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Bioelectrocatalytic Oxidation of Alkanes in a JP-8 Enzymatic Biofuel Cell

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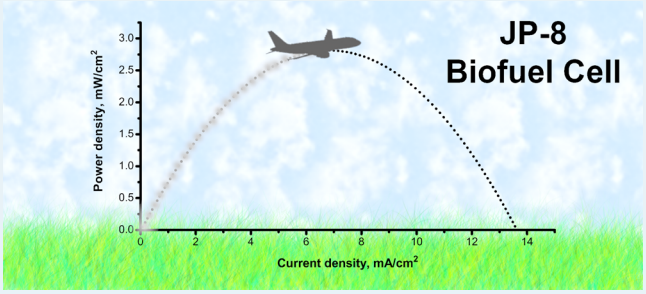
Supporting Information

ABSTRACT: Alkanes are attractive fuels for fuel cells due to their high energy density, but their use has not transitioned to biofuel cells. This paper discusses the development of a novel enzyme cascade utilizing alkane monooxygenase (AMO) and alcohol oxidase (AOx) to perform mediated bioelectrocatalytic oxidation of hexane and octane. This was then applied for the bioelectrocatalysis of the jet fuel JP-8, which was tested directly in an enzymatic biofuel cell to evaluate performance. The enzymatic catalysts were shown to be sulfur tolerant and produced power densities up to 3 mW/cm^2 from native JP-8 without desulfurization as opposed to traditional metal catalysts, which require fuel preprocessing.



KEYWORDS: *bioelectrocatalysis, biofuel cells, JP-8, alkane oxidation, alkane monooxygenase, alcohol dehydrogenase, alcohol oxidase*

INTRODUCTION

JP-8 is a kerosene-based jet fuel that is used in military supply chains. It has been designed to operate in a variety of conditions from subzero temperatures to extreme deserts and is safe due to a lack of flammability. However, the current methods for converting JP-8 to energy are insufficient. The dominant mechanism for decomposing JP-8 is through a standard internal combustion engine or tactical quiet generator (TQG). There are numerous issues with this method including low efficiency (<30% at peak load and significantly lower at nonoptimal loads) and large thermal and acoustic signatures. Because of these shortcomings, a solution for electrochemical oxidation of JP-8 is highly desirable. There has been significant research into the use of solid oxide fuel cells (SOFCs) for conversion of JP-8 into electrical energy.[1] However, the metal catalysts in a traditional SOFC are poisoned by the sulfur inherent in JP-8, which can vary from 400 to 1600 ppm depending on source. In order to use the SOFCs, a reformer needs to be placed in front of the fuel cell, adding complexity and weight to the overall solution. The SOFC catalysts also require very high temperatures (>500 °C).[1b]

Enzymatic biofuel cells are a low-temperature electrochemical alternative to SOFCs. They can operate at ambient temperature ranges (from −20 to 60 °C depending on the enzyme cascade), tolerate impurities such as sulfur, and are not easily passivated,[2] but alkanes have never been explored as fuels for enzymatic biofuel cells. The common fuels for enzymatic biofuel cells are sugars (i.e., sucrose,[3] trehalose,[4] glucose,[5] and fructose[6]) and alcohols (methanol[7] and ethanol[8]). Although there are a variety of other fuels being studied (lactate,[9] pyruvate,[10] hydrogen,[11] etc.), alkanes have never been evaluated for enzymatic biofuel cells, even though they are frequently considered for production of biofuels. There are a number of reasons for this: most alkane-based fuels are mixtures, and therefore, promiscuous enzymes are needed with broad substrate specificity of alkanes of different carbon chain length. Furthermore, in order to derive sufficient energy from an alkane fuel, an enzyme cascade is needed to deeply oxidize the alkane fuel.

In this paper, we evaluate the use of an enzyme cascade of AMO and an alcohol oxidizing enzyme (either alcohol oxidase or dehydrogenase) for the bioelectrocatalysis of hexane, octane, and JP-8. The reaction scheme for the proposed cascade is shown in Figure 1. AMO is an oxidoreductase enzyme which converts alkanes to alcohols. AOx is an FAD-containing enzyme that is rarely used for bioelectrocatalysis. More commonly used enzymes for alcohol oxidation are NAD-dependent alcohol dehydrogenase and PQQ-dependent alcohol dehydrogenase (PQQ-ADH). NAD-dependent alcohol dehydrogenase is very specific, whereas PQQ-ADH is promiscuous;[12] however, it is a membrane-bound protein that is relatively unstable in solution of alkanes or alcohols in high

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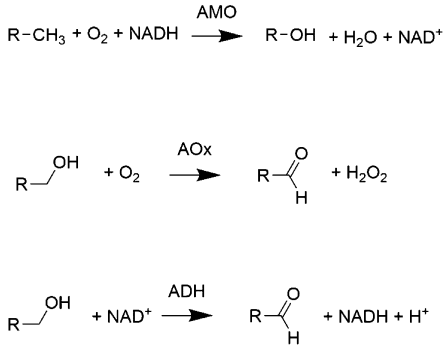

Figure 1. Reaction scheme for the enzyme cascade.

concentrations.[13] For this study, we chose to test both AOx and NAD-dependent alcohol dehydrogenase to determine which produces larger bioelectrocatalytic current in a cascade with AMO. Although the promiscuity of AOx has not been previously explored, in this paper, we show it is a stable and promiscuous enzyme that is ideal for this application. The two-enzyme cascade of AMO and AOx was then incorporated into a JP-8 biofuel cell.

EXPERIMENTAL SECTION

Methods and Materials. All chemicals employed in the preparation and testing of the enzymatically modified electrodes were purchased from Sigma-Aldrich (St. Louis, MO) and utilized as received unless specified otherwise. Electrode materials were obtained from Buckeye Composites (Dayton, OH) for multiwall carbon nanotube buckypaper (MWNT-BP) and Cheaptubes (Cambridgeport, VT) for single and multiwall carbon nanotubes (SWNTs and MWNTs). The enzymes alcohol oxidase (AOx, 20 U/mg: 47 U/mL, from *Candida boidinii*, EC 1.1.3.13) and alcohol dehydrogenase (ADH, 500 U/mg, from *Bakers yeast*, EC 1.1.1.1) were obtained from Sigma-Aldrich (St. Louis, MO) and Calzyme Laboratories (San Luis Obispo, CA), respectively. Additionally, a sample of AOx was obtained from MP Biomedical (Santa Ana, CA) for performance comparison purposes.

***Pseudomonas oleovorans* Growth.** *Pseudomonas oleovorans* (ATCC 8062) was rehydrated in nutrient broth (NB, 3 g/L beef extract and 5 g/L peptone). Several drops of this suspension were used to inoculate a nutrient agar plate (3 g/L beef extract, 5 g/L peptone, and 15 g/L agar). Plates were incubated at 26 °C for 48 h until small white colonies were observed. A 50 mL aliquot of NB was inoculated from the plate and 1% hexane was added. After 24 h, this culture was added to 6 L of NB containing 1% hexane and 1% octane. After 48 h, the cells were collected by centrifugation and stored at −80 °C until further use.

AMO Isolation. AMO from *P. oleovorans* was isolated and purified according to a literature procedure.[14] Frozen cells were resuspended in 50 mM pH 7.4 Tris buffer containing 1 mM ethylenediaminetetraacetic acid (EDTA) (4 mL per gram of cells). The mixture was stirred for 30 min at room temperature and then centrifuged at 10 000*g* for 30 min. The supernatant was discarded, and the pellet was resuspended in 50 mM pH 7.4 Tris buffer containing 10 mM mercaptoethanol and RNase and DNase (50 μg per mL). Cells were disrupted by sonication on ice at 90% amplitude with 6−7 pulses (30 s pulses at 1 min intervals). The mixture was centrifuged at 10 000*g* for 30 min. Streptomycin was added to the supernatant to a final concentration of 5% and the mixture was stirred for 15 min. After centrifugation at 10 000*g* for 15 min, the pellet was discarded, and the supernatant was subjected to ammonium sulfate precipitation. Ammonium sulfate was added in increasing amounts, and the precipitate at each concentration was collected by centrifugation at 20 000 rpm for 30 min. The precipitate collected below 25% ammonium sulfate was discarded. Precipitates that formed between 25% and 35% were collected, while the precipitate from 45% ammonium sulfate was discarded. The precipitate was resuspended in minimal 50 mM pH 7.4 Tris buffer with 10% glycerol.

AMO Purification. The crude extract was clarified at 100 000*g* for 1 h. The enzyme was purified on a Sepharose CL-6B gel filtration column (2.5 cm × 60 cm) equilibrated with 0.1 M pH 7.4 Tris buffer containing 50 mM KCl and 10% glycerol. The column was eluted with the same solution at 1 mL/min. Fractions were collected and assayed for AMO activity. Typically four peaks were observed, and the first three peaks showed activity to hexane. Active fractions were stored at −20 °C.

Specific Activity of AMO. A 15 μL aliquot of 12 mM NADH and 100 μL of 10 mM octane were added to 865 μL of 50 mM pH 7.4 Tris buffer. A 20 μL aliquot of enzyme was added and mixed briefly. The absorbance at 340 nm was recorded. The activity of the enzyme (in U/mL) was calculated using the following equation:

$$\frac{\mathrm{U}}{\mathrm{mL}} = \frac{\left(\frac{\Delta \mathrm{Abs}}{\mathrm{min}}\right)(\text{dilution factor})(\text{total volume})}{(6.22\ \mathrm{mM^{-1}cm^{-1}})(\text{volume of enzyme})}$$

The specific activity is then calculated by dividing by the protein concentration (in mg/mL) which was determined using a Pierce BCA protein assay kit.

MWNT-BP Electrode Preparation. A piece of MWNT-BP (1 × 1 cm) was first washed with isopropyl alcohol (IPA), followed by a thorough rinsing with ultrapure (18 MΩ) water and then drying with nitrogen gas. Poly(methylene green) (PMG) films were then electrochemically deposited onto the surface of MWNT-PB electrode as per a procedure modified from refs 15 and 16. The electrodes were submerged in 5 mL of solution containing 0.5 mM methylene green (MG), dissolved in 0.05 M potassium phosphate buffer (pH 7) with 0.1 M potassium nitrate. Cyclic voltammetry (CV) was employed for electrodeposition of PMG by cycling the potential from −0.5 to 1.3 V vs sat. Ag/AgCl for 10 cycles at a scan rate of 50 mV/s. Coiled platinum wire was utilized as a counter electrode, and the deposition solution was purged with nitrogen gas for the duration of polymerization. Once PMG was deposited onto the electrode, it was first washed with copious amounts of ultrapure water in order to remove any unreacted MG from the electrode surface and then dried with N_2 gas.

Enzymatic Ink Preparation. A 1 wt % COOH-MWNTs (dia <8 nm, 0.5−2.0 μm length, >95% w% purity) ink solution containing 0.1 wt % tetrabutylammonium-bromide-modified Nafion (TBAB-Nafion) (in ethanol/water mixture) was prepared following a previously described procedure.[17] Enzymatic inks were prepared by addition of the enzyme (either powder or solution) to the above-mentioned ink. For alcohol (ethanol, hexanol, octanol, and dodecanol) testing, enzymatic inks were prepared by adding 1 mg of ADH and 33.3 μL of ultrapure water to 16.7 μL of MWNT ink for a total volume of 50 μL. The ink was gently vortexed, evenly drop-cast onto the electrode, and allowed to dry at 4 °C overnight. A

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similar procedure was employed for the preparation of AOx-modified electrodes with few modifications: no PMG was deposited on the MWNT-BP prior to the addition of the enzymatic ink, and 21.3 μL of AOx enzyme and 12 μL of ultrapure water were added to 16.7 μL of MWNT ink for a total of 50 μL enzymatic ink volume that was deposited onto the electrode surface. Total enzyme loading for either ADH or AOx was held constant at 1 mg/cm^2. Casting and drying procedures remained unchanged.

Alcohol Oxidizing Enzymatic Electrode Testing. A three-electrode electrochemical test cell was employed for all enzymatic electrode characterization. The MWNT-BP enzyme-modified electrode was submerged in 4.28 mL of 0.1 M Tris buffer (pH 7.4) with either 5 mM nicotinamide adenine dinucleotide (NAD^+) or 10 mM hydroquinone (HQ) for ADH or AOx alcohol testing, respectively. Platinum coiled wire and sat. Ag/AgCl were utilized as the counter and reference electrodes. Open circuit potential (OCP) was measured by a multichannel potentiostat (VMP3, BioLogic Inc.) until a stable value was reached (approximately 1 h). Amperometry was utilized in order to develop a concentration profile. Constant potential of 0.3 V vs AglAgCl was applied to the electrode, and the current was monitored as a function of time. Aliquots of various alcohols (63.2 mM stock in Tris buffer) were added to the electrochemical cell, incrementally, and the peak current was used to plot current versus concentration. Specifically for dodecanol, testing was carried out under elevated temperature conditions of 37°C because dodecanol is a solid at room temperature. These experiments were performed in triplicate, except that octanol with AOx was tested four times and hexanol with AOx was tested five times. The plotted data show the averages of the measurements.

AMO Ink Preparations. Electrodes were prepared such that each AMO sample being studied had the same 8.2 μg/cm^2 enzymatic loading. The values for the various components of the enzymatic ink are summarized in Table 1. The enzymatic ink was deposited onto 1 cm^2 MWNT-BP electrode that was modified with PMG prior to the addition of the ink and allowed to dry at 4 °C overnight.

Table 1. Tabulated Summary of Enzymatic Ink Components Employed for the Fabrication and Electrochemical Testing of Electrodes Modified with Several AMO Samples

electrode Name	AMO volume (μL)	AOx volume (μL)	MWNT ink volume (μL)	water volume (μL)	total volume (μL)
crude AMO 1	16.89	21.3	16.7	0	54.89
crude AMO 2	1.01	21.3	16.7	11	50.01
AMO 1	33.3	21.3	16.7	0	71.3
AMO 2	0.78	21.3	16.7	11.2	49.98
AMO 3	1.01	21.3	16.7	11	50.01

Electrochemical characterization was performed in a three-electrode cell with platinum wire and sat. Ag/AgCl as counter and reference electrodes, respectively. The cell was filled with 5 mL of 0.1 M Tris buffer (pH 7.4) with 5 mM NAD^+ and 10 mM hydroquinone. Some of the NAD^+ is reduced by hydroquinone to NADH which can then be used for the enzymatic reaction. For amperometric measurements under 0.3 V perturbation, the test solution was spiked with 1.69 mM octane. For the sample that was tested with JP-8 fuel, the fuel concentration was 0.0109 g/mL, and 79 μL of this fuel was added to the cell for testing.

Complete Enzymatic Fuel Cell Preparation and Testing. *Bioanode Preparation.* Carbon felt (Alfa Aesar) of 5.226 cm^2 geometric surface area and 1.27 cm thickness was modified with PMG following the same procedure described above. Solution of MWNT ink with AMO and AOx was prepared by scaling the previously described parameters in order to deposit a total of 850 μL of the enzymatic ink solution. Casting and drying procedures remained unchanged.

Cathode Preparation. The Prussian Blue cathode was prepared in a similar procedure as described in ref 18 by adding 6 mg of iron(III) chloride, 6 mg of potassium ferricyanide, 6 g of carbon fiber, and 12 g of carbon black (Vulcan XC-72R) to about 10−12 mL of 1-butyl-3-methylimidazolium chloride (BMIMCl) ionic liquid, and the preparation was ground well to obtain a thick paste mixture.

Fuel Cell Testing. The bioanode was fitted into polycarbonate hardware along with a piece of platinum wire, used for electrical connection. The cathode ionic liquid paste was placed into the cathodic plate hardware and sealed with a Nafion 212 film (Ion Power). Two cathodes were employed for the fabrication of the fuel cell. The schematic in Figure 2

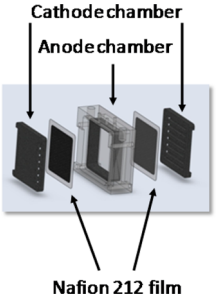

Figure 2. Representative schematic of polycarbonate hardware employed for testing of a complete biofuel cell.

depicts the hardware setup. The cell was filled with 2% (by vol.) JP-8 mixed with 0.1 M Tris buffer (pH 7.4) with 5 mM NAD^+ and 10 mM HQ. Constant load discharge (CLD) technique was employed for fuel cell testing where a series of resistances (from 5 MΩ to 5 Ω) were applied to the cell while current and voltage were monitored as a function of time. Complete cell power and current density curves were generated for the enzymatic fuel cell. All electrochemical studies were performed under ambient laboratory conditions. The average of three trials is reported, and the uncertainties correspond to the standard deviation of those triplicate measurements.

RESULTS AND DISCUSSION

JP-8 is a kerosene-based fuel made of a series of hydrocarbons between C6 and C16. Therefore, hexane and octane were used to test the ability of AMO to act as an enzyme within the JP-8 cascade. AMO is an enzyme responsible for converting an alkane to a primary alcohol, which can then be oxidized to an aldehyde by either AOx or ADH. We evaluated the ability of AMO to function bioelectrocatalytically with the addition of octane and hexane. Amperometry was performed at a poly(methylene green)-coated electrode with AMO dissolved in solution and the substrate (octane or hexane) added in increments. Supplemental Figure S1 shows representative amperometric data for an AOx-modified electrode with

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injections of octanol and in solution containing AMO with injections of hexane. Results for control electrodes with no enzyme are also shown. Figures S2 and S3 show the ability of AMO in solution to do mediated bioelectrocatalysis of octane and hexane. The current densities are much higher for hexane than octane, but both systems show NAD/NADH-mediated bioelectrocatalysis. Because octane is probably a better simulant for JP-8, we evaluated the current density of crude AMO (specific activity 0.071 mU/mg) and three different column purification aliquots (AMO1, specific activity of 3.3 mU/mg; AMO2, specific activity of 0.77 mU/mg; AMO3, specific activity of 0.20 mU/mg) after the addition of 1.69 mM octane. Table 2 shows the current density at 0.3 V vs Ag/AgCl for each

Table 2. Summary of Amperometric Test Results at 0.3 V vs Ag/AgCl for Various AMO Sample Electrodes

enzyme/substrate	protein concentration (mg/mL)	specific activity (U/mg)	current density (μA/cm^2)
crude AMO/octane	59.5	0.071	0.26 ± 0.04
AMO1/octane	0.245	3.3	0.4 ± 0.2
AMO 2/octane	1.37	0.77	0.3 ± 0.2
AMO 3/octane	8.07	0.20	0.3 ± 0.1
AMO1/JP-8	0.245	3.3	0.45

of the AMO samples utilizing octane as substrate. The purified AMO yields 2× higher current density. Therefore, we tried the purified sample (AMO1) with JP-8 and found similar current densities for JP-8 compared to octane.

The second step of the enzyme cascade is to take the resulting alcohols formed from the alkanes and oxidize those alcohols to aldehydes. Because JP-8 is not a pure source of alkanes, there is a need for a promiscuous enzyme that can oxidize a variety of long chain, primary alcohols. We investigated both NAD-dependent ADH and AOx. ADH has the benefit of using the same cofactor as AMO, but Figure 3

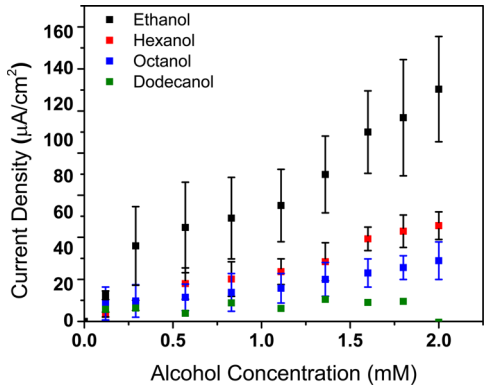

Figure 3. Representative calibration curves for ADH-modified electrodes in various alcohols: ethanol, hexanol, and octanol.

shows that the bioelectrocatalysis of ethanol, hexanol, and octanol is very substrate-dependent, and no activity was observed for decanol or dodecanol. On the other hand, AOx has broad substrate specificity as shown by the bioelectrocatalysis in Figure 4. There is no statistical difference between the performance of the AOx bioanodes utilizing ethanol (the natural substrate), hexanol, or octanol. Longer chain alcohols (i.e., dodecanol) did show a decrease in activity, but still

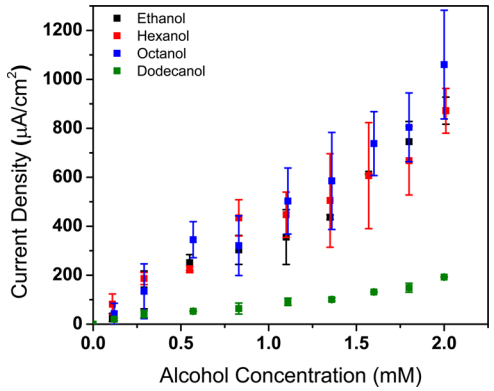

Figure 4. Representative calibration curves for AOx-modified electrodes in various alcohols: ethanol, hexanol, and octanol.

showed significant enzymatic activity as compared to ADH-modified electrodes.

After deciding AOx was the preferred enzyme for the enzyme cascade, we evaluated two different commercial sources of AOx (Sigma-Aldrich and MP Biomedical). As shown in Figure 5,

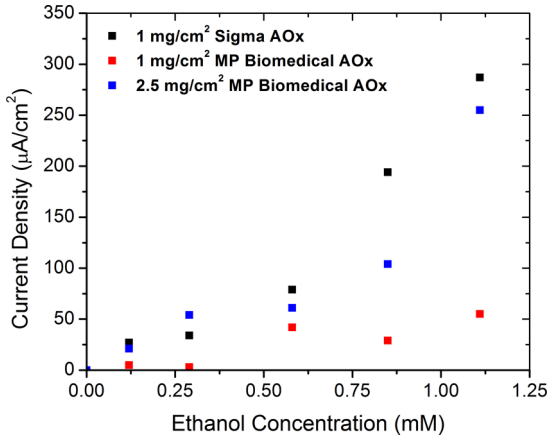

Figure 5. Comparison of biocatalytic performance of AOx, obtained from different vendors, toward ethanol.

there is better performance for the Sigma-Aldrich enzyme than the MP Biomedical enzyme. We need twice as much MP Biomedical enzyme for the same bioelectrode sensitivity. We also evaluated the difference between immobilizing in tetrabutylammonium-bromide-modified Nafion and the proprietary CFDRC immobilization matrix (CFDRC/CNT ink) which contains carbon nanotubes, polyethylenimine, and a cross-linker.[19] The results in Figure 6 show similar performance between the two immobilization matrixes. This shows that both immobilization strategies allow for large active surface area and large enzyme loading and that neither immobilization strategy negatively impacts transport in the system.

One of the major issues facing current methods of energy production from JP-8 is the intolerance to sulfur. To evaluate whether sulfur would cause a decrease in power production by the enzymatic fuel cell, activity assays with the AMO were performed in the absence and presence of sulfate. As shown in Figure S4, there is no change in octane oxidation when sulfate is added so the presence of sulfur in the JP-8 should not affect the power output. The sulfate concentrations used (5 and 10 mM) fall within the range of sulfur concentrations found in JP-8.

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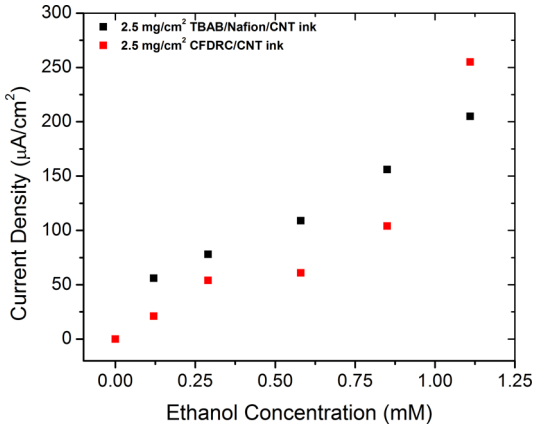

Figure 6. Comparison of enzyme immobilization techniques: evaluation of biocatalytic performance of AOx immobilized in TBAB-modified Nafion matrix (black) and CFDRC carbon nanotube ink (red) toward ethanol.

Finally, we evaluated the enzyme cascade in a complete fuel cell with JP-8 fuel. Initial tests were performed with immobilized AOx at the bioanode with AMO in solution and a Prussian Blue cathode previously used in biobatteries.[18] Figure S5 shows polarization curves and power curves for a 2% by volume JP-8 fuel cell. The maximum current and power densities of 11.4 mA/cm^2 and 3 mW/cm^2, respectively, were obtained for the enzymatic fuel cell testing with JP-8 fuel, while 0.05 mW/cm^2 and 1.2 mA/cm^2 values were obtained for the control cell (tested without JP-8 fuel). Open circuit potentials were between 600 and 630 mV. The current densities are significantly higher compared to the amperometric results with individual alkanes. These experiments were repeated using an anode with both AMO and AOx immobilized and the average results of three trials are shown in Figure 7. With both enzymes

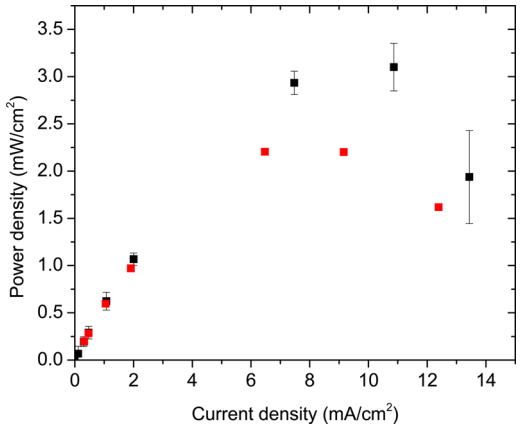

Figure 7. Power density curves for enzymatic fuel cell tested utilizing 2 vol % JP-8 (in black) and 8 vol % JP-8 (in red).

immobilized, the maximum current and power densities are ~14 mA/cm^2 and 3.1 mW/cm^2, respectively, with an OCV of ~600 mV. Representative data for a fuel cell tested with 10 vol % JP-8 is also included. No significant change in OCV or power or current density is observed with the higher fuel concentration. JP-8 consists of a mixture of alkanes, and the concentrations of these alkanes are unknown. The high currents can be attributed to the fact that the enzymes are able to react with many of these alkanes, and the concentrations may be higher than those used in the amperometry experiments. The shape of the polarization curve indicates that the biofuel cell suffers from mass transport limitation, which is to be expected as one of the enzymes and the mediator are free in solution.

CONCLUSIONS

This paper describes the first ever bioelectrocatalysis of alkanes. A two-enzyme cascade of AMO and AOx was used to oxidize a variety of alkanes and then used to oxidize JP-8, a jet fuel consisting of a mixture of alkanes. This enzyme cascade is efficient and produces power densities up to 3 mW/cm^2 in a JP-8 enzymatic biofuel cell without preprocessing of the fuel, which is comparable to high power density sugar and alcohol biofuel cells.[7a,8,20]

ASSOCIATED CONTENT

Supporting Information

The following files are available free of charge on the ACS Publications website at DOI: 10.1021/cs500802d.

Experimental procedures, amperometric data for enzymatic electrodes with substrate injections, calibration curves for enzymatic electrodes with several substrates, colorimetric activity assay results in the absence and presence of sulfate, and fuel cell results with AMO in solution (PDF).

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Notes

The authors declare no competing financial interest.

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