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Reagentless Mediated Laccase Electrode for the Detection of Enzyme Modulators

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We have investigated aerobic mediation of electron transfer to a laccase enzyme by the solution redox couples $[Os(bpy)_2Cl_2]^{+/0}$ and $[Os(bpy)_2(MeIm)Cl]^{2+/+}$, where bpy is 2,2-bipyridine and MeIm is *N*-methylimidazole. The factors that influence the homogeneous mediation reaction are investigated and discussed. Investigation of ionic strength, pH, and temperature effects on the kinetics of intermolecular electron transfer elucidates the governing factors in the mediator−enzyme reactions. Coimmobilization of both enzyme and an osmium redox mediator in a hydrogel on glassy carbon electrodes results in a biosensor for the reagentless addressing of enzyme activity, consuming only oxygen present in solution. Thus, these immobilized enzyme biosensors can be utilized for the detection of modulators of laccase activity, such as the inhibitor sodium azide. The enzyme inhibition biosensor can detect levels of azide as low as 2.5×10^{-6} mol dm^{-3} in solution.

In recent years, we have investigated the development of reagentless sensors of enzyme activity, using osmium-based mediators of oxidoreductase enzymes.[1,2] These sensors can be used to detect modulators of enzyme activity, such as inhibitors or activators. The use of these enzyme electrodes can be regarded as a sensitive and selective method for the detection of enzyme modulators because of the amplifying nature imparted by substrate turnover and the specificity of the biological recognition.[1−3]

The detection of toxic enzyme modulators, such as the respiratory poisons cyanide, CO, and azide, which function by inhibiting the cyrochrome oxidase enzyme complex, is of considerable importance. Previous research on enzyme electrodes has focused on cytochrome oxidase, horseradish peroxidase (HRP), or tyrosinase-based enzyme sensors which are inhibited by the respiratory poisons.[1−6] Albery and co-workers[3] investigated a sensing strategy based on the coimmobilization of cytochrome oxidase and its natural cofactor, cytochrome *c*. However, the lack of stability of the immobilized enzyme and the complex requirements of the system limit the application of this sensor. Smit and Cass[4] utilized a dual-electrode strategy for the electrochemical generation of the substrate (H_2O_2) of an immobilized HRP at one electrode and detection of the solution mediator, ferrocene, at the second electrode. Recently, Tatsume and Oyama[5] modified this approach using a single HRP-modified graphite electrode and a potential pulsing routine for the detection of cyanide based on its inhibition of direct charge transfer from the HRP. In an innovative approach, Smit and Rechnitz[6] reported that the natural substrate for the copper-containing tyrosinase enzyme could be replaced with an electrochemically regenerable redox species, ferrocyanide, that can act as an electron donor in the enzymatic reduction of oxygen. We have recently reported on an immobilized reagentless sensor for enzyme modulators using the tyrosinase enzyme covalently attached to a redox hydrogel deposited on electrode surfaces.[1] The immobilization scheme, pioneered by Heller's group,[7] results in an enzyme inhibition sensor sensitive to azide and cyanide at micromolar levels. During these studies, we discovered that the enzyme laccase has a higher affinity for the respiratory poisons compared to tyrosinase and that its natural substrates can also be replaced by inorganic or organic redox species, thus making it a candidate for the development of sensitive and selective enzyme inhibition sensors.

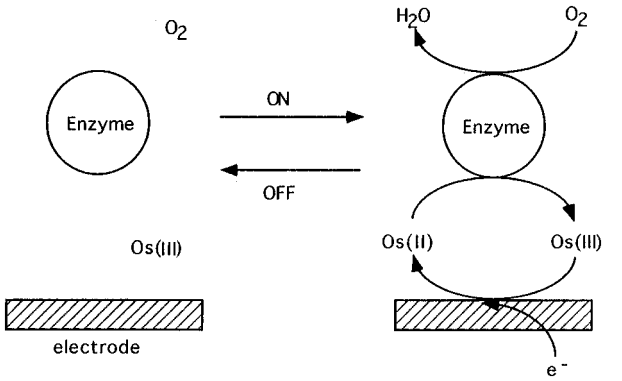

Figure 1. Catalytic scheme for the activity of the coimmobilized laccase and osmium redox mediator in a hydrogel on a glassy carbon electrode surface.

Here we report on the study of a laccase enzyme for the development of a reagentless sensor for detection of enzyme modulators. The sensor reaction scheme is depicted in Figure 1. The system is based on the coimmobilization of laccase with a redox polymer, $[Os(bpy)_2(PVI)_{10}Cl]Cl$ (OsPVI), where bpy is the 2,2′-bipyridine ligand and $(PVI)_{10}$ is poly(*N*-vinylimidazole), using the method of Ohara et al.[7] The redox couple $[Os(bpy)_2Cl_2]^{+/0}$ (Osbpy) and a model monomeric compound of the polymeric system $[Os(bpy)_2(MeIm)Cl]^{+/2+}$ (OsMeIm), where MeIm is *N*-methylimidazole, are used as solution mediators for the investigation of factors that affect the homogeneous mediation kinetics. Investigation of the effect of factors, such as temperature, pH, and ionic strength, on the enzyme mediation of oxygen

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reduction by these compounds may help clarify the structure surrounding the active site of the enzyme and lead to an optimized enzyme inhibition sensor. The coimmobilized enzyme−mediators are shown to retain their inhibition and mediation activities and thus can be used for the sensing of enzyme modulators. The respiratory poison azide is selected as a model inhibitor to demonstrate the feasibility of the approach.

EXPERIMENTAL SECTION

Materials and Methods. Electrochemical experiments were implemented in either 0.05 mol dm^{-3} phosphate (PB) or acetate buffers (AB) at room temperature, unless otherwise stated, using either a Pine Instruments AFCBP1 bipotentiostat connected to a BioAnalytical Systems (BAS) *X*−*Y* recorder or a BAS 100B/W electrochemical analyzer. One-compartment cells of either 0.5 or 2 cm^3 volume were used for the electrochemical studies. The electrochemical cell consisted of a Ag/AgCl reference electrode (BAS, 3 mol dm^{-3} KCl), a Pt wire counter electrode (Aldrich), and a 3 mm diameter glassy carbon working electrode purchased from BAS or constructed in-house by embedding a glassy carbon rod (V-25, Atomergic Chemetals) in a glass tube using Spurr low-viscosity epoxy (Polyscience) and contacting the unexposed side to a copper wire with silver epoxy (Epoxy-Technology). Electrodes were abraded with successively finer grades of SiC paper and polished to a "mirrorlike" finish with 0.3 and 0.05 μm alumina slurry on microcloth pads (Buehler).

Isozyme II of purified laccase (benzenediol:oxygen oxidoreductase EC 1.10.3.3) from *Trametes versicolor* was obtained from the Pulp and Paper Research Institute of Canada (PAPRICAN, Montréal, PQ). Enzyme concentrations were measured by UV−visible spectrophotometry at 610 nm using an extinction coefficient[8] of 4900 M^{-1} cm^{-1}. Enzyme activities were evaluated periodically, using syringaldazine as a substrate, and monitoring absorbance at 530 nm, to check the stability of our enzyme solution. No decrease in activity of a lacasse solution (1×10^{-3} mol dm^{-3}) stored at 4 °C over a period of three months was observed.

Stock solutions of the mediators were freshly prepared weekly in buffer. The $[Os(bpy)_2Cl_2]Cl$ and $[Os(bpy)_2(MeIm)Cl](PF_6)$ complexes were prepared and purified according to literature procedures.[9,10] The oxidized form of $[Os(bpy)_2(MeIm)Cl](PF_6)$ was prepared immediately before use by oxidation of a suspension of the reduced complex in buffer with PbO_2, followed by filtration to remove the oxide. Poly(*N*-vinylimidazole) was prepared by bulk free-radical polymerization of vacuum-distilled *N*-vinylimidazole (Polysciences) using azoisobutyronitrile (AIBN, Aldrich) as the initiator. Synthesis and characterization of the $[Os(bpy)_2(PVI)_{10}Cl]$-Cl was carried out according to literature methods.[10]

Redox polymer-modified electrodes containing covalently bound laccase were prepared using poly(oxyethylene)bis(glycidyl ether) (PEG400, Sigma) as a linker by pipetting 2 μL of a freshly mixed solution of equal volumes of the redox polymer (5 mg/mL in water), laccase (1.23×10^{-5} mol dm^{-3} in AB) and PEG (2 mg/mL in water) onto the surface of a glassy carbon electrode and allowing the subsequent hydrogel to dry for at least 48 h. A conservative estimate of 0.50 cents per film produced is an indication of the relative cost-effectiveness of this method.

RESULTS AND DISCUSSION

Laccase. Laccase is a "blue" copper oxygenase that oxidizes many aromatic substrates coupled to a 4-electron reduction of dioxygen to water. The broad range of substrates oxidized by laccase include phenols, aromatic amines, methoxy-substituted monophenols, and organic and inorganic electron mediators, such as 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonate) (ABTS) and ferrocyanide.[11,12] Although the copper centers are similar for all fungal laccases, significant differences in the thermodynamic and kinetic properties of the laccase enzymes exist. For example, the redox potential of the *Polyporus versicolor* laccase is almost 300 mV higher than that found for the *Rhus vernicifera* enzyme, and both laccase enzymes exhibit varying degrees of activity toward their substrates.[13] We have replaced the natural substrates for the isozyme II of a laccase isolated from *Trametes* (= *Coriolus* = *Polyporus*) *versicolor* white-rot fungus with osmium-based complexes that function as electron mediators. Investigation of the activity of laccase toward different mediators, and the effect that parameters such as pH, ionic strength, and temperature have on this activity, could help elucidate structure−function relationships in copper-based enzymes. Electrochemical monitoring of enzyme activity for the mediated reactions is also shown to be useful for the evaluation of enzyme inhibition. Coimmobilization of enzyme and mediator in a redox hydrogel on electrode surfaces is demonstrated to yield a highly effective reagentless sensor for the detection of these inhibitors, which include toxic poisons, such as cyanide and azide.

Homogeneous Enzyme Reactions. The type I copper of the *P. versicolor* laccase enzyme has a reduction potential (vs Ag/AgCl) of ∼570 mV in 0.1 mol dm^{-3} PB at pH 6.0,[14] while direct reduction of oxygen at the glassy carbon electrodes utilized in this study begins at ∼−200 mV (vs Ag/AgCl). Thus, useful electron donors should have a reduction potential between −100 and +500 mV. We have investigated the interaction between the enzyme and the electron donors Osbpy and OsMeIm. These osmium complexes are soluble in AB in their oxidized forms and exhibit reversible, pH-independent, voltammetry with formal redox potentials of −20 and +130 mV (vs Ag/AgCl), respectively. The osmium complexes were selected for investigation for the following reasons.

(i) The synthetic flexibility of these complexes: The synthesis of a broad range of complexes of varying charge, shape and formal potential would allow us to probe structure−function relationships for oxidoreductase enzymes.

(ii) The ability to replace the chloride ligands with polymeric pyridine or imidazole pendant groups,[7,10] yielding a redox polymer that can be adsorbed on electrode surfaces: We have selected the poly(vinylimidazole) system for immobilization of both enzyme and mediator using a diepoxide linker that is reactive toward imidazoles and amino lysines. Thus, the OsMeIm complex is a monomeric model of the polymeric mediator and is used for investigation of homogeneous reaction parameters.

Typical slow-scan cyclic voltammograms (CVs) of 1×10^{-4} mol dm^{-3} OsMeIm (A) and Osbpy (B) in oxygenated AB, pH 4.7, at

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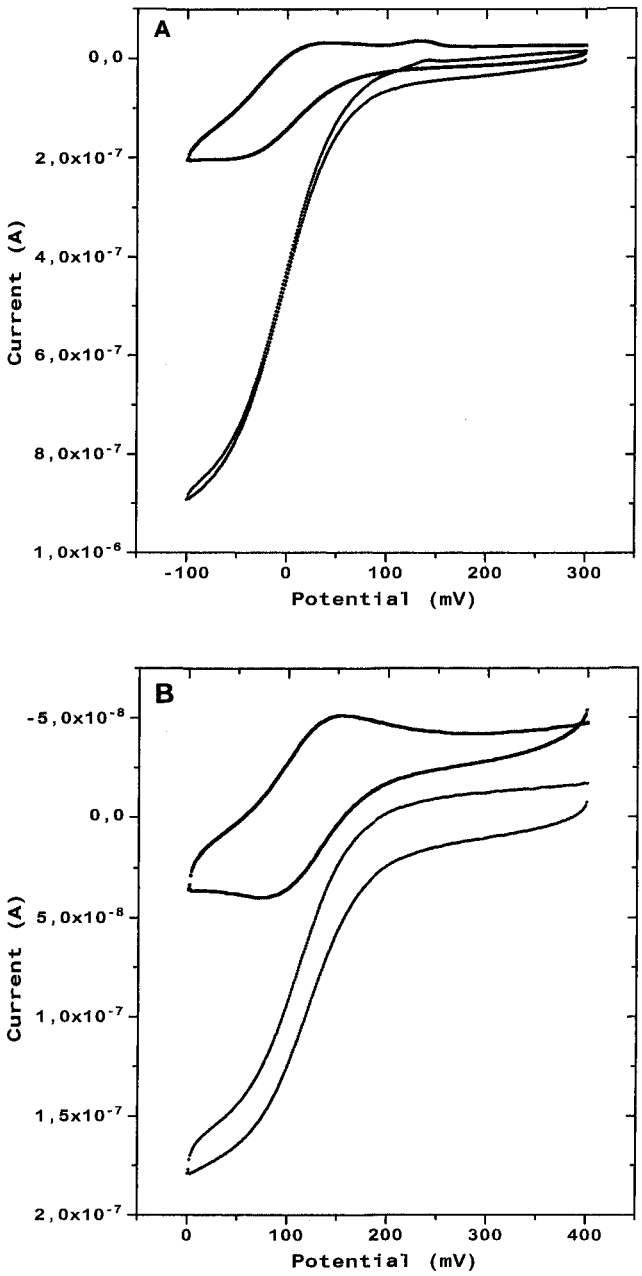

Figure 2. Slow-scan (2 mV/s) cyclic voltammograms of 1×10^{-4} mol dm^{-3} OsMeIm (A) and Osbpy (B) redox couples both before (tailing) and after (sigmoid) the addition of 1.2×10^{-7} mol dm^{-3} laccase to the 0.05 mol dm^{-3} acetate buffer electrolyte (pH 4.7).

a glassy carbon electrode both before and following the addition of laccase are shown in Figure 2. The enzyme is added to a solution of the oxidized mediator with the electrode poised at an oxidizing potential. Switching of the system to an active state is achieved by reduction of the redox complex, which can then mediate electron transfer to the enzyme, as depicted in the scheme in Figure 1, resulting in a catalytic current for the enzyme-mediated reduction of oxygen. Removal of oxygen from solution, by outgassing the electrolyte with argon or nitrogen (not shown), or the addition of excess inhibitor (azide, vide infra), decreases the catalytic current (i_{cat}), confirming the biocatalytic nature of the reaction.

Homogenous second-order rate constants (k_{med}) for the interaction between the redox complexes and laccase were evaluated

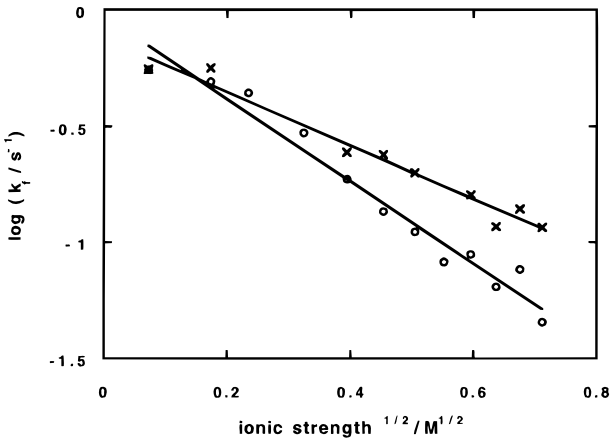

Figure 3. Pseudo-first-order rate constant vs square root of ionic strength for the reaction between laccase and the Osbpy (○) and OsMeIm (×) mediators. Ionic strength was adjusted with NaCl. Other conditions as in Figure 2.

by cyclic voltammetry using the approach of Cass et al.[15] based on the theory of Nicholson and Shain[16] for steady-state voltammetry. Values for the rate constant obtained for rapid kinetics (greater than 1×10^4 dm^3 mol^{-1} s^{-1}) are assumed to be accurate within 1 order of magnitude.[17] Rate constants of $(2.2 \pm 0.1) \times 10^6$ and $(3.3 \pm 0.6) \times 10^6$ M^{-1} s^{-1} were evaluated ($n = 3$) for the Osbpy and OsMeIm mediators, respectively. The rate constants obtained are indicative of rapid electron transfer, are comparable to the rates obtained for cytochrome *c* and blue copper electron transport proteins, such as plastocyanin and azurin, and are 5 orders of magnitude more rapid than that obtained for electron transfer between cytochrome *c* and a *R. vernicifera* laccase.[18] The k_{med} obtained for the model monomeric complex, OsMeIm, is similar, within experimental error, to that obtained for the Osbpy redox couple. The slight increase in rate constant could be attributed to the effect of the increased positive charge on ther monomer, increased thermodynamic driving force because of the lower formal potential for the OsMeIm redox couple, the interaction between the aromatic imidazole, or, more likely, a combination of all of these factors.

The ionic strength dependence of the reaction rate constant can help determine whether electrostatic interactions play a role in these enzymes reactions. The sweep rate-independent psuedo-first-order rate constant k_f was evaluated for the reaction between laccase and the osmium mediators in solutions of increasing ionic strength (pH 4.7), and the results are plotted in Figure 3. The isoelectric point for a *P. versicolor* laccase has been reported to be ~3.2; thus, at pH 4.7 laccase is negatively charged. The relation between rate constants and ionic strength can be expressed as[18]

$$\log k = 1.0182\sqrt{\mu Z_i Z_j} \qquad (1)$$

where μ is the ionic strength and Z_i and Z_j are the charges of the reactions *i* and *j*. Taking the fixed charges of the mediators as

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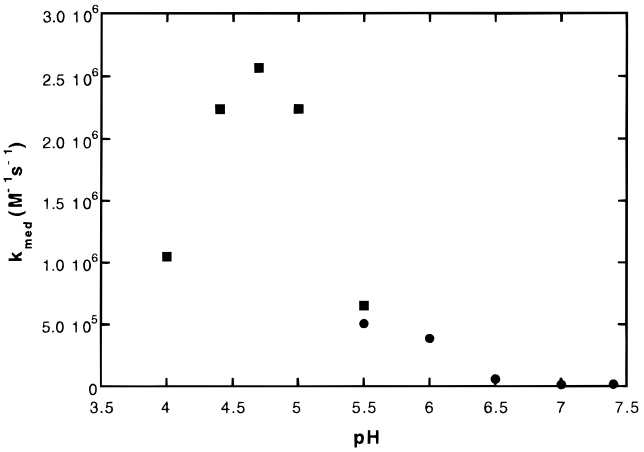

Figure 4. Effect of pH on the magnitude of the bimolecular rate constant for electron transfer between laccase and Osbpy in both acetate (●) and phosphate (■) buffer (0.05 mol dm^{-3}).

+2 (OsMeIm) and +1 (Osbpy) gives a net charge of approximately −0.58 and −1.78 for the laccase in the respective reactions, indicating an electrostatic association between the positively charged mediators and negatively charged sites on the enzyme. Indeed, Sakurai[18] reported that electron transfer between the positively charged cytochrome *c* and a positively charged *Rhus* laccase is favored by electrostatic interactions ($Z_iZ_j = -0.9$ at pH 6.0), leading him to speculate on the presence of a negatively charged amino acid near the substrate binding site. The reasons for the differences in the calculated charge for the laccase using the two osmium mediators in our study remain to be elucidated, possibly by examination of the ionic strength dependence of the laccase reaction with similarly sized osmium mediators of varying charge.

The effect of pH on the mediation of laccase by the osmium complexes was also examined. The k_{med} was evaluated in both phosphate and acetate buffers (0.05 mol dm^{-3}) at various pH's, and the curve is shown in Figure 4. The phosphate anion is reportedly a weak inhibitor of laccase.[8] The k_{med} remains relatively constant at pH 5.5 upon switching from a phosphate- to an acetate-based buffer system, demonstrating either the similarity in their inhibition affinities for laccase or the lack of inhibition of enzyme activity by these anions under these conditions. The redox potential for the laccases is reported to be pH sensitive, changing from a value of 380 mV (NHE) at a pH of 9.0 to 460 mV (NHE) at a pH of 4 for the *Rhus* laccase, for example.[18] Thus, the increase in reaction rate may be a result of increased thermodynamic driving force for electron transfer with decreasing pH, while a p*I* of pH 3.2 for laccase could explain the rapid decrease in enzyme mediation by these positively charged complexes when the pH is decreased to below 4.4. Other factors such as acid−base equilibria near the medator binding site, determined to be important for these complexes, can also be invoked to explain the pH dependence of these reactions. Yarpolov et al. suggested that the active site of laccases contain at least two acid−base groups of pK_a's close to 5.5.[11] The optimum pH for both Osbpy and OsMeIm mediation was 4.4−4.7.

The temperature dependence of the reaction between laccase and the osmium mediators was examined. An Arrhenius type plot of ln(k_f) vs reciprocal temperature yielded "activation energies" of 49 and 48 kJ mol^{-1}, respectively, for the OsMeIm and Osbpy complexes, demonstrating the similarity in their thermodynamic reaction with the laccase. The laccase activity rapidly decreases at temperatures above ∼55 °C, which is consistent with the temperature stability of other fungal laccases.[11]

Laccase Michaelis constant, K_m, values in an oxygen-saturated buffer of $(40 \pm 10) \times 10^{-6}$ and $(70 \pm 10) \times 10^{-6}$ mol dm^{-3} were obtained from the steady-state catalytic currents of OsMeIm and Osbpy, respectively, using the graphical method of Cornish-Bowden.[19] These K_m values are similar to those obtained for these osmium mediators at the tyrosinase enzyme[1] and for the organic mediator ABTS at several fungal laccases.[11] The higher affinity of the laccase enzyme for OsMeIm compared to Osbpy, reflected in the lower K_m value, may be due to increased electrostatic and/or hydrophobic interactions in the enzyme substrate entry channel with the more positively charged, hydrophobic OsMeIm mediator.

Homogeneous inhibition of laccase was investigated using the OsMeIm redox couple as a mediator in 0.05 mol dm^{-3} AB pH at 4.7. Small volumes of concentrated inhibitor solutions were added successively to a solution of the osmium mediator (1×10^{-4} mol dm^{-3}) and enzyme. The solution was magnetically stirred (300 rpm) for 1 min following each addition, and slow-scan (2 mV s^{-1}) cyclic voltammograms were recorded following a 1 min equilibration period. The addition of increased concentrations of fluoride, azide, and cyanide inhibitors resulted in a measurable decrease in the slow-scan rate catalytic currents. Reproducible normalized inhibition curves were obtained by plotting $(i_{cat} - i_{inh})/(i_{cat} - i_d)$ vs inhibitor concentration, where i_{inh} is the current observed in the presence of inhibitor, and i_d is the current observed in the absence of mediation (O_2 cosubstrate removed from the solution by degassing with nitrogen). From these curves, approximate IC_{50}'s of 2.5×10^{-3}, 0.5×10^{-3}, 0.5×10^{-3}, and 1.4×10^{-5} mol dm^{-3} were obtained for inhibition by sodium iodide, sodium fluoride, sodium cyanide, and sodium azide, respectively.

We have also investigated the selectivity of the system by adding possible inhibitors and interferents to the cell. Three classes of interferents are possible: (i) direct electrochemical reaction of an interferent at the electrode surface in the potential window of interest, (ii) mediated reduction or oxidation of the interferent by the osmium redox couple, or (iii) inhibition or activation of the laccase activity. No change in the catalytic currents for laccase were observed upon addition of up to 5×10^{-3} mol dm^{-3} of ClO_4^-, NO_3^-, SO_4^{2-}, Cl^-, Pb^{2+}, or Na^+ to the electrochemical cell. Concentrations above 5×10^{-3} mol dm^{-3} were not investigated because of decreased catalytic currents upon increase in the ionic strength of the electrolyte (vide supra). The catalytic currents increased upon addition of Fe^{2+}/Fe^{3+} to the cell, possibly because of the mediated reduction of Fe^{3+} by the osmium redox couple, as reported previously for the osmium polymers.[20] An increase of 32% in the catalytic current was apparent for the addition of 1×10^{-3} mol dm^{-3} Fe^{2+}. Additions of low ($<1 \times 10^{-3}$ mol dm^{-3}) concentrations of Hg^{2+} resulted in an initial decrease in the catalytic currents, whereas further addition resulted in increased currents due to direct reduction of the mercury at the glassy carbon electrode.

Immobilized Reagentless Enzyme Inhibition Electrode. The coimmobilization of both enzyme and electron donor in a stable film on the electrode surface is necessary for the application of these enzyme electrodes as portable, "reagentless" biosensors.

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Coimmobilization of both laccase and the osmium mediator can be achieved using an approach devised by Ohara et al.[7] for the construction of mediated glucose biosensors. In this approach, the osmium redox polymer and the enzyme are cross-linked with PEG in a buffer on the electrode surface. The cross-linker reacts with both the free imidazole nitrogen of the polymer and the enzyme amino groups to produce a stable hydrogel on the electrode surface. Slow drying of the drop-coated electrode in a water-saturated atmosphere for a period of at least 48 h was found to yield stable, active films of the immobilized enzyme−electron donor system. The formal redox potential for the immobilized osmium redox couple in AB, pH 4.7, was 220 mV (vs Ag/AgCl). The response observed for the enzyme−mediator immobilized electrode remains stable on the experimental time scale provided sufficient oxygen is present in solution. We have tested the overnight stability of these modified electrodes in buffer by recording a slow-scan (5 mV/s) CV each hour. An 8% loss in signal on the second scan (hour), with respect to the first scan, was apparent. A gradual decrease in mediated currents of ∼1.1%/h was observed for subsequent scans with a 25% difference between the initial and final (16 h) scan. A gradual decrease in the response for the mediated reduction of oxygen by the modified electrode is also observed upon continuous cycling in buffer. This is attributed to current limitation by decreasing oxygen concentrations in solution, as the signal returned to the initial level if scanning was interrupted and the potential poised at +0.4 V for several minutes. Modified electrodes prepared by drop-coating the redox polymer alone, or the redox polymer and the PEG cross-linker on the electrode surface, did not exhibit catalytic currents for the reduction of oxygen.

The inhibition of the enzyme-modified electrode catalytic current in the presence of sodium azide was investigated by cyclic voltammetry. While constant-potential amperometric monitoring of enzyme activity for continuous inhibition sensors is more attractive, at a fixed applied potential of 0.0 V a gradually decreasing current due to the depletion of oxygen cosubstrate was observed. Amelioration of this deterioration in signal is under investigation by addressing the enzyme activity using a potential pulsing strategy,[6] thus minimizing oxygen depletion. Representative cyclic voltammograms of an immobilized enzyme electrode before and after successive additions of the inhibitor, sodium azide, are shown in Figure 5. The decrease in the reduction current apparent upon addition of inhibitor indicates that enzyme activity is retained upon immobilization and that the mediation of electron transfer from the polymeric osmium redox couple to the enzyme active site occurs in the hydrogel. The shape of the modified electrode cyclic voltammetric waves also changes from that of a sigmoidal shape, normally observed for electrocatalytic reactions before addition of inhibitor, to a wave shape normally observed for a surface-modified redox polymer upon increasing the inhibitor concentration, again indicating inhibition of catalytic activity. Changes in the catalytic current induced by inhibition of the laccase enzyme by concentrations of azide as low as 2.5×10^{-6} mol dm^{-3} can be easily detected (Figure 5), thus demonstrating the sensitivity of the reagentless sensors to the presence of enzyme modulators.

The reversibility of the sensor was investigated by placing the inhibited electrode in distilled water for a period of time. The catalytic current decreased to 50% of its initial (no inhibition) height in CV upon addition of 3.5×10^{-4} mol dm^{-3} of sodium azide to the cell. No return to the initial (no inhibition) current level was evident even after prolonged washing with water, indicating irreversible inhibition of the sensor by azide. The sensor could, however, be partially regenerated following inhibition with sodium cyanide. Inhibition with 1.42×10^{-3} mol dm^{-3} produced a decrease in the catalytic current to 66% of its initial (no inhibition) level. Washing in water for 40, 75, and 120 min resulted in an increase in the catalytic current to 71%, 78%, and 83% of its initial (no inhibition) level, respectively. Given that a loss of ∼10% of the signal occurs during the first 2 h of operation, from the sensor stability studies, this level could represent the catalytic current expected for an noninhibited electrode.

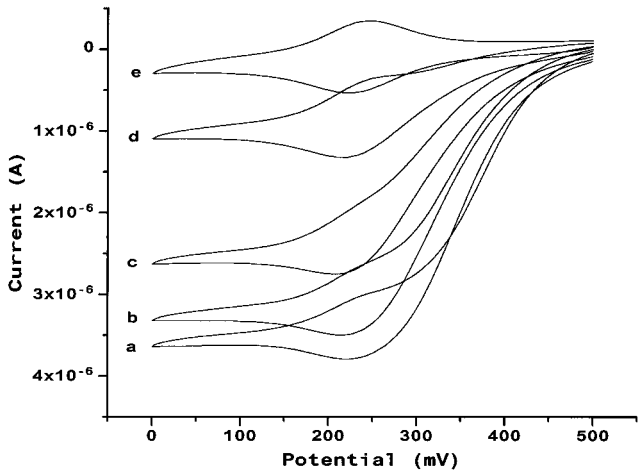

Figure 5. Slow-scan (2 mV/s) cyclic voltammograms obtained at a modified electrode upon addition of 0 (a), 2.5×10^{-6} (b), 7.5×10^{-6} (c), 3.25×10^{-5} (d), and 5×10^{-3} (e) mol dm^{-3} sodium azide to the acetate buffer (pH 4.7, 0.05 mol dm^{-3}) electrolyte.

CONCLUSIONS

The investigation of mediation of electron transfer to laccase by osmium complexes can help elucidate structure−function relations controlling enzyme activity. Further research using a wide variety of such mediators possessing different functionalites and formal potentials will clarify electron-transfer reactions in oxidoreductase enzymes such as laccase.

The "reagentless" detection of enzyme modulators can be achieved using an immobilized enzyme−mediator-modified electrode. The use of the electrode as an enzyme inhibition sensor is demonstrated by the detection of azide. Future research will focus on optimization of electrolyte parameters, polymer/enzyme ratios, and determination of inhibiton kinetics in the modified electrode for optimum response to inhibitors.

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