A Biopolymer Composite that Catalyzes the Reduction of Oxygen to Water

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A biopolymer composite consisting of polypyrrole, ABTS, and laccase (PAL) was electrodeposited onto the surface of an electrode and was shown to catalyze the reduction of dioxygen to water under acidic conditions. The catalytic activity of this biopolymer composite is highest at pH 4, decreasing with increasing pH. The activity of laccase immobilized within this polymer composite was found to be higher than laccase dissolved in solution when methanol was present or at elevated temperatures.

Introduction

Laccase, a copper oxidase, catalyzes the oxidation of a variety of aromatic compounds (e.g., phenol derivatives) by dioxygen to produce reactive aromatic radicals and water.[1,2] Substitution of the natural substrate with the redox mediator, ABTS (2,2′-azinobis (3-ethylbenzothiazoline-6-sulfonate) diammonium salt), enables the coupling of this reaction to the cathode of a biofuel cell to generate electrical power.[3] The redox mediator facilitates, by diffusion, the transfer of electrons from the cathode to the active site of laccase at a potential near that of dioxygen.

Several studies have shown that immobilized laccase is more stable than laccase in solution. For example, laccase immobilized in a gelatin matrix retains 90% of its activity after 4 months of storage at 4 °C compared to laccase in solution, which retains only 50% of its activity when stored under the same conditions.[4] Other examples include immobilization of laccase onto different types of glass beads (e.g., round, porous).[5−7] In general, when laccase is immobilized onto porous glass beads treated first with 3-aminopropyltriethoxysilane followed by glutaraldehyde, both the immobilization yields (100%) and retention of activity (90%) are very high. Although this method of immobilization does not change the optimal pH and temperature,[5] the specific activity of immobilized laccase is higher than that of the enzyme in solution, suggesting that the enzyme was purified further during the immobilization process.[8]

Immobilization also stabilizes laccase in the presence of organic solvents. For example, when laccase was immobilized on sepharose CL-6B, the gel−enzyme composite showed good stability in organic solvent.[9] In another study, laccase was immobilized on porous glass beads silanized with glutaraldehyde and its activity was evaluated in water-miscible organic solvents such as ethylene glycol or methoxyethanol. The immobilized laccase retained 95% activity after 7 months storage at 4 °C, while laccase in solution retained only 40% of its original activity.[10] Laccase immobilized in an Os(III/II) redox polymer was an active cathodic catalyst (compared to Pt) in a direct methanol fuel cell when the buffered catholyte contained as much as 10 M methanol.[11]

In this study, polypyrrole was examined as an immobilization matrix for laccase and ABTS for the purpose of using this biopolymer composite in the cathode compartment of a biofuel cell. Polypyrrole was chosen as an immobilization matrix for several reasons including its electrical conductivity, polycationic nature, and water solubility of its monomer.[12−14] Although previous studies have used polypyrrole as an immobilization matrix for enzymes[15−21] and other

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biomolecules,[22−32] to the best of our knowledge, this study demonstrates the first example of a biopolymer composite consisting of both an oxidoreductase and redox-active mediator co-immobilized in polypyrrole. The significance of this demonstration is twofold. First, enzymes immobilized in polypyrrole in the absence of a redox-active mediator show sluggish electron-transfer kinetics.[33] Inclusion of a redox-active mediator significantly improves electron-transfer kinetics between polypyrrole and the active site of an oxidoreductase. This enhancement of kinetics is demonstrated herein with laccase and ABTS as a specific example. Second, polypyrrole can be prepared via chemical,[34] enzymatic,[35] and electrochemical[36] methods. As such, polypyrrole is easy to prepare and when prepared via electrosynthesis, tremendous flexibility and control is possible with regard to film characteristics such as morphology, thickness, dopant, and location of electrodeposited film. Elegant examples of enzymes entrapped in redox-active polymers have been demonstrated.[37,38] The preparation of redox-active polymers, however, often requires more advanced synthetic skills (i.e., monomer synthesis) with little control over the film characteristics stated above. In this study, the properties of a biopolymer composite consisting of polypyrrole, ABTS, and laccase (PAL) are reported in terms of its ability to reduce dioxygen to water as a function of pH, temperature, and concentration of methanol.

Experimental Section

Chemicals. The laccase used in this study was a generous gift from Wacker Consortium fur Elektrochemische Industrie GmbH and was used as received. The redox mediator, 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonate) diammonium salt (ABTS), pyrrole, and buffering salts were purchased from Aldrich and were used without further purification.

Equipment. A three-neck single-compartment cell, configured with a glassy carbon working electrode (0.1257 cm^2), a platinum-gauze counter electrode, and a saturated silver/silver chloride reference electrode or SCE (saturated calomel electrode) reference electrode, was used for all electrochemical experiments. An EG&G potentiostat/galvanostat (model 273A) was used both to electrodeposit films of PAL onto glassy carbon electrodes and to measure the catalytic current density produced at the resulting PAL-coated electrodes. All solutions were purged with N_2 during electrodeposition and were blanketed with N_2 during measurements of bioelectrocatalytic activity. Scanning electron micrographs were obtained with a LEO 1530 field emission scanning electron microscope.

Electrosynthesis of Films of PA, PAL. *Constant Current Method.* Films were electrodeposited onto a glassy carbon electrode (0.1257 cm^2) at constant current (400 μA) for 10 min prior to measurement of bioelectrocatalytic activity. Films of polypyrrole doped with ABTS (PA) were electrosynthesized from an aqueous solution containing 200 mM pyrrole and 25 mM ABTS whereas films of polypyrrole doped with both ABTS and laccase (PAL) were electrosynthesized from the same solution with the addition of laccase (6 mg mL^{-1}).

Potential Cycling Method. Films were electrodeposited onto a glassy carbon electrode (0.1257 cm^2) by cycling the potential between 0 and 700 mV for 100 cycles prior to measurement of bioelectrocatalytic activity as a function of methanol concentration or temperature. Lower concentrations of ABTS (2 mM) and laccase (3 mg mL^{-1}) were used to prepare these films compared to films prepared via the constant current method. We distinguish films prepared via potential cycling from those prepared via constant current by the addition of an asterisk to the acronym (e.g., PA*, PAL*).

The thickness of the electrodeposited films of PA and PAL was measured with a Dektak profilometer. On average, 0.4 μm of PA and 1 μm of PAL were electrodeposited for each minute of applied potential. On the basis of eq 1, 2.49 μmol of charge is transferred during a 10-min electrodeposition

$$\frac{I \times t}{F} = \frac{400\ \mu\text{A} \times 600\ \text{s}}{96485\ \text{C/nmol}} = 2.49\ \mu\text{mol} \qquad (1)$$

where I is the current used for electrodeposition, t is the time of electrodeposition, and F is the Faraday constant. For every molecule of pyrrole oxidized and incorporated into a growing strand of polypyrrole, 2.50 electrons are released.[39] (Others have proposed that 2.25−2.33 electrons are released per pyrrole monomer during the electrodepostition of polypyrrole[40,41] or that 2 electrons are released per pyrrole monomer during electropolymerization.[42]) Assuming a coverage of 1×10^{-9} mol cm^{-2} represents a perfect monolayer of pyrrole and each molecule of pyrrole is ∼4 Å wide, then the thickness of a film corresponding to the transfer of 2.49 μmol of charge should be ∼3.2 μm thick. Compared with profilometry data (0.4 μm of PA or 1 μm of PAL/minute), not all of the charge transferred converts to lengthening of the polypyrrole chains. In fact, most of the charge transferred is due to the oxidation of ABTS (at a constant current of 400 μA, the potential at the electrode is ≥500 mV vs Ag/AgCl). In addition, some pyrrole is oxidized to dimers and trimers that diffuse away from the surface of the electrode prior to its capture at a growing strand of polypyrrole.[43]

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Electrochemical Measurements. *(a) Bioelectrocatalytic Activity toward the Reduction of Dioxygen to Water*. Linear sweep voltammetry (LSV) was used to characterize the bioelectrocatalytic activity of electrodes coated with PA and PAL. The electrolyte was sodium acetate buffer (pH 4) or citrate-phosphate buffer (pH range from 4 to 7) saturated with N_2 or O_2 and maintained at 25 °C. A fresh film was made for each experiment.

(*b) Bioelectrocatalytic Activity toward the Reduction of Dioxygen to Water in the Presence of Methanol.* Equivalent aliquots of a stock solution containing 2 mM ABTS and 3 mg mL^{-1} laccase (AL) in 0.2 M sodium acetate buffer (pH 4) were spiked with five different concentrations of methanol (0, 2.5, 5, 7.5, and 10 M). A linear sweep voltammogram (from 650 mV to 300 mV vs SCE) was taken of each aliquot saturated with O_2 to measure the effect of methanol on the bioelectrocatalytic activity of nonimmobilized laccase. Background currents were measured after saturating each aliquot with N_2. Similar experiments were performed on a freshly prepared film of PAL*, which was immersed in equivalent aliquots of a stock solution containing 10 mM ABTS in 0.2 M sodium acetate buffer (pH 4) spiked with five different concentrations of methanol (0, 2.5,, 5, 7.5, and 10 M). A linear sweep voltammogram was taken of each aliquot saturated with O_2. Background currents were measured after saturating each aliquot with N_2.

(*c) Bioelectrocatalytic Activity toward the Reduction of Dioxygen to Water as a Function of Temperature.* Four samples consisting of 3 mg mL^{-1} laccase dissolved in 0.2 M sodium acetate buffer (pH 4) were prepared. Each sample was held at a different temperature (25 °C, 40 °C, 60 °C, and 80 °C) for 5 min. Subsequently, 2 mM ABTS was added to each sample and then was purged with O_2. A linear sweep voltammogram was taken of each sample by sweeping the potential from 650 mV to 300 mV versus SCE. A background voltammogram was obtained in a similar manner after purging each sample with N_2. For comparison, four samples of PAL were subjected to a heat shock (25 °C, 40 °C, 60 °C, and 80 °C) for 5 min. Subsequently, each sample was immersed in 0.2 M sodium acetate buffer (pH 4) containing 10 mM ABTS and was purged with O_2 at the corresponding temperature. A linear sweep voltammogram of each sample was measured. A background voltammogram was obtained for each sample after purging the buffer solution with N_2. ABTS leaches out of the polymer composite over the course of several days until equilibrium with the buffer solution is obtained. The rate of loss of mediator can be slowed using a buffer solution that contains ABTS (which was not done for the studies reported herein) or can be eliminated by using a polymeric version of ABTS.[44]

Results and Discussions

Bioelectrocatalytic Activity of PAL. Polypyrrole in its conductive state is cationic, with approximately one cation per three to five pyrrole subunits.[36,45−49] Electrodeposition of pyrrole in the presence of dianionic ABTS, therefore, results in a polymer composite with an approximate composition of 10 pyrrole units per each molecule of ABTS (Figure 1).[35] As such, the resulting polymer composite of

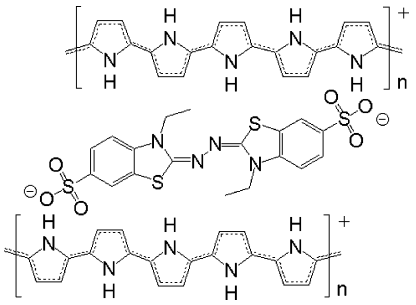

Figure 1. Molecular structure of polymer composite consisting of polypyrrole and ABTS (PA).

polypyrrole and ABTS (PA) is both conductive and redox active and recently was shown to be a viable cathode material in a polymer-based battery.[50]

Electrodeposition of pyrrole in the presence of both ABTS and laccase results in the entrapment of laccase within a polymer matrix that is both electrically conductive and redox-active. Entrapment of laccase within the polypyrrole−ABTS matrix involves the entanglement of the two macromolecules (polypyrrole and laccase) to form ionic interactions between anionic substituents on laccase (pI (isoelectric point) ∼ 3)[51−55] and the cationic backbone of conductive polypyrrole.

Illustrated in Figure 2 is the pathway for electron transfer between a cathode coated with PAL and dioxygen and the corresponding thermodynamic potentials of each constituent in the overall reaction. Electrons are transferred from the cathode along the conductive fibers of polypyrrole to reduce $ABTS^{\bullet}$ to ABTS. After 4 equiv of ABTS have transferred their electrons to the four Cu(II) ions in the active site of laccase (thus regenerating $ABTS^{\bullet}$), dioxygen binds across two of the four Cu(I) ions[56] and subsequently is reduced to 2 equiv of water. Bioelectrocatalysis continues once $ABTS^{\bullet}$ is available to accept additional electrons from the cathode and the active site is fully oxidized (i.e., four Cu(II) ions).

Linear sweep voltammetry (LSV) was used to measure the bioelectrocatalytic current generated at three different electrode configurations: (1) a bare glassy carbon electrode, (2) an electrode coated with PA (i.e, no laccase), and (3) an electrode coated with PAL (Figure 3a). Fresh films of PA and PAL were electrodeposited onto a bare glassy carbon electrode for 10 min prior to voltammetric measurements, which were performed on electrodes immersed in a sodium acetate buffer (pH 4) purged with either Ar or O_2. In the absence of dioxygen (i.e., buffer purged with Ar), reductive current was not observed at any of the electrode configurations. When dioxygen is present, however, reductive current is observed only at the electrode coated with PAL. Rotation of the electrode coated with PAL results in a corresponding increase in current density because of an increase in the

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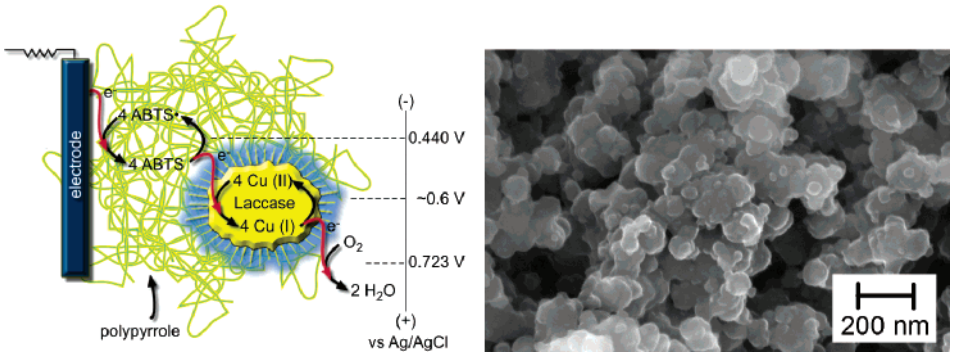

Figure 2. (left) Schematic of bioelectrocatalytic reduction of dioxygen to water at an electrode coated with PAL, (right) scanning electron micrograph of PAL electrodeposited onto glassy carbon revealing the nanonodular structure of the biocomposite.

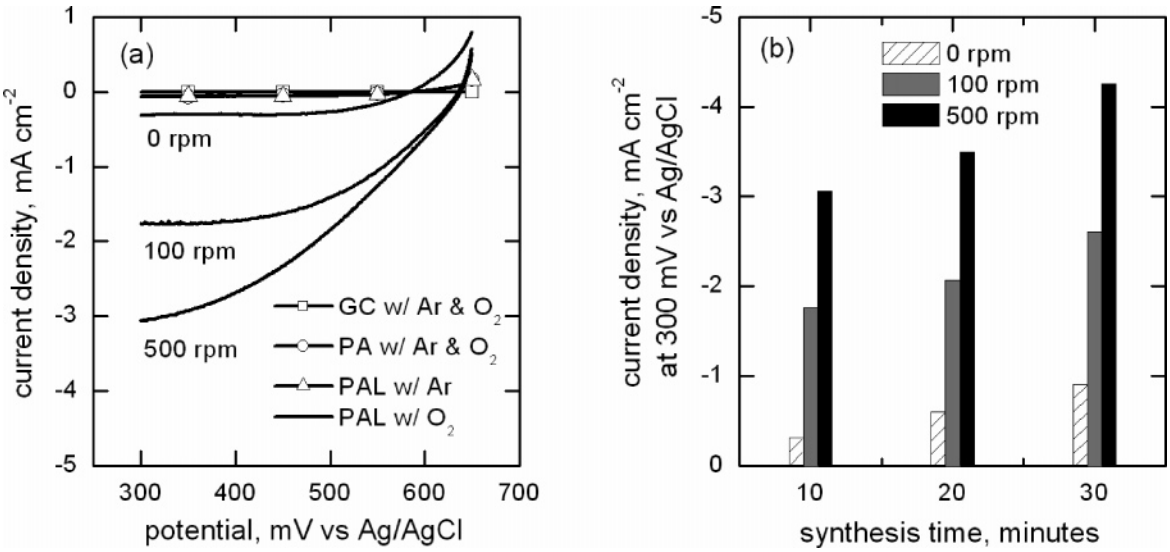

Figure 3. (a) Linear sweep voltammograms of a glassy carbon (GC) electrode or a GC electrode coated with PA or PAL in the presence or absence of dioxygen. Current associated with the reduction of dioxygen is not observed at either an uncoated GC electrode or a GC electrode coated with PA. (b) Reductive current density at 300 mV generated by an electrode coated with PAL as a function of electrodeposition time.

transport of dioxygen to the biocatalytic sites. Films that did not contain ABTS did not exhibit any bioelectrocatalytic activity indicating that (1) ABTS, in addition to laccase, is required for oxygen to be reduced by the polymer composite, (2) electron transfer does not occur directly from the electrode or polypyrrole to the active site of laccase, and (3) decomposition products from polypyrrole, if present, did not participate in electron transport.

Increasing the electrodeposition time increases the amount of PAL deposited onto an electrode, which in turn increases the reductive current measured at an electrode coated with PAL. For example, shown in Figure 3b are the reductive currents measured at 300 mV at an electrode coated with PAL after 10, 20, and 30 min of electrodeposition. The reductive current increases by only 0.5 mA cm^{-2} (~16% increase) with each additional 10 min of electrodeposition regardless of rate of rotation used. This limited increase indicates that the efficiency of charge transfer decreases with increasing film thickness, which may be due to a decrease in conductivity of the polypyrrole matrix.[57]

Bioelectrocatalytic Activity of PAL as a Function of Electrodeposition Conditions. Electrodeposition of doped polypyrrole can be achieved using several methods including the application of a constant potential, a constant current, or via cyclic voltammetry. When constant potential is used, the amount of polypyrrole deposited is difficult to control accurately on the basis of the variability in the current response from sample to sample. In contrast, when constant current is used, the amount of polypyrrole deposited can be monitored accurately by the amount of charge passed. When doped polypyrrole is electrosynthesized via potential cycling, the resulting films are homogeneous and compact (e.g., PAL*).[58] These films, however, yield lower current densities than films electrodeposited via application of constant current. Nevertheless, films that are more homogeneous produce more consistent results.

Bioelectrocatalytic Activity of PAL as a Function of pH. The activity of laccase is known to be pH-dependent.[59] Within a pH range of 2−7, the activity of laccase is highest at pH 4 or pH 3 when the buffer is sodium acetate or citrate-phosphate, respectively. This pH-dependent activity is a consequence of a shift in the potential of dioxygen as a function of pH and competitive inhibition of dioxygen

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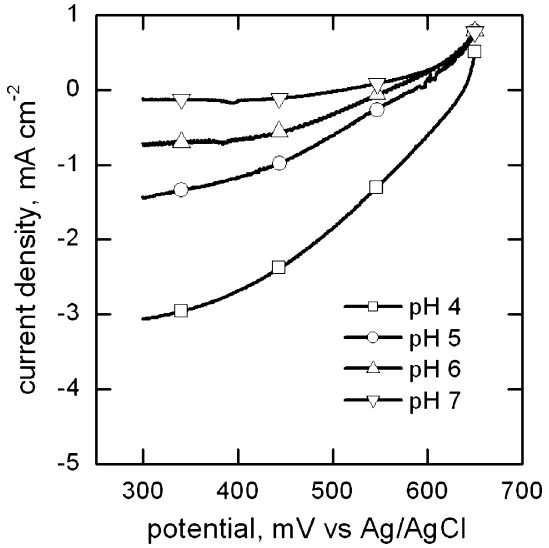

Figure 4. Current density as a function of pH produced by an electrode coated with PAL immersed in sodium acetate buffer and rotated at 500 rpm. A freshly prepared film of PAL (i.e., 10-min electrodeposition) was used for each pH.

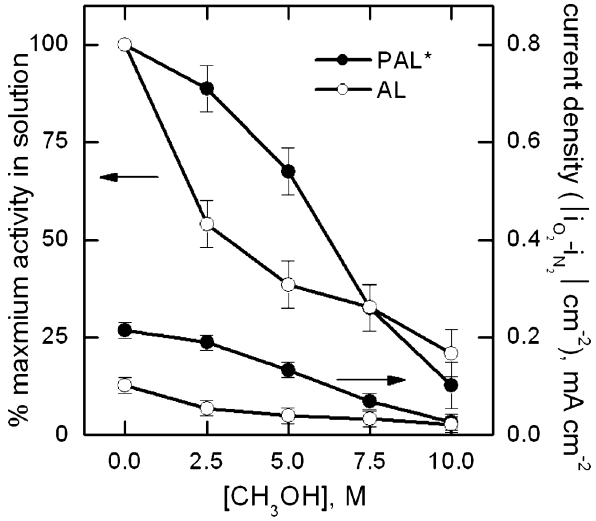

Figure 5. Activity of laccase relative to its maximum activity in solution as a function of methanol concentration (left scale). The error bars indicate the range of data from three samples. Current density from faradaic processes (i.e., $|i_{O2} - i_{N2}|$ cm^{-2}) produced by laccase, both immobilized (PAL*) and in solution (AL), as a function of methanol concentration (right scale).

binding by hydroxide ion.[60] Shifting the pH of the buffer to higher values shifts the potential of dioxygen to more negative values, eventually making the laccase-catalyzed reduction of dioxygen by ABTS a reaction that is unfavorable thermodynamically. Because the activity of laccase is pH-dependent, the current density that results from the bioelectrocatalytic reduction of dioxygen by an electrode coated with PAL is expected to be pH-dependent. Shown in Figure 4 is the current density generated at an electrode coated with PAL as a function of pH. For each pH, a fresh film of PAL was electrodeposited onto a glassy carbon electrode for 10 min, was subsequently rinsed with water, was immersed in sodium acetate buffer of a given pH, and was rotated at 500 rpm while sweeping the potential from 650 mV to 300 mV. As the potential is swept negative, reductive current is observed. The reductive (negative) current is highest in value at pH 4, the pH where the activity of laccase is at a maximum in sodium acetate buffer. Increasing the pH of the buffer results in a corresponding decrease in reductive current until eventually no reductive current is observed at pH 7, a pH where this isozyme of laccase has little or no biocatalytic activity.

Bioelectrocatalytic Activity of PAL as a Function of Methanol Concentration. Methanol crossover is a known problem for direct methanol fuel cells (DMFC) that use a noble metal as the cathodic catalyst.[61] This problem is a consequence of the concentration gradient across the polymer electrolyte membrane (PEM), which limits how much methanol can be added to the anolyte. Typically, less than 10% methanol (<2.5 M) is used in DMFC.[62] When methanol diffuses into the cathode compartment, it is oxidized at the platinum cathode, which reduces both the voltage of the fuel cell and its efficiency at converting chemical energy into electrical energy. Several approaches to solving the problem with methanol crossover have been employed, including lower methanol concentration in the anolyte,[63] thicker separation membranes,[64] new membrane materials,[65−70] and more selective cathodic catalysts, including biocatalysts.[71] Using a cathodic biocatalyst in a DMFC has several advantages over platinum including high selectivity, resistance to poisoning, low cost, and renewability. Several disadvantages remain, however, including low turnover numbers and low thermal stability.

The affect of methanol concentration on the bioelectrocatalytic activity of laccase in solution (AL) and immobilized laccase (PAL*) is shown in Figure 5. The activity of laccase, both in solution and immobilized in doped polypyrrole, was obtained by subtracting the current density in the absence of dioxygen from the current density in the presence of dioxygen, both at 300 mV. The activity of laccase decreases with increasing concentration of methanol, regardless of whether the enzyme is in solution or immobilized. Methanol is known to denature enzymes by disrupting the hydrogen-bonded network of water in contact with the enzyme.[72] The activity of immobilized laccase, however, decreases less than that of laccase in solution when the concentration of methanol is below 7.5 M. For example, in 2.5 and 5 M methanol, PAL* retains 90% and 70% of its maximum activity, respectively,

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while AL retains 60% and 40%, respectively. In 7.5 M methanol, both PAL* and AL show a similar decrease in activity, and at 10 M methanol, the activity of PAL* decreases more than that of AL. At 10 M methanol, the activity of PAL* is only 12.5% of its maximum. If the film is rinsed with acetate buffer (pH 4), however, the activity of the film increases to 80% of its original activity in the absence of methanol.

Bioelectrocatalytic Activity of PAL as a Function of Temperature. Laccase in its natural environment (white rot fungi) typically functions at temperatures between 20 and 80 °C.[73] Up to a point, increasing temperature increases the kinetics of enzyme-catalyzed reactions.[73] At some temperature threshold, however, the enzyme becomes unstable and unfolds, resulting in catastrophic loss of activity. Several approaches have been employed to stabilize purified enzymes (i.e., separated from other cellular components) against increases in temperature including cross-linking reactions,[74] crystallization,[75] site-directed mutagenesis,[76] and polymer entrapment.[77] All of these approaches are thought to stabilize the enzyme by increasing the number of covalent and noncovalent interactions of the folded state, which consequently raises the activation barrier for denaturation.[78] Immobilization of laccase within doped polypyrrole should likewise improve the stability of this enzyme to increases in temperature relative to the enzyme in solution.

The effect of temperature on the bioelectrocatalytic activity of laccase in solution (AL) and immobilized laccase (PAL*) is shown in Figure 6. Activity was measured using the same procedure as that used for measuring activity in the presence of methanol. On the basis of the data shown, the activity of laccase immobilized in doped polypyrrole is always higher than laccase in solution at all temperatures studied except at 25 °C where the activities of both samples are the same. Moreover, the maximum activity of immobilized laccase is at 40 °C (∼20% increase over 25 °C) whereas the maximum activity of laccase in solution is at 25 °C. This result indicates that immobilization of laccase in doped polypyrrole increases its thermal stability so that increased enzyme kinetics at higher temperature can be realized. As the temperature is increased to 60 °C, the activity of immobilized laccase decreases to a value matched by the activity of laccase in solution at 40 °C; as the temperature is increased further to 80 °C, the activity of laccase both immobilized and in solution decreases to less than 50% of its maximum values.

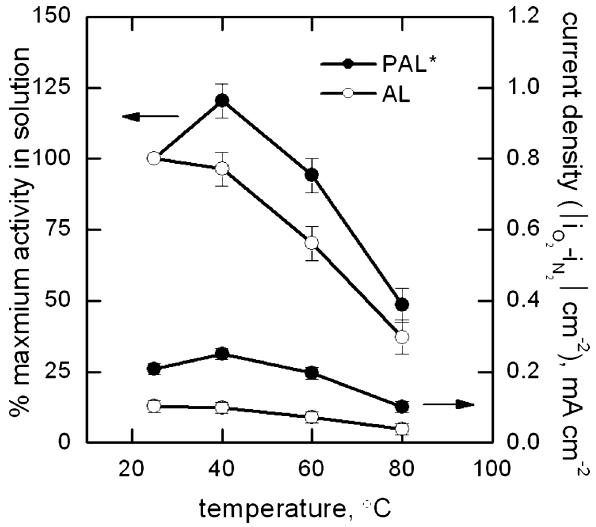

Figure 6. Activity of laccase relative to its maximum activity in solution as a function of temperature (left scale). The error bars indicate the range of data from three samples. Current density from faradaic processes (i.e., $|i_{O2} - i_{N2}|$ cm^{-2}) produced by laccase, both immobilized (PAL*) and in solution (AL), as a function of temperature (right scale).

Conclusion

In this work, a biopolymer composite was synthesized by electrodepositing polypyrrole in the presence of the redox mediator, ABTS, and the enzyme, laccase. This biopolymer composite bioelectrocatalytically reduces dioxygen to water under acidic conditions. Four factors that affect the bioelectrocatalytic activity of PAL were analyzed: film thickness, pH, concentration of methanol, and temperature. Optimal activity was observed in films of PAL that were 1-μm thick and that were immersed in an acetate buffer (pH 4) at 40 °C. Moreover, the activity of PAL was higher than that of laccase in solution with increasing temperature. Finally, the activity of PAL was resistant to methanol poisoning up to 7.5 M, above which its activity was similar to that of laccase in solution. Additional stabilization of laccase against changes in pH, temperature, and methanol concentration may be achieved by modification of the biopolymer composite to include a polymer of ABTS, which will be the subject of future studies.

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Supporting Information Available: Profilometry data of films. This material is available free of charge via the Internet at http://pubs.acs.org.

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