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A novel leaflet-selective fluorescence labeling technique reveals differences between inner and outer leaflets at high bilayer curvature

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ABSTRACT

Understanding the differences in the physical properties of the inner and outer leaflet of membranes and how the leaflets are coupled to each other requires methods that can selectively label both the outer and inner leaflets. In this report we introduce a combined chromatography/cyclodextrin method for selective labeling of the inner leaflet. Combining this method with selective labeling of the outer leaflet, we are able to show that there is a distinct difference in polar headgroup physical properties of the inner and outer leaflet headgroups in small unilamellar vesicles composed of a wide variety of phosphatidylcholines and a phosphaticylcholine/sphingomyelin mixture. It appears that the inner leaflet headgroups are more tightly packed than those of the outer leaflet. This differential packing disappears when vesicle size increases, showing that it is a consequence of membrane curvature. Differential packing is also reduced as acyl chain length is decreased. In the future, selective leaflet labeling is likely to be a powerful tool for investigating the properties of asymmetric lipid vesicles.

1. Introduction

Lipid vesicles (liposomes) of various sizes have been extensively used in biophysical research to model the complexity of biological membranes and, in particular, of the plasma membrane. Thanks to recent advances, it is now also possible to readily assemble asymmetric vesicles that mimic the lipid asymmetry found in cells [1,2]. The increased availability of such asymmetric model membranes brings about also the need for independent labeling of each leaflet, in order to make use of bulk fluorescence techniques like e.g. fluorescence resonance energy transfer (FRET). While labeling of the outer leaflet is a simple and established procedure, the confinement of a fluorescent dye in the inner leaflet is more complex. The methods so far available are based on the labeling of the whole bilayer and the subsequent chemical bleaching (e.g. nitrobenzoxadiazole (NBD) bleaching by sodium dithionite [3]) or photophysical quenching (e.g. by iodide) of the dye in the outer leaflet. This methodology can have several drawbacks: the quenching might not be permanent, undesired molecules that may perturb the properties of a solution must be added, and, in some cases, even penetrate the inner lumen of the vesicles (e.g. at high temperature), defeating their use. Most importantly, the approach is limited to a very small subset of fluorescent probes. As an alternative to quenching, the fluorescent dye in the outer leaflet can be removed exploiting its spontaneous inter-bilayer transfer to unlabeled acceptor vesicles [4,5] or through binding to bovine serum albumin [6-8]. If the extracted dye interferes with the measurement, it needs to be separated from the rest of the sample using e.g. multiple centrifugation steps before further analysis.

Following an analogous principle, this paper presents a simple procedure to label the inner leaflet of lipid vesicles with a fluorescent molecule. The fluorescent probe in the outer leaflet is, in this case, removed by gel chromatography following a short incubation with low concentrations of methyl-beta-cyclodextrin (mβCD). This method does not require any time-consuming vesicle separation procedure or any quencher left in solution during the actual measurement time; also, it is very fast (less than ~10 min) and is more general because the only requirement is the amphiphilic nature of the fluorescent probe to allow extraction from a membrane.

As an example of how the selective labeling can be used, we examined the effect of curvature on establishing a bilayer asymmetry in small unilamellar vesicles (SUV). Membrane curvature is involved in several biological processes like membrane fusion [9], the function of tubules and small vesicles [10] and protein binding [11-13].

Abbreviations: 6-NBD-PC, 2-(6-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)hexanoyl-1-hexadecanoyl-sn-glycero-3-phosphocholine; bSM, porcine brain sphingomyelin; DLS, dynamic light scattering; DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; mβCD, methyl-beta-cyclodextrin; NBD, nitrobenzoxadiazole; NBD-DPPE, N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine triethylammonium salt; NR12S, N-[3-[[9-(diethylamino)-5-oxo-5H-benzo[a]phenoxazin-2-yl]oxy]propyl]-N-methyl-N-(3-sulfopropyl)-1-dodecanaminium; PBS, phosphate buffered saline; PC, phosphatidylcholine; SOPC, 1-stearoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; SUV, small unilamellar vesicles; T_m, mixing temperature; TMA-DPH, 1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene *p*-toluenesulfonate

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Fluorescence studies have addressed this issue but, so far, they have been limited to investigating the properties of either the outer leaflet or the whole bilayer [14,15], since the inner leaflet could not be labeled with fluorophores in a selective manner. Using SUVs labeled with the fluorescent probe NR12S in either the inner or the outer leaflet, we show here that in the inner leaflet the probe is in a less hydrated/polar environment than the outer leaflet. According to the results of a recent simulation study [16], this effect seems to originate from simple steric constraints in a highly curved geometry and the resulting larger distances between the polar headgroups of the outer leaflet.

2. Materials and methods

2.1. Materials

All lipids were purchased from Avanti Polar Lipids (Alabaster, AL). Lipids were stored in chloroform at −20 °C and their concentrations were determined by dry weight. N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine triethylammonium salt (NBD-DPPE), 1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene *p*-toluenesulfonate (TMA-DPH) and 2-(6-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)hexanoyl-1-hexadecanoyl-sn-glycero-3-phosphocholine (6-NBD-PC) were from Invitrogen (Eugene, OR). The Nile Red-based probe N-[3-[[9-(diethylamino)-5-oxo-5H-benzo[a]phenoxazin-2-yl]oxy]propyl]-N-methyl-N-(3-sulfopropyl)-1-dodecanaminium (NR12S) was synthesized as described in Ref. [17]. The chemical structures of used fluorescent lipids and probes are drawn in Fig. S1. Methyl-beta-cyclodextrin and sodium dithionite were from Sigma-Aldrich (St. Louis, MO). The gel for the chromatography columns was the Sephacryl S-300 from Pharmacia Biotech (now GE Healthcare), Piscataway, NJ.

2.2. Model membrane vesicle preparation

Ethanol dilution small unilamellar vesicles were prepared similarly to as described previously [18]. Lipids (and fluorophores when needed) were mixed in glass tubes, dried under N_2, dissolved in ethanol and then dispersed in phosphate buffered saline (PBS, pH 7.4, Bio-Rad, Hercules, CA) at room temperature or at 70 °C for lipids with higher melting temperature (i.e. higher than ~30 °C), for a final lipid concentration of 100 μM and an ethanol concentration of 2 vol%. The concentration of lipids in ethanol was 5 μmol/mL unless otherwise noted. Lower lipid concentrations were obtained by diluting the SUV suspension prepared with the above-described procedure. 200 μM SUV samples were obtained by doubling the total amount of ethanol (final ethanol concentration 4 vol%). To prepare large extruded unilamellar vesicles, the dried lipids were dispersed in PBS at room temperature. Final samples contained 200 μM lipids. The so-obtained suspension was then subjected to 11 passages at room temperature through a mini-extruder (Avanti Polar Lipids) using 100 nm filters and diluted if needed. All the samples were then measured with dynamic light scattering (DLS) (DynaPro, Wyatt Technologies, Santa Barbara, CA) to determine the vesicle radius. All the DLS measurements were performed 6 times in two independent samples and averages ± average deviations are reported. In order to label the outer leaflet of the vesicles, the fluorophores were added from a concentrated ethanol stock solution to a final molar concentration (compared to the lipids) between 0.1 and 0.16%, at room temperature or at 70 °C for lipids with higher melting temperature.

2.3. Fluorescence measurements

Fluorescence was measured on a SPEX Fluorolog 3 or a SPEX Tau 2 operating in steady state mode (Jobin-Yvon, Edison, NJ) using quartz semi-micro cuvettes. Fluorescence anisotropy was measured using a SPEX automated Glan-Thompson polarizer accessory. TMA-DPH anisotropy values were calculated from the fluorescence intensities with polarizing filters set at all combinations of horizontal and vertical orientations. The following equation was used: $A = [((I_{vv} \times I_{hh})/(I_{vh} \times I_{hv})) - 1]/[((I_{vv} \times I_{hh})/(I_{vh} \times I_{hv})) + 2]$ where A is anisotropy and I_{vv}, I_{hh}, I_{vh}, and I_{hv} are the fluorescence intensities with the excitation and emission polarization filters, respectively, set in the vertical (v) and horizontal (h) orientations [19], after subtraction of fluorescence intensity in background samples lacking fluorophore. TMA-DPH fluorescence was measured with an excitation λ of 364 nm and the emission was monitored between 400 and 460 nm (426 nm for anisotropy measurements). NBD fluorescence was measured at an excitation λ of 460 nm and the emission was monitored between 515 and 560 nm. NR12S fluorescence was measured at an excitation λ of 529 nm and the emission was monitored between 590 and 650 nm. In order to carefully measure the peak position for the emission of the NR12S when comparing different samples, binding curves were measured with varying lipid concentrations (see Fig. S2). For all the samples, the background signal was measured and subtracted. Slit bandwidths for fluorescence intensity measurements were set to 5 nm for both excitation and emission, while the slit-width was set to the maximum (10 nm) for fluorescence anisotropy measurements. All the measurements were performed on duplicate samples and at room temperature, unless otherwise specified.

2.4. Extraction of fluorophores from vesicles by MβCD

To measure the MβCD concentration dependence of fluorophore extraction from the outer leaflet of vesicles at room temperature, the outer leaflet was labeled as described above. After a 15–30 min incubation, the peak intensity of the fluorescence spectrum of each sample was measured. Next, an aliquot of MβCD from a 360 mM stock solution in water was added and, after a 5 min incubation, the fluorescence intensity was remeasured. This was repeated for a series of aliquots of MβCD. Controls showed that extraction by MβCD reached equilibrium within 2 min for vesicles of various lipid compositions and that higher temperature did not affect the extraction process significantly.

3. Results and discussion

3.1. Gel filtration partially extracts fluorophores from the outer leaflet of SUVs

In order to obtain selective labeling of the inner leaflet, the most straightforward approach is to first produce vesicles labeled in both leaflets and, subsequently, remove the fluorescent label from the outer leaflet only. It is implicit in this approach that the dye has to have a very slow rate of transverse diffusion (flip-flop) between the inner and outer leaflets. To characterize the extraction of fluorescent labels from the outer leaflet, SUVs produced via ethanol dilution were labeled in the outer leaflet only using the following dyes: 6-NBD-PC, NR12S and TMA-DPH. The dye 6-NBD-PC is an acyl-modified nitrobenzoxadiazole phospholipid probe often used in FRET and FRAP studies [20,21]; NR12S is a modification of the solvatochromic dye Nile Red to which a lipid anchor is added [17]. TMA-DPH is a cationic modification of diphenylheatriene whose fluorescence lifetime and anisotropy signals depend on membrane environment [22,23]. It is worth noting that some of these fluorophores (i.e. NBD and NR12S) could be quenched chemically, thus allowing selective labeling of the inner leaflet [24]. Nevertheless, they were chosen in this context to illustrate how this new method for selective leaflet labeling works in general, also when use of chemical quenchers is not possible or desirable. The above mentioned fluorescent probes are amphiphilic and, therefore, can be delivered to the

outer leaflet of vesicles in aqueous suspension by simply adding a small volume (1–20 μL) from an ethanol stock solution directly to the vesicle-containing PBS. The large (or charged) polar moiety anchors the molecule in the outer leaflet, strongly interacting with water molecules and with the hydrophilic interface of the bilayer, thus significantly slowing the flip-flop rate of the dye [17,25]. Within 30 min after the addition of the dyes, the SUV samples were chromatographed on a small high-resolution Sephacryl S-300 column, with a base radius of ~1 cm and a height between 5.5 and 8 cm. Since the exclusion limit of the gel (1500 kDa) is smaller than the smallest vesicle weight, and therefore size,[1] the SUVs are almost totally eluted in the void volume. Thus, the SUVs can be collected in the early fractions (i.e. collected during the first ~3 min), being just slightly diluted. The fluorescence intensity measured in samples with different dyes in the outer leaflet was measured after the gel chromatography (choosing the fraction containing the highest SUV concentration) and compared with that measured before injecting the sample in the column. The fluorescence intensity measured after the filtration (I) is in general lower than the one measured before (I_0). The ratio between the two intensities is determined by i) how much the vesicles were diluted and ii) the amount of fluorophore which was lost in the buffer solution during the procedure. The former factor depends on vesicle size, composition and the volumes of the sample and of the column. The latter depends on the specific fluorescent label used and, in particular, its partition between the bilayer, the water and unspecific interactions with the gel material. To normalize for vesicle dilution during chromatography, we used SUVs labeled in both leaflets, with the fluorescently labeled phospholipids NBD-DPPE, a lipid too tightly bound to the membrane to be extracted because of its long saturated acyl chains (see below). $I_{\text{NBD-DPPE}}/I_{0\ \text{NBD-DPPE}}$ should be lower than 1 due just to vesicle dilution. This value of I/I_0 corresponds therefore to a full (100%) retention of the dye in the bilayer. Fig. 1 shows, for two lipid compositions (i.e. 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) and a 1:4 mixture of DOPC and porcine brain sphingomyelin (bSM)) and four different fluorophores, the amount of dye which is retained in the outer leaflet of the SUVs, compared to the amount measured before the gel filtration after normalization for vesicle dilution using the formula:

$$\%\,\text{dye retention in the vesicle} = 100\% * \left(I_{\text{dye}}/I_{0\ \text{dye}}\right)/\left(I_{0\ \text{NBD}-\text{DPPE}}/I_{\text{NBD}-\text{DPPE}}\right)$$

This experiment clearly shows that up to almost 90% of the TMA-DPH in the outer leaflet is removed during the passage through the column. Of interest, also NR12S and 6-NBD-PC can be extracted, albeit to a lower extent (up to ~40% of the initial value). The efficacy of the removal of TMA-DPH probably derives from its charged group and increased hydrophilicity, which favor the dissolution of the dye into the water phase while the SUVs travel through the gel column. The non-specific interaction binding the fluorescent molecule to the beads constituting the gel also appears to play a role in extraction. Importantly, this causes a small contamination of the column with fluorescent material (data not shown). For measurements that are extremely sensitive to fluorescent contaminants, it is recommended to use fresh columns for chromatography (or better, fresh columns through which SUV lacking probes have been chromatographed to saturate irreversible lipid binding sites and so reduce SUV loss). Finally, the physical state of the membrane does not seem to be an important factor: the extraction of the fluorescent dye does not decrease significantly even when the bilayer is strongly ordered due to the presence of 80% bSM.

[1] ~$2 \cdot 10^4$ KDa for a vesicle with external radius of 30 nm, bilayer thickness 3 nm, lipid molecular weight of 750 Da and headgroup surface area of 0.7 nm^2.

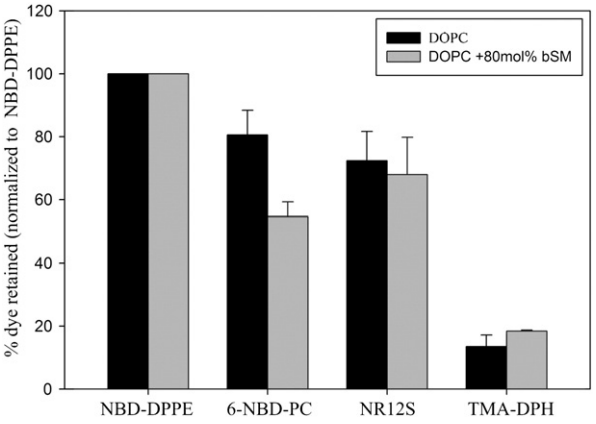

Fig. 1. Gel-filtration of SUVs partially extracts the fluorophores from the outer leaflet. The percentage of dye left in the outer leaflet of SUVs after a passage through a ~7 cm S-300 gel column is shown as a function of different lipid compositions at room temperature. The values are relative to the measurements for NBD-DPPE, which is assumed to be fully retained in the vesicles. The concentration of the SUVs before the filtration was 100 μM (sample volume was 1 mL) and the concentration of the dye, added to the outer leaflet only, was 0.1 mol% (NR12S and TMA-DPH) or 0.16 mol% (NBD-DPPE and 6-NBD-PC). 1 mL fractions were collected from the column and, for analysis, the peak fraction was used. The error bars represent the average deviations between duplicate experiments.

3.2. mβCD extracts fluorophores from the outer leaflet of SUVs

The selective inner leaflet labeling method is easily improved by treating the vesicles with a small amount of mβCD for 2–5 min at room temperature before the gel filtration. mβCD is a cyclic oligosaccharide that is able to bind hydrophobic molecules and lipids and extract them from a membrane. At low concentration (e.g. 1–10 mM), mβCD can extract only molecules which are not too hydrophobic and not too strongly interacting with the lipid bilayer. For example, few mM mβCD can bind cholesterol (and thus extract it from or deliver it to a lipid membrane) [26]. In this experiment, DOPC SUVs were labeled in the outer leaflet with several fluorescent molecules (i.e. NBD-DPPE, NR12S, 6-NBD-PC and TMA-DPH), exactly as described in the previous paragraph. In this case though, instead of performing a gel chromatography, the extraction of the fluorophore was monitored as a function of mβCD concentration. Fig. 2 shows the fluorescence peak intensity of the dye in a sample containing 100 μM DOPC SUVs without mβCD (0 mM) and how it decreases in response to mβCD addition. The fluorescence intensity decrease is due to the fact that, in general, the quantum yield of most membrane dyes is much lower in an aqueous environment than when embedded in the bilayer: at very low mβCD concentrations the dye is mostly in the membrane and the measured fluorescence signal is high; the more mβCD is added to the sample, the more dye is extracted from the hydrophobic membrane environment, thus decreasing the measured fluorescence signal. In agreement with the results of the previous paragraph and its significant hydrophilicity, TMA-DPH is easily extracted from the membrane by the action of mβCD. As little as 1 mM mβCD is enough to cause a 50% decrease in fluorescence intensity for a sample containing DOPC SUVs labeled with TMA-DPH in the outer leaflet. In the case of NR12S (or 6-NBD-PC), much higher mβCD concentrations are needed to obtain a similar decrease in fluorescence signal. The expected decrease depends on the quantum yield that each fluorophore has when it is completely extracted from the membrane and fully bound to mβCD in the aqueous solution. The dashed lines in Fig. 2 represent the fluorescence measured when the corresponding dye is dissolved in a solution containing 10 mM mβCD but no lipid vesicles. Such a measurement is an approximation for the case in which the dye is completely extracted from the outer leaflet. It is clear that, while the TMA-DPH is almost non fluorescent outside the lipid bilayer, NR12S and 6-NBD-PC maintain a fairly

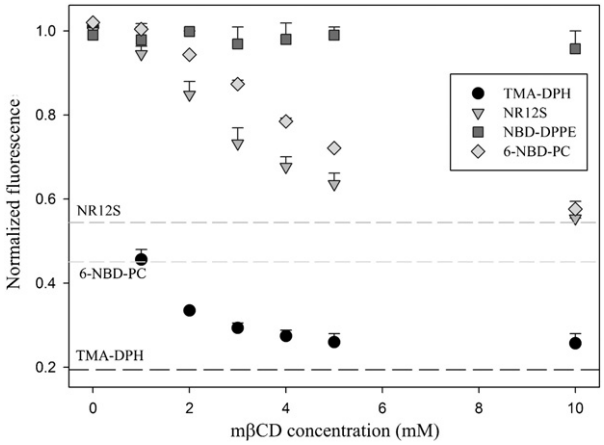

Fig. 2. mβCD extracts fluorophores from the outer leaflet. Fluorescence intensity of each dye in the outer leaflet of DOPC SUVs is plotted as a function of mβCD concentration and normalized to the value measured before the addiction of the mβCD. The concentration of the SUVs was 100 μM and the concentration of the dye, added to the outer leaflet only, was 0.1 mol% (NR12S and TMA-DPH) or 0.16 mol% (NBD-DPPE and 6-NBD-PC). The error bars represent the average deviation of the values measured in duplicate experiments. The horizontal dashed lines represent the intensity measured when the same amount of each dye (with the exception of NBD-DPPE) was dissolved in a sample containing 10 mM mβCD without lipid vesicles. This value represents the expected fluorescent intensity that would be measured if the dye was totally extracted from the outer leaflet. The 0 mM mβCD point of each curve is slightly shifted for a better visualization.

high quantum yield, thanks to the interaction with mβCD molecules. Based on this, Fig. 2 shows that 10 mM mβCD is sufficient to almost completely extract the NR12S from the vesicles, notwithstanding the fact that the fluorescence intensity decreased by a factor of only ~2. It is also clear that the mβCD concentration required for 50% dye removal is higher for NR12S and 6-NBD PC than for TMA-DPH, consistent with their ease of removal from membranes via chromatography and that NBD-DPPE cannot be extracted by mβCD. Similar mβCD extraction results were observed using SUVs composed of DOPC with 80 mol% bSM and when performing the extraction at ~70 °C (data not shown).

The experiments above illustrate a simple and fast procedure to selectively label the outer or the inner leaflet of lipid vesicles. The only two prerequisites (often linked) are that the fluorescent membrane dye i) is at least slightly soluble in aqueous solution and ii) has an amphiphilic anchor that slows inter-leaflet diffusion (flip-flop). Such a fluorescent molecule can be dissolved in a concentrated stock solution (e.g. ethanol) and delivered to the outer leaflet of already formed liposomes by adding a small aliquot into solution. On the other hand, inner leaflet labeling can be easily achieved by producing the SUV with the dye initially in both leaflets and then performing mβCD-driven extraction followed by gel chromatography. The advantages of using both the approaches on the same samples are that a very large amount of membrane dye can be removed from the outer leaflet of the vesicles and that the final sample will have no mβCD (since it is a low molecular weight compound that it will be separated from the SUVs during the gel filtration), while keeping the duration of the whole operation under ~10 min.

3.3. Membrane curvature causes differential hydration of inner and outer leaflets

To illustrate how this method can be used to study the different properties of outer and inner leaflets in SUVs and how they depend on the bilayer curvature, we have investigated the environment in the polar headgroup region of the membrane.

By selective leaflet labeling of SUVs with different lipid compositions, it was possible to monitor the bilayer hydration/polarity (using the environment sensitive probe NR12S) and the acyl chain order (measuring the anisotropy of TMA-DPH) independently in the two leaflets. This was done as a function of temperature. Fig. 3A shows the anisotropy of TMA-DPH either in the inner or in the outer leaflet of SUVs composed of 1-stearoyl-2-oleoyl-*sn*-glycerol-3-phosphocholine (SOPC) or bSM:DOPC 2:1. At low temperatures, the anisotropy measured in either leaflet of the bSM:DOPC SUVs is higher than that measured in the SOPC vesicles. This reflects the presence of ordered (gel) phase in the SUVs containing bSM. Increasing temperature causes a decrease of anisotropy due to the increasing disorder of the bilayer and the consequent increased motional freedom of the fluorescent dye. While the decrease is small and gradual for SOPC vesicles, being in the disordered (liquid) state at all the experimental temperatures, a steep decrease in anisotropy was observed for bSM:DOPC, reflecting melting of ordered gel state bilayer at the melting/mixing temperature (T_m) range (20–40 °C). The anisotropy difference between the two leaflets for a single lipid composition is small compared to the average deviation between duplicate experiments, especially in the case of vesicles in the liquid state.

Fig. 3B shows the maximum emission wavelength for NR12S, a probe of the lipid headgroup region, either in the inner or in the outer leaflet of SUVs composed of SOPC or bSM:DOPC 2:1. The emission wavelength of NR12S is an indicator of the state of the bilayer, with blue-shifted fluorescence in a less polar and less hydrated environment [17]. As a consequence, the peak position of the emission spectrum is sensitive to several factors, including lipid composition. As evident in Fig. 3B, it was observed that in membranes composed of SOPC at room temperature the probe detects a more hydrated/polar environment than in those composed of bSM:DOPC. This is not

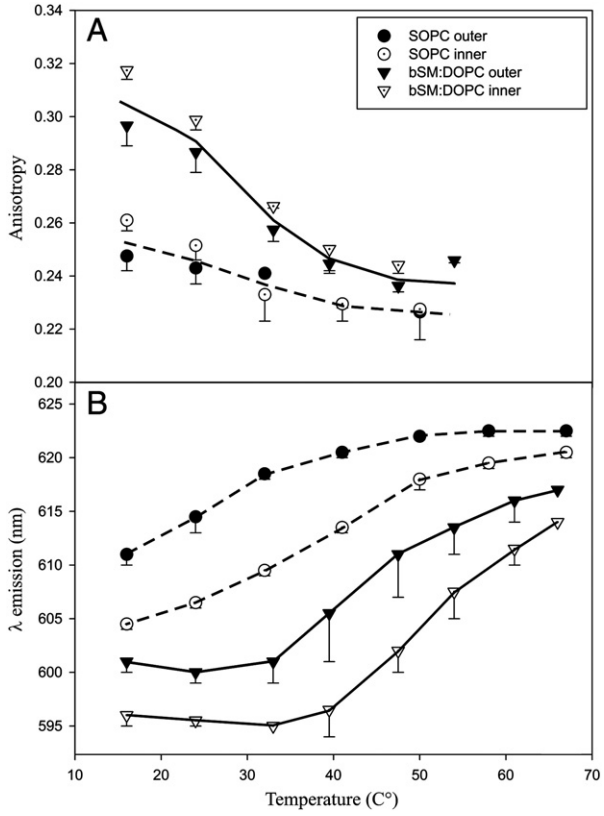

Fig. 3. TMA-DPH anisotropy and NR12S emission wavelength as a function of temperature and leaflet localization. Panel A shows the anisotropy of TMA-DPH, either in the inner or outer leaflet of SUVs composed of SOPC or a mixture of bSM and DOPC (2:1 molar). Panel B shows the emission peak wavelength of NR12S, either in the inner or in the outer leaflet of SUVs composed of SOPC or a mixture of bSM and DOPC (2:1 molar). The error bars represent the average deviations of the values measured in independent duplicate experiments. For sake of clarity, only the lower halves of the error bars are shown. Lines are guide to the eye.

surprising as the latter composition should exhibit coexistence of ordered gel and disordered domains until above the T_m of the mixture, with NR12S partitioning in both lipid phases. Fluorescence bulk measurements can probe only the average behavior of the fluorophore and will therefore report in general a less polar environment compared to a homogenous liquid bilayer (since ordered domains are less hydrated than a disordered liquid phase [27]). Increasing the temperature weakens the lipid–lipid (packing) interactions for all lipid compositions and, in particular, causes the melting of the ordered domains in the bSM:DOPC mixture. In both cases, as a consequence of the increased distance between the polar heads of the lipids, more water molecules can interact with the polar heads and the lipid backbone, thus increasing membrane hydration. This effect is evident in the red-shift of the emission spectrum of NR12S for both lipid composition at higher temperature, with the values measured for the bSM:DOPC SUVs approaching those of the SOPC vesicles. Also, in correspondence of the T_m of the bSM:DOPC bilayers, a change in slope of the corresponding curves can be observed: below the T_m, the hydration sensed by the probe is almost constant; above the T_m, the bilayer becomes progressively more hydrated as temperature increases. A similar behavior was observed for different lipid compositions, i.e. 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine, 1,2-distearoyl-*sn*-glycero-3-phosphocholine and bSM (data not shown).

However, the most striking feature of the data presented in Fig. 3B is the difference observed between the inner and outer leaflet of SUVs of each lipid mixture. Both for SOPC and for bSM:DOPC SUVs in fact, it is evident that the NR12S emission peak for the inner leaflet is ~5 nm lower than the one measured for the outer leaflet of the same SUVs at every temperature. A similar difference was observed for several lipid compositions (see below), when using a different vesicle preparation method (i.e. sonication, data not shown) and with a different inner leaflet labeling procedure, i.e. bleaching the outer leaflet NR12S using sodium dithionite (data not shown). At this regard, it is worth noting that the sodium dithionite inner leaflet labeling approach could be used only at room temperature: increasing temperature caused a strong decrease in NR12S fluorescence, probably due to increased permeation of sodium dithionite into the vesicle lumens.

The difference in the emission spectrum on NR12S in the inner and in the outer leaflet of SUVs is ascribed to the high curvature of these bilayers: in the inner leaflet the lipid head groups are closer together (i.e. they have a higher surface density) and less hydrated. The opposite is observed in the outer leaflet, where the positive-curvature bending of the monolayer creates packing defects that bring the polar heads farther apart, increasing their solvation. The effect of curved geometry on the packing of lipids has been object of early work [15] but, so far, the outer leaflet could be compared only to the bilayer as a whole, since selective labeling of the inner leaflet was not easily achievable. Further studies concluded that curvature affected the mobility of the dye microenvironment in the shallow regions of the outer leaflet of small vesicles [14]. The general idea of asymmetry in the structure of curved bilayer was confirmed by X-ray scattering data [28], but direct information about the increased packing density in the inner leaflet could be obtained only by computer-simulation studies [16]. In that case, for example, the area per lipid in the inner leaflet of DPPC liposomes was reported to be 60% of that in the outer leaflet. The present study shows, for the first time, a direct experimental comparison between the packing/hydration properties of the inner and outer leaflet. It should be noted that we cannot rule out that the response of the probe to changes in headgroup packing could partly reflect a change in the depth of the fluorescent group in the membrane. If looser headgroup packing resulted in a shallower NR12S depth within the bilayer, that would also result in red-shifted fluorescence. However, this seems unlikely, as looser packing would be expected to allow NR12S to penetrate deeper into the bilayer. In any case, the underlying phenomenon, namely differential headgroup packing, is the same.

In order to determine whether the differential hydration depends on lipid shape, SUVs made of phosphatidylcholines (PC) with different chain lengths were analyzed at room temperature. Fig. 4 shows the difference between the NR12S peak emission in the outer and inner leaflet as a function of the number of carbon atoms in the acyl chains of the lipids. All the examined lipids in this case had a PC polar head and identical acyl chains in the *sn*-1 and -2 positions, with a *cis* double bond near the center of the acyl chains. Previous studies proposed that, in small highly curved vesicles, lipids with intrinsic negative curvature (i.e. inverted cone-shape with small polar heads and voluminous hydrophobic moieties) would pack well in the inner leaflet [16,29]. Vice-versa, lipids with intrinsic positive curvature would prefer the outer leaflet, since they can fill the voids in the bent outer monolayer. In a similar way, lipids with shorter acyl chain might be able to prevent the packing defects in the outer leaflet, thus decreasing the hydration of the outer leaflet and making it more similar to the inner monolayer. In line with this view, we observed a decrease of the differential hydration between the leaflets for SUVs made of short-chain lipids. As an alternative explanation for these data, bilayers made of short-chain lipids (i.e. thinner bilayers) have a relatively small bending rigidity [30]. This would allow such membranes to release the mechanical stress associated with the high curvature via local thermal fluctuations, thus decreasing also the curvature-induced differential hydration. Control experiments exclude a third possibility: that the larger difference between inner and outer leaflets observed for thicker bilayers (i.e. composed of lipids with longer acyl chains) may be caused by a smaller vesicle size. In fact, the mean vesicle radius increased with increasing acyl chain length from ca. 30 nm (diC14:1 PC) to ca. 50 nm (di C24:1 PC). The implicit assumption motivating this control is that if vesicle size is strictly connected to the curvature of the bilayer, vesicles with large size (i.e. with a curvature small compared to the size of a lipid) should not exhibit a trans-bilayer asymmetry in terms of leaflet properties. It was in fact previously reported that the outer leaflet of vesicles with a radius of 125 nm is less hydrated compared to 11 nm radius vesicles [15]. In order to confirm the relation between size and hydration, SOPC vesicles with different sizes were prepared. The NR12S was in either the inner or the outer leaflet at room temperature. Fig. 5 shows the shift between the emission peaks in the inner and outer leaflet and its dependence on the size of the vesicles.

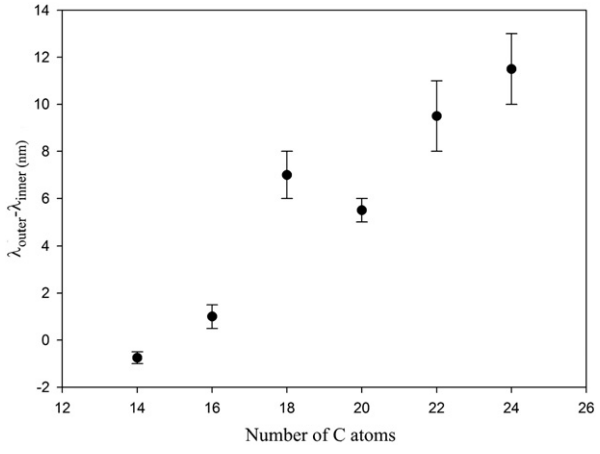

Fig. 4. Differential hydration between inner and outer leaflets depends on lipid chain length. The difference between the emission peak wavelengths of NR12S in the outer leaflet and in the inner leaflet is measured in SUVs composed of synthetic PC lipids having in both *sn*-1 and -2 positions identical acyl chains with different number of C atoms and a double *cis* bond in each acyl chain. The error bars represent the average deviations of the values measured in independent duplicate experiments. From shortest to longest acyl chains, the lipids used were dimyristoleoyl PC (di C14:1 PC), dipalmitoleoyl PC (di C16:1 PC), DOPC (di C18:1 PC), dieicosanoyl PC (di C20:1 PC), dierucoyl PC (di C22:1 PC), and dinervonoyl PC (di C24:1 PC).

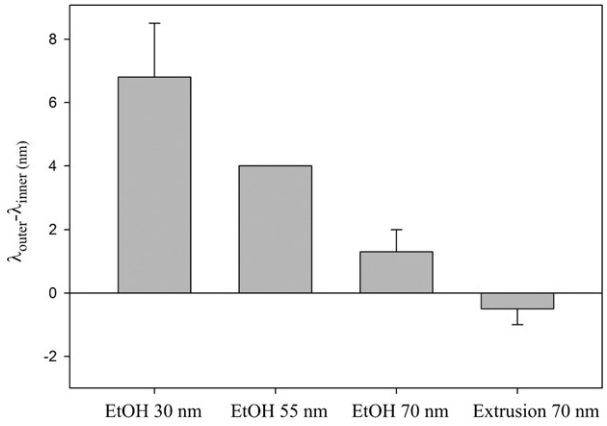

Fig. 5. Differential hydration of inner and outer leaflet depends on vesicle size. The difference between the maximum emission wavelength of NR12S in the outer and in the inner leaflets of SOPC vesicles is shown as a function of vesicle preparation method (EtOH = ethanol dilution, Extrusion = extrusion through 100 nm pore membranes) and approximate average size as calculated via DLS. Error bars represent the average deviation between duplicate experiments.

Vesicles with varying sizes were obtained using two different methods: extrusion and ethanol dilution. Extrusion through 100 nm pores yielded vesicles with average radius of 71 ± 4 nm. On the other hand, the size of the vesicles obtained via the ethanol dilution method depends on the concentration of the lipids in ethanol [31]. For all the experiments described above, only a concentration of 5 μmol/mL was used and that resulted in vesicles with average radius of 32 ± 3 nm. In order to obtain vesicles with intermediate size, two additional concentrations (i.e. 20 and 40 μmol/mL) were also used. In this case, the average vesicle radii were 54 ± 6 and 70 ± 11 nm respectively. The results clearly show that increasing the size of the liposomes causes a reduction of the difference between the emission peak of NR12S in the inner and outer leaflets. Such a reduction is due both to a size-dependent blue shift of the emission peak of NR12S in the outer leaflet and a concomitant red shift of the emission peak of NR12S in the inner leaflet, although the former contribution seems more significant (see Fig. S3). Liposomes with an average radius of ~70 nm appear to be almost completely symmetric in terms of their lipid packing, and this is at least largely independent of the vesicle preparation method used. It appears clear then that the leaflet-associated shift in NR12S fluorescence emission is due to the high curvature of the bilayer and the consequent decreased packing of lipid polar heads in the outer leaflet compared to a non-curved bilayer. We also observed that the high curvature caused an increase in the density of the lipid polar heads in the inner leaflet but, in line with simulation studies [16], this effect is small compared to that on the outer leaflet. Increasing the size of the vesicles releases the curvature of the membrane and restores its trans-bilayer symmetry.

4. Conclusions

This paper reports a novel simple approach useful for the leaflet-targeted labeling of model membranes. The protocol could easily be modified to use serum albumin or other cyclodextrins, with the best choice of extracting agent being made based on binding specificity and spectroscopic interference, which would depend on the specific experiment. The method is based on the extraction of the dye selectively from the outer leaflet of vesicles using sequentially low concentrations of mβCD and a quick gel chromatography step. Suitable fluorescence dyes include – but are not limited to – TMA-DPH and NR12S, which can be used to investigate the structural and dynamic properties of the membrane. The method might be easily applied to monitor the separate behavior of the leaflets, even as a function of temperature, in complex asymmetric model vesicles [1]. Its application could also be extended to supported lipid bilayers to monitor, for example, the lateral organization of a fluorescent probe exclusively in the surface-proximal leaflet. In that case, the chromatography step would be simply substituted by a thorough washing of the sample.

This method was then used to evaluate differences between the two monolayers of SUVs using the leaflet-targeted labeling with NR12S. The small dimensions of the vesicles (radius <~70 nm) and the consequent high curvature of the bilayer cause the lipids in the outer leaflet to be further away from each other compared to those in the inner leaflet. As a consequence, the NR12S in the inner leaflet senses a more hydrophobic, less hydrated environment than that in the outer leaflet. The difference between the two leaflets becomes smaller in presence of lipids with short acyl chains or when the vesicles are larger. Both observations point to the importance of the local curvature in influencing the properties of the single monolayers of the membrane, perhaps also in a biological context. It is well known, in fact, that high curvature can be observed in some natural biomembranes and examples include synaptic vesicles, tubules or viral envelopes [10]. Therefore, these observations may have biological significance. Furthermore, high curvature of the membrane can influence lipid–protein interactions [11,12] and it is possible that the underlying molecular mechanisms include those described in this work.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.bbamem.2012.02.005.

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