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Single bilayer liposomes prepared without sonication

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SUMMARY

Single bilayer liposomes, indistinguishable from those obtained by sonication, can be prepared by injecting an ethanolic solution of phospholipid into water. The dilute suspension is easily concentrated by ultrafiltration and the mild conditions allow neither degradation nor oxidation of the phospholipid.

Single bilayer liposomes are usually prepared by either of two methods of sonication: a high-energy probe is immersed directly into an aqueous dispersion of phospholipids[1] or the phospholipid dispersion is placed in a glass vial suspended in a low-energy ultrasonic cleaning bath[2]. Both procedures produce vesicles of about 25-nm diameter consisting of a single phospholipid bilayer shell. These liposomes have proved very useful as models of biological membranes but there are several disadvantages to the preparative methods. High-energy sonication often causes oxidation and degradation of phospholipid[3], although this can be minimized by rigorously controlled conditions[4], and may damage solute molecules it is desired to capture in the interior space. Titanium particles erode from the probe and, with radioactive material, a potentially hazardous aerosol is inevitably produced. These latter difficulties are avoided by low-energy sonication in a closed system but, in our experience, that procedure varies in its efficiency, frequently requires long periods of sonication, can also be destructive to phospholipid molecules, and cannot be used to prepare large quantities of liposomes. For these reasons we have developed a procedure for the preparation of single bilayer liposomes that avoids sonication.

An ethanol solution containing 20–40 μmoles of egg lecithin/ml (Lipid Products, South Nutfield, England) was rapidly injected through a Hamilton syringe into 0.16 M KCl to give a maximum of 7.5% ethanol. All solutions had been purged with N_2. As much as 60 ml of suspension was concentrated to 1–2 ml in 30–60 min on an Amicon

(Lexington, Mass.) ultrafiltration device using a 43-mm diameter, XM-100A membrane with rapid stirring under N_2 pressure of 10 lb/inch^2. If larger volumes were to be concentrated the membrane was changed and the concentrated samples were pooled. Some preparations were concentrated to 80–100 μmoles/ml and were only faintly opalescent. As determined with radioactive phospholipids, less than 2% of the phospholipid passed through the filter and none was adsorbed to it. Rapid stirring and low pressure were necessary to avoid formation of larger, more heterogeneous liposomes and the concentration of phosphatidylcholine in ethanol could not exceed 40 mM. Equally good results were obtained when water or 1% sodium phosphotungstate were substituted for 0.16 M KCl, and when the phospholipid contained 5–10% stearylamine as in the experiments discussed in this paper.

The preparative procedure had no degradative effect on the phosphatidylcholine detectable by thin-layer chromatography of 100 μg of phospholipid, and the absorption spectra of the liposomes and of the phosphatidylcholine after redissolving the liposomes in ethanol showed no evidence of oxidation[5] (Fig. 1). Electron microscopy of unfractionated liposomes (Fig. 2A) revealed a reasonably homogeneous preparation of vesicles of average diameter about 26.5 nm. The single bilayer aspect of the vesicles was best illustrated when the liposomes were prepared by injecting the phospholipid directly into

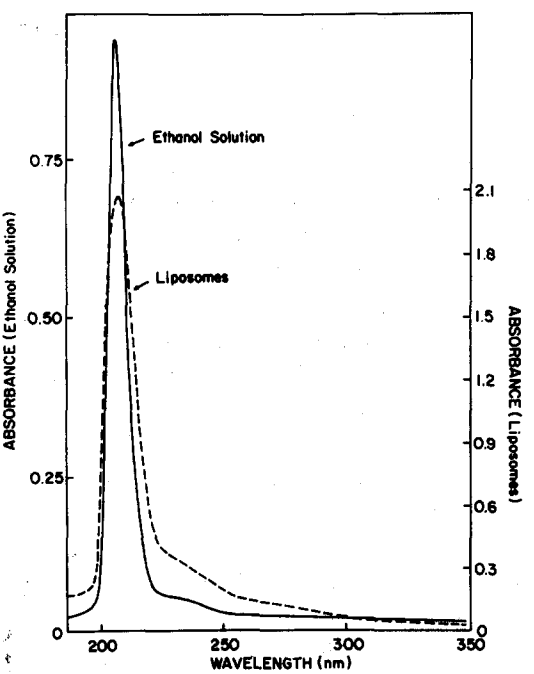

Fig. 1. Absorption spectrum of a liposome preparation (0.73 mM phosphatidylcholine, 0.07 mM stearylamine) and of an ethanol solution (0.64 mM phosphatidylcholine, 0.06 mM stearylamine) of the liposomes. The ethanol spectrum is identical to that obtained for an ethanol solution of the starting material. The absence of oxidation[5] is shown by the low ratio of $A_{233\,nm}/A_{215\,nm}$.

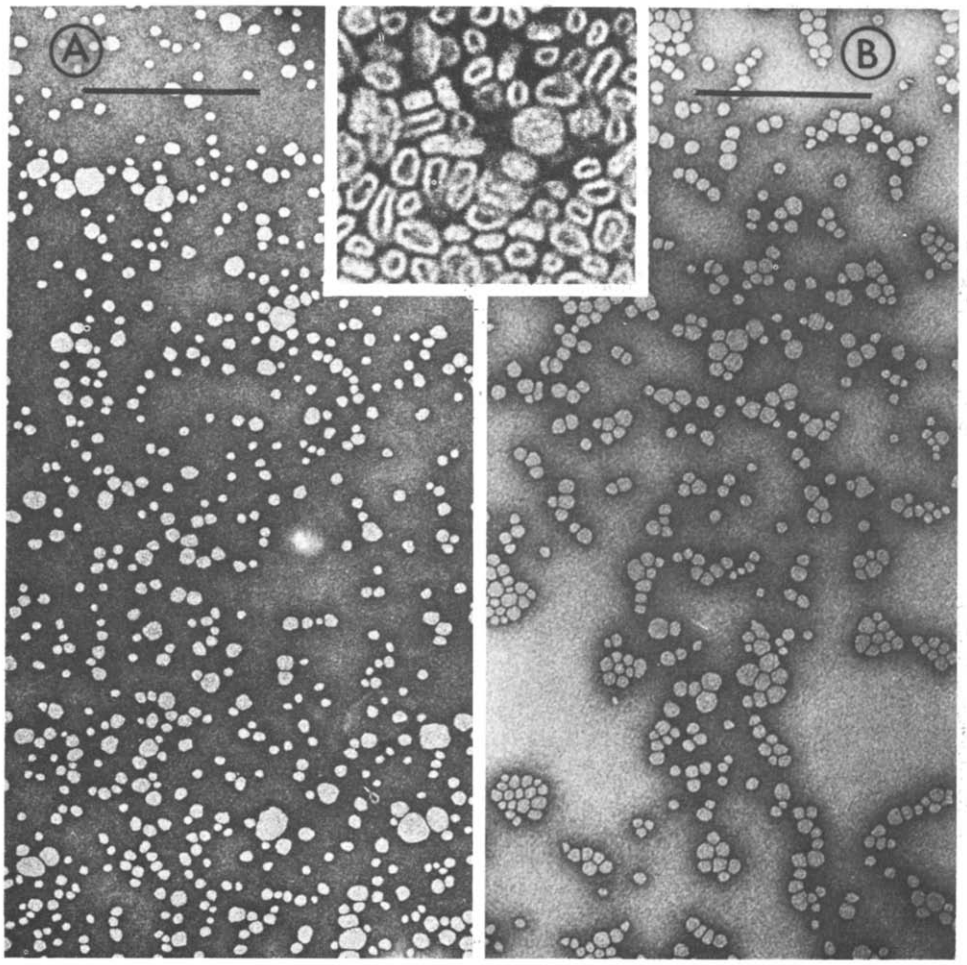

Fig. 2. Negatively stained liposomes. A, Representative field of unfractionated liposomes prepared by injecting 1.5 ml of 36.5 mM phosphatidylcholine–3.5 mM stearylamine in ethanol into 30 ml of 0.16 M KCl. B, A similar preparation after fractionation on Sepharose 4B (Fraction 23, Fig. 4). Samples were mixed with 1% sodium phosphotungstate at a phospholipid concentration of 1 mM and sprayed on a carbon-coated collodion grid. Insert: Liposomes were prepared by injecting 0.5 ml of 9.1 mM phosphatidylcholine–0.9 mM stearylamine in ethanol directly into 5 ml of 1% sodium phosphotungstate. Micrographs were taken at an instrumental magnification of 36200. The bars in A and B are 400 × 57 920. The insert is magnified about 109 000 ×.

1% sodium phosphotungstate (Fig. 2, insert). NMR spectroscopy of unfractionated liposomes showed well-defined peaks (Fig. 3) with resolution at least as good as reported for sonicated liposomes[6]. A simple aqueous dispersion of the lipid mixture gave no NMR spectrum under these conditions.

When a liposome preparation was separated on Sepharose 4B (refs 1, 4) without prior removal of any large vesicles by centrifugation[4] approximately 6% of the applied

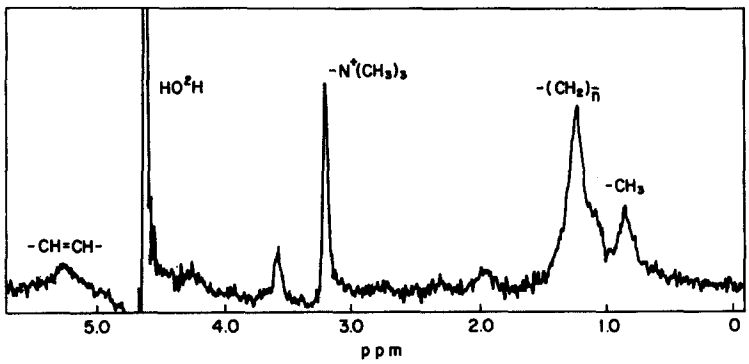

Fig. 3. 100 MHz NMR spectrum of liposomes (14 mM phosphatidylcholine–1 mM stearylamine) prepared in 0.16 M KCl in ^2H_2O. Temperature, 31 °C.

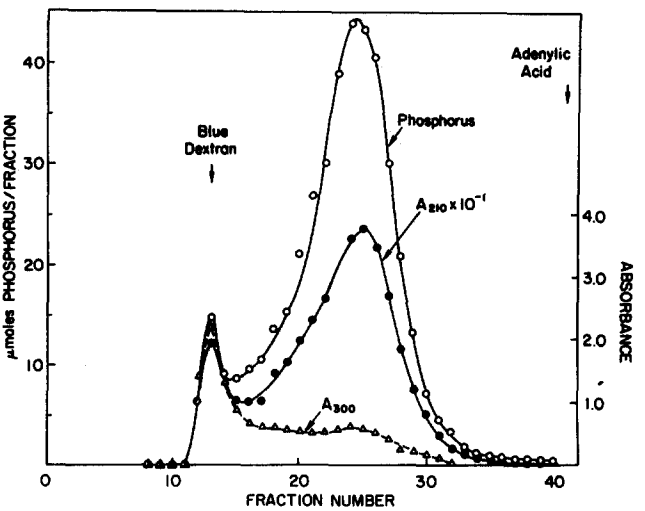

Fig. 4. Gel filtration of liposomes on Sepharose 4B. A liposome suspension (7 ml of 64 mM phosphatidylcholine–6 mM stearylamine) was applied to a 2.5 cm × 40 cm column of Sepharose 4B and eluted with 0.16 M KCl. Fractions were monitored at 300 nm and 210 nm, and analyzed for lipid phosphorus[7]. This preparation was not centrifuged before applying it to the column.

phospholipid was eluted in the void volume and about 80% of the applied phospholipid was recovered in an included fraction (Fig. 4). Negative staining electron microscopy of the included material showed a relatively uniform preparation of small vesicles (Fig. 2B).

The internal aqueous volume of the liposomes was determined by preparing them in a solution containing radioactive glucose and measuring the amount of trapped glucose in liposomes separated by chromatography on Sephadex G-50 (ref. 7). An internal volume of $5.2 \cdot 10^{-4}$ ml/μmoles of phospholipid was found compared to the calculated value of $3.9 \cdot 10^{-4}$ ml/μmole of phospholipid for liposomes with an external diameter of 26.5 nm

assuming a single bilayer of thickness 4.5 nm (ref. 2) and a surface area of 0.72 nm^2/phospholipid molecule. This internal volume is somewhat larger than the volume of $3.1 \cdot 10^{-4}$ ml/μmole reported for sonicated liposomes of 25-nm diameter[2]. The differences could be explained by the presence of very few relatively large liposomes in this unfractionated preparation or by an average diameter about 17% greater than the diameter of the sonicated liposomes[2].

By all the above criteria liposomes prepared by the procedure described in this paper are indistinguishable from those prepared by sonication. The procedure is rapid, highly reproducible, and allows preparation of unlimited amounts of liposomes. Chemical degradation is avoided and oxidation is controlled simply by working under N_2. The liposomes can be used immediately[2] or stored for days. However, two potential disadvantages are inherent in the procedure. First, the liposome suspension will contain ethanol but this can be removed by dialysis or by washing on the ultrafilter. Second, since large volumes of aqueous solution are used, it will be necessary to use relatively large amounts of any solute it is desired to trap within the liposome. It should usually be possible, however, to recover the water-soluble material from the ultrafiltrate.

REFERENCES

1 Huang, C. (1969) *Biochemistry* 8, 344–352
2 Johnson, S.M., Bangham, A.D., Hill, M.W. and Korn, E.D. (1971) *Biochim. Biophys. Acta* 233, 820–826
3 Hauser, H. (1971) *Biochem. Biophys. Res. Commun.* 45, 1049–1055
4 Huang, C. and Charlton, J.P. (1972) *Biochem. Biophys. Res. Commun.* 46, 1660–1666
5 Klein, R.A. (1970) *Biochim. Biophys. Acta* 210, 486–489
6 Finer, E.G., Flook, A.G. and Hauser, H. (1972) *Biochim. Biophys. Acta* 260, 49–58
7 Ames, B.N. and Dubin, D.T. (1960) *J. Biol. Chem.* 235, 769–777
8 Johnson, S.M. and Bangham, A.D. (1969) *Biochim. Biophys. Acta* 193, 82–91
9 Small, D.M. (1967) *J. Lipid Res.* 8, 551–557