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THE LOWER LIMIT TO THE SIZE OF SMALL SONICATED PHOSPHOLIPID VESICLES

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The effective hydrodynamic radius of small sonicated phospholipid vesicles has been measured by photon correlation laser light scattering. It is found that the minimum radius obtained for these vesicles is within the range 10.25±0.55 nm independent of the phospholipid hydrocarbon chain length for synthetic phosphatidylcholines in the even numbered series of 12 to 18 carbons per hydrocarbon chain. The minimum radius of vesicles of egg yolk phosphatidylcholine is 10.7±0.3 nm.

Introduction

The mechanisms which determine the structure of biological membranes are the subject of much speculation. Despite an enormous volume of information on the structure and physical properties of membrane systems, few observations are available which unambiguously test these ideas. In a series of articles Israelachvili et al. [1] have developed the ideas of Tanford [2] in proposing that the equilibrium structure of phospholipid bilayers is determined to a major extent by geometrical constraints associated with the phospholipid hydrocarbon chains. It has been suggested that small phospholipid vesicles represent the equilibrium state of phospholipid bilayers in water and that their diameter will be strongly dependent on the hydrocarbon chainlength of the phospholipid used in their formation [3]. In the present communication we examine this model by measuring the size of small phospholipid vesicles prepared from a variety of phosphatidylcholines with hydrocarbon chainlengths varying from 12 to 18 carbons in length. The polar moiety is of course unchanged and thus any variation in vesicle diameter may be attributed directly to variations in chainlengths.

Two approaches have been developed to select for the smallest diameter vesicles that will form from each of the lipids studied here. The first approach has been to follow the decrease in the apparent vesicle diameter for vesicle suspensions which have been subjected to increasing periods of centrifugation. The larger sized vesicles sediment out of solution leaving behind a progressively narrower range of sizes of smaller vesicles. The smallest diameter is then determined from the extrapolation of the measured diameter to very long periods of centrifugation.

A second approach has been to follow the growth in the apparent vesicle size for a solution prepared using a single long period of centrifugation. In this case the minimum vesicle diameter is determined from extrapolating the measured diameter back to the time corresponding to the completion of centrifugation.

Materials and Methods

Dilauroylphosphatidylcholine (DLPC), dimyristoylphosphatidylcholine (DMPC), di-

palmitoylphosphatidylcholine (DPPC), egg yolk phosphatidylcholine (egg yolk PC) and distearoylphosphatidylcholine (DSPC) were purchased from Calbiochem, La Jolla, U.S.A. and used without further purification. The phosphatidylcholine was judged to be pure by thin-layer chromatography both prior to and following ultrasonic irradiation.

Vesicles were prepared by sonicating 5-ml quantities of solutions of 2 mg of lipid per ml of triple filtered, glass distilled water for 15 min using a Branson sonifier operating at 20 kHz and approx. 50 W into a 1-cm diameter macroprobe. Samples were sonicated under oxygen-free dry nitrogen at a temperature of approx. 20°C above the main transition temperature of the phosphatidylcholine being studied. For each phospholipid, several samples were prepared and combined prior to transferring the solution to 8×2 ml cellulose nitrate centrifuge tubes. The solutions were centrifuged at 47000 rpm in a Ti50 Beckman Rotor. In one series of experiments the centrifuge was stopped at regular intervals and one of the tubes removed and replaced by a water counter balance. Measurements of the vesicle radii were made within 3–5 min of their removal from the centrifuge, by placing the centrifuge tube so that the upper fraction of the supernatant was in the focused beam of a He-Ne Spectral Physics type 125, 50 mW laser and observing the 90° scattered light using an E.M.I. type 9863B/100 photomultiplier. The photocount correlation function measurement was made with a Langley Ford Instruments Correlator connected to an HP9835A computer using a sample interval of 5 or 10 μs. At least five measurements were taken at each time interval. With the exception of DLPC, the vesicle solutions were spun and their radius was measured at 4°C. DLPC vesicles were found to be insufficiently stable at 4°C to permit the measurement of their minimum size. However, by elevating the centrifugation and measurement temperature to 20°C, the vesicles achieved a stability comparable to those formed from other phosphatidylcholines. The measurements of vesicle growth were made at 20°C.

To determine the minimum radius the vesicle size was plotted against the time for which the sample had been spun. The radius was found to plateau as the centrifugation time was increased. Assuming an exponential form for the relationship between the apparent vesicle radius and the time for which the solution had been centrifuged, the final estimate of the vesicle radius was obtained from a minimised absolute differences computer fit of the apparent vesicle size to an expression of the form $Ce^{-t/\tau} + D$, where the value of D was taken as the limiting size of the vesicle radius.

In a second series of experiments the freshly sonicated vesicle solutions were centrifuged continuously for 120–180 min and the subsequent growth of the vesicles remaining in the supernatant measured at regular intervals over the following 10–20 h. The subsequent growth of the apparent vesicle size was fitted at short times to an expression of the form $Ae^{t/\tau} + B$. The minimum vesicle radius was taken as B.

Plots were also made of the time dependence of the intensity of scattered light and of the polydispersity index. The polydispersity index is the normalised variance in the decay rate of the photocount correlation function given by

$$\text{Polydispersity} = \frac{\int_0^\infty (\Gamma - \bar{\Gamma})^2 G(\Gamma) \mathrm{d}\Gamma}{\bar{\Gamma}^2}$$

where $G(\Gamma)$ is the distribution of decay rates present in the autocorrelation function. Γ is directly related to the radius (R) of a spherical scatterer by

$$\Gamma = K^2(kT/6\Pi\eta R)$$

for scattering vector K and viscosity η. The intensity-weighted value of $\bar{\Gamma}$ (and hence the radius) and the polydispersity are determined directly by cumulants analysis [4] of the correlation function using a second order fit. At centrifugation times in excess of 120 min at which time the scattered light intensity is reduced to 5000 counts/s or less, the polydispersity increases. In order to establish the source of the high polydispersity we have carried out comparative measurements as a function of concentration on solutions of latex spheres known to be monodisperse from data collected at higher concentrations. At count rates down to 2000 counts/s we observe little variation in the mea-

sured radius though a loss of precision occurs at the lowest rates. However, a significant increase in the apparent polydispersity was measured at count rates below about 5000 counts/s. We attribute this to the effect of residual dust which is relatively more significant at low sample concentrations.

In the far longer data collection periods rquired at these low concentrations, a dust particle has a greatly increased probability of diffusing into the scattering volume and distorting the correlation function. In view of the close similarity between the observations on monodisperse latex solutions and our vesicle preparations, we suggest that the polydispersity values at long centrifugation times do not reflect the size distribution of the vesicles and that the data presented in Table I correspond to substantially monodisperse solutions at the limit of long centrifugation times.

Results

Typical plots of the variation in apparent vesicle radius during and following centrifugation are shown in Figs. 1 and 2. Table I shows the estimated minimum radius for the five phosphatidylcholine studied.

The vesicle radii shown in Table I were in all cases a little larger when determined from the growth curve than from the sedimentation behaviour. This suggests that we have not started with the smallest size vesicle when following the vesicle growth. The smallest size when estimated from the sedimentation data is determined by extrapolating to infinitely long periods of centrifugation. Thus although the two sets of data provide very similar results we consider the sizes determined from the sedimentation data to be more realistic and are taken as the present estimate of the minimum vesicle radius. The two data sets have not been pooled. An analysis of variance of these data showed that the only difference found to be significant to a 95% confidence level is that DMPC is smaller than egg yolk PC or DSPC.

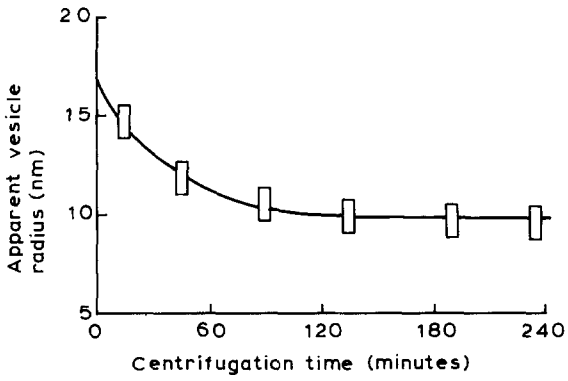

Fig. 1. An example of the fall in the apparent vesicle radius with increasing periods of centrifugation. The smooth curve drawn through the data points is a computer-determined line of best fit as described in the text. The lipid employed in this case was DMPC.

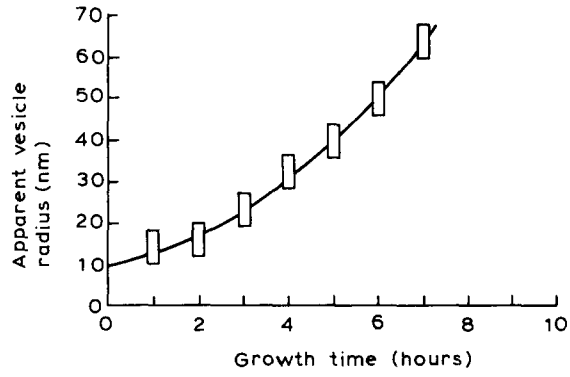

Fig. 2. An example of the growth in the apparent vesicle radius following a single long period of centrifugation, typically 120 min. Again the smooth curve drawn through the data points is a computer-determined line of best fit as described in the text. The lipid employed in this case was DSPC.

TABLE I

THE ESTIMATED MINIMUM RADII OF VESICLES PREPARED FROM A VARIETY OF PHOSPHATIDYLCHOLINES

Figures are presented as mean ± 1 S.D. Number of experiments in parentheses.

Phosphatidylcholine	Minimum radius (nm) obtained	
	Time dependence of vesicle size during centrifugation	Growth of the apparent vesicle size following centrifugation
DLPC	10.15±0.31 (11)	11.60±0.92 (14)
DMPC	9.70±0.15 (14)	10.08±0.45 (8)
DPPC	10.15±0.55 (3)	10.50±0.76 (3)
Egg yolk PC	10.7 ±0.30 (2)	10.90±0.10 (7)
DSPC	10.70±0.3 (3)	10.90±0.30 (2)

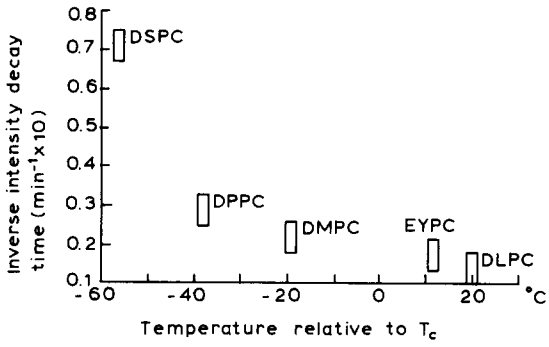

Fig. 3. The vesicle sedimentation rate during centrifugation for the various lipids studied here plotted as a function of the deviation in temperature from the lamellar phase main transition temperature (T_c). The sedimentation rate has been obtained by inverting the time constant, τ, derived from a computer fit of the decrease in the scattered light intensity as a function of the time of centrifugation to an expression of the form $Ce^{-t/\tau} + D$. Examples of this data are shown in Fig. 5.

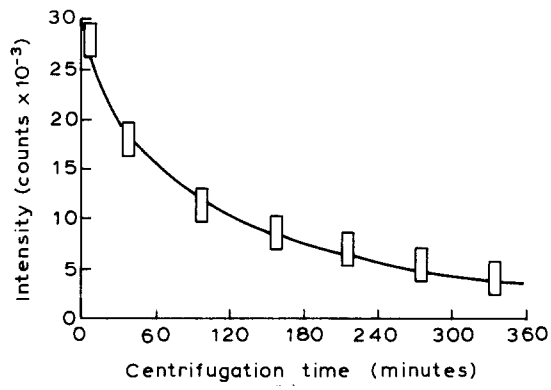

Fig. 5. An example of the loss of intensity of scattered light from, in this case, a solution of egg yolk PC vesicles plotted as a function of time of centrifugation. The smooth curve drawn through the data points is a computer fit to an expression of the form $Ce^{-t/\tau} + D$. The background scattered light intensity, D, was typically 1000–2000 counts/s.

Different chain length lipids formed vesicles with different sedimentation rates during centrifugation. The sedimentation rate is influenced by many factors, the most important in the present context being the vesicle density and size. As the densities of the lipid were all very similar at the centrifugation temperatures employed here [5] the major source of the different sedimentation rates arises from the different sized lipid aggregates present in the freshly sonicated dispersions or those formed during centrifugation. Likewise the growth rate of the apparent vesicle size following the completion of centrifugation was found to differ for the different lipids studied here. As a guide to the source of this variation in sedimentation and growth rate Figs. 3 and 4 show these as a function of the temperature relative to the main lamellar phase transition temperature of the lipid

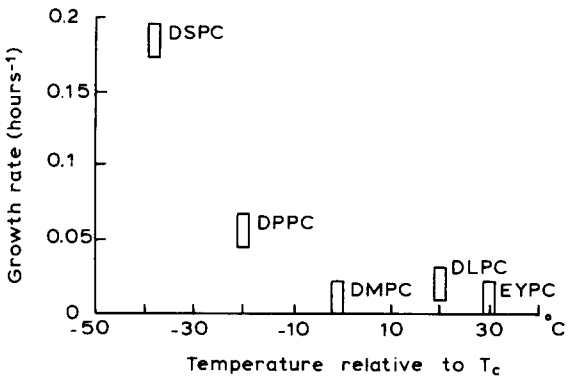

Fig. 4. The vesicle growth rate following centrifugation for the various lipids studied here, also plotted as a function of the deviation in temperature from the lamellar phase main transition temperature (T_c). The growth rate has been obtained by inverting the time constant, τ, derived from a computer fit of the growth in apparent vesicle size to an expression of the form $Ae^{t/\tau} + B$. Examples of these data are shown in Fig. 2.

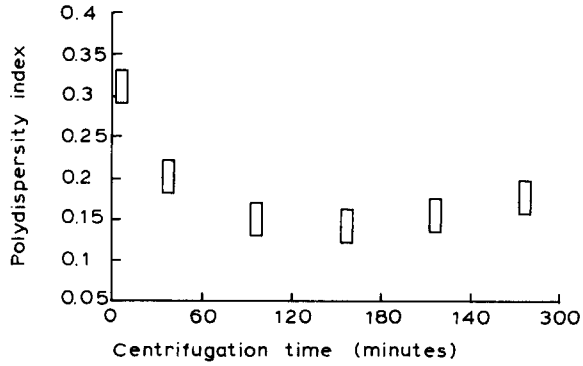

Fig. 6. An example of the time dependence of the polydispersity index during centrifugation. The increase in polydispersity at times longer than approx. 150 min has been shown to result from the scattered light intensity derived from the vesicles falling to a level that is comparable to that of the intensity of light scattered from filtered water. This data was obtained on the same egg yolk PC dispersion and at the same time as the data shown in Fig. 5.

concerned. In both cases the trend is for the vesicles centrifuged or stored below the main lamellar phase transition temperature to sediment or grow more rapidly.

Examples of the loss of scattered light intensity I_0 and polydispersity index are shown in Figs. 5 and 6 as a function of centrifugation times.

Discussion

We have previously [6] shown that vesicles formed from DMPC do not vary in size by more than 3% on being cooled through the temperature range including the main fluid to gel lamellar phase transition temperature. This observation has been explained [7] by a model in which the spherical geometry of the smallest vesicles formed during sonication prevents the changes in area and bilayer thickness normally found in a lamellar phase system. A number of articles [8,9] have now reported the progressive loss of stability of vesicles stored below the lamellar phase transition temperature of their composite lipids. This effect on the vesicle stability is seen in Figs. 3 and 4 and influences both the sedimentation rate and growth rate of the apparent vesicle size following centrifugation. Thus vesicles prepared from DSPC and DPPC aggregate and sediment more rapidly than do DMPC, DLPC or egg yolk PC. The minimum vesicle radius taken from the sedimentation data shown in Table I reveals only a slight dependence on the lipid chainlength. The only statistically significant result is that vesicles of DMPC have a smaller radius than vesicles of DSPC or egg yolk PC. This trend is not continued however with DLPC.

The restricted range of radii of the minimum size vesicle which can be produced from phospholipids with hydrocarbon chain lengths ranging from 12 to 18 carbon bonds, suggests that it is unlikely that this limiting size is determined by a geometrical constraint associated with the hydrocarbon chain length of the lipid as suggested by Israelachvili et al. [3]. We have previously proposed that for phosphatidylcholine vesicles, the dominant size constraint is a result of the polar group interactions on the vesicle interior [10]. These groups are of course, the same for each of the lipids studied here, which is consistent with the observed insensitivity of the vesicle radius to the lipid chain length.

Acknowledgements

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References

1 Israelachvili, J.N., Marcelja, S. and Horn, R.G. (1980). Q. Rev. Biophys. 13, 121–200
2 Tanford, C. (1973) in The Hydrophobic Effect. Formation of Micelles and Biological Membranes, pp. 74–80, John Wiley, New York
3 Israelachvili, J.N., Mitchell, D.J. and Ninham, B.W. (1977) Biochim. Biophys. Acta 470, 185–201
4 Koppel, D.E. (1972) J. Chem. Phys. 57, 4814–4820
5 Nagle, J.F. and Wilkinson, D.A. (1978) Biophys. J. 23, 159–175
6 Cornell, B.A., Middlehurst, J., Fletcher, G.C. and Separovic, F. (1981) Biochem. Biophys. Acta 642, 375–380
7 Cornell, B.A., Middlehurst, J. and Separovic, F. (1980) Chem. Phys. Lett. 73, 569–571
8 Larrabee, A.L. (1979) Biochemistry 18, 3321–3326
9 Lichtenberg, E., Freire, E., Schmidt, C.F., Barenholz, Y., Felger, P.G. and Thompson, T.E. (1981) Biochemistry 20, 3462–3467
10 Cornell, B.A., Middlehurst, J. and Separovic, F. (1980) Biochim. Biophys. Acta 598, 405–410