Biochimica et Biophysica Acta 1818 (2012) 2860–2867

Contents lists available at SciVerse ScienceDirect

Biochimica et Biophysica Acta

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

Ganglioside GM1 forces the redistribution of cholesterol in a biomimetic membrane

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ARTICLE INFO

Article history:
Received 26 March 2012
Received in revised form 5 July 2012
Accepted 17 July 2012
Available online 22 July 2012

Keywords:
Floating membrane
GM1 ganglioside
Lipid membrane
Langmuir–Blodgett
Neutron reflectivity
Cholesterol

ABSTRACT

Neutron reflectivity has been applied to investigate different mixed asymmetric lipid systems, in the form of single "supported + floating" bilayers, made of phospholipids, cholesterol and GM1 ganglioside (Neu5Acα2-3(Galβ1-3GalNAcβ1-4)Galβ1-4Glcβ1Cer)) in bio-similar mole ratios. Bilayer preparation was carried out layer-by-layer with the Langmuir–Blodgett Langmuir–Schaefer techniques, allowing for compositional asymmetry in the system buildup. It is the first time that such a complex model membrane system is reported. Two important conclusions are drawn. First, it is experimentally shown that the presence of GM1 enforces an asymmetry in cholesterol distribution, opposite to what happens for a GM1-free membrane that, submitted to a similar procedure, results in a full symmetrization of cholesterol distribution. We underline that natural cholesterol has been used. Second, and most interesting, our results suggest that a *preferential* asymmetric distribution of GM1 and cholesterol is attained in a model membrane with biomimetic composition, revealing that a true *coupling* between the two molecular species occurs.

1. Introduction

The structural complexity of biomembranes, based on their heterogeneity in composition, dramatically involves the asymmetric disposition of different components, from lipids to proteins, in the transverse and longitudinal directions. Furthermore, inhomogeneities in the two leaflets of a membrane can couple, constituting the basis for the structural stabilization and modulation of functional domains involved in transmembrane signaling. An example is constituted by L_o domains, commonly referred to as lipid rafts [1].

They both organize in a different structure as compared to the surrounding membrane, due to their selected lipid composition, and host special proteins or receptor molecules suited for such lipid environment [2]. Among lipid rafts are those enriched in gangliosides; glycosphingolipids reside only in the outer layer of the membrane. The transmembrane asymmetry in the disposition of gangliosides has been largely invoked to provide the structural basis for the third-dimension static deformation of caveolae [3], due to their huge headgroup hindrance as compared to phospholipids. Another feature that seems to make sphingolipids unique under the perspective of membrane structure regulation is their special liaison with cholesterol [4]. Cholesterol and sphingolipids show cooperative effects on many biological processes, not limited to lipid-driven membrane organization. For example, they are thought to synergically act as regulators of the function of transmembrane receptors [5] either by direct interaction or by modulating the structure of their environment. Remarkably, it is becoming clear that their concentrations at different cellular sites are subject to a tight regulation in a very narrow range [6], compositional asymmetry being strictly mastered. On the basis of the lipid composition of isolated lipid rafts, a strong enrichment in cholesterol is expected in the inner leaflet of biological membranes, while the outer leaflet ends up highly enriched in lipids like gangliosides and phosphatidylcholines [7]. Ganglioside–cholesterol asymmetric distribution in the cytofacial/exofacial leaflets of membranes may reflect in asymmetric fluidity and molecular partition. In this view, gangliosides and cholesterol could constitute a collective-pair acting as a structural unit across the membrane.

Despite the claimed importance of asymmetry in biological membranes, the experimental study of asymmetric model membranes is rare, due to the difficulty in preparing artificial membranes with the desired defined heterogeneous composition. However some attempts have been made, for example by preparing phospholipid unilamellar vesicles (LUVs) containing small amounts of gangliosides only in their outer layer. The mechanical properties of the membrane were found to be strongly affected by the doping ganglioside, producing a softening that turns into hardening in the case of symmetric redistribution of molecules [8]. The aim of this work is the study of the structural effects brought by the presence of the monosialo GM1 ganglioside to a cholesterol-containing lipid membrane, and the eventual cholesterol–GM1 coupling. We have used an experimental model with a single macroscopic bilayer floating at 1.5–2 nm on top of a supported one, adhering to a silicon flat surface, prepared by a combination of Langmuir–Blodgett and Langmuir–Schaefer techniques. The system so built has led in the past to stable and reproducible floating bilayers [9]. This step-building technique allows the preparation of layers of different

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doi:10.1016/j.bbamem.2012.07.010

compositions. In particular we studied "supported + floating" bilayer systems composed of phospholipids and cholesterol in bio-similar mole ratios (11:2.5 mol:mol) and their structural response to the addition of the monosialo ganglioside GM1.

The wavelength of the neutron beam, of the order of the tenth of nanometer, makes them ideal tools for the structural characterization of lipid bilayers. Moreover, biological membrane components, like most soft materials, are rich in hydrogen, so that neutrons, with their unique capability of being scattered differently by hydrogen and deuterium, can be profitably used. It is possible to choose the suitable H_2O/D_2O water composition "optically" matching different portions of the sample, with the well known *contrast variation* technique. It is also possible to accentuate or annihilate the scattering from individual parts of a complex system, for example, by specific deuterium labeling.

The neutron reflectivity technique applied to the "supported + floating" bilayers could reveal structural details of this complex system to the Ångstrom scale.

2. Materials and methods

Both H-lipids and fully deuterated lipids were used. Cholesterol, in its natural molecular species, was purchased from Sigma-Aldrich Co. Fully deuterated 1,2-distearoyl-*sn*-glycero-3-phosphatidyl-choline (d_{85}-DSPC) and fully deuterated 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidyl-choline (d_{75}-DPPC) were from Avanti Polar Lipids Co. The GM1 ganglioside was extracted and purified according to Tettamanti et al. [10].

According to a well assessed standard protocol [11,12], cholesterol and phospholipids were independently dissolved in chloroform (99%) to a final concentration of 1 mg/ml. GM1 ganglioside was dissolved in the organic solvent mixture chloroform: methanol 2:1, vol:vol. Mixed lipid systems were obtained by mixing appropriate amounts of single-lipid solutions. Lipids were then deposited on the surface of a Langmuir trough (NIMA, UK), equipped with a Wilhelmy plate for pressure sensing, filled with pure water, processed in a Milli-Q system (Millipore, Bedford, MA) to a resistivity of 18 MΩ cm, and kept at T = 18 °C (±0.5). After spreading the solutions, organic solvents were left to evaporate completely for 15 min. Before deposition, monolayers were compressed to a surface pressure of 40 mN/m, similar to the lipid pressure in real systems [9], while recording the corresponding pressure–area (π–A) isotherms. In these conditions, all of the prepared monolayers were in the liquid condensed phase. They were then layer-by-layer deposited on a silicon substrate, as described in great detail in the following section. Asymmetric bilayers were realized by completely substituting the monolayer on top of the surface of the Langmuir trough with a new one with the desired composition.

A GM1 micellar sample was also prepared, by suspending the dry powder in pure Milli-Q water to a final concentration of 1 mg/ml (6.4×10^{-4} M), well above the critical micelle concentration, *cmc* (2×10^{-8} M) [13]. This solution was used for ganglioside incubation on a preformed floating membrane.

Depositions were all carried out in H_2O, while for the reflectivity measurements three contrast solutions were used, H_2O (Milli-Q system), D_2O (99% pure, provided by ILL) and Silicon Matched Water, that is, a mixture of H_2O and D_2O with the same scattering length density of Silicon (SMW i.e. 0.62:0.38 H_2O:D_2O volume fractions). When necessary, the contrast solution (2 ml) was changed directly in the measuring cell, by slowly flushing with the new solvent by means of a slow pumping system (20 μl/s) for 20 min. The low *cmc* value of all of the used lipids guarantees the stability of the system against single-monomer loss during the solvent-exchange procedure.

Substrates were single crystals of silicon ($5\times5\times1.5$ cm^3) polished on one large face (111). They were cleaned before use in subsequent baths of chloroform, acetone, ethanol, and water and treated with UV-ozone for 30 min [14].

2.1. Neutron reflectivity

Reflectivity measurements were performed on the D17 [15] reflectometer at ILL, Grenoble, France (TOF mode, variable resolution = 1–10%, λ range between 2 and 20 Å, with two incoming angles of 0.7 and 4°). The cell was oriented vertically and kept in position while changing solvents and temperature. Measurements were performed at the silicon–water interface, the beam coming from the silicon block side.

In a neutron reflectivity experiment, the ratio between the intensities of the reflected and incoming beams, R, is measured as a function of q, the momentum transfer perpendicular to the interface [16].

Reflectivity is related to the scattering length density across the interface by the approximate relation:

$$R(q_z)\approx\frac{\left(16\pi^2\right)}{q_z^2}|\rho(q_z)|^2$$

valid in the Born approximation [17]. $\rho(q_z)$ is the Fourier transform of the scattering length density profile $\rho(z)$ along the normal to the interface, giving information about the composition of each layer and about its structure. The scattering length density is given by:

$$\rho(z)=\sum_j b_j n_j=\sum_j b_j n_j$$

where n_j is the number of nuclei per unit volume and b_j is the scattering length of nucleus j.

The method of analysis often used for specular reflection data involves the construction of a model of the interface that may be represented by a series of parallel layers of homogeneous material. Each layer is characterized by an average scattering length density, weighted on all of its non-water components, and a thickness. These parameters are used to calculate a model reflectivity profile by means of the optical matrix method [17]. The interfacial roughness between any two consecutive layers may also be included in the model by the Abeles method. The calculated profile is compared to the measured profile and the quality of the fit is assessed by using χ^2 in the least-squares method.

Data were analyzed using the software Motofit [18], allowing simultaneous fit of data sets referred to the same sample in different contrast conditions, using the SLDs reported in Table 1. Measurements on a bare silicon substrate were performed, in different solvents (H_2O, D_2O, SMW), to measure the characteristics of the silicon oxide layer formed at the silicon surface.

3. Biomimetic sample preparation: floating bilayer build-up

For all of the samples, the bilayer adhering to the silicon support was made of the long chain phospholipid d_{85}-DSPC, being in gel phase all over the investigated temperature range, from 22 °C to 49.5 °C [23,24]. This guarantees the compactness and stability of the supporting bilayer. For the floating bilayer, d_{75}-DPPC was used as lipid matrix, DPPC being a common lipid in membrane domains enriched in cholesterol and sphingolipids [25]. A deuterated matrix was chosen in order to enhance the visibility of the H-species embedded cholesterol and GM1.

Double bilayer depositions were done in water, coupling the Langmuir–Blodgett [26] and Langmuir–Schaefer Techniques [27], as follows. Initially, the silicon substrate was immersed in water at 18 °C in the Langmuir trough. A d_{85}-DSPC solution, was spread on the water surface and progressively compressed to 40 mN/m, optimal for saturated long chain phosphocholines [9]. The silicon substrate was then slowly withdrawn for the entire length, to form the first adsorbed layer on the surface, and subsequently dipped (speed of withdrawing and dipping, 5 mm/min) across the monolayer, while keeping the pressure constant. This way, two facing monolayers were adsorbed onto the silicon surface,