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Induced-fit binding of laccase to gold and carbon electrodes for the biological fuel cell applications

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ABSTRACT

Analogues of laccase natural substrates (syringic, veratric, ferulic, vanillic, isovanillic, 3,5-dimethoxybenzoic aldehydes) were employed to bind and orient laccase molecules in a way which facilitates adsorption of the catalyst molecules and their electrical connection with the conductive support. Laccase was bound efficiently to these substrates both on gold and carbon electrodes forming, respectively, 2D and 3D films sensitive to oxygen. Gold electrodes were used for the surface plasmon resonance evaluation of the extent of laccase binding. Glassy carbon electrodes covered with single-wall carbon nanotubes (SWCNTs) covalently modified with laccase were shown to lead to higher catalytic reduction currents than the bare carbon nanotubes deposited in a similar film at the electrode. SWCNTs with ferulic group chosen for the practical application of the biocathode in the air-zinc biobattery allowed to improve its performance both in terms of power and stability in time. The open circuit potential of the cell was 1.71 ± 0.05 V and the maximum power density achieved was 5.1 mW/cm^2 at 0.6 V.

1. Introduction

Laccase – a multicopper enzyme capable of oxidizing phenolic compounds reduces oxygen to water in a 4ē process [1–3]. The active site of laccase contains four copper ions divided into three groups distributed within the protein. The copper ion of the T1 site is located on one side of the protein, at the bottom of the hydrophobic cavity. The other three copper ions constitute T2 and T3 sites and are located about a nanometer (1.2 nm) from the T1 site. The substrate molecule is oxidized on T1 site and the electron is transferred to T2/T3 cluster, where the reduction of oxygen takes place [1–10]. Appropriately designed chemical groups can facilitate the contact between the T1 centre surrounded by the hydrophobic environment formed by different aminoacids, and the electrode, and provide the path for direct electron transfer to the T1 site [6–8]. A most suitable group would be one that consists of the natural substrates in the catalytic reactions involving laccase, which show the highest affinity towards the enzyme.

The aim of this study was to provide an induced-fit interaction between laccase and electrode surface in order to improve the communication between the active site of laccase and the electrode by using the natural substrates of laccase and their analogues. Surface plasmon resonance (SPR) is a convenient tool for the observation of the interactions between molecules in the real time [11–13]. The gold surfaces modified with thiolated compounds which mimic natural substrates for laccase were studied using SPR. While one type of the molecules is immobilized on the surface of the SPR sensor, the other one is in the solution flowing along the surface. The changes of the resonance angle depend on many factors including binding of biomolecules. In particular, they allow to determine the surface concentration of compound – in our case laccase, bound to the surface and relate it to the properties of the group attached to the electrode surface and responsible for addressing the enzyme.

The practical utility of the gold electrode covered with a monolayer of the thiol compound binding molecules of laccase is limited due to a small population of catalyst present on the surface [14,15].

The 3-dimensional network of modified carbon nanotubes on the electrode is able to accommodate and accumulate huge amount of catalyst [16–19]. In our recent papers [20–22, and citations therein] we demonstrated that arylated carbon nanotubes (phenylated, naphthylated or terphenylated) provide more efficient pathways for the transfer of electrons between the active centre of laccase and the conducting support. The groups bound to the nanotubes allow stable and durable adsorption of the enzyme and increase the amount of enzyme molecules in a suitable orientation for the exchange of electrons without the need of adding low

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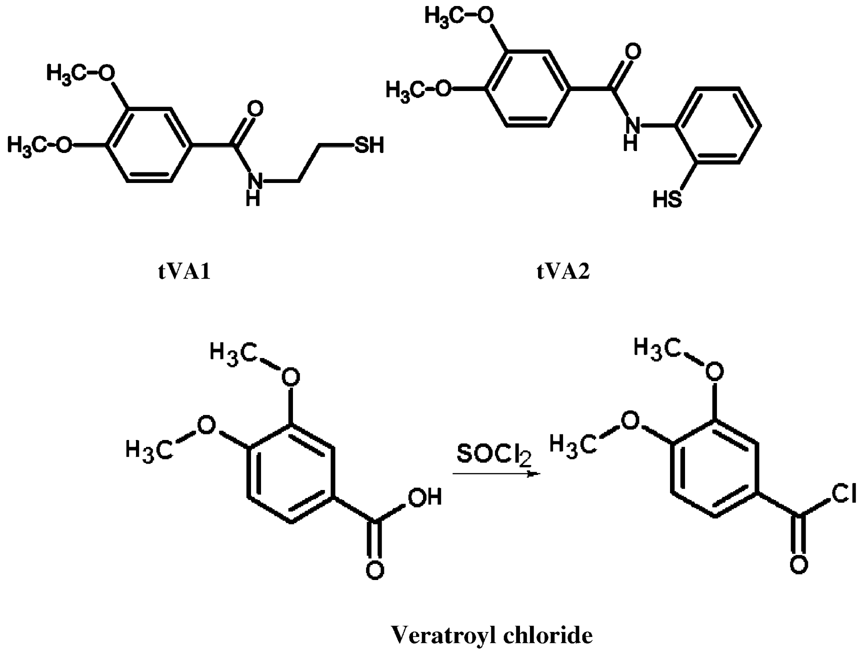


Scheme 1. Veratric acid thiolated derivatives.

molecular mass mediators. Natural substrates of laccase attached to these nanotubes should optimize the fit and improve the contact of laccase active site with the conductive support.

Formerly, we presented the synthesis, purification and characterization of arylated SWCNTs [22]. In the present paper, we describe the synthesis of SWCNTs equipped with laccase natural substrates that correspond to the lignin monomers. These lignin-related aromatic compounds can be produced in fungi during ligninolysis [23] as well as in de novo synthesis from glucose [24]. We prove that these materials assure accommodation of a larger amount of catalyst at the electrode. They retain the laccase binding feature of the arylated carbon nanotubes but are advantageous because they fit better to the cavity surrounding the T1 site of the enzyme as in the case of the natural laccase substrates employed in the enzymatic reactions in the solution.

2. Experimental

2.1. Materials and chemicals

All reagents were of analytical grade; aqueous solutions were prepared using Milli-Q water. SWCNTs and MWCNTs were from CheapTubes, Brattleboro, USA (purity > 90%); or from Nanocyl, Sambreville, Belgium.

Laccase *Cerrena unicolor* C-139 was obtained from the culture collection of the Regensburg 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) and lyophilized on Labconco (Kansas City, USA, FreeZone 12 Lyophiliser) in clear stoppering chambers [25,26].

Laccase activity was measured spectrophotometrically with syringaldazine as the substrate. The protein content was determined according to Bradford with bovine albumin as the standard [27]. The concentration of isolated and frozen (−18 °C) enzyme was $C_{lacc} = 178\ \mu g/cm^3$. After lyophilizing, the laccase activity in each vial containing 1 mg of protein after dilution in 1 ml of water was 256 U/ml.

2.2. Compounds used for the modification of gold and carbon electrodes

Chloride of 3,4-dimethoxybenzoic acid (veratroyl chloride) was obtained in the following way: 364 mg (2 mmol) of the acid was treated with thionyl chloride (2 ml) and 0.01 ml dry pyridine. The chloride dissolves immediately. The solution was kept at 50 °C for 4 h. Next, the volatiles were removed under reduced pressure and the solid residue was dissolved in 2 ml of dry toluene. The solvent and traces of thionyl chloride were removed in vacuum. Yield ~410 mg (100%), m.p. 68–72 °C (Scheme 1).

The thiolated derivative, tVA1 was synthesized by adding 200 mg (~1 mmole) of the above veratroyl chloride to solution of cysteamine hydrochloride (170 mg, 1.5 mmole) and pyridine (1 ml) in 5 ml of ice cooled water. The mixture was vigorously stirred maintaining the temperature close to 0 °C for 4 h. Then, the mixture was acidified and the product was extracted with ethyl acetate. The solid product obtained upon removal of the solvent was triturated with saturated sodium bicarbonate solution, filtered off and washed with water. Yield ~70%. Thiolated derivative tVA2 was obtained by reacting veratroyl chloride (200 mg) with 1.1 mmole of o-aminothiophenol in water and in the presence of pyridine as above. The product was isolated as in the previous experiment. Yield ~70%.

2-Aminoethyl side-functionalized SWCNTs (SWCNT-$(CH_2)_2$-NH_2) [22] were used for covalent immobilization of lignin monomers in form of N-acyl derivatives, Schiff bases and their reduction products.

The mixtures of SWCNT-$(CH_2)_2$-NH_2 and syringic, veratryl, vanillic, isovanillic, ferulic aldehydes or 3,5-dimethoxybenzaldehyde were sonicated for 4 h and during 8 hrs rotated in a rotator (Neolab, Heidelberg, Germany) at 10 rpm. Next, the samples were centrifuged at 10,000 × *g* force for 8 min, washed with MilliQ water and centrifuged under the same as above conditions. The reduction of the Schiff bases was done by adding $NaBH_4$ under 1 h rotation at 10 rpm. After centrifugation at

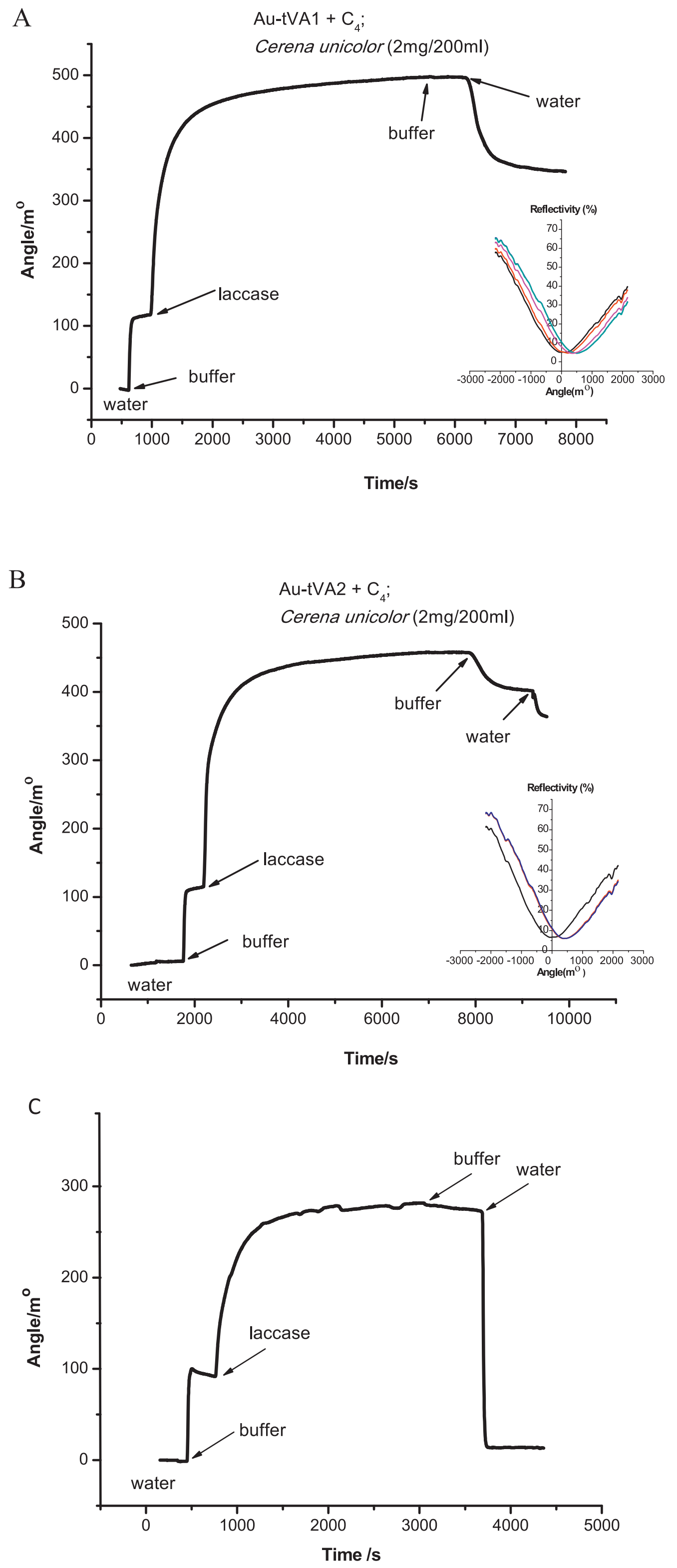


Fig. 1. SPR sensograms performed using the Au slide covered with (A) tVA_1 and butanethiol and (B) tVA_2 and butanethiol (1:9) monolayers (C) unmodified gold sensor following injection of: water, buffer solution, enzyme solution, buffer solution and water.

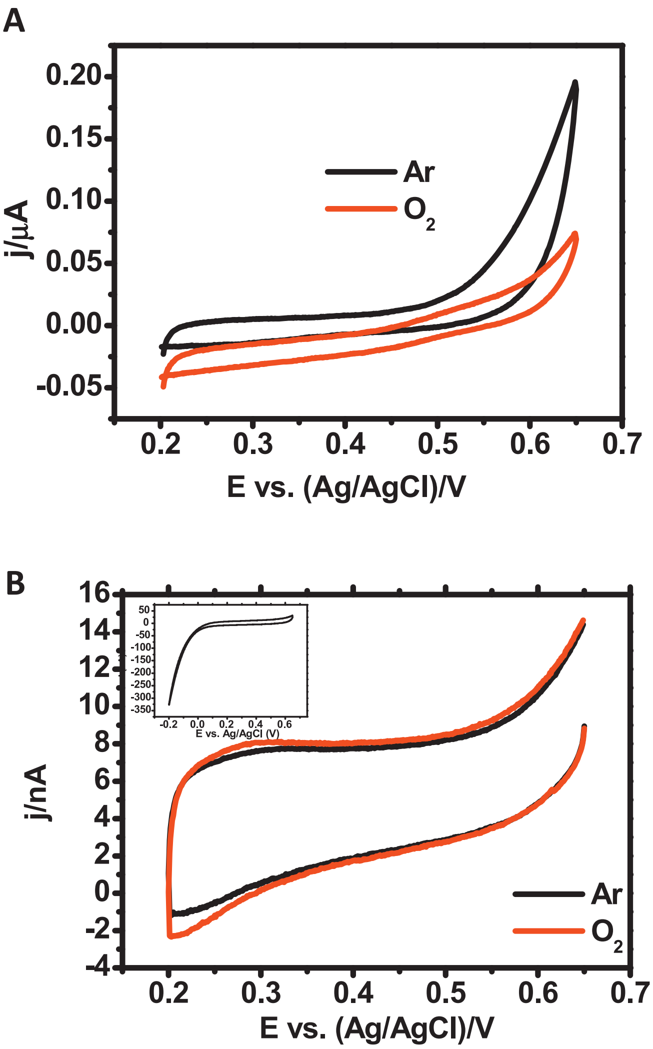
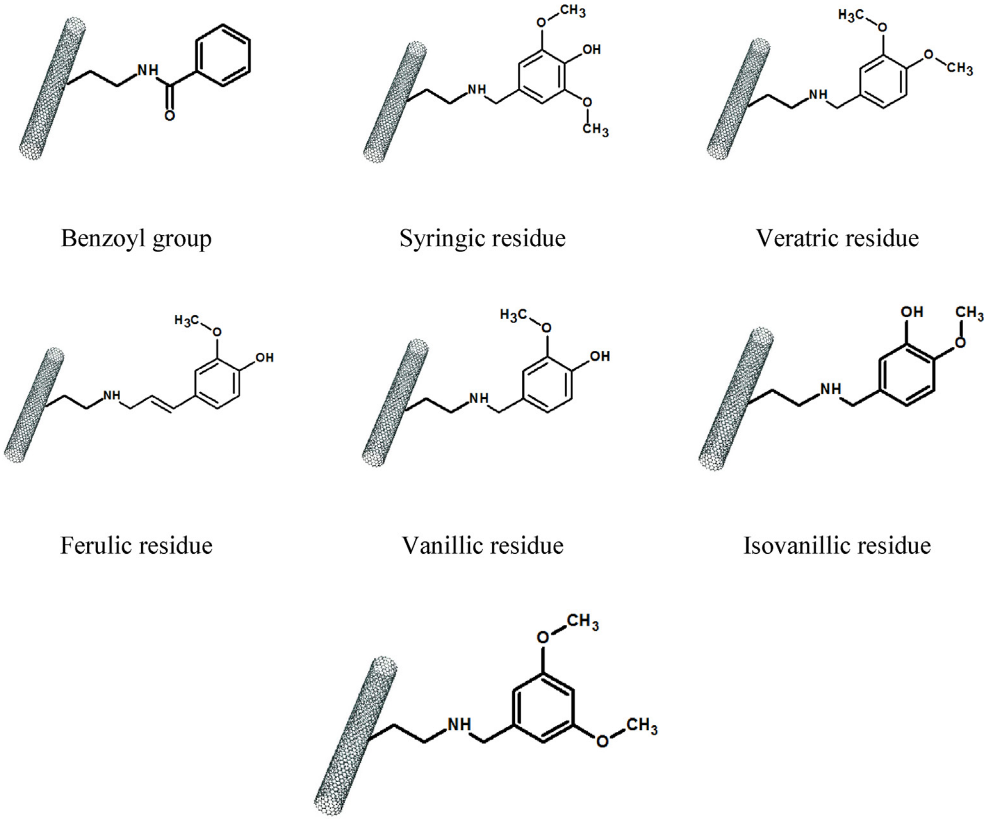


Fig. 2. Cyclic voltammograms recorded using (A) Au electrode covered with tVA_2 and butanethiol (1:9), (B) Au unmodified electrode. Experiments were conducted in oxygen (red line) or Ar (black line) saturated 0.15 M McIlvaine buffer solution, pH 5.3, scan rate was 1 mV/s. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

10,000 × g force for 5 min, the 10 mM acetic acid (1 ml) was added to decompose the excessive sodium borohydride and rotated for 15 min. Next, the modified carbon nanotubes were washed with 0.1 M McIlvaine buffer, pH 5.3 and centrifuged at 10,000 × g (5 min). The washing procedure was repeated 3 times. Then, the laccase solution (50 U) was added to the suspension of carbon nanotubes in 0.1 M McIlvaine buffer, pH 5.3 and allowed to react 12 h under rotation at 10 rpm. The washing conditions of the obtained materials were as above.

2.3. Instrumentation and sensor/electrode modification procedures

Surface plasmon resonance measurements were carried out in 0.04 M McIlvaine buffer (pH 5.4) at 25 ± 2 °C on the Autolab–Springle (Metrohm Autolab B.V., Netherlands) automated system using slides with 50 nm of gold (Ssens, Netherlands). The gold sensor was covered with thiolated veratric acid derivatives and butanethiol 1:9 (v/v) mixture by self-assembly from ethanolic solution for 24 h and running buffer containing laccase of different concentrations (2, 1 or 0.5 mg) of protein/200 mL in 0.04 M McIlvaine buffer, pH 5.4.



Benzoyl group

Syringic residue

Veratric residue

Ferulic residue

Vanillic residue

Isovanillic residue

N-3,5-Dimethoxybenzyl residue

Scheme 2. Structures of SWCNTs with analogues of lignin monomer residues and benzoyl group.

The cyclic voltammetry experiments were performed using a CHI 700B bipotentiostat in a three-electrode arrangement with a calomel (SCE) or Ag/AgCl reference electrode and a platinum sheet as the counter electrode. The glassy carbon electrode (GCE) was first covered with a suspension of bare MWCNTs (90 μl of suspension prepared from 8 mg of MWCNTs and 12 ml of ethanol) and then with 60 μl water suspension of the modified SWCNTs unless stated elsewhere. The electrode was left for 12 h in the *C. unicolor* laccase solution prepared by dissolving 2 mg of enzyme in 2 ml McIlvaine buffer (pH 5.3). The electrodes prepared using this procedure were tested in the hybrid cell with zinc wire anode (zinc wire from Goodfellow – purity 99.9%). Variable loads, in the range from 10 MΩ to 1 kΩ were applied between the anode and the cathode to determine the cell voltage (U). All current densities and power densities are reported using the geometrical area of the GCE (BAS) ($A = 0.071\ \text{cm}^2$). Experiments with the hybrid fuel cell were carried out using PICOTEST multimeter M3500A. All electrochemical measurements were done at 23 ± 2 °C.

3. Results and discussion

3.1. Surface plasmon resonance monitoring of the enzyme interactions with thiolated veratric acid derivatives

Surface plasmon resonance (SPR) is used here to measure the change in refractive index due to binding of laccase to the gold surface or modified gold surface. From the change in SPR signal (Δ angle, m°) the mass of the bound molecules, m, was calculated according to the equation given in the Autolab instrument manual:

$$122m^\circ = 100\ \text{ng/cm}^2$$

Gold sensors bare and covered with the thiolated veratric acid derivatives tVA1 or tVA2 were placed on the prism using matching oil and water was allowed to flow over it. Next, the angle was set to zero and buffer was injected to the cell (Fig. 1, point – buffer).

Table 1
Comparison of catalytic currents of oxygen reduction recorded at electrodes modified with SWCNTs modified with the natural substrate analogues.

Residue	I_{cat} (μA cm^{-2})	$I_{cat} - I_{beg}$ (measured in 0.0 V) (μA cm^{-2})	K_m (mM) [30]
Unmodified SWCNTs	−425.35	−367.60	–
N-3,5-Dimethoxybenzyl	−592.48	−275.76	0.0780
Vanillic	−672.94	−467.75	0.1720
Isovanillic	−745.72	−498.20	–
Veratric	−751.73	−460.72	0.0427
Ferulic	−929.42	−668.24	0.0887
Syringic	−749.36	−524.18	0.0803

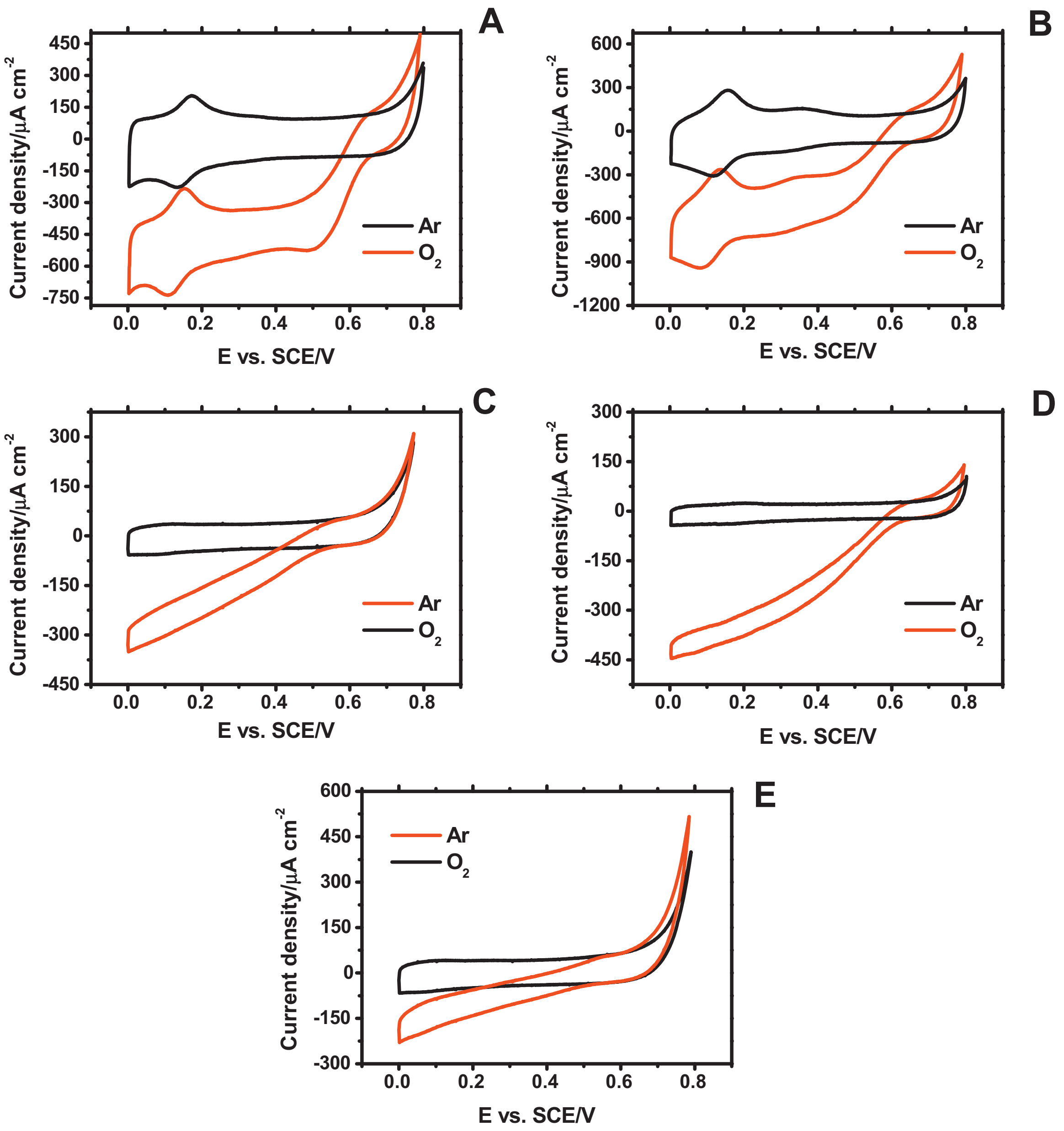


Fig. 3. Cyclic curves of GCE covered with MWCNTs and modified SWCNTs with syringic (A), ferulic (B), veratric (C) benzoyl (D) residue and (E) unmodified SWCNTs. Experiments were conducted in oxygen (red line) or Ar (black line) saturated 0.15 M McIlvaine buffer solution, pH 5.3, scan rate was 1 mV/s. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

After stabilization of the baseline, the solution of enzyme was injected to the cell (Fig. 1, point – laccase). Laccase undergoes adsorption on the monolayer modified surface as seen on the sensogram (Fig. 1). The shape of the sensograms reflects increased adsorption of the enzyme on the slide bare covered with the compounds studied compared to bare Au surface. The extent of the change in resonance angle compared to that obtained for bare electrode points to this extra affinity of the nanostructured surface towards laccase molecules. When binding step is completed a stable line on the sensogram (plateau) is obtained. Next, the slide was washed with buffer and water (Fig. 1, points: buffer and water). In our experiments desorption of the enzyme was not observed even when water was used in the washing step. The only difference of the resonance angle was connected with removing of the buffer from the SPR cell – laccase remained strongly attached to the modified surface. This is not the case for the unmodified gold sensor – laccase is quantitatively removed from the surface when water is used to wash the adsorbed protein.

The surface concentrations of the enzyme on the layers of thiolated veratric acid derivatives covering the slide were 286.4 ± 5.4 ng/cm^2 and 285.5 ± 5.7 ng/cm^2 for tVA1 and tVA2, respectively. It should be noted, that laccase could be adsorbed on a bare Au surface, or covered by alkanethiol, however, the surface concentrations were much smaller: 151.5 ± 4.3 ng/cm^2 and 133.3 ± 7.7 ng/cm^2, respectively.

3.2. Oxygen reduction at electrodes modified with analogues of natural laccase substrates

3.2.1. Gold electrodes modified with thiolated veratric acid derivatives

The coverage of the gold slide with a single component, thiolated veratric acid derivative tVA$_2$ calculated from the desorption curve in alkaline medium was 3.72×10^{-10} mol/cm^2. The SPR experiments indicated that the monolayer is suitable to bind laccase and the coverage of the electrode with laccase was 285.5 ng/cm^2.

Assuming the protein mass of ca. 60 kDa, we calculated that the protein coverage is very low – two orders of magnitude smaller than the coverage of the slide with tVA_2, the difference in their molecular sizes has to be also taken into consideration [2,28].

To check whether laccase attached to the SPR slide retains its activity towards oxygen, the electrochemical experiments in the absence and presence of oxygen were performed (Fig. 2).

When the buffer solution is saturated with oxygen, the voltammogram revealed the presence of a small catalytic wave at 0.55 V, proving the activity of adsorbed laccase. The amount of the enzyme is, however, much larger in the case of electrodes modified with SWCNTs possessing the same groups. For comparison the voltammograms recorded using bare gold in the same solutions are provided (Fig. 2).

3.2.2. Carbon electrodes modified with SWCNTs conjugates with the analogues of chosen laccase natural substrates

Studies using the SPR technique reassured us that the groups mimicking the natural laccase substrates show high affinity towards the enzymatic active site. On the other hand, the low population of laccase molecules adsorbed in the right orientation to be addressed from the electrode make the 2D system not favourable for practical applications. We switch therefore to the similar headgroups but placed on linkers attached covalently to carbon nanotubes, thus forming a 3D layer on the electrode surface and enhancing the electrode working surface and allowing a lot more laccase molecules to be adsorbed on it. Electrochemical experiments were performed to compare several types of modified carbon nanotubes including the analogues of common laccase substrates linked by the same linker. Structures of the modified SWCNTs are collected in Scheme 2. The currents of catalytic oxygen reduction are significantly larger than in the case of bare SWCNTs or those modified with benzoyl groups (Fig. 3).

This is not unexpected since the structure of these compounds makes them fit better to the laccase active site, so that they are able to get closer to it, which facilitates the direct electron transfer. As can be seen, some of the groups are electroactive (Fig. 3A and B) and undergo electrode processes at ca. +0.1 V. Nevertheless, the large catalytic reduction appears at more positive potentials, 0.55 V, which corresponds to the potential of laccase electrode processes.

All groups representing natural substrates are favourable for binding laccase.

It was expected, that since Michaelis–Menten constant value determined in solution studies is the lowest for the syringic and veratryl groups, the nanotubes modified with these groups would give the largest catalytic oxygen reduction currents. However, as shown in Table 1, the catalytic current and the difference between catalytic current and background current were largest for the nanotubes modified with the ferulic residues. The affinities of fungal laccases to substrates significantly depend on the source of enzyme and on the kind of substrates [25,29]. The length of the linker and its degree of unsaturation are also important for the adsorption of laccase and as described by Armstrong et al. for the possibility of achieving suitable orientation for the electron exchange with the electrode [3].

The electrodes prepared using the described procedure were tested in a hybrid cell with zinc wire anode. Plots of power density vs. current density and voltage vs. current density for the best modification – electrodes modified with ferulic groups are presented in Fig. 4.

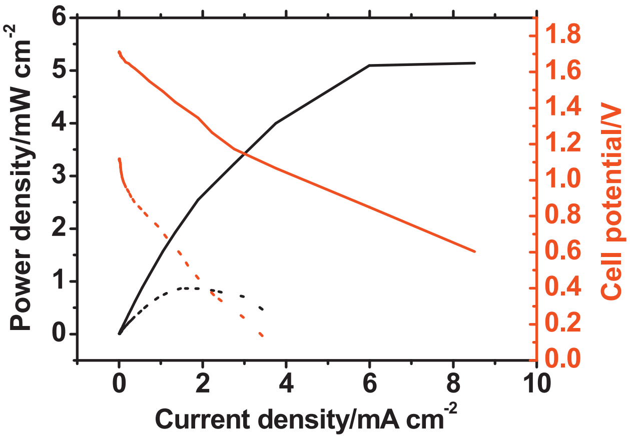


Fig. 4. Plots of power density vs. current density and voltage vs. current density for GCE covered ferulated SWCNTs: (dotted lines) unbiomodified and (solid lines) following storage in laccase suspension for 12 h at 4 °C which leads to adsorption of the enzyme on the electrode.

The open circuit potential measured for systems containing ferulated SWCNTs was ca. 1.71 V. The maximal power density was large, ca. 5.1 mW/cm^2 at 0.6 V, which is significantly larger than the power density of the system with the cathode covered with SWCNTs but without laccase (Fig. 4).

4. Conclusions

We show that by improving the fit of groups anchored to gold substrates or carbon nanotubes to the hydrophobic pocket of the enzyme, the population of laccase molecules appropriately oriented towards the conducting support and in direct electrical contact with it can be increased. Using the surface plasmon resonance approach we show that the surface concentration of laccase binding to gold surface modified with veratric acid derivatives is twice larger compared to that adsorbed on clean gold surface. In case of mixed monolayer on gold containing veratric acid derivative and butanethiol, the number of sites responsible for binding laccase is very low, still, the catalytic effects towards oxygen could be recognized. On the other hand, using a three-dimensional film composed of carbon nanotubes modified with the same analogues of laccase natural substrates lead to high activities of the electrode towards oxygen reduction. The largest catalytic currents of oxygen reduction and highest potential were obtained using ferulated SWCNTs, therefore, these analogues were employed for modifying the bioelectrode for the application in the biobattery. The open circuit potential measured for systems containing SWCNTs covalently modified with ferulic residues was ca. 1.71 V and power density attained 5.1 mW/cm^2 at 0.6 V. 1.71 V and power density attained 5.1 mW/cm^2 at 0.6 V. These values are relatively large compared to Zn bio-batteries published previously by our group as well as others [20,22,31 and references therein].

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