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BILAYER ASYMMETRY IN LYSOPHOSPHATIDYLCHOLINE/CHOLESTEROL (1:1)VESICLES. A PHOSPHORUS-31 NMR STUDY

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Prolonged sonication (3 h) of equimolar amounts of lysophosphatidylcholine (lysoPC) and cholesterol (chol) produces small unilamellar vesicles. Phosphorus-31 NMR (32.20 MHz) of the vesicles gave rise to a single peak (40.5 ppm) which was split upon addition of lanthanide ions. An additional, more intense signal appeared downfield near 51.0 ppm due to 2.4 mM Pr^{3+}, upfield near 34.3 ppm due to 5 mM Yb^{3+}. The more intense signals responsive to paramagnetic ions were assigned to lysoPC located in the outer vesicle leaflet; the signal not shifted by the ions was assigned to inside lysoPC. Based on peak intensities, an outside-to-inside lysoPC ratio ($R_{o/i}$) of 6.5-6.6 was determined. Essentially the same $R_{o/i}$ values (6.6-6.8) were obtained when Pr^{3+} was present only in the vesicle interior or when Pr^{3+} was on the inside and Pr^{3+} and Yb^{3+} were on the outside. Ion leakage did not occur. Our data demonstrate that lysoPC/chol (1:1) vesicles are drastically asymmetric and that lysoPC shows a distinct preference for the outer bilayer leaflet.

Lysophosphatidylcholine (lysoPC) by itself produces spherical micelles in aqueous medium (1,2), an arrangement that is quite consistent with the inverted cone shape of the lysoPC molecule (3). In contrast, equimolar mixtures of lysoPC and cholesterol (chol) were early recognized to form "myelinic figures as does lecithin alone" (4). X-ray diffraction (5), electron spin resonance (6) and electron microscopy (6) have provided evidence that lysoPC and cholesterol combine in stoichiometric proportion (7). A cylindrical shape has been proposed for the lysoPC/chol (1:1) molecular complex (5) consistent with the observation that equimolar mixtures of lysoPC and cholesterol form lamellar structures in aqueous medium (4-10).

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Abbreviations: lysoPC, lysophosphatidylcholine; PC, phosphatidylcholine; chol, cholesterol; SUV, small unilamellar vesicles; NMR, nuclear magnetic resonance; TLC, thin-layer chromatography.

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In the present study, we have prepared small, unilamellar vesicles (SUV) of uniform size (30 nm diameter) by prolonged sonication of equimolar amounts of lysoPC and cholesterol and we have measured the outside/inside distribution ($R_{o/i}$) of lysoPC using phosphorus-31 NMR and lanthanide shift reagents.

MATERIALS AND METHODS

1-O-Hexadecanoyl-sn-glycero-3-phosphocholine (lysoPC) was obtained from Avanti Polar Lipids (Birmingham, AL) and was pure as judged by thin-layer chromatography (TLC; developing solvent A, $CHCl_3/CH_3OH/H_2O$, 65:35:8, by vol). LysoPC was characterized by ^{13}C NMR (11) using $CDCl_3/CD_3OD/D_2O$, 50:50:15 (by vol) as solvent (11,12). Cholesterol was purchased from Nu-Chek Prep (Elysian, MN) and was shown to be pure by TLC (developing solvent B, hexane/diethyl ether/acetic acid, 70:30:1, by vol). Deuterated solvents ($CDCl_3$, 99.8% d; CD_3OD, 99.5% d; D_2O, 99.8% d) were from KOR Isotopes (Cambridge, MA). Praseodymium chloride (99.9%) and ytterbium chloride (99.9%) were from Aldrich Chemical Company (Milwaukee, WI). Stock solutions of the shift reagents were prepared in D_2O and small aliquots (20 µl) were directly added to the lipid dispersions.

Micellar solutions of lysoPC were prepared by vortexing 50 µmoles of lysoPC in 2.5 ml of 10 mM Tris/HCl buffer in D_2O containing 0.15 M KCl until the solutions appeared clear.

Small unilamellar lysoPC/chol vesicles were prepared from equimolar mixtures of lysoPC and chol (50 µmoles each). The lipids were dissolved in $CHCl_3/CH_3OH$ (1:1) using a 25-ml round-bottom flask, the solvent was removed at room temperature on a rotary evaporator, and the sample was dried in vacuo for at least 6 h. The lipid film was dispersed by vortexing in 2.5 ml of 10 mM Tris/HCl buffer in D_2O containing 0.15 M KCl, and the dispersion was sonicated under nitrogen for 3 h in an ice-bath (Branson tip sonifier, Model 350; output setting, 4). Debris was removed at 105,000 x g for 1 h using a Beckman Model L5-75 ultracentrifuge. Aliquots of the sonicated dispersions, before and after NMR, were routinely analyzed to assure the integrity of the lipid components. For this purpose, lipids were extracted (13) and then analyzed by TLC using developing solvents A and B (see above).

Electron microscopy was used to assess the homogeneity of the preparations and to measure vesicle size. Samples were placed on an inverted 200-mesh carbon-coated copper grid, negative-stained with 2% aqueous phosphotungstic acid and examined under a JEOL 100S electron microscope at 60 KV. Micrographs were taken at magnifications of up to 40,000x and then enlarged to up to 100,000x.

Dialysis was done using Spectrapor membrane tubing (15.9 mm diameter, up to 14,000 Dalton; Spectrum Medical Industries, Inc., Los Angeles, CA) to remove "outside" Pr^{3+}. Approximately 2.5 ml of the vesicle preparation was placed into the tubing, the tubing was clamped at both ends, and the sample was dialyzed twice for 12 h by immersing into 200 ml of stirred D_2O buffer.

Phosphorus-31 NMR spectra were recorded at 32.203 MHz on a Varian FT-80A Pulse Fourier Transform instrument equipped with a broadband probe. Spectra were measured at 37±1°C on vesicle samples containing about 20 µmoles of lysoPC/ml (10 mm o.d. sample tube, 5,000 transients, 4 KHz sweepwidth, 8 K data points). For measuring outside/inside lysoPC ratios ($R_{o/i}$), spectra were recorded with and without proton noise decoupling; identical data were obtained by either mode. Spin-lattice relaxation (T_1) data were measured by

the inversion-recovery method using a pulse delay greater than 5 T_1. All measurements were done at least in duplicate.

RESULTS AND DISCUSSION

Lysophosphatidylcholine forms spherical micelles in aqueous medium. When 1-O-hexadecanoyl-sn-glycero-3-phosphocholine was dispersed in Tris/HCl buffer (20 μmol/ml), ^{31}P NMR showed a sharp, isotropic signal near 40.5 ppm ($\nu_{1/2}$ 1.8 Hz; T_2^* 0.16 s; T_1 1.98 s). Upon addition of 2.4 mM Pr^{3+}, the signal was shifted downfield by 10.5 ppm. A shift of the entire phosphorus signal due to Pr^{3+} is consistent with the structure of a lysoPC micelle in which all phospholipid polar headgroups are accessible to the ions.

Lysophosphatidylcholine in the presence of equimolar amounts of cholesterol forms lamellar structures. Yet, the type of lamellae formed is very much dependent on the mode of dispersion. One-hour sonication of lysoPC/chol (1:1) in Tris/HCl buffer produced extended, stack-like lamellae about 4 nm thick. In contrast, sonication of lysoPC/chol (20 μmol each/ml) for 3 h yielded unilamellar, spherical vesicles which were uniform in size and averaged 30 nm in diameter. The structures were characterized by electron microscopy after negative-staining. ^{31}P NMR of lysoPC/chol (1:1) SUV gave rise to a single peak near 40.5 ppm (Fig. 1A, bottom). The signal was somewhat broader ($\nu_{1/2}$ 6.3 Hz; T_2^* 0.05 s) than that of lysoPC micelles and its spin-lattice relaxation was somewhat faster (T_1 0.65 s) indicating that average lysoPC headgroup motion in lysoPC/chol (1:1) unilamellar vesicles is slower than it is in micellar lysoPC.

Upon addition of lanthanide ions to lysoPC/chol (1:1) vesicles, the phosphorus signal was split. Next to the 40.5 ppm signal, a more intense peak appeared downfield near 51.0 ppm when 2.4 mM Pr^{3+} was added (Fig. 1A, top). Upon addition of Yb^{3+} (5 mM) to lysoPC/chol (1:1) SUV, the more intense signal appeared upfield near 34.3 ppm (Fig. 1C, top). The shifts were dose-dependent (up to 12 mM tested). The more intense signals responsive to paramagnetic ions were assigned to lysoPC located in the outer vesicle leaflet; the signal not shifted by the ions was assigned to lysoPC located in the inner vesicle

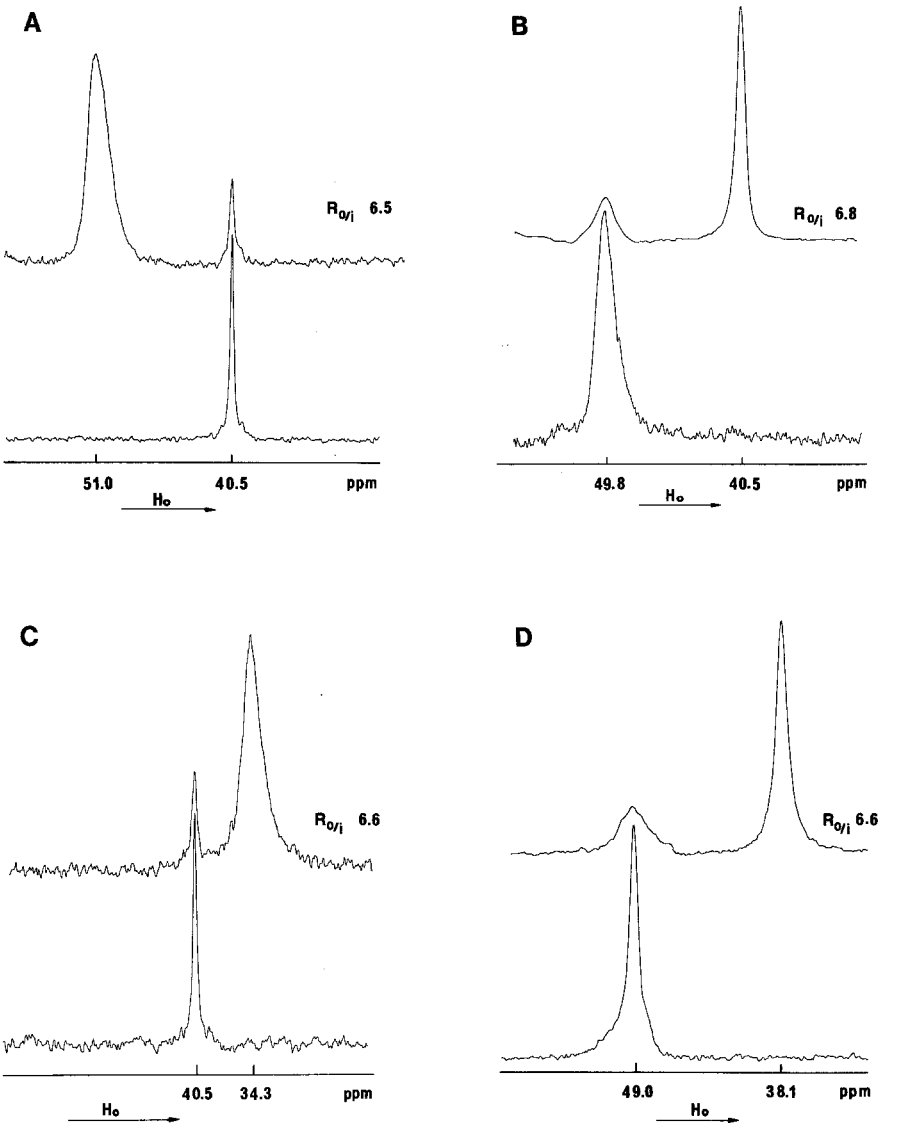

Figure 1. Distribution of lysoPC between the outer and inner bilayer leaflet ($R_{o/i}$) of lysoPC/chol (1:1) vesicles as determined by phosphorus-31 NMR (32.20 MHz) in the presence of lanthanide shift reagents. $R_{o/i}$ values were measured by integration of respective peak areas. (A) LysoPC/chol (1:1) vesicles before (bottom) and after addition of 2.4 mM Pr^{3+} (top). The signal near 51.0 ppm represents lysoPC phosphorus shifted by Pr^{3+} (outside lysoPC); the signal near 40.5 ppm is due to lysoPC in the absence of Pr^{3+} (inside lysoPC in top spectrum; $R_{o/i}$ 6.5). (B) LysoPC/chol (1:1) vesicles prepared in the presence of 2.4 mM Pr^{3+} before (bottom) and after removal of outside ions by dialysis (top). The signal near 49.8 ppm represents lysoPC phosphorus shifted by Pr^{3+}; the signal near 40.5 ppm (top) is due to lysoPC in the absence of Pr^{3+} (outside lysoPC; $R_{o/i}$ 6.8). (C) LysoPC/chol (1:1) vesicles before (bottom) and after addition of 5 mM Yb^{3+} (top). The signal near 34.3 ppm represents lysoPC phosphorus shifted by Yb^{3+} (outside lysoPC); the signal near 40.5 ppm is due to lysoPC in the absence of Yb^{3+} (inside lysoPC in top spectrum; $R_{o/i}$ 6.6). (D) LysoPC/chol (1:1) vesicles prepared in the presence of Pr^{3+} before (bottom) and after addition of 5 mM Yb^{3+} (top). The signal near 49.0 ppm represents lysoPC phosphorus shifted by Pr^{3+} (inside lysoPC in top spectrum); the signal near 38.1 ppm represents lysoPC shifted by Pr^{3+} and Yb^{3+} (outside lysoPC; $R_{o/i}$ 6.6).

shell. Based on integrated peak intensities (measured under proton noise-decoupled and under proton-coupled conditions) with Pr^{3+} as shift reagent (Fig. 1A), an outside-to-inside lysoPC ratio ($R_{o/i}$) of 6.5 was determined. With Yb^{3+} as paramagnetic ion, $R_{o/i}$ was 6.6 (Fig. 1C). No spectral changes occurred when the vesicle preparations were kept at room temperature for extended periods of time (up to five days) indicating that small unilamellar lysoPC/chol (1:1) vesicles are, for practical purposes, impermeable to lanthanide ions.

We considered it desirable to provide complementary experimental proof for the pronounced preference of the lysophospholipid for the outer vesicle shell. Therefore, lysoPC/chol (1:1) was sonicated in the presence of Pr^{3+} and outside ions were removed by dialysis. As one would expect, the vesicles having Pr^{3+} at the inside and at the outside (Fig. 1B, bottom) produced a single downfield peak (49.8 ppm). However, when outside ions were removed by dialysis (Fig. 1B, top), an additional more intense signal occurred upfield at 40.5 ppm characteristic of lysoPC in the absence of paramagnetic ions. The less intense downfield signal (49.8 ppm) was assigned to inside lysoPC, the upfield signal to outside lysoPC. Based on respective peak intensities, an $R_{o/i}$ value of 6.8 was determined.

Finally, Yb^{3+} (5 mM) was added to lysoPC/chol (1:1) vesicles which had been sonicated in the presence of Pr^{3+} (2.4 mM). This gave rise to a distinct downfield signal for inside lysoPC shifted to 49.0 ppm by Pr^{3+} and to an upfield signal for outside lysoPC shifted to 38.1 ppm due to the additive effects of both paramagnetc ions (Fig. 1D). The outside-to-inside lysoPC ratio determined by this method was 6.6.

Our data clearly show that small unilamellar lysoPC/chol (1:1) vesicles are drastically asymmetric in respect to lysoPC which has a distinct preference for the outer bilayer leaflet. The outside-to-inside phospholipid ratio of 6.7 $\pm$ 0.2 which we determined for small unilamellar lysoPC/chol (1:1) vesicles is much greater than that ($R_{o/i}$ 2.2 $\pm$ 0.6) reported for phosphatidylcholine (PC) vesicles (14-16) or PC/chol (1:1) vesicles (16,17).

On the other hand, PC vesicles containing a small percentage of lysoPC (5-15%) have previously been shown to preferentially accommodate the lysophospholipid in the outer vesicle shell (16,18). The distinct preference of lysoPC for the outer bilayer leaflet of lysoPC/chol (1:1) vesicles is likely to reflect the geometric requirements of the lysoPC molecule.

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