Chapter 8

REDUCTION OF LIPOSOME SIZE AND PREPARATION OF UNILAMELLAR VESICLES BY EXTRUSION TECHNIQUES

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I. INTRODUCTION

The term “liposome extrusion” has become synonymous with the process whereby multilamellar liposomes are forced through filters with defined pore sizes to obtain a liposome population with a mean diameter that reflects the diameter of the filter pore. It is currently one of the most commonly encountered procedures for reducing liposome size and producing unilamellar vesicles on a research scale.

This popularity is the result of several developments in the application of the technology to the field of model membrane research. As originally practiced by Olson et al., extrusion involved the sequential extrusion under low pressures (<80 lb/in.), through a series of filters with decreasing pore size. This procedure was made more convenient by the demonstration by Hope et al. that large unilamellar vesicles (LUV) with a mean diameter of 90 nm could be prepared directly from multilamellar vesicles (MLV) by employing medium pressures (<800 lb/in.) to force MLVs through polycarbonate filters with a pore size of 100 nm. This was a considerable practical advance for preparing LUVs. Techniques such as sonication result only in “limit size” vesicles, which are subject to lipid packing constraints and suffer other limitations including lipid degradation, heavy metal contamination, and limited trapping efficiencies. Reverse-phase evaporation (REV) procedures, which involved dilution from organic solvents such as ether or ethanol, or the formation of aqueous/organic emulsions followed by solvent evaporation, can produce LUVs with large trapped volumes and improved trapping efficiencies. However, these methods are restricted by lipid solubility in solvent or solvent mixtures: moreover, removal of residual solvent can be tedious. Detergent dialysis techniques are also subject to similar practical difficulties associated with lipid solubility and complete removal of detergent.

The advantages of the extrusion technique are that it can be applied to a wide variety of lipid species and mixtures, works directly from MLVs, and is rapid (preparation time in the order of 10 min). Problems related to removal of organic solvents or detergents from final preparations are eliminated, and it is possible to make homogeneous populations of LUVs in the size range of 40 to 150 nm. In addition, the popularity of extrusion as a preparative procedure for unilamellar vesicles can also be attributed to the low-cost equipment required.

II. LIPOSOME EXTRUSION

Multilamellar liposomes usually exhibit broad size distributions ranging from 0.5 to 10 µm in diameter. The degree of lamellarity also varies and is dependent upon the method of hydration and lipid composition. The appli-
cation of multilamellar liposomes to many areas of model membrane research is restricted by a number of factors. As discussed in detail elsewhere. For example, only a small proportion of the total lipid for a highly multilamellar system will be exposed to the external medium (< 10%), compared to a large unilamellar system in which 50% of the lipid will be located in the outer monolayer. Other limitations include the large diameter of MLVs, the heterogeneity of size distribution, multiple internal compartments, low trap volumes, and inconsistencies from preparation to preparation. Extrusion is one of several techniques developed to overcome some of these problems.

Using moderate pressures, a liquid crystalline MLV suspension can be forced through filters with defined pore sizes. As the concentric layers of the multilamellar liposome deform to pass through the pore, a breaking and resealing of the membranes occurs. If a liposome preparation is repeatedly cycled through a filter, this process gives rise to a liposome population with a mean diameter that reflects that of the filter pore. Consequently, extrusion is a rapid and convenient way to decrease the size heterogeneity exhibited by most MLV preparations. The heterogeneity of liposome size (as determined by laser-based quasi-elastic light scattering, for example) decreases as the filter pore size decreases. Therefore, a more homogeneous size distribution is obtained for liposomes sized through smaller pore diameters.

Extrusion can be performed using a hand-held syringe fitted with a standard sterilization filter holder. Using this technique, a dilute suspension of liposomes (composed of liquid crystalline lipid) can be passed sequentially through filters of decreasing pore size. This method, however, is limited by the back pressure that can be tolerated by the syringe and filter holder, as well as the pressure that can be applied manually. Generally, phospholipid concentrations have to be less than 20 mg/ml. and the filter pore diameter greater than 200 nm in order to comfortably extrude liposomes manually. Moreover, in order to pass a preparation through a filter with a 200-nm pore size, it must first be sequentially sized through filters with larger pores. Even then, back pressure is too high to further process the liposome suspension through pores with diameters of 100 nm and less. These limitations apply to a typical laboratory preparation of 1 to 5 ml of liposomes using a 10-ml plastic syringe and Luer-lok filter holder containing a 25-mm-diameter polycarbonate filter with straight-through pores, such as the type supplied by Nuclepore. Of course, customized devices can be envisaged that would enable the manual pressure applied to a plunger to be magnified at the filter surface, allowing for much higher extrusion pressures. However, the surface area of filter and volume of sample usually have to be proportionately decreased and, thus, become limiting factors.

Another approach taken by some researchers is to employ low-pressure devices (normally limited to a maximum operating pressure of 100 lb/in. *) with operating volumes in the range of 1 to 10 ml or steel filter holders.
designed for in-line sterilization applications and capable of operating at higher pressures. A drawback associated with this type of equipment is the inconvenience experienced in cycling a liposome preparation through the filters, which is a necessary step in getting a homogeneous size distribution.\textsuperscript{3,4,10} Other laboratories have custom-made filter holders that are better suited to liposomal extrusion; some of these devices are now commercially available. An example is shown in Figure 1. The extruder is a stainless steel filter holder which can operate up to pressures of 800 lb/in.\textsuperscript{2} A quick-fit sample port assembly allows for the rapid and convenient cycling of preparations through the filter holder. The operating pressure range of 100 to 800 lb/in. means that the sequential use of filters to reduce back pressure is not necessary, and large multilamellar systems can be extruded directly through filter pore sizes as small as 30 nm. The equipment shown in Figure 1 is also fitted with a water-jacketed sample holding barrel which enables the extrusion of lipids that have gel-liquid crystalline phase transitions that are above room temperature (see Section IV of this chapter). Subsequent data described and shown in this article have been obtained using filtration equipment similar to that shown in Figure 1.

A variety of filters suitable for reducing the mean diameter of liposome preparations are available from scientific suppliers. The most commonly used are standard polycarbonate filters (with straight-through pores). Other filter materials can be used, but the polycarbonate type has proven to be reliable, inert, durable, and easy to apply to filter supports without damage. Pore density influences the back pressure encountered during extrusion. Our experience has been that there is very little variation between filters from the same supplier; consequently, extrusion conditions are generally reproducible for the same lipid composition and buffer. Another type of filter often used for sterilization and filtration is the tortuous path type. These differ from the straight-through pore previously described in that the pores trace a tortuous path through the filter membrane. The pore diameter is not as well defined as the straight-through type, and back pressures tend to be higher when using these filters for liposome extrusion. However, adequate size reduction can still be achieved.

III. SIZE REDUCTION AND UNILAMELLAR VESICLES

As we have indicated in the previous sections, to obtain a narrow size distribution, it is necessary to cycle a liposome suspension several times through the filter membrane. This is indirectly demonstrated in Figure 2, in which we present data on the lamellarity of a liposome preparation repeatedly passed through a variety of pore sizes. One of the most accurate and straightforward procedures for determining the lamellarity of phospholipid dispersions is to use \textsuperscript{1P}-nuclear magnetic resonance (NMR) to monitor the phospholipid
FIGURE 1. This commercially available filter device (Lipex Biomembranes Inc., Vancouver, B.C.) is more suitable for liposome extrusion than most similar equipment because of the wide pressure range at which it can operate, the temperature-controlled sample holder, and the quick-release sample pore assembly that allows for rapid recycling.

phosphorus signal intensity. In particular, adding an impermeable paramagnetic or broadening reagent to the external medium will decrease the intensity of the initial $^{31}$P-NMR signal by an amount proportional to the fraction of lipid exposed to the external medium. The data shown in Figure 2 demonstrate that liposomal structure changes dramatically in the first five passes of a liposomal suspension through two (stacked) polycarbonate filters, but very little change can be detected between five to ten passes. This is consistent with a rapid size reduction in the first few extrusion cycles, reducing lamellarity and increasing the proportion of lipid exposed to the external medium. As the liposome diameter approaches that of the filter pore, less membrane
FIGURE 2. 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. $^{31}$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. (From Mayer L. D. et al.. Biochim. Biophys. Acta. 858.161.1986. With permission.)

deformation must occur to allow passage through the filter. After five passes, the majority of liposomes pass through without any change in diameter. This is confirmed by results obtained if quasi-elastic light scattering is employed.
to monitor mean diameter and size distribution as a function of the number of repetitive extrusions (data not shown).

Unilamellar vesicles are the most popular model membrane systems. The well-defined aqueous compartment enclosed by a single bilayer enables their use in a wide range of applications. Repeated extrusion of MLVs through two stacked polycarbonate filters of 100 nm pore size results in a homogeneous population of LUVs with a mean diameter of approximately 90 nm. We have employed a combination of \(^{31}\)P-NMR, freeze-fracture electron microscopy, and trapped volume measurements to characterize extruded liposome preparations to determine the conditions that will give rise to a population of largely unilamellar vesicles. \(\ldots\) The signal intensity data shown in Figure 2A reveal that only after multiple passes through pore sizes of 100 nm or less does the proportion of lipid exposed to the external medium approach 50%. It can be calculated that the proportion of lipid in the external monolayer of a unilamellar vesicle with a diameter of 100 nm will be approximately 50%. This fraction will increase as the vesicle diameter decreases because of the increasing ratio of external to internal membrane surface areas. This is observed for liposomes sized through 50- and 30-nm pore sizes (Figure 2A).

Subjecting MLVs to freeze-thaw cycles increases the proportion of unilamellar vesicles in a particular preparation. We estimate that as much as 90% of vesicles passed through a filter with a pore size of 200 nm are unilamellar if prepared from FATMLVs frozen and thawed multilamellar vesicles (FATMLVs). \(^{4,10}\) Freeze-fracture electron microscopy gives a more qualitative indication of lamellarity than \(^{31}\)P-NMR signal intensity measurements. This technique provides a unique view of internal lamellae when cross-fracturing occurs. Figure 3A is a freeze-fracture micrograph of an egg phosphatidylcholine (PC) multilamellar liposome which has cross-fractured, thus demonstrating the close apposition and large number of internal bilayers associated with a typical MLV. Dramatic structural disruption occurs during repeated freezing and thawing (Figure 3C). Vesicles within vesicles and vesicles between lamellae are commonly observed. This type of morphology is not seen in the precursor MLVs and is presumably due to physical disruption of lamellar structure, possibly by ice crystals formed during the freezing process. This is supported by the observation that addition of cryoprotectants prior to freezing-thawing inhibits the morphology change (Figure 3B). The improved swelling associated with FATMLVs is reflected by a large increase in trapped volume for these systems. \(^{2,11}\) The disruption of internal lamellae and the increased separation of bilayers caused by internal vesiculation during the freeze-thaw process results in a greater proportion of unilamellar vesicles produced when FATMLVs are extruded. Figure 2B shows that the mean diameter of extruded FATMLVs reaches pore diameter in a fewer number of passes than normal MLVs (Figure 2A). and that FATMLVs sized through a 200-nm-pore size filter exhibit a much greater proportion of lipid exposed to
FIGURE 5. Freeze-fracture electron micrographs of FATML's passed through 100-nm-pore size filters. (A) Vesicles extruded at 400 mg egg PC ml of aqueous buffer; (B) vesicles from (A) diluted to 100 mg lipid/ml; (c) vesicles extruded at 100 mg lipid/ml. All micrographs are at the same magnification, and the bar = 150 nm. (From Hope, M. J. et al., Chem. Phys. Lipids. 40, 189, 1986. With permission.)

often in mixtures with cholesterol (Chol). In our experience, all liquid-crystalline, bilayer-forming phospholipids (in isolation or as complex mixtures) are amenable to the extrusion technique. The rate of extrusion, or the operational pressure required to force liposomes through filters, varies with charge, acyl composition, pH, ionic strength, and the presence of interacting ions such as $\text{Ca}^{2+}$ or $\text{Mg}^{2+}$. However, these variations are not usually significant and do not prevent extrusion.

Perhaps the most important compositional factor in liposome extrusion is the gel-liquid crystalline phase transition temperature (Tc) of the membrane.
viscosity above $T_c$. However, when saturated systems are extruded (above $T_c$), size reduction and the formation of unilamellar vesicles proceeds normally, with some exceptions.

First, the freeze and thaw protocol described in the previous section, in which MLV swelling is improved by repeatedly freezing and thawing a liposome preparation, is only effective if the sample is thawed at temperatures above its $T_c$. This is demonstrated by the trapped volume data presented in Figure 7. MLVs composed of DSPC exhibit a typical trapped volume for a multilamellar system composed of PC. However, the trapped volume of this system only increases when the liposomes are thawed at 65°C. If thawing is carried out at 37°C, repeated freezing and thawing has no effect (Figure 7A). In the presence of cholesterol, however, the increase in trapped volume (which correlates with the morphological changes shown in Figure 3C) is not temperature dependent. A second point of note when extruding long-chain unsaturated liposomes is that the sized systems are often unstable when cooled below $T_c$. For example, limit-size vesicles produced by extrusion of the longer chain PCs (DSPC and diarachidoyl phosphatidylcholine. DAPC) through 30-nm-pore size filters are metastable. These vesicles tend to aggregate and fuse when incubated at 4 or 20°C. This is likely due to the gel-liquid-crystalline phase transition which is associated with a large decrease in molecular surface area as lipid enters the gel state. This reduced surface area (perhaps as much as 40 to 50%) may be expected to lead to vesicle instability, particularly for limit-size vesicles. It might also be responsible for the abnormal freeze-fracture morphology observed for DSPC systems. As shown in Figure 8, angular fracture planes are observed in DSPC vesicles (Figure 8a to c), but not in DSPC/Chol vesicles (Figure 8d to f). The absence of angular fracture planes and the increased stability observed for DSPC/Chol vesicles is consistent with the ability of cholesterol to prevent the phospholipid from forming a cooperative, all-trans, gel-state configuration, thus reducing any changes in surface area as the temperature is decreased.

For obvious practical purposes, extrusion of saturated systems is limited to lipids with a $T_c$ below 100°C. We have successfully extruded PCs with chain lengths ranging from 14 to 22 carbons, the latter (dibehenoyl phosphatidylcholine) is extruded at 100°C (unpublished observation). Because of the high viscosity associated with the membranes of long-chain saturated lipids, especially if extrusion occurs at or near the $T_c$, back pressure tends to be high and extrusion rates slow.

So far, we have restricted our discussion to phospholipid bilayer systems. Unsaturated phosphatidylethanolamines (and other lipids under specific conditions) do not adopt the bilayer configuration, but prefer an inverted macroscopic organization such as the hexagonal $H_{II}$ phase. Lipid in this phase does not form vesicular structures; consequently, even when this type of lipid
FIGURE 7. Trap volume measurements of DSPC (A) and DSPC/Chol(55:45 mole ratio); (B) MLVs frozen and thawed from 37°C (○) or 65°C (■). DSPC and DSPC/Chol MLVs (40 mg/ml) were prepared in 150 mM NaCl, 20 mM HEPES buffer (pH 7.4) containing $[^{3}H]$inulin ($1 \mu$Ci/ml). The MLVs were subjected to freeze-thaw cycles employing liquid N$_2$ and 37°C and 65°C before each freezing cycle. Untrapped $[^{3}H]$inulin was removed by washing the MLVs in $[^{3}H]$inulin-free buffer and centrifugation at 12000 x g for 15 min. This sup was repeated three times to ensure that all the untrapped $[^{3}H]$inulin was removed. Aliquots of the MLV pellets were then assayed for radioactivity and lipid phosphorus. (From Nayar, R. et al., Biochim Biophys. Acta. 986, 200, 1989. With permission.)

structure is extruded. It rapidly aggregates to reform a bulk nonbilayer phase (results not shown). Of more interest is the extrusion of lipids other than phospholipids. Bilayer-forming mixtures of galactosyl and glucosyl lipids have been extruded, as have mixtures of phospholipid and neutral lipids such as diglycerides, aiglycerides, and cholesterol esters (unpublished observations).
FIGURE 8. Freeze-fracture electron micrographs of DSPC and DSPC/Chol (55:45) DSPC MLVs were sequentially extruded ten times through 100-, 50-, and 30-nm pore size polycarbonate filters at 6X. Freeze-fracture was performed on these samples in the presence of 25% glycerol using standard procedures. All panels exhibit the same magnification: the bar in panel (f) represents 200 nm. (From Nayar, R. et al., Biochim. Biophys. Acta, 986, 203, 1989. With permission.)

V. APPLICATIONS

Of the techniques available for producing unilamellar vesicles, extrusion is compatible with most applications. Liposomal systems are widely used, from industrial food technology, 16 cancer chemotherapy, and the cosmetics industry, to every facet of basic research into membrane structure and function.
Conceptually, extrusion can be easily scaled up to manufacture liposomes in large quantities for industrial and medical applications. The simplicity of the process means that complex equipment is not needed and sterility can be maintained. For example, research-scale equipment (Figure 1) can be sterilized, depyrogenated, and operated in a sterile environment for drug delivery research. A scaled-up extrusion process can be accomplished in a number of ways, but the two most straightforward designs either use inert gas pressure, similar to the research-scale equipment described earlier, or use a pump to drive liposome suspensions through in-line filter holders.

Although extrusion is limited to producing unilamellar vesicles in the diameter range of 40 to 150 nm, this is a most useful mode! membrane size. For instance, 100-nm LUVs appear to have the optimum diameter for drug delivery to cells in vitro. Such vesicles readily pass down gel filtration columns without clogging, enabling the rapid exchange of external buffers. This property, coupled with the rapid preparation time, is particularly useful in permeability studies or research in which large numbers of vesicle preparations are required. It is quite possible to make as many as 100 separate vesicle preparations in a day, which is virtually impossible by any other technique. The ability to prepare vesicles with a variety of well-defined diameters in a reproducible manner is also useful and enables research into the effects of bilayer curvature on various membrane properties as studies on vesicle interactions with cell or viral surfaces and intracellular organelles.

Extruded vesicles have also been successfully employed in the study of lipid-protein interactions. Madden has used extrusion to size vesicles reconstituted with cytochrome oxidase. This procedure is particularly interesting because extrusion was carried out in the presence of detergent before final dialysis to obtain reconstituted systems with well-defined diameters. Others have taken advantage of the reproducibility of extrusion to prepare vesicular substrates for lipid enzyme studies.

VI. SUMMARY

Liposome extrusion has become one of the most popular procedures for making unilamellar vesicles. It is not restricted by lipid solubility or lipid composition. Its low cost and flexibility mean that it is suitable for a wide range of applications. The simplicity of the process suggests that scale-up for industrial use will be straightforward. At the research level, standard laboratory filtration equipment can often be adapted to enable liposome extrusion. Alternatively, low-cost devices can be purchased that are better suited to recycling samples through filters necessary to achieve optimal sizing.
REFERENCES