ACS Catalysis

Research Article

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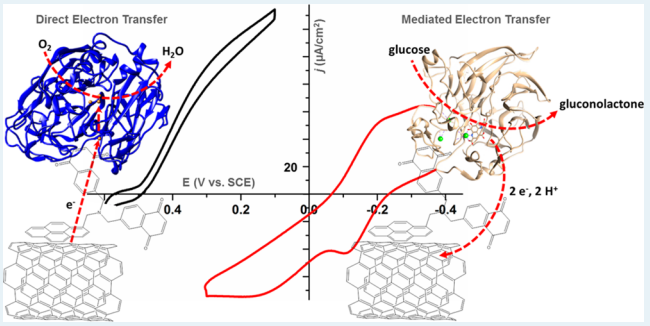
Simplifying Enzymatic Biofuel Cells: Immobilized Naphthoquinone as a Biocathodic Orientational Moiety and Bioanodic Electron Mediator

Fabien Giroud,[†,§] Ross D. Milton,[†] Bo-Xuan Tan,[†] and Shelley D. Minteer*[,†,‡]

[†]Department of Chemistry, [‡]Department of Material Science and Engineering, University of Utah, Salt Lake City, Utah 84112, United States

Supporting Information

ABSTRACT: An elegant method to perform bioelectocatalysis with different oxidoreductases at the cathode and at the anode of an enzymatic biofuel cell is presented. Noncovalent functionalization of multiwalled carbon nanotubes (MWCNTs) was accomplished via $\pi-\pi$ interactions of pyrene derivatives. 1-[Bis(2-naphthoquinonyl)aminomethyl]pyrene was synthesized and successfully immobilized on MWCNTs. The incorporation of the quinone-modified MWCNTs within enzymatic bioelectrocatalytic applications was evaluated. The hydrophobic nature of the naphthoquinone aided orientation of laccase and bilirubin oxidase toward the electrode, which enhanced their ability to undergo the direct bioelectrocatalysis of oxygen. In contrast, the electrochemical properties of the quinone were used at the bioanode to mediate electrons from the bioelectrocatalytic oxidation of glucose by pyrroloquinoline quinone-dependent glucose dehydrogenase. This method demonstrates how the smart modification of MWCNTs can develop materials, which can be used simultaneously at both electrodes of enzymatic biofuel cells.



KEYWORDS: *bioelectrocatalysis, biofuel cells, pyrene, quinones, glucose oxidase, multicopper oxidases*

INTRODUCTION

A key consideration in the development of enzymatic biofuel cells is the mechanism by which the proteins communicate with electrodes. In this aspect, the nanostructured organization of electrodes plays a large role. Although many reports have shown the direct electrochemistry of "blue" multicopper oxidases (MCOs) to achieve high performance for biocathodes in biofuel cells (for oxygen reduction),[1] most of the oxidoreductases used at the bioanodes are "wired" with a redox mediator to afford bioelectrocatalysis.[2] Typically, different immobilization or orientation strategies are used at the bioanodes and biocathodes of enzymatic biofuel cells to achieve high performance, although this increases the complexity of such devices.

A trend in surface modifications for MCO-based biocathodes incorporates the use of polycyclic molecules. First proposed by Armstrong and co-workers, a wide range of polyaromatic compounds (anthracene, anthraquinone, naphthalene) are now used to modify surfaces to aid the orientation of the enzymes (via their hydrophobic substrate pocket) close to the electrode.[3] Because they are able to interact strongly with the T1 site, they are able to stabilize and enhance the bioelectrocatalytic activity of the enzymes. Different strategies have since been proposed for immobilizing these moieties on surfaces for direct electron transfer (DET) of MCOs. Among them, a popular choice is the use of pyrene derivatives. Pyrenes, like other aromatic compounds, are known to noncovalently bind to carbon nanotubes (CNTs) or graphene surfaces through $\pi-\pi$ stacking.[4] This property permits a wide variety of surface functionalizations[5] and enzyme immobilization strategies via covalent binding of pyrenes to proteins.[6]

We report the synthesis of a disubstituted pyrene derivative for the functionalization of MWCNTs to be applied for bioelectrocatalysis. Recently, research published by Cosnier et al. demonstrated that disubstituted pyrenes with two anthraquinone groups displayed a 2.5-fold increase in electrocatalytic currents compared with monosubstituted pyrenes.[7] The presence of the naphthoquinone (NQ) was thought to favorably orientate MCOs toward the conductive surface of the electrode (promoting their DET by acting as a hydrophobic orientational aid) and to enable the mediation of oxidizing enzymes[8] such as pyrroloquinoline quinone-dependent glucose dehydrogenase (PQQ-GDH) or glucose oxidase (GOx), as a result of the relatively low redox potential of the quinone groups. Furthermore, catalytic currents at low potentials are desired for fuel-oxidizing electrodes in BFC applications, which can increase open-circuit potentials (OCPs). This, in turn, can theoretically increase maximum achievable power densities. The smart modification of electrodes is critical to obtain a high potential difference between the cathode and the anode of a

Received: December 4, 2014
Revised: January 10, 2015
Published: January 13, 2015

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DOI: 10.1021/cs501940g
ACS Catal. 2015, 5, 1240−1244

biofuel cell while undergoing bioelectrocatalysis with minimal overpotential, thus minimizing voltage losses. The ability of NQ-modified pyrene to aid bioelectrocatalytic oxygen reduction and glucose oxidation was assessed individually for each enzyme/pyrene couple using cyclic voltammetry. Thus, the simultaneous use of this molecule at the biocathode and bioanode of a biofuel cell is reported, where the redox potential of the NQ-modified pyrene (pyr-$(NQ)_2$) is utilized at the bioanode while having only orientational (no electrochemical) use at the biocathode.

EXPERIMENTAL SECTION

Materials and Methods. 1-Pyrenemethylamine hydrochloride (1-PMA), laccase from *Trametes versicolor* (EC 1.10.3.2), tributylamine, sodium citrate and Nafion perfluorinated resin solution, and 2,2′-Azobis(2-methylpropionitrile) (AIBN) were purchased from Sigma-Aldrich, USA. 6-Methyl-1,4-naphthoquinone was obtained from Santa Cruz Biotechnology, USA. Solvents were purchased from Sigma-Aldrich and used as received. PQQ-dependent glucose dehydrogenase (PQQ-GDH, EC 1.1.5.2) and bilirubin oxidase (BOx, EC 1.3.3.5) were purchased from Amano Enzyme Inc. Toray paper TGP-H-060 (nonwet proofed) was received from Fuel Cell Earth, USA. Citric acid was purchased from Macron Chemicals. Multiwalled carbon nanotubes (MWCNTs) were received from www.cheaptubes.com, USA (o.d. = 10−20 nm, length = 10−30 μm). Tetrabutylammonium bromide-modified Nafion suspension was prepared as previously described.[9]

Synthesis of Pyrene Derivative. *Synthesis of 6-Bromomethyl-1,4-naphthoquinone (6-BM-1,4-NQ).* The synthesis of 6-BM-1,4-NQ was adapted from a previous study.[10] *N*-Bromosuccinimide (NBS, 356 mg, 2 mmol) was added to a solution of 6-methyl-1,4-naphthoquinone (230 mg, 1.3 mmol) and 50 mL of dry benzene. The mixture was vigorously stirred and warmed. AIBN (45 mg, 0.27 mmol) was added, and the resulting solution was heated to reflux for 4 h. The completion of the reaction was monitored by thin layer chromatography (silica). After completion, the solution was filtered to remove NBS from the product, and the solvent was removed under vacuum. The product was purified over a silica gel column with acetone/hexane (20:80). Yield, 63%; ^{1}H NMR ($CDCl_3$, 500 MHz): δ = 8.10−7.75 (3H, m), 7.0 (2H, s), 4.55 ppm (2H, s, $-CH_2-$Br).

Synthesis of 1-[Bis(2-naphthoquinonyl)aminomethyl]-pyrene (pyr-$(NQ)_2$). The synthesis of pyr-$(NQ)_2$ was adapted from research published by the Cosnier group.[7] Briefly, 6-BM-1,4-NQ (58.6 mg, 0.23 mmol), potassium carbonate (0.55 mmol), and 1-pyrenemethylamine (23.1 mg, 0.1 mmol) were dissolved in CH_3CN (50 mL). The mixture was stirred under reflux and nitrogen flow overnight. The solution was cooled to room temperature and filtered. The crude product was purified over a silica gel column with CH_2Cl_2 to remove unwanted reactants and products. The product was finally collected using CH_2Cl_2/methanol (90:10). Yield, 36%; ^{1}H NMR ($CDCl_3$, 500 MHz) δ = 8.24 (2H, d), 8.20−7.80 (15H, m), 7.65 (2H, br), 6.81 (4H, s), 4.25 (2H, br, N−CH_2−pyr), 3.73 ppm (4H, br, N−CH_2−NQ).

Electrode Preparation. *Modification of MWCNTs with Pyrene Moieties.* Unmodified MWCNTs (100 mg) were dispersed by sonication in CH_2Cl_2 (40 mL) for 30 min. Subsequently, pyr-$(NQ)_2$ (10 mg in CH_2Cl_2) was added to the CNT dispersion. The solution was sonicated for a further 30 min. The resulting dispersion was stirred for 24 h at room temperature, and the modified MWCNTs were filtered and washed thoroughly with CH_2Cl_2 to remove any free pyrene compounds from the dispersion.

Pyr-$(NQ)_2$/MWCNT-Modified Electrodes. Modified (pyr-$(NQ)_2$)/MWCNTs (7.5 mg) were dispersed in 75 μL of 50 mM citrate buffer (pH 4.5) by successive sonication and vortex steps (1 min each, repeated 4 times). A TBAB-modified Nafion suspension (25 μL) was used as an immobilizing agent and added to the solution. The final mixture was briefly vortexed and sonicated. Toray paper electrodes were painted with the resulting black ink and left to dry at room temperature for 2 h.

Multicopper Oxidase-Based Bioelectrodes. Laccase and BOx were tested as bioelectrocatalysts. Modified (pyr-$(NQ)_2$)/MWCNTs (7.5 mg) were mixed with a MCO suspension (1.5 mg) in 75 μL of 50 mM citrate buffer (pH 4.5 and pH 6.5 for laccase and BOx, respectively) by successive sonication and vortex steps (1 min each, repeated 4 times). A TBAB-modified Nafion suspension (25 μL) was added to the solution. The resulting mixture was briefly vortexed and sonicated to yield a black bioink. The bioink was painted onto the Toray paper electrodes (1 cm^2) to form a visually homogeneous film and left to dry open to the air for 2 h.

PQQ-Dependent Glucose Dehydrogenase-Based Bioelectrodes. Modified (pyr-$(NQ)_2$)/MWCNTs were dispersed in DI water at a concentration of 26.6 mg/mL by successive steps of vortex-mixing and sonication (1 min each, repeated twice). A solution of PQQ-GDH (75 μL from a 10 mg/mL in 20 mM MOPS, 6 mM $CaCl_2$, 20 mM KCl stock solution at pH 6.5) was added to 75 μL of the resulting MWCNTs dispersion, and the solution was further homogenized by successive steps of vortex-mixing and sonication (1 min each, repeated twice). A TBAB-modified Nafion suspension (50 μL) was incorporated into the mixture, and the resulting solution was vortex-mixed for 1 min, followed by 15 s of sonication. Finally, 50 μL of the bioink was drop-cast onto the Toray paper electrodes (1 cm^2) and left to dry open to the air for 2 h.

RESULTS AND DISCUSSION

The noncovalent immobilization of the MWCNTs was achieved by $\pi-\pi$ stacking interactions between the pyrene moieties (the 1-[bis(2-naphthoquinonyl)aminomethyl]pyrene, or pyr-$(NQ)_2$, Figure 1) and the walls of the MWCNTs. Pyr-$(NQ)_2$/MWCNTs electrodes were characterized by cyclic voltammetry; a layer of pyr-$(NQ)_2$/MWCNTs was deposited on a Toray paper electrode, and voltammograms were recorded in 50 mM citrate buffer at pH 4.5 at different scan rates (Supporting Information Figure S3) to evaluate the immobilization and electrochemical behavior of the pyr-$(NQ)_2$. As expected, a reversible redox peak was observed at $E_{1/2}$ = −0.042 ± 0.003 V, confirming the grafting on the MWCNTs. Furthermore, the reduction and oxidation peak currents are linearly dependent on the scan rate, supporting a surface-controlled process and, thus, the successful immobilization of the pyrene molecule. Integration of the charge under the naphthoquinone reduction peak was utilized to determine the surface coverage (Γ) of pyr-$(NQ)_2$ at the surface of the electrode, according to Laviron's equation, $I_{pf} = n^2F^2A\Gamma\upsilon/4RT$, where n is the number of electrons involved in the process, F is Faraday's constant, A is the surface area of the electrode, R is the gas constant, T is the temperature, and ν is the scan rate. The average surface coverage (Γ) of the pyr-$(NQ)_2$ was 2.48 ± 0.77 nmol/cm^2, which was not optimized further. This value was from 5 to 11-fold higher than different quinone-derivative

DOI: 10.1021/cs501940g
ACS Catal. 2015, 5, 1240−1244

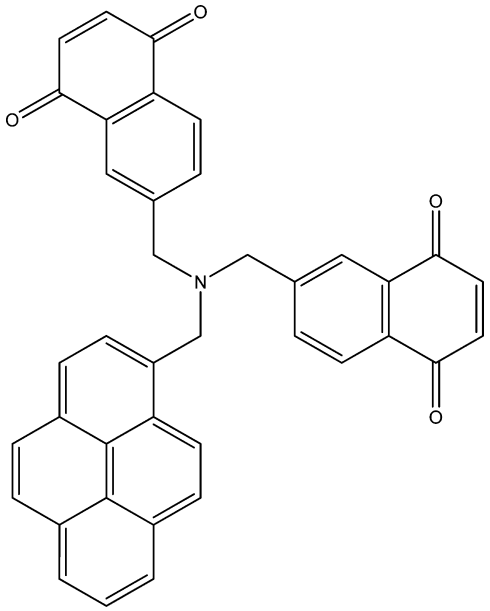

Figure 1. Structure of the pyrene derivative, 1-[bis(2-naphthoquinonyl)aminomethyl]pyrene (pyr-$(NQ)_2$).

monolayers demonstrated by other research groups (0.22 and 0.5 nmol/cm^2), indicating the beneficial use of the pyrene-MWCNTs coupling.[11]

Next, we considered the bioelectrochemical response of the construct for the oxygen reduction reaction (ORR) in the presence of the two different MCOs. Laccase/pyr-$(NQ)_2$ and BOx/pyr-$(NQ)_2$ electrodes were evaluated in 50 mM citrate buffer at pH 4.5 and 6.5, respectively (Figure 2). In the absence of enzymes, the electrodes did not show any significant redox

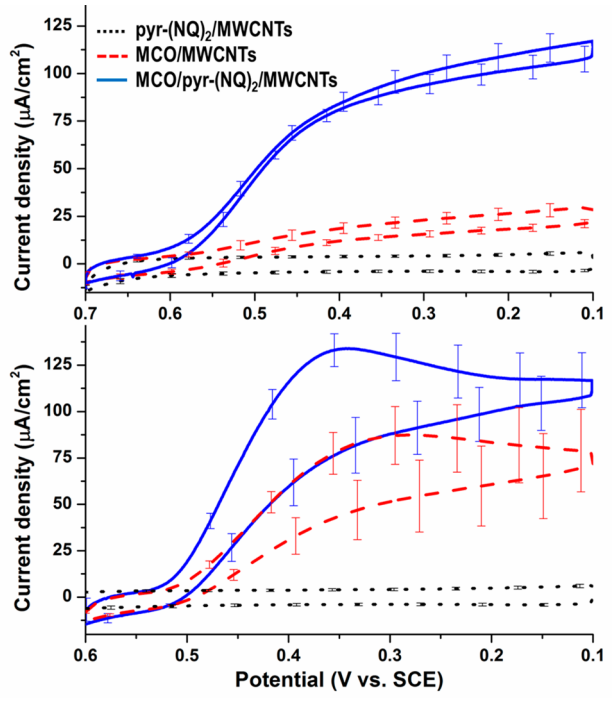

Figure 2. Representative cyclic voltammograms of MCO-based bioelectrodes: (top) laccase in 50 mM citrate buffer (pH 4.5) and (bottom) BOx in 50 mM citrate buffer (pH 6.5) in quiescent solutions, recorded at 1 mV/s.

activity in the potential window of interest (i.e., 0.7−0.1 V). In the absence of pyr-$(NQ)_2$ at the electrodes (but with enzymes present), direct electron transfer was observed at unmodified MWCNTs, although in the presence of pyr-$(NQ)_2$, both the laccase- and BOx-modified electrodes showed an increase in catalytic current (4.3- and 1.5-fold enhancement for laccase- and BOx-modified bioelectrodes, respectively). This demonstrates and confirms the ability of the pyr-$(NQ)_2$ to aid the orientation of these MCOs to the modified electrode surface and improve their direct bioelectrocatalytic activities as a promoter. Specific activities of both enzymes were calculated by UV−vis spectrophotometric assays using 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) as the substrate. Both laccase and BOx demonstrated similar specific activity toward ABTS (laccase, 8.9 ± 0.5 U/mg solid; BOx, 8.4 ± 0.1 U/mg solid), implying that the difference in enhancement was due to structural properties of the two enzymes, such as different binding pocket conformations[12] or hydrophilicity.[12,13]

The current efficiency per mole of docking agent J_{cat}/Γ was investigated by comparing $J_{cat} = J_{cat,functionalized} - J_{cat,unmodified}$. Table 1 compares the pyr-$(NQ)_2$ modification and similar literature reports involving the direct modifications of the walls of CNTs or an anthraquinone-based assembly through $\pi-\pi$ stacking of a pyrene moiety. Because experimental conditions differed greatly among the experiments (pH, type of modification, CNTs, etc..), currents were taken from available data at 0.155 or 0.3 V and from CVs recorded at 1 mV/s or from amperometric measurements under saturated oxygen conditions.[3d,7] Although electrocatalytic currents obtained only from CNT functionalization are slightly lower than comparable literature reports, the construct seems more favorable in terms of efficiency per molecule of docking agent, outperforming CNTs that were chemically modified with anthraquinone on the walls and CNTs functionalized with a pyrene-anthraquinone derivative.

Because the 3D structure of the molecules was shown to help orientate the two MCOs favorably (enhancing their DET at CNT-modified electrodes, at relatively high potentials), we further investigated if the presence of the quinone could be used to mediate fuel oxidation through the use of another oxidoreductase. The formal potential of pyr-$(NQ)_2$ was calculated to be $E_{1/2}$ = −0.143 ± 0.002 V at pH 6.0. PQQ-GDH and GOx have formal potentials ranging from −0.120 to −0.233 V[14] and from −0.292 to −0.444 V[15] (respectively), depending on the experimental conditions and the organism from which the enzyme is extracted. This implies that the two enzymes could be theoretically "wired" by the quinone groups.

PQQ-GDH and GOx were immobilized with unmodified MWCNTs and pyr-$(NQ)_2$-functionalized MWCNTs. As shown from voltammograms given in Figure 3, in the absence of pyr-$(NQ)_2$, an irreversible peak corresponding to the reduction of oxygen on the MWCNTs was observed at −0.375 V. At more positive potentials, PQQ-GDH began to oxidize glucose at around +0.15 V, with small catalytic currents of −6.0 ± 0.8 μA/cm^2 (background-subtracted) at +0.3 V vs SCE in a 20 mM MOPS buffer containing 20 mM KCl and 6 mM $CaCl_2$ (pH 6.5).

When the MWCNTs were functionalized with the pyrene derivative, a quasi-reversible system was observed, which was attributed to the quinones. The reduction currents were marginally higher than the oxidative currents, which could be due to the catalytic reduction of O_2 by the NQ. In the presence of glucose, the reduction peak intensity decreased while the

DOI: 10.1021/cs501940g
ACS Catal. 2015, 5, 1240−1244

Table 1. Comparison of Electrocatalytic Currents for O_2 Reduction Obtained from Different Surface Functionalizations under Saturated O_2 Conditions[a]

	$J_{cat,unmodified}$ (μA/cm^2)	$J_{cat,functionalized}$ (μA/cm^2)	J_{cat} (μA/cm^2)	Γ (nmol/cm^2)	J_{cat}/Γ (μA/nmol)
laccase/AQ-side SWCNT (at 0.155 V vs SCE)[3d]	27 ± 5	215 ± 15	187 ± 12	297.0 ± 3.7	0.63
laccase/Pyr-$(AQ)_2$/MWCNT (at 0.3 V vs SCE)[7]	180 ± NA	900 ± NA	720 ± NA	123 ± 15	5.9
laccase/Pyr-$(NQ)_2$/MWCNT	29.1 ± 1.9	189.5 ± 22.4	160.4 ± 22.5	2.5 ± 0.8	64.7 ± 22.4
BOx/Pyr-$(NQ)_2$/MWCNT	161.2 ± 35.2	277.4 ± 52.6	116.2 ± 63.3	2.5 ± 0.8	46.9 ± 29.2

[a]AQ, anthraquinone; NQ,naphthoquinone; Pyr, pyrene.

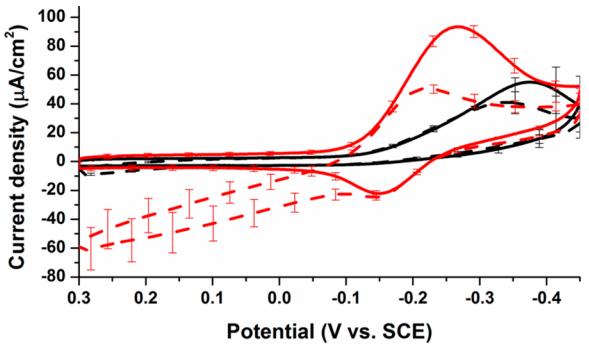

Figure 3. Representative cyclic voltammograms of PQQ-GDH/MWCNTs (black) and PQQ-GDH/pyr-$(NQ)_2$/MWCNTs (red) TPs electrodes recorded at 1 mV/s in 20 mM MOPS, 20 mM KCl and 6 mM $CaCl_2$ buffer (pH 6.5). Solid CVs were recorded without glucose inside the electrolyte; dashed CVs were obtained in the presence of 10 mM substrate.

oxidation peak currents increased at potentials as low as −0.130 V and reached −55.7 ± 13.9 μA/cm^2 at +0.3 V. The onset potential was lowered by ∼300 mV in comparison with unfunctionalized electrodes. Further experiments were performed to confirm that the bioelectrocatalytic behavior of PQQ-GDH was due to mediation by the pyr-$(NQ)_2$.

PQQ-GDH/pyr-$(NQ)_2$- and PQQ-GDH-based electrodes were tested in the presence of glucose and with different concentrations of free PQQ in solution, ranging from 10 to 50 μM. As shown in Supporting Information Figure S4, the addition of free PQQ to the electrolyte modified the electrocatalytic response of the electrodes when tested in the presence of 10 mM glucose. In the presence of 50 μM free PQQ, electrocatalytic currents were as high as −124.5 ± 0.4 and −166.2 ± 2.7 μA/cm^2 at 0 and 0.3 V, respectively. Under the same conditions, electrodes without pyrene functionalizations produced only −15.1 ± 6.2 and −34.6 ± 10.3 μA/cm^2 at 0 and 0.3 V, respectively. Bioelectrodes prepared with pyr-$(NQ)_2$-functionalized MWCNTs displayed a larger increase in glucose oxidative currents than unmodified MWCNTs. The increase in catalytic current in the absence of pyr-$(NQ)_2$ at the electrodes could suggest that free PQQ could be used as a mediator to "wire" the holoenzyme, as was previously discussed.[16] Furthermore, the large increase in catalytic current obtained at naphthoquinone-modified electrodes could be a result of not all of the immobilized GDH in its holo form but, rather, in its apo form where the PQQ was not bound to the protein.

Finally, the results supported that the pyr-$(NQ)_2$ was able to act as a mediator for the bioelectrocatalytic conversion of $PQQH_2$ back to PQQ (the active form of the cofactor used for glucose oxidation). Nevertheless, it is clear that the presence of the pyr-$(NQ)_2$ moieties significantly aids MET from PQQ-GDH. Mediated bioelectrocatalysis was not obtained at GOx/pyr-$(NQ)_2$-modified electrodes. Cyclic voltammograms presented the characteristic redox wave assigned to the NQ at about −0.190 V in 0.1 M phosphate/0.1 M nitrate buffer at pH 7.0; however, upon successive additions of glucose, no catalytic response was measured at oxidative potentials (Supporting Information, Figure S5).

Because of the physical and electrochemical properties demonstrated within this article, pyr-$(NQ)_2$ is appropriate for use both at the biocathode to aid direct bioelectrocatalytic O_2 reduction and at the bioanode to oxidize glucose via MET. Thus, we combined the two different electrodes (Lc/pyr-$(NQ)_2$ and PQQ-GDH/pyr-$(NQ)_2$ or BOx/pyr-$(NQ)_2$ and PQQ-GDH/pyr-$(NQ)_2$), resulting in a complete membraneless glucose/O_2 biofuel cell (BFC). Half cells were tested independently in their specific electrolytes (Supporting Information, Figure S6). In 20 mM MES, 20 mM KCl, and 6 mM $CaCl_2$ (pH 5.5), CVs of laccase biocathodes showed a 35% decrease in performance compared with the same electrodes in 50 mM citrate buffer at pH 4.5. This diminution is clearly due to the instability of the enzyme at higher pH.[17] In contrast, the BOx-based biocathodes showed improved O_2 reduction in the 20 mM MOPS, 20 mM KCl, and 6 mM $CaCl_2$ bulk solution, as opposed to in 50 mM citrate buffer (both at pH 6.5), with current densities reaching 92.6 ± 9.9 μA/cm^2 at 0.3 V in quiescent solutions. For the PQQ-GDH-based bioanodes, results were similar regardless of the buffer or its pH value.

Finally, the complete biofuel cells were characterized with linear polarization to investigate whether the pyr-$(NQ)_2$ can effectively be used simultaneously at a biocathode and a bioanode. To confirm this theory, the current and the power densities of BFCs composed of enzyme/pyr-$(NQ)_2$ bioelectrodes were compared with those obtained for BFCs, which did not incorporate pyr-$(NQ)_2$ at the bioelectrodes. As demonstrated in Figure 4, the BFCs containing the synthesized pyr-$(NQ)_2$ had higher OCPs and were able to generate more electrical power than their counterparts, which did not incorporate pyr-$(NQ)_2$ immobilized on the MWCNT (Supporting Information, Figure S7). Current and power densities were 50−80 μA/cm^2 and 12−20 μW/cm^2, respectively.

CONCLUSION

This paper describes the synthesis of a pyrene-modified naphthoquinone. The molecule was immobilized on MWCNTs and was used simultaneously in a biofuel cell to efficiently orientate MCO oxidases to promote DET for the ORR and to mediate electron transfer of PQQ-GDH for glucose oxidation. It is envisioned that greater current and power densities could be achieved because the BFCs were not optimized (quinone loading, enzyme loading, ionic strength). This system shows promising results because it greatly simplifies the construction of biofuel cells.

DOI: 10.1021/cs501940g
ACS Catal. 2015, 5, 1240−1244

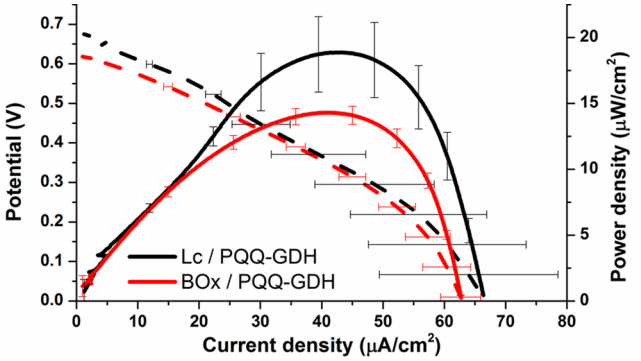

Figure 4. Polarization (dashed line) and power curves (solid line) obtained from laccase/PQQ-GDH-based biofuel cells in 20 mM MES, 20 mM KCl, and 6 mM $CaCl_2$ buffer (pH 5.5) (black) and from BOx/PQQ-GDH-based biofuel cells in 20 mM MOPS, 20 mM KCl, and 6 mM $CaCl_2$ buffer (pH 6.5) (red) with enzymes immobilized on pyr-$(NQ)_2$/MWCNT in the presence of 10 mM glucose. Polarization curves are recorded at 1 mV/s. All experiments were recorded under quiescent conditions.

■ ASSOCIATED CONTENT

Supporting Information

The following file is available free of charge on the ACS Publications website at DOI: 10.1021/cs501940g.

Detailed experimental conditions and additional figures (PDF)

■ AUTHOR INFORMATION

Corresponding Author

*Phone: 801-587-8325. E-mail: minteer@chem.utah.edu.

Present Address

[§](Fabien Giroud) Département de Chimie Moléculaire, Université de Grenoble, Saint Martin d'Hères, France.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

The authors thank the National Science Foundation (Grant #1158943) for financial support. We thank Prof. Matthew S. Sigman and his group for critical synthesis discussion.

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DOI: 10.1021/cs501940g
ACS Catal. 2015, 5, 1240−1244