# Direct, Electrocatalytic Oxygen Reduction by Laccase on Anthracene-2-methanethiol-Modified Gold

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ABSTRACT Laccase, a multicopper oxidase, catalyzes the four-electron reduction of oxygen to water. Upon adsorption to an electrode surface, laccase is known to reduce oxygen at overpotentials lower than the best noble metal electrocatalysts usually employed. Whereas the electrocatalytic activity of laccase is well established on carbon electrodes, laccase does not typically adsorb to better defined noble metal surfaces in an orientation that allows for efficient electrocatalysis. In this work, we utilized anthracene-2-methanethiol (AMT) to modify the surface of Au electrodes and examined the electrocatalytic activity of adsorbed laccase. AMT facilitated the adsorption of laccase, and the onset of electrocatalytic oxygen reduction was observed as high as $1.13 V_{RHE}$. We observed linear Tafel behavior with a 144 mV/dec slope, consistent with an outer sphere single electron transfer from the electrode to a Cu site in the enzyme as the rate-determining step of the oxygen reduction mechanism.

SECTION Surfaces, Interfaces, Catalysis
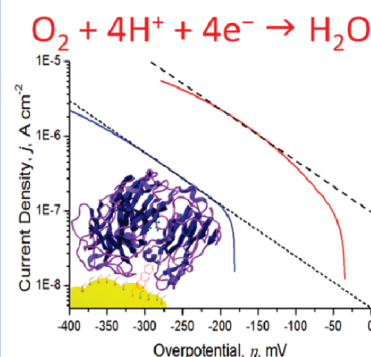
Although the 4 e$^-$ electroreduction of dioxygen to water is highly favored thermodynamically with a reversible reduction potential of 1.23 V versus the reversible hydrogen electrode (RHE), this potential is rarely realized because the reaction is kinetically slow and proceeds only at significant overpotentials (ca. -300 mV), even with the best Pt-based catalysts. The slow kinetics of the oxygen reduction reaction (ORR) is one of the major fundamental challenges to the widespread application of fuel cells, and electrocatalysis of the ORR is the subject of extensive research efforts.[1]

Some multicopper oxidases, particularly certain types of laccase (Lc), reduce O$_2$ at significantly smaller overpotentials than Pt and have been utilized as cathode electrocatalysts in enzyme-based fuel cell applications.[2] Lc functions as an electrocatalyst on carbon electrodes because it adsorbs in an orientation that allows direct electron transfer (DET) from the electrode to a type 1 Cu site; subsequent internal electron transfer transports the electron to a mixed type 2/type 3 tricopper cluster where O$_2$ is activated and reduced.[3] Although poor durability and low current densities are typical, significant improvements are realized upon diazonium modification of graphite and high surface area carbon electrodes to incorporate anthracene moieties on the surface prior to the adsorption of Lc.[4,5] Anthracene purportedly mimics a natural substrate of the enzyme and penetrates the hydrophobic pocket near the T1 Cu site, which properly orients the enzyme on the surface, facilitates electron transfer to the T1 Cu site, and reduces dissolution of enzyme off of the electrode surface. A key parameter in the characterization of electrocatalysts is the slope of a Tafel plot; however, Lc-modified carbon electrodes

exhibit nonlinear Tafel behavior.[4] Characterization of Lc on a better defined noble metal surface such as Au is desired to provide a potentially superior analytical platform.

On Au, Lc does not adsorb in the proper orientation for direct electrocatalysis at low overpotentials.[3] Prior investigations of Lc on Au electrodes examined a variety of strategies including adsorption of the enzyme to bare and thiol-modified Au and covalent attachment to thiol-modified Au.[3,6–10] However, none of these motifs allowed observation of the direct electrocatalytic reduction of O$_2$ at low overpotentials. Direct electrocatalysis has been reported for a low potential (high overpotential) Lc.[11] Recently, it was also reported that adsorption of Lc to 50 nm Au nanoparticles allows the observation of direct electrocatalysis.[12] Herein we report that modification of a roughened Au surface with anthracene-2-methanethiol (AMT) to orient and bind Lc from *Trametes versicolor* (*Tv*Lc) enables the direct electrocatalytic reduction of O$_2$ at low overpotentials. (See Figure 1.) *Tv*Lc was chosen for study because of its intrinsically low overpotential for the ORR relative to other laccases.[3] Additionally, we report successful observation of linear Tafel behavior.

A self-assembled monolayer (SAM) of AMT on polished Au was characterized electrochemically and by scanning tunneling microscopy (STM). As seen in Figure 2, a hexagonal closest packed overlayer is formed by the AMT on the Au(poly) surface with nearest-neighbor spacing of ca. 0.4 nm. Similarly,
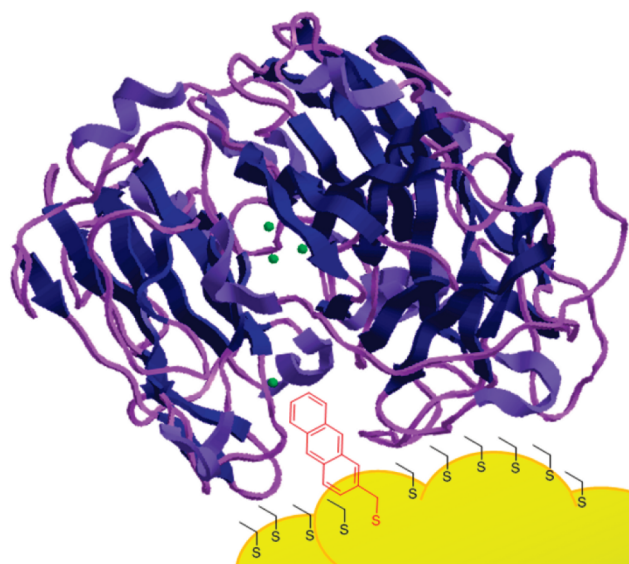
Figure 1. Schematic representation of Lc from *Trametes versicolor* properly oriented for electrocatalytic reduction of O_2 at an electrochemically roughened Au surface modified with a mixture of anthracene-2-methanethiol (AMT) and ethanethiol (ET). The AMT is shown in the hydrophobic pocket, where it facilities electron transfer from the electrode surface to the T1 Cu site. After an internal electron transfer, the 4 e⁻ reduction of O_2 to water occurs at the T2/T3 tricopper cluster near the center of the enzyme.[13]
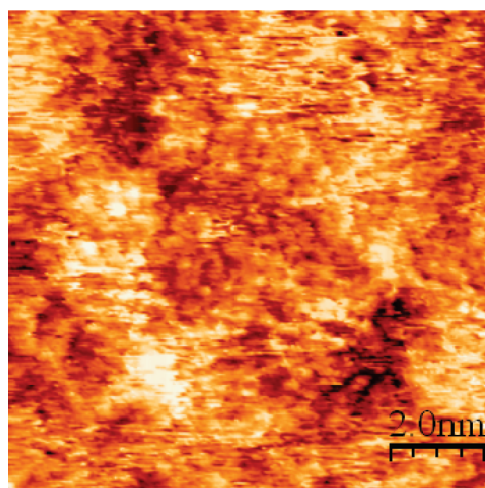
Figure 2. STM micrograph of hexagonal closest packed AMT monolayer formed on a polycrystalline Au surface. Nearest-neighbor spacing is ca. 0.4 nm within each domain (V_{bias} = 150 mV, i_t = 2.00 nA).

($\sqrt{3} \times \sqrt{3}$)R30° lattices of benzyl mercaptan and 4-biphenylmethanethiol monolayers form on Au(111) surfaces with nearest-neighbor spacing of 0.49 ± 0.01 nm.[14] On the basis of theoretical work done by Sellers et al., the methylene spacer likely promotes a vertical geometry, allowing for strong intermolecular interactions between the AMT molecules.[15] However, isolated anthracene moieties need to protrude from the surface to access the hydrophobic pocket of Lc and orient the enzyme. Indeed, we found that the ORR activity of *Tv*Lc at a polished Au electrode modified with an AMT SAM was not
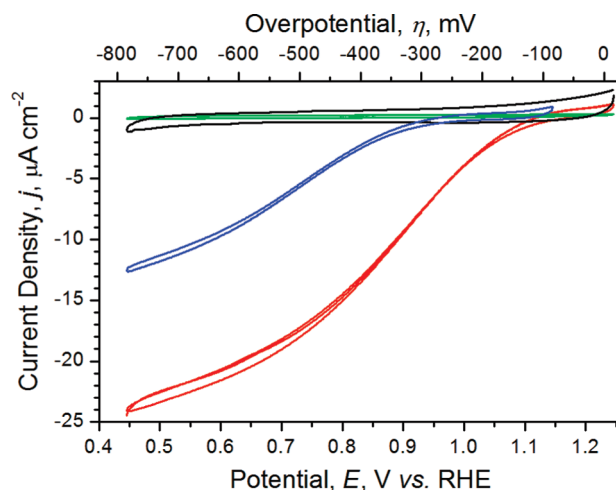

Figure 3. Cyclic voltammograms showing the electrocatalytic activity of *Tv*Lc adsorbed on a roughened Au electrode modified with: an AMT/ET mixture (red), AMT only (blue), and HDT (green) in O_2-saturated electrolyte; and Lc on the AMT/ET modified electrode in Ar-saturated solution (black). Data recorded at 5 mV/s in 0.1 M citric acid buffer adjusted to pH 4.00 with 0.2 M Na_2HPO_4.

significant and was comparable to prior observations made with other thiols. (See the Supporting Information.)
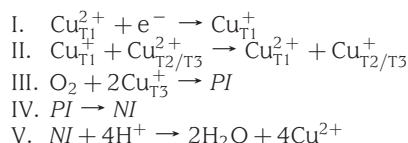
To disrupt the SAM and create isolated anthracene moieties accessible to the enzyme, we electrochemically roughened the Au surface forming rounded asperities on the order of ca. 100 nm in diameter, as observed by atomic force microscopy (AFM). (See the Supporting Information.) *Tv*Lc adsorbed on the roughened Au surface modified with AMT exhibited direct electrocatalytic reduction of O_2 at potentials approaching 1 V versus RHE (Figure 3). Use of a mixed monolayer of AMT and ethanethiol (ET) on a roughened surface (Figure 1) increased the onset of the ORR to 1.13 V versus RHE and yielded current densities approaching $-25 \mu A/cm^2$ in quiescent solution (Figure 3). An AMT/ET ratio of 1:3 was chosen arbitrarily for this study; future work will seek further increases in current density and ORR onset potential that may be realized upon optimization of the thiol mixture. We attribute the improved activity to the formation of an increased number of anthracene moieties accessible to *Tv*Lc in the mixed monolayer.

Sparging the electrolyte with Ar leads to the disappearance of the O_2 reduction current (Figure 3). On the basis of the electrocatalytic current densities, we estimate that the expected current density from e⁻ transfer to the Cu sites in the absence of O_2 is too small to observe (Supporting Information) because of the low surface concentration of *Tv*Lc due to the low number of accessible anthracene moieties, even with roughening and utilization of a mixed monolayer. Figure 3 also shows data obtained at a roughened electrode modified with hexadecanethiol (HDT) in place of AMT; the absence of O_2 reduction currents indicates that the anthracene moiety is required to orient the enzyme properly. Electrodes prepared with *Tv*Lc on roughened gold without a thiol modification or modified with benzyl mercaptan also did not show any significant ORR activity. (See the Supporting Information.)
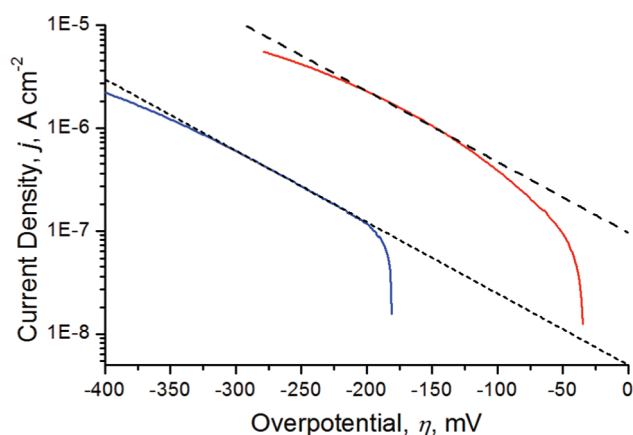
Figure 4. Tafel plots of the O_2 reduction activity of *Tv*Lc adsorbed on roughened Au electrodes modified with AMT (blue) and an AMT/ET mixture (red) in O_2-saturated pH 4.00 citrate-phosphate buffer and linear fits equivalent to Tafel slopes of 144 mV/dec (short dashes) and 145 mV/dec (long dashes). Data recorded during a 5 mV/s cathodic potential scan.

Tafel plots of the ORR at the electrodes utilizing AMT only and the AMT/ET mixture to bind *Tv*Lc have linear regions with slopes of ca. 144 mV/dec (Figure 4). The similar slope in both systems indicates that the ORR mechanism does not change as a function of the number of active *Tv*Lc sites on the electrode surface. *Tv*Lc reduces oxygen following a ping-pong-type mechanism that couples the single electron oxidation of four substrate molecules to the four-electron reduction to water as represented below

I. $Cu_{T1}^{2+} + e^- \rightarrow Cu_{T1}^+$
II. $Cu_{T1}^+ + Cu_{T2/T3}^{2+} \rightarrow Cu_{T1}^{2+} + Cu_{T2/T3}^+$
III. $O_2 + 2Cu_{T3}^+ \rightarrow PI$
IV. $PI \rightarrow NI$
V. $NI + 4H^+ \rightarrow 2H_2O + 4Cu^{2+}$

where *PI* and *NI* designate the peroxy intermediate and native intermediate, respectively.[16] For a multistep multielectron reaction, the relationship between the transfer coefficient (α) and the reaction mechanism is described by eq 1

$$\alpha = \left(\frac{\gamma}{\nu} + r\beta\right) \quad (1)$$

where γ is the number of electrons transferred prior to the RDS, ν is the stoichiometric number (the number of times the step occurs per cycle of the overall reaction), r is the number of electrons transferred during the RDS, and β is the symmetry factor (usually ~0.5).[17] If step I (the outer sphere single electron transfer from the electrode to the T1 Cu site) is the RDS, then $\gamma = 0$ and $r = 1$; therefore, $\alpha = \beta$. If step II is the RDS, then $\gamma = 4$, $\nu = 3$, and $r = 0$; therefore, $\alpha = 4/3$. If any of steps III−V is the RDS, then $\gamma = 4$, $\nu = 1$, and $r = 0$; therefore, $\alpha = 4$. The value of α is determined from the Tafel slope using eq 2.[17]

$$\frac{dE}{d\log i} = \frac{2.3RT}{\alpha F} \quad (2)$$

The observed Tafel slope of 144 mV/dec yields $\alpha = 0.41$, which is most consistent with step I as the RDS and $\alpha = \beta = 0.41$, which is within the normal range expected for the

symmetry factor. This is consistent with the suggestion that the first electron transfer is the RDS for the oxidation of certain substrates with Lc (based on the observation that the catalytic efficiency of various laccases depends on the redox potential of the T1 Cu site).[3] It also illustrates the remarkable ability of the tricopper T2/T3 site to activate the O═O bond, the typical RDS with other electrocatalysts (including Pt).[1]

Linear Tafel behavior is expected only when current densities at overpotentials greater than ca. 118 mV are free from mass-transport effects.[18] Therefore, the Tafel plot of the electrode with AMT only is more linear than the electrode with the AMT/ET mixture, and the still higher geometric current density *Tv*Lc-modified carbon electrodes reported by Blanford et al. do not have linear Tafel behavior.[4] The lack of linearity may be due to mass-transport effects accompanying the higher current densities or the heterogeneous nature of the carbon surface.

The observation of linear Tafel behavior from laccase on a modified Au surface provides an analytical platform with which to characterize further different laccase systems. Whereas our system is suitable for the characterization of Lc at a Au surface, systems employing high surface area carbons remain at this time more practical for enzyme-based fuel cells because of the accessibility of higher current densities. Optimization of laccase binding site density on Au may provide for further increases in current density in the future.

EXPERIMENTAL METHODS

Working electrodes were 10 mm diameter polycrystalline Au disks polished with 9.5 μm alumina powder (Buehler), sonicated in 18.2 MΩ water (Milli-pore), and wiped clean with a Kimwipe (wiping was found to improve roughening consistency). Electrochemical roughening was performed using a series of oxidation/reduction cycles as previously described, except that 0.5 M KCl was employed as the electrolyte and resulted in a dark reddish brown surface.[19,20] The roughened electrode was rinsed with water and then ethanol and then immersed in the appropriate thiol solution (0.5 mM AMT in EtOH or 0.5 mM AMT + 1.5 mM ET in EtOH) for 1 h. The excess thiol was then removed by rinsing with EtOH, and the electrode was immersed in a *Tv*Lc solution prepared from 30 μL of a >500 U/mL *Tv*Lc stock solution and 2970 μL of pH 4.0 citrate/phosphate buffer. The electrode was allowed to soak for 1 h at ambient temperature prior to use (or could be soaked for 24 h at 5 °C without any difference in activity). The electrode was rinsed with copious amounts of water to remove unbound Lc prior to prompt transfer to the electrochemical cell. Allowing the surface to dry results in the loss of ORR activity.

Electrochemical measurements were performed in a standard three-electrode configuration using a CHI760C or CHI 760D potentiostat (CH Instruments). The working electrode was placed in hanging meniscus contact with the electrolyte. A Pt gauze counter electrode was separated from the working electrode by a porous glass frit, and a "no leak" Ag/AgCl reference electrode (Cypress Systems) was isolated with a Luggin capillary. The headspace of the cell was closed and either Ar or O_2 atmosphere was employed. The electrolyte

consisted of 0.1 M citric acid adjusted to pH 4.00 with 0.2 M Na_2HPO_4.

STM measurements were carried out using a Nanoscope III E (Digital Instruments). The gold substrate was clamped to the stage using a Teflon cell. Pt−Ir STM tips (0.25 mm in diameter) were purchased from Veeco Instruments.

SUPPORTING INFORMATION AVAILABLE Synthesis and characterization of AMT, *Tv*Lc purification procedure, electrode roughening procedure, additional characterization data, and more extensive discussion of the absence of voltammetric features under inert atmospheres. This material is available free of charge via the Internet at http://pubs.acs.org.

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