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LARGE VESICLE CONTAMINATION IN SMALL, UNILAMELLAR VESICLES

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Summary

Small, unilamellar phospholipid vesicles have been prepared using a new, high-powdered cup sonifier that avoids contact of the sample with a titanium probe. These vesicles have been characterized by gel filtration chromatography both before and after fractionation by high-speed centrifugation. Plots of the turbidity of centrifuged vesicles between 300 and 650 nm against the reciprocal fourth power of the scattering wavelength were linear with zero intercepts (extrapolated to infinite wavelength). In the presence of minute quantities of large, multilamellar vesicles, these plots remained linear but had intercepts quantitatively proportional to the amount of contaminating large vesicles. Since this measurement requires only a standard spectrophotometer and very small quantities of lipid, this method is suggested as a useful assay for determining contamination of small vesicle preparations by large vesicles. Two applications of this method as well as a practical limitation are discussed.

Introduction

The use of small, unilamellar phospholipid vesicles in biochemistry has increased considerably in recent years both because of their potential application as vehicles for drug delivery [1] and because of their use as model membranes [2]. The preparation of well-defined populations of these vesicles has recently been made much more convenient by the introduction of a high-speed

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Abbreviations: C_{24} sphingomyelin, DL-erythro-*N*-lignoceryl sphingosine phosphorylcholine; $DC_{15}PG$, 1,2-dipentadecanoyl-3-*sn*-phosphatidylglycerol; Tes, 2-{[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino} ethanesulfonic acid.

centrifugation technique for removal of large vesicle species as demonstrated by autocorrelation light scattering and gel filtration chromatography [3]. Unfortunately, autocorrelation light scattering is time consuming and requires expensive instrumentation of limited availability. In addition, gel chromatography requires fairly large quantities of lipid and subjects the sample to contamination on the column and to dilution during the column run. Thus, neither of these techniques is particularly useful for routine monitoring of small, unilamellar vesicle preparation intactness.

We describe here a simple method, using a standard spectrophotometer, for quantifying the amount of large, multilamellar vesicles in preparations of the small vesivles. Since many experimental treatments of small, unilamellar vesicles (e.g., storage below the phospholipid phase transition [4] and exposure of charged vesicles to multivalent cations [5]) produce larger structures, this method provides a quick assessment of the stability of a small, unilamellar vesicle preparation during experimental manipulations.

Finally, we outline here a new sonication procedure and characterize the resultant vesicle populations. This procedure uses the Heat Systems Cup Horn Sonicator in order to avoid exposure of the lipid to a titanium probe, thereby eliminating contamination by titanium. For this reason, it would seem indispensable for the sonication of charged lipids or of low-pH samples. Unfortunately, our initial experience with this new instrumentation, as well as the experience of several other investigators (Barenholz, Y., Pagano, R. and Litman, B., personal communications), resulted in very poor production of small, unilamellar vesicles. For this reason, we feel it important to report the conditions we have found appropriate for the efficient production of small vesicles with this device.

Methods and Materials

Hen egg lecithin as well as 1,2-dimyristoyl-3-*sn*-phosphatidylcholine and 1,2-dipalmitoyl-3-*sn*-phosphatidylcholine were prepared as previously described

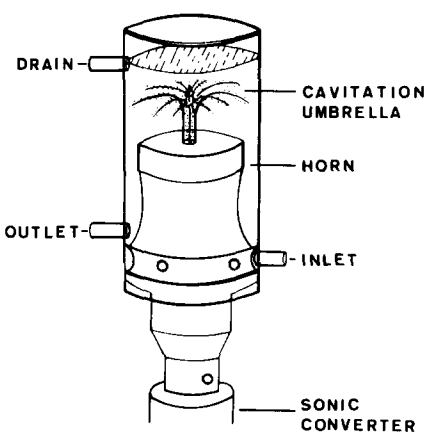


Fig. 1. Cup Horn Sonicator (Heat Systems-Ultrasonics, Inc., Plainview, NY) illustrating the proper umbrella-shaped pattern of cavitation within the cup.

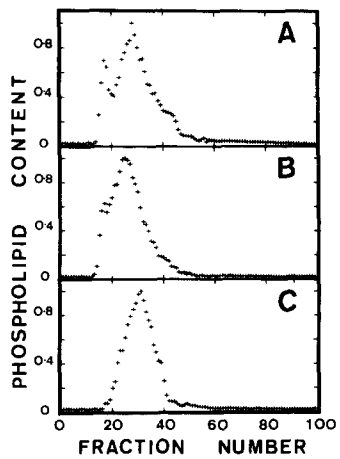
[6]. C_{24} sphingomyelin (DL-erythro-*N*-lignoceryl sphingosine phosphorylcholine) was prepared, purified, and donated by Dr. Y. Barenholz. Phosphatidylglycerol (1,2-dipentadecanoyl-3-*sn*-phosphatidylglycerol, $DC_{15}PG$) was purchased from Avanti Biochemical (Birmingham, Alabama) and purified by recrystallization from acetone [6].

The large vesicles were prepared at temperatures above relevant lipid phase transitions by the method of Bangham [7] with the refinements of Schwartz and McConnell [8]. The small vesicles were prepared by sonication with a Heat Systems W350 Sonicator equipped with a cup horn (see Fig. 1). Water was circulated through the cup horn from an external thermoregulated bath. All sonications were carried out at a temperature at least 8°C above the phase transition of the lipid to be dispersed. Lipids were dried onto the interior surface of thin-walled, glass ampoules (A.H. Thomas, 1088-D44) from a chloroform solution by means of a stream of argon and the resulting thin film further dried in vacuo for 6—12 h. Buffer was added directly to the ampoule at the sonication temperature and the sample was focussed in the cavitation 'umbrella' of the cup horn (see Fig. 1). The power of the instrument was adjusted for optimal operation (setting of 3—4 on our instrument) and the sample was sonicated for three times longer than necessary to achieve visual clarity (usually 45—60 min total on our instrument). A highly homogeneous vesicle population was obtained by centrifuging the sonicate at 168 000 × *g* for 1 h, as described by Barenholz et al. [3].

Spectrophotometric measurements were performed with a GCA/McPherson Series 700 Modular System equipped with a temperature-controlled cell holder in order to maintain the vesicles above their phase transition. Corroborating results were obtained with the Hitachi Model 100-20 and Beckman Model 25 spectrophotometers. The precise concentrations of all the vesicle suspensions studied were determined within ±2% by a modification of the phosphate assay of Chen et al. [9]. The absorbance of each sample was recorded against a buffer blank at wavelength intervals from 250 to 600 nm. The data was plotted during the course of measurement by means of a Hewlett Packard 9815 S calculator equipped with a 7225A plotter. This evaluation of the small, unilamellar vesicle sample required approx. 10—15 min, after which the sample could be returned for use in other experiments.

Results and Discussion

We have attempted to determine the conditions that would optimize the production of small, unilamellar vesicles using the Cup Horn sonication technique. We have identified three crucial parameters: bath fluid composition; cross-sectional area of the sonication vessel; and focussing of the sample in the cavitation 'umbrella' (see Fig. 1). In the temperature-controlled mode, the Heat Systems cup horn uses the fluid from an external circulating bath as a medium for transmitting sonic energy from the horn to the sample vial. Bath fluids containing ethylene glycol (50, 33, or 25%) or other surface-tension lowering agents (e.g., detergents) resulted in greatly reduced sonication efficiency. Fortunately, we have found that the sample temperature can be maintained below 4°C (adequate for most applications) using only pure water as coolant. The



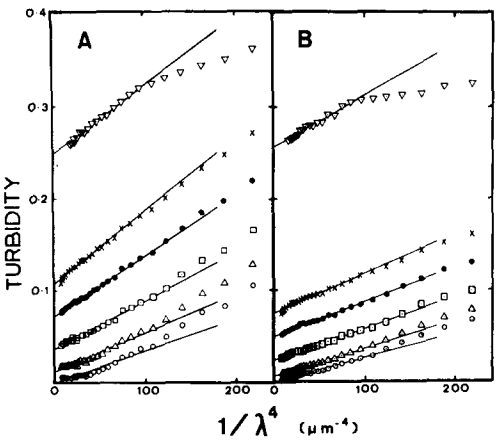


Fig. 2. Gel permeation column profiles of egg phosphatidylcholine vesicles sonified for 1 h with the Cup Horn, A; or with the titanium probe for six 1-min pulses interspersed with 1-min pauses, B. These samples were microfuged for 15 min at 15 000 × *g* before application to the column. The profile in C was obtained by centrifuging the titanium probe-sonicated sample for 60 min at 168 000 × *g* in a Beckman Ti 50 rotor [3] before application to the column. An identical profile was obtained when the Cup Horn-sonicated sample was similarly centrifuged. The ordinate in these figures represents relative phospholipid content normalized to the peak fraction of each profile. A 0.9 cm Glenco column was packed with Sepharose CL 4B and run with an upward flow driven by an infusion pump. The void volume and total volume were at fractions 17 and 43, respectively. Samples were prepared and column runs made with pH 7.8 Tes buffer (0.01 M Tes/0.1 M NaCl/0.02% NaN_3) at 45°C.

Fig. 3. Turbidity vs. $1/\lambda^4$ measured at 25°C for egg lecithin, A; and at 47°C for 1,2-dipalmitoyl-3-*sn*-phosphatidylcholine, B. Symbols: ○, pure small, unilamellar vesicles; ▽, pure large, unilamellar vesicles. Additions of the following amounts of appropriate large, unilamellar vesicles were made to 3 ml of 0.5 mM small, unilamellar vesicles; △, 12.5 nmol; □, 37.5 nmol; •, 75 nmol; ×, 125 nmol. Included are least-squares linear regression fits to the data from 320 to 560 nm.

second parameter found to affect the small vesicle production was the cross-sectional area of the sonication vessel. Sonication efficiency increased, in our experience, as the cross-sectional area of the vessel increased, as long as the depth of the lipid suspension within the vessel was maintained in the range of 2—4 cm. It should be noted that we have explored only flat-bottomed glass vessels of cylindrical shape (scintillation vials, ampoules, shell vials) and cannot comment on the parameters of vessel material or shape. The parameter found to be most crucial to successful use of the cup horn was proper positioning of the sample vessel. When bath fluid was flowing properly through the horn assembly, an 'umbrella' of cavitation was clearly visible if illuminated from above the apparatus (Fig. 1). Maximum efficiency was achieved when the vesicle sample was placed within the 'cap' of the 'umbrella'. For a sample that filled the sonication ampoule to a depth of 3 cm, best results were obtained when the 'cap' was positioned 2 cm above the base of the ampoule. When used in this fashion, the cup horn technique produced populations of vesicles comparable to those produced by the immersed-horn method, as illustrated in the case of egg phosphatidylcholine in Fig. 2. If there were differences between the populations produced by these two methods, it was that the cup horn procedure produced a somewhat smaller population of vesicles as indicated by the

profiles of Fig. 2. In studies of charged phospholipids, we have obtained inconsistent results in assays for phospholipid asymmetry [10] with vesicles prepared by the immersed-horn procedure, while Cup-Horn-produced vesicles have yielded reproducible and consistent results.

For small, unilamellar vesicle samples prepared as described above and isolated by high-speed centrifugation [3], the sample turbidity varied linearly with the reciprocal fourth power of the wavelength in the range of 300 to 600 nm. Below 290 nm, slight positive deviations from linear behavior were recorded, as illustrated in Fig. 3 for egg lecithin and 1,2-dipalmitoyl-3-*sn*-phosphatidylcholine small vesicles. Similar results were obtained with small vesicle samples prepared from 1,2-dimyristoyl-3-*sn*-phosphatidylcholine, C_{24} sphingomyelin and mixtures of $DC_{15}PG$ with 1,2-dimyristoyl-3-*sn*-phosphatidylcholine. In all cases, least squares fitting of the data in the linear range predicted intercepts within 1 S.D. of zero absorbance. These results are as would be expected for populations of Rayleigh scatterers, i.e., isotropic particles of diameters which are small relative to the wavelength of light [11]. It has been previously noted that properly prepared small, unilamellar vesicles behave as Rayleigh scatterers [3]. With the addition of very small quantities of large, multilamellar vesicles, however, the intercepts of these turbidity plots increased significantly from zero absorbance, as illustrated in Fig. 3 for egg lecithin and 1,2-dipalmitoyl-3-*sn*-phosphatidylcholine. The increase in intercept was directly related to the mass of contaminating large vesicles added to the small vesicle samples, with proportionality constants of 2.47 ± 0.03 and 2.33 ± 0.04 absorbance units per mM per cm for egg lecithin and 1,2-dipalmitoyl-3-*sn*-phosphatidylcholine, respectively, as determined with the optical configuration of the GCA/McPherson spectrophotometer. Because no standard spectrophotometer can make an idealized turbidity measurement, these values will vary for different instruments. For comparison, the same instrument was used to directly determine the specific turbidities of large multilamellar vesicle preparations at several wavelengths between 300 and 600 nm. These values varied little with wavelength but extrapolated at $1/\lambda^4 = 0$ to values of 2.5 ± 0.1 and 2.6 ± 0.2 absorbance units mM per cm for egg lecithin and 1,2-dipalmitoyl-3-*sn*-phosphatidylcholine, respectively. These values agree roughly with the proportionality constants relating the $1/\lambda^4$ turbidity intercepts to large vesicle contamination. This suggests a simple assay for contamination of small unilamellar vesicle preparations by large, multilamellar vesicle-type structures. In this procedure, a turbidity intercept for a small, unilamellar sample is first obtained from a plot such as Fig. 3. Using the same spectrophotometer and phospholipid, the specific turbidity intercept of the large vesicles may be determined. This value along with the small vesicle turbidity intercept may then be used to quantitate the contamination of the small vesicle sample. This procedure is illustrated here by using the data contained in Fig. 3 and the large vesicle turbidities quoted above. Expected and known contaminations are compared in Fig. 4. Linear regression analysis showed a correlation between these data of 0.9998 or better for both egg lecithin and 1,2-dipalmitoyl-3-*sn*-phosphatidylcholine.

The usefulness of this procedure is as an assay for the integrity of small, unilamellar vesicle preparations used under conditions suspected of introducing structural changes in these small vesicles. One such condition has been reported

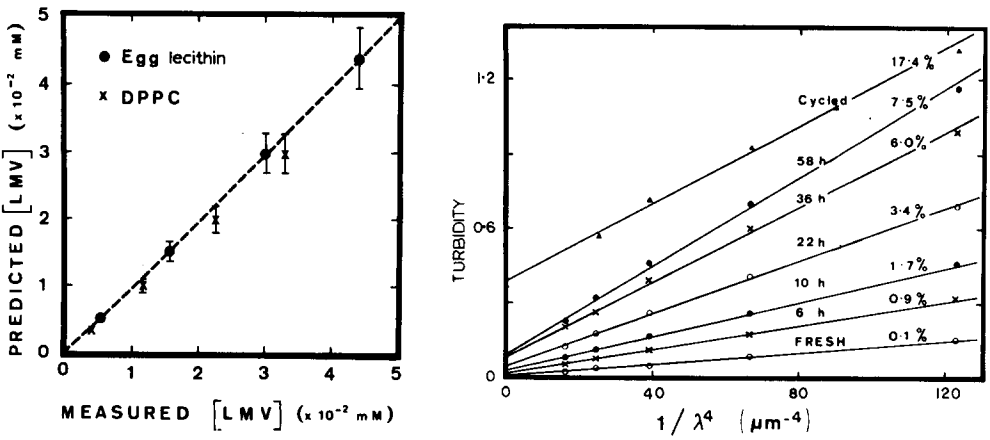


Fig. 4. Predicted concentration of contaminating large multilamellar vesicles (LMV) (calculated from extrapolated $1/\lambda^4 = 0$ values) is plotted against the actual concentration of large, multilamellar vesicles added for egg lecithin (•) and 1,2-dipalmitoyl-3-*sn*-phosphatidylcholine (DPPC) (X). The dashed line represents perfect correlation.

Fig. 5. Turbidity vs. $1/\lambda^4$ at 25°C for 3.7 mM 1,2-dipalmitoyl-3-*sn*-phosphatidylcholine vesicles stored at 4°C for the lengths of time indicated. The uppermost data are for a 4.6 mM sample which was cycled 13 times from 4—55°C over a period of 9 h. Also included are estimates of percentage large, multilamellar vesicle contamination (see text).

to be storage below the liquid crystalline phase transition [4]. Small, unilamellar vesicle preparations, cycled through their phase transition, have been observed to change their thermotropic behavior at a rate related in a first-order fashion to the time spent at temperatures below the phase transition [4]. To illustrate one application of the proposed light-scattering procedure, we have examined 1,2-dipalmitoyl-3-*sn*-phosphatidylcholine small, unilamellar vesicles prepared as described above and stored for various lengths of time at 4°C. Turbidity plots for these vesicles are presented in Fig. 5, along with an indication of storage time. Linear regression intercepts have been used to calculate the large, multilamellar vesicle-type contaminations which are indicated in Fig. 5. Analysis of these contamination data according to a simple first-order model gave a $t_{1/2}$ for the production of these large vesicles of about 600 h. There were insufficient data to distinguish meaningfully between a first- and second-order model. It should be noted that the characteristic decay time obtained from this experiment was much longer than that derived from the cycling experiment of Suurkuusk et al. [4], who reported a decay constant of 5.4 h. This could be because our light-scattering method was measuring different structural changes than those detected by the calorimetric methods of Suurkuusk et al. It could also be because storage at 4°C affected the small vesicle preparations differently than did cycling. In an attempt to distinguish between these two possibilities, we have examined a 1,2-dipalmitoyl-3-*sn*-phosphatidylcholine small vesicle sample that was cycled 13 times between 4 and 55°C over a 9 h period. The turbidity plot for this sample is recorded in Fig. 5. It is noteworthy that the intercept corresponds to a much larger contamination by large vesicles than that observed in the 4°C sample even after 58 h of storage. In analogy with

Suurkuusk et al., we can derive an approximate decay constant of 22 h for the first-order production of large vesicles during cycling. This is much more in agreement with the result of Suurkuusk et al. It appears, therefore, that passage through the phase transition is more crucial to small vesicle degradation than is storage below the phase transition.

Another condition which has been shown to result in fusion of small, unilamellar vesicles to larger structures is the presence of multivalent metal ions in negatively charged vesicle preparations [5]. We have used the turbidity assay to control for such events during studies of europium (Eu^{3+}) binding to $DC_{15}PG$/1,2-dimyristoyl-3-*sn*-phosphatidylcholine vesicles (Sarasua and Lentz, unpublished results). Here we found that small vesicles of 25% $DC_{15}PG$/75% 1,2-dimyristoyl-3-*sn*-phosphatidylcholine began to show significantly increased intercept absorbance values at Eu^{3+} concentrations of around $5 \cdot 10^{-4}$ M. However, small vesicles of 10% $DC_{15}PG$/90% 1,2-dimyristoyl-3-*sn*-phosphatidylcholine were not altered by the addition of the same amount of Eu^{3+} as determined by this assay.

While we have pointed out the usefulness of this assay in monitoring the integrity of small vesicle preparations, we must also make clear its limitations. Precisely stated, the assay measures the extent to which a small, unilamellar vesicle preparation behaves as a population of Rayleigh scatterers. Further, if the deviation from the Rayleigh behavior is caused by large, multilamellar vesicle structures, the assay provides an estimate of the amount of large vesicle structures present. However, if structures with specific turbidities different from that of the large vesicles are present, their concentration in the sample cannot be accurately estimated. Finally, the assay will not detect vesicles which are small enough to behave as Rayleigh scatterers (e.g., diameters of approx. 500 Å) but which should not be appreciably present in a properly prepared small, unilamellar vesicle sample [3]. Thus, the vesicle sample having the size distribution shown in Fig. 2B was predicted by the turbidity assay to have a large, unilamellar vesicle contamination of only 0.3%. This may well be an accurate estimate since sonicated but unfractionated vesicle samples haven been shown to contain very few large, multilamellar vesicle-type structures, but significant quantities of 500–1000 Å single lamellar structures [12]. These structures would produce the void volume peak shown in Fig. 2B but still behave more as Rayleigh scatterers than as large, unilamellar vesicles in their light scattering. Not surprisingly, the slopes of the turbidity plots obtained with unfractionated (Fig. 2B) and fractionated (Fig. 2C) vesicles were considerably different (2.16 and 0.57 absorbance units per μm^{-4}/M, respectively), indicating qualitatively different populations of Rayleigh scatterers.

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