

Catalytic activity of oxidases hosted in lipidic cubic phases on electrodes

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Abstract

The monoolein-based liquid crystalline cubic phase was used as the matrix to incorporate redox enzymes — glucose (GOx), pyranose (PyOx) oxidases and laccase. Thin layer of the cubic phase embedding GOx or PyOx activated glucose oxidation in the presence and absence of appropriate mediators. The electrodes exhibited unchanged voltammetric response to glucose for not less than six days. The potentials and ratio of catalytic to diffusion currents could be modified by choosing appropriate electroactive probes as mediators. Ferrocenecarboxylic acid and $\text{Ru}(\text{NH}_3)_6^{2+}$ provided contact between the electrode and the enzyme. The sensitivity to glucose for glucose oxidase was 0.4 ± 0.05 , $11 \pm 3.1 \mu\text{A}/\text{cm}^2/\text{mM}$ without mediator and with ferrocenecarboxylic acid respectively and 0.9 ± 0.06 , $31 \pm 5.6 \mu\text{A}/\text{cm}^2/\text{mM}$ for pyranose oxidase without and with mediator. The system based on glucose oxidase and $\text{Ru}(\text{NH}_3)_6^{2+}$ as mediator was found useful due to the most negative potential of the process. The catalyses of oxygen reduction by two laccases: *Cerrena unicolor* and *Trametes hirsuta* embedded in the cubic phase together with 2,2'-azino-bis-3-ethylbenzothiazoline-6-sulfonate (ABTS) as the mediator were found efficient and the reduction potential was positive enough to be considered in the application of lyotropic liquid crystals as a material for biofuel cells.

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1. Introduction

Cubic phases based on monoacylglycerols form readily and attract interest due to their ability to incorporate and stabilize proteins, and to their low toxicity permitting their use as drug delivery systems [1–6]. Cubic liquid crystals with a number of different structures have been reported [7–9]. These are usually grouped into two main types, namely the discrete and bicontinuous structures, which can be of the normal or reverse kind [10].

Retaining enzymes in functionally-active forms on the electrode surface is a challenging and difficult task. Ideally the immobilization of protein should be performed under conditions that provide membrane like environment in which all the normal interactions of the proteins are preserved. Providing electronic contact of the protein molecules with the conducting substrate by means of a biocompatible medium is even more difficult.

The lipidic cubic phase can be characterized as a curved bilayer forming a three-dimensional crystallographically well-ordered structure that is interwoven by aqueous channels (Fig. 1).

In the binary system used in our studies, i.e. 1-monooleoyl-*rac*-glycerol/water at 20 °C, two reversed bicontinuous cubic phases, belonging to the space groups *Ia3d* and *Pn3m*, are present, as determined by X-ray spectroscopy [11–14].

Homogeneity and transparency are advantages of the lipidic cubic phases as matrix material. Because the phases are isotropic and optically transparent they are ideal matrices for UV-visible and other spectroscopic investigations by which the functionality of the protein can be probed.

Recently an alternative to glycerol monooleate, a corresponding ether was reported to form liquid crystalline material having the advantage of enhanced stability also against hydrolytic enzymes e.g. lipases due to lack of ester bond in the molecule [15–18]. For monoolein, the *Pn3m* phase formed at hydration over 20%, is stable in the presence of excess of water which is important when the cubic phase is considered as an electrode modifying material [11,19]. Due to high viscosity, the cubic phases can be simply smeared over solid substrates such as electrodes and used to host enzymes and synthetic

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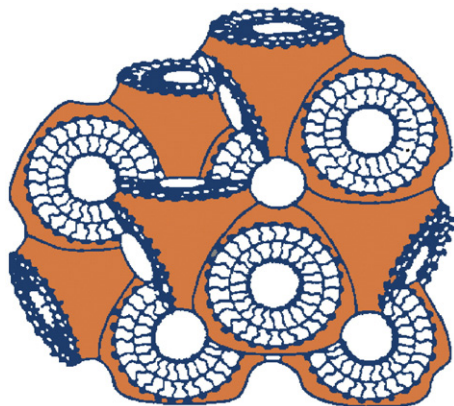


Fig. 1. Structure of the cubic phase www.msri.org/publications/sgp/SGP/index.html.

catalysts leading to new types of catalytically active modified electrodes. Examples of cubic phase modified electrodes have been described in our recent papers [11,19–22]. We have shown that cubic phase modified electrodes are useful for the determination of cholesterol, CO_2 and O_2 .

Monoolein (MO) is used in the present work to prepare the cubic phase by mixing according to the procedure of Rummel et al. [23]. We follow the behaviour of an extensively studied enzyme glucose oxidase in the cubic phase matrix with the ultimate goal of finding a useful anode for biofuel cells. Glucose oxidase is also the most widely used enzyme in the biosensors field [24–27]. It catalyses oxidation of β -D-glucose by oxygen to D-glucono-1,4-lactone and to hydrogen peroxide and this reaction forms basis for the determination of glucose in different media [24,25 and citations therein]. Glucose oxidase electrodes may be constructed in many different ways, the main problem is connected with the large gap between the active site and the electrode surface which makes the transfer of electron between the enzyme and electrode slow or even impossible to follow [26–29]. Therefore, a small mediating compound is usually needed and various one-electron acceptors have been proposed [25] bound to the electrode or freely diffusing to the enzyme either in solution or embedded in different matrices.

Heller et al. described two types of GOx electrodes — based on redox mediator attached to the enzyme, and hydrogels

containing cross-linked adduct of GOx and a redox polymer [30–33]. The redox center of the polymer e.g. the $\text{Os}^{2+/3+}$ complex is bound to the polymer backbone through a long and flexible spacer arm which enables to collect efficiently electrons from GOx. The recent generation of glucose sensors from Heller's laboratory is coulometric sensors containing GOx and the mediator in a very small volume and dissolved in the blood at the moment of measurement [34].

Razumas et al. [35] and Nylander et al. [36] estimated amperometrically diffusion coefficients of glucose in the monoolein-based cubic phase. This approach was based on the determination of H_2O_2 produced in the process of catalytic glucose oxidation. Some assumptions concerning enzyme activity had to be made and since the method was indirect (based on the H_2O_2 oxidation currents) the authors found advantageous the direct measurements of glucose diffusion by the holographic laser interferometry or NMR methods. Results obtained by the latter techniques indicated that diffusion was not perturbed by macroscopic defects in the cubic phase but was likely to be controlled by transport through the water channels [37]. We compare here the efficiency of both glucose and pyranose oxidases embedded in the cubic phase, to collect electrons from the electrode without and with the help of mediators freely diffusing through the cubic phase channels. In a recent work [22] we described the application of the cubic phase to hold different laccases and to compare their activities using as the substrate — hydroquinone and measuring changes in its oxidation current. The mechanism of laccase interactions with oxygen and its immobilization on electrodes are important also from the viewpoint of its possible application in the biofuel cells [37–47]. Several mediators have been studied to connect electrically the enzyme with the electrode [43]. In the present work, the monoolein cubic phase is used to host ABTS (2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonate) diammonium salt), the mediator and laccase, the oxygen-to-water reduction catalyst.

2. Experimental

2.1. Reagents and equipment

Monoolein (1-oleoyl-rac-glycerol) (MO), glucose oxidase (E.C. 1.1.3.4 from *Aspergillus niger*), pyranose oxidase (E.C.

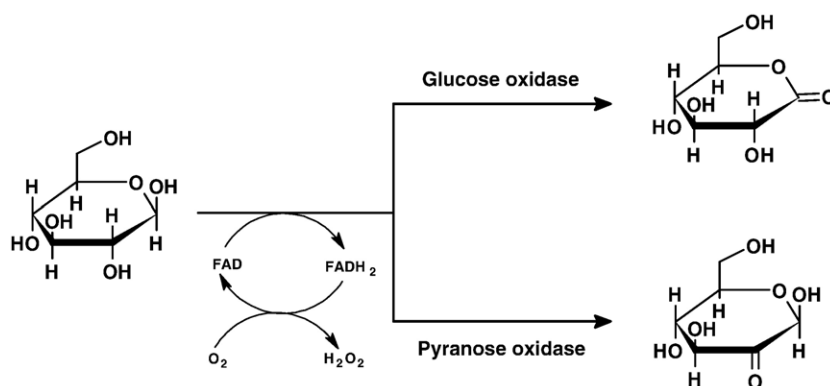


Fig. 2. Conversion of glucose by glucose and pyranose oxidases.

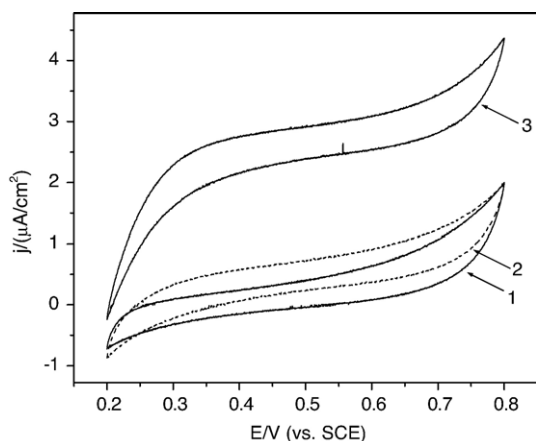


Fig. 3. Cyclic voltammograms recorded using Pt electrode modified with the monoolein containing glucose oxidase in solutions containing: (1) 0 or (3) 1 mM glucose. (----) cyclic voltammogram recorded using Pt electrode modified with the monoolein cubic phase without glucose oxidase in solution containing 3 mM glucose. Oxygenated buffer solution containing 100 mM Na_2HPO_4 and KH_2PO_4 , pH=7 scan rate=1 mV/s.

1.1.3.10 from *Coriolus* sp.), ferrocenecarboxylic acid, $\text{Ru}(\text{NH}_3)_6\text{Cl}_2$, 2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonate) diammonium salt (ABTS), D-(+)-glucose were purchased from Sigma and were used as received. Laccases *Cerrena unicolor* and *Trametes hirsuta* have been kindly provided by professor Jerzy Rogalski from Marie Curie University in Lublin and by professor Lo Gorton from the University in Lund. Na_2HPO_4 , KH_2PO_4 and citrate acid were from POCh (Polish Chemicals Co.). All solutions were prepared using Milli Q water ($18.2 \text{ M}\Omega \cdot \text{cm}^{-1}$), Millipore, Bedford, MA, USA. Stock solutions of D-(+)-glucose were prepared at least 24 h before the experiment to 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 glassy carbon electrode (GCE) or platinum electrode as the working electrodes.

2.2. Preparation of the phase

The cubic phases were prepared by melting monoolein in a small glass vial (about 10 mg) and appropriate amount of water or enzyme solution was added. The ratio of components was chosen on the basis of phase diagram for the monoolein-water system and it corresponds to diamond type of cubic phase [11,19]. 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.

The "empty" cubic phase can be stored in closed vial for several months, but in the case of cubic phase "with enzyme" stored in room temperature, the activity of GOx remains about 90% of initial value for 7 days.

2.3. Electrode modification procedures

The Pt electrode ($A=0.03 \text{ cm}^2$) was first activated in 0.5 M H_2SO_4 and its physical surface area was estimated on the basis of charge of the hydrogen ion reduction peak. Before experiment, the GCE ($A=0.07 \text{ cm}^2$) and Pt electrodes were polished with aluminum oxide powder (grain size down to $0.05 \mu\text{m}$) on a wet pad, rinsed with water and ethanol, dried at room temperature, and then modified with the cubic phase. The electrode was weighed before and after application of cubic phase to determine the amount of cubic phase on the electrode. This amount was usually from 9 to 12 mg per electrode. Cubic phase was deposited on electrode surface using spatula. The thickness of the layers was changed in the range 0.34–2 mm.

The electrode modified with cubic phase was immersed in the deoxygenated supporting electrolyte and was kept in this solution for additional 20 min before each experiments, this time was needed for equilibrating the gas concentration between the cubic phase and solution.

In the case of experiments with mediators, the appropriate amount of each compound was dissolved in the supporting electrolyte solution following its deoxygenation with all

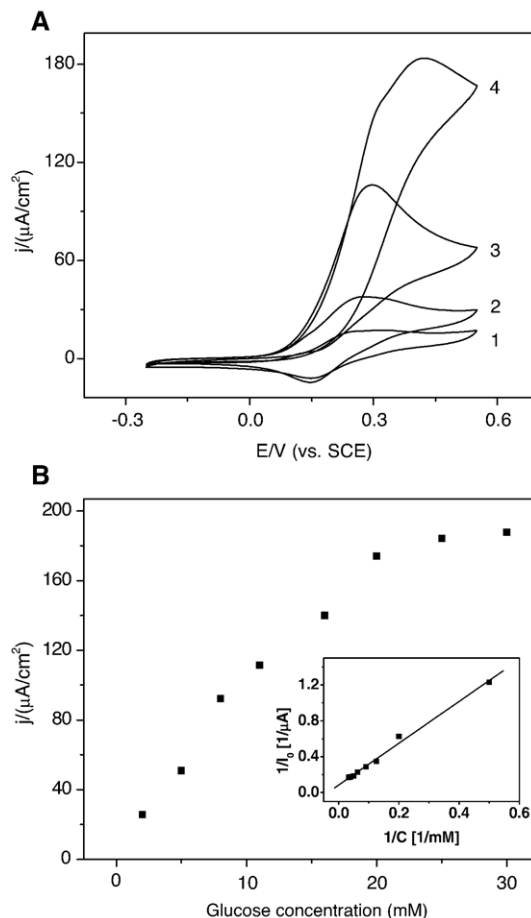


Fig. 4. A. Voltammograms recorded using Pt electrode modified with cubic phase containing 300 ng pyranose oxidase. Deoxygenated 100 mM phosphate buffer, pH=7.0, with 1 mM ferrocenecarboxylic acid, containing: (1) 0; (2) 2; (3) 6; (4) 10 mM glucose. Scan rate: 5 mV/s. B. Current density vs. glucose concentration; inset: $1/I$ vs. $1/C_{\text{glucose}}$ plot.

Table 1
Parameters of the catalysed glucose oxidation process

Enzyme, mediator	K_M [mM]	j_{max} ($\mu\text{A}/\text{cm}^2$)	Sensitivity ($\mu\text{A}/\text{cm}^2/\text{mM}$)
GOx without mediator	5.3 ± 1.3	6.7 ± 1.5	0.4 ± 0.05
GOx with mediator: FcCOOH	27.7 ± 2.3	383 ± 50	11 ± 3.1
PyOx without mediator	4.5 ± 1.2	30 ± 3.3	0.9 ± 0.06
PyOx with mediator: FcCOOH	2.5 ± 0.5	193 ± 40	31 ± 5.6

electrodes including the cubic phase modified electrode immersed in the solution. The electrode was left in the solution for 60 to 120 min, depending on the nature of probe for equilibrating the concentration of electroactive probe in the solution and in the cubic phase. An important advantage of cubic phases is the very efficient diffusion of hydrophilic probes [20]. The diffusion coefficients of the electrochemical probes are significantly larger in the lipidic cubic phase than in other types of matrices. For example, for $[\text{Ru}(\text{NH}_3)_6]^{3+}$ it is $2.17 \pm 0.44 \times 10^{-6} \text{ cm}^2/\text{s}$ [20], while in Nafion it is reported to be $2 \times 10^{-10} \text{ cm}^2/\text{s}$ [48].

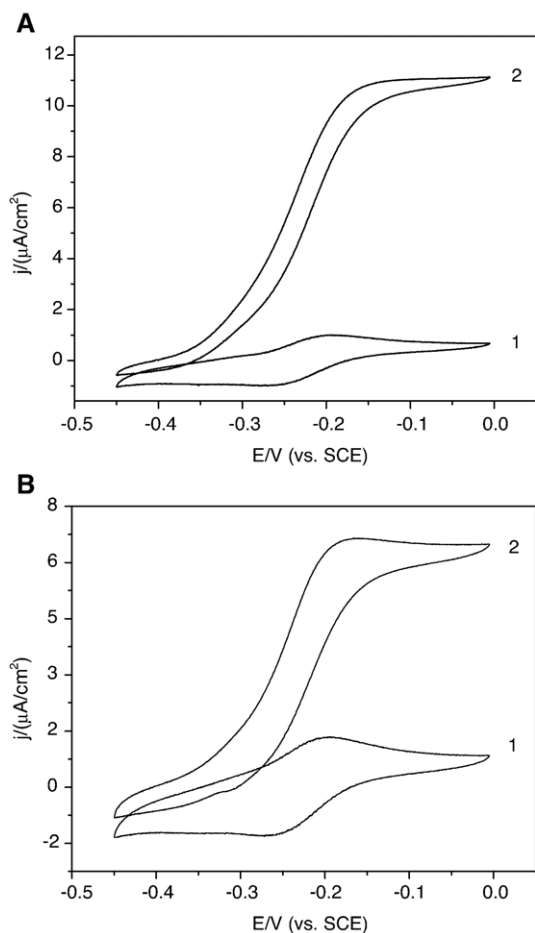


Fig. 5. Voltammograms recorded using GCE modified with cubic phase containing 300 ng glucose oxidase. Deoxygenated 0.1 M phosphate buffer solution, pH=7.0 with 1 mM $\text{Ru}(\text{NH}_3)_6^{2+}$ and (1) 0; (2) 30 mM glucose. Scan rate (A) 0.5 mV/s (B) 1 mV/s.

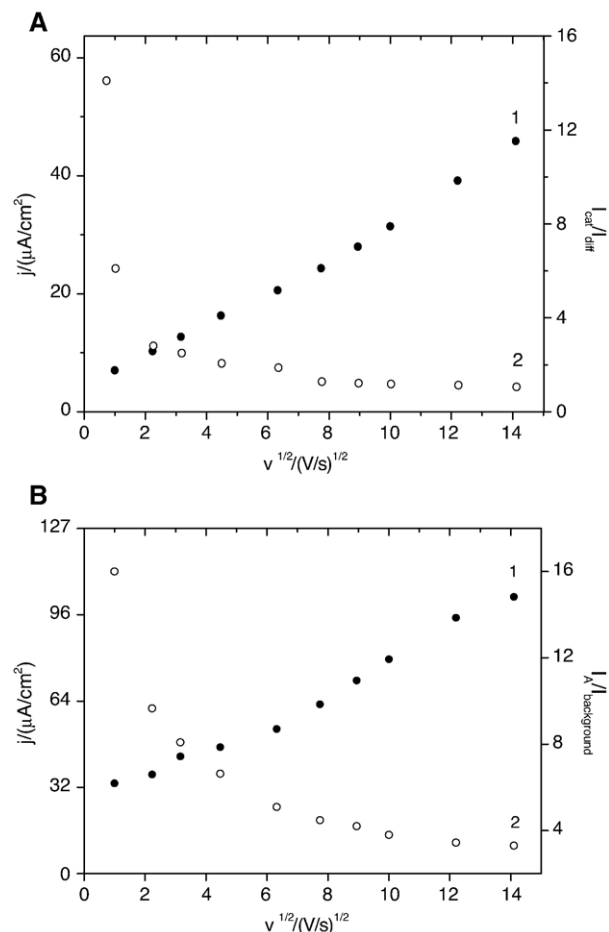


Fig. 6. Current density vs. $v^{1/2}$ and (1) I_{cat}/I_{diff} vs. $v^{1/2}$ (2) $I_a/I_{background}$ vs. $v^{1/2}$. Plots obtained for GOx modified electrode in 0.1 M phosphate buffer, pH=7.0 containing 30 mM glucose (A) deoxygenated buffer containing 1 mM $\text{Ru}(\text{NH}_3)_6^{2+}$, (B) buffer saturated with O_2 without any mediators.

2.4. Immobilization of enzymes

The appropriate amounts of enzymes (glucose or pyranose oxidases) were weighed (usually 4–5 mg) in a vial and water was added (about 100 μl). Lyophilised laccase was dissolved in 1 ml of water to obtain 1 mg/ml solution of laccase. These solutions were used for the cubic phase preparation. Taking into account the cubic phase volume, the estimated concentrations of the enzymes in the cubic phase were equal to $0.38 \mu\text{mol}/\text{dm}^3$ for GOx, $0.11 \mu\text{mol}/\text{dm}^3$ for PyOx and $0.31 \mu\text{mol}/\text{dm}^3$ for laccases. In part of experiments, a suitable electroactive probe was added to mediate the electron transfer between the enzyme and electrode.

3. Results and discussion

Glucose and pyranose oxidases catalyse oxidation of D-glucose to gluconic acid or 2-keto-glucose respectively (Fig. 2).

Glucose oxidase catalyses C1-oxidation anomer β -D-glucose while pyranose oxidase catalyses C2-oxidation α and β anomer of glucose. Hydrogen peroxide is the product of both reactions. Its oxidation can be detected amperometrically using the

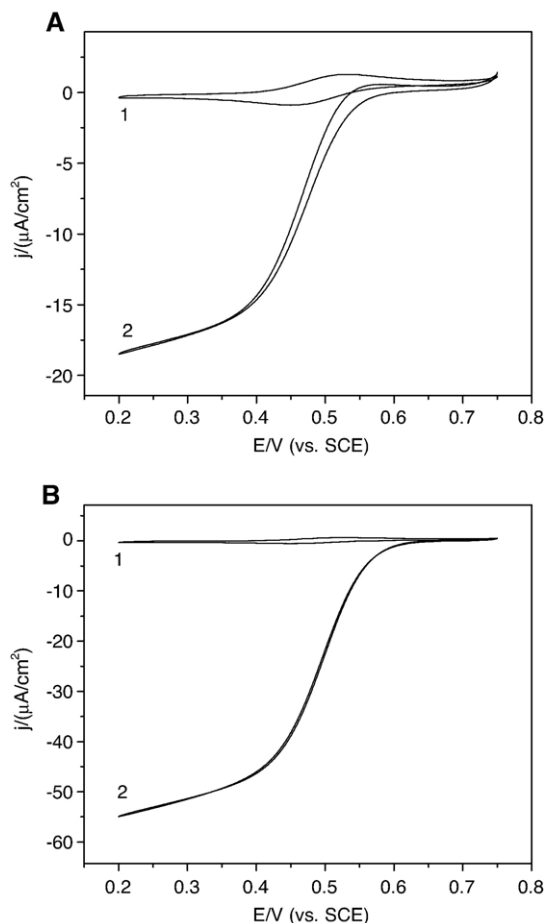


Fig. 7. Voltammograms recorded using GCE modified with cubic phase containing 300 ng laccase (A) *Cerrena unicolor*, (B) *Trametes hirsuta*. McIlvaine buffer, pH=5.6 containing 1 mM ABTS: (1) deoxygenated; (2) oxygenated. Scan rate 1 mV/s.

platinum electrode. In the presence of oxygen and glucose oxidase, the oxidation of H_2O_2 , the product of catalytic reaction, in the cubic phase was observed at 0.3 V (Fig. 3 — curve 3). Without glucose oxidase no oxidation current was seen at these potentials (Fig. 3 — curve 2).

The oxidation current increases with the increase of the glucose concentration (data not shown). Linear dependence of limiting current on glucose concentration was in the range 1×10^{-3} M to 2×10^{-2} M for glucose oxidase. More work is still needed to optimise the conditions of our procedure. The current increases relative to the background for lower scan rates.

Ferrocenecarboxylic acid and $\text{Ru}(\text{NH}_3)_6\text{Cl}_2$ were used next for improving electric contact of enzymes with electrode surface. The desired lower working potential could be achieved especially in case of the latter. In these experiments oxygen interfered at low glucose concentrations, thus deaeration was required.

The voltammograms recorded using and electrodes modified with a layer of cubic phase containing pyranose oxidase are shown in Fig. 4.

The sensitivities to glucose exhibited as the slopes of the calibration curves were 11 ± 3.1 and 31 ± 5.6 $\mu\text{A}/\text{cm}^2/\text{mM}$ for

glucose oxidase and pyranose oxidase, respectively. These values are ca. thirty times larger than without the mediator, ferrocenecarboxylic acid. Above 2×10^{-2} M for glucose oxidase and 1×10^{-2} M for pyranose oxidase the current becomes constant and from the reciprocal of current vs. reciprocal of glucose concentration, the K_M constants were evaluated (Fig. 4B). The constant characterizes here the enzyme electrode and not the enzyme intrinsic property. It takes into account the constraints applied on substrates by the presence of film. All currents were measured at very low scan rates. The inset of Fig. 4B shows Lineweaver–Burk plots allowing the determination of kinetic parameters for the enzyme in the liquid-crystalline lipid matrix. The constant K_M and maximal current I_{max} are calculated based on equation:

$$1/I_0 = K_M/I_{\text{max}} \times 1/[S] + 1/I_{\text{max}} \quad (1)$$

where I_0 — is maximal current for given glucose concentration, $[S]$ — glucose concentration.

The characteristics of the catalytic processes are shown in Table 1.

Based on H_2O_2 oxidation current, the I_{max} value without mediators was found to be 0.214 ± 0.05 μA , turnover number is 0.6 ± 0.05 s^{-1} and K_M is 5.3 ± 1.3 mM for glucose oxidase. For pyranose oxidase, I_{max} value is 0.9 ± 0.1 μA , turnover number is 8.5 ± 2.3 s^{-1} and K_M is 4.5 ± 1.2 mM. In the presence of ferrocenecarboxylic acid as the mediator and glucose oxidase, the value of I_{max} is 12 ± 1.5 μA , turnover number 33 ± 1.5 s^{-1} and the K_M value is 27.7 ± 2.3 mM. When the same mediator is used but pyranose oxidase is embedded in the cubic phase, the K_M value drops to 4.5 ± 1.2 , the I_{max} is 6.2 ± 1.3 μA and turnover number is 56 ± 10 s^{-1} . This turnover number is somewhat smaller than the value from spectroscopic measurements equal to 71 s^{-1} reported by Leitner et al. [49].

The voltammograms recorded using electrodes modified with a layer of cubic phase containing glucose oxidase placed in

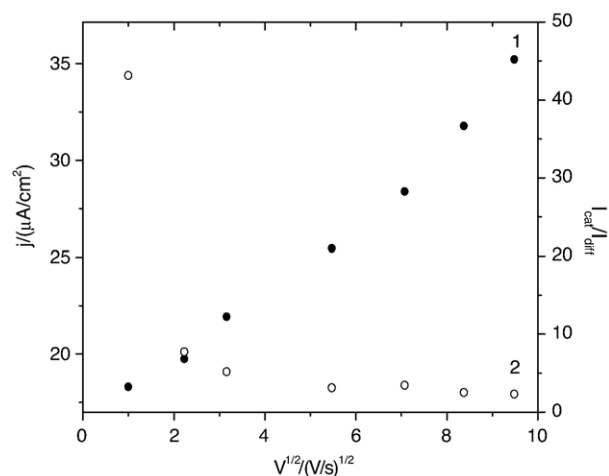


Fig. 8. Oxidation current density vs. $v^{1/2}$ (1) and $I_a/I_{\text{background}}$ vs. $v^{1/2}$ (2) plots recorded for laccase *Trametes hirsuta* modified electrode in McIlvaine buffer, pH=5.6 solution containing 1 mM ABTS.

1 mM $\text{Ru}(\text{NH}_3)_6\text{Cl}_2$ deoxygenated solution show reversible behaviour of this probe in the absence of glucose (Fig. 5).

In the presence of glucose, catalytic oxidation can be clearly recognized. The solution was carefully deoxygenated and the oxidation of glucose was monitored at the potentials of Ru complex oxidation ($E_{\text{pa}} = -200$ mV). Thus, the working potential is shifted to more negative values. In the presence of glucose and glucose oxidase, upon decreasing scan rate, the typical peak shape of the voltammetric curve turns to sigmoidal, and the ratio of catalytic to diffusion current ratio, $I_{\text{cat}}/I_{\text{diff}}$ increases. This ratio at different scan rates for $\text{Ru}(\text{NH}_3)_6^{2+}$ is shown in Fig. 6.

In our previous paper [22] we have shown that the cubic phase is useful as the matrix for comparing the activities of various laccases and for monitoring the levels of oxygen. Fig. 7 presents the catalytic reduction of oxygen in the presence of ABTS as the mediator for two types of laccases *C. unicolor* and *T. hirsuta*.

The current density vs. $v^{1/2}$ and $I_{\text{cat}}/I_{\text{diff}}$ ($I_{\text{catalytic}}$ to $I_{\text{diffusion}}$ ratio) vs. $v^{1/2}$ plots for laccase *C. unicolor* — ABTS are shown in Fig. 8.

Both laccases are highly efficient as catalysts in the cubic phase and can be further considered together with the cubic phase layers with oxidases in the biofuel cell device.

4. Conclusion

The enzymatic processes taking place in the cubic phase are shown in the presence of both organic and inorganic electroactive probes as the mediators. The potential and efficiency of the catalytic process can be tuned by choosing a suitable electroactive probe. The most negative potential for glucose oxidase is obtained using $\text{Ru}(\text{NH}_3)_6\text{Cl}_2$ as the mediator. Most stable system as the electrode modifier is the one based on ferrocenecarboxylic acid. Using glucose oxidase and $\text{Ru}(\text{NH}_3)_6\text{Cl}_2$ as the mediator, the working potential of the system is shifted to -0.2 V while high efficiency and the highest mobility of the mediator in the cubic phase are retained. When $\text{Ru}(\text{NH}_3)_6\text{Cl}_2$ is used as the mediator, the glucose oxidation appears at -0.2 V, hence with low overpotential and high catalytic efficiency is observed. Using reduced forms of the probes is more convenient since it allows to switch on and off the catalytic process by applying appropriate potential to the electrode.

In summary, monoolein cubic phase was shown to be a suitable material for the electrode modification with glucose or pyranose oxidases and the catalytic processes are very efficient. Laccase incorporated in the cubic phase retains its activity and catalyses 4e reduction of oxygen. The enzymes are held close to the electrode surface and in functional forms. The monoolein cubic phase:

- allows fast diffusion of small hydrophilic mediators in the cubic phase;
- is stable in contact with water, viscous and transparent;

The enzymatic process can be observed in the presence of both organic and inorganic electroactive probes as the mediators

and the potential of the catalytic process can be tuned by selecting the appropriate probe. These properties allow to consider the monoolein cubic phase as a convenient matrix for holding enzymes and for its application in biocathodes and bioanodes for electrochemical sensing and biofuel cells.

Acknowledgements

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