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PHASE TRANSITIONS IN PHOSPHOLIPID MODEL MEMBRANES OF DIFFERENT CURVATURE

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Summary

1. Nuclear magnetic resonance, light scattering and freeze fracturing electron microscopic techniques were used to characterize the size of unilamellar phospholipid vesicles of 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine.

2. Differential scanning calorimetric and light scattering analyses showed that very small unilamellar vesicles obtained by the sonication method exhibit a downward shifted, largely broadened phase transition with a slightly decreased enthalpy change when compared with multilayered liposomes.

3. The phase transition of vesicles with variable diameter as obtained by injection methods resembled the pattern of multilayered liposomes the more the diameter was increased.

4. Repeated cycling through the lipid phase transition was shown to have a progressive effect on a fusion process. This effect was strongly increased when the osmolarity of the medium was enhanced (e.g. by the addition of cryoprotectors).

Furthermore it was shown that ice-water transitions of the systems caused abrupt fusion of the lipid structures.

5. Controversial results in the literature on the thermotropic behaviour of vesicles could be explained in terms of these fusion processes.

Introduction

Both multilayered liposomes and unilamellar vesicles are commonly used as model membranes because they are chemically well defined systems. Multilayered liposomes are easily obtained, but they are very heterogeneous in size and have an unknown and variable outer surface, which complicates the interpretation of, for example, permeability studies on these systems. Unilamellar

vesicles have a rather narrow size distribution and consequently a well defined outer and inner surface, and are therefore, from a morphological point of view, preferable to multilayered liposomes.

However, the strong curvature of the vesicle may affect the packing of the molecules in the interface in such a way that the physico-chemical properties are significantly different from a planar bilayer. It is in this respect that published data on lipid phase transitions in the two systems are somewhat controversial. Differential scanning calorimetric analyses carried out in our laboratory revealed no differences between vesicles and liposomes [1]. On the other hand clear distinctions in thermotropic behaviour have been reported by other groups. The phase transition of vesicles was found to be shifted to a lower temperature by 5—10°C and to be significantly broader when compared to liposomes [2—4]. The enthalpy change of the vesicle phase transition was found to be 10—20% [2,3], 50% [5] or even 85% [4] lower than the value found for liposomes. In the present study it will be shown that such factors as differences in vesicle size due to methods of preparation and various fusion processes are important in the explanation of the observed discrepancies.

Materials and Methods

1,2-Dimyristoyl-*sn*-glycero-3-phosphorylcholine (14 : 0/14 : 0-glycerophosphocholine) and 1,2-dipalmitoyl-*sn*-glycero-3-phosphorylcholine (16 : 0/16 : 0-glycerophosphocholine), were synthesized by Mrs. A. Lancée-Hermkens according to the method of van Deenen and de Haas [6]. Multilayered liposome structures were obtained by dispersion of 100 mg of lipids in 3.0 ml 25 mM Tris/acetate, 100 mM NaCl, pH 7.0, at temperatures above the phase transition. Unilamellar vesicles were derived from the multilayered structures by subsequent ultra-sonication under nitrogen (Branson sonifier, range 4, maximal output) for periods of 15 s, with 1 min intervals, until the suspension became translucent (2—3 min total sonication time). The sonicate was centrifuged for 60 min at 37 000 × *g* in order to remove titanium fragments and residual multilayered structures. All manipulations starting with the dispersion of the lipid were carried out at 35°C, or 45°C, in the case of 14 : 0/14 : 0- and 16 : 0/16 : 0-glycerophosphocholine, respectively.

Unilamellar vesicles were also obtained by injecting lipids, dissolved in ethanol, into a water system [7]. By varying the lipid concentration in the ethanol and standardizing the injection velocity as described by Kremer et al. [8] monodisperse populations of vesicles with variable diameter could be obtained.

Freeze fracture electron microscopy was performed as described previously [9]. To prevent freeze damage, glycerol was added to the vesicles. The samples were quenched from above the phase transition temperature of the phospholipid.

^{31}P NMR measurements were performed at 35°C using a Bruker WH-90 spectrometer [1]. To the sample $^{2}H_2O$ was added to give a final concentration of 20% (v/v). Peak intensities were measured with respect to an external triphenylphosphine standard using gated decoupling. From a comparison of the total intensity of the signal from the vesicles with the intensity of the un-

shifted inside resonance in the presence of 3 mM Nd^{3+} the outside-inside distribution was determined, which enabled the vesicle size to be calculated.

Vesicle size was also measured by angular-dependent light scattering, using a FICA light scattering photometer and techniques recently described by Kremer et al. [8]. The light scattering recorded at 90° versus temperature also enabled us to analyse the phase transition. In these experiments the temperature of the cuvet was raised or lowered at a speed of 5°/h.

Differential scanning calorimetric measurements were carried out using a Perkin-Elmer 2 calorimeter cooled by a Perkin-Elmer Intracooler 1. The expansion chamber of the Intracooler is in direct contact with the sample holder enclosure block which is thereby kept at −50°C; in this way the sample compartments can be cooled if necessary. The advantage of using the Intracooler instead of cooling by liquid nitrogen will be discussed. Heating and cooling rates were 5°C/min. Calibration of the temperature and the peak area and the calculation of the heat content of the transition were performed as described previously [10].

Results and Discussion

Vesicle size

The phospholipid distribution over the inside and outside of the vesicle could be determined by means of ^{31}P NMR. In an earlier study [1] an inside/outside ratio of 0.38 was found for 14 : 0/14 : 0-glycerophosphocholine vesicles. In that study both the unshifted inside signal and the shifted outside signal (corrected for the Nuclear Overhauser Effect by a factor derived from experiments with egg lecithin) were used. In the present experiments the total intensity without shift reagent and the inside intensity after adding shift reagent were used in the calculation. This gave an inside/outside ratio of 0.32 ± 0.01, which we think is a more reliable value. Assuming an identical packing density for the inside and outside layer and a membrane thickness of 35 Å [11] it can be calculated [1] that the sonicated vesicles have an average diameter of 220 ± 10 Å. Considering this value it should be noted that in the absence of shift reagent only 84—88% of the total amount of lipid phosphate was observable in the spectrum as a narrow signal with a linewidth of 9 Hz. This can be explained if the remaining fraction of the phospholipid molecules was present as larger vesicles with a ^{31}P signal which was too broad to be observed under the conditions employed. This interpretation is supported by the observation that upon chromatography of the vesicles over a Sepharose 4B column about 15% of the phosphorus appeared in the void volume, indicating the presence of a fraction consisting of larger structures.

Typical results of the light scattering analyses are given in Fig. 1, in which the reciprocal value of the Rayleigh ratio at angle θ (corrected for the use of unpolarised light) is plotted against $\sin^2 \theta/2$. As discussed elsewhere [8] the intercept obtained by extrapolation of the experimental curve to angle $\theta = 0$ gives approximately $1/M$, in which M is the total lipid molecular weight of the vesicle. At lower angles small fractions of larger vesicles cause deviations from linearity, and extrapolation is therefore made from high angles [8]. Using M and assuming an area per molecule of 60 $Å^2$ and a bilayer thickness of 35 Å

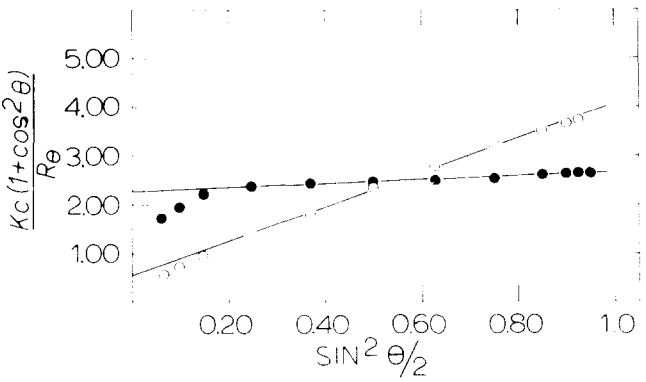

Fig. 1. Angular dependence of the scattered light as determined for the various 14 : 0/14 : 0-glycerophosphocholine preparations. •———•, Vesicles obtained by sonication, after chromatography over a Sepharose 4B column; o———o, vesicles of 800 Å diameter prepared by the injection technique.

[11] the diameter of the vesicle was calculated to be 280 ± 20 Å.

Considering the assumptions that had to be made it can be concluded that the NMR and light scattering data are in reasonable agreement. The results could also be confirmed by electron-microscopy. Fig. 2A shows a typical freeze fracture electron micrograph of a sonicated 14 : 0/14 : 0-glycerophosphocholine vesicle preparation. From such pictures it could be calculated that the majority of the sonicated vesicles had a diameter between 200 and 300 Å.

In agreement with the results of Kremer et al. [8], the injection method of Batzri and Korn [7] produced vesicles with larger and variable diameters, dependent on the lipid concentration in the alcohol solution. As the method gives diluted dispersions, the vesicle size could only be determined by the light scattering method. We injected 1-ml samples of 14 : 0/14 : 0-glycerophosphocholine in ethanol into 10 ml of buffer. Concentrations of 10 mg lipid per ml yielded vesicles with an average diameter of 540 Å and concentrations of 15 mg/ml vesicles with a diameter of 700 Å. For reasons which are not entirely clear, one of the 540 Å preparations apparently underwent fusion, to give a population of 800 Å vesicles, after storage in the refrigerator. A typical plot of the angular dependence of the scattered light of these vesicles, obtained by the injection method, is shown in Fig. 1 for the vesicles of 800 Å. From the linearity of the plot it was concluded that the vesicles were monodisperse.

Analyses of the phase transition

Multilayered liposomes of 14 : 0/14 : 0-glycerophosphocholine studied by differential scanning colorimetry exhibited a thermotropic behaviour as shown in Figs. 3A and 3B. The main transition occurred at 23.5°C and involved an enthalpy change of 6.9 ± 0.1 kcal/mol in agreement with earlier reported values [1,2,4,10]. When a sample of sonicated 14 : 0/14 : 0-glycerophosphocholine vesicles was placed in the sample compartment and cooled to 2°C, heating and cooling curves were observed as shown in Figs. 3C and 3D, respectively. A broad peak is seen ranging from 16 to 28°C with a ΔH value of 5.6 ± 0.4 kcal/mol, in agreement with the observations of Suurkuusk et al. [2].

Sonicated vesicles prepared from 16 : 0/16 : 0-glycerophosphocholine also

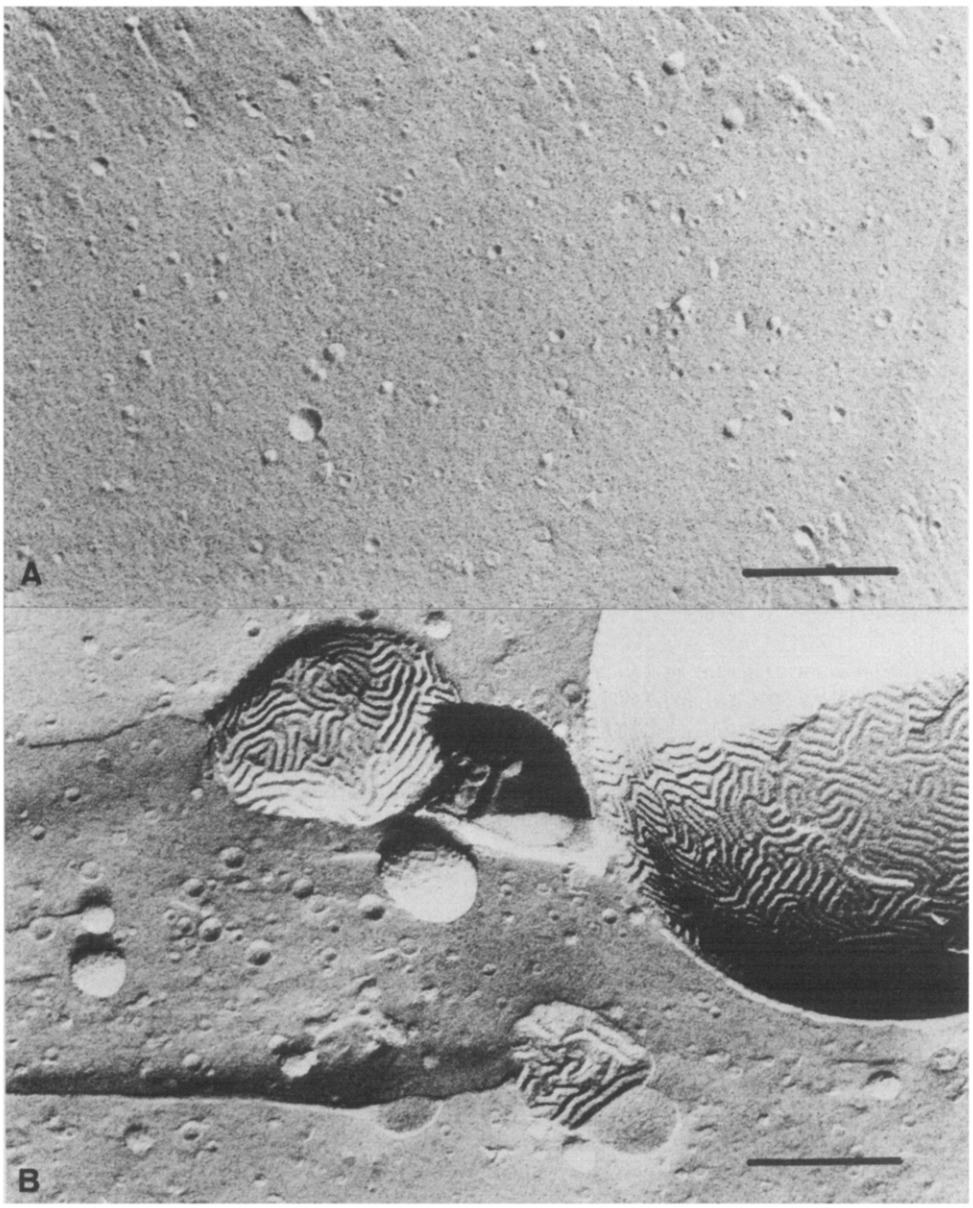

Fig. 2. Electron micrographs of 14 : 0/14 : 0-glycerophosphocholine sonicated vesicles. A, before and B, after passing the ice-water transition. Glycerol was added to the samples (final concentration 30 vol%). to prevent freeze damage, before they were quenched from 35°C. The bars in the figures represent 0.25 μm.

showed a broadened peak (32—43°C) and a decrease in ΔH value (from 8.8 ± 0.2 to 7.5 ± 0.3 kcal/mol) when compared with the corresponding liposomes. As Lawaczek et al. [12] reported that vesicles prepared below the transition temperature are unstable and fuse readily to form larger structures we also studied the calorimetric behaviour of 14 : 0/14 : 0- and 16 : 0/16 : 0-glycero-

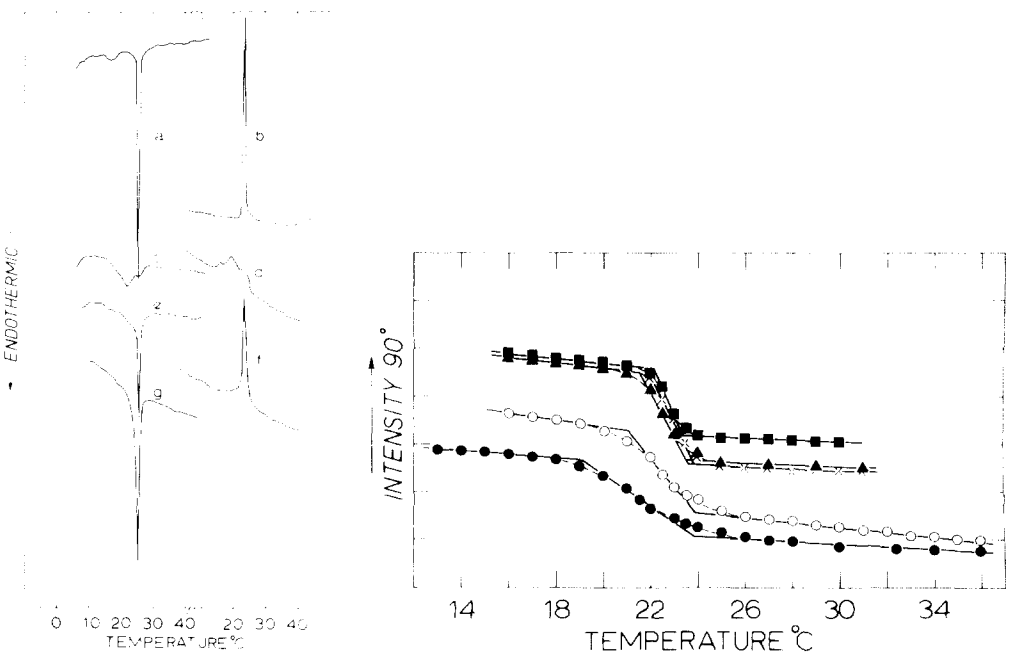

Fig. 3. Thermotropic properties of 14 : 0/14 : 0-glycerophosphocholine bilayers. Liposomal preparation: a, heating scan; b, cooling scan. Sonicated vesicles: c, heating scan; d, cooling scan. Sonicated vesicles after freezing: e, heating scan; f, cooling scan. Sonicated vesicles in 50 vol% ethyleneglycol: g, heating scan.

Fig. 4. Temperature dependency of the 90° scattered light as observed for various 14 : 0/14 : 0-glycerophosphocholine preparations a, sonicated vesicles, cooling scan (●); b—d, vesicles with defined diameters; b, 540 Å diameter, cooling scan (○); c, 700 Å diameter, cooling scan (X), heating scan (▲); d, 800 Å diameter, heating scan (■).

phosphocholine vesicles obtained by sonication at 4 and 23°C, respectively. Although the transition peaks displayed a more pronounced sharp shoulder (10—15% of the total peak area) the preparations exhibited a transition broadness and an enthalpy change typical of vesicles.

As the vesicle preparations obtained by the injection methods were too diluted to be studied by differential scanning calorimetry, Fig. 4 shows a comparative analysis of the phase transitions as recorded by the light scattering technique. It can be concluded that the very small vesicles exhibit a very broad transition, but that the temperature range over which the scattering changes is rapidly reduced when the diameter of the vesicles is increased. The light scattering tracing of the sonicated vesicles suggests a somewhat smaller temperature range of transition (18—25°C) when compared with the differential scanning calorimetric analyses (16—28°C). This may be explained by the fact that the larger vesicles make a greater contribution to light scattering intensity during the phase transition [8].

In understanding the anomalous behaviour of the small vesicles the following may be of importance: (i) It has become clear recently that in multilayered liposomes the main transition is from a $L\alpha$ to a $P\beta'$ phase whereas at the pretransition a change from $P\beta'$ to $L\beta$ occurs [11,14]. Since in vesicles a $P\beta'$ phase is very unlikely it can be speculated that the broadened peak represents

a transition from Lα directly to Lβ or Pβ. This may explain the downward shift of the transition temperature. (ii) The broad transition may be explained by a distortion of the packing of the phospholipid molecules due to the high curvature of the vesicles. It has been concluded, however, that the molecular motion and the order parameters of different chain positions in vesicles is very similar to those in liposomes [1,13,15] and that there are no changes in relative number of trans and gauche isomers in both systems [16]. On the other hand NMR studies of Chan et al. [17] indicated a more disordered lipid packing in vesicles while interchain interactions were affected by sonication as deduced from Raman studies [16]. (iii) Another possibility which would account for the broad transition is a decrease in cooperativity due to the size of the vesicles. On theoretical grounds it has been argued by Marsh et al. [18], that the size of the cooperative unit per perimeter molecule at the centre of the transition is in the same range as the vesicle radius. The fact that there is only a small decrease in energy content of the phase transition of the vesicles compared to liposomes may favor this explanation.

Fusogenic conditions

During this study it appeared that a number of experimental conditions greatly affect the apparent results of the differential scanning calorimetric analyses and explain earlier findings [1].

In the braod transition of the sonicated vesicles as shown by differential scanning calorimetry (Figs. 3C and 5A) a slight shoulder could normally be observed, which we think is due to the small fraction of larger structures present in the preparation. The intensity of this shoulder increased slowly upon repeated scanning. In one of the experiments the intensity of the shoulder increased from 5.6 to 36% of the total peak area after 25 cycles through the transition (Fig. 5B and insert). The dynamic change of the liquid crystalline into the gel state and the return must be responsible for this effect, for it appeared that incubations at constant temperature, chosen in, above or below the phase transition region (Figs. 5B, 5C and 5D), did not give a strong enhancement of the shoulder peak in the subsequent analyses. It is possible that variations in the vesicle volume as a consequence of changes in membrane area, due to the phase transition, cause osmotic forces that break up the continuous bilayer and create conditions favourable for fusion. This view is supported by the finding that cryoprotectors greatly enhance the rate of fusion upon passing the phase transition. In our differential scanning calorimetric analyses of vesicles prepared in 50 vol% ethyleneglycol a pattern completely comparable to that of liposomes was found (Fig. 3G). The hypothesis that vesicles prepared in 50 vol% ethyleneglycol fuse upon passing through the phase transition can also be deduced from the disappearance of the sharp signal seen by ^{31}P NMR (Cullis, P.R., unpublished results).

By the use of an Intracooler instead of liquid nitrogen as employed in earlier studies [1], the equilibration of the vesicle sample at 2°C, prior to the start of the differential scanning calorimetry scan, could carefully be controlled and any freezing effects prevented. To observe the effect of an ice-water transition in the system, a vesicle sample was cooled in order to induce such an ice-water transition prior to differential scanning calorimetric analyses. The results given

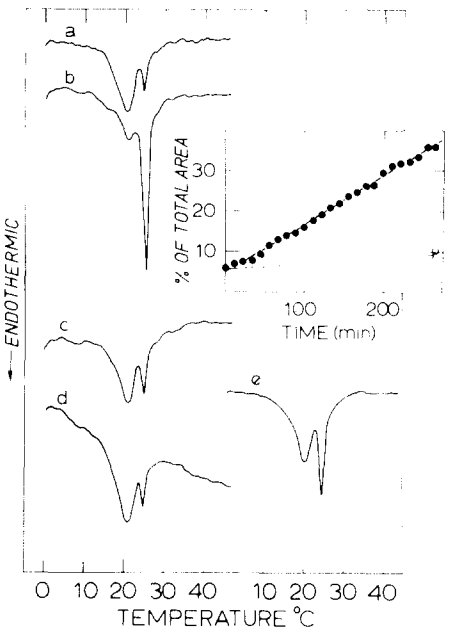

Fig. 5. Thermotropic properties of sonicated 14 : 0/14 : 0-glycerophosphocholine vesicles under various conditions. a, heating scan taken immediately after preparation; b, heating scan after 25 cycles through the transition region (heating rate 5°C/min, cooling rate 40°C/min; scanning range 2—47°C; c, heating scan of the vesicles after incubation for the same period of time as b at 35°C (265 min); d, heating scan of the vesicles after incubation for the same period of time as b at 4°C (265 min); e, heating scan of the vesicles after incubation for 90 min at 20°C. In the insert the intensity of the sharp shoulder relative to the total peak area is plotted against the time of incubation. •, Cycling through the transition region; ○, incubation at 4°C; X, incubation at 35°C.

in Figs. 3E and 3F shows a sharp peak at around 24°C with a ΔH of 6.9 kcal/mol in agreement with observations of Suurkuusk et al. [2]. Electron microscopy on this sample (compare Fig. 2B) showed that under these conditions the vesicles had been fused to predominantly multlayered liposomes. Conventional quenching is not rapid enough to prevent the transition from the Lα to the Pβ' phase [19]. Therefore the multilayered liposomes display the typical band pattern.

Acknowledgements

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