Anal Bioanal Chem (2008) 391:1569–1578
DOI 10.1007/s00216-008-2149-y

REVIEW

Cubic phases in biosensing systems

Ewa Nazaruk · Renata Bilewicz · Göran Lindblom · Britta Lindholm-Sethson

Received: 22 November 2007 / Revised: 16 April 2008 / Accepted: 18 April 2008 / Published online: 17 May 2008

Abstract Incorporation of membrane proteins with retained activity in artificial membranes for use in membrane-based sensors has attracted scientists for decades. This review briefly summarises general concepts on relevant cubic phases with and without incorporated proteins and provides some insight into the development of biosensors where bicontinuous cubic phases are used for incorporation of an enzyme. Some new data on impedance characterisation of a supported cubic phase are also shown. An efficient membrane-based electrochemical biosensor requires that the analyte has free access to the immobilised membrane protein and that regeneration of the catalysing enzyme is fast. Long-term stability of the system is also necessary for the biosensor to find applications outside the research laboratory. These basic concepts are discussed in the review along with presentation of those biosensing systems based on cubic phases that are reported in the literature.

Keywords Biosensors · Cubic phase · Membrane-based sensors

E. Nazaruk · R. Bilewicz
Faculty of Chemistry, University of Warsaw,
ul. Pasteura 1,
02093 Warsaw, Poland

G. Lindblom · B. Lindholm-Sethson (✉)
Department of Chemistry, Biophysical Chemistry,
Umeå University,
901 87 Umeå, Sweden
e-mail: britta.sethson@chem.umu.se

B. Lindholm-Sethson
Centre for Biomedical Engineering and Physics, Umeå University,
901 87 Umeå, Sweden

Introduction

Model bilayer lipid membranes have been constructed for precise investigations of biological processes and have provided deep and detailed information at a cellular level of, for example, ligand–receptor interactions, viral attacks, and cellular signalling events [1]. These artificial lipid membranes are also suggested as useful templates for membrane proteins in biosensor applications. There are several suggested approaches to achieve stable lipid membranes on electrode surfaces and the most important approaches are described briefly below.

1. Solid supported lipid bilayers, SLB. In these the surface should be smooth, clean, and hydrophilic for formation of a high-quality fluid film. The lipid layer is separated from the solid support with a thin water film, ~10 Å, enabling some mobility of the lipids in the lower leaflet. There is, however, no room for bulky transmembrane proteins [2–6].
2. Polymer cushioned bilayers, PCB. In these a thin polymer cushion is formed to mimic the extracellular matrix and provide space for transmembrane proteins [7–14].
3. Hybrid lipid bilayers, HLB. In these the first layer is a self-assembled monolayer, SAM, of a thiol or silane with a long carbon chain, providing the first leaflet of an artificial bilayer. The second layer is added with the same methods as for SLB, i.e. vesicle fusion and/or Langmuir–Blodgett transfer [15–18]. A hydrophilic spacer is sometimes introduced at the base of the alkanethiol–silane to form an aqueous reservoir between the bilayer and the solid substrate to make room for transmembrane proteins in the bilayer [19–21].
4. The classical black lipid membrane, BLM. This is easily formed by painting a lipid solution across a small aperture [22]. This type of artificial lipid bilayer is,

however, fragile and only useful for fundamental research. The stability has however increased considerably with the introduction of nano-BLMs that are readily produced on porous alumina substrates from lipid solutions [23, 24].

So far there is no super-stable model membrane that can be kept in the laboratory drawer for several months before use, although some measures have been proposed for further stabilisation of these fragile systems [14]. Most model membranes as referred to above would delaminate immediately on exposure to air. However, there is one promising exception—the lipid cubic phase is stable for months and is easily prepared [25]. It consists of a 3D bilayer lipid membrane where membrane proteins can be inserted with retained activity [26]. Their unique structure has inspired to several different application areas such as:

1. Biofuel cells based on the cubic phase can easily be miniaturized and stacks of cells could be constructed. There is a possibility of constructing biofuel cells without the bulk solution phase since the cubic phase allows elimination of separating membranes. Moreover, it has already been demonstrated that the monoolein cubic phase is a suitable matrix for immobilisation of enzymes as a holder of the catalyst at either the cathode or anode, or both, in a biofuel cell [27].
2. Cubic phases are also good matrices for controlled release and drug delivery in pharmacological applications. Lipidic cubic phases are biocompatible, digestible, and bioadhesive, and the pore structure provides a diffusion pathway for controlled release. Due to presence of bicontinuous water and amphiphilic channels there is a possibility of incorporation of water-soluble or lipid-soluble molecules, for example proteins, vitamins, and small drugs. Moreover, the cubic phase have been shown to improve the topical/transdermal delivery of relatively small molecules such as nicotine, salbutamol, acylovir cyclosporin A(CysA), and aminolevulinic acid esters [28–32]. The diffusion of these molecules depends on the size of the molecules and the dimension and/or charge of the aqueous channels. Applying lipids with different acyl chains to the lipid mixture that comprises the cubic phase will modify the water channels, which in turn modulates the rate of transport.
3. The bicontinuous cubic phase also allows for crystallization of membrane proteins "in cubo" and has resulted in well-ordered crystals that enabled determination of high-resolution structures of several membrane proteins [33, 34].
4. Åkerman et al. have shown that the bicontinuous cubic phase can be used as a matrix for electrophoresis of both membrane-bound fluorescent probes and oligonucleotides. Since both the bilayer lipid membrane and the aqueous phase are continuous throughout the crystal both the hydrophobic and the hydrophilic probes can migrate through the same bicontinuous cubic crystal. A size separation capability was demonstrated for nanosized hydrophilic molecules with a strong retardation [35, 36]. However, migration of both types of probe were slow and therefore it was concluded that the cubic phase is not a feasible replacement for conventional gels.
5. Another suggested and challenging application is to use the lipid cubic phase to host membrane proteins in biosensing applications since it gives a unique option of depositing a stable lipid bilayer on an electrode surface. Water-soluble proteins can accommodate successfully with extended lifetime in the ca 5-nm aqueous channels of the cubic phase close to the electrode surface, for example, use of glucose or pyranose oxidase to determine sugars [37–39]. The analyte in the surrounding aqueous phase will have free access to both the electrode surface and the inserted protein, which is easily wired to the electrode with an electroactive mediator.

No commercial membrane-based biosensor has yet been prepared by anyone, so this particular approach remains a great challenge and inspiring subject for biosensor-oriented analytical chemists. The background and requisites for this type of biosensor are discussed in this paper.

Background of liquid crystalline cubic phases

Lipids, like most other amphiphiles, can form a variety of lyotropic liquid crystalline phases. The lamellar phase with its multibilayer structure is well known and the different nonlamellar structures, occurring for a number of lipids, have gradually entered the scene. The lipid aggregate units can have different shapes like spheres, rods, or lamellae. Lamellar liquid crystalline phases exhibit one-dimensional periodicity, i.e. lamellar units of infinite extension are stacked regularly, and hexagonal liquid crystalline phases exhibit a two-dimensional periodicity with rod-like aggregates of infinite length packed into a hexagonal lattice.

One particular class of liquid crystalline structure has cubic symmetry. Similar to lamellar and hexagonal phases they possess long-range order with (short range) disordered hydrocarbon chains. The aggregates formed by the lipids are situated in a cubic three-dimensional lattice. In contrast to the lamellar and hexagonal phases, which are optically birefringent, the cubic phases are optically isotropic. Cubic phases might in some cases be difficult to discover experimentally due to their isotropic properties. Furthermore, they are very viscous, often waxy or in some cases jelly-like and can also be extremely metastable. The cubic

phases are classified according to their space group obtained by X-ray diffraction. The number of different cubic structures might be quite many, but the space groups carefully determined for cubic phases containing membrane lipids are still limited to a few such as *Ia3d* and *Im3m* with body-centred space groups and *Pn3m*, *Pm3n* with primitive cubic lattices [25, 40, 41].

The first observations of cubic liquid crystalline phases were made in the 1960s. Since then these phases have been paid intense attention and knowledge of cubic phases has accumulated substantially. The most important and exhaustive reviews are found in Refs. 25, 40 and 41.

Discussion here is restricted to systems containing membrane lipids in order to keep it to a reasonable size. These lipids may be divided into two groups:

1. water soluble micelle-forming amphiphiles; and
2. water insoluble swelling amphiphiles.

An example of the first group is lysolecithin while lecithin belongs to the second group. However, there is not always a sharp boundary between the two groups since an insoluble swelling lipid at room temperature may be soluble and form micelles at a higher temperature. Cubic phases for binary systems containing a swelling amphiphile, for example monoolein, usually form in equilibrium with an L_α phase on one side and with excess water on the other side (Fig. 1). Another possibility is that the cubic phase is in equilibrium with an H_{II} phase, which in turn is in equilibrium with excess water.

Cubic phases form in several lipid systems and their existence often critically depends on temperature and composition, as seen in phase diagrams of lipid–water systems. Determination of phase diagrams is usually very tedious work using classical methods and there are only a limited number of phase diagrams available. NMR techniques were therefore developed since they are more rapid for such investigations. These elegant and convenient methods, based generally on ^{2}H and ^{31}P NMR, are frequently utilized in the laboratory at Umeå University. The positions in the phase diagram of the various phases often give very helpful information about physicochemical properties and, in particular for cubic phases, about the aggregate structure building up the phases. Hitherto a cubic phase has been observed between almost every other phase in the phase diagram, which definitely shows that the cubic phase is not an abnormality.

Methodologically it is convenient to divide the cubic phases into two fundamentally different groups:

1. cubic structures having regions which are continuous with respect to *both* polar (water) and nonpolar (hydrocarbon) components, *called bicontinuous*; and
2. cubic structures built up either with discontinuous hydrocarbon regions but with continuous water regions (for example micelles in water) or with discontinuous water regions but with continuous hydrocarbon regions (for example reversed micelles in liquid hydrocarbon).

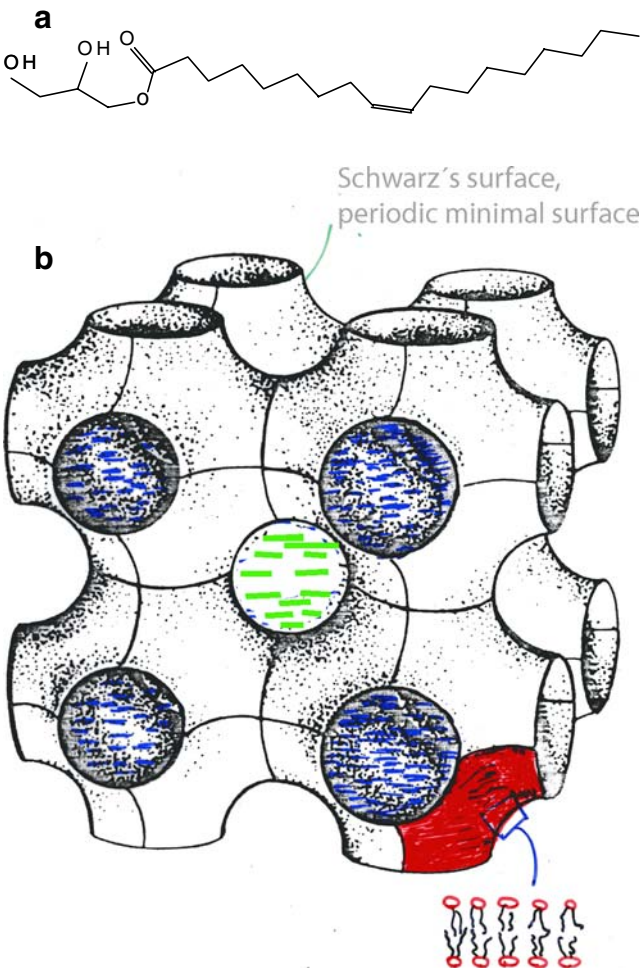


Fig. 1 (**a**) Structure of monoolein. (**b**) Typical structure of a cubic phase

There is a remarkable difference in viscosity between the two types of structure—all the bicontinuous phases are extremely viscous whereas the phases built up of globular aggregates usually are less viscous and have a more jelly-like consistency. Most of the cubic phases formed by membrane lipids have been shown to be bicontinuous. This group of cubic phases is also the most attractive approach for biosensor applications since they allow transport of both hydrophilic and hydrophobic substrates throughout the crystal [35].

Characterisation of lipid cubic phases

Structure

Theoretical treatment of the formation and structure of cubic phases has advanced considerably during recent years. This has been possible mainly due to the application

of differential geometry to the description of the complicated surfaces building up many cubic structures. However, more than one physical method are required to solve the complex structure of the cubic phases. Thus X-ray diffraction [42] alone will not be able to give a conclusive answer. One promising candidate for further complementary information is transmission electron microscopy (TEM) that has recently been applied to membrane lipid systems [43].

NMR techniques have shown to be particularly powerful for this purpose and, above all, the NMR pulsed-field gradient method. A large number of cubic phases formed by membrane lipids have been investigated with this method for the determination of translational diffusion coefficients of both the lipid and water components [44]. Since the molecular motion is isotropic the NMR spectra of cubic phases exhibit narrow highly resolved peaks, where one is from the lysolipids. The NMR signals of cubic phases are therefore similar to those of a micellar solution and are consequently not sensitive to details of the structures of isotropic phases. Therefore, for cubic phases the diffusion coefficient can be measured directly without using probe molecules and no model-dependent assumptions have to be made. From comparison of the lipid translational diffusion coefficients in the cubic and lamellar liquid crystalline phases, it is possible to differentiate between the two fundamentally different types of cubic phase, namely the bicontinuous ones and those built up of closed micellar aggregates [45–47].

Protein interactions with cubic phases

Remarkable amounts of proteins in their native forms can be incorporated in a cubic phase without loss of the cubic structure. This property provides the fundamental requirements for development of biosensors based on immobilisation of membrane proteins in cubic phases. Some of these are discussed below.

Fontell et al. investigated a cubic monoacylglycerol–protein–water phase with low-angle X-ray diffraction. A ternary phase diagram of the monoolein–lysozyme–water system was constructed and it was proposed that the protein molecules were located in the water channel systems [26]. It was concluded that high repulsive electrostatic forces facilitated the formation of this cubic phase.

Cytochrome c (cyt c) forms a cubic phase with monoolein and water, and is an interesting model of the inner mitochondrial membrane. The lipid–protein interactions in this system were investigated with FT-IR spectroscopy, differential scanning calorimetry, and electrochemical techniques by Razumas et al. [48, 49]. A structural rearrangement in the polar head group region and a higher conformational order of the acyl chains was found which is related to the presence of the protein. In order to avoid phase transitions the water content was around 38–41%, which ensured that the cubic phase remained in space group Pn3m irrespective of protein content [48] up to a concentration of at least 5% *w/w*. In this cubic phase the inner core of the lipid bilayer forms a diamond-type infinite surface. An increase in the unit cell dimension was observed at high protein loadings or on addition of buffer solution. The diffusion coefficient of the entrapped protein was estimated with electrochemical techniques and showed very restricted mobility of the protein. This is the first report on direct redox conversion of an entrapped protein in a cubic phase.

Later, Kraineva et al. investigated the MO–cyt c–water system in order to characterise the lipid phases and their stability at various temperatures and pressures with small-angle X-ray scattering. In this case the water content was around 20% and an increase of cyt c concentration above 0.2% *w/w* in the bicontinuous cubic phase promoted a phase transition. It was shown that protein interaction with the bilayer membrane affects not only the conformation of the protein itself but also lipid membrane curvature [50].

Razumas et al. also investigated interactions in the vitamin A–monoolein–water cubic phase system and found, from cyclic voltammetry, in this case also, rather slow mobility of the entrapped vitamin. The phase diagram of the binary system was not affected at a concentration below 1% *w/w* vitamin A [51].

In a recent publication Rogalska et al. investigated hydrophobin interactions with various lipid layers, i.e. lipid monolayers at the air–water interface and in lamellar and monoolein cubic phases [52]. Interestingly the adsorption of a slightly modified Cu^{II}-chelating *Schizophyllum commune* hydrophobin, SC3, in the cubic phase results in open-circuit accumulation of copper ions in the protein–lipid system. The preconcentrated Cu^{II} can be readily detected with cyclic voltammetry and it is suggested that this could open the way for electrochemical detection of copper. The impact of the added hydrophobin on the cubic phase structure was studied using SAXS. A slight decrease in the lattice parameter was found compared with pure monoolein cubic phase. The cubic phase was not damaged by SC3 as are lipid monolayers [52], again indicating the robustness of the cubic phase.

These observations are indeed promising for biosensing applications since it has been shown that membrane proteins can be incorporated in the cubic phase to rather large concentrations. It has also been shown that an electroactive protein is readily accessible and can be oxidised or reduced within the cubic phase. It should be stressed that the water content of the phase, the temperature, the pressure, and lipid composition are of importance to the stability of the cubic phase when increasing the protein loading. A high protein loading might promote a

phase transformation to a lamellar phase and this would substantially decrease the efficiency of the biosensor because of the loss of water channels.

Electrochemical characterisation

The suggested use of a cubic phase as a supporting matrix for membrane proteins in biosensor applications motivates the characterisation of the system with electrochemical methods. First, the membrane protein (either oxidase or reductase) should be incorporated readily into the cubic phase in its native form with retained activity for prolonged time and without rupture of the cubic structure. Second, the mass transport of the substrate into the cubic phase should not be restricted in order to ensure efficient oxidation or reduction. Third, regeneration of the membrane protein should be rapid. In an optimum biosensor the mass and charge-transport properties should harmonize with the heterogeneous and homogeneous rate constants for oxidation or reduction reactions so that the whole volume of the cubic phase is active in the recognition event. It was recognised early in research on polymer film electrodes for electrocatalytic purposes that in many cases only the outermost layer of catalysts was active and the limiting step was charge transport through the film. The original theory and background are found in Ref. [53]. The same logic should also be valid for biosensors based on cubic phases with immobilised membrane proteins.

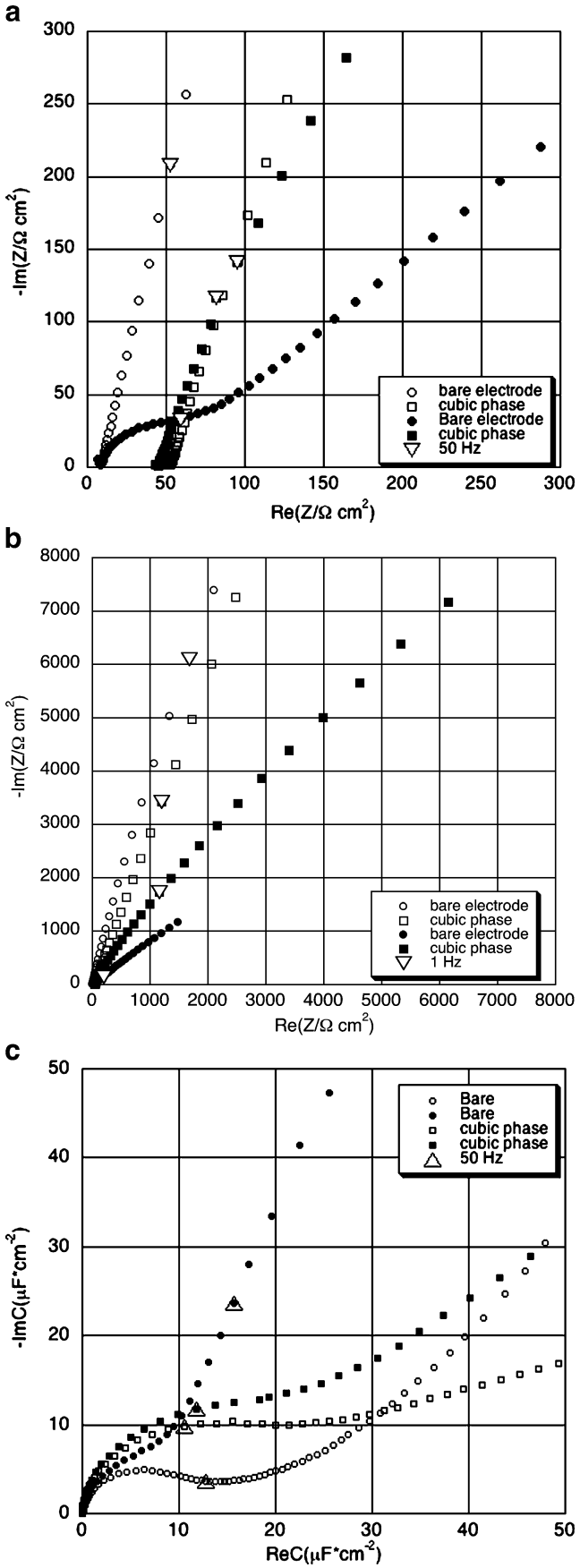
Rowinski et al. [54] investigated mass transport properties of hydrophilic probes in a monoolein–water cubic phase with cyclic voltammetry and chronocoulometry. The diffusion coefficients for $Ru(NH)_6^{3+}$, benzoquinone, and ferrocene carboxylic acid were found to be 5×10^{-6}, 1×10^{-6}, and 4×10^{-6} cm^2 s^{-1}, respectively, where the first two values are factors of four and two smaller than those in pure solution and the last value is similar to that in pure solution. Similarly, Kostela et al. [55] have investigated diffusion of hydrophilic, hydrophobic, and amphiphilic redox probes. They used chronocoulometry and found that the hydrophilic probes where retarded by a factor of 2–4, in good agreement with Rowinski [54], whereas the hydrophobic probes ferrocene and TMV (amphiphilic viologen) where slowed down by a factor of 50.

Interestingly, Kumar et al. have recently investigated three redox probes in a similar system, i.e. a lyotropic hexagonal liquid crystalline phase based on Triton X-100 and water. They found that the diffusion coefficients for the hydrophilic probes, i.e. ferro/ferricyanide and hexaammineruthenium(II/III) were not much retarded whereas the diffusion coefficients for the hydrophobic probe ferrocene/ferrocenium was slowed down by a factor of two orders of magnitude [56]. This is surprisingly in good agreement with results from cubic phases, but can be explained by the fact that the hexagonal and cubic phases are polycrystalline so the ion mobility is not restricted to the aqueous channels in the two different types of crystalline phase.

The monoolein–water system is at present investigated with electrochemical impedance techniques by the authors. Here we want to present some of the unpublished data. In the complex impedance plane a capacitive line was observed in the case of no electroactive couple in solution. Surprisingly, the presence of a cubic phase gave rise, in most cases, to an increase in solution resistance of approximately 40–50 Ω cm^2 (Fig. 2a,b). In Fig. 2c the impedance data are displayed in the complex capacitance plane. The diameter of the high-frequency semicircle is in this case identical with the double-layer capacitance and a simple graphical estimation gave $C_{dl}=9.6\pm0.8$ μF cm^{-2} on the bare Pt electrode whereas with cubic phase on the electrode surface C_{dl} was increased to 22.4 ± 0.8 μF cm^{-2}. A similar increase was also observed in cyclic voltammetry. Interestingly, this is in contrast with the case when lipid bilayers are deposited on an electrode surface where the membrane capacitance at high frequencies decreases to, for instance, 0.58 μF cm^{-2} as reported in Ref. [57]. The reason is the special structure with water channels within the cubic phase that increases the charge storage capacitance at the interface.

With $Fe(CN)_6^{3-}/Fe(CN)_6^{4-}$ in solution the effect of the cubic phase was considerable. A constant phase element with ca 45° slope at medium and low frequencies was identified as a Warburg line, Fig. 2a [58]. In this frequency range mass transport of the ferro/ferri cyanide couple is the rate-limiting step and the apparent diffusion coefficient, D_{app}, was calculated from the Warburg coefficient of 5.3×10^{-6} cm^2 s^{-1} obtained at the bare electrode, which is in accordance with literature values, whereas the mass transport was much retarded in the cubic phase with a diffusion coefficient of 2.5×10^{-8} cm^2 s^{-1}. On the bare electrode a semicircle was observed in the high-frequency range related to the slow heterogeneous electron transfer, whereas with the cubic phase at the electrode surface the semicircle disappeared.

The observations from electrochemical investigations clearly show that the cubic phase is permeable to ions although mass transport is retarded. Interestingly, the diffusion coefficient of ferro/ferricyanide is smaller when determined with impedance spectroscopy than with chronocoulometry. Chronocoulometry is a dynamic method in which much energy is introduced into the system when the potential is stepped from fully oxidised to fully reduced, or vice versa, and migration effects cannot be excluded. In impedance spectroscopy the experiment is performed near equilibrium and the driving force is mainly thermodynamic. The latter situation is more relevant in the case of a mediated electrocatalytic reaction in a biosensor.



◀ **Fig. 2** Impedance spectroscopy at a planar Pt electrode, A=0.031 cm^2, f=0.01–100,000 Hz logarithmically distributed. Peak to peak amplitude=10 mV Thickness of cubic phase=1 mm. *Filled symbols*: with 0.5 mmol L^{-1} $Fe(CN)_6^{3+}$ and 0.5 mmol L^{-1} $Fe(CN)_6^{4+}$; *Unfilled symbols*: without added redox couple. Complex impedance plane, measurement at 50 Hz is *highlighted*. Complex impedance plane, measurement at 1 Hz is *highlighted*. Complex capacitance plane, measurement at 50 Hz is *highlighted*

State of the art in biosensor applications

Razumas et al. pioneered work on biosensors based on cubic phases, and in 1994 they presented the first report on membrane-based biosensors for determination of glucose, lactate, urea, and creatinine. The enzymes were trapped in a thin layer of a cubic phase of monoolein–water–protein that was coated with a dialysis membrane. Anodic biocatalytic currents due to oxidation of H_2O_2 were recorded at the amperometric enzyme electrodes [37].

In an attempt to produce an electroactive cubic phase Kostela et al. introduced different types of ferrocene amphiphiles into a melted mixture of mono and diglycerides and then an appropriate amount of water was added [59]. After centrifugation and storage for two weeks the samples where investigated with SAXD measurements, visual polarization microscopy, and electrochemistry. Phase diagrams where constructed and the electrochemical measurements revealed a 100–200-fold decrease in diffusion coefficient as compared with that is found in acetonitrile solutions for the ferrocene amphiphile. The idea of assembling a liquid crystalline cubic phase based on lipid bilayers with built in electroactive moieties is indeed challenging, since it would provide an appealing model system for studies of electron transfer processes in biomembranes. If more rapid electron transport could be provided by, for instance, electron hopping between the ferrocene amphiphiles, the system would provide a means of efficient regeneration of an inserted membrane protein serving as the recognition element in a biosensor. However, many problems remain to be solved.

Ropers et al. investigated two different cubic phases in the development of a cholesterol biosensor [60]. Thus, monoolein and a fluorinated derivative of an ethoxylated alcohol were used to produce two types of reverse bicontinuous cubic phases in excess water for immobilisation of cholesterol oxidase, ChO. The protein was efficiently entrapped in the water channels of the two cubic phases, and a slight increase in the dimensions of the unit cell was observed. The diffusion of the analyte, i.e. cholesterol, was much slower in the MO–water phase than in the fluorinated cubic phase; this enabled a fast response in the latter system. The reduction peak in cyclic voltammetry was used to investigate the concentration-dependence of the biosen-

sor based on a fluorinated cubic phase and a linear range of 0.01–5 mmol L^{-1} cholesterol in solution was found. The ChO–MO–water system was more stable but was not usable for biosensing purposes because of the retarded transport of analyte into and out of the cubic phase.

There are a few reports in which laccase oxidoreductase was entrapped in a cubic phase for catalysis of O_2 reduction following the original work from 1994 of Razumas [37]. In 2004 Rowinski et al. investigated two systems based on a MO-H_2O cubic phase where laccases from either *Trametes sp* (fungal) or *Rhus vernicifera* (higher plant) were inserted [61]. Cyclic voltammetry and chronoamperometry were employed to characterise the modified electrode. Hydroquinone was added to the solution to serve as a mediator for regeneration of the oxidised laccase following the catalysed reduction of oxygen. The formed quinone is continuously regenerated at the electrode surface (Fig. 3). The reduction current was found to be proportional to the O_2 content of the solution with a linear range between 0.09 and 0.2 mmol L^{-1} O_2 (Fig. 4) [61]. Moreover, the overpotential for oxygen reduction was decreased and no poisoning of the electrode surface was observed.

In another paper the same group reports on two laccases that were immobilised in a liquid-crystalline cubic phase, i.e. native laccase from *Trametes hirsuta*, and a modified version of the same enzyme to render it more hydrophobic. The mediator used was 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonate) diammonium salt ($ABTS^{2-}$). The system was investigated with Raman spectroscopy and cyclic voltammetry [62]. The mediated enzymatic processes were found to be effective.

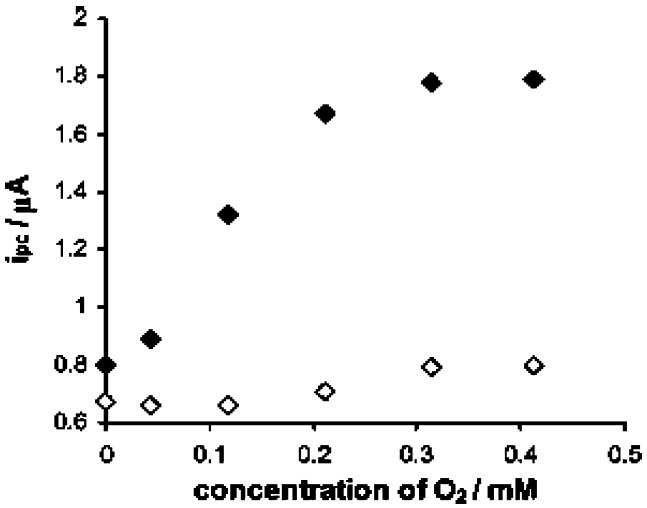


Fig. 3 Chronoamperometric current versus concentration of dioxygen. The current value was measured at −0.3 V, 300 s after applying this potential to the electrode, recorded in 0.05 mol L^{-1} phosphate buffer, pH 6. 1/1 mmol L^{-1} 1,4-benzoquinone: (*filled diamonds*) laccase-modified electrode and (*empty diamonds*) enzyme-free electrode. Quantity of *Trametes* sp. Laccase immobilized on the electrode, 180 µg. Figure 7 in Ref. [61]. Reprinted with kind permission from the American Chemical Society

The same group employed two different oxidases, i.e. glucose oxidase and pyranose oxidase, that were immobilised in the monoolein cubic phase and the oxidation of glucose was monitored with and without mediator [38]. Both oxidases catalyse the oxidation of glucose, with hydrogen peroxide as the product of both reactions. The concentration of glucose could therefore be determined amperometrically by oxidation of H_2O_2 at a platinum electrode. The linear range of this system was 1–20 mmol L^{-1} for glucose oxidase. In an attempt to improve the biosensing system either ferrocene or ruthenium hexamine was used as a mediator. This did increase the sensitivity considerably but not the linear range.

Implanted biocell fuel cell electrodes are often readily poisoned by biological components from body liquids. Thus, the electrodes of an O_2 cathode for medical implants are negatively affected by serum urate at the working potential and therefore methods have to be developed in order to exclude urate from the cathode. This is also the objective in a recent report from Rowinski et al. in which bilirubin oxidase was wired to the electrode with a copolymer of acrylamide and *N*-vinlyimidazole [63]. The wired bilirubin oxidase film was then coated by a layer of 1,2-dioleoyl-*sn*-glycero-3-phosphate-doped monoolein cubic phase in order to exclude the urate from the cathode. Doping with phospholipids changes the charge on the channel walls inside the cubic phase and thus the anion-to-cation permeability ratio. Thus, the permeation of the unwanted contaminant urate decreased even further when making the aqueous channel anionic. The dopant was also found to improve the stability by increasing the shear strength of the film [39] which made possible an investigation of the catalytic system at a rotating-disk electrode. Rowinski et al. have also reported on the formation of a lyotropic liquid crystalline cubic phase that is stabilized by adding a newly synthesized amphiphile with lyotropic properties and vitamin E acetate [63]. Similar to the previous case glucose oxidase was wired to the carbon electrode with a redox polymer that was subsequently coated with the cubic phase. The linear range of this sensor was 2–30 mmol L^{-1} which is most appropriate for glucose control of diabetic patients. However, the response time is much retarded because of the slow diffusion of glucose in the cubic phase.

Wiyaratn et al. used cyclic voltammetry, chronoamperometry, and rotating disk experiments to investigate the catalysis of the reduction of organohalides at a cubic phase with immobilised Zn(II) porphyrin. The detection limit of organohalides was decreased to 0.1 µmol L^{-1}, which is one order of magnitude lower than in an electro-polymerised

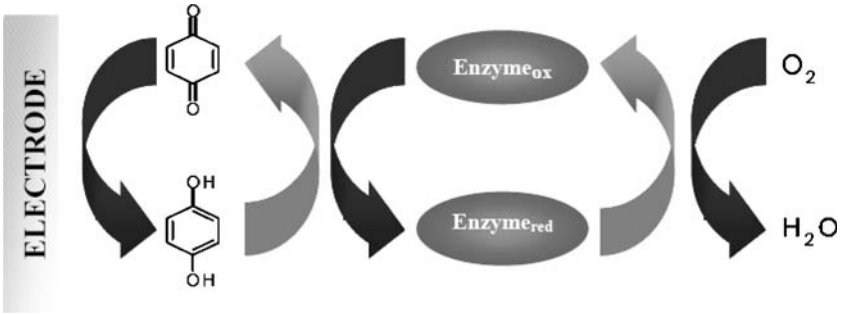


Fig. 4 Quinone/hydroquinone redox process catalyzed by a laccase. Scheme 1 in Ref. [61]. Reprinted with kind permission from the American Chemical Society

film. The authors suggest that the reason is the open structure of the cubic phase that allows effective mass transport of the substrate [64]. This system is certainly not a true biosensor but shows that rapid mass transport of the substrate within the cubic phase is an important parameter that can substantially increase the detection limit in a cubic phase-based sensor.

In an interesting approach to protein biochip development it has been suggested that self-assembled nanochannel networks can be formed from a mixture of monoolein and maleimide (triethylene glycol) ether lipid. In this highly ordered structure, sensor protein fragments, human blood proteins, were inserted and long-term stability in a broad temperature range was demonstrated [65]. Furthermore, in a later publication the same group employed freeze fracture electron microscopy and synchrotron X-ray diffraction to investigate the protein-induced porous soft matter cubosomic nanopattern, such as nanoridges and open nanochannels [66]. In these papers the authors investigated how protein inclusion affects cubic phase morphology and the stability of the nanostructures. However, no data were provided about the functionality of the captured or entrapped biomolecules, whereas it was concluded that the proteins were immobilized in the cubosome. More investigations are needed, with, for instance, variation of the charge of the analyte to increase/reduce transmembrane communication and exchange. This might pave the road for a new class of immunological biosensor assays if only for a limited number of low-molecular-weight molecules.

The search for a robust cubic phase that can be used in rugged biosensing applications or in drug delivery systems has motivated Tiberg et al. to investigate the aqueous phase behaviour of glycerol monoloeate, GMO, and glyceryl monooleyl ether, GME, at various mixing ratios [67]. GMO is known to increase chemical stabilisation and to reduce susceptibility to hydrolysis. The two lipids are totally miscible with each other and when a PPO–PEO triblock copolymer, e.g. "Pluronic F127", was added a dispersion of cubic phase nanoparticles was formed. Three ternary phase diagrams were constructed based on results from X-ray diffraction and ^{2}H NMR.

Concluding remarks

The cubic phase seems to be promising in biosensor design. A stable three-dimensional lipid bilayer can be deposited on the electrode surface where membrane proteins can be inserted with retained activity. The inserted redox proteins are readily recovered by mediators that shuttle electrons between the electrode surface and the protein. The mediator can, for example, be dissolved in the electrolyte or be linked to the lipid three-dimensional structure as inserted electroactive amphiphiles.

The advantage the cubic phase sensors can provide compared with those based on supported planar lipid membranes is twofold: i.e. the cubic phase has a better stability than the supported bilayer and it can also hold a larger surface concentration of protein on the electrode surface. The large amount of membrane protein that can be accommodated inside the cubic phase will give increased sensitivity provided mass and charge transport is rapid within the cubic phase. The signal-to-noise ratio can also be substantially improved by modification of the charge at the walls in the water channels. This is an advantage compared with other electrochemical sensors based on different enzyme immobilization approaches.

Other major advantages are the non-toxicity of the cubic phase and the ability of biodegradation in organisms. However, if the cubic phase is intended for use directly in a physiological fluid, the reaction with lipases may interfere. Under these conditions either a lipase-impermeable membrane should be used to cover the cubic phase film or monoacylglycerol cubic phase can be substituted by compounds not containing acyl moieties e.g. phytantriol. Improved characteristics of three-component liquid-crystalline matrices for the cathode and anode have been demonstrated recently by Heller and coworkers [39]. In general, the use of a ternary cubic system seems promising to further improve the stability and the shear strength of the lipid phase, which is also the inclination in several other recent publications [56, 63, 65–67].

Also the physical parameters of the cubic phase are of importance for improved performance of the biosensing system. Here, experience from drug delivery research

provides important information on how to fine-tune the diameter of the water-channels and/or the charge on the walls by substituting amphiphiles in the phase. The aim with this research is of course primarily to control the release of a particular drug, but it also provides information on how to exclude contaminants from entering the cubic phase, to increase the sensitivity and specificity of a biosensor. Moreover ongoing research concerning biofuel cells and crystallisation of membrane proteins provides important information for development of a stable and sensitive biosensing system.

Surprisingly there are only a few biosensors reported in the literature so far that are based on a cubic phase on an electrode surface. It should be recalled that when the theory concerning kinetics of electrochemical reactions mediated by redox polymer films was launched by Saveant et al. [53] in the early 1980s a great interest arose in the scientific community to utilize this elegant theory. The aim was to improve the efficiency of this particular electrochemically mediated system. Thus, the work on finding stable cubic phases that can be characterised with a rotating disc electrode in order to fully elucidate the electrode kinetics is promising and also necessary for future developments. It might, for instance, be useful to decrease the film thickness to increase the sensitivity of the biosensor if the diffusion of the analyte and/or the regenerating charge transport for re-reduction of an inserted reductase is/are retarded. A theory that considers the mass and charge transport properties and the electrochemical kinetics both at the redox protein and at the electrode surface is essential to fully optimise the system.

References

1. Castellana E, Cremer P (2006) Surf Sci Rep 61:429–444
2. Brian A, McConnell H (1984) Proc Natl Acad Sci U S A 81:6159–6163
3. Zhang L, Spurlin T, Gewirth A, Granick S (2006) Phys Chem Lett B 110:33–35
4. Purrucker O, Hillebrandt H, Adlkofer K, Tanaka M (2001) Electrochim Acta 47:791–798
5. Forstner MB, Yee CK, Parikh AN, Groves JT (2006) J Am Chem Soc 128:15221–15227
6. Puu G, Gustafsson I, Artursson E, Ohlsson P-Å (1995) Biosens Bioelectron 10:463–476
7. Rehfeldt F, Tanaka M (2003) Langmuir 19:1467–1473
8. Goennenwein S, Tanaka M, Hu B, Moroder L, Sackmann E (2003) Biophys J 85:646–655
9. Baumgart T, Offenhausser A (2003) Langmuir 19(5):1730–1737
10. Morigaki K, Schonherr H, Frank C, Knoll W (2003) Langmuir 19:6994–7002
11. Vikholm-Lundin I (2005) Langmuir 21:6473–6477
12. Merzlyakov M, Li E, Gitsov I, Hristova K (2006) Langmuir 22:10145–10151
13. Lindholm-Sethson B, Gonzalez JC, Puu G (1998) Langmuir 14:6705–6708
14. Albertorio F, Diaz A, Yang T, Chapa V, Kataoka S, Castellana E, Cremer P (2005) Langmuir 21:7476–7482
15. Vikholm I, Peltonen J, Teleman O (1995) Biochim Biophys Acta 1233:111–117
16. Vikholm I, Viitala T, Albers W, Peltonen J (1999) Biochim Biophys Acta 1421:39–52
17. Andersson M, Keizer H, Zhu C, Fine D, Dodabalapur A, Duran R (2007) Langmuir 23:2924–2927
18. Wilkop T, Xu DK, Cheng Q (2007) Langmuir 23:1403–1409
19. Wagner ML, Tamm LK (2000) Biophys J 79:1400–1414
20. Tamm LK, Crane J, Kiessling V (2003) Curr Op Struct Biol 13:453–466
21. Krishna G, Schulte J, Cornell B, Pace R, Osman P (2003) Langmuir 19:2294–2305
22. Mueller P, Rudin D, Tien H, Wescot W (1962) Nature 194:979–980
23. Romer W, Lam Y, Fischer D, Watts A, Fischer W, Goring P, Wehrspohn R, Gosele U, Steinem C (2004) J Am Chem Soc 126:16267–16274
24. Danelon C, Perez J, Santschi C, Brugger J, Vogel H (2006) Langmuir 22:22–25
25. Lindblom G, Rilfors L (1989) Biochim Biophys Acta 988:221–256
26. Ericsson B, Larsson K, Fontell K (1983) Biochim Biophys Acta 729:23–27
27. Nazaruk E, Michota A, Bukowska J, Shleev S, Gorton L, Bilewicz R (2007) J Biol Inorg Chem 12:335–344
28. Carr MG, Corish J, Corrigan OI (1997) Int J Pharm 157:35–42
29. Helledi LS, Schubert L (2001) Drug Del Ind Pharm 27:1073–1082
30. Bender J, Ericson MB, Merclin N, Iani V, Rosen A, Engstrom S, Moan J (2005) J Contr Release 106:350–360
31. Lopes LB, Lopes JLC, Oliveira DCR, Thomazini JA, Garcia MTJ, Fantini MCA, Collett JH, Bentley MVLB (2006) Eur J Pharm Biopharm 63:146–155
32. Clogston J, Caffrey M (2007) J Contr Release 107:97–111
33. Qutub Y, Reviakine I, Maxwell C, Navarro J, Landau EM, Vekilov PG (2004) J Mol Biol 343:1243–1254
34. Chi ML, Nollert P, Loewen MC, Belrhali H, Pebay-Peyroula E, Rosenbusch JP, Landau EM (2000) Acta Crystallogr D 56:781–784
35. Carlsson N, Winge A-S, Engström S, Å, kerman B (2005) J Phys Chem B 109:18628–18636
36. Carlsson N, Sanandaji N, Voinova M, Åkerman B (2006) Langmuir 22:4408–4414
37. Razumas V, Kanapieniene J, Nylander T, Engström S, Larsson K (1994) Anal Chim Acta 289:155–162
38. Rowinski P, Kang C, Shin H, Heller A (2007) Anal Chem 79:1173–1180
39. Rowinski P, Rowinska M, Heller A (2008) Anal Chem 80:1746–1755
40. Orädd G, Lindblom G, Fontell K, Ljusberg-Wahren H (1995) Biophys J 68:1856–1863
41. Lindblom G, Larsson K, Johansson L, Fontell K, Forsén S (1979) J Am Chem Soc 101:5465–5470
42. Kraineva J, Narayanan RA, Kondrashkina E, Thiyagarajan P, Winter R (2005) Langmuir 21:3559–3571
43. Danino D, Kaplun A, Lindblom G, Rilfors L, Orädd G, Hauksson JB, Talmon Y (1997) Chem Phys Lipids 85:75–89
44. Lindblom G, Orädd G (1994) Prog Nucl Magn Reson Spectrosc 26:483–516
45. Lindblom G, Larsson K, Johansson L, Fontell K, Forsén S (1979) J Am Chem Soc 101:5465–5470
46. Eriksson P-O, Lindblom G, Arvidson G (1985) J Phys Chem 89:1050–1053
47. Eriksson P-O, Lindblom G, Arvidson G (1987) J Phys Chem 91:846–853
48. Razumas V, Larsson K, Miezis Y, Nylander T (1996) J Phys Chem 100:11766–11774
49. Razumas V, Talaikyt Z, Barauskas J, Miezis Y, Nylander T (1997) Vib Spectrosc 15:91–101

50. Kraineva J, Narayanan RA, Kondrashkina E, Thiyagarajan P, Winter R (2005) Langmuir 21:3559–3571
51. Caboi F, Nylander T, Razumas V, Talaikyte Z, Monduzzi M, Larsson K (1997) Langmuir 13:5476–5483
52. Corvis Y, Brezesinski G, Rink R, Walcarius A, Heyden A, Mutelet F, Rogalska E (2006) Anal Chem 78:4850–4864
53. Andrieux CP, Saveant JM (1982) J Electroanal Chem 134:163–166
54. Rowinski P, Korytkowska A, Bilewicz R (2003) Chem Phys Lip 124:147–156
55. Kostela J, Elmgren M, Kadi M, Almgren M (2005) J Phys Chem B 109:5073–5078
56. Kumar SV, Lakshminarayanan V (2007) Langmuir 23:1548–1554
57. Lindholm-Sethson B (1996) Langmuir 12:3305–3314
58. Bard AJ, Faulkner LR (2001) Electrochemical Methods: Fundamentals and Applications, 2nd ed. John Wiley & Sons Inc., p. 380
59. Barauskas J, Razumas V, Talaikyte Z, Bulovas A, Nylander T, Tauraite D, Butkus E (2003) Chem Phys Lipids 123:87–97
60. Ropers MH, Bilewicz R, Stebe MJ, Hamidi A, Miclo A, Rogalska E (2001) Phys Chem Chem Phys 3:240–245
61. Rowinski P, Bilewicz R, Stebe MJ, Rogalska E (2004) Anal Chem 76:283–291
62. Nazaruk E, Bilewicz R (2007) Bioelectrochemistry 71:8–14
63. Nazaruk E, Michota A, Bukowska J, Shleev S, Gorton L, Bilewicz R (2007) J Biol Inorg Chem 12:335–344
64. Wiyaratn W, Somasundrum M, Surareungchai W (2005) Analyst 130:626–631
65. Angelova A, Ollivon M, Campitelli A, Bourgaux C (2003) Langmuir 19:6928–6935
66. Angelova A, Angelov B, Papahadjopoulos-Sternberg B, Bourgaux C, Couvreur P (2005) J Phys Chem B 109:3089–3093
67. Popescu G, Barauskas J, Nylander T, Tiberg F (2007) Langmuir 23:496–503