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Laccase-Catalyzed Reduction of Oxygen at Electrodes Modified by Carbon Nanotubes with Adsorbed Promazine or Acetosyringone

Bartłomiej Olszewski and Krzysztof Stolarczyk *

Faculty of Chemistry, University of Warsaw, Pasteura 1, 02-093 Warsaw, Poland; bartlomiej.j.olszewski@student.uw.edu.pl

* Correspondence: kstolar@chem.uw.edu.pl; Tel.: +48-2282-26351

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Abstract: One of the problems with the use of enzymes as catalysts in biofuel cells is to achieve good contact between the enzyme and the electrode surface. One solution to this problem is the use of various nanostructures such as carbon nanotubes, fullerenes, graphene derivatives, gold nanoparticles, as well as mediators for the construction of electrodes. Acetosyringone and promazine adsorbed on glassy carbon electrodes (GCEs) covered with multiwall carbon nanotubes (MWCNTs) and laccase were used as biocathodes. These mediators showed very efficient adsorption on modified glassy carbon electrodes covered with MWCNTs and enabled efficient and stable adsorption of laccase, which acts as the bioelectrocatalyst. Very good electrical contact between the electrode surface and the laccase enzymatic active sites made it possible to increase the catalytic current density of oxygen-reduction by about 82% compared to electrodes without mediators. Application of acetosyringone and promazine used in the construction of biocathode also improved the current and power of the biobattery ca. twice comparing to the system without mediators. The device output reached the power that equaled approximately 2 mW/cm^2 at 0.8 V and open circuit potential (OCP) was 1.6 V. The systems elaborated proved also useful in oxygen sensing and allowed to determine lower oxygen concentrations in solution compared to the GCE modified with MWCNTs and laccase alone. The electrode showed also better stability in long-timescale measurements.

Keywords: multiwall carbon nanotubes; bioelectrocatalysis; laccase; oxygen reduction; oxygen sensor; biobattery

1. Introduction

More and more often, biofuel cells are used as alternative energy sources, supplying devices that require a small power consumption for their work [1–6]. Biofuel cells are currently considered to be one of the most promising future energy sources and self-integrated bioelectrochemical devices [7–9]. Unfortunately, this research field encounters serious problems, including obtained catalytic currents, the power of biofuel cells are too small, and the working time is not long enough to supply them in order to consider their possible applications [10]. Biofuel cells consist of two electrodes that are able to convert chemical energy into electricity. They are a special kind of fuel cell, in which the sources of chemical energy are reduction and oxidation processes of naturally occurring renewable compounds present in the environment or in living organisms [10,11]. In enzymatic biofuel cells, enzymes are used as catalysts. The catalysts of anodic processes are enzymes that catalyze oxidation reactions, e.g., lactate dehydrogenase, glucose dehydrogenase, alcohol dehydrogenase, glucose oxidase, fructose dehydrogenase, and the catalysts used most often to construct the cathode are laccase, bilirubin oxidase or cytochrome oxidase, which catalyze the reduction of oxygen to water [1,12–15].

The sources of fuel for these biofuel cells may be metabolites (e.g., glucose, lactate) and oxygen present in the body fluids.

The biggest obstacle arising during the use of the enzymatic catalysts is the difficulty to establish a good contact between the active center of the enzyme and the electrode surface. The electron transport between the active center of the enzyme and the electrode surface is carried out according to two main mechanisms: direct electron transfer (DET) and electron transfer using a mediator (MET) [1]. The direct electron transfer between the enzyme and the electrode surface is difficult in most cases due to the poorly accessible location of the enzyme active center inside the protein structure. Direct electron transfer is only possible when the active center of the enzyme is located near the enzyme surface. One of the methods to support direct electrical contact between the enzyme and the electrode surface is the use of various nanostructures: such as carbon nanotubes, nanospheres, grapheme, or nanoparticles [16–22]. Nanomaterials act as very efficient materials for enzyme immobilization, because of their characteristics: small size, very good electron transport or the ability to react with different functional groups, thus allowing the covalent immobilization of enzymes or mediators [16,18,23,24]. The studies of such systems have shown that the introduction of nanostructures creates a favorable micro-environment, providing efficient contact between the enzyme active center and the electrode surface, and the enzyme activity can be maintained for a long time. It is assumed that materials having big surface areas and good conductivity can influence the kinetics of electron transfer, in a way to improve the performance of the cell. In this context, the nano-engineering and design of new materials play important roles in the technology of enzymatic biofuel cells.

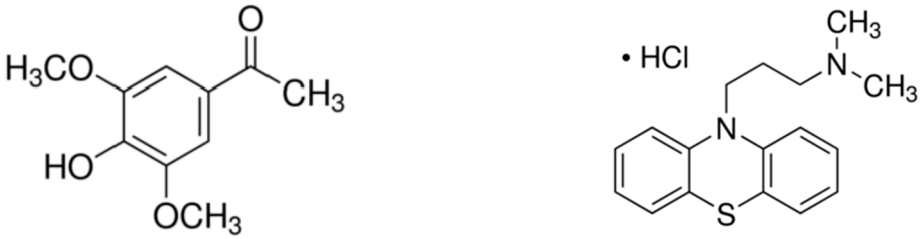
In a situation where an effective electrical contact of the enzyme with the electrode cannot be obtained, due to the too large distance of the active site from the protein surface, inappropriate orientation of the enzyme towards the electrode or partial denaturation resulting from direct contact with the electrode surface, small molecule intermediates called mediators are used. Then, the method of electron transport is called mediated electron transfer (MET) [25]. Mediated electron transfer uses small molecules as electron transfer mediators between active centers of the redox enzymes and the electrode. The use of the mediator molecule allows to increase the efficiency of electron transfer, which results in higher current densities that are obtained from the biofuel cell [26].

Laccase is a commonly used enzyme for the cathodic reaction in a biofuel cell, where the fuel i.e., oxygen is reduced to water [27]. From the environmental point of view this is the perfect enzymatic catalyst for a bioelectrode. It has been used by our group for many years. For the studies of direct electron transfer between the active center of laccase and the electrode surface, we used various materials including boron-doped diamonds, carbon particles, fullerenes. For mediated electron transfer between the active center of laccase and the electrode we used different mediators [19,22,26,28]. Among the mediators used at the cathode, two of them deserve special attention. One of the best mediators of the oxygen reduction process using laccase and bilirubin oxidase is ABTS (2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) [29]. It is characterized by very good solubility and stability. It is a diffusion mediator and its use requires the introduction of a membrane to prevent electron transfer of electrolyte directly from the anode to the cathode. The next mediator used quite often at the cathode is the osmium complex, in which the redox center $Os^{3+/2+}$ is stabilized by ligands containing heterocyclic atoms, e.g., nitrogen, and directly connected to a polymer chain composed of more than 10 carbon atoms and heteroatoms, i.e., nitrogen [30,31]. Osmium complexes are characterized by very good stability and the ability to undergo quick and reversible redox reactions.

In this work, at the glassy carbon electrode covered with carbon nanotubes, laccase and acetosyringone (3′,5′-dimethoxy-4′-hydroxyacetophenone) or promazine (10-(3-dimethylaminopropyl) phenothiazine hydrochloride) mediators (Scheme 1) were immobilized and such modified electrodes were applied in the catalytic reduction of oxygen. The efficiency of catalyzed oxygen reduction on GCE electrodes modified with MWCNTs, laccase and acetosyringone (ASYR) and promazine (PRZ) was compared to the one corresponding to GCE electrodes modified only with MWCNTs and laccase. The studied systems were applied in the construction of biobattery and the powers of such prepared

systems were compared. In addition, the tested electrodes were used as sensors for the measurements of oxygen concentration. Promazine, an aliphatic derivative of phenothiazine, is a neuroleptic drug with moderate sedative and antipsychotic activity, and it also acts moderately anti-emetic [32,33]. The drug is indicated for the treatment of psychomotor agitation and anxiety in the elderly. Acetosyringone, a phenolic natural product, is involvemed in plant-pathogen recognition [34]. Promazine and acetosyringone were used to adsorb laccase on glassy carbon electrodes by Fernandez-Sanchez and coworkers [35]. Both mediators and laccase adsorbed on glassy carbon electrodes were used for catalytic reduction of oxygen. Unfortunately, the GCE electrode was characterized by low porosity, therefore the currents achieved for such systems were small, as well as the stability of such systems in the long term was poor. To overcome these problems, in our research, we used carbon nanotubes. The systems containing carbon nanotubes, are appropriate to be used not only for biobattery or biofuel cell construction but also as sensors for oxygen concentration measurements.

(A) (B)



Scheme 1. Structural formula of (**A**) acetosyringone (3′,5′-dimethoxy-4′-hydroxyacetophenone), (**B**) promazine (10-(3-dimethylaminopropyl) phenothiazine hydrochloride).

2. Results and Discussion

2.1. Electrochemical Studies

Electrochemical oxygen reduction occurs with a large overpotential on a glassy carbon electrode at the potential −0.6 V versus Ag/AgCl electrode at pH 7 [36]. The application of oxygen reduction reactions to the construction of a biocathode in a biofuel cell requires a reduction of its overpotential. To solve this problem, many research groups use various nanostructures, for example carbon nanotubes. Carbon nanotubes have been increasingly used in electrochemistry in the last few years due to the fact that placing them on the electrode increases the physical surface of the electrode, as well as improves its conductivity. Oxygen reduction on carbon electrodes modified only with carbon nanotubes starts at a potential of around −0.2 V versus saturated calomel electrodes (SCE) [37]. After immobilization of the laccase enzyme, the oxygen reduction potential can be shifted to a potential of about 0.6 V against Ag/AgCl at pH near 5 [22,26]. In our research, at the beginning, we performed oxygen reduction experiment on glassy carbon electrodes modified only with multiwall carbon nanotubes and laccase enzyme. Carbon nanotubes were adsorbed on glassy carbon electrodes by dropping a suspension of carbon nanotubes and left to dry in air. In order to immobilize the laccase enzyme, the prepared electrodes were cycled in the potential range from 0.8 V to 0.0 V with 0.1 V/s scan rate in deoxygenated McIlvaine buffer of pH 6.0 containing laccase. The electrodes were then rinsed with plenty of water and transferred to a clean McIlvaine buffer (pH 6.0) that did not contain laccase dissolved in solution. Modified electrodes underwent cyclic voltammetry measurements in deoxygenated and oxygenated McIlvaine buffer of pH 6.0 with 5 mV/s scan rate in the potential range from 0.8 V to 0 V (Figure 1). During the measurements carried out in the deoxygenated buffer, no signals on voltammetric curves were observed, while in the oxygenated solution catalytic oxygen reduction signals were observed. The catalytic oxygen reduction wave began at a potential of about 0.6 V versus Ag/AgCl electrode. This shape of the voltammetric curve indicates that the laccase effectively catalyzed the reduction of

oxygen to water. The wave was not observed when the measurement was carried out in deoxygenated solution due to the absence of oxygen, which is the substrate of the catalytic reaction.

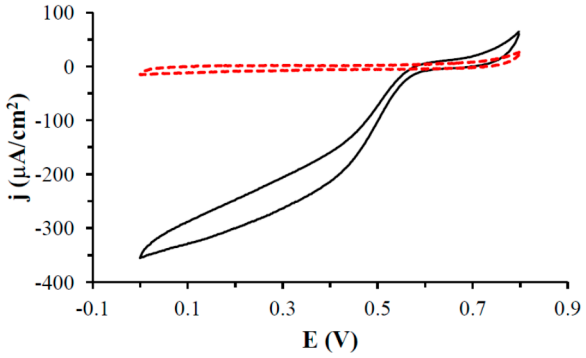


Figure 1. Cyclic voltammetry curves recorded on the GCE electrode modified with MWCNTs and laccase in deoxygenated (red curve, dashed line) and oxygenated (black curve, continuous line) McIlvaine buffer (pH 6.0), 5 mV/s scan rate.

Table 1 presents the current densities of the studied systems in the deoxygenated and oxygenated solutions and their difference, measured from voltammetric curves at the potential equal to 0.0 V. In the tested system, the densities of catalytic oxygen reduction currents of approximately -346 $\mu A/cm^2$ were obtained. The results confirm the effectiveness of immobilization of laccase on the MWCNTs modified GCE electrode. Introduction of laccase to the system leads to a potential shift, at which oxygen reduction takes place, towards much smaller overpotentials. The catalytic process of oxygen reduction begins at potential equal to +0.6 V vs. Ag/AgCl, thus reaching a value close to the formal potential of the laccase. However, this process is very slow, clear catalytic curves are formed only at very low rates of potential change. Due to the modification of the glassy carbon electrodes with nanotubes, the electrodes capacities were increased. In addition, the immobilization of MWCNTs on the electrode surface increased significantly the active surface of the electrode, what allowed the attachment of a larger number of enzyme molecules on the electrode surface and the generation of significant catalytic currents without the participation of a mediator.

Table 1. Current densities calculated from measurements carried out at potential equal to 0.0V versus Ag/AgCl electrode (KCl_{sat}) in deoxygenated (j_{bcg}) and oxygenated (j_{cat}) McIlvaine buffer (pH 6.0) using GCE electrodes modified with MWCNTs, promazine (PRZ) or acetosyringone (ASYR) and laccase (Lc).

Electrode GCE	j_{bcg} ($\mu A/cm^2$)	j_{cat} ($\mu A/cm^2$)	$j_{cat} - j_{bcg}$ ($\mu A/cm^2$)
MWCNTs/Lc	-16.0 ± 0.8	-346.0 ± 8.0	-330.0 ± 8.2
MWCNTs/PRZ/Lc	-57.7 ± 7.1	-480.8 ± 30.4	-423.0 ± 27.0
MWCNTs/ASYR/Lc	-128.3 ± 8.3	-629.6 ± 8.0	-501.4 ± 16.2

In another experiment, the possibility of adsorption of PRZ and ASYR on MWCNTs modified GCE electrodes, was investigated and the effect of such modification on the achieved catalytic oxygen reduction currents was measured. Typically, the use of mediators improves catalytic process by improvement contact of the enzyme with the electrode and increases the densities of catalytic currents. First, MWCNTs were adsorbed on the GCE electrode and allowed to dry, followed by adsorption of the redox compounds—the mediators PRZ and ASYR. Different adsorption procedures were used for PRZ- and ASYR-MWCNTs-modified glassy carbon electrodes.

In the case of PRZ, the best method of modification was to cycle (five cycles) the modified electrode in deoxygenated McIlvaine buffer (pH 6.0) containing 1 mM PRZ in the potential range from -0.2 V to 1.0 V following the electroadsorption at the potential equal to -0.2 V for 60 s, and then cycling

additional 5 cycles in the adsorption solution, as at the beginning, because after such preparation the highest current densities of catalytic process of oxygen reduction in the presence of laccase were obtained. On the voltammetric curves, a system of irreversible peaks corresponding to the process of PRZ oxidation was observed (Figure 2A). The oxidation peaks at the potential of about 0.7 V and 0.9 V decreased. Probably the product of electrooxidation of the drug adsorbs at the electrode surface.

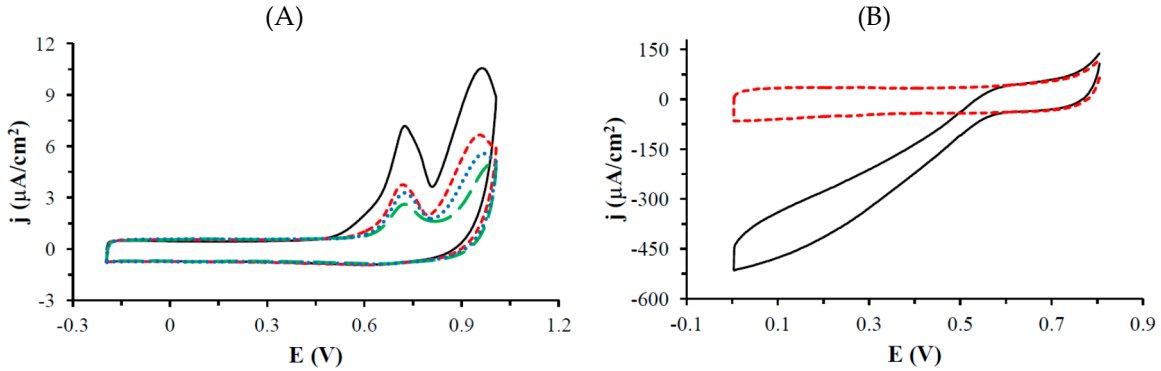


Figure 2. (**A**) Cyclic voltammetry curves recorded on the GCE electrodes modified MWCNTs in deoxygenated McIlvaine buffer (pH 6.0) containing 1mM PRZ, 1st scan (black continuous line curve), 2nd scan (red curve short-dashed line), 5th scan (blue curve dotted line), 10th scan (green curve long-dashed line), scan rate 0.1 V/s. (**B**) Cyclic voltammetry curves recorded on GCE electrodes modified with MWCNTs/PRZ/laccase in deoxygenated (red curve dashed line) and oxygenated (black curve continuous line), McIlvaine buffer (pH 6.0), scan rate 5 mV/s.

After adsorption process the electrodes were rinsed thoroughly with distilled water and one cycle at 0.1 V/s was recorded to adsorb the laccase on the modified electrode, followed by a subsequent second cycle at the scan rate 5 mV/s in deoxygenated McIlvaine buffer (pH 6.0) containing laccase. The potential range was from 0.8 to 0.0 V. In the next step, the electrodes were washed and the curves in deoxygenated and oxygenated McIlvaine buffer (pH 6.0) were recorded at the scan rate of 5 mV/s (Figure 2B). No signals were observed on the voltammetric curves registered in the deoxygenated McIlvaine buffer, while in the oxygenated McIlvaine buffer, a catalytic wave beginning at a potential equal to about 0.6 V was observed. This is a proof that the catalytic reduction of oxygen in the presence of laccase immobilized on GCE electrode modified with carbon nanotubes and PRZ occurs. The ratio of catalytic current to diffusion current increases with the decreasing scan rate, which confirms the catalytic nature of oxygen reduction in the presence of laccase. From the studied curves, both registered in the oxygenated and deoxygenated McIlvaine buffer, the reduction currents were measured at 0.0V potential and the results are presented in Table 1. In the deoxygenated solution, a current density of -57.7 μA/cm^2 and in oxygenated solution of -480.8 μA/cm^2 were obtained, respectively. Both current densities, in deoxygenated and oxygenated McIlvaine buffer, are much higher than the current density measured on electrodes modified only with carbon nanotubes and laccase—about 260% in deoxygenated solution and about 40% in an oxygenated solution. The increase in reduction currents in the deoxygenated solution is associated with the increase of the electrode capacity, what confirms the effective adsorption of PRZ. In the oxygenated solution, the increase in the reduction current is related to the effective adsorption of the laccase enzyme on the modified electrode surface. The use of the mediator system, like PRZ on the electrode, caused an increase in the electron transport rate between the electrode and the enzyme.

We also carried out another experiment, in which we used ASYR as the redox system. Analogically to the previous experiment, first GCE electrodes were modified with MWCNTs, and then the ASYR redox compound was adsorbed. Shortly, MWCNTs were dropcast on the electrode surface and left to dry, and then the as-prepared electrodes were cycled in deoxygenated McIlvaine buffer (pH 6.0) containing 1mM ASYR in the potential range from -0.2 V to 1.0 V. On voltammetric curves,

peaks corresponding to the oxidation and reduction process of ASYR were observed (Figure 3A). It is visible that the oxidation and reduction peaks at about 0.3 V increased, what indicates the effective ASYR adsorption on MWCNTs-modified GCE electrodes. Oxidation peaks at 0.6 V and 0.7 V potential decreased indicating polymerization ASYR on the modified electrode. Phenolic derived systems are known that they can polymerize and are used as the pseudocapacitive systems in energy storage [38–40].

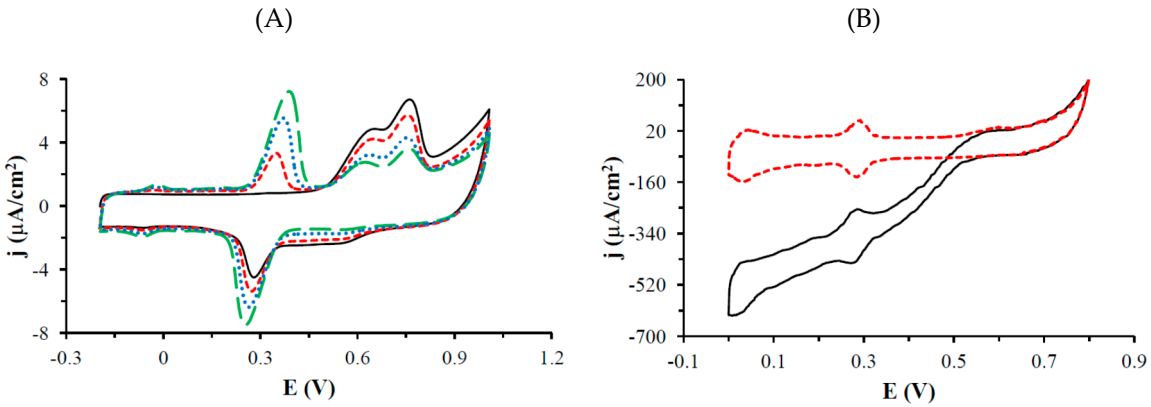


Figure 3. (**A**) Cyclic voltammetry curves recorded on the GCE electrodes modified with MWCNTs in deoxygenated McIlvaine buffer (pH 6.0) containing 1 mM ASYR: 1st scan (black curve continuous line), 2nd scan (red curve short-dashed line), 5th scan (blue curve dotted line), 10th scan (green curve long-dashed line), scan rate 0.1 V/s, (**B**) Cyclic voltammetry curves registered on the GCE electrodes modified with MWCNTs/ASYR/laccase in deoxygenated (red curve dashed line) and oxygenated (black curve continuous line), McIlvaine buffer (pH 6.0), scan rate 5 mV/s.

After the adsorption, the electrodes were rinsed thoroughly with distilled water. Then one cycle with 0.1 V/s scan rate was recorded with the use of modified electrodes, followed by a subsequent cycle at a scan rate of 5 mV/s in deoxygenated McIlvaine buffer (pH 6.0) containing laccase in the potential range from 0.8 V to 0.0 V. In the next stage, the electrodes were rinsed with distilled water and voltammetric curves were recorded in deoxygenated and oxygenated McIlvaine buffer (pH 6.0) without the presence of laccase in the solution in the potential range from 0.8 V to 0.0 V (Figure 3B). On the voltammetric curves registered in the deoxygenated McIlvaine buffer, signals corresponding to the mediator's redox processes adsorbed on the electrode were observed. In the oxygenated McIlvaine buffer, catalytic waves beginning at potential equal to about 0.6 V were observed. This proves the occuring catalytic reduction of oxygen in the presence of laccase immobilized on the surface of a GCE electrode modified with carbon nanotubes and ASYR. In addition, as for the deoxygenated solution, redox signals corresponding to the redox reactions of the mediator were observed on the catalytic wave. From the studied curves, both registered in the oxygenated and deoxygenated McIlvaine buffer, the reduction currents were measured at 0.0 V potential and are presented in Table 1. The densities of reduction currents in the deoxygenated solution were -128.3 μA/cm^2 and in the oxygenated solution -629.6 μA/cm^2. Both current densities in the deoxygenated and oxygenated McIlvaine buffer are much higher (about 700% in the deoxygenated solution and about 82% in the oxygenated solution) than the current densities measured on electrodes modified only with carbon nanotubes and laccase, what proves that not only the adsorption of ASYR but also of laccase are effective on such modified electrodes. The use of ASYR contributed to the improvement of the efficiency of the work of the biocathode, compared to the GCE electrode modified only with MWCNTs and laccase, in particular, the increase in the densities of catalytic currents, which is the result of improved efficiency of electron exchange between the electrode and the enzyme. Of the three systems studied, the largest reduction currents measured at 0.0 V were observed on MWCNTs, ASYR and laccase modified GCE electrodes, because ASYR and also laccase molecules were most effectively adsorbed on MWCNTs modified GCE

surface. The use of carbon nanotubes contributed to the improvement of the efficiency of individual biocathodes (200 times higher current densities), in comparison to those described in the literature on the GCE electrode without carbon nanotubes [35].

2.2. Biobattery Studies

Modified electrodes were tested in a biobattery system in which a hopeite layer modified zinc plate acted as the anode. Zinc anode is a very convenient system in tests because its potential does not change during measurement in both deoxygenated and oxygenated solutions; we have shown this in previous publications [41]. This is the reason why such a system is very good for studying the characteristics of a biocathode, because any changes in the system are determined by its behavior. As biocathode, the following GCE electrodes modified with: (a) MWCNTs and laccase, (b) MWCNTs, PRZ and laccase, and (c) MWCNTs, ASYR, and laccase characterized previously in electrochemical studies were used. The dependence of power density and potential of the cell on the current density was plotted by recording the voltage drop of the working system after connecting it to the decreasing variable resistance. The dependences of the power density (P) on the current (j) density and the cell potential (E) on the current density (j), during the load of the system with subsequent resistance, are shown in Figure 4. The curves have different characteristics for all tested biocathodes. It was observed that the power of the system along with increasing current initially increased to a certain level, reached its maximum and then decreased. The border resistance, below which the power of biobatteries decreased was 5 kΩ. This is probably due to too low oxygen concentration near the surface of the electrode. In the case of biobatteries in oxygenated solutions—for the same current—higher values of power, compared to deoxygenated solutions, were observed. This is due to the presence of oxygen as the fuel of the enzymatic reaction for the biocathode. The increase in oxygen concentration increases the power, current density and open circuit potential. The values of power and current density were calculated based on the measured cell potential at a resistance of 5 kΩ and the results are shown in Table 2. For the bioelectrode covered with MWCNTs and laccase the lowest values of power and current densities were obtained. For this system, the maximum power density equaled 822.1 $\mu W/cm^2$ and the current density was at the level of 1521.7 $\mu A/cm^2$ with an external applied resistance of 5 kΩ, what corresponded to cell potential of 0.540 V. Under zero-current conditions, OCP of the system was characterized by potential of 1.570 V.

Another biocathode, which was tested, was GCE electrode modified with MWCNTs adsorbed PRZ and laccase. Higher power densities, current densities, at the same load as for the previous electrode, were achieved for this system. This is due to the adsorption of the mediator on the MWCNTs modified GCE surface. The presence of a mediator results in the increased amount of enzyme molecules adsorbed on the prepared biocathode and it also improves the contact of the enzyme with the electrode surface, which can be seen both, as the increase of the power density, as well as the current density. In the tested system, the power density of 1307.2 $\mu W/cm^2$, the current density of 1918.3 $\mu A/cm^2$ and the potential of the cell of 0.681 V were obtained at 5 kΩ load and OCP equal to 1.544 V. These values of power are about 40 percent higher compared to biobattery with a biocathode consisting of GCE electrode modified only with MWCNTs and laccase.

The next system of our studies was a biobattery containing a biocathode composed of a GCE electrode modified with MWCNTs and adsorbed ASYR. And, in the next step, it was also modified with laccase. In the applied system, at a load of 5 kΩ of external resistance, the obtained power density, current density, the cell potential and the OCP were equal to 1853.9 $\mu W/cm^2$, 2284.3 $\mu A/cm^2$, 0.811 V and 1.637 V, respectively. The power of this system is much higher compared to the biobattery with GCE electrode modified with MWCNTs, PRZ and laccase. Compared to the biobattery with GCE electrode modified only with MWCNTs and laccase, the power is higher more than two times. From electrochemical studies and adsorption curves, it is visible that this system was the most efficiently adsorbed on modified electrodes, resulting in higher catalytic oxygen reduction currents than

the other two systems. The ASYR mediator proved to be the most beneficial to use in the preparation of the biocathode, because all the determined biobattery parameters showed the highest values.

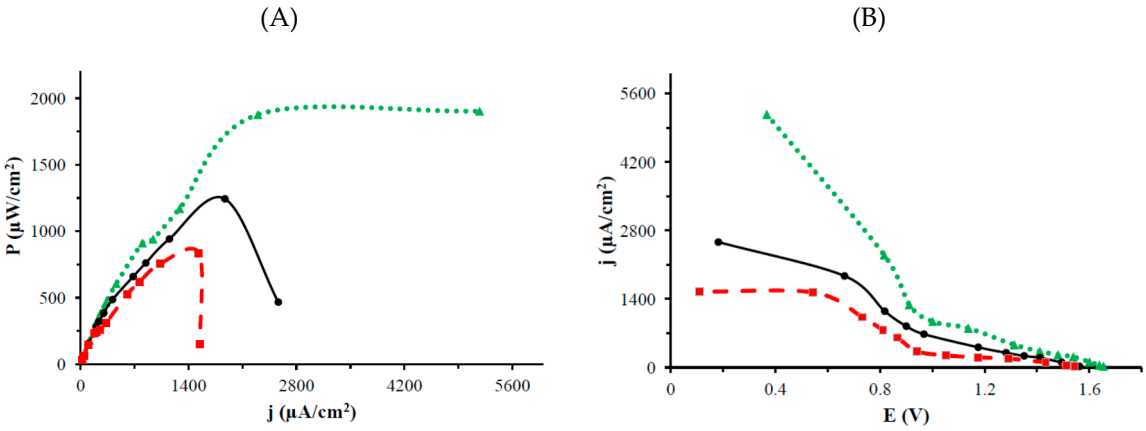


Figure 4. The dependence of (**A**) power density and (**B**) potential of the cell on the current density for the biobattery: anode-zinc plate, biocathode: GCE covered with: (1) (red curve dashed line) MWCNTs and laccase, (2) (black curve continuous line) MWCNTs/PRZ and laccase, (3) (green curve dotted line) MWCNTs/ASYR and laccase in oxygenated McIlvaine buffer (pH 6.0).

Table 2. Biobattery characteristics: anode-zinc plate, biocathode-GCE covered with MWCNTs/with or without mediator/laccase, in (A) oxygenated and (B) deoxygenated McIlvaine buffer (pH 6.0).

(**A**)					
Electrode GCE	**P (μW/cm²)**	**J (μA/cm²)**	**E (V)**	**R (kΩ)**	**OCP (V)**
MWCNTs/Lc	822.1 ± 17.0	1521.7 ± 15,8	0.540 ± 0.006	5	1.570 ± 0.012
MWCNTs/PRZ/Lc	1307.2 ± 90.0	1918.3 ± 66.2	0.681 ± 0.024	5	1.544 ± 0.035
MWCNTs/ASYR/Lc	1853.9 ± 125.3	2284.3 ± 77.5	0.811 ± 0.028	5	1.637 ± 0.032
(**B**)					
Electrode GCE	**P (μW/cm²)**	**J (μA/cm²)**	**E (V)**	**R (kΩ)**	**OCP (V)**
MWCNTs/Lc	2.9 ± 0.8	89.7± 3.7	0.032 ± 0.005	5	0.230 ± 0.061
MWCNTs/PRZ/Lc	6.0 ± 1.2	129.5 ± 12.7	0.046 ± 0.005	5	0.215 ± 0.025
MWCNTs/ASYR/Lc	22.1 ± 4.0	248.9 ± 22.6	0.088 ± 0.008	5	0.186 ± 0.011

2.3. Oxygen Sensor

Biocathodes based on oxygen reduction were used to prepare a sensor for measuring the oxygen concentration in a solution. Three GCE electrodes modified first with MWCNTs, followed by (A) laccase, (B) PRZ and laccase, (C) ASYR and laccase were used in the research. The research was carried out using a chronoamperometry. First, based on voltammetric curves registered with the use of modified electrodes in oxygenated McIlvaine buffer (pH 6.0), the potential for measuring the currents of oxygen reduction was selected. This potential was equal to +0.2 V versus the Ag/AgCl reference electrode. Figure 5A shows the amperometric curves of oxygen reduction after multiple addition of 2 mL of oxygenated McIlvaine buffer (pH 6.0) (about 1.1 mM oxygen concentration) to 20 mL of deoxygenated McIlvaine buffer (pH 6.0) (0.0 mM oxygen concentration). The measurement was carried out every 60 s after the current stabilized, and then 2 mL of oxygenated McIlvaine buffer (pH 6.0) was added. In Figure 5B the dependence of the measured catalytic current density on the oxygen concentration in the solution is shown. Analyzing the curves (Figure 5) for the tested electrodes, a linear dependence of the catalytic current density of oxygen reduction on oxygen concentration in McIlvaine buffer (pH 6.0) can be observed for the concentration range from 0 to 0.3 mM oxygen in the solution. For MWCNTs-modified GCE electrodes covered with mediators and laccase, relatively higher catalytic current densities (about 80% at 0.3 mM oxygen concentration)

were obtained at the same levels of dissolved oxygen concentration compared to MWCNTs-modified GCE electrodes and laccase only. It allows to measure lower oxygen concentrations in solution in both cases of mediated systems. GCE electrode modified with MWCNTs, PRZ and laccase showed higher stability during measurements compared to GCE electrode modified with MWCNTs, ASYR and laccase, which can be explained by better interactions between the laccase and PRZ adsorbed on the electrode.

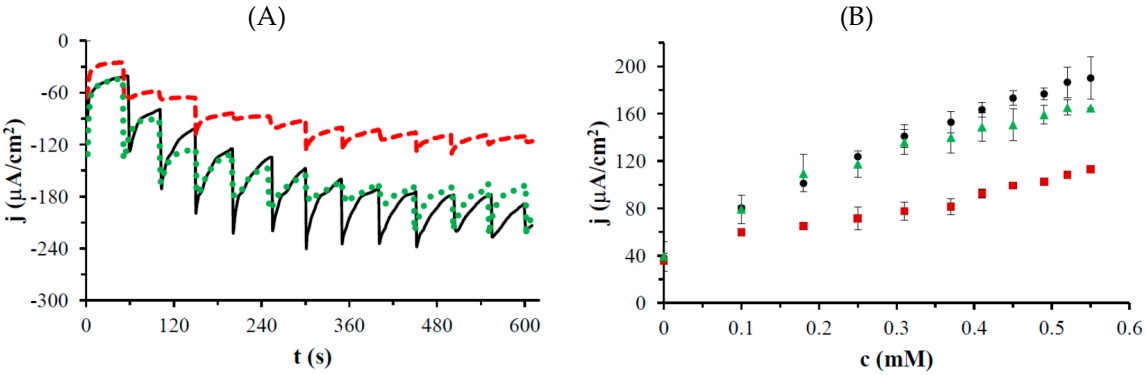


Figure 5. (**A**) Chronoamperometric curves recorded with GCE electrode covered with (a) MWCNTs and laccase (red curve dashed line), (b) MWCNTs/PRZ and laccase (black curve continuous line) and (c) MWCNT/ASYR and laccase (green curve dotted line) in McIlvaine buffer (pH 6.0) with different oxygen concentrations. (**B**) Dependence of catalytic current densities of oxygen reduction (j_{cat}) on concentration of dissolved oxygen for tested electrodes (a) MWCNTs and laccase (red squares), (b) MWCNTs/PRZ and laccase (black dots) and (c) MWCNTs/ASYR and laccase (green triangles).

3. Materials and Methods

3.1. Materials and Chemicals

The Cerrena unicolor C-139 laccase lyophilized samples in vials were obtained from the Department of Biochemistry (Maria Curie-Sklodowska University, Poland) [42]. The activity of laccase stock solution after dissolving the protein powder (from vial) in 1 mL of Milli-Q water was equal to 128 U per mg of protein.

Nanopure water (resistance 18.2 MΩ/cm) was distilled and passed through Milli-Q purification system (Millipore Corporation, Bedford, MA, USA). The inorganic and organic reagents were obtained from POCh (Gliwice, Poland) and Sigma-Aldrich (Saint Louis, MO, USA), and were used without further purification. Oxygen and argon (99.5%) were used from Air Products (Kielce, Poland). Multi-walled carbon nanotubes (MWCNTs Nanocyl Thin MWCNT 95+% C purity with 9.5 nm average diameter and 1.5 μm length) were purchased from Nanocyl (Sambreville, Belgium).

3.2. Electrochemical Instrumentation and Procedures

The unmodified MWCNTs suspension was prepared by adding 8 mg of nanotubes to 12 mL of ethanol. Next, the mixture of nanotubes in ethanol was placed in an ultrasound bath for 30 min.

GCE/MWCNTs electrode preparation, a GCE surface was modified with MWCNTs by dropping 30 μL of as-prepared suspension of nanotubes on the electrode surface and was left to dry in air.

The working laccase solution was prepared by dissolving 1.6 mg of enzyme in 20 mL of McIlvaine buffer (pH 6.0).

McIlvaine buffer solution pH 6.0 was prepared by mixing 0.1 M citric acid and 0.2 M disodium phosphate to obtain the desired pH, under the control of Mettler Toledo pH-meter.

In order to immobilize the laccase enzyme, the electrodes modified with MWCNTs with/without mediators were cycled in the potential range from 0.8 V to 0.0 V, one cycle with scan rate 0.1 V/s

following one cycle with scan rate 5 mV/s in deoxygenated McIlvaine buffer of pH 6.0 containing laccase. The electrodes were then rinsed thoroughly with water.

Promazine immobilization on the electrode modified with MWCNTs consisted of adsorption of promazine by cycling previously modified electrodes in the potential range from −0.2 V to 1.0 V with 0.1 V/s scan rate (5 cycles), then the electroadsorption of the compound at the potential equal to −0.2 V for 60 s and lastly cycling 5 times with 0.1 V/s scan rate in the range of potentials from −0.2 V to 1.0 V in deoxygenated McIlvaine pH 6.0 containing 1 mM promazine. The electrodes were then rinsed thoroughly with water.

Acetosyringone immobilization on the electrode modified with MWCNTs included adsorption of acetosyringone by cycling the modified electrodes in the potential range from −0.2 V to 1.0 V with 0.1 V/s scan rate (10 cycles), and at the end the electroadsorption at the potential equal to −0.2 V for 60 s in deoxygenated McIlvaine pH 6.0 containing 1 mM acetosyringone. The electrodes were then rinsed thoroughly with water.

Electrochemical experiments were performed using three electrode arrangement with Ag/AgCl (KClsat.) reference electrode, platinum foil as the counter electrode and glassy carbon electrode with geometric surface area of 0.071 cm^2 (GCE, BASi) as the working electrode. Before surface modification, GCE electrode was cleaned to eliminate possible contaminants. For this purpose, the electrode was polished on a polishing disc with Al_2O_3 powder (diameter of 0.3 μm). The electrode was then rinsed twice with distilled water until the Al_2O_3 was completely washed away. Finally, the electrode was washed twice with ethanol and wiped with a dust-free tissue (Kimberly-Clark) leaving no fibers on the electroactive surface.

Cyclic voltammetry (CV) and chronoamperometric (CA) experiments were carried out using ECO Chemie Autolab potentiostat. All electrochemical measurements were performed at 22 °C. All current densities were calculated using geometric area of the electrode. Electrochemical measurements were recorded in an anaerobic environment obtained by passing argon through the solution for about 20 min. In the case of cathode activity tests, the solution was saturated with oxygen by passing oxygen through the solution for about 20 min. During the measurements, the appropriate gas was passed over the solution. The current values obtained were converted to the current density, dividing the obtained value by the size of the geometrical surface of the working electrode. The potential of oxygen sensor used for recording the CA oxygen reduction current was set to 0.2 V.

The biobattery parameters were examined in two electrode system: anode/cathode. For the system, open circuit potential (OCP) and the cell potential (E), were measured in all experiments. The cell potential (potential between anode and cathode) was recorded for different loads in the resistance range from 10 MΩ to 1 kΩ. The measurements after each load were restricted to 5 s to minimize the power loss caused by oxygen depletion during the testing of the cathodes. The anode for biobattery was zinc plate (Goodfellow) coated with a hopeite layer, which was formed during the Zn electrode oxidation. The surface area of the anode was the same as surface area for cathode. The cell potential was converted into current using Ohm's law. Next, the current value was divided by the surface of the electrode, obtaining the current density. The power density of biobattery was calculated from P = j * E dependence. The dependences P (j), E (j) were determined, and indicate the efficiency of the cell in the range of given resistance.

4. Conclusions

Comparison of the results for oxygen reduction on the three studied systems revealed that, the drugs used were good mediators and lead to significant enhancement of oxygen reduction currents. The data obtained prove that not only the adsorption of mediators, but also of the laccase on these electrodes was very efficient. All recorded oxygen reduction waves were well-developed and started at the potential of +0.6 V vs. Ag/AgCl ($KCl_{sat.}$). The best-electrode was that covered with multiwall carbon nanotubes, ASYR, and laccase. The use of mediators as expected, leds to the improvement of the efficiency of the work of the biocathode, compared to the GCE electrode modified only with

MWCNTs and laccase. Carbon nanotubes increase the electrode surface and provided good contact with ASYR, improving electron exchange between the electrode and the enzyme.

The tested biocathodes were also effective in the biobattery system. In all systems, the achieved open circuit potential was close to 1.6 V. For biobattery with bioelectrode covered with MWCNTs and laccase, the lowest power and current density values were obtained, while the largest for biobattery with bioelectrode covered with MWCNTs, ASYR, and laccase. In the latter case, the obtained values of power density were 1.85 mW/cm^2 of current density 2.3 mA/cm^2 and the potential was 0.811 V for the biobattery loaded with an external resistance of 5 kΩ and the OCP was 1.6 V. The ASYR mediator proved to be the most beneficial for the preparation of biobatteries.

Both mediators were useful for oxygen-sensing devices since electrodes covered with mediators and laccase, gave about 80% larger catalytic current densities at 0.3 mM oxygen concentration compared to MWCNTs-modified GCE electrodes with laccase alone. The GCE electrode modified with MWCNTs, PRZ, and laccase showed higher stability during measurements compared to the GCE electrode modified with MWCNTs, ASYR, and laccase, revealing stronger interaction of adsorbed laccase with an MWCNTs-PRZ-modified electrode.

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