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Electroreduction of laccase covalently bound to organothiol monolayers on gold electrodes

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Abstract

Cerrena unicolor laccase was immobilized on the gold electrode by covalent bonding to self-assembled monolayers of mercaptoundecanoic or mercaptopropionic acids. STM images of immobilized laccase proved high population of the laccase molecules on the monolayer modified electrode. The SERS experiments in concert with resonance Raman effect confirmed that the structure at the "blue" copper site of the immobilized protein remained intact. The accessibility of individual copper sites for electron exchange with the gold electrode surface was investigated by voltammetry. The electrode behavior of laccase is different in the presence and absence of oxygen, showing that the immobilized enzyme is reactive towards oxygen. Addition of two common mediators improved the electrical connectivity of the enzyme with the electrode, increased the catalytic efficiency of immobilized laccase and switched the onset of catalytic current to the potentials of the mediator. Immobilization of laccase on well-organized mercaptoundecanoic acid separates efficiently the enzyme from the electrode and does not allow easy access of mediators to the surface. Attachment of the enzyme at smaller distance from the electrode by means of significantly shorter spacer molecule—mercaptopropionic acid improved the efficiency of catalytic reduction of oxygen on the monolayer modified electrode.
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1. Introduction

Laccases, EC 1.10.3.2, *p*-diphenol: dioxygen oxidoreductase, are a part of a larger group of multicopper enzymes, including, e.g. ascorbic acid oxidase and ceruloplasmin. Laccase was first described by Yoshida [1], and was characterized as a metal containing oxidase by Bertrand [2]. Laccases are widely distributed in nature being produced by plants, fungi and insects [3]. Laccases of plant origin are reported to play an important role in wound response and lignin synthesis [4] or degradation [5]. In addition to the strictly biological functions, laccases are being investigated for a variety of practical applications, i.e. electrocatalytic reduction of dioxygen [6–9] and oxidation of phenolic substrates [10–12].

Laccase is capable of oxidizing phenols by reducing molecular oxygen to water by a multicopper system. The enzyme active site contains four copper atoms of types I (blue copper), II, and III, which play different roles in the enzymatic process. Substrates are oxidized at the T1 site, and the electrons are transferred to the T2/T3 cluster, where molecular oxygen is reduced [3,13]. A fascinating character of the direct four-electron reduction of oxygen to water is another important application of laccase in the cathode compartment of biofuel cells [14–25].

It has been recognized for many years that the enzymatic reduction of oxygen to water could provide an alternative to noble metal electrocatalysis for fuel cell cathodes. Unfortunately, many redox enzymes do not take part in direct electron transfer with conductive supports, and therefore a variety of electron mediators are used for the electrical wiring of the biocatalyst to the electrode [26]. An important goal for biotechnology is to understand the electrochemical reactions of redox proteins and control their interactions with metal substrates. One of the crucial steps in the development of electrodes for biofuel cells would

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be the appropriate immobilization since its biological activity as well as electrical contact have to be preserved [27–32]. This goal has been achieved by covalently attaching the protein to an electrode through a self-assembled monolayer, thereby preventing diffusion of the protein away from the electrode. Long-range electron-transfer processes through such monolayers are also well known [33,34].

The aim of this paper is to study the electron transfer in a biomolecular assembly consisting of laccase molecules covalently bound to gold surface by means of alkanethiol monolayer. The procedure of covalent immobilization of laccase used in this work is similar to reported earlier [35–38]. Properties of electrodes modified with *Cerrena unicolor* laccase attached monolayers of 11-mercaptoundecanoic acid and mercaptopropionic acid are compared. STM images and SERS spectra of immobilized laccase prove high population of the laccase molecules on the monolayer modified electrode and native structure of the enzyme. The electrode behavior of laccase is different in the presence and absence of oxygen. Addition of common mediators and using shorter spacer organothiol molecules lead to improvement of the catalytic efficiency of immobilized laccase.

2. Experimental section

2.1. Reagents

C. unicolor C-139 was obtained from the culture collection of the Regensberg University and deposited in the fungal collection of the Department of Biochemistry (Maria Curie-Sklodowska University, Poland) under the strain number 139. Laccase from the fermentor scale cultivation was obtained according to already reported procedure after ion exchange chromatography on DEAE-Sepharose (fast flow) [39] and lyophilized on Labconco (Kansas City, USA, FreeZone Lyophilizer). Enzyme activity was measured spectrophotometrically with syringaldazine as the substrate for laccase [40]. The protein content was determined according to Bradford with bovine albumine as the standard [41]. The concentration of isolated and frozen (−18 °C) enzyme was C_{lacc} = 178 μg cm^{-3} and activity 186,000 nkat dm^{-3}. After lyophilizing the laccase activity dissolved in 1 ml of water was 1,150,110 nkat dm^{-3} and C_{lacc} = 1.18 mg cm^{-3}.

1,1′-Ferrocenedimethanol, trifluoroacetic acid, 11-mercaptoundecanoic acid (MUA), 3-mercaptopropionic acid (MPA), 3,4-dimethoxyphenolic acid, 1-ethyl-3-(3-dimethyl-aminopropyl) carbodiimide (EDC), *N*-hydroxysucinimide (NHS), 2,2′-azino-bis-3-ethylbenzothiazoline-6-sulfonate (ABTS) were purchased from Sigma. Na_2HPO_4, and citrate acid were from POCh. All reagents were analytical grade and used as received. Ultra pure water was obtained from a Milli Q purification system (nominal resistivity of 18.2 MΩ cm^{-3} at 25 °C) and used to prepare all the stock solutions.

2.2. Electrode preparation

Gold of 200 nm evaporated on glass with a pre-layer of 2–4 nm of chromium (Gold Arrandee, Gmbh) were used in all the experiments as the electrode material. The monolayers were self-assembled on gold substrates. Before the monolayer deposition, the substrates were flame annealed and then cooled to room temperature. Such a pretreatment results in the formation of atomically flat Au (1 1 1) terraces. In our case the width of the terraces was in the range of 100–200 nm. After this procedure gold samples were transferred to the coating solution. In order to avoid the formation of bilayers, the adsorption of the 11-mercaptoundecanoic acid was carried out from 1 mM ethanol solution, which contained 2% (v/v) of trifluoroacetic acid [42]. Gold samples were soaked for 24 h and then modified substrates were rinsed with ethanol and water.

Subsequently, the modified substrates were used as a matrix for laccase immobilization. Although laccase can electrostatically interact with thiol molecules terminated with $–NH_2$ or –COOH groups, in order to increase the number of immobilized molecules as well as to improve the stability of such system we bound the enzyme to the electrode covalently. Laccase was immobilized by formation of amide bond between amino group from the enzyme and carboxylic group from the 11-mercaptoundecanoic or mercaptopropionic acids. In order to activate the terminal carboxylic groups the electrode was immersed in aqueous solution containing 75 mM EDC and 25 mM NHS for 30 min. We checked that this kind of activation of carboxylic group does not affect the monolayer structure itself. After rinsing with water and drying in argon, the electrode was placed in 2.5 cm^3 McIlvaine buffer solution of pH 4.8 containing 400 μg laccase and left at 4 °C for a whole night. After rinsing, the electrode was immersed in phosphate buffer solution to remove the unbound enzyme, dried and used in the electrochemical experiment.

2.3. Electrochemistry

Cyclic voltammetry were performed using the CHI 750B potentiostat (CH Instrument, Austin, USA) in a three-electrode arrangement, with a saturated Ag/AgCl electrode as the reference electrode and platinum foil as the counter electrode. The working electrodes were the gold electrodes (Arrandee), modified according to procedures described above. All measurements were performed in McIlvaine buffer solution (0.1 M citric acid + 0.2 M NaH_2PO_4) at pH 5.2, degassed with pure argon or saturated with medical oxygen.

2.4. Scanning tunneling microscopy

STM experiments were performed using Nanoscope IIIa (Digital Instruments, Santa Barbara, CA) equipped with low current converter. We used commercially available Pt–Ir tips (Nanotips, Santa Barbara, CA). All images were taken in air at ambient conditions.

2.5. Raman spectroscopy

Raman experiments with laccase immobilized on the Au surface modified by 3-mercaptopropionic acid (3-MPA) monolayers were performed. Before thiol attachment, the gold surface was electrochemically roughened to obtain a sufficiently

enhanced intensity of the SERS bands. The gold electrode was roughened in a separate electrochemical cell by applying the successive positive–negative cycles in 0.1 M KCl aqueous solution from −0.6 to +1.2 V versus SCE at a sweep rate of 0.5 V s^{-1}. Self-assembled monolayers of 3-MPA were obtained by immersion of the substrates for 3 h into 10 mM solution of thiols, buffered to pH 5.2. In order to activate the carboxyl groups of the deposited 3-MPA a solution of EDC/NHS was slowly injected onto the thiol-coated gold surface. After 20 min, the substrates were immersed into the solution containing laccase, pH 5.2. The surfaces coated with laccase, were washed with buffer solution, dried and immediately used in Raman experiment with the use of confocal Raman microscope. Resonance Raman (RR) spectra of laccase solutions were recorded with a Jobin–Yvon Raman spectrometer equipped with a Kaiser holographic notch filter, 600 grooves mm^{-1} holographic grating, 1024 × 256 pixels nitrogen cooled CCD detector, and an Olympus BX40 microscope with a 50× long-distance objective. A Laser-Tech model LJ-800 mixed Ar/Kr laser provided excitation radiation of 647.1 nm or 514.5 nm.

3. Results and discussion

3.1. STM results

The STM images shown in Fig. 1 present the monolayer-modified substrates obtained according to above procedure. Dark features visible on both images are single-atom deep Au vacancy islands, which are indicative for the adsorption of thiol molecules on gold surface. Molecular resolution image indicates that 11-mercaptoundecanoic acid forms well-ordered assembly with the distance between the neighboring molecules being equal 5 ± 1 Å. It is also evident that self-assembly procedure used here leads to formation of single molecular layer of 11-mercaptoundecanoic acid since the features characteristic for bilayer were not observed at the STM images [42].

Fig. 2 shows the STM images taken over two regions with different surface concentration of laccase. Bright spots represent single laccase molecules immobilized on the top of 11-mercaptoundecanoic acid monolayer. It is evident that the laccase is not evenly distributed and its surface coverage varies in different regions. Obviously this may be caused by short time of incubation of the substrates in laccase solution. We also found that at low coverage regions the enzyme is very often immobilized at the edges of the gold terraces (see Fig. 2B). This observation suggests that the disordered parts of 11-MUA monolayer are favored during the immobilization process. Fig. 3 represents the STM image of the sample, which was incubated in laccase solution overnight. In this case we have observed higher surface density of immobilized molecules on the surface, which proves that longer immersion time leads to higher surface coverage and more compact structure of the enzyme overlayer. The size distribution of laccase molecules immobilized on the surface is shown in Fig. 4. We found that the diameter of the highest population of the molecules was 6.5 nm, which is close to the diameter of laccase reported in the literature (5 nm) [43,44]. Small difference may be caused by the tip-broadening effect.

STM images of mercaptopropionic acid were also obtained. Substrates used in the experiment were prepared following the aforementioned procedure. Fig. 5 A demonstrates the image of gold electrode modified with the densely packed MPA monolayer. Single-atom deep Au vacancy islands characteristic for the thiols can be seen. Formation of bilayers was not observed.

Image taken after an overnight incubation of the modified MPA sample in laccase *C. unicolor* is shown in Fig. 5B. The enzyme shows a tendency to form regular linear aggregates. The average width of the linear features is 6.5 nm, which is very close to the size of the single protein. We have also found that in some regions immobilized laccase generates irregular aggregated forms. According to their size, between 13 and 35 nm, we can conclude that the protein aggregates contain from two to six molecules.

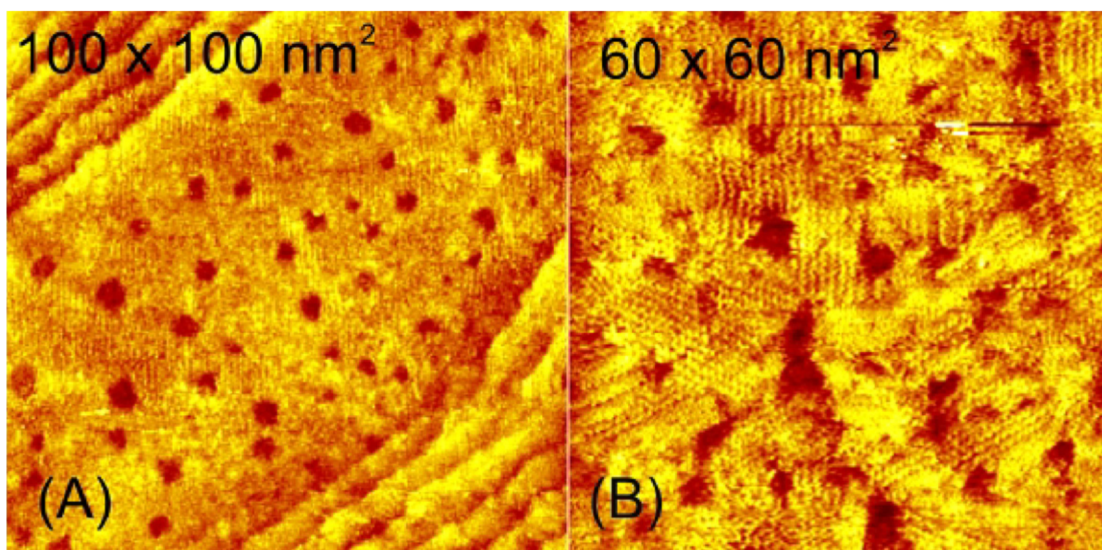

Fig. 1. STM images of 11-mercaptoundecanoic acid monolayer adsorbed on gold. The imaging conditions: tunneling current 10 pA, bias voltage 1 V.

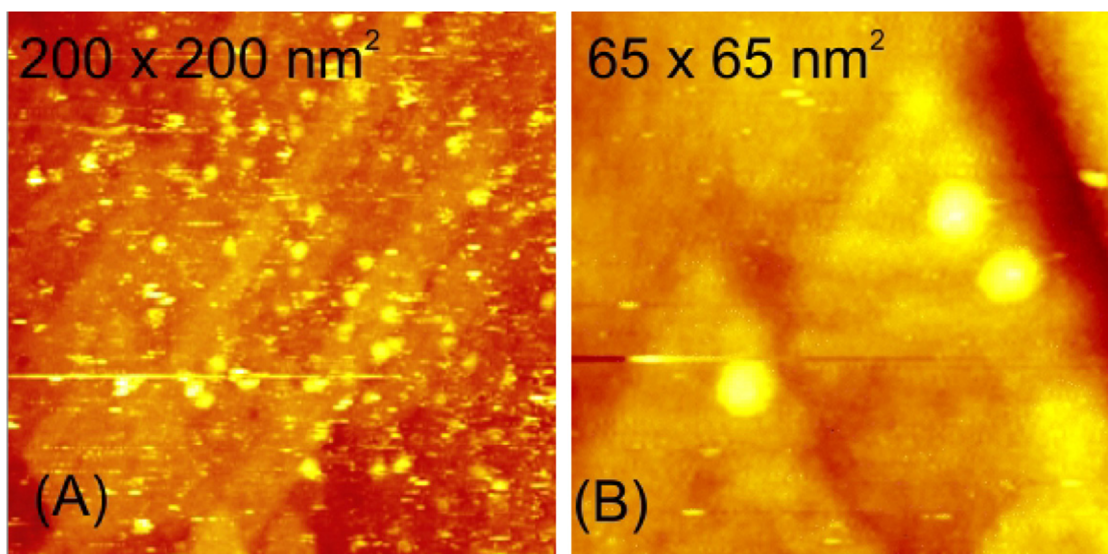

Fig. 2. STM images of *Cerrena unicolor* laccase immobilized on 11-mercaptoundecanoic acid monolayer on gold. The incubation time in the laccase solution was 30 min. The imaging conditions: tunneling current 1.5 pA, bias voltage 1 V.

3.2. Raman spectroscopy experiments

Absorbance spectra of *C. unicolor* laccase exhibits electronic band, typical for blue copper proteins, centred near 610 nm [45]. A 647.1 nm laser line lies within this broad band, quite close to its maximum. Thus, excitation of the Raman spectrum of this laccase at λ = 647.1 nm, provides resonance enhancement of the Raman scattering. For non-blue laccases, a strong fluorescence background in the Raman spectrum and lack of the resonance enhancement results in very poor quality of the spectra. The resonance Raman (RR) spectrum of native laccase sample shown in Fig. 6 is characterized by two intense bands at 384 and 405 cm^{-1}, a band of moderate intensity at 263, 332 and 468 cm^{-1}. This spectrum generally agrees with that reported for tree laccase (*Rhus vernicifera*), differing in relative intensities of some bands, particularly in the intensity of the 420 cm^{-1} band reported by Nestor et al. [46]. This band in our spectrum is very weak. As follows from the literature data assignment of the spectrum reproduced in Fig. 6 is unequivocal [45,46]. A medium-intensity band near 263 cm^{-1} was assigned to Cu–S (cysteine) [45] or to Cu–N (imidazole) stretching vibrations [46], while the bands in the 400 cm^{-1} region were attributed to the Cu–N stretching modes (from a peptide bond or an amide side chain) [45] or to ν(Cu–S) (cysteine) vibration with contribution of the cysteine torsional motion [46]. High relative intensity of the 384 and 405 cm^{-1} bands in the RR spectrum of laccase in solution points out to the presence of T2 copper, since removal

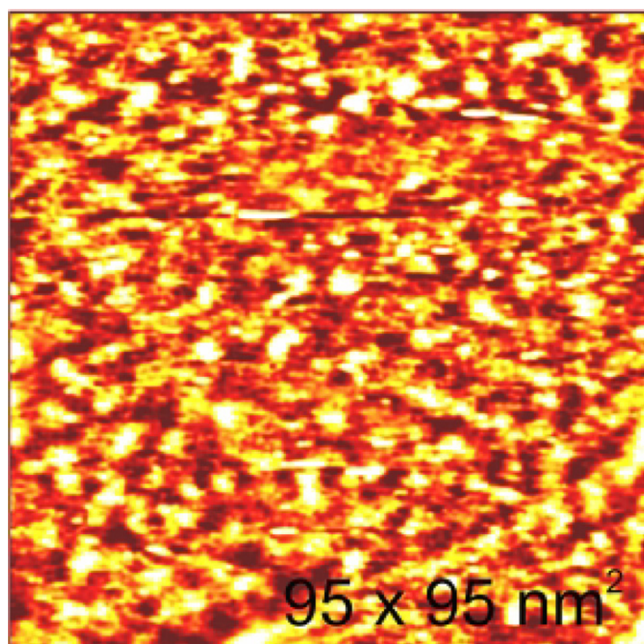

Fig. 3. STM images of *C. unicolor* laccase immobilized on 11-mercaptoundecanoic acid monolayer on gold. The sample was incubated in laccase solution overnight. The imaging conditions: tunneling current 0.5 pA, bias voltage 1.2 V.

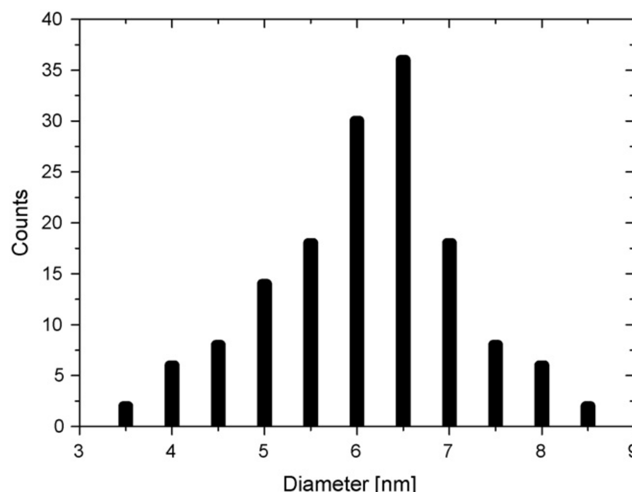

Fig. 4. The distribution of the laccase diameters found from STM images.

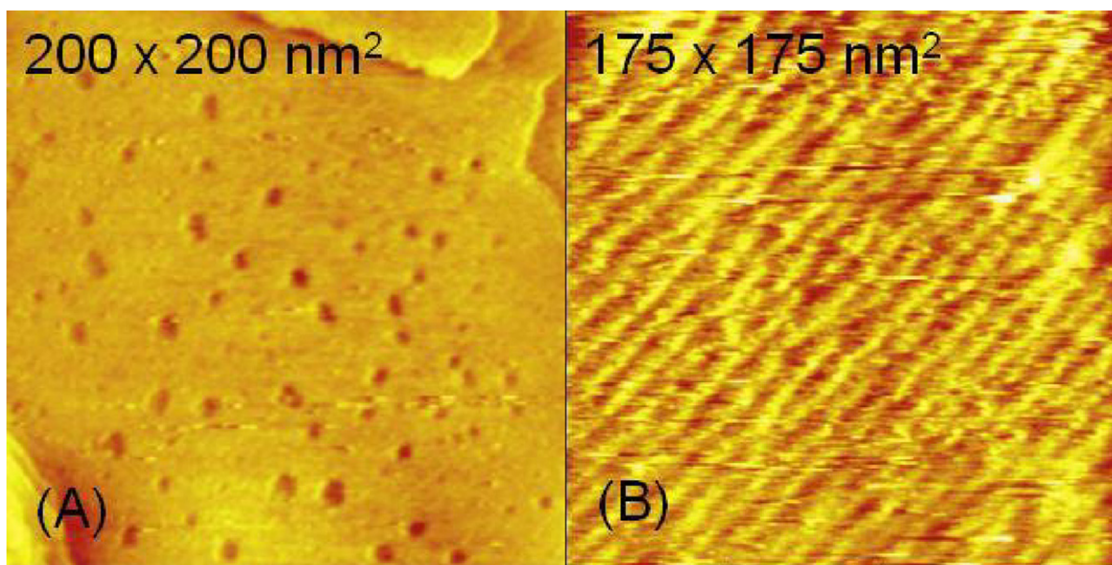

Fig. 5. (A) STM image of 3-mercaptopropionic acid monolayer adsorbed on gold. The imaging conditions: tunneling current 3 pA, bias voltage 0.7 V, $\Delta z = 2$ nm; (B) STM image of *C. unicolor* laccase immobilized on 3-mercaptopropionic acid monolayer on gold. The sample was incubated in laccase solution overnight. The imaging conditions: tunneling current 0.35 pA, bias voltage 1.2 V, $\Delta z = 5$ nm.

of the type 2 Cu^{II} considerably reduces the intensities of these bands [46].

3.3. Laccase immobilized on thiol modified Au surface

Fig. 7 (spectrum A) shows typical SERS spectrum within the range of 200–1550 cm^{-1} of 3-MPA monolayer formed on the Au surface from 10 mM MPA buffer solution at pH 5.2 for 3 h. Two Raman bands at 645 and 724 cm^{-1} are attributed to the C–S stretching vibrations of adsorbed 3-MPA molecules corresponding to gauche and trans conformers of MPA, respectively [47–50]. Strong band at 935 cm^{-1} is due to the C–COO^- stretching vibration of the MPA molecules containing deprotonated carboxylic groups [50].

In the Raman spectrum (Fig. 7, spectrum B) recorded after immobilization of laccase onto the MPA monolayer several new bands appeared (at 270, 302, 326, 394, 402 and 464 cm^{-1}) typical for laccase, clearly indicating immobilization of laccase on the surface. The general similarity of the SERRS (surface enhanced resonance Raman scattering) spectrum of the surface-bound laccase to the RR spectrum of the bulk sample of the enzyme suggests that the structure of the "blue" copper sites in

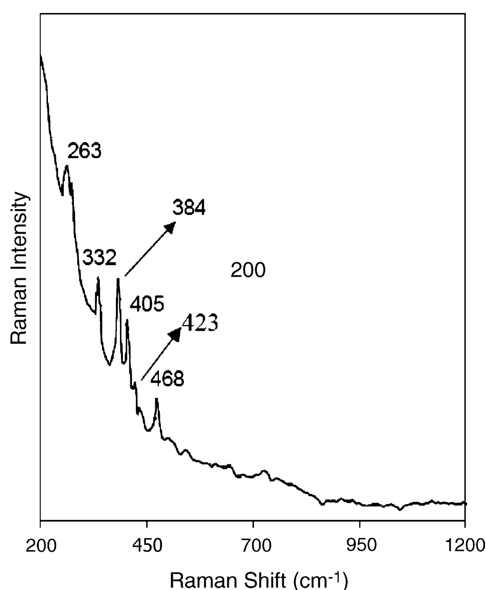

Fig. 6. Resonance Raman spectrum of native *C. unicolor* laccase in aqueous solution, pH 5.2.

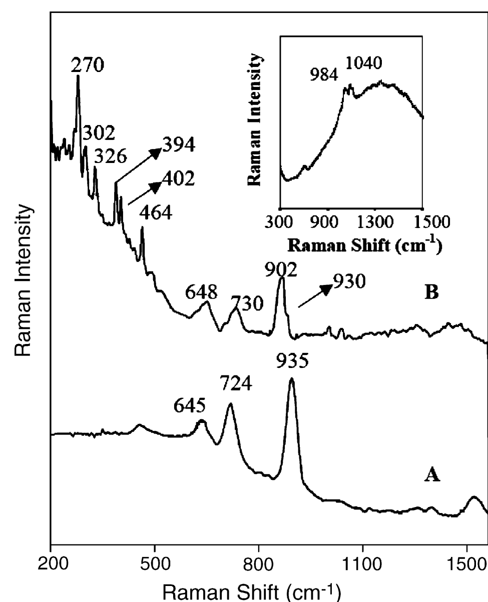

Fig. 7. (A) SERS spectrum of MPA on the Au in buffer solution at pH 5.2. (B) SERS spectrum after immobilization of laccase on the MPA monolayer. *Inset*: resonance Raman spectrum of syringaldazine in contact with laccase immobilized on MPA monolayer.

the immobilized enzyme is conserved. The small changes of the band frequencies ascribed to the mixed Cu–N and Cu–S stretching vibrations are caused by rather minor changes in geometry in the immediate vicinity of the active copper sites. Simultaneously, some changes in the SERS spectrum of MPA monolayer are observed, namely the band corresponding to $C–COO^-$ stretching vibrations at $935\,cm^{-1}$ practically disappear pointing out to binding of laccase to MPA molecules. Instead, the $902\,cm^{-1}$ band due to the C–COOH stretching vibrations appears indicating protonation of the carboxylic groups of MPA molecules, probably at least in part, unbonded with laccase.

Enzymatic activity of the surface immobilized laccase was checked by recording resonance Raman (RR) spectra, of the coloured product (red) of oxidation of syringaldazine by laccase in the presence of molecular oxygen. A visible spectrum of the oxidized syringaldazine (quinone methide) exhibits strong absorption band with maximum about 530 nm. Thus, the resonance Raman effect for this compound may by observed for 514.5 nm excitation line. With this excitation the bands corresponding to both MPA adlayer on Au and immobilized laccase are not enhanced, thus we are able to observe only the RR spectrum of oxidized syringaldazine. This simple method that allows monitoring bioactivity of laccase immobilized on thiol-coated metal supports was recently developed in our laboratory [51]. Therefore, to test enzymatic activity of laccase, the 0.5 mM ethanol solution of syringaldazine was dropped onto the surface bearing the immobilized enzyme, thoroughly washed with water. The RR spectrum was recorded with confocal Raman microscope by focusing the laser beam on the surface of the molecular film. As can be seen in Fig. 7 (inset) two new bands characteristic for quinone methide appeared at 984 and about $1040\,cm^{-1}$, thus confirming that laccase immobilized on the surface retained its enzymatic activity. These experiments were done using MPA modified electrode since in case of much longer MUA spacer the distance of laccase from the electrode surface was too large which affected the quality of the spectra.

3.4. Electrochemical results

Comparison of the voltammetric curves for the electrodes covered with mercaptoundecanoic acid (MUA)–laccase monolayer in deaerated and oxygen saturated solutions Fig. 8 reveals the presence of poorly developed c1/a1 couple of peaks ascribed to the few laccase molecules in direct electrical contact with the electrode. In the absence of oxygen the potentials of c1 and a1 peaks are ca. 0.02 V (0.22 V versus NHE) and 0.25 V (0.45 V versus NHE), respectively. On the other hand, the large peak-to-peak separation indicates that under these conditions the electron transfer reaction is irreversible. The c1/a1 peaks increase linearly with scan rate proving surface character of this reaction. The voltammetric peaks become much better developed and much more reversible in the presence of oxygen (Fig. 8, solid line). In oxygen saturated solution the reduction of laccase appears at 0.110 V (0.310 V versus NHE) thus at a potential 90 mV more positive (peak c1′) than under anaerobic conditions. The corresponding a1′ appears at 0.156 V (0.356 V versus NHE) and

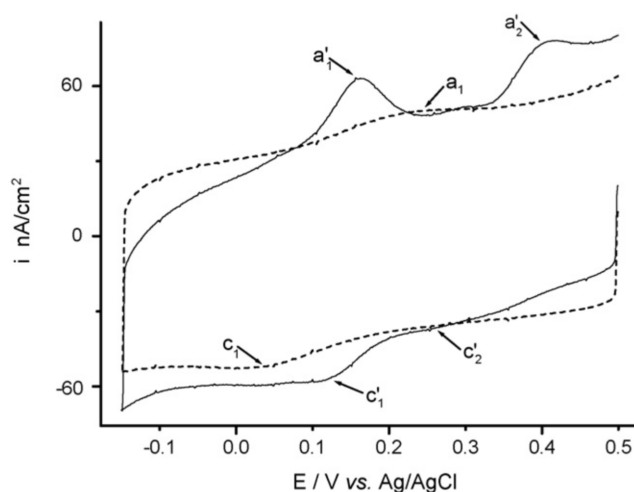

Fig. 8. Comparison of cyclic voltammograms for gold electrode modified with MUA with *C. unicolor* laccase attached in deoxygenated (dashed line) and oxygen saturated (solid line) McIlvaine buffer solution pH 5.2. Scan rate $20\,mV\,s^{-1}$.

is much higher. In addition, a new system of peaks is formed at much more positive potentials. The peak c2′ at 0.3 V (0.5 V versus NHE) would correspond to the reduction of a different copper center—probably the T1 center. Clearly, oxygen makes the reduction at T2 easier and opens the access of electrons to the T1 center. Thus in the presence of oxygen, laccase immobilized on the MUA monolayer becomes electroactive and “visible” in the voltammetric experiment. Electrocatalytic properties of laccase can be seen by the wave-shape of the c1′ signal at low scan rates. At the T_2 center reduction of oxygen to water takes place [20,22,32]. The shift of the cathodic c1′ peak to less negative potential reveals that chemical reaction follows the electrode process. Reoxidation peak is seen without any mediator at 0.6 V versus NHE. On the other hand using MPA as the spacer the electrode is not blocked efficiently and at these positive potentials the laccase voltammetric peaks are overlapped by the final gold oxidation current.

In order to increase the catalytic efficiency of the monolayer immobilized laccase, a common mediator has been added to the solution in order to help in the regeneration of the reduced Cu^+ form of the enzyme. ABTS is chosen to make the electron transfer at the T1 site more efficient. When ABTS is added in small concentration, 1×10^{-6} M, the system of peaks ascribed to the mediator appears at $E_{pa} = 0.461$ V and $E_{pc} = 0.356$ V, in agreement with the literature data. The catalytic reduction of oxygen starts in the region of ABTS processes revealing communication of the laccase centers. In the absence of laccase, no increase of current in the ABTS electroactivity region is observed. With decreasing scan rate the oxygen catalytic reduction current is higher and the onset of current appears at much more positive potentials than that of the c1′ peak.

Using MPA instead of MUA improves the efficiency of the catalytic oxygen reduction process. Enhancement of the catalytic current in this case was $i_{cat}/i_{diff} = 7.1$ in the presence of 5×10^{-6} M ABTS in the solution (Fig. 9). Clearly shorter spacer improves the electrical contact between the active site of the enzyme and the gold substrate.

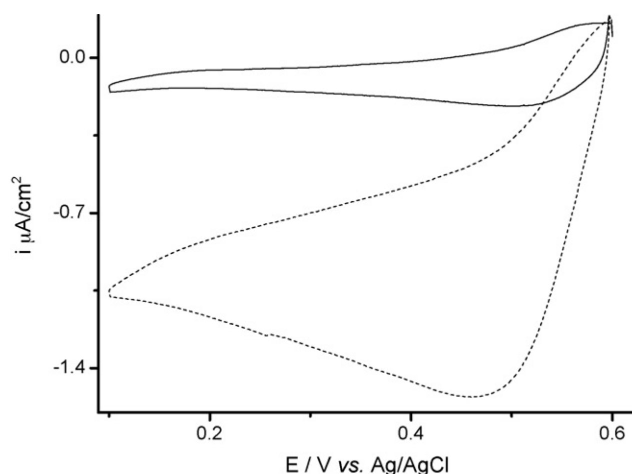

Fig. 9. Voltammograms recorded using Au electrode modified with MPA and *C. unicolor* laccase in McIlvaine buffer solution pH 5.2 containing 5×10^{-6} M ABTS. Solution deoxygenated with argon (solid line) and saturated with oxygen (broken line). Scan rate: 1 mV s^{-1}.

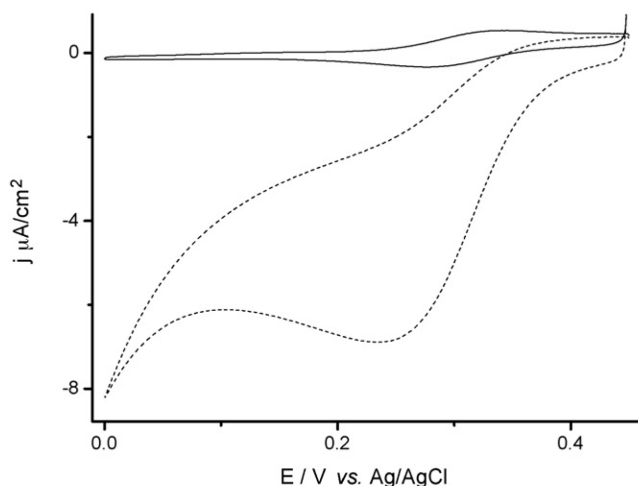

Fig. 10. Voltammograms recorded using Au electrode modified with MPA and *C. unicolor* laccase in McIlvaine buffer solution pH 5.2 containing 10^{-5} M 1,1′-ferrocenedimethanol. Solution deoxygenated with argon (solid line) and saturated with oxygen (broken line). Scan rate: 1 mV s^{-1}.

The onset of the oxygen reduction current can be shifted by changing the mediator. Curve 1 (solid line) on Fig. 10 is recorded using the laccase modified electrode in the deoxygenated solution containing 10^{-5} M 1,1′-ferrocenedimethanol as the mediator. Electrode processes of 1,1′-ferrocenedimethanol take place at potentials ca. 0.25 V. In solutions saturated with oxygen, i_{cat}/i_{diff} ratio was 20.

4. Conclusions

The mechanism of mediatorless electrode processes of a different type of laccase *Trametes hirsuta* (with formal potential equal to 0.780, and 0.410 V versus NHE for the T1 and T2 copper sites, respectively) has been recently investigated by Shleev et al. [20,32] using either a bare gold electrode and laccase in the solution or a very short thiol monolayer on the electrode to anchor the enzyme. The authors ascribed the less positive signals (ca. 0.4 V versus NHE) observed in the presence of oxygen far away from the T1 site potential to the reduction of oxygen to hydrogen peroxide instead of water.

We compared the processes at a well-ordered undecanoic acid monolayer with those at the electrode covered with a short spacer—mercaptopropionic acid. In both cases *C. unicolor* laccase was bound to the monolayer by means of a covalent bond. In the former case, a densely packed monolayer is obtained inhibiting the oxidation of gold and decreasing efficiency of catalytic processes in the presence of oxygen. On the other hand, it enabled detection of laccase electrode processes. The population of laccase on the monolayer was high as shown by the STM images and the size of attached molecule was in agreement with the size of laccase proving that aggregation of the molecules was unfavorable. In deaerated solution, a single pair of peaks could be hardly resolved, however, upon saturation of the solution with oxygen, two regions of laccase electroactivity could be easily resolved. The more positive signals indicated that the T1 center could gain electrical contact with the electrode surface, the more negative—that the remaining centers are also connected electrically with the surface. The wave shape of the reduction signal at more negative potentials under conditions of low scan rates may reflect the contribution of oxygen reduction to peroxide instead of water as postulated by Shleev et al. [20,32].

The catalytic effect is not large and to improve the efficiency of bound laccase two mediators were added in small concentrations allowing still to observe both regions of potentials where laccase is electroactive. ABTS was found to improve the catalytic efficiency of bound laccase both in terms of current and reduction potential in the positive region of potentials. In the presence of ABTS, the onset of catalytic oxygen current at the potentials of the mediator processes. Under these conditions, all centers become cooperative and the reduction of oxygen can proceed all way down to water similarly to be observed using boron doped diamond electrodes where no mediators are needed [52]. Moreover, using thiols with shorter alkyl chains (MPA) as the binding molecules resulted in the increased efficiency of the catalytic process. This shows that a compromise has to be achieved between the attempt to eliminate direct contact of the enzyme with the electrode surface and, on the other hand, unfavorable increase of the distance separating the enzyme active center from the electrode.

Acknowledgement

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