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PREPARATION OF GIANT LIPOSOMES

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A method is described for the preparation of giant unilamellar lipid vesicles that are stable in electrolyte solution. In general, it involves dialysis of lipid and indifferent solute in a water-miscible organic solvent against an aqueous buffer. During dialysis the concentration of organic solvent decreases so that vesicles form under conditions where their internal contents are continuously hyperosmotic. Interlamellar attractive forces are neutralized, even between bilayer membranes with no net charge, and giant vesicles are generated in large numbers. The population is heterogeneous but most large vesicles have diameters between 10 and 100 μm. The method is simple. One procedure involves dialysis for a day or more of a methanol solution of phosphatidylcholine, supersaturated with methylglucoside, against an aqueous phase containing up to 1 M univalent electrolyte. The procedure is effective over a wide range of temperature and pH.

Introduction

Artificial phospholipid vesicles or liposomes have been studied extensively as models for natural membranes. The classical liposome preparations have been made by mechanical shaking (multilamellar vesicles) or by sonication of a lipid suspension (small unilamellar vesicles). Although the vesicles generated by the latter method can be unilamellar, they are also extremely small. Because many applications of liposomes are difficult, tedious, or inefficient, given vesicles with small trapped volumes, a number of laboratories have developed methods for the preparation of large unilamellar vesicles [1–9]. Vesicles produced by these methods may be up to a micron or so in diameter, but although they represent a substantial increase in volume, they are still small in comparison with cells. Truly large vesicles are needed if liposomes are to be used for models of cells such that they can be easily observed under the light microscope, manipulated mechanically, and impaled with microelectrodes. Reeves and Dowben [10] described a procedure to make large vesicles that was subsequently modified [11] to enable preparation of vesicles of the requisite size (up to tens of microns in diameter). The procedure, unfortunately, requires that electrolytes be essentially absent from the aqueous phase, a restriction which obviously severely limits its usefulness. Recently, an ingenious double emulsion process has been described by Kim and Martin [12], that generates very large vesicles which are stable in electrolyte solutions. The method has an advantage of producing populations that are quite uniform; however, compared with the methods we describe here, it has the disadvantages of (1) being much more complex and time-consuming, (2) less versatile, requiring the presence of particular lipids in certain proportions, and (3) yielding vesicles that are not as large. Large vesicles can also be prepared by precipitation of certain acidic phospholipid with calcium followed by chelation of the calcium [8].

Abbreviation: Mops, 4-morpholinepropanesulfonic acid.

Large vesicles are produced by the procedure of Deamer and Bangham [1] and, judging from Fig. 8 of Hargreaves and Deamer [13], some giant vesicles are produced as well.

Here we describe a very simple method for preparing giant unilamellar vesicles * from lipids soluble in alcohols. It works well over a wide range of electrolyte concentration, pH and temperature.

Materials and Methods

Pure and crude egg yolk phosphatidylcholine and 1-*O*-methyl-α-D-glucoside were purchased from Sigma Chemical Co., St. Louis, MO. Calcein was from HACH Chemical Co., Ames, IA. [^{14}C]Methylglucoside was from New England Nuclear, Boston, MA. *N*,*N'*-Dioctadecyloxacarbocyanine-*p*-toluenesulfonate was from Eastman Organic Chemicals, Rochester, NY. Preparation of liposomes was as follows: 200 mg of 1-*O*-methylglucoside were dissolved in 200 μl of methanol and poured into a suspension of lipid (10 mg lipid/200 μl of methanol). This mixture was then dialyzed against Mops-buffered saline (pH 7.4) plus 0.1 mM calcein for 2 days. The supersaturated solution of methylglucoside in methanol was prepared by heating the mixture above the boiling point in heavy-wall, screw-cap culture tubes. The hot tubes are under pressure and the obvious precautions should be taken. It is of no consequence if crystallization of the methylglucoside occurs after mixing with the lipid solution, although transfer to dialysis bags is easier if it is done before crystallization occurs. We therefore work rapidly and often use slightly warm solutions.

The volume inside the liposomes was determined according to a method described elsewhere [14]. In brief, 20 μl of sample was diluted with 480 μl of Mops-buffered saline and the fluorescence intensity was measured before and after addition of 2 μl of 10 mM $CoCl_2$. Cobalt ion quenches the fluorescence of the calcein that is accessible to it, i.e., that which is outside the liposomes, so that the fraction of the fluorescence (corrected for background fluorescence) remaining after cobalt addition corresponds to the fraction of the total volume that is encapsulated. Background fluorescence (due to incomplete quenching) is a few tenths of a percent of the total and was determined for each sample by lysing the vesicles with 25 μl of 10% Triton X-100 after the cobalt addition. The permeability of the vesicles to calcein and/or cobalt ion is gotten by determining the rate of change of fluorescence that occurs after the first sharp drop due to quenching of external fluorescence. The permeability is expressed as percent loss of internal fluorescence per minute. The concentration of phospholipid was obtained by phosphate assay [15].

Results and Discussion

Uncharged or weakly charged phospholipids such as phosphatidylcholine, phosphatidylethanolamine, and sphingomyelin are soluble in methanol. When the methanol is diluted to about 50% with aqueous phase, lipids come out of solution and form typical multilayered liposomes. If the methanol is saturated with a hydroxylic solute that is moderately soluble in alcohols, such as methylglucoside, the lipid remains in solution. When these solutions are slowly diluted with an aqueous phase, the lipid again comes out of solution. In the latter case, however, many of the vesicles formed are found to be vastly larger than those produced from organic phases lacking solute. Another striking difference is that these giant vesicles are only rarely multilamellar (Fig. 1). The giant vesicles in Fig. 1 are composed of crude egg yolk lecithin (mostly phosphatidylcholine with smaller amounts of other lipids such as phosphatidylethanolamine and sphingomyelin) and were formed during dilution of the lipid/methylglucoside/methanol mixture by dialysis against Mops-buffered saline. Dilution by dialysis avoids dilution of the lipid vesicles and affords the forming vesicles protection from hydrodynamic shear stresses. In addition, it provides for elimination of the methanol and methylglucoside.

The apparent difference between liposomes

* Following nomenclature that is becoming widely accepted, we suggest GUV (giant unilamellar vesicles) as the acronym for vesicles larger than 10 μm in diameter. LUV (large unilamellar vesicles) has been used to describe vesicles up to about 1 μm in diameter.

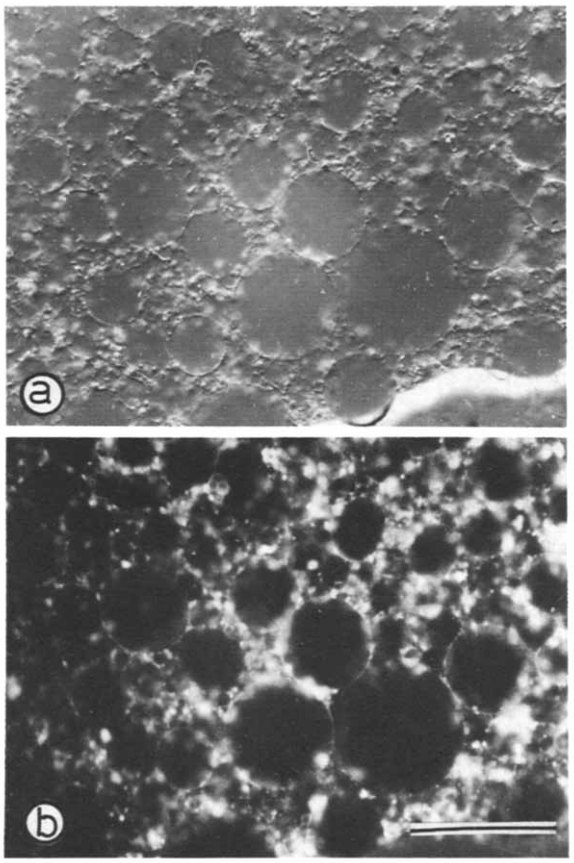

Fig. 1. Light micrograph of giant unilamellar vesicles. This mixture was dialyzed against Mops-buffered saline for 2 days. 200 mg of 1-*O*-methylglucoside were dissolved in 200 μl of methanol and mixed with 10 mg egg yolk phospholipids plus 0.05 mg *N,N'*-dioctadecyloxacarbocyanine-*p*-toluenesulfonate dissolved in 200 μl of methanol. The upper photograph (a) was taken through differential interference contrast (Nomarski) optics. The lower photograph (b) was taken with fluorescence illumination. Bar: 100 μm.

formed from an organic phase with additional solute and those formed from an organic phase without additional solute is their internal osmolarity, which is greater in the former liposomes. Since untrapped solute is lost from the dialysis bag much faster than solute is lost from liposomes, the latter will experience an osmotic influx of water for much of the dialysis period. It seems likely that such an 'osmotic inflation' process contributes significantly to the formation of giant vesicles. The generation of larger vesicles from small would reduce the osmotic stress because of the favorable increase in the ratio of area to volume. The high concentration of solute in the organic phase, however, may also reduce the van der Waals attractive forces between the lipid bilayers and, thus, the tendency to form multilamellar vesicles by increasing the polarizability of the aqueous phase [16]. As dialysis proceeds, the concentration of solute outside of the vesicles is reduced, diminishing its effect on van der Waals forces, and it is possible that smaller vesicles expanding within the confines of the dialysis bag may come into contact and fuse. Although it may not be a prerequisite for the formation of giant vesicles, a very high concentration of polyhydroxylic solute is also likely to have some effect on the structure of the lipid bilayer by affecting parameters such as interfacial tension or molecular packing.

As would be expected from the appearance of large vesicles during the dialysis process described above, the volume encapsulated by liposomes so generated greatly exceeds that of multilayered vesicles generated by the common hand shaking

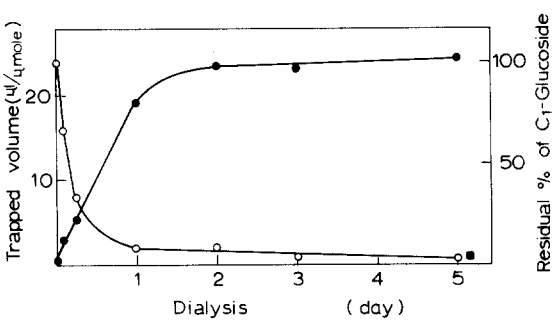

Fig. 2. Dialysis removes methylglucoside and leads to the generation of giant unilamellar vesicles. Solutions were prepared as described in the legend for Fig. 1 except that 1 μCi of [^{14}C]methylglucoside was included. The lipid/methylglucoside/methanol mixture was divided into seven parts and each was dialyzed against 10 mM Mops-buffered saline containing 0.1 mM calcein. At the indicated times, 20 μl of the suspension in the bag was taken for determination of the proportion of encapsulated calcein (trapped volume) and its rate of release. Another 20 μl was taken for phosphorus assay. A portion of 20 μl was used to determine residual methylglucoside by liquid scintillation counting. Multilamellar vesicles were prepared by shaking 10 mg egg yolk phospholipids in 2 ml of a solution as used for dialysis. The trapped volume of these liposomes is given by the filled square (lower right).

procedure. As shown by the data of Fig. 2, the trapped volume, measured by the proportion of calcein that is protected from quenching of its fluorescence by cobalt ions, rapidly increased over the course of the first day's dialysis. Concomitantly, most of the methylglucoside was lost. The methylglucoside more slowly released over the course of the next several days was presumably entrapped within liposomes. Although we have not assayed for elimination of methanol directly, the permeability of the vesicles to calcein is rather high during the first six hours of dialysis, i.e., about 5% of the trapped calcein is released per minute at room temperature. After the first six hours of dialysis, the rate stabilizes at slightly under 2%. We would ascribe the permeability difference to the presence of methanol in the vesicles. It may take a few hours for the methanol to be eliminated but it will certainly be lost much more rapidly than methylglucoside.

Although the lipid used for most of our experiments has a small negative charge (zeta potential of about −10 mV in 50 mM NaCl), electrostatic repulsion is not responsible for the absence of multilayered vesicles in these preparations. Purified egg yolk phosphatidylcholine with no net charge also forms giant vesicles by the procedures described. These vesicles tend to be slightly smaller,

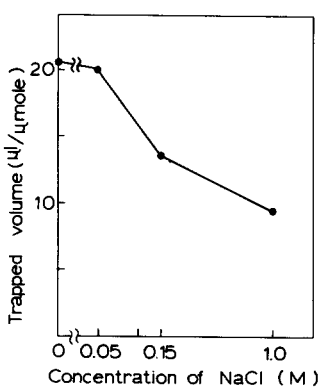

Fig. 3. Effect of NaCl on trapped volume of giant unilamellar vesicles. Mixtures of egg yolk phospholipids and methylglucoside in methanol were prepared as described in the other legends and dialyzed against Mops-buffered saline containing concentrations of NaCl as shown in the figure. After 2 days, aliquots of the suspension within the dialysis bags were taken for assay of trapped volume and phosphate.

on the average, than mixed lipid vesicles but the difference did not exceed a factor of two (Fig. 3). Vesicles prepared from purified egg yolk phosphatidylcholine did, however, adhere to each other noticeably more than those prepared from crude egg yolk lipids.

The pH of the dialysis solution was varied from 4 to 10. As monitored by light microscopy, large numbers of giant vesicles were generated throughout this range. Giant unilamellar vesicles were also generated at the same efficiency when the lipid-methylglucoside-methanol complex was dialyzed at 0°C or at 37°C as when dialysis was done at room temperature.

Our initial survey of a variety of different sugars revealed that methylglucoside and methylgalactoside were most satisfactory for the generation of giant vesicles. Most of our preparations have been made with the former. Small numbers of giant vesicles were observed when the solute was sucrose, glucose, galactose or arabinose, but virtually none were found with solutions of xylose, inositol or lactose. There appeared to be some correlation between the efficacy of the sugar in facilitating giant vesicle formation and the ease with which it dissolved in methanol. In this regard it should be noted that glycerol, the only carbohydrate tested that is miscible with methanol, also supported the formation of giant vesicles. Use of this solute simplifies the procedure somewhat but the vesicles so generated are slightly smaller, by perhaps a factor of 2 or 3, than those formed from methylglucoside in methanol. A difference in the reflection coefficient of bilayers for the two solutes may contribute to the disparity.

Acidic lipids such as phosphatidylserine and phosphatidylinositol, as well as cholesterol, are only slightly soluble in methanol. Lipid mixtures containing substantial proportions of these lipids may therefore generate vesicles whose composition is not representive of the original mixture. It is possible to modify or change the solvent so that it dissolves both lipids and solute to an extent comparable to that obtained with phosphatidylcholine/methylglucoside/methanol. When such mixtures imbibe water, however, solubilities change and selective precipitation of the bilayer or other phase may still occur. For example, one may replace methanol with tetrahydrofuran and induce

cholesterol to go into solution along with egg yolk phospholipid and methylglucoside. Nevertheless, after dialysis of the mixture, a few crystals of cholesterol may be seen. Undoubtedly, the bilayers present do contain some cholesterol, but its amount and uniformity of distribution is not known. Similarly, acidic lipids are soluble in tetrahydrofuran and dioxane, but less so given the additional presence of saturating concentrations of methylglucoside. A precipitate is seen when phosphatidic acid containing lipid mixtures are prepared for dialysis in the usual way. We would advise caution when dealing with complex mixtures, even with an organic solvent that dissolves all components of a mixture. Indeed, it would appear that any method for liposome formation that involves lipid mixtures in an organic solvent [1–4] has the potential for giving rise to some segregation of lipids of complex mixtures. Heterogeneity of composition of vesicles is not easily measured, especially in the case of the small vesicles generated by injection methods, and most investigators have not concerned themselves with this possibility.

Using typical values for the area per molecule of phospholipids, we calculate the encapsulated volume of lipid vesicles to be approximately $60r$ μl/μmol as phospholipid, where r is the vesicle radius in μm. Since we find trapped volumes of about 20 μl/μmol, it is clear that vesicles larger than 10 μm in diameter cannot represent more than 7% of the lipid. This must reflect the presence of substantial numbers of quite small vesicles and not a selection of a minor component of egg phospholipid, because similar results are obtained with chromatographically purified phosphatidylcholine. Small vesicles are indeed seen by microscopy, although it is difficult to estimate their proportion. Reeves and Dowben [10] describe a centrifugation procedure for purifying large vesicles and this may well enable enrichment of giant vesicles prepared by our procedure. Applications of giant vesicles that involve observation or manipulation of individual vesicles should be adequately served by the method as described; the yield of 10 μm and larger vesicles is in excess of 10^7/mg lipid. Our interest, for example, is in the morphological response of vesicles to their mutual adhesion or fusion and to their treatment with bilayer modifying agents, and the procedure meets our needs for such investigations.

It is obvious that a number of variations on the procedures outlined here are possible. It seems likely that a systematic variation of the parameters of the procedure would give rise to a higher yield of giant vesicles. What appears to us to be most significant and to augur well for the understanding and, thus, control of liposome formation, is the fact that van der Waals forces that normally give rise to multilayered vesicles can be overcome by the rather simple artifice of generating vesicles under conditions of gradually diminishing osmolarity.

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References

1 Deamer, D. and Bangham, A.D. (1976) Biochim. Biophys. Acta 443, 629–634
2 Kremer, J.M.H., Van den Esher, M.W.J., Pathmamanoharan, C. and Wiersema, P.H. (1977) Biochemistry 16, 3932–3935
3 Schieren, H., Rudolph, S., Finkelstein, M., Coleman, P. and Weissmann, G. (1978) Biochim. Biophys. Acta 542, 137–153
4 Cafiso, D.S., Petty, H.R. and McConnell, H.M. (1981) Biochim. Biophys. Acta 649, 129–132
5 Milsmann, M.H.W., Schwendener, R.A. and Weder, H.G. (1978) Biochim. Biophys. Acta 512, 147–155
6 Enoch, H.G. and Strittmatter, P. (1979) Proc. Natl. Acad. Sci. U.S.A. 76, 145–149
7 Zumbuehl, O. and Weder, H.G. (1981) Biochim. Biophys. Acta 640, 252–262
8 Papahadjopoulos, D., Vail, W.J., Jacobson, K. and Poste, G. (1975) Biochim. Biophys. Acta 394, 483–491
9 Szoka, F.C. and Papahadjopoulos, D. (1978) Proc. Natl. Acad. Sci. U.S.A. 75, 4194–4198
10 Reeves, J.P. and Dowben, R.M. (1969) J. Cell Physiol. 73, 49–57
11 Boroske, E., Elwenspoek, M. and Helfrich, W. (1981) Biophys. J. 34, 95–109
12 Kim, S. and Martin, G.M. (1981) Biochim. Biophys. Acta 646, 1–9
13 Hargreaves, W.R. and Deamer, D.W. (1978) Light Transducing Membranes. Structure, Function and Evolution (Deamer, D.W., ed.), pp. 23–59, Academic Press, New York
14 Oku, N., Kendall, D.A. and MacDonald, R.C. (1982) Biochim. Biophys. Acta 691, 332–340
15 Johnston, P.V. (1971) in Basic Lipid Methodology, Special Publication 19, College of Agriculture, University of Illinois at Urbana-Champaign
16 Rand, R.P. (1981) Annu. Rev. Biophys. Bioeng. 10, 277–314