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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