Building a biomimetic membrane for neutron reflectivity investigation: complexity, asymmetry and contrast.

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Abstract

The preparation and investigation of model membranes is deserving growing interest both for the physics of complex systems, and for biology. The need of simplified models should preserve mimicking the qualifying characteristics of biological membranes, and keep non-invasive and detailed description. As a main feature, biological membranes are non-homogeneous in the disposition of components, both in the lateral and in the transverse direction. We prepared asymmetric supported membranes containing GM1 ganglioside in biomimetic proportion according to different protocols. Then, we studied their internal structure by neutron reflectivity, providing few-Angstrom sensitivity in the cross direction meanwhile avoiding radiation damage. This technique can also be profitably applied to study interactions at the membrane surface. The best protocol has proven to be the Langmuir-Blodgett/Langmuir-Schaefer. Notably, also the simpler and most accessible protocol of vesicle fusion was found to be suitable for straightforward and good quality deposition of compositionally asymmetric membranes.

Introduction

The realization and structural characterization of model membrane systems is deserving growing interest both for the physics of auto-aggregating complex systems and for membrane biology. However, because of membranes complexity, the research in this field is far from trivial. Biological membranes are in fact composed by thousands of different components, whose disposition is inhomogeneous both in the lateral and in the transverse direction [1,2]. The need of dealing with simplified models should match the opportunity to mimic the qualifying characteristics of biological membranes, in different respects. As an typical example, the so-called Glycosphingolipid Enriched Microdomains (GEMs) [3] are membrane domains with prominent structural and functional roles. Besides the distinctive lipidic composition, a qualifying aspect for GEMs biomimesis is compositional asymmetry. In fact, glycolipids reside only in the outer leaflet of the cell [4,5]. Besides model construction, detailed and resolved structural observation is then required. In this respect, the use of neutron spectroscopy offers the unique advantage of visibility modulation, via the isotopic H-D substitution, with no significant impact on the physico-chemistry of the membrane, meanwhile avoiding radiation damage. These features have been largely exploited, by using neutron scattering and diffraction in the study of structural and dynamic properties of colloidal systems, including biocolloids [6,7,8]. Within this favourable frame, the neutron reflectometry technique has been implemented to the membrane biology edge and now it is increasingly employed to access the local structural properties of biomimetic single membranes [9-13] and in particular to achieve information about phospholipid membranes asymmetry [14,15].

In this context, we studied the structural characteristics of model supported membranes containing GM1 ganglioside, prepared according to different protocols. In fact, being GM1 a very important component of functional membrane domains, the deposition of model membranes bearing GM1 asymmetry and suitable for structural and morphological investigation, is of great interest. The features of bicomponent model systems with asymmetric GM1 distribution have been studied by means of molecular dynamics simulations [16]. On the other hand, building up experimental models with controlled asymmetric disposition of components is not trivial. The laborious layer by layer Langmuir Blodgett/Langmuir-Schaefer [17,18] deposition technique ensures ganglioside asymmetry, while the easier and most commonly used vesicle-fusion technique was never shown, although hypothesized [19], to allow for asymmetric membranes.

The study of the lateral pressure of mixed d_{75}DPPC-GM1 as a function of the area-per-molecule (π-A Langmuir isotherms) evidences that the mutual interactions between lipids of different species reflect in the surface arrangement of molecules, known as 'umbrella effect'. Supported membranes allow the investigation of membrane transverse structure by neutron reflection. We built supported single membranes (50 Å thickness) with macroscopic lateral extension (25 cm^2 area) by either layer-by-layer Langmuir-Blodgett/Langmuir-Schaefer deposition or vesicle fusion. The macroscopic extension of the single membrane allows for significant statistics, while keeping high detail in the description of the cross section. Neutron spectroscopy takes also advantage from the isotopic H-to-D substitution to modulate the visibility of different components admixed in the membrane. We verified that either protocols are suitable for a good deposition of mixed systems, concerning in particular the macroscopic membrane integrity.

Materials and methods

d_{62}-DPPC, d_{75}-DPPC and d_{83}-DSPC were from Avanti Polar Lipids Co.. GM1 ganglioside was extracted and purified according to [20]. D_2O ($\geq$ 99% purity) was purchased by ILL.

Different membranes were been prepared by different protocols.

Langmuir films for isotherms and layer-by-layer membranes build-up

Phospholipids were dissolved in chloroform to a final concentration of 1mg/ml, GM1 ganglioside was dissolved in chloroform:methanol (2:1 vol:vol) to the final 1mg/ml concentration. Mixed systems were obtained by mixing appropriate amounts of the different organic solvent lipid solutions. 60 µl of the desired lipid solution were then deposited on the 450 cm^2 surface of a Langmuir trough filled with water kept at T = 22°C. Monolayers were compressed up to collapse ($\sim$ 60 mN/m), while recording the corresponding (π-A) isotherms. For membrane deposition, layers were collected from the surface at 40 mN/m. All of the used monolayers are in the solid phase in these conditions.

Lipid vesicles and micelles

Unilamellar vesicles (roughly 100 nm diameter) were obtained by the following procedure: lipid powders (d_{75}-DPPC or d_{62}-DPPC:GM1 10:1 mol) were weighted in glass ball-shaped containers, dissolved the appropriate organic solvent, then evaporated under continuous rotation so that lipid films were deposited over the balloons surface. Chloroform evaporation was completed under vacuum for 30 minutes. Then the films were submitted to a gentle stream of humidified nitrogen for 30 minutes, to disentangle multilayer compact stacks. Finally, 150 mM NaCl water solution was added, to the final concentration of 0.5 mg/ml. The d_{62}-DPPC:GM1 10:1 mol system spontaneously forms unilamellar vesicles, whereas the d_{75}-DPPC multilamellar system was extruded through twinned polycarbonate filters (800 Å porosity) with a manual extrusor (LiposoFast, Avestin Inc.). Samples were then stored at 45°C, above the chain gel-to-fluid transition, to ensure vesicle stability.

GM1 micelles were prepared by dissolving 1 mg of GM1 powder in 1ml of pure water (MilliQ).

Membranes deposition

Solid supports were single crystals of silicon (5 x 5 x 1 cm^3) polished on one large face (111), cleaned before use with chloroform, acetone, ethanol and pure water in the sequence, and then treated with plasma cleaner. Supported membranes A and B were obtained by vesicle fusion, widely used for the deposition of membranes of selected phospholipids, applicable to neutron reflectivity measurements [11]. Vesicle solutions were incubated in the measuring cell during 40 minutes at 45°C (d$_{75}$-DPPC) or 50°C (d$_{62}$-DPPC:GM1 10:1). After 40 minutes the cell was thoroughly rinsed with deionized water to flush the excess vesicles and NaCl. Membrane A was then incubated with GM1, by injecting 25 μl of the GM1 micellar solution in the measuring cell. After 12 hours at T=55°C, the cell was flushed with solvent. Supported membrane C and floating membrane D were layer-by-layer deposited on the silicon substrate by the Langmuir-Blodgett and Langmuir-Schaefer techniques. Asymmetric bilayers were built by completely removing and replacing the monolayer in the Langmuir trough in between different steps.

Pressure-area isotherms

Pressure-area (π-A) isotherms were recorded on a Nima Langmuir-Blodgett trough, using a Wilhelmy plate for pressure sensing. All (π-A) experiments were carried out at 22°C (±0.5), below the melting temperature of the used lipids. Water for the subphase was processed in a Milli-Q system (Millipore, Bedford, MA), to a resistivity of 18 MΩ·cm. Each lipid solution was spread over the water subphase and the organic solvent was allowed to evaporate completely, over 15 minutes. All isotherms were recorded using a barrier speed of 25 cm^2/min. Stability and reproducibility of Langmuir films were verified by performing various compression-expansion cycles on two different Nima Langmuir troughs.

Neutron reflectometry

In a neutron reflectivity experiment a neutron beam is sent at grazing angle to a stratified sample and the specular reflected beam is collected as a function of q=(4π sinθ)/λ, where θ is the incident angle and λ the neutron wavelength. The technique allows recovering the neutron scattering length density profile ρ(z) of a membrane along the transverse direction. Compositional asymmetry can be investigated by the use of selective deuteration. Measurements were performed on FIGARO [21] horizontal reflectometer at ILL (FR), in TOF mode, dq/q = 8% , reflection angles 0.8° and 3.2° or 0.8° and 2.8°.

Data were analyzed by the software Motofit [22], that allows describing membrane layers in terms of thickness, scattering length density (then mean composition), coverage and roughness. Experiments performed on the same membrane in different contrast solutions (H$_2$O and D$_2$O) allows decoupling thicknesses from compositional information. For data analysis, each membrane was modeled as a 4-layers system: internal and external polar portions, and internal and external hydrophobic portions. The scattering length densities used to fit the data are reported in Table S1 of the Supplementary Material.

Results

Langmuir monolayers

The present goal is to investigate the feasibility and reliability in building model membranes with bio-similar asymmetric composition, then assessed by neutron reflectometry, by different commonly used protocols. One is the Langmuir-Blodgett/Langmuir Schaefer layer-by-layer deposition. The Langmuir technique allows to study the phase behavior of lipid monolayers at the air-water interface and to get information about the lateral packing at different lateral pressures and in mixtures. The optimal monolayer conditions for deposition on macroscopic supports can be determined.

To this scope, we recorded the pressure-area (π-A) curve of the Langmuir monolayers at the air-water interface of the monocomponent lipid systems d_{75}-DPPC and GM1 and of a proper (biomimetic) mixture of the two. For d_{75}-DPPC and the mixed system the subphase was pure water, whereas for pure GM1 the subphase was a 150mM NaCl water solution. The (π-A) curve of the mixed system, shew the effect of the addition of GM1 ganglioside on deuterated DPPC, from the gas to solid phase and, in particular, at the 40 mN/m pressure, used for model membrane deposition. The corresponding curves are reported in Figure 1. The monolayers are stable in time and the pressure-area results are reproducible both upon compression-expansion cycles and after monolayer re-spreading.
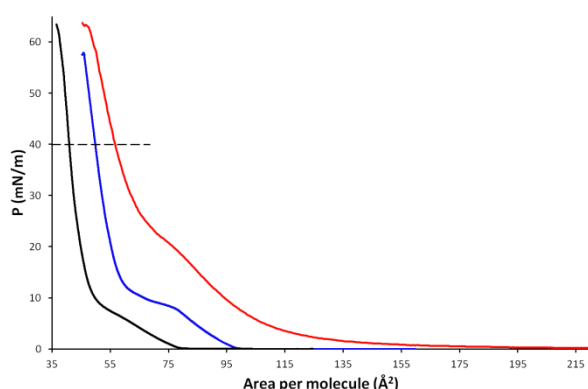

Figure 1. (π-A) curves of d_{75}-DPPC (blue), GM1 ganglioside (red) and d_{75}-DPPC: GM1 10:1 mol (black) at T=22°C. The curve relative to GM1 ganglioside was recorded in the presence of 150mM NaCl water solution as a subphase

The addition of GM1 to d_{75}-DPPC results in decreasing the occupied average area per molecule. At 40 mN/m, the area per molecule is 50 Å^2 for pure d_{75}-DPPC, 57 Å^2 for pure GM1, and only 41 Å^2 for the d_{75}-DPPC:GM1 10:1 mixture, that is, 10 Å^2 less than expected for ideal mixing. This can be due both to GM1 headgroup protrusion from the "average layer surface" in the mixed system and to the spacer effect played by phospholipids among the bulky dissociable ganglioside heads. There is a well known interplay between DPPC and GM1 ganglioside which could affect the structure of the membranes deposited with different protocols.

Neutron reflectometry

The creation of asymmetric single membranes suitable for structural investigation is a delicate matter and requires specific investigation according to the molecular asymmetry to be mimicked. Within the presented work, we tried and compared different techniques and protocols to reproduce glycolipid asymmetry in a phospholipid membrane. In particular, we addressed DPPC:GM1 membranes with fully asymmetric GM1 disposition (see Figure 2). The use of deuterated phospholipids was aimed to improve the visibility of the H-containing GM1 in the two membrane leaflets, a unique opportunity offered by neutron investigation.
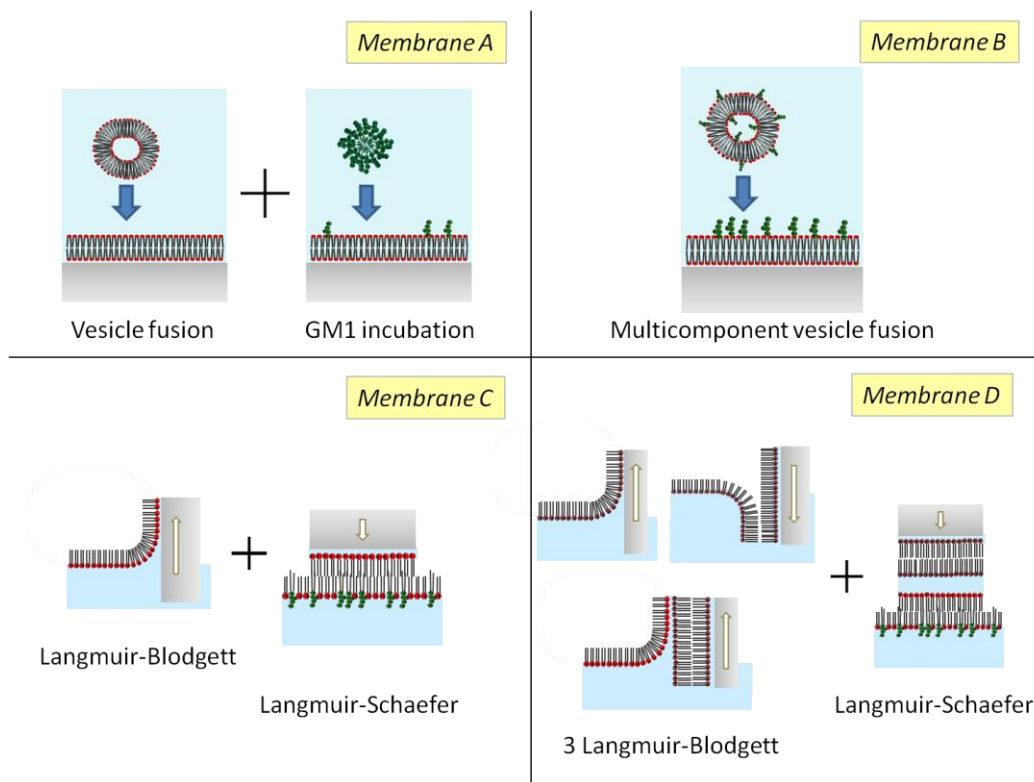
Figure 2. Scheme of the four asymmetric model systems prepared and investigated by neutron reflection.

Supported membrane A - DPPC vesicles fusion + GM1 micelles incubation

The first model membrane was built by a two-steps preparation. First, a d_{75}-DPPC model membrane was deposited by vesicle fusion, and after bilayer adsorption on the silicon support, GM1 was incubated by injecting micelles in the bulk solvent within the measuring cell. Then GM1 asymmetrically enters the exposed leaflet of the membrane, as sketched in Figure 2 and described in the experimental section. After incubation, the membrane was characterized in D_2O and H_2O at T=25°C. The reflectivity curves, together with the best fits and the corresponding scattering length density profiles are reported in the Supplementary Material. In Table 1, the membrane parameters used to contemporary fit the spectra collected in D_2O and H_2O are reported.

Table 1. Structural parameters of Membrane A (*DPPC vesicles fusion + GM1 micelles incubation*) obtained by combined data fitting in two solvents contrasts. Chains and heads 'in' refer to the layer facing the silicon block, chains and heads 'out' refer to the external layer, exposed to the bulk solvent. ρ_{lip} refers to the scattering length density contribution of the lipid components, not the solvent.

	Thickness (Å)	$\rho_{lip}(10^{-6}$ Å$^{-2})$	Solvent (%vol)
heads in	6±1	4.98±0.06	35±5
chains in	16±1	7.91±0.05	25±5
chains out	15±1	6.98±0.05	30±5
heads out	6±1	4.67±0.06	40±5

We observe that, among the considered protocols, this is simple to carry out, but it is time consuming and does not result in good mixed membranes. In fact, GM1 incubation caused membrane worsen, as seen by high interlayers roughness (8 Å), high solvent penetration, and low membrane thickness (43±2Å), see Table 1. The estimated amount of embedded GM1 was 11% in volume. A water layer 2Å thick was detected between the membrane and the silicon support.

Supported membrane B - bicomponent DPPC-GM1 vesicles fusion

The second model membrane was built-up by one-step preparation. A solution containing bicomponent d_{62}-DPPC:GM1 10:1 mol:mol vesicles was injected in the measuring cell kept at T = 45°C onto a naked silicon block, completely replacing the preexisting solvent, and left during 40 minutes to allow for membrane fusion, as described in the Experimental section. Figure 3 reports the partial spectra, restricted to the low grazing angle configuration, recorded at different delays from mixed vesicles injection. It can be seen that the fusion process onset occurs after a time lag of about 20 minutes, then rapidly evolving to a final state. After fusion, the cell was flushed with H_2O. Reflectivity was measured at 25°C in H_2O, then replaced by D_2O, and the corresponding spectra were fitted in parallel to get membrane parameters, as shown and reported in the Supplementary Material. We notice that, after solvent exchange from H_2O to D_2O, the membrane roughness increased from 2-3 Å to 5-7 Å and the water layer trapped between the membrane and the solid support became thicker, from 4 Å to 7 Å, all the other structural parameters being maintained, as reported in Table 2. The total membrane thickness was 54±2Å. We underline that an increase of the membrane roughness after solvent exchange procedure, suggests some membrane fragility which is not surprising, since in hosts a significant amount of GM1 ganglioside.
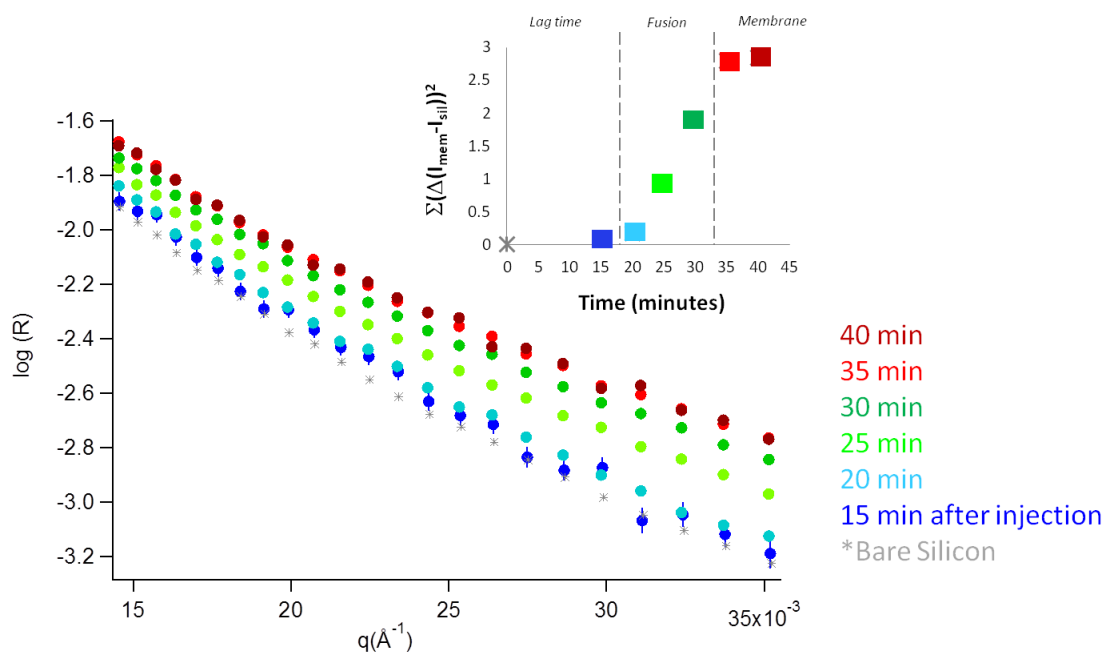

Figure 3. Series of reflectivity spectra (low grazing angle) collected at 5 minutes intervals (from bottom to top) from injection of the d_{62}-DPPC:GM1 10:1 mol:mol mixed vesicles solution. The spectrum corresponding to the naked silicon block is also reported. In the insert the sum of the squared point by point differences of the intensities of the curves with respect to that of bare silicon are reported. The onset of membrane formation on the support is seen after ~20 minutes, then rapidly reaching a final state.

Table 2. Structural parameters of Membrane B (*bicomponent DPPC-GM1 vesicles fusion*) obtained by combined data fitting in two solvents contrasts. Chains and heads 'in' refer to the layer facing the silicon block, chains and heads 'out' refer to the external layer, exposed to the bulk solvent. ρ_{lip} refers to the scattering length density contribution of the lipid components, not the solvent.

	Thickness (Å)	$\rho_{lip}(10^{-6}\ \text{Å}^{-2})$	Solvent (%vol)
heads in	7±1	1.75±0.07	35±5
chains in	16±1	7.91±0.05	25±4
chains out	20±1	6.42±0.05	25±4
heads out	11±1	1.84±0.07	35±5

Notably, data analysis reveals that total GM1 asymmetrisation occurred during fusion, that is, although incubating randomly mixed vesicles, in the resulting supported membrane all the GM1 is hosted in the outer layer, to an external leaflet composition DPPC:GM1 9:2 mol:mol. In Figure 4, the experimental reflectivity curve and scattering length density profile of Membrane B in H_2O at T=25°C, are compared to the ones for a hypothetic membrane, with the same structural parameters symmetrically hosting GM1 in the two leaflets. The difference is evident. The observation of GM1 asymmetrisation during fusion is a very important result concerning single membranes deposition for structural and morphological investigations, sometimes hypothesized [19] but never seen before. GM1 redistribution during fusion, passing from spontaneously-curved mixed vesicles to a flat geometry, with strong GM1 content and unbalance, could contribute to membrane stress, inducing the observed response upon solvent exchange.
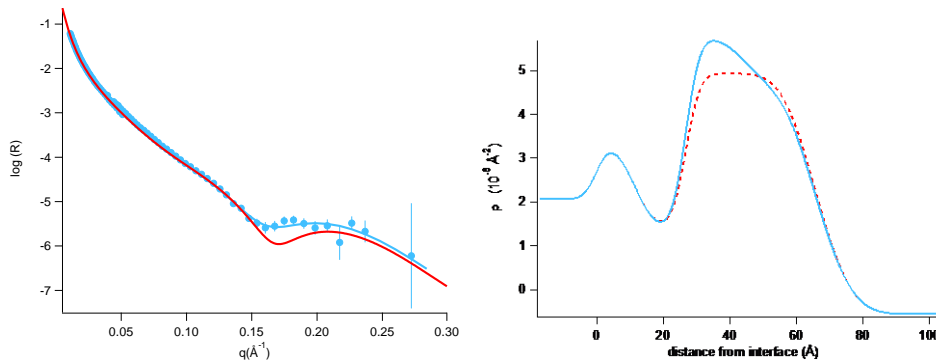

Figure 4. Left panel: reflectivity curve (blue dots) and relative fit (blue line) of Membrane B in H_2O at T=25°C. The red line corresponds to the simulated spectrum of a hypothetic membrane with the same structural parameters of Membrane B, but symmetrically hosting the total amount of GM1 in the two leaflets. Right panel: scattering length density profile of membrane B in H_2O (blue line). The simulated symmetric one (red dashed) is reported for comparison.

We observe that this procedure for membrane preparation is, among the investigated, the most time saving and technically less complicated to perform. Moreover, the asymmetric membrane so obtained can be considered 'good enough' for neutron investigation and as a target for macromolecules-membrane interaction studies. Both lipids-silicon interaction, and lipid-lipid interactions and different lipid packing are likely to play a role in the kinetics of membrane adsorption to the solid surface and in asymmetric

redistribution of components. These aspects will be further investigated in the future, by considering complex membranes of different composition.

Supported membrane C - Langmuir Blodgett + Langmuir Schaefer

A supported asymmetric membrane was deposited by coupling one Langmuir Blodgett deposition of d_{75}-DPPC and one Langmuir Schaefer deposition of d_{75}-DPPC:GM1 10:1 mol:mol layer, then submitted to the whole neutron reflectivity characterization, as reported in the Supporting Material.

We observe that the use of the LB/LS technique for membrane deposition requires both the presence of a Langmuir trough equipped for multilayers deposition on macroscopic supports in the vicinity of the experimental beamline, and experienced technical skill. Moreover, a single membrane deposition takes roughly 2 hours. Nonetheless, the membranes obtained by this technique showed to be the best in terms of coverage (see Table 3). The total membrane thickness is 51±2Å and the interlayer roughness is 3Å. No detectable water layer was found between the membrane and the silicon support.

Table 3. Structural parameters of Membrane C (*Langmuir Blodgett + Langmuir Schaefer*) obtained by combined data fitting in two solvents contrasts. Chains and heads 'in' refer to the layer facing the silicon block, chains and heads 'out' refer to the external layer, exposed to the bulk solvent. ρ_{lip} refers to the scattering length density contribution of the lipid components, not the solvent.

	Thickness (Å)	$\rho_{lip}(10^{-6}$ Å$^{-2})$	Solvent (%vol)
heads in	7±1	4.98±0.06	22±5
chains in	16±1	7.91±0.05	11±5
chains out	19±1	7.16±0.05	12±5
heads out	9±1	4.21±0.06	22±5

Floating membrane D - Langmuir Blodgett + Langmuir Schaefer

Last, a floating asymmetric membrane was deposited by coupling three Langmuir Blodgett depositions (two d_{83}-DSPC layers to form the decoupling supported membrane and one layer of d_{75}-DPPC) and a Langmuir Schaefer deposition of the most external d_{75}-DPPC:GM1 10:1 mol:mol layer. Reflectivity at 25°C was measured immediately after preparation and 24 hours later. The collected reflectivity spectra with their fits and relative scattering length density profiles are reported in Figure 5. The system evolves on that timescale. The floating membrane got thicker (from 44±2 to 51±2 Å) and its roughness increased from 5-9 Å to 9 Å (see Table 4, fit parameters of the freshly prepared membrane are reported in Table S2 of the Supplementary Material). On the contrary, the roughness of the supporting DSPC membrane decreased from 5 Å to 3-4 Å. A slight decrease in the floating membrane solvent penetration was also detected. These results suggest that membrane components diffuse and organize in time, finding a better way to optimize their distribution and packing and to stabilize the membrane structure. In fact, the mature membrane displays a more compact structure than when freshly prepared, a 'time annealing' process in place of the usual thermal annealing. Thickening of the floating membrane can partially be due to increased undulations.
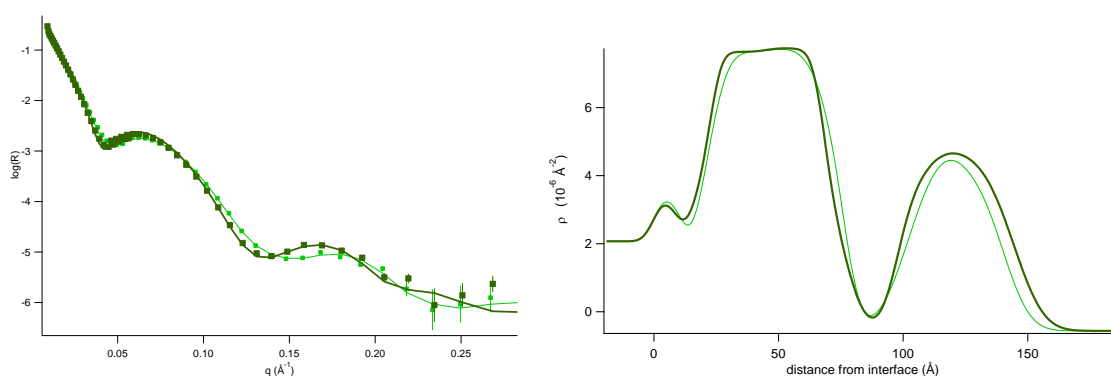
Figure 5. Left panel: reflectivity curves (dots) and corresponding fits (lines) of double-membrane system D in H₂O collected just after preparation (light green) and 24 hours after preparation (dark green). Right panel: scattering length density profiles of the double-membrane D just after preparation (light green) and 24 hours after preparation (dark green). T=25°C.

Table 4. Structural parameters of the double membrane system D at 24 hours delay from preparation obtained by combined data fitting in two solvents contrasts. Chains and heads 'in' refer to the layer facing the silicon block, chains and heads 'out' refer to the external layer, exposed to the bulk solvent. ρ_{lip} refers to the scattering length density contribution of the lipid components, not the solvent.

		Thickness (Å)	$\rho_{lip}(10^{-6}$ Å$^{-2})$	Solvent (%vol)
Supporting d₈₃DSPC	heads in	8±1	4.98±0.06	15±5
	chains in	22±1	7.96±0.05	4±5
	chains out	23±1	7.96±0.05	3±5
	heads out	8±1	4.98±0.06	15±5
water		21		
Floating d₇₅DPPC:GM1	heads in	9±1	4.98±0.06	45±5
	chains in	16±1	7.91±0.05	34±5
	chains out	18±1	7.16±0.05	34±5
	heads out	8±1	4.21±0.06	45±5

General considerations and comparison

The collected results can be revisited according to different criteria, as for ease and length of preparation procedure, requirement for additional specific instrumentation, quality of the obtained membrane, visibility and adaptation to the investigation focus. Figure 6 reports a comparison among the scattering length density profiles of the four prepared mixed asymmetric membranes, relative to H₂O. Membrane A (red line) displays significant solvent penetration following GM1 incubation. The asymmetry of membrane B (blue line) seems to be more marked, but this is due to the high ganglioside content in the outer layer, twice the others. Moreover, its thickness seems to be lower than the others, but this is an "optical effect"

as the lipid heads of membrane B are not deuterated, therefore less contrasting in H_2O. It appears to be of good quality both for structural investigation and as a candidate for mimicking membrane interaction with approaching molecules. Membrane C (green line) appears to be the most contrasting in H_2O, that is, the one less affected by solvent penetration and shows the best structure and compactness. Solvent penetration in the floating membrane of system D (black line) is high too, but floating membranes have the advantage to be decoupled from the rigid silicon support. The following considerations can be resumed concerning sample preparation. Membrane A presents important disadvantages, namely lengthy sample preparation, not compensated by the quality of the mixed membrane. As for membrane B, the 'mixed vesicle' fusion protocol used for deposition, is easy to perform and does not require the presence of particular instrumentation. This technique is widely used to deposit raft-mimic membranes. Here we show that effective ganglioside asymmetry is obtained, a feature that had never been investigated before. Membrane C, the supported membrane obtained by Langmuir-Blodgett/Langmuir-Schaefer technique, shows the best structure and compactness, but requires the availability *in loco* of a Langmuir trough suitable for membrane deposition on macroscopic supports. The same holds for the floating membrane of system D. Free floating is a required and unique feature when the aim is to mimic biological membranes also in terms of mobility of components or as models for the study of membrane-macromolecule interaction (with drugs, proteins, peptides, nanoparticles,...).
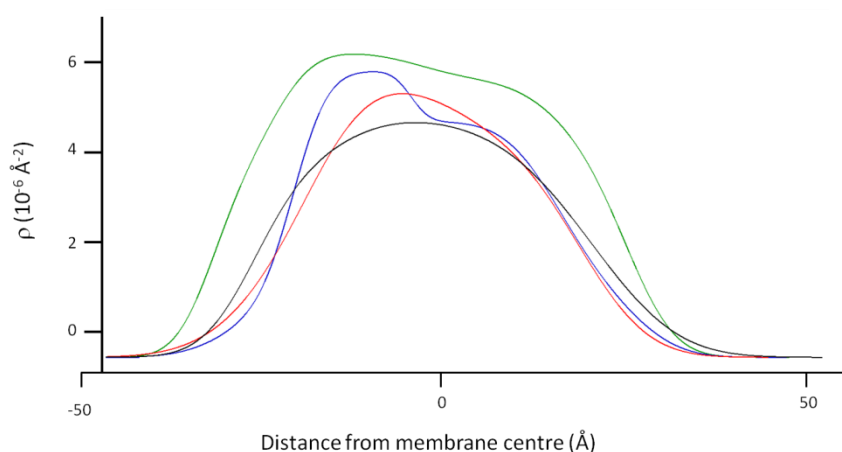

Figure 6. Scattering length density profiles of the investigated membranes in H_2O. For better comparison, the SiO_2 contribution has been canceled by simulating the presence of a wide 50 Å water layer between the membrane and the silicon block. Membrane A: red, Membrane B: blue, Membrane C: green, Membrane D: black. The positive distances from the membrane center, set as zero, refer to the leaflet exposed to the bulk solvent, hosting GM1.

Conclusions

The structure of single phospholipid membranes containing GM1 ganglioside, prepared by different protocols, has been studied by neutron reflectivity. In view of biological significance, it is of primary importance both to reproduce the basic membrane characteristics, in terms of composition and disposition of components, and to select the preparation protocol allowing for the optimal membranes in terms of compactness and stability. The best protocol depends of course on membrane composition.

Asymmetric distribution is a distinctive feature of ganglioside-containing membranes. We examined some of the most used procedures for the deposition of lipid membranes in excess solvent.

The first membrane (Membrane A) was obtained by d_{75}-DPPC vesicles fusion on the silicon support and GM1 was *a posteriori* incubated in the phospholipid membrane. This membrane was not optimal, having lower thickness than expected, high solvent penetration and roughness. It was anyway evident that GM1 insertion in the outer membrane layer occurred.

The second supported membrane (Membrane B) was obtained by the fusion of d_{62}-DPPC:GM1 vesicles with spontaneous-mixing asymmetry. The resulting bilayer displayed better coverage and higher thickness. Notably, total GM1 asymmetrization occurred. In fact, the membrane adsorbed on the silicon support was completely asymmetric in the ganglioside distribution, fully hosted in the external leaflet of the membrane. This result is of great importance when the aim is to mimic the typical biological membranes asymmetry. Besides, membrane B appeared to be of good quality both for structural investigation and as a candidate for mimicking membrane interaction with approaching molecules.

The third supported membrane (Membrane C) was deposited by the Langmuir-Blodgett/Langmuir-Schaefer technique and asymmetry was created by changing the film at the air-water interface between depositions. The first layer was composed by d_{75}-DPPC and the second by d_{75}-DPPC:GM1 10:1 mol. The membrane obtained by this technique was very good and stable.

Also the fourth membrane (Membrane D) was obtained by the Langmuir-Blodgett/Langmuir-Schaefer technique, with the same composition of Membrane C but floating over a d_{83}-DSPC cushion membrane. Also this membrane was nicely stable, and we observed that upon 24 hours ageing components diffuse and redistribute at room temperature, making the floating membrane more stable and thick. Although not the best in terms of bilayer compactness and coverage, this system provides a floating membrane decoupled from the silicon support and therefore very interesting as biomimic.

We conclude that all of the considered protocols result in the deposition of mixed asymmetric lipid systems, giving reasonable results concerning in particular the macroscopic membrane integrity. Although, as expected, the best protocol has proven to be the Langmuir-Blodgett/Langmuir-Schaefer, allowing for both control in the distribution of mixed components and physical decoupling from the rigid support, nonetheless, and notably, also the mixed vesicle fusion was found to be suitable for straightforward deposition of asymmetric raft mimes. It constitutes a good compromise between easy and prompt preparation and sample quality, if decoupling from the solid support is not required.

The possibility to access the internal structuring of customized complex membranes is an opening, both for the design of appropriate models for structural and morphological investigations and for the detailed study of biosimilar membranes response to external stimuli such as approaching macromolecules.

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