Multilayered Vesicles Prepared by Reverse-Phase Evaporation: Liposome Structure and Optimum Solute Entrapment

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ABSTRACT: Liposome structure and solute entrapment in multilayered vesicles (MLVs) prepared by reverse-phase evaporation (REV) were studied. MLV-REV vesicles prepared from ether/water emulsions have high entrapment. Entrapment depends on drug, drug concentration, lipid, lipid concentration, and the container used to prepare the vesicles. By use of 300 μL of aqueous phase and 100 mg of phosphatidylcholine (PC), vesicles prepared in a test tube 25 mm × 175 mm have higher entrapment than vesicles prepared in a 100-mL round-bottom or pear-shaped flask. By use of a test tube, 100 mg of PC, and 300 μL of aqueous phase containing sucrose (1–50 mg/mL), >90% sucrose entrapment was obtained. Increasing lipid content to 150 mg of PC decreased entrapment to ~80%. Neutral PC MLV-REV vesicles have optimum entrapment. Mixing negatively charged lipids or cholesterol (CH) with PC to make MLV-REV vesicles results in decreased entrapment compared to using only PC. Preparing vesicles with the solid lipid dipalmitoylphosphatidylcholine (DPPC) or DPPC/CH mixtures ($0 \leq$ mol % CH ≤ 50) results in ~30–40% entrapment when diethyl ether is used to make the MLV-REV emulsion. Substituting diisopropyl ether for diethyl ether and heating the MLV-REV emulsion during vesicle formation generate DPPC/CH vesicles that entrap 60% of added solutes. The high entrapment found for MLV vesicles prepared from water/organic solvent emulsions depends on maintaining a core during the process of liposome formation. A method to calculate the fraction of water residing in the liposomes' core is presented and used to compare multilayered vesicles prepared by different processes. X-ray diffraction data demonstrate that a heterogeneous distribution of lipid may exist in multilayered vesicles prepared by the REV process.

Lipids dispersed in excess water assemble into spherical lipid particles called liposomes. Methods to disperse lipids can generate either single or multilayered liposomes. The REV[1] procedure is experimentally the preferred method for preparing single-layered vesicles if high solute entrapment is desirable (Szoka & Papahadjopoulos, 1978; Szoka et al., 1980). Recently the REV method has been modified to form two types of multilayered vesicles; MLV-REV vesicles (Pidgeon et al., 1986) and SPLV vesicles (Gruner et al., 1985). MLV-REV vesicles have entrapment values near 65% and SPLVs of 35–40%. We have further increased solute entrapment of the MLV-REV process and characterized the structure of MLV-REV vesicles. The structure of MLV-REV vesicles is compared to the structure of multilayered vesicles prepared by the SPLV process.

Structure elucidation of the vesicles was accomplished by comparing the known aqueous volume trapped by planar bilayers (Small, 1967; Ladbrooke et al., 1968; LeNeveu et al., 1977; Lis et al., 1982; Rand et al., 1981) to the aqueous trapped volume of liposomes. Our X-ray diffraction measurements demonstrate that long spacing in MLV-REV and SPLV vesicles is similar to the long spacing of planar bilayers. Because of this, the interlamellar trapped volume of these liposomes is similar to the trapped volume in planar bilayers. When egg phosphatidylcholine is deposited on the floor of a container, planar bilayers exist when as little as 15 wt % water for head group hydration is added (Small, 1967). Increasing water content causes the bilayers to separate or swell to a maximum separation at ~50 wt % water [e.g., Lis et al. (1982), Ladbrooke et al. (1968), and Small (1967)]. Although 50 wt % water is imbibed by PC at maximum bilayer swelling, head group hydration water is unavailable for solute dissolution. For instance, 100 mg of PC imbibing 100 μL of water (50 wt %) contains 25 μL of hydration water and 75 μL of water available for solutes. Interlamellar trapped water in PC/water mixtures corresponds to a lamellar encapsulation ratio (E^r) of 1 μL of H_2O/μL of PC, or 0.75 μL of H_2O/μL of PC corrected for head group hydration water, which is unavailable to the aqueous space markers capable of measuring E^r. This represents the lower limit of liposomal trapped volume and occurs for vesicles with virtually all of the entrapped water

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[1] Abbreviations: MLV, multilayered vesicle; MLV-REV, MLV prepared by the reverse-phase evaporation method; SPLV, stable plurilamellar vesicle; REV, reverse-phase evaporation (a process used to make REV vesicles); PC, phosphatidylcholine; PS, phosphatidylserine; PA, dipalmitoylphosphatidic acid; PG, dipalmitoylphosphatidylglycerol; DPPC, dipalmitoylphosphatidylcholine; CH, cholesterol; PBS, phosphate-buffered saline; CPW, continuous-phase water; w/o, water/organic solvent; LSS, long-spacing signature; fw^{core}, trapped volume fraction residing in the liposomes' core; E^{ratio}, encapsulation ratio of the entire liposome population in microliters of water per microliter of lipid (includes lamellar water and core water); $fw^{lamellae}$, trapped volume fraction residing in the liposomes' lamellae; E^r_n, encapsulation ratio of an individual lamella; E^r, encapsulation ratio of all the lamellae; d_w, interlamellar water thickness; d_l, bilayer thickness; D_p, long spacing ($d_w + d_l$) in planar membranes; D_{MLV}, long spacing in multilayered liposomes.

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in the repeating lamellae instead of the liposomes' core. For liposome suspensions, aqueous space markers measure the encapsulation ratio E^{ratio} of the entire liposome population instead of only that of the lamellae. However, calculating E^{ratio} is not useful when optimum solute entrapment is desirable. E^{ratio} characterizes the vesicles after the vesicles are formed and reflects the carrying capacity of the liposome particles themselves. If $E^{ratio} = 1$, then 100 mg (or $\sim$100 μL) of PC liposomes will carry 100 μL of internal aqueous volume. The carrying capacity of the particles, however, does not reflect the efficiency of the process used to make the vesicles. Thus, our solute-trapping experiments utilized percent entrapped to characterize process variables, whereas E^{ratio} is used to elucidate liposomal structure. From experimental and computer-generated data we demonstrate that maintaining a large volume fraction of water in the liposomes' core during the REV process is necessary for high entrapment. Under these conditions, E^{ratio} exceeds the limiting value obtained for planar bilayers. Computer-generated data are given to compare the volume trapped in planar membranes to the volume trapped in curved membranes (liposomes) with identical long spacings.

We have partitioned the trapped volume in MLV-REV vesicles into the lamellar trapped volume and core volume. The fraction of water trapped in the repeating bilayers was calculated from $0.95E^{r}/E^{ratio}$, where E^{ratio} is the encapsulation ratio of the entire liposome population and therefore reflects the total trapped water. The factor 0.95 corrects for the small decrease in trapped volume elicited by liposomes that contain curved membranes (and finite bilayer numbers) compared to the trapped volume of planar membranes. The fraction of water trapped in the liposomes, core was calculated from $1 - 0.95E^{r}/E^{ratio}$.

Materials and Methods

Chemicals and Solutions. Egg L-α-phosphatidylcholine (PC), bovine brain phosphatidylserine (PS), and dipalmitoylphosphatidylcholine (DPPC) were purchased from Avanti Polar Lipids, Inc., Birmingham, AL. DL-α-Dipalmitoylphosphatidic acid (PA), dipalmitoyl-1-α-phosphatidyl-*dl*-glycerol (PG), and cholesterol (CH) were purchased from Sigma Chemical Co., St. Louis, MO. PA was extracted with 0.1 N HCl and stored in $CHCl_3$. Phospholipid purity was periodically checked by thin-layer chromatography on silica gel plates in $CHCl_3$–CH_3OH–H_2O (65:35:4). When lysolecithin or degradation products were observed, the lipid was not used.

Calcium- and magnesium-free Dulbecco's phosphate-buffered saline 10× (Grand Island Biological Co.) was diluted with sterile water for injection to make isotonic PBS. A stock solution of sucrose, 90 mg/mL (350 mOsm), was diluted with isotonic PBS to obtain sucrose solutions between 10 and 50 mg/mL. PBS–sucrose (1 mg/mL sucrose) was prepared by adding sucrose directly to PBS. Tobramycin sulfate (supplied by Eli Lilly and Co.; lot HO4291), 50 mg/mL, was prepared by dissolving 500 mg of tobramycin in 5 mL of sterile water followed by $\sim$3.5 mL of HCl, 1 N, to pH 7.4. The solution was diluted to 10 mL with sterile water for injection to give isotonic (290 mOsm) tobramycin (50 mg/mL), pH 7.4.

L-α-Dipalmitoyl[*methyl*-^{3}H]phosphatidylcholine ([^{3}H]-DPPC; 51 Ci/mmol; lot 1782-187) was purchased from New England Nuclear, Boston, MA. [U-^{14}C]Sucrose (360 mCi/mmol; 9777801) was purchased from ICN, Irvington, CA. α-Amino-[α-^{3}H]butyric acid (22.2 Ci/mmol; lot 1618-251) and L-[*ring*-2,6-^{3}H]tyrosine (34.7 Ci/mmol; lot 1618-167) were also purchased from New England Nuclear. All other chemicals and reagents were used as received. Osmotic strength was measured in a freezing point depression apparatus (advanced osmometer 67-31LAS).

Liposome Preparation. Conventional MLV vesicles were prepared by hydrating with excess PBS lipid films that had been predried onto the bottom of 100-mL round-bottom flasks; SPLV vesicles were prepared as described (Gruner et al., 1985), except 10 mL instead of 5 mL of diethyl ether was used for the emulsion (Pidgeon et al., 1986). For solid lipid mixtures (DPPC/CH 9:1), SPLVs were also prepared from diisopropyl ether and the vesicles were heated to 50 °C during or after liposome formation. SPLV and MLV liposomes were washed and purified by centrifugation as described under MLV-REV Preparation: Fluid Lipids.

MLV-REV Preparation: Fluid Lipids. Fluid lipids used to prepare MLV-REV liposomes utilized egg phosphatidylcholine (PC) as the predominant membrane-forming lipid; CH, PG, and PA were also mixed with PC. MLV-REV vesicles were prepared essentially as described previously (Pidgeon et al., 1986). Thus, ether solutions (10 mL) of lipid were emulsified with PBS solutions (0.3 or 0.5 mL) of drug by 1–2 min of sonication under nitrogen. Emulsion ether was removed at 30 °C in two stages. Stage 1 required 9 ± 3 min at $\sim$400 mmHg to form a gel. The vacuum was broken, and the gel was vortexed for about 5 s. Gel inversion to liposome formation, stage 2, required lowering the vacuum at 50-mmHg increments (2 min/interval) from 400 to $\sim$100 mmHg; approximately 16 ± 2 min was needed to form the vesicles. In some experiments 50–300 μL of sterile water was added to the gel before stage 2 was initiated. We denote this extra nonemulsion water as continuous-phase water (CPW) for reasons discussed under Results. It is difficult to stop the MLV-REV process at the gel stage to add CPW. Frequently breaking the vacuum causes spontaneous gel inversion as evidenced by a relatively clear gel turning white. The flask was rapidly rotated during the rotoevporation of the emulsion at stages 1 and 2. After stage 2, the viscous liposome suspension was diluted to $\sim$1.5 mL with PBS and transferred to microfuge tubes. Liposomes were separated from unentrapped aqueous space markers by 10–15 min of centrifugation in a microfuge (12000 rpm). Liposomes containing CH, PS, PA, and PG required 20–25 min of centrifugation. Usually liposomes pelleted, and the supernatant containing unentrapped drug or markers was discarded. Occasionally vesicles floated, and for these samples the subphase was discarded. After centrifugation, vesicles were suspended in excess PBS and centrifuged again. This vesicle purification process was performed at least 3 times for all vesicles. For some experiments vesicles were pelleted at 12000 rpm in a Sorvall LL-3 automatic centrifuge instead of a microfuge.

Radiolabeled drug markers were used to quantitate the amount of drug entrapped. The amount of drug entrapped was always corrected for lipid recovery, and therefore, drug entrapment values reflect the theoretical values if 100% of the lipid was recovered throughout the purification process. Typically, lipid losses were less than 10%.

Containers used to make MLV-REV vesicles included a 100-mL round-bottom flask, a 50-mL pear-shaped flask, and a 25 mm × 175 mm test tube. The test tube had a 24/40 ground-glass joint. This test tube is the same container we used to prepare single-layered REV vesicles (Pidgeon et al., 1986).

MLV-REV Preparation: Solid Lipids. DPPC was used as a model compound to study drug entrapment in MLV-REV vesicles prepared from lipids with a phase transition temperature (T_c) above room temperature. The T_c of DPPC is 41.5

°C, and diethyl ether has a bp^{760} of 34.6 °C. Because liposomes must be heated above the T_c of the highest melting lipid in the liposome membrane (Chapman & Fluck, 1966; Ladbrooke et al., 1968; Szoka & Papahadjopoulos, 1981), MLV-REV liposomes composed of DPPC or DPPC/CH were prepared by two methods. Method I heated the liposomes after emulsion ether was removed, i.e., after lipid assembly into membranes, whereas method II heated the liposomes during lipid assembly into membranes. Method I is identical with the procedure used for fluid liposomes (above) except the gel suspension was heated to 50 °C for 1 h. For method II, diisopropyl ether, bp^{760} 68–69 °C, was substituted for diethyl ether as in Method I, and the MLV-REV process was performed at 50 °C instead of 30 °C. For method II diisopropyl ether was evaporated in two stages and, as with Method I, when most of the organic solvent was removed, a gel suspension formed. DPPC/CH mixtures were reconstituted with either 1 or 10 mL of PBS at 25 °C. Vesicle purification and drug entrapment were performed as described above. In studying fluid lipids by dispersion method I, we have found ~90 μL of water/10 mL of diethyl ether to be lost from a diethyl ether/water azeotrope (Pidgeon et al., 1986). For this reason diisopropyl ether was saturated with water by equilibrating overnight (25 °C) 50/50 v/v mixtures of diisopropyl ether/water. This is similar to earlier studies of REV vesicles composed of DPPC whereby diisopropyl ether was washed just prior to use to remove peroxides (Düzgünes et al., 1985).

X-ray Diffraction. Multilayered vesicles were pelleted by microfuge, supernatants were discarded, and 25–50 μL of PBS was added to the pellet. With a syringe, an aliquot of the concentrated liposome pellet was pipetted into the center of a 1.5-mm quartz capillary tube and sealed. Lipid concentrations of MLV-REVs, SPLVs, and conventional MLVs were ~125 mg/mL. Vesicles were stored in the refrigerator until diffraction patterns were obtained. Diffraction data were collected by using Cu Kα radiation, a double-mirror focusing camera (Franks, 1955; Harrison, 1968) with an order-to-order resolution of ~1500 Å, and an Elliott GX20 rotating-anode generator with a 0.15 mm × 2.5 mm focal spot. Exposure times were usually 2–3 h at an X-ray power of 35 kV and 28 mÅ. For negatively charged SPLV vesicles, diffraction patterns were absent at 2–3-h exposure; 15-h exposure times were required. Diffraction patterns were recorded on Kodak DEF-5 (X-ray) film with a sample-to-film distance of 300 mm. An Optronics C-4100 film scanner was used to digitize the optical densities on a 50-μm raster.

The center of each diffraction pattern was determined by using the symmetry of the pattern. Then the average optical density at each radius was calculated by performing a circular integration over 25-μm shells. The radial intensity distribution on each film was obtained from these circularly averaged data. Liposome long spacings are the average value measured from both the first- and second-order diffraction peaks. For the asymmetric diffraction pattern of DPPC/CH (9/1), the largest long spacing is reported. Diffraction patterns of negatively charged SPLV and conventional MLV vesicles were typically too diffuse to obtain accurate long-spacing measurements.

Results

Earlier we reported that the MLV-REV gel is clear compared to the REV gel that generates single-layered vesicles. This observation was based on our earlier work in which vesicles were prepared from PC or mixtures of PC containing small amounts of PA. The physical appearance of the gel, however, depends on the type of lipid mixture. Adding negatively charged lipids (PA, PS, PG) slightly increased the opacity of the PC MLV-REV gel, but using DPPC or DPPC/CH mixtures generates a gel that appears to be precipitated lipids. This is particularly apparent if substantial dehydration of the PC/water mixture occurs during removal of the organic solvent from the starting emulsion.

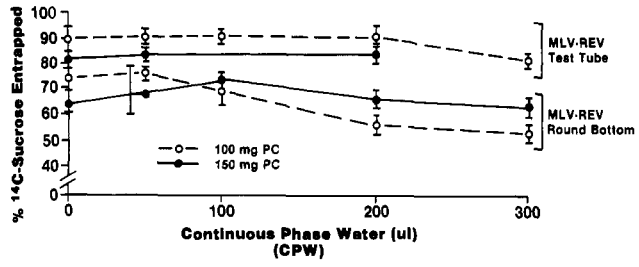


FIGURE 1: Dependence of MLV-REV solute entrapment on container. MLV-REV vesicles were prepared in a 100-mL round-bottom flask or a 25 mm × 175 mm test tube. The MLV-REV emulsion was made by cosonicating 10 mL of ether, containing egg PC, with 0.3 mL of PBS containing 1 mg/mL sucrose. At the gel stage, various amounts of continuous-phase water (CPW) were added, followed by brief vortexing. The MLV-REV process was then continued to generate multilayered vesicles. The percent entrapment was measured by using [^{14}C]sucrose as a tracer. Entrapment values are corrected for lipid recovery and reflect the mean ± SD for $n > 3$.

MLV-REV vesicles are prepared from water droplets emulsified in ether/PBS solutions. Initially, ether is the continuous phase, but evaporation of the ether generates a gel followed by water becoming the continuous phase; i.e., a gel inversion occurs. During gel inversion water movement causes liposomal core water to become extraliposomal bulk water. Water movement as gel inversion proceeds should be accompanied by solute movement from inside the incompletely formed liposomes to outside the liposomes. Water and solute (including drug) movement will thus cause drug entrapment to decrease in the final liposome population. We attempted to optimize drug entrapment by adding continuous-phase water (CPW) (bulk water) to the MLV-REV gel. Supplying bulk water to the gel reduces the need for core water to move and become bulk water.

Optimum entrapment from the MLV-REV process depends on the volume of emulsion water, the type and amount of lipid, and the container used to prepare the vesicles. Figure 1 shows that, without the addition of CPW, MLV-REVs prepared in a test tube entrap more sucrose (~18% increase) than those prepared in a round-bottom flask. MLV-REV vesicles prepared in a pear-shaped container yield entrapment values similar to liposomes prepared in a round-bottom flask (C. Pidgeon, unpublished observations). In agreement with our earlier findings, 100 mg of PC is more efficient than 150 mg of PC with regard to entrapment for both test tube and round-bottom flask MLV-REVs (Pidgeon et al., 1986). Adding small volumes of CPW to MLV-REVs prepared in a test tube has little effect on entrapment. However, 300 μL of CPW decreased entrapment ~10%. In contrast, a slight increase in sucrose entrapment occurs for PC MLV-REVs prepared in a 100-mL round-bottom flask when 50–100 μL of CPW is added. Further increase in CPW causes entrapment to decrease. During vesicle formation ~90 μL of water is lost by a diethyl ether/water azeotrope (Pidgeon et al., 1986). Adding 50–100 μL of CPW to replace azeotropic water loss can increase entrapment. However, there is also an (undetermined) optimum emulsion–PBS concentration. When MLV-REVs are prepared in a round-bottom flask from 100 mg of PC and 500 μL of PBS, addition of 50–300 μL of CPW to the gel decreases drug entrapment from 75 ± 3.0% (n = 3) to ~50% (n = 12). The dependence of CPW on entrap-

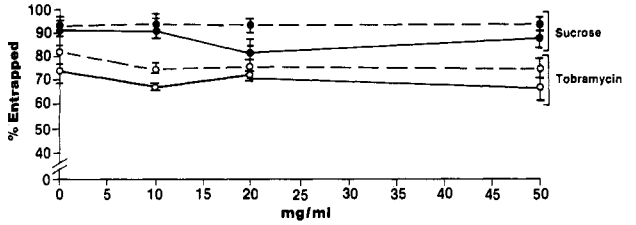


FIGURE 2: Effect of solute concentration on drug entrapment. MLV-REV vesicles were prepared from 100 mg of PC and 10 mL of diethyl ether. Emulsion water was 300 μL and contained either sucrose or tobramycin at concentrations from 1 to 50 mg/mL. MLV-REV vesicles were prepared with (---) or without (—) 100 μL of continuous-phase water. Entrapment values corrected for lipid recovery are the mean ± SD for $n \geq 3$.

Table I: Sucrose Entrapment in MLV-REV Liposomes Prepared from Phosphatidylcholine and Negatively Charged Lipids or Cholesterol

lipid mixture[b]	w/w (mg/mg)	mol % PC	% entrapped[a] for continuous-phase water (μL)[b] 0.0	100
PC	100	100	90.5 ± 4.2	90.9 ± 5.3
PC/PS	100/8.3	93	83.9	86.7
PC/PA	100/4	95	84.2 ± 5.0	90.9 ± 2.2
PC/PG	100/10	89	60.8 ± 6.0	90.0 ± 1.7
PC/CH	100/10	83	57.5 ± 3.7	91.1 ± 2.2
PC/CH	94/15	75	87.4 ± 2.0	83.8 ± 6.6
PC/CH	80/20	66	70.0 ± 17.5	64.8 ± 10.9

[a] Values are mean ± SD for $n > 3$, except for PC/PS, which is the average of two experiments. [b] The MLV-REV gel was inverted to form liposomes with or without 100 μL of continuous-phase water.

ment is thus variable and depends on the exact experimental conditions used to form the liposomes. Because drug entrapment is optimum when MLV-REV vesicles are prepared in a test tube, the remaining experiments were done with test tube MLV-REVs unless explicitly stated.

Increasing the drug concentration in the MLV-REV emulsion water causes only a small decrease in the amount of drug entrapped (Figure 2). MLV-REV vesicles were prepared from 0.3 mL of sucrose and tobramycin solutions at concentrations from 1 to 50 mg/mL (Figure 2). At all concentrations tested, sucrose and tobramycin were more efficiently entrapped when 100 μL of CPW was added at the gel stage of the MLV-REV process. At 50 mg/mL tobramycin entrapment increased from 67.9 ± 4.0% to 75.6 ± 3.3% and sucrose increased from 88.6 ± 2.1% to 93.0 ± 2.2%. In other experiments whereby 15–20 populations of MLV-REV vesicles containing tobramycin were pooled, drug entrapment was 55% for vesicles prepared in a round-bottom flask and 72% for vesicles prepared in a test tube (C. Pidgeon, unpublished experiments). This study and Figure 2 clearly demonstrate that the container in which liposomes are prepared and CPW substantially affect drug entrapment.

Drug entrapment in MLV-REVs depends on lipid composition. Adding negatively charged lipids PS, PG, and PA to egg PC decreased sucrose entrapment unless 100 μL of CPW was used during vesicle preparation (Table I). Adding cholesterol to egg PC also decreased sucrose entrapment. With low membrane cholesterol (~17%), adding 100 μL of CPW increased drug entrapment from 57.5 ± 3.7% to 91.1 ± 3.2%. For high membrane cholesterol (25–35 mol % CH), using CPW had little effect or decreased entrapment. We have found the incorporation of small concentrations of CH into PC MLV-REV vesicles to have variable effects on the MLV-REV gel and also percent entrapment in the final liposome population. For example, PC/CH 100/10 w/w has an entrapment of 57.5 ± 3.7% and 91.1 ± 2.2% with and without 100 μL of CPW, respectively (Table I). Variability in percent entrapped when CPW is added is due in part to the difficulty in identifying the precise time to stop the MLV-REV process and add CPW. As stated earlier, we make the assumption that the amount of drug entrapped in the final liposome population reflects the solute permeability characteristics from the core to bulk solution during liposome formation. For incompletely formed liposomes, different lipid mixtures can be expected to elicit different barriers to solutes. Thus the solute and lipid mixture are important variables in trying to optimize entrapment. However, if gel inversion has begun, adding CPW probably does not influence drug entrapment.[2]

Table II: Drug Entrapment in Neutral MLV-REV Liposomes Prepared in a Test Tube

	% entrapped (PC)[a] for continuous-phase water (μL) 0	100
α-aminobutyric acid	52.3	61.7
tobramycin	74.2	83.7
poly(I)·poly(C)	83.5	65.8
tyrosine	65.2	70.3

[a] Every value is the mean of two determinations. The range never exceeded 10%.

Table II gives entrapment values for four aqueous soluble solutes. For these experiments MLV-REV liposomes were prepared from emulsion–PBS containing 1 mg/mL drug. All aqueous solutes tested had high entrapment, but entrapment was always less than that predicted from sucrose (Figures 1 and 2). Except for poly(I)·poly(C), 100 μL of CPW added to the MLV-REV gel suspension slightly increased drug entrapment. Frequently drug entrapment in conventional liposomes is estimated from radiolabeled aqueous space markers like [^{14}C]sucrose, [^{14}C]glucose, etc. In conventional MLVs solute equilibration across all bilayers is assumed and the amount of entrapped drug is frequently calculated from the amount of entrapped radiolabeled aqueous space marker. This assumption is questionable for MLV-REV vesicles prepared in a test tube, which routinely entrap >90% sucrose without CPW (Figure 1, Table I). The drug-entrapment studies in Table II usually do not entrap 90% of added drug. Even when CPW is added, entrapment does not always reach the value of 90%. This suggests that entrapment values based on [^{14}C]sucrose will overestimate the amount of drug entrapped, and the drug of interest should be assayed directly if accurate entrapment values are to be obtained. Quantitative or near-quantitative drug entrapment may affect the solute distribution in the final liposome population. The MLV-REV process begins with an aqueous core containing an aqueous soluble drug. If the ether/water emulsion supplies bulk water for the final liposome population (viz., core water), then quantitative drug entrapment would result in a liposomal drug concentration higher than the drug concentration in the initial aqueous solution used to prepare the vesicles. In addition, the core would contain the highest drug concentration with the outer lamella being depleted of drug. Thus quantitative drug entrapment may create a solute gradient in MLV-REV vesicles

[2] Sucrose, 75 mg/mL, in the emulsion buffer stabilized the gel and prevents gel inversion. We have not evaluated the effect of CPW on gels containing 75 mg/mL sucrose.

Table III: Sucrose Entrapment in MLV-REV Vesicles Prepared from Mixtures of Dipalmitoylphosphatidylcholine and Cholesterol (DPPC/CH) by Using Diethyl Ether/PBS or Diisopropyl Ether/PBS[a]

DPPC/CH (mg/mg)	mol % CH	diethyl ether/PBS (10 mL/0.3 mL)[c]		diisopropyl ether/PBS (10 mL/0.5 mL)[c,d]	
		% entrapped	E^{ratio}	% entrapped	E^{ratio}
100/0	0	31.95 ± 4.8	0.95	61.9 ± 2.95	3.0
94/6	10	49.7 ± 17.3	1.49	49.0 ± 0.5	2.45
88/12	17	29.1 ± 7.1	0.87	44.3 ± 5.9	2.21
81/19	33	31.7 ± 7.6	0.95	55.5 ± 2.8	2.77
74.26	40	42.8 ± 9.1	1.28	59.4 ± 1.7	2.97
65.35	52	31.7 ± 14.4	0.95	57.3 ± 4.3	3.00

[a] E^{ratio} was calculated by assuming a lipid density of unity. [b] Values are mean ± SD for $n = 3$, except for 10% CH, where $n = 6$. [c] Except for 10 mol % CH, the MLV-REV gel was opaque and inverted rapidly to a white precipitated lipid mixture. [d] Vesicles were prepared at 50 °C from isopropyl ether saturated with water.

whereby the core has the largest concentration of drug.

Sucrose entrapment in MLV-REV vesicles prepared from lipids with a phase transition temperature above room temperature is shown in Table III for DPPC/CH mixtures. The MLV-REV process in the presence of DPPC/CH mixtures generates distinct physical changes depending on the emulsion–organic solvent. Thus, for dispersion method I, DPPC/CH mixtures are insoluble (25 °C) in diethyl ether/PBS (10/0.3 v/v) but sonication to form an emulsion generates a clear solution. As diethyl ether is evaporated, the solution becomes opaque. However, removing diethyl ether forms a gel that is almost clear. Upon gel inversion the mixture appears similar to a wet precipitated lipid except when 40–50 mol % CH is present. At ~40–50 mol % CH, the T_c of DPPC is eliminated, the lipid is fluid, and gel inversion forms a creamy white gel suspension containing liposomes. In contrast, DPPC/CH mixtures dispersed by method I containing ~10% mol % CH generate a distinct, relatively stable clear gel. This concentration of cholesterol also elicited the highest entrapment for lipid dispersions by method I. DPPC/CH MLV-REV vesicles prepared by method I have entrapment values near 30–40% except at 10 mol % CH, where entrapment was 50%. Method I removes emulsion diethyl ether at 30 °C and heats the gel suspension to 50 °C. Method I thus heats the lipid mixture above the phase transition temperature after lipid assembly into membranes is essentially complete. From computer-generated data (next section and Discussion) high entrapment at 10 mol % CH probably reflects the ability of this particular lipid mixture to maintain a stable core during liposome formation. Core stability reflects the ability of the core to sequester drug in the incompletely formed liposomes during the MLV-REV process.

Unlike method I, which heated the DPPC/CH gel suspension for 1 h at 50 °C, method II heated the lipids during lipid assembly into membranes; in addition, diisopropyl ether was substituted for diethyl ether. DPPC/CH mixtures were insoluble in diisopropyl ether/PBS (10/0.5 v/v) at all concentrations tested. During sonication, this organic solvent/water mixture never became clear as with method I. Evaporation of the organic solvent at 50 °C never formed a gel, but the gel suspension was similar to that obtained from method I after gel inversion; i.e., a white, wet precipitated lipid mixture was found when most of the organic solvent was removed. However, [^{14}C]sucrose entrapment in DPPC/CH vesicles was 50–60% except for 17% CH, which was 45%. These values are higher than entrapment values obtained from method I.

Table IV: Percent Sucrose Entrapped in DPPC/CH (9:1) MLV-REV and SPLV Vesicles Prepared with and without Heat during Liposome Formation[a]

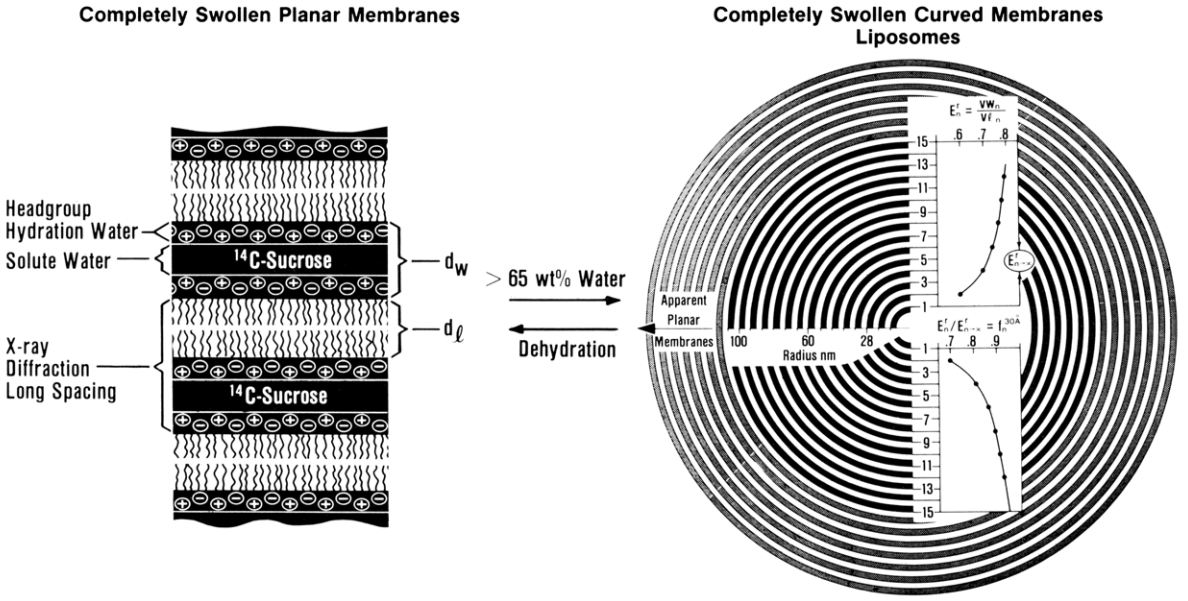
emulsion	thermal conditions (°C) during lipid assembly into membranes	thermal conditions (°C) after lipid[b] assembly into membranes	% [^{14}C]sucrose entrapped MLV-REV	% [^{14}C]sucrose entrapped SPLV
diethyl ether/PBS (10/0.3 v/v)	25	25[b]	17.8 ± 12.4[c]	22.57 ± 3.17
diethyl ether/PBS (10/0.3 v/v)	30	50[d]	49.7 ± 17.0[c]	3.8 ± 2.3
diisopropyl ether/PBS (10/0.5 v/v)	50	25[b]	49.0 ± 0.5	1.5 ± 0.7

[a] Liposome formation includes lipid assembly into closed bilayer membranes and vesicle purification. Heat was applied during lipid assembly or after lipid assembly but prior to vesicle purification. All vesicle purification and washes were performed at 25 °C. [b] The gel suspension was suspended in 1.0 mL of PBS, 25 °C, and the vesicles were washed 3× and isolated as a pellet. [c] Values are the mean ± SD for $n = 6$; all other experiments had $n = 3$. [d] The gel suspension was heated to 50 °C for 1 h prior to washing and vesicle purification.

When conventional MLVs are prepared, it is recommended to heat the vesicle above the T_c of the highest melting lipid in the membrane to anneal the membranes (Szoka & Papahadjopoulos, 1981). Unannealed vesicles may contain "cracks" in the membrane. These cracks could allow solutes to leak from the liposome during vesicle purification if the membranes are not annealed during or after the process of lipid assembly into liposomes. In contrast to this, SPLV liposomes are reported not to require heating with solid lipids (Lenk et al., 1985). Table IV compares SPLV and MLV-REV vesicles prepared from DPPC/CH (9:1) with and without heating. High entrapment in MLV-REVs depends on whether or not the vesicles were heated before the suspension was washed; heating before or after lipid assembly into membranes does not affect entrapment (Table IV). This suggests that any disclinations in the membrane must be annealed before the unentrapped aqueous space markers are separated by centrifugation (i.e., washing the vesicles). Optimum entrapment in SPLVs requires the vesicles not be heated. Heating the SPLV w/o emulsion during evaporation of the ether generated only 1.5 ± 0.7% entrapped; heating the SPLV gel suspension generated only 3.8 ± 2.3% entrapped. Entrapment trends for SPLV and MLV-REV vesicles may depend on lipid composition, emulsion water, and other experimental variables. Because of this, different lipid mixtures may require different experimental conditions for optimum entrapment.

Water Distribution in Multilayered Liposomes. Water trapped inside liposomes can be partitioned into two compartments; interlamellar water and core water. The fraction of entrapped water residing in the repeating bilayers can be obtained by comparing the long spacing of completely swollen planar membranes to the long spacing in liposomes. If long spacing remains constant when planar membranes are compared to curved liposomal membranes, then the volume of water per milligram of lipid imbibed by completely hydrated and swollen lipids (i.e., planar membranes) is the same as the trapped volume per milligram of lipid in the repeating bilayers of liposomes. This assumes that membrane curvature does not increase or decrease the interlamellar trapped volume in the vesicles.

Scheme I: Structure and Volume-Trapping Characteristics of Planar Membranes and Liposomes[a]



[a] The liposome is drawn to scale from d_w = 30 Å, d_l = 35 Å, and core diameter of 300 Å. The planar bilayers are not drawn to scale. Inside the liposome, graphs were plotted to demonstrate when membrane curvature affects the volume-trapping characteristics of the membranes. The phosphatidylcholine head group is denoted by ⊖⊕.

X-ray diffraction measurements of lipid/water mixtures have demonstrated that 40–50 wt % interlamellar water is imbibed by various lipid mixtures (Small, 1967; Ladbrooke et al., 1968). Few liposomes exist in this mixture because sufficient bulk water is not available to suspend all the lipid into vesicles. However, after the lamellae are completely swollen, adding more water may cause some membrane curvature; undoubtedly this transition must occur for dried lipid films to ultimately become closed membranes as in liposomes. Although some membrane curvature may exist at maximum bilayer swelling, the volume-trapping characteristics of the lipid will not vary much beyond that expected from completely swollen planar membranes. Thus, with regard to interlamellar volume trapping, it is reasonable to compare the long spacing obtained from concentrated lipid/water mixtures to the long spacing of liposomes.

The volume of water between swollen lamellae equals the interlamellar water thickness d_w times the membrane area A_m, and the volume of lipid needed to form the lamella equals $d_l A_m$, where d_l is the bilayer thickness. The volume of water per volume of lipid in the repeating planar membranes is $(d_w/d_l)_p$ because membrane area cancels.[3] In the transition from planar membranes to curved liposomal membranes, bilayer number, core size, membrane curvature, and water thickness in the repeating bilayers determine when the liposome lamellae trap volumes characterized by $(d_w/d_l)_{planar}$. However, regardless of these variables, if bilayer number (n) approaches infinity, then liposome membranes are functionally equivalent to planar membranes at least with respect to volume trapping because liposome membranes would not have curvature at the molecular level. Thus

$$(d_w/d_l)_{planar} = (d_w/d_l)^{n=\infty}_{liposome} \quad (1)$$

[3] Since water molecules are present in the lipid head group region, d_l and d_w do not actually equal any real distances in the structure. The values of d_l and d_w are the thickness of water and lipid if each layer were compressed into slabs. From a volume-trapping viewpoint this is unimportant. Franks and Lieb (1981) discussed this problem with respect to lamella structure.

We used computer-simulated liposome populations to evaluate how d_w, n, and core diameter affect when individual liposome lamellae imbibe as much water as planar membranes. In other words, we identified the structural features necessary for individual liposomal lamellae to have an encapsulation ratio E^r_n similar to the encapsulation ratio $E^r_{n=\infty}$ expected from planar membranes. In eq 2, E^r_n is the encapsulation ratio of

$$E^r_n = Vw_n/Vl^n \quad (2a)$$

$$E^r_{n=\infty} = (d_w/d_l)^{n=\infty}_{liposome} = (d_w/d_l)_{planar} \quad (2b)$$

the nth lamella, Vw_n is the volume of water trapped in the nth liposome lamella, and Vl_n is the volume of lipid needed to form the nth liposome lamella. Because Vw_n and Vl_n were calculated from equations for spheres, the effect membrane curvature has on trapped interlamellar water volume is taken into consideration. The fraction f_n given by

$$f_n = E^r_n/E^r_{n=\infty} \quad (3)$$

allows direct comparison of the volume trapped per milligram of lipid in liposome bilayers to the volume trapped per milligram of lipid of planar bilayers. The relationships described by eq 2a, 2b, and 3 are shown in the graphs in the liposome of Scheme I. The liposome in Scheme I was drawn to scale with a 300-Å core, a 30-Å water thickness, and a 35-Å bilayer thickness; however, several computer simulations were performed [available as supplementary material (see paragraph at end of paper regarding supplementary material)]. For 300-Å core liposomes, approximately 9–10 bilayers are needed for liposomal lamellae to contain >90% of the water between planar membranes of the same repeat distance. This is true for d_w of 20 Å and d_w of 74 Å. Increasing the core diameter to 2000 or 4400 Å causes all lamellae, even the second bilayer, to contain 90–97% of the water trapped between planar membranes. The reduced curvature of a 2000- or 4400-Å core is responsible for the second lamella to trap volumes close to the limiting value found in planar bilayers.

Regardless of the method of preparation, multilayered liposome populations have diameters from ~0.1 to >1.0 μm (Szoka & Papahadjopoulos, 1981). Both SPLV and MLV-

Table V: Calculation of Core Water in Computer-Generated Heterogeneous Liposome Populations Containing Variable Core Diameters[a]

population[b]	% trapped core water calculated from lamellar repeat distance: $fw^{core} \times 100\ [(1 - E^r_{n\to\infty}/E^{ratio}) \times 100]$	$fw^{core} \times 100^c\ [(1 - 0.95E^r_{n\to\infty}/E^{ratio}) \times 100]$	actual core volume[d] (%)
A	12	16.0	18.0
B	2.7	7.5	10.0
C	7.4	12.0	14.0
D	15.4	19.7	21.4
E	<2	<2	<1.0
F	14.8	19.1	19.8
G	44.6	47.4	47.9

[a] Liposome populations were generated similarly to our previous computer simulations on liposome populations (Pidgeon & Hunt, 1981; Pidgeon et al., 1986). [b] Populations A–D contained vesicles with cores ranging from 800 to 4400 Å, but a constant bilayer number (n = 15) and therefore variable liposome diameters exist in the population. Populations A–D contained some liposomes with each of the following internal core diameters: 800, 1200, 1600, 2000, 3600, and 4400 Å. Population A contained an equal number of liposomes, i.e., subpopulations, with each of those cores. All the liposomes in population A were generated from d_w = 74 Å. Populations B–D were derived from population A by weighting the subpopulations. This is equivalent to generating liposome populations with skewed frequency distributions. Weighting factors for population B–D were the sets B = {10, 8, 6, 4, 2, 1}, C = {2, 4, 8, 8, 4, 2}, and D = {1, 2, 4, 6, 8, 10}. Liposome populations E–G were generated from d_w = 30 Å and d_l = 35 Å. For all of these populations, bilayer number varied from 10 to 24. All liposomes in population D contained a 300-Å core. All liposomes in population E contained a 2000-Å core. All liposomes in population F contained a 4400-Å core. [c] The factor 0.95 is a correction factor to account for the volume-trapping characteristics of curved membranes in liposomes containing a finite number of bilayers. [d] The actual core volume in each artificial liposome population was calculated directly from the computer simulations. This was done by summing all the water in the core of every individual liposome in the population and comparing it to all the trapped water in the population.

REV vesicles have mean diameters >0.6 μm (Gruner et al., 1985; Pidgeon et al., 1986). Typically the mean vesicle diameter of unextruded multilayered vesicles is 0.6–0.8 μm. Since liposome membranes function as planar membranes after 0.2 μm, then virtually all of the lipids in multilayered liposomes function as apparent planar membranes. For this reason partitioning liposomal trapped water between the lamellae and core, on the basis of X-ray diffraction measurements of long spacing, can be done as follows.

Since all individual liposome lamellae trap water as efficiently as planar bilayers, the total interlamellar trapped water of multilayered vesicles can be calculated. In multilayered liposome populations the fraction of water trapped only in the repeating bilayers is

$$fw^{lamellae} = 0.95E^r_{n=\infty}/E^{ratio} \quad (4)$$

where the fraction 0.95 has been introduced to account for the small decrease in trapped volume elicited by liposome membranes compared to planar membranes (Table V). The fraction of trapped water residing in the cores of a heterogeneous liposome population can be calculated from

$$fw^{core} = 1 - fw^{lamellae} \quad (5)$$

We have verified eq 5 by simulating populations of liposomes that contain, within the population, individual liposomes with variable core diameters, water spacing, and bilayer numbers. Table V shows seven liposome populations generated from d_w = 30 or 74 Å and d_l = 37 or 35 Å. In all populations tested a good estimate of core water was obtained from eq 5. The estimate is particularly good when the lamellar trapped volume is corrected for membrane curvature by the factor 0.95. Thus,

Table VI: Core Water in MLV-REV and SPLV Multilayered Liposomes[a]

lipid mixture	MLV-REV fw^{core}	MLV-REV E^{ratio}	SPLV fw^{core}	SPLV E^{ratio}
PC	0.62	2.04 ± 0.42[c]	0.40	1.27 ± 0.14[c]
PC[b]	0.48	1.46 ± 0.18[d]	0.30	1.08 ± 0.17[d]
PC/PA (9:1)	0.21	0.95[e]	0.05	0.74[e]
PC/PA/CH (5:1:4)	0.13	0.86 ± 0.07[c]	0.05	0.67 ± 0.02[e]

[a] f^{core} was calculated from our previous published experiments, which measured E^{ratio} (Pidgeon et al., 1986). For each lipid composition, f^{core} is the fraction of water in all the cores of the liposomes in a given population compared to the total trapped volume (core water + lamellar water) of the population. [b] [^{14}C]Inulin was used to measure E^{ratio} for these liposome preparations. For all other preparations [^{14}C]sucrose was used to measure E^{ratio}. [c] n = 3. [d] n = 5. [e] n = 1.

the fraction of water in the core, and the fraction of water in the lamellae, of multilayered liposome populations can be calculated. We calculated core and lamellar water for SPLV and MLV-REV liposomes, and this is described below.

Table VI compares the fractional trapped core volume in MLV-REV and SPLV liposomes.[4] For every lipid mixture tested MLV-REV vesicles have a larger volume fraction residing in the liposomes' core. SPLVs are sonicated during liposome formation, and because of this we postulated that SPLVs undergo core collapse during vesicle formation (Pidgeon et al., 1986); Table VI is in agreement. Conventional MLVs are prepared by hydrating in excess buffer lipids dried on the flow of a container. These vesicles have encapsulation efficiencies between 1 and 4 μL/μmol of lipid (Szoka & Papahadjopoulos, 1981). Since E^r = 0.75, conventional MLVs also contain a core where a large fraction of the particle's water volume resides. In our laboratory we always obtain an encapsulation efficiency of 2–2.5 μL/μmol for conventional MLVs.

X-ray Diffraction of MLV, MLV-REV, and SPLV Vesicles. X-ray diffraction is a well-established method to obtain the repeat distances in lipid bilayers. The lamellar repeat distance D is the sum of the water thickness, d_w, and the bilayer thickness, d_l. Observed changes in D can correspond to changes in either d_w or d_l. When excess water is not present, X-ray diffraction measurements of planar membranes enable d_w and d_l to be calculated if the weight fraction and mass density of both lipid and water are known in the specimen (Luzzati, 1968). Under conditions of excess water, as in multilayered liposome suspensions, it is not possible to calculate d_l and d_w directly. However, bilayer thickness will vary little, if any, during the transition from completely swollen planar membranes to liposomes. Estimates of d_l from concentrated lipid/water mixtures at maximum bilayer swelling can thus be extrapolated to liposome populations of the same lipid mixture with confidence. To use eq 4 and 5, we need to compare the repeat distance in MLV-REV vesicles (D_{MLV}) to the repeat distance (D_p) in lipid membranes at maximum swelling. If $D_{MLV} = D_p$, the volume trapped inside the liposome lamella is similar to the volume trapped between planar membranes at maximum bilayer swelling. In most lipid mixtures maximum bilayer swelling occurs at 30–45 wt % water, which corresponds to interlamellar trapped volumes between 75 and 90 μL/100 mg of lipid (Small, 1967; Lad-

[4] All entrapment data in Table VI were taken from our earlier experiments (Pidgeon et al., 1986) whereby both MLV-REV and SPLV vesicles were prepared in a 100-mL round-bottom flask. In these experiments gel inversion was facile, and entrapment in MLV-REV vesicles was 65%. We believe solute equilibration occurred during this experiment and calculating core water is justified.

Table VII: Lamella Structural Parameters of MLV-REV Vesicles

liposomal lipids[a] (molar ratio)	long spacing,[b] $D = d_w + d_l$ (Å)	membrane thickness,[c] d_l (Å)	interlamellar water thickness,[d] d_w (Å)	E^r (d_w/d_l)[e]
MLV-REV vesicles				
egg PC	64.8 ± 0.06	35.1[f]	29.7	0.85
egg PC/CH (50/50)		40.3[g]	25.2[g]	0.62
egg PC/PA (93/7)	66.3	35.1[h]	31.2	0.89
egg PC/PS (95/5)	66.0	35.1[h]	30.9	0.88
MLV-REV vesicles, diethyl ether/PBS emulsion[i]				
DPPC	63.7	44.2[g]	19.5	0.44
DPPC/CH (90/10)	80.6	45.0[g]	35.6	0.79
DPPC/CH (50/50)	66.2	37.7[g]	28.5	0.76
MLV-REV vesicles, diisopropyl ether/PBS emulsion[j]				
DPPC	63.5	44.2	19.3	0.44
DPPC/CH (90/10)	78.7	45.0	33.7	0.75
DPPC/CH (50/50)	66.5	37.7[g]	28.8	0.76

[a] MLV-REV liposomes were prepared from lipid mixtures described below. [b] Lamella long spacing is the sum of the membrane thickness, d_l, and the interlamella water spacing, d_w. X-ray exposure times were 3–4 h at 25.5 ± 0.5 °C. [c] For planar membranes bilayer thickness, d_l, is independent of water content after maximum lipid hydration. Thus d_l obtained from completely swollen or separated planar membranes was used as an approximation for d_l in the MLV-REV population. Values of d_l for particular lipid mixtures were taken from the references denoted in footnotes *f* and *g* of this table. [d] When the long spacing ($d_w + d_l$) was measured, d_w was derived from $D = d_w + d_l$. For lipid mixtures where the long spacing was not measured, both d_w and d_l are taken from the references in footnote f or g of this table. [e] E^r is the ratio of interlamellar water thickness/bilayer thickness. This value should be corrected for membrane curvature, the head group hydration, to estimate the maximum interlamella solute water present in the final liposome population. [f] Parsegian, 1979. [g] Lis, 1982. [h] d_l for this mixture was assumed to be equal to d_l for pure egg PC. [i] MLV-REV vesicles were prepared from ~100 mg of lipid and 10/0.3 diethyl ether/PBS emulsion. The gel suspension was heated to 50 °C for 1 h. [j] MLV-REV vesicles were prepared from ~100 mg of lipid and 10/0.3 diisopropyl ether/PBS emulsion. Emulsion diisopropyl ether was removed at 50 °C. The gel suspension was reconstituted with isotonic PBS at 25 °C.

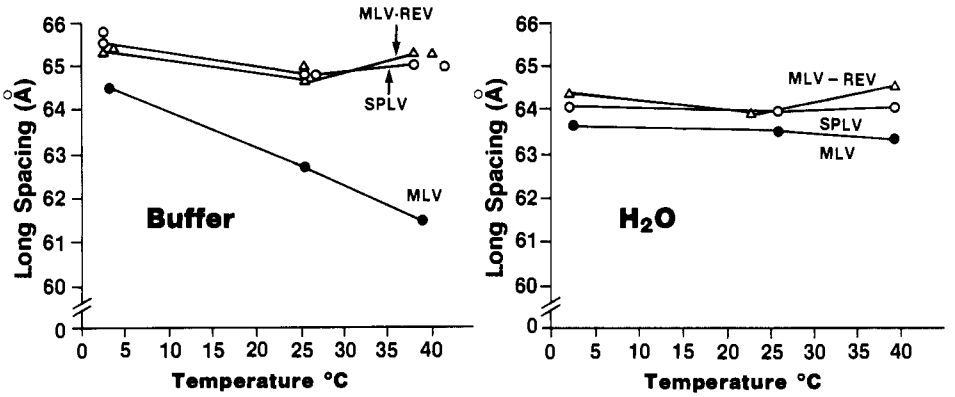


FIGURE 3: Effect of temperature on long spacing in MLV, MLV-REV, and SPLV liposomes prepared in water or buffer.

brooke et al., 1968; Lis et al., 1982). Table VII shows long spacings for MLV-REV vesicles prepared from various lipid mixtures. D_{MLV} values of MLV-REV vesicles are in excellent agreement with previously reported D_p values for all lipid mixtures tested. For example, X-ray diffraction studies of planar membranes composed of DPPC/water mixtures at 50 wt % water show the long spacing to be 64.5 Å. Addition of CH up to 7.5 mol % increases the long spacing to 81 Å. Further addition of CH causes the long spacing to approach 64.5 Å at ~50% CH (Ladbrooke et al., 1968). Similar long spacings were obtained for MLV-REV vesicles containing the same cholesterol content. Because D_{MLV} for MLV-REV vesicles is similar to D_p, d_l estimated for planar membranes is similar to that for liposome membranes, and the values are listed in Table VII. Calculating d_w by the difference between D_{MLV} and d_l gives the liposomes' d_w. The encapsulation ratio $(d_w/d_l)_{liposome} = E^r$ for the liposome lamellae can be estimated directly from Table VII to show the expected trapping characteristics of the lamellae in liposome membranes. For most of the lipid mixtures in this study, E^r is 0.75–0.89 μL of water/μL of lipid. This range of E^r includes hydration water.

In the comparison of conventional MLV vesicles to SPLV vesicles, a characteristic temperature-dependent long spacing for each of these vesicles has been reported (Gruner et al., 1985). This feature of the vesicles was described as a long-spacing signature (LSS) capable of distinguishing SPLV from conventional MLV vesicles. Figure 3 shows the LSS for conventional MLV, MLV-REV, and SPLV vesicles prepared in isotonic buffer and water. We have confirmed that SPLV vesicles can be distinguished from MLV vesicles because they have a lamellar repeat distance that remains relatively constant over the temperature range of 3–40 °C whereas conventional MLVs elicit a substantial decrease in long spacing. MLV-REV vesicles prepared in buffer are similar to SPLVs as expected because both MLV-REVs and SPLVs are prepared from w/o emulsions; conventional MLVs are prepared from dried lipid films.

The difference in LSS between SPLV and MLV vesicles prepared in buffer has been attributed to a homogeneous solute distribution for SPLVs compared to a heterogeneous solute distribution for MLVs. This hypothesis was given because when SPLV and MLV vesicles are prepared in the absence of solutes (i.e., only water), the long-spacing signatures are identical. We have confirmed that MLV and SPLV vesicles have similar LSSs when the vesicles are prepared in water (Figure 3); Figure 3 also shows that the temperature-dependent long-spacing change for MLV-REV vesicles is similar to that for MLVs and SPLVs.

Changes in bilayer thickness as the temperature is changed are a consequence of the lipid molecular packing of the hy-

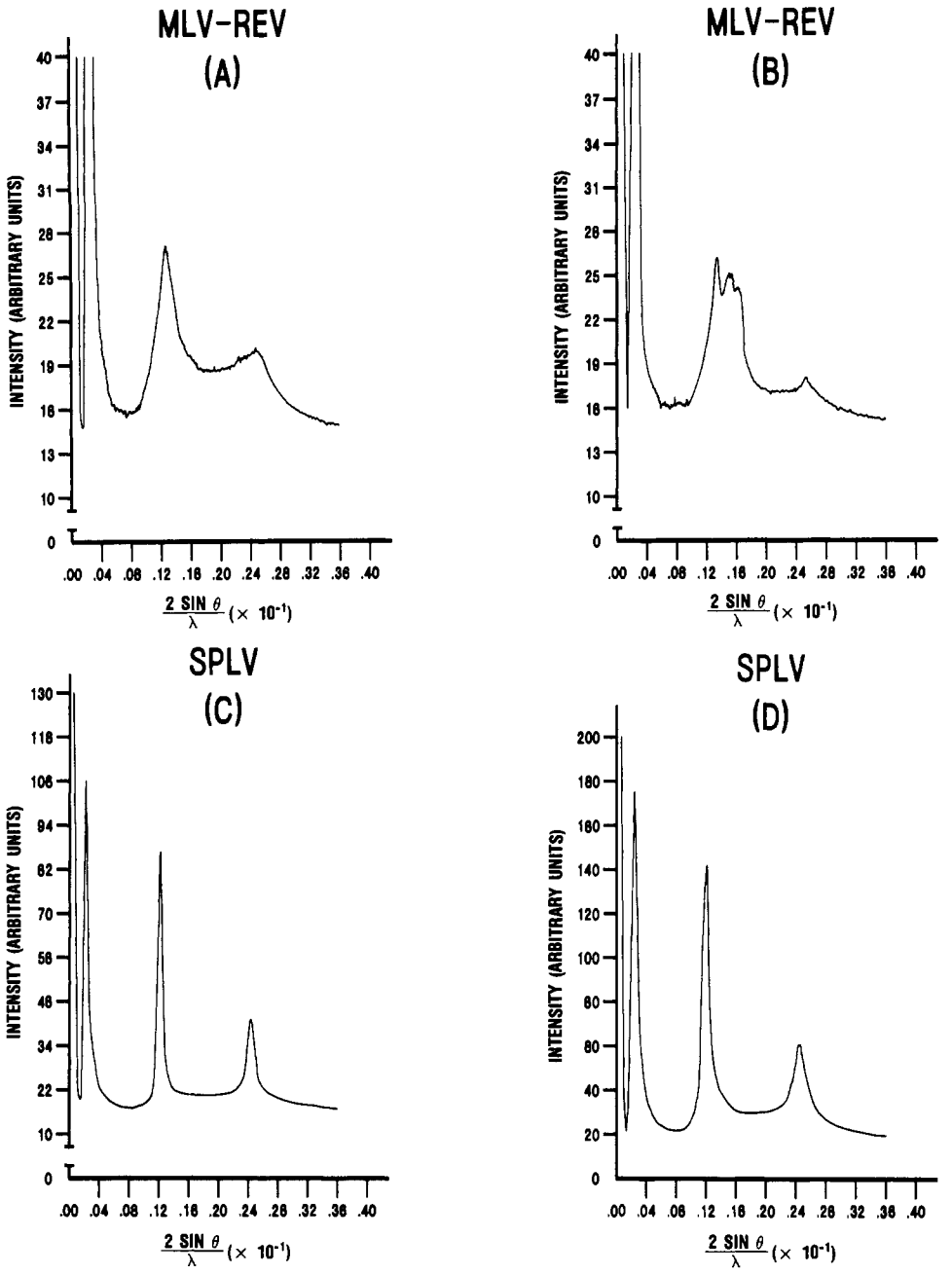


FIGURE 4: X-ray diffraction patterns of DPPC/CH (9:1) MLV-REV and SPLV liposomes. Liposomes for (A) and (C) were prepared from diethyl ether/PBS (10/0.3 v/v), and the vesicles were heated to 50 °C for 1 h after the organic solvent was evaporated. Liposomes for (B) and (D) were prepared from diisopropyl ether/PBS (10/0.3 v/v), and the organic solvent was evaporated at 50 °C. For SPLV vesicles [(C) and (D)] the organic solvent is removed with concurrent sonication of the emulsion. All X-ray diffraction patterns were obtained at 26 ± 0.05 °C with 3-h exposure times.

drocarbon chains in the membrane (Tardieu et al., 1973). Thus the long-spacing signature can be used to compare qualitatively the hydrocarbon packing in the vesicle. From Figure 3, the hydrocarbon packing in multilayered vesicles prepared in buffer is MLV-REV = SPLV ≠ MLV, and that in multilayered vesicles prepared in water is MLV-REV = SPLV = MLV.

X-ray diffraction measurements also demonstrate that the manner in which the lipids are dispersed affects the final liposome structure. In this regard, processes used to prepare liposomes can create lamellae with an irregular repeating lamella. Lipid mixtures that allow long spacing to vary substantially are most susceptible to irregular distances in the repeating lamella of the final liposome population. We studied two lipid mixtures that allow D_p and $D_{liposome}$ to vary several angstroms. Lipid compositions tested were (i) DPPC/CH at ~10 mol % CH and (ii) PC/PA or PC/PS. Negative charges imparted on the membrane by PA and PS allow the membranes to separate well beyond the ~30-Å separation expected from neutral PC bilayers (Rand, 1981). Thus if the process used to make liposomes substantially disrupts the bilayers during lipid assembly into liposomes, then negatively charged vesicles will have heterogeneous repeat distances. For composition i, long spacing increases 15 Å between DPPC and DPPC/CH (9:1) liposomes (Table VII). Long spacings of DPPC/CH mixtures are thus very sensitive to the membrane concentration of CH, particularly near 10 mol % CH. If processes used to make multilayered liposomes from mixture i create heterogeneous lipid distributions in the final vesicle population, one can expect heterogeneous long spacings. If all of the repeating distances are irregular, then one will not obtain a diffraction pattern from the vesicles. Figure 4 shows

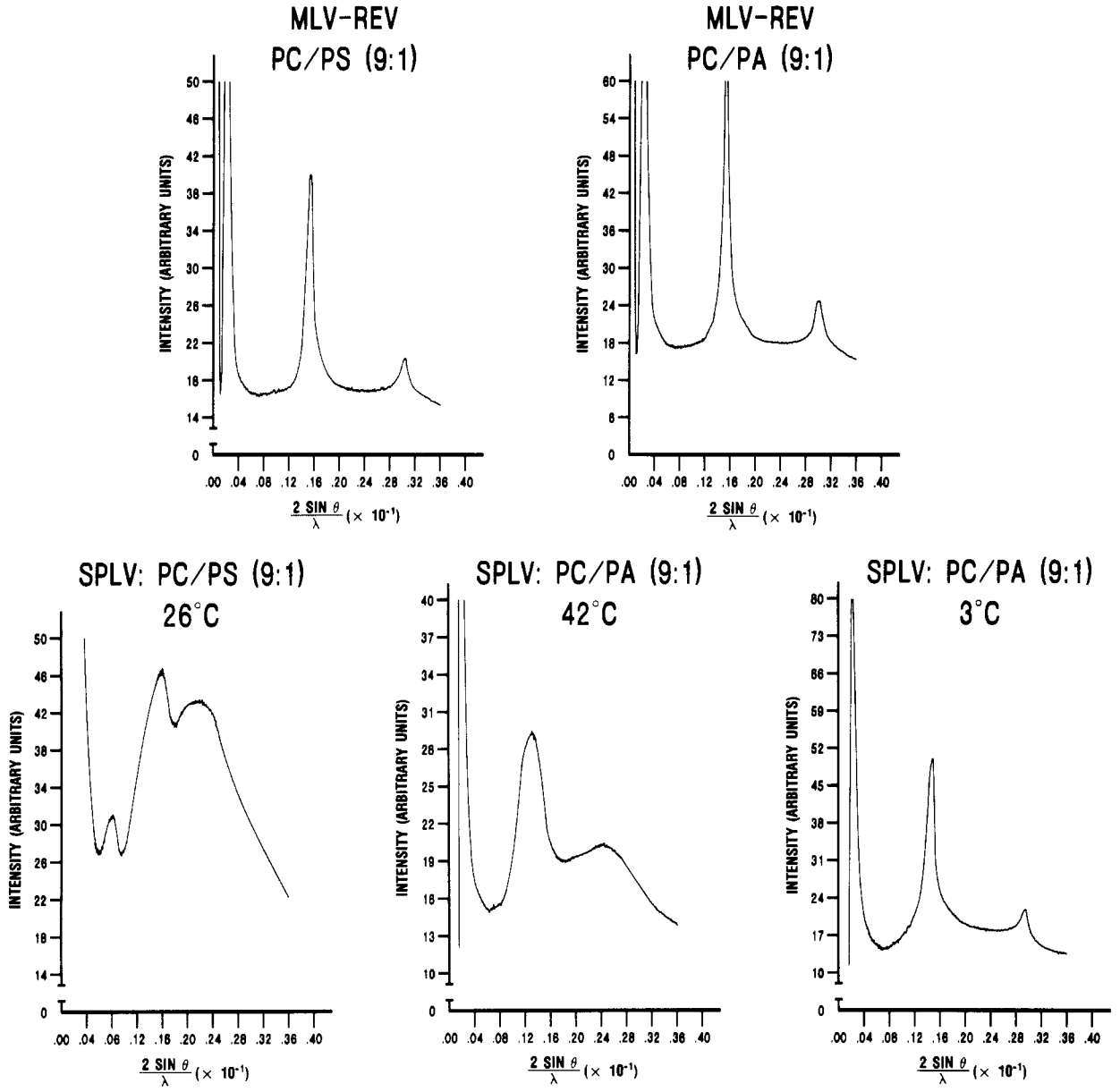


FIGURE 5: X-ray diffraction patterns of MLV-REV and SPLV vesicles with negatively charged membranes. All vesicles were prepared from diethyl ether/PBS (10/0.3 v/v). MLV-REV X-ray diffraction patterns were performed at 25.5 ± 0.5 °C with a 2-h exposure time. SPLV specimens were exposed for 16 (PC/PS) or 3 h (PC/PA).

that irregular repeating distances in MLV-REV vesicles are generated from DPPC/CH (9:1) mixtures but not in SPLV vesicles of the same mixture. This occurred when heat was applied during or after lipid assembly into membranes for both processes. In two additional experiments DPPC/CH (9:1) MLV-REV vesicles elicited only integral orders of diffraction, indicating a homogeneous lipid distribution with vesicles. Thus homogeneous mixing of the lipids may or may not occur during vesicle formation by the MLV-REV process. For all SPLV preparations tested only integral orders of diffraction were found; CH is homogeneously distributed throughout the lamella of SPLV vesicles composed of DPPC/CH (9:1). Except for 10 mol % CH, MLV-REV vesicles gave diffraction patterns similar to those of SPLVs in Figure 4, and the long spacings are shown in Table VII. Only DPPC/CH (9:1) MLV-REV vesicles show evidence of a heterogeneous lipid distribution in the vesicles. SPLVs composed of DPPC/CH (9:1) always give symmetric integral orders of diffraction with a repeat distance corresponding to 82.2 Å. This compares favorably with MLV-REVs of the same composition (Table VII).

X-ray studies of negatively charged liposomes also demonstrate that heterogeneous repeat distances can occur, depending on the method used to assemble the lipids. X-ray diffraction patterns of negatively charged MLV-REV vesicles are shown in Figure 5. The X-ray diffraction pattern of negatively charged MLV-REV vesicles depends on the history of the sample. Integral orders of diffraction are obtained for PC/PA and PC/PS MLV-REV vesicles only if the liposomes are prepared and maintained at 4–25 °C. Heating the vesicles to 40 °C causes broad diffraction patterns. In contrast, negatively charged SPLV vesicles generate either no diffraction pattern or a diffuse diffraction pattern ($n = 6$) if the vesicles were prepared and maintained between 4 and 40 °C. However, the SPLV X-ray diffraction pattern obtained at 3 °C showed evidence of homogeneous bilayer spacing for negatively charged vesicles (Figure 5). Thus, even though the X-ray diffraction patterns are diffuse, bilayer repeat distances are relatively homogeneous. The SPLV vesicles composed of PC/PA (9:1) in Figure 5 gave long spacings of 80.7 and 68.4 Å at 42 and 3 °C, respectively. It is unreasonable to expect

the bilayer thickness to decrease 12.3 Å, and thus the water thickness in the lamella is changing. This large change in long spacing suggests that substantial rearrangement of the bilayer occurs for negatively charged multilayered liposome membranes exposed to temperature changes. These data support the hypothesis that sonication during membrane assembly into liposomes (SPLV process) allowed the interlamellar distances to be heterogeneous for negatively charged membranes.

Discussion

The structure and solute entrapment characteristics of multilayered vesicles prepared from w/o emulsions depends on (i) the aqueous volume in the emulsion, (ii) the amount of emulsion lipid, and (iii) the volume trapped in the liposome core compared to the volume trapped in the liposome lamella. Our earlier work (Pidgeon et al., 1986) addressed (i) and (ii), and this report addresses (iii).

The computer-generated data (Table V), X-ray diffraction data (Table VII, Figures 4 and 5), and the relationship between core and lamellar water (Table VI) are conveniently discussed with reference to Scheme I. Scheme I compares the formation and structure of both planar and liposome membranes. When egg phosphatidylcholine is deposited on the floor of container, planar membranes exist when as little as 15 wt % water is added. Increasing water content causes the bilayers to separate or swell to a maximum separation at 40–50 wt % water. Interlamellar water reflects the microliters of trapped volume (similar to the liposomal trapped volume) and is constant for a given type and amount of lipid. Although ~50 wt % water is imbibed by PC/water mixtures, ~15 wt % water is needed to hydrate the PC head groups. Thus 100 mg of PC imbibing 100 μL of water (~50 wt %) contains ~25 μL of hydration water and ~75 μL of water available for solutes. Solute water volume is the only volume of water available for drug dissolution. The total interlamellar trapped water volume in PC/water mixtures at 50 wt % water is 0.75 μL/μmol of PC, which corresponds to an E^{ratio} of ~1.0 μL/μL of PC because the density of lipids is ~1. Subtracting hydration water from total trapped water gives the available solute trapped volume per micromole (or microliter) of lipid. Under this limiting condition E^{r} = 0.75 μL of H_2O/μL of PC. Since long spacing remains constant during the transition from completely swollen planar bilayers to liposome populations for neutral vesicles (Table VII), water between the liposome lamellae will remain constant for a given weight of lipid. That is, any given liposome population composed of PC should contain ~50 wt % water between the lamellae. In addition, the smallest allowable E^{ratio} for any MLV population equals E^{r} because the lamellae are completely swollen. Experimentally measured trapped volumes using radiotracers do not measure hydration water, and E^{r} corrected for hydration water becomes the lower limit of the liposome population E^{ratio}. The lamellar E^{r} corrected for hydration water reflects the available volume (microliters) per milligram or microliter of lipid that is accessible to solutes. Some investigators have corrected for hydration water in liposomes to account for observed entrapment being less than expected from volume measurements (Adrian & Huang, 1979).

In the transition from planar membranes to liposome particles (Scheme I), the precise water content necessary to suspend all the lipid into liposome particles is not clearly defined. X-ray diffraction measurements to determine bilayer separation, or long spacing, for various lipids are typically performed at ~50 wt % water, which "always provides an excess of water" (Ladbrooke, 1968) in the mixture. Although excess water exists, insufficient water is available to form liposome particles. Consider 100 mg of PC imbibing 90 μL of water (~45 wt % water). Twenty microliters of bulk water added to this mixture is not enough water to suspend the 190 μL of planar membranes into closed liposome particles.[5] However, maximum bilayer separation exists, and excess water is present. Small (1967) estimated that, at approximately 65 wt % water, floating myelin structures (liposomes) appear in PC/water mixtures. For this reason >65 wt % water is denoted in Scheme I as the lower limit of water needed for vesicle formation.

A significant finding from this study is that water inside multilayered liposomes can be partitioned between the core and the lamellae. The liposomes' core contains excess water whereas the lamellae are completely swollen. Thus, with regard to hydration and dehydration of lipid mixtures (Scheme I), the liposomes' core is formed after the lamellae are formed (during hydration), but the liposome's core is destroyed before the lamellae are destroyed (during dehydration). In contrast, for MLV-REV and SPLV vesicles, the core is formed first and the lamellae are established after the organic solvent is removed from the w/o emulsion. However, as with conventional MLVs, dehydration of MLV-REV or SPLV vesicles should cause the liposomes' core to collapse before the lamellae (because lamellae will remain completely swollen as long as sufficient water is available in the PC/water mixture). This is in agreement with our previous work that postulated that as one dehydrates the MLV-REV gel to less than 45 wt % water before adding excess buffer, the core of the particle collapsed, resulting in decreased entrapment (Pidgeon et al., 1986).

When planar membranes are compared to liposome membranes, curvature may or may not affect the volume-trapping characteristics of the liposomes' lamellae. The repeating bilayers shown in Scheme I are drawn to scale. The volume-trapping characteristics of bilayers 1–15 are shown in Scheme I. For liposome membranes to trap water similar to planar membranes, $E^{r} = E^{r}_{n=\infty} = d_w/d_l$. This occurs after ~15 bilayers when d_w = 30 Å and core diameter = 300 Å. However, membrane curvature is more important than bilayer number or core diameter. Membrane curvature is related to the radius of the particular lamella in question. Liposome membranes with >1000-Å radius trap volumes similar to planar membranes. This is evident in Scheme I. Thus, large core diameters make interlamellar trapping insensitive to membrane curvature and bilayer separation at least up to $d_w \leq 74$ Å (supplementary material).

Recently, a method to form multilayered vesicles has been reported (Gruner et al., 1985). Liposome formation by this method involves continuously sonicating excess water/ether emulsions containing lipids while purging them with nitrogen. When the organic solvent is removed, SPLV vesicles are formed. We have found SPLV vesicles to have E^{ratio} values of 0.67–0.74 μL of H_2O/μL of PC for negatively charged lipids and 1.08–1.27 μL of H_2O/μL of PC for neutral vesicles. From these low E^{ratio} values it was postulated that SPLV cores contain a small volume fraction of trapped water in their cores (Pidgeon et al., 1986). This is in agreement with Table VI, which compares the fraction of water trapped in the liposomes' core for SPLV and MLV-REV vesicles. For all lipid mixtures tested, SPLV vesicles have a smaller fraction of water trapped

[5] PC lipid/water phases at 50–65 wt % water generate myelin structures. Myelin structures have been photographed and studied by Shew and Deamer (1985), Sakurai (1985), and Sakurai and Kawamura (1984). Myelin structures are cylindrical and contain repeating lamellae throughout the cylinder.

in the liposomes' core in comparison with MLV-REVs.

Because negatively charged SPLV vesicles have heterogeneous interlamellar repeat distances, calculating f^{core} and $f^{lamellae}$ was done with caution. However, since $E^{ratio} < E^{r}$ of planar membranes, we believe the vesicles have a small core. Had E^{ratio} of negatively charged SPLVs been substantially greater than E^{r}, it would be difficult to speculate on how water is partitioned throughout the particle. We believe that maximizing solute entrapment during the MLV-REV process depends on maintaining a core throughout the process. Maintaining a core will assure an entrapment of >0.9 μL/μL of PC, which is the optimum trapped aqueous volume for interlamellar PC phases. Because interlamellar water is ~1.0 μL/μL of PC, 100 mg of PC will entrap ~100 μL of water if the core collapses during the process. Entrapment under conditions of core collapse will be 33%/100 mg of lipid if 300 μL of emulsion water is used; entrapment will increase to 45%/100 mg of lipid if 100 μL of azeotropic water is lost during the process. Our experiments with solid lipid mixtures of DPPC/CH (Table IV) support the idea that core collapse can occur during the MLV-REV process. Preparation of solid liposomes whereby the gel suspension is heated (method I) results in low drug entrapment because one is merely heating concentrated DPPC/CH–water mixtures; entrapment is 35–40%. Heating the membranes as the membranes are forming (method II) increased entrapment to ~55%, which suggests that a core was maintained throughout the process. The increased entrapment with method II is particularly significant under the experimental conditions that used 500 μL of emulsion water. When emulsion water is increased from 300 to 500 μL, entrapment typically decreases for the MLV-REV process (Pidgeon et al., 1986). This occurs when MLV-REV vesicles are prepared in a round-bottom flask or test tube.

DPPC/CH mixtures, 0 ≤ mol % CH ≤ 50, sequesters 30–40 wt % water (Ladbrooke et al., 1968). Thus 80–90 μL of water/100 mg of lipid is sequestered between planar lamellae composed of DPPC/CH mixtures. In agreement with planar membranes, MLV-REV vesicles composed of DPPC/CH mixtures with 0, 13, 17, and 32 mol % CH prepared by method I show E^{ratio} values of ~0.90 μL of H_2O/μL of lipid. This corresponds to 95–100 μL of H_2O trapped/100 mg of lipid. Since planar membranes entrap similar volumes, "core collapse" occurred during liposome formation by method I. The higher drug entrapment found for 10 mol % CH, however, supports the idea that a core was maintained throughout the process. E^{ratio} for DPPC/CH (9:1) was 1.49, which corresponds to 149 μL of trapped volume, which exceeds the trapping ability of the lamellae. Thus 10 mol % CH was able to maintain a core and/or keep solutes sequestered during liposome formation to increase entrapment. Ladbrooke et al. (1968) reported that 7.5 mol % CH represents the maximum amount of CH that can be accommodated into the hexagonal lattice of DPPC without seriously disturbing the hydrocarbon chains. Intercalation of ~10 mol % CH may be sufficient to promote a stable core during the process of liposome formation. It is difficult to determine what the membrane concentration of CH is at the core because this mixture had a heterogeneous distribution of CH throughout the lamellae. Heating the membrane during liposome formation from solid lipids (method II) gives E^{ratio} values between 2 and 3 (Table III). From the computer-generated data, this suggests that a core was maintained during method II of the MLV-REV process for all DPPC/CH mixtures.

Conclusions

The method of assembling lipids into liposomes affects entrapment and particle structure. Multilayered liposomes prepared from w/o emulsions have high entrapment. Sonicating the w/o emulsion while concurrently evaporating the organic solvent (SPLV process) causes core collapse and facilitates lipid equilibration throughout the lamella. Sonication also causes negatively charged SPLV vesicles to elicit heterogeneous repeat distances. Evaporating the organic solvent from the w/o emulsion without sonicating (MLV-REV process) can generate a heterogeneous distribution of lipid between individual lamellae in the liposomes (Figure 4). This is probably due to the solubility of different lipids in the organic solvent. Negatively charged MLV-REV vesicles have homogeneous repeat distances when the vesicles are prepared and maintained at room temperature. This indicates that interlamellar water spacings were not disrupted during liposome formation.

Supplementary Material Available

Table giving computer-generated data comparing the trapped volume in the lamellae to the total liposomal trapped volume in multilayered liposomes with small and large cores and finite bilayer numbers (4 pages). Ordering information is given on any current masthead page.

Registry No. PG, 4537-77-3; DPPC, 2644-64-6; PA, 5129-68-0; CH, 57-88-5; Et_2O, 60-29-7; i-Pr_2O, 108-20-3; $EtCH(NH_2)CO_2H$, 80-60-4; L-Tyr, 60-18-4; poly(I)·poly(C), 24939-03-5; tobramycin, 32986-56-4; tobramycin sulfate, 49842-07-1; sucrose, 57-50-1.

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Lipid Mobility and Order in Bovine Rod Outer Segment Disk Membranes. A Spin-Label Study of Lipid–Protein Interactions

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ABSTRACT: Lipid–protein interactions in bovine rod outer segment disk membranes have been studied by using a series of eight stearic acid spin-label probes which were labeled at different carbon atom positions in the chain. In randomly oriented membrane dispersions, the electron spin resonance (ESR) spectra of the C-8, C-9, C-10, C-11, C-12, C-13, and C-14 atom positional isomers all apparently consist of two components. One of the components corresponds closely to the spectra obtained from dispersions of the extracted membrane lipids, and the other, which is characterized by a considerably greater degree of motional restriction of the lipid chains, is induced by the presence of the protein. Digital subtraction has been used to separate the two components. The proportion of the motionally restricted lipid component is approximately constant, independent of the position of the spin-label group, and corresponds to 30–40% of the total spin-label spectral intensity. The hyperfine splitting of the outer maxima in the difference spectra of the motionally restricted component decreases, and concomitantly, the line widths increase with increasing temperature but change relatively little with increasing distance of the spin-label group from the polar head-group region. This indicates that the corresponding chain motions of the protein-interacting lipids lie in the slow-motion regime of spin-label ESR spectroscopy ($\tau_R \sim 10^{-8}$ s) and that the mobility of these lipids increases with increasing temperature but does not vary greatly along the length of the chain. The data from the hyperfine splittings also suggest the existence of a polarity gradient immediately adjacent to the protein surface, as observed in the fluid lipid regions of the membrane. The more fluid lipid component is only slightly perturbed relative to the lipids alone (for label positions 5–14, inclusive), indicating the presence of chain motions on the nanosecond time scale, and the spectra also reveal a similar polarity profile in both lipid and membrane environments. ESR spectra have also been obtained as a function of magnetic field orientation with oriented membrane samples. For the C-14 atom positional isomer, the motionally restricted component is observed to have a large hyperfine splitting, with the magnetic field oriented both parallel and perpendicular to the membrane normal. This indicates that the motionally restricted lipid chains have a broad distribution of orientations at this label position. A motionally restricted lipid component is also detected with the C-5 atom positional isomer in oriented membranes. It is concluded that the spin-labeled lipids in direct association with rhodopsin are not highly ordered and display motional restriction along the entire length of their chains, with rotational correlation times in the range of 10 ns.

Electron spin resonance (ESR)[1] spectroscopic measurements on a variety of different biological membranes and reconstituted lipid–protein systems have revealed the presence of two populations of spin-labeled lipids, differing in their molecular mobility. One population corresponds to the fluid environment typical of lipid bilayers, and the other, more motionally restricted component, is assigned to the lipid population interacting directly with the integral membrane proteins [see, e.g., Marsh & Watts (1982a)]. The effective number of motionally restricted lipids per protein is independent of the total lipid to protein ratio, if allowance is made for protein–protein contacts at low lipid/protein ratios. In this sense, the motionally restricted lipid may be defined as a first-shell or boundary layer (Jost et al., 1973a) of lipids interacting directly with the intramembranous surface of the protein.

The ESR spin-label method has proved very useful in analyzing both the stoichiometry and specificity of the lipid interactions with the surface of integral proteins in a wide variety of membrane systems. The effective number of

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[1] Abbreviations: ESR, electron spin resonance; ROS, rod outer segment(s); *n*-SASL, *n*-(4,4-dimethyloxazolidine-*N*-oxyl)stearic acid; *n*-PCSL, 1-acyl-2-[*n*-(4,4-dimethyloxazolidine-*N*-oxyl)stearoyl]-*sn*-glycero-3-phosphocholine; BHT, butylated hydroxytoluene; EDTA, ethylenediaminetetraacetic acid; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane.

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