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THE PREPARATION OF LARGE SINGLE BILAYER LIPOSOMES BY A FAST AND CONTROLLED DIALYSIS

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Summary

A new method is described for the preparation of large, homogeneously sized, single bilayer phospholipid vesicles. This method, based on a fast and controlled dialysis of sodium cholate from phosphatidylcholine/cholate mixed micelles, has the advantage of high yield of homogeneous vesicles avoiding any dilution and mechanical stress during preparation.

Physicochemical properties of these vesicles are examined by several techniques and compared with those prepared with other methods.

Introduction

Phospholipid vesicles are one of the most extensively studied systems as models for biological membranes. The major aspects of studies on these model membranes involve the entrapment of different molecules, either to study their permeation through the bilayer or to use the vesicles as drug carriers for biological and therapeutical applications [1].

Aqueous liposome dispersions can be prepared by several methods. Mainly three methods are described: (a) ultrasonic irradiation [2—4], (b) rapid injection of an organic phospholipid solution into an aqueous system [5—7], and (c) the lately described gel chromatography method [8], which consists in solubilizing unsonicated phosphatidylcholine dispersions with sodium cholate and removing the detergent from the mixed phosphatidylcholine/cholate micelles by gel filtration. In all these methods of preparation several disadvantages are encountered such as vesicle size heterogeneity, mechanical stress, many-fold dilution, and some difficulties for entrapment of small molecules into the vesicles.

The purpose of this publication is to describe a new preparation method which is based on solubilization of phosphatidylcholine dispersions with sodium cholate followed by fast and controlled removal of the detergent by

dialysis. This procedure yields large homogeneous bilayer liposomes under prevention of most of the above-mentioned disadvantages.

Methods of preparation and Characterization

Phosphatidylcholine was isolated from fresh chicken egg yolks by the aluminia column method [9] and the oxidation index [10] was found to be 0.11 indicating a slight oxidation of 0.6%. Sodium cholate (Merck 99%), cholic [^{14}C]carboxylic acid ([^{14}C]cholate, NEN Chemicals) with a specific activity of 45 mCi/mmol and L-α-dipalmitoyl-(2-palmitoyl-[9,10-^{3}H]phosphatidylcholine (Appl. Sci. Lab. Inc., State College, Pa., U.S.A.) with 13 Ci/mmol specific activity were used. All other chemical reagents were of analytical grade. Phosphatidylcholine dispersions were prepared in 1 mM phosphate buffer, pH 7.3, adjusted to 0.16 ionic strength. This buffer was used throughout all further experiments. Mixed micelles were prepared by adding variable amounts of solid sodium cholate ^{14}C labeled to the ^{3}H-labeled phosphatidylcholine dispersions (1—2% (w/v) = 13—27 mM) to obtain molecular ratios of phosphatidylcholine/cholate in the range 0.1—1.0 according to Shankland [11]. For routine preparations a ratio of 0.625 (18.87 mM phosphatidylcholine/30.20 mM cholate) was found to be optimal on behalf of micelle formation. To avoid autoxidation and chemical degradation, the solutions were kept under nitrogen atmosphere. The clarified mixed micelle solutions were dialysed against the buffer for 20—24 h at 22°C. Fig. 1 shows the dialysis system used. It is a modified equilibrium Teflon cell (modified by Diachema Ltd., Rüschlikon/Zürich) with two flow compartments and a stirred reaction compartment with an internal volume of 6.5 ml. A membrane of pure cellulose with a molecular weight cut-off of 10 000 was used. Applying optimal experimental conditions as suitable stirring speed (75 rev./min) a membrane with reproducible permeability and an appropriate and constant flow rate the bilayer liposomes could be prepared with high reproducibility. The course of dialysis was followed by measuring the [^{14}C]cholate activity determined on a liquid scintillation counter (Searle Mark III, PCS scintillation cocktail) using a variable quench program with a ^{14}C efficiency of 93% and a 0.25% standard deviation of the net count rate at 95% confidence level. After completed dialysis the remaining cholate and the actual

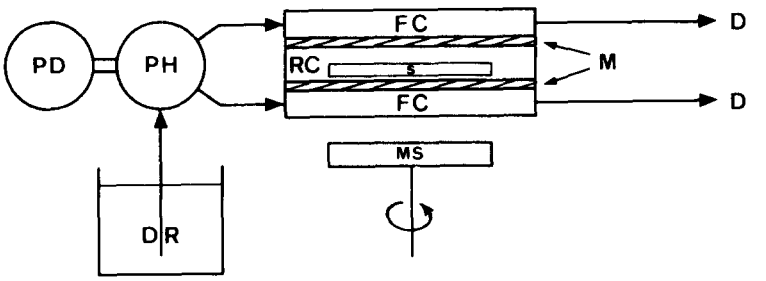

Fig. 1. Stirred three-compartment dialysis cell. RC, stirred reaction compartment with stirring bar S; FC, flowthrough compartments; M, membranes; DR, dialysis fluid (buffer) reservoir; PD, pump drive; PH, pump head; D, diffusate.

phosphatidylcholine concentrations were determined with $^3H/^{14}C$ dual label counting program. The remaining cholate concentration in the retentate was less than 0.5%, whereas the loss of phosphatidylcholine during dialysis was usually less than 5%. The remaining amount of cholate in the vesicles after dialysis was less than 0.03 mol % of the total lipids. Following dialysis, the liposome dispersions were centrifuged for 30 min at 100 000 × *g* (TGA ultracentrifuge, Al-65 fixed angle rotor) to remove any contaminations such as dust particles (especially when using the sample for laser light scattering measurements). The experimentally determined recovery of phospholipids in the supernatant was higher than 98%. This supernatant was used for the following physicochemical characterization studies.

Homogeneity, vesicle size and particle weight were determined by analytical ultracentrifugation (MSE Analytical Mark 2), dynamic laser light scattering, electron microscopy, and gel filtration on Sepharose 4B. For electron microscopy the liposomes (10 mg phosphatidylcholine/ml were cryofixed in a propane jet (Müller et al., unpublished) without the addition of a cryoprotectant. Freeze-fracturing was performed at −165°C in a Balzers BAF 300 apparatus equipped with electron guns. The platinium/carbonreplicas were examined in a Philips EM 301.

Diffusion coefficient and particle diameter were also determined by dynamic laser light scattering. The basic principles are briefly summarized: For a large number of identical spherical scatterers the normalized second-order correlation function of the photon count of the scattered light is given by [12]

$$q^{(2)}(\tau) = 1 + e^{-2D_T q^2 \tau} \tag{1}$$

where q is the scattering vector and D_T the translational diffusion coefficient. The Einstein-Stokes equation defines the hydrodynamic particle radius

$$r_h = \frac{kT}{6\pi\eta D_T} \tag{2}$$

where η is the viscosity, T the temperature and k the Boltzman constant. The polydispersity of the system can be described by a distribution function $F(\Gamma)$ of the decay rates

$$\Gamma = D_T q^2 \tag{3}$$

The deviation from a single exponential function can be characterized by the second moment [13]

$$\mu_2 = \int F(\Gamma)(\Gamma - \overline{\Gamma})^2 \, d\Gamma \tag{4}$$

where

$$\overline{\Gamma} = \int F(\Gamma)\Gamma \, d\Gamma \tag{5}$$

The quality parameters for polydispersity is defined by

$$Q = \frac{\mu_2}{\overline{\Gamma}^2} \tag{6}$$

The light scattering measurements were carried out with an argon ion laser (514.5 nm, Spectra Physics) scattering photometer at a scattering angle of 90° and a temperature of 23°C. The liposome concentration varied between 10 and 13 mg phosphatidylcholine/ml. All further experimental details are summarized in the legends to the figures and tables.

Results and Discussion

The hydrodynamic properties of the vesicles determined by diffusion and sedimentation velocity measurements yielded the size and weight properties listed in Table 1.

Fig. 2a shows the sedimentation Schlieren pattern of the vesicles prepared by the described method indicating a single component of high homogeneity with a corresponding S value of 6.7 S. The diffusion Schlieren pattern illustrated in Fig. 2b shows high symmetry. From diffusion data fitted by least squares an apparent diffusion coefficient D^{20}_{app} of $0.72 \cdot 10^{-7}$ cm^2/s was calculated. From these data a Stokes radius of 300 ± 25 Å resulted. Comparing this vesicle radius with those prepared with the other methods a 2-fold vesicle radius increase could be attained.

The dynamic laser light scattering results confirm the data obtained by the ultracentrifugal diffusion studies (see Table I). The Q factor (see eqn. 6) characterizing the polydispersity of a system was found to be in the range 0.04–0.08, indicating an extremely high monodispersity. Ideal scatterers as highly homogeneous latex spheres give corresponding Q values of 0.05. The slightly decreased particle radius compared to the one from the ultracentrifugal diffusion measurements is hard to explain. It is possibly a concentration effect which we observed. Decreasing vesicle concentration results in larger particle radii striving towards the Stokes radius of 300 Å.

The apparent vesicle radius was also confirmed by electron microscopy (see Table I). The mean apparent vesicle radius of 285 ± 32 Å was statistically evaluated from 250 particles from photographs of negatively stained liposomes

TABLE I

SIZE AND PHYSICOCHEMICAL PROPERTIES OF PHOSPHATIDYLCHOLINE VESICLES PREPARED BY FAST AND CONTROLLED DIALYSIS

Property		Method used
Diffusion coefficient	$0.72 \cdot 10^{-7}$ cm^2/s	Analytical ultracentrifuge [a]
Stoke's radius	300 ± 25 Å	Calculated from a
Sedimentation coefficient	6.52 ± 0.38 S	Analytical ultracentrifuge [b]
Particle weigth	$1.981 \cdot 10^7$ dalton	Calculated from a and b [c]
Diffusion coefficient	$0.81 \cdot 10^{-7}$ cm^2/s	Dynamic laser light-scattering [d]
Particle radius	266 ± 15 Å	Dynamic laser light-scattering [d]
Apparent vesicle radius	285 ± 32 Å	Electron microscopy

[a] Using a double sector synthetic boundary cell, 6000 rev./min, 20°C, $c_{phosphatidylcholine}$ = 6.5 mg/ml.

[b] $s_{20,w}$, 42 000 rev./min, 20°C, $c_{phosphatidylcholine}$ = 13 mg/ml, $s^0_{20,w}$ = 7.84 S.

[c] Using partial specific volume $\bar{v}_{20}$ = 0.984 cm^3/g [14].

[d] $c_{phosphatidylcholine}$ = 13 mg/ml.

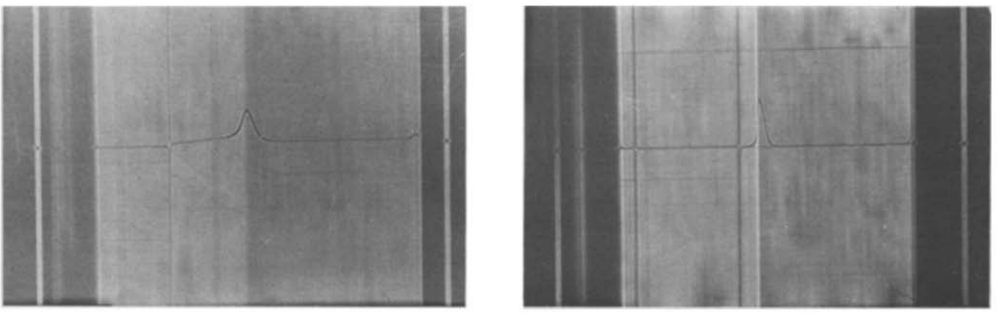

Fig. 2. Ultracentrifugal sedimentation and diffusion Schlieren patterns of dialysis method at 20°C. (a) 13 mg/ml, single sector cell, 40 000 rev./min, 4460 s after starting the experiment. (b) 6.5 mg/ml, double sector synthetic boundary cell, 6000 rev./min, 640 s after starting the experiment.

modified staining technique, Müller, M., unpublished). Fig. 3 shows again the homogeneity of the liposomes prepared by the dialysis method.

Fig. 4 shows the gel filtration pattern of bilayer liposomes prepared with our dialysis method. The elution pattern consists of only one narrow symmetrical peak without preceding elution of multilamellar structures as found by ultrasonic irradiation [4] and injection methods [5—7] as the elution pattern of ultrasonic irradiated liposomes demonstrates (open circles in Fig. 4). In order to

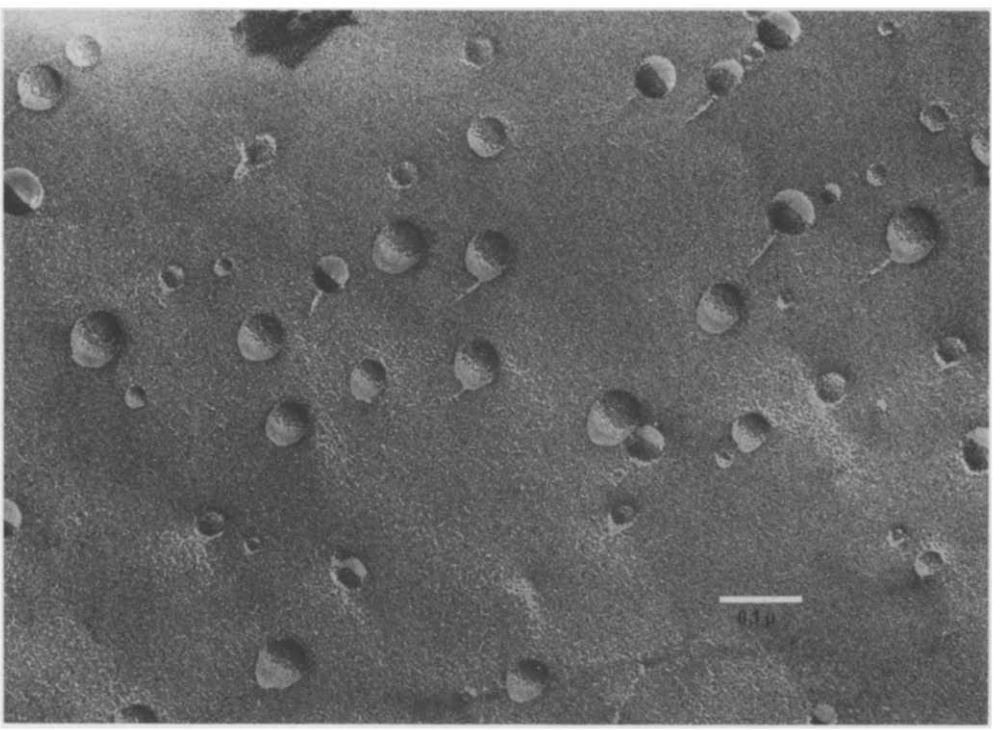

Fig. 3. Freeze-fracture electron microscopy photograph of vesicle preparation obtained by the dialysis method. The instrumental magnification was approx. 30 000, bar = 0.1 μm.

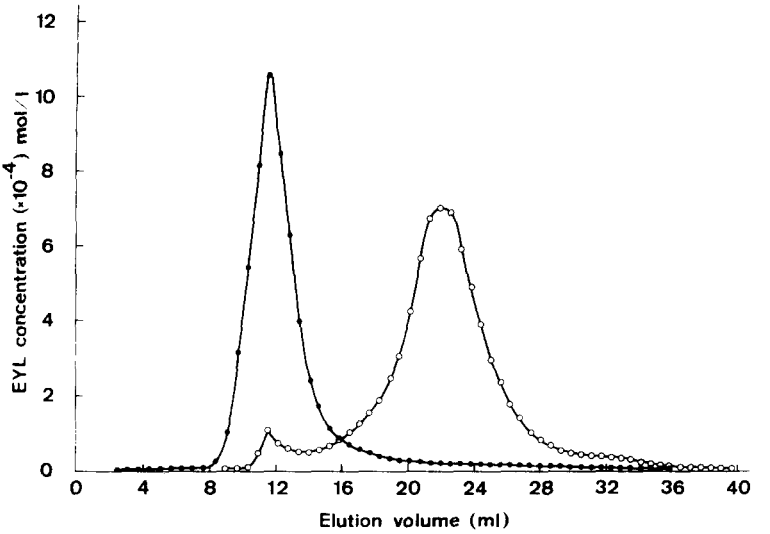

Fig. 4. Separation of a 1 : 1 mixture (mol/mol) vesicles and liposomes prepared by dialysis on Sepharose 4B, •, elution pattern of [^{3}H]phosphatidylcholine-labeled dialysed liposomes of 600 Å diameter, ○, elution pattern of [^{14}C]phosphatidylcholine (specific activity 50 Ci/mol, NEN)-labeled sonicated liposomes of 250 Å diameter. In the void volume (V_0 = 11.3 ml) the large single bilayer liposomes and some multilamellar structures of the sonicated preparation are eluted. Column dimensions 0.8 × 55 cm, flow rate 7.5 ml/h, T = 22°C.

elucidate the vesicle formation during dialysis, we studied the kinetics of the detergent removal. The upper curve (open circles) in Fig. 5 represents the permeation of cholate from a pure cholate solution (30.2 mM). At the beginning the dialysis process is of higher order due to the presence of micelles and possibly higher aggregates. After 90 min the cholate concentration in the reaction compartment has reached the critical micelle concentration. Below the critical micelle concentration the diffusion of the cholate is no longer perturbed by micelles and is, therefore, following first-order kinetics. The corresponding diffusion constant (K_D) evaluated from the time interval 90—200 min was $8.26 \cdot 10^{-3}$ cm/min.

In contrast to this, the diffusion of cholate from phosphatidylcholine/cholate mixed micelles leads to a quite different permeation pattern (see Fig. 5, filled circles). Obviously the diffusion is much slower and the process can be separated into three different sections. The first section covering the time interval from start to 50 min, the second from 51 to 90 min and the third from 91 to 200 min, possibly representing different states in the formation of liposomes. Thus we analyzed the described system at different times of dialysis over the entire liposome preparation period. The data in Table II allow the following interpretation of vesicle formation: During the first 30 min a single component with an apparent S value of 2.0 appears, still representing mixed micelles. After about 55 min a second component with a very high apparent S value of 17.2 can be seen. After 76 min these components reach their maximal apparent S values, illustrated in Fig. 6. In the subsequent dialysis course the first component disappears, whereas the second decreases to the final apparent S value of the formed liposomes. This formation is obviously a very

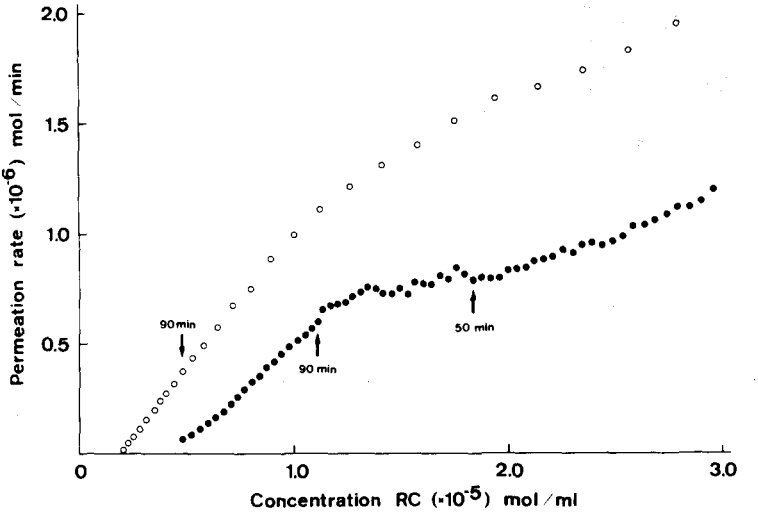

Fig. 5. Permeation rate of cholate in the presence (●) and in the absence (○) of phosphatidylcholine mixed micelles during the first 200 min of dialysis. Arrows indicate the time periods from which the apparent diffusion constants were calculated.

complex process which cannot be definitely described with our results. Nevertheless, the final formation process seems to be of second order as shown in Fig. 7. The extrapolated intercept on the time coordinate shows that the formation of liposomes with a corresponding apparent S value of 6.5 is completed after 580 min. Further dialysis is only necessary to remove the remaining cholate. To summarize the described liposome preparation method is presented a brief count of the advantages of this method in comparison with the usual preparations. It is an excellent method with the advantages of yielding large and homogeneous vesicles with high reproducibility and low expenditure of work. The indispensable concentrating procedures connected with the gel filtration and injection methods can be avoided. Furthermore, the liposome preparation does not contain any organic solvent residues and undefined

TABLE II

FORMATION OF VESICLES FROM MIXED PHOSPHATIDYLCHOLINE/CHOLATE MICELLES SOLUTIONS ANALYZED IN TERMS OF SEDIMENTATION COEFFICIENTS OF THE TWO OCCURING COMPONENTS $S_{1(app)}$ AND $S_{2(app)}$

Time of dialysis (min)	Sedimentation coefficient of component in Svedberg units		Remaining cholate in the reaction compartment (%)	Phosphatidylcholine/cholate ratio
	$S_{1(app)}$	$S_{2(app)}$		
37	2.0	—	67.3	0.886
55	2.5	17.2	51.3	1.162
76	3.5	21.3	41.4	1.439
100	—	17.6	34.0	1.753
150	—	13.4	16.8	3.550
360	—	8.6	3.5	16.980
1440	—	6.5	<0.5	>120

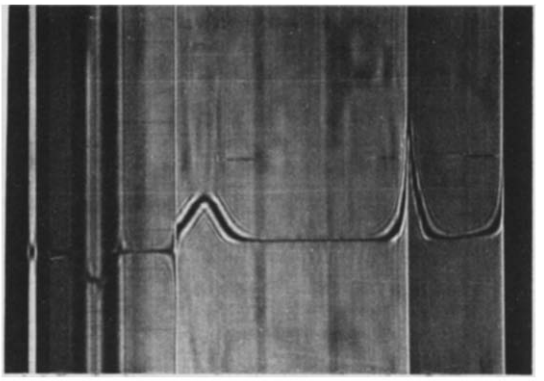

Fig. 6. Ultracentrifugal sedimentation Schlieren patterns of the phosphatidylcholine/cholate system after 76 min of dialysis. Sedimentation from left to right showing the two components $S_{1(app)}$ = 3.5 S and $S_{2(app)}$ = 21.3 S.

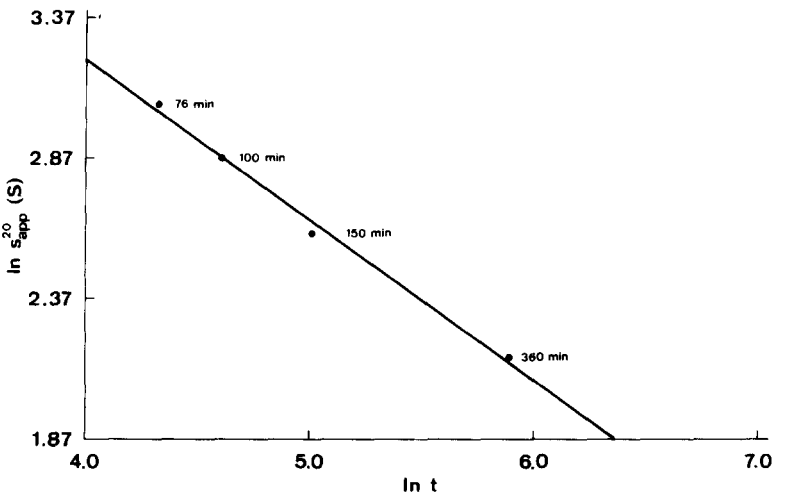

Fig. 7. Apparent sedimentation coefficients of the "liposome-forming component S_2" at different times of dialysis (cf. Table II).

multibilayer structures. It also seems to be a feasible method for the entrapment of labile biological and pharmacological active molecules, particularly in respect to the "smooth" way of preparation.

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