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Nanostructured Carbon Electrodes for Laccase Catalyzed Oxygen Reduction without Added Mediators

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Abstract

Reduction of dioxygen catalyzed by laccase was studied at carbon electrodes without any added mediators. On bare glassy carbon electrode (GCE) the catalytic reduction did not take place. However, when the same substrate was decorated with carbon nanotubes or carbon microcrystals the dioxygen reduction started at 0.6 V vs. Ag/AgCl, which is close to the formal potential of the laccase used. Four different matrices: lecithin, hydrophobin, Nafion and lipid liquid-crystalline cubic phase were employed for hosting fungal laccase from *Cerrena unicolor*. The carbon nanotubes and nanoparticles present on the electrode provided electrical connectivity between the electrode and the enzyme active sites. Direct electrochemistry of the enzyme itself was observed in deoxygenated solutions and its catalytic activity towards dioxygen reduction was demonstrated. The stabilities of the hosted enzymes, the reduction potentials and ratios of catalytic to background currents were compared. The boron-doped diamond (BDD) electrodes prepolarized to high anodic potentials exhibited behavior similar to that of nanotube covered GCE pointing to the formation of nanostructures during the anodic pretreatment. BDD is a promising substrate in terms of potential of dioxygen reduction, however the catalytic current densities are not large enough for practical applications, therefore as shown in this paper, it should be additionally decorated with carbon particles being in direct contact with the electrode surface.

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1. Introduction

The interest in designing and use of various nanostructured carbon electrodes for fuel cells and sensing systems is connected with their large physical surface area, good conducting properties and ease of modification [1-5]. Elaborating efficient catalytic surfaces for the 4e reduction of dioxygen to water, working under room temperature, normal pressure and in pH close to physiological, is crucial for the development of biofuel cell technology [6-9]. The main goal for practical applications is to maximize the charge transfer rate by decreasing the high overpotential of dioxygen reduction on carbon electrodes while maintaining high current density. This has been achieved using redox enzymes of the oxidoreductase class [10-12]. One of the interesting redox enzymes involved in dioxygen reduction is the multicopper oxidase - laccase. It is able to oxidize organic and inorganic substrates with concomitant reduction of dioxygen directly to water without formation of reactive oxygen intermediates [13]. This feature makes it together with bilirubin oxidase interesting as the biofuel cell catalysts [14]. Heller et al. [8,14,15] and Ikeda et al. [16-18] demonstrated the practical utility of these enzymes for the construction of mediated enzymatic electrodes. In 2001 Heller et al. presented the miniaturized glucose-dioxygen biofuel cell based on a carbon electrode with the enzyme immobilized by crosslinking in a redox hydrogel with high density of $Os^{2+/3+}$ complex playing the role of mediator delivering electrons from the electrode to the active centres of the enzyme [7-9,15]. The active site of laccase 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 electrons to the T2/T3 cluster, where dioxygen is reduced to water. Laccases are widely investigated for a variety of practical reasons ranging from use in the pulp and paper industry to their possible use in bioremediation, phenolic drug and pollutant analysis, and in organic synthesis. Furthermore, since laccase is electrochemically active at different electrodes, and its oxidation is linked to the dioxygen reduction, the enzyme was often employed in the construction of biosensors [19,20]. In a recent communication [21] we have shown the catalytic efficiency of *Cerrena unicolor* laccase towards dioxygen reduction on boron-doped diamond (BDD) electrode modified with a layer of liquid-crystalline cubic phase.

Monoolein cubic phases were useful as hosting layers for modifying electrodes with selected enzymes [22] and also synthetic catalysts [23]. Using carbon substrates,

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such as GCE [24-28] requires, however, a mediator to be employed in order to connect electrically the enzyme with the electrode. On the other hand, mediators often affect the stability of the enzymes, lifetime of the fuel cell, biocompatibility of the systems and possibility of their *in situ* application [29-31]. Interestingly, at BDD substrates, laccase catalyzed dioxygen reduction appeared at highly positive potentials and without any purposely added mediator. The dynamics of electron and counter ions transport in the layer is another important factor for the efficiency of the bioelectrocatalytic system [32]. In the present paper we explore this interesting aspect comparing different decorated carbon materials (using nanotubes, microcrystals) and selected matrices: lecithin, hydrophobin, Nafion and lipid liquid-crystalline cubic phase from the viewpoint of their applicability for cathodes used for catalyzed dioxygen reduction.

Hydrophobins are small, highly tensioactive proteins consisting of 110 amino acids, secreted by fungi [33-35]. They mediate the interactions of the fungi with the environment by assembling at the fungal cell walls and other interfaces into amphipathic layers [33]. Hydrophobins assemble as well at water/hydrophobic fluid and water/solid interfaces [35,36]. It has been shown also that SC3 hydrophobin allows immobilizing enzymes, both negatively and positively charged, on a hydrophobic glassy carbon electrode [37,38]. Lecithin is a zwitterionic phospholipid with two alkyl tails. When small quantity of water is added to lecithin, the micelles grow axially into flexible cylinders. The micellar system of ternary mixtures of the type lecithin/water/oil is known as wormlike, threadlike or polymer-like structures [39]. Currently, there is much interest in using reverse micelles as host for enzymes [40,41]. Nafion is an ion-exchange polymer with a high concentration of sulfonic acid groups. Such membranes are known to provide appropriate environment for the immobilization of laccase [42].

2. Experimental

Cerrena 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)

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[43] and lyophilised on Labconco (Kansas City, USA, FreeZone Lyophiliser). Enzyme activity was measured spectrophotometrically with syringaldazine as the substrate for laccase [44]. The protein content was determined according to Bradford with bovine albumin as the standard [45]. The concentration of isolated and frozen (−18 °C) enzyme was C_{lacc} = 178 g cm^{-3} and activity 186,000 nkat dm^{-3}. After lyophilising, the laccase activity dissolved in 1ml of water was 1,150,110 nkat dm^{-3} and C_{lacc} = 1.18 mg cm^{-3}.

SC3 hydrophobin purified from the culture medium of *Schizophyllum commune* as described previously [46] was obtained from E. Rogalska, University of Nancy. Prior to use, freeze-dried SC3 protein was dissolved in 100% trifluoroacetic acid, which was then gently removed by evaporation in a stream of filtered air. The dried monomeric SC3 was dissolved in Millipore water without agitation.

Lecithin (3-sn-Phosphatidylocholine from fresh egg yolk) was from Fluka, Nafion from Aldrich (5% in mixture alcohol/water), and monoolein (1-Oleoyl-rac-glycerol) from Sigma. All other chemicals were of the highest purity available commercially and were used without additional 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) as the reference electrode, platinum foil as the counter electrode and boron-doped diamond substrates (BDD, doping level of boron 10 ppm, gift from Prof. G. Swain) or glassy carbon electrode (GCE, BAS) as the working electrode. Cyclic voltammetry experiments were carried out using ECO Chemie Autolab potentiostat. All electrochemical measurements were done at 22 ± 2 °C. All current densities were calculated using geometrical area.

Unless otherwise stated, prior to use in electrochemical experiments, BDD electrodes were activated by cycling the potential in aqueous 1 M HNO_3 between 0 and +5 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.

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, followed by centrifugation for 30 min at 3000 g. Laccase solutions of concentrations ranging from

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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 electrodes were modified with cubic phase by spreading it on the electrode surface under a microscope (10 times magnification) 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. The reproducibility of dioxygen catalytic current is 20% on 10 different electrodes in one oxygenated solution.

GCE or BDD electrodes covered with unmodified nanotubes (gift from Prof. Olszyna from Warsaw University of Technology) or carbon microcrystals (MO-300, average particle size 20 μm, from Carbon GmbH) were prepared by dropping mixture of nanotubes or carbon microparticles in chloroform. After drying, 10 - 20 microliters of enzyme/matrix casting solution were pipetted onto the electrode and allowed to dry. Images of these electrodes were done using scanning electron microscopy (SEM LEO 1530) or optical microscopy (Nikon Eclipse LV150).

Lecithin/laccase mixture was prepared by mixing lecithin (5 mg lecithin in 1ml methanol) and laccase (1 mg in 0.4 ml water). Nafion/laccase mixture was prepared by mixing Nafion (0.2 ml 5% Nafion in methanol) and laccase (1 mg in 0.2 ml water). Before using, Nafion was modified using the procedure described elsewhere [42]. Hydrophobin/laccase mixture was prepared by mixing hydrophobin (0.5 mg hydrophobin in 0.5 ml water) and laccase (1 mg laccase in 0.5 ml water) according to the earlier described procedure [36,37,46].

3. Results and discussion

In order to use laccase for catalytic reduction of dioxygen usually mediators either soluble or bound to the electrode surface are required. Without them, at unmodified GCE the reduction of dioxygen 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 our recent paper [21] when BDD is used as the electrode substrate a catalytic signal of dioxygen reduction is observed in the presence of laccase in the solution (Fig. 1). The reduction of dioxygen starts at a very positive potential ca. +0.590 V vs. Ag/AgCl electrode that is +0.790 V vs. NHE. The $E_{1/2}$ of the catalytic wave is 0.405 V vs.

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Ag/AgCl (0.605 V vs. NHE). The waves are highly reproducible and well developed although no mediator is present in the solution. Thus, the electrode itself is able to mediate electrons as far as to reach the copper centres of laccase although no mediators have been added. The wave is not present on GCE substrates. It is not present without laccase or without dioxygen. This proves its assignment to mediatorless laccase catalyzed dioxygen reduction. The catalytic current increases with increasing laccase and dioxygen concentrations in the solution (Fig. 1 inset).

Good electrical contact between laccase and BDD electrode surface is connected with the species or structures formed on the surface during activation of the electrode since without this pretreatment the electrode is catalytically inactive. Before each experiment the BDD electrodes were activated in 1 M HNO_3 by recording 10 voltammetric cycles in the range 0 V to 5 V at 0.1 V/s. The activation curve is shown in Fig. 2. Upon magnification, two systems of peaks are seen at ca. 0.5 V and 1.5 V. The less positive may be attributed to the quinone/hydroquinone couple, the nature of the more positive at 1.5 V is unknown. In this range, nanostructures improving electrical connectivity between BDD and laccase in solution are probably inducing favorable orientation of the enzyme towards the electrode substrate. Similar effects were seen in the presence of cytochrome c [3].

In deoxygenated solutions, a small poorly developed signal was observed but only in the first cycle (Fig. 3). The current increasing at ca. 0.5 V probably corresponds to the reduction of oxidized laccase but it disappears in the next cycles.

In order to improve the communication of laccase with the GCE and BDD electrode, it was covered with carbon microcrystals or unmodified nanotubes. The suspension of those nanoparticles in chloroform was applied to the electrode surface and dried in air. Next, the electrode was immersed in oxygenated solution containing the enzyme. The nanotubes do not form a layer of uniform thickness as shown on SEM image (Fig. 4A). It is rather a porous 3-dimensional structure built of carbon threads on the underlying GCE substrate. However the catalytic reduction of dioxygen is clearly seen (Fig. 5A). The reduction wave starting at 0.6 V is connected with the presence of the carbon nanotubes since without them - on bare GCE the catalytic mediatorless reduction of dioxygen is not observed. The voltammetric curve is flat at these potentials when the solution is deoxygenated. The reduction current onset, half-wave potential,

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current density and ratio of current to background current (without dioxygen) are depicted in Table 1.

Table 1. Characteristics of the catalytic reduction of dioxygen at different nanostructured carbon electrodes in McIlvaine buffer solution (pH 5.2) containing 0.047 mg/ml laccase and saturated with dioxygen

Electrodes	E_{onset}[a]	$E_{1/2}$ [V]	j [$\mu A/cm^2$] [at 200mV]	i_{cat}/i_{bcg} [at 200mV]
Boron doped diamond (BDD)	0.590	0.405	0.40	2.1
Boron doped diamond (BDD) modified with nanotubes	0.603	0.383	1.21	2.9
Glassy carbon electrode (GCE) modified with nanotubes	0.600	0.408	5.01	2.9
Glassy carbon electrode (GCE) modified with carbon microparticles	0.590	0.383	28.20	5.5

[a] potential of onset of the catalytic dioxygen reduction current

At the electrodes modified with nanotubes, the current densities are larger than on BDD in part due to the larger physical surface area in the presence of nanotubes. With time the nanotubes pass to the solution since only weak nonpolar-nonpolar interactions retain them at the electrode. The nanotubes were also lost upon transfer of the electrode through the water-air interface.

Largest current densities are recorded for GCE decorated with carbon microcrystals. Microcystals are distributed over the whole surface. The largest aggregates of microcrystals are seen in the optical microscopy images (Fig. 4B). The voltammetric curves have clearly a wave shape and the catalytic to background current ratio is almost 6 with the onset of current at 0.590 V (Fig. 5B). The current density is almost 70 times larger than obtained using unmodified BDD surface. This indicates that distribution of carbon particles on the surface is of importance for the catalytic reduction of dioxygen. At more polar, compared to GCE, prepolarized BDD surface the number of nonpolar nanotubes or microcystals is smaller, hence the dioxygen reduction current densities are smaller. Also the stability of the nanotube modified surface is worse in case of the BDD surface.

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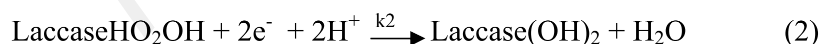
Using laccase for practical applications requires that the enzyme is also immobilized on the solid supports. However, simple immobilization methods allowing stabilization of enzyme activity and reproducibility of the results are wanting. Our strategy for stable laccase immobilization [22-24] is to use a liquid crystal matrix with cubic symmetry. In the voltammograms recorded in deoxygenated McIlvaine buffer solutions, pH 5.2 using BDD electrodes modified with cubic phases and laccase, only in the first cycle, the reduction process of laccase itself can be seen, with the onset of current at ca. 0.6 V. This behavior is similar to observed for solution resident laccase (Fig. 3). The need of dioxygen to get reproducible reduction waves in the successive scans is an interesting and yet not well understood phenomenon. In the voltammograms recorded in carefully deoxygenated solutions, using BDD electrodes modified with cubic phases and laccase, only in the first cycle, the reduction process of laccase itself can be seen. Maybe the traces of dioxygen species bound to the BDD surface get involved in the process – when consumed the wave diminishes. It should be mentioned that on other carbon-based electrodes, such as spectrographic graphite, HOPG (edge plane) and plastic formed carbon electrode [12,47] laccase electrode processes were not seen even in the first scan without purposely added mediators or nanostuctures. It is known that enzyme molecules adsorbed on the electrode and properly oriented are able to conduct direct electron transfer [48-50]. This would indicate that on BDD surface dioxygenated species may play role in the electron transfer process to laccase active sites.

The process of bioelectrocatalytic dioxygen reduction by laccase can be presented by the following scheme which takes into account recent views on the mechanism of enzymatic catalysis [51] and includes: formation of the enzyme–substrate complex (1), step of synchronous transfer of the first two electrons (2) and dissociation of enzyme–substrate complex with formation of products of the reaction (3):

$$\text{Laccase} + O_2 + H_2O \xrightarrow{k1} \text{LaccaseHO}_2\text{OH} \quad (1)$$



$$\text{LaccaseHO}_2\text{OH} + 2e^- + 2H^+ \xrightarrow{k2} \text{Laccase(OH)}_2 + H_2O \quad (2)$$

$$\text{Laccase(OH)}_2 + 2e^- + 2H^+ \xrightarrow{k3} \text{Laccase} + 2H_2O \quad (3)$$

The catalytic dioxygen reduction wave using BDD as substrate was seen both with laccase present in solution and in the cubic phase layer (Fig. 6). On the BDD electrode

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modified with cubic phase without laccase no faradaic current was observed. Without additional mediators added to the solution or film, the reduction of dioxygen started at potential +0.62 V.

The potential value for the dioxygen reduction was close to the redox potential of the laccase used. Shleev et al. and Tarasevich el al. [10,52,53] have investigated the mechanism of electron transfer properties of laccase at different kinds of electrodes and concluded that at Au electrodes modified with self-assembled monolayers the observed voltammetric response could be attributed to the redox process of T2 copper ion, while those at the carbon-based electrodes could be ascribed to the redox process of T1 copper ion in laccase. The high potential observed at BDD would be in agreement with this statement.

A two-step preparation of the electrodes was used in order to immobilise both the nanotubes and laccase on the electrode surface. In the first step of the procedure, the nanotubes suspension in chloroform was placed on the electrode surface and allowed to dry. In the second step, laccase was coadsorbed on this nanostructured surface and the electrode was left to dry. The current density of dioxygen reduction attains in this case values over 40 $\mu A/cm^2$ (Fig. 7A), however, it decreases with time pointing to leaching of laccase from the layer to the solution. It is laccase that is desorbed to the solution since a new portion of laccase can be adsorbed on the same surface and gives a new catalytically active surface thus proving that nanotubes remain attached to the substrate. Other matrices were, therefore, employed for nanotube and laccase immobilization on the electrode surface. The mixture of laccase either in Nafion, lecithin or hydrophobin was placed on the nanotubes covered electrode. These mixtures were liquid and not as viscous as the cubic phase hence during this deposition step the nanotubes remained directly in contact with the electrode surface. In case of the cubic phase, the nanotubes lost contact with the electrode surface when this highly viscous material was smeared over the electrode surface. The characteristics of dioxygen reduction at these electrodes are presented for comparison in Table 2.

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Table 2. Characteristics of the catalytic reduction of dioxygen at different modified electrodes containing ca. 40 μg laccase in the matrix. McIlvaine buffer solution (pH 5.2)

Electrodes	E_{onset}[a]	$E_{1/2}$	j [μA/cm^2] [at 200 mV]	i_{cat}/i_{bcg} [at 200 mV]
BDD with laccase in cubic phase film	0.620	0.368	0.7	3.6
GCE/nanotubes with adsorbed laccase	0.620	0.441	58.2	3.7
GCE/nanotubes with laccase in hydrophobin film	0.599	0.465	17.2	6.6
GCE/nanotubes with laccase in lecithin film	0.611	0.494	40.6	5.0
GCE/nanotubes with laccase in Nafion film	0.611	0.448	110.0	10.6

[a] potential of onset of the catalytic dioxygen reduction current

Fig. 7 shows representative voltammograms of dioxygen reduction recorded for these matrices. The hydrophobin films (Fig. 7B) formed via strong, noncovalent interactions are highly insoluble and cannot be removed from hydrophobic surfaces even in hot sodium dodecyl sulfate [54]. We showed recently that hydrophobin could be used as an agent permitting immobilization of small, electroactive molecules for example (azobenzene, coenzyme Q_0), and the long hydrocarbon chain ubiquinone Q_{10} on hydrophilic and hydrophobic electrodes [55]. The interactions involved in molecule immobilization were supposed to be nonpolar. They are not denaturing for the enzymes adsorbed on the film formed with SC3. In the absence of dioxygen, the system of peaks at 0.459 V is due to the electrode processes of laccase itself (Fig. 7B). Upon oxygenation, the catalytic reduction of dioxygen starts at 0.599 V. The current density measured at 0.2 V is 17.2 μA/cm^2.

The same 2-step procedure used for the lecithin matrix leads to higher current densities of dioxygen reduction (Fig. 7C). Laccase peaks are well developed at potential 0.453 V in solutions not containing dioxygen. In dioxygen saturated solution, the reduction process starts at +611 V and the current density measured at 0.2 V is 40.6 μA/cm^2. The electrode modified this way shows also the best stability.

Largest current densities were obtained for the mixtures of neutralized Nafion and laccase deposited on the nanotube layer as shown in Fig. 7D. The onset of dioxygen reduction is at 0.611 V and current density attains value of 110.0 μA/cm^2 .

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4. Conclusions

The BDD electrode is envisaged to be an interesting substrate for the investigations of interfacial electron transfer of multicopper oxidases as well as for the development of biosensors and biofuel cells. The decrease of overpotential is ca. 1.1 V compared to the noncatalytic process. In the present study it is clearly shown that appropriately designed BDD possesses unique structural and electronic properties different from other kinds of carbon-based materials which are especially important for the catalyzed dioxygen reduction. Catalytic activity of laccase on BDD electrode is due to activation of boron-doped diamond electrodes surface by cycling 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 dioxygen, making the surface increasingly hydrophilic [56]. Pretreated in such a way BDD is a highly active composite material that may provide the optimum orientation of the enzyme molecules for the direct bioelectrocatalysis - where direct electrical contact is needed. Laccase active centres of molecules immobilized on anodically pretreated BDD surface should be, hence, in closer contact with the hydrophilised BDD electrode than e.g with GCE even after anodic pretreatment of the latter. The facilitated electron transfer between laccase and BDD and the catalytic activity of the laccase/BDD modified electrode toward O_2 reduction at the laccase formal potential makes the electrodes promising as a cathode in biofuel cells. Our thinking should be directed now on how to increase the current density of catalyzed dioxygen reduction at the positive potentials. Higher densities were obtained in the presence of nanotubes and microcrystals at GCE, however, these electrodes are also known to be not very reproducible. When laccase is incorporated into the surface layer of Nafion, lecithin, hydrophobin or cubic phase, the stability and obviously utility of the electrodes are improved. It may be proposed that nanostructures are produced at the BDD surface upon application of 5 V and are responsible for the laccase catalyzed dioxygen reduction without added mediators. The experiments performed with all 3 matrices indicate that among different modifying procedures the 2-step procedure for preparing the nanotubes-laccase modified electrodes leads to most stable and efficient layer. Hence nanotubes remain attached to the electrode surface and transfer electrons to the molecules of laccase in the matrix. The crucial point in further development of these electrodes is the efficient contact of all nanotubes with the surface – maybe even growing the nanotubes directly on the electrode material. Also providing

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largest possible contact of laccase molecules with the nanotube active parts without deactivation of laccase would lead to further improvement of current efficiency of dioxygen reduction at these highly positive potentials.

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References

[1] W. Johnston, M.J. Cooney, B.Y. Liaw, R. Sapra, M.W.W. Adams, Enzyme Microbial Techn. 36 (2005) 540.

[2] R.H. Baughman, A. Zakhidov, W.A. de Heer, Science 297 (2002) 787.

[3] F. Marken, C.A. Paddon, D. Asogan, Electrochem. Commun. 4 (2002) 62.

[4] B.S. Sherigara, W. Kutner, F.D'Souza, Electroanal. 15 (2003) 753.

[5] A. Guiseppi-Elie, C. Lei, R.H. Baughman, Nanotechnology 13 (2002) 559.

[6] S.C. Barton, H.H. Kim, G. Binyamin, Y. Zhang, A. Heller, J. Am. Chem. Soc. 123 (2001) 5802

[7] S.C. Barton, H.H. Kim, G. Binyamin, Y. Zhang, A. Heller, J. Phys. Chem. B 105 (2001) 11917.

[8] N. Mano, J.L. Fernandez, Y. Kim, W. Shin, A.J. Bard, A. Heller, J. Am. Chem. Soc. 125 (2003) 15290.

[9] E. Katz, A.F. Buckmann, I. Willner, J. Am. Chem. Soc. 123 (2001) 10752.

[10] I.V. Berezin, V.A. Bogdanovskaya, S.D. Varfolomeev, M.R. Tarasevich, A.I. Yarapolov, Dokl. Akad. Nauk SSSR 260 (1978) 615.

[11] M.R. Tarasevich, A.I. Yarapolov, V.A. Bogdanovskaya, S.D. Varfolomeev, J. Electroanal. Chem. 104 (1979) 393.

[12] C.W. Lee, H.B. Gray, F.C. Anson, B.G. Malmstrom, J. Electroanal. Chem. 172 (1984) 289.

[13] E.I. Solomon, U.M. Sundaram, T.E. Machonkin, Chem. Rev. 96 (1996) 2563.

[14] N. Mano, H.H. Kim, A. Heller, J. Phys. Chem. B 106 (2002) 8842.

[15] A. Heller, Phys. Chem. Chem. Phys. 6 (2004) 209.

[16] S. Tsujimura, K. Kano, T. Ikeda, J. Electroanal. Chem. 576 (2005) 113.

[17] S. Tsujimura, H. Tatsumi, J. Ogawa, S. Shimizu, K. Kano, T. Ikeda, J. Electroanal. Chem. 496 (2001) 69.

[18] S. Tsujimura, M. Kawaharada, T. Nakagawa, K. Kano, T. Ikeda, Electrochem. Commun. 5 (2003) 138.

[19] R.S. Freire, N. Duran, L.T. Kubota, Talanta 54 (2001) 681.

[20] B. Haghighi, L. Gorton, T. Ruzgas, L.J. Jonsson, Anal. Chim. Acta 487 (2003) 3.

[21] K. Stolarczyk, E. Nazaruk, J. Rogalski, R. Bilewicz, Electrochem Commun. 9 (2007) 115.

[22] R. Bilewicz, P. Rowiński, E. Rogalska, Bioelectrochem. 66 (2005) 3.

ACCEPTED MANUSCRIPT

[23] P. Rowinski, R. Bilewicz, M. J. Stebe, E. Rogalska, Anal. Chem. 74 (2002) 1554.

[24] E. Nazaruk, A. Michota, J. Bukowska, S. Shleev, L. Gorton, R. Bilewicz, J. Biol. Inorg. Chem. 12 (2007) 335.

[25] M. Klis, E. Maicka, A. Michota, J. Bukowska, S. Sek, J. Rogalski, R. Bilewicz, Electrochim. Acta 52 (2007) 5591.

[26] S. Shleev, M. Klis, Y. Wang, J. Rogalski, R. Bilewicz, L. Gorton, Electroanal. 19 (2007) 1039.

[27] W. Nogala, E. Rozniecka, I. Zawisza, J. Rogalski, M. Opallo, Electrochem. Commun. 8 (2006) 1850.

[28] I. Zawisza, J. Rogalski, M. Opallo, J. Electroanal. Chem. 588 (2006) 244.

[29] A. Ramanavicius, A. Kausaite, A. Ramanaviciene, Biosens. Bioelectron. 20 (2005) 1962.

[30] J. Frebortova, K. Matsushita, H. Arata, O. Adachi, Biochim. Biophys. Acta 1363 (1998) 24.

[31] I. Willner, G. Arad, E. Katz, Bioelectrochem. Bioenerg. 44 (1998) 209.

[32] A. Markoci, Microchim. Acta 152 (2006) 157.

[33] J.G.H. Wessels, Trends Plant Sci. 1 (1996) 9.

[34] H.A.B. Wosten, M. Richter, J.M. Willey, Fungal Genet. Biol. 27 (1999) 153.

[35] J.G. Wessels, Fungal Genet. Biol. 27 (1999) 134.

[36] H.A.B. Wosten, T.G. Ruardy, H.C. van der Mei, H.J. Busscher, J.G.H. Wessels, Coll. Surf. B 5 (1995) 189.

[37] Y. Corvis, A. Walcarius, R. Rink, N.T. Mrabet, E. Rogalska, Anal. Chem. 77 (2005) 1622.

[38] Y. Corvis, K. Trzcinska, R. Rink, P. Bilkova, E. Gorecka, R. Bilewicz, E. Rogalska, J. Phys. Chem. C 111 (2007) 1176.

[39] S.H. Tung, Y.E. Huang, S.R. Raghavan, J. Am. Chem. Soc. 128 (2006) 5751.

[40] Z.D. Knezevic, S.S. Siler-Marinkovic, L.V. Mojovic, Appl. Microbiol. Biotechnol. 49 (1998) 267.

[41] D. Madamwar, A. Thakar, Appl. Biochem. Biotechnol. 118 (2004) 361.

[42] S. Topcagic, S.D. Minteer, Electrochim. Acta 51 (2006) 2168.

[43] G. Janusz, PhD Thesis, UMCS, Lublin, 2005, p. 222.

[44] A. Leonowicz, K. Grzywnowicz, Enzyme Microb. Technol. 3 (1981) 55.

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[45] M.M. Bradford, Anal. Biochem. 72 (1976) 248.
[46] L.G. Lugones, H.A.B. Wosten, J.G.H. Wessels, Microbiology 144 (1998) 2345.
[47] S. Tsujimura, T. Nakagawa, K. Kano, T. Ikeda, Electrochem.72 (2004) 437.
[48] F.A. Armstrong, G.S. Wilson, Electrochim. Acta 45 (2000) 2623.
[49] Y. Tian, T. Ariga, N. Takashima, T. Okajima, L. Mao, T. Ohsaka, Electrochem. Commun. 6 (2004) 609.
[50] L.J.C. Jeuken, Biochim. Biophys. Acta 67 (2003) 1604.
[51] M.R. Tarasevich, V.A. Bogdanovskaya, A.V. Kapustin, Electrochem. Commun 5 (2003) 491.
[52] S. Shleev, A.E. Kasmi, T. Ruzgas, L. Gorton, Electrochem. Commun. 6 (2004) 934.
[53] M.R. Tarasevich, Y.G. Chirkov, V.A. Bogdanovskaya, A.V. Kapustin, Electrochim. Acta 51 (2005) 418.
[54] O.M.H. De Vries, M.P. Fekkes, H.A.B. Wösten, J.G.H. Wessels,. Arch. Microbiol. 159 (1993) 330.
[55] R. Bilewicz, J. Witomski, A. Van der Heyden, D. Tagu, B. Palin, E. Rogalska, J. Phys. Chem. B 105 (2001) 9772.
[56] B. Marselli, J. Garcia-Gomez, P.A. Michaud, M. A. Rodrigo, Ch. Comninellis, J. Electrochem. Soc. 150 (2003) D79.

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Figure Captions

Fig. 1. Cyclic voltammograms recorded for catalyzed dioxygen reduction using BDD in McIlvaine buffer solution (pH 5.2) containing 0.047 mg/ml laccase: (——) deoxygenated and (— —) saturated with dioxygen, scan rate 5 mV/s. (Inset. Dependence I_{O_2}/I_{Ar} current on dioxygen concentration measured at 0.2 V).

Fig. 2. Cyclic voltammograms recorded during the pretreatment procedure of BDD in 1 M HNO_3, scan rate 100 mV/s (Inset: voltammogram recorded in narrower potential range).

Fig. 3. Cyclic voltammograms recorded using BDD electrode in deoxygenated solution of McIlvaine buffer (pH 5.2) containing 0.047 mg/ml laccase (——) first cycle (— —) second cycle, scan rate 100 mV/s.

Fig. 4. Images of nanostructured GCE. A) SEM image of electrode covered with carbon nanotubes and B) optical microscopy of carbon electrode modified with carbon microparticles.

Fig. 5. Cyclic voltammograms for catalyzed dioxygen reduction in McIlvaine buffer solution (pH 5.2) containing 0.047 mg/ml laccase: (——) deoxygenated and (— —) saturated with dioxygen. GCE electrode modified with carbon A) nanotubes and B) microparticles. Scan rate: 5 mV/s.

Fig. 6. Cyclic voltammograms recorded in McIlvaine buffer solution (pH 5.2) using BDD electrode modified with cubic phase containing laccase: (——) deoxygenated, (— —) dioxygen saturated solution (Inset. Dependence of current on dioxygen concentration measured at 0.2 V), scan rate: 5 mV/s.

Fig. 7. Cyclic voltammograms for catalyzed dioxygen reduction in McIlvaine buffer solution (pH 5.2): (——) deoxygenated and (— —) saturated with dioxygen. GCE electrodes modified with nanotubes and laccase (A) adsorbed on the surface, and in B) hydrophobin, C) lecithin, D) Nafion layer, scan rate: 5 mV/s.

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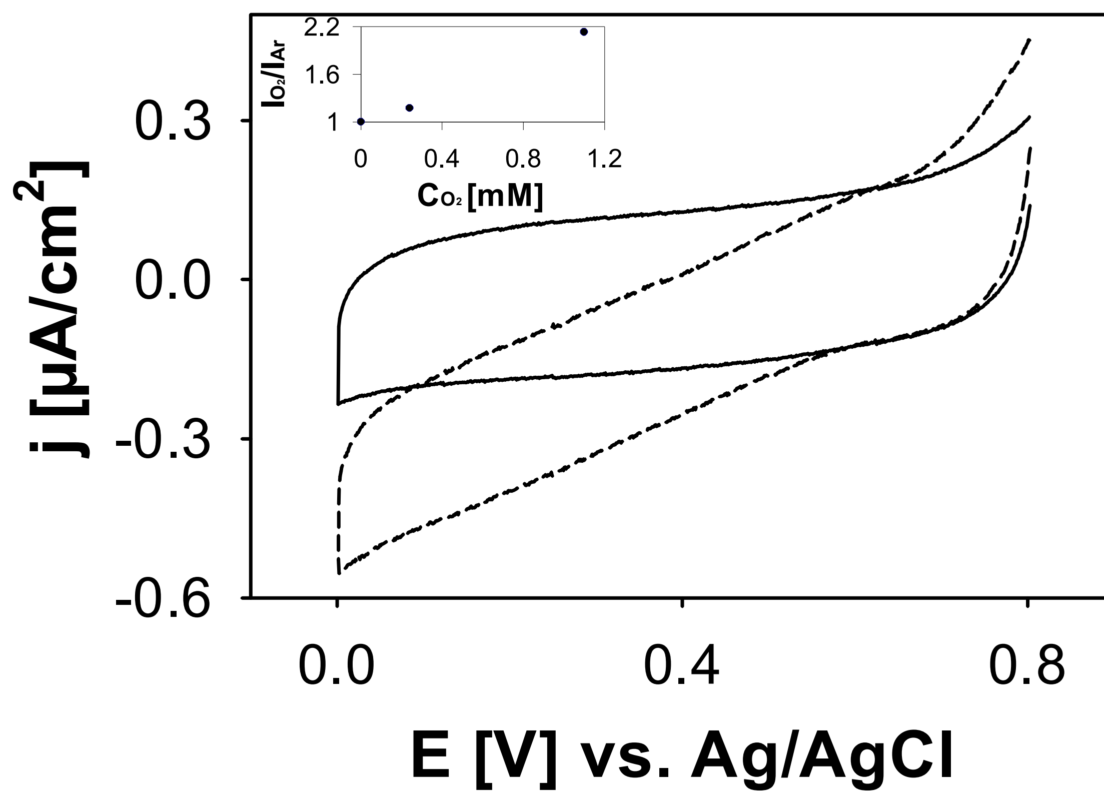

Fig.1.

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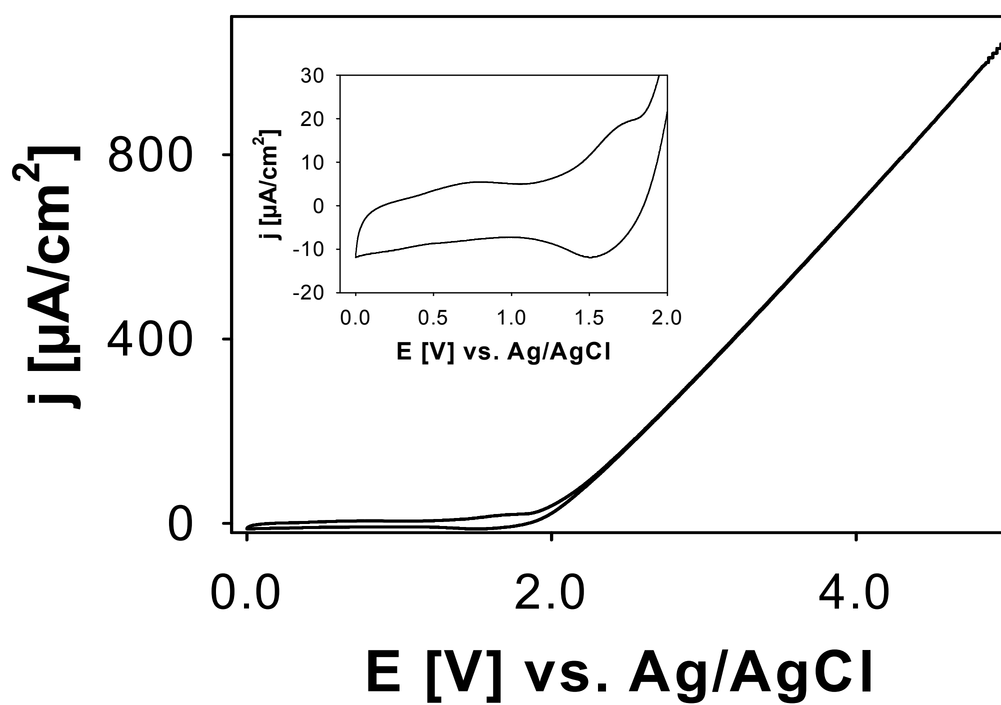

Fig. 2.

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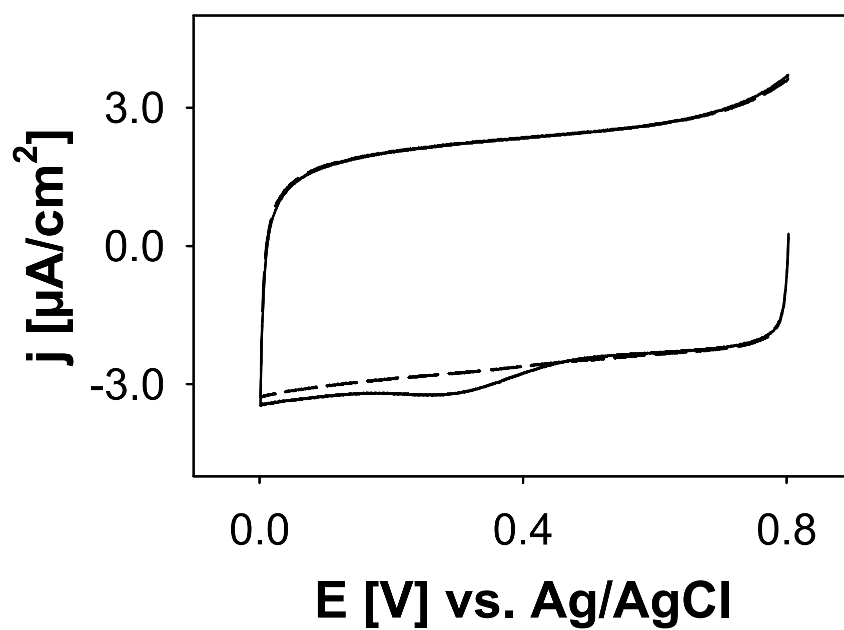

Fig.3

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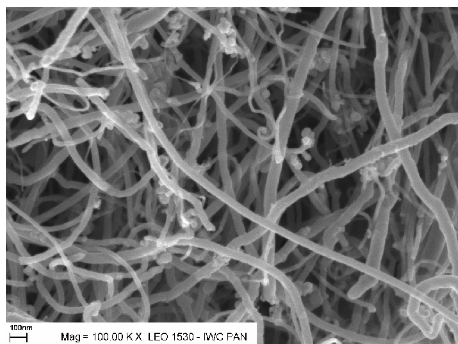
A)



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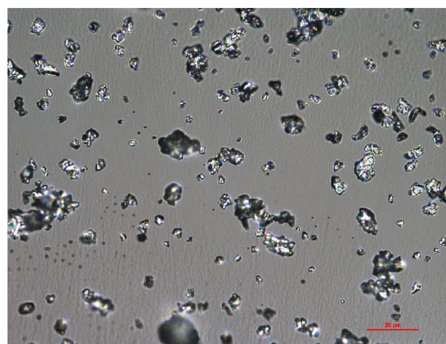

Fig.4.

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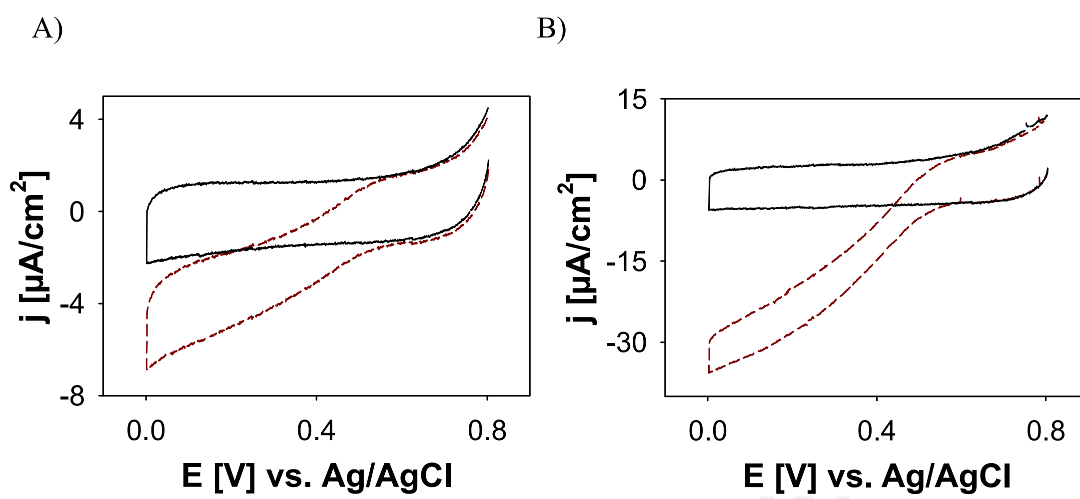

Fig. 5.

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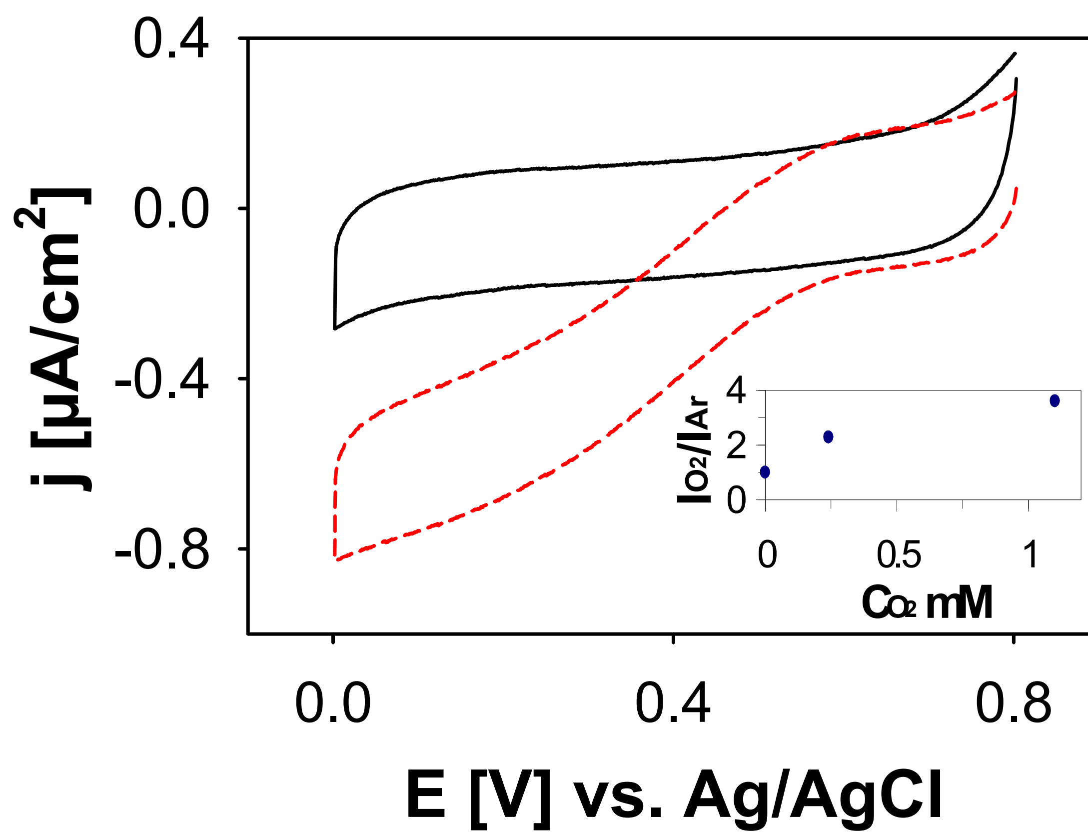

Fig.6.

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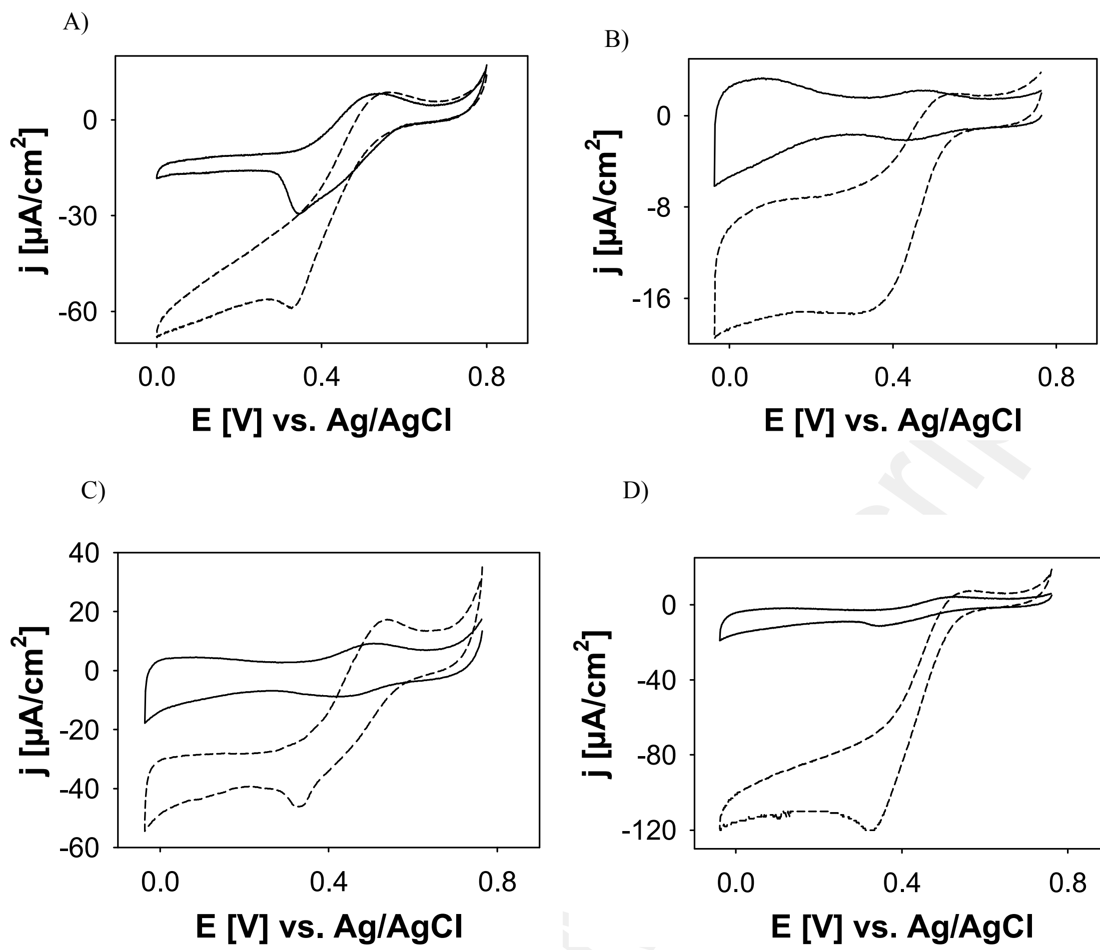

Fig.7.