Enhanced direct electron transfer of laccase at hierarchical carbon nanotubes. Comparison of three enzyme immobilization methods

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Abstract: Three immobilization protocols were investigated for direct electron characteristics of laccase at 3D-hierarchical carbon nanotubes composite materials (3D-CNT/CMF). Electrochemical comparison between randomly adsorbed laccase molecules and those possessing site-directed covalent binding to the support are presented. The high current densities measured are suggesting an excellent compatibility of biocomponent and supporting electrode, leading to a high loading and optimum orientation of laccase onto 3D-CNT/CMF for efficient electron communication between them.

Keywords: laccase, CNT, biofuel cell, biocathode, ORR

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Introduction

In the present context of biofuel cell development intensive efforts are gathered [1, 2] towards fulfilling of key limiting parameters [3], not only in terms of current densities and extended cell potential, but also regarding the adaptation to physiological conditions [4]. From an implantable biocathode point of view that catalyzes the oxygen reduction to water, the maximum output that can be feasibly obtained is limited by both the oxygen concentration and the potential provided by the biocathode. The latter one can be maximized using a high-redox potential laccase, whose T1-site is approximately +750 mV vs. NHE, connected to the cathode. On the other hand, the maximum oxygen concentration is approximately 200 μM and depends on the specific tissue diffusion coefficient [5]. As a consequence, the efficiency of a biocathode can be improved by addressing an optimized electrocatalytic reduction of oxygen at the electrode surface via electrode-enzyme direct electron transfer [6, 7] and by securing a maximum oxygen supply [3]. Implementation of hierarchical carbon materials on micro- and nanoelectrodes' surface can multiply both the efficient diffusion profile of oxygen and the biocatalyst packing. Such hierarchical carbon materials consist on multiwall carbon nanotubes (CNT) grown onto carbon microfibers (CMF), which in turn are synthesized *via* chemical vapor deposition (CVD) onto a supporting electrode of glassy carbon, graphite rod or carbon paper. This three-dimensional composite material (3D-CNT/CMF) was recently fabricated [8] and utilized in bioelectrocatalysis of horseradish peroxidase (HRP) for H_2O_2 reduction at +600 mV vs. Ag|AgCl [9]. The main advantages for using the 3D-CNT/CMF composite electrode as immobilization surface for laccase consists on: i) avoiding the use of a redox hydrogel film as matrix for the electronic communication between enzyme and electrode, thus eliminating additional limitations such as the electron hopping step [10] and a further drop in the cell potential [11–13]; and ii) reduction of the large number of interfacial cascades in-between the individual CNT produced when they are simply dropped onto a supporting electrode in presence or not of a

mediator/crosslinking polymeric net [14–16], which requires extensive optimization protocols and characterization methods, such as SECM [17, 18].

In the present study, the properties of the 3D-CNT/CMF hierarchical structure synthesized onto a graphite rod electrode, as depicted in Figs 1A-C, are tested for three specific methods for laccase immobilization (as seen in schemes from Figs. 2A-C).

Trametes hirsuta laccase was covalently bound to the nano-structured electrodes modified with an amino phenyl monolayer by forming amide bonds *via* carboxylic groups from its aspartic/glutamic residues (method A – see Fig. 2A), or by forming imino bonds *via* aldehyde groups generated at its sugar residues (method B – see Fig. 2B). Both types of residues exist around the T1-site of this laccase, which is located near the protein surface and herein is the site of electron exchange with redox donor molecules [19–21]. Direct electron transfer between the enzyme and the electrode requires correct orientation of the immobilized laccase with the T1-site in proximity of the electrode surface [6, 22].

On the other hand, for method C – see Fig. 3C, 1-pyrenehexanoic acid (PHA) molecules are adsorbed *via* π -stacking onto the carbon hierarchical structure [23]. Therefore, by the insertion of carboxyl groups at low coverage density, the CMF/CNT structure is increasing its hydrophilic degree and thus favors an improved stability of the adsorbed laccase, although with a random orientation. This method was recently applied for the immobilization of laccase onto CNTs, although using an amino terminated pyrene derivative [24].

Material and Methods

Hierarchical 3D-carboneous material was fabricated at Ruhr University Bochum, Bochum Germany (RUB) in accordance to a protocol previously presented elsewhere [9, 8]. Bioelectrocatalytic experiments were performed in two separate laboratory facilities, thus covalent bindings of laccase (methods A and B) were investigated at CSIC, Madrid, Spain, whereas its physical adsorption (method C) was studied at RUB.

Enzyme: *Trametes hirsuta* laccase from the basidiomycete strain *T. hirsuta* 56 was obtained from the laboratory collection of the Moscow State University of Engineering Ecology following the purification procedure previously reported [25]. 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 [26]. 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 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) as substrate and its value was 340 units/mg laccase. Two different batches of *T. hirsuta* were involved and the added amount of enzyme was normalized for finally reaching the same unit loadings onto the electrode material.

Electrochemical measurements were performed in a thermostated electrochemical cell with of three-dimensional hierarchical carbon nanotube electrodes fitted into a MSR electrode rotator from Pine Instruments (the geometric electrode area exposed to the solution was 0.073 cm²). An Ag|AgCl|3 M KCl reference electrode from Bioanalytical Systems (all potential values reported are versus this electrode) and a platinum wire counter electrode were used. Cyclic voltammograms and chronoamperograms were controlled by an Autolab (PGSTAT 12 or PGSTAT 30) potentiostat. All measurements were carried out at 27 °C in 50 mM acetate buffer, pH 4.2, containing 100 mM NaClO₄. Electrocatalytic activity of the laccase electrodes was measured with solutions saturated with air or pure O₂ (Air Liquide). Blank cyclic voltammograms in absence of oxygen were measured within a MBraun glovebox with an oxygen content inferior to 1 ppm and the electrochemical solution was purged with pure nitrogen (Alphagaz®, Air Liquide). Chronoamperometry 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.

Electrode preparation was performed using three individual methods: methods A) and B) imply covalent attachment of laccase, whereas method C) proceeds *via* a non-covalent physical adsorption to the three-dimensional hierarchical carbon nanotube electrodes.

For covalent attachment of laccase, the electrodes were initially modified with an amino phenyl monolayer as reported [22]. Further, two different procedures were used for forming covalent bonds between *T. hirsuta* laccase molecules with the amino groups of the electrode surface (Fig 1A) and thus establishing two distinctive orientations of laccase onto the electrode, with a long electron distance between T1-active center and electrode in case of method A) and a short electron distance between T1-site and electrode in the case of method B).

In **method A** the modified electrode was coated with 22 μl of a 7.5 mg ml^{-1} laccase solution for 20 minutes at ambient temperature to favor 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 22 μl drop of a 17 mM N-hydroxysuccinimide (NHS, Fluka) in 10 mM phosphate buffer, pH 6.0, and immediately a 22 μl drop of a 36 mM 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide (EDC, Sigma-Aldrich) in 10 mM phosphate buffer, pH 6.0. Amide bonds formation between **carboxylic groups** of the enzyme and amino groups of the electrode (Fig. 2A) was allowed to carry on during 2 h at ambient temperature with the electrode surface covered with an Eppendorf tube to avoid evaporation. Finally, the modified electrode was washed in 50 mM acetate, 100 mM NaClO_4 , pH 4.2 buffer solution for 10 min with stirring to remove any unbound compounds.

In **method B**, the **hydroxyl groups of the sugar residues** of *T. hirsuta* laccase were oxidized to aldehyde groups by adding 450 μl of 47 mM NaIO_4 (Panreac) to 22 μl of 7.5 mg ml^{-1} laccase solution. After 30 minutes of reaction, 1.8 ml 100 mM phosphate buffer, pH 7.0 were added and a electrode modified with an aminophenyl layer was immersed into the solution during 90 min for formation of imino bonds between the amino groups of the electrode surface and the aldehyde

groups of the enzyme (Fig. 2B). Finally, the modified electrode was washed in 50 mM acetate, 100 mM NaClO₄, pH 4.2 buffer solution for 10 min with stirring to remove any unbound compounds.

Method C consists in a non-covalent adsorption of native laccase from *T. hirsuta* onto 3D-CMF/CNT modified graphite rod electrode (Fig. 2C). Before addition of enzyme solution the electrode surface was modified by insertion for 90 min in 10 mM 1-pyrenehexanoic acid, (PHA) (Sigma) in N,N-dimethylformamide (DMF) (Sigma). After that, the modified electrode was washed with DMF for removal of unbound PHA, followed by additional washing in acetate buffer as above. Then, once the electrode was dried, 10 µl of 15 mg ml⁻¹ *T. hirsuta* laccase solution (equal units added per electrode as in methods A and B) was added onto the disk of electrode (0.073 cm²) and the adsorption of enzyme was allow to proceed in water saturated vapors for a mild evaporation. Finally the electrode was washed throughout for acetate buffer to remove any weak adsorbed enzyme molecules.

Results and Discussion

The electrocatalytic reduction of O_2 in the absence of redox mediators of the laccase-modified electrodes was measured by cyclic voltammetry with an electrode rotator. Electrochemical experiments demonstrate the successful immobilizations of laccase onto the modified surfaces of hierarchical carbonaceous material irrespective of the involved method. However, the biocatalytic currents and transport of substrate (oxygen) to laccase differs significantly in respect to the immobilization method applied. Further comparison of the three methods has mainly to consider the qualitative terms, since the real electrode area is, for obvious reasons, not identical between each electrode. In addition, the multiple steps for surface activation and enzyme anchoring yield significant experimental deviations in enzyme loading for a solid quantitative evaluation. In order to compare only the real contribution of the bioelectrocatalytic current and minimizing the role of the capacitive current (proportional to the real electrode area), the related cyclic voltammograms were plotted after subtracting the background CV recorded in absence of oxygen (by purging inert gas such as N_2 or Ar). The individual CVs without background subtractions are available as supporting information.

Fig. 3A shows the cyclic voltammograms obtained with a laccase electrode in which the laccase was covalently bound *via* amide bonds (method A). The catalytic waves measured under pure O_2 at 0 rpm and 1000 rpm were quite similar. Furthermore, the plateau current values measured in stirred solutions purged with air or O_2 were almost the same.* [27] Therefore, these results clearly suggest that the electrocatalytic reduction of O_2 is not rate-limited by the substrate transport to the electrode. This is also supported by the evidences of extended potential intervals (of approx. +370 mV) of kinetic limited domains, starting from the onset potential at around +620 mV and reaching the plateau current around +250 mV. Besides, in this case the catalytic current increased two-fold when 0.2 mM ABTS was added to the solution as redox mediator for accessing also the unfavorable oriented enzymes (results not shown). Thus, method A of coupling *via* carboxylic groups of acid

residues is leading to a large proportion of immobilized laccase molecules with an unfavorable orientation for direct electron transfer (DET). This conclusion is supported also by the fact that the catalytic plateau currents ($\sim 500 \mu\text{A cm}^{-2}$) are smaller when the laccase is bound *via* its carboxylic groups (using method A), in comparison to method B as seen from Fig. 3B.

Fig. 3B, corresponding to method B, shows the voltammograms obtained with an electrode in which laccase was bound *via* imino bonds, by a Schiff base reaction between the amino groups of the electrode surface and the sugar shell of the laccase. The bioelectrocatalytic reduction of oxygen is clearly observed in the subtracted voltammograms, recorded either in the presence of air or at saturated concentration of oxygen. Meritor is to emphasize the very high onset potential of biocatalytic process for oxygen reduction at approximately +700 mV which is more than 100 mV higher than the redox potential of the T1-site of *T. hirsuta* [25]. Contrary to method A, method B of immobilization leads to a significant smaller potential interval of the kinetic limited domain, reaching the diffusional limitation already at potentials around +500-550 mV. This is visible also by occurrence of an oxygen reduction peak at 0 rpm, most probably due to an initial accumulation of oxygen within the 3D-CNT/CMF structure. The electrocatalytic current increases significantly (almost 3 times) after the electrochemical solution was saturated with pure O₂. In this case, when the enzyme seems to be favorably oriented for an efficient DET, the additional electrode rotation up to 1000 rpm contributes to a significant augment of plateau current. This confirms limitations due to the oxygen diffusion to address the entire enzymatic loading onto the 3D-structures. Thus, under strong convective transport of oxygen towards the electrode structure, a catalytic current density (measured after subtracting the control voltammogram under N₂) of 1.5 mA/cm^2 was obtained at +0.2 V, considering the geometric area of the electrode. This value is much higher than those previously reported for the same laccase covalently bound to non-nanostructured graphite electrodes [22]. This increase of the catalytic current is undoubtedly explained by the enhancement of the electroactive surface area upon formation of the three dimensional hierarchical carbon nanotube structures on the

carbon electrode [8]. This should allow a notable increase of the laccase coverage on the electrode in active form, meaning in a full electric contact with the supporting graphite electrode and in consequence an increase of electroenzymatic O₂ reduction. No increase of current was measured in presence of the redox mediator ABTS, which suggests that all immobilized active laccase molecules are correctly oriented for DET.

These results suggest that method B of immobilization leads to a larger number of laccase molecules with adequate orientation for fast DET with the electrode than by method A. Indeed the crystal structure of *T. hirsuta* laccase indicates that several of the aminoacids with carboxylic groups that could establish covalent bonds with the modified electrodes are located opposite to the T1-site [29]. Therefore, laccase molecules covalently bound *via* these glutamic and aspartic aminoacid residues can participate in mediated electrocatalysis but not in good extent in the direct electrocatalysis. Their contribution to DET is however feasible only due to a compensation of a longer electron tunneling distance by a higher overpotential for the reduction of T1-site. Oppositely, most of the sugar residues of the laccase are located in regions of the protein surface that should favor correct orientation of the T1-site for direct electron transfer upon covalent immobilization *via* these residues [19]. This explains why addition of ABTS to the electrochemical solution in that case does not contribute to a further increase of the catalytic current.

Finally, method C of immobilization based on a random physical adsorption of laccase molecules onto the electrode surface modified with a pyrene derivative containing carboxylic groups. The resulting CVs in air and oxygen are demonstrating similar non-diffusional limitations, judging that the electrode rotation at 1000 rpm does not contribute to any significant increase (not more than 2 %- data not shown) of the bioelectrocatalytic current at low potentials. However, what differs to method A, is a rather narrow kinetic limited domain in between the onset potential of +650 mV and the potential of reaching the current plateau at around +400 mV. This can be rationalized due to a multitude of orientations of laccase molecules onto the electrode surface which are not fixed to the

initial position as in the case of covalent binding, but which hypothetically adjust their initial orientation to a more favorable one once the applied potential is swept towards low potentials. In addition, it can be speculated that taking into account the pH value and the protonated/deprotonated state of carboxylic groups from the laccase and from the electrode, the proximity of two negative domains from the enzyme and from the electrode might be electrostatically hindered and therefore favoring the presence of the T1-site in the vicinity of the electrode. Despite the fact that method C is leading to lower values of biocatalytic plateau currents compared to method B, however it shall be emphasized the simplicity and still the high current densities that are achieved with laccase onto this recently introduced composite material as electrode support in bioelectrochemistry.

When facing physiological conditions laccase electrodes for implantable biofuel cells should overcome chloride concentration of 150 mM, which is a strong inhibitor of laccase native activity. The operational stability of covalently bound laccase electrode was studied by chronoamperometry with magnetic stirring of the solution. For this study, only method A was selected for testing, due to several reasons: i) amide bonds are more resistant towards hydrolysis than imino bonds and therefore the measurement will reflect better the effect on enzymatic activity rather than stability of enzyme link; and ii) specific covalent binding can be easily attributed to a certain property, instead of a random orientation *via* method C which cannot provide a particular unique interpretation. Fig. 4 shows the evolution of the catalytic current of O₂ reduction at + 200 mV against time and the effect of the concentration of chlorides in solution. Fig. 4 shows that covalent binding of laccase *via* amide groups to the surface of the nanostructured electrode provides a rather resistant bioelectrocatalytic activity towards Cl⁻ inhibition. Thus, there was only a 16 % loss of the catalytic current at a concentration of Cl⁻ near to the physiological one. By contrast, a considerable decrease of the catalytic current was measured upon addition of only 30 mM fluoride, which is known to induce an irreversible inhibition of biocatalytic activity of laccase [30, 31, 28]. Similar results were reported previously with this laccase bound to a non-nanostructured graphite electrode, where a possible

explanation was proposed for the different behavior of the electrocatalytic activity towards the two halides [22].

Conclusions

In summary, this study demonstrates the compatibility of hierarchical carbon composite electrode with laccase for development of a biocathode based on direct electron transfer. Three methods of laccase immobilization were tested, leading to the following general conclusions: i) covalent binding of laccase using its carboxylic groups via amide bond leads mainly to an unfavorable orientation onto electrode, demonstrated by a mixed DET and MET activity in presence of ABTS as redox mediator, although it also shows a high resistance towards Cl^- inhibition; ii) covalent binding of laccase using activated hydroxyl groups from sugar residues of laccase *via* imino bond shows an excellent biocatalytic activity with 100 % DET participation (T1-site in proximity of the electrode), which is demonstrated by no effect upon ABTS addition, although with a lower operational stability; and iii) simply physical adsorption of laccase onto pyrene-carboxylic modification of hierarchical carbon composite electrode provides a robust bioelectrocatalytic effect for laccase, however at a rather random orientation of laccase onto the electrode surface.

In conclusion, this study predicts a wide range of applications in bioelectrochemistry of various redox enzymes using hierarchical carbon composite material as conductive support. In particular, they are potentially interesting for biofuel cell development as it is the case of herein, laccase, or previously published horseradish peroxidase (HRP) [9] and HRP/glucose oxidase [32] -based biocathodes capable to generate high current densities at physiological conditions.

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Figure Legends

Fig. 1. SEM pictures of 3D-CNT/CMF material on graphite rod electrode as supporting electrode.

Fig. 2. Schemes of covalent immobilization of *T. hirsute* laccase *via* amide bonds (A) or imino bonds (B) to electrodes modified with amino phenyl groups, and its physical adsorption onto pyrenecarboxylic-modified hierarchical CNT/CMF electrode (C).

Fig. 3. Cyclic voltammograms under air or O₂ of hierarchical CNT/CMF electrodes with immobilized *T. hirsuta* laccase. A) Electrode modified with amino phenyl groups and laccase covalently bound *via* amide bonds. B) Electrode modified with amino phenyl groups and laccase covalently bound *via* imino bonds. C) Electrode modified with 1-pyrenehexanoic acid and laccase physically adsorbed. Measured in 50 mM acetate buffer, pH 4.2, 100 mM NaClO₄. Scan rate was 10 mV/s, rotation rate was 0 or 1000 rpm and the temperature was 27°C.

Fig. 4. Chronoamperometric measurement of an electrode covalently modified with *T. hirsuta* laccase *via* amido bonds and effect of halides concentrations on the catalytic current. The electrolyte solution contained 50 mM acetate buffer, pH 4.2, 100 mM NaClO₄. The applied potential was +200 mV, the temperature was 27°C, the solution was bubbled with 1 atm O₂ and was stirred with a magnetic bar.

Fig. 1

Fig 2.

Fig. 3

Fig. 4