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Enzymes and mediators hosted together in lipidic mesophases for the construction of biodevices

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ABSTRACT

Self-assembled, highly viscous, and optically transparent lipidic cubic phases are employed as matrices for enzyme-glucose dehydrogenase and vitamin K derivatives differing in hydrophobicity: phylloquinone ($VitK_1$), menaquinone ($VitK_2$), and menadione ($VitK_3$). The lipidic cubic mesophase has been employed to hold these electroactive biological molecules in close vicinity of the electrode surface in order to study their behavior in the lipid environment by electrochemical methods. Liquid–crystalline properties of the analyzed samples of non-doped and doped phase were identified using X-ray and polarized microscopy. Incorporation of the enzyme together with the mediator in the lipidic matrix results in the formation of a catalytically active and stable film on the electrode surface and makes the modified electrode useful for the development of biosensors. Single-walled carbon nanotubes were employed to nanostructure the electrode surface in order to increase the working area of the electrode.

1. Introduction

Lipids can form a variety of lyotropic liquid–crystalline phases. When placed in water they spontaneously organize into liquid–crystalline structures, that is, lamellar, hexagonal, or cubic phases. While the lamellar or hexagonal phase exists in broad temperature and concentration range, formation of cubic phases often critically depend on these parameters as often seen in the phase diagrams of lipid/water systems [1]. The cubic phases are classified according to their symmetry that can be deduced from X-ray diffraction. For our studies, monoolein/water (MO/water) system was chosen as the phase diagram for this system is well known and cubic phases formed in such system are commonly used in protein crystallization, drug delivery, and also in biosensing or biofuel cell construction [2–4]. At MO/water phase diagram, a number of different cubic structures may be formed, but the space groups carefully determined for cubic phases containing membrane lipids are limited, for example, *Ia3d* and *Im3m* with body-centered space groups and *Pn3m* with primitive cubic lattices [1–6].

Cubic phase can be characterized as a curved, non-intersecting bilayer with two water channels. Cubic phase is highly viscous often waxy or in some cases jelly-like, at hydration over 20% stable in contact with water. In contrast to lamellar or hexagonal phase, the cubic phase is optically isotropic, so its experimental identification may be more difficult. Cubic phase formed in the monoolein/water system is non-toxic, biocompatible, and biodegradable and shows good chemical and physical stability of incorporated drugs especially macromolecular drugs. These materials exhibit phase stability and can be stored for a month without phase transition. Due to the presence of bicontinuous water and amphiphilic channels, it can host water- or -lipid soluble molecules, for example proteins, vitamins, or drugs. Liquid–crystalline phases formed by polar lipids in aqueous media can be used as model matrices to mimic biological processes. Cubic phases have been shown to improve the topical/transdermal delivery of relatively small molecules such as nicotine, salbutamol, acyclovir, cyclosporine A (CysA), and aminolevulinic acid esters [7–11]. The diffusion of these molecules depends on the size of the molecules and the rate of transport can be modulated by applying lipids with different acyl chains to change the charge of the water channels. Cubic phase found application for membrane protein crystallization and allowed to determine protein structure with high resolution [3,4,12,13].

The presence of 5 nm wide water channels enable incorporation of water soluble proteins. 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 system [14]. Razumas et al. presented the first report on membrane based biosensors for the determination of glucose, lactate, urea, and creatinine where the lipid membrane was a cubic liquid crystalline phase film in which the corresponding enzymes were

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trapped. Oxidation of H_2O_2, the product of enzymatic reaction, was monitored on Pt electrode [15]. MO/cytochrome c/water system was characterized with the use of X-ray diffraction, FT-IR, differential scanning calorimetry, and electrochemistry [16]. The presence of the protein brought about a structural rearrangement in the polar head group region and a higher conformational order of the acyl chains although the cubic phase remained in space group *Pn3m* [17]. Razumas et al. also investigated the effect of VitK1 in the MO/water cubic phase and based on cyclic voltammetry experiments found in this case rather slow mobility of the entrapped vitamin. The MO/water cubic phase can accommodate up to 1 wt.% VitK1 without structural changes. It was shown that incorporation of vitK1 at concentrations higher than 1 wt.% leads to the appearance of reversed hexagonal phase already at room temperature, which in case of non-doped binary system MO/H_2O at the same ratio, is formed at temperature above 90 °C [18].

Recently, we have demonstrated that liquid–crystalline cubic phase is a convenient matrix for biocatalysts immobilization since it holds enzymes in their active forms close to the electrode surface. It also facilitates transport of substrates and products of the enzymatic process through the film and provides biocompatible environment for the reactions. Cubic phases were employed for the immobilizing laccase, glucose oxidase, and pyranose oxidases [19–22]. In the present study, cubic phase formed in MO/H_2O system is employed to immobilize enzymes and vitamins belonging to the vitamin K group. In our experiments, ternary systems consisting of MO/VitK/H_2O at the ratio 64/1/35 wt.% and MO/enzyme/H_2O containing 1.2 wt.% of enzyme were used. Structural and electrochemical studies were performed to monitor the behavior of glucose dehydrogenase and 2-methyl-1, 4-naphthoquinone derivatives: menadione (VitK3), phylloquinone (VitK1), and menaquinone (VitK2) in the lipidic cubic phase environment. Vitamin K is the essential compound in blood clotting, when used in topical delivery, it increases the rate of bruise absorption. Since it was found to reduce redness, it was used in treatment of laser induced redness and also has influence on the absorption of bruising that occur intradermally or subcutaneously [23–25]. Applying monoolein based liquid crystalline phases results in 3-fold increase in effectiveness of topical vitamin K absorption.

NAD^+ dependent enzymes are oxygen insensitive and demonstrate applicability for the oxidation of a large variety of substrates, for example, glucose and ethanol, which makes the systems potentially useful for biosensors or biofuel cells. Formal potential of the redox couple NAD^+/NADH at pH 7 and at room temperature is −0.560 V (vs. SCE) [26]. On the other hand, the direct NADH oxidation at bare electrodes proceeds with large overpotential of ca. 1 V [27]. Recently, it was shown that deposition of single walled carbon nanotubes at the electrode surface can lead to a shift of NADH oxidation to ca. 0.2 V [28] For further reduction of the overpotential of NADH oxidation phenoxazine dyes or quinones and their derivatives, or metal nanoparticles are employed [29–31].

In the present work, dehydrogenase is used for glucose oxidation with the aim of applying such systems in the enzymatic biofuel cell or bio-batteries. NAD^+ dependent glucose dehydrogenase was entrapped into the lipidic matrix. Derivatives of vitamin K were used as mediators in the oxidation reaction. The properties of all doped systems were studied by cyclic voltammetry. Cubic symmetry of applied systems was confirmed with X-ray diffraction and polarizing microscope. VitK1 and VitK2 both having long alkyl chains are distributed mainly within the lipidic layer of the cubic phase, and even after several hours, they are fully retained in the matrix. On the contrary, VitK3, which is most hydrophilic of these vitamins, is mainly located in the water channels of the cubic phase. The rate of diffusion of these molecules out of the matrix depends on the structure of the vitamin and on the presence of the hydrophobic alkyl chains.

2. Materials and methods

Monoolein (1-oleoyl-rac-glycerol) (MO) ⩾99%, vitamins K1 (purity not specified), K2 (purity not specified), K3 (98%) (Scheme 1), glucose dehydrogenase from *Pseudomonas sp*. 297U/mg, D-(+)-glucose, diaphorase from *Clostridium kluyveri* (5.96 U/mg), and NAD^+ (⩾96.5%) were purchased from Sigma and were used as received.

McIlvaine buffer was prepared by mixing 0.1 M citric acid (analytically pure) and 0.2 M disodium phosphate (analytically pure) (both 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. All solutions were prepared using Milli Q water (18.2 MΩ cm^{-1}), Millipore, Bedford, MA, USA.

Electrochemical measurements were performed using an ECO Chemie Autolab potentiostat with GPES software in a three-electrode arrangement with an Ag/AgCl reference electrode and a platinum foil as the counter electrode. Working electrode was glassy carbon electrode (GCE) modified with monoolein cubic phase film. Before the experiments, the electrode was polished on alumina (0.1 and 0.05 μm) on a polishing cloth. The electrodes were then rinsed with water in an ultrasonic bath rinsed with water and left to dry. Glassy carbon electrodes were covered with cubic phase layer. For each type of the cubic phase, experiments were repeated three times.

To identify liquid–crystalline properties of the analyzed samples, the X-ray diffraction with Bruker GADDS system working with Cu Kα radiation and Bruker Nanostar system working with Cu Kα radiation equipped with Vantec 2000 area detector were used. For polarized microscopy, Nikon Eclipse E400 microscope equipped with a LINKAM THMS 600 heating/cooling stage was applied.

To prepare non-doped cubic phase, monoolein was melted in small glass vial, and then appropriate amount of water was added. The ratio of components was chosen on the basis of phase diagram for the monoolein/water system. The glass vial was centrifuged in the aim of mixing the components until transparent and highly viscous cubic phase was obtained. Cubic phases doped with vitamin K were prepared in a mixture containing VitK/MO/H_2O used at the ratio 1/64/35. Vitamins were melted in lipid and then water was added. Sample was centrifuged to obtain homogenous viscous, jelly-like sample that was stored in tightly closed vial at room temperature. Cubic phases doped with vitamins K, glucose dehydrogenase (GDH), and diaphorase (DI) were prepared in similar way, but instead water, enzyme solution (20 mg GDH, 5 mg DI dissolved in 300 μl H_20) was added to the melted lipid.

3. Results and discussion

High viscosity and transparency due to the lack of birefringence are typical for liquid crystals of cubic symmetry. Therefore, cubic symmetry can be detected by visual inspections. To evaluate the effect of addition of enzyme or derivatives of 2-methyl-1, 4-naphthoquinone, that is, menadione (VitK3), phylloquinone (VitK1) and menaquinone (VitK2) on the cubic phase structure, X-ray diffraction and polarizing microscope were employed.

3.1. Polarizing light microscope

Other than cubic mesophases are birefringent, which is related to anisotropy of molecular arrangement. In contrary to anisotropic hexagonal or lamellar liquid crystals, the cubic phase is isotropic, which makes it difficult to characterize with optical methods. Fig. 1A displays microscopic texture of non-doped cubic phase, full