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High Performance Glucose/O_2 Biofuel Cell: Effect of Utilizing Purified Laccase with Anthracene-Modified Multi-Walled Carbon Nanotubes

Michael Minson,[a,*] Matthew T. Meredith,[a] Alexander Shrier,[a] Fabien Giroud,[a,] David Hickey,[b,*] Daniel T. Glatzhofer,[b] and Shelley D. Minteer[a,**,z]**

[a]*Department of Chemistry, University of Utah, Salt Lake City, Utah 84112, USA*
[b]*Department of Chemistry and Biochemistry, University of Oklahoma, Norman, Oklahoma 73019, USA*

Laccase, a blue multicopper oxidoreductase enzyme, is a robust enzyme that catalyzes the reduction of oxygen to water and has been shown previously to perform improved direct electron transfer in a biocathode when mixed with anthracene-modified multi-walled carbon nanotubes. Previous cathode construction used crude laccase enzyme isolated as a brown cell extract powder containing both active and inactive proteins. Purification of this enzyme, yielding a blue solution, resulted in greatly improved enzyme activity and removed insulating protein that competed for docking space in this cathodic system. Cyclic voltammetry of the purified biocathodes showed a background subtracted limiting current density of 1.84 (±0.05) mA/cm^2 in a stationary air-saturated system. Galvanostatic and potentiostatic stability experiments show that the biocathode maintains up to 75% and 80% of the original voltage and current respectively over 24 hours of constant operation. Inclusion of the biocathode in a glucose/O_2 biofuel cell using a mediated glucose oxidase (GOx) anode produced maximum current and power densities of 1.28 (±0.18) mA/cm^2 and 281 (±50) μW/cm^2 at 25°C and 1.80 (±0.06) mA/cm^2 and 381 (±33) μW/cm^2 at 37°C, respectively. Enzymatic efficiency of this glucose/O_2 enzymatic fuel cell is among the highest reported for a glucose/O_2 enzymatic fuel cell.
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Laccase is an oxidoreductase enzyme from a class of multicopper oxidases (MCO) that catalyzes the four-electron reduction of molecular oxygen to water. Laccase has four copper atoms integrated into its two catalytic active sites: a tri-nuclear cluster responsible for the reduction of molecular oxygen, and a mononuclear Cu atom responsible for scavenging electrons from a variety of nonspecific aromatic substrates through one-electron oxidation and radical product formation.[1,2] Laccase is relatively thermostable and has a high turnover rate, making it an ideal target in the field of bioelectrocatalysis.[3,4]

Biofuel cells allow for the harnessing of electrical energy that is available from a chemical reaction through the use of bioelectrocatalysts, and oxidoreductase enzymes are common bioelectrocatalysts considered for efficient energy conversion. For this purpose, a large amount of research effort has been put forth developing materials and methods to enhance the electrical connection of catalytic oxidoreductase enzyme active sites to electrode surfaces.[5–8] Two primary methods of electron transfer exist for connecting the enzyme active sites to a conductive electrode surface: mediated electron transfer (MET) and direct electron transfer (DET). MET focuses on using a reversible redox species as a shuttle for electrons from the active site of the enzyme to the electrode surface. This method is suitable for enzymes whose active sites are buried deep inside the insulating protein shell and are not very accessible to pass electrons directly to a conductive surface. Typically, MET employs a polymer matrix to immobilize the enzyme on the electrode surface while the mediator can be bound to the immobilization polymer,[9–14] immobilized into a separate layer of the electrode,[15–17] or dissolved in the substrate solution.[18–20] The primary drawback to this type of system is that the presence of a mediator couples the reaction of the mediator to the reaction of the enzyme, adding an additional source of instability and lowering the total reaction potential. This means that there is a built-in loss in the operating and open circuit potentials of the enzymatic biofuel cell.[21]

The second method for electron transfer, DET, wires the enzyme active site directly to the electrode surface, thereby eliminating the need for a mediator. While DET is a desirable goal for electrode fabrication, achievement of significantly high current density is dependent upon factors such as enzyme structure, enzyme orientation to minimize the electron tunneling distance, and density of electrically connected enzymes. Certain enzymes undergo DET with more difficulty due to the fact that the active site is buried deep within the protein, like glucose oxidase, making it difficult to wire to metallically conducting electrode surfaces.[22] Some enzymes have their active sites near to the surface of the protein, making DET possible based on proximity to the electrode alone. Other enzymes, like fructose dehydrogenase, have cofactors such as heme groups or quinones that can pass electrons to the electrode by simple adsorption.[23] Many methods of enzyme immobilization simply mix the enzyme in a casting solution or polymer to achieve a homogenous distribution of enzyme for coating on an electrode. This results in a random orientation of the enzymes, many of which may not be oriented favorably for DET. Modified electrode surfaces have been fabricated to increase the conductive surface area that comes into contact with the enzyme by including nanomaterials such as carbon nanotubes, carbon nanoparticles, and gold nanoparticles, thereby increasing the likelihood that more enzymes can come into contact with the conductive surface in a favorable orientation.[24–28] A noteworthy effort made by Ueda et al. utilized UV light and ozone to increase the surface area of a nanostructured carbon films, which improved the DET catalytic current density of bilirubin oxidase electrode by thirty times than that of a two dimensional film.[29] Random enzyme orientation has also been surmounted by simple methods of encapsulation such as liquid induced shrinkage of nanotube forests around the enzyme[28] or high pressure compression of nanotube and enzyme mixtures to achieve current densities higher than 1 mA/cm^2.[30] These examples demonstrate that high performance electrodes can be fabricated by simple adsorption or by the mixing of enzymes with conductive nanomaterials.

Other techniques for achieving DET have focused on covalently attaching enzymes to conductive surfaces such as gold, graphite, and carbon nanotubes.[31,32] These techniques sacrifice enzyme activity, which is typically decreased during covalent attachment, for a higher concentration of correctly oriented enzymes. A favorable alternative to covalent enzyme attachment is utilizing non-covalent interactions to effectively orient the enzymes for wiring.[33–35] Work done by Armstrong et al. has showed that covalent attachment of polycyclic groups to electrode surfaces increases the bioelectrocatalytic reduction of molecular oxygen to water with MCO enzymes like laccase and bilirubin oxidase.[34–36] It is hypothesized that the attachment of these groups mimics the natural substrates of the enzymes and binds to the pocket surrounding their T1 Cu active site. This hypothesis was further strengthened in a recent study by our group, which involved the synthetic attachment of anthracene groups to multi-walled carbon nanotubes to properly orient the active sites of laccase enzymes

*Electrochemical Society Student Member.
**Electrochemical Society Active Member.
zE-mail: minteer@chem.utah.edu

for DET. This allowed for large enzyme loadings to be electrically connected to the biofuel cell.[37]

The previous study with anthracene-modified multi-walled carbon nanotubes (MWNTs) showed that only ∼2% of the theoretical maximum of current density was achieved based on the enzyme loading.[37] Much of the protein content loaded onto the electrode was therefore either denatured or somehow not electrically connected. We hypothesized that excess crude protein or inactive/denatured enzymes could compete with active laccases for docking space. An enzyme that may be properly oriented for DET but has no catalytic activity cannot contribute to the bioelectrocatalysis and adds insulative resistance to the encapsulation matrix. In this work we show that purification of laccase in our system seems to increase the number of active enzymes that are electrically connected by lowering the amount of crude protein and increasing the activity of the enzyme solution that is cast onto the electrode. This results in much higher current densities, which are critical for the fabrication of high performance biofuel cells.

Experimental

Chemicals and solutions.— Glucose oxidase from *Aspergillus niger* (EC 1.1.3.4, type X-S, 157 units/mg of solid, 75% protein), diethylaminoethyl (DEAE) Sephacel, Nafion© EW1100 suspension, 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), glucose, and solvents and salts were purchased from Sigma-Aldrich and used as received. Ethylene glycol diglycidyl ether (EGDGE) was purchased from Polysciences Inc., and used as received. MWNTs were purchased from cheaptubes.com and used as received. Stock solutions of glucose were allowed to mutarotate over 24 hours and stored at 4°C. Laccase from *Trametes versicolor* (EC 1.10.3.2, 20 units/mg of solid) was purchased from Sigma-Aldrich and purified as previously reported and stored at 4°C[38]. Anthracene-modified MWNTs (An-MWNTs), the redox polymer $FcMe_2$-C_3-LPEI, and tetrabutylammonium bromide (TBAB)-modified Nafion© were all prepared as previously reported.[12,37,39]

Purification of laccase.— Laccase from *Trametes versicolor* (EC 1.10.3.2, 20 Units/mg of solid) was purchased from Sigma-Aldrich and purified and assayed as previously reported.[38] A purified laccase enzyme activity assay with the substrate 2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) was carried out using a Thermo Scientific© Evolution 260 Bio UV-Visible Spectrophotometer at 25°C. Activity was determined to be 230 U/mg.

Enzyme electrode fabrication.— Purified laccase biocathodes were prepared as follows: An-MWNTs (15.0 mg) were added to 75 μL of purified laccase solution and 75 μL of 1 mM phosphate buffer (pH 7.0), and the solution was briefly sonicated and vortexed. 50 μL of TBAB-modified Nafion© was added to this mixture and then vortexed and sonicated in one minute intervals for ten minutes. The mixture was painted onto 1 cm^2 pieces of Toray carbon paper (thickness 0.168 ± 0.012 mm, measured with a Vernier caliper) and allowed to dry overnight at room temperature. From previously reported optimization experiments, the weight percent of the An-MWNTs, TBAB-modified Nafion,© and the total enzyme casting solution volume were found to be optimum at 15.0 mg, 50 μL, and 200 μL respectively.[37] Further optimization experiments of the purified laccase electrodes varied the amount of purified laccase solution while holding the total volume of the enzyme and buffer solutions to 150 μL. Mediated glucose oxidase bioanodes were prepared according to a previous protocol[12] and 25 μL of the enzyme casting solution was drop-coated onto 1 cm^2 Toray carbon paper electrodes and set to dry in a low humidity environment at room temperature overnight. The dry volume of the cathodic cast solution was found to be 0.0021 (±0.0003) cm^3 by subtraction of the thickness of the Toray carbon paper from that of the fabricated electrodes (thickness 0.190 ± 0.014 mm).

Electrochemical measurements.— Cyclic voltammetry experiments were carried out with a CH Instruments model 650A potentiostat (Austin, TX) or a Pine WaveNow potentiostat/galvanostat using a three-electrode set up with a saturated calomel reference electrode and a platinum mesh counter electrode. Purified and crude laccase cathodes were tested in 50 mM citrate buffer (pH 4.5). Galvanostatic and potentiostatic 24-hour operational stability experiments were carried out at RT on a Pine WaveNow potentiostat/galvanostat or a Digi-IVY DY2023 biopotentiostat.

A glucose/O_2 fuel cell was constructed using an H-cell design to hold the two compartments of the cell at a different pH. The two-compartment cell was fabricated by stacking four 5 cm × 5 cm × 1 cm pieces of acrylic glass with the two center pieces hollowed out and separated using a Nafion© 212 proton exchange membrane. The anodic compartment of this cell was filled with 10 mL of 100 mM phosphate + 200 mM NaCl and 100 mM glucose at pH 7.4. The cathodic compartment contained 10 mL of 150 mM citrate at pH 4.0. The fuel cell setup allowed for the placement of the electrodes to be separated by a distance of ∼1 cm. Each cell was short-circuited 3 times and the OCV was allowed to stabilize prior to polarization. Electrochemical measurements for each cell were made in triplicate at 25°C and 37°C. All fuel cell solutions were stationary and air saturated. Reported error corresponds to one standard deviation.

Results and Discussion

Cyclic voltammetry of purified laccase/An-MWNT cathodes.— Based on observations made during the initial optimization and characterization of the An-MWNTs with laccase, it was hypothesized that a purification of the laccase enzyme could serve two purposes: one, to remove insulating protein from the enzyme/nanotube/polymer matrix, which would increase the conductivity while allowing for more oxygen to diffuse through the matrix, and two, increase the density of active enzymes contributing to catalytic current density. Cyclic voltammetry of the purified biocathodes showed a background subtracted current density of 1.84 (±0.05) mA/cm^2 in a stationary air-saturated system at RT. This is among the highest DET current densities seen for two-dimensional enzyme cathodes under stationary air-saturated conditions.[26,28,30,31,33,40–43] It is important to note that other methods of fabrication that are more complicated than simple mixing of nanotubes and enzyme have been able to achieve exceptionally high current outputs, but those methods require high pressures[30] or liquid-induced shrinkage[28] for enzyme immobilization. Figure 1 shows a representative cyclic voltammogram of the purified

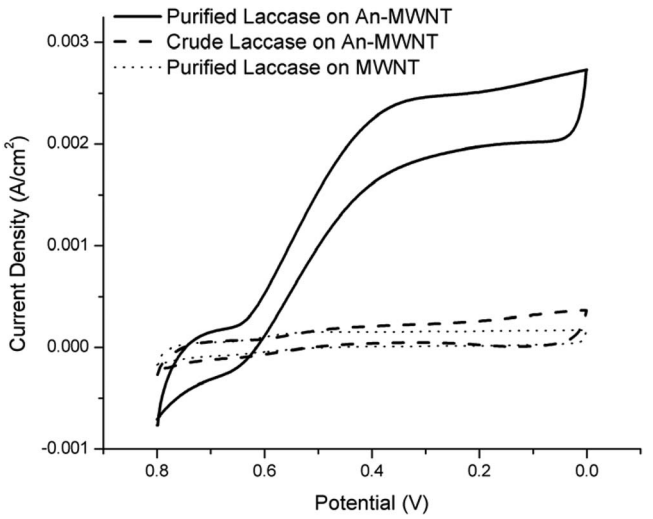

Figure 1. Representative cyclic voltammograms of laccase cathodes at 10 mV/s in stationary air-saturated 50 mM citrate buffer (pH 4.5). Purified laccase on An-MWNTs (black) is shown with the control of purified laccase on unmodified MWNTs (dotted) and crude laccase on An-MWNTs (dashed). Purified laccase activity was 230.5 U/mg

laccase cathode on anthracene-modified MWNTs, purified laccase on unmodified MWNTs, and a crude laccase cathode as previously reported (using anthracene-modified MWNTs).[37] It is interesting to note that the purification resulted in a tenfold increase in enzyme activity (crude enzyme activity was 20 U/mg and purified enzyme activity was 230 U/mg) and a subsequent tenfold increase in current density. This suggests that the removal of non-active material present in the crude enzyme allows for many more active laccase enzymes to become electrically connected to the cathode.

The onset potential of the purified laccase electrodes occurred at about 0.65 V, which is 50 mV higher than that of the crude laccase electrodes at 0.60 V. This is consistent with a lower film resistance, but this surprisingly did not lower the overall capacitance of the electrode, suggesting a higher film resistance. This phenomenon is counter intuitive and its cause is under further study.

This performance increase resulting from enzyme purification also gives insight into the ability of these nanomaterials to wire laccase enzymes to the electrodes. In our previous study, we used the amount of enzyme (in units) coated on each electrode and the known turnover rate for laccase to calculate a theoretical current output, and concluded that the biocathode was operating at a mere 2% efficiency, meaning that 98% of the active laccase enzymes in the casting solution were not involved in current production.[37] In the current study, there was a ∼10-fold increase in the units of enzyme deposited on the electrode due to the enzyme purification procedure. While this increase corresponded to a 10-fold increase in actual current density, it also corresponded to a 10-fold increase in *theoretical* current density, meaning that the efficiency of the electrode system did not change, and remained at ∼2%. This means, again, that 98% of the active enzymes present in the casting solution are not electrically connected to the cathode, likely due to improper orientation or enzyme denaturing in the fabrication process, and further investigation of this low efficiency is a future focus of our research.

Investigation of the purified laccase loading.— Purified laccase concentration was varied by mixing various volumes of the purified laccase solution and 1 mM phosphate buffer, (pH 7.0) keeping the total volume at 150 μL while holding the mass of the An-MWNTs and TBAB modified Nafion polymer constant in the fabrication of the biocathodes. Figure 2 shows the background subtracted current densities versus the enzyme loadings (in wt%) used in the fabrication of the biocathodes. Approximately 14 wt% of enzyme was the maximum amount of enzyme added without showing a decrease in performance. This result is consistent with our previous laccase loading study (using the crude enzyme), showing that this cathodic system reaches a maximum protein allowance of ∼14 wt%.[37] Loadings above 14% resulted in lower catalytic currents, likely due to an increase in the resistance of the nanotube/enzyme/polymer matrix from insulating protein. When the electrodes performed very well, as in the case of the ∼10 and ∼14 wt% loadings, the standard deviations of the maximum current density are between 150 and 160 μA/cm^2, as shown in Figure 2. The reasons for these large standard deviations are unclear at this time, but may be due to slight, unintentional variations in the fabrication procedure, as the electrode films are painted on manually.

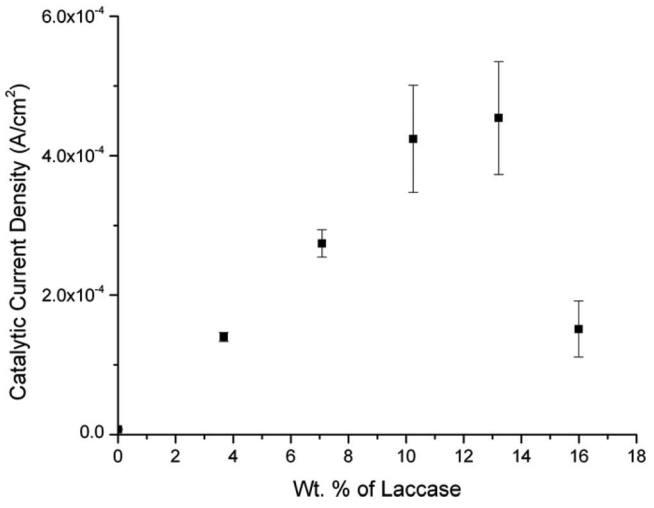

Figure 2. Investigation of purified laccase loading in AN-MWNT electrodes. Background-subtracted steady-state current densities of biocathodes measured with varying amounts of laccase enzyme (86.7 U/mg activity). Measurements made in air-saturated 50 mM citrate buffer, pH 4.5 using a cyclic voltammetry scan rate of 10 mV/s (the scan window was 0.8 → 0.0 V vs. SCE). Current values were determined by subtracting the steady state current at 0.7 V from the current at 0.3 V. All measurements made in triplicate; error bars correspond to the standard deviation.

The decrease in performance observed after adding more than 14 wt% enzyme may also be due to a saturation of binding sites for the enzyme, which would indicate that catalytically inactive portions of the laccase enzymes can overlap onto more than one anthracene group on the An-MWNTs. From the discussion of electrode efficiency above, it would seem that a certain percentage of the nanotube docking sites are blocked by protein, regardless of whether or not that protein is active enzyme. When enzyme solutions of higher activity are used, a larger percentage of the overall protein content in that solution is active enzyme, and therefore more active enzymes can be docked and contribute to the catalytic current. However, the same *percentage* of enzymes (as a percentage of the total protein content) are able to be connected. Therefore, even if the enzyme/An-MWNT casting solution contained only anthracene-modified nanotubes and 100% pure, active enzymes, the electrode would still operate at 2% efficiency. It would seem that a docking procedure that would provide a stronger method of enzyme orientation is necessary to improve the electrode efficiency. In addition, immobilized enzyme assays will be carried out in the future to ensure that the enzymes that are cast are still fully active even if they are not electrically connected.

Stability of the biocathode.— Operational stability of bioelectrodes is a large concern with their use in enzymatic biofuel cells. Both galvanostatic and potentiostatic stability experiments were performed on the biocathode to study the potential or current as a function of time under constant operation: 0.4 V (vs SCE) for 24 hours and measuring the current discharge as a function of time, or 300 μA for 24 hours and measuring the change in potential as a function of time. Figure 3 shows galvanostatic stability to decrease linearly with time for the first 12 to 15 hours losing only 25% of the original voltage. For the remaining 12 hours, it operates at or above 75% of the original voltage. Potentiostatic stability shows a 20% loss in current output over the 24 hours. The small fluctuations in the data may be due to small temperature changes in the room. These data show there is no significant difference in the operational stability of the purified laccase cathode in comparison to the stability of the crude laccase cathodes. This means that the stability of the An-MWNT/laccase system[37] is independent of protein activity. In conjunction with the loading experiment, it can be seen that both electrochemical stability and optimal protein loading of the biocathode are not dependent on the purity of the protein, but are likely inherent properties of this specific bioelectrode architecture.

Enzymatic biofuel cells.— To test the performance of the purified laccase DET cathode, it was used in a glucose/O_2 enzymatic biofuel cell with a mediated glucose oxidase (GOx) anode which uses a dimethylferrocene-based redox polymer to electrically connect the enzyme active site to the electrode surface. In a previous study, this redox polymer was shown to enable the production of up to 2 mA/cm^2 on a smooth glassy carbon electrode,[12] so the cathode and anode should have similar maximum current density capabilities. In order to maintain a different pH for the operation of the glucose/O_2 fuel cell, a H-type cell was used, with the two compartments separated by a Nafion© 212 proton exchange membrane. This cell was tested both at 25°C and 37°C. Characteristic polarization and power curves are shown in

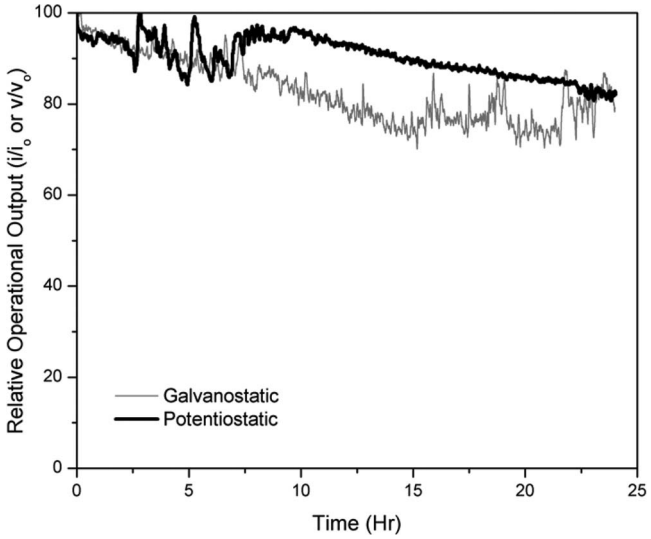

Figure 3. Operational stability of the cathode. Potentiostatic (black): Cathode held at 0.4 V vs SCE in 50 mM citrate buffer, pH 4.5 at 25°C for 24 h (stationary air-saturated solution), and the current was measured as a function of time. Galvanostatic (gray): Under the same conditions, 300 μA of current was passed through the electrode for 24 h, and the voltage change was measured as a function of time.

Figure 4. The open circuit potential for this cell was 0.807 (±0.004) V at 25°C and 0.864 (±0.007) V at 37°C. The potential difference between the two experiments is expected due to the change in the Gibbs free energy with the temperature increase. The maximum current densities and power densities were 1.28 (±0.18) mA/cm^2 and 281 (±50) μW/cm^2 at 25°C and 1.80 (±0.06) mA/cm^2 and 381 (±33) μW/cm^2 at 37°C, respectively. The cell voltage at maximum power did not significantly change between the two temperatures, and was 0.293 (±0.003) V at 25°C and 0.290 (±0.018) V at 37°C.

On the representative RT polarization curve (Figure 4A) at low voltage, it can be seen that the current reaches a maximum at and then curls back to intersect the X-axis at ∼0.00118 A/cm^2. This crossover phenomenon is not observed at the higher temperature, which suggests that at RT, oxygen levels at the electrode surface become locally depleted at low voltage and the current is diffusion-limited. Interestingly, at 37°C, the crossover behavior was not seen even though the concentration of oxygen was lower due to the higher temperature. Therefore, the increase in the flux of oxygen due to the higher temperature was more significant than the decrease in O_2 concentration due to the higher temperature and as a result, the crossover was not seen. Future studies will focus on the use of oxygen-saturated solutions and/or a rotating biocathode to evaluate this hypothesis.

While the theoretical catalytic efficiency of the biocathode as a function of biocatalyst loading did not increase over our previous study, it is desirable to compare the efficiency of the overall cell to other glucose/O_2 enzymatic biofuel cells. To this end, the volume of the nanotube/enzyme composite layer deposited on the electrode surface of the modified cathode was calculated to be 0.0021 (±0.0003) cm^3. In order to assess the efficiency of the glucose/O_2 enzymatic biofuel cell in comparison to the literature, the volumetric current density was calculated to be 600 (±130) mA/cm^3 and the specific current density was 1010 (±150) mA/g of cathode. Cell efficiency as a function of aqueous biocatalyst loading was 42.8 (±6.3) μA/U. Compared to the recently published glucose/O_2 enzymatic biofuel cell from the work of Zebda et al., (3.6 mA/cm^3, 4.2 mA/g, and 1.96 μA/U), our cell shows significant increases in the efficiency of cathodic bioelectrocatalysis.[30] The important advantage of this biocathode is the increase in current per unit of active enzyme, because enzyme is the most costly portion of the enzymatic biofuel cell design.

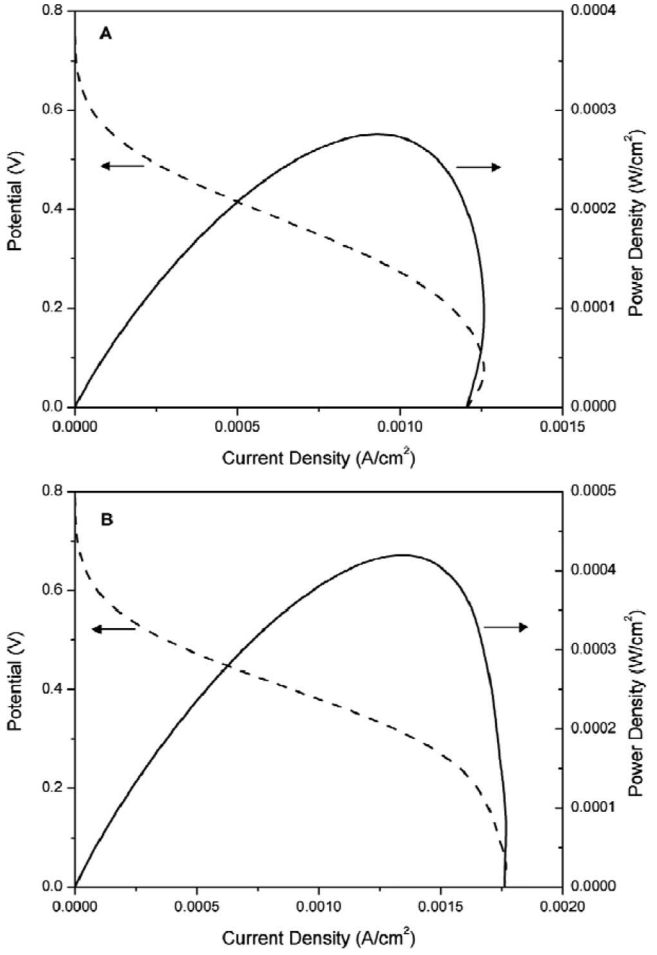

Figure 4. Power and polarization curves for the glucose/O_2 enzymatic fuel cells at 25°C (A) and 37° (B). This compartmentalized cell operated with the anodic compartment filled with 10 mL of 100 mM phosphate + 200 mM NaCl and 100 mM glucose at pH 8.4 and the cathodic compartment contained 10 mL of 150 mM citrate at pH 4.0. Both anodic and cathodic compartments were air-saturated and quiescent and the cells were polarized at 1 mV/s. Purified laccase activity was 230.5 U/mg [prior to immobilization] At room temperature, O_2 concentration was about 8.3 mg/L, and at 37 C, oxygen concentration was about 6.8 mg/L.

Conclusions

Purification of the laccase enzyme greatly enhanced the current and power output of the biocathode. This performance enhancement carried over into its inclusion in a biofuel cell. Background subtracted current density from cyclic voltammetry showed 1.84 (±0.05) mA/cm^2 in an air saturated non-convective solution at room temperature. It is hypothesized that such a great improvement over use of the crude enzyme is directly related to an increase in the loading of active laccase enzyme. Current production efficiency (calculated using the theoretical current production as a function of biocatalyst loading) was ∼2%, which shows that enzyme purification does not affect the mechanism by which laccase enzymes are wired to the electrode. This is further supported in that enzyme loading optimization for the purified laccase electrode was similar to the optimal loading found for crude laccase. Stability of the biocathode showed a 25% potential loss at 300 μA over 24 hours and 20% current decrease at 0.4 V vs. SCE over 24 hours, similar to the crude laccase/An-MWNT system. This shows that performance increase (but not stability) is dependent on the specific activity of the catalytic enzyme species.

This high performance biocathode was included in a biofuel cell system containing a mediated glucose/O_2 cell. Maximum current and power densities of 1.28 (±0.18) mA/cm^2 and 281 (±50) μW/cm^2 at

25°C and 1.80 (±0.06) mA/cm^2 and 381 (±33) μW/cm^2 at 37°C were reached for the glucose/O_2 biofuel cell. Efficiency of this glucose/O_2 fuel cell is superior to previous studies in literature.

It is possible to increase performance of the electrode by increasing oxygen concentration or convection in the system, but such a modification is counterintuitive since doing so requires energy input, and the problem is only temporarily solved. Instead, future research will focus on increasing the concentration of the laccase enzyme that can be loaded onto the electrode while not increasing resistance or inhibiting oxygen diffusion. Modifications that allow for more rapid diffusion of oxygen under stationary conditions may optimize the performance of the cathode in real world application conditions.

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