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Solute distributions and trapping efficiencies observed in freeze-thawed multilamellar vesicles

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It has recently been observed (Gruner, Lenk, Janoff and Ostro (1985) Biochemistry, in the press) that mechanical dispersion of dry lipid in an aqueous buffer to form multilamellar vesicle (MLV) systems does not result in equilibrium trans-membrane distributions of solute. In particular, the entrapped buffer exhibits reduced solute concentrations. Here we demonstrate that egg phosphatidylcholine MLV systems dispersed in the presence of Mn^{2+} also exhibit non-equilibrium solute distributions, and that repetitive freeze-thawing cycles can remove such solute heterogeneity. Further, the resulting freeze-thawed MLVs exhibit dramatically enhanced trapped volumes and trapping efficiencies. At 400 mg phospholipid per ml, for example, the trapping efficiencies can be as high as 90%. This is associated with a remarkable change in MLV morphology where large inter-bilayer separations are commonly observed.

Liposomes or multilamellar vesicles (MLVs) are usually formed by mechanical dispersion of dried lipid in an aqueous buffer [1]. It is commonly assumed that this procedure results in an equilibrium interlamellar distribution of solutes present in the buffer. However, it has recently been demonstrated [2] that the trapped buffer may have reduced solute concentrations resulting in osmotic imbalances between exterior and interior environments. Equilibrium solute distributions can be achieved by techniques involving dispersion of the lipid in mixtures of organic solvent and aqueous buffer, where the organic solvent is subsequently removed under reduced pressure [2].

In this communication we demonstrate that effects corresponding to such non-equilibrium ion distributions are also observed for lipid dispersed in the presence of Mn^{2+}. It is shown that repetitive freeze-thaw cycles can result in equilibrium transmembrane Mn^{2+} concentrations, and that such freeze-thaw cycles result in an appreciable increase in the trapped volume and trapping efficiencies. This is associated with remarkable changes in liposome morphology as detected by freeze-fracture.

The first series of experiments were aimed at demonstrating that non-equilibrium transmembrane distributions of the paramagnetic cation Mn^{2+} are obtained on dispersion of egg phosphatidylcholine in an aqueous buffer containing the cation. Mn^{2+} is a 'broadening' agent which quenches the ^{31}P-NMR signal arising from available phospholipids. As shown in Fig. 1(A), when the buffer contains no Mn^{2+}, the usual 'bilayer' [3] ^{31}P-NMR lineshape is observed. Alternatively, when the lipid is dispersed in a buffer containing 0.5 mM Mn^{2+}, a slightly broadened bilayer ^{31}P-NMR lineshape is observed, with a much broader underlying component (Fig. 1(B)). That the Mn^{2+} is not equally accessible to all phospholipids in this dispersion is indicated by the spectrum of Fig. 1(C) obtained after subjecting the sample of Fig. 1(B) to five freeze-thaw cycles. This broad feature-

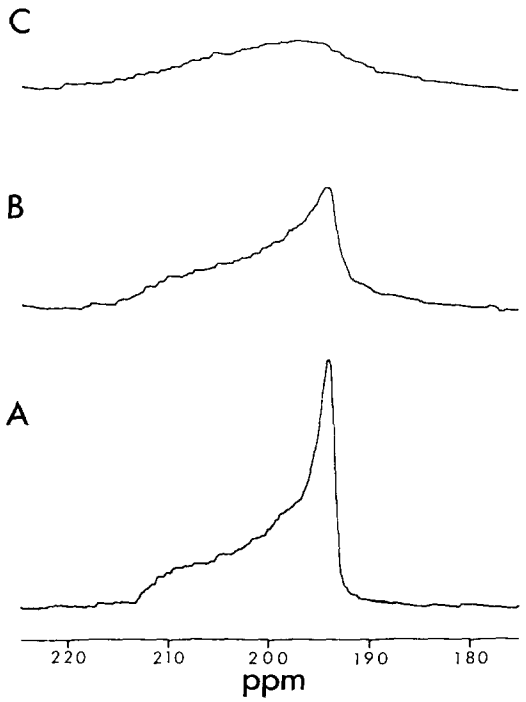

Fig. 1. 81.0 MHz ^{31}P-NMR spectra of egg phosphatidylcholine MLVs dispersed (a) in the absence of Mn^{2+} and (b) in the presence of 0.5 mM Mn^{2+}. The spectrum of part (c) was obtained from the MLVs dispersed in the presence of 0.5 mM Mn^{2+} which were subsequently subjected to five freeze-thaw cycles. The phosphatidylcholine was isolated from egg yolks employing standard procedures and was more than 99% pure as indicated by thin-layer chromatography. The MLVs were prepared by adding 2 ml of buffer (150 mM NaCl, 20 mM Hepes, pH 7.5) to 200 mg of lipid. This dispersion was vortexed intermittently (2 min vortexing, 3 min interval) over 20 min. The ^{31}P-NMR spectra were collected at 20°C employing a Bruker WP 200 spectrometer employing a 20 kHz sweep width, 2-s interpulse delay and broad band proton decoupling. The freeze-thaw cycles were performed employing liquid N_2.

less spectrum is of similar intensity and width as that obtained on addition of Triton X-100 (10%) to the sample of Fig. 1(B) (data not shown).

The requirement for a number of freeze-thaw cycles to obtain equilibrium distributions of Mn^{2+} is indicated in Fig. 2. This shows the ^{31}P-NMR signal intensity (integrated over the range 180–220 ppm) as a function of freeze-thaw cycles for samples initially dispersed in the presence of 0.5 mM and 5 mM Mn^{2+}. Three or more freeze-thaw steps are required to reduce the signal intensity to levels obtained on solubilization of the sample in Triton, where a micellar organization presumably results in equal availability of phospholipid to Mn^{2+}.

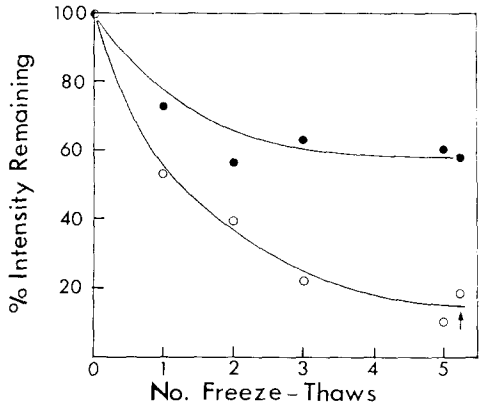

Fig. 2. Influence of the number of freeze-thaw cycles on the ^{31}P-NMR signal intensity of egg phosphatidylcholine MLV systems prepared in the presence of 0.5 mM Mn^{2+} (●) or 5 mM Mn^{2+} (○). For details of sample preparation, see legend to Fig. 1. The signal intensities were obtained by cutting and weighing the normalized spectra. The arrow indicates the signal intensity obtained after addition of sufficient Triton X-100 (10%, w/v) to solubilize the sample.

The freeze-thaw protocol results in a dramatic change in liposome morphology as indicated by the freeze-fracture electron micrographs of Fig. 3. Before freeze-thawing the samples exhibit the tightly packed 'onion skin' arrangements of concentric bilayers normally associated with liposomal systems. After five freeze-thaw steps, however, new structures are observed (Fig. 3(B)) where the interlamellar spaces are much increased, and where closed lamellar systems can be intercalated between bilayers. It must be emphasized that the structures of Fig. 3(B) are representative of the entire fracture face. In particular, no tightly packed systems similar to those of Fig. 3(A) were detected.

The electron micrographs of frozen and thawed MLVs (FATMLVs) clearly suggest that the trapped volume should be significantly greater than for their non-freeze-thawed counterparts. Thus the trapped volumes (per μmol phospholipid) were determined employing ^{22}Na as an aqueous marker. As shown in Table I, the FATMLVs exhibited trapped volumes more than an order of magnitude larger than non-freeze thawed MLVs (5.27 μl/μmol phospholipid as compared to 0.47 μl/μmol phospholipid at 100 mg/ml phosphatidylcholine concentrations). Further, remarkably high trapping efficiencies (measured as the percentage of the total aqueous marker which was entrapped) of 66% were achieved at 100 mg/ml lipid, which

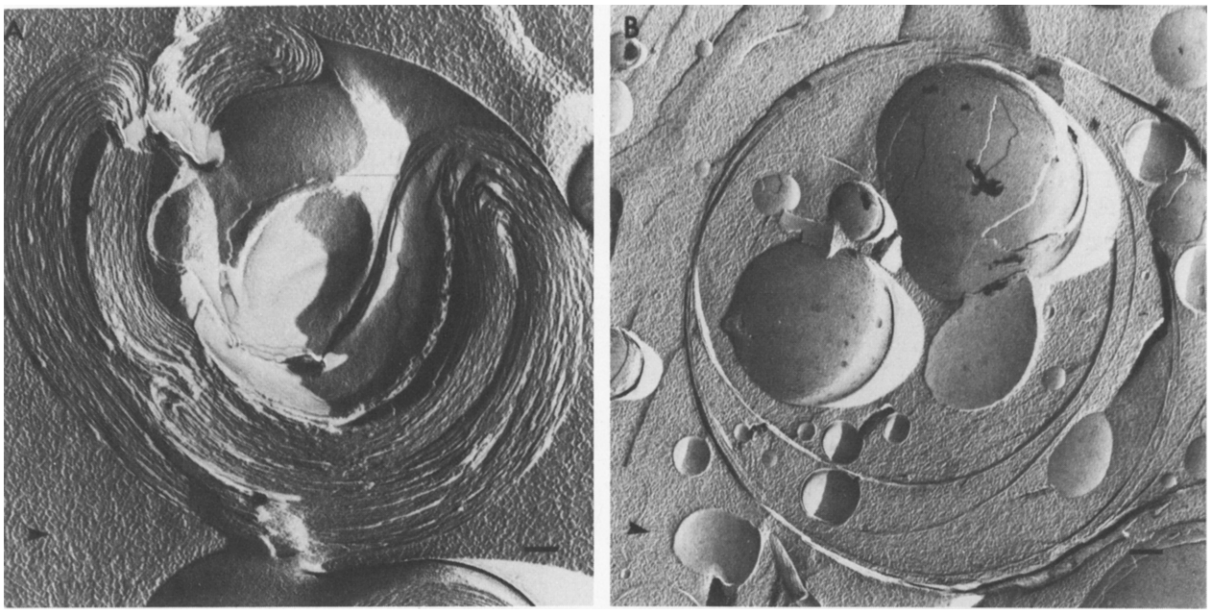

Fig. 3. Freeze-fracture electron micrographs of MLVs before (A) and after (B) five freeze-thaw cycles. The phospholipid concentration was 100 mg/ml. For other details of sample preparation, see the legend to Fig. 1. The arrow indicates the direction of shadowing and the bar represents 140 nm.

TABLE I

TRAPPED VOLUMES AND TRAPPING EFFICIENCIES OF FROZEN AND THAWED MLVs (FATMLVs) AT VARIOUS LIPID CONCENTRATIONS

Sample	Lipid concn. (mg/ml)	Trapped volume (μl/μmol lipid)	Trapping efficiency (%)
MLV	100	0.47 ± 0.03	5.8
FATMLV	50	5.02 ± 0.04	31.3
FATMLV	100	5.27 ± 0.17	65.9
FATMLV	200	3.07 ± 0.05	76.7
FATMLV	400	1.77 ± 0.09	88.6

Egg PC MLVs were prepared as described in the legend to Fig. 1. The FATMLVs were prepared employing five freeze-thaw cycles. Trapped volumes and trapping efficiencies were determined by including ^{22}Na (1 μCi/ml) in the buffer in which the lipid was dispersed. After the MLVs or FATMLVs were formed, aliquots were assayed for lipid phosphorus and ^{22}Na. Untrapped ^{22}Na was then removed by washing the MLVs in ^{22}Na-free buffer employing low speed centrifugation. This procedure was repeated until supernatant counts were reduced to background levels. Aliquots of the pellet were then assayed for ^{22}Na and lipid phosphorus. Trapping efficiencies were calculated as the ratio of the cpm per μmol lipid after and before removal of untrapped ^{22}Na. Standard deviations were calculated from results obtained from three samples.

could be increased to 88.6% at 400 mg/ml phosphatidylcholine (Table I). It may be noted that the trapped volume per μmol lipid decreases at the higher lipid concentrations, which can be attributed, at least in part, to the lack of available buffer.

Two important aspects of these results concern the transbilayer distributions of solute in MLV systems and their utility in applications requiring the efficient capture of water-soluble compounds. First, the demonstration that a dispersion of phosphatidylcholine in an $MnCl_2$ buffer does not result in equilibrium interlamellar concentrations of Mn^{2+} supports previous results indicating low solute concentrations in the aqueous compartments of MLV systems [1]. This is clearly a finding of fundamental importance, and indicates that further processing (such as freeze-thawing) must be performed before all lipids of MLVs can be considered to experience the same environment. This is in accord with previous observations [4,5] that freeze-thaw cycles result in more homogeneous MLV-local anaesthetic dispersions. In the absence of such processing, the resulting ion gradients may lead to membrane potentials, transbilayer pH gradients and deformations due to osmotic forces.

The second point concerns the use of MLV

systems in applications such as drug delivery. Previously, the low trapped volume and trapping efficiency of MLV systems has presented difficulties, leading to development of other procedures for making liposomal systems, many of which involve the use of organic solvents or detergents [6]. The freeze-thaw protocol offers an attractive alternative, in that the procedure is simple, relatively gentle, and allows very high trapping efficiencies. The fact that this technique does not require the use of potentially toxic solubilizing agents is an important additional benefit.

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