Generation of large unilamellar vesicles from long-chain saturated phosphatidylcholines by extrusion technique

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Extrusion of multilamellar vesicles under moderate pressures through filters of defined pore size is a convenient method for generation of large unilamellar vesicles of variable size (Hope et al. (1986) Chem. Phys. Lipids 40, 89–108). To date, this technique has been applied primarily to unsaturated phospholipids in the liquid-crystalline state. In this work we extend this procedure to include saturated phosphatidylcholines of chain lengths varying from C_{14} (dimyristoylphosphatidylcholine) to C_{20} (diarachidoyl phosphatidylcholine). It is shown that whereas gel-state lipids cannot be extruded at convenient pressures, systems incubated at temperatures above the gel-to-liquid-crystalline transition (T_c) can be readily extruded through filters with pore sizes ranging from 30 nm to 200 nm to produce homogeneously sized systems. The presence of cholesterol (45 mol%) slightly facilitates extrusion at temperatures below T_c and results in reduced extrusion rates above T_c. Vesicle systems containing long-chain saturated lipids have potential in applications where highly stable large unilamellar vesicles are required.

Introduction

General procedures for the production of large unilamellar vesicles (LUVs) composed of long-chain saturated phospholipids are not presently available. Techniques such as sonication [1,2] result only in 'limit size' vesicles and suffer other limitations, including lipid degradation and limited trapping efficiencies. Techniques involving dilution from organic solvents such as ether [3] or ethanol [4] must often be modified because of the limited solubility of long-chain saturated lipids in these solvents, whereas detergent dialysis procedures [5–7] have problems inherent in lipid solubilization and detergent removal.

In previous work we have shown that extrusion under moderate pressures can generate LUVs of variable size from multilamellar precursors [8,9]. These studies largely employed unsaturated lipids in the liquid-crys-talline state. In the current investigation we apply extrusion to systems composed of saturated phosphatidylcholines (PCs). It is shown that LUVs can be efficiently generated for saturated PC with acyl chains as long as 20 carbons (diarachidoyl-PC), provided the extrusion temperature is greater than the gel-to-liquid-crystalline transition temperature (T_c) of the lipid. Further, the effects of cholesterol on extrusion and the influence of freeze-thaw on the trapping properties of the resulting LUVs are characterized.

Materials and Methods

Lipids and chemicals

Egg phosphatidylcholine (egg PC), dimyristoylphosphatidylcholine (DMPC), dipalmitoylphosphatidylcholine (DPPC), distearoylphosphatidylcholine (DSPC), and diarachidoylphosphatidylcholine (DAPC) were obtained from Avanti Polar Lipids (Birmingham, AL). Chromatographic grade cholesterol (> 99% pure) was obtained from Sigma and used without further purification. The [*methoxy-*^{3}H]inulin was obtained from New England Nuclear (Mississauga, Ontario). All other chemicals were obtained from BDH (Vancouver, B.C.) and were of analytical grade.

Vesicle preparation

Lipid films were made by drying the lipid in a chloroform solution under nitrogen. The use of chloroform/methanol mixtures was avoided because phase separation of cholesterol frequently occurred during evaporation. Large multilamellar vesicles (MLVs) were prepared by hydrating (vortex mixing) the dry lipid film in 150 mM NaCl, 20 mM Hepes (pH 7.4), maintained at a temperature that was approximately ten degrees above the gel-to-liquid-crystalline phase transition temperature (T_c) of the phospholipid. Egg PC and egg PC/chol (55:45 mol ratio) were hydrated at 21°C; DMPC and DMPC/chol (55:45 mol ratio), at 30°C; DPPC and DPPC/chol (55:45 mol ratio), at 50°C; DSPC and DSPC/chol (55:45 mol ratio), at 65°C; and DAPC and DAPC/chol (55:45 mol ratio), at 85°C. Lipid concentrations of 50 mg · ml^{-1} were routinely employed. The frozen and thawed MLV systems (FATMLVs) were obtained by freezing the MLVs in liquid nitrogen and thawing the sample in a water bath at the same temperature used for hydration.

MLVs and FATMLVs were extruded using a stainless steel extrusion device (Lipex Biomembranes, Vancouver, B.C.) equipped with a 10-ml water-jacketed 'thermobarrel' attached to a circulating water bath, which allowed extrusion at elevated temperatures. The lipid suspensions were equilibrated at the appropriate temperature for at least 15 min prior to extrusion. Extrusions were performed through two (stacked) polycarbonate filters (25 mm diameter) of pore sizes ranging from 200 nm to 30 nm in diameter at nitrogen pressures of up to 5600 kPa (800 p.s.i.). The vesicle recovery after the extrusion cycles was typically found to be greater than 95% after ten extrusion cycles.

Size determination by quasi-elastic light scattering

The size distribution of the extruded liposomal systems was determined by quasi-elastic light scattering (QELS) analysis utilizing a Nicomp Model 370 submicron laser particle sizer (Pacific Scientific, MD), which was equipped with a 5 mW Helium-Neon Laser at an excitation wavelength of 632.8 nm. The Nicomp QELS uses digital autocorrelation to analyze the fluctuations in scattered light intensity generated by the diffusion of vesicles in solution. The measured diffusion coefficient is used to obtain the average hydrodynamic radius and hence the mean diameter of the vesicles. For the saturated lipid systems, values obtained in the solid-particle mode were taken as the vesicle size, since these values were similar to the vesicle sizes determined by freeze-fracture electron microscopy. This correspondence is clearly illustrated in Table I, where the sizes of DPPC, DSPC, and DAPC vesicles, which have been extruded ten times through 100 nm pore-size filters, were determined by freeze-fracture electron microscopy

TABLE I

Comparison of vesicle size by freeze-fracture and QELS analysis

Lipid [a]	Freeze-fracture analysis (nm ± S.D.)	QELS analysis	
		solid particle (nm ± S.D.)	vesicles (nm ± S.D.)
DPPC	67 ± 19	68 ± 30	117 ± 52
DSPC	66 ± 15	74 ± 40	176 ± 95
DAPC	60 ± 15	71 ± 33	133 ± 63

[a] Lipid vesicles were extruded ten times through 100-nm pore-size filters.

techniques and by QELS analysis in the vesicle and solid-particle modes.

Determination of trapped volumes

Trapped volumes of the vesicles were determined using [*methoxy*-^{3}H]inulin as the aqueous trap marker. Phospholipid vesicles were hydrated and dispersed in the presence of trace amounts of [*methoxy*-^{3}H]inulin (1 μCi · ml^{-1}). After extrusion, the vesicles were passed down a 5-ml Sepharose-4B column to remove untrapped marker. Aliquots of the vesicle-containing fraction were assayed for lipid phosphorus [10] and monitored for radioactivity using a Beckman Model 3801 liquid scintillation counter. Trapped volumes were calculated from the specific activity of [*methoxy*-^{3}H]inulin and expressed as μl of aqueous trapped volume per μmol of total lipid following removal of free [*methoxy*-^{3}H]inulin. Untrapped marker was removed from the MLV preparations by centrifuging the vesicles for 15 min at 12000 × g. The resulting pellet was washed three times by resuspension in buffer and centrifugation in order to remove all the untrapped marker.

Freeze-fracture electron microscopy

Vesicle preparations were mixed with glycerol (25% v/v) and frozen from 20°C in a Freon slush suspended in liquid nitrogen. The DSPC vesicles were incubated at 60°C for 30 min in order to ensure equal distribution of glycerol across the vesicle membrane. Samples were fractured and replicated employing a Balzers BAF 400D apparatus, and micrographs of replicas were obtained using a Joel 1200 electron microscope. Distributions of vesicle size were determined according to van Venetie et al. [11].

Results

Effect of temperature on extrusion of DSPC and DSPC/chol MLVs

In the first series of experiments, the feasibility of extruding DSPC and DSPC/chol (55:45 mol ratio) MLVs through two (stacked) 100 nm pore-size filters at temperatures above or below the T_c of DSPC (55°C)
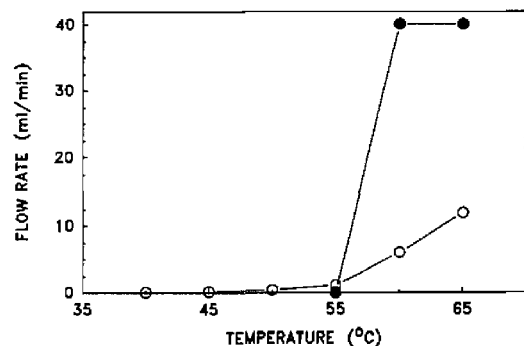
202

Fig. 1. Effect of temperature on extrusion of DSPC (●) and DSPC/chol (55:45 mol ratio) (○) MLVs. MLVs were initially hydrated at 15 mg·ml⁻¹ in 150 mM NaCl, 20 mM Hepes buffer (pH 7.4) at 65°C and equilibrated for 15 min. Aliquots (2 ml) were equilibrated for 15 min at the appropriate temperature in the extrusion device and subsequently extruded at 3500 kPa.

was investigated. The lipid MLV suspension (15 mg·ml⁻¹ lipid, 2 ml) was equilibrated for 15 min at temperatures above or below the T_c and then extruded at 3400 kPa using polycarbonate nuclearpore filters of 25 mm diameter. Fig. 1 illustrates the flow rates of DSPC and DSPC/chol (55:45 mol ratio) MLVs extruded at different temperatures. Pure DSPC MLVs could only be extruded above 55°C; at lower temperatures pressures as high as 5600 kPa did not result in lipid extrusion. The DSPC/chol mixture could be extruded below the T_c of DSPC but at significantly lower flow rates than observed when extrusion was carried out above the T_c. The flow rate for extrusion of DSPC/chol (55:45 mol ratio) MLVs at 3400 kPa was approximately 0.06 ml·min⁻¹ at 40°C, compared to 12 ml·min⁻¹ at 65°C and 24 ml·min⁻¹ at 85°C (data not shown). Similar effects were also observed for the other saturated lipid mixtures (results not shown). These results clearly indicate that lipids in gel-state cannot be extruded and the presence of cholesterol, which abolishes the cooperative gel-liquid-crystalline transition [12], does not result in acceptable extrusion rates at temperatures below the T_c. Thus, in subsequent experiments, extrusions were carried out at temperatures approximately 10 C° above the T_c of the saturated phospholipid being used. It should be noted that the flow rate of extrusion is also dependent on the surface area of the filter.

Generation of vesicles of various sizes and lipid compositions

The ability to generate vesicles of different sizes and lipid compositions was examined using dimyristoyl-PC (DMPC), dipalmitoyl-PC (DPPC), distearoyl-PC (DSPC), and diarachidoyl-PC (DAPC) in the presence or absence of 45 mol% cholesterol. The temperatures and the pressures required to extrude lipid suspensions

(50 mg·ml⁻¹) of egg PC, DMPC, DPPC, DSPC, and DAPC through different filters with and without 45 mol% cholesterol are shown in Tables II and III, respectively. MLVs were prepared as indicated in Materials and Methods and were extruded ten times through two stacked 100-nm pore-size filters at temperatures above the T_c of the phospholipid. Subsequently, aliquots of the 100-nm extruded vesicles were extruded ten times through filters of 50 nm or 30 nm pore size.

Extrusion of all the vesicle systems composed of different saturated phospholipid species through the 100 nm filters, and subsequently the 50-nm filters, was readily achieved at moderate pressures of 2100 kPa to 3400 kPa, provided the extrusion temperature was at least 5 C° to 10 C° above the T_c of the phospholipid. Following extrusion through filters with 50 nm pore size, vesicles with a mean diameter of 60 nm were obtained according to QELS analysis. Extrusion through the 30-nm filters resulted in a slight decrease in vesicle size to approximately 50 nm diameter. However, extrusion of lipid suspensions containing DSPC and DAPC through the 30-nm filters resulted in unstable preparations, which appeared to aggregate and fuse into vesicles with diameters exceeding 600 nm in diameter when incubated at room temperature or at 4°C. This result is seen in the freeze-fracture electron micrograph for DSPC

TABLE II

Parameters for generating egg PC, DMPC, DPPC, DSPC, and DAPC vesicles of various sizes [a]

Lipid	Filter pore size (nm)	Pressure (kPa)	Temperature (°C)	Size QELS [b] (nm ± S.D.)
egg PC	100	2100	21	107±27
	50	3100	21	59±17
	30	3500	21	56±14
DMPC	100	2100	30	100±23
	50	3100	30	59±15
	30	3500	30	49±14
DPPC	100	1750	50	83±34
	50	2100	50	70±16
	30	2800	50	42±15
DSPC	100	1750	65	120±63
	50	2500	65	49±20
	30	4200	65	38±16
DAPC	100	1750	85	106±40
	50	2500	85	106±40
	30	3500	85	35±15

[a] Lipid concentrations of 50 mg·ml⁻¹ were used for extrusion. The MLVs were hydrated in buffer at the appropriate temperature for 15 min. The sample was then extruded ten times through 100-nm pore-size polycarbonate filters and aliquots subsequently extruded ten times through 50-nm or 30-nm pore-size polycarbonate filters.
[b] Quasi-elastic-light scattering measurements were made using the solid-particle mode at sensitivity setting of approximately 190.
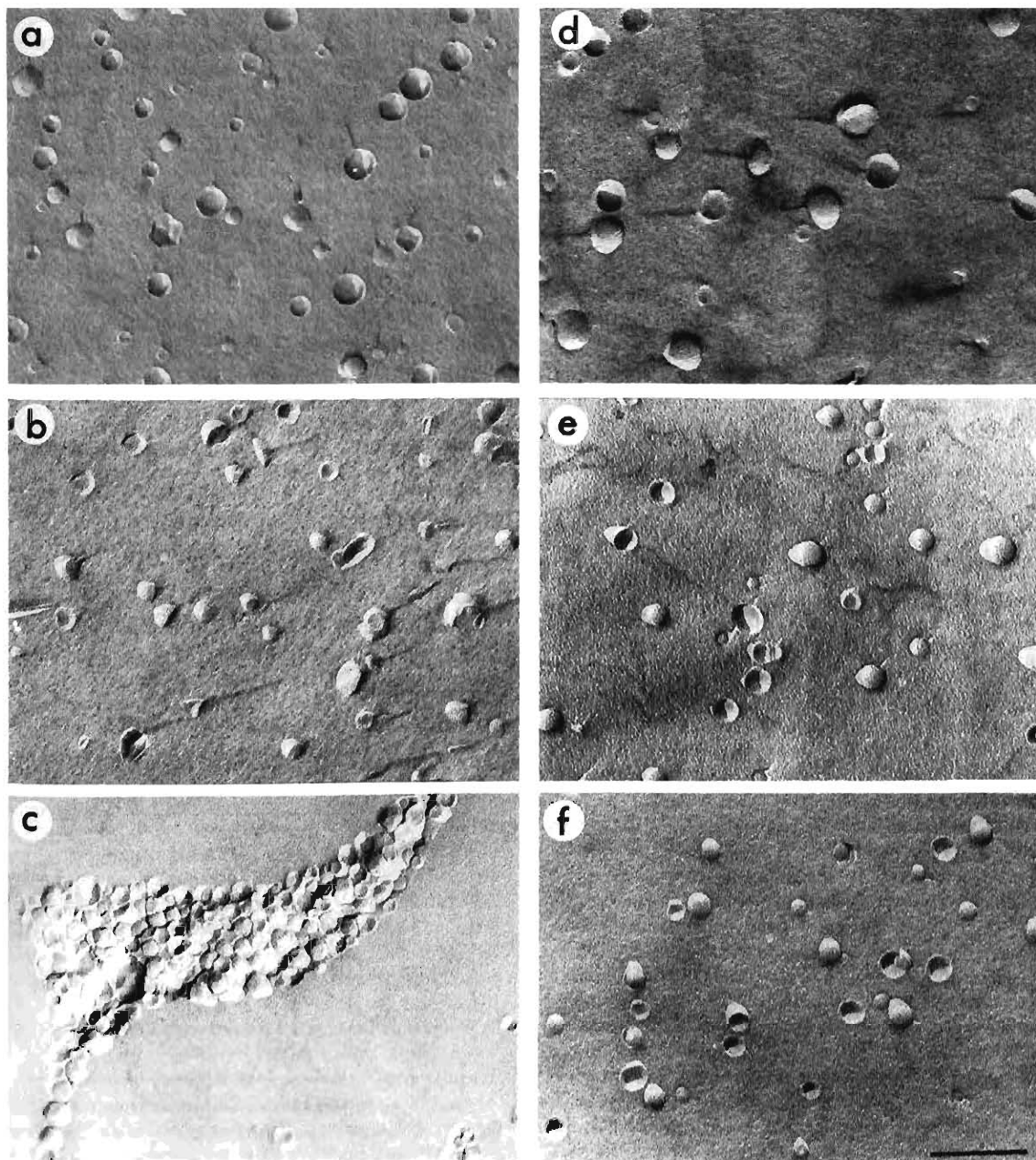
Fig. 2. Freeze-fracture electron micrographs of DSPC and DSPC/chol (55:45). DSPC MLVs were sequentially extruded ten times through 100-nm (a), 50-nm (b), and 30-nm (c) pore-size polycarbonate filters at 65°C. Similarly, DSPC/chol (55:45 mol ratio) MLVs were extruded sequentially through the 100-nm (d), 50-nm (e), and 30-nm (f) pore-size filters at 65°C. Freeze-fracture was performed on these samples in the presence of 25% glycerol using standard procedures. All panels exhibit the same magnification; the bar in panel (f) represents 200 nm.

30-nm vesicles shown in Fig. 2c, where extensive aggregation is observed. This result is consistent with previous findings [13,14] that have shown the inherent instability of small sonicated vesicles composed of saturated phosphatidylcholines to aggregate and fuse to form larger vesicles below their T_c. In contrast, the cholesterol-containing systems did not aggregate, and appeared to maintain their size for at least 30 days at

TABLE III

Parameters for generating egg PC/chol, DMPC/chol, DPPC/chol, DSPC/chol, and DAPC/chol vesicles of various sizes [a]

Lipid	Filter pore size (nm)	Pressure (kPa)	Temperature (°C)	Size QELS [b] (nm ± S.D.)
egg PC/chol	100	2100	21	129 ± 35
	50	2800	21	59 ± 19
	30	4200	21	55 ± 17
DMPC/chol	100	2100	30	125 ± 56
	50	2800	30	70 ± 23
	30	3200	30	49 ± 21
DPPC/chol	100	2500	50	122 ± 43
	50	3500	50	62 ± 22
	30	4500	50	59 ± 26
DSPC/chol	100	2100	65	107 ± 31
	50	2500	65	61 ± 18
	30	4900	65	59 ± 17
DAPC/chol	100	2100	85	74 ± 22
	50	3500	85	69 ± 16
	30	4900	85	54 ± 21

[a] Lipid concentrations of 50 mg·ml^{-1} were used for extrusion. The MLVs at 55 : 45 phospholipid-to-cholesterol molar ratio were hydrated in buffer at the appropriate temperature for 15 min. The sample was then extruded ten times through 100-nm pore-size polycarbonate filters and aliquots subsequently extruded ten times through one 50-nm or 30-nm pore-size polycarbonate filters.

[b] Quasi-elastic-light scattering measurements were made using the solid-particle mode at sensitivity setting of approximately 190.

4°C as determined by QELS and freeze-fracture analysis (results not shown).

The morphologies of DSPC and DSPC/chol (55 : 45 mol ratio) vesicles sized through the 100-nm, 50-nm, and 30-nm pore-size filters are shown in Fig. 2. DSPC vesicles generated by extrusion through 100- and 50-nm pore-size filters appeared non-spherical, with angular fracture planes when examined in freeze-fracture micrographs. Similar features have previously been observed for sonicated vesicles composed of DPPC using small-angle X-ray diffraction techniques [15] and in freeze-fracture studies on DPPC vesicles prepared by reverse-phase evaporation techniques [16]. Freeze-fracture of vesicles composed of DPPC and DAPC extruded through the 100-nm and 50-nm pore-size filters showed similar morphology (results not shown). The morphologies of DSPC/chol vesicles sized through the 100-nm, 50-nm, and 30-nm pore-size filters are shown in Fig. 2d and e. All three preparations exhibited smooth fracture faces consistent with those observed for liquid-crystalline vesicles.

Trapping efficiency of saturated lipid systems

It has previously been demonstrated that the trapping efficiencies of egg PC lipid vesicles can be significantly enhanced by freezing and thawing the multi-

lamellar vesicles [17]. The freeze-thaw procedure also ensures a more complete solute distribution between internal lamellae [17,18]. To determine whether trapping efficiencies of DSPC and DSPC/chol MLVs were dependent on temperatures, freeze-thawing cycles were performed below the T_c of DSPC at 37°C or above the T_c at 65°C. As shown in Fig. 3A, higher trap volumes for DSPC MLVs were achieved only when the lipid was cycled above the T_c. Freeze-thawing DSPC MLVs from below their T_c did not affect the trap volume, which remained at approximately $1.7 \mu l \cdot \mu mol^{-1}$ lipid, whereas five cycles of freeze-thawing at 65°C resulted in more than a 2-fold increase in the trapping of [methoxy-^{3}H]inulin. In contrast, DSPC/chol MLVs did not exhibit temperature dependence; trap volumes increased to the same extent upon freeze-thawing at either 37°C or 65°C, as shown in Fig. 3B. The values of trap volume obtained, after various freeze-thaw cycles of
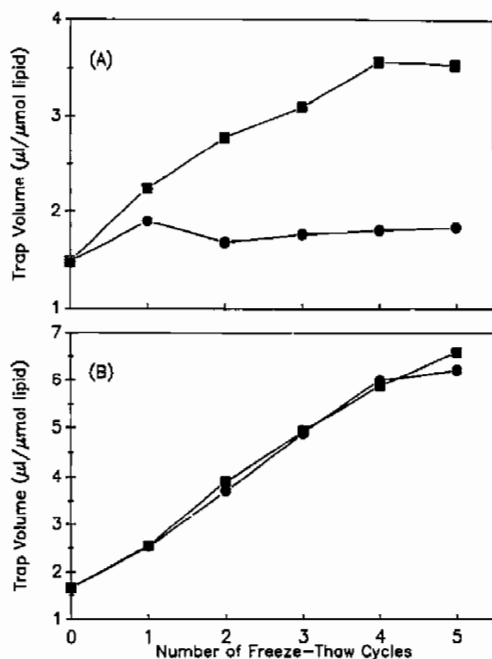

Fig. 3. Trap volume measurements of DSPC (a) and DSPC/chol (55 : 45 mol ratio) (b) MLVs frozen and thawed from 37°C (●) or 65°C (■). DSPC and DSPC/chol MLVs (40 mg·ml^{-1}) were prepared in 150 mM NaCl, 20 mM Hepes buffer (pH 7.4) containing [methoxy-^{3}H]inulin (1 μCi·ml^{-1}). The MLVs were subjected to freeze-thaw cycles employing liquid N$_2$ and 37°C and 65°C water baths. Samples were allowed to reach the appropriate temperature by being equilibrated for at least 15 min at either 37°C at 65°C before each freezing cycle. Untrapped [methoxy-^{3}H]inulin was removed by washing the MLVs in [methoxy-^{3}H]inulin-free buffer ar ntrifugation at 12000 × g for 15 min. This step was repeated three times to ensure that all the untrapped [methoxy-^{3}H]inulin was removed. Aliquots of the MLV pellets were then assayed for radioactivity and lipid phosphorus.

TABLE IV

Physical characteristics of saturated vesicles freeze-thawed and extruded through filters of various pore sizes [a]

Sample		Filter pore size			
		200 nm	100 nm	50 nm	30 nm
egg PC/chol	$\mu l/\mu mol$ [b]	1.09	0.65	0.53	0.35
QELS	nm ± S.D.	214 ± 62	129 ± 35	73 ± 24	68 ± 31
DMPC/chol	$\mu l/\mu mol$	0.97	0.82	0.51	0.30
QELS	nm ± S.D.	343 ± 187	149 ± 48	95 ± 32	79 ± 37
DPPC/chol	$\mu l/\mu mol$	0.96	0.87	0.53	0.37
QELS	ns ± S.D.	272 ± 117	137 ± 45	83 ± 29	73 ± 35
DSPC/chol	$\mu l/\mu mol$	1.00	0.72	0.54	0.38
QELS	nm ± S.D.	230 ± 54	99 ± 36	77 ± 15	69 ± 24
DAPC/chol	$\mu l/\mu mol$	1.17	0.62	0.53	0.45
QELS	nm ± S.D.	202 ± 52	91 ± 26	78 ± 18	78 ± 30

[a] MLVs were freeze-thawed three times in liquid nitrogen and thawed to above the T_c of the lipid and sequentially extruded ten times through two polycarbonate filters.
[b] Trap volume measurements were determined using [*methoxy*-^{3}H]inulin as the aqueous trap marker.

DSPC/chol MLVs (55:45 mol ratio), are comparable to published values for egg PC MLVs that had undergone freeze-thaw cycling [17].

Trapped volumes and mean diameters for a series of vesicles composed of saturated phospholipids and 45 mol% cholesterol are shown in Table IV. The physical characteristics of these preparations are comparable to those obtained for unsaturated egg PC/chol vesicles.

Discussion

This work shows that extrusion techniques can be readily applied to generate LUVs of defined size from long-chain saturated phosphatidylcholines. Particular points of interest are the generality of the extrusion technique and the characteristics of the resulting LUV systems, the mechanism of extrusion, the unity of freeze-thaw procedures to increase trap volumes, and the applications of LUVs composed of long-chain saturated lipids. These areas are discussed in turn.

As discussed elsewhere [19], extrusion under moderate pressures provides a convenient and general method for generating LUVs from 50 nm to 200 nm in diameter without the use of organic solvents, detergents, or harsh procedures such as sonication. The results of the present work show that these techniques can be extended to long-chain saturated PCs (and presumably other saturated phospholipid species) providing the extrusion temperature is above T_c. The inability to extrude below T_c is presumably related to the deformation process that must accompany extrusion of the multilamellar systems through the filter pores. In particular, gel-state systems exhibit significantly greater membrane viscosities (as reflected by much-reduced lipid lateral diffusion rates [20]) than do liquid-crystalline systems.

The ability of cholesterol to facilitate extrusion below T_c but reduce extrusion rates above T_c also correlates with cholesterol's ability to increase fluidity (decrease viscosity) below T_c and decrease fluidity above T_c.

Extrusion at temperatures above T_c provides the first general method for producing LUVs of defined size from long-chain saturated PCs. To our knowledge, no previous techniques have allowed the generation of LUVs from very long-chain PCs such as DAPC. The LUV systems produced exhibit several interesting physical features. First, limit-size vesicles produced by extrusion of the longer chain PCs (DSPC, DAPC) through 30-nm pore-size filters are metastable. The aggregation and fusion observed of these limit-size vesicles on incubation at 4°C or 20°C is likely related to production of the vesicles in the liquid-crystalline state and subsequent incubation at temperatures below the T_c. It is well-known that the gel-to-liquid transition is associated with a large increase in the area per molecule (A_m) at the lipid-water interface [21,22]. With DPPC, for example, this transition can result in a change in A_m from 40 Å^2 below T_c to 60 Å^2 above T_c [21]. The reduction in A_m, which is associated with an extended all-*trans* configuration of the acyl chains [22], may be expected to lead to vesicle instability below T_c, particularly for limit-size vesicles. Second, the effective decrease in the surface area of vesicles resulting from the decrease in A_m below the T_c is likely related to the abnormal freeze-fracture morphology observed for the DSPC systems. As shown in Fig. 2, the angular fracture planes are only observed in the DSPC vesicles (Fig. 2a–c) and not in the DSPC/chol vesicles (Fig. 2d–f). This type of morphology could be expected as a consequence of a 40–50% reduction in the surface area of the vesicles caused by the reduction in A_m of the saturated

DSPC molecules below their T_c. Third, this is also consistent with the observation that inclusion of cholesterol might compensate for the A_m of the saturated DSPC molecules and thereby allow production of stable spherical LUV systems as shown in Figs. 2d–f. Fourth, an additional factor that could contribute to the stability of cholesterol-containing vesicles is the ability of cholesterol to undergo relatively rapid trans-bilayer diffusion. This effect could help to relax asymmetrical stresses that would otherwise be created as the two constituent monolayers of a small vesicle undergo a temperature-dependent decrease in mean molecular area (which will happen even in cholesterol-containing vesicles).

LUVs composed of long-chain saturated lipids such as DSPC or DAPC are likely to be of use in applications where liposome integrity and stability are of importance. In the area of liposome drug delivery, for example, the use of DSPC systems as opposed to egg PC systems is associated with greatly enhanced liposome stability in vivo [23,24]. The enhanced trapped volumes obtained by freeze-thaw procedures are also of importance for drug delivery. As detailed elsewhere [17], in conjunction with reasonably high lipid concentrations, freeze-thaw protocols can result in trapping efficiencies approaching 80% for MLV systems. Extrusion of these systems through 100-nm pore-size filters can result in LUV systems exhibiting trapping efficiencies in the range of 50%. A limitation of utilizing vesicles containing long chain saturated lipids such as DSPC and DAPC could be encapsulation of temperature-sensitive compounds. In summary, the results presented here indicate that the extrusion technique can be generally applied to unsaturated and saturated lipid systems, in both the presence and the absence of cholesterol, provided extrusion is performed above the gel-to-liquid-crystalline transition temperature. The technique provides a convenient method of generating well-characterized vesicle systems of variable sizes.

Acnowledgements

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