# Oxygen cathode based on a layer-by-layer self-assembled laccase and osmium redox mediator

R. Szamocki [a,1], V. Flexer [a], L. Levin [b], F. Forchiasin [b], E.J. Calvo [a,*,1]

[a] INQUIMAE-DQIAyQF, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, 1428 Buenos Aires, Argentina
[b] Micología Experimental, Departamento de Biodiversidad y Biología Experimental. Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, 1428 Buenos Aires, Argentina

## ARTICLE INFO

## ABSTRACT

*Trametes trogii* laccase has been studied as biocatalyst for the oxygen electro-reduction in three different systems: (i) soluble laccase was studied in solution; (ii) an enzyme monolayer was tethered to a gold surface by dithiobis *N*-succinimidyl propionate (DTSP), with a soluble osmium pyridine-bipyridine redox mediator in both cases. The third case (iii) consisted in the sequential immobilization of laccase and the osmium complex derivatized poly(allylamine) self-assembled layer-by-layer (LbL) on mercaptopropane sulfonate modified gold to produce an all integrated and wired enzymatic oxygen cathode. The polycation was the same osmium complex covalently bound to poly-(ally-lamine) backbone (PAH-Os), the polyanion was the enzyme adsorbed from a solution of a suitable pH so that the protein carries a net negative charge. The adsorption of laccase was studied by monitoring the mass uptake with a quartz crystal microbalance and the oxygen reduction electrocatalysis was studied by linear scan voltammetry.

While for the three cases, oxygen electrocatalysis mediated by the osmium complex was observed, for tethered laccase direct electron transfer in the absence of redox mediator was also apparent but no electrocatalysis for the oxygen reduction was recorded in the absence of mediator in solution.

For the fully integrated LbL self-assembled laccase and redox mediator (case iii) a catalytic reduction of oxygen could be recorded at different oxygen partial pressures and different electrolyte pH. The tolerance of the reaction to methanol and chloride was also investigated.

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## 1. Introduction

Laccase (EC 1.10.3.2; benzenediol: oxygen oxidoreductase) is an extracellular blue copper enzyme in plants and fungi which catalyses the oxidation of biphenols and the four-electron reduction of molecular oxygen to water. It contains four copper atoms, denoted T1, T2 and T3 according to their spectroscopic properties. The copper center T1 can be reduced at high potential by phenolic compounds, redox mediators and direct electron transfer from electrodes. While substrates are oxidized at T1, further internal electron transfer leads to the reduction of molecular $O_2$ at the T2/T3 cluster [1–3].

The catalytic ability of laccases to activate the four-electron reduction of oxygen at unprecedented high electrode potentials (between 0.35 and 0.50 V vs. Ag/AgCl) has promoted their study in cathodes for bio-fuel cells and an extensive literature on the electro-chemistry of laccases from different sources has followed in recent years [4–7].

The electrocatalysis of oxygen reduction by soluble *Trametes versicolor* laccase mediated by 2,2′-azinobis-3-ethylbenzothiazoline-6-sulfonate (ABTS) in solution has been studied by D'Amore et. al. [8], who reported kinetic constant values such as $k_{cat}$, $k_{ABTS}$, $K_M$, $K_S$ and the soluble mediator's diffusion coefficient, $D_{ABTS}$.

The direct electron transfer from electrodes to laccase has been extensively reported: the first laccase modified electrode was introduced by Tarasevich et al. [9–11] who reported the direct adsorption of laccase on carbon black particles. There have been other reports on the enzyme adsorption on other materials [12,13]; in particular, Armstrong et al. have recently reported an impressive catalysis of $O_2$ reduction by attachment of substrate-like antracene-based units to the surface of pyrolytic graphite [14].

Mediated electron transfer to laccase via redox mediators has been demonstrated with different redox shuttles such as ABTS [5,8,15–19], osmium complexes [6,7,20–25], etc.

Different immobilization techniques such as adsorption, tethering to electrode surfaces by chemical bond or entrapping the enzyme in hydrogels has been reported. Among the chemical mod-

ification, covalent attachment of laccase to electrode surfaces via carboxylic functional groups using EDC/NHS to form peptide bonds [16,17], oxidation of the carbohydrate moieties of the laccase by $NaIO_4$ to aldehyde sites and post-funtionalization via reaction of a chemisorbed amino-thiol to form a Schiff base on the electrode surface [26] have been reported. Also, hydrogels formed by condensation reaction of glutaraldehyde with the amine sites of the enzyme [27,28], crosslinking on a layered double hydroxide electrode with the mediator ABTS by intercalation between the layers of a Zn–Cr hydroxide as reagentless cathode were reported. [19]. In particular, D'Amore et al. and Opallo et al. [15], entrapped laccase into microporous silica matrices [18]. In both reports the mediator ABTS was co-immobilized together with the enzyme and could freely diffuse in the porous inorganic matrix. Other types of immobilization used for oxygen cathodes based on laccase were organic polymer hydrogels such as Nafion® and poly(ethyleneimine) [29–31]. Blanchard [32] attached laccase to Zr-phosphate modified electrodes by coordination of the enzyme carboxylic groups to the metal centers.

An alternative technique to obtain well organized enzyme films with redox mediator integrated into the system is the layer-by-layer (LbL) self-assembly of redox polyelectrolytes of opposite charge, introduced by Decher [33] and co-workers which allows the fabrication of electroactive nanometer thin films on electrode surfaces with a fine tuning of film thickness and a precise organization of the molecular components in space. The technique was extended to the adsorption of proteins [34,35], in particular we have introduced this LbL technique to the immobilization of "wired" enzyme glucose oxidase by redox polyelectrolytes in organized multilayers [36–38].

In the present communication we extend the previous work with GOx to *Trametes trogii* laccase [39] self-assembled layer-by-layer by sequential electrostatic adsorption of PAH-Os and GOx on mercapto-propane sulfonate (MPS) thiolated gold surfaces. A recent report of Lvov [40] described the LbL of laccase with (non-redox) poly(dimethylammonium chloride) multilayers on cellulose micro-fiber surfaces to fabricate bioactive composites.

Furthermore we compare the electrocatalysis of $O_2$ reduction by laccase in a self-assembled multi-layer with soluble laccase and tethered laccase monolayer by DTSP on gold electrode [41] with soluble osmium mediator, respectively.

## 2. Experimental

### 2.1. Reagents

#### 2.1.1. Organism

The enzyme laccase from *Trametes trogii* has been employed in this study [39]. Strain 463 (BAFC: Mycological Culture Collection of the Department of Biological Sciences, Faculty of Exact and Natural Sciences, University of Buenos Aires) of *Trametes trogii* (*Funalia trogii*) (Polyporaceae, Aphyllophorales, Basidiomycetes) was used in these experiments. Stock cultures were maintained on malt extract agar slants at 4 °C.

#### 2.1.2. Culture conditions

Medium for fungal culture (GA medium) contained glucose, 20 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; $KH_2PO_4$, 0.5 g; $K_2HPO_4$, 0.6 g; $MnCl_2 \cdot 4H_2O$, 0.09 mg; $H_3BO_3$, 0.07 mg; $Na_2MoO_4 \cdot H_2O$, 0.02 mg; $FeCl_3$, 1 mg; $ZnCl_2$, 3.5 mg; thiamine hydrochloride, 0.1 mg; asparagine monohydrate, 3 g; distilled water up to 1 l, supplemented with 1 mM copper sulfate. Initial pH of the medium was adjusted to 6.5 with NaOH 1N. 500 ml Erlenmeyer flasks with 50 mL of medium were inoculated with four 25-mm² surface agar plugs

from a 7-day-old culture grown on malt agar (1.3% malt extract, 1% glucose, agar 2%). Incubation was carried out statically at $28 \pm 1$ °C. Cultures were harvested at day 22 and filtered through a filter paper using a Büchner funnel, the culture supernatants were used as enzyme sources.

#### 2.1.3. Enzyme activity

In this preliminary work the enzyme was used as a filtered crude and ionic exchange chromatography has shown some ABTS-inactive protein contamination in the yellow enzyme crude. Enzyme activity was measured spectrophotometrically in sodium acetate buffer pH 5 at 25 °C using ABTS [42]. Oxidation of ABTS was determined by the increase in $A_{420}$ ($\varepsilon_{420} = 36$ mol cm$^{-1}$ mL). Laccase activity is expressed in International Units (U), as the amount of enzyme needed to release 1 μmol of product *per* min. The activity of the crude enzyme solution was determined to 124 U mL$^{-1}$.

##### 2.1.3.1. Chemicals.
Dithiobis-*N*-succinimidyl propionate (DTSP), 3-mercaptopropyl sulfonate (MPS), potassium nitrate, $Na_2HPO_4$, $NaH_2PO_4$ and DMSO were purchased from Sigma. 2,2′-Azinobis-3-ethylbenzothiazoline-6-sulfonate (ABTS), poly (allylamine) (PAH), poly(sodium vinyl sulfonate) (PVS), sodium acetate and acetic acid (100%) were from Fluka. All reagents were analytical grade and used without further purification except PAH and PVS that were dialyzed against Milli-Q® water. 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.

The complex [Os(bpy)$_2$Cl(PyCOH)]Cl (where PyCOH is pyridinecarbaldehyde, in this article called "Os complex") and osmium poly(allylamine) (PAH-Os) were synthesized as described elsewhere [43]. This polymer solution was purified by dialysis against water for 3 days. The osmium content was evaluated spectrophotometrically at $\lambda = 475$ nm ($\varepsilon = 8100$ M$^{-1}$ cm$^{-1}$). Soluble [Os(bpy)$_2$Cl (PyCOOH)]$^+$ was prepared as described elsewhere [44].

### 2.2. Electrode preparation

#### 2.2.1. Tethered laccase

Gold disc electrodes ($d = 5$ mm embedded in KelF®) were first polished using alumina particles (1, 0.3 and 0.05 μm; Buehler, USA), sonicated for 30 s in water to remove resting alumina particles and rinsed with Milli-Q® water. To remove further impurities on the surface the gold was cycled 200 times between 0 and 1.6 V) in 2 M $H_2SO_4$ at scan rate of 10 V s$^{-1}$. Then the cleaned electrodes were immersed in a solution of 4 mM DTSP in DMSO for 1 h. After thoroughly rinsing the so prepared electrodes were dipped into a solution of 124 U mL$^{-1}$ laccase in 0.1 M phosphate buffer pH6 overnight. After rinsing with Milli-Q® water the gold discs were characterized electrochemically.

#### 2.2.2. LbL self-assembly

The gold electrodes were polished and cleaned as described above. Then in a first step a monolayer of MPS was formed by immersing the electrodes in a solution of 0.02 M MPS in 0.01 M $H_2SO_4$ for 30 min. After rinsing with Milli-Q® water 100 μL of a solution of $4.4 \times 10^{-4}$ M PAH-Os of pH8 were deposited on the electrode surface and allowed to assemble for 10 min. Then 100 μL of 124 U mL$^{-1}$ laccase in 0.1 M acetate buffer pH 4.7 was dropped on the same electrode and after 10 min of self-assembly the laccase solution was removed and the modified gold disc was rinsed with Milli-Q® water. These deposition steps were repeated until the desired number of polymer and enzyme layers were deposited. In

every case we covered the LbL self-assembled electrode structures with a topmost layer of PAH-Os.

### 2.3. Electrochemistry

Cyclic voltammetry was performed using an Autolab PGSTAT 30 potentiostat in a three-electrode cell with a platinum gauze as counter electrode and Ag/AgCl/(Cl$^-$ 3 M) as reference electrode (unless otherwise stated, all electrochemical potentials in this work are referred to this system). The working electrodes were gold discs ($d = 5$ mm) embedded in KelF® polymer. All measurements were performed in 0.1 M acetate buffer containing 0.2 M KNO$_3$.

Before measurements all solutions were degassed with pure argon or saturated with gas mixtures of argon/oxygen in different ratios. In order to control the oxygen partial pressure, the argon/oxygen ratio of this gas mixture was controlled by means of precision flow meters and flow regulators (G. Bruno Schilling, Argentina). Calibration of the O$_2$/Ar gas mixtures was performed with the rotating disc electrode (RDE) convective-diffusion limiting current density.

### 2.4. Quartz crystal microbalance measurements

The quartz crystal resonator at 10 MHz was used as a quartz crystal microbalance (QCM) fully described in previous publications [38]. The experimental setup allowed for the assessment of viscoelastic losses and measurement of the resonant frequency.

## 3. Results and discussion

### 3.1. Laccase in solution

In the absence of redox mediator a solution containing laccase shows no electrochemical activity on a gold electrode in the range of +550 to +50 mV potential. Upon addition of soluble redox mediator to the solution, for example [Os(bpy)$_2$Cl(PyCOH)]Cl, the characteristic cyclic voltammetry features of the Os(III)/Os(II) redox system are apparent with a half wave potential around 0.3 V. Further saturation of the solution with oxygen leads to a catalytic wave tuned to the osmium redox potential. Fig. 1 shows the current-potential curves for a bare gold electrode in a solution of 11 U mL$^{-1}$ laccase and 0.4 mM [Os(bpy)$_2$Cl(PyCOH)]Cl in 0.1 M acetate buffer of pH 4.7 containing 0.2 M NaClO$_4$ supporting electrolyte. Notice that under argon atmosphere only the reversible redox couple of the Os(III)/Os(II) can be observed while under aerobic conditions ($P_{O_2} = 1$ atm) a catalytic wave with half potential at ca. 0.30 V can be seen with a maximum steady state current density of 110 µA cm$^{-2}$. This catalytic current arises from the electrochemical reduction of the Os(III) that is continuously formed during the enzymatic reduction of oxygen.

Furthermore, increasing the concentration of the soluble Os complex the catalytic current at oxygen saturation follows, as expected, a Michaelis–Menten type behavior (shown in Fig. 1B). The effect of the O$_2$ concentration at constant Os complex concentration, however, has a more complex pattern (see below).

### 3.2. Tethered laccase and soluble mediator

Laccase was tethered covalently to the gold surface via post-functionalization of an activated carboxylate tethered to the Au surface by a dithiol (DTSP) forming a peptide bond with surface NH$_2$ at the surface of laccase as shown in Fig. 2A [41,45]. Fig. 2B shows the QCM uptake of laccase by the activated carboxylate gold modified surface during adsorption. A saturation enzyme coverage of 0.7 µg cm$^{-2}$ has been found, which corresponds to 13 pmol cm$^{-2}$

and is consistent with the expected value for a monolayer of close packed laccase on a flat surface of 4.4 pmol cm$^{-2}$.

In oxygen-free buffer solution, linear scan voltammetry shows a reversible redox peak couple at 0.220 V with 80 mV peak separation at 5 mV s$^{-1}$ as depicted in Fig. 3A. The peaks are better developed in O$_2$ saturated buffer as previously reported by Bilewicz [17]. However, no oxygen electrocatalysis could be observed in O$_2$ saturated solution in the absence of redox mediator, since the anodic and cathodic current peaks are very similar . It should be noted that the potential of this redox process does not correlate with that of the T1 copper center of *Trametes trogii* laccase, i.e. 0.59 V [46]. The redox potential in Fig. 3A ca. 0.22 V is very close to the typical potential of the T2 copper center, i.e. 0.19 V as has been measured for *Rhus vernicifera* laccase by Reinhammer et al. [47,48]. Since it has been established that all laccases have a very similar structural environment for the T2 copper it can be assumed that they also have similar redox potentials.

Therefore the O$_2$ reduction electrocatalysis at the T2/T3 cluster is not favored by the tethering of the enzyme to the Au surface, in spite of the evidence for direct electron transfer pathway for the native enzyme from the electrode to T2 site. Shleev et al. [13] have suggested that high potential laccases adsorb only on carbon electrodes in a configuration that facilitates the direct electron transfer to the T1 site with further internal electron transfer to the T2/T3 cluster reducing oxygen. On Au, laccase adsorbs in an orientation on the surface that the electron transfer from the electrode to the laccase goes directly through the T2 copper center thus preventing the reduction of O$_2$ [49]. In order to efficiently reduce O$_2$ all four copper sites in laccase must get first into the reduced Cu(I) state [50] therefore the electro activity of T2/T3 sites is not enough condition for oxygen catalysis.

Addition of a soluble redox mediator to this electrode demonstrates that the catalytic O$_2$ reduction site is active if proper access to the electron acceptor site in the enzyme is achieved by the mediator. Fig. 3B depicts the catalytic response of tethered laccase on gold in a solution of 0.4 mM Os complex in oxygen saturated and degassed buffer. As expected for the O$_2$-free buffer only the signal for the redox mediator was observed, but for the O$_2$ saturated solution a catalytic reduction of oxygen starting at 0.35 V can be clearly seen with a maximum catalytic current of 105 µA cm$^{-2}$. It should be noticed that this value is some ten times higher than the catalytic current observed with ABTS as mediator, 12 µA cm$^{-2}$, (see Fig. 3C for comparison); however the oxygen reduction starts at more positive potential, ca. 0.50 V in the latter case. We therefore conclude that the outer sphere one-electron osmium complex is efficient in reducing oxygen catalyzed by laccase and its redox potential can be increased closer to the T1 enzyme redox potential by suitable choice of ligands.

### 3.3. Laccase and mediator immobilized by LbL self-assembly

In the two previous sections we presented systems where at least one component was in solution. However, for most applications, especially for biosensors or biofuel cell cathodes, there is a need for all components of the catalytic chain to be immobilized on the electrode. Here we introduce for the first time the co-immobilization of laccase and a redox polyelectrolyte via LbL electrostatic self-assembly technique. This method has been extensively employed by our group and by others to form "wired" glucose oxidase multilayer films using redox polyelectrolytes [35,36]. Using cationic redox polyelectrolytes and adsorbing polyanion enzyme solution at a pH value where the protein carries a net negative charge, all-integrated enzyme–mediator system can be formed [37,38].
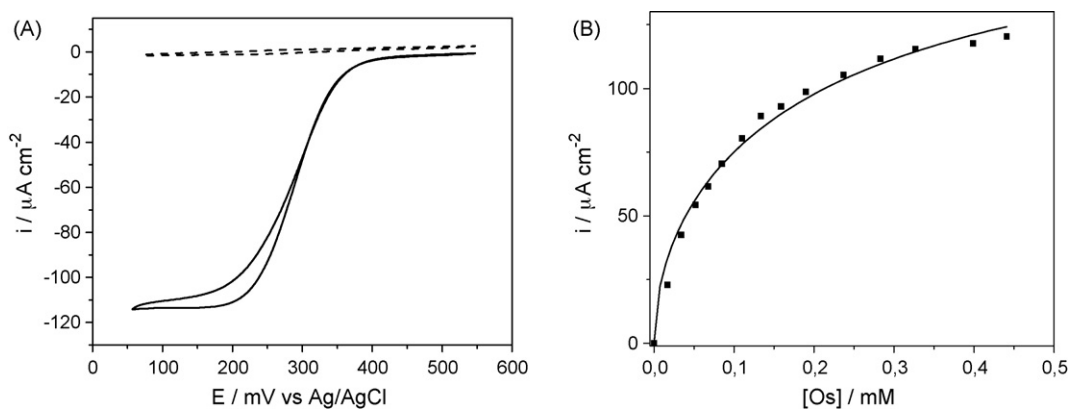
**Fig. 1.** (A) Cyclic voltammetries of an gold electrode in a solution of 11 U mL$^{-1}$ laccase, 0.4 mM Os complex and 0.2 M NaClO$_4$ in 0.1 M acetate buffer pH 4.7; saturated with argon (dashed line) and oxygen (solid line), scan rate 5 mV s$^{-1}$. (B) Dependence of the catalytic current of oxygen saturated solution on the Os concentration.

The adsorption of laccase on the PAH-Os layers was monitored by QCM measurements as a function of time (see Fig. 4A). A saturation value was found after 10 min with 0.5 μg cm$^{-2}$ mass gain per adsorption step, which corresponds to a laccase surface concentration of $9.0 \times 10^{-12}$ mol cm$^{-2}$. This is slightly lower than the surface concentration of laccase immobilized with DTSP but it was found that in every adsorption step roughly the same amount of laccase was adsorbed (see Fig. 4A).

Cyclic voltammetry of these electrodes in O$_2$-free solutions reports only the Os(III)/Os(II) redox couple at 0.30 V, while in O$_2$ saturated buffer a catalytic reduction wave clearly develops at ca. 0.45 V (Fig. 4B), thus a reagentless catalysis is observed. Furthermore, the catalytic oxygen reduction current increases linearly with the number of PAH-Os/laccase bilayers as depicted in Fig. 4C. It should be emphasized that not only the number of bilayers, but also the nature in the topmost layer has a strong influence on the catalytic properties of this laccase cathode. If the capping layer is positively charged PAH-Os the catalytic current density is larger, while for an LbL structure terminated in an additional PVS polyanionic layer the catalytic activity of the electrode is completely lost (not shown). However, adding another PAH polycationic layer onto this structure results in a partial recover of the catalytic activity. This behavior has already been found by our group for a similar system of PAH-Os/GOx [51] and has been related to the structure and electron transport properties of these layers.

Since it is well known that laccase shows maximum activity at pH 4.5 the effect of the external electrolyte pH on the O$_2$ reduction activity of the laccase LbL multilayer has been studied. It should be borne in mind that poly(allylamine) matrix itself has buffer capacity. The result is shown in Fig. 5A with an optimum pH in the range from pH 3.5 to pH 4.5 and a decay of activity above pH 6. This result is in good agreement with previous reports of laccase in different environments [8,18,19,22,27].

Changes of pH can lead to protonation/deprotonation of poly(allylamine) primary NH$_2$ and changes in film structure due to charge repulsion [52]. For instance, if a PAH-Os/laccase cathode is immersed in a buffer of optimal pH of 4.7 and then in buffer of pH 6, where no activity is observed, upon immersion back in a buffer of pH 4.7 a larger activity is observed (see Fig. 5B). In the sequence, the catalytic current increases some 20% which might be due to a change in the film structure and a better wiring of the enzyme by the osmium redox film.

Thus, by virtue of the organized enzyme and redox polyelectrolyte film one can gain fine control of properties by simply adjusting the pH or adding a topmost layer of pre-determined charge.
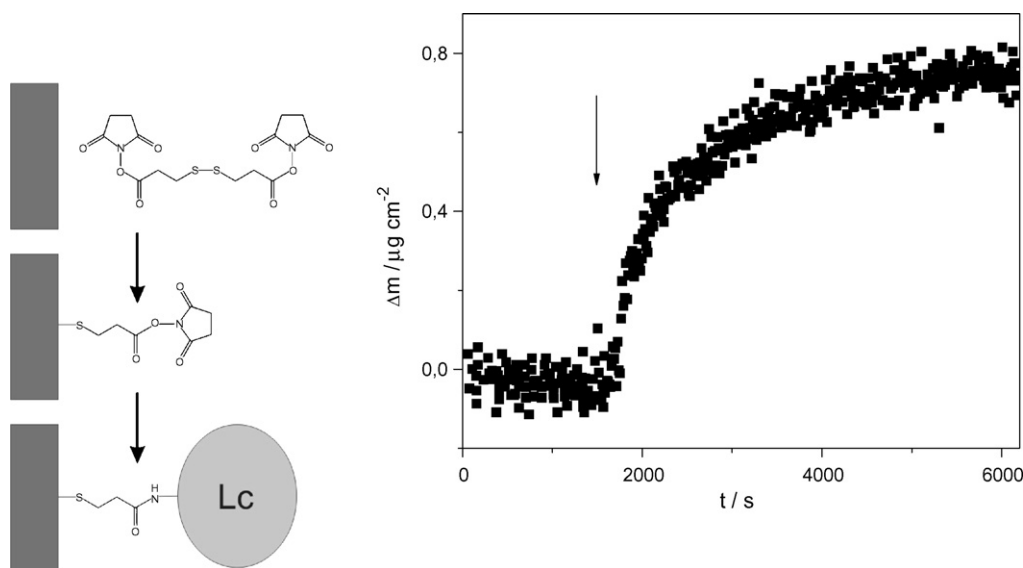


**Fig. 2.** (A) Schematic illustration of laccase immobilization with DTSP. (B) QCM measurement during the immobilization of laccase on a DTSP monolayer from a solution of 124 U mL$^{-1}$ laccase in 0.2 M phosphate buffer pH 6. Addition of laccase is marked by the arrow.
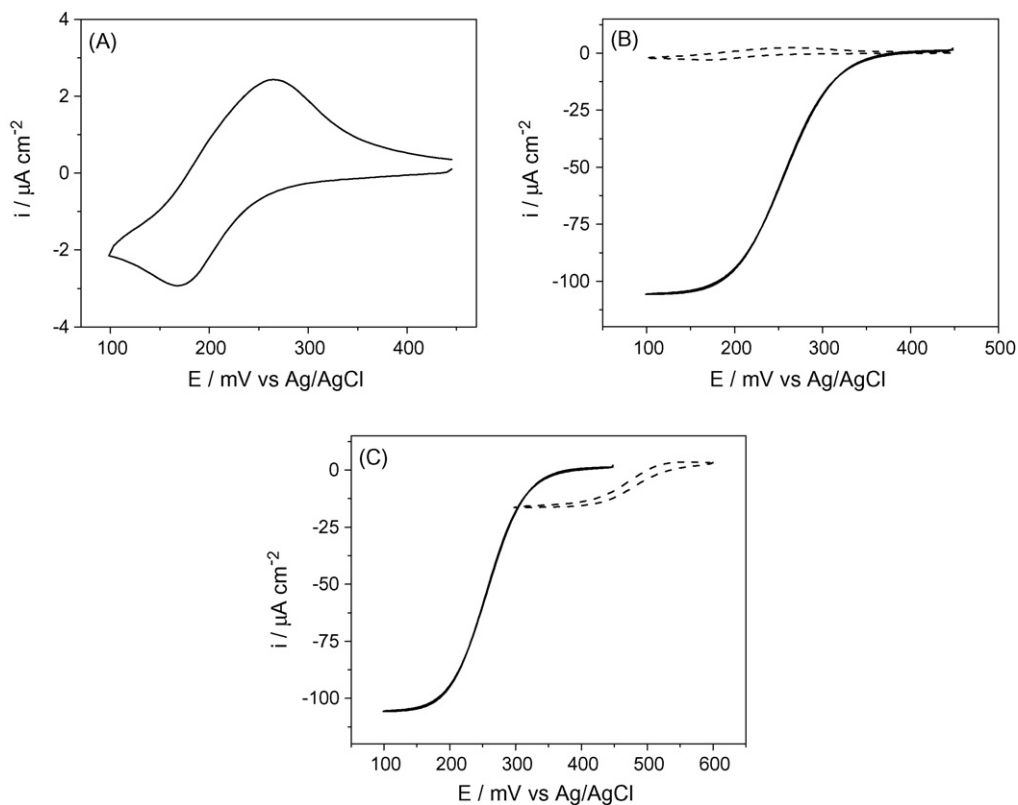
**Fig. 3.** (A) Cyclic voltammetry of a gold electrode modified with tethered laccase in oxygen saturated electrolyte. (B) Cyclic voltammetry of the same electrode in a solution containing 0.1 M Os complex saturated with argon (dashed line) and oxygen (solid line). (C) Comparison of the signals of 0.1 M Os complex (solid line) and 0.1 M ABTS (dashed line) in oxygen saturated electrolyte. Electrolyte of all experiments was 0.2 M $KNO_3$ in 0.1 M acetate buffer pH 4.7, scan rate $5\,mV\,s^{-1}$.
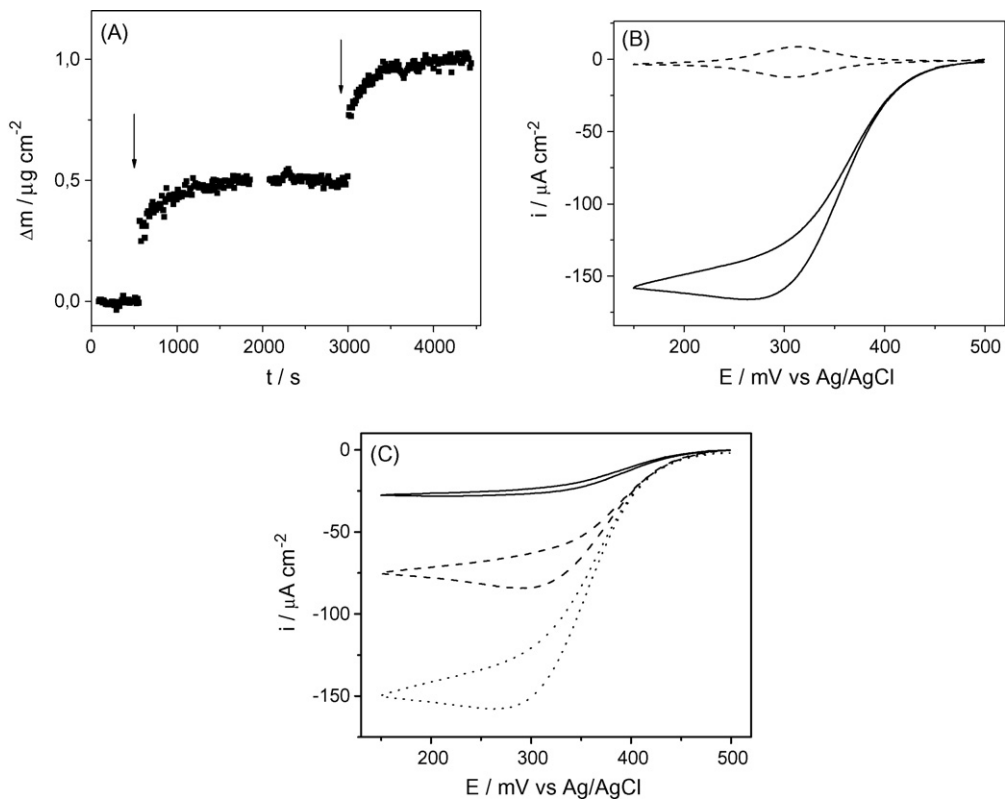


**Fig. 4.** (A) QCM measurement during the laccase adsorption of two laccase layers by LbL self-assembly from a solution of $124\,U\,mL^{-1}$ laccase in 0.1 mM acetate buffer pH 4.7. Adding of laccase is marked with arrows. (B) Cyclic voltammetries of an electrode modified with eight layers of PAH-Os and seven layers of laccase (labeled with (PAH-Os)$_8$(Lc)$_7$) by LbL self-assembly in electrolyte saturated with argon (dashed line) and oxygen (solid line). (C) Signals of (PAH-Os)$_2$(Lc)$_1$, (PAH-Os)$_4$(Lc)$_3$ and (PAH-Os)$_8$(Lc)$_7$ electrodes in oxygen saturated electrolyte. Electrolytes for the electrochemical experiments: 0.2 M $KNO_3$ in 0.1 M acetate buffer pH 4.7; scan rate: $5\,mV\,s^{-1}$.
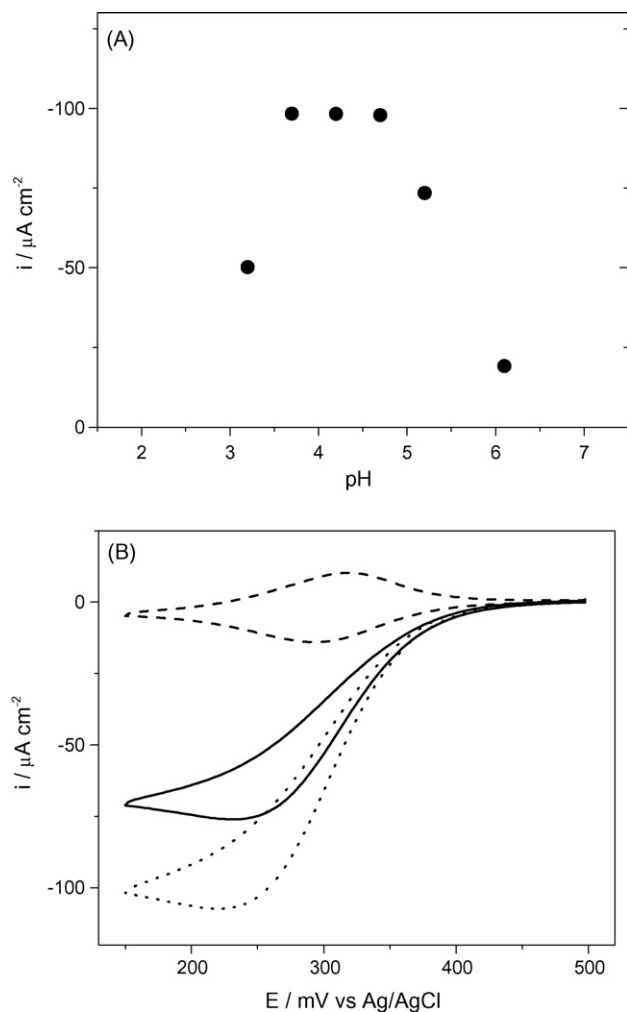
**Fig. 5.** (A) Dependence of the catalytic current of an electrode of (PAH-Os)$_4$(Lc)$_3$ from the pH of the solution. (B) Cyclic voltammetry of the same electrode in buffer pH 4.7 as prepared (solid line), put in buffer pH 6 afterwards and measured (dashed line) and in a third step measured again in buffer pH 4.7 (dotted line). Electrolytes for all measurements: 0.2 M NaClO$_4$ in 0.1 M acetate buffer, saturated with oxygen; scan rate: 5 mV s$^{-1}$.



**Fig. 6.** (A) Cyclic voltammetry of a (PAH-Os)$_4$(Lc)$_3$ electrode at different methanol concentrations. Methanol concentrations: 0 M (solid line), 100 mM (dashed line) and 1 M (dotted line). (B) Catalytic current of the same electrode in function of the chloride concentration. All measurements were performed in 0.2 M KNO$_3$ in 0.1 M acetate buffer pH 4.7.

Another important factor for a biofuel cell cathode is the tolerance to methanol in direct methanol fuel cells (DMFC). Because of the crossover effect due to membrane permeability to methanol that can hamper the oxygen catalyst, it is interesting to find oxygen reduction catalysts tolerant to methanol. We have studied the wired laccase cathodes up to a 1 M methanol solutions and the results are shown in Fig. 6A. A great tolerance to methanol for the oxygen cathode comprised of self-assembled laccase and osmium polymer has been found.

Another important factor in laccase activity is the concentration of chloride in solution since chloride binds as a ligand to the T2/T3 copper of the enzyme [53] and inhibits the internal electron transfer from T1 to the tri-nuclear copper cluster and therefore the enzyme activity decays in chloride containing solutions. As shown in Fig. 6B we have found, however, that for the LbL laccase and osmium films the catalytic current decreases with the chloride concentration when the chloride concentration is higher than 1 mM. Notice that even at a NaCl concentration of 100 mM the catalytic current drops by 30%. Surprisingly, below 1 mM NaCl the enzymatic reaction is not inhibited and even an increase of 20% is recorded for 1 mM as compared to chloride-free solution. This effect is seen when the concentration of chloride in solution is below the concen-
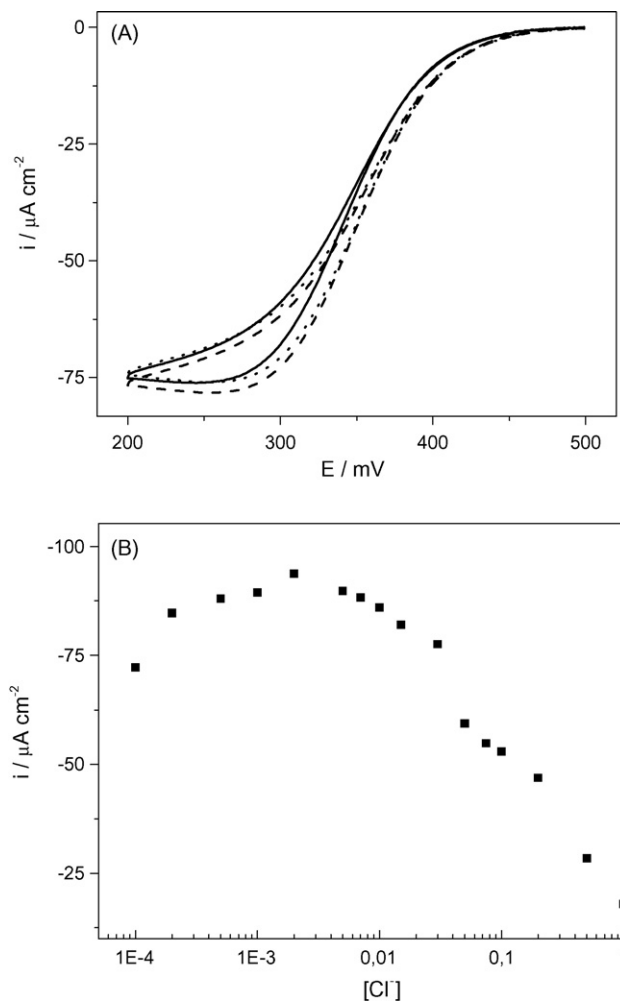
tration of chloride bound to positively charged groups in the film (i.e. NH$_3^+$).

The effect of oxygen partial pressure on the enzyme activity towards the electro-reduction of molecular O$_2$ was carefully measured by varying the composition of the gas in equilibrium with the testing buffer solution at a potential of 0.15 V, where oxygen reduction at bare gold does not take place. The results are shown in Fig. 7.

For a two-substrate enzyme reaction one expects a Michaelis–Menten type curve in response to both the redox mediator and to the co-substrate oxygen. While this is observed for the osmium concentration dependence in Fig. 1B, for O$_2$ concentration a Michaeli–Menten type curve is only observed above 0.5 mM. Below this oxygen concentration the observed current density increases almost linearly with O$_2$ concentration in all three experiments: (i) soluble mediator and enzyme; (ii) tethered enzyme with soluble mediator and (iii) all integrated enzyme and mediator in the film at the electrode. Therefore, at low oxygen concentration the catalytic current is insensitive to the concentration of enzyme and the concentration of mediator in three different experiments which suggests that the oxygen concentration is the limiting factor. It seems that the osmium-wired enzyme reduces oxygen with a Michaelis–Menten mechanism above a threshold
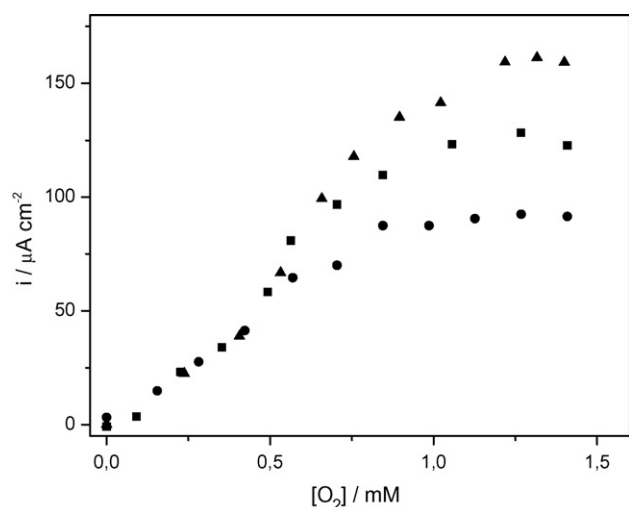
**Fig. 7.** Catalytic currents of three different gold electrodes: (1) bare gold in a buffer solution containing 11 U mL$^{-1}$ laccase and 0.4 M Os complex (▲), (2) DTSP/laccase modified gold in 0.4 M Os complex containing buffer (●) and (3) gold modified by LbL self-assembly with (PAH-Os)$_6$(Lc)$_5$ (■). All experiments were performed in 0.2 M KNO$_3$ in 0.1 M acetate buffer pH 4.7.

O$_2$ concentration of 0.5 mM; below which another O$_2$ reduction mechanism may be operative for laccase and the one-electron osmium mediator. This abnormal oxygen concentration kinetics has not been observed for ABTS as a redox mediator of laccase, but there are no detailed O$_2$ concentration dependence studies with one electron mediators such as osmium complexes.

Careful examination of Fig. 7 suggests a current–concentration curve that resembles an allosteric behavior with oxygen as the allosteric effector [54,55]. In these three experiments the electrode potential was kept at 0.15 V where all the osmium is present at the surface as Os(II); therefore in the absence of oxygen the enzyme is in its fully reduced state. Alternatively, depletion of oxygen in the lower oxygen concentration solutions may lead to lower currents than expected. Further studies with rotating disc electrode are being carried out at present to resolve this unusual behavior.

## 4. Conclusions

We have shown for the first time the electrocatalytic behavior of a LbL laccase and a redox polymer mediator modified electrode, all integrated in a multilayer built on a gold electrode surface. Laccase reduces oxygen at the potential of the redox mediator which wires efficiently the enzyme to the underlying gold electrode. A comparison with the electrocatalysis of all soluble enzyme and mediator and with tethered enzyme and soluble mediator has also been presented. Tolerance to methanol and chloride in LbL laccase films has been shown; the increase of catalytic current at very low chloride concentration resembles results reported by Daigle et al. for hydrogels of laccase and osmium polymers at pH 5 [20].

The catalytic current increases with the number of adsorption steps of enzyme and redox mediator polyelectrolyte up to seven enzyme–redox polyelectrolyte bilayers; however, it is expected for thicker films a change in kinetic case from a linear dependence with number of layers to thickness independence when the kinetics of the enzyme–mediator reaction are limited within the film as shown for glucose oxidase [56].

The catalytic current densities for the enzymatic reduction of oxygen mediated by osmium bipyridine-pyridine-chloro modified poly(allylamine) molecular wire for seven enzyme–polymer bilayers show the expected potential dependence and reach a limiting

current level of 150 μA cm$^{-2}$. This can be compared to the much lower catalytic currents for un-mediated laccases [57,58] with the exception of the very large current densities reported by Armstrong [14] for laccase attached to carbon surfaces by an anthracene tether. For co-immobilized laccase with osmium mediators in thick hydrogels, Leech and co-workers have reported much lower current densities [21]. Calabrese Barton, on the other hand, have reported catalytic current densities as high as 2.5 mA cm$^{-2}$ for a 600-nm thick hydrogel of a poly(vinylimidazol)-bipyridine osmium with redox potential 0.44 V (vs. Ag/AgCl 3 M Cl$^-$) and electron diffusion coefficient 2.3 × 10$^{-9}$ cm$^2$ s$^{-1}$ [59]. For the LbL system reported here with an ellipsometric film thickness of 55 nm (3 enzyme layers) we find 80 μA cm$^{-2}$ for $D_e \sim 10^{-9}$ cm$^2$ s$^{-1}$ and 0.3 V which is consistent with a lower catalytic current density.

Detailed studies of the effect of the number of layers, enzyme stability and the extraction of kinetic data from experimental results using a relaxation and simplex mathematical algorithms applied to the study of steady-state electrochemical responses of LbL-immobilized enzyme electrodes [60] are in progress with highly purified laccase form *Trametes troggi* and will be reported in the near future.

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