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Grazing Incidence X-ray Diffraction and Brewster Angle Microscopy studies on domain formation in phosphatidylethanolamine/cholesterol monolayers imitating the inner layer of human erythrocyte membrane

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ABSTRACT

In this work the properties of monomolecular films composed of 1-stearoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine (SOPE) and cholesterol, differing in lipid proportion, were investigated in the context of domain formation in the inner leaflet of membrane. To perform comprehensive analysis of the studied model systems the Langmuir monolayer experiments were performed in combination with Brewster angle microscopy (BAM) and Grazing Incidence X-ray Diffraction (GIXD) techniques. The analysis of the collected data proved non-ideal behavior of the investigated films. It was found that cholesterol at its lower concentration in the system (10%) is of disturbing influence on SOPE film. Further addition of cholesterol into phospholipids film (33, 50, and 67% of cholesterol) induces an ordering effect on SOPE *acyl* chains and provokes the formation of sterol-poor and sterol-rich domains which stoichiometry is independent of monolayer composition. The foregoing findings allow one to conclude that in cytosolic leaflet of membrane the lipids may segregate into domains of various cholesterol contents which depending on their composition may play different roles in membrane functioning.

1. Introduction

Phosphatidylethanolamines (PEs) are, after phosphatidylcholines (PCs), the most abundant phospholipids in mammalian membranes [1]. This group of glycerophospholipids displays wide biological functions, which are related for example to their metabolism in the heart and liver as well as their role as precursors in the synthesis of neurotransmitters (e.g. Anandamide) and glycosylphosphatidylinositol anchors for various proteins [2]. In cellular membranes of human erythrocytes PEs are localized mainly in the inner leaflet where they cover a significant mass of total phospholipids [3,4]. As the components of membrane PEs play a significant role in membrane fusion and they are important structural elements of bilayer [2,5]. Therefore, there are performed the investigations on the PE-containing artificial systems dedicated to explore the role of these phospholipids in estimation of the organization of various cellular membranes. The studies involve both the mixtures composed of PEs and other phospholipids imitating e.g. bacterial membranes [6] as well as PE/cholesterol model systems imitating the inner layer of e.g. mammalian membrane [7]. The latter systems are investigated mainly from the point of view of the effect of cholesterol on the organization and domain formation in cytosolic membrane layer. At this point it should be noted that the reports concerning the foregoing PE/cholesterol systems are much rarer as compared to those for mixtures composed of cholesterol and the outer layer lipids (phosphatidylcholine — PC, sphingomyelin — SM). On the other hand this is a very interesting task because the composition of both membrane leaflets is different and the major phospholipids in each membrane layers (PC — in the outer layer versus PE in the inner layer) differ significantly in their structure. The ability of ethanolamine moiety to interact via hydrogen bonds causes more favorable PE–PE interactions as compared to chol–PE forces and thus the mixing in PE/Chol system is limited [8] and the interactions of cholesterol in PE mixtures differ from those in PC-containing systems. This is directly connected with the structure of both phospholipids and undoubtedly may influence the organization of both membrane layers.

As it was widely evidenced the incorporation of cholesterol into PC or SM or PC/SM membranes induces the formation of liquid ordered (*lo*) phase [9] and it was postulated that *lo* domains in the outer layer are connected to those existing in the inner layer [10]. Although it is believed that domains are formed also in the inner layer, their nature and compositions are explored much less accurately as compared to the morphology of the outer layer [10–13]. As indicated the results presented in literature *lo* domains are formed in various cholesterol/PE mixtures [7,8,14,15]. It was also evidenced that the properties of these systems e.g. the interactions and miscibility of cholesterol with PEs in model systems, as well as the influence of cholesterol on the chain-melting phase transition temperature of the host PE bilayer and organization of membrane [7,16] are determined by various factors, e.g. the proportion of lipids in the mixture, structure of PE chains as

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well as temperature. For example considering the solubility limit of cholesterol in PEs the X-ray diffraction experiments evidence the formation of crystalline cholesterol domains in mixtures with egg phosphatidylethanolamine (egg PE) or with dielaidoyl phosphatidylethanolamine (DEPE) at 35–40% of cholesterol, at 43% in DMPE [17] and at 51% in POPE-containing systems [18]. In general cholesterol was found to reflect better miscibility with PE possessing mono- and polyunsaturated chains as compared to the fully saturated [9].

From the point of view of membrane organization and considering inconsistencies in the results obtained for various Chol/PE systems postulated in literature [15] it is required to investigate the ability of cholesterol to form *lo* phase in PE environment and to analyze the properties of the formed domains. The investigations involving saturated PEs proved that they do not form complexes with cholesterol [19]. On the other hand recent investigations performed on monolayers and vesicles evidenced that 1-stearoyl-2-linoleoyl-sn-glycero-3-phosphatidyl-ethanolamine (SLPE) is able to form specific complexes with cholesterol, which may form nanoscale assemblies in the inner membrane layer. It was also suggested that other PE molecules containing unsaturated chains may form domains in the inner leaflet [14]. The aims of this work were to verify the formation of domains in cholesterol/SOPE films of various sterol contents and to characterize the properties of these domains and their composition. In these studies the Langmuir monolayer technique together with BAM was applied to investigate the miscibility and interactions between molecules in the mixtures as well as the domain formation in the studied films. To gain insight into the properties of the domains formed in the foregoing system GIXD experiments were performed.

2. Experimental

2.1. Materials

The investigated phospholipids, namely 1-stearoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine (SOPE) as well as cholesterol (Chol) were synthetic products of high purity (≥99%) purchased from Avanti Polar Lipids, Inc. (700 Industrial Park Drive Alabaster, Alabama 35007-9105, USA). To prepare spreading solutions the lipids were dissolved in chloroform/methanol (4:1 v/v) mixture (both chloroform and methanol were purchased from Sigma-Aldrich, Poland, HPLC grade, ≥99.9%). From the respective stock solutions the mixed solutions were prepared and desirable volume of the latter was deposited onto the water subphase with the Hamilton micro syringe, precise to 1.0 μL. The volume of the spreading solutions was varied between 150 and 200 μL. Measurements were performed at 20 °C ± 0.1 °C and the temperature was controlled thermostatically by a circulating water system. Ultrapure Milli-Q water used as the subphase in the monolayer experiments at 20 °C ± 0.1 °C has surface tension of 72.6 mN/m and resistivity of 18.2 MΩ·cm.

2.2. Methods

Brewster angle microscopy experiments were performed with ultraBAM instrument (Accurion GmbH, Goettingen, Germany) equipped with a 50 mW laser emitting *p*-polarized light at a wavelength of 658 nm, a 10× magnification objective, polarizer, analyzer and a CCD camera. The spatial resolution of the BAM was 2 μm. The experiments were carried out with KSV 2000 Langmuir trough (KSV Instruments Ltd., Helsinki, Finland) (total area = 870 cm^2) equipped with two movable barriers. The microscope and the film balance were placed on a table (Standa Ltd, Vilnius, Lithuania) with active vibration isolation system (antivibration system VarioBasic_40, Halcyonics, Göttingen, Germany). The surface pressure was measured with the accuracy of ± 0.1 mN/m using a Wilhelmy plate made of filter paper (ashless Whatman Chr1) connected to an electrobalance. After spreading, the monolayers were left for solvent evaporation for 10 min and then the compression was initiated with the barrier speed of 5 cm^2/min (2.5 $Å^2 molec^{-1}\ min^{-1}$).

X-ray scattering experiments were performed at the BW1 (undulator) beamline at the HASYLAB synchrotron source (Hamburg, Germany) using a dedicated liquid surface diffractometer [20] with an incident X-ray wavelength λ ≈ 1.304 Å. A Teflon thermostatted Langmuir trough (Riegler & Kirstein, Potsdam, Germany), equipped with a movable barrier for monolayer compression, was placed in a gastight container and mounted on the diffractometer. After spreading the solution onto the subphase, at least 40 min was allowed for the trough container to be flushed with helium to reduce the scattering background and to minimize beam damage during X-ray scans. Then, the monolayers were compressed to the surface pressure of 32.5 mN/m (the surface pressure at which the properties of monolayer can be compared with those of bilayers in the natural membrane [21,22]), at which the X-ray experiments were performed. As far as the GIXD experiments are concerned, the X-ray scattering theory and the liquid diffractometer construction have been described previously [23–25].

3. Data analysis

To verify and compare the state of the investigated films and to obtain information on molecular ordering in monolayer from the isotherm data points the compression modulus values at a given monolayer composition were calculated according to Eq. (1) [26].

$$C_S^{-1} = -A\ (d\pi/dA)_T \tag{1}$$

where A is area per molecule at a surface pressure π. The ordering effect of cholesterol was analyzed by comparison of the variations of the compression modulus values with the addition of sterol at a given surface pressure.

The analysis of the condensing effect of cholesterol on phospholipid film was based on the values of the excess area per molecule in the mixed monolayer calculated as follows [27,28]:

$$A^{Exc} = A - A^{id} \tag{2}$$

wherein A is the mean area per lipid molecule in the mixed monolayer determined form isotherm at a given surface pressure whereas A^{id} denotes the molecular area resulting from the assumption of ideal mixing of the film components at the same π value.

The values of A^{id} for binary mixed monolayers were calculated on the basis of the equation:

$$A^{id} = X_1 A_1 + X_2 A_2 \tag{3}$$

A_1, A_2 are mean molecular areas of the respective lipids in their pure films at a given surface pressure and X_1, X_2 are the molar fractions of components 1 and 2 in the mixed film.

GIXD experiments provided the information on the lateral ordering of the samples. The scattered intensity was measured by scanning over a range of horizontal scattering vectors Q_{xy}:

$$Q_{xy} \approx \frac{4\pi}{\lambda} \sin\left(2\theta_{xy}/2\right) \tag{4}$$

where $2\theta_{xy}$ is the angle between the incident and diffracted beam projected on the liquid surface. The GIXD intensity resulting from a powder of 2D crystallites can be represented as Bragg peaks, resolved in the Q_{xy} direction, by integrating the scattered intensity over the Q_z direction, which is measured by the position-sensitive detector placed perpendicular to the air-water interface. Conversely,