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Revisiting direct electron transfer in nanostructured carbon laccase oxygen cathodes

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ABSTRACT

The biocatalytic electroreduction of oxygen has been studied on large surface area graphite and Vulcan® carbon electrodes with adsorbed *Trametes trogii* laccase. The electrokinetics of the O_2 reduction reaction (ORR) was studied at different electrode potentials, O_2 partial pressures and concentrations of hydrogen peroxide.
Even though the overpotential at 0.25 mA·cm^{-2} for the ORR at T1Cu of the adsorbed laccase on carbon is 0.8 V lower than for Pt of similar geometric area, the rate of the reaction and thus the operative current density is limited by the enzyme reaction rate at the T2/T3 cluster site for the adsorbed enzyme. The transition potential for the rate determining step from the direct electron transfer (DET) to the enzyme reaction shifts to higher potentials at higher oxygen partial pressure.
Hydrogen peroxide produced by the ORR on bare carbon support participates in an inhibition mechanism, with uncompetitive predominance at high H_2O_2 concentration, non-competitive contribution can be detected at low inhibitor concentration.

1. Introduction

Among multicopper enzymes, laccase (EC1.10.3.2) is an extracellular blue copper enzyme in plants and fungi which catalyzes the oxidation of biphenols and the four-electron reduction of molecular oxygen to water. This enzyme contains four copper atoms, denoted by T1, T2 and two T3 according to their spectroscopic properties. In high potential laccases the copper center T1 can be reduced by phenol compounds [1], one-electron redox mediators [2–4] and also by direct electron transfer from electrodes [5–8]. T1Cu site is located close to the enzyme surface at a hydrophobic pocket where enzyme substrates bind; the oxygen binding site is the trinuclear T2/T3 cluster separated by 13 Å from T1. Reduction of T1 Cu(II) site proceeds with further internal electron transfer to the trinuclear T2/T3 cluster [9,10] where the reduction of molecular oxygen to water takes place.

The catalytic ability of laccases to activate the O_2 4-electron reduction under physiological conditions at unprecedented high electrode potentials (c.a. 0.60 V vs. Ag/AgCl) has encouraged their study in cathodes for bio-fuel cells and an extensive literature on the electrochemistry of laccases from different sources has followed in recent years [3,11–13].

The first evidence of direct electron transfer (DET) from an electrode to T1Cu of laccase was reported by Tarasevich [14] on carbon black electrodes. Several other reports on DET to laccase biocatalyst of the oxygen reduction reaction (ORR) have followed [5,13,15–30].

Based on the molecular structure and size of laccases the maximum surface concentration of the enzyme monolayer on electrode surfaces has been estimated 2–8 × 10^{-12} mol·cm^{-2}. [31] The orientation of the T1Cu center close to the enzyme surface with respect to the electrode is critical for the catalysis and since the population of enzyme molecules at tunneling distance is very low, the current densities reported for flat electrodes with enzyme monolayers are small, typically below 1 μA·cm^{-2}. Therefore large surface area carbon and gold electrodes have been employed in DET studies.

Laccase biocathodes have been intensively studied for biofuel cells [30,32]. Our research group demonstrated in 2010 that laccase can be inhibited by self-produced H_2O_2 [4]. Later it was shown that biofuel cells based on a laccase cathode suffer an important loss in performance due to the H_2O_2 produced by the glucose oxidase immobilized at the anode [34]. The inhibition mechanism of H_2O_2 on laccase electrodes was unclear until recently when it was demonstrated that a non-competitive inhibition takes place for Os-complex [35] and ABTS-mediated [36] biocathodes. On the other hand, inhibition on DET laccase cathodes was reported to follow an uncompetitive mechanism [34,36].

The present paper revisits the effect of the interplay of the direct electron transfer from carbon electrodes to adsorbed laccase and the

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enzymatic electrocatalysis ORR biocathodes, and the inhibition of the cathodic reaction by hydrogen peroxide produced by the ORR on carbon. The low rate of the enzymatic activation of oxygen by electrodes modified with laccase limits the current that can be drawn from these biocathodes in spite of the high potential for direct electron transfer to the enzyme T1Cu site.

2. Experimental section

2.1. Materials

Purified enzyme laccase from *Trametes trogii* has been employed in this study. Strain 463 (BAFC: Mycological Culture Collection of the Department of Biological Sciences, Faculty of Exact and Natural Sciences, University of Buenos Aires) of *T. trogii* (*Funalia-trogii*) (Polyporaceae, Aphyllo phorales, Basidiomycetes) was used in these experiments. Stock cultures were maintained on malt extract agar slants at 4 °C. Details of culture conditions and purification of the enzyme laccase have been reported elsewhere [4]. Vulcan® Carbon XC-72 was obtained from Cabot Corporation. H_2O_2, H_2SO_4, HNO_3, KNO_3 Methylpyrrolidone (NMP), poly(ethylenimine) solution (PEI) and poly(vinyl chloride) (PVC) were purchased from Sigma-Aldrich Argentina. Sodium acetate and acetic acid (100%) were obtained from Fluka. All reagents were analytical grade and used without further purification. Ultra pure water was obtained from a Milli-Q® purification system (nominal resistivity 18.2 MΩ at 25 °C) and used to prepare all solutions.

2.2. Preparation of carbon electrodes

Vulcan® carbon powder was cleaned with nitric acid before use. A suspension of carbon (90%) in poly(ethylenimine) solution (PEI) or poly(vinyl chloride) (PVC) (10%) in H_2O or methylpyrrolidone (NMP) respectively was prepared and diluted five times in their respective solvent and sonicated for 30 min. A gold disk electrode (diameter 5 mm) was first polished with alumina powder and tested in H_2SO_4 (1 M). A drop of the carbon suspension is deposited on the gold electrode and allowed to dry. Before adsorption of the enzyme a cyclic voltammetry was performed in order to measure the capacity of the electrode. It is worth mentioning that this step is crucial for the successive adsorption of laccase. For Vulcan carbon supported laccase, no catalysis has been observed in the case of direct adsorption of laccase on carbon without cycling the electrode in acetate buffer. This confirms the importance of surface state of the electrode for the adsorption of laccase. The spectrographic carbon electrode consists of high purity graphitic carbon rods (d = 0.5 cm) (National Carbon, USA). To fabricate the carbon disk electrode of a RDE, a rod was embedded in epoxy resin, shaped in the form of cylinder using a lathe, and provided with the adequate electrical connections. These electrodes were cleaned by sequential sonication in ethanol, isopropanol and water. The electroactive area for both systems was estimated from the capacitive current and varies between 100 and 2000 cm^2. Adsorption of laccase was carried out by depositing a drop of a purified laccase solution in H_2O (0.3 $mg.mL^{-1}$) for 30 min, followed by thorough rinsing with water. The successful laccase adsorption was identified by measuring the OCP (open circuit potential), being higher than 0.5 V in presence of active laccase.

2.3. Electrochemistry

Cyclic voltammetry and impedance spectroscopy were performed using an Autolab PGSTAT 30 potentiostat in a three-electrode cell with a platinum gauze as counter electrode and Ag/AgCl (3 M KCl) as reference electrode (all potentials herein are referred to this reference electrode). The working electrodes were the previously described gold disks modified with the Vulcan® carbon and the spectrographic carbon disk electrodes. All measurements were performed in 0.1 M acetate buffer of pH 4.7 containing 0.2 M KNO_3. In some experiments a rotating disk electrode (RDE) has been employed as described elsewhere [4].

Before measurement, all solutions were degassed with pure nitrogen or saturated with gas mixtures of nitrogen/oxygen in different ratios controlled by means of precision flow meters and flow regulators (G. Bruno Schilling, Argentina).

2.4. Inhibition study

For the inhibition study, the working electrodes were rotating gold disks (d = 5 mm) embedded in KelF® polymer. The electrode was prepared as explained previously with successive deposition of the Vulcan® carbon suspension and laccase. A chrono-amperometry at 0.3 V was used in order to obtain the catalytic currents used for the inhibition mechanism study. All measurements were performed in 0.1 M acetate buffer (pH = 4.7) with KNO_3 (0.2 M) under rotation (6 Hz) with successive additions of freshly prepared hydrogen peroxide solution.

3. Results and discussion

3.1. Catalytic oxygen reduction on carbon by laccase

In this study we have employed very large surface area electrodes in order to magnify the biocatalytic current response and eventually to improve the orientation between the electrode and T1Cu site of laccase by the carbon nanostructures. Panels A and B in Fig. 1 depict scanning electron micrographs of a composite Vulcan® XC-72 carbon with adsorbed laccase (a) and of the graphite electrode surface where graphitic planes are clearly seen (b).

Based on the molecular structure and size of laccase the maximum surface concentration of the enzyme monolayer on electrode surfaces has been estimated 2–8 × 10^{-12} $mol \cdot cm^{-2}$ [31] and based on the maximum enzyme rate, ≥2000 M^{-1} s^{-1}, [10] one would expect current densities in the order of few $mA \cdot cm^{-2}$. However most studies of ET with laccases report much lower currents as is the case in the present report, which can be accounted for by partial inactivation of the adsorbed enzyme or less favorable orientation of the T1Cu site in a population of enzyme with respect to the electrode surface.

Fig. 2 depicts current potential curves for Vulcan® carbon adsorbed laccase cathode in nitrogen saturated and oxygen saturated electrolyte respectively. The large capacitive charging current, $i_{cap} = C \cdot dV/dt$ observed in cyclic voltammetry is due to a large surface area of the dispersed Vulcan® carbon as seen in Fig. 1A. Comparison between the capacitance obtained from the charging current at different potential sweep rates and the standard value for HOPG capacitance, c.a. 2 $\mu F \cdot cm^{-2}$ we obtain a surface roughness of 1500 for Vulcan® carbon electrodes and 3100 for the graphite electrode surface. Notice that the current densities referred to the real surface area are in the $nA \cdot cm^{-2}$, and therefore only a small population of adsorbed laccase molecules are electrochemically active.

A clear onset of the bio-catalytic O_2 reduction current at 0.65 V (Ag/AgCl, 3 M KCl) is observed in good agreement with the high potential of *T. trogii* laccase T1 copper site, i.e. 0.79 V (NHE) [37].

The addition of fluoride as inhibitor of laccase (see figure in the Supporting information) confirms the biocatalytic nature of the cathodic currents in oxygen containing solutions since a complete offset of the catalysis has been observed.

From the electrocatalytic response two potential regions can be distinguished: i) a potential dependent current, independent of the oxygen partial pressure at low overpotentials, and ii) a low potential region, with current strongly dependent on the oxygen concentration at high overpotentials. It should be noted that the current plateau at high overpotentials is well below the convective-diffusion controlled current since experiments with a rotating disk electrode yield catalytic currents below the convective-diffusion currents for oxygen reduction at 9 Hz, i.e. 2.25 $mA \cdot cm^{-2}$ calculated with the Levich equation for

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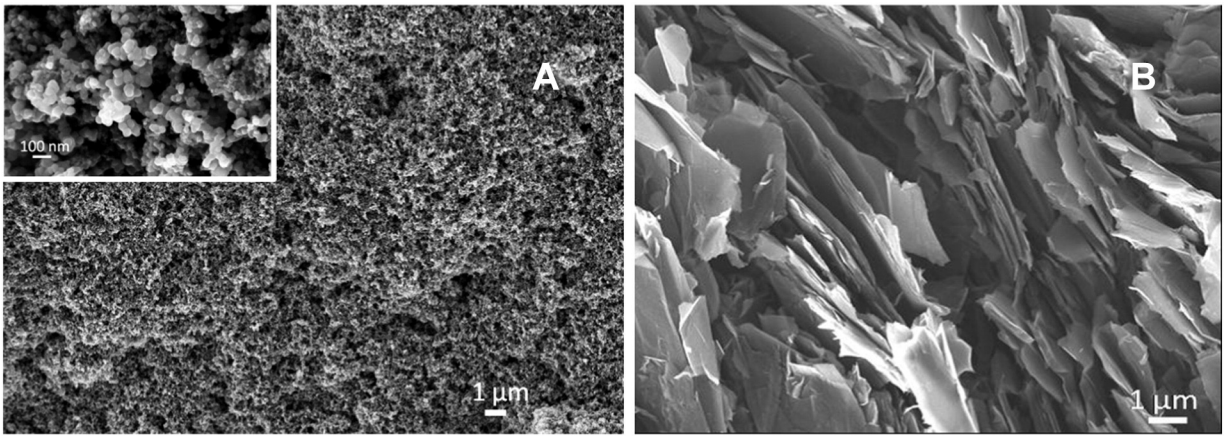

Fig. 1. SEM micrographs of Vulcan® carbon-PVC deposited on gold (A) and of the spectrographic carbon (B).

1 atm O_2 saturated electrolyte and a diffusion coefficient $D_{O2} = 1.41 \times 10^{-5}\ cm^2 \cdot s^{-1}$ in ref. [4]. Furthermore, comparison of the biocatalytic currents at 0 and 9 Hz in the Supporting information demonstrates that there is no concentration polarization of oxygen.

In Fig. 2 it can be seen some current slope below 0.35 V which can be ascribed to some DET to the T2/T3 cluster for a fraction of the enzyme molecules oriented with T2/T3 cluster towards the carbon surface. [28] This is also offset by addition of fluoride, (see Fig. SI).

Fig. 3 shows the polarization curves in solutions saturated with different oxygen partial pressures after substracting the capacitive charging current in N_2 saturated solution. It is of note two distinct behaviors: at the high onset potential of the ORR the biocatalytic currents are potential dependent but insensitive to the oxygen partial pressure, while at higher overpotentials a very weak potential dependence is observed and the currents depend on the oxygen partial pressure. The biocatalytic oxygen reduction current dependence on the oxygen partial pressure and the electrode potential is consistent with the mechanism described by Solomon which can be represented by Eqs. (1)–(4):

$$Lac\left(Cu^{II}_{T1}\right) + e \rightarrow Lac\left(Cu^{I}_{T1}\right) \quad (1)$$

$$Lac\left(Cu^{I}_{T1}\right) + Lac\left(Cu^{II}_{T2/T3}\right) \rightarrow Lac\left(Cu^{II}_{T1}\right) + Lac\left(Cu^{I}_{T2/T3}\right) \quad (2)$$

with two-electron oxidation of the fully reduced intermediate and formation of a peroxide intermediate, with $k = 2 \times 10^6\ M^{-1} \cdot s^{-1}$ [38]:

$$Lac\left(Cu^{I}_{T1}\right) + 3Lac\left(Cu^{I}_{T2/T3}\right) + O_2 \rightarrow Lac\left(Cu^{I}_{T1}\right) + Lac\left(Cu^{I}_{T3}\right)O_2 Lac\left(Cu^{II}_{T3}\right) + Lac\left(Cu^{II}_{T2}\right) \quad (3)$$

and further two-electron internal redox conversion into the native intermediate, with $k > 350\ s^{-1}$ [38]:

$$Lac\left(Cu^{I}_{T1}\right) + Lac\left(Cu^{I}_{T3}\right)O_2 Lac\left(Cu^{II}_{T3}\right) \rightarrow Lac\left(Cu^{II}_{T1}\right) + 2Lac\left(Cu^{II}_{T3}\right) + 2H_2O \quad (4)$$

The direct electron transfer (DET) from carbon to T1Cu atom represented by Eq. (1) is dependent on the electrode potential and is insensitive to the oxygen partial pressure.

$$k_{DET} = k^0_{DET} e^{-\frac{\alpha F}{RT}\left(E - E^0_{T1}\right)} \quad (5)$$

where E^0_{T1} is the formal redox potential of CuT1 center in *T. trogii* laccase, 0.79 V vs. NHE [37] and α the charge transfer coefficient.

According to Solomon et al. [10] the internal electron transfer from T1Cu site to the tri-nuclear copper cluster T2/T3 across 1.3 nm (Eq. (2)) is fast, then both T1Cu site and the T2/T3 Cu(I) which reacts with molecular oxygen according to Eq. (3) are in equilibrium. The enzymatic reaction is insensitive to the electrode

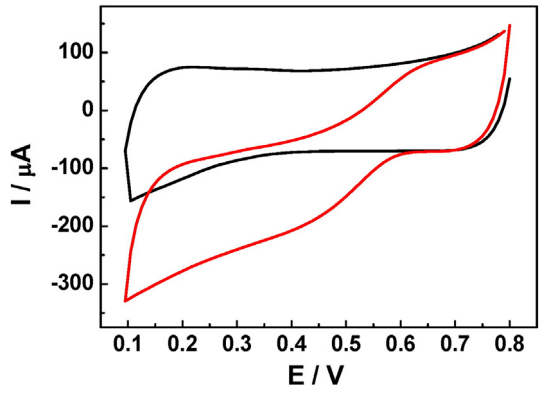

Fig. 2. Cyclic voltammograms of the catalytic reduction of O_2 by laccase adsorbed on Vulcan® carbon-PVC deposited on the gold electrode in acetate buffer (pH = 4.7) with 0.2 M KNO_3 in N_2 saturated electrolyte (black line) and in O_2 saturated electrolyte (red line) at 25 mV/s. Electrode area 0.2 cm^2. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

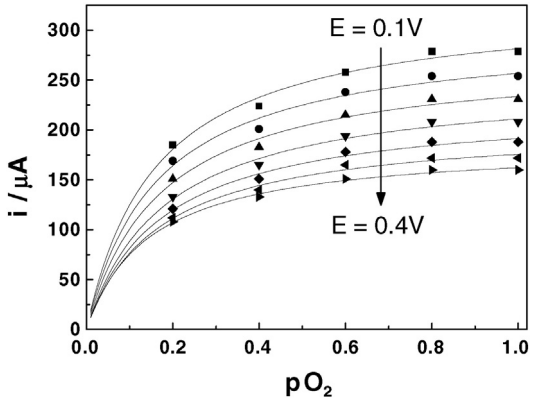

Fig. 3. Catalytic O_2 reduction currents at different electrochemical potentials (E = 0.1, 0.15, 0.2, 0.25, 0.3, 0.35 and 0.4 V). Lines are best fit to Eq. (6).

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potential and has a Michaelis–Menten dependence on oxygen partial pressure, p_{O2}:

$$k_{ENZ} = \frac{k_{max} p_{O2}}{K_M + p_{O2}} \quad (6)$$

The experimental data at different oxygen partial pressures are depicted in Fig. 3 for different electrode potentials and show a Michaelis–Menten behavior. However the apparent Michaelis–Menten constant increases at high overpotential as shown in Table 1 and reaches $K'_M = 0.16$ for 0.1 V, this is due to the coupling of ET and enzyme kinetics [31].

The interplay between DET to T1Cu and the enzymatic ORR at T2/T3 site has a balance point around 0.55 V for $p_{O2} = 1$, as can be seen from the impedance Nyquist plots for a Randles circuit with a minimum of the charge transfer resistance, c.a. 10 kΩ, around that potential (see Fig. 4).

For the sequential two step reaction mechanism of DET to T1Cu followed by the enzymatic Michaelis–Menten reduction of O_2 described by Eqs. (1)–(3), the total bio-catalytic current can be expressed as a sum of the reciprocals of DET and enzyme kinetics [31]:

$$\frac{i}{i_{max}} = \frac{1}{1 + \exp\left[\frac{F}{RT}\left(E - E^0_{T1}\right)\right] + \frac{k_{ENZ}}{k^0_{DET}} \exp\left[\frac{\alpha F}{RT}\left(E - E^0_{T1}\right)\right]} \quad (7)$$

where $i_{max} = -4Fk_{ENZ}\Gamma_{lac}$ dependent on p_{O2} and Γ_0 represents the surface concentration of electrochemically active laccase. Eq. (7) assumes fast intra molecular electron transfer between T1Cu and T2/T3 cluster.

Panels A and B in Fig. 5 show experimental data of the O_2 biocatalytic current (corrected for capacitive charging currents) at different oxygen partial pressures and different electrode potentials for Vulcan® XC-72 and graphite electrodes, respectively.

Best fits to Eq. (7) are shown as continuous lines and data collected in Table 2. For low p_{O2}, an almost Nernstian behavior is observed with α close to 1, while the experimental data fit can be explained by quasi reversible and irreversible kinetics as the oxygen partial pressure increases since k_{ENZ} increases with p_{O2}.

The full biocatalytic current dependence can be explained by the interplay of direct electron transfer to T1Cu site and further O_2 activation at the trinuclear T2/T3 cluster. Notice that T1Cu has a very high potential ($E^0_{DET} = 0.57$ from fit), much higher than the best ORR electrocatalysts like Pt; however the rate of reaction becomes limited by the enzymatic rate quite below the diffusion regime.

This is illustrated in Fig. 6 with a comparison of graphite (1), laccase catalyzed ORR on graphite (2) and a Pt electrode (3) of similar geometric area. While the onset of the O_2 reduction is 0.8 V more positive for laccase on carbon than for the platinum electrode, the enzymatic limiting current is very small as compared to platinum. Therefore, the enzyme turnover and surface concentration of properly oriented T1Cu sites limits the ORR biocatalytic current which never reaches the values observed for platinum at higher overpotential.

The transition from DET Reaction (1) to enzyme kinetics Reaction (3) as rate determining step can be clearly seen from a normalized plot to the limiting current in Fig. 7 as suggested by Ulstrup et al. [31]. As the oxygen partial pressure increases, k_{ENZ}/k^0 increases from 0.72 to 1.18 and the inflection point for the change in rate determining step (rds) moves towards larger overpotentials as predicted by Eq. (7) [31].

Table 1
Apparent Michaelis–Menten constants at different electrode potentials.

E/V	0.1	0.15	0.2	0.25	0.3	0.35	0.4
K_m	0.16	0.16	0.17	0.18	0.18	0.17	0.15

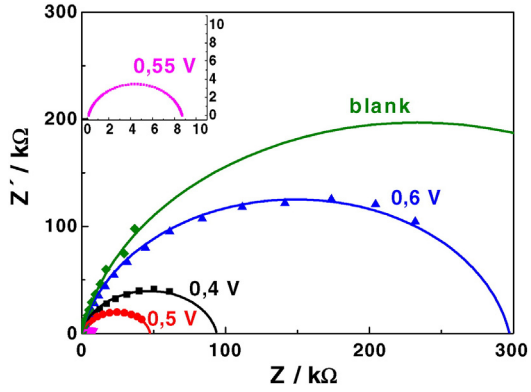

Fig. 4. Impedance electrochemical analysis: Nyquist plots of laccase on Vulcan® carbon-PEI deposited on gold at different potential values in acetate buffer (pH = 4.7) with 0.2 M KNO_3 at $pO_2 = 1$ atm. Amplitude = 5 mV, frequencies between 10 kHz and 5 mHz. Lines are best fits to Randles equivalent circuits extracted with ZView (Scribner Associates Inc.). The blank measurement is the Nyquist plot of the electrode without laccase at a potential of 0.55 V.

3.2. Inhibition of the catalytic activity by hydrogen peroxide

Another limitation of laccase biocathodes on carbon electrodes is that the ORR on carbon is a two electron process producing hydrogen peroxide, which inhibits the O_2 cathode as has been shown for both mediated [4] and direct electron transfer [36] mechanisms.

The reversible inhibition effect of hydrogen peroxide added into the electrolyte at increasing concentrations can be seen in Fig. 8 for the biocatalytic current dependence with oxygen.

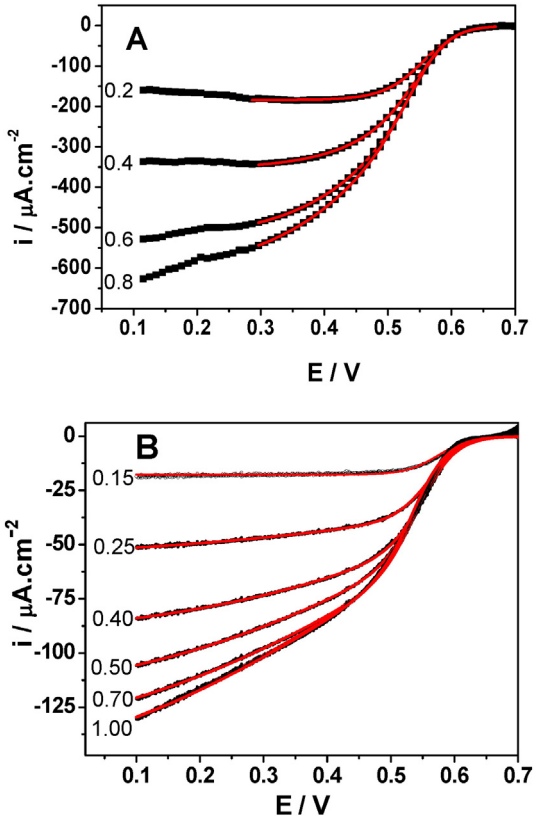

Fig. 5. Polarographic curves of the ORR catalysis by laccase on gold electrodes modified with Vulcan® carbon-PVC (A) and on spectroscopic carbon (B) at different pO_2. Lines are best fit to Eq. (7).

Table 2
Best fit parameters of data in Fig. 5 to Eq. (7).

p_{O2}	E^0_{T1}	α	k_{ENZ}/k^0_{DET}
0.2	0.57	0.72	0.72
0.4	0.55	0.45	0.95
0.6	0.54	0.28	1.07
0.8	0.53	0.21	1.18

Parallel double reciprocal linear Lineweaver–Burk plots (Fig. 9) for the ORR with DET of laccase electrodes are indicative of uncompetitive inhibition mechanism, unlike mediated mechanism. Moreover, Eadie–Hofstee plots (Fig. 10) suggest non-competitive inhibition mechanism with parallel lines for low H_2O_2 inhibitor concentration, but at higher H_2O_2 concentrations non linear plots are apparent. This result indicates the presence of a mixed inhibition mechanism, with predominance of uncompetitive inhibition, especially at high H_2O_2 concentration. This mechanism corresponds to the preferred binding of H_2O_2 to the enzyme-substrate complex and the results are consistent with similar findings from Milton and Minteer for laccase adsorbed on multiwall carbon nanotubes [36].

4. Conclusions

The direct electron transfer to *T. trogii* T1Cu of laccase takes place at the usual high potential, c.a. 0.6 V vs. Ag/AgCl, 3 M KCl. However the bio-catalytic reduction of oxygen at the cluster T2/T3 is limited by the turnover of the process and the surface coverage of properly oriented enzyme molecules (active on the surface). This is very important since the enzyme turnover limits the rate of oxygen reduction at high overpotential.

Therefore, even though T1Cu site exhibits a very high potential, close to the thermodynamic O_2/H_2O couple, the enzyme kinetics cannot withstand high current densities to compete with platinum under similar conditions.

The ORR on carbon support produces H_2O_2 which leads to reversible uncompetitive inhibition of the enzyme.

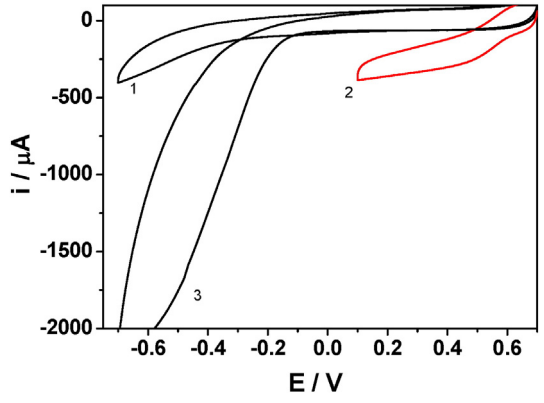

Fig. 6. Cyclic voltammograms of the ORR for graphite with laccase in N_2 saturated electrolyte (1), O_2 saturated electrolyte (2) and Pt electrode (3) in acetate buffer (pH = 4.7) with 0.2 M KNO_3.

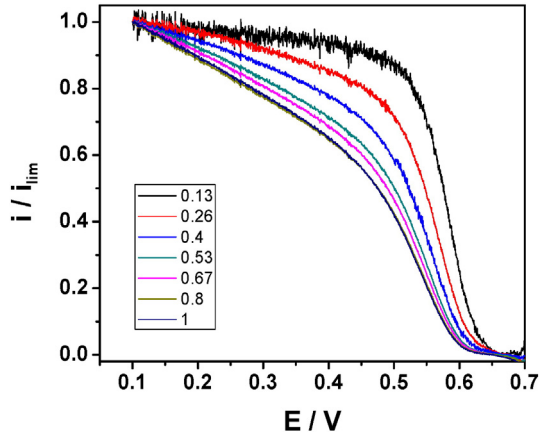

Fig. 7. Normalized plots from Fig. 5 to the limiting current at $p_{O2} = 1$.

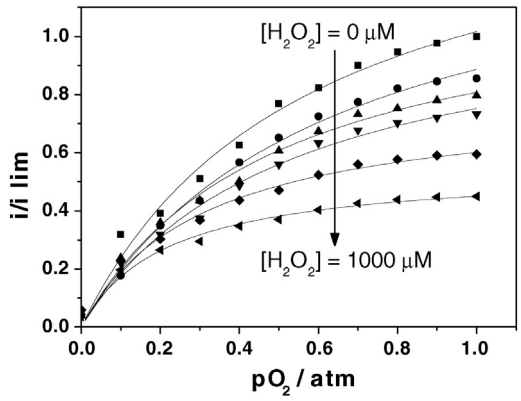

Fig. 8. Normalized O_2 reduction catalytic currents at different hydrogen peroxide concentrations (0, 10, 50, 100, 500 and 1000 µM) at E = 0.3 V, W = 6 Hz. Lines are best fit to Eq. (6).

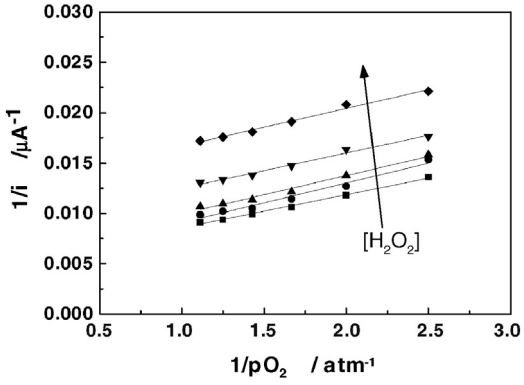

Fig. 9. Lineweaver–Burk plot for data in Fig. 8.

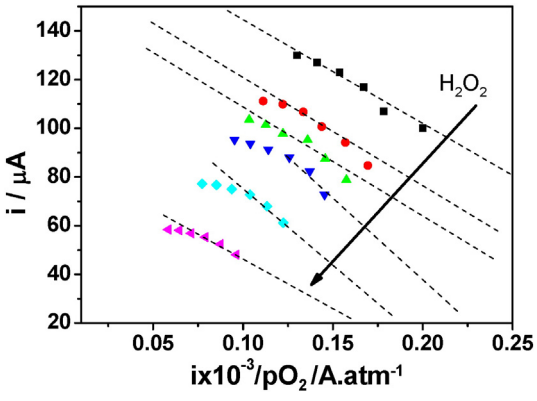

Fig. 10. Eadie–Hofstee plot for data in Fig. 8.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bioelechem.2016.01.007.

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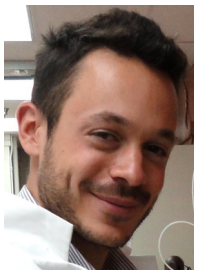

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