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POXC laccase from *Pleurotus ostreatus* : a high-performance multicopper enzyme for direct Oxygen Reduction Reaction operating in a proton-exchange membrane fuel cell

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Abstract: In this work, POXC, a laccase from *Pleurotus ostreatus* was immobilized on carbon nanotubes (CNTs) modified by electrografting of anthraquinone and naphthoate diazonium salts. Thanks to a favorable interaction between laccase and covalently-modified CNTs, this laccase exhibits high-potential/high current ORR performances, surpassing the performances of the well-known laccase from *Trametes versicolor* (TvLAC) immobilized on the same nanostructured electrodes. Furthermore, immobilized POXC demonstrates high ORR activity over a wide range of pH (2 to 8), being also highly active at gas-diffusion electrode (GDE). Finally, thanks to these unique performances, this enzyme was able to operate at the interface of the microporous layer, humidified air and a polymer electrolyte, ie nafion[®], in a conventional H₂/air proton-exchange membrane fuel.

The development of hydrogen fuel cells, in particular proton-exchange membrane fuel (PEMFC), at an industrial scale requires to tackle major challenges. The 4H⁺/4e⁻ Oxygen Reduction Reaction (ORR) occurs at the cathode side. For now, ORR requires a high amount of platinum catalysts to be efficient. And no competitive, base-metal catalysts have been proven to represent a viable alternative yet.^[1,2] One of this alternative is the development of ORR catalysts based on multicopper oxidases (MCOs)^[3-5] or their synthetic models.^[6-8] In enzymatic fuel cells, a subcategory of fuel cells in which electrocatalytic reactions are performed by immobilized enzymes, ORR mostly relies on MCOs.^[3,5,9-11] Thanks to high potential ORR, laccases and bilirubin oxidases have been most exclusively used in enzymatic fuel cells. The MCO active site is based on a set of two copper-containing centres: a Type 1 (T1) copper centre located at the surface of the protein and at which the phenolic substrate is activated and a trinuclear copper centre (TNC) at which O₂ is activated and reduced into H₂O. In most cases where MCOs are immobilized on an electrode, electrons are transferred to the TNC via the T1 relay centre, which has to be at electron-tunneling distance of the surface of the electrode in order to achieve a so-called Direct Electron Transfer (DET). In particular, different strategies have been studied in order to maximize amount of electroactive enzymes and minimize distance

between the T1 centre and the surface of the electrode. Several studies have been performed on laccase from *Trametes versicolor* (TvLAC), which exhibits one the lowest overpotential towards ORR in the MCO family and comparable to Pt/C, around 100 mV. F. A. Armstrong and colleagues have shown that grafted polycyclic aromatics were able to promote the favorable immobilization of TvLAC on modified graphitic electrodes.^[12,13] In order to increase the enzyme surface coverage, this strategy was transferred to nanostructured electrodes. Carbon nanotubes (CNTs) or graphene sheets were modified by non-covalent^[14-17] and covalent^[18-21] methods to introduce anthraquinone, anthracene or naphthalene molecules to favour TvLAC orientation. This strategy has led to the design of highly-efficient oxygen-reducing biocathodes for fuel cell applications. However, the ORR studies of these enzymes have been exclusively limited to near-neutral-pH buffered solutions, at which the enzymatic activity is optimal. However, a functional PEMFC has to rely on solid, "unbuffered" and acidic polymer electrolytes such as nafion[®]. The enzymatic activity of most enzymes is strongly inhibited in such acidic and low-water content medium. The only example of such enzyme operating in a PEMFC has been shown by Minter and colleagues using laccase from *Rhus vernificera* entrapped in neutralized tetrabutylammonium-bromide-modified-Nafion[®] and operating in H₂/O₂ fuel cell.^[22] Unfortunately, the fuel cell exhibited low cell voltages of 0.6 V despite high current densities, which were reached at low voltages under O₂.

In this work, we studied the direct electrochemistry of POXC, a laccase from *Pleurotus ostreatus*, which has not been investigated on electrodes yet. POXC is the most abundantly secreted laccase under all growth conditions examined until now, and seems to play a major role during vegetative growth.^[23] The enzyme has been extensively characterized in solution: it is a high redox potential laccase with an acidic pl.^[24,25] Multi-walled carbon-nanotube (MWCNT)-coated glassy carbon and gas-diffusion electrodes (GCE and GDE respectively) were modified by electrografting of 6-carboxynaphthalenediazonium and 2-diazoniumanthraquinone tetrafluoroborate salts according to previously-described procedures.^[26] We have already demonstrated that such functionalization strategy leads to the formation of a thin organic layer on the surface of CNT sidewalls. The synergistic effect of grafted functional groups and CNT graphene sidewalls induces a favorable and specific interaction with metalloenzymes such as bilirubin oxidases^[26,27], promoting direct electron transfer between MWCNTs and the enzyme active site or electron relay. Here, we show that this covalent functionalization strategy favors both POXC immobilization and DET. The behavior of this enzyme was, for the first time, exhaustively investigated towards ORR by Cyclic Voltammetry (CV) and electrochemical models. Furthermore, the comparison between POXC and TvLAC at the same functionalized electrodes was investigated, underlining the

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similar overpotentials towards ORR for both laccases and the higher current densities obtained for POXC as compared to *TvLAC*. Finally, high current densities over a wide range of pH (between 2 and pH 8) make POXC, a promising enzyme able to operate at the triple phase boundary between a polymer electrolyte, i.e. nafion[®], the MWCNT-coated GDE and the gas phase in a conventional PEMFC.

Direct Electrochemistry of POXC on covalently-functionalized MWCNTs

POXC was immobilized on pristine MWCNTs and MWCNTs modified with 6-carboxynaphthalenediazonium and 2-diazoniumanthraquinone tetrafluoroborate salts according to a previously-described procedure.^[26] Figure 1 displays the 3D representation of the enzyme and CV performed under saturated O₂ purging for pristine MWCNT, naphthoate-modified MWCNT and anthraquinone-modified MWCNT electrodes ([O₂] = 0.72 mmol L⁻¹)

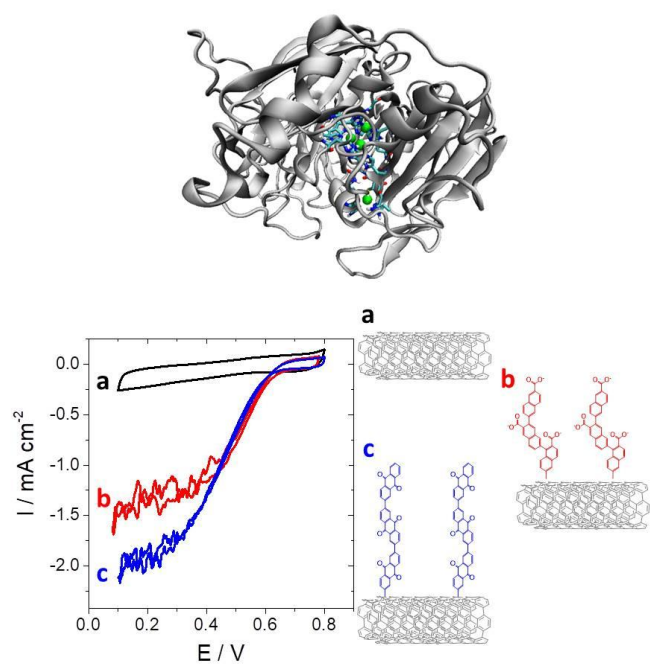


Figure 1. 3D representation of POXC and CV of POXC-functionalized MWCNT electrodes under O₂ for (a) pristine MWCNTs, (b) naphthoate-modified MWCNTs and anthraquinone-modified MWCNTs (stirred and oxygen-purged citrate-phosphate buffer pH 3, $v = 10$ mV s⁻¹).

For all MWCNT electrodes, an irreversible electrocatalytic reduction wave is unambiguously attributed to the electroenzymatic reduction of O₂ into H₂O by immobilized POXC. For naphthoate- and anthraquinone-modified MWCNT, a respective 23-fold and 35-fold increase, as compared to pristine MWCNTs, are observed in terms of current density, reaching 2 mA cm⁻². As it has been demonstrated for *TvLAC* in the case of

anthraquinone-modified electrodes^[13], this can be attributed to the favorable hydrophobic interactions between anthraquinone and the hydrophobic substrate cavity of POXC. Furthermore, this is the first time that a similar observation can be made with naphthoate groups for these enzymes. However, this type of functional groups has already been shown to improve DET for bilirubin oxidases from *Myrothecium verrucaria*, owing to a combination of hydrophobic interactions and electrostatic interactions via negatively-charged carboxylate groups.^[26,28] In order to confirm these hydrophobic interactions and to directly compare POXC with *TvLAC* in the same conditions, the same experiments were performed by using commercially-available *TvLAC*. As expected, modification of MWCNTs with hydrophobic molecules such as anthraquinone or naphthoate groups also led to an important increase in current density for *TvLAC*, reaching 0.7 mA cm⁻² at pH 5 (figure S1). According to previous studies,^[13,14,21] this is in good agreement with the presence of a favorable interaction between the cavity of both POXC or *TvLAC* and naphthoate- and anthraquinone-modified MWCNTs.

Long-term stability experiments were performed by one-hour chronoamperometric discharge at 0.2 V almost every day during 20 days (figure S2). POXC immobilized on both anthraquinone- or naphthoate-modified MWCNTs shows similar and excellent activity over weeks. The bioelectrodes still achieve 65% of their activity after 7 days and 38 % after 20 days, namely 0.4 mA cm⁻². This stability is superior to bioelectrodes based on entrapment of laccases in redox hydrogels (66 % of the activity is lost after 5 days for laccase from *Coriolus hirsutus*)^[29,30] and falls in the same range as previously-described nanostructured electrodes based on laccases from *Trametes sp.*^[14,31] *Trametes hirsuta* laccases immobilized at gold nanoparticle-based electrodes exhibited maximum current densities of 1 mA cm⁻², with a 40% loss of activity after 5 days of continuous operation.^[32] The same laccase was immobilized at anthraquinone-modified CNTs which exhibited current densities up to 3.5 mA cm⁻² with 21 % loss of activity after 7 days.^[18] Pyrene-modified laccases from *Trametes sp C30* immobilized at both MWCNT and gold-nanoparticle-based electrodes exhibited maximum current density of 3 mA cm⁻², losing 60 % of its activity after one month.^[31] *TvLac* immobilized at adamantane- or pyrene-modified MWCNTs^[14,17] exhibited maximum current densities of 2 mA cm⁻² keeping 66% and 50 % of its initial activity after one month. *TvLAC* immobilized at Anthracene-modified MWCNTs exhibited maximum current density of 140 μ A cm⁻² accompanied with loss of activity of 25 % after 24 h.^[19]

Kinetic analysis

In order to get more insights into the difference between nonmodified MWCNTs, naphthoate- and anthraquinone-modified MWCNTS towards POXC and to extract kinetic parameters, the model developed by Armstrong and Léger was applied to the faradaic electrocatalytic wave.^[33,34] This model takes into account the random distribution of redox-active enzyme on the electrode surface. This distribution of orientation is taking into account in the βd_0 tunneling factor. βd_0 is the product of the exponential decay constant β and d_0 , the minimal distance between the enzyme electron entry point or electrochemical relay centre and the electrode. This factor accounts for the existence of an apparent cone of electron

transfer rate distribution, arising from different statistical orientations of POXC. Figure 2 displays the experimental background-subtracted CV curve accompanied by its simulated counterpart after fitting of the CV curve for each MWCNT electrodes under O_2 at pH = 3. It is noteworthy that, beyond immobilization of the enzyme, mass transfer contributions were minimized through stirring the solution and maintaining a constant oxygen flow. By increasing stirring speed (or by increasing Rotating Disk Electrode rate above 1000 rpm^[31]), no increase of electrocatalytic currents (indicative of mass-transport limitations) was observed with any of the tested bioelectrodes.

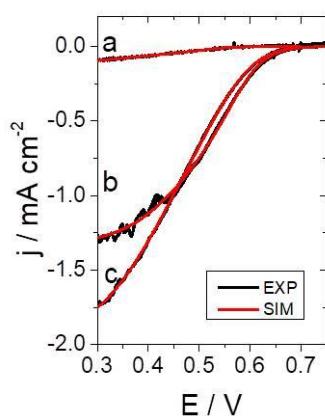


Figure 2. Background-subtracted CV of POXC-functionalized (a) MWCNT, (b) naphthoate-MWCNT and (c) anthraquinone-MWCNT electrodes under O_2 . The simulated red trace takes into account the average between the backward and forward scan.

It is noteworthy that this model takes into account that the electrocatalysis has to be limited by the kinetics of the electroenzymatic reaction. In this case, diffusion limitations, arising from the highly porous structure of the MWCNT film, might influence the electrocatalytic response, especially at high overpotentials. This underlines the fact that this model has to be considered as an approximation of the experimental response. However, models fit well with experimental curves for all tested conditions with R^2 of 0.99 in most cases (see supplementary materials for details). This fit gives access to constant parameters, i.e. the catalysis redox potential (E_{cat}), the ratio between catalytic rate constant k_{cat} and heterogenous electron transfer rate constant at minimal distance k_0^{max} and the electron transfer tunneling factor βd_0 . High βd_0 value is observed for pristine MWCNTs ($\beta d_0 = 26$), arising from the high randomization of orientation on MWCNT sidewalls. As expected, much smaller values of 5.0 and 5.3 are observed for anthraquinone-modified and naphthoate-modified MWCNTs respectively, underlining that the orientation of POXC is favored at these modified surfaces. As previously mentioned, this effect has been already observed for $TvLAC$ on different type of functionalized electrodes^[13,14,19,35] and can be extended to POXC. The electrocatalytic waveshape for ORR performed by POXC was also modeled between pH 2 and pH 8 for MWCNTs modified

with anthraquinone and naphthoate groups (figure 3). First, these fits allow the access to E_{cat} between pH 2 and 8, allowing the calculation of the dependence of E_{cat} towards pH (figure 3C). This dependence was well fitted using eq. (1):

$$E_{cat} = E_{cat(acid)} + \frac{2.3RT}{nF} * \log\left(1 + \frac{[K_a]}{[H^+]}\right)$$

where R is the gas constant, T the temperature, F the Faraday constant and n the number of electron involved in the redox system. K_a is the proton dissociation constant for the oxidized form of the enzyme and $E_{cat(acid)}$ is the limiting value of E_{cat} at pH below pKa. Using $n = 1$, the fit gives access to an $E_{cat(acid)}$ of 0.57 ± 0.01 V and a pKa of 5.1 ± 0.2 ($R^2 = 0.96$). This equation accounts for a $1e^-/1H^+$ accompanied with an acid-base couple with a pKa of 5.1. First, the redox potential of E_{cat} of 0.55 V vs. Ag/AgCl (or 0.75 V vs. NHE) at pH 5 matches very well with the redox potential of POXC measured by redox titration (0.74 V at pH 5^[36]). This unambiguously confirms that the electrochemical redox centre is controlled by DET to the T1 copper centre. Furthermore, this particular pH dependence has already been observed for other copper enzymes such as azurin^[37], CueO^[38] or BODs.^[39] The mono-electronic T1 Cu(II)/Cu(I) proton-coupled electron transfer (PCET) is thus influenced by the acid/base couple of an amino acid such as histidine which induces a structural rearrangement of the T1 copper centre upon the reduction/oxidation process.^[38,40] This also implies that this protonation causes an increase in ORR overpotentials below pH 5 when comparing with the evolution of the standard redox potential of the O_2/H_2O couple (gray line, figure 3C). This dependence was also compared with the evolution of the catalytic potential of $TvLAC$ obtained by the same procedure. The E_{cat} for $TvLAC$ follows a typical linear trend towards pH (slope of 52 mV, red line, figure 3C) corresponding to a typical $1e^-/1H^+$. By comparing the two laccases, it is noteworthy that $TvLAC$ and POXC exhibit closed redox potential above pH 5. This implies that $TvLAC$ and POXC likely share the same electrocatalytic mechanism involving electron transfers at the T1 centre, the only striking difference being the involvement of an amino acid protonation step below pH 5 in the case of POXC. When looking at maximum current densities for both types of modified MWCNT electrodes, POXC shows an electrocatalytic activity over a wide pH range between pH 2 and pH 8, reaching maximum current density of 1.97 mA cm^{-2} at pH 3. Naphthoate- or anthraquinone-modified MWCNTs exhibit similar behavior over this pH range. The decrease of current densities below pH 3 for naphthoate might be attributed to an influence of the protonation of naphthoate groups (pKa ~ 4) on a partial desorption of POXC or on an unfavorable orientation of the enzyme.

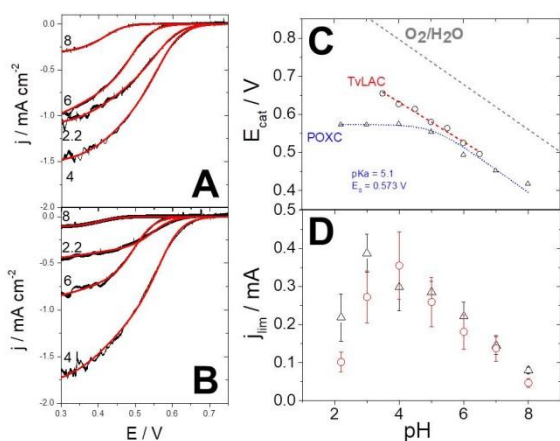


Figure 3. Background-subtracted CV of POXC-functionalized (A) anthraquinone-MWCNT and (B) naphthoate-MWCNT between pH 2 and pH 8. The simulated red traces take into account the average between the backward and forward scan. (C) Evolution of E_{cat} and (D) j_{lim} estimated from simulated CV curves performed between pH 2.2 and 8.

Laccase-based PEMFC

POXC was integrated in a GDE. GDE is used in PEMFC technology, allowing the catalyst to operate at the interface between the catalytic layer, the humidified gas phase and the polymer electrolyte. We and others have successfully adapted this technology for the integration of enzymes in fuel cells.^[41,42,42–47] Anthraquinone was electrografted on a GDE modified with a thin layer of MWCNTs. Half-cell experiments were performed at pH 3 under Ar, humidified air and O_2 (figure 4A). POXC exhibits similar behavior at both MWCNT-coated GDE and GCE. This confirms that POXC can operate at the interface between the electrolyte, the gas phase and the electrode. Maximum current densities of 1.2 and 1.9 $mA\ cm^{-2}$ were obtained under air and O_2 at MWCNT-coated GDE respectively in quiescent solution. Similar current densities between GCE and GDE indicate that electrocatalysis is likely limited by the kinetics of the enzymatic reaction. The performance of this POXC-based GDE is among the best performances for a laccase-based GDE, surpassing GDE based on laccases from *Trametes hirsuta* or *Rhus vernificera*, which delivers maximum current densities of 0.5 to 1 $mA\ cm^{-2}$ under O_2 .^[42,43]

Since one of the main advantage of POXC is its ability to efficiently operate at acidic pH, this prompted us to integrate POXC in a conventional PEMFC. Figure 4C displays the polarization and power curves measured for this POXC/Pt PEMFC, obtained by successive constant current discharges. A commercial Pt/C GDE was used at the anode for H_2 oxidation. The fuel cell was operated at 25°C with humidified streams of H_2 and air at atmospheric pressure. The fuel cell delivers a maximum power density of 0.12 $mW\ cm^{-2}$ at 0.38 V, accompanied with an open-circuit voltage (OCV) of 0.90 V. High cell voltage of 0.90 V confirms that POXC is still active at the interface between the MWCNT layer, air and the polymer electrolyte.

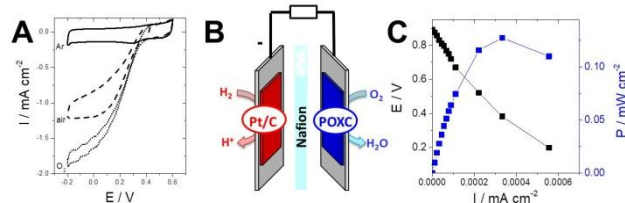


Figure 4. CV of a POXC-functionalized AQ-MWCNT GDE under humidified Argon, air and O_2 ($v = 10\ mV\ s^{-1}$, pH 3, 25°C); (B) Schematic representation of the H_2 /air fuel cell; (C) polarization (▲, black) and power (□, blue) curves for the fuel cell.

The presence of an important slope in the ohmic region of the polarization curve is indicative of the fact that proton conduction through the nafion® membrane is hindered by the low amount of produced water. However, the OCV of 0.90 V of this POXC/Pt PEMFC is closed to the OCV (around 1.0 V) observed for conventional Pt/Pt PEMFC, despite lower power densities^[1,48]. In a control experiment in which the cathode is based on pristine MWCNTs (without immobilization of POXC), low voltages of 0.7 V and power densities of 45 $\mu W\ cm^{-2}$ underline the poor catalytic activity of MWCNTs (figure S3). For comparison, a full Pt/C PEMFC delivers 100 $mW\ cm^{-2}$ approximately in the same test cell at 60°C^[48]. Furthermore, this is the highest OCV reported so far for a fuel cell based on an enzyme operating in a nafion®-based PEMFC. A previous example has been shown by Minter and colleagues with TvLAC. However, low OCV of 0.6 V were observed for this enzyme, despite high current densities of 10 $mA\ cm^{-2}$ at low voltages of 0.2 V. Furthermore, we have recently shown that bilirubin oxidase from *Myrothecium verrucaria* was particularly efficient in a non-noble metal fuel cell running in a pH 5 phosphate buffer but was not able to operate in nafion®-based PEMFC.^[48]

This work thus demonstrates that DET of POXC is promoted at naphthoate- and anthraquinone-modified MWCNTs. Thanks to its efficient wiring on MWCNT electrodes, direct electrochemistry of POXC can be exhaustively investigated at these electrodes. POXC demonstrates efficient ORR performances over a wide range of pH, surpassing performances of the most well-known laccases towards ORR at both GCE and GDE. This acidophilic laccase is able to work at a three-phase boundary between a solid electrolyte, CNT sidewalls and humidified air. This work demonstrated that the study of novel enzymes by direct electrochemistry can led to the discovery of highly-efficient biocatalyst able to operate in metal-catalyst-based conventional systems which, by design, are unconventional for enzymes. This is the reason why its activity might be hindered by the strong acidic nature of sulfonated groups and the low water content at the interface, despite gas humidification. Therefore, there is still room for improvement, especially in order to design enzyme-specific proton-exchange membranes that would increase water production and retention at the interface between the enzyme, the electrode and the humidified gas. Furthermore, such copper biocatalysts that perform low-overpotential ORR in a PEMFC also pave the way for the development of novel molecular

copper catalysts that would perform low-overpotential ORR in a fuel cell.^[6,8]

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Keywords: laccase • oxygen reduction • fuel cells • diazonium • carbon nanotubes

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