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Laccase electrode for direct electrocatalytic reduction of O₂ to H₂O with high-operational stability and resistance to chloride inhibition

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ABSTRACT

Laccase from *Trametes hirsuta* basidiomycete has been covalently bound to graphite electrodes electrochemically modified with phenyl derivatives as a way to attach the enzyme molecules with an adequate orientation for direct electron transfer (DET). Current densities up to 0.5 mA/cm² of electrocatalytic reduction of O₂ to H₂O were obtained in absence of redox mediators, suggesting preferential orientation of the T1 Cu centre of the laccase towards the electrode. The covalent attachment of the laccase molecules to the functionalized electrodes permitted remarkable operational stability. Moreover, O₂ bioelectroreduction based on DET between the laccase and the electrode was not inhibited by chloride ions, whereas mediated bioelectrocatalysis was. In contrast, fluoride ions inhibited both direct and mediated electron transfers-based bioelectrocatalytic reduction of O₂. Thus, two different modes of laccase inhibition by halides are discussed.

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1. Introduction

Laccases are a family of enzymes that catalyse four electron reduction of O₂ to H₂O coupled to the one electron oxidation of different substrates. In nature these electrons are supplied by several phenols, amines and lignins, as well as inorganic ions (Yaropolov et al., 1994; Solomon et al., 1996; Morozova et al., 2007). It is now known that the catalytic activity of these proteins involves four copper ions grouped into three types named T1, T2 and T3. T1 and T2 are similar to planar Cu²⁺ complexes, whereas T3 is a binuclear Cu^{2+/1+}Cu^{2+/1+} complex (Solomon et al., 1996; Morozova et al., 2007). The T2 and T3 sites form a trinuclear Cu cluster where O₂ is reduced to H₂O with four electrons transferred from the T1 site, which is the site of electron exchange with the redox donor molecules (Shleev et al., 2005a).

The redox potential of the T1 site for different laccases ranges between +430 and +790 mV vs. NHE (Shleev et al., 2004, 2005a). The possible factors that tune their redox potentials values have been recently revised (Quintanar et al., 2007). The laccase isolated from *Trametes hirsuta* has been considered of high value for applications based on enzymatic electrodes, i.e. biosensors and biofuel

cells, due to its lower carbohydrate content and to its high stability, activity and redox potential of the T1 site (Yarasevich et al., 2002; Barriere et al., 2004; Shleev et al., 2004). This later characteristic is important because the T1 site is directly involved in the electron transfer between polarized electrodes and the laccases molecules in solution, as it has been demonstrated by cyclic voltammetry, potentiometry and spectroelectrochemistry (Shleev et al., 2005a,b,c). The high redox potential of the T1 site (780 mV vs. NHE, pH 6.5) permitted measurement of direct electrocatalysis of O₂ reduction by laccase adsorbed on bare graphite electrodes at low overpotentials compared to the thermodynamic equilibrium of the O₂/H₂O couple (Shleev et al., 2005c). Adsorption of the laccase on bare gold or covalent immobilisation of the enzyme on gold electrodes modified with a self-assembled monolayer of amino-thiophenol molecules has also been reported (Shleev et al., 2006; Pita et al., 2006). However, in these later cases bioelectrocatalytic reduction of O₂ was measured at more than 250 mV lower potentials compared to that of the T1 site, and a fraction of the laccase molecules were producing H₂O₂ instead of H₂O (Pita et al., 2006).

Very recently Armstrong and co-workers have reported an efficient high potential direct reduction of O₂ to water at anthracene-modified pyrolytic graphite 'edge' electrodes with fungal laccases adsorbed in a proper orientation (Blandford et al., 2007). The rational behind this study was the oriented immobil-

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isation of laccase molecules by affinity interaction of anthracene molecules anchored on the modified surface with hydrophobic pockets on the enzyme pointing to the T1 site. The stability of the electrodes with *Pycnoporus cinnabarinus* laccase were tested discontinuously under storage conditions, showing a higher stability than the unmodified pyrolytic graphite electrodes used as controls (Blandford et al., 2007). However, no results on the stability of the designed biocathodes under working operating conditions were reported, a critical issue for the practical use of enzymatic electrodes in biofuel cells (Katz and Willner, 2003; Heller, 2004; Barton et al., 2004; Kamitaka et al., 2007). Another issue concerning the application of laccase electrodes in biofuel cells working under physiological conditions is their inhibition by Cl^- (Spira-Solomon et al., 1986).

We have shown previously that 4-aminophenyl monolayers generated *in situ* by electroreduction of diazonium salts are robust enough to anchor a monolayer of hydrogenase molecules, covalently bound to the amino groups, for extended periods of time (Rüdiger et al., 2005; Alonso-Lomillo et al., 2007). Therefore, we have investigated the covalent immobilisation of a laccase onto carbon electrodes that incorporate functional groups as potential inducers of efficient direct electron transfer (DET) to the carbon base and at the same time allow anchoring the enzyme by stable covalent bonds. In the present work we describe the fabrication and characterization of highly stable, efficient and chloride resistant biocathode based on low-density graphite electrodes (LDG) with covalently bound high redox potential fungal laccase.

2. Materials and methods

2.1. Chemicals

The reagents 4-nitrobenzene diazonium salt, sodium nitrite, 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide (EDC), 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), catechol, potassium ferricyanide and sodium fluoride were purchased from Sigma–Aldrich. 2-Nitro-4-aminophenol was from Acros Organics and *N*-hydroxysuccinimide (NHS) was from Fluka. Buffer solutions were prepared with Na_2HPO_4 , NaH_2PO_4 , NaCH_3COO and CH_3COOH in deionized water (Milli-Q grade, Millipore). Bu_4NBF_4 (Sigma–Aldrich), NaClO_4 (Sigma–Aldrich) and KCl (Panreac) were used as electrolytes. Sulphuric acid, ethanol and acetonitrile were supplied by Panreac. Acetonitrile was made anhydrous prior to use with molecular sieves (Sigma–Aldrich), whereas all other reagents were used as received.

2.2. Enzyme

The experiments reported in this work were done with *T. hirsuta* laccase from the basidiomycete strain *T. hirsuta* 56 obtained from the laboratory collection of the Moscow State University of Engineering Ecology following the purification procedure previously reported (Shleev et al., 2004). The enzyme was homogeneous as judged from SDS-PAGE and HPLC. The highly concentrated preparations of *T. hirsuta* laccase were stored in 0.1 M phosphate buffer, pH 6.5, at -20°C . The concentration of the enzyme in stock solution was measured using BIO-RAD (Bio-Rad Laboratories) according to the Bradford method (Bradford, 1976). The calibration curve was done using bovine serum albumin (BSA) as the protein standard. The specific activity of the enzyme was measured spectrophotometrically using 1 mM ABTS as substrate. Two different batches of purified laccase were used with specific activities of 260 and 150 units/mg laccase.

2.3. Electrochemical measurements

Electrochemical measurements were performed in a thermostated electrochemical cell of 20 ml with mild magnetic stirring using stationary 3 mm diameter planar working electrodes of low-density graphite (Sigma–Aldrich). Prior to surface modification the electrodes were polished with emery paper and cleaned by sonication in Milli-Q water for 5 min. An Ag|AgCl|3 M KCl reference electrode from Bioanalytical Systems and a platinum wire counter electrode were used. Cyclic voltammograms and chronoamperograms were controlled by an Autolab (PGSTAT 12) potentiostat. All measurements with laccase electrodes were carried out at 27°C in 50 mM acetate buffer, pH 4.2, containing 100 mM NaClO_4 . Amperometric measurements in stirred solutions were performed by applying a potential of +0.2 V (vs. Ag|AgCl|3 M KCl) and allowing the steady-state current to be reached.

2.4. Modification of LDG electrodes with a 2-aminophenol monolayer

The procedure was based on the method described by Nassef et al. (2006). 1 ml of a 0.09 M sodium nitrite solution was added to a stirred 3 ml ice-cold 0.03 M solution of 2-nitro-4-aminophenol, both solutions prepared in 2 mM HCl. Afterwards, the mixture was stirred for 1.0 h in an ice bath, the pretreated electrode was immersed in the mixture and the potential cycling was carried out between 0.6 and 0.0 V (vs. Ag|AgCl|3 M KCl) (10 cyclic voltammograms at 100 mV s^{-1}) to electrochemically reduce the “*in situ*” generated 2-nitrophenol diazonium salt. The electrode was then transferred to a previously deoxygenated 0.1 M H_2SO_4 solution and subjected to 10 redox potential scans between 0.0 and -0.85 V (vs. Ag|AgCl|3 M KCl) at 50 mV s^{-1} for complete reduction of the nitro groups of the attached aryl rings, forming a grafted film of 2-aminophenol on the electrode surface (Theodoridou et al., 1981; Leitner et al., 2004). The modified electrode was finally washed with water to remove physically adsorbed compounds.

2.5. Modification of LDG electrodes with aminophenyl monolayer

The procedure was based on the method developed by Pinson and co-workers (Delamar et al., 1992; Allongue et al., 1997). 2.5 mg of 4-nitrobenzene diazonium salt were dissolved in 5 ml of 0.1 M Bu_4NBF_4 prepared in anhydrous CH_3CN . The pretreated electrode was immersed in 3 ml of the above solution and the potential was cycled two times between 0.75 and -0.05 V (vs. Ag|AgCl|3 M KCl) at 200 mV s^{-1} . The electrode was then transferred to a 1/9 $\text{EtOH/H}_2\text{O}$ solution (containing 0.1 M KCl) deoxygenated previously and subjected to two cyclic voltammograms between 0 and -1.4 V (vs. Ag|AgCl|3 M KCl) at 10 mV s^{-1} for reduction of the nitro groups of the attached phenyl rings.

2.6. Laccase immobilisation on LDG electrodes

The monolayer-modified electrode was coated with $5\ \mu\text{l}$ of a $0.72\ \mu\text{g}\ \mu\text{l}^{-1}$ laccase solution (prepared in 10 mM phosphate buffer, pH 6.0). The drop of enzyme solution was maintained for 20 min at ambient temperature to favour concentration and orientation of enzyme molecules on the electrode surface. The electrode was covered with an eppendorf tube in order to avoid a quick evaporation of the drop. Then, amide coupling was activated by depositing on the electrode a $4.5\ \mu\text{l}$ drop of a 20 mM NHS solution (in 10 mM phosphate buffer, pH 6.0) and immediately a $5.5\ \mu\text{l}$ drop of a 40 mM EDC solution (in 10 mM phosphate buffer, pH 6.0). Covalent immobilisation of the enzyme was allowed to carry on during 2 h at ambient temperature with the electrode surface covered with an eppendorf

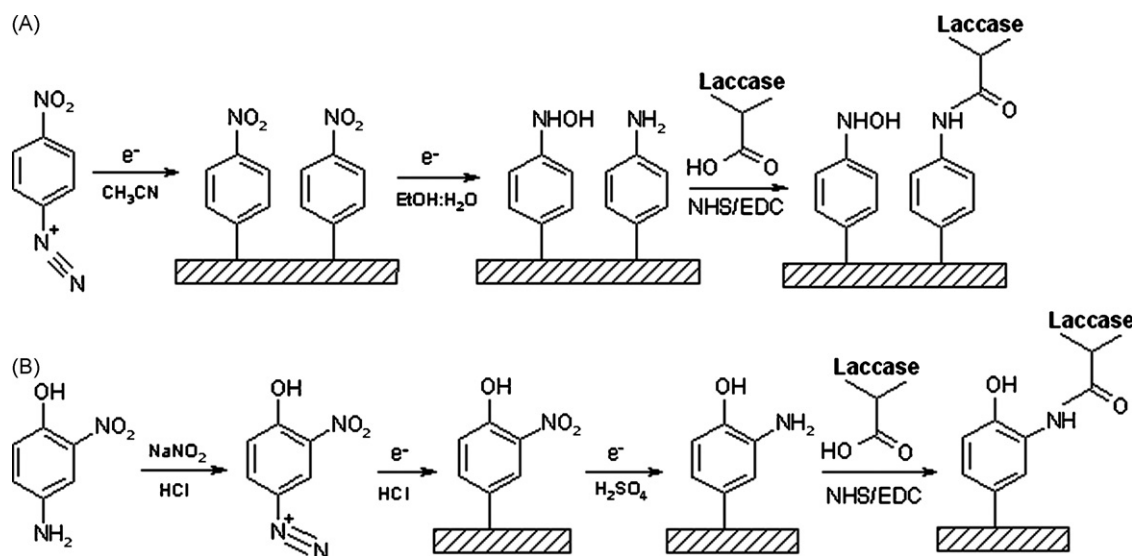


Fig. 1. Scheme of electrode modification with laccase via aminophenyl groups (A) or 2-aminophenol groups (B).

tube. Finally, the modified electrode was soaked in the electrochemical measurement buffer solution for 10 min with stirring to remove any unbound compounds.

3. Results

3.1. Modification of the electrode surface with aryl groups

Functionalization of LDG electrodes was done by electrochemical reduction of 4-nitrophenyl diazonium salt in acetonitrile. This reductive strategy, developed by Pinson and co-workers, allows covering carbon surfaces with highly stable covalently bonded aryl groups (Delamar et al., 1992; Allongue et al., 1997). Further electrochemical reduction of the nitro groups generated a mixed monolayer of hydroxylaminophenyl and aminophenyl groups (Fig. 1A). Hydroxylaminophenyl grafted groups give rise to a well-defined redox process at -300 mV vs. Ag|AgCl|3 M KCl observed by cyclic voltammetry (Ortiz et al., 1998; Brooksby and Downard, 2004). By integration of its reduction or oxidation wave and integration of the voltammetric waves measured during reduction of the initial nitro groups, the global coverage of the mixed monolayer can be estimated as $2.5 \pm 0.8 \times 10^{-9}$ mol cm $^{-2}$ with a 1:3 ratio of amino to hydroxylamine groups (Supplementary Fig. S1). This coverage was calculated considering the electroactive area of each electrode, measured by chronocoulometry of ferrocenemethanol (on average 1 ± 0.3 cm 2), and not the geometric area, as this material has a high roughness factor. Thus, the coverage is somewhat higher than the theoretical value for a compact monolayer of phenyl rings, 1.35×10^{-9} mol cm $^{-2}$ (Pinson and Podvorica, 2005). Alternatively, diazonium salts were prepared by reaction of 4-amino-2-nitrophenol with sodium nitrite. Electrochemical reduction of the resulting diazonium salt promoted the covalent attachment of 2-nitrophenol rings to the electrodes, which were subsequently irreversibly reduced in a second electrochemical step to 2-aminophenol rings (Fig. 1B). A coverage of $1.3 \pm 0.4 \times 10^{-10}$ mol cm $^{-2}$ was measured from integration of the voltammetric wave observed at -170 mV, which corresponds to the reversible oxidation of 2-aminophenol to 2-quinoneimine (Nassef et al., 2006). This result is roughly equivalent to 10% of a compact monolayer of phenyl rings (Supplementary Fig. S2).

3.2. Direct vs. mediated electrocatalysis of O₂ reduction at laccase electrodes

T. hirsuta laccase was covalently bound to the modified electrodes by forming ester and/or amide bonds between carboxylic groups of the protein surface and hydroxyl and/or amino groups, respectively of the modified electrodes (Fig. 1). The currents of O₂ electrocatalytic reduction in the absence of redox mediators in solution were measured by cyclic voltammetry. Fig. 2 shows the voltammograms obtained for each type of electrode monolayer after laccase immobilisation. In both cases high currents of direct electrocatalytic reduction of O₂ were measured and the half wave of the process occurred approximately at the redox potential of the T1 site of *T. hirsuta*, as has been reported for the same laccase absorbed directly on bare graphite electrodes (Shleev et al., 2005c). Furthermore, chronoamperometric experiments showed that the direct electrocatalytic (DEC) current outputs from *T. hirsuta* modified graphite electrodes polarized at $+200$ mV vs. Ag|AgCl|3 M KCl were stable and showed approximately half of the signal compared to the mediated electrocatalytic (MEC) currents measured with the same electrodes in presence of the electron donor (ABTS) in solution (55% and 43% for the aminophenyl and 2-aminophenol electrodes, respectively, Fig. 3).

Control experiments were done in which (i) laccase was not deposited on the electrode, (ii) the electrode was not modified with an aryl monolayer and (iii) EDC/NHS was not added during the immobilisation procedure. In the absence of laccase neither DEC nor MEC reduction of O₂ were detected. Fig. 4 shows the DEC/MEC ratio measured for the other control experiments in comparison with the completely modified amino phenyl electrodes. In these control experiments the MEC currents were similar to those of the completely modified laccase electrodes. This indicates that active laccase adsorbed both to bare and amino phenyl LDG electrodes. Nevertheless, the DEC/MEC ratio was much higher for the experiments done with amino phenyl LDG electrodes, which suggests that the presence of amino groups on the electrode surface favoured direct electron transfer between the T1 site of the laccase (either adsorbed or covalently bound) and the electrode.

The effect of ionic strength and pH during the immobilisation step was studied in order to investigate the incidence of electrostatic interactions during the enzyme immobilisation step on the

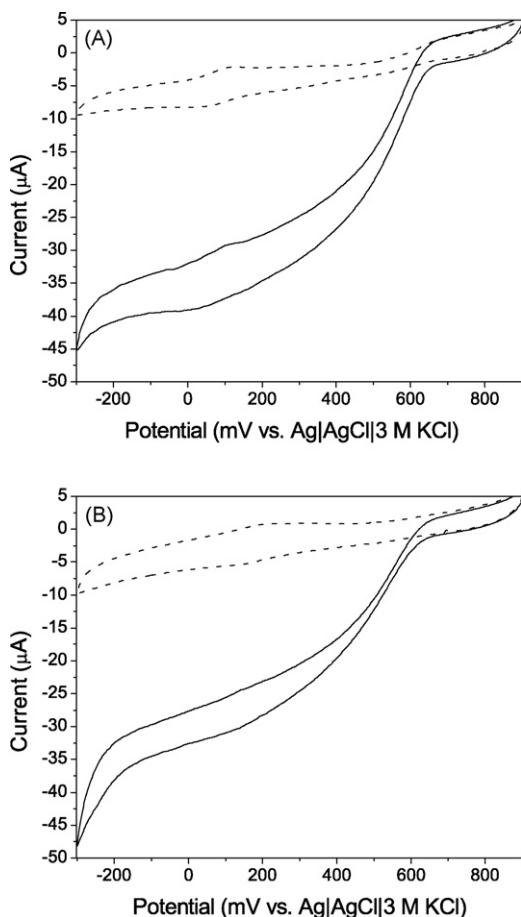


Fig. 2. Cyclic voltammograms under N_2 (dashed line) and under O_2 (straight line) of an aminophenyl electrode (A) and a 2-aminophenol electrode (B) covalently modified with *T. hirsuta* laccase. Measured in 50 mM acetate buffer, pH 4.2, 100 mM $NaClO_4$. Scan rate was 10 mV s^{-1} and the temperature was 27°C .

performance of the laccase electrode. The MEC currents decreased significantly at neutral and basic pH values. The optimal pH for DEC was 5.1, as the highest values were measured for the absolute current (0.5 mA/cm^2 considering the geometric area of the graphite electrode) and the ratio to the MEC current. However, the DEC/MEC ratio was considerably high at all pH values measured and a clear trend cannot be established (Supplementary Table S1). At high ionic strength and pH 5.7 the catalytic currents of O_2 reduction decreased, and also the DEC/MEC ratio, which was half of that obtained at low ionic strength and same pH (Supplementary Fig. S3). Thus, we conclude that electrostatic interactions influence laccase orientation on the electrode during immobilisation, although other types of interactions may also be important.

3.3. Stability of the electrodes under operating conditions

The stability under operating conditions of a LDG with laccase covalently bound to the aminophenyl monolayer was followed throughout 2 weeks by chronoamperometry at $+200\text{ mV vs. Ag|AgCl|3 M}$, 27°C with pure O_2 bubbled through the electrolyte solution. The initial DEC current was lower than in previously shown experiments because a new batch of enzyme was used that had lower specific activity. About 75% of the initial DEC current was retained after 10 days of continuous operation of the electrode as shown in Fig. 5. Cycles of saturation of the solution with Ar/O_2 or addition/removal of ABTS did not affect the stability of the electrode (Fig. 5). The control experiments of laccase adsorbed to bare LDG or

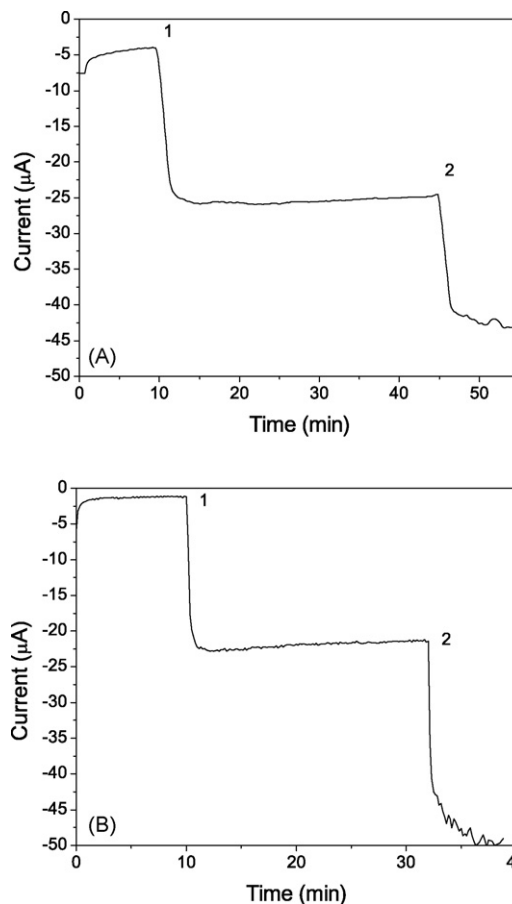


Fig. 3. Chronoamperometric measurements of an aminophenyl electrode (A) and a 2-aminophenol electrode (B) covalently modified with *T. hirsuta* laccase. At time 1 the anaerobic solution was bubbled with 1 atm O_2 and at time 2 0.2 mM ABTS was added. The electrolyte solution contained 50 mM acetate buffer, pH 4.2, 100 mM $NaClO_4$. The applied potential was $+200\text{ mV vs. Ag|AgCl|3 M KCl}$, the temperature was 27°C and the solution was stirred with a magnetic bar.

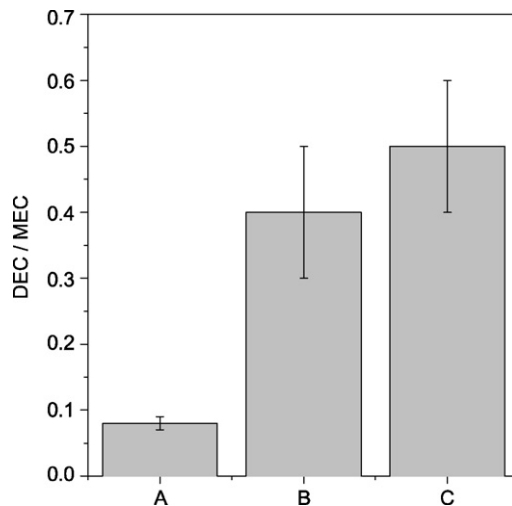


Fig. 4. DEC/MEC ratio of O_2 reduction by *T. hirsuta* laccase directly adsorbed on bare LDG (A), immobilized on aminophenyl LDG in absence of NHS/EDC (B), or covalently bound to aminophenyl modified LDG (C). Measurement conditions were the same as in Fig. 3. The bars represent the average value measured from four sets of experiments.

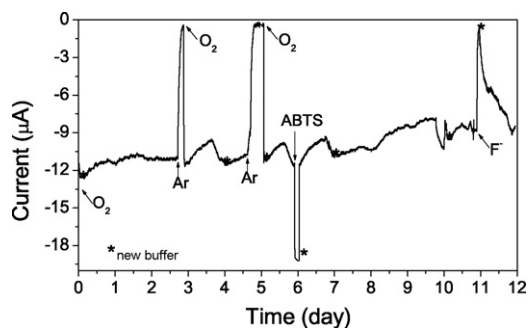


Fig. 5. Stability of *T. hirsuta* laccase covalently bound to an aminophenyl electrode under continuous operation. Measurement conditions were the same as in Fig. 3.

to LDG modified with amino phenyl groups indicated that in those cases the laccase activity is significantly less stable (Supplementary Table S2).

3.4. Inhibition studies of laccase/LDG electrodes

The high stability of O_2 reduction by the LDG electrodes covalently modified with laccase allowed us to investigate the inhibitory effect of halides on its activity. The repetitive addition of Cl^- seldom affected DEC of O_2 reduction, as shown in Fig. 6A. As expected, an inhibitory effect of Cl^- on the MEC activity with ABTS was clearly observed, as after addition of 150 mM Cl^- the electrocatalytic current decreased to the DEC level (Fig. 6B). Oppositely, fluoride did inhibit completely the electrocatalytic activity of the immobilized laccase, as it also affected the DEC current (Figs. 5 and 6B). The inhibitory effect of Cl^- was not specific for ABTS as substrate, as it

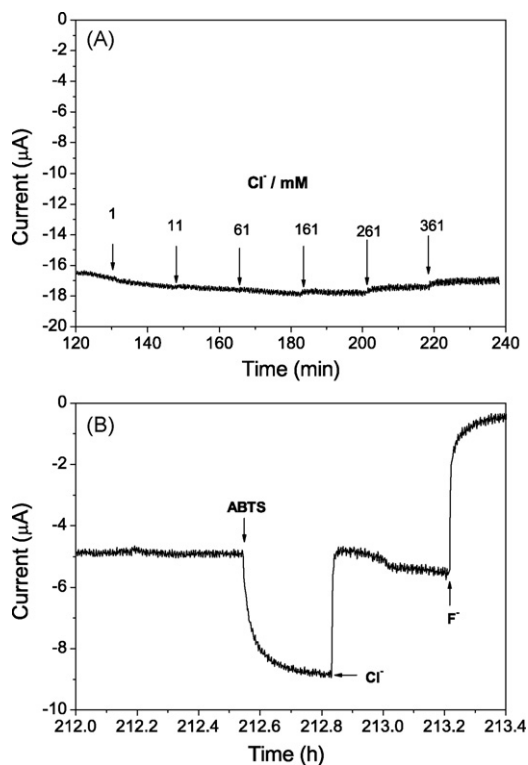


Fig. 6. Effect of halide inhibitors on O_2 reduction by an aminophenyl electrode covalently modified with *T. hirsuta* laccase: (A) effect of different concentrations of chloride on DEC, (B) effect of 150 mM chloride and 13 mM fluoride on MEC (with 0.2 mM ABTS) and DEC, respectively. Measurement conditions were the same as in Fig. 3.

was also observed with other redox mediators such as ferricyanide or catechol (Supplementary Fig. S4).

4. Discussion

Electrochemically controlled functionalization of LDG electrodes with aminophenyl groups in this work has produced a coverage between 1 and 2 compact monolayers of phenyl rings on an electrode surface. Thus, it is possible that multilayers of aminophenyls rings have been formed in some regions of the electrode (Kariuki and McDermott, 2001; Brooksby and Downard, 2004). Nevertheless, covalent immobilisation of *T. hirsuta* on these modified electrodes has led to high DEC of O_2 reduction. As the rate of DET between redox proteins and electrodes decreases exponentially with the distance, we can conclude that a great proportion of the covalently bound laccase molecules were fixed at small distances from the graphite surface (Heering et al., 1998; Leger et al., 2002). By contrast, the coverage of 2-aminophenol groups obtained on LDG electrodes was only 10% of a compact monolayer of phenyl rings. In spite of this, the MEC and DEC currents of O_2 reduction measured with this monolayer under optimal conditions were almost equivalent to those measured with the electrodes modified with aminophenyl groups. This result is not surprising because the coverage of a compact monolayer of phenyl rings is approximately three orders of magnitude higher than that of enzymes on electrodes (Pinson and Podvorica, 2005). Thus, 10% of a compact monolayer of functionalized aryl rings should be enough for anchoring a compact monolayer of laccase molecules.

We do not have a direct measurement of the amount of laccase immobilized on our electrodes, but the MEC currents should be proportional to the total amount of active laccase assuming that the enzyme did not drastically change its specific activity during the immobilisation procedures (Mege and Bourdillon, 1985; Bourdillon et al., 1994). As ABTS is able to diffuse and exchange electrons between the electrode and the T1 site of immobilized laccase molecules, the rate of MEC should be independent of the orientation of the later on the LDG surface. By contrast, only the immobilized laccase molecules that have a redox site close to the electrode surface should give DEC. In this work DEC is most probably associated to DET via the T1 site because the cyclic voltammetry experiments show that it takes place at the redox potential of this Cu site. The redox potential applied in the chronoamperometric experiments was much higher than the reported one at which incomplete reduction of O_2 to H_2O_2 was measured with the same laccase immobilized on gold electrodes (Pita et al., 2006). The authors of that work suggested that this was due to direct electron transfer via the T2 Cu site. Furthermore, the DEC currents were completely inhibited by F^- , which is also a clear indication that we are only measuring the native enzymatic activity of reduction of O_2 to H_2O (Pita et al., 2006). Therefore, our DEC currents must be proportional to laccase molecules properly oriented with the T1 site facing the LDG surface. As can be seen from Fig. 4 a very small fraction of laccase molecules had adequate orientation for DEC when adsorbed directly on the bare LDG electrode, whereas much more laccase molecules were properly oriented towards the aminophenyl modified electrodes via the T1 site for DET-based O_2 electroreduction.

Electrostatic interactions between the positively charged amino groups of the electrode monolayer and the negatively charged regions of the protein surface may control the orientation of laccase molecules during immobilisation. In fact, the crystallographic structure of *T. versicolor* laccase, which is highly homologous to the one used in this work, indicated that the T1 site is surrounded by a negatively charged pocket, due to carboxylic residues that are

non-protonated at pH values above 4–5 (Piontek et al., 2002). The DEC/MEC ratio decreased by a factor of two when laccase immobilisation was done at very high ionic strength. Therefore, we conclude that electrostatic interactions favour preferential orientation of immobilized laccase with the T1 site facing the electrode. Nevertheless, other type of interactions may also favour this orientation because DEC currents were still measured when laccase immobilisation was done at very high ionic strength or at high pH, at which the amino groups of the electrode surface are not charged (Rüdiger et al., 2005). Other possible interactions could be hydrogen bonds between conserved aspartate and histidine residues of the T1 site of laccases and the functional groups of the electrode monolayer, or hydrophobic interactions between the aromatic rings of the electrode monolayer and of some amino acids forming the cavity of the T1 site. Similar interactions of laccases with organic substrates have been proposed previously by other authors based on crystallographic structures (Bertrand et al., 2002; Enguita et al., 2004) and from the study of laccase adsorption onto a carbon electrode modified with an anthracene monolayer (Blandford et al., 2007).

The maximum DEC current density measured in this work (0.5 mA/cm^2 at $+0.2 \text{ V vs. Ag/AgCl (3 M)}$) is much higher than those reported for laccases covalently bound to modified gold electrodes (Johnson et al., 2003; Gupta et al., 2004; Pita et al., 2006; Kliis et al., 2007). A higher DEC current density than the one obtained in this work has been reported very recently for laccase adsorbed on a carbon electrode. However, in all likelihood the achieved 10 mA/cm^2 density current can be attributed to the very high specific area of the electrode used rather than to optimised orientation of laccase on the electrode surface (Tsujimura et al., 2007).

Without any doubt the high operational stability of the laccase electrodes prepared in the present work is related to enzyme immobilisation via covalent bonds, as it was much lower for the control experiments in which laccase was adsorbed to the bare or functionalized graphite surface. Laccase electrodes prepared by adsorption onto modified carbon surfaces by other authors have been reported to be stable under storage conditions (Blandford et al., 2007; Liu et al., 2007) or under operational conditions with low rates of O_2 mass transfer (Tsujimura et al., 2007). However, in the present work we have studied the stability under continuous operation with high rates of O_2 mass transfer, which is more demanding. The operational stability of our electrode improves also that reported for laccase entrapped within a polymer-modified carbon electrode (Servat et al., 2007). Therefore, covalent linking of enzymes via target functional groups of their surface to electrodes is an adequate strategy for obtaining the high operational stability required for different applications, as shown in this work for laccase and in our previous work for a hydrogenase (Alonso-Lomillo et al., 2007).

A very interesting result of the present study is the ability to differentiate the inhibition modes of fungal laccases by halides. It is widely accepted that F^- binds to the T2/T3 copper cluster of laccases and blocks the intra-molecular electron transfer from the T1 site to the trinuclear copper site (Naki and Varfolomeev, 1980; Spira-Solomon et al., 1986; Xu et al., 1998). This explains why both DEC and MEC of O_2 reduction were abolished by F^- in the present study. Most likely, DEC of O_2 reduction was not suppressed by the bulkier Cl^- ions due to restricted access to the T2/T3 catalytic site, and therefore may not block intra-molecular electron transfer. Contrary to F^- inhibition, Cl^- ions may block the access of the redox mediators to the T1 sites, thus suppressing MEC but not DEC, as in the later case the electrons travel directly from the electrode to the Cu of the T1 site. These results are in excellent agreement with former kinetic experiments of laccase inhibition by halide ions reported previously. It was shown that Cl^- is a competitive inhibitor, whereas F^- is non-competitive, with respect to the electron donor (Naki and Varfolomeev, 1981). Moreover, I^- binding

to the T1 site of a fungal laccase has also been suggested and its slow enzymatic oxidation by the T1 site of the enzyme has been reported (Xu, 1996). Further studies beyond the scope of this work are required to demonstrate which site of laccases is the Cl^- binding centre. Nevertheless, the observed resistance of the designed high redox potential laccase-based biocathodes to Cl^- inhibition is very important for biofuel cell applications. A possible niche of application of fuel cells based on biological electrocatalysts is for devices that can efficiently and steadily operate under physiological conditions (Heller, 2004; Barton et al., 2004; Barriere et al., 2006). In this work we show that laccase-modified electrodes that reduce O_2 to H_2O , without the need of redox mediators, are highly active and stable in the presence of very high concentrations of Cl^- . This is an important step for their use in potentially implantable biodevices.

5. Conclusions

LDG electrodes electrochemically modified with a monolayer of aminophenyl or 2-aminophenol groups are adequate platforms for covalent bonding of *T. hirsuta* laccase favouring orientation for DEC of O_2 to H_2O . Probably, these aryl derivatives with polar functional groups interact favourably with the laccase region that surrounds the T1 site, allowing fast electron transfer between this Cu site of the enzyme and the electrode. The laccase-based electrodes gave high catalytic currents of O_2 reduction in absence of redox mediators (up to 0.5 mA/cm^2) with remarkable operational stability.

We have shown that the inhibition mode of laccases by Cl^- is different from that by F^- . The later affected both DEC and MEC of O_2 reduction, whereas the first only affected MEC. We suggest that the smaller halides penetrate within the laccase structure blocking the intra-molecular electron transfer pathway between the Cu centres, which impedes enzymatic reduction of O_2 to H_2O . In contrast, the larger halides most probably cannot penetrate so deep into the protein and may bind to the surface region to which the redox donor substrate interacts, blocking inter-molecular electron transfer.

Finally, the functional properties of designed laccase-based electrodes (high density currents by DET, high stability and resistance to Cl^- inhibition) are very suitable for their possible application as a cathode in biofuel cells.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bios.2008.05.002.

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