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Preparation and characterization of monodisperse unilamellar phospholipid vesicles with selected diameters of from 300 to 600 nm

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A method has been developed for making large unilamellar vesicles (LUV) with low polydispersity. The LUV, constituted of dioleoylphosphatidic acid (DOPA), 300 nm in diameter are made by a modification of the pH adjustment technique (Hauser, H. and Gains, N. (1982) Proc. Natl. Acad. Sci. USA 79, 1683–1687). This size is 10 times that (30 nm) of vesicles prepared by prolonged sonication. Vesicle size is increased stepwise by adding cholesterol (to a maximum of 40 mol% cholesterol) to form vesicles in 0.15 M KCl with up to 600 nm diameter. The vesicle size is measured by photon correlation spectroscopy, electron microscopy, and by measurement of the internal volume with cyanocobalamin while calculating the number of DOPA molecules per vesicle. Vesicles are stable for at least three weeks. Sepharose 4B column chromatography of the preparation yields a peak of fractions with the same polydispersity as the original sample and shows that 30 to 40% of the original lipid in a sample is recovered as LUV. Less than 2% of the sample forms small unilamellar vesicles (SUV) (diameter = 30 nm), which emerge from the column in a separate peak. Since the remaining lipid is not suspended in the buffer during vesicle formation, for most purposes the vesicles may be used immediately after titration so that they can be prepared in less than 40 min.

Introduction

Ever since the classic experiments of Chapman [1] which clearly described the transition temperature in lamellar sheets of membrane lipids, investigators have been seeking methods of making model lipid bilayers that resemble natural biological membranes. Bangham and co-workers [2] first succeeded in making lipid preparations (multilayered liposomes) that entrapped aqueous compartments and were osmotically active. An important requirement was that the liposome be above the transition temperature of lipids. A unilamellar liposome (vesicle) of uniform size (30 nm) was soon prepared by Huang [3] using extensive sonication. Huang's vesicles were curvature limited with each phospholipid head group on the exterior surface occupying 0.74 nm^2 and on the interior surface, 0.61 nm^2 [4]. In recent years methods have been developed for preparing large 'uniform' vesicles (LUV's) which are generally made at solvent-water interfaces [5–9] or by detergent removal methods [10–14]. See Ref. 10 for a more complete review of LUV preparation procedures. In a careful review of these procedures Parente and Lentz [10] found those made at solvent-water interfaces to be mixed with multilamellar vesicles

Abbreviations: DLPA, dilauroylphosphatidic acid; DOPA, dioleoylphosphatidic acid; EM, electron microscopy; LUV, large unilamellar vesicle; PA, phosphatidic acid; PC, phosphatidylcholine; PCS, photon correlation spectroscopy; SUV, small unilamellar vesicle; TLC, thin-layer chromatography; Tris, tris(hydroxymethyl)aminomethane hydrochloride.

and not reliably reproducible. They found that cholate-prepared vesicles left significant amounts of cholate as a contaminant in the preparation, and octylglucoside-prepared vesicles required long dialysis (3 days) in order to produce a uniform preparation of detergent-free unilamellar vesicles in the size range reported here. Vesicle preparations that are not monodisperse LUV's are not appropriate for precise osmotic swelling experiments because the forces are different in different sizes in the same solution [6]. In surprising contrast to attempts to make LUV's with low polydispersity, several vesicle preparations derived from biological membranes are uniform in size. The preparations of mitochondrial inner membrane vesicles (submitochondrial particles) [15] or bacterial membrane vesicles [16] turned out in later work to be relatively uniform in size. Although submitochondrial particles are uniformly 100 nm, the latter preparation was from 500 to 1500 nm in diameter. Both are inside-out in orientation of the bilayer as compared to that of the biological system. Prokaryote cells are approximately 2 μm in length whereas eukaryote cells are 20–30 μm. The properties (including size) of cell-derived vesicles are presumably dominated by the proteins in the membrane as each preparation has its unique size range and the isolated lipids do not spontaneously form the same size vesicles. Uniform size is important for many physical studies and transport studies and innumerable attempts have been made to prepare monodisperse vesicles in the size range obtained from biological sources.

Dynamic light scattering provides a rapid and accurate technique for determining the diffusion coefficients of vesicles in solution [17–20]. From diffusion coefficients, one can easily determine the vesicle size. Photon correlation spectroscopy (PCS) measures the digital correlation function of photoelectric pulses. As a digital technique it is well suited to computer analysis. The techniques of dynamic light scattering have been extensively discussed in the literature [21].

Recently, Gains and Hauser [22–25] reported a new method of vesicle preparation in which uniform vesicles with a diameter of 30 nm (SUV) were produced together with a nonuniform mixture of vesicles in a larger size range (LUV). In this method, a dispersion of phosphatidic acid (PA) (either DLPA or PA derived from egg lecithin) with or without PC was subjected to a sudden increase of pH. Using this technique and applying principles developed regarding the dynamics of both the hydrocarbon domain [26] or the headgroup domain [27] together with the simplicity and speed of a photon correlation spectroscopic assay of vesicle size, polydispersity, and amount, the pH adjustment method has been honed to enhance the size, uniformity and yield of vesicles. We find that we can produce uniformly sized vesicles reproducibly in minutes with a size of our choice between 300 and 600 nm.

Experimental

Materials. L-α-Dioleoylphosphatidic acid (DOPA) was procured from Avanti Polar Lipids, Inc. (Birmingham, AL). Its purity was monitored with thin-layer chromatography (TLC) on Silica gel 60 plates (EM Laboratories, Elmsford, NY) using $CHCl_3/CH_3OH/H_2O$ (65 : 35 : 5, v/v/v) as a solvent system. Ultrapure sucrose was purchased from Schwarz/Mann (Orangeburg, NY). Sepharose 4B was obtained from Pharmacia Fine Chemicals (Piscataway, NJ). The vitamin B-12 was procured from Sigma (St. Louis, MO). Formvar solution (0.5%) in ethylene dichloride was obtained from Ladd Research Industries, Inc. (Burlington, VT). The uncoated copper grids were procured from Polaron Equipment Ltd. (Watford, U.K.). Cholesterol (98% pure) was procured from Aldrich Chemical Company, Inc. (Milwaukee, WI). All chemicals were reagent grade unless otherwise indicated. All solvents were redistilled before use.

Preparation of vesicles. L-α-Dioleoyl phosphatidic acid (disodium salt, 8 mg) was dissolved in 2 ml of chloroform. In order to convert the DOPA largely to the acidic form, this solution was washed four times with 2 ml of chloroform/methanol/0.2 M HCl (3 : 48 : 47, v/v/v). The upper phase was discarded each time. The resulting lower phase was washed with a 2 ml mixture of chloroform/methanol/water (3 : 48 : 47, v/v/v). When cholesterol-incorporated DOPA vesicles were made, chloroform containing cholesterol was added in this lower phase. The lower phase solvent was then removed by rotary evaporation in a round bottom flask to form a phospholipid film.

The phospholipid film was dried under a water aspirator at room temperature for about 10 min. In order to establish that this procedure did not result in hydrolysis of DOPA, the dried film was examined with TLC (see Materials). It was found to migrate as a single spot with no free fatty acid or lysophosphatidic acid present. The dried film was then dispersed in 3.0 ml of either a sucrose or KCl solution (both having pH = 7.55) depending on the desired medium for vesicles. Both sucrose (0.25 M) and KCl (0.15 M) solutions with 2 mM Tris and 0.02% sodium azide had the same osmolality. The suspension was stirred for 30 min at room temperature in order to suspend the lipid film in the solution. The final concentration of DOPA in the suspension was 1.0 mg/ml.

Sonicated vesicles. The crude vesicle preparation (pH was about 4.6 ± 0.4) was adjusted to pH 7.55 with 0.1 M NaOH in the Tris buffer in sucrose solution. The resulting suspension was then sonicated with a Branson sonicator at a sonication power of 50 watt.

pH adjustment vesicles. This suspension (pH was about 4.6 ± 0.4) was titrated with 0.1 M NaOH to a pH of 7.55–11.0. The pH of the sample was readjusted to 7.55 with 0.1 M HCl.

The vesicle preparation of pH adjustment was chromatographed on a Sepharose 4B column (47×0.9 cm) at room temperature. The elution rate was 18–20 ml/h. An automatic fraction collector was used to collect 0.3 ml fractions.

Photon correlation spectroscopy (PCS). The size of the vesicles was determined by using photon correlation spectroscopy. The apparatus used is described in Ref. 28. Light from an argon ion laser at 488 nm was focussed onto the vesicle sample in a glass cuvette maintained at constant temperature (20°C) by a Lauda water circulator. The intensity of the scattered light was detected at 90° to the incident beam with a Hamamatsu (Middlesex, NJ) photomultiplier tube (PMT).

The radius of the vesicles in a sample is calculated from the correlation function. In PCS, one measures the second order correlation function $g^{(2)}(\tau)$, where τ is the delay time. For particles in Brownian motion, the correlation function decays exponentially with the delay time according to the following expression:

$$g^{(2)}(\tau) = B(1 + A \cdot e^{-\Gamma \cdot \tau})$$

where B is the base line, Γ is the inverse correlation time and the A is a constant between 0 and 1 determined by the optical system. Γ is given by $2Dq^2$, where D is the translational diffusion coefficient and q is the scattering vector. D is related to the particle radius (R) by the Einstein-Stokes expression:

$$D = kT/6\pi\eta R$$

k = Boltzmann's constant, η = viscosity of the solution and T = absolute temperature. By fitting a single exponential through the correlation data, one can determine the diffusion coefficient, D and from this the particle radius (R).

If the vesicles in a sample have a distribution of sizes, the correlation function does not exhibit the simple exponential decay indicated above, but is a superposition of exponentials instead. It is then possible to extract the mean decay time Γ_0 and μ_2, the second moment of Γ about Γ_0. By a cumulants analysis technique, the average radius (actually the z-average radius) is found from Γ_0 while the ratio μ_2/Γ_0^2 is an index of polydispersity. For vesicles whose size distribution is gaussian with a mean radius R_0 and a standard deviation σ, the fractional width of the distribution is related to the index of polydispersity (μ_2/Γ_0^2) by:

$$\sigma/R_0 = x\mu_2/\Gamma_0^2$$

where x is an empirical number between 1 and 4 determined experimentally. Our comparison of electron micrograph measurements of the diameter and standard deviation of uniform polystyrene spheres (Dow Chemical Co., Indianapolis, IN) with the polydispersity obtained from PCS on samples having diameters 85 nm, 126 nm and 557 nm yield $x = 4$, 3, and 1, respectively. For DOPA vesicles having diameter 50 nm, and 500 nm the values for x are 1.5 and 3, respectively. Several workers have developed techniques for extracting the complete molecular weight distribution of polydisperse samples from correlation data by sophisticated computer analysis, but these techniques are still controversial. The cumulants method, on the contrary, is generally reliable and has been widely applied [29].

The correlation function was accumulated in a 60 channel photon autocorrelator and initially dis-

played on an oscilloscope. An A/B ratio greater than 0.5 indicates a good signal to noise ratio. The sampling time was adjusted to obtain exponentially decaying correlation functions which were collected by a DEC PDP-8 minicomputer and subsequently analyzed by the technique of multiple linear regression (cumulants analysis) on a VAX 11/780 mainframe computer (DEC, Maynard, MA).

The average diffusion coefficient and standard deviation were calculated by averaging eleven successive autocorrelation functions, each with the same bin (sampling) time. The count rate was essentially constant in most of the samples which implies that the vesicle sample was not highly polydisperse. Occasional samples with widely fluctuating count rates were discarded. In addition to the diffusion coefficient, the analysis yields an index of polydispersity of the sample which was determined by the cumulants analysis method [29].

The scattering vector $q = 4\pi n \sin(\theta/2)/\lambda$, where n is the refractive index of the solution, θ is the scattering angle and λ is the vacuum wavelength of the incident light. To determine D and R from the measured Γ, it is thus necessary to accurately determine n and η. Refractive indices of sucrose and KCl solutions were determined by measuring the angle of minimum deviation at a wavelength of 488 nm. The refractive index was measured at various concentrations and the desired value was evaluated from a linear fit through the data. The viscosity, η, of sucrose and KCl solutions were directly determined with an Ostwald viscometer using water as a standard.

The light scattering apparatus was calibrated with a standard, monodisperse sample of polystyrene latex spheres (diameter 85 nm). Whether or not samples were chromatographed prior to examination by light scattering, they were diluted 10 times to bring the photon count rate within the range of 5000 to 10000 counts per second. They were filtered through Millipore (Bedford, MA) membrane filters with a pore size of 3 μm. Sucrose and KCl Tris buffers were prefiltered through a 0.25 μm pore size filter.

Negative staining electron microscopy. Vesicle sizes were also studied with a Philips 300 EM at 60 kV. Formvar coated grids were prepared with a 0.5% formvar solution on uncoated grids. A drop of solution containing phospholipid vesicles was placed on the Formvar grid. The excess solution was adsorbed with a No. 1 filter paper after 15 s. A drop of uranyl acetate solution (5%) was placed on the grid. After 5 s, the excess was absorbed with filter paper. The grid was then allowed to dry in air for 10 min prior to examination with the electron microscope.

Trapped volume determination. The trapped volume of the vesicles was determined by preparing the vesicles in the presence of vitamin B-12 (7.0 mM in 0.15 M KCl solution). External vitamin B-12 was removed by passage over a Sepharose 4B column. The total trapped marker was determined on a spectrophotometer at 361 nm ($\varepsilon = 2.81 \cdot 10^4$). The internal volume was calculated from the amount of trapped vitamin B-12, its concentration in the initial medium and the amount of phospholipid. The latter was assayed by the procedure of Ames and Dubin [30]. A DOPA molecule was assumed to occupy 0.6 nm^2 on both the inner and outer surface of the bilayer.

Results

Column chromatography of vesicles

The pH adjustment method of Hauser and Gains [22] yielded uniform small unilamellar vesicles of dilauroylphosphatidic acid (DLPA) of about 30 nm diameter. These vesicles came off a Sepharose 4B column as the major peak with some large vesicles containing a wide variation of size coming off with the void volume.

In our experiments (Fig. 1) the pH adjustment vesicles produced from DOPA also yielded two peaks, the principal one, however, came off the column near the void volume. The SUV peak was minor. Three methods were used to quantitate the results. First, phosphate analysis (Fig. 1) was used to determine the amount of phospholipid per fraction [30]. Second, the total counts per fraction (Fig. 1) were measured with photon correlation spectroscopy. Third, the absorbance of vitamin B-12 at 361 nm (not shown) was measured during the entrapped volume determination. All results gave the same pattern (Fig. 1), which shows the utility of light scattering for assaying vesicles in column fractions (as well as measuring vesicle radii) since it is so rapid and convenient.

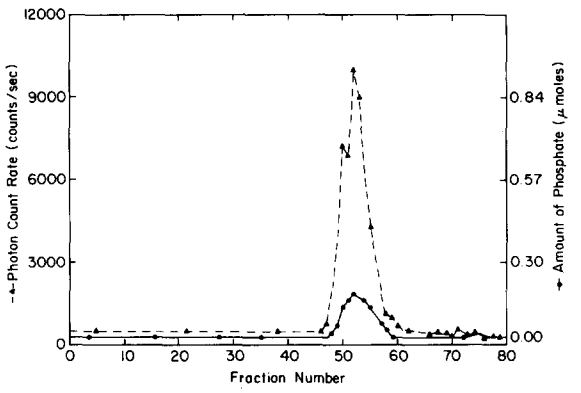

Fig. 1. Elution profile of DOPA vesicles from a Sepharose 4B column (47 × 0.9 cm). Phospholipid was assayed by the method of Ames and Dubin [30] and total counts were measured by PCS. The vesicles were made by the pH adjustment method (maximum pH 9.00) in 0.15 M KCl-Tris buffer. The original vesicle suspension in the buffer solution (1.0 ml) was loaded onto the column. Fractions (0.3 ml) were collected with an elution rate of 18–20 ml/h at room temperature. The void volume is approximately at fraction 40, the total volume is off scale at about fraction 110.

No difference was noted in vesicle size when isoosmolal solutions of sucrose (0.25 M) or KCl (0.15 M) were used with buffer (Table I).

The addition of cholesterol to DOPA did not increase the size of the vesicles made in sucrose solution.

Huang [3] and others [22–25] have found that the passage of phospholipid vesicles through a Sepharose 4B column results in adsorption of phospholipids onto the column. It was suggested that this problem might be avoided by first saturating the column with phospholipids. In order to explore the nature of this adsorption, we examined vesicles that were passed through a column twice. The vesicle size was found to be smaller for vesicles prepared in sucrose after a second passage through the column (Table II) and the polydispersity was found to increase. This suggested that the adsorbed phospholipids may be derived directly from the vesicles reducing their average diameter. For vesicles prepared in a KCl-Tris buffer, the size did not change on passing the sample twice through the column.

TABLE I

A COMPARISON OF VESICLES PREPARED IN SUCROSE AND KCl SOLUTIONS HAVING THE SAME OSMOLALITY.

The maximum pH of KCl solution was 9.00 and that of sucrose solution was 9.05. Standard deviation (σ) refers to the error in measurement (not to the vesicle size).

Sepharose 4B column	Buffer containing	Radius $\pm \sigma$ (nm)	% Index of polydispersity
+	0.15 M KCl	125.0 ± 6.8	6
+	0.25 M sucrose	127.6 ± 3.5	8

TABLE II

THE EFFECT OF PASSAGE THROUGH THE SEPHAROSE 4B CHROMATOGRAPHIC COLUMN ON THE VESICLE SIZES

The vesicles were prepared with the pH adjustment method in a sucrose-Tris buffer. The maximum pH was 8.3. Standard deviation (σ) refers to the error in measurement (not to the vesicle size).

Sepharose 4B column	Radius $\pm \sigma$ (nm)	% Index of polydispersity
once	77.0 ± 0.8	4
twice	62.7 ± 1.2	8

The size of sonicated vesicles

The size of vesicles prepared in sucrose by the sonication method was found to decrease with the duration of sonication up to a period of 30 min. At this point, the vesicles had a radius of 27 ± 0.2 nm with a standard deviation of 20% of vesicle size. It should be noted that Huang [3] obtained curvature-limited vesicles with a radius of 12.5 nm displaying low polydispersity after 160 min sonication.

The size of pH adjustment vesicles

Routine size measurements were made by photon correlation spectroscopy. Because the results were different from those of Hauser and Gains [22] we reexamined the effect of varying the maximum pH (Fig. 2) and the pH adjustment time (Table III). Gains and Hauser [24] examined how variation of maximum pH affected the relative amount of LUV and SUV on columns. Our interest was in maximizing the uniformity and the size of the LUV's.

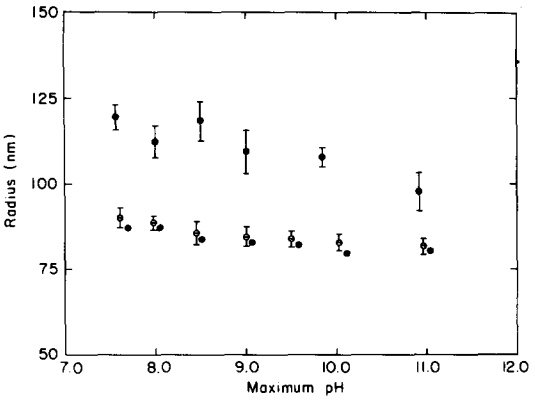

Fig. 2. The size of DOPA vesicles made by the pH adjustment method in 0.25 M sucrose-Tris buffer in which the maximum pH is varied. The initial pH was 4.80 for ● and 5.20 for both ⊖ and ⊕. (The latter are separate measurements of duplicate experiments). Vesicle size was measured with photon correlation spectroscopy (PCS). Standard deviation refers to the error in measurement (not to the vesicle size).

The variation of vesicle size, prepared in a sucrose-Tris buffer, with the magnitude of pH adjustment is shown in Fig. 2. We found that there was not a significant change in the mean vesicle size of the LUV's prepared in sucrose with variation in maximum pH. Vesicles prepared in both sucrose and KCl by the pH adjustment method were found to be stable for at least 3 weeks. When cholesterol was incorporated into the lipid preparation of vesicles prepared in 0.15 M KCl-Tris buffer by the pH adjustment method, the ensuing vesicles were found to be larger than those without cholesterol. The variation of vesicle sizes with the amount of cholesterol in DOPA is shown in Fig. 3.

TABLE III

VARIATION OF VESICLE SIZE WITH THE CHANGE IN pH ADJUSTMENT DURATION

Vesicles were prepared in a 0.25 M sucrose-Tris buffer at room temperature. The magnitude of the maximum pH was 10.0 and the sample was filtered through 0.8 μm pore size filter. Standard deviation (σ) refers to the error in measurement (not the vesicle size).

Sepharose 4B column	pH adjustment duration	Radius $\pm \sigma$ (nm)	% Index of polydispersity
−	1 s	75.6 ± 0.9	8
−	1 min	71.5 ± 0.5	7
−	5 min	74.4 ± 1.0	8
+	1 s	76.2 ± 0.9	6
+	1 min	77.0 ± 0.8	4

Electron microscopy

Negative stained electron micrographs of vesicles made both by sonication and by pH adjustment are shown in Fig. 4. The uniformity of the vesicles is apparent in both pictures. Below each micrograph is a distribution of the size as measured from the photomicrographs. The standard deviation of the size is shown above the bar graph. Both the sonicated and the pH adjusted vesicles are shown to have standard deviations representing approx. 20% of the size. This approach was checked by examining polystyrene spheres of 85 nm, 126 nm, and 557 nm size. We found the same standard deviation as those provided by Dow Chemical Co., who also used electron microscopy to measure the size and determine the standard deviation. The size of the vesicles

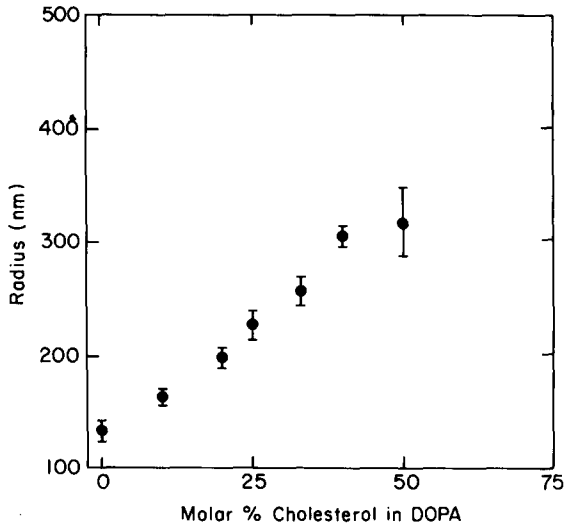

Fig. 3. Size of DOPA vesicles made by the pH adjustment method in 0.15 M KCl-Tris buffer (maximum pH 9.00). The initial pH was 4.50. Chloroform containing the cholesterol was added in the lower phase after acid washing of the disodium salt of DOPA. The acidified phosphatidic acid/cholesterol mixture was dried in vacuo, and the buffer was added to the dried film. The size was measured with photon correlation spectroscopy (PCS). Standard deviation refers to the error in measurement (not to the vesicle size).

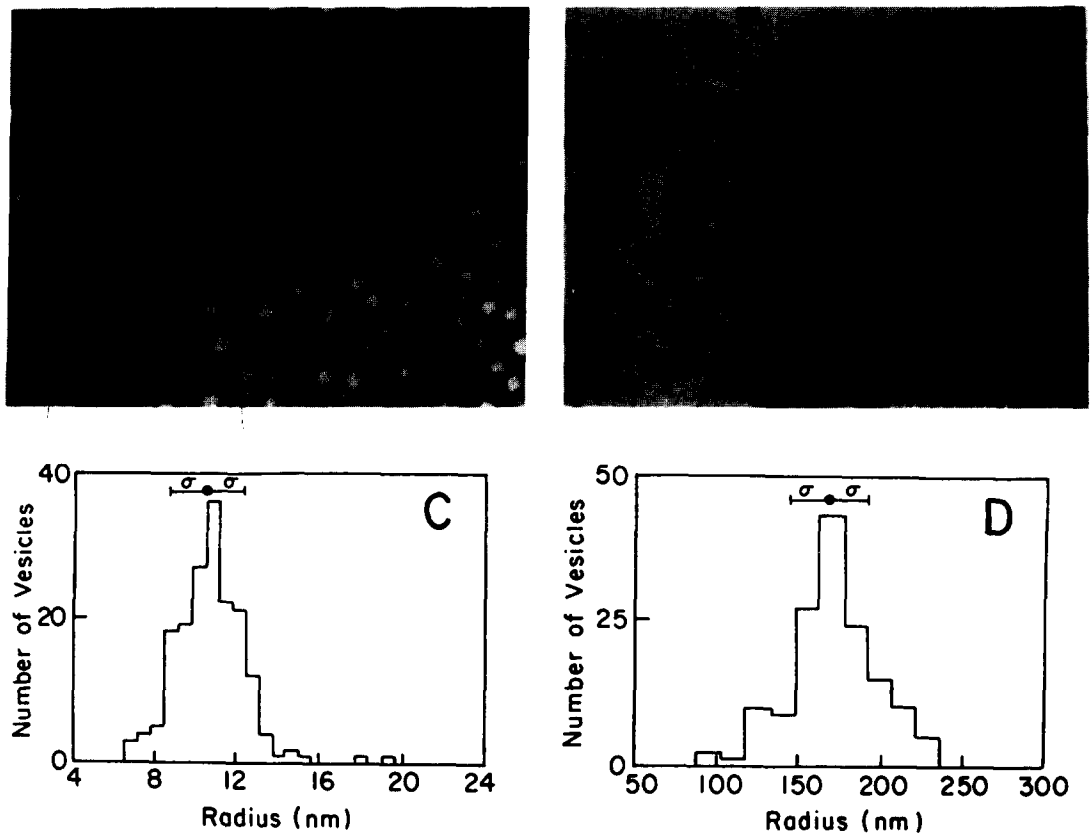

Fig. 4. Negative stained electron micrographs of vesicles made by sonication (A) and pH adjustment (B). The DOPA vesicles were both made in 0.15 M KCl-Tris buffer. Maximum pH for the vesicles made by pH adjustment was 9.00. Sonication time for the sonicated vesicles was 30 min. Grids were examined on a Philipis 300 EM at the magnification ×33000 and ×13000 for (A) and (B), respectively. Uranyl acetate solution (5%) was used for negative staining. (C) and (D) are vesicle size distributions for (A) and (B), respectively. They were the measurements of vesicle size on the electron micrographs. The size of sonicated vesicles on electron micrograph (A) were smaller than those obtained from photon correlation spectroscopy (PCS) presumably because the negative staining procedure shrunk the vesicles slightly. The standard deviation (σ) of the vesicle size is shown above the bar graph.

found in the electron micrographs is smaller than those obtained by light scattering.

Encapsulation of vitamin B-12

For internal volume determination, vesicles were prepared in cyanocobalamin. The sizes of vesicles entrapping cobalamin do not significantly differ from those without cyanocobalamin as measured by photon correlation spectroscopy. Vesicles prepared in the presence of cyanocobalamin displayed a mean radius of 80.2 ± 1.4 nm (compared to a control of 87.7 ± 0.6 nm) with an index of polydispersity of 8%. The trapped volume determination yielded a mean radius of 90.4 nm which is comparable to that obtained by light scattering. Vesicles containing cyanocobalamin were found to be unstable over several hours.

Discussion

The formation of monodisperse large unilamellar vesicles of a desired size between 300 and 600 nm diameter is described. The preparation is made of dioleoylphosphatidic acid in 0.15 M KCl-Tris buffer with varying amounts of cholesterol. The method is a further development of the pH adjustment technique of Hauser and Gains [23]. The ionic strength is apparently critical since addition

of cholesterol to the vesicles made in sucrose-Tris buffer did not produce larger vesicles. Important differences allowed us to make vesicles 10-times larger and yet with the same standard deviation in vesicle size as is found for small unilamellar vesicles. These differences include a high-salt concentration (0.15 M KCl), the choice of dioleoylphosphatidate as opposed to dilauroylphosphatidate, and the use of cholesterol to further separate the headgroups, which we believe to be largely acid-anions [27], during vesicle formation. It should be noted that vesicles are approximately the same size when they are made in 0.25 M sucrose, 0.15 M KCl or 0.3 M mannitol [25].

The vesicle sizes and polydispersity were determined by several methods which gave approximately the same results. We were able to verify the manufactures size and standard deviation of polystyrene spheres by both electron microscopy and photon correlation spectroscopy. This gives us confidence in the use of photon correlation spectroscopy as a more reliable method for the determination of vesicle size than electron microscopy for the measurement of vesicle radii where the vesicles (two molecules thick) must first be stained with uranyl acetate which concentrates on the surface. We reason that polystyrene spheres are less susceptible to distortion in electron microscopy and the fact that vesicles are found to be slightly smaller in electron micrographs is taken as evidence that the photon correlation spectroscopy results are more reliable. The measurement of vesicle size by entrapment of B-12 with its accompanying assumptions about the area occupied by a phospholipid molecule is useful in that it validates the size range of the vesicles. Its estimate is slightly larger than that of photon correlation spectroscopy. Photon correlation spectroscopy was shown in these studies to yield mean radii, measure approximate polydispersity and compare relative amounts of vesicles in fractions (Fig. 1) in a few minutes for each sample.

Parente and Lentz [10] have prepared LUV's in the 80–100 nm diameter size range by using octylglucoside dialysis. Their preparation is made from many synthetic lipids, all with saturated chains. Their results have demonstrated clearly the difficulties associated with removing trace impurities from detergent dialysed sample. In the present method the principal contaminants expected to be introduced would be in the original solvent. The lipid was found by TLC to be unhydrolysed after preparation for vesicle formation; this includes acid-washing.

The attempt to enhance the size of the LUV's and maintain uniformity was based on a combination of principles derived from two theoretical views on the hydrocarbon domain [26] and the headgroup domain [27] of lipid bilayers. In general it was reasoned that a large vesicle would be derived from a more planar array of phospholipids. Such an array would be maximized by balancing the average attractive and repulsive interactions between neighboring headgroups. Attractive interactions are considered to be acid-anion dimer interactions (acidic H-bonding) [27] which average on a rapid timescale over the entire bilayer and repulsive interactions are due to adjacent anions. In this view, the pK values of phosphatidic acid in the bilayer may not be predicted from pK values measured in the absence of a bilayer. The pK of such a group as phosphate in the bilayer results not only from the functional group's measured pK values but also from the forces that hold adjacent groups in close proximity allowing acid-anion formation. Thus a membrane lipid containing two negative charges would display pK values including those of both acid-anions [27].

A significant difference between the present work and that of Hauser [22–25] is the use of 150 mM KCl and of dioleoylphosphatidic acid, which has a single *cis* double bond in each chain. It is presumed that pi-pi interactions [26] maintain at least the double bond of the secondary ester nearly perpendicular to the plane of the bilayer. This statement is consistent witht he data of Seelig and Waespe-Sarceic [31] who determined the orientation of the double bond of oleic acid in phosphatidylcholine by deuterium quadrupole coupling and infrared dichroism to be nearly normal to the plane of the bilayer. The unsaturated phospholipid is expected to thin the bilayer and spread the headgroups as compared to a saturated chain lipid. The *cis* double bond of the secondary ester chain, which is closer to the headgroup plane than the *cis* double bond in the primary ester chain, is expected to perturb the primary ester chain between its ester group and the double bond. This would

orient the *cis* double bond on the primary ester chain so that it is significantly more parallel to the plane of the bilayer, thereby thinning it.

The separation of the headgroups provides, in this view, a favorable balance of attractive and repulsive forces for planar formation because less hydrogen bonding (on the average) occurs. The addition of cholesterol was reasoned to even further spread the headgroups and reduce the H-bonding during the pH adjustment. A limiting value of 40 mol% cholesterol was found at which point the vesicle reached a size of 600 nm diameter.

Although the predicted success of this protocol is encouraging with regard to the practical applications of the theories, their general usefulness will have to await developments.

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