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Vesicles of Variable Diameter Prepared by a Modified Injection Method†

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ABSTRACT: A modified injection method is described, which enables the formation of single bilayered vesicles of variable diameter (15–50 nm) in a controlled way. The vesicles produced have been characterized by light scattering, electron microscopy, and laser light scattering.

The contribution of individual membrane components to biological processes like permeation and fusion is difficult to assess from studies on "intact" membranes. Therefore it seems useful to complement these studies with measurements on simple "artificial" membranes. Model membranes such as vesicles or liposomes, prepared from components that occur naturally in biological membranes, offer possibilities of studying a system of intermediate complexity. The use of pure lipids with well-defined chain length and polar head groups is necessary if one is to understand the physical chemical behavior of these molecules in biological processes from a more fundamental point of view. However, it will first be necessary to characterize the model systems used and to find a method of obtaining these systems in a reproducible and reliable way.

Vesicles (single bilayered liposomes), which have proved useful as models for biological membranes, commonly are prepared by sonication (Huang, 1969) which entails the danger of damaging the lipid molecules (Klein, 1970; Hauser, 1971), although the damage can be minimized by carefully controlled conditions (Huang and Charlton, 1972).

An alternative way to prepare vesicles is the injection of an ethanolic solution of lipid into an aqueous (salt) solution (Batzri and Korn, 1973). This procedure has no degrading effect on the phospholipid and gives a reasonably homogeneous preparation of vesicles with a diameter of about 26.5 nm.

In this paper we show that the injection method can be used to obtain fairly monodisperse vesicles of variable size (25–120 nm) by varying the injection conditions. The presence of ethanol is a disadvantage, but this can easily be removed by dialysis. Moreover, characterization of the vesicles by light scattering shows no differences between dialyzed and nondialyzed samples.

Materials and Methods

The lecithins used were dimyristoylphosphatidylcholine (DMPC)[1] and dipalmitoylphosphatidylcholine (DPPC) obtained from Koch and Light Laboratories, Coinbrook, Bucks, England (art. 2203t, batch No. 65508 and art. 3409t, batch No. 67082, respectively). To remove a fusogenic impurity, DMPC was purified by column chromatography on a silica column (Merck, Kieselgel 60 reinst, 70–230 mesh) using a continuous gradient of chloroform and methanol (up to 70%) as eluent. Fractions were analyzed by thin-layer chromatography with a chloroform–methanol–water (65:35:4) mixture as developer. The first and last fractions showing the presence of lecithin were discarded to avoid contamination of small amounts of impurity, that would not be visible with this low sensitivity analysis. The lipid thus obtained was colorless and

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[1] Abbreviations used: DMPC, dimyristoylphosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; Tris, tris(hydroxymethyl)aminomethane.

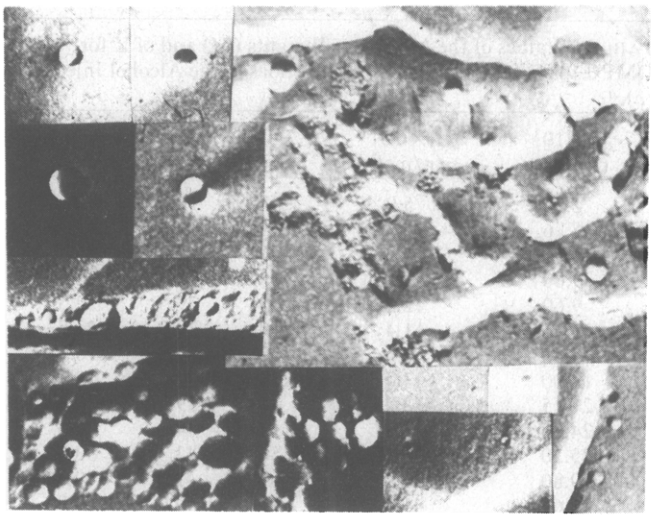

FIGURE 1: Freeze-etching electron microscopy photographs of different vesicle preparations obtained by the modified injection method.

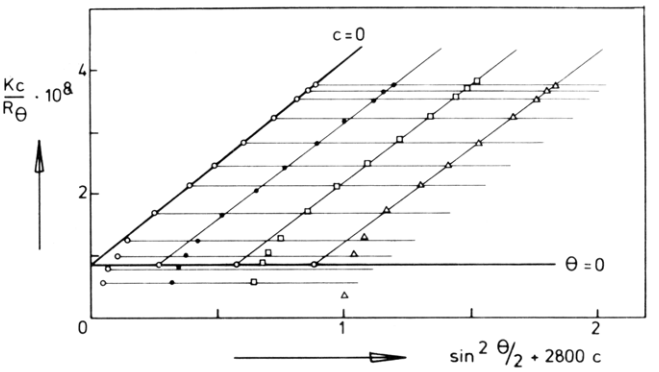

FIGURE 2: Zimm plot for a vesicle solution of DMPC in buffer for concentrations of 0.3445 (△), 0.1746 (□), and 0.1065 (●) mg/mL.

kept in ethyl alcohol at −20 °C.

Aqueous lipid solutions were prepared by injecting an ethanolic solution of 2 to 50 μmol of lecithin per mL of alcohol through a Hamilton syringe into 10 mL of a magnetically stirred buffer solution, giving a 2.5–7.5% ethanolic solution. The solution was contained in a cylindrical vessel of 2-cm diameter and the actual injection took place about 2.5 cm below the liquid surface. Stirring was accomplished by a standard magnetic stirrer. The injection velocity was varied between 10^{-4} and 5×10^{-2} mL/min. During injection, the temperature was kept at 35 and 55 °C for DMPC and DPPC, respectively, well above the phase transition temperature of the lipid used. The buffer solution used throughout this work was NaCl (0.1 M, BDH analar) in Tris-HCl (0.01 M, BDH) adjusted to pH 7 and filtered through a Millipore filter (25 nm). All aqueous solutions were prepared with doubly distilled water.

The aqueous lipid solution was again filtered through a Millipore filter (450 nm) directly into the measuring cells used for light scattering experiments, while keeping the temperature constant. The amount of lipid lost in this procedure was about 2 mg per Millipore filter as determined by phosphor analysis.

Light scattering measurements were done with a FICA light scattering photometer at angles between 30° and 150° with light of 546 nm at 30 °C and 50 °C for DMPC and DPPC, respectively. The refractive index increment for DMPC was measured with a Rayleigh interferometer (λ = 546 nm) at 30 °C and was found to be 0.132 ± 0.002 mL per g. For DPPC we used a value of 0.1255 mL per g (Yi and MacDonald, 1973).

Diffusion coefficients were determined from quasi-elastic light scattering (Berne and Pecora, 1976) using an argon ion laser (λ = 546 nm). With this technique one monitors the fluctuations in the scattered light intensity caused by the Brownian movement of the scattering particles and relates these fluctuations to the diffusion coefficient to be determined.

A further, and more direct, check on the nature of the particles produced can be obtained from freeze-etching electron microscopy. Due to the low concentration of lipid used in this work ($c \leq 1.5$ μmol/mL solution), many photographs had to be taken. A composition picture of some differently prepared vesicle samples is given in Figure 1. From this figure it is clear that the vesicles consist of only one, spherical bilayer. The exact size and degree of polydispersity can of course not be ascertained from these photographs, due to the unknown plane at which the vesicles are cut.

The removal of the ethanol in the solution was accomplished by dialyzing the samples against pure buffer solutions. The refractive index of the samples was measured as a test for the presence of alcohol. Typically, after about 8 h, no ethanol could be observed with this method, which detects ethanol at a concentration of about 0.01% by volume. Samples studied by light scattering at this stage showed no changes as compared with freshly injected samples containing ethanol. Even after dialyzing for 24 h no differences in the Zimm plots for fresh and dialyzed preparations could be observed. We conclude that small amounts of ethanol remaining in the dispersion do not affect the vesicle size.

Results and Discussion

A typical Zimm plot of the light scattering experiments is shown in Figure 2 for a solution of DMPC at 30 °C. It is clear from these graphs that at low angles the correlation is nonlinear, which may be caused by a small fraction of large particles. As shown in the Appendix, extrapolation from higher angles to $\theta = 0$, $c = 0$ gives the molecular weight, M, of the smaller particles. From the dependence of R_θ, the Rayleigh ratio, on angle, we have determined the outer radius, R_Z, of the vesicles, using eq 10, given by Pecora and Aragon (1974). In this calculation, a bilayer thickness of 3.75 nm has been used.

The molecular weight and the radius of the vesicles, produced by the injection method, may depend on the injection velocity, the final alcohol concentration in the buffer, the lipid concentration in the buffer, and the lipid concentration in the alcohol. Also the rate of stirring of the mixture during injection and the size of the vessel could be of importance. A study of the influence of these factors showed that the injection velocity did not influence the molecular weight and radius of the lipid particles. However, a faster injection rate combined with a high rate of stirring lead to less curvature in the Zimm plots, indicating a smaller polydispersity. The size of the reaction vessel also appears less crucial. In preparing larger quantities of vesicles we used a larger vessel and a larger amount of lipid–alcohol solution, obtaining virtually identical results. Only the lipid concentration in the injected ethanol influenced the molecular weight and radius of the lipid particles markedly.

In Tables I and II we report the values of M and the dissymmetry Z, obtained by extrapolation from higher angles, and R_Z for DMPC and DPPC, respectively, as a function of the lipid concentration in the alcohol injected. From the observed molecular weights, M, the outer radii of the vesicles can

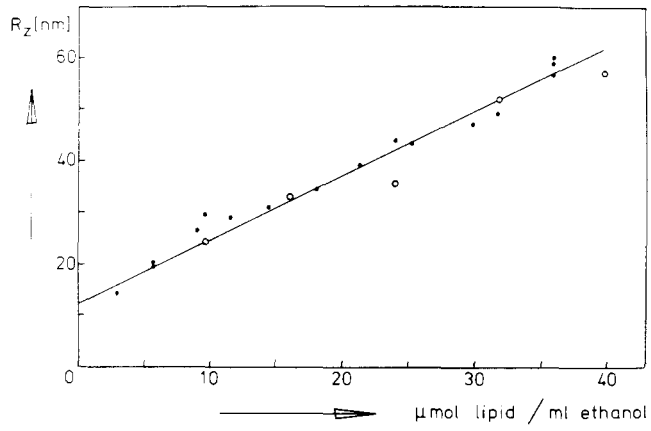

FIGURE 3: Outer radius (R_Z), as defined in Table I, of DMPC at 35 °C (●) and DPPC at 50 °C (○) vesicles as a function of the lipid concentration in the alcohol injected.

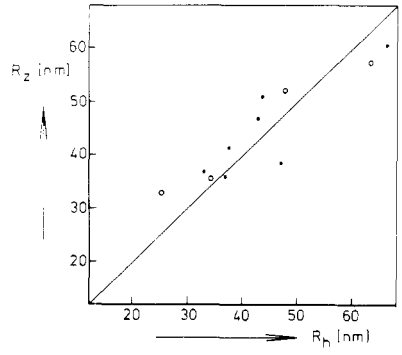

FIGURE 4: Outer radii (R_Z) for DMPC (●) and DPPC (○) vesicles compared with radii obtained from diffusion coefficients (R_h).

again be calculated, using the bilayer thickness and the surface area per monomer (0.6 nm^2). These values are reported as R_M in Tables I and II. It is seen that R_M and R_Z, calculated from independent measurements, agree rather well. These results confirm the electron microscopy photographs, showing that the particles are indeed single bilayered vesicles. The extrapolated radii calculated from the dissymmetry are plotted in Figure 3 as a function of the lipid concentration in the alcohol for both DMPC and DPPC. It is seen that one single smooth curve describes the results of both lipids. The extrapolated minimum radius (~10 nm) is of the same order as that found for sonicated lipid solutions. The effect of increased stirring rate is merely to shift these curves downwards somewhat, thus yielding slightly smaller particles for the same lipid:alcohol ratio. From these figures we conclude that decreasing the lipid:alcohol ratio yields smaller particles.

The reason for this experimental observation must be sought in the difference in solubility of the lipid in alcohol and water. Addition of more alcohol to a given quantity of lipid will increase the solubility of the lipid in the mixture. The injected lipid solution will then form smaller lipid containing droplets, which will yield smaller vesicles after reorientation of the lipid monomers. This explanation is confirmed by the observation that addition of alcohol to the buffer solution before injection does yield smaller vesicles, as compared with injection in a pure buffer solution. Also, injection of a larger amount of lipid–alcohol into pure buffer solutions gives a larger polydispersity, caused by the different sizes of lipid droplets formed in this case. However, these effects can be minimized by a faster stirring and injection rate.

The difference between the radii R_M and R_Z (see Tables I and II) may be caused by a small fraction of larger particles present. Such a fraction appears to be present even in well sonicated samples (Andrews et al., 1975). As shown in the Appendix we expect in this case $R_Z > R_M$ which is indeed observed. In a few cases, e.g., for a DMPC concentration of 9.0 × 10^{-3} mol/L, R_M is exceptionally low compared with R_Z, which we ascribe to a larger degree of polydispersity in these dispersions.

As a further independent check on our results, we have determined diffusion coefficients of the vesicles prepared by our procedure via laser light scattering.

With the Stokes–Einstein relation a hydrodynamic radius, R_h, can be calculated from these diffusion coefficients. The results for DMPC and DPPC are shown in Figure 4, compared with values obtained from the dissymmetry (R_Z). As can be seen, the two values are approximately the same.

In summary we may conclude that the modified injection method, as described in this paper, offers the possibility to make single bilayered vesicles of different radius in a reproducible and reliable way. The only important factor in determining their final size is the concentration of the lipid in the alcohol injected in the buffer solution.

TABLE I: Values of the Molecular Weights (M) and of Z for DMPC for Different Lipid Concentrations in the Alcohol Injected (c).[a]

$c \times 10^3$ (mol/L)	$M \times 10^{-6}$ (g/mol)	Z	R_Z (nm)	R_M (nm)
2.88	5.0	1.02	14.0	15
5.76	7.6	1.07	20.0	17.5
5.76	6.25	1.06	19.3	16.2
9.00	2.6	1.16	26.5	12.5
9.60	12.2	1.20	29.5	22.5
11.50	11.4	1.19	29.0	21.5
14.40	6.6	1.22	31.0	16.7
18.00	23.4	1.28	34.5	23.2
21.15	23.25	1.38	39.0	30.2
24.00	24.1	1.54	44.2	30.5
25.20	28.6	1.50	43.3	33.0
29.80	43.7	1.62	47.0	40.7
31.65	41.6	1.71	49.0	39.8
36.00	83.4	2.35	60.0	55.5
36.00	74.2	2.11	56.5	52.5
36.00	71.5	2.19	58.0	50.7

[a] Z was taken as the ratio of R_{45} and R_{135}, obtained from the straight line, extrapolated from higher angles for $c = 0$. Radii calculated from M and Z are given as R_M and R_Z, respectively. Errors are about 10% for M, 1% for Z, and 15% for R_Z.

TABLE II: The Same Data as in Table I but for DPPC.

$c \times 10^3$ (mol/L)	$M \times 10^{-6}$ (g/mol)	Z	R_Z (nm)	R_M (nm)
9.57	18.6	1.12	24.0	26.2
15.96	20.9	1.25	33.0	28.0
23.93	21.9	1.30	35.5	28.2
31.91	44.6	1.84	52.0	41.5
39.89	71.4	2.15	57.3	49.6

Appendix

The light scattering of a dilute solution of monodisperse particles of molecular weight M is given by (Stacey, 1956):

$$R_\theta = KcMP(\theta) \quad (1)$$

where R_θ is the Rayleigh ratio at angle θ to the incident beam,

c is the weight concentration of the solute, and $P(\theta)$ a correction factor, accounting for intraparticle interference. The constant K is defined as:

$$K = 2\pi^2 n_0^2 (dn/dc)^2 N_{av}^{-1} \lambda_0^{-4} \quad (2)$$

with n_0 the refractive index of the solution, dn/dc the refractive index increment, N_{av} Avogadro's number, and λ_0 the wavelength of the incident light in vacuo.

For a polydisperse system eq 1 is generalized to:

$$R_\theta = K \sum_i c_i M_i P_i(\theta) \quad (3)$$

and in a Zimm plot the quantity

$$\frac{Kc}{R_\theta} = \frac{K \sum_i c_i}{K \sum_i c_i M_i P_i(\theta)} \quad (4)$$

is plotted vs. angle for different concentrations. Assuming for simplicity that only two fractions, small and large particles, are present in concentrations c_1 and c_2, respectively, and that at higher angles $c_1 M_1 P_1(\theta) \gg c_2 M_2 P_2(\theta)$, we obtain, using eq 4 (Kratochvil, 1965):

$$\frac{Kc}{R_\theta} = \frac{c_1 + c_2}{c_1 M_1 P_1(\theta)} \quad (5)$$

Thus extrapolation of Kc/R_θ from high angles to $\theta = 0$, $c = 0$ gives approximately $1/M_1$. The values for M_1 obtained in this manner will be smaller than the "true" M_1 by a factor $c_1/c = c_1/(c_1 + c_2)$ which is hopefully close to unity. Hence we expect the radius R_M, calculated from M_1 obtained by the extrapolation procedure, to be smaller than R_Z, calculated from the angular dependence of the scattered light intensity.

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