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Development and Characterization of a Liposome Preparation by a pH-Gradient Method

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Abstract—A pH gradient across liposome bilayers was established in order to load a model drug (orciprenaline sulphate) into liposome vesicles. This method of liposome loading resulted in yields as high as 80–85% encapsulation. An eight-step process was designed to scale-up the process and was evaluated. In this process a diafiltration technique was successfully used to remove the excess orciprenaline sulphate present in the external medium. Finally, drug-loaded liposomes were lyophilized using lactose as an internal and external liposomal cryoprotectant. Five-month stability data for the liposomes is reported. An HPLC technique was used to determine the drug concentration and a laser light-scattering technique was employed to determine the liposome vesicle size and polydispersity factor. Liposomes prepared by the pH-gradient method showed high encapsulation efficiency. Upon storage at 2–8°C the vesicle size increased and encapsulation efficiency decreased with time. These phenomena are attributed to gradual fusion of liposomes and loss of drug to the extra-liposomal media.

Liposomes are microscopic vesicles consisting of membrane-like lipid bilayers surrounding an aqueous media. The lipid vesicles are formed spontaneously when phospholipids are hydrated in aqueous media (Bangham et al 1974). Because of their entrapping ability, liposomes are being investigated as drug-carrying structures or vesicles (Gregoriadis 1976; Stamp & Juliano 1979; Juliano 1980; Chien 1982; Vemuri et al 1991). Since liposomes are phospholipid bilayer structures, which resemble naturally occurring biological membranes, they may well provide compatibility and an acceptable safety profile for human use. The aqueous medium entrapped in lipid vesicles may contain a variety of drugs (Poste 1983). Variations in liquid concentration can yield liposomes of different physicochemical characteristics, such as drug encapsulation ability, surface charges, and permeability. Recent review articles (Talsma & Crommelin 1992 a, b) have emphasized the importance of large-scale production, characterization, and stabilization of liposomes. The differences in structure of vesicles cause differences in in-vitro behaviour of liposomes (Gruner et al 1985).

It has been shown that many different polar compounds can be entrapped in liposomes (Gregoriadis 1976). A water-soluble drug, orciprenaline sulphate, which has a short biological half-life of about 4 h was selected as a model drug for this study. It has already been shown that pH gradients are helpful in loading monoamines into liposomes (Deamer et al 1972; Nichols & Deamer 1976). Further, pH gradient-loading techniques have been reviewed by Mayer et al (1993).

A variety of techniques has been used to produce liposomes of various sizes and lamellarity. These vesicular structures can be prepared with diameters ranging from 80 nm to 100 μm. When phospholipids are dispersed in an aqueous phase, hydration of the polar head groups of the lipid results in a heterogeneous mixture of structures. A detailed discussion of several methods for preparation of liposomes has been given by Hope et al (1986). The complexity of sterile manufacturing of liposomes has been discussed in book chapters (Martin 1990; Brandl et al 1990; Amselem et al 1993).

The laboratory techniques used for processing the liposomes have tended to limit their commercial development because the methods are not generally suitable for large scale production. On the commercial scale, consistency and reproducibility of the process are important to meet Good Manufacturing Practice guidelines and Investigated New Drug and New Drug Applications requirements, as well as to achieve the desirable pharmacokinetic properties for particular drugs. This topic does not appear to have attracted the attention which it deserves, except for a few reports (Vemuri et al 1990, 1991).

The drug encapsulation ability and the stability of liposomes can be changed either by altering the lipid compositions of the bilayer or by altering the process conditions. Often it is not possible to change the lipid composition in the down-stream process development work since the early development defines the composition of lipids used to make liposomes. This study was aimed at optimizing process conditions to improve encapsulation efficiency. The objectives of the study were to increase encapsulation (greater than 90%) by using pH gradients established across the liposome membranes and to develop and evaluate a process scheme for scale-up.

Materials and Methods

Materials

Egg phosphatidylcholine, egg phosphatidylglycerol (Avanti Polar Lipids, Inc., Birmingham, AL), cholesterol (Croda, Inc., Mill Hall, PA), and orciprenaline sulphate (Vinchem, Inc., Chatham, NJ), were obtained and used as received. (±)-α-Tocopherol, disodium phosphate dibasic heptahydrate, lactose monohydrate and edetate disodium were obtained from Sigma Chemicals (St Louis, MO). Sodium

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phosphate monobasic monohydrate, USP and chloroform were supplied by Mallinckrodt Chemical Company.

Preliminary investigations

Liposomes (5 mL) were prepared by the Luvet method (Hope et al 1985) using 0·1 M phosphatidylcholine/phosphatidylglycerol 9 : 1. The liposomes were prepared in 0·1 M phosphate buffer, at pH 4·7, and 10 mM orciprenaline sulphate was either present at the start or added later, depending on the experimental conditions. Gradients were produced by adjusting the pH of the external buffer to near 9·0 with 1·0 M potassium hydroxide.

Liposomes were then separated from the external medium by centrifugation at 100 000 *g* in a Beckman Ultracentrifuge for 30 min, and the distribution of orciprenaline inside and outside was determined spectrophotometrically by UV absorbance at 270 nm. A small amount of the detergent 1% Triton-X was added to clarify the suspension and minimize light scattering effects.

Scale-up experiments

Orciprenaline was encapsulated and preserved in lipid bilayers using eight different unit operations as follows.

Roto-evaporation. A Rotovapor (Buchi/Brinkmann), was used. Egg phosphatidylcholine (30 mg, EPC), egg phosphatidylglycerol (6 mg, EPG), cholesterol (12 mg), and (±)-α-tocopherol (0·4 mg) were mixed with 50 mL chloroform. Mixing was continued until a clear solution was obtained. The chloroform solution of the lipids was placed in a 100 mL round-bottom flask which was connected to the Rotovapor and the chloroform removed under vacuum at a temperature of 37°C. As the chloroform evaporated, a film of lipid layer resulted on the rotating flask. To ensure complete evaporation of the chloroform, the Rotovapor was maintained at vacuum and 37°C for an additional 10 min after complete removal of organic solvent as indicated by visual observation.

Preparation of aqueous drug solution. We prepared 100 mL orciprenaline sulphate (100 mg mL^{-1}) in 0·1 M phosphate buffer with 5% lactose, pH 9·5, and then filtered the solution through a 0·2 μm membrane filter.

Hydration of dried lipids. The dried lipid film was hydrated with 40 mL 0·1 M phosphate buffer containing 5% lactose, pH 4·5. Lipids and buffer were then mixed on the Rotovapor at a moderate speed (25% of the full speed) for 1 h or until the lipid had completely dispersed in the aqueous phase.

Mixing. The drug-lipid dispersion in a container was mixed with a homogenizer (Ross Mixer) at high speed for 1–2 min to break up any undispersed lipids.

Sizing. The liposome suspension was sized to around 0·2 μm mean diameter by a membrane filtration technique (Szoka & Papahadjopoulos 1978). A series of filter membranes with an average pore diameter of 8–0·2 μm was used to size the liposomes. Nucleopore capillary pore membranes were used for the filtration. The pressure required during filtration to push the liposome dispersion was gradually increased as the filter membrane pore size decreased. The average liposome vesicle size distribution after 0·2 μm filtration was measured on a Nicomp Particle Size Analyzer.

pH-gradient loading. Equal volumes of hydrated lipid suspension (inside liposomes pH 4·5) and drug solution, pH 9·5, were mixed in a beaker and allowed to approach equilibrium for 30 min. If a pH gradient exists, orciprenaline inside (C_{in}) is less than C_{out} and drug accumulates inside as the protonated species. A schematic representation of drug-loading into liposomes is shown in Fig. 1.

Separation of liposomes from free-drug by diafiltration. Liposomes may be washed during a concentration procedure by simply adding diluent to the recirculating fluid at the same rate as the filtration rate. We required 5–20 wash volumes to remove greater than 95% of the small molecules from a preparation. As the liposome solution concentrates in the flask, a partial vacuum in the head space of the flask will draw dialysate into the sidearm at the same rate as filtration proceeds. The dialysate mixes with the concentrate, and filtration proceeds as the free drug in the concentrate is replaced with the buffer salts from the dialysate.

In this study, a tangential flow, ultrafiltration system (Minitan, Millipore Corp. Bedford, MA) and a polysulfone filter membrane (mol. wt cut-off 10 kDa) were used to remove free drug present in the external media. The membrane pores selected are such that smaller molecules can pass through while larger aggregates (liposomes) were retained and concentrated. The concentrated material was returned to the reservoir, and the filtrate was collected in a

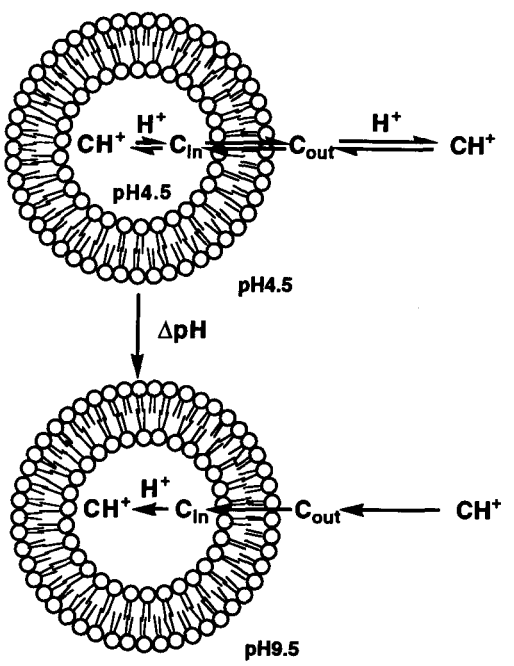

FIG. 1. A schematic representation of liposome loading with a water soluble drug by pH-gradient method is shown. Key: orciprenaline sulphate disassociates and yields orciprenaline (C) and sulphate ion. If a pH gradient exists, acid inside, C_{in} is less than C_{out} and orciprenaline accumulates inside liposomes as a protonated species. For every pH unit of gradient, there is a tenfold concentration of orciprenaline, according to Henderson–Hasselbach theory.

separate container. The initial volume of the drug-lipid suspension was noted and then pumped through the ultrafiltration system. The suspension was re-circulated through the system at a flow rate of 50 mL min^{-1} and a back pressure of 10–15 lb in^{-2}. During this process, samples of retentate were collected at 50 and 120 min. The process was stopped at 120 min. The resultant liposome suspension was highly encapsulated whereas the concentration of free drug in external media was low. As a final step of this unit operation, liposomes were concentrated by stopping the diluent (dialysate) flow while continuing the filtration process.

Lyophilization. Three millilitres of the liposome suspension was filled in each of the 5-mL vials and loaded into the lyophilizer (Dura Dry, FTS Systems Inc, Stone Ridge, NY). Freeze-drying was carried out under 150 millitorr vacuum at −30°C for 20 h. The lyophilized samples were stored in a refrigerator until analysis.

Percent encapsulation determination
The freeze-dried cake was reconstituted and diluted with 50 mL physiological saline to minimize possible damage to liposomes due to osmotic pressure changes. A 1-mL portion of the above suspension was spun at 100 000 rev min^{-1} (4°C) for 30 min (Ultracentrifuge, Model TL100, Beckman Instruments, Inc., Palo Alto, CA). The supernatant and the pellet were separated by decantation. The supernatant was further diluted to 50 mL with saline. This diluted solution was then analysed by HPLC. The pellet which remained in the centrifuge tube was diluted to 10 mL with saline. Equal portions of the suspended pellet and 0·5% Triton-X in saline were mixed to obtain a clear solution. The final clear solution was also analysed by HPLC. The percentage of drug encapsulated was calculated by the ratio of the drug in the pellet to the drug recovered.

HPLC system. The orciprenaline content of various samples was assayed by an HPLC system which consisted of an IBM (Danbury, CT) model LC/9560 and a variable wavelength UV detector (LC/9563) equipped with an autosampler (LC/9505). The output of the detector signal was fed to an IBM 9000 computer system to generate the chromatogram. A Whatman C-18 ODS analytical column, 10 μm particle size, was used. The flow rate was 1 mL min^{-1}. The mobile phase consisted of 70% phosphate buffer (pH = 7·0) and 30% methanol. The UV detector's wave length was set at 278 nm to detect orciprenaline. The drug concentration in a given sample was determined by comparing the absorbance values against the standard curve.

Drug release measurement
The reconstituted liposome cake was diluted 1 : 10 with saline and aliquots were dispensed into vials and placed into the chambers of an end-over-end tumbler device (Van-Kel Industries, Edison, NJ). These samples were then rotated at a pre-selected rate for various lengths of time. At various time intervals (0–24 h), triplicate vials were collected for analysis. Each suspension was subjected to centrifugation at 100 000 rev min^{-1} for 30 min to pellet the liposome, in an ultracentrifuge. The supernatant and pellet were collected and analysed as described above.

Liposome size distribution
Liposome size distribution was determined by dynamic light scattering analysis, using a Nicomp 270 submicron particle sizer.

Electron microscopy
The liposome concentrate samples were suspended in an aqueous solution of 20–30% glycerol. The suspension was then frozen in Freon 22 and fractured at 10^{-6}–10^{-7} torr vacuum. The freeze-fracture was performed on a Balzer Freeze-Etching System BAF 400D. The micrographs were obtained using a JEM-1200 EX electron microscope. Details of this technique can be found elsewhere (Mayer et al 1985).

Results and Discussion

In preliminary experiments, the pH-gradient hypothesis was tested by adding orciprenaline sulphate in pH 9·5 buffer or a pH sensitive dye (pyranine) to a liposome preparation; we observed the effect on the changes in fluorescence of pyranine. Fig. 1 shows a schematic representation of pH-gradient loading of orciprenaline sulphate into liposomes. Under the experimental conditions the drug disassociates into orciprenaline and sulphate ions. If orciprenaline permeates readily it would be expected to cause rapid decay of pH gradients as it moves across the liposome membrane. This initial study demonstrated that pH gradient was in fact discharged in less than 1 min by orciprenaline (pH 9·5) addition. This observation suggests that orciprenaline permeates sufficiently to result in enhanced accumulation as the protonated species in liposomes as a result of pH gradients.

As a logical extension of the preliminary study, another experiment was carried out to elucidate the extent of orciprenaline accumulation by pH gradients. A centrifugal gel filtration technique (1 × 5 cm Biogel P-6 gels) was used to separate liposomes from the free drug. The gels were subjected to centrifugation at 100 000 *g* in a Beckman Airfuge. The liposomes were prepared as usual with an acidic buffer (pH 4·5) inside the vesicles but orciprenaline sulphate solution was added to it afterwards. pH gradients were then established by adding 1·0 M potassium hydroxide. This yielded an approximate external pH of 9·5. The liposomes were incubated for various lengths of time and then subjected to centrifugation. The amount of drug in the liposome vesicles was then measured by an HPLC technique. It was found that the drug accumulated in liposomes very rapidly and then slowly leaked out to a level of 65%. These two preliminary investigations demonstrated that the pH gradients of 5 pH units offered a large driving force and accumulated about 75–80% of model drug, orciprenaline sulphate, into liposomes. In accordance with the Henderson–Hasselbach theory one might expect a 100% drug accumulation in liposomes under the study conditions; however, we have observed only about 80% drug accumulation, at best. This finding can perhaps be attributed to the osmotic pressure differential over the bilayers immediately after drug accumulation in liposomes. It was reported by others (Fransen et al 1986) that when the liposomes were exposed to hyperosmotic conditions in the external media there was a significant increase in rate of leakage of

Table 1. Characteristics of liposome vesicles produced by a pH-gradient method. Degree of drug encapsulation, concentration of orciprenaline outside liposomes and vesicle size distribution is shown at various stages of processing.

Processing step	Encapsulation %	Drug concn	Vesicle size (nm)	Polydispersity %
Mixing	20·5	128·5	2371	49
Sizing (0·2 μm)	26·8	125·2	203	34
Retentate, t = 50 min	82·7	77·4	197	33
Retentate, t = 120 min	91·7	38·1	195	29
End of diafiltration	89·5	33·8	197	42
Lyophilized cake	75·5	33·1	215	42

fluorescent dye from liposomes as compared with the iso-osmotic situation.

Since the preliminary experiments showed high drug encapsulation by the pH-gradient method, an attempt was made to scale-up the process to a 10 g batch that can easily be translated to a much larger batch size. An eight step process was designed. Dried lipid layers were produced in a Rotovapor and hydrated with low pH (4·5) buffer to yield an acidic pH inside the liposomes. The sized liposomes were then incubated for 30 min with orciprenaline sulphate solution at high pH (approx. 9·5). This experimental condition afforded the pH gradients of 5 pH units. The liposome suspension was then subjected to diafiltration to remove the free drug from the external medium. Table 1 shows a decline of drug concentration in the external medium as the process progresses and an increase in the percent encapsulation during that time. The diafiltration step was used to remove the free drug from the external medium. The time course of change in drug concentration in the external medium was monitored and is shown in Fig. 2. This technique required as little as 120 min to remove most of the free drug from the external medium. The permeate was collected at 10 mL min^{-1} (1200 mL permeate in 120 min; 12 volume exchanges with the drug suspension). Within 2 h of the diafiltration process, most of the drug in the external medium was removed either by drug accumulation into liposomes or to waste. The diafiltration process was stopped at 3 h and the liposomes concentrated using the same equipment. The diafiltration technique satisfactorily removed free drug from the liposome preparation. Evaluation of other techniques (Storm et al 1985), such as the use of adsorbents to remove positively charged drugs, was not in the scope of this study.

Fig. 3 illustrates the kinetics of drug release for liposomes prepared by the pH-gradient method and compared with liposomes prepared by another method (control). There was no difference in total amount of drug released over a 24 h period. However, there is an initial lag in drug release observed when the liposomes were prepared by the pH-gradient method.

Table 1 shows the average liposome vesicle size at various stages of the process. Vesicle size data show uniformity of vesicle size during the process after the sizing step. The polydispersity factor (% variability) fluctuated during the process (30–50%). The diafiltered and concentrated liposomes were stored in a refrigerator overnight before lyophilization. During that period the polydispersity factor of liposomes increased. No change in polydispersity factor was noted after lyophilization of samples. This finding suggests that the liposomes were not fused during the lyophilization process when lactose is present on the inside and outside of the formulation. Lactose appears to offer the necessary cryoprotective effect but not total protection by keeping the liposomes apart during the lyophilization process. There is 10–15% encapsulation loss due to lyophilization, however. This observation is in agreement with an earlier

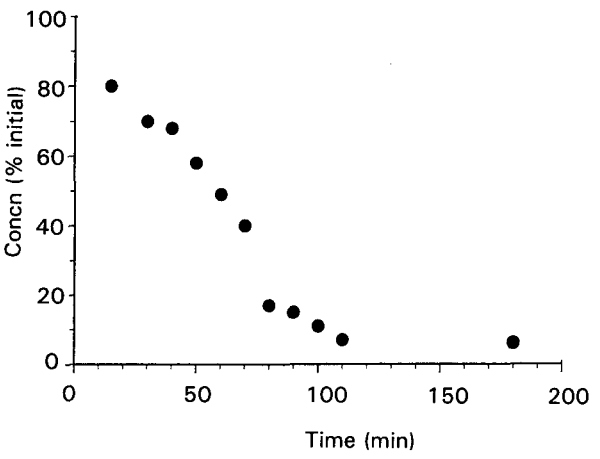

FIG. 2. Removal of drug (orciprenaline) from the external medium of a liposome preparation by diafiltration technique. Drug removal kinetics is shown.

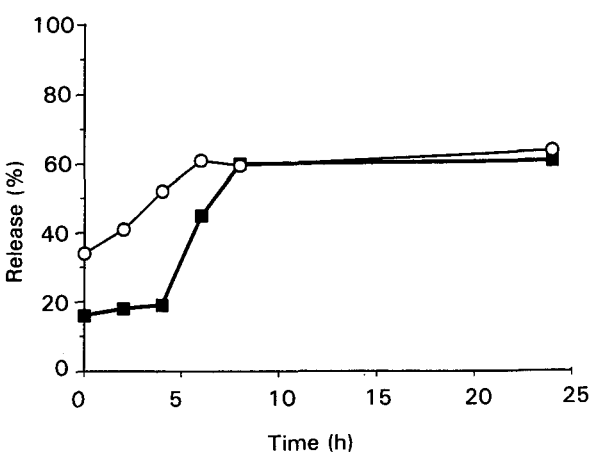

FIG. 3. Drug release rates of a liposome formulation prepared by two different methods. A pH-gradient method (■) was compared with a control method (○).

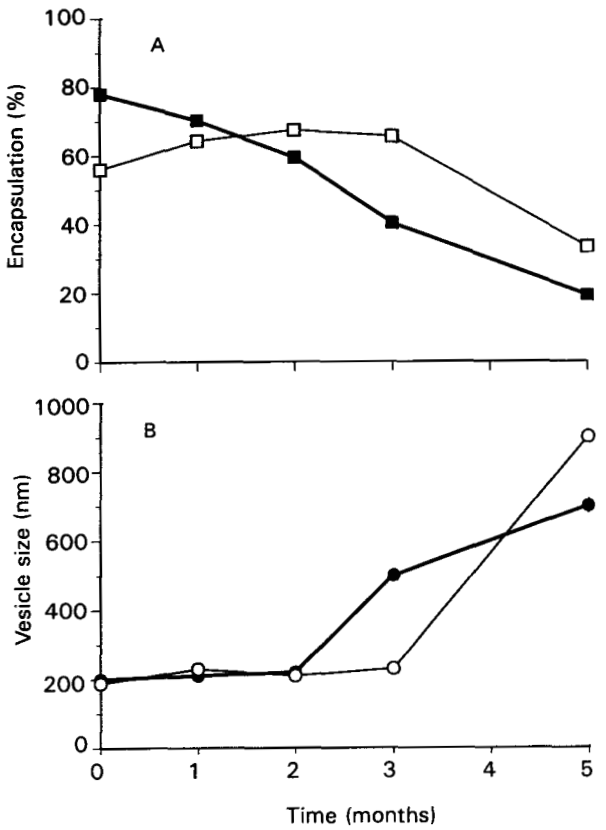

FIG. 4. Stability of a lyophilized liposome preparation by pH-gradient method. Stability samples were stored at 2–8°C. A. Percent encapsulation. B. Vesicle size. □○ Controls, ■● pH gradient.

report (Vemuri et al 1991) which also showed 15–20% encapsulation loss upon lyophilization in the presence of cryoprotectants.

Lyophilized samples were stored in a refrigerator and tested for percent encapsulation and vesicle size every month for a period of five months. The results are shown in Fig. 4. As shown in Fig. 4A, a significant loss in drug encapsulation was noted during five months storage. Encapsulation loss was associated with an increase in size, which is evident in Fig. 4B.

Fig. 5 shows electron micrographs of liposomes before and after the sizing step. The cross-sectional view of an unsized liposome in the right panel shows several layers which is characteristic of a multilamellar vesicle. The vesicles in the left panel are uniform in size and appear to consist of one- or two-layer vesicles. These two micrographs clearly demonstrate that the sizing step in the process made the liposome preparation uniform in size and yielded unilamellar vesicles.

This study had demonstrated that pH gradients can be used to load and enhance the encapsulation of water-soluble drugs into liposomes. Percent encapsulation was as high as 75–80% with the model drug orciprenaline sulphate. The desired encapsulation of near 100% was not achieved. Diafiltration proved to be an efficient processing step in removing the free drug from the external medium. The lyophilization step in the process did not alter the liposome vesicle size, but reduced the percent encapsulation by 15%. Drug leaked rapidly from lyophilized samples. The drug leakage observed was associated with an increase in vesicle size. The change in vesicle size was attributed to the fusion of liposomes evident by a growth in liposome size.

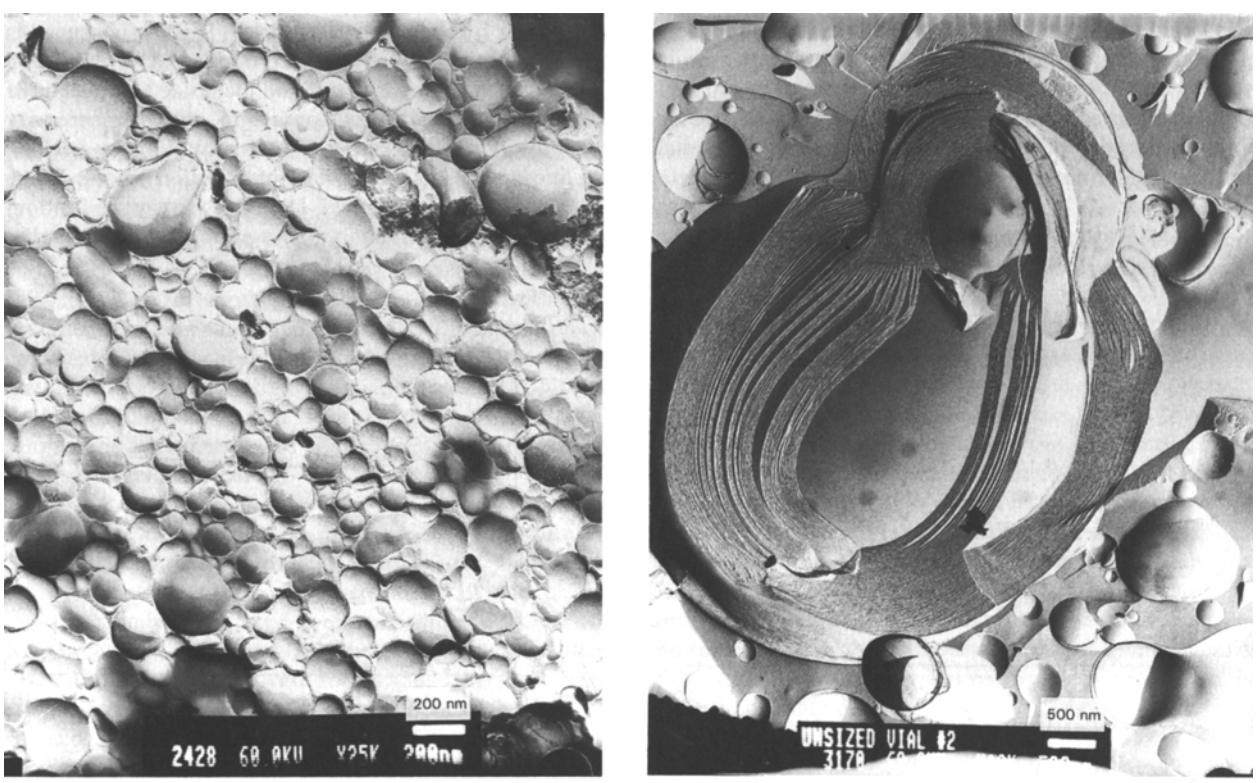

FIG. 5. Freeze-fracture electronmicrographs of liposomes before and after the sizing step. The right panel shows a multilamellar vesicle in an unsized preparation, while the left panel shows uniformly distributed unilamellar vesicles.

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