Vesicles of variable sizes produced by a rapid extrusion procedure

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Previous studies from this laboratory have shown that large unilamellar vesicles can be efficiently produced by extrusion of multilamellar vesicles through polycarbonate filters with a pore size of 100 nm (Hope, M.J., Bally, M.B., Webb, G. and Cullis, P.R. (1985) Biochim. Biophys. Acta 812, 55–65). In this work it is shown that similar procedures can be employed for the production of homogeneously sized unilamellar or plurilamellar vesicles by utilizing filters with pore sizes ranging from 30 to 400 nm. The unilamellarity and trapping efficiencies of these vesicles can be significantly enhanced by freezing and thawing the multilamellar vesicles prior to extrusion. This procedure is particularly applicable when very high lipid concentrations (400 mg/ml) are used, where extrusion of the frozen and thawed multilamellar vesicles through 100 and 400 nm filters results in trapping efficiencies of 56 and 80%, respectively. Freeze-fracture electron microscopy revealed that vesicles produced at these lipid concentrations exhibit size distributions and extent of multilamellar character comparable to systems produced at lower lipid levels. These results indicate that the freeze-thaw and extrusion process is the technique of choice for the production of vesicles of variable sizes and high trapping efficiency.

Introduction

The term 'liposome' was originally introduced [1] to describe aqueous dispersions of multilamellar vesicle (MLV) systems produced by mechanical agitation of an aqueous medium in the presence of a dry lipid film. In current usage it is a generic term for hydrated lipid dispersions which may be large or small and of unilamellar or multilamellar nature. A large number of subclasses of liposomes therefore exist, produced by an equally large variety of techniques. Small unilamellar

vesicles (SUVs) can be produced by sonication [2,3] or French press [4] techniques, large unilamellar vesicles (LUVs) via dilution from organic solvent [5–7], detergent dialysis [8–10] or extrusion of MLVs [11], and multilamellar vesicles with superior hydration and trapping properties can be obtained by techniques involving organic solvents [12] and freeze-thaw [13] procedures.

All of these 'liposomes' have particular advantages and drawbacks, and it is often difficult to determine the optimum procedure for a particular application, as no procedure of general utility has been available. Features required of such a general procedure would be the absence of organic solvents or detergents (which are difficult to remove subsequently), an ability to generate unilamellar or multilamellar vesicles with a variety of well-defined and homogeneous size distributions,

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Abbreviations: MLV, multilamellar vesicle; SUV, small unilamellar vesicle; LUV, large unilamellar vesicle; LUVETs, large unilamellar vesicles produced by extrusion techniques; PC, phosphatidylcholine; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

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an ability to obtain high trapping efficiencies for hydrophilic solutes, and last but not least, a relatively straightforward production procedure. In previous work [11] we have demonstrated that repetitive extrusion of MLV systems through filters with a 100 nm pore size results in the straightforward production of homogeneously sized LUV systems (approx. 90 nm diameter) which can exhibit high trapping efficiencies. We have also demonstrated that freeze-thawing of MLV systems can result in markedly enhanced trapped volumes and trapping efficiencies [13]. In this study we combine the freeze-thaw and extrusion protocols and employ filters with a variety of pore sizes in the range 30–400 nm. We demonstrate that predominantly unilamellar systems with variable but homogeneous size distributions, which exhibit high trapping efficiencies (at least 50%), can be generated employing filters with 200 nm pore size or less. Filters with larger pore sizes give rise to larger systems with higher trapping efficiencies and increasingly multilamellar character. These results indicate that the freeze-thaw extrusion technique more closely satisfies the demands of an optimum protocol for preparing liposomes than previously available procedures.

Materials and Methods

Egg phosphatidylcholine (egg PC) was purified from hen egg yolks according to established procedures and was chromatographically pure. ^{22}Na and [^{14}C]inulin were obtained from New England Nuclear.

Multilamellar vesicles were produced by dispersing dry lipid in 150 mM NaCl, 20 mM Hepes (pH = 7.5) with vortex mixing. The frozen and thawed MLV systems [13] were obtained by freezing the MLVs in liquid nitrogen and thawing in a 40°C water bath, where the freeze-thaw cycle was repeated five times. Extrusion of the MLV or frozen and thawed preparations through two (stacked) polycarbonate filters of the various pore sizes (30–400 nm) was performed employing nitrogen pressures of up to 800 lb/in^2.

^{31}P NMR spectra of egg PC liposomes were obtained employing a Bruker WP-200 spectrometer operating at 81.0 MHz. Free induction decays corresponding to 1000 transients were accu-

mulated utilizing 15 μs 90° radiofrequency pulse, gated proton decoupling and a 20 KHz sweep width. An exponential multiplication corresponding to a 40 Hz line broadening was applied prior to Fourier transformation. Signal intensities were determined by cutting and weighing spectra.

The size distributions of the extruded liposomal systems were determined by freeze-fracture microscopy and quasi-elastic light scattering. Vesicle preparations to be used for freeze-fracture were mixed with glycerol (25% by volume) and frozen in a freon slush. Samples were fractured and replicas obtained employing a Balzers BAF400D apparatus, and micrographs of the replicas were produced using a Phillips' 400 electron microscope. Vesicle size distributions were estimated by measuring the diameter of fractured vesicles exhibiting 50% shadowing according to the procedure of van Venetie et al. [14]. Size distributions were determined by quasi-elastic light scattering analysis, performed utilizing a Nicomp Model 200 Laser Particle Sizer with a 5 mW Helium-Neon Laser at an exciting wavelength of 632.8 nm. Quasi-elastic light scattering, also referred to as dynamic light scattering or photon correlation spectroscopy, employs digital autocorrelation to analyze the fluctuations in scattered light intensity generated by the diffusion of vesicles in solution. The measured diffusion coefficient is used to obtain the average hydrodynamic radius and hence the mean diameter of the vesicles.

Vesicle-trapped volumes were determined as follows: phospholipid vesicles were hydrated and dispersed in the presence of tracer amounts of ^{22}Na or [^{14}C]inulin (1 μCi/ml). Subsequent to the extrusion process the vesicles were diluted to 100 mg/ml (when necessary) and passed down a Sephadex G-50 or Ultrogel (LKB AcA-34) column to remove untrapped ^{22}Na or [^{14}C]inulin, respectively. Aliquots of the vesicle-containing fraction were assayed for lipid phosphorus [15] and monitored for ^{22}Na utilizing a Beckman 8000 gamma counter or [^{14}C]inulin using a Phillips' PW-4700 liquid scintillation counter. Trapped volumes were calculated and expressed as μl of aqueous trapped volume per μmol of phospholipid. Trapping efficiencies were calculated as the dpm/μmol phospholipid after gel filtration divided by the dpm/μmol phospholipid before the gel filtration step.

Nomenclature: as indicated in Results, a variety of unilamellar and multilamellar vesicles of differing sizes can be generated by extrusion of MLVs through polycarbonate filters with different pore sizes. It is convenient to introduce the general term 'VETs' to indicate 'vesicles by extrusion techniques' with a numerical subscript to indicate the pore size employed. Thus a VET_{50} system indicates vesicles extruded through filters with 50 nm pore size, whereas a VET_{400} system indicates extrusion through 400 nm pore size filters.

Results

In previous work [11] we have demonstrated that repetitive extrusion of MLV systems through two stacked polycarbonate filters with 100 nm pore size results in LUV systems (LUVETS) exhibiting a relatively homogeneous size distribution centred about 90 nm diameter. It is clearly of interest to determine whether this technique can be extended to filters with different pore sizes and to ascertain the size and lamellarity of the vesicles produced. Vesicle lamellarity can be conveniently determined employing ^{31}P NMR techniques [11], where the addition of external Mn^{2+} 'quenches' the ^{31}P NMR signal of phospholipids in the outermost monolayer by broadening the resonance beyond detection. Thus, for LUVs, the addition of Mn^{2+} should result in a 50% reduction in the ^{31}P NMR signal intensity. For smaller systems, a reduction of more than 50% would be expected as the amount of phospholipid in the external monolayer is greater than that found in the inner monolayer due to the highly curved nature of the vesicle. Unilamellar vesicles of 50 nm diameter, for example, contain 54% of total lipid in the outermost monolayer assuming a bilayer thickness of 4 nm.

The influence of external Mn^{2+} on the ^{31}P NMR signal intensity of egg PC MLV systems (100 mg/ml) extruded through polycarbonate filters with pore sizes in the range 30 nm to 400 nm is illustrated in Fig. 1A. Employing the nomenclature indicated in Methods, the signal intensity of the VET_{100} systems decrease to 50%, indicating unilamellar character, after eight passes through two stacked filters. Previously [11], only four passes were found to be required to obtain
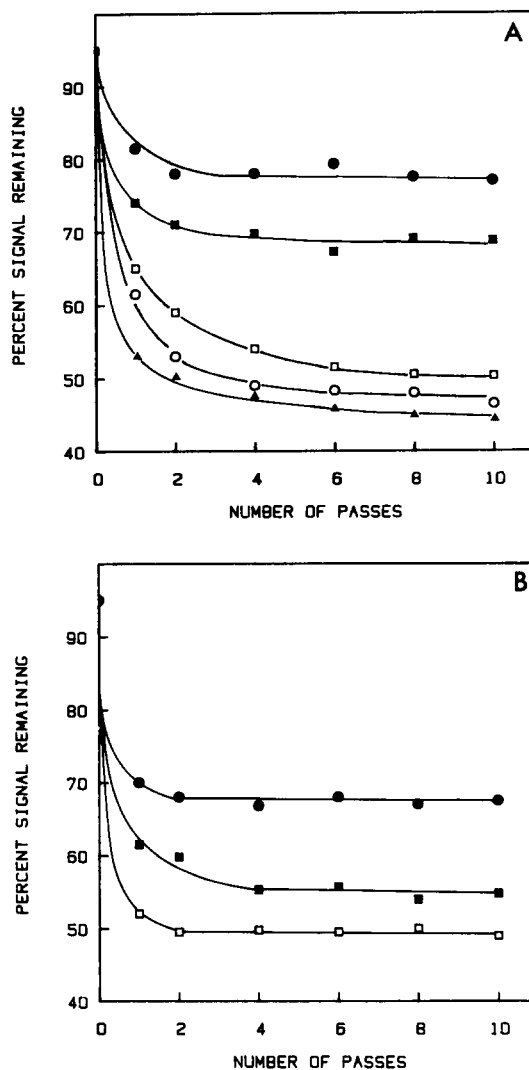

Fig. 1. Percent ^{31}P NMR signal intensity remaining after Mn^{2+} addition for MLVs (A) and frozen and thawed MLVs (B) passed through polycarbonate filters of defined pore sizes. MLVs and frozen and thawed MLVs were prepared at 100 mg egg PC/ml as described in Materials and Methods. ^{31}P NMR signal intensities were determined before and after addition of Mn^{2+} (final concentration 5 mM) for vesicles passed the indicated number of times through 400 (●), 200 (■), 100 (□), 50 (○) and 30 (▲) nm pore size filters.

predominantly unilamellar character, and this difference is presumably due to the higher lipid concentrations employed in the present study (100 mg/ml as opposed to 50 mg/ml or less). The VET_{50} and VET_{30} systems obtained on extrusion

through the 50 and 30 nm pore size filters exhibit residual ^{31}P NMR intensities of 48 and 44%, respectively (after eight passes), consistent with a unilamellar population of smaller vesicles.

The MLV systems extruded through the 200 nm and 400 nm filters retain multilamellar character as the residual ^{31}P NMR signal intensities are 70 and 78%, respectively. This indicates that the proportion of lipid in the outermost bilayer for these systems is 60 and 44%, respectively. If it is assumed that the multilamellar vesicle population contains only two bilayers which are tightly packed (e.g., separated by 10 nm or less), then this would indicate that approx. 20% of the VET$_{200}$ systems are unilamellar and approx. 80% bilamellar. If the average number of lamellae is more than two, the proportion of unilamellar vesicle will be higher. Similar calculations for the VET$_{400}$ systems are consistent with nearly all the vesicles exhibiting bilamellar character, or with a small proportion of unilamellar vesicles which will increase for higher proportions of multilamellar vesicles with three or more bilayers. These sorts of calculations are of interest with regard to our recent observation [13] that MLV systems subjected to freeze-thaw cycles (frozen and thawed MLVs) exhibit significantly larger interlamellar spacings and much larger trapped volumes. It may therefore be expected that VET$_{400}$ and VET$_{200}$ systems prepared from frozen and thawed MLVs should exhibit a somewhat higher unilamellar character due to the reduced fraction of tightly packed lamellae in the frozen and thawed MLV precursors. This appears to be the case as illustrated in Fig. 1B, where it is shown that the residual ^{31}P NMR intensities (after addition of Mn^{2+}) for the VET$_{400}$ and VET$_{200}$ systems are 68 and 56%, respectively. This would correspond to an average of approximately 20% unilamellar and 80% containing two (tightly packed) lamellae for the VET$_{400}$ systems and a substantial population of unilamellar vesicles (at least 75%) for the VET$_{200}$ systems when prepared from frozen and thawed MLVs. It may also be noted that fewer passes are required to generate the unilamellar LUVET$_{100}$ systems from frozen and thawed MLVs than from non-freeze thawed MLVs. Similar effects were observed for the VET$_{50}$ and VET$_{30}$ preparations (results not shown).

Assuming a similar size distribution, the in-

TABLE I

SIZE DISTRIBUTIONS OF EXTRUDED VESICLES

MLVs (100 mg egg PC/ml) were frozen and thawed five times prior to the extrusion process. These frozen and thawed MLVs were then passed 20 times through two (stacked) polycarbonate filters of the indicated pore size. Size distribution analysis of vesicles employing freeze-fracture electron microscopy were completed as described in Materials and Methods. Mean diameters and S.D. were determined by measuring at least 150 vesicles. Size distributions of vesicles employing quasi-elastic light scattering were completed as described in Materials and Methods utilizing Nicomp distribution and Gaussian analysis for mean diameter and standard deviation determinations, respectively. n.d., statistical analysis of the data yielding low fit error could not be accomplished, a mean vesicle diameter value is therefore not reported.

Filter pore size (nm)	Mean diameter ± S.D. (nm)	
	freeze-fracture electron microscopy	quasi-elastic light scattering
400	243 ± 91	n.d.
200	151 ± 36	179.9 ± 55
100	103 ± 20	138.7 ± 36
50	68 ± 19	73.8 ± 18
30	56 ± 17	63.1 ± 17

creased unilamellar character of the VET$_{400}$ and VET$_{200}$ systems prepared from frozen and thawed MLVs suggests that the trapped volume (ex-
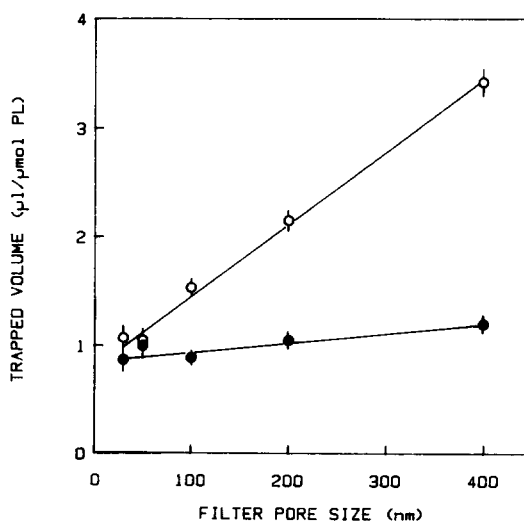

Fig. 2. Aqueous trapped volumes of MLVs (●) and frozen and thawed MLVs (○) passed 20 times through polycarbonate filters of the indicated pore size. Vesicles were prepared at 100 mg lipid/ml of buffer containing [^{14}C]inulin. The error bars represent the S.D. determined from three samples.

pressed as μl trapped/μmol phospholipid) of freeze-thawed, sized vesicles should be significantly increased. That this is the case is illustrated in Fig. 2, where the trapped volume of the vesicles prepared from frozen and thawed MLVs increases from 1 μl/μmol to 3.6 μl/μmol phospholipid as the pore size is increased from 30 to 400 nm. This contrasts strongly with the trapped volumes of the VETs prepared from MLV precursors, which increase by 20% or less for the same range of pore sizes. It is interesting to note that whereas the trapped volumes of the VET_{30} and VET_{50} vesicles are the same for frozen and thawed MLV and MLV precursors, the trapped volumes of the $LUVET_{100}$ systems increase by 50% (to 1.5 μl/μmol) when frozen and thawed MLV's are employed. Comparable results were observed when [14C]inulin was used as the aqueous trap marker

rather than ^{22}Na. Given that the ^{31}P NMR data indicate unilamellar character for frozen and thawed MLV and MLV systems extruded through filters with 100 nm pore size or less, it would not be expected that the trapped volumes observed should be sensitive to the freeze-thaw procedure. It is possible that the effects for the $LUVET_{100}$ vesicles are related to a slightly larger size distribution coupled with complete removal of a very small fraction of multilamellar systems which could account for the enhanced trapped volumes.

It is clearly of interest to correlate the size of the vesicles produced by extrusion with the pore size of the filters employed and the trapped volumes observed. Two convenient techniques for determining vesicle size distributions are freeze-fracture electron microscopy [14] and quasi-elastic light scattering [16], and we employed these proce-
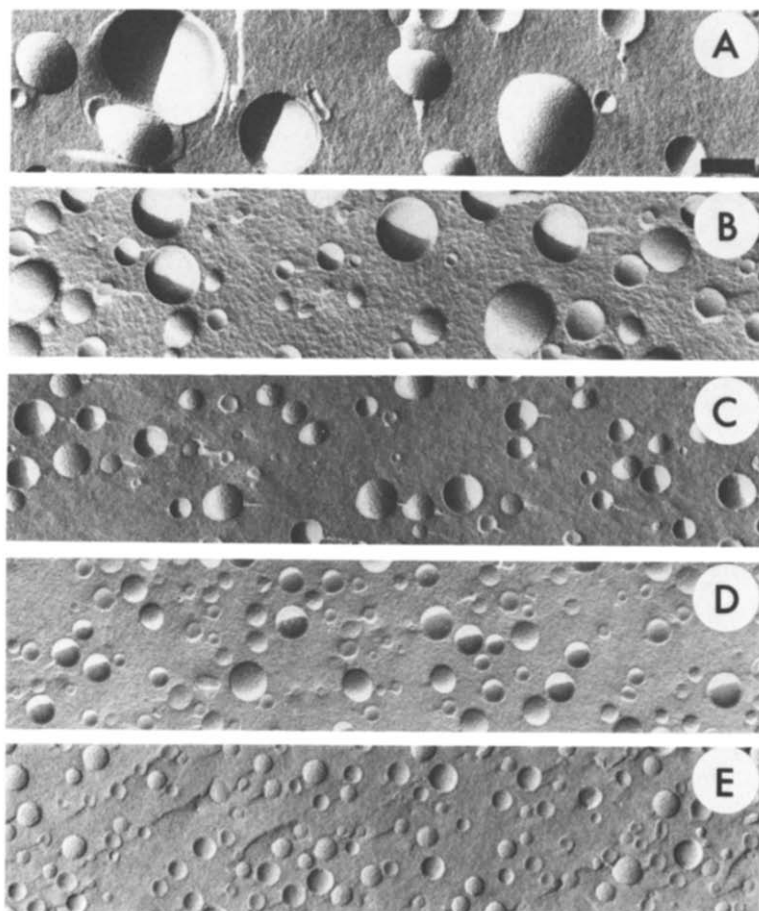

Fig. 3. Freeze-fracture electron micrographs of frozen and thawed MLVs passed 20 times through filters of various pore sizes. Vesicles were prepared (100 mg egg PC/ml) and fractured as described in Materials and Methods. The pore size of the filters employed were 400 (A), 200 (B), 100 (C), 50 (D) and 30 (E) nm. The bar in panel A represents 150 nm and all panels exhibit the same magnification.
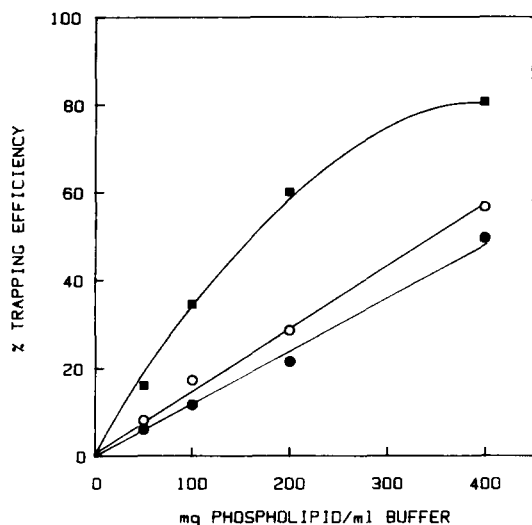
Fig. 4. Percent trapping efficiency of frozen and thawed MLVs passed through 400 (■), 100 (○) and 50 (●) nm pore size filters as a function of phospholipid concentration. Vesicles were prepared in the presence of ^{22}Na as an aqueous marker. The values given represent the amount of ^{22}Na remaining associated with the vesicles after removal of free ^{22}Na relative to the total amount of ^{22}Na in the initial lipid dispersion.

dures for determining the size distributions of the VET systems prepared from frozen and thawed MLV precursors and extruded through filters with 400 nm pore size or less. Freeze-fracture electron micrographs of vesicles prepared at 100 mg/ml lipid concentrations are illustrated in Fig. 3. The corresponding size distributions calculated by the procedure of Van Venetie et al. [14] are given in Table I and reveal relatively homogeneous vesicle populations whose mean sizes are either somewhat smaller than the filter pore size (e.g., the VET_{400} and VET_{200} systems) or of the same size or larger than the filter pore size (e.g., the VET_{50} and VET_{30} systems). The size of the VET_{200} and VET_{400} vesicles may be related to a non-Gaussian size distribution which is slightly skewed toward smaller systems, whereas the sizes of the VET_{50} and VET_{30} vesicles may reflect a limiting size of the VET systems. The absence of cross-fractures in the system extruded through 30, 50, and 100 nm filters is consistent with their unilamellar char-
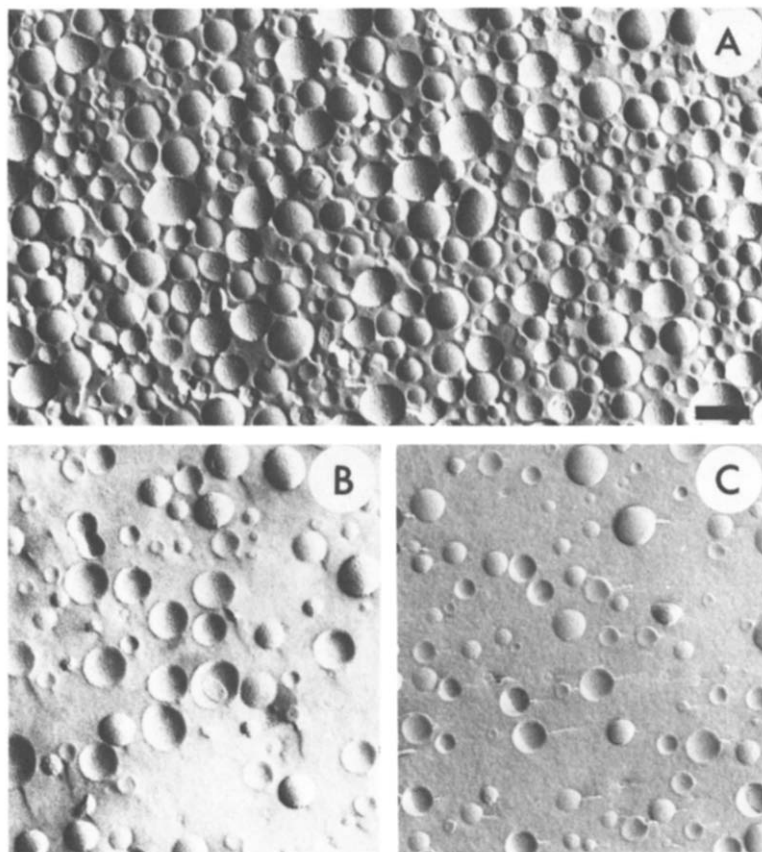

Fig. 5. Freeze-fracture electron micrographs of frozen and thawed MLVs passed through 100 nm pore size filters. Panel A represents vesicles extruded at 400 mg egg PC/ml of aqueous buffer. Panel B represents the vesicles from panel A diluted to 100 mg egg PC/ml. Panel C represents vesicles extruded at 100 mg egg PC/ml. All panels exhibit the same magnification and the bar in panel A represents 150 nm.

acter indicated by ^{31}P NMR. It is interesting to note that the occasional cross-fractures observed for the VET_{200} systems usually reveal three or more lamellae per multilayer vesicle. If this is true of all of the multilayer systems in the VET_{200} preparation, then at least 90% of the vesicles are unilamellar.

The vesicle sizes determined by quasielastic light scattering techniques are also given in Table I. Unimodal size distributions with mean values somewhat larger than those obtained from the freeze-fracture studies are observed. The reasons for the larger sizes reported by the scattering techniques are not understood in detail, but may be related to the type of analysis applied to the raw scattering data.

The results to this stage indicate that homogeneously sized vesicles of LUV or MLV character can be readily generated by extruding MLVs or frozen and thawed MLVs through polycarbonate filters of appropriate pore size. For many applications an important additional parameter concerns the trapping efficiency (defined as the percentage of the total aqueous volume that is entrapped) that can be achieved. In previous work [11] it has been shown that by employing high lipid concentrations (300 mg/ml) trapping efficiencies as high as 35% are attainable employing normal MLVs as precursors to $LUVET_{100}$ systems. We have also observed [13] that the frozen and thawed MLVs can exhibit trapping efficiencies as high as 88% for lipid concentrations of 400 mg/ml. It was therefore reasoned that extrusion of frozen and thawed MLVs prepared at high lipid concentrations should result in high trapping efficiencies for the smaller, sized systems. That this is the case is illustrated in Fig. 4, where trapping efficiencies of 80, 56 and 50% are obtained for frozen and thawed MLVs (prepared at 400 mg/ml) extruded through 400 nm, 100 nm and 50 nm filters, respectively. These trapping efficiencies are clearly remarkable, particularly for the smaller VET_{100} and VET_{50} systems, and it may be suspected that extrusion of high lipid concentrations results in somewhat different systems than at lower (e.g., 100 mg/ml) concentrations. That this is not the case is illustrated in Fig. 5 which presents freeze-fracture micrographs of VET_{100} systems obtained at 400 mg/ml (Fig. 5a) and then diluted to 100 mg/ml

(Fig. 5b). It is clear that the size distribution is similar to that observed for the $LUVET_{100}$ systems prepared at 100 mg/ml (Fig. 5c), and the low frequency of cross-fractures supports a unilamellar character.

Discussion

In previous work we have emphasized the advantages of unilamellar systems produced by extrusion of MLV systems through polycarbonate filters with 100 nm pore size [11]. These advantages include the absence of organic solvents or detergents, a homogeneous size distribution and a straightforward means of preparation. The work presented here indicates such advantages are also exhibited by frozen and thawed MLVs extruded through filters with pore sizes of 30 to 200 nm, with the added features that different vesicle size distributions are available and extremely high trapping efficiencies can be achieved. The extrusion technique would therefore appear to be the protocol of choice for producing unilamellar model membrane systems of size 50 to 180 nm, with applications ranging from permeability studies [17,18] to protein reconstitution [19].

The VET systems exhibiting high trapping efficiencies and controlled lamellarity would also appear to have significant advantages for packaging and possibly delivering biologically active agents in vivo. This applies particularly to the small VET systems and the larger VET systems. Small unilamellar vesicles, for example, exhibit longer lifetimes [20] and enhanced stability [21] in the circulation in comparison to larger LUV or MLV systems, and appear to deliver their contents more readily to 'target' cells [22–24]. The fact that similar features are exhibited by VET_{30} systems (Sommerman, E. and Cullis, P.R., unpublished data) together with the added benefit of efficient production and loading suggests them to be a most appropriate system for targeting protocols. Alternatively, VET systems exhibiting various degrees of multi-lamellarity would be expected to exhibit different leakage rates of entrapped solutes, which have potential in the use of liposomes for controlled release of encapsulated materials.

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