Biosensors and Bioelectronics 100 (2018) 437–444

Contents lists available at ScienceDirect

Biosensors and Bioelectronics

journal homepage: www.elsevier.com/locate/bios

Lipidic liquid crystalline cubic phases for preparation of ATP-hydrolysing enzyme electrodes

Martina Zatloukalová[a,b], Ewa Nazaruk[a], David Novák[b], Jan Vacek[b,*], Renata Bilewicz[a,*]

[a] Faculty of Chemistry, University of Warsaw, Pasteura 1, 02-093 Warsaw, Poland
[b] Department of Medical Chemistry and Biochemistry, Faculty of Medicine and Dentistry, Palacký University, Hněvotínská 3, 775 15 Olomouc, Czech Republic

ARTICLE INFO

Keywords:
Lipid cubic phase
Sodium potassium pump
Enzyme activity
Protein biosensor
Voltammetry
ATPase

ABSTRACT

The lipidic liquid-crystalline cubic phase (LCP) is a membrane-mimetic material useful for the stabilization and structural analysis of membrane proteins. Here, we focused on the incorporation of the membrane ATP-hydrolysing sodium/potassium transporter Na^+/K^+-ATPase (NKA) into a monoolein-derived LCP. Small-angle X-ray scattering was employed for the determination of the LCP structure, which was of Pn3m symmetry for all the formulations studied. The fully characterized NKA-LCP material was immobilized onto a glassy carbon electrode, forming a highly stable enzyme electrode and a novel sensing platform. A typical NKA voltammetric signature was monitored *via* the anodic reaction of tyrosine and tryptophan residues. The *in situ* enzyme activity evaluation was based on the ability of NKA to transform ATP to ADP and free phosphate, the latter reacting with ammonium molybdate to form the ammonium phosphomolybdate complex under acidic conditions. The square-wave voltammetric detection of phosphomolybdate was performed and complemented with spectrophotometric measurement at 710 nm. The anodic voltammetric response, corresponding to the catalytic ATP-hydrolysing function of NKA incorporated into the LCP, was monitored at around + 0.2 V *vs.* Ag/AgCl in the presence or absence of ouabain, a specific NKA inhibitor. NKA incorporated into the LCP retained its ATP-hydrolysing activity for 7 days, while the solubilized protein became practically inactive. The novelty of this work is the first incorporation of NKA into a lipidic cubic phase with consequent enzyme functionality and stability evaluation using voltammetric detection. The application of LCPs could also be important in the further development of new membrane protein electrochemical sensors and enzyme electrodes.

1. Introduction

Membrane proteins, located in lipid bilayers, are involved in important cellular processes, such as the transport of ions and low-molecular weight compounds, the conversion of energy, signal transduction, and cell-cell interactions. Understanding these processes at a molecular level requires knowledge of the structure of these proteins and the retention of their activity under the selected experimental conditions. The incorporation or reconstitution of membrane proteins into lipid systems, such as linear lipid monolayers, supported lipid bilayers, liposomes, lipid nanodiscs, or more complex systems such as lipidic cubic phases, enables them to retain their activity and global or local structural integrity. The above point is an important prerequisite for subsequent structural/functional analysis and the investigation of membrane protein biomolecular interactions (Caffrey, 2015; Landau and Luisi, 1993; Landau and Rosenbusch, 1996; Razumas et al., 1994; Van'T Hag et al., 2016; Zabara et al., 2014). From the methodological point of view, current research focuses on the development of stable lipid membrane-mimetic systems and versatile sensing devices and biosensors useful for the characterization and trace analysis of membrane proteins and their lipid membrane associates. This study is devoted to the immobilization of the lipidic liquid crystalline cubic phase (LCP), as a suitable accommodation matrix for the ATP-hydrolysing transmembrane protein sodium-potassium pump, onto a carbon electrode surface. In this way, a functional and highly stable enzyme electrode was developed.

LCPs are suitable materials for protein immobilization, since they are stable in excess water, biocompatible, and can accommodate relatively large loads of host material due to their high surface area of *ca.* 400 $m^2\ g^{-1}$ (Angelov et al., 2003; Kulkarni et al., 2011; Luzzati et al., 1968). Cubic phases are formed upon mixing lipids with water in a certain ratio and generally occur in two different arrangements, the closed discontinuous micellar array and the bicontinuous cubic phase, which exhibits a three-dimensional, continuous membrane bilayer. The structural parameters of LCPs depend on the composition of the aqueous part and on the selected lipid. Frequently used lipids for the

* Corresponding authors.
E-mail addresses: jan.vacek@upol.cz (J. Vacek), bilewicz@chem.uw.edu.pl (R. Bilewicz).

http://dx.doi.org/10.1016/j.bios.2017.09.036
Received 27 May 2017; Received in revised form 17 September 2017; Accepted 18 September 2017
Available online 20 September 2017
0956-5663/

formation of LCPs are monoacylglycerols, specifically 1-monoolein (MO) (Qiu and Caffrey, 2000). The highly structured reverse LCPs, which are composed of curved lipid bilayers surrounded by two identical, non-intersecting aqueous channels, exhibit interesting properties as the host materials of biologically important molecules. Because of their internal structure, LCPs can incorporate hydrophilic, amphiphilic, and hydrophobic drugs, enzymes, and other bioactive molecules of various sizes and molecular weights. LCPs have therefore been investigated as drug carriers (Boyd et al., 2006; Fong et al., 2016; Nazaruk et al., 2015) and as hosting matrices for redox enzymes, while the deposition of these catalytic films on electrode surfaces has also been studied (Nazaruk et al., 2014, 2008; Razumas et al., 1994).

Embedding the transmembrane proteins in the cubic phase has often been used for the crystallization of these proteins (Landau and Rosenbusch, 1996); however, only a few studies have been devoted to their functions and the evaluation of their activity when immobilized in this biocompatible environment. The activity of photosynthetic protein transporters, (Katona et al., 2003) heme-copper oxidases, (Li and Caffrey, 2011) the ion channel KvLm, (Santos et al., 2012) outer membranes proteins, and the enzyme DgaK were also investigated in LCPs (Tiefenbrunn et al., 2011).

Here we present for the first time the cubic phase as a suitable environment for the immobilization of the transmembrane protein sodium-potassium pump in its fully functional form on the electrode surface. The sodium-potassium pump (EC 3.6.3.9), also known as Na^+/K^+-ATPase (NKA), is a transmembrane plasma protein that actively transfers sodium and potassium ions against their electrochemical potential gradients. The energy necessary to transport ions is obtained from the hydrolysis of ATP. During this process, NKA changes structure between two conformation states, E1 and E2. In the E1 state, NKA has a high affinity for sodium ions and ATP; therefore, the channel is opened toward the cytoplasm side. In the E2 state, the cation binding site is oriented towards the extracellular space, and NKA has a high affinity for potassium ions (Kaplan, 2002). Structurally, the sodium potassium pump is a heterodimeric protein, consisting of an α-subunit (109 kDa) and a smaller β-subunit (55 kDa). The α-subunit is responsible for the catalytic function of ATP hydrolysis, and the β-subunit is necessary for the proper maturation of the enzyme in the plasma membrane (Geering, 2001; Toyoshima et al., 2011). Both subunits, together with the gamma unit, are highlighted in the molecular visualization (3WGV) in Scheme 1.

For the evaluation of NKA activity, the transformation of ATP to ADP accompanied by free phosphate (P_i) release is monitored. As previously described, the determination of the release of phosphates can be monitored by spectrophotometry based on the reaction with ammonium molybdate (Bartolommei et al., 2013; Sarkadi et al., 1992), BIOMOL Green reagent (Juel et al., 2013), or Malachite Green (Benabdelhak et al., 2005), or alternatively by using labelled phosphate for liquid scintillation analysis (Dalla et al., 2013; Ferrandi et al., 1996) or fluorimetric methods (Johansson et al., 2000). To the best of our knowledge, the quantitative electrochemical evaluation of NKA's function has not yet been reported.

The aims of our studies were to **(a)** prepare a stable LCP with incorporated NKA enzyme, (b) cover the carbon electrode surface with the NKA-LCP layer, **(c)** evaluate NKA functionality (ATP-hydrolysing activity) *in situ* using the electrochemical monitoring of free phosphate and complement the electrochemical data with spectrophotometric results, and **(d)** estimate the ATP-hydrolysing activity (enzyme stability) of NKA embedded in LCP over a 14-day period.

2. Experimental section

2.1. Reagents

Chemicals were purchased from Sigma Aldrich (St. Louis, MO, USA), BioRad Laboratories (Hercules, CA, USA), or POCh (Polish Chemicals Co.). Monoolein was purchased from Hampton Research (Aliso Viejo, CA, USA). All solutions were prepared using Milli-Q water (18.2 MΩ cm^{-1}), (Millipore, Bedford, MA, USA). The sample preparation and analyses were performed under aerobic conditions.

2.2. Protein isolation, solubiliation, and purification

Two NKA samples, based on different isolation and solubiliation protocols, were used. The first one was commercially available NKA (Sigma-Aldrich), which was solubilized in 50 mM TRIS/HCl buffer (pH 7.4) containing 4.5 mg ml^{-1} octaethylene glycol monododecyl ether ($C_{12}E_8$) detergent (*sample A*). The second one was NKA isolated from fresh pig kidney (donated by Makovec Co., Czech Republic). The method of Klodos et al. (2002) was used for isolation. Briefly, after several homogenization and centrifugation steps on kidney tissue in ISE buffer (25 mM imidazole, 250 mM sucrose, 1 mM ethylenediaminetetraacetic acid, pH 7.4), the corresponding amount of sodium dodecyl sulphate (0.24 mg SDS/mg protein) was used for protein solubiliation (*sample B*).

The protein concentration was determined by Bradford or bicinchoninic acid protein assays (Bradford, 1976). The protein purity was verified by SDS-PAGE carried out on 10% polyacrylamide gels with Coomassie Brilliant Blue staining, and the specific enzyme activity was determined by Baginski assay (Baginski et al., 1967). Both the solubilized *samples A* and *B* were pipetted into small aliquots and stored at − 80 °C.

2.3. Preparation of LCPs

LCPs without protein were prepared by mixing the appropriate amount of molten monoolein (MO) and buffered solutions of 50 mM TRIS/HCl pH 7.4 with 1.12 mg ml^{-1} $C_{12}E_8$ (for *sample A*) or ISE with 0.08 mg ml^{-1} SDS (for *sample B*) at room temperature in small glass vials. The ratio of components was chosen on the basis of the phase diagrams for the MO/water system (Qiu and Caffrey, 2000). The composition of the non-doped LCPs was 60.2/39.8 (w/w) % for MO/buffer. LCPs with incorporated NKA were prepared similarly as described above, first NKA *sample A* or *B* was diluted in buffer solution and mixed with the appropriate amount of molten MO. The final composition of the NKA-doped LCPs was 60/0.2/39.8 (w/w) % for MO/NKA/buffer. Samples were stabilized for at least 48 h to obtain transparent, viscous, and homogenous LCPs. Samples were stored in tightly closed vials at room temperature in the dark. Their stability was confirmed by macroscopic observation of the samples and by small-angle X-ray scattering (SAXS) measurements.

2.4. SAXS analysis

The phase identity and structural parameters of the lipidic samples were determined by SAXS. The experiments were carried out with a Bruker Nanostar system equipped with a Vantec-2000 area detector using CuKα radiation (λ = 1.54 Å). LCP formulations were injected into 1.5-mm diameter quartz capillaries sealed with epoxy glue (UHU). The temperature in the sample cell was kept at 22 °C and was regulated by a Peltier element, while the scattered intensity was collected over 20 min. The scattering intensities I(q) were represented as a function of the magnitude of the scattering vector $q = (4\pi/\lambda)\sin(\theta)$, where 2θ is the scattering angle and λ is the wavelength of the incoming X-ray beam. The 2D images were integrated to produce 1D plots representing the scattering intensity I *vs.* q, called scattering profiles. The lattice parameter (a) for Pn3m LCP was calculated using the following equation:

$$a = \frac{2\pi}{q}\sqrt{h^2 + k^2 + l^2} \quad (1)$$

, where q is the scattering vector, and *h*, *k*, and *l* are the Miller indices of the Bragg peaks.