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COMMUNICATION

Selective electroenzymatic oxyfunctionalization by alkane monooxygenase in a biofuel cell

Mengwei Yuan,[a] Sofiene Abdellaoui,[a] Hui Chen,[a] Matthew J. Kummer,[a] Christian A. Malapit,[a] Chun You[b] and Shelley D. Minteer*[a]

Abstract: Aliphatic synthetic intermediates with high added value are generally produced from alkane sources (e.g., petroleum) by inert carbon-hydrogen (C-H) bond activation using classical chemical methods (high temperature, rare metals). As an alternative approach for these reactions, alkane monooxygenase from *Pseudomonas putida* (alkB) is able to catalyze the difficult terminal oxyfunctionalization of alkanes selectively and under mild conditions. Here, we report an electrosynthetic system using an alkB biocathode which produces alcohols, epoxides, and sulfoxides through bioelectrochemical hydroxylation, epoxidation, sulfoxidation, and demethylation. The capacity of the alkB binding pocket to protect internal functional groups, a lucrative capability, is also demonstrated. By coupling our alkB biocathode with a hydrogenase bioanode and using H_2 as a clean fuel source, we have developed and characterized a series of enzymatic fuel cells capable of oxyfunctionalization while simultaneously producing electricity.

Petroleum, the combustion of which accounts for 45% of all carbon dioxide emissions in the U.S., will find increasing utility as a source of chemical synthons while its use in other sectors is being replaced by renewable energy sources.[1] As the petroleum industry carries out this transition, it is of critical importance to improve sustainability and minimize the negative environmental impact of petroleum chemical conversions which currently involve the use of high temperatures (200-600℃), rare metals, and organic solvents.[2] Such conditions are required due to the inertness of carbon-hydrogen (C-H) bonds in alkane compounds, a major component of petroleum. In addition to these conventional methods being energy-consuming, alkane chemical conversions typically have poor selectivity.[3] Particularly, the selective activation of the terminal position of alkanes is a class of reactions with a particular interest in the production of commodity chemicals.[4] Internal sites are more readily functionalized due to their weaker bond dissociation energies (BDE: 94.6 kcal/mol vs. 104 kcal/mol for n-decane), leading to the generation of unwanted by-products.[5]

The design of more efficient synthesis routes with high regioselectivity, clean oxidants (i.e.,O_2), aqueous media, and mild conditions is achievable through the use of biocatalysts.[6] Alkane monooxygenase (alkB) from *Pseudomonas putida* GPO1, an integral membrane, nonheme, diiron enzyme, has uniquely high selectivity for the oxidation of terminal carbons of gasoline-range alkanes (C5-C12) and aromatic compounds; therefore, it has been identified as an attractive enzyme for petroleum-derived chemical conversions. Natively, alkB belongs to a system comprised of nine proteins coded by a gene cluster alkBFGHJKLST which enables *P. putida* to assimilate alkanes as a carbon source. The crucial first step of this pathway, the conversion of alkanes to fatty alcohols, involves three proteins: the monooxygenase alkB, a soluble electron transfer protein called rubredoxin (alkG), and a soluble NADH rubredoxin reductase (alkT). AlkG is reduced by alkT using NADH as an electron donor, and the reduced alkG transfers electrons to the diiron cofactor of alkB, forming a high-valent iron-oxo species upon complexation with O_2. This complex enables the catalytic hydrogen extraction from an alkane yielding a radical alkane intermediate and an iron hydroxide, which is subsequently attacked by the radical to generate an alcohol by the following reaction:[7]

$$C_nH_{2n+2} + O_2 + NADH + H^+ = C_nH_{2n+1}OH + H_2O + NAD^+ \; n=5\text{-}12$$

Furthermore, alkB catalyzes other terminal-position reactions including the epoxidation of olefins, sulfoxidation of thioethers, and demethylation of branched methyl ethers.[8] These reactions are lucrative for the synthesis of many building blocks widely used in fine chemical and pharmaceutical industries, such as: 1,2-epoxyoctane, a highly reactive intermediate in organic synthesis; cyclohexanol, a molecule further converted to nylon; 2-phenylethanol, used in the synthesis of flavor additives or fragrances; and 1-alkanols, used in cosmetics, food, industrial solvents, and detergents.[6, 9] Despite the versatile activity of alkB, a primary constraint for its use in such chemical conversions is the continuous and costly consumption of NADH; it is therefore preferable to replace this cofactor with a mediated electrochemical system.[7a]

Finally, enzymatic fuel cells (EFCs) are devices using redox enzymes to catalyze different reactions under ambient conditions and are highly desirable alternatives for chemical production with the spontaneous generation of power.[10] Our group has demonstrated that the combination of a hydrogenase anode with cathodes containing nitrogenase or aldehyde deformylating oxygenase can be used to produce ammonia or alkanes, respectively, oxidizing H_2 as a clean fuel while generating electricity.[11]

In this work, we present a biocathode in a series of EFCs capable of four types of oxyfunctionalization. Toluidine blue O (TBO) serves as an electrochemical mediator continuously supplying electrons to an alkB/alkG biocathode, thereby replacing both NADH and alkT. Further, C-H bond activation is demonstrated with varied substrates. Octane, cyclohexane, and

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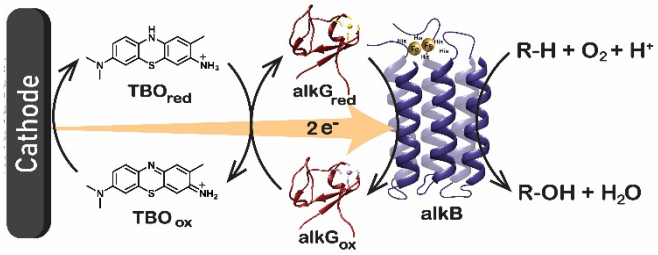
ethylbenzene are functionalized as a model linear alkane, cyclic alkane, and aromatic-containing alkane, respectively. Next, the epoxidation of terminal olefins, sulfoxidation of thioethers, and demethylation of branched methyl ethers are demonstrated using 1-octene, methyl n-octyl sulfide, and 2-methoxyoctane, respectively. To demonstrate the unique regioselectivity and ability of alkB to protect internal functional groups, the activity of alkB towards 4-octene was examined. Finally, by combining an alkB/alkG biocathode with a hydrogenase anode, we constructed EFCs capable of generating power while producing alcohols, epoxides, and sulfoxides. This work demonstrates a potential platform for the utilization of a broad range of alkane hydroxylases in future biosynthetic applications.



Scheme 1. Biocathode design utilizing alkane monooxygenase (alkB; structure adapted from reference[12]), rubredoxin (alkG; PDB ID: 1S24) and TBO for C-H activation.

Initially, the ability of TBO to serve as an electrochemical mediator for the alkB/alkG biocathode was investigated (Scheme 1). Figure 1 presents the voltammograms obtained with the alkB/alkG biocathode in the absence (black) and presence (red) of octane (see Supporting Info for details). Redox peaks are observed at -0.12 V and -0.20 V *vs.* SCE and correspond to the two-proton coupled, two-electron transfer processes between oxidized and reduced TBO species (Figure S1). A catalytic wave (red curve) is observed after the addition of octane, exhibiting enzyme activity. Schematically, reduced TBO is oxidized by alkB/alkG as the electroenzymatic conversion of octane to 1-octanol occurs through C-H activation. A control experiment in the absence of alkB (Figure S2) displayed no catalytic response. The result is consistent with chronoamperometry performed at -0.5 V *vs.* SCE under anaerobic conditions. After an injection of octane and air, twice the current response was observed in the presence of alkB (Figure 1, inset) compared to its absence; the background signal observed is due to the reduction of O_2 by TBO.

To confirm the formation of the expected products, bulk electrolysis experiments were performed at -0.5 V *vs.* SCE for 1 hour followed by GC-MS analysis. The alkB/alkG biocathode produces 1-octanol from octane, and this production is not observed in the absence of alkG, alkB, or TBO (Figure S3). A small quantity of 1-octanal likely generated by overoxidation is also formed (Figure S3E).[8] No secondary alcohols such as 2-octanol were detected, showing that the alkB/alkG biocathode catalyzes this reaction selectively. The headspace air content and alkB/alkG ratio were optimized (Figure S4). Under these conditions, the alkB/alkG biocathode was able to produce 1-octanol at the rate of 1.77 ± 0.01 μmol h^{-1} per mg of alkB. A 25 ± 1% faradaic efficiency is observed as a consequence of electrochemical O_2 reduction by reduced TBO, which competes with C-H activation in octane.

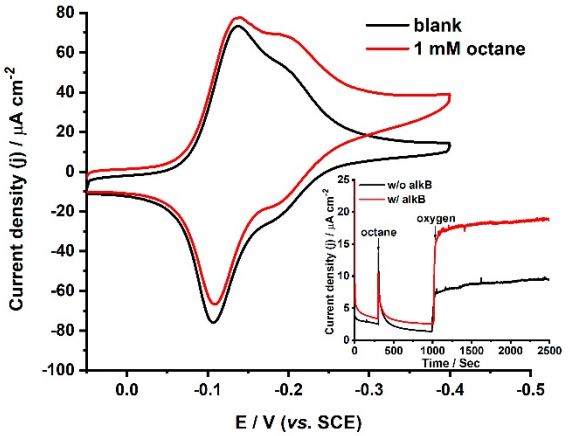


Figure 1. Representative CVs of the alkB/alkG biocathode without (black) and with (red) 1 mM octane. Inset: representative chronoamperometric responses of the alkB/alkG biocathode with the injection of octane (1 mM final concentration) and air in the presence (red) or absence (black) of alkB. See Supporting Info for experimental details.

Table 1. Product quantification, faradaic efficiency and rate of product generation for C-H activation of octane, cyclohexane, and ethylbenzene.

Substrate	Product	Product detected (nmol h^{-1})	Faradaic efficiency (%)	Rate (μmol h^{-1} mg^{-1} of alkB)
Octane	1-Octanol	265 ± 4	25 ± 1	1.77
Cyclohexane	Cyclohexanol	29 ± 2	2.6 ± 0.3	0.19
Ethylbenzene	2-Phenylethanol	71 ± 5	5.1 ± 0.2	0.47

Cyclic and aromatic alkanes that fit the active site of alkB can also be converted by the enzyme.[13] Cyclohexane and ethylbenzene are chosen as model cyclic and aromatic alkane substrates due to the wide potential uses of the corresponding synthetic alcohols, cyclohexanol and 2-phenylethanol. The product quantification, faradaic efficiencies, and rates of product generation during the bulk electrolysis of octane, cyclohexane, and ethylbenzene using the alkB/alkG biocathode are summarized in Table 1. The high binding affinity of aliphatic hydrocarbons compared to these bulky substrates is responsible for the decreased rate of product formation.

Further, the ability of the binding pocket of alkB to protect functional groups and react exclusively at the terminal position was investigated using 4-octene as the substrate. Here, epoxidation of this substrate to 2,3-dipropyloxirane is simply facilitated by metal catalysts through a high-valent oxo complex with the C=C bond.[14] Allylic C-H bonds are relatively reactive (BDE: 83.4 kcal/mol), and thus allylic C-H hydroxylation can be performed using chalcogen-based oxidants.[15] In contrast, the terminal positions of alkenes (BDE: 100.1 kcal/mol) are difficult to hydroxylate, and the products of this reaction are desirable for the generation of compounds including insect pheromones with costs

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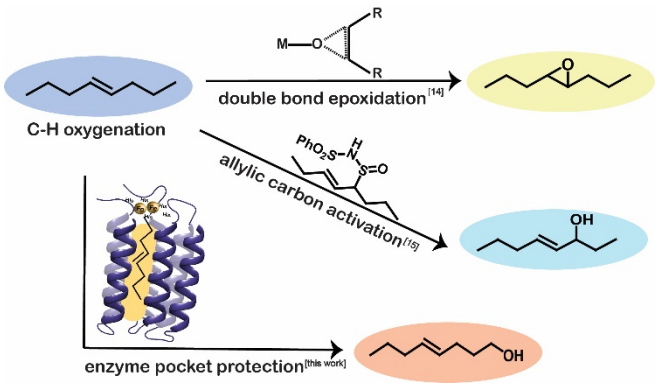
as high as $14,000/kg.[16] After bulk electrolysis of 4-octene, GC-MS analyses show that alkB catalyzes terminal C-H activation, forming 4-octen-1-ol as the exclusive product (Figure S5); this is due to the unique enzyme binding pocket which protects the internal carbon-carbon double bond, a feature which other types of catalysts do not possess (Scheme 2).



Scheme 2. Illustration of three pathways for oxygenation of an internal alkene by a metal catalyst, allylic sulfonamide intermediate, and alkB.

Table 2. Product quantification, faradaic efficiency, and rate of product generation for the epoxidation, sulfoxidation and demethylation of 1-octene, methyl n-octyl sulfide, and 2-methoxyoctane, respectively.

Substrate	Product	Product detected (nmol h^{-1})	Faradaic efficiency (%)	Rate (μmol h^{-1} mg^{-1} of alkB)
1-Octene	1,2-Epoxyoctane	144 ± 8	16 ± 2	0.96
Methyl n-octyl sulfide	1-Methane-sulfinyloctane	110 ± 10	8.7 ± 0.3	0.36
2-Methoxyoctane	2-Octanol	241 ± 5	18 ± 1	1.61

As previously mentioned, alkB catalyzes other reactions beyond C-H activation which are of interest in the field of biocatalysis; the wide range of substrates utilized by alkB confers a significant potential for the synthesis of other aliphatic chemical intermediates. Here, 1-octene, methyl n-octyl sulfide, and 2-methoxyoctane are readily available substrates to demonstrate epoxidation of a terminal olefin, sulfoxidation of a methyl thioether, and demethylation of a branched methyl ether by the alkB/alkG biocathode, respectively. Bulk electrolysis experiments were conducted following the same procedures as above. Chromatograms from GC after bulk electrolysis show the formation of 1,2-epoxyoctane, 1-methanesulfinyloctane, and 2-octanol (Figure S6). Product quantification, faradaic efficiencies, and rates of product generation are shown in Table 2.

We next evaluated methyl viologen (MV) as an electron donor to support H_2 oxidation on a hydrogenase bioanode in an EFC. As shown in the chronoamperometric curve (red) of Figure S7, when holding an oxidative potential for MV (-0.45 *vs.* SCE), an increase in oxidative current while bubbling H_2 is observed; this corresponds to an increase in reduced MV concentration as hydrogenase is turned over compared to the negative control (black) in the absence of hydrogenase.

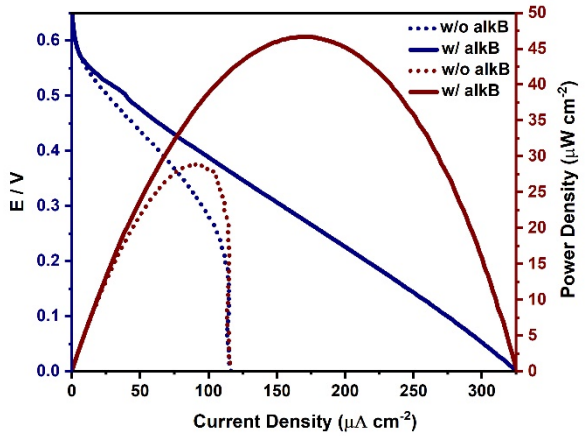
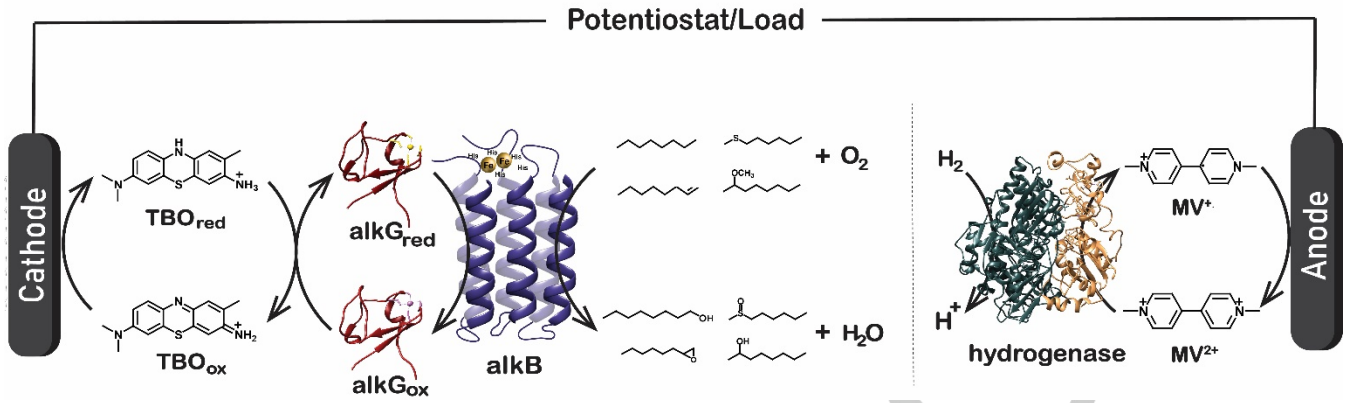


Figure 2. Representative polarization curves (blue lines) and power curves (red lines) for the H_2/octane EFC. Experiments were conducted with (solid lines) and without (dashed lines) alkB by linear sweep polarization at 0.5 mV s^{-1}. See Supporting Info for experimental details.

Finally, 4 different types of EFCs (Scheme 3) were developed to simultaneously generate power and valuable products: H_2/octane EFC to produce 1-octanol, H_2/1-octene to produce 1,2-epoxyoctane, H_2/methyl n-octyl sulfide to produce 1-methanesulfinyloctane, and H_2/2-methoxyoctane to produce 2-octanol. As displayed in Figure 2, the H_2/octane EFC yielded an OCP of 0.65 ± 0.01 V and generated maximum current (I_{max}) and power densities of 318 ± 7 μA cm^{-2} and 45 ± 1 μW cm^{-2}, respectively. After 1 hour of EFC operation (Figure S8), 172 ± 9 nmol of 1-octanol were detected, corresponding to a faradaic efficiency of 23 ± 1%. A control experiment was performed with the addition of the same amount of air and octane in the absence of alkB to demonstrate the oxygen reduction effect by reduced TBO. This resulted in a lower EFC efficiency (OCP: 0.631 ± 0.008 V, I_{max}: 110 ± 6 μA cm^{-2}, and P_{max}: 28 ± 1 μW cm^{-2}; See Figure S9 for control EFCs without aliphatic substrates). Similar EFC results were found for H_2/1-octene, H_2/methyl n-octyl sulfide, and H_2/2-methoxyoctane (Figure S10). Table 3 exhibits the summarized OCP, P_{max}, I_{max}, product quantification, and faradaic efficiency results for 4 types of EFCs. All tested substrates demonstrated comparable EFC performances.



Scheme 3. The designed EFC with an alkB/alkG biocathode (alkG: PDB ID: 1S24; alkB structure adapted from reference[12]) and Nafion-separated hydrogenase bioanode (representative structure: PDB ID: 1FRV) for hydroxylation, epoxidation, sulfoxidation, and demethylation.

Table 3. OCP, I_{max}, P_{max}, product quantification, and faradaic efficiencies for H_2/octane, H_2/1-octene, H_2/methyl n-octyl sulfide, and H_2/2-methoxyoctane EFCs.

Cathode substrate	OCP (V)	Maximum current density (μA cm^{-2})	Maximum power density (μW cm^{-2})	Product quantification (nmol cm^{-2})	Faradaic efficiency (%)	Rate (μmol h^{-1} mg^{-1} of alkB
Octane	0.65 ± 0.01	318 ± 7	45 ± 1	690 ± 34	23 ± 1	1.15
1-Octene	0.657 ± 0.009	190 ± 16	36 ± 3	230 ± 12	15 ± 1	0.38
Methyl n-octyl sulfide	0.647 ± 0.002	335 ± 3	50 ± 5	220 ± 16	9.0 ± 0.4	0.18
2-Methoxyoctane	0.663 ± 0.004	280 ± 26	47 ± 4	420 ± 56	17 ± 2	0.70

In summary, enzymatic C-H activation of the terminal position of inert alkanes by an alkB/alkG biocathode was demonstrated. Electrolysis experiments showed a maximum production of 1-octanol at the rate of 1.77 μmol h^{-1} mg^{-1} of alkB from octane with a faradaic efficiency of 25%. The ability of alkB to protect internal functional groups, a very rare and lucrative capability, was demonstrated by the generation of 4-octen-1-ol as the exclusive product from 4-octene. Four EFCs (H_2/octane, H_2/1-octene, H_2/methyl n-octyl sulfide, and H_2/2-methoxyoctane) were constructed utilizing the alkB/alkG biocathode and a hydrogenase bioanode. The resulting H_2/octane EFC (producing 1-octanol), representative of all EFCs constructed, generated electrical energy with an OCP of 0.65 V, a maximum current density of 318 μA cm^{-2}, and a faradaic efficiency of 23%. This bioelectrochemical system represents an alternative, environmentally conscientious approach to petrochemical conversions with high added value. Future work will focus on developing a TBO-based redox polymer and immobilization technique of alkB/alkG to improve enzyme stabilities and faradaic efficiencies. In addition, alkB performs stereoselective reactions that have not been fully explored, and this system will serve as an ideal platform for further studies in this area. The displayed capabilities of alkB inspire its use in systems with greater feasibility for practical electrosynthesis of these valuable products, such as bioreactors.

Acknowledgements

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Keywords: electroenzymatic catalysis • alkane monooxygenase • alkane • oxyfunctionalization • biofuel cell

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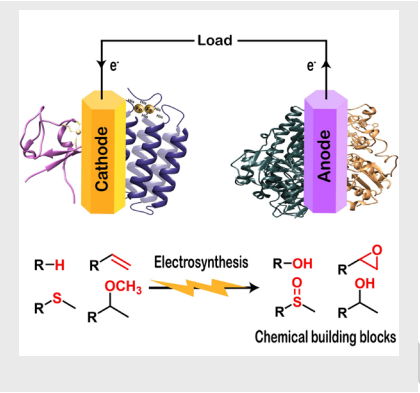
COMMUNICATION

Regioselective hydroxylation, epoxidation, sulfoxidation, and demethylation of petroleum-derived chemicals in a H_2-fueled biofuel cell.



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