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PHYSICOCHEMICAL CHARACTERIZATION OF LARGE UNILAMELLAR PHOSPHOLIPID VESICLES PREPARED BY REVERSE-PHASE EVAPORATION

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Properties of large unilamellar vesicles (LUV), composed of phosphatidylcholine and prepared by reverse-phase evaporation and subsequent extrusion through Unipore polycarbonate membranes, have been investigated and compared with those of small unilamellar vesicles (SUV) and of multilamellar vesicles (MLV). The unilamellar nature of the LUV is shown by ^{1}H-NMR using Pr^{3+} as a shift reagent. The gel to liquid-crystalline phase transition of LUV composed of dipalmitoylphosphatidylcholine (DPPC) monitored by differential scanning calorimetry, fluorescence polarization of diphenylhexatriene and 90° light scattering, occurs at a slightly lower temperature (40.8°C) than that of MLV (42°C) and is broadened by about 50%. The phase transition of SUV is shifted to considerably lower temperatures (mid-point, 38°C) and extends over a wide temperature range. In LUV a well-defined pretransition is not observed. The permeability of LUV (DPPC) monitored by leakage of carboxyfluorescein, increases sharply at the phase transition temperature, and the extent of release is greater than that from MLV. Leakage from SUV occurs in a wide temperature range. Freeze-fracture electron microscopy of LUV (DPPC) reveals vesicles of 0.1–0.2 μm diameter with mostly smooth fracture faces. At temperatures below the phase transition, the larger vesicles in the population have angled faces, as do extruded MLV. A banded pattern, seen in MLV at temperatures between the pretransition and the main transition, is not observed in the smaller LUV, although the larger vesicles reveal a dimpled appearance.

Introduction

Multilamellar vesicles (MLV) prepared by mechanical shaking of fully hydrated phospholipid bilayers, and small unilamellar vesicles (SUV), produced from the former by ultrasonic irradiation have been used as models for biological membranes. The structure, permeability and phase transition characteristics of such vesicles made of a large variety of phospholipids and their mixtures with cholesterol have been investigated extensively (reviewed in Refs. 1 and 2). These studies have been very useful for our understanding of how the lipid bilayer component may influence the structure and function of biological membranes. Never-

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theless, the large number of stacked bilayers in MLV and the highly curved bilayer of SUV limit the utility of these systems as models for biological membranes. Vesicles with a single bilayer of low curvature would be more suitable as model membranes. Such vesicles would also have more internal aqueous volume which would be useful for permeability measurements and the encapsulation of drugs and macromolecules.

Several methods have been described recently for the preparation of large unilamellar vesicles (LUV) (reviewed in Ref. 3). These include detergent dialysis [4–7], swelling of hydrated phospholipids in nonelectrolytes [8], fusion of small vesicles [9], ethanol injection [10] or ether injection [11]. Another technique, reverse-phase evaporation, has been used recently to produce large unilamellar vesicles with high encapsulation efficiency [12]. A uniform size distribution may be achieved in LUV preparations by extruding the vesicles through polycarbonate membranes of defined pore diameter [13,14]. In this communication we describe in detail the morphology, phase transition characteristics, and permeability of LUV prepared by reverse-phase evaporation and subsequent extrusion and compare them with those of MLV and SUV.

Materials and Methods

Materials. Dipalmitoylphosphatidylcholine (DPPC) was purchased from Sigma and purified further by high-performance liquid chromatography [15]. Egg phosphatidylcholine was prepared according to Papahadjopoulos and Miller [16]. The lipids were kept in chloroform in sealed ampules under argon at −40°C. Carboxyfluorescein (Eastman Kodak) was purified by chromatography on Sephadex LH-20. Diphenylhexatriene and deuterium oxide were from Aldrich. L-Histidine and *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (Tes) were obtained from Sigma. Water was double distilled, the second time in an all-glass apparatus.

Preparation of vesicles. LUV composed of DPPC were prepared by reverse-phase evaporation [12] as follows: DPPC (10 μmol) was dried in high vacuum and dissolved in 1 ml diisopropyl ether which had been washed with water just prior to use to eliminate any peroxides. 0.34 ml of the aqueous solution (either 100 mM NaCl, 2 mM L-histidine, 2 mM Tes, 0.1 mM EDTA (pH 7.4, Buffer A), or 50 mM carboxyfluorescein (sodium salt), 2 mM L-histidine, 2 mM Tes, 0.1 mM EDTA (pH 7.4, Buffer B) was added and the mixture was sonicated under argon in a bath-type sonicator (Laboratory Supplies Co., Hicksville, N.Y.) at 45–50°C for 5 min. The time of sonication, however, is not critical, as long as a stable emulsion is obtained either by brief sonication or vigorous mechanical shaking. The ether was removed slowly from the emulsion in a rotary evaporator at 45–50°C. The vacuum was maintained at 360–560 mmHg during this stage. The resulting gel was broken up by vortex mixing twice or three times during the procedure, and the ether was evaporated further at higher vacuum (160–260 mmHg) until an opalescent aqueous suspension was obtained. An additional 0.66 ml of the aqueous solution was added and any residual ether was evaporated at 10 mmHg for 20 min. The absence of any ether in the final preparation was ascertained by NMR spectroscopy. The vesicle suspension was centrifuged at 10000 × *g* for 20 min at 25°C. Unless indicated otherwise the supernatant, containing approx. 90% of the initial lipid, was extruded under N_2 pressure at 45–50°C through a polycarbonate membrane (Unipore, Bio-Rad) with a pore diameter of 0.2 μm to achieve a uniform size distribution of vesicles [13,14].

LUV composed of egg PC were prepared with diethyl ether rather than diisopropyl ether at a temperature of 30–35°C; extrusion was carried out at room temperature.

Multilamellar vesicles (MLV) were prepared by drying the DPPC in high vacuum, hydrating with the appropriate buffer (either buffer A or buffer B) and vortex mixing for 10 min under argon at 45–50°C. Small unilamellar vesicles (SUV) were prepared by sonicating the MLV for 1 h at 45–50°C under argon [3], centrifuging for 1 h at 100000 × *g* and resonicating the supernatant just before the measurements. Vesicles were kept under argon and used the same day of the preparation. Storage temperature was either 50°C (DPPC) or 4°C (egg PC).

NMR spectroscopy. ^{1}H-NMR spectra were obtained at 150 MHz on a spectrometer built by

Vladimir Basus and Joseph Murphy (Dept. Pharmaceutical Chemistry, UCSF) and equipped with a Nicolet 1180 computer system. Vesicles were prepared in ^2H_2O containing salts and buffer as above. $PrCl_3$ (Alfa) was added to a final concentration of 10 mM. Each spectrum was the result of 500 accumulations with an acquisition time of 4 s and a delay time of 4 s.

Differential scanning calorimetry. MLV were concentrated by centrifugation at $10000 \times g$ for 10–20 min and LUV by centrifugation at $234000 \times g$ for 3 h at 25°C. The pellets were transferred to aluminum pans which were sealed hermetically, each pan containing 1–2 μmol lipid. Thermograms were obtained in a Perkin-Elmer DSC-2 instrument at a scan speed of 5 K/min and a sensitivity setting of 2 mcal/s in a temperature range of 10–70°C. In some experiments the LUV were concentrated by vacuum dialysis; the DSC scans of these preparations were identical to those of the centrifuged preparations. The enthalpy of the phase transition was determined from the area under the endotherm using indium as a standard, and the lipid concentration was determined by phosphate analysis [17] after dispersing the contents of the pans in 1.0 ml water.

Light scattering. 90° light scattering experiments were carried out in a Perkin-Elmer MPF 43 or SLM 4000 fluorometer set at 450 nm in both excitation and emission monochromators. The temperature was adjusted by circulating water through a jacket around the cell, and recorded by a thermocouple in the cuvette. The scan speed was about 1 K/min. Lipid concentration was 1 μmol/ml.

Fluorescence polarization. These measurements were performed in a Perkin-Elmer MPF 43 fluorometer with polarization accessory. An aliquot of a 2 mM solution of diphenylhexatriene in tetrahydrofuran was added to the vesicle suspension (1 μmol lipid/ml) at a diphenylhexatriene/DPPC molar ratio of 1 : 750 and allowed to equilibrate for 2 h at 45°C. Polarization was calculated as before [18] with a correction factor for the $I_\perp$ to account for the intrinsic polarization of the instrument [19]. The excitation wavelength was 357 nm and the emission wavelength was set at 430 nm. Readings were taken at fixed temperatures. Temperature was controlled and determined as described above.

Freeze-fracture electron microscopy. Vesicles (10 μmol lipid/ml) were incubated in 30% glycerol for at least 2 h before freezing. Samples (5–10 μl) were frozen either from 25°C or 50°C in Freon cooled by liquid N_2, and kept in liquid N_2. They were fractured in a Balzers freeze-fracture apparatus at −115°C, replicated with platinum-carbon shadowing and observed in a Siemens 101 electron microscope.

Release of carboxyfluorescein. Vesicles were prepared in buffer B. LUV and SUV were freed of unencapsulated materials by chromatography on Sephadex G-75 (Pharmacia) at 25°C with buffer A as the elution medium, and kept on ice. MLV were separated from non-encapsulated carboxyfluorescein by repeated washing and centrifugation at 25°C. Lipid concentrations were determined by assaying for phosphate. Carboxyfluorescein fluorescence was followed in an SLM 4000 or Perkin-Elmer MPF 43 fluorometer with the excitation wavelength set at 430 nm and the emission wavelength at 525 nm. The vesicle suspension (0.05 μmol lipid/ml) was stirred continuously. The temperature was increased at a rate of 1 K/min by circulating water around the cuvette and was recorded with a thermocouple. Maximal fluorescence was determined by lysing the vesicles with 0.1% Triton X-100 (Sigma) at 25°C; the fluorescence at each temperature is given as a percentage of this value (i.e. uncorrected for the temperature dependence of fluorescence, which decreases by about 6% at 35°C and 20% at 45°C from its value at 25°C).

Results

^{1}H-NMR of egg phosphatidylcholine vesicles

The distribution of phospholipids in the inner and outer monolayers of SUV (PC) has been determined previously by ^{1}H-NMR measurements [20]. Paramagnetic shift reagents interacting with the outer monolayer of the vesicles cause the choline methyl signal to split. We used Pr^{3+} as a shift reagent [21,22] and confirmed the asymmetric distribution of egg PC across the SUV bilayer. The ratio of the peak intensities corresponding to the outer and inner choline methyl groups was 2.0. A similar experiment was performed with the large vesicles composed of egg PC and prepared by

reverse-phase evaporation and subsequent extrusion (Fig. 1). Here, the ratio of the areas under the peaks of the outer and inner monolayer $N^+(CH_3)_3$ signals was 1.1. For very large unilamellar vesicles we would expect a ratio of 1.0, and for vesicles in the range of 0.1–0.2 μm diameter a ratio of 1.1. Our results indicate, therefore, that these vesicles are unilamellar. Vesicles made of DPPC were found to be permeable to Pr^{3+} and therefore were not used for such experiments.

Differential scanning calorimetry

The gel-liquid crystalline phase transition of MLV (DPPC) has been investigated extensively by differential scanning calorimetry [23–25]. Studies with small (approx. 250 Å diameter) unilamellar vesicles have shown that the main transition is shifted to lower temperatures and is broadened [25,26]. Similar observations were made with dimyristoylphosphatidylcholine (DMPC) vesicles [27].

Fig. 2 shows the differential scanning calorimetry thermograms of a preparation of LUV composed of DPPC. Vesicles, not first centrifuged at 10000 × *g*, were sedimented at 234000 × *g* for 3 h. The pellet displayed a main peak centered at 40.8°C with a high-temperature shoulder (Scan 1). If the initial preparation was centrifuged first at 10000 × *g* for 20 min, the high-speed pellet of the supernatant had a single peak centered at 40.8° (Scan 2). The pellet of the low-speed centrifugation (less than 10% of the lipid) had an endotherm with a peak at 42°C and a low-temperature shoulder (Scan 3). This material presumably represents a fraction of MLV and some larger unilamellar vesicles in the preparation. The pre-transition of MLV at about 37°C (Scan 4) was not particularly apparent in the thermograms of the centrifuged LUV (Scan 2), although a broadened endotherm was discernible below the main transition. A pretransition could be observed in the pellet of the low-speed centrifugation (Scan 3).

The thermogram of LUV (DPPC) could be converted to that of MLV by repeated scanning through the T_c. Fig. 3 shows the thermograms of MLV (DPPC) (Scan 1), LUV from which the MLV fraction was removed by low-speed centrifugation and concentrated by high-speed centrifugation (Scan 2) and LUV after one cycle through the T_c (Scan 3). A high temperature peak appeared

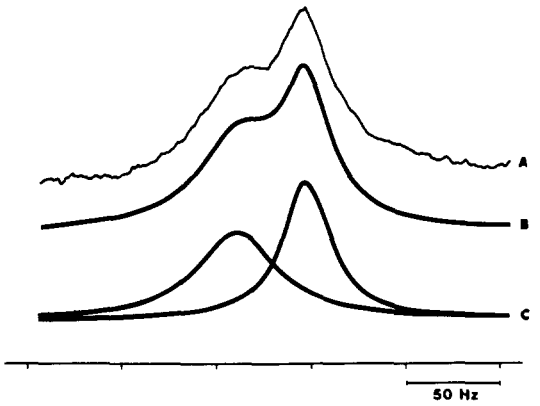

Fig. 1. 150 MHz ^{1}H-NMR spectrum (A) of the choline methyl groups of egg PC LUV of 0.2 μm diameter in ^2H_2O and 10 mM $PrCl_3$ at 25°C. Curve B is the sum of the two curves shown in C, generated by a curve-fitting routine. The downfield peak corresponds to the outer choline methyl groups shifted by the Pr^{3+}.

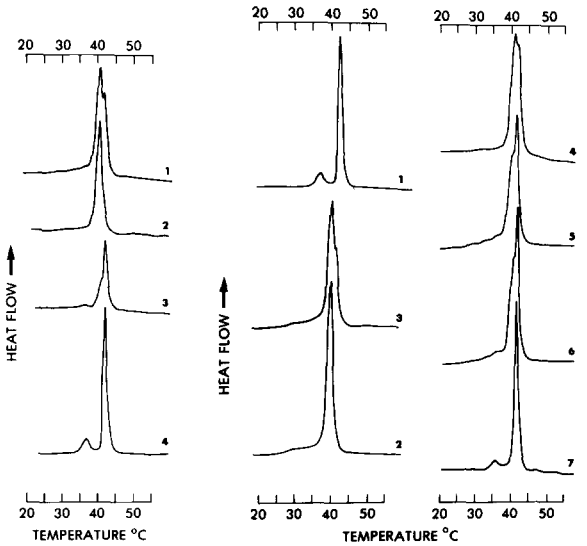

Fig. 2. Differential scanning calorimetry thermograms of DPPC LUV and MLV. 1, LUV preparation before low-speed centrifugation (10000 × *g* for 20 min at 25°C); 2, LUV low-speed supernatant; 3, LUV low-speed precipitate; 4, MLV.

Fig. 3. Differential scanning calorimetry thermograms showing the conversion of LUV (0.2 μm diameter) to MLV by repeated scanning through the phase transition. 1, MLV; 2, LUV; 3, LUV, after 1 scan through the T_c; 4–6, LUV after 10, 20, and 30 cycles through the T_c, respectively; 7, LUV after 100 scans through the T_c.

after the first cycling. This second peak became more pronounced as the cycling was repeated (Scans 4–6) until at 100 cycles a thermogram characteristic of the MLV was obtained (Scan 7). Such a transformation to structures producing thermograms characteristic of MLV has also been observed in the case of SUV [25]. The nature of this transformation is not known at present; it is likely that the LUV fuse to form larger and/or multilamellar vesicles during the repetitive cycling through the T_c.

LUV preparations extruded through polycarbonate membranes of varying pore diameter (0.1, 0.2, and 0.4 μm) produced superimposable DSC scans (data not shown). The width at half-maximum of the endotherm of the LUV (2°C) was slightly broader than that of MLV (1.3°C), implying that the cooperative unit of the transition consists of a smaller number of molecules [24]. The enthalpy of the LUV transition endotherm was 8.9 ± 0.2 kcal/mol. The reported values for the enthalpy of the main transition of MLV range from 8.2 to 9.7 [24,25,28,29].

90° Light scattering

To avoid problems of aggregation and fusion that may occur in concentrated samples necessary for calorimetry, we investigated the phase transition characteristics of LUV by 90° light scattering [27,30,31]. The main transition of LUV made of DPPC took place at a slightly lower temperature than that of the MLV (Fig. 4) in accordance with the results obtained by scanning calorimetry. A similar observation was made with LUV prepared from DMPC (data not shown). A striking feature of the scans with MLV was the drop in light scattering at a temperature corresponding to the pre-transition seen by calorimetry scans, in agreement with earlier observations [30]. Similar results have been obtained with turbidity measurements [32]. No drop in scattering intensity was observed with an initial (uncentrifuged) preparation of LUV, but rather a slight increase (Fig. 4, LUV [a]). This increase was eliminated by centrifuging the LUV suspension at $10000 \times g$ for 20 min; in this case, only the main transition was observed (Fig. 4, LUV [b]). As the MLV were extruded sequentially through polycarbonate membranes, the pretransition had a complex behavior (Fig. 5). The contributions of the refractive index and anisotropy of the membrane to the scattered light [31] are likely to be a complex function which also depends on the size of the scattering particle. When the vesicles were extruded through filters of 0.2 μm pore diameter, the scattering scan was similar to those of uncentrifuged LUV (cf Fig. 4, LUV [a]). The en-

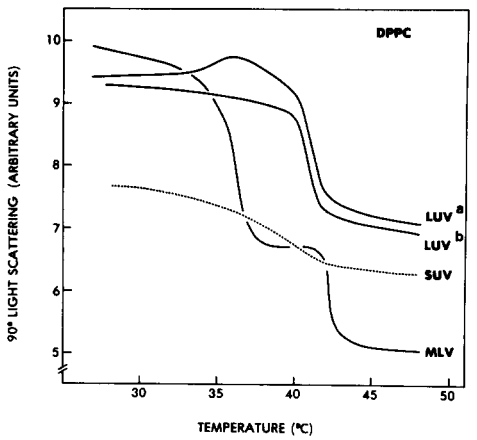

Fig. 4. 90° light scattering scans of DPPC, LUV, MLV, and SUV. LUV [a] designates the scan of a preparation of reverse-phase evaporation vesicles extruded through 0.2 μm membranes but not initially centrifuged at $10000 \times g$. LUV [b] is the scan of a preparation initially centrifuged at $10000 \times g$ for 20 min at 25°C.

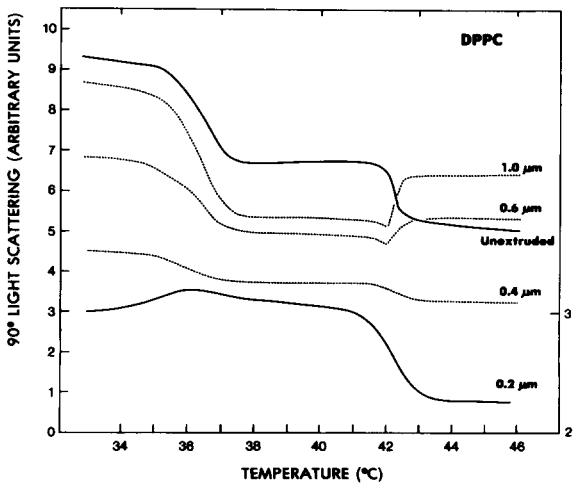

Fig. 5. 90° light scattering scans of DPPC MLV and MLV extruded sequentially through polycarbonate membranes of decreasing pore diameter. The 0.2 μm samples was scanned at a 3-fold higher sensitivity setting that the others; the scale for this scan is given on the right-hand ordinate.

capsulated volume of MLV extruded through 0.2 μm filters, based on entrapment of carboxyfluorescein (see below), was approx. 5 μl/μmol phospholipid, which is similar to the values obtained for unilamellar vesicles of this diameter. These observations raise the possibility that extrusion of MLV through polycarbonate membranes produces oligolamellar or unilamellar vesicles. This method could prove useful for encapsulating molecules sensitive to detergents or solvents used in other methods of vesicle formation.

Fluorescence polarization

The fluorescence polarization of diphenylhexatriene may be used to detect the phase transition and fluidity of phospholipid bilayers [18,25,33–35]. Fig. 6 shows the comparison of the phase transitions of various vesicles composed of DPPC. In agreement with the results of scanning calorimetry, the LUV exhibited a slightly lower transition temperature, T_c, than the MLV: The mid-point of the transition of LUV was 40.7°C (onset of the transition 39.5°C, end: 42.2°C) and that of MLV 42.0°C (onset: 40.9°C; end: 43°C). The transition of the SUV was broad and occurred at even lower temperatures, as observed previously [25,25,34].

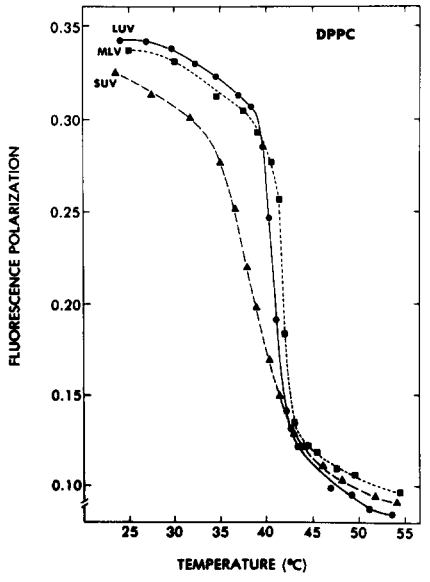

Fig. 6. Fluorescence polarization of diphenylhexatriene embedded in DPPC MLV, LUV and SUV as a function of temperature.

Permeability at the phase transition

The permeability of liposomes made of saturated phospholipids increases sharply at the T_c [36,37]. This has been interpreted to be the result of an increased permeability of the membrane at domain boundaries between gel phase and liquid-crystalline phase lipid domains which coexist at the T_c.

The temperature dependence of carboxyfluorescein efflux from LUV, MLV, and SUV composed of DPPC is shown in Fig. 7. LUV released about 50% of their contents as the temperature was increased through the T_c at a rate of 1 K/min. The increase in the permeability of the membrane corresponded closely to the temperature of the phase transition of LUV. LUV composed of DMPC released about 90% of their contents when heated through their T_c (data not shown), suggesting that they are more unstable than DPPC vesicles. MLV (DPPC) showed considerably less leakage of contents at the phase transition, and the increase in the carboxyfluorescein release occurred at a higher temperature than with the LUV, consistent with the higher T_c of MLV. Another smaller discontinuity in carboxyfluorescein release was observed at about 37°C, which is within the pre-transition endotherm of MLV (DPPC) (Fig. 2). The release of carboxyfluorescein from SUV started at a lower temperature compared to the other vesicle types and continued over a broad temperature range. When the temperature was increased at faster rates, the extent of release from SUV was less (data not shown).

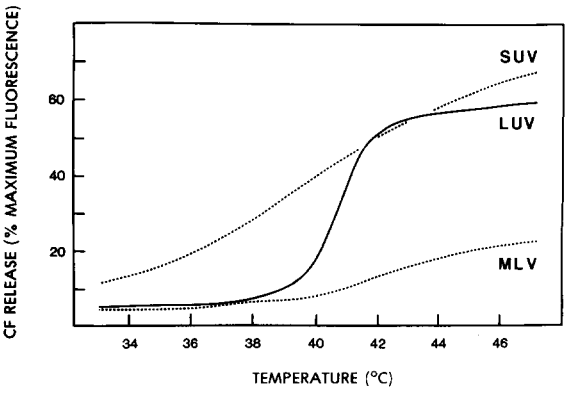

Fig. 7. Release of encapsulated carboxyfluorescein from DPPC LUV, MLV and SUV as a function of temperature.

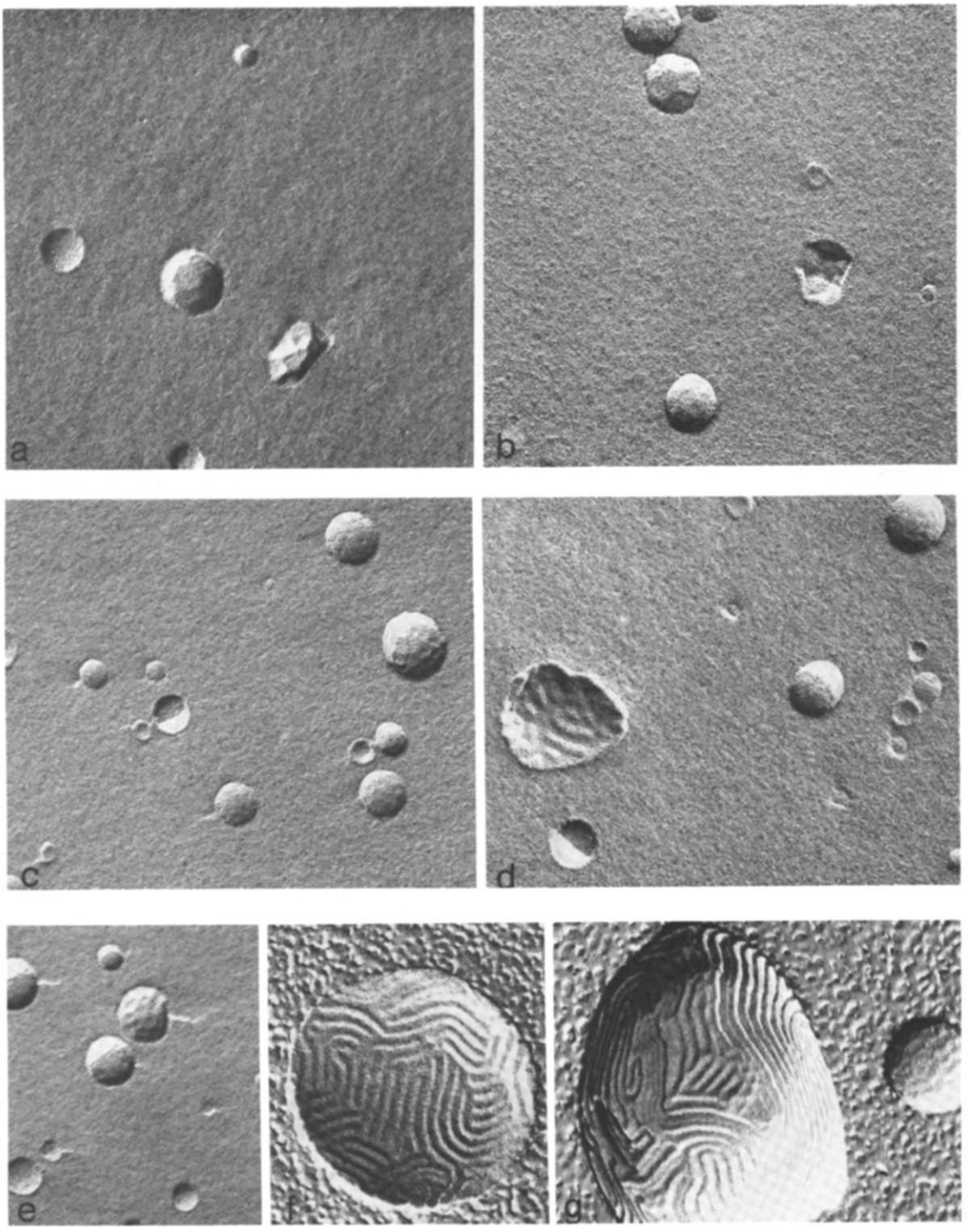

Fig. 8. Freeze-fracture electron micrographs of LUV and MLV composed of DPPC and extruded through polycarbonate filters of 0.2 μm pore diameter. (a,b) LUV quenched from 25°C; (c,d,e,) LUV quenched from 50°C; (f,g,) MLV quenched from 50°C. Magnification: 100000×.

Freeze-fracture electron microscopy

Fig. 8 shows freeze-fracture replicas of extruded LUV and MLV made of DPPC. The diameter of the LUV was in the range of 0.1–0.2 μm. When quenched from 25°C the LUV in general had smooth fracture faces (panels a and b). However, many vesicles, especially the larger ones in the population, displayed an angled structure. Such angled structures were prevalent in MLV quenched from 25°C (Fig. 9). The size of the facets on MLV was considerably larger than those on LUV. In order to investigate the structure of the bilayer between the pre-transition and the main transition, vesicles were also quenched from 50°C. It has been shown before that in order to visualize the banded pattern characteristic for MLV between the two transitions, the preparations have to be frozen from approx. 50°C, freezing rates being not sufficiently fast to prevent a temperature drop during quenching [27,38–40]. Panels c, d, and e of Fig. 8 show LUV (DPPC) frozen from 50°C. At this temperature, the smaller vesicles in the population showed smooth fracture faces. The larger vesicles, however, revealed a conspicuous, dimpled structure, similar to the surface of a golf-ball. A vestige of the banded pattern was discernible in the largest vesicles of the preparations (panel d). Panels f and g show some of the larger vesicles in an extruded MLV preparation quenched from 50°C. The banded pattern was clearly seen in these vesicles. It should be noted that the smaller vesicles in the extruded MLV preparation (see panel g) did not show the banded pattern but rather the golf-ball structure. It is not known whether the absence of the $P_{\beta'}$ phase in these vesicles is due to their small size or to their possible unilamellar character, as noted above.

Fig. 9. Freeze-fracture electron micrograph of MLV made of DPPC, extruded through polycarbonate membranes of 0.2 μm pore diameter, and quenched from 25°C.

Discussion

This paper presents results on the characterization of large unilamellar phospholipid vesicles prepared by reverse-phase evaporation and extrusion through polycarbonate membranes. The unilamellar nature of these vesicles is demonstrated by ^{1}H-NMR measurements, in which Pr^{3+} was used as a paramagnetic shift reagent. The observed ratio of the peak intensities corresponding to the outer and inner monolayer of the vesicles (Fig. 1) was identical to the theoretical value for unilamellar vesicles with a diameter of approx. 0.2 μm. The internal aqueous volume of the vesicles (5 l/mol lipid), based on the encapsulation of carboxyfluorescein, closely corresponds to the expected value and, thus, also demonstrates the unilamellar nature of the liposomes. Additional evidence for the unilamellar character of vesicles prepared by reverse-phase evaporation has been obtained previously by others, using a number of techniques: (1) Periodate oxidation of phosphatidylglycerol-containing vesicles [14]. (2) Reduction of the ^{31}P-NMR signal to 0.5 by Mn^{2+} [41]. (3)Trinitrobenzenesulfonate labeling of vesicles containing small amounts of phosphatidylethanolamine [41]. (4) Non-lytic degradation of the outer monolayer of PC vesicles by phospholipase A_2 (Wilschut, J., unpublished data).

An initial preparation of DPPC vesicles formed by reverse-phase evaporation may contain a low percentage of MLV. We have employed a low-speed centrifugation step to remove these MLV. Usually only 5–10% of the lipid sedimented during this centrifugation, the pellet consisting of a mixture of MLV and relatively large unilamellar vesicles. When charged lipids, such as phosphatidylserine, are included in the vesicles, the fraction of the lipid that sediments during the low-speed centrifugation step is even less than 5%.

The gel to liquid-crystalline phase transition temperature, T_c, of LUV is about 1 K lower than

that of MLV, as shown by differential scanning calorimetry, 90° light scattering and fluorescence polarization, and the temperature range of the transition is broader by about 50%. The phase transition of SUV, however, occurs at a considerably lower temperature and over a much wider temperature range (Figs. 4 and 6; Refs. 25 and 26). Thus, LUV, whose phase behavior is closer to that of MLV, are not affected appreciably by the curvature of the bilayer. For DMPC vesicles, Raman spectroscopy of acyl chain methylene CH stretch intensities indicates that the order of acyl chain packing decreases from MLV to LUV to SUV [42]. This sequence is in agreement with the sequence of phase transition temperatures of the three vesicle types reported in the present study.

The permeation of encapsulated carboxyfluorescein through the membranes of LUV (DPPC) and MLV exhibits a substantial difference (Fig. 7). In either case the temperature at which increased release occurs, corresponds to the phase transition temperature as observed by other methods, but leakage from LUV is considerably more extensive. This more extensive release may be explained on the basis of the permeability of carboxyfluorescein through the single bilayer of LUV in contrast to its permeability through multibilayers. In addition, the phase transition of LUV is wider and therefore less cooperative than that of MLV, which would imply that domain boundaries between coexisting solid and fluid bilayer areas are more abundant in the LUV. It has been proposed that permeation of water-soluble molecules at the phase transition temperature occurs preferentially at these domain boundaries [36]. The relatively high permeability of the LUV at the T_c is consistent with this concept. The phase transition of SUV is very broad and, likewise, release is also extensive and occurs over a wide temperature range. The presence of equimolar cholesterol in the LUV membrane completely inhibits the increase in carboxyfluorescein permeability at the phase transition temperature of the pure DPPC membrane (data not shown), consistent with the abolition of the endothermic phase transition in the presence of cholesterol [28].

When observed by freeze-fracture electron microscopy the LUV (DPPC) preparation shows a fairly uniform particle-size distribution. Many of the vesicles have a smooth surface, irrespective of the temperature before quenching being 25°C or 50°C. When quenched from 50°C the larger vesicles in the preparation have a dimpled appearance much like the surface of a golf-ball and the largest vesicles display the beginnings of a banded pattern. When extruded MLV are quenched from 50°C the well-documented banded pattern is observed on the larger vesicles in the population. This banded pattern has been associated with the $P_{\beta'}$ phase, where the hydrocarbon chains are tilted with respect to the plane of the membrane which in turn undulates, and occurs at temperatures between the pretransition and the main transition [39,40,43,44]. The smaller vesicles in the MLV preparation, however, revert to the golf-ball appearance. These observations suggest that an extended surface area is necessary for the formation of the banded pattern in LUV or extruded MLV. The golf-ball appearance of the larger LUV may represent an intermediate structure between a smooth membrane surface and the banded pattern. The absence of a pre-transition, and thus of the $P_{\beta'}$ phase, in unilamellar vesicles formed by fusion of SUV (DPPC) [45,46] or by ethanol injection and subsequent gel chromatography [47] is likely to be due to the small size of these vesicles. The apparent absence of the pre-transition in 90° light scattering and DSC scans of LUV after low speed centrifugation (Fig. 2, Scan 2, and Fig. 4, LUV [b]) is in agreement with the observation that there are very few vesicles displaying the banded pattern in freeze-fracture replicas. The transition from the faceted structure at 25° to the golf-ball structure at 50° presumably has a very low enthalpy and is not accompanied by any significant change in optical properties. The possibility cannot be excluded, however, that the $P_{\beta'}$ phase arises from interbilayer interactions and that the large vesicles displaying the banded pattern are oligolamellar, a property which freeze-fracture electron microscopy would not reveal readily. Electron diffraction studies of multibilayers and single bilayers formed on electron microscope grids have suggested that the $P_{\beta'}$ phase does not exist in single bilayers [48]. Further studies on very large unilamellar vesicles are needed to resolve this question.

When quenched from 25°C some of the vesicles in the LUV (DPPC) preparation show angled faces.

Extruded MLV display extended planar domains forming a faceted structure. The formation of facets is likely to arise from the long range order created by the packing of the phospholipid molecules. The all-*trans* configuration of the acyl chains below the T_c [44,50–52] could force the membrane to form flat domains and edge dislocations [49,53]. A faceted structure has been observed by X-ray diffraction of SUV (DPPC) below the phase-transition temperature [49]. The facets in SUV will obviously be smaller than those in LUV as the constraints on the bilayer imposed by the size of the former type of vesicle are more stringent than those of the latter.

In the present study we have concentrated on LUV made of DPPC in order to compare their thermotropic properties with those of SUV and MLV. Reverse-phase evaporation and extrusion through polycarbonate filters has been used successfully with many other phospholipids and their mixtures [14,54–59]. Reconstitution of membrane proteins resistant to organic solvents has also been achieved with this technique [60]. The thermotropic behavior of LUV prepared by reverse phase evaporation is similar to that of MLV. This property constitutes a significant advantage for the use of LUV over SUV as models for biological membranes, since the high curvature of the SUV affects the packing and motion of phospholipids in the bilayer [61–65]. For example, SUV and LUV have different susceptibilities to divalent cation-induced membrane fusion [66] or aggregation [67]. The high encapsulation efficiency and large internal volume of LUV render this vesicle system especially suitable for studies on ion and non-electrolyte permeability through phospholipid membranes and for entrapment of drugs and macromolecules for intracellular delivery or targeting to specific cells [68].

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