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Small-volume extrusion apparatus for preparation of large, unilamellar vesicles

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The design and performance of a filter holder which enables convenient preparation of volumes of up to a milliliter of large, unilamellar vesicles formed by extrusion (LUVETs) from multilamellar vesicles (MLVs) are described. The filter holder provides for back-and-forth passage of the sample between two syringes, a design that minimizes filter blockage, eliminates the need to change filters during LUVET preparation and reduces preparation time to a few minutes. Replicas of slam-frozen LUVETs in the electron microscope are unilamellar and reasonably homogeneous with an average diameter close to the pore size of the filters used to extrude them. Extrusion per se does not destabilize the vesicles, which trapped a fluorescent dye only when they were disrupted on freeze-thawing and during the first extrusion when most of the MLVs were apparently converted to LUVETs.

Introduction

Large, unilamellar vesicles produced by extrusion techniques (LUVETs) present various advantages for studies involving model membranes. They are free of organic solvent and detergent; the arrangement of their constituent lipids is at equilibrium and features no destabilizing constraints on the constituent lipids; they are relatively homogeneous in size and structure; they resemble cell membranes in being unilamellar and able to contain relatively large volumes; and they can be prepared quickly and easily from dried lipid in less than 1 h. To extend the applicability of the method of LUVET preparation, pioneered by Olson et al. [1] and made practicable by Hope et al. [2], we have designed and constructed a hand-driven, extrusion apparatus with a capacity of less than a milliliter, which does not require gas under pressure, is easily made at low cost and meets the needs of investigators who use only small amounts of LUVETs. Our extruder has the added advantage over presently used designs of a self-cleaning feature; because the sample passes back and forth through the filter, blockage is greatly reduced. Examination of the vesicles produced with this hand-driven extruder indicated them to be comparable to those produced with a large volume, commercially available apparatus. We have also measured the uptake of fluorescent, aqueous phase marker added to the vesicles at various stages in the extrusion process and thereby afforded some insight into the mechanism of LUVET generation from multilamellar, large vesicles (MLV).

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Abbreviations: LUVET, large, unilamellar vesicle produced by extrusion technique; MLV, large, multilamellar vesicle; MBSE, 0.15 M NaCl/0.01 M Mops (pH 7)/0.1 mM EDTA; Mops, 4-morpholinepropanesulfonic acid.

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Materials and Methods

Preparation of LUVETs

Egg phosphatidylcholine (Avanti Polar Lipids, Alabaster, AL) in chloroform was dried under nitrogen and kept under high vacuum for at least 1 h. The lipid was hydrated at a concentration of 6–30 mM in MBSE and freeze-thawed ten-times in a solid CO_2/ethanol bath to ensure solute equilibration between trapped and bulk solutions [3,4]. The multilamellar vesicles were extruded through polycarbonate filters (Nuclepore, Pleasanton, CA) mounted in the mini-extruder (Fig. 1)

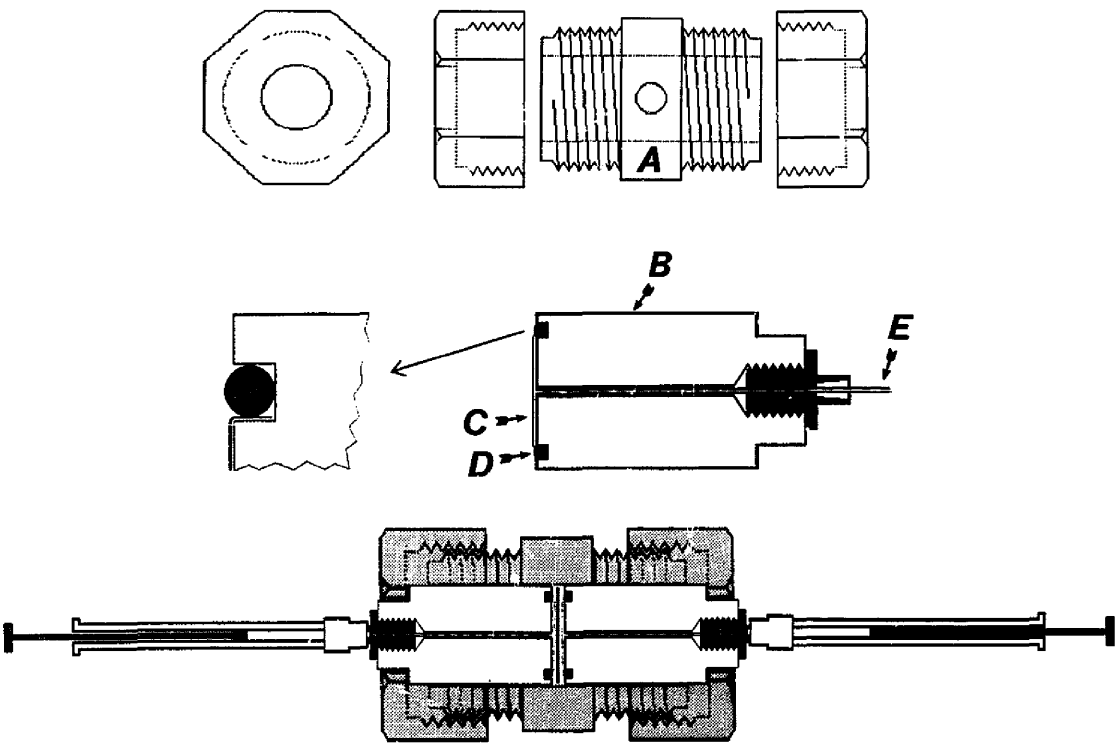

Fig. 1. Extrusion device. The housing (A), with its two end caps, provides for confining and pressing together the membrane supports (B; two required) which have woven nylon screen (40 mesh is adequate) (C) on their surfaces to allow distribution of the suspension over the filter (or two tandem filters) and "O" rings (D) to retain the screen and prevent leakage of the sample. The stainless steel capillary (E) has a narrow bore to minimize hold-up volume. It is a tight press fit into the membrane support. The projecting end of the capillary is rounded and of appropriate length to fit into the teflon inner portion of a Luer lock microsyringe. The black threaded portion is a female Luer Lock bulkhead fitting to provide mechanical support for the syringes. A diameter for the supports (B) of between 0.5 and 1.0 inch is desirable and the filter diameter should be a few thousandths of an inch smaller in diameter than the supports. The size and proportions of the components are not critical. They may be fabricated from inert metal or plastic; nylon is satisfactory for all components except the capillary tubing. To use the extruder, one filter support is placed in the housing, from which one end cap has been removed, with the Luer connector projecting through the other end cap. The filters are placed on the screen surface of the filter support. The second filter support is placed on the other surface of the filters and the support is sealed against the filter by screwing on the second end cap. The assembled device is shown at the bottom of the figure.

fitted with two 0.25 ml Hamilton syringes (Hamilton, Reno, NV). Usually we subjected samples to 19 passes through two filters in tandem as recommended in Ref. 2. In some cases, a single filter was used and the number of passages was reduced by half without markedly different results. An odd number of passages was performed to avoid contamination of the sample by large vesicles which might not have passed through the filter.

Preparation of lipid vesicle replicas for electron microscopy

5 μl of LUVET suspension was pipetted onto a 2 mm slab of gelatin mounted on an aluminum disc (Medvac, St. Louis, MO [5]). The vesicles, free of chemical fixative or cryoprotectant, were rapidly-frozen by dropping the discs onto a liquid nitrogen-cooled copper block wih the bounce-free, Gentleman Jim quick-freeze system (Pelco, Inc.; Tustin, CA [6]). The frozen samples were fractured with a knife in a Cressington CFE 40 freeze-fracture machine (Cressington Scientific, Walford, U.K.) at a vacuum $> 5 \cdot 10^{-7}$ mbar and a temperature of $-135°C$. Immediately after fracture, the specimens were shadowed with platinum/carbon at an angle of 45°. Replicas were reinforced with carbon evaporated obliquely from above, cleaned in chloroform/methanol, commercial bleach and water and examined on uncoated 400-mesh thin bar hexagonal grids at 100 kV in a

JEOL 100 CX Temscan electron microscope. In the case of negative stained preparations, we followed standard procedures [7].

Sizing of vesicles

Vesicle diameters were measured from electron micrographs taken at a magnification of 72000-times the original size. Only replicas of concave pieces of vesicles with a shadow were measured. In one experiment (see Fig. 2) the measured diameters were corrected for non-equatorial fracture of vesicles, the replicas with shadows covering more or less than 50% of their areas. The formula devised and used is

$$r = [(d/2 - x)^2 + d^2/4]^{1/2}.$$

where d is the actual diameter of the vesicle, r is the radius of the crater in the replica that formed over that vesicle and x is the width of the shadowed region of the crater at its midpoint.

Measurement of uptake of external aqueous phase during various stages of LUVET preparation

The extent of communication between the vesicle compartment and the external phase during extrusion was measured according to a previously described assay in which the fluorescent dye, calcein, serves as an aqueous phase volume marker [8,9]. The fluorescence of a suspension of vesicles is determined before and after addition of cobalt ion, which quantitatively quenches the fluorescence of calcein in the external phase but has no effect on that trapped in the vesicles. The fraction of the total fluorescence which is quenched is thus equal to the fraction of the total calcein in the external phase. Since the total volume is easily measured, the volume of the external phase which is internalized is readily calculated. At various stages in the preparation of LUVETS, 2 μl of 10^{-2} M calcein (Hach, Ames, IA), adjusted to pH 7 with NaOH, was added to 0.2 ml of vesicles in MBSE. The vesicle suspension was then processed through one or more subsequent steps in the preparation of LUVETS. One μl of the vesicle suspension at the desired stage of preparation (given in Table II) was diluted in 0.36 ml MBSE and read in a fluorometer before (F_t) and after (F_u) addition of 5–10 mM $CoCl_2$. The excitation monochromator was set at 490 nm, the emission monochromator at 520 nm and band-pass interference filters were used at both positions to eliminate contributions from light scattering. The background fluorescence given by $CoCl_2$-quenched samples in the presence of 1% Triton X-100 (F_b) was subtracted from both measurements. The internal volume that became equilibrated with the external phase (effectively a 'trapped volume', although the total internal volume does not change) was taken to be $[(F_u - F_b)/(F_t - F_b)]$ × (volume of lipid suspension). Lipid was determined by assay of inorganic phosphorus [10] in order to relate internalized volume to amount of lipid, i.e., as μl internalized per μmol lipid.

Results and Discussion

Design of the mini-extruder

The essential features of the extrusion device are (a) support for both sides of the membrane so that time-saving back and forth extrusion can be employed and the filter is self-cleaning, (b) a small hold-up volume so that extrusion of 0.25–0.5 ml samples is possible and (c) ability to withstand the pressures necessary for extrusion without leakage or membrane rupture. These features have been incorporated into the design of the device shown in Fig. 1 *. The filter is pressed between two supports (turned from appropriate rod stock) which contain O rings to prevent leakage. The O rings hold in place pieces of nylon mesh which distribute the sample over the surface of the filter. The supports have connections for syringes and are pressed together by tightening threaded caps on the housing. An inspection hole is drilled through the housing to facilitate centering the filter supports and to check for absence of leaks around the filter during the extrusion process. The housing need be threaded only at one end, but we have found it expeditious to adapt commercially available tubing connectors (tube to tube union), which are threaded on both ends, rather than to machine a housing de novo. The typical tubing connector (available at hardware stores, plumbing shops and industrial supply companies) must be bored out to the diameter of the tubing support. It may also be necessary to modify the end caps slightly to accommodate the protruding portion of the filter support. If the two membrane supports rotate relative to one another during tightening, the filter may tear. This is easily prevented by placing a dowel pin in a keyway drilled along the junction of the membrane support and housing and parallel to the axis of the extruder. Only one side need be modified; the end cap on the side with the pin is that which is tightened. Although a functional extruder can be fabricated from a variety of materials, we have found nylon to be quite satisfactory.

We have used extruders holding 1/2 inch or 3/4 inch diameter filters (punched from bulk filter sheet, Nuclepore). For extrusion through 100 nm pore filters, either size is convenient; however, with 1/2 inch filters, extrusion through 30 nm pore filters requires a prohibitively large amount of muscular effort which is not required for the larger diameter filter.

* We will send detailed drawings and instructions to anyone who includes a self-addressed envelope with his or her request.

The extrusion device was designed to be used primarily with two 250 or 500 μl syringes. The Teflon Luer lock type with Teflon plungers (Hamilton) is satisfactory. The syringes with threaded terminations that we have tested invariably failed under the pressures required for extrusion. It should be appreciated that the pressures generated are significant. A pound of force on the plunger is equivalent to about 100 lb/inch^2 on the sample and one typically applies several pounds of force when using the device. (We have not had a syringe barrel fail, but prudence would call for wearing safety glasses when using the device.) Indeed, the small diameter of the syringe is precisely what makes the device practical. With larger diameter syringes, the average person cannot apply enough force to extrude vesicles through 100 nm pores at a practical rate. Teflon-tipped plungers are essential to avoid leakage between plunger and barrel. The seal between the syringe and the extruder is not based on the usual mating surfaces of the Luer lock connectors. Normal Luer lock connections have rather large dead volumes and they frequently begin to leak after a few months of use due to the compression of the Teflon inner member. Instead, the seal is made by insertion of the stainless steel capillary tubing of the filter support into the Teflon portion of the Luer connection. It is necessary to drill out the orifice of the Teflon inner portion of the Luer lock connector so that its mating with the end of the capillary is a tight push fit. The capillary thus has the dual role of providing a low volume path from the syringe to the filter and a leak-tight connection to the syringe. Capillaries of 1/16 inch o.d. are commonly available with 0.020 or 0.010 inch i.d. With the latter, the total internal volume of the extruder is about 8 μl, so at most, 4 μl escapes filtration. Our capillary was purchased from Small Parts, Miami, FL. The female Luer lock fitting is attached to the extruder primarily to support the syringe; the capillary is only available in soft temper and it would bend without a rigid connection to the extruder. Nylon and metal fittings are available from syringe manufacturers and some tube fitting supply companies. We have used fittings from Ark-Plas Products, Flippin, AR.

Although reusable syringes provide the most secure seal for the extrusion device, we have found that at least one brand of 1 ml, disposable plastic syringes (Terumo), if used only a few times, seal sufficiently well that they may be used to extrude phospholipid vesicles through 100 nm pore membranes at concentrations at least as high as 20 mg/ml.

Properties of the extruded vesicles

Fig. 2 consists of electron micrographs of replicas of LUVETs slam-frozen, fractured and shadowed as described under Materials and Methods. LUVETs in Fig. 2a were extruded through filters with 100 nm pores, whereas LUVETs in Fig. 2b were extruded through filters with 30 nm pores. The LUVETs extruded through filters of both pore sizes in our apparatus are evidently essentially all single-layered, as indicated by the insig-

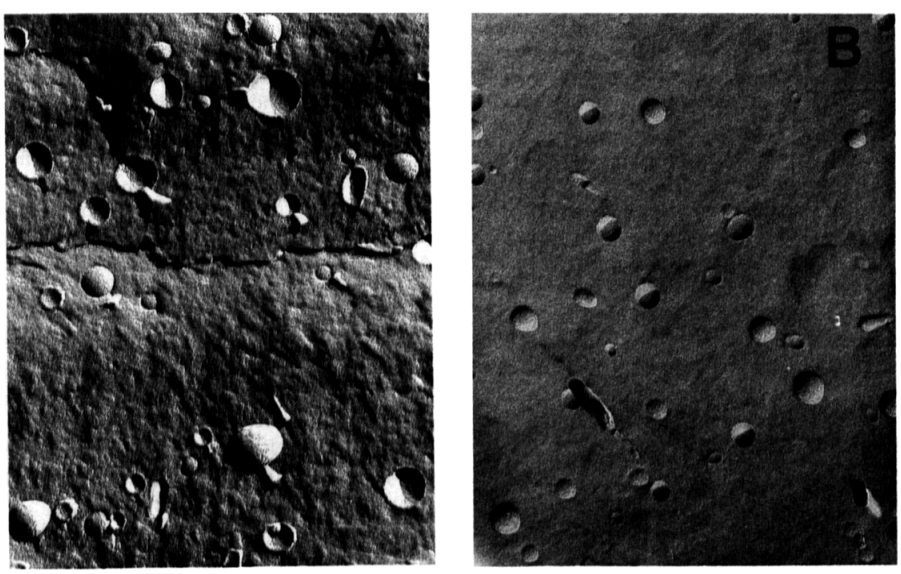

Fig. 2. Electron micrographs of replicas of rapid-frozen extruded vesicles. (A) Vesicles extruded through 100 nm pore filter. (B) Vesicles extruded through 30 nm pore filter. The lipid in both cases was egg phosphatidylcholine suspended in buffered 0.15 M NaCl. The heights of the letters A and B correspond to 120 nm.

nificant fraction of cross-fractured vesicles with inner lamellae. Although the slam-freeze method has not often been used to rapidly freeze aqueous suspensions of lipid vesicles without fixative or cryoprotectant (an exception is Ref. 11, reviewed in Ref. 12), the present results show that the ultrastructural preservation by slam-freezing is at least as good as that obtained with spray- [13–17], plunge- [18,19] and jet-freezing [20,21]. As seen in Fig. 2, well-frozen specimens lacked evidence of ice crystal formation, i.e., the areas between the vesicles were smooth. The near-50% shadowing of most replicas indicates that most vesicles were fractured equatorially. Olson et al. [1] came to the same conclusion on the grounds that the size distributions of their vesicles produced by extrusion and then negatively stained, agreed with those of vesicles similarly produced and then freeze-fractured. Likewise, our comparison of the diameters of freeze-fractured and negative stained (data not shown), extruded vesicles – data for the latter adjusted for their flattened disposition – yielded similar results.

Table I gives the diameters of replicas of vesicles extruded from our apparatus as described under Materials and Methods. The data from two experiments agree well – i.e., 69 and 80 nm from 100 nm pore filters and 47 and 52 nm from 30 nm pore filters. The diameters and standard deviations of vesicles filtered through pores of 100 nm diameter are the same as those reported by Hope et al. [2], who used a large-capacity extruder, now available commercially. Before addressing the disparity between the measured and expected diameters, we should point out that correcting the measured diameters for non-equatorial fracture of the vesicles, as described in Materials and Methods and indicated by asterisks in Table I, had no effect on the average diameters but did have some effect on the standard deviations. This essentially negligible effect is expected since the diameter of even a shadowless replica

TABLE I

Diameters of replicas of extruded vesicles slam-frozen, fractured and shadowed

Each measurement is given as the mean ± S.D. of the number of vesicles given in parentheses. The phosphatidylcholine concentration was 6 mM in Expt. I and 30 mM in Expt. II.

	100 nm pore filter	30 nm pore filter
Expt. I	80.0 ± 24.7 (26)	47.5 ± 12.4 (36)
Expt. II	69.3 ± 38.7 [a] (111)	53.7 ± 19.8 [a] (78)
	69.3 ± 18.8 (111)	52.2 ± 10.2 (78)

[a] These measurements were corrected for non-equatorial fracture of the vesicle, as judged from the shadow extending 50% beyond the radius of the replica, so that the diameter of the replica was less than the diameter of the vesicle. The correction was made according to the equation given in the Materials and Methods.

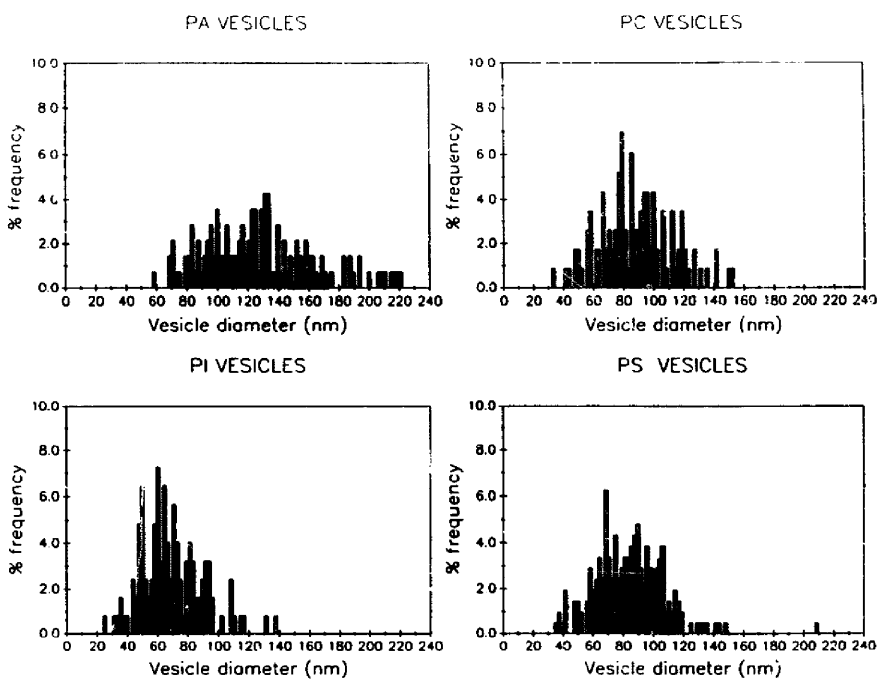

Fig. 3. Size distributions of vesicles of different phospholipids. The size distributions of vesicles composed of phosphatidic acid, phosphatidylserine, phosphatidylinositol and phosphatidylcholine were obtained from electron micrographs of the negatively stained vesicles. The compositions of the vesicles, which were extruded through a filter of 100 nm pore diameter, are indicated in the figure.

should be about $(1/2)^{1/2}$- or 0.7-times the diameter of the original vesicle [22].

Fig. 3 presents histograms of sizes of vesicles of lipids other than phosphatidylcholine, all extruded through filters with 100 nm pore diameters. These vesicles were extruded eleven times and were sized from electron micrographs of negative stained specimens. As may be seen, the diameters of phosphatidylserine and phosphatidylcholine vesicles are distributed around 80 nm, which is very close to that of the phosphatidylcholine vesicles sized by freeze-fracture. The phosphatidylinositol vesicles appear to be somewhat smaller than the phosphatidylcholine vesicles, whereas the phosphatidic acid vesicles are considerably larger. Moreover, the size distribution of the latter is considerably broader than that of any other phospholipid. It is possible that the phosphatidic acid vesicles were affected by the negative stain treatment in a way that the other lipids were not, but we suspect otherwise, since other vesicles (egg phosphatidylcholine extruded through 30 nm pore filters, see above; dipalmitoyl phosphatidylcholine extruded through 100 nm pore filters, see below) are clearly larger than the pores of the filter through which they were extruded. It thus seems likely that the same phenomenon is operative, but the threshold for an effect is lower with phosphatidic acid.

Vesicles may be prepared at temperatures other than ambient by performing the extrusion process either under water of the desired temperature or in a partially closed oven, after allowing thermal equilibration of the extruder with its surroundings. The option of extruding at higher than ambient temperatures may be necessary in the case of lipids which at ambient temperatures are in the gel phase and, therefore, difficult to extrude [23]. We have not sized vesicles of such lipids but we have supplied an extruder to Dr. Barry Lentz of the University of North Carolina, who has done so. According to his results, vesicles of dipalmitoylphosphatidylcholine, extruded above the phase transition temperature through pores of 100 nm diameter, have diameters of 125 nm measured by quasi-elastic light scattering at room temperature. This is nearly the same size as vesicles prepared by Lentz with the commercially available extrusion device used by Nayar et al. [23].

Characteristics of the extrusion process

The transformation of an MLV into a LUVET during extrusion may entail the simple pinching off of vesicles from liposome tubes stretched into the filter pore; however, the process cannot involve the disintegration of tubes into vesicles of the same diameter, for vesicle sizes do not necessarily correspond to the pore diameter. In the case of the egg phosphatidylcholine vesicles shown in Fig. 2, filters with pores of 100 nm diameters gave LUVETs with an average diameter of 75 nm, whereas filters with pores of 30 nm diameters gave LUVETs with an average diameter of 50 nm. This difference is not attributable to a difference in pore depth between the 100 nm and 30 nm pore filters, since filters of both pore sizes are 600 nm thick (Nuclepore). Furthermore, filters of the same pore size can generate vesicles with average diameters smaller or larger than the pore diameters, as occurred with 100 nm pore filters which gave phosphatidylcholine LUVETs with an average diameter of 80 nm but phosphatidic acid LUVETs with an average diameter greater than 100 nm (Fig. 3).

TABLE II

Uptake of calcein at different stages in the preparation of LUVETs

2 μl of 10^{-2} M calcein (pH 7) was added to 0.2 ml egg phosphatidylcholine vesicles in MBSE at the stage in the procedure indicated. Calcein within the LUVETs recovered at the end of the procedure was determined in duplicate by measuring the fluorescence of a 1 μl sample diluted in 0.36 ml MBSE before and after addition of 5–10 mM $CoCl_2$. A background value given by a $CoCl_2$-containing sample in the presence of 1% Triton X-100 was subtracted from both of these measurements. All extrusions were through two 100 nm pore filters. The data are expressed as trapped volumes, i.e., μl trapped per μmol lipid. Lipid was determined by assay of inorganic phosphorus [10].

Calcein added	Internalized volume (μl/μmol lipid)	
	Expt. I	Expt. II
(1) Before freezing-thawing. Vesicles then freeze-thawed and extruded 19 times	2.0	-
(2) After freeze-thawing but before extrusion. Vesicles extruded 19 times	0.9	1.9
(3) After one extrusion. Vesicles extruded 10 times subsequently	-	0.45
(4) After 5 or 10 extrusions. Vesicles extruded 10 times subsequently	0.15	0.31

To determine at which stage in the process of LUVET formation vesicle contents communicate with the external phase, we added the fluorescent dye, calcein, to lipid suspensions at various stages of the extrusion process. The results, expressed as volumes of extravesicular solution internalized per μmol of lipid, are given in Table II. The net gains in uptake of the dye were 1.1 μl/μmol phospholipid due to freeze-thawing (Expt. I, row 1 minus row 2), 1.45 μl/μmol due to the first extrusion (Expt. II, row 2 minus row 3) and 0.14 μl/μmol due to subsequent extrusions (Expt. II, row 3 minus row 4). Data from both experiments indicate that the membranes are not permeable during extrusion per se, since the volumes trapped are negligible when the dye is introduced after, instead of prior to, the first extrusion. Thus, the vesicles become permeable to and trap calcein primarily when the MLVs undergo rupture into smaller vesicles during the first extrusion. The uptake of calcein during freezing and thawing of lipid vesicles has been

observed previously [8]. Incidentally, the trapped volume of 2 μl/μmol for vesicles extruded through 100 nm pore filters (Table II) is that expected for 80 nm diameter vesicles. The close correspondence of this calculated diameter to the measured values (Table I), attests to the unilamellar configuration of extruded vesicles, also indicated by electron microscopy.

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