Use of nonimmobilized enzymes and mediators achieved high power densities in closed biobatteries

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Abstract
The immobilization of redox enzymes on the surface of electrodes is a typical practice for enzymatic fuel cells (EFCs) but enzyme immobilization usually results in low retaining enzymatic activities and low power densities. Here, we investigated an alternative solution – the use of nonimmobilized thermostable enzymes (e.g., NAD-based glucose 6-phosphate dehydrogenase and flavin-containing diaphorase) and a nonimmobilized mediator (i.e., benzyl viologen or 9,10-anthraquinone-2,7-disulphonic acid, AQDS) for achieving high power densities from glucose 6-phosphate in closed EFCs (called biobatteries). At the same enzyme loading, power densities of biobatteries increased in an order from the case of immobilized enzymes and immobilized vitamin K3 to the case of nonimmobilized enzymes and immobilized vitamin K3 to the case of nonimmobilized enzymes and nonimmobilized AQDS. The maximum power density of the biobattery based on nonimmobilized enzymes and AQDS was 1.1 mW cm⁻² of anode at room temperature, 34-times of the biobattery based on immobilized enzymes and immobilized vitamin K3. When enzyme loading was increased by another 10-fold, the highest power density of biobattery was increased to 2.4 mW cm⁻² at 37°C. The biobattery based on nonimmobilized enzymes and AQDS retained 60% of its initial current density after running for 2 h. When repeatedly being used for 6 rounds, the biobattery had 35% of its initial current density after 12 days. Our results suggest that closed biobatteries equipped with nonimmobilized thermostable enzymes and mediators feature simple system configuration and have high power densities.

Introduction
Enzymatic fuel cells (EFCs) can convert a number of fuels to electricity directly by using enzymes as low-cost catalysts instead of platinum, suitable for powering portable electronics [1]. When gaseous (low-energy in terms of volumetric density) hydrogen is used, fuel cells usually run under a continuous flow-through regime and the catalyst hydrogenase or platinum must be immobilized on the surface of electrode [2]. However, when high-energy density organic compounds (e.g., sugar, methanol) are used in biological fuel cells, these fuel cells can be operated in a “closed” batch or fed-batch mode [3–5].

Enzyme immobilization becomes a common practice in biosensors and most EFCs because it not only increase the stability of mesophilic enzymes but also facilitate...
electron transfer from immobilized enzymes to electrodes. Also, immobilized enzymes on the surface of electrodes enable easy separation of biocatalysts from the fuel and reuse of the electrode, especially important when fuel cells are run in a flow-through mode. However, enzyme immobilization has several inherent disadvantages, including decreased enzyme activities, poor mass diffusion, electrode fouling due to enzyme leakage, and possibly bad reproducibility due to complicated enzyme immobilization procedures [5–8].

Electron transfer from redox enzymes to electrodes in EFCs is classified by two mechanisms: direct electron transfer (DET) and mediated electron transfer (MET) [9]. In DET, redox enzymes (e.g., glucose oxidase and laccase) must be immobilized on the surface of electrodes to ensure efficient electron transfer [10, 11]. In MET, one or several small molecular weight, redox-active compounds, are introduced to shuttle electrons between the enzyme active site and electrode, such as, NADH, vitamin K₃, and benzyl viologen (BV) [4, 5, 12]. In order to retain the mediators in flow-through bioelectrochemical systems, costly mediators are usually immobilized on electrodes. Redox mediators can be immobilized through a variety of means, such as adsorption [13–15], polymerization [16–19], entrapment [20, 21], or covalent linking [22]. Another advantage of immobilizing mediators is a decrease in the electron shuttling distance of the mediator to the electrode, resulting in fast electron transfer rates [23]. For example, low-water solubility vitamin K₃ (2-methyl-1,4-naphthoquinone) adsorbed on the surface of anode is a widely used mediator for NAD-dependent redox enzymes thanks to its fast kinetics and small thermodynamic loss [5, 12, 24–27]. In contrast, highly water-soluble mediators, such as BV, AQDS, vitamin K₃, poly-l-lysine (PLL, MW ~70–150 kDa), poly- (NAD+), poly- L-lysine (PLL, MW ~70–150 kDa), and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC), and N-hydroxysulfosuccinimide (NHS) were purchased from Sigma-Aldrich (St. Louis, MO). Carbon paper (AvCarb MGL200) as the anode supporter was purchased from Fuel Cell Store (San Diego, CA). COOH-functionalized multiwall carbon nanotubes (MWCNTs) with an outer diameter of <8 nm were purchased from Cheap Tubes.com (Brattleboro, VT).

Electrochemical measurements
The “i-cell” set-up of EFCs was shown in Figure 1A. Nafion 212 membrane was used to separate an anode and a

Increasing power density of biobatteries is one of the most important R&D priorities for the development of practical biobatteries powering numerous portable electronic devices. In this study, we re-investigated the feasibility of the use of nonimmobilized enzymes and mediators in biobatteries for retaining the maximum enzyme activity and increasing mass transfer in systems, featuring drastic increases in power outputs and simple system configuration. To address the stability of the nonimmobilized enzymes, we used home-made thermostable enzymes, instead of commercially available mesophilic enzymes, as well as a stable electron mediator.

Experimental
Reagents
All chemicals including glucose 6-phosphate, vitamin K₃ (VK₃), BV, AQDS, nicotinamide adenine dinucleotide (NAD⁺), poly-l-lysine (PLL, MW ~70–150 kDa), and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC), and N-hydroxysulfosuccinimide (NHS) were purchased from Sigma-Aldrich (St. Louis, MO). Carbon paper (AvCarb MGL200) as the anode supporter was purchased from Fuel Cell Store (San Diego, CA). COOH-functionalized multiwall carbon nanotubes (MWCNTs) with an outer diameter of <8 nm were purchased from Cheap Tubes.com (Brattleboro, VT).

Preparation of enzymes
Geobacillus stearothermophilus 10 diaphorase (DI), G. stearothermophilus 10 glucose 6-phosphate dehydrogenase (G6PDH) were produced and their activities were measured as described previously [27].

Preparation of bioanodes
Enzymes were immobilized on the carbon paper electrode as previously described, through successively casting of PLL, MWCNTs, EDC, NHS, enzymes, and VK₃ [12]. For nonimmobilized-enzyme anodes, the anodes without enzymes casted were submerged in the electrolyte containing the same enzyme loading as used for the immobilized system. For nonimmobilized-enzyme-and-mediators EFCs, the same anodes were used without casted by VK₃. Instead, soluble BV or AQDS was used as the mediator.

Electrochemical measurements
The “i-cell” set-up of EFCs was shown in Figure 1A. Nafion 212 membrane was used to separate an anode and a
cathode. An air-breathing carbon cathode was coated with 0.5 mg cm$^{-2}$ Pt. All electrochemical measurements were conducted using a 1000B Multi-Potentiostat (CH Instruments Inc., Austin, TX) interfaced to a PC. Linear sweep voltammetry was performed at a scan rate of 1 mV sec$^{-1}$.

To compare immobilized and nonimmobilized enzymes in the EFC, the same amount of enzyme was either immobilized on anodes or soluble in the solution. The electrolyte contained 10 mmol/L G6P, 2 mmol/L NAD$^+$, 100 mmol/L HEPES (pH 7.3), 10 mmol/L MgCl$_2$, 0.5 mmol/L MnCl$_2$, and 100 mmol/L NaCl. Six mL of the electrolyte was stirred at 600 rpm during measurement at the room temperature.

Cyclic voltammetry was performed at different scan rates (1–100 mV sec$^{-1}$) to compare immobilized VK$_3$ and nonimmobilized BV or AQDS using a 3-electrode system with a Ag/AgCl reference electrode and a Pt wire counter electrode. The anolyte contained 5 mmol/L mediator, 20 mmol/L G6P, 8 mmol/L NAD$^+$, 100 mmol/L HEPES (pH 7.3), 10 mmol/L MgCl$_2$, 0.5 mmol/L MnCl$_2$, and 100 mmol/L NaCl. Twelve units of G6PDH and 16 units of DI were soluble in the 6 mL solution.

Various concentrations (0.2–5 mmol/L) of mediators (VK$_3$, BV or AQDS) were used to study the impact of the mediator concentration on the performance of the EFCs. Other components of the electrolyte were the same as above. Linear sweep voltammetry at the scan rate of 1 mV sec$^{-1}$ was performed.

Various temperatures (23, 37, or 50°C) were optimized when AQDS was chosen as the optimal mediator in the EFCs, where concentrations of several reaction elements were increased such as 40 mmol/L G6P, 20 U mL$^{-1}$ G6PDH and 27 U mL$^{-1}$ DI to achieve high a power density.

To test the long-time performance of the biobatteries, an external load of 150 Ω was applied and the current density was recorded for 2 h. The electrolyte contained 5 mmol/L mediator, 20 mmol/L G6P, 8 mmol/L NAD$^+$, 100 mmol/L HEPES (pH 7.3), 10 mmol/L MgCl$_2$, 0.5 mmol/L MnCl$_2$, 100 mmol/L NaCl, 2 U mL$^{-1}$ G6PDH, and 2.7 U mL$^{-1}$ DI. After every 3 days when all the G6P in the batch was consumed, a 20 mmol/L of fresh G6P substrate was supplemented and the current density was recorded for another 2 h to find out it maximum value. This experiment was repeated for 6 rounds and 12 days.

**Results and Discussion**

To increase power density of biobatteries, we studied the performance of the glucose 6-phosphate-powered biobatteries equipped with glucose 6-phosphate dehydrogenase (G6PDH) and diaphorase (DI), which represent the most important part of the sugar-oxidation and electron-transfer chain in the complete sugar-oxidation biobattery [5]. The biobattery based on two immobilized enzymes through covalent binding along with immobilized vitamin

![Figure 1. Scheme of i-cell configuration biobattery (A), the anode compartment based on two immobilized enzymes (i.e., G6PDH and DI) and the immobilized mediator – VK$_3$ (B), the anode compartment based on two nonimmobilized enzymes and the immobilized mediator – VK$_3$ (C), and the anode compartment based on two nonimmobilized enzymes and the non-immobilized mediator (i.e., benzyl viologen, BV; or 9,10-anthraquinone-2,7-disulfonic acid, AQDS) (D). G6P, glucose 6-phosphate; 6PG, 6-phosphogluconate; NAD$^+$/NADH, nicotinamide adenine dinucleotide; G6PDH, glucose 6-phosphate dehydrogenase; DI, diaphorase.](image-url)
K₃ (VK₃) was tested (Fig. 1B) in comparison with the one based on the same amount of non-immobilized enzymes along with immobilized VK₃ (Fig. 1C). VK₃ was immobilized on the carbon nanotube-casted electrode via simple physical adsorption. The power curves showed that the immobilized-enzyme system presented a maximum power density of 0.032 mW cm⁻², approximately a third of the one based on the nonimmobilized enzymes (Fig. 2A). This difference was due to that the covalent binding decreased the apparent activity of immobilized enzymes, resulting in decreased power densities [8]. The mass transport region of the nonimmobilized-enzyme biobattery exhibited a higher current density than that of the immobilized-enzyme one (Fig. 2B). The negative control reaction that the biobattery contained no enzyme generated a power density of 0.0013 mW cm⁻² only.

The use of nonimmobilized enzymes in EFCs were not new [4, 35–38]. However, these studies were often overlooked because of rapid deactivation of nonimmobilized mesophilic enzymes and enzyme wash-out concern. With the discovery of more and more thermostable enzymes from thermophiles and use of engineered enzymes with increased stability, the lifetime of enzymes can be extended greatly even without enzyme immobilization [39]. The advantages of the use of nonimmobilized thermoenzymes in EFCs were the maximum retaining-enzyme activities and great homogeneous mass transfer between nonimmobilized enzymes and soluble substrates. When organic fuels were completely oxidized to CO₂ [4, 5, 34], the closed biobatteries enabled to retain nonimmobilized enzymes in the devices without wash-out concerns.

To further increase the power density, we examined whether a nonimmobilized mediator can be used in biobatteries based on nonimmobilized enzymes (Fig. 1D) because adsorbed VK₃ was easily desorbed on the surface of the electrode [5]. Two water-soluble mediators –BV and AQDS– were compared to immobilized VK₃ for nonimmobilized-enzyme biobatteries. The redox wave could be ascribed to the redox reaction of VK₃ (Fig. 3A), BV (Fig. 3B), and AQDS (Fig. 3C) in the anolyte, based on cyclic voltammetry with three different scan rates from 1 to 100 mV sec⁻¹ applied. Based on the average of the peak potentials of the redox couple at the scan rate of 1 mV sec⁻¹, the midpoint potentials were estimated to be −0.10 V, −0.15 V, and −0.25 V versus Ag/AgCl in a 100 mmol/L HEPES buffer (pH 7.3) for VK₃, BV, and AQDS respectively. The linear dependence of the peak current on the square root of the scan rate (R² = 0.99 for all three mediators) as shown in insets of Figure 3A, B, and C indicated that all three mediators regardless of nonimmobilized or immobilized could shuttle the electrons in biobatteries and they were all diffusion-control processes. The immobilized VK₃ was actually diffused in the immobilized layer, in accordance with Sony's previous finding [40]. A background scan using the electrolyte in the absence of mediators did not have any redox peak (Fig. 3D).

The effect of mediator loading was investigated on maximum current densities obtained from polarization curve scanning (Fig. 4A). Increasing the mediator concentration from 0 to 0.2 mmol/L drastically increased current densities. A further increase of mediator concentration from 0.2 to 1 mmol/L slightly increased in the maximum current densities by 10–20% and then levelled off when the mediator concentrations were more than 1 mmol/L. Nonimmobilized AQDS exhibited the highest maximum current densities, followed by immobilized VK₃ and non-immobilized BV.

Polarization curves were presented at 5 mmol/L of the mediator loading in order to further compare three mediators (Fig. 4B). The AQDS-mediated biobattery showed the highest open circuit potential due to its higher redox potential as compared to BV and VK₃. The highest current density of 3.6 mA cm⁻² and power density of 1.1 mW cm⁻² were obtained from the AQDS-mediated biobattery (Fig. 4C). Both BV- and VK₃-mediated biobatteries had a similar maximum power density of

![Figure 2](image-url)
0.45 mW cm\(^{-2}\) whereas the maximum current density of the VK\(_3\)-mediated one was nearly 1.7-fold that based on nonimmobilized BV (Fig. 4C). These data were approximately 10-fold higher than the negative control without any mediators. According to the polarization curve (Fig. 4C), the BV-mediated biobattery had a significant mass transport loss in the high current density region, implying that the electron-transfer via BV to anode became rate-limiting at this region. It was found that DI had the specific activity of 3.0 U mg\(^{-1}\) on VK\(_3\), 5.2 U mg\(^{-1}\) on AQDS, and 0.5 U mg\(^{-1}\) on BV, indicating that the same amount of DI was the least efficient to transfer electrons base on BV. Therefore, at the high current density region DI activity may not be high enough to transfer electrons from it to BV, resulting in a dramatic mass transport loss according to its polarization curve. As for the other two nonimmobilized mediator-based biobatteries, the dominating voltage loss was Ohmic loss rather than mass transport loss. More details regarding voltage losses will be investigated through a comprehensive analysis on the biobattery resistance and mathematic modeling in the future.
The reaction condition was further optimized to enhance power density of such biobatteries based on non-immobilized enzymes and mediators. The amount of the substrate and enzymes was increased by 2- or 10-fold and the reaction temperature was further optimized at 23, 37, and 50°C (Fig. 5). At 23°C, the high enzyme-loading biobattery had a power density of 1.4 mW cm⁻². At 37°C, the highest power density of 2.4 mW cm⁻² was obtained. The power density at 50°C was dramatically decreased to 0.5 mW cm⁻², possibly due to the deactivation of the enzymes and the cofactors. This result suggests another threefold increase in the maximum power density compared to our previous result (i.e., 0.8 mW cm⁻²) [5]. This biobattery were among highest-power-density EFCs reported [41–43].

The biobattery equipped with nonimmobilized enzymes and a nonimmobilized mediator AQDS utilized thermoenzymes such as DI and G6PDH to minimize the enzyme deactivation issue because both enzymes had a long lifetime of several weeks at the room temperature [27]. Long time performance based on the current generation was evaluated for up to 12 days or 6 rounds of reusability testing. In all tests, an external load of 150 Ω was applied and the current density was recorded for 2 h. The biobattery based on non-immobilized enzymes and AQDS retained 60% of its initial current density, the one based on VK₃ or BV could retain 65–70%, and the one based on immobilized enzymes retained more than 75% (Fig. 6A). When repeatedly being used for six rounds, after 12 days, the biobattery based on AQDS still had 35% of its initial current density, while the one based on immobilized enzymes retained 65% (Fig. 6B). Battery based on nonimmobilized enzymes and mediators produced more power densities that the based on nonimmobilized enzymes and mediators although the latter had better stability.

The use of nonimmobilized enzymes for high-power EFCs is not a new concept but this practice was often overlooked. For example, Whitesides and his coworkers used three nonimmobilized cascade NAD-based redox enzymes in a closed methanol fuel cell achieved a power density of 0.67 mW cm⁻² [4]. Fujita et al. demonstrated that a refuelable mediated biofuel cell based on nonimmobilized enzymes that were entrapped in a hierarchical porous carbon electrode exhibited a power density of 1 mW cm⁻³ through 10 refuelling cycles [44]. Another high-power EFC based on glucose oxidase achieved a power density of 1.30 mW cm⁻², where glucose oxidase was entrapped with carbon nanotubes without any chemical covalent linking [43].

Our comparative study between the two nonimmobilized mediators (Fig. 4) suggested that AQDS was better than BV in terms of power outputs. AQDS, which has been used in an organic-inorganic aqueous flow battery [30] and microbial fuel cells [45], is inexpensive, highly soluble, stable, and undergoes extremely rapid two-electron redox reaction. This new biobattery configuration
exhibited more than 50 times higher power outputs compared to those based on immobilized VKs, the same amount of immobilized enzymes, and the same electrode material [5, 12, 27]. Our data suggested that the use of nonimmobilized enzymes and mediator could be an alternative solution to achieve high power density and high potential for biobatteries and simplify the configuration of biobatteries.

**Conclusions**

A biobattery equipped with two nonimmobilized G6PDH and DI as well as nonimmobilized AQDS as a mediator was demonstrated to achieve high power densities as high as 2.4 mW cm\(^{-2}\). Recent advances in the complete oxidation of organic fuels and the development of thermostable enzymes made it possible to use nonimmobilized enzymes and mediators in closed biobatteries. Further advances in thermostable enzymes, high-activity redox enzymes, and high-surface area and high-conductivity 3-D electrodes could lead to high-power, low-cost, and long-lasting biobatteries with simple fuel cell configuration.

**Conflict of Interest**

None declared.

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