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LIPOSOME FILTRATION

DEPENDENCE ON TRANSITION TEMPERATURE

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Summary

Liposomes formed by vortexing and passed through polycarbonate surface retention membranes showed appreciable differences in filtration behavior depending on the temperature of filtration relative to the liposome gel-liquid crystal transition temperature. Below transition, liposomes were filterable and size distributions could be determined; the cumulative volume distributions were log-normal. Above transition, liposomes were not filterable: smaller liposomes were formed until a limiting size was reached. These results suggest that liquid crystal liposome size distributions cannot be determined by filtration. This filtration behavior is a physical property of liposomes, related to the gel-liquid crystal transition, not previously reported. This property could be exploited as a new method for controlling liposome size distributions, but the implications for lipid membranes, including biological membranes, are general.

Liposomes are artificial vesicles composed of phospholipid bilayer membranes encapsulating an aqueous phase [1, 2]. They are useful as models of biological membranes, and have pharmacological potential as drug delivery vehicles, since water-soluble substances may be sequestered within the internal aqueous space, while lipid-soluble species may be dissolved in the membranes [1, 2].

In performing serial filtrations originally designed to determine liposome size distributions, we found that liposome filtration properties depend on the temperature of filtration relative to the liposome gel-liquid crystal transition temperature. These results are important because: (1) they increase

understanding of the physicochemical properties of lipsosomes as model biological membranes; (2) they may be similar to those for liposomes traversing capillaries in vivo; (3) they suggest the need for care in the use of filtration as a preparative step in liposome production; and (4) they indicate the conditions which permit liposome size distributions to be determined by filtration.

Liposome preparation: Liposomes were prepared from egg yolk phosphatidylcholine, dilauroylphosphatidylcholine, dimyristoylphosphatidylcholine, or dipalmitoylphosphatidylcholine. Dilauroylphosphatidylcholine was purchased from Calbiochem; all others were obtained from Sigma. Lipids were used without further purification. Nominal purities were 99% for dipalmitoylphosphatidylcholine and 98% for dimyristoylphosphatidylcholine; egg phosphatidylcholine was chromatographically prepared by the manufacturer. The aqueous phase consisted of 0.01 M Tris-HCl buffered 0.9% saline, pH 7.1.

Typically, 60 mg of lipid, in 3 ml of chloroform, was put into a 20 × 150 mm test tube. The tube was coupled to a rotary evaporator, and the chloroform evaporated by a stream of nitrogen while the tube rotated. The tube contents were then subjected to vacuum for at least 30 min. Buffer (20 ml) was added and the tube placed in a 37° C(dipalmitoylphosphatidylcholine, 50° C) water bath for 30 to 60 min. The tube was vortexed twice (one min bursts); between vortexings, the tube contents were returned to bath temperature. The liposomes were either allowed to cool to 22° C or left at bath temperature, and the suspension brought to final volume by the addition of 10 ml of buffer. Sonicated dipalmitoylphosphatidylcholine liposomes were formed by sonication of vortexed liposomes in a 50° C water bath for 4 h.

Filtration: Polycarbonate surface retention membrane filters (Nuclepore) were used throughout. Nominal pore sizes were 8, 5, 3, 1, 0.8, 0.6, 0.4, 0.2, 0.1 and 0.08 μm. The smallest pore sizes were not used in all experiments. The liposome suspension was placed in a syringe or Antlia hand pump (Schleicher and Schuell) and sequentially filtered under pressure through a membrane of each pore size. According to the manufacturer's specifications, maximum pressure obtainable with the Antlia pump is 7.8 atm. Pressures were not measured during filtrations, but were judged qualitatively by the resistance of the syringe plunger or the number of piston strokes applied to the Antlia pump required for flow initiation. Each filter was inspected after filtration; no breakage or damage was observed. Flow rates during filtration were qualitatively determined by inspection. For adsorption controls, liposomes were filtered through five 8 μm and then through five 5 μm filters. For filtration above 22° C, the entire filter apparatus was immersed in a heated water bath. For filtration below 22° C, the apparatus was placed in an ice-water bath. In both cases, the filtrate was conveyed outside the bath through plastic tubing.

Phosphorus assay: The phosphorus assay was essentially that of Chen et al, [3]. Two 250 μl aliquots of the liposome suspension and each of its filtrates were taken for the assay.

In the course of obtaining size distributions of liposomes by filtration, a

qualitative difference in the filtration behavior of dipalmitoylphosphatidylcholine vs. egg phosphatidylcholine liposomes was observed. Although both dipalmitoylphosphatidylcholine and egg phosphatidylcholine liposome populations appeared to have similar size distributions as judged by optical microscopy, and no aggregation was observed in the liposome suspensions or their filtrates, the egg phosphatidylcholine liposomes passed through filters as though they were considerably smaller than the dipalmitoylphosphatidylcholine liposomes. Substantially lower back pressures and higher flow rates were observed for egg phosphatidylcholine liposomes than for dipalmitoylphosphatidylcholine liposomes. As shown in Fig. 1A, the serial filtration of

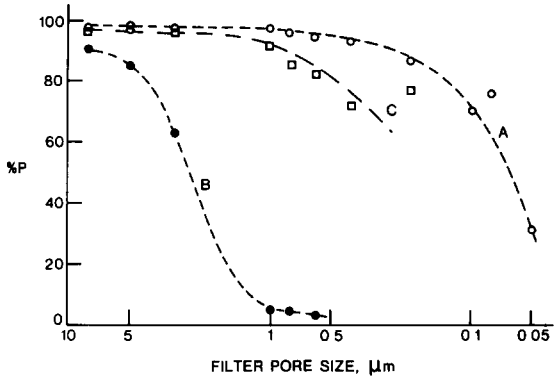

Fig. 1. Phosphorus profiles of liposomes filtered above or below transition temperature. A, Egg phosphatidylcholine liposomes filtered at 22° C (mean of two experiments); B, dipalmitoylphosphatidylcholine liposomes filtered at 22° C; and C, dipalmitoylphosphatidylcholine liposomes (same preparation as in B) filtered at 50° C. %P, filtrate phosphorus as percent of unfiltered liposome suspension phosphorus.

egg phosphatidylcholine liposomes at 22° C resulted in the recovery in the filtrate of 95—100% of the phosphorus present in the unfiltered egg phosphatidylcholine liposomes, for filter pore sizes as small as 0.4 μm. In contrast, for dipalmitoylphosphatidylcholine liposomes filtered at 22° C, the percent of phosphorus remaining in the filtrate decreased considerably: 60—70% for the 3 μm and 5—10% for the 1 μm filter (Fig. 1B). The gel-liquid crystal transition temperature of egg phosphatidylcholine liposomes is —15 to —7° C, while that of dipalmitoylphosphatidylcholine liposomes is 41° C [4]. At 22° C, egg phosphatidylcholine liposomes are in the liquid crystal state, while dipalmitoylphosphatidylcholine liposomes are in the gel state. We attributed the difference in filtration properties to this difference in the physical state of these two preparations. To test this hypothesis, dipalmitoylphosphatidylcholine liposomes were filtered at 50° C, at which temperature they are liquid crystals. The filtration behavior of liquid crystal dipalmitoylphosphatidylcholine (Fig. 1C) resembles that of liquid crystal egg phosphatidylcholine liposomes. The percent phosphorus in the filtrate was about 96% at 3 μm, 90% at 1 μm, and 70% at 0.4 μm.

Additionally, dimyristoylphosphatidylcholine liposomes were filtered above (37° C) and below (5° C) their transition temperature of 23° C [4]. The filtration behavior at 37° C was similar to that of dipalmitoylphosphatidyl-

choline liposomes filtered at 50° C, while that at 5° C was like that of dipalmitoylphosphatidylcholine liposomes filtered at 22° C. Dilauroylphosphatidylcholine liposomes (transition temperature, 0° C) [4] were also serially filtered at 22° C. Results were similar to those for egg phosphatidylcholine liposomes filtered at 22° C and for dipalmitoylphosphatidylcholine liposomes filtered at 50° C (unpublished data).

Back pressures for liquid crystal liposome filtration were consistently perceptibly less than for gel liposomes. Conversely, flow rates for liquid crystal liposome filtration were markedly higher than for gel liposomes. The repeated filtration at 22° C of egg phosphatidylcholine (Fig. 2A) and dipalmitoylphosphatidylcholine (Fig. 2B) liposomes through 8 or 5 μm filters caused no decrease in the percent of phosphorous in the filtrate. Therefore, non-specific adsorption of liposomes to the filters did not contribute to the observed filtration behavior.

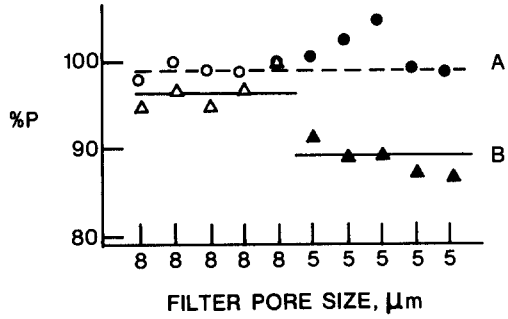

Fig. 2. Repeated filtration of liposomes. Liposomes were filtered 5 times through 8 μm membranes and then 5 times through 5 μm membranes: A, Egg phosphatidylcholine, and B, dipalmitoylphosphatidylcholine liposomes; both at 22° C. %P, filtrate phosphorus as percent of unfiltered liposome suspension phosphorus.

The filtration behavior of the dipalmitoylphosphatidylcholine and dimyristoylphosphatidylcholine gel-state liposomes is most likely the product of the heterogeneous size distribution of non-sonicated vortex-formed liposomes. Transformed to probit coordinates, the filtration characteristic of the dipalmitoylphosphatidylcholine gel liposomes (Fig. 1B) becomes linear. Since phosphorus content is proportional to volume for vortexed (multilamellar) liposomes, the cumulative volume distribution for these liposomes was log-normal. Sonicated dipalmitoylphosphatidylcholine liposomes, which are known to be smaller than those produced by vortexing [1, 2], were not retarded by the large pore filters: phosphorus assays showed 95—100% recovery for pore sizes as small as 0.4 μm. This suggests that liposomes in the gel state are unable to pass through pores smaller than the liposome diameter.

Differences between liquid crystal and gel liposome filtration characteristics could be due to one or more of four mechanisms: (1) the passage of the 'soft' liquid crystal liposomes through pores smaller than the liposome diameter by deformation, with no change in post-filtration liposome size; (2) the formation of smaller liposomes by passage through pores, either by shearing or by repacking (permanent reduction of the internal aqueous space

with reforming of the lipid bilayers in a smaller liposome volume); (3) the aggregation of small liposomes below transition, with decreased aggregation above transition; and (4) decreased liposome diameters above transition independent of filtration. Optical microscopy, however, revealed no aggregation of liposomes in either the original preparations or the filtrates. No size differences between gel and liquid crystal liposome suspensions were apparent by microscope. Microscopy showed that filtrate liposomes were smaller than parent liposomes. To distinguish between the remaining two alternatives, (1) and (2), liquid crystal dipalmitoylphosphatidylcholine liposomes were filtered only to 1 μm (prefiltration); they were then allowed to cool to 22° C (gel state) and filtered through the entire filter battery (Fig. 3A). A fraction of the original suspension was filtered at 22° C only (Fig. 3B). Phosphorus recovery in the 8 through 3 μm 22° C filtrates of the prefiltered liposomes (Fig. 3A) was nearly complete, while the control (Fig. 3B) showed

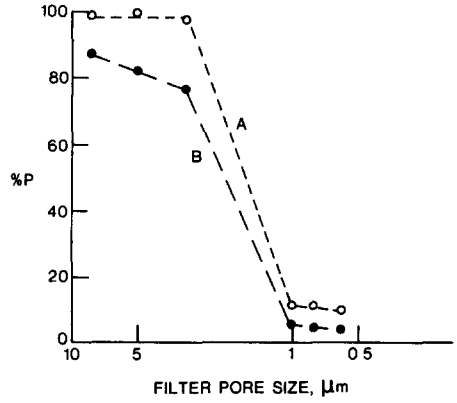

Fig. 3. Effect of filtration on the size of liquid crystal liposomes. A, Prefiltered (50° C, to 1 μm) dipalmitoylphosphatidylcholine liposomes filtered at 22° C. B, Control: dipalmitoylphosphatidylcholine liposomes from same preparation filtered at 22° C only. %P, filtrate phosphorus as percent of unfiltered liposome suspension phosphorus.

considerable reduction in filtrate phosphorus in this range. The results indicate that liquid crystal liposomes are indeed made smaller by filtration. The 22° C control filtration (Fig. 3B) showed that 20% of the phospholipid was in liposomes of 3 μm or greater diameter. Nearly all the phosphorus present in the unfiltered liposome suspension was recovered in the 1 μm filtrate of the 50° C prefiltration, in agreement with the results shown in Fig. 1C. Therefore the results (Fig. 3A) of the filtration at 22° C of the 50° C prefiltered liposomes were not due to retardation of the large liposomes during the prefiltration. Also, the results are not in accord with those expected if liquid crystal liposomes were significantly smaller than gel liposomes, instead of being reduced in size by passage through the filter pores. In that case, filtration would not change the size distribution of the liquid crystal liposomes, and cooling of the liposomes to 22° C would have restored the size distribution to the control results (Fig. 3B). Since the size distribution (Fig. 3A) obtained by filtration at 22° C after the 50° C prefiltration showed little retention of phospholipid in the 8 through 3 μm filters, and since almost

no phospholipid was retained in the 50°C prefiltration, the results suggest that filtration of liquid crystal liposomes causes a reduction of the liposome diameters. However, some deformation of large liposomes may occur, since the prefiltered gel liposomes (Fig. 3A) showed a sudden decrease in percent phosphorus going from 3 μm to 1 μm, rather than from 1 μm to 0.8 μm. In a similar experiment, the dipalmitoylphosphatidylcholine liposomes were prefiltered at 50°C through the filter battery to the 0.6 μm pore size filter. These prefiltered liposomes were allowed to cool to 22°C and were then serially filtered. As a control, a portion of the liposome suspension not subjected to the prefiltration was filtered at 22°C only. The results of this second prefiltration experiment were entirely consistent with those illustrated in Fig. 3. Phosphorus recovery at 22°C in the prefiltered liposomes was nearly 100% to the 0.8 μm filtrate, fell to about 90% at the 0.6 μm filtrate, and then dropped to about 10% in the 0.4 μm filtrate. Phosphorus recovery in the control was similar to that of Fig. 3B: about 5% of the phosphorus present in the unfiltered suspension was recovered in the 1 μm filtrate. About 90% of the phosphorus present in the unfiltered liposome suspension was recovered in the 0.6 μm filtrate of the 50°C prefiltration, in agreement with the results shown in Fig. 1C. Therefore the 50°C prefiltration did not simply retard liposomes larger than 0.6 μm, since the 22°C control filtration showed that almost all the phosphorus was contained in liposomes larger than 0.6 μm.

To account for the possibility that the filtration results obtained for liquid crystal liposomes were due to manipulation of the liposomes above the transition temperature, dipalmitoylphosphatidylcholine liposomes were subjected to repeated simulated (no membrane in the filter holder) 'filtration' at 50°C. The liposome suspension was then allowed to cool to 22°C and serially filtered. The phosphorus distribution obtained was identical to the 22°C control (Fig. 3B). Therefore, the liquid crystal liposome size distribution was not altered by the manipulations (for example, passing the liposomes through the Luer tip or membrane support grid of the filter holder and gentle mixing of suspensions by inversion before aliquots were taken) to which the liposomes were subjected in the course of filtration. Since liposomes can be formed or reformed only above the transition temperature [1, 2], manipulations of the gel-state liposomes are not likely to result in changes in size distribution. Because the filtration results obtained at 22°C for these liposomes, which had been maintained in the liquid crystal state (50°C) for over 1 h in the course of the simulated 'filtration', were identical to the control (Fig. 3B), liposome fusion at temperatures above transition was not significant in this system. Liposome fusion above transition, if it did occur in our liposome suspensions, did not alter the nearly complete recovery of phosphorus in the filtrates of the liquid crystal liposomes. If the filtration results for the gel state liposomes were due to fusion at temperatures below transition, and both liquid crystal and gel state liposome filtrations were interpreted as simple sieving, giving actual liposome size distributions, then the diameters of nearly all liquid crystal (e.g. egg phosphatidylcholine or dilauroylphosphatidylcholine at 22°C) liposomes formed by vortexing would be at or below the resolution limit of the light microscope. However, size

distributions observed with the light microscope appeared to be identical for liquid crystal and gel liposomes. Also, an experiment designed to detect liposome fusion at 22° C gave negative results. Dipalmitoylphosphatidylcholine liposomes were serially filtered at 22° C to 3 μm. About 77% of the phosphorus in the unfiltered liposome suspension was recovered in the 3 μm filtrate, in agreement with the results shown in Fig. 1B. This 3 μm filtrate was stored for 12 days at 22° C, and then again serially filtered. Phosphorus recovery in this second filtration was 96 to 100% for the 8 through 3 μm filtrates, and fell to about 5% in the 1 μm filtrate. These results are inconsistent with the generation of large liposomes that would accompany any significant degree of fusion in these gel-state liposomes. We conclude that the filtration behavior of the liquid crystal liposomes was the result of the passage of the liposomes through pores, with concomitant reductions in diameter of liposomes larger than the pore diameter, while the filtration behavior of the gel state liposomes was due to retardation of liposomes larger than the pore diameter by the filters.

Only a few observations of the filtration behavior of liposomes have been reported. The results reported here for liquid crystal liposomes are in agreement with the observation of Szoka and Papahadjopoulos for small liquid crystal liposomes formed by reverse phase evaporation and filtered through surface retention membranes [5]. The results of Rahman et al. [6] and of Schullery and Garzaniti [7] for large liquid crystal liposomes passed through depth retention filters are in apparent disagreement with each other and those reported here. Rahman et al. [6] found that a substantial volume fraction of very large (greater than 5 μm diameter) egg phosphatidylcholine liposomes passed through a 1.2 μm pore size Millipore filter. Schullery and Garzaniti [7] reported both nonspecific adsorption and filtration by size for the passage of egg phosphatidylcholine liposomes through a series (8 through 0.45 μm pore sizes) of Millipore filters. It is not clear why such different results have been obtained. Some differences may be due to the filter type (depth retention rather than surface retention) used or to the liposome composition (including aqueous phase components). We have observed that several substances can alter liposome filtration properties (unpublished data).

The dependence of liposome filtration properties on transition temperature has immediate bearing on liposome production methods. The reduction in size of the liquid crystal liposomes without loss of lipid suggests a new way to custom tailor liposome dimensions while avoiding sonication. On a more basic level, a previously unreported physical property of liposomes has been found. The further implications of this finding with respect to liposomal and biological membranes remain to be considered.

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Recently, Olson et al. [8] reported on the 'Preparation of liposomes of defined size distribution by extrusion through polycarbonate membranes'. They primarily studied liposomes composed of phosphatidylserine, phosphatidylcholine, and cholesterol, in a fluid state without clearly defined transition temperature. In addition, they reported a few results with pure dipalmitoyl phosphatidylcholine liposomes. These results are in essential agreement with ours, with some exceptions. They report clogging of membranes and filter breakage, when dipalmitoyl phosphatidylcholine liposomes are filtered below the transition temperature. We observed no such behavior, but instead found liposome retardation as reported above. The differences are probably attributable to a difference in technique.

References

1 Tyrrell, D.A., Heath, T.D., Colley, G.M. and Ryman, B.E. (1976) Biochim. Biophys. Acta 457, 259—302
2 Kimelberg, H.K. and Mayhew, E.G. (1978) Critic. Rev. Toxicol. 6, 25—79
3 Chen, P.S., Toribara, T.Y., and Warner, H (1956) Anal. Chem. 28, 1756—1758
4 Chapman, D. (1975) Q. Rev. Biophys. 8, 185—235
5 Szoka, F. and Papahadjopoulos, D. (1978) Proc. Natl. Acad. Sci. U.S.A. 75, 4194—4198
6 Rahman, Y.E., Rosenthal, M.W., Cerny, E.A. and Moretti, E.S. (1974) J. Lab. Clin. Med. 83, 640—647
7 Schullery, S.E. and Garzaniti, J.P. (1973) Chem. Phys. Lipids 12, 73—95
8 Olson, F., Hunt, C.A., Szoka, F.C., Vail, W.J. and Papahadjopoulos, D. (1979) Biochim. Biophys. Acta 557, 9—23