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Preparation of multilamellar vesicles of defined size-distribution by solvent-spherule evaporation

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A novel method of preparing multilamellar vesicles is described. The process involves dispersing in aqueous solutions small spherules of volatile hydrophobic solvents in which amphipathic lipids are dissolved. The lipids form vesicles when the solvents are evaporated in the proper manner. The resulting vesicles have been characterized morphologically with light microscopy and electron microscopy. The method yields multilamellar vesicles with a defined size distribution which can be adjusted by varying the duration of mechanical agitation of the spherules and by varying the concentration of amphipathic lipids in the solvents. This is the first fundamentally new method of multilamellar vesicle preparation since Bangham's report in 1965 (Bangham, A.D., Standish, M.M. and Watkins, J.C. (1965) J. Mol. Biol. 13, 238–252).

Introduction

Bangham and co-workers first reported preparation of multilamellar vesicles in the 1960's [1,2], and the method has proven to be very popular. As far as we are aware, all multilamellar vesicles to date have been prepared by Bangham's method of hydrating dried amphipathic lipids. A major difficulty with Bangham's method is the control of the vesicle size distribution. Attempts have been made to overcome this problem by filtration through membrane filters with uniform pores [3]. The process, however, requires a series of four to five passages through filters of decreasing pore size, is able to produce only smaller vesicles, and is always accompanied by loss of lipids and aqueous solution to filter surfaces and dead spaces.

We describe in this report a novel method for rapid preparation of multilamellar vesicles with a defined and adjustable size distribution. This method is quite versatile in that variations of it can give unilamellar or multivesicular vesicles with certain modifications as described in previous reports [4,5].

Materials and Methods

Lipids and other materials. Cardiolipin, DMPG, and DMPC were purchased from Avanti, Birmingham, AL, and cholesterol was from Sigma, St. Louis, MO. All lipids gave a single spot on silica gel thin-layer chromatography. Sudan III was from Harleco, Philadelphia, PA; Ponceau S was from Reeve Angel, London, U.K.; amphotericin B was a gift from Squibb, Princeton, NJ; doxorubicin

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Abbreviations: DMPG, dimyristoylphosphatidylglycerol; DMPC, dimyristoylphosphatidylcholine; DOPC, dioleoylphosphatidylcholine.

with lactose was from Adria, Columbus, OH; reagent grade glucose, NaCl, anhydrous diethyl ether, and chloroform were from Mallinkrodt, St. Louis, MO; and D-[^{14}C]glucose was from Schwartz/Mann, Orangeburg, NY. One-dram glass vials were from Acme Glass, Los Angeles, CA. The mechanical shaker was a vortex machine purchased from American Scientific Products, (Catalog number S8223), McGaw Park, IL, and manufactured by Scientific Industries Inc., Bohemia, NY. The circuitry was modified so that the agitation could be controlled by the toggle switch without having to depress the shaker head. All mechanical agitations were performed at the maximum speed setting.

Preparation of chloroform-diethyl ether spherules. The process involves dissolving amphipathic lipids in chloroform-diethyl ether solvent and then making an 'oil-in-water' emulsion consisting of small spherules of the lipid-containing solvents. Controlled evaporation of the solvents produces multilamellar vesicles.

In a clean one-dram glass vial (1.4 cm diameter $\times$ 4.5 cm height in external dimensions), 1.5 ml of 5% (w/v) glucose was placed. In another container, 3–48 μmol of amphipathic lipids and any lipid-soluble substance to be incorporated into vesicles were completely dissolved in 50 μl of chloroform and 50 μl of diethyl ether. The lipid phase was placed under the surface of 5% glucose solution in the vial with a glass capillary pipet. The vial was immediately closed with an aluminum foil-lined screw cap, attached horizontally to the head of the vortex machine with a piece of an adhesive paper tape and agitated for 5–75 s to produce small chloroform-diethyl ether spherules suspended in 5% glucose solution.

Evaporation of chloroform and diethyl ether to form vesicles. On the bottom of a 50 ml Erlenmeyer or filtration vacuum flask (bottom diameter 5 cm), 1.5 ml of 5% glucose solution and 0.5 ml of 0.9% NaCl solutions were placed. A stream of nitrogen gas at 5 l/min was introduced into the flask via a piece of glass tubing protruding 3 cm into the mouth of the flask. The chloroform-diethyl ether spherules suspended in 5% glucose solution were then taken up into a Pasteur pipet and carefully instilled into the flask one drop at a time over a 5 min period. Throughout the evaporation, the flask was gently swirled, care being taken to keep the bottom of the flask covered with the liquid. The flask was kept in a water bath at 37°C. The solvent evaporation was then allowed to proceed for 2 more min. No odor of chloroform or diethyl ether was detectable at this time. Water-soluble substances to be incorporated into the vesicles were added to the 1.5 ml of 5% glucose solution at the chloroform-diethyl ether spherule preparation step and to the aqueous solution in the flask at the solvent evaporation step. The concentrations of the substances were the same in both steps.

Size distribution. The multilamellar vesicle preparations were wet mounted without any dilution on a hemacytometer and photographed on a Zeiss photomicroscope. The negatives were projected onto a piece of paper by using a photographic enlarger, with the magnification adjusted to 1000 $\times$. Diameters (d_i) of 395–1247 vesicles were measured for each preparation and assigned to the nearest 1 μm-size group (i). The volume (V_i) occupied by vesicles in each size group was obtained by multiplying the number (n_i) of vesicles in each size group by the volume of a sphere ($\pi d^3/6$) of the same diameter. The vesicle volume of each size group (V_i) was then divided by the sum of the volumes ($\Sigma_i V_i$) and multiplied by 100 to obtain percent of total volume, $v_i = (V_i/\Sigma_i V_i) \cdot 100$. From volume-normalized size distribution (v_i versus d_i), the mean diameter ($\bar{d}$) and standard deviations were calculated.

Optical volume. A useful parameter is the amount of vesicle volume produced by a given mass of lipid. One measure of this is the captured volume per mass of lipid (vide infra). Another is the volume based upon the optical measurement of the size distribution. We thus define the optical volume (V_{op}) as the quotient of the sum of the vesicle volumes above ($\Sigma_i V_i$) (extrapolated to the total preparation) and the mass of lipid (M) used in a given preparation.

$$V_{op} = \left(\sum_i V_i\right) \cdot (V_t/V_h) \cdot (1/M)$$

where V_t is the total volume of the vesicle preparation, (suspending medium plus vesicles) and V_h is the volume of the hemacytometer. V_{op} quantitates the volume occupied by vesicles that are visible under the light microscope (greater than about 1

μm in diameter) per unit amount of lipid used.

Excluded volume. To study the collective physical volume occupied by all vesicles, both visible and invisible to light microscopy, we measured the 'excluded volume' (V_{ex}), which was obtained by determining the amount of radiolabeled glucose excluded by the vesicles when radiolabeled glucose was added to a suspension of vesicles prepared in the absence of labeled glucose. In detail, liposomes were prepared with the lipid combination DOPC/cholesterol/DMPG at 9:9:2 molar ratio at the lipid concentration of 30 mg in 100 μl of solvents. The duration of mechanical agitation was 15 s. At the end of the solvent evaporation, ^{14}C-labeled glucose was added to the preparation and mixed with 9-fold volume of 0.3 M sucrose solution. The liposomes were then floated to the top of the centrifuge tube in a Beckman preparative ultracentrifuge (Model L5-75B) at 61000 × *g* for 30 min. The clear suspending medium in the bottom was separated from the floating liposome fraction with a syringe and hypodermic needle piercing the centrifuge tube. The excluded volume was calculated from the equation:

$$V_{ex} = \frac{C_m - C_l}{C_m} \cdot \frac{V_l}{M}$$

where C_m is the counts of [^{14}C]glucose in 50 μl of suspending medium, C_l is the count in 50 μl of liposome fraction (pellet), V_l is the volume of the liposome fraction, and M is the amount of lipid in the liposome fraction.

Captured volumes. To determine captured volumes (Table I), vesicles were pelletted in a centrifuge at 1500 × *g* for 10 min and washed with a solution containing 4.3% (w/v) glucose and 0.13% (w/v) NaCl. The quantities of materials in the pellet and in the supernatant were used to calculate the percentage of material incorporated. The very small vesicles that were too small to be pelletted at this *g*-force and the materials unincorporated into the vesicles are removed with the supernatant. The captured volume is the portion of the original aqueous volume remaining after the washing divided by the amount of lipid used. For glucose incorporation, DOPC/cholesterol/DMPG (9:9:2 molar ratio) lipid combination was used, with identical parameters as for Fig. 3c. The vesicles of this lipid combination were floated to the top of centrifuge tubes in Beckman preparative ultracentrifuge at 61000 × *g* for 30 min after mixing the liposome preparation with 9-fold volume of 0.3 M sucrose solution. The liposomes were washed twice with 0.3 M sucrose solution.

TABLE I

VARIOUS MATERIALS INCORPORATED INTO VESICLES

11.3 mg (in 100 μl of solvents) of the lipid combination DMPC/DMPG (1:1 molar ratio except for amphotericin B for which it was 7:3) were used in each preparation and the mechanical agitation was for 5 s. V_{ex} and V_{op} are defined in the text.

Materials incorporated	Concn.	% of total volume	Volumes [c]	
			μl/mg	l/mol
Ponceau S	17 μg/ml	1.3	4.0	2.7
Glucose [a]	5% (w/v)			
captured vol.		0.6	0.5	0.3
optical vol. (V_{op})		2.9	2.6	1.6
excluded vol. (V_{ex})		3.8	3.3	2.0
Doxorubicin	67 μg/ml	56	170	120
Sudan III	0.45 μg/mg [b]	74	n.a.	
Amphotericin B	89 μg/mg [b]	73	n.a.	

[a] 30 mg (in 100 μl of solvents) of DOPC/cholesterol/DMPG (9:9:2 molar ratio) were used with 15 s mechanical agitation (identical parameters as Fig. 3c). The difference in the captured volume compared to the encapsulation of glucose above was the result of entirely different experimental conditions in which the duration of mechanical shaking, the type and the amount of lipids used were different.

[b] Micrograms of material to be incorporated per milligram of lipids used. n.a., not applicable, the material is insoluble in water.

[c] Captured volumes unless otherwise specified.

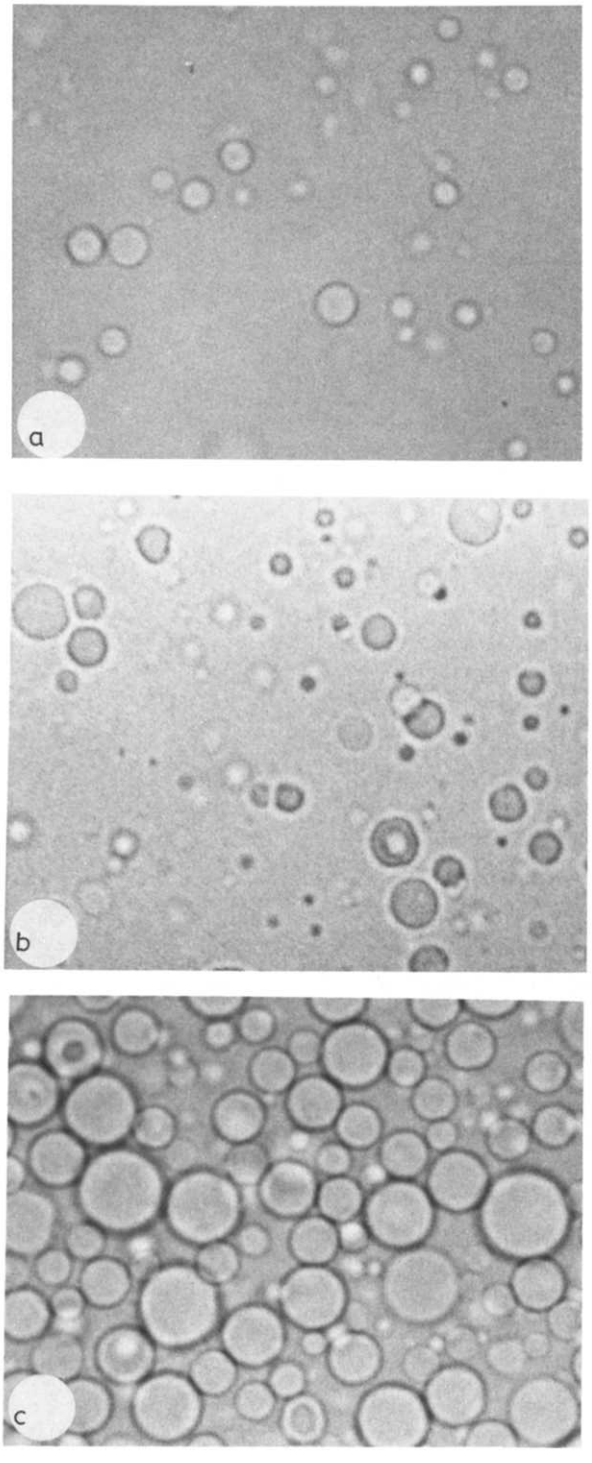

Fig. 1. Light micrographs of three separate preparations of vesicles with increasing concentration of lipids used. See text for experimental details. (a) 3.3 mg lipid/100 μl of solvent; (b) 10 mg/100 μl; (c) 30 mg/100 μl. In all cases the lipids were

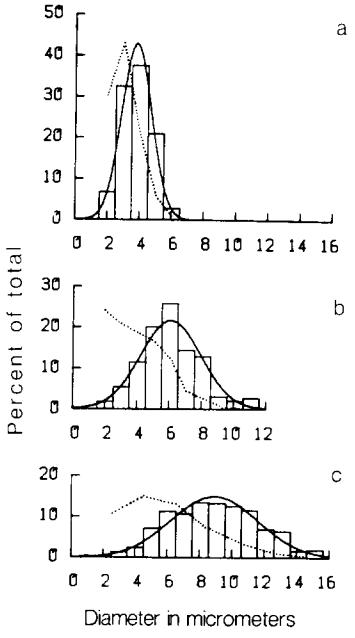

Fig. 2. Size distributions of three separate preparations of vesicles with increasing lipid concentration (3.3 mg, 10 mg, and 30 mg of lipids in 100 μl of solvents for a, b and c, respectively). The bars and solid curves are the volume-normalized distributions, for which the ordinate represents the percentage of total vesicular volume. The bars are the experimental data (derived in part from the photos of Fig. 1) and the solid curves are Gaussian distribution functions. The dotted curves show the relation between the fraction of vesicles in a given size group (expressed as percentage of total number) and vesicle diameter.

Ponceau S and doxorubicin were quantitated spectrophotometrically at 510 and 480 nm, respectively, after clarification with 1% Triton X-100. Radiotracer was used to quantitate the glucose-captured volume. Sudan III and amphotericin B were spectrophotometrically quantitated in 90% methanolic solution at 508 and 406 nm, respectively. For each milligram of amphotericin B, 25 μl of dimethylsulfoxide was used to dissolve amphotericin B before adding to the diethyl ether/chloroform lipid phase at the beginning of the vesicle preparation.

Electron microscopy. To make electron micro-

DOPC/cholesterol/DMPG at 9:9:2 molar ratio, and mechanical agitation was for 15 s. The size distributions of the vesicles of each micrograph are shown in Fig. 2. The scale bar is 10 μm.

graphs, the vesicle preparations were mixed in 0.7% phosphotungstic acid or 1.5% ammonium molybdate and spread on carbon-coated copper grids. The excess liquid was drawn off with a piece of filter paper, dried in air for 1 h, and then viewed on a JEOL 100C.

Results

The aqueous solutions used were chosen after testing various combinations of solutions of physiological osmolality of different ionic strengths. Control of ionic strength is critical to prevent clumping and/or deformation of the vesicles. The optimal aqueous solution for the preparation of the chloroform-diethyl ether spherules is one with minimum ionic strength. Increase in the ionic strength results in increased fusion of the chloroform-diethyl ether spherules. For the evaporation of the chloroform and diethyl ether step, at least a small amount of salt was required to bring the ionic strength above 0.005 to prevent deformation of the vesicles.

Several lipid combinations form vesicles satisfactorily (Figs. 1, 3 and 7, and Table I). The constraints on the lipid combinations that can be used were that a small amount of negatively charged amphipathic lipid be present (to prevent

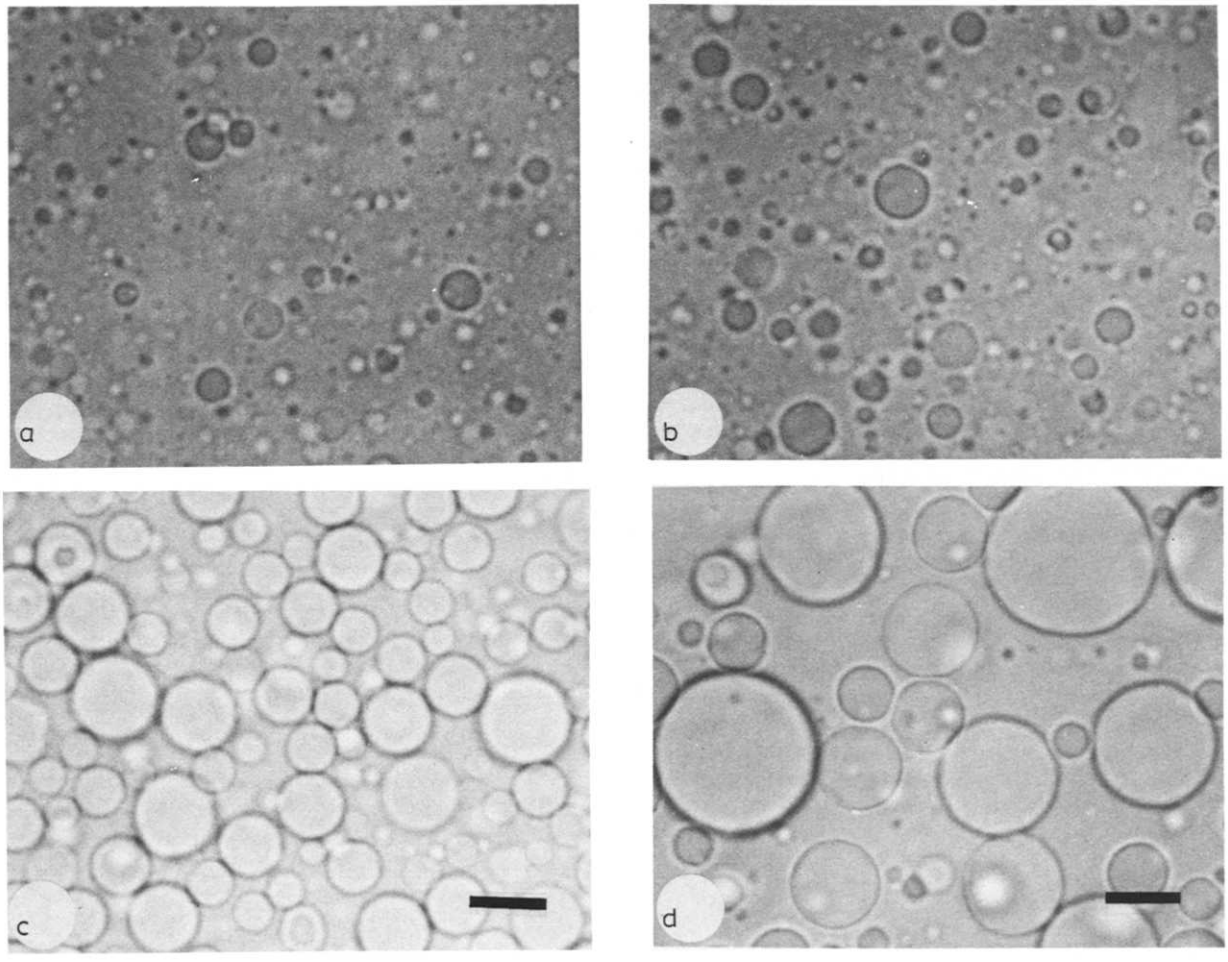

Fig. 3. Light micrographs of four separate preparations of vesicles with decreasing duration of mechanical agitation. (75, 45, 15 and 5 s for 1, b, c and d, respectively). Lipids used were DOPC/cholesterol/DMPG at 9:9:2 molar ratio and the concentration was kept constant at 30 mg in 100 μl of solvents. The scale bar is 10 μm.

coalescence of chloroform-diethyl ether spherules) and that the lipids be soluble in the chloroform-diethyl ether mixture. In contrast to the previously published methods of solvent-spherule evaporation, which leads to unilamellar and multivesicular vesicles [4,5], no triacylglycerol or other neutral lipids were required for the formation of multilamellar vesicles. The amount of solvent remaining after the solvent evaporation process was not measured in the present instance, but previous measurements on multivesicular vesicle preparations (which start with much larger quantities of solvents) were shown to contain no detectable residual solvents [5].

Morphologically, the multilamellar vesicles prepared by the solvent-spherule evaporation process are refractile spheroids under the light microscope (Figs. 1 and 3). The multilamellar structure of the vesicles is revealed with negative-stain electron microscopy (Fig. 7).

The average vesicle size is affected by the concentration of amphipathic lipids in the chloroform-diethyl ether spherules. Micrographs of vesicle preparations of various mean sizes are shown in Fig. 1. Fig. 2 shows the size distributions of vesicles whose average sizes were varied by adjusting the concentration of the amphipathic lipids while keeping the duration of mechanical agitation constant. Fig. 5 shows the relationship between the concentration of amphipathic lipids and the mean vesicle volume ($\pi\bar{d}^3/6$). As expected, the mean vesicle volume (as calculated from the mean vesicle diameter) was directly proportional to the concentration of the amphipathic lipids used.

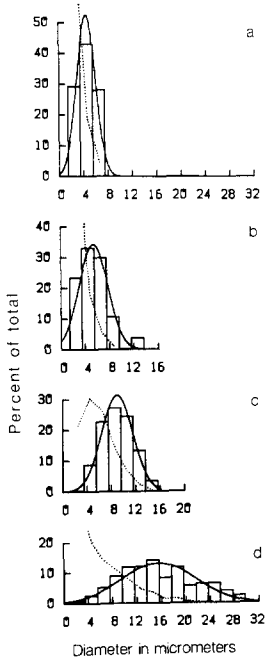

Fig. 4. Size distribution of vesicles prepared by varying the duration of mechanical agitation. The distributions were determined in part from the photographs shown in Fig. 3. The panel letters correspond to those of Fig. 3. The mean diameters increase as the duration of the agitation is reduced. See Fig. 2 for the details of the presentation of the distributions.

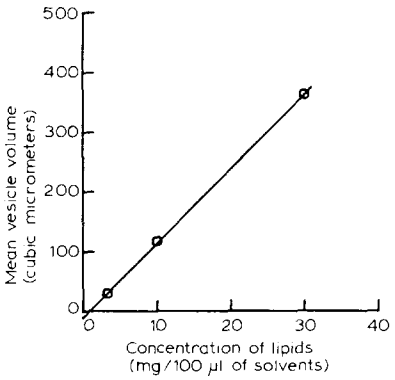

Fig. 5. The effect on the mean vesicle volume of increasing the concentration of lipids in the hydrophobic solvent. Note the linear relationship.

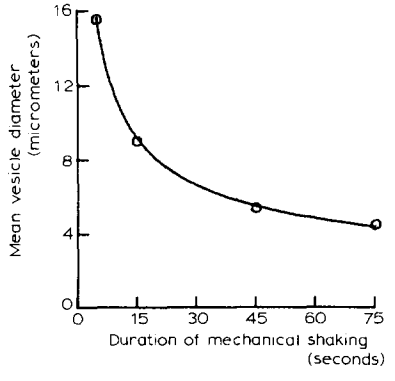

Fig. 6. The effect on mean vesicle diameter of increasing the duration of mechanical agitation. The experimental points are derived from Fig. 4. The curve is the least-squares fit of the data to the equation $D = a + bT^{-1/2}$ where D is the diameter and T is the duration of agitation.

As is the case with the two other types of vesicles prepared by the solvent-spherule evaporation method [4,5], the size of the multilamellar vesicles can be varied by changing the size of the chloroform-diethyl ether spherules which in turn is controlled by the duration of mechanical agitation. It was found that the volume-normalized average diameter is inversely proportional to the square root of the duration of mechanical agitation, as shown in Fig. 3, 4 and 6.

Table I shows that a variety of materials can be incorporated into vesicles. The hydrophobic substances, such as Sudan III and amphotericin B, were incorporated at very high efficiency, while

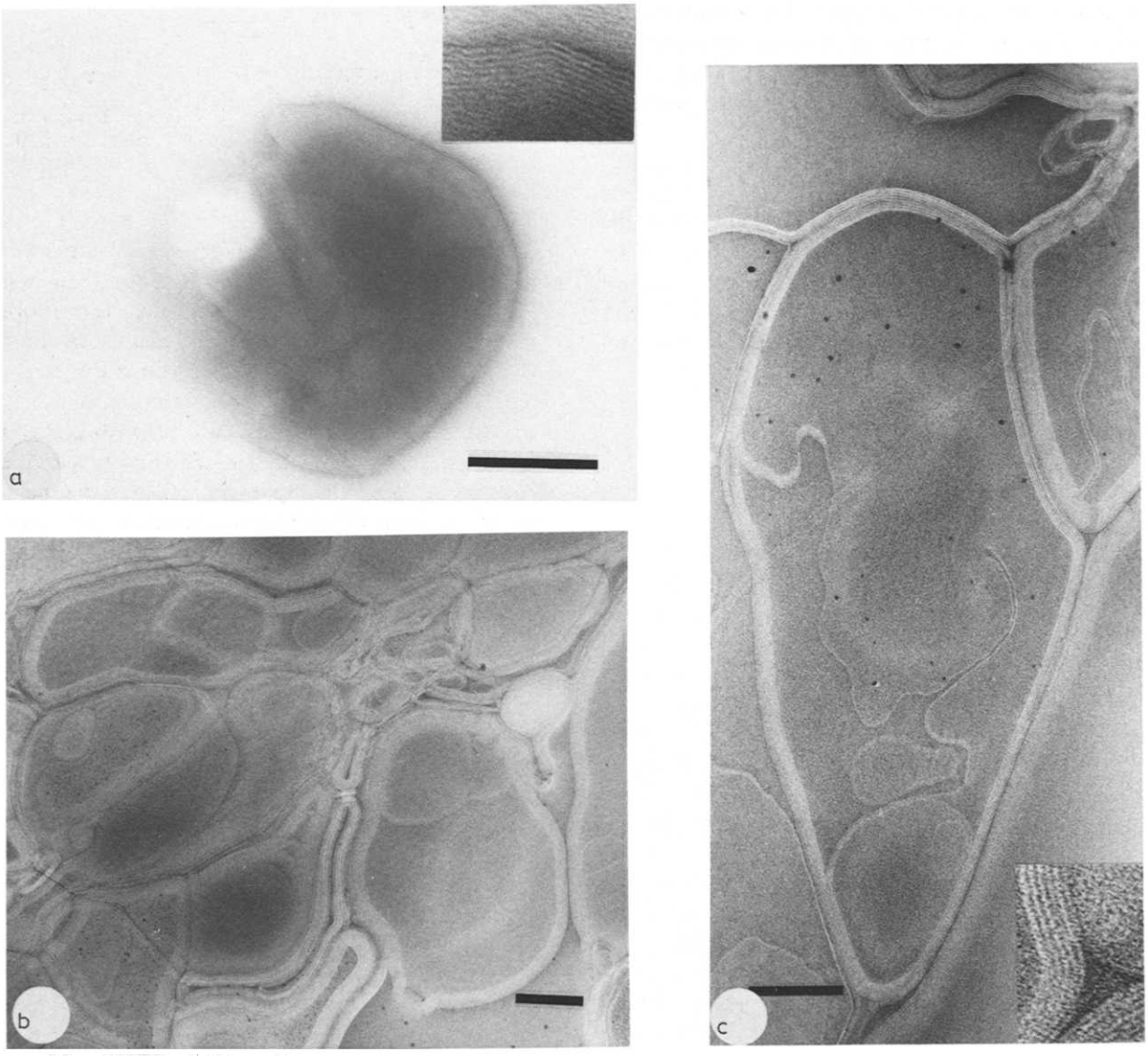

Fig. 7. Negative stain electron micrographs of multilamellar vesicles prepared by the solvent-spherule evaporation method. The vesicles were prepared with 11.5 mg of lipid (DDPC/cardiolipin = 176 : 1) dissolved in 100 μl of solvent. The scale bars = 0.2 μm. (a) 15 s of mechanical agitation. Stained with 0.7% phosphotungstic acid. (b and c) 60 s of mechanical agitation. Stained with 1.5% Ammonium molybdate. Because of the staining procedure, it is unlikely that the detailed structures seen here are exactly the same as the native vesicles. However, the electron micrographs do show that the lipids form multilamellar vesicular structures.

water-soluble substances, glucose and Ponceau S dye, were very poorly incorporated except for substances such as doxorubicin that interact strongly with the lipids [6].

We use in this report two quantities that are useful for understanding of the nature of our multilamellar vesicles: optical volume (V_{op}) and the excluded volume (V_{ex}) (see Materials and Methods). V_{op} estimates the maximum volume occupied by vesicles visible under the light microscope. V_{ex} estimates the total volume occupied by all vesicles regardless of visibility in the light microscope. As seen in Table I, V_{op} is about 80% of the V_{ex}, indicating that about 4/5 of vesicles (weighted by volume) are visible and about 1/5 are too small to be seen by light microscopy. The captured volume, which gives an estimate of the aqueous space within the vesicles, is only about 15% of the excluded volume, indicating that most of the volume within the vesicles is filled with multiple bilayers with a minimal amount of aqueous space within.

Discussion

This is the third in a series of reports on solvent-spherule methods of vesicle preparation [4,5]. Because all three methods use the same basic process of solvent evaporation from lipid/solvent spherules dispersed in water, we propose to call these 'solvent-spherule evaporation' methods. Since all three types of vesicles, i.e., unilamellar, multivesicular and now the multilamellar, can be made by this method, it appears to be a versatile and effective technique for vesicle preparation. The essential procedural step that determines which type of vesicle will form is the control of the amount of water dispersed within the spherules. Zero, single or multiple water droplets lead, respectively, to multilamellar, unilamellar or multivesicular vesicles. An additional advantage is that the solvent-spherule varporation process is a very rapid method of vesicle preparation. Once the simple apparatus and the reagents are set up, it takes only about 15–30 min in our hands to prepare each batch of multilamellar vesicles. The most likely mechanism of vesicle formation by this method is conceptually simple. The 'oil-in-water' emulsion, which consists of small spherules of lipid-containing solvents, has a monolayer of amphipathic lipids at the surface of each spherule with the hydrophilic 'head groups' facing the water and the hydrophobic 'tails' dissolved in the solvent. As the solvents evaporate, the dissolved amphipathic lipids come out of solution and 'precipitate' as bilayer membranes.

In general, the multilamellar vesicles are ideal for encapsulation of hydrophobic materials, since there are plenty of lipid bilayers in each vesicle. Conversely, the multilamellar vesicles encapsulate hydrophilic materials very poorly in view of the very small aqueous compartments between lipid bilayers. Compared to the vesicles prepared by Bangham's method, the capture efficiency of hydrophilic substance by the present method is even lower (Table I). This is probably due to a mechanism of vesicle formation in which there is minimal disruption of bilayers once they are formed. In support of this hypothesis, filtration through 'Nuclepore' membrane filters, which results in partial disruption of bilayer membranes, increases the capture efficiency significantly [3]. Unilamellar or multivesicular vesicles made by solvent spherule evaporation have much larger aqueous compartments and are more efficient for encapsulation of hydrophilic materials [4,5]. A possible disadvantage of the solvent-spherule evaporation method is the presence of solvents during encapsulation which may preclude encapsulation of materials sensitive to organic solvents. The amount of solvent remaining after the solvent evaporation process was not measured in the present instance, but previous measurements of multivesicular vesicle preparations (which start with much larger quantities of solvents) were shown to contain no detectable residual solvents [5].

Like the unilamellar and multivesicular vesicles made by the solvent-spherule evaporation method, the most significant advantages of multilamellar vesicles made by the present method are the defined size distribution and the adjustibility of the vesicle diameter. If a way could be found to control more closely the size distribution of the solvent spherules, it might be possible to make the size distribution narrower.

Acknowledgments

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