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Enzymatic biofuel cell based on electrodes modified with lipid liquid-crystalline cubic phases

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ABSTRACT

Two glassy carbon electrodes modified with enzymes embedded in lyotropic liquid-crystalline cubic phase were used for the biofuel cell construction. The monoolein liquid-crystalline film allowed to avoid separators in the biofuel cell. Glucose and oxygen as fuels, and glucose oxidase and laccase as anode and cathode biocatalysts, respectively were used. The biofuel cell parameters were examined in McIlvaine buffer, pH 7 solution containing 15 mM of glucose and saturated with dioxygen. A series of mediators were tested taking into account their formal potentials, stability in the cubic phase and efficiency of mediation. Most stable was the biofuel cell based on tetrathiafulvalene (TTF) and 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonate) (ABTS) as anode and cathode mediators, respectively. The open-circuit voltage was equal to 450 ± 40 mV. The power densities and current densities were measured for all the systems studied.

1. Introduction

The role of enzymatic biofuel cell is to convert the chemical energy into electrical current using the redox enzymes as biocatalysts. The power output generated by such system is high enough to supply microelectronics systems, microdevices and pace makers that require relatively low power [1–15]. The future goals include the use of these devices in physiological media and to power implanted medical devices. For these applications the biofuel cells have to be biocompatible, safe but also cheap since the enzyme film in the devices should be easily replaced.

The main advantage of this type of fuel cell is the application of natural compounds, e.g. glucose or ethanol, as fuels and the ability to operate under mild condition (at temperature of 20–40 °C and at pH near to neutral). These properties make biofuel cells attractive for applications where generating high temperature is difficult or where severe reaction conditions are unfavourable. Most of the biofuel cells rely on the primary alcohols and sugars as the substrates and alcohol or glucose dehydrogenases or glucose oxidase as the anode biocatalysts. Glycerol was found a convenient alternative since better power densities could be achieved than for common ethanol biofuel cells and swelling the matrix (common difficulty with the Nafion matrix) was avoided [16]. One of the critical challenges in developing direct biofuel cells is inefficient electron conduction between biocatalysts and electrodes. Using carbon substrates, usually a mediator to be employed in order to connect electrically the enzyme with the electrode and provide high catalytic efficiency of the system [17–19].

Recently proposed biocathodes and bioanodes are often based on metal electrodes, e.g. platinum or on employing osmium complex-linked polymers as the mediators. Examples of application of osmium complexes in biofuel cells are collected in Table 1 [8].

On the other hand, attempts have been made to both avoid Pt electrodes, and osmium complexes as mediating moieties. Osmium complexes rise some concern among the medical community in case of applications in living organisms. Using organic biocompatible mediators or new ways to eliminate the necessity of mediators would bring a solution to these problems. Vitamin K_3-immobilized polymers were found useful as bioanode mediators [20]. Only few direct electron transfer-type (DET-type) biofuel cell have been presented in the literature [21,22]. A notable fructose/dioxygen bio-

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Table 1
Biofuel cells based on osmium complexes

System (oxidation/reduction)	Anode biocatalysts/electrodes	Cathode biocatalysts/electrodes	Open-circuit voltage (V)	Power density ($\mu W\,cm^{-2}$)[a]	Reference
MET glucose/MET O_2	GOx with redox polymer containing [Os(*N*,*N*-dialkylated-2,2-bis-imidazole)$_3$]$^{2+/3+}$ centres	BOx with redox copolymer of polyacrylamide and poly(*N*-vinyl-imidazole) with [Os(4,4-dichloro 2,2-bipyridine)$_2$Cl$_2$]$^{+/2+}$	0.84	432 $\mu W\,cm^{-2}$ (at 0.52 V) 0.85 $\mu A\,cm^{-2}$	[9]
MET glucose/MET O_2	GOx with redox polymer PVI-[Os(4,4-diamino-2,2-bipyridine)$_2$Cl]$^{+}$/$^{2+}$	BOx with redox copolymer of polyacrylamide and poly(*N*-vinyl-imidazole) with [Os(4,4-dichloro-2,2-bipyridine)$_2$Cl$_2$]$^{+}$/$^{2+}$	0.68	50 $\mu W\,cm^{-2}$; 0.2 $\mu A\,cm^{-2}$	[10]
MET glucose/O_2	GOx with redox polymer (PVP-[Os(*N*,*N*-dialkylated-2,2-bisimidazole)$_3$]$^{2+/3+}$)	BOx with redox polymer(PAA-PVI-[Os(4,4-dichloro-2,2-bipyridine)$_2$Cl]$^{+/2+}$)	0.8	440 $\mu W\,cm^{-2}$ (at 0.52 V)	[7]
MET glucose/O_2	GOx with redox polymer poly(4-vinylpyridine)[Os(*N*,*N*-dimethyl-2,2-bisimidazole)$_3$]$^{2+/3+)}$	Laccase with redox polymer composed of an osmium complex Os[(4,4-dimethyl-2,2-bipyridine)$_2$ (4-aminomethyl-4methyl-2,2-bipyridine)] reacted to form amides with *N*-(5-carboxypentyl) pyridinium functions of poly(4-vinylpyridine)	1.0	350 $\mu W\,cm^{-2}$	[2]
MET glucose/MET O_2	GOx, with redox polymer poly(4-vinylpyridine)[Os(*N*,*N*′-dimethyl-2,2′-bisimidazole)3]$^{2+/3+}$)	Laccase with redox polymer composed of Os[(4,4_-dimethyl-2,2-bipyridine)$_2$ (4-aminomethyl-4-methyl-2,2-bipyridine)]	0.880	350 $\mu W\,cm^{-2}$ (J_{sc} = 0.4 mA cm^{-2})	[11]
MET glucose/O_2	GOx, redox polymer [Os(4,4′-diamino-2,2′-bipyridine)$_2$(poly{*N*-vinylimidazole})-(poly{*N*-vinylimidazole})$_9$Cl]Cl	Laccase, redox polymer [Os(phenanthroline)$_2$(poly{*N*-vinylimidazole})$_2$-(poly{*N*-vinylimidazole})$_8$] Cl_2	0.4	16 $\mu W\,cm^{-2}$	[12]

[a] Larger values are obtained under stirring conditions as described in the text.

fuel cell based on DET-type of bioelectrocatalysis was presented by Kamitaka et al. [22]. D-Fructose dehydrogenase and laccase were used as biocatalysts for the anode and cathode, respectively. The maximum power density of 850 $\mu A\,cm^{-2}$ was obtained at 410 mV of the cell voltage under stirring. Since in the common practical devices stirring (to enhance the current densities) will not be used, the current densities under stirring are just given for comparison with other electrode systems, while the results in unstirred solutions are much more useful.

The aim of our work was to check the utility of the electrodes modified with enzymes embedded in cubic phase matrices for the construction of a biofuel cell. We have shown in our recent papers that cubic phase-modified electrodes are useful for immobilizing enzymes on the electrode surface in their active forms, and can be employed for immobilizing laccase, glucose oxidase and pyranose oxidase [17,23–26]. Improved characteristics of the liquid-crystalline matrices for the cathode and anode have been demonstrated recently by Heller and coworkers [27,28].

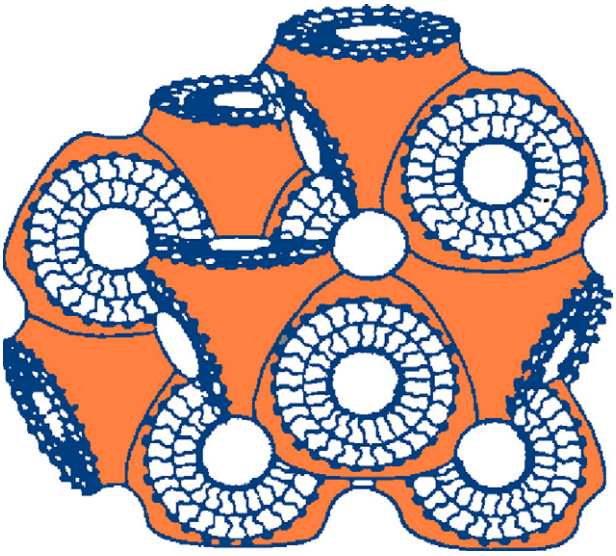
In this report we present the construction and characteristics of a membrane-less biofuel cell based on electrodes modified with liquid-crystalline cubic phase film.

Liquid-crystalline phases formed by polar lipids in aqueous media can be used as model matrices to mimic biological process. Lyotropic cubic phase have a well-defined and reproducible structure determined, e.g. by small angle X-ray spectroscopy [29–32]. From the electrochemical point of view it is important that amphiphilic enzyme molecule remains attached to the lipid bilayer, while water channels allow the enzymatic substrates and products to diffuse freely through the system while the enzyme remains anchored in the nanostructure [26,33] (Scheme 1).

Moreover, the cubic phases are viscous, so they can be easily smeared onto solid supports, and they are stable in the presence of excess of water. One of the main advantages of lipid cubic phases is their non-toxicity and ability of biodegradation in organisms. If the cubic phase film is used directly in the physiological fluid, the reaction with lipases may interfere and hence under these conditions either lipase impermeable membrane should be used or the monoacylglycerol cubic phase can be substituted, e.g. by phytantriol [28].

The liquid-crystalline cubic phase resembles also natural environment for membrane proteins and can be used for their crystallization. Doping monoolein (MO) with 1,2-dioleoyl-*sn*-glycero-3-phosphate (DOPA) can increase the shear strength of the MO/H_2O cubic phase and renders the walls of the aqueous channels anionic allowing more stable immobilization of cationic mediators inside the cubic phase due to electrostatic interactions [27].

Recent papers on development of biofuel cells are often based on glucose as fuel and dioxygen as an oxidant [34]. In such system glucose is oxidized at the anode by glucose oxidase (GOx) producing



Scheme 1. Structure of the cubic phase.

protons and electrons whereas at the cathode dioxygen is reduced by laccase or bilirubin oxidase, generating water. Laccase used in our biofuel cell (*p*-diphenol:oxygen oxidoreductase, EC 1.10.3.2) is a multi-copper oxidase that catalyzes the four-electron reduction of dioxygen directly to water with the concomitant oxidation of various aromatic compounds. The metal centre in laccase contains four copper atoms classified in accordance with their spectroscopic characteristics as a T1, T2 and T3 sites. The T1 site of the enzyme is involved in binding of substrate, its oxidation, and transferring of the electron to the T2/T3 cluster, where dioxygen is reduced to water [35–38]. Glucose oxidase (1.1.3.4), a dimeric protein which catalyzes the oxidation of β-D-glucose into D-glucono-1,5-lactone which then hydrolyses to gluconic acid was employed as the anode enzyme.

2. Experimental

Laccase from *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) [39] and lyophilised on Labconco (Kansas City, USA, FreeZone Lyophiliser). Enzyme activity was measured spectrophotometrically with syringaldazine as the substrate for laccase [40]. The protein content was determined according to Bradford with bovine albumin as the standard [41]. 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 1 ml of water was 1,150,110 nkat dm^{-3} and $C_{lacc} = 1.18\,mg\,cm^{-3}$.

The mutant of filamentous fungi *Aspergillus niger* AM-11 from the culture collection of the Department of Industrial Microbiology (M.C. Sklodowska University, Lublin, Poland) was used as a source of glucose oxidase (GOD) [42,43]. The strain was stored at 4 °C on malt agar slants. The culture was run in liquid stationary cultures on media which contained the following components ($g\,l^{-1}$): glucose, 80.0; peptone, 3.0; $NaNO_3$, 0.5; KH_2PO_4, 1.0; $CaCO_3$ (sterilized separately) 10.0. After inoculation with 2% spore suspension of *A. niger* AM-11, the cultures were incubated for 10 days at 30 °C in non-agitated 500 ml Erlenmeyer flasks containing 150 ml of culture medium. The mycelium was then separated by centrifugation at 7500 rpm, using Sigma centrifuge-type 4K10 (Germany). The concentrated fraction was subsequently desalted by gel filtration chromatography on Sephadex G-50 (2.8 cm × 50.0 cm). The column was eluted with a 5 mM phosphate buffer, pH 6.0. The fractions containing GOD activity were collected and lyophilized using Labconco lyophylizer-type 77535 (USA).

Step	Total activity (U)	Specific activity (U mg^{-1} protein)	Purification factor	Yield (%)
Crude lyophilisate	1651.95	6.1	1	100
High Q lyophilisate	1530.15	36.8	6.03	92.62

The activity of GOD was determined according to Fiedurek and Gromada [43]. One unit of enzyme activity was defined as the amount of protein that produces 1 μmol of H_2O_2 min^{-1} at 37 °C. The reaction mixture contained 1 ml suitably diluted enzyme and 2 mg glucose, 0.02 mg *o*-dianisidine, 0.06 mg peroxidase in 2 ml of Tris–phosphate–glycerol buffer pH 7.0 was incubated for 30 min at 37 °C. The reaction was stopped by adding 4 ml of 5N HCl. The absorbance at 525 nm was measured against blank composed and incubated as describe above but without the enzyme. Reducing sugars were analyzed by the Somogyi-Nelson method [44,45]. Protein content was determined with the method of Bradford [41] using bovine serum albumin as the standard.

Monoolein (1-oleoyl-rac-glycerol) (MO), ferrocenecarboxylic acid, tetrathiafulvalene (TTF), methylene blue (MB), 2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulfonate) diammonium salt (ABTS), D-(+)-glucose were purchased from Sigma and were used as received. Na_2HPO_4, KH_2PO_4 and citrate acid were from POCh (Polish Chemicals Co.). All solutions were prepared using Milli Q water (18.2 MΩ cm^{-1}), Millipore, Bedford, MA, USA. Stock solutions of D-(+)-glucose were prepared at least 24 h before the experiment to reach equilibrium between α and β anomers.

Cyclic voltammetry experiments were performed using an ECO Chemie Autolab potentiostat with GPES software in a three-electrode arrangement with a saturated calomel reference electrode (SCE), a platinum sheet as a counter electrode, and a cubic phase film-modified glassy carbon electrode (GCE) as the working electrode.

The undoped cubic phase was prepared by melting monoolein and appropriate mediator in a small glass vial (about 10 mg) and appropriate amount of water or enzyme solution were added as described elsewhere [23–26]. The ratio of components was chosen on the basis of phase diagram for the monoolein–water system and it corresponded to the formation of a diamond type of cubic phase.

The glass vial was tightly closed and centrifuged for 15 min at 4500 rpm in the aim of mixing the components. After centrifugation, transparent and highly viscous cubic phase was obtained. The stability of system was confirmed by macroscopic observations of the sample viscosity and clarity. The cubic phase was weighed to determine the amount of enzyme in the system.

To remove any possible traces of H_2O_2 in the anodic electrode film catalase was added, however, it did not affect either the stability or the parameters of the cell.

The 1,2-dioleoyl-*sn*-glycero-3-phosphate-doped MO/H_2O cubic phase was prepared according to the published method [27]. 1,2-Dioleoyl-*sn*-glycero-3-phosphate was weighed and then monoolein and appropriate mediator was added to obtain 8 mol% 1,2-dioleoyl-*sn*-glycero-3-phosphate-containing monoolein. To avoid phase separation the lipids and mediator were dissolved in chloroform, which was then evaporated, then appropriate amount of enzyme solution was added. The mixture was then centrifuged for 30 min at 4500 rpm. The stability of system was confirmed by macroscopic observations of the sample viscosity and clarity.

3. Results and discussion

An enzyme-based glucose/O_2 biofuel cell was constructed applying glucose oxidase and laccase as biocatalysts. Catalytic efficiencies of the enzymes in the cubic phase films were determined using cyclic voltammetry. To enhance the electron transfer between the enzyme and the electrode surface several electron mediators were tested. As the bioanode we applied a glassy carbon disk modified with the cubic phase containing glucose oxidase isolated from *A. niger* and tetrathiafulvalene, methylene green (MG) or ferrocenecarboxylic acid (FcCOOH) as the mediators.

First, the electrochemical behavior of different mediators on the electrode modified with cubic phase was studied using cyclic voltammetry. The voltammograms for mediators are shown in Fig. 1.

Fig. 1A presents the voltammogram recorded for ABTS, common mediator used for oxygen reduction catalyzed by laccase. ABTS exhibits reversible electrochemistry, the advantage of ABTS is that its formal potential is close to the formal potential of laccase. The potential of ABTS of 0.49 V (vs. Ag/AgCl) is in accordance with the

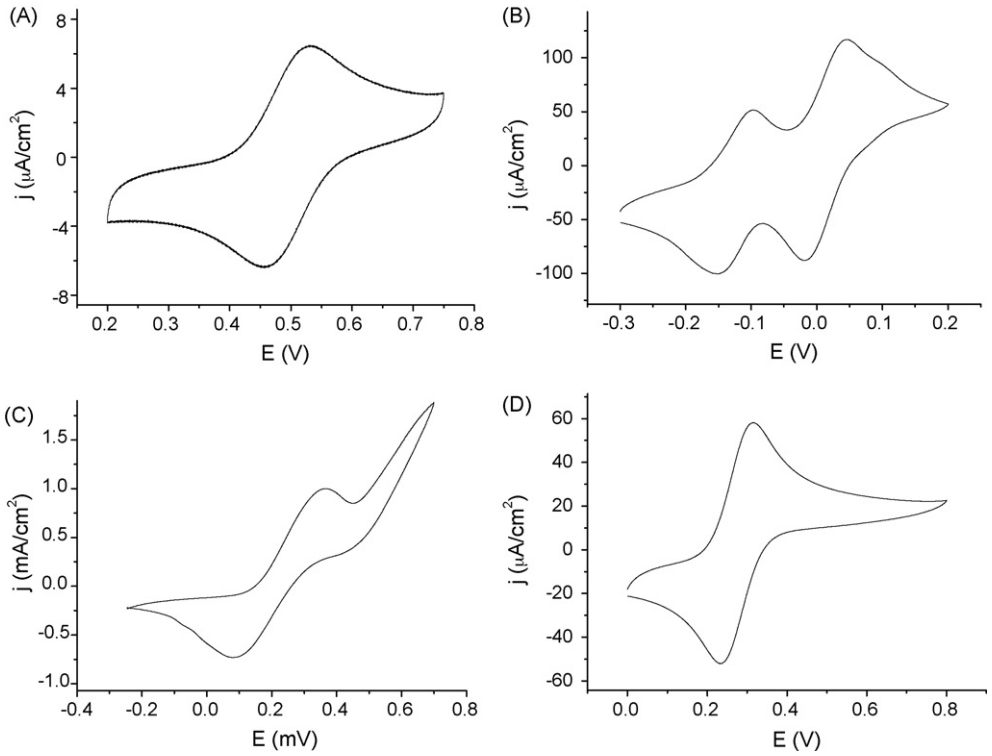


Fig. 1. Voltammograms recorded in McIlvaine buffer solution, pH 7, using GCE modified with cubic phase with 60:37:3 wt%: (A) MO/H_2O/ABTS, (B) MO/H_2O/MG, (C) MO/H_2O/TTF, and (D) MO/H_2O/FcCOOH. Scan rate: 20 mV s^{-1}.

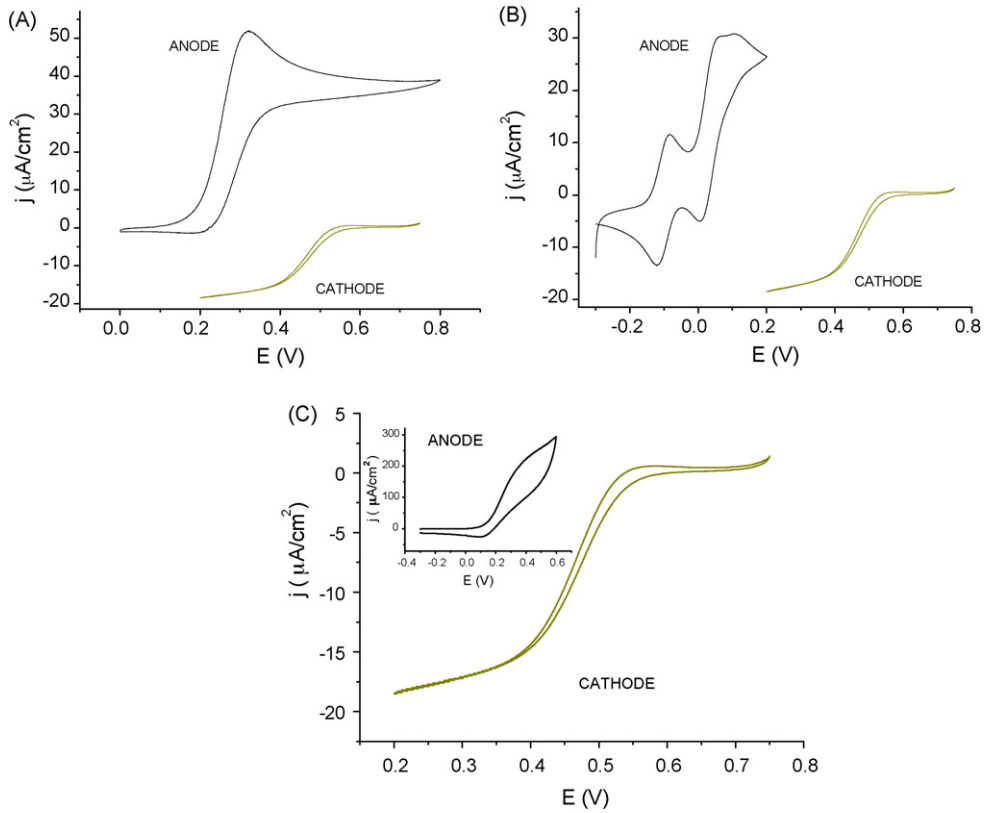


Fig. 2. Cathode: oxygen reduction catalyzed by laccase *Cerrena unicolor* and ABTS as mediator, MO/H_2O/ABTS 60:37:3 wt%. Anode: catalytic glucose oxidation on GCE modified with cubic phase with glucose oxidase from *Aspergillus niger* with 60:37:3 wt%: (A) MO/H_2O/MG, (B) MO/H_2O/TTF, and (C) MO/H_2O/FcCOOH, as mediators. Scan rate: 1 mV s^{-1}.

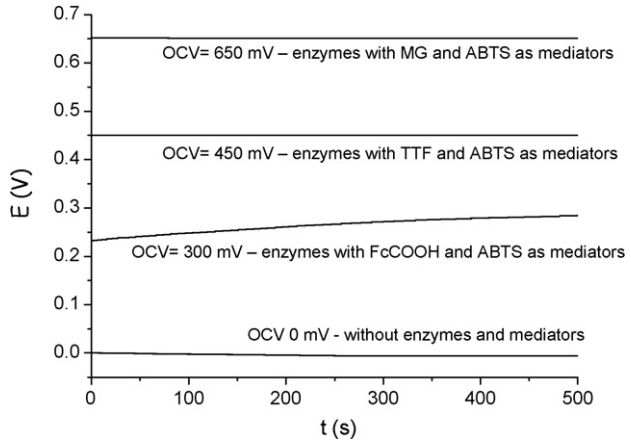
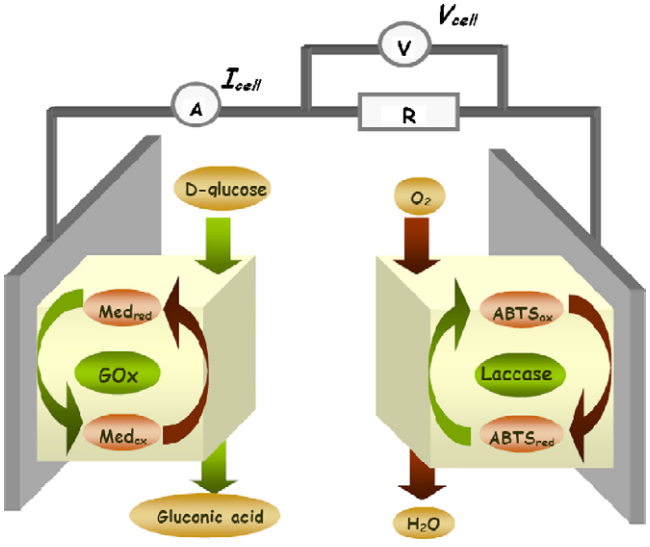


Fig. 3. Open-circuit voltage.

values reported in the literature. The electrode material used for the biocathode was always glassy carbon. Its surface was modified with the cubic phase and laccase from *C. unicolor* and ABTS. Cyclic voltammograms for laccase catalyzed reduction of oxygen at the cathode are shown in Fig. 2D. Recently, we have reported the kinetic parameters for this system [17,23].

For the anodic process, three different mediators were tested by embedding them in cubic phase film. Results of these experiments are presented in Fig. 1B–C. The value of formal redox potential for ferrocenecarboxylic acid (FcCOOH) is $E_f = 0.330$ V versus Ag/AgCl, for tetrathiafulvalene is $E_f = 0.222$ V and for methylene green $E_{f1} = 0.03$ V, $E_{f2} = 0.123$ V. The anodic current increases and the shape of the curve changes after adding glucose into the solution, implying biocatalytic glucose oxidation. Cyclic voltammograms for the glucose oxidase catalyzed oxidation of glucose on the anode and laccase catalyzed reduction of oxygen at the cathode are shown in Fig. 2.

When MG is used instead of FcCOOH as the mediator, the potential of glucose oxidation is shifted to ca. 0.03 V, hence to more negative potentials. Potential is favourable for the application in the biofuel cell, which is confirmed by the value of open-circuit potential (Fig. 3).



Scheme 2. Schematic representation of the biofuel cell circuit.

On the other hand, the processes involving MG in the cubic phase are not so clear and in addition the stability in time is not as good as in case of TTF or FcCOOH. Much better stability in the cubic phase is achieved when the MG mediator is adsorbed on carbon nanotubes forming a network within the film [46].

The biofuel cell used in our experiments was fabricated from glass and solution volume was 20 ml. Scheme 2 shows the configuration of the biofuel cell.

Open-circuit voltage was determined for each system (Fig. 3). Applying mediators with a formal potential close to the potential of the FAD in glucose oxidase results in the increase of the open-circuit voltage of the cell. Mediators with formal potentials close to that of the enzyme improved the performance of the cell.

The variable loads, in the range from 10 kΩ to 10 MΩ, were applied between the anode and the cathode to determine the cell current (I_{cell}) and the cell voltage (V_{cell}). Current and cell voltage were measured after the stabilization of system. The cell voltage was determined by the value of formal potential of the mediator used in the anodes and cathode films.

The biofuel cell parameters were examined in phosphate buffer solution, pH 7 containing 15 mM of glucose and saturated with dioxygen. Parameters evaluated for each system are collected in Table 2.

The most stable system was obtained when TTF and ABTS were used as mediators. The open-circuit voltage was then equal to 450 ± 40 mV. Upon increasing the external load, the current decreases and is almost 0 at external load of 1 MΩ. The cell voltage increases, and at ca. 3000 kΩ, it levels of to a constant value of ca. 400 mV.

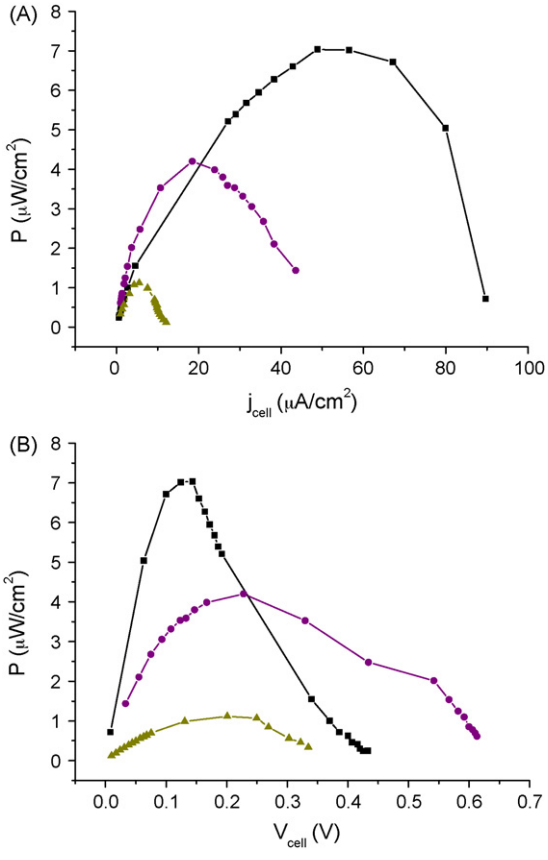


Fig. 4. Power density of the biofuel as a function of (A) current density (j_{cell}), (B) cell voltage (V_{cell}), (FcCOOH/ABTS, ▲; MG/ABTS, ●; TTF/ABTS, ■).

Table 2
Characteristics of the biofuel cells

Mediators cathode/anode	OCV (mV)	J_{cell} ($\mu A\,cm^{-2}$)	V_{cell} (mV)	Power density ($\mu W\,cm^{-2}$)
FcCOOH–ABTS	300 ± 40	5 ± 0.7	200 ± 20	1.12 ± 0.4
MG–ABTS	650 ± 50	20 ± 5	250 ± 30	4.18 ± 0.6
TTF–ABTS	450 ± 40	60 ± 10	125 ± 30	7.09 ± 0.9

Fig. 4 displays dependence of power density on the current density and cell voltage for each tested system. We obtained the maximum performance of our cell when TTF and ABTS were used as mediators. A maximum power density of ca. 7 $\mu W\,cm^{-2}$ was obtained with ca. 0.125 V potential output.

4. Conclusions

A membrane-less biofuel cell based on the application of monoolein cubic phase film has been prepared. Selectivity of the enzymes used and the immobilization of both the enzyme and the mediator in the thin liquid-crystalline film on the electrode allow the construction of bioanode and biocathode without the necessity of additional separation of the biofuel cell electrodes. Hence, both cubic phase film electrodes can be immersed in one solution without any semi-permeable membranes. In the cell proposed only organic mediators are employed to contact the biocatalyst with the conductor and the electrodes are made of glassy carbon. The cell based on cubic phase matrix [47] is environmentally safe and biocompatible. The cell works reproducibly in neutral solutions and the parameters $7 \pm 0.9\,\mu W\,cm^{-2}$ at ca. 125 mV. Maximum current density of value $60 \pm 10\,\mu A\,cm^{-2}$ at external load of 10 kΩ was obtained. To improve these parameters a glucose oxidase mediator with a more negative formal potential would be useful. Nanotubes and mediators bound covalently to carbon nanotubes are promising since they increase the electrode working area and improve the conductivity of the system. Work on miniaturization of the device and increasing the number of electrodes in the system is underway in our laboratory.

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