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Ganglioside GM1 forces the redistribution of cholesterol in a biomimetic membrane

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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 (Neu5Ac α 2-3(Gal β 1-3GalNAc β 1-4)Gal β 1-4Glc β 1Cer)) 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.

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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_0 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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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 H₂O/D₂O 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 Ångström 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 (d₈₅-DSPC) and fully deuterated 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidyl-choline (d₇₅-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 H₂O, while for the reflectivity measurements three contrast solutions were used, H₂O (Milli-Q system), D₂O (99% pure, provided by ILL) and Silicon Matched Water, that is, a mixture of H₂O and D₂O with the same scattering length density of Silicon (SMW i.e. 0.62:0.38 H₂O:D₂O 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 × 5 × 1.5 cm³) 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:

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

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 (H₂O, D₂O, 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 d₈₅-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, d₇₅-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 d₈₅-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,

Table 1
Theoretical values of SLDs for the compounds used.

Material	SLD (10^{-6} \AA^{-2}) ^a
Si	2.07
SiO ₂	3.41
H ₂ O	−0.56
D ₂ O	6.36
Cholesterol C ₂₇ H ₄₆ O	0.22
GM1 chains C ₃₈ H ₇₈ (gel phase)	−0.41
GM1 chains (fluid phase)	−0.33
GM1 head C ₄₁ H ₆₅ N ₃ O ₃₁	2.22
Phospholipid D-head C ₈ D ₉ H ₅ O ₈ PN	4.87
Phospholipid D-chains 2[(CD ₂) _{n−1} (CD ₃)] (gel phase)	7.66
Phospholipid D-chains (fluid phase)	7.15

^a SLD values have been theoretically calculated from [19], [20], [21] and [22].

constituting the adhering (“supported”) bilayer. Then, the water surface was thoroughly cleaned with an aspiration pipette, the Wilhelmy balance was appropriately set to 0 mN/m, and a different solution was spread, with the composition desired for the inner side of the floating membrane. The third layer was then deposited by rising the block again while keeping the surface pressure at 40 mN/m, according to the same procedure. To prepare asymmetric floating bilayers, the surface of the trough was cleaned again and a solution with the composition desired for the outer side of the floating membrane was spread and compressed to 40 mN/m. The fourth closing monolayer was adsorbed onto the others by rotating the substrate by 90°, in the Langmuir–Schaefer configuration, and lowering it carefully onto the surface, strictly parallel to it. The block was then closed in a Teflon holder and fixed into an aluminum thermostated cage. The Teflon holder is provided with filling ports to allow for solvent substitution or addition of solutes directly in the one-millimeter-thick bulk water in contact with the deposited layers.

We underline that the 4-layer deposition procedure is long and laborious, taking itself about 3 h. During this time, the in-progress sample is alternatively dipped twice into water or exposed to air for long times, which is necessary for monolayer removal and replacement, although in a protected and controlled environment. Nonetheless, the final system is impressively well done and stable, apart from sporadic events.

4. Results and discussion

The aim of this work is the study of the structural effects brought by the presence of GM1 ganglioside to a cholesterol-containing lipid membrane, and the eventual cholesterol–GM1 coupling. In fact, it is often claimed that GM1 and cholesterol constitute a collective-pair affecting the structural properties of their environment in membrane microdomains [4].

In biological membrane microdomains, the three components phospholipid:sphingolipid:cholesterol are found approximatively in a 10:1: 2.5 molar ratio [28]. Besides sphingolipids, exclusively residing in the outer leaflet, a cross asymmetry is claimed also for cholesterol. A good approximation to simulate the composition of a biomembrane microdomain considers 70% of the total cholesterol in the inner (cytoplasmic) layer of the membrane, and 30% in the outer [25].

In our floating membrane model, the third and fourth layers of the deposition are meant to represent the inner and outer leaflets of the membrane, respectively.

The experimental design was drawn on the following samples:

- a1) a floating membrane, composed of fully deuterated d₇₅-DPPC and cholesterol in the mole ratios expected to be found in real membranes (see Fig 2, Sample A), was prepared and examined;

- a2) the same membrane was incubated with GM1, administered as a micellar solution added to the overhanging solvent in an appropriate amount and allowed to interact with the membrane for many hours (20 h), and then examined;
- b) another floating membrane was prepared, depositing all of the cholesterol in the “internal” layer and all of the GM1 directly in the “external” layer during the Langmuir–Blodgett Langmuir–Schaefer procedure (see Fig. 2, Sample B), and examined.

We underline that Sample A and Sample B are both prepared from the beginning, starting from the cleaned silicon block. As already pointed out, we applied selective deuteration to enhance or minimize the visibility of different components of the membrane under study. In particular, deuterated d₇₅-DPPC was used, in order to highlight cholesterol and GM1 with respect to the phospholipid matrix. All of the membranes were, at some point, observed in three solvents, H₂O, D₂O and SMW.

4.1. Sample A

As a first step, a floating asymmetric bilayer composed of fully deuterated d₇₅-DPPC and cholesterol (see Fig. 2) was examined. It was placed under the neutron beam as it came from the deposition procedure, without any annealing. It was thermostated at 22 °C, and four subsequent reflectivity measurements were taken, covering 7 h from preparation. The four spectra overlap, showing that the system is stable in these conditions and over these times. Then, it was subjected to a temperature increase of 49.5 °C (the procedure currently referred to by “annealing”) and then back to 22 °C. Reflectivity was measured both at 49.5 °C and at 22 °C after annealing.

Experiments at 22 °C, after annealing, were performed in the three contrast solutions (H₂O, D₂O and SMW) and constituted a starting point in analyzing the data. The “supported + floating” system has shown to be stable against solvent exchange, consisting of a progressive substitution under continuous flow. This confirms the possibility to use a biologically relevant solution in a true biomimetic perspective.

Fits of the experimental data were obtained with Motofit, using an 11-layer model to account for the silicon oxide, a water layer, the first bilayer (composed of 4 layers: heads, chains, chains, heads), a sandwiched water layer and the floating bilayer (composed of 4 layers: heads, chains, chains, heads). Where applicable, we performed a simultaneous fit of the three-solvents-systems, gaining a better resolution for the complex structure. Errors are estimated from the maximum variation in the acceptable fit subject to the constraints of space filling and stoichiometry. Parameters for the oxide layer were fixed by previous measurements of the bare substrate. Previous results on single adsorbed bilayers made of long chain saturated phosphocholines and the good reproducibility guaranteed by this protocol with these molecules, allowed to put strong constraints on the fitting parameters relative to the adsorbed d₈₅-DSPC bilayer. The simultaneous fit of three solvent–water contrasts (H₂O, D₂O and SMW) for each system, allowed the reduction of the number of possible models fitting the data. Finally, the adoption of physically meaningful values, space filling and stoichiometry rules allowed the selection of a unique set of parameters that best describe the reflectivity profiles (Table 2).

As we recently found and reported [29], the annealing procedure on the DPPC-cholesterol sample results in the symmetric redistribution of cholesterol in the floating bilayer, despite the asymmetry imposed during deposition. After annealing, 50% of the total amount is found in the third layer chains, 50% in the fourth [29].

4.2. Ganglioside incubation on Sample A

Once annealed, the sample was heated again at 49.5°, in order to facilitate GM1 insertion into the membrane, namely into the (fourth)

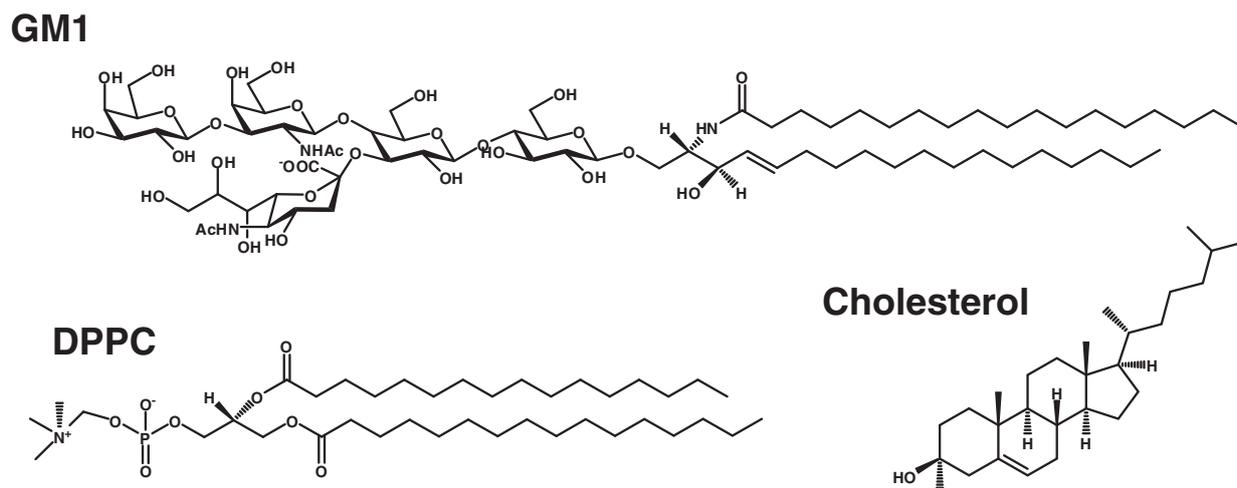


Fig. 1. From top, in clockwise direction: GM1 ganglioside, cholesterol and DPPC molecular structures.

d75-DPPC:cholesterol layer. 20 μ l of GM1 solution was injected into the cell through the port, without moving it from the beamline. The expected final mole ratio was d75-DPPC:GM1 = 10:1, largely within the limits of GM1 absorption by DPPC membranes [30]. Reflectivity was measured before ganglioside addition and 20 h after the incubation of GM1.

In Fig. 3 we show the reflectivity spectra *before* and *after* GM1 insertion.

The *supported* bilayer is stable against GM1 addition, as expected and as shown in Table 2, reporting the corresponding *before* and *after* parameters. An overall increase in its roughness is the sole appreciable variation, presumably due to the fact that the sample has been kept at 49.5 $^{\circ}$ C for 24 h.

In Fig. 4 the contrast profiles of the *floating* bilayer are reported, before and after GM1 incubation. They have to be reconstructed with a reasonable distribution of different components, including solvent, under general constraints preserving physical consistency. Comparison of the fitting results shows that an overall thickening of the bilayer occurs following incubation. The thickening is almost entirely paid by the external layer, both in its hydrophobic and hydrophilic regions, as reasonably expected due to the insertion of longer GM1 amphiphiles (see Fig. 1). Reasonably, also the roughness increases, and the contrast of the 4th layer changes, consistently with the insertion of length-mismatched and H-rich GM1.

Rather, the intriguing effect is that the contrast of the third layer is lowered in its hydrophobic part, indicating an increase in its hydrogen

content. This feature has to be reconstructed by a reasonable model for the redistribution of components. In our experiment, hydrogen comes either from water, GM1 or cholesterol (see Table 1). Below, we discuss two extreme but reasonable scenarios, which could result in such an experimental observation.

4.2.1. Extreme Model 1

The less speculative situation we could imagine is that, as GM1 enters the external layer of the floating membrane, some water penetration occurs in the inner (third) layer (partial layer leakage) to compensate for the unbalanced volume increase that would otherwise provoke an outward curvature of the bilayer.

In this model, no cholesterol migration takes place, and there is no water penetration in the fourth layer. The contrast profile is reconstructed by assuming that 15 vol.% of water penetrates the inner layer of the membrane as 17 vol.% of GM1 enters the external layer of the floating membrane (see Table 3).

4.2.2. Extreme Model 2

An alternative extreme situation considers that the unbalanced volume is recovered by cholesterol migration and pushed down to the inner layer by GM1 insertion. Cholesterol is assumed to be moved, rather than d75-DPPC, because of its small polar group. Assuming full cholesterol migration to the inner leaflet of the membrane, some water penetration (+5%) has still to be allowed in the same layer in order to recover the best fit contrast values. We

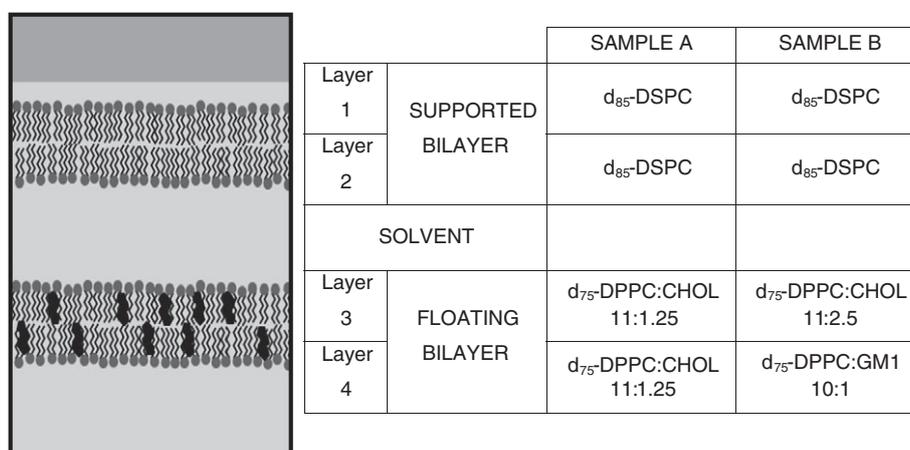


Fig. 2. Scheme of sample construction. Mole ratios given correspond to the initial values as prepared. The pictorial sketch on the left refers to preparation of Sample A.

Table 2
Physical parameters of the supported bilayer as calculated from the spectra relative to Sample A, before and after GM1 incubation. The corresponding values for the supported bilayer of Sample B are the same within the experimental errors. Water content takes into account headgroup hydration and incomplete coverage. We underline that in the single-component supported membrane $\rho_{lip}(z)$ is equivalent to the SLD of DSPC.

Layer	Supported d85-DSPC bilayer – Sample A – 49.5 °C before incubation of GM1				Supported d85-DSPC bilayer – Sample A – 49.5 °C after incubation of GM1			
	T (Å)	$\rho(z)$ ($\times 10^{-6} \text{ \AA}^{-2}$)	W (vol.%)	r (Å)	T (Å)	$\rho(z)$ ($\times 10^{-6} \text{ \AA}^{-2}$)	W (vol.%)	r (Å)
SiO2	13	3.41	5	2	13	3.41	5	2
Heads 1	9	4.87	22	6	4.87	16	2	2
Chains 1	22	7.66	19	2	23	7.66	11	2
Chains 2	19	7.66	17	6	7.66	20	2	2
Heads 2	7	4.87	19	6	6	4.87	30	6
Water	21	–	100	6	3	–	100	3

T: layer thickness ($\pm 1 \text{ \AA}$).

$\rho_{lip}(z)$: average scattering length density of the lipid components of the layer ($\pm 0.05 \times 10^{-6} \text{ \AA}^{-2}$).

W: solvent content of the layer ($\pm 5\%$ in volume).

r: roughness between the layer and the previous one ($\pm 2 \text{ \AA}$).

also assumed that the same amount of water penetrated the fourth layer, together with the strongly hydrated GM1 that enters the outer layer in 9.3 vol.% amount (see Table 4).

4.2.3. Biomimetic model

Our suggestion is that the real situation is neither of the two, but the one that finally better biomimicks the cross distribution of ganglioside and cholesterol in membrane microdomains, which intermediate between the two extreme cases. GM1 enters the external layer; the solvent penetration increases all over the floating membrane and some cholesterol migrates from the outer to the inner layer of the membrane, heading for the asymmetry of real membrane microdomains. Cholesterol migration is then forced to the inner layer by collective coupling with GM1, resulting in a preferential asymmetric disposition of the two components in the membrane (see Table 5).

As the membrane keeps its flatness, the redistribution of components is accordingly balanced both in volume and in contrast.

By assuming 0.9/10 mole fraction of GM1/lipid entering the external layer (consistent with the added amount, the biosimilar), in the presence of 2.5/11 mole fraction of cholesterol/lipid, and 1000 \AA^3 being the hydrophobic volume of GM1 [31], we evaluate that for each $0.9 \times 1000 = 900 \text{ \AA}^3$ of added chains, half of the same volume (450 \AA^3) of cholesterol has to be pushed down in order to reach a balance. Being $\sim 630 \text{ \AA}^3$ its molecular volume [32], this means that

0.7/11 mole fraction redistribution of cholesterol occurs. In this hypothesis, the final mole fraction distribution of cholesterol is 1.95/11 (the 78% of total cholesterol content) in the inner layer of the membrane, and 0.55/11 (the 22% of total cholesterol content) in the outer layer of the floating membrane, where GM1 is placed.

The same analysis has been done after bringing the sample back to 22 °C in the gel phase of the lipid chains and fit results, obtained following the biomimetic model interpretation, are reported in the left panel of Table 6.

4.3. Sample B

To test for the soundness of our hypothesis, that is, that a true coupling between GM1 and cholesterol occurs, we prepared Sample B, where GM1 is included in the deposition protocol, and with a complete nominal asymmetry. In fact, over the DSPC supported bilayer, a floating bilayer is deposited, the overall composition of which is the biosimilar, as before (DPPC:GM1:cholesterol = 10:1:2.5), but including all of the cholesterol and all of the GM1 in opposing monolayers, the third and the fourth, respectively. This floating bilayer displays, then, two important features in view of data analysis and interpretation of the results. On one hand the ganglioside content of the external (fourth) layer is well known, as it is fixed at deposition. This

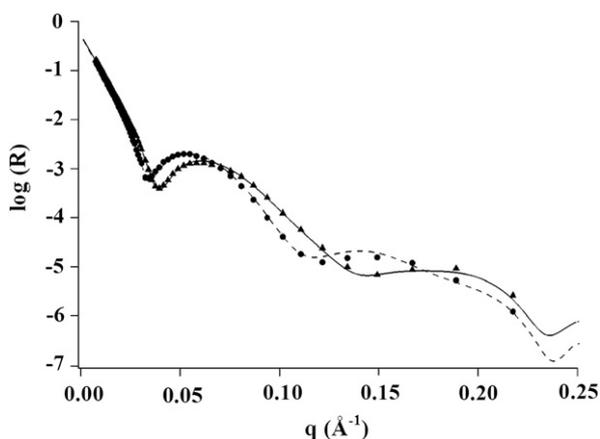


Fig. 3. Modification of the neutron reflectivity spectrum induced by the incubation of GM1 (before: triangles, after: circles) in a d75-DPPC + cholesterol bilayer. Straight and dashed lines represent the curves obtained from the parameters used to fit the data (see Tables 2 and 3). The solvent is H₂O and T = 49.5 °C. Error bars are smaller than graphical symbols.

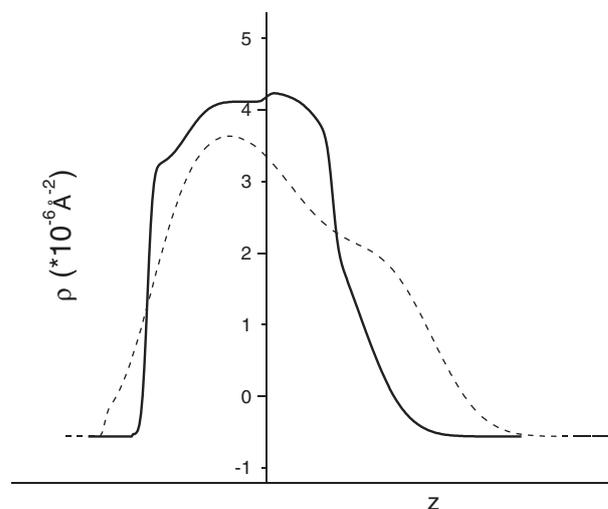


Fig. 4. Contrast profiles, $\rho(z)$, of the floating bilayer of Sample A at 49.5 °C in H₂O. Full line: before GM1 incubation. Dashed line: after GM1 incubation. In the figure the vertical axis is placed roughly at the center of the floating bilayer, to guide the eye. The contrast profile account for lipid components, included solvent and roughness.

Table 3

Physical parameters of the *floating bilayer* of Sample A before and after GM1 incubation, as calculated from the contrast profiles of Fig. 4, following the *Extreme Model 1* assumptions. Water content takes into account headgroup hydration and incomplete coverage. We underline that in the mixed floating membrane $\rho_{lip}(z)$ refers to the weighted average of non-aqueous components.

Layer	Floating mixed bilayer – Sample A – 49.5 °C starting point				Floating mixed bilayer – Sample A – 49.5 °C after incubation of GM1 – <i>Extreme Model 1</i>			
	T (Å)	$\rho_{lip}(z)$ ($\times 10^{-6} \text{ \AA}^{-2}$)	W (vol.%)	r (Å)	T (Å)	$\rho_{lip}(z)$ ($\times 10^{-6} \text{ \AA}^{-2}$)	W (vol.%)	r (Å)
Heads 3	9	4.87	40	2	9	4.87	50	7
Chains 3	18	6.64	35	4	23	6.64	50	10
Chains 4	15	6.64	33	2	17	2.53	33	10
Heads 4	6	4.87	39	2	14	3.39	39	10

T: layer thickness ($\pm 1 \text{ \AA}$).

$\rho_{lip}(z)$: average scattering length density of the lipid components of the layer ($\pm 0.05 \times 10^{-6} \text{ \AA}^{-2}$).

W: layer solvent content ($\pm 5\%$ in volume).

r: roughness between the layer and the previous one ($\pm 2 \text{ \AA}$).

Table 4

Physical parameters of the *floating bilayer* of Sample A before and after GM1 incubation, as calculated from the contrast profiles of Fig. 4, following the *Extreme Model 2* assumptions. Water content takes into account headgroup hydration and incomplete coverage. We underline that in the mixed floating membrane $\rho_{lip}(z)$ refers to the weighted average of non-aqueous components.

Layer	Sample A 49.5 °C starting point				Floating mixed bilayer – Sample A – 49.5 °C after incubation of GM1 – <i>Extreme Model 2</i>			
	T (Å)	$\rho_{lip}(z)$ ($\times 10^{-6} \text{ \AA}^{-2}$)	W (vol.%)	r (Å)	T (Å)	$\rho_{lip}(z)$ ($\times 10^{-6} \text{ \AA}^{-2}$)	W (vol.%)	r (Å)
Heads 3	9	4.87	40	2	9	4.87	45	7
Chains 3	18	6.64	35	4	23	6.21	38	10
Chains 4	15	6.64	33	2	17	3.60	38	10
Heads 4	6	4.87	39	2	14	3.77	50	10

T: layer thickness ($\pm 1 \text{ \AA}$).

$\rho_{lip}(z)$: average scattering length density of the lipid components of the layer ($\pm 0.05 \times 10^{-6} \text{ \AA}^{-2}$).

W: layer solvent content ($\pm 5\%$ in volume).

r: roughness between the layer and the previous one ($\pm 2 \text{ \AA}$).

Table 5

Physical parameters of the *floating bilayer* of Sample A before and after GM1 incubation, as calculated from the contrast profiles of Fig. 4, following the *biomimetic model* assumptions. Water content takes into account headgroup hydration and incomplete coverage. We underline that in the mixed floating membrane $\rho_{lip}(z)$ refers to the weighted average of non-aqueous components.

Layer	Sample A 49.5 °C starting point				Floating mixed bilayer – Sample A – 49.5 °C after incubation of GM1 – <i>biomimetic model</i>			
	T (Å)	$\rho_{lip}(z)$ ($\times 10^{-6} \text{ \AA}^{-2}$)	W (vol.%)	r (Å)	T (Å)	$\rho_{lip}(z)$ ($\times 10^{-6} \text{ \AA}^{-2}$)	W (vol.%)	r (Å)
Heads 3	9	4.87	40	2	9	4.87	41	7
Chains 3	18	6.64	35	4	23	6.39	35	10
Chains 4	15	6.64	33	2	17	2.95	35	10
Heads 4	6	4.87	39	2	14	4.67	45	10

T: layer thickness ($\pm 1 \text{ \AA}$).

$\rho_{lip}(z)$: average scattering length density of the lipid components of the layer ($\pm 0.05 \times 10^{-6} \text{ \AA}^{-2}$).

W: layer solvent content ($\pm 5\%$ in volume).

r: roughness between the layer and the previous one ($\pm 2 \text{ \AA}$).

Table 6

Physical parameters of the floating bilayer of Sample B (right side) as compared to those of Sample A after GM1 incubation, with the *biomimetic model* hypothesis (left side). Water content takes into account headgroup hydration and incomplete coverage. We underline that in the mixed floating membrane $\rho_{lip}(z)$ refers to the weighted average of non-aqueous components.

Layer	Floating mixed bilayer – Sample A – after annealing and GM1 incubation – <i>biomimetic model</i> – T = 22 °C				Floating mixed bilayer – Sample B – after annealing – T = 22 °C			
	T (Å)	$\rho_{lip}(z)$ ($\times 10^{-6} \text{ \AA}^{-2}$)	W (vol.%)	r (Å)	T (Å)	$\rho_{lip}(z)$ ($\times 10^{-6} \text{ \AA}^{-2}$)	W (vol.%)	r (Å)
Water	21	–	100	8	22	–	100	4
Heads 3	9	5.70	24	3	9	5.70	15	8
Chains 3	23	6.65	22	8	21	6.70	14	7
Chains 4	19	7.10	18	2	17	7.05	7	2
Heads 4	15	5.30	21	2	16	5.30	16	2

T: layer thickness ($\pm 1 \text{ \AA}$).

$\rho_{lip}(z)$: average scattering length density of the lipid components of the layer ($\pm 0.05 \times 10^{-6} \text{ \AA}^{-2}$).

W: layer solvent content ($\pm 5\%$ in volume).

r: roughness between the layer and the previous one ($\pm 2 \text{ \AA}$).

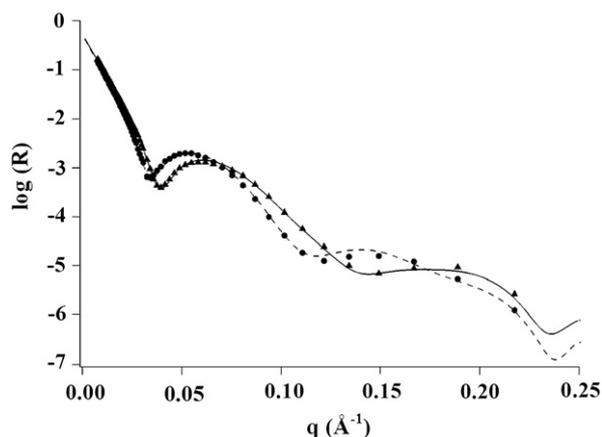


Fig. 5. Modification of the neutron reflectivity spectrum induced by annealing Sample B (before: triangles, after: circles). Straight and dashed lines represent the curves obtained from the parameters used to fit the data (see Table 6). The aqueous phase is H₂O and T = 22 °C. Error bars are smaller than graphical symbols except for highest q, as shown.

reduces the number of free parameters in component distribution assessment. On the other hand, the initial complete asymmetry of cholesterol distribution (all in the internal, third layer) gives maximum quantitative visibility to its eventual migration. Sample B was placed under the neutron beam, thermostated at 22 °C, then heated to above the chain melting temperature and then back to 22 °C. The reflectivity profile was determined at each of the mentioned steps and data were fitted by using Motofit. The supported bilayer is stable, as well as the thickness (~22 Å) of the water layer between the supported and the floating bilayers.

In Fig. 5 we show the reflectivity spectra at 22 °C before and after annealing of Sample B.

In Fig. 6 and Table 6, the contrast profiles and the structural parameters of the floating bilayers at 22 °C are shown, compared to the 'biomimetic case' of the previous analysis.

The contrast profiles of Fig. 6 are astonishingly similar, despite the different preparation procedures of the two samples, for what

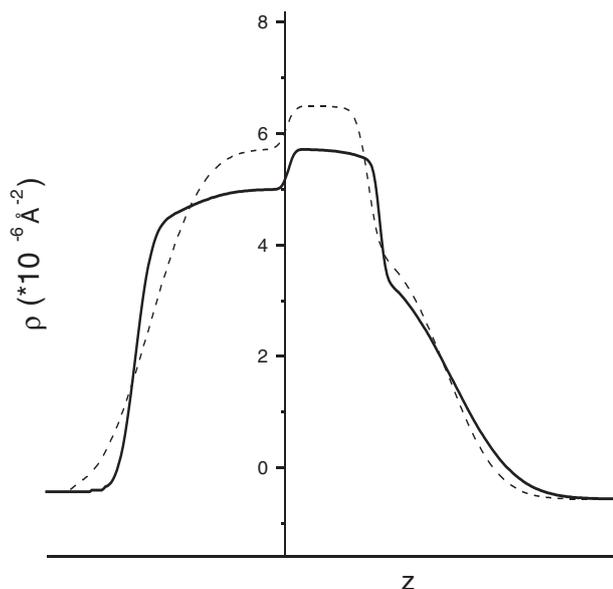


Fig. 6. Contrast profiles of the floating bilayers of the two samples at T = 22 °C in H₂O. Straight line: Sample A, incubated; dashed line: Sample B (see text). In the figure the vertical axis is placed roughly at the center of the floating bilayer, to guide the eye. The contrast profile account for lipid components, included solvent and roughness.

concerns the presumed distribution of components. Reversely, we recall that the overall composition of the two samples is identical. The overall slight area difference under the contrast profile curve can be recovered by solvent penetration. The solvent content, higher in the case of the sample where GM1 has been incubated, is very likely to depend on the different treatments the two samples were submitted to. In both cases, GM1 is prevented from flip-flopping to the third layer because of steric hindrance/amphiphilicity reasons (see Fig. 1). We underline that, in Sample A, GM1 was not in the membrane from its formation, so it did not undergo compression during the Langmuir–Blodgett–Langmuir–Schaefer membrane deposition, but inserted later, disturbing an existing equilibrium. In the second case, GM1 underwent compression together with d₇₅-DPPC, being allowed to eventually assume specific lateral distribution.

Table 6 reports the output of the fit, compared to the 'biomimetic case' of the previous analysis. The first two columns of each section (in bold) reveal the close similarity of the structure of the two floating bilayers, for what concerns thicknesses and lipid composition.

Finally, also in the floating bilayer of Sample B, cholesterol redistributes between layers: a certain amount migrates from its original place, the third layer, to the opposing layer of the membrane, where GM1 is hosted. The redistribution is allowed and completed, at least in short times, during annealing. Moreover, the amount of migrated cholesterol is "exactly" the same that is kept by GM1 in "its monolayer" while inserting in Sample A, as shown in Fig. 7. In fact, in both cases, roughly 80% of the total amount of cholesterol (84% in Sample A after GM1 incubation, and 81% in Sample B) is in the third, inner, layer of the membrane, and roughly 20% (16% and 19%, respectively) is in the outer, together with GM1. This clearly proves that cholesterol redistribution does not result from a trivial volume re-balance, but obeys coupling ratios with GM1, consistent with the biomimetic lipid composition.

5. Conclusions

We prepared complex mixed asymmetric membranes, containing phospholipid, ganglioside and cholesterol in biosimilar proportion, by the Langmuir–Blodgett–Langmuir–Schaefer techniques, and we characterized their structure by neutron reflectivity.

Three important conclusions are drawn:

- i) it is experimentally possible to build complex bilayer systems that mimic the lipid composition of biomembranes. These models are stable once components redistribute in the fluid phase. External stimuli can be applied, like solvent change.
- ii) it is experimentally shown that the presence of GM1 forces asymmetry in cholesterol distribution. This is clearly opposite to what happens to a similar bilayer where an asymmetric (70:30) distribution of cholesterol is realized during the deposition of a GM1-free membrane (with the same mole ratio lipid:cholesterol = 11:2.5) that is then submitted to a similar procedure of annealing. As shown in the left panel of Fig. 7, in that case, annealing results in a full symmetrization of cholesterol distribution, as previously found [28].
- iii) most interestingly, it is suggested from experimental data that a preferential asymmetric distribution of GM1 and cholesterol is realized in a model membrane with biomimetic composition, as shown in the central and right panels of Fig. 7, as for a true coupling between the two molecular species.

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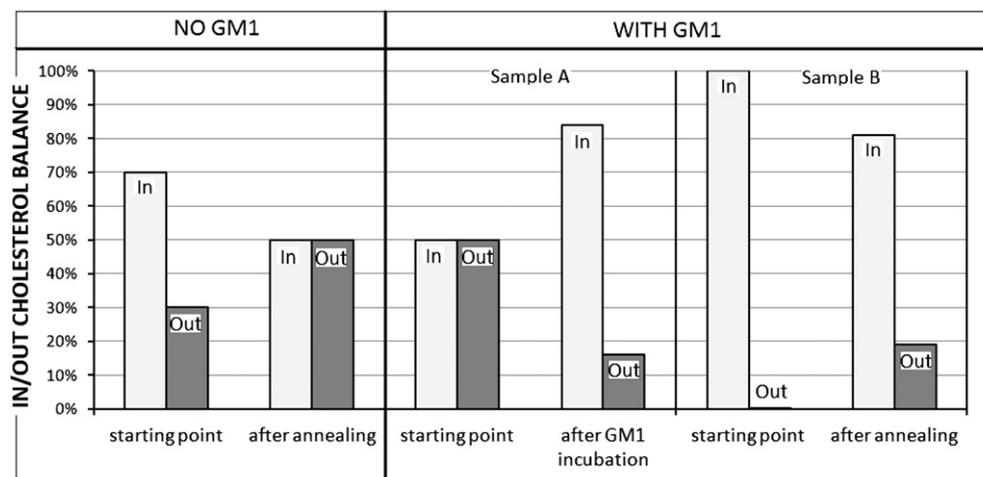


Fig. 7. Cholesterol disposition in the inner (light gray) and outer (dark gray) leaflets of the floating membrane. Left panel: without GM1, casted asymmetric disposition of cholesterol is destroyed by annealing [29]. Central and right panels: GM1 forces redistribution of cholesterol in an optimal coupling ratio. Sample A: GM1 is inserted a posteriori into a pre-casted membrane where cholesterol is symmetrically distributed; Sample B: GM1 participates from deposition to the outer layer of a membrane where cholesterol is initially hosted only in the inner layer.

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