(A)** and the one-compartment biosensor **(B)** systems used in this study. In **(A)**, the anode was graphite felt (0.0235 cm^2) modified with the CMC–NR–NAD^+–fumarate reductase complex, and the cathode was Fe(III)–graphite (0.785 m^2). In **(B)**, the inside diameter and height of the reactor were 1 cm and 0.6 cm, respectively. The thicknesses of the ceramic membrane and anode were 1 mm and 0.6 cm, respectively.

1 mm and 0.6 cm, respectively. The electrodes and septum were placed in a rubber stopper that was carved out to make them fit.

Electrochemical Reaction for Succinate Detection

Succinate standard solutions (0, 1, 5, 50, 100, 200, 300, and 400 μ*M* in buffer A) were used to determine the lowest succinate concentration that can be detected by our bioelectrocatalyst. Using the system shown in Fig. 3A, the current (*A*) and potential (*V*) were measured simultaneously after 10 mL of a succinate solution had been continuously flowed through

the system. The electrical system was under closed-circuit configuration. Succinate standards were used in order of increasing concentration. Succinate standard solutions (1–10 m*M* in buffer A) were used to determine the catalytic activity of the graphite felt electrode modified with CMC–NR–NAD^+–fumarate reductase. The potential was measured in the open-circuit configuration after having continuously circulated (5 mL/min) a succinate standard (starting with the lowest concentration) through the system for 5 min. The current was then measured by changing to a closed-circuit configuration. The highest current value was recorded, the flow of the succinate solution was stopped, and the circuit was maintained in closed configuration until the current returned to zero. Similar measurements were then performed with succinate standards of increasing concentration.

Electricity Production Coupled to Succinate Oxidation by the Graphite Electrode (Anode) Modified With the CMC–NR–NAD^+–Fumarate Reductase Complex

This experiment used the two-compartment system shown in Fig. 3B *(right)*. Buffer A, containing 17 m*M* succinate, was the anolyte; 200 m*M* potassium phosphate (pH 7.0) containing 200 m*M* NaCl was the catholyte. Succinate and fumarate concentrations, as well as potential (in the open-circuit configuration) and current (in the closed-circuit configuration) were monitored over time.

Electrical Reduction of Fumarate to Succinate by the Graphite Electrode (Cathode) Immobilized With CMC–NR–NAD^+–Fumarate Reductase

This experiment used the two-compartment system shown in Fig. 3B *(left)*. Buffer A, containing 44 m*M* fumarate, was the catholyte; 200 m*M* potassium phosphate (pH 7.0) containing 200 m*M* NaCl was the anolyte. Fumarate reduction by the graphite felt electrode modified with the CMC–NR–NAD^+–fumarate reductase complex (cathode) coupled to electricity consumption was measured. A constant 2 V dc potential was provided (potentials above 3 V led to water electrolysis); the current oscillated between 8 and 10 mA. Two control tests were performed. In the first control, the graphite felt electrode modified with the CMC–NR–NAD^+–fumarate reductase complex was used, but no electricity was supplied; in the second control, 1 m*M* NADH was added to the reactor, but no electricity was supplied.

Chemical Analysis

Fumarate and succinate were quantitatively analyzed by high-performance liquid chromatography (HPLC) (Waters model) equipped with an Aminex Fast Acid column (100 mm × 7.8 mm; Bio-Rad, Hercules, CA).

Results

Before immobilizing the CMC on graphite felt, we first tested our immobilization protocol using a Petri dish as the template instead of graphite felt. A CMC solution in water was poured in the plate and dried. The CMC could then be peeled off the plate surface as a film. This film was transferred to a glass beaker, where it was treated with dicyclohexylcarbodiimide and NR as described in steps d–f of the CMC–NR immobilization protocol outlined earlier. As opposed to a CMC film that would dissolve in water, the resulting CMC–NR film was completely insoluble in methanol and in boiling water. For this reason, we were confident that our protocol would allow the CMC–NR complex to be immobilized on graphite felt. On graphite felt, the CMC–NR film intricately covers the graphite felt fibers, and it cannot be stripped off the electrode, even after prolonged boiling in water.

Figure 1 shows a diagram of the graphite felt electrode linked to CMC–NR–NAD^+–fumarate reductase. To determine how much protein was bound to the graphite felt electrode, we determined how much protein remained unbound in solution (i.e., 1.285 mg/mL). According to this calculation, after immobilization, the electrode contained 0.07% (w/v) membrane protein. The amount of NAD^+ linked to the electrode (250 μmol/g electrode) was calculated from the results of experiments described later in this article. Figure 2 illustrates the systems used for oxidation–reduction reactions with the graphite felt electrode modified with CMC–NR–NAD^+–fumarate reductase.

Our graphite felt electrode modified with the fumarate reductase–NAD^+–NR complex (i.e., the bioelectrocatalyst) was first tested for its sensitivity in succinate detection. In this experiment, the bioelectrocatalyst was immobilized onto the anode (*see* Fig. 2, *left*), which was placed into the biosensor system (*see* Fig. 3A). Figure 4 shows the potential and current produced by increasingly concentrated succinate solutions. Both potential and current increased linearly with succinate concentration from 5 to 400 μ*M*. Detailed bioelectrocatalyst stability studies were not performed, but identical results were obtained after the electrode was stored in buffer A for 24 h at 4°C. Figure 5 shows the potential and current produced when higher succinate concentrations (1–10 m*M*) were used. Both potential and current also varied linearly in the 1- to 10-m*M* succinate concentration range. We did not test succinate concentrations above 10 m*M*.

Our next series of experiments examined the enzyme on the anode with respect to serving in a biofuel cell and as a chemical catalyst for oxidizing succinate to fumarate. For these experiments, we used the biofuel system depicted in Fig. 3B *(right)*. As shown in Fig. 6, in the open-circuit configuration, succinate oxidation to fumarate and potential produced by the bioelectrocatalyst reached maxima of 5 m*M* fumarate and nearly 0.6 V, respectively (*see* Fig. 6A). These maxima appear correlated to the amount of NAD^+ initially present in the system (i.e., immobilized on the electrode)

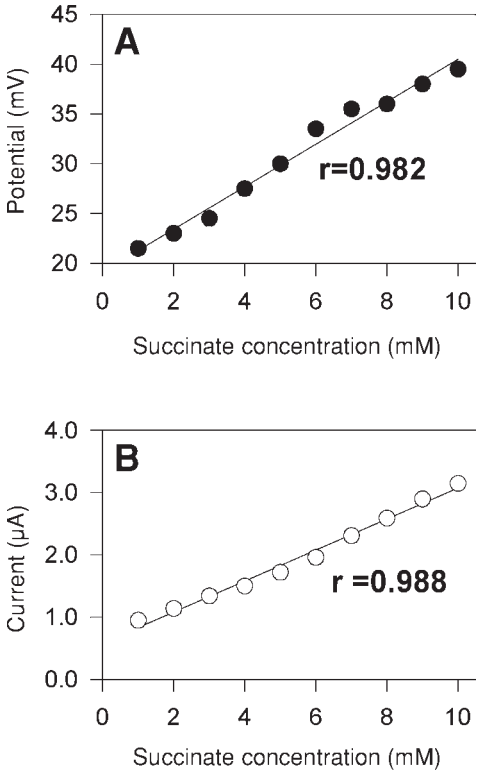

Fig. 4. Potentiometric **(A)** and amperometric **(B)** responses of the CMC–NR–NAD^+–fumarate reductase electrode sensor complex at pH 7.0 and 26°C. This experiment used the system shown in Fig. 1B. The potential was measured in the open-circuit configuration after having continuously circulated (5 mL/min) each succinate solution through the system for 5 min. The current was measured in the closed-circuit configuration.

that can be reduced to NADH. Because the production of 1 mmol fumarate requires the reduction of 1 mmol NAD^+, the production of 5 m*M* fumarate in the 15-mL anolyte suggests that 250 µmol NAD^+ was immobilized per gram of electrode (i.e., 75 µmol on a 0.3-g electrode). In the closed-circuit configuration (*see* Fig. 6B), all of the succinate was stoichiometrically converted to fumarate and electricity. The maximal power produced was calculated at 1-h incubation, when the current was maximal. At that time, the current generated was 0.35 mA. The theoretical potential was calculated using the fact that the current was measured in a closed circuit containing an external 1000-Ω resistance (i.e., theoretical potential = 0.35 mA × 1000 Ω = 0.35 V). Under these conditions, the maximum power generated was 122 µW (with 0.086 µW/cm^2) and the current density was 0.25 µA/cm^2. Between the 10-h and 12-h time-points, the bioelectrocatalyst produced an average current of 43 µA. We used this value to calculate the quantity of electricity produced during the 12-h incubation. The bioelectrocatalyst produced almost 2 C of electricity (i.e., 43 µA × 12 × 3600 s).

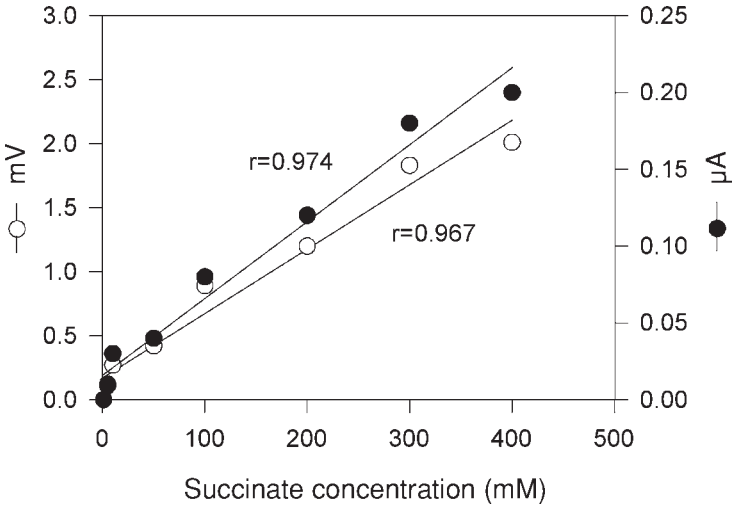

Fig. 5. Lowest detection limit for succinate by the bioelectrocatalyst (i.e., CMC–NR–NAD^+–fumarate reductase complex immobilized on a graphite felt electrode). This experiment used the system shown in Fig. 1B. The potential (mV) and current (µA) were measured in the closed-circuit configuration without external resistance. The lowest detection limit was confirmed to be 5 µ*M* succinate.

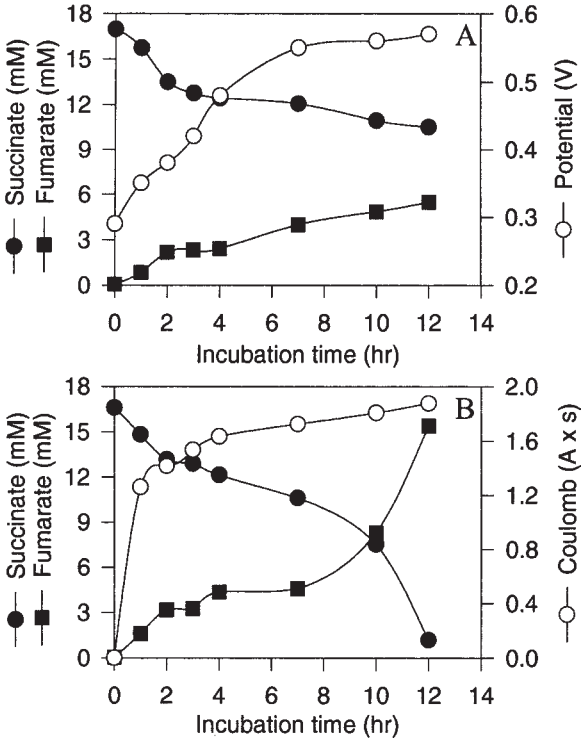

Fig. 6. Succinate oxidation to fumarate by the bioelectrocatalyst coupled to electricity production in open-circuit **(A)** and a closed-circuit **(B)** biofuel cell systems. This experiment used the system shown in Fig. 1A (left). The anode was graphite felt modified with the CMC–NR–NAD^+–fumarate reductase complex, and the cathode was Fe(III)–graphite. The anode surface area was 0.141 m^2 (0.3 g electrode at 0.47m^2/g).

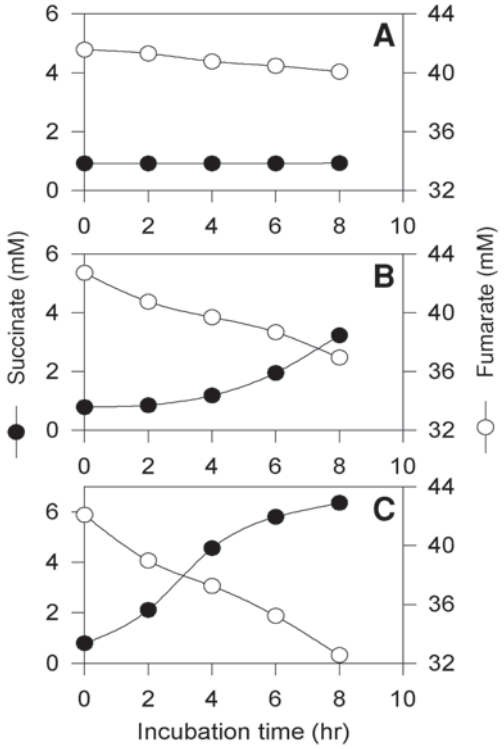

Fig. 7. Fumarate reduction to succinate by the CMC–NR–NAD^+–fumarate reductase complex immobilized on a graphite felt cathode. This experiment used the system shown in Fig. 1A (right). The anode was Fe(III)–graphite. **(A)** Neither electricity nor NADH was supplied; **(B)** 1 m*M* NADH was added as the reducing power without electricity; **(C)** 2 V dc power was supplied (8–10 mA). The cathode surface area was 0.141 m^2 (0.3 g electrode at 0.47m^2/g).

In our last series of experiments, we immobilized the bioelectrocatalyst onto the cathode and examined electrical reduction of fumarate to succinate. Figure 7A shows that little fumarate was consumed and no significant succinate was formed in the absence of added electricity or NADH. Adding 1 m*M* NADH to the fumarate reductase complex enabled about 2 m*M* succinate to be produced (*see* Fig. 7B). Figure 7C shows that electricity greatly enhanced the rate of fumarate reduction to succinate and about 6 m*M* product was formed.

Discussion

Our data demonstrate that NR can electrochemically recycle fumarate reductase electron-transfer cofactors (i.e., menaquinone and/or NAD) during the utilization of the oxidoreductase as a biosensor, biofuel cell, or synthetic biocatalyst. The inability to recycle cofactors or the expense of cofactor recycling has limited the industrial use of oxidoreductases *(2)*. By our new method, a bioelectrocatalyst is engineered (viz. an oxidoreduc-

tase [e.g., fumarate reductase] an electron mediator [e.g., neutral red], and a cofactor [e.g., NAD] are covalently linked to CMC, which is bound onto a woven graphite electrode). The bioelectrocatalyst can then serve as an anode or cathode for biochemical synthesis or detection, and as a fuel cell.

Much more work needs to be completed to optimize our bioelectrocatalyst method. For example, we need to test and maximize stability and we need to understand the relationship between component juxtaposition and concentration ratio with bioelectrocatalyst activity and stability. We also need to replace NR with other electron mediators that can electrically oxidize or reduce NAD(H), such as Mn *(30)*, Meldola blue *(9)*, or phenothiazin derivatives *(5)* to see how they compare to NR. More studies are also needed to determine the breadth of applicability of this method. We are currently attempting to engineer alcohol dehydrogenase and lactate dehydrogenase into additional bioelectrocatalysts using this method. Other new methods for electrical recycling of cofactors are under development elsewhere *(13,15)*. For example, NADH was electro-oxidized by use of Meldola blue, methylene green, or riboflavin adsorbed onto zirconium phosphate-modified electrodes *(15)*.

On-line sensors for succinate detection during fermentation processes are not available *(31)*. Our bioelectrocatalyst sensor allowed continuous measurement of succinate and was linear from 1 to 10 m*M* succinate. Succinate is present at this concentration range in fermentation broths of diverse organisms, including *Escherichia coli* and *A. succinogenes (32)*. Sensors for detecting many other kinds of chemical have been reported previously *(4,10,34)*. For example, an enzyme sensor for glycerol was developed by immobilizing glycerol dehydrogenase and NAD in a carbon paste electrode under a poly(0-phenylene diamine) film *(35)*.

Recently, enzymatic fuel cells have been developed *(14)* and miniaturized for providing electricity in implanted medical devices *(6)*. Katz et al. *(36)* developed a novel one-compartment fuel cell comprised of an anode with immobilized glucose oxidase, FAD, and pyrroloquinoline, and a cathode with immobilized cytochrome-*c*. The fuel cell converted glucose to gluconic acid and generated 4 nW maximum power. Katz et al. *(34)* also developed a lactate-sensing device in which the anode consisted of lactate dehydrogenase, NAD^+, and pyrroloquinoline quinone immobilized on an Au electrode; the cathode was a crosslinked cytochrome-*c*/cytochrome oxidase Au electrode. The device could sense lactate in the range 1–80 m*M*. Our bioelectrocatalyst fuel cell using the fumarate reductase generated 122 µW of power, which is much higher than reported previously *(6,14,36)* for enzyme-based fuel cells. Chen et al. *(6)* developed an enzyme fuel cell that generated 600 nW at 37°C, enough to power small silicon-based microelectronics. In this system, the anode contained immobilized osmium salts, copolymer, and glucose oxidase; the cathode contained immobilized osmium salts copolymer and laccase. These new methods could perhaps be applied to further improve our bioelectrocatalyst engineering method.

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