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Mediatorless catalytic oxygen reduction at boron-doped diamond electrodes

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Abstract

Most approaches to electron conduction from electrode to the enzyme requires the use of mediators – molecular relays which can take electrons from the electrode and deliver them to the redox sites of the enzyme. In the present paper, the biocatalytic reduction of oxygen to water in the presence of laccase is shown to proceed on the boron-doped diamond at highly positive potentials and without any additional mediator. The onset of catalytic reduction current appears at 0.805 V vs. NHE in solutions of pH 5.2. Laccase is either dissolved in the solution or trapped on the BDD electrode in a thin film of lipidic cubic phase. The remarkable stability of the modified electrode, avoiding the use of mediators and positive potential of the dioxygen reduction process make the BDD–laccase system especially interesting for applications in electrochemical sensing and microbiofuel cells.

Keywords: Boron-doped diamond; Laccase; Dioxygen reduction; Electrocatalysis; Biocathode

1. Introduction

Direct, unmediated electron transfer (ET) between a redox protein and an electrode has been achieved for several combinations of proteins and electrode surfaces over the past 25 years [1,2]. We have shown recently that monoolein cubic phases are useful as hosting layers for modifying electrodes with selected enzymes [3–5] and also synthetic catalysts [6]. Liquid crystalline phases formed by polar lipids in aqueous media can be used as model matrices to mimic biological proceses [7]. Lyotropic cubic phase have a well-defined and reproducible structure determined e.g. by small angle X-ray spectroscopy [3,8,9]. From the electrochemical point of view it is important that amphiphilic enzyme molecule remains attached to the lipid bilayer, while water channels allow the enzymatic substrates and products to diffuse freely through the system. The advantage of cubic phases is also the more efficient diffusion of hydrophilic probes, comparing to other commonly used molecular matrices [10,11]. Moreover, the cubic phases are viscous, so they can be easily smeared onto solid supports, and they are stable in the presence of excess of water.

One of the interesting redox enzymes is the multicopper oxidase–laccase. It is able to oxidize organic and inorganic substrates with concomitant reduction of oxygen directly to water without formation of reactive oxygen intermediates [12]. This feature makes it together with bilirubin oxidase especially interesting as the biofuel cell catalysts. The active site of the enzyme contains four copper atoms classified in accordance with their spectroscopic characteristics as T1, T2 and T3 sites. The T1 site of the enzyme is involved in binding of substrate, its oxidation, and transferring of the electron to the T2/T3 cluster, where oxygen is reduced to water [12]. Laccases are widely investigated for a variety of practical reasons ranging from use in the pulp and paper industry to their

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possible use in bioremediation, phenolic drug and pollutant analysis, and in organic synthesis. Furthermore, since laccase is electrochemically active on different electrodes and its oxidation is linked to the dioxygen reduction, the enzyme was often employed in the construction of biosensors [13,14] and cathodes for a biofuel cell [15,16].

In the present communication we show the catalytic efficiency of *Cerrena unicolor* laccase towards oxygen reduction on boron-doped diamond (BDD) and glassy carbon electrodes (GCE). It was demonstrated earlier [17,18] that the laccase immobilized through adsorption at various carbon materials (carbon black, activated carbon, pyrographite) may perform a mediatorless (direct bioelectrocatalysis) oxygen electroreduction in weakly acidic solutions. It was pointed out that only a negligible part (10%) of the immobilized enzyme molecules takes part in the bioelectrocatalytic reaction due to unfavorable orientation of most laccase molecules relative to the electrode [19]. The composite materials based on enzymes differ considerably from the electrocatalysts of metallic nature: first, the enzymes show no electronic conductivity; second, the size of biocatalysts is comparable to the size of the other structural units of the composite material. Therefore, in the case of the nanostructured material formation, one should take into account the nature of the carbon carrier (the dispersion degree, type and amount of the functional groups at the carbon material surface), which provide the maximum coverage and favorable spatial orientation of the enzyme molecules relative to the carbon surface. Boron-doped diamond has been studied intensely over the recent years [20–22]. Applications included anodic waste destruction, sonoelectroanalysis, HPLC electrochemical post column detection, and sonoelectrosynthesis. Due to its mechanical and chemical robustness, diamond is superior to many other carbon-based electrode materials, especially under extreme conditions encountered using very positive applied potentials, corrosive solution environments, the presence of power ultrasound, or processes in liquid ammonia. BDD is a relatively new electrode material, and extremely useful for electroanalysis due to the wide working potential window, low background current, favorable electron transfer kinetics and high stability [23–26]. Diamond electrodes are inert chemically, and are thus resistant to poisoning by insulating deposits. The hydrophilicity or hydrophobicity of the electrode can be easily controlled by oxidation or hydrogenation of the surface, to promote interactions with polar or nonpolar proteins, respectively. All of these features are particularly relevant to the problems that are commonly experienced in direct protein electrochemistry. Diamond has good optical transparent properties from the near-UV to the far-IR making it possible to combine electrochemical measurements with UV and IR spectroscopies. Here we show the unique properties of BDD as the electrode substrate for the laccase catalyzed reduction of molecular oxygen.

2. Experimental

All chemicals were of the highest purity available commercially and were used without any purification steps. Water was distilled and passed through Milli-Q purification system.

Electrochemical experiments were performed in three-electrode system with Ag/AgCl (1 M KCl) or SCE as the reference electrode, platinum foil as the counter electrode and boron-doped diamond substrates (BDD, Windsor Scientific, Slough, Berkshire) or glassy carbon electrode (GCE, BAS) as the working electrodes. Cyclic voltammetry experiments were carried out using ECO Chemie Autolab potentiostat. All electrochemical measurements were done at 22 ± 2 °C.

Prior to use in electrochemical experiments, boron-doped diamond electrodes were activated by (1) boiling in concentrated HNO_3 (for 10 min) or (2) cycling the potential in aqueous 1 M HNO_3 between 0 and $+5$ V or (3) cycling the potential in aqueous 1 M HNO_3 between 0 and -3 V vs. Ag/AgCl until stable reproducible following curves were obtained (10 cycles with 0.1 V/s scan rate). Before each experiment, GCE electrode was polished with aluminum oxide powder (grain size down to 0.05 μm) on a wet pad, rinsed with water and ethanol, and then dried at room temperature. After that the working electrode was modified with the cubic phase.

Cubic phases were prepared by mixing monoolein (0.60–0.64 MO weight fraction) and pure water or laccase solution in a small glass vial with a spatula, followed by centrifugation for 30 min at 3000 *g*. Laccase solutions of concentrations ranging from 10 to 50 mg/ml were prepared using pure water, pH 6.0–6.5. After centrifugation, a transparent and highly viscous cubic phase was obtained. BDD and GCE electrodes were modified with cubic phase [27] by spreading it on the electrode surface under a microscope (magnification $10\times$) using a spatula; the layer thickness was adjusted to 2 mm. The electrode modified with the cubic phase was immersed in the deoxygenated supporting electrolyte and was kept in it for 20 min before each experiment to equilibrate gas concentration between the cubic phase and the solution.

3. Results and discussion

In order to use laccase for catalytic reduction of oxygen usually mediators either soluble or bound to the electrode surface are required. Without them, at unmodified GCE the reduction of oxygen proceeds with large overpotential (at potentials ca. -0.6 V) both in the absence and presence of laccase in the solution. However, as shown in Fig. 1 when BDD is used as the electrode substrate a catalytic signal of oxygen reduction is seen in the presence of laccase in the solution. The reduction of oxygen starts at a very positive potential ca. $+0.605$ V vs. Ag/AgCl electrode that is $+0.805$ V vs. NHE. The $E_{1/2}$ of the catalytic wave is 0.380 V vs. Ag/AgCl (0.580 V vs. NHE). Such

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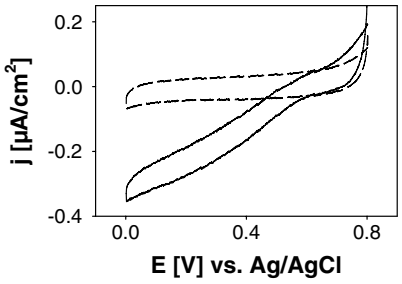

Fig. 1. Voltammograms recorded in (——) oxygen saturated and (- - -) deoxygenated McIlvaine buffer solution (pH 5.2) containing 0.047 mg/ml laccase using BDD electrode, scan rate: 0.001 V/s.

catalytic O_2 wave, at similarly positive potentials can be seen without mediators only on specially structured carbon surfaces e.g. superdispersed colloid graphite or acetylene carbon black as demonstrated in Tarasevich et al. papers [17–19,28]. In order to possess these favourable properties BDD has to be pretreated in a special way. Following the procedure used by Marken et al. [25] for the studies of direct cytochrome c electrochemistry at BDD we checked several surface preparation procedures to find the best performance of the BDD surface for the catalytic reduction of oxygen. As shown in Fig. 2, the catalytic activity of laccase towards electroreduction of oxygen is exhibited when the BDD electrode is activated by cyclic polarization in the range 0–5 V in 1 M HNO_3.

Using laccase for practical applications requires that the enzyme is active, stable and kept at high concentration in close vicinity of the electrode surface. Our strategy for stable laccase immobilization is to use the lipid liquid crystalline matrix with cubic symmetry. In these experiments two types of carbon materials were employed: glassy carbon electrode and boron-doped diamond. Typical cyclic voltammograms obtained in oxygenated and deoxygenated McIlvaine buffer solution (pH 5.2) using BDD and GCE covered with thin film of cubic phase containing laccase are compared in Fig. 3. Fig. 3 shows another feature of laccase as the oxygen reduction catalyst. In the presence of fluorides in the solution (Fig. 3a – dotted line) the enzyme

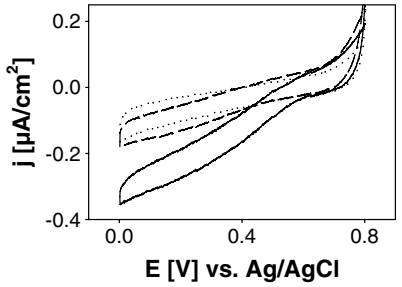

Fig. 2. Voltammograms recorded in oxygen saturated McIlvaine buffer solution (pH 5.2) containing 0.047 mg/ml laccase using BDD electrode activated by (·····) boiling in concentrated HNO_3, (- - -) cycling the potential in aqueous 1 M HNO_3 between 0 and −3 V and (——) cycling the potential in aqueous 1 M HNO_3 between 0 and +5 V, scan rate: 0.001 V/s.

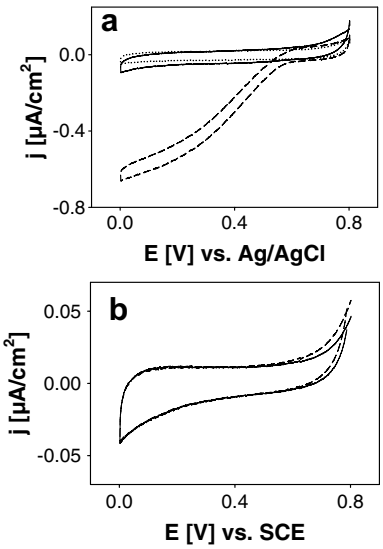

Fig. 3. Voltammograms recorded in (——) deoxygenated and (- - -) oxygen saturated McIlvaine buffer solution (pH 5.2) using: (a) BDD and (b) GCE electrode modified with cubic phase containing laccase. (·····) Voltammogram recorded in oxygen saturated McIlvaine buffer solution (pH 5.2) using BDD electrode modified with cubic phase containing laccase in the presence of 10 mM F^- in the solution, scan rate: 0.001 V/s.

is completely inhibited and no catalytic oxygen reduction at 0.620 V is seen. The curve almost retraces that recorded in the absence of laccase confirming the role of laccase in the process of oxygen reduction.

In the presence of laccase in the cubic phase mediatorless reduction of oxygen is seen using BDD as the electrode substrate contrary to using GCE as the electrode (Fig. 3b). Mediatorless reduction of oxygen was reported earlier using more complex carbon materials e.g. superdispersed colloid graphite, acetylene carbon black (pH 4.0) or glassy carbon electrode modified with nanotubes (pH 6.0) [17–19,28,29]. It should be stressed that without laccase no reduction of oxygen was seen in the mentioned range of potentials as shown for BDD in Fig. 4.

Various pretreatment procedures, including chromic acid activation were tried for GCE but without results-no oxygen reduction at potentials positive to 0 V was seen. Efficient reduction of oxygen on GCE modified with cubic phase containing laccase required the use of media-

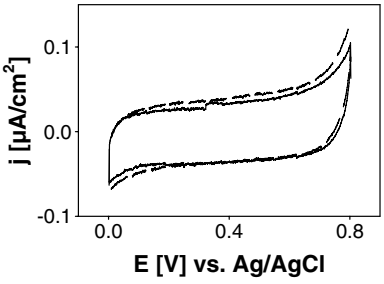

Fig. 4. Voltammograms recorded using BDD electrodes modified with cubic phase in (——) deoxygenated, (- - -) oxygen saturated McIlvaine buffer solution (pH 5.2), scan rate: 0.001 V/s.

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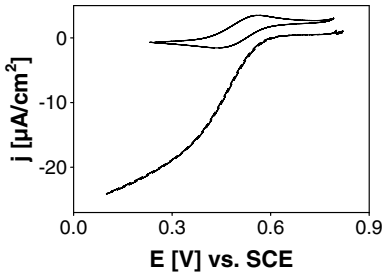

Fig. 5. Voltammograms recorded using BDD electrode modified with cubic phase containing laccase in (——) deoxygenated, (- - -) oxygen saturated McIlvaine buffer solution (pH 5.2) with 0.5 mM $ABTS^{2-}$, scan rate: 0.001 V/s.

tor e.g. hydroquinone or $ABTS^{2-}$ (2,2′-azino-bis-3-ethylbenzothiazoline-6-sulfonate) as shown elsewhere [5,6,27]. On the other hand, using these mediators was reported to lead to major instability and fast deactivation of the biocatalyst [30]. Fig. 5 demonstrates the efficient oxygen reduction catalyzed by laccase in the cubic phase film in the presence of $ABTS^{2-}$. Reversible cyclic voltammogram of $ABTS^{2-}$ in the cubic phase containing laccase is also shown in Fig. 5. In the presence of $ABTS^{2-}$ and laccase in the cubic phase film the oxygen reduction wave appears at the formal potential of the $ABTS/ABTS^{2-}$ mediator system thus less positive than without the mediator and the catalytic current densities are similar for the GCE and BDD.

4. Conclusions

High catalytic activity of laccase on BDD electrode is due to activation of boron-doped diamond electrodes surface by cycling the potential in aqueous 1 M HNO_3 between 0 and +5 V vs. Ag/AgCl. The anodic polarization of BDD surface causes the addition of surface oxygen, making the surface more and more hydrophilic. On the other hand GCE cannot be pretreated in order to show efficient catalysis of oxygen reduction by laccase without the addition of appropriate mediator. Activated BDD is hence a highly active composite material providing the appropriate contact of the enzyme molecules with the electrode surface and leading to direct bioelectrocatalysis of the dioxygen reduction. Hence, BDD possesses unique structural and electronic properties that are very different from other common carbon materials. The cubic phase provides a convenient biocompatible matrix to hold the enzyme in the close vicinity of the electrode. The facilitated electron transfer at the laccase modified BDD electrodes and the mediatorless catalyzed reduction of O_2 proceeding at a positive potential close to the laccase formal potential points to the utility of the BDD based system as the cathode in oxygen monitoring devices and biofuel cells.

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