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Improved operational stability of a laccase-based electrode applicable in biofuel cells

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To ensure high electrocatalytic activity and operational stability of immobilized laccase enzyme (isolated from the basidiomycetes *Trametes pubescens*), different immobilization protocols on various electrode materials (graphite, gold and glassy carbon) were studied. The physical methods of immobilization – physisorption or entrapment of the enzyme in a composite layer, did not yield bioelectrodes with long lasting activity, whilst the immobilization approach based on covalent attachment of the enzyme to the electrode surface was found to produce a bioelectrode with extended operational stability. The basic electrode material was found to play an important role in bioelectrode's performance. The comparative studies carried out with two different electrode materials – polycrystalline gold and glassy carbon, modified with electrodeposited gold nanoparticles, with identically immobilized laccase on them showed much higher current density for the second type of enzyme electrode. An extended operational stability of ca. 3 weeks was achieved for the laccase immobilized on gold-nanoparticles modified glassy carbon electrode. The value of the open circuit voltage (over 450 mV *vs*. Ag/AgCl, 3M KCl at pH = 4.5) of the laccase based electrode suggests that it is a good candidate for cathode when engineering biofuel cells.

Keywords: Laccase, Immobilization, Self-assembled monolayers, Biocathode.

INTRODUCTION

There are two multicopper oxidases – the enzymes laccase and bilirubin oxidase, that are capable of performing the oxygen reduction even more efficiently than platinum and, hence, they are often used for the development of cathodes for biofuel cells applications [1-4]. Type of laccase immobilization is of key importance in the latter case because it has to guarantee that the enzyme is electrochemically active [5, 6], i.e. capable of exchanging electrons with the underlying electrode surface, and possesses long-lasting operational stability [7] which is one of the most important electrode characteristics when constructing biofuel cells.

One of the main drawbacks of the laccase-based bioelectrodes is the extremely short lifetime of the immobilized enzyme – depending on the immobilization type it does not exceed 24-48 hours. In this connection, the objective of the present work is to find a suitable immobilization approach, which ensures an extended lifetime of the immobilized laccase together with high electrochemical activity of the immobilized enzyme.

EXPERIMENTAL

Materials

Laccase (Lac) (E.C. 1.10.3.2) from *Trametes pubescens* (kindly provided by Dr R. Ludwig, BOKU University, Vienna, Austria) was with homogeneous activity of 46 U mg^{-1} (1 U oxidises 1.0 μmole of ABTS per min at pH 4.5 at 25 °C).

Cysteamine (Acros), chitosan (Acros), glutaric aldehyde (Fisher), ABTS-2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid (Acros), citric acid monohydrate and sodium citrate (Fluka); $HAuCl_4.H_2O$ (Acros) were of analytical grade and used as received.

The working electrodes were discs from polycrystalline gold (d = 1.0 mm, CHI, USA), glassy carbon (d = 3.0 mm, CHI, USA) or graphite (d = 3.0 mm, RWO, Germany).

Buffer solutions (0.05 M) were made of citric acid and sodium citrate dissolved in double distilled water with pHs 4.5, and 4.0, adjusted with a pH meter pH 211 (Hanna Instruments, USA). To increase the ionic strength of the buffer solutions, $NaClO_4$ was added to the buffer to 0.1 M concentration.

Apparatus and measurements

All electrochemical experiments were performed in a conventional three-electrode cell (working volume 25 ml). Ag/AgCl (3M KCl) was used as a reference electrode, graphite, glassy carbon or polycrystaline gold as working electrode, and a platinum wire as an auxiliary electrode, connected to a computer-controlled electrochemical workstation PalmSens (Palm Instruments BV, The Netherlands).

The biocatalytic activity of the immobilized laccase was tested by constant-potential amperometry, by adding aliquots of 1 mM aqueous solutions of ABTS (freshly prepared before measurements) to 10 ml of buffer (pH=4.0 or

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pH=4.5) with simultaneous registration of the current at a constant potential of -0.1 V. The solution was stirred at 460 rpm during the measurements with a magnetic stirrer (IkaMag RCT, Ika, Germany). Cyclic voltammetry (CV) at scan rates from 5 to 50 mV s^{-1}; differential pulse voltammetry (DPV) at a scan rate of 7 mV s^{-1} with 30 mV amplitude and square wave voltammetry (SWV) with an amplitude of 25 mV, at a frequency of 2-5 Hz, were also used in the studies. When necessary, the solutions were purged with either Ar gas or air.

Five different immobilization protocols were tested for laccase immobilization: i) Physical adsorption from laccase solution (16 mg mL^{-1}) on graphite; ii) Sorption of Lac onto graphite modified with electrodeposited gold; iii) Entrapment of laccases in chitosan layer, deposited on graphite; iv) Immobilisation of laccases onto graphite modified with chitosan- gold particles composite; v) Covalent binding of laccase to cysteamine moieties self-assembled on gold using a bifunctional reagent (glutaric aldehyde).

Covalent immobilization was done on both smooth polycrystalline gold electrode and a glassy carbon electrode with electrodeposited thin gold layer (up to 100 nm thick). Before modification both gold and glassy carbon electrodes were polished with 0.3 and 0.05 μm alumina slurry on a polishing cloth (LECO, USA), water-rinsed and cleaned by ultrasonication in water for 2-3 min.

The working surface of the cleaned and polished glassy carbon electrodes was modified through direct electrodeposition of gold. The metal particles were grown onto the electrode surface by electroreduction of tetrachloroaurate ion from electrolyte containing 2% $HAuCl_4$, dissolved in 0.1 M HCl, by applying a constant potential of -155 mV (*vs*. Ag/AgCl, 3M KCl) for 5 s.

Prior to the enzyme immobilization both electrode types – smooth gold and gold-modified GC electrodes were cleaned electrochemically in 0.5 M H_2SO_4 by cyclic voltammetry (CV, scan rate 0.1 V s^{-1}) over the potential range from 0 to 1.7 V (*vs*. Ag/AgCl, 3 M KCl) for at least 10 cycles, then thoroughly rinsed with double distilled water. The self-assembly of cysteamine was carried out under static conditions by immersing the electrodes in solutions containing 20 mg ml^{-1} cysteamine dissolved in distilled water. The duration of the adsorption process was varied from 1 to 24 h. After completing the chemisorption the loosely bound cysteamine was removed from the surface by soaking the electrode in water for ca. 0.5 h at room temperature and then rinsed thoroughly. Then a 9 μL drop of laccase solution (16 mg mL^{-1}) was cast on the electrode surface and 3 μL of glutaric aldehyde (45 mM aqueous solution) was mixed with it and allowed to react for 30 min at ambient temperature. So prepared enzyme electrode was stored in 0.05 M sodium citrate buffer of pH=5.6 in a refrigerator at a temperature of 4 ^{o}C until measurements.

RESULTS AND DISCUSSION

Several protocols for laccase (Lac) attachment to the electrode surface, using physical methods for immobilization (physisorption, entrapment in composite layer and sorption on porous surface) were explored in order to choose the optimal one guaranteeing high electrocatalytic activity, resp. high current density, low noise level, and extended operational stability.

The ability of laccase to perform mediated electron transfer in the presence of substituted aromatic compounds was used as a tool for measuring the apparent enzymatic activity of the immobilized enzyme. Since 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid, also known as ABTS, is routinely used to determine the homogeneous catalytic activity of laccase enzyme, it was chosen as the redox mediator (electron shuttle) in these studies for optimization of the experimental conditions and as a basis to compare the electrocatalytic activity of differently immobilized laccases.

Chronoamperometric records of the electrode response (Fig. 1A) to ABTS at a constant potential of – 100 mV (*vs*. Ag|AgCl, 3 M KCl) and under oxygenated conditions suggest that the maximum current variation upon addition of the substrate is achieved with the electrode prepared by usual physical adsorption of the enzyme onto the graphite surface. This electrode preparation shows also the lowest noise level and the widest dynamic range, which motivated us to choose this immobilization protocol for further studies. On Fig.1 B are compared the CVs of electrodes with laccase immobilized by methods 1, 2 and 4 at pH = 4.0; v = 10 mV s^{-1}; 25 ^{0}C. These curves differ in the values of the capacitive current, suggesting big alterations in the electrochemically accessible surface area of so modified electrodes. The graphite electrode with adsorbed laccase is characterized with the smallest surface area producing in the same time the largest response in ABTS present. From these findings it could be deduced that the electrode with physisorbed laccase ensures the highest activity of the immobilized enzyme, thus further studies shall be continued with this type of electrode fabrication.

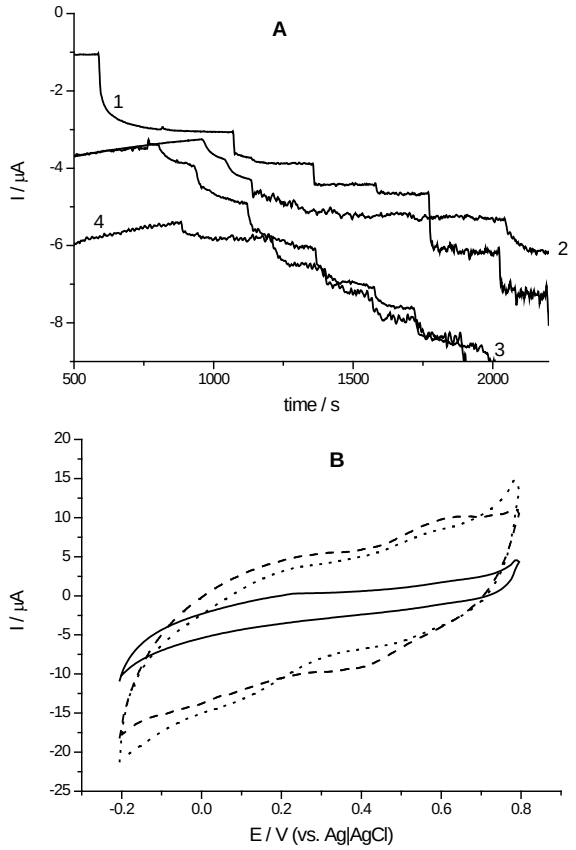


Fig. 1. A) Chronoamperometric record of the laccase electrode response to ABTS in presence of oxygen. Laccase enzyme immobilized through: physisorption (1); Lac adsorbed on chitosan-gold particles composite (2); laccase entrapped in a chitosan layer (3) and sorption on gold- modified graphite (4); pH = 4.0; E = -100 mV; 25 ^{0}C; B) CVs of TP laccase electrodes with the enzyme immobilized through physisorption (solid); sorption on gold-modified graphite (dotted) and laccase adsorbed on chitosan-gold particles composite (dashed); pH = 4.0; v = 10 mV s^{-1}; 25 ^{0}C.

The CVs of graphite electrodes with adsorbed laccase in buffer solution differ considerably from the CV of the bare graphite electrode (not shown). The possibility to record reproducible voltammograms upon continuous cycling of the laccase electrodes suggests that the enzyme adheres firmly to the graphite surface.

In order to evaluate the effect of the buffer composition on laccase electrocatalytic activity, the dependences of the electrode response on the mediator concentration were recorded in 0.05 M citrate and 0.05 M acetate buffer solutions, at E = -100 mV, over the pH range of 4.0 – 5.0). To maintain a constant ionic strength, sodium perchlorate was added to both buffer solutions to a concentration of 0.1 M. On the next figure (Fig. 2A) the calibration plots for ABTS on a laccase electrode are compared in the two buffer solutions. The bioelectrocatalytic activity of the laccase was found a bit higher in the citrate than in the acetate buffer solution. In all cases, the reaction kinetics obeys the mechanism of Michaelis – Menten. As it could be seen from Fig. 2B the current variation upon addition of an aliquot of ABTS is much more distinct at pH 4.5 than at pH 4.0, which motivated us to perform all further studies at the former pH.

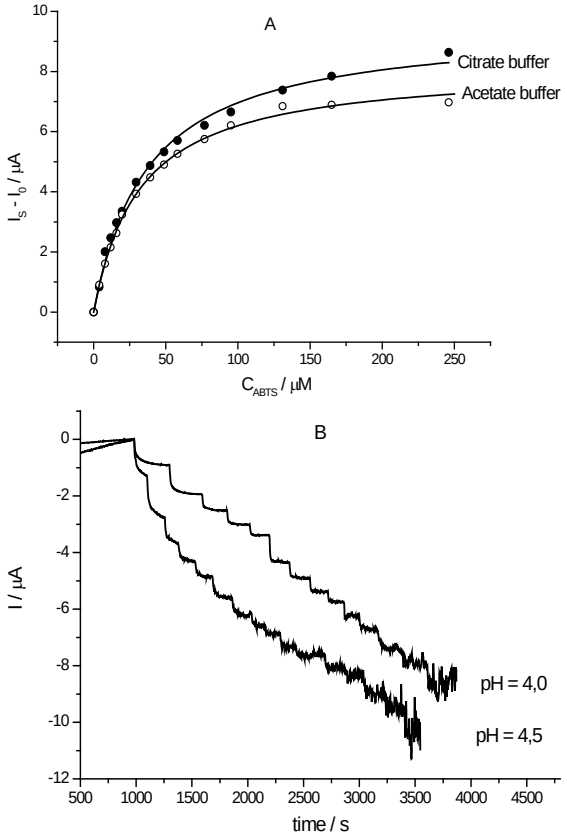


Fig. 2. A) Dependence of the electrode response on ABTS concentration at a constant potential E = -100 mV, pH = 4.0; in citrate buffer and in acetate buffer. B) Chronoamperometric record of the electrode response (with adsorbed laccase) to aliquots of 2 mM ABTS stock solution; air-saturated 0.05 M citrate buffer, pH = 4.0 and pH = 4.5; E = -100 mV; 25 ^{0}C.

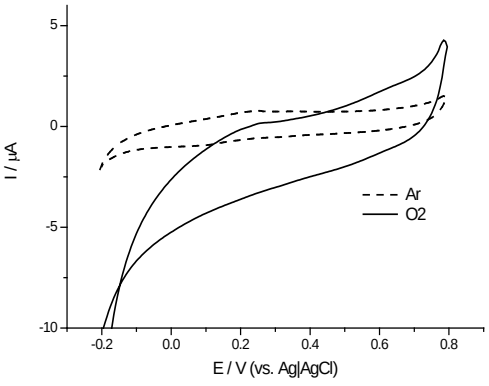


Fig. 3. CVs of a laccase (*Trametes pubescens)* electrode in de-aerated with Ar (dashed) and oxygenated (solid) 0.05 M citrate buffer, containing 0.1 M $NaClO_4$, pH = 4.5; v = 10 mV s^{-1}; 25 ^{0}C.

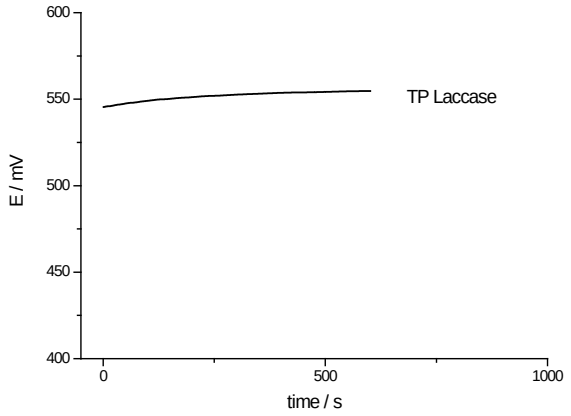
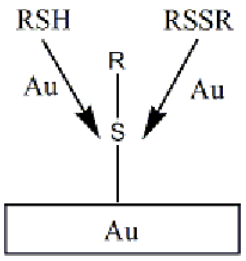


Fig. 4. Open circuit potential of the laccase electrode under oxygenated conditions (0.05 M citrate buffer + 0.1 M $NaClO_4$), pH 4.5.

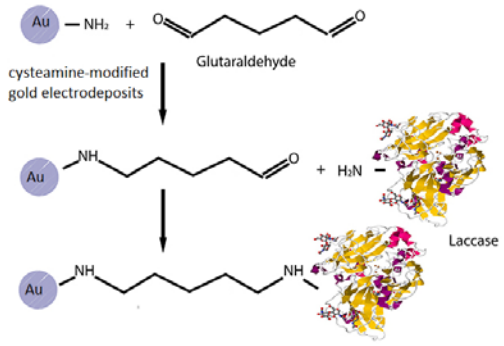
The voltammograms presented on Fig. 3 recorded in de-oxygenated and air-saturated buffer testify that Lac is very efficient in the direct bioelectrocatalytic reduction of O_2. In addition, the open circuit potential (OCP, Fig. 4.) of the latter electrode measured under oxygenated conditions at pH 4.5 was found to be ca. 540 mV, i.e., Lac is a promising electrocatalyst for oxygen reduction.

Unfortunately, the stability of so obtained enzyme electrodes was found fragile – all the attempts to repeatedly use the enzyme electrodes failed. This could be due to the electrode fouling with the products from side reactions, e.g. from mediator oxidation when testing its biocatalytic activity, but could be also a result of rapid enzyme desorption from the graphite surface. Therefore, our further studies were directed towards stabilization of the enzyme layer through covalent attachment of the laccase to electrode surface. For that purpose two types of electrode materials were tested: smooth gold electrode (SGE) and glassy carbon electrode (GCE), modified with thin (below 100 nm thick) electrodeposited gold layer. The availability of gold on the electrode surface is of key importance for the immobilization procedure as its surface can be easily functionalized with sulfur-containing organics [8] using the so-called gold-thiol chemistry, (Scheme 1).



Scheme 1. Surface functionalization of gold surface with sulfur-containing organic compounds – thiols or disulfides.

The formation of cysteamine self-assembled monolayer onto the gold surface plays a double function in this particular immobilization protocol: i) it serves as a tool for spatial orientation of the enzyme upon immobilization: its terminal amino groups are positively charged at the working pH and hence they will attract electrostatically the negatively charged enzyme active site; and ii) it is an anchor to which the enzyme is linked *via* bi-functional reagent (glutaric aldehyde, Scheme 2.).



Scheme 2. Covalent binding of laccase to cysteamine self-assembled on gold using glutaric aldehyde.

Following the already discussed experimental procedure for testing the biocatalytic activity, the responses to ABTS for both types of enzyme electrodes (SGE and gold-modified GCE with covalently attached laccase) were examined. Chronoamperometric records of the response to ABTS at a constant potential of –100 mV (*vs.* Ag|AgCl, 3 M KCl) for the enzyme-bearing SGE under oxygenated conditions show much smaller current variation upon addition of the substrate than for the equivalently prepared laccase electrode based on gold-modified GCE (Fig. 5A).

Comparison of the CVs of the two electrode types (Fig. 5B) reveals that the electrochemically accessible surface area of the SGE is about twice as low as the surface of the gold-modified GCE with covalently immobilized enzyme, whilst in the same time the catalytic current differs more than 10 times (being bigger for the latter electrode type). The obtained much bigger current density with the second electrode type suggesting considerable activity of the immobilized enzyme motivated all further studies to be implemented with this particular type of electrode fabrication.

The record of the open circuit potential with time done in both de-oxygenated and air-saturated buffer, presented on Fig. 6, suggests that covalently immobilized laccase is still an efficient biocatalyst of the direct bioelectrocatalytic reduction of O_2.

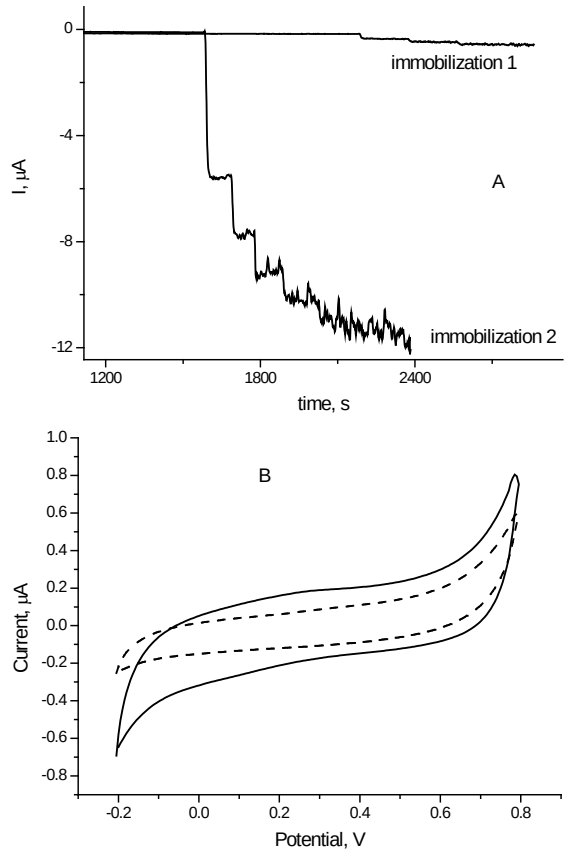


Fig. 5. A) Chronoamperometric record of the laccase electrode response to ABTS in presence of oxygen. Laccase enzyme immobilized covalently on gold (curve 1) and gold-thin film -modified glassy carbon (curve 2); pH = 4.5; E = -100 mV

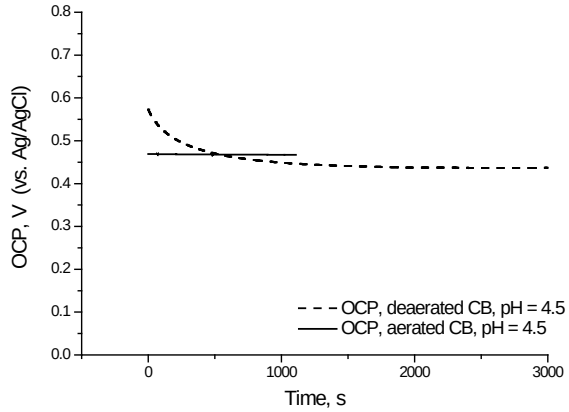


Fig. 6. Open circuit potential of the laccase electrode in oxygenated buffer solution (0.05 M citrate buffer + 0.1 M $NaClO_4$), pH 4.5; partly deaerated buffer (dashed line); continuously aerated buffer (solid line).

Its open circuit potential at pH 4.5 and under oxygenated conditions exceeds 460 mV. The slight decrease in OCP as compared with the value measured with laccase physisorbed on graphite electrode can be assigned to a lesser conformational "comfort" of the enzyme due to the covalent immobilization accompanied by crosslinking with the glutaric aldehyde.

DPVs of laccase electrode prepared freshly and used continuously over 1 week period indicate a gradual decrease in the intensity of the current maximum representing the rate of the electron exchange between the enzyme active site and the electrode surface (not shown). However, the activity of the immobilized Lac remains high enough to ensure proper functioning of the bioelectrode. As depicted in Fig. 7, the normalized *versus* the initial response electrode activity decreases gradually to ca. 30% of the initial one after 21 days (3 weeks) that is more than 21 times increase in operational stability of the immobilized enzyme.

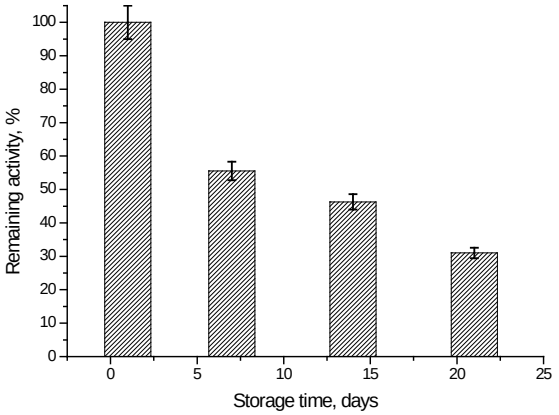


Fig. 7. Remaining activity of the laccase electrode as a function of storage time (days). The remaining activity was normalized *vs.* the response of the freshly prepared electrode.

Summarizing, the present studies show that physical adsorption of laccase on graphite electrodes provides high electrochemical activity of the immobilized enzyme, however, the lifespan of such a bioelectrode is rather short. Alternatively, when covalently immobilized, the enzyme exhibits more than 20 times higher operational stability, manifesting in the same time high enough electrochemical activity. Prospects are to test the so produced biocathode together with a microbial anode operating in a less acidic pH medium and to fully characterize the so constructed biofuel cell.

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