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Review

Modified electrodes based on lipidic cubic phases

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Abstract

The lipidic cubic phase can be characterized as a curved bilayer forming a three-dimensional, crystallographical, well-ordered structure that is interwoven by aqueous channels. It provides a stable, well-organized environment in which diffusion of both water-soluble and lipid-soluble compounds can take place.

Cubic phases based on monoacylglycerols form readily and attract our interest due to their ability to incorporate and stabilize proteins. Their lyotropic and thermotropic phase behaviour has been thoroughly investigated. At hydration over 20%, lipidic cubic phases *Ia3d* and *Pn3m* are formed. The latter is stable in the presence of excess water, which is important when the cubic phase is considered as an electrode-modifying material. Due to high viscosity, the cubic phases can be simply smeared over solid substrates such as electrodes and used to host enzymes and synthetic catalysts, leading to new types of catalytically active modified electrodes as shown for the determination of cholesterol, CO_2, or oxygen.

The efficiency of transport of small hydrophilic molecules within the film can be determined by voltametry using two types of electrodes: a normal-size electrode working in the linear diffusion regime, and an ultramicroelectrode working under spherical diffusion conditions. This allows determining both the concentration and diffusion coefficient of the electrochemically active probe in the cubic phase.

The monoolein-based cubic phase matrices are useful for immobilizing enzymes on the electrode surface (e.g., laccases from *Trametes* sp. and *Rhus vernicifera* were employed for monitoring dioxygen). The electronic contact between the electrode and the enzyme was maintained using suitable electroactive probes.

Keywords: Lipidic cubic phase; Lyotropic liquid crystals; Diffusion coefficient; Laccase; Cholesterol oxidase oxygen sensing

1. Introduction

Retaining enzymes in functionally active forms on the electrode surface is a challenging and difficult task. Ideally, the immobilization of proteins 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. We review here recent studies on the application of liquid crystalline phases formed by polar lipids in aqueous media as model matrices for hosting both synthetic catalysts and biocatalysts. Monoolein (MO), monopalmitolein (MP), and other natural lipids or synthetic surfactants can be used to prepare the cubic phases according to the procedure of Rummel et al. [1].

Cubic liquid crystals with a number of different structures have been reported [2–4]. These are usually grouped into two main types, namely the discrete and bicontinuous structures, which can be of the normal or

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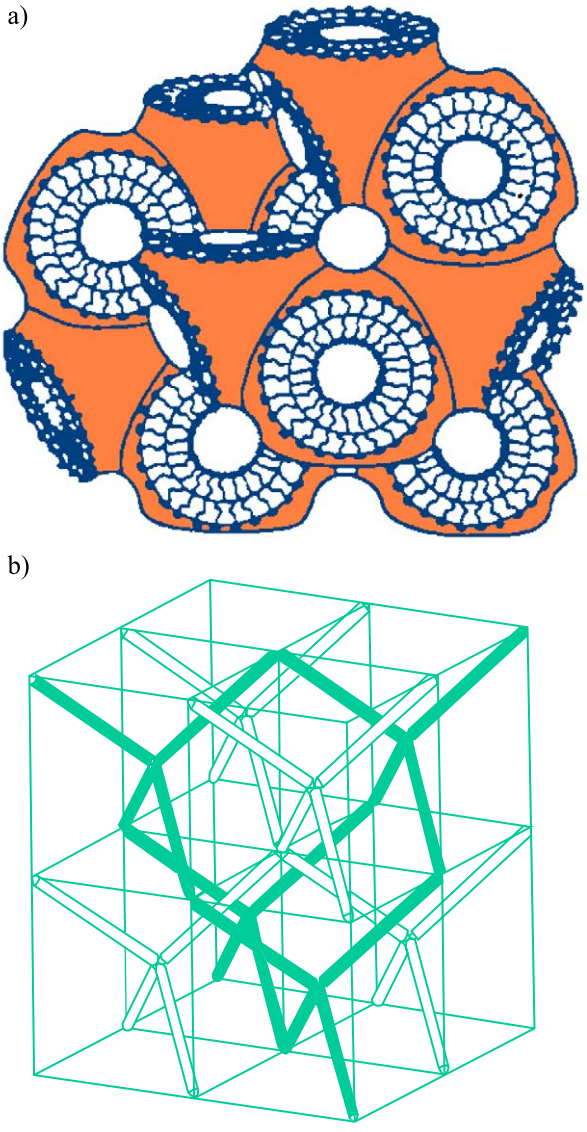
reverse kind [5]. Within the two main groups of cubic structures, several spatial arrangements are possible, leading to a number of different space groups [4]. In the binary system used in our studies (i.e., 1-monooleoyl-rac-glycerol/water at 20 °C), two reversed bicontinuous cubic phases belonging to the space groups *Ia3d* and *Pn3m* are present [6–8], as determined by X-ray spectroscopy [9]. The water channels of diameters around 50–60 Å can easily accommodate hydrophilic proteins of similar sizes without modifying the structure of the matrix.

The lipidic matrices are characterized by a curved bilayer extending in three dimensions, forming a crystallographically well-ordered structure that is interwoven by aqueous channels [1,10] (Scheme 1).

Lateral diffusion of hydrophobic proteins along the curved bilayer was demonstrated [10] and exploited for the crystallization of a number of membrane proteins: bacteriorhodopsin, halorhodopsin, two photosynthetic reaction centers, and light-harvesting complex [11–13]. Small solutes and water-soluble proteins diffuse within the network of aqueous channels, allowing crystallization from this compartment [14]. Thus, lipidic cubic phases may provide a matrix for the reconstitution of proteins under conditions, which preserves functionally important hydrophobic interactions at the lipid–water interface. Because of the cubic symmetry, the orientations of integral membrane proteins in the lipidic phase are equivalent with no inside/outside distinction as in vesicles. This allows unrestricted access of soluble proteins to membrane proteins, for example.



Scheme 1. Structure of the cubic phase: (a) scheme showing the bilayer structure; (b) interconnected rods model.

Homogeneity and optical 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 with which the functionality of the protein can be probed.

Examples of cubic phase-modified electrodes have been described in recent papers [14,15,16,17]. We have shown that cubic phase-modified electrodes are useful for the determination of cholesterol, CO_2, and O_2 [6,18,20].

2. Preparation of the phase

The cubic phases are prepared by weighing monoolein in a small glass vial (ca. 10 mg), then water or enzyme (e.g., laccase solution) is added [6,18,20]. The ratio of components is chosen on the basis of the phase diagram for the monoolein–water system, and corresponds to the diamond-type cubic phase.

The glass vial is tightly closed and centrifuged for 15 min at 4500 rpm to mix the components. After centrifugation, a transparent and highly viscous cubic phase should be obtained. The stability of the system is confirmed by macroscopic observations of the sample viscosity and clarity.

The enzyme-free cubic phase can be stored in closed vial for several months, but in the case of cubic phase with laccase stored in temperature below 0 °C, the activity of laccase remained at about 90% of the initial value for 2 days and, after longer storage, it decreased gradually. Cubic phases have a well-defined and reproducible structure, which is determined by X-ray spectroscopy [6,19,20].

3. Electrode modification procedures

The glassy carbon electrode is polished on Buehler plates with 0.3 μm alumina, then rinsed with water and dried in the air. The GCE are weighed before and after application of cubic phase in order to determine the amount of cubic phase on the electrode. This amount was usually from 9 to 12 mg per electrode. The cubic phase can be deposited on the

electrode surface using a spatula. The thickness of the layer was in the range 1–2 mm [19,20].

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

In the case of experiments with mediators, the appropriate amount of each compound was dissolved in the supporting electrolyte after deoxygenation of the cubic phase. The electrode was left in the solution for 60–90 min, depending on the nature of the probe [19,20]; this time is needed for equilibrating the concentration of the electroactive probe in the solution and in the cubic phase.

4. Diffusion in the cubic phase matrix

The diffusion of compounds in the cubic phase is usually studied using nuclear magnetic resonance or holographic laser interferometry [21]. Holographic laser interferometry was found to be a powerful technique for monitoring the diffusion processes in porous media, since it not only allowed to measure the concentration profiles due to diffusion but also revealed any distortions during the diffusion process [22]. Razumas et al. [15] and Nylander et al. [22] estimated amperometrically the diffusion coefficients of glucose in the monoolein-based cubic phase. This approach relied on catalytic glucose oxidation, leading to the formation of electroactive H_2O_2 oxidized next on the Pt electrode. Some assumptions concerning enzyme activity had to be made, and since the method was indirect (based on H_2O_2 oxidation currents), the authors found advantageous the direct measurements of glucose diffusion by the holographic laser interferometry or nuclear magnetic resonance (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 [21].

The approach used in our studies was based on the application of two electrodes of different sizes operating in different diffusion regimes. The normal-size electrode operated under conditions of linear diffusion where the peak current is proportional to the square root of diffusion coefficient [23–25], while the ultramicroelectrode is under conditions of spherical diffusion, where the limiting current is proportional linearly to the diffusion coefficient [26–28]. Based on the currents recorded using these two electrodes parallelly in one solution, we could solve a system of two equations and determine both the actual concentration of the redox probes and their diffusion coefficients in the cubic phase present on the electrode. We did not need any assumptions concerning the concentration of the electroactive compound based on density measurements, which is required in any electrochemical approach based on a single technique as described for the characterization of diffusion of vitamin K [16]. The layer thickness is always much bigger compared to the diffusion layer width.

Typical voltamograms for $Ru(NH_3)_6^{3+}$ are shown in Fig. 1.

The peak current recorded using the normal-size electrode for the conditions of linear and infinite diffusion is given by the equation [23]:

$$I_p = 2.69 \times 10^5 n^{3/2} A C_{\text{cubic phase}} v^{1/2} D^{1/2}, \quad (1)$$

where I_p is the peak current (A), n is the number of electrons, A is the electrode area (cm^2), C^o is the concentration (mol/cm^3), v is the scan rate (V/s), and D is the diffusion coefficient (cm^2/s).

In case of ultramicroelectrode, the conditions of spherical diffusion prevail except for very short timescales. The steady-state current recorded voltametrically is described by the equation [29,30,31]:

$$i_{ss} = 4nFDC_{\text{cubic phase}} r, \quad (2)$$

where r is the radius of the ultramicroelectrode.

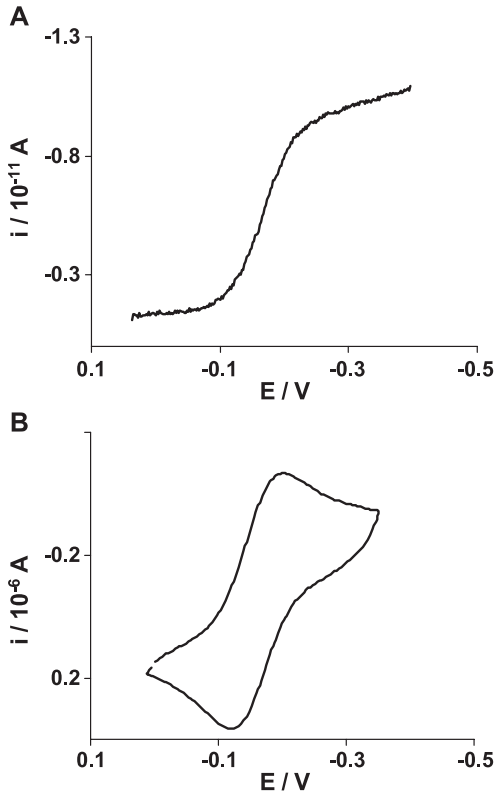


Fig. 1. Determination of diffusion coefficients in the cubic phase: voltammograms recorded using the electrode modified with cubic phase in 0.5 M KCl solution containing about 1 mM $Ru(NH_3)_6^{3+}$. The thickness of the cubic phase layer on the electrode was ca. 2 mm. (A) Pt ultramicroelectrode (r=5 μm); scan rate is 0.02 V/s. (B) GCE (A=0.07 cm^2); scan rate is 0.01 V/s.

An important advantage of cubic phases over other matrices was the more efficient diffusion of hydrophilic probes [19]. The diffusion coefficients of the electrochemical probes are significantly larger in the lipidic cubic phase than in Nafion layers. For example, for $[Ru(NH_3)_6]^{3+}$, it is $2.17\pm0.44\times10^{-6}$ cm^2/s, while in Nafion, it is reported to be only 2×10^{-10} cm^2/s [32]. The concentration in the solution was 9.8×10^{-4} M, and the calculated value based on the system of equations was 8.5×10^{-4} M. The lower value of diffusion coefficient compared to that for the bulk solution may, in part, be a geometric effect due to the excluded volume in the cubic phase environment. Currently, we are trying to determine the contribution of this effect.

These results, confirmed for several electrochemically active probes [19], indicated that enzymes incorporated in the cubic phase could efficiently communicate with the electrode surface by means of small mobile redox mediators simultaneously incorporated in the phase. This property of the cubic liquid crystalline phases is useful also in view of practical applications of such modified electrodes in sensing and electrocatalysis, since small mediators, substrates, and products can readily access both the electrode surface and the catalytic centers of the modifying layer.

5. Immobilization of enzymes

Cubic phases based on monoolein were used as matrices to immobilize the *Rhodococcus* sp. cholesterol oxidase. The enzyme trapped in these systems remained catalytically active for at least a week. This technique allows determination of the conditions under which the cholesterol oxidase can function as a sensing element of the electrode-modifying layers for cholesterol detection [6] based on reactions shown in Scheme 2.

In Fig. 2, the voltamograms recorded using the electrode modified with cholesterol oxidase in the cubic phase are shown. The curves are obtained in the presence and absence of cholesterol and differ in peaks a_1 and c_1, which can be ascribed to the electrode processes of H_2O_2 formed in the catalytic process (Scheme 2).

The direct electrochemical detection of hydrogen peroxide formed in the presence of the sterol is the basis for a sensitive cholesterol sensor.

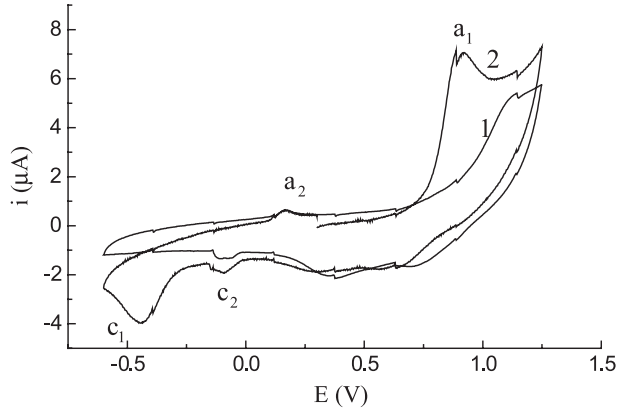


Fig. 2. Cyclic voltammograms recorded on gold electrode modified with the monoolein cubic phase containing the enzyme in monoolein in the absence and presence of 0.2 mM cholesterol. Buffer solution containing 50 mM Na_2HPO_4 and KH_2PO_4, and 0.1 M KCl (pH=7.8; scan rate=0.1 V/s). Electrode area is 0.07 cm^2 and the cubic phase layer thickness is 2 mm.

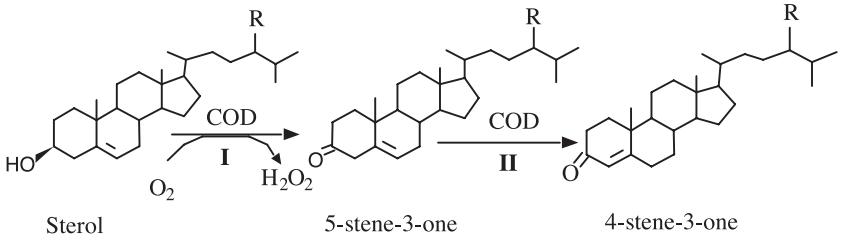
Monoolein cubic phase was also employed for the immobilization of another enzyme, laccase, on solid supports.

Laccases oxidize *o*- and *p*-benzenediols to appropriate quinones, with concomitant reduction of oxygen to water [33–38]. The enzyme active site contains four copper atoms of types I, II, and III, which play different roles in the enzymatic process. When hydroquinone is used as the laccase substrate, the enzymatic process is described by Eqs. (1) and (2):

$$2Cu^{2+} + \text{hydroquinone} \rightarrow 2Cu^{+} + \text{quinone} + 2H^{+}$$

$$2Cu^{+} + 1/2O_2 + 2H^{+} \rightarrow 2Cu^{2+} + H_2O \qquad (4)$$

Laccases are exceptionally versatile enzymes, catalyzing one basic reaction from which all its activities spring. In addition to the strictly biological functions, laccases are increasingly being investigated for a variety of practical applications ranging from use in the pulp and paper industry to their possible use in bioremediation, analytical processes, and organic synthesis [34–38]. The redox potential for type I copper in *Rhus vernicifera* was estimated to be 432 mV at pH 6. Since laccase oxidation is linked to dioxygen reduction, it was also employed in constructing cathodes for a biofuel cell [39,40].



Scheme 2. Conversion of sterols (5-stene-3β-ol) to 4-stene-3-one by cholesterol oxidase (COD).

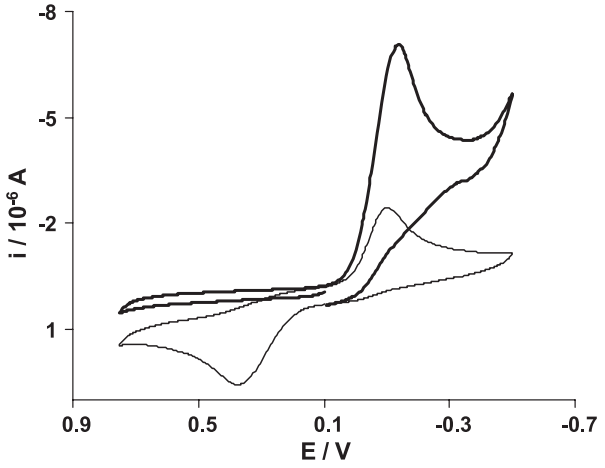


Fig. 3. Voltammograms recorded on GCE modified with cubic phase in oxygenated (bold line) and deoxygenated (thin line) 0.05 M phosphate buffer (pH=6.3) containing 1 mM hydroquinone. Scan rate=0.05 V/s. Solution saturated with oxygen. Laccase from *Trametes* sp. Quantity of enzyme on the electrode: 0.1 mg.

Laccase was stored at about −18 °C and taken to the room temperature at about 30 min before the experiment. Laccase is soluble in water, and a homogenous solution is obtained and used for the cubic phase preparation [20]. The contact between the electrode and the enzyme is maintained by means of a suitable electroactive probe, which is oxidizable by the enzyme. The voltamograms recorded using electrodes modified with cubic phase containing the laccase and hydroquinone (Fig. 3) couple indicate decreasing anodic peak due to consumption of hydroquinone in the catalytic reaction.

This system was found suitable for controlling oxygen in aqueous solutions at 10^{-5}–10^{-4} M concentration levels [20] (Fig. 4).

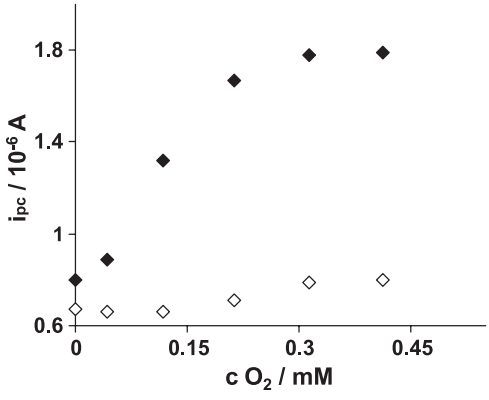


Fig. 4. The dependence of chronamperometric current value vs. dioxygen concentration in the solution. Current value measured at −0.3 V, after 300 s from applying this potential to the electrode, was recorded in phosphate buffer solution (pH=6.1) containing 1 mM 1,4-benzoquinone. Diamonds—electrode with enzyme; squares—electrode without enzyme. Quantity of laccase from *Trametes* sp. was 0.18 mg per electrode.

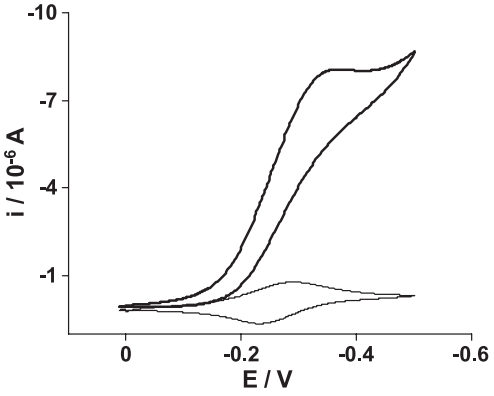


Fig. 5. Voltammograms recorded on GCE modified with cubic phase with laccase from *Trametes* sp. (0.4 mg of enzyme per electrode) in 0.05 M phosphate buffer (pH=6.0) containing 1 mM $Ru[(NH_3)_6]Cl_3$. Scan rate=0.005 V/s. Bold line—oxygenated solution; thin line—deoxygenated solution.

Laccase enzymatic process can be observed in the presence of both organic and inorganic electroactive probes. The potential of the catalytic process can be tuned by choosing a suitable electroactive probe (Figs. 3 and 5).

Using oxidized forms of this probe allows to switch on and off the catalytic process by applying appropriate potential to the electrode, which generates the form actually undergoing reaction with laccase [20].

In summary, monoolein cubic phase is a suitable material for electrode modification since:

- It holds laccase and other catalysts close to the electrode surface and in a functional form.
- Diffusion of small hydrophilic probes in the cubic phase is fast. It is stable in contact with water, viscous, and transparent.
- Laccase enzymatic process can be observed in the presence of both organic and inorganic electroactive probes, and the potential of the catalytic process could be tuned by changing the electroactive probe. The electrode modified with laccase in the cubic phase catalyzes the oxygen reduction process.

6. Conclusions

The research on electrodes modified with cubic phases containing enzymes and synthetic catalysts demonstrates their high application potentiality. Indeed, recent results show that cholesterol, carbon dioxide, and dioxygen levels can be monitored using these systems. Compared to other electrodes, cubic phase-based electrodes show different advantages, as discussed in the original papers. Research on the interactions between the matrix, the immobilized molecules, and the analytes, currently underway, should give more insights into the mechanisms governing the functioning and structure of the cubic-based systems and allow their use as sensors.

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