A NEW METHOD FOR PREPARATION OF PHOSPHOLIPID VESICLES (LIPOSOMES) – FRENCH PRESS

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1. Introduction

Recently there has been a growing interest in phospholipid vesicles (liposomes) not only as model membranes but also as carriers of drugs and as a tool for the introduction of various substances into cells or in the whole animal [1–3]. At least 5 different methods have been developed for preparation of liposomes. Ultrasonic irradiation leads to the formation of small (~250 Å diam.) unilamellar vesicles (SUV) [4–6]. Vesicles of a similar average size are formed when aqueous mixed dispersions of phospholipids and bile salts are passed through a Sephadex G-50 column [7].

Dialysis of detergents from phospholipid–detergent dispersions constitute another method for the preparation of vesicles. This procedure, which leads to the formation of heterogeneous population of liposomes with av. ~ 1200 Å diam. is especially suitable for reconstitution experiments in which proteins are included in the formed large unilamellar vesicles (LUV) [8–10]. Even larger vesicles are formed when mixtures of lipids in organic solvents are injected into heated aqueous solutions [11–13]; the size of these liposomes indeed depends on the solvent, rate of injection and temperature of the aqueous solution. Very large vesicles (~1 μm diam.) are formed when etheric 12 or petroletheric [13] solutions of phospholipids are injected into aqueous solutions at 70°C. Liposomes of similar sizes are also obtained from phosphatidylserine (PS) containing SUV when they are subjected to Ca^{2+}-induced fusion, followed by washing of the Ca^{2+} [14].

One of the most important properties of the various liposome preparations is their stability. In virtue of their very curved surface, the SUV are probably the least stable vesicles, in terms of their 'fusibility' [15]. Moreover, for some proteins, ultrasonic irradiation on one hand and detergents on the other, might be destructive. However, the tendency of SUV to fuse with cell membranes might be advantageous for their use as carriers. It is therefore an advantage to produce vesicles of small sizes without the use of detergents or ultrasonic irradiation.

It has been mentioned [1] that Hamilton and Goerke had prepared SUV by injecting aqueous suspension of phospholipids thorough the small orifice of a French press. No details of the method of preparation and no characterization of the formed vesicles are given.

Here it is shown that homogeneous preparations of quite small (315–525 Å diam.) unilamellar vesicles can be obtained by the use of a Power laboratory press (French press). In this apparatus, dispersions can be subjected to high hydraulic pressure. It is frequently used for distintegrating chloroplast material, blood cells and other cells with rigid walls such as yeast cells. Its application for vesicle preparation is presented.

2. Materials and methods

A dispersion of multilamellar liposomes of egg

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phosphatidylcholine (Makor Chemicals, PO Box 6750, Jerusalem) in 100 mM NaCl or 50 mM KCl was prepared as in [16]. Egg PC dispersion (5–25 ml) at 5–100 mM were used for the preparation of lipid vesicles. For NMR experiments the solutions were made in D_2O. The formed multilamellar PC dispersions were placed in a 1 in. diam. cylinder of the Aminco French pressure cell (Am. Instr. Co.) at 4°C. The pressure was raised to 20 000 p.s.i. and while keeping it constant, the valve at the bottom of the cylinder was slowly opened, to enable the dispersion flow out of the cylinder at ~1 ml/min. The effluent which was much clearer than the multilamellar liposomes was collected at 4°C. It was divided into two parts; the first was centrifuged for 4 h at 160 000 × *g* (Ti-65 fixed-angle rotor). Samples for negative staining [17] were taken before and after the centrifugation. The second part of the lipid dispersion was retreated by the French press, 3 more times, then a sample was taken for negative staining. Negative staining was performed using 1% phosphotungstate.

Trapping experiments were performed using two different systems:

1. Lipid vesicles were made using 20 mM egg PC in 5 ml 100 mM NaCl containing 5 μCi $^{45}Ca^{2+}$ (Amersham). External $^{45}Ca^{2+}$ was removed using repeating dialysis against 100 vol. NaCl.
2. Lipid vesicles were made using 10 mM egg PC in 100 mM potassium salt of 6-carboxy fluorescein (6 CF) (Eastman Kodak). The 6 CF was purified as in [18]. Free 6 CF was removed using gel filtration on Sephadex G-50 columns [18].

Sedimentation coefficients velocity were measured as in [13]. The areas of the bands were measured by weighing paper cutouts of blown-up photographs.

^1H NMR spectra were recorded on a Jeol MH 100 instrument, at probe temp. 27°C. Linewidths ($\nu_{1/2}$) were measured using a spectral width of 2 Hz/cm and a 1 Hz/s sweep rate. Experimental variabilities (which are mainly due to baseline determination) depend upon the widths and were estimated as amounting to ±10% for samples in which narrow lines ($\nu_{1/2} < 25$ Hz) of the paraffinic protons were observed and up to ± 50% of the measured $\nu_{1/2}$ for much broader lines. The linewidths were corrected for field inhomogeneity by subtracting the HOD linewidth from the experimental values

$$\Delta\nu_{1/2} = \nu_{1/2}\,(\text{signal}) - \nu_{1/2}\,(\text{HOD})$$

Spectra were also measured after the addition of $PrCl_3$. Intensity ratios in these measurements were detected by the MH-100 built-in integrator. Experimental errors in the measurements of these ratios were up to ± 0.1.

3. Results and discussion

The dispersions prepared by the French pressure cell, using one treatment only, included in most cases 2–4 species of PC aggregates, as evident from the Scheleiren bands in the fluc. Electron micrographs show also the presence of small multilamellar liposomes (fig.1A). When the dispersions were centrifuged for 1–4 h at 160 000 × *g* at 4°C, the supernatant, which always included > 60% of the phospholipid, contains only single lamellar vesicles of 250–500 Å diam. (EM). This differential high speed centrifugation gave rise to one band in the analytical ultracentrifuge, indicating that the resultant preparations were rather homogeneous in terms of the size of the formed PC aggregates. After 4 treatments by the French press (without centrifugation) the electron micrographs show a vesicles population of 260–630 Å diam. with ~94% of the vesicles 315–525 Å diam. (see fig.1B, fig.2).

In another experiment we prepared these aggregates in the presence of radioactively labelled Ca^{2+}. After preparation, the sample was dialyzed against 100 vol. 100 mM NaCl. Changes of the dialysis medium (5–6) and total time of 13–16 h was required to reach a constant value of radioactivity. Almost no change in the radioactivity was obtained when dialysis was continued against 100 mM NaCl for 30 h at 4°C, indicating the integrity of the formed membranes. In a control experiment, almost no $^{45}Ca^{2+}$ remained in the dialysis bag when it was added to a pre-made dispersion of French press aggregates, indicating entrapment (rather than binding) of the $^{45}Ca^{2+}$.

Similar results were obtained using 6 CF. The use of the latter enables one to perform very convenient experiments of leakage through the lipid bilayers. Comparison with SUV of egg PC show that under iso-osmotic conditions the leakage rate of 6 CF through the vesicle made by the French press is much

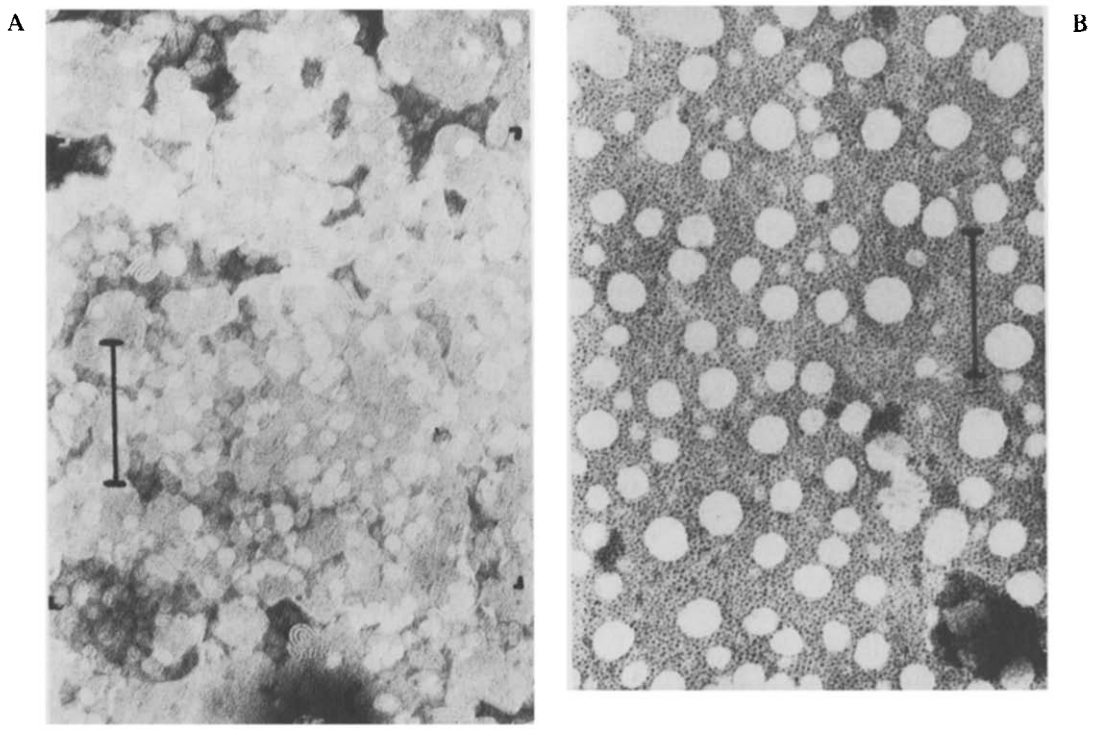

Fig.1. Electron micrographs of negatively-stained liposomes [17] made of egg phosphatidylcholine. After 1 treatment (1A) and 4 treatments (1B) by the French press. Final magnification was 105 000 ×; a 2000 Å measure is shown on both micrographs.

slower than through the bilayer of vesicle made by ultrasonic irradiation. Half-life time of 6 CF release is 1 day for the SUV and about 14 days for the new liposomes. The latter are more sensitive to change in osmotic pressure than the SUV as measured by the release of entrapped 6 CF upon transfer to hypotonic media.

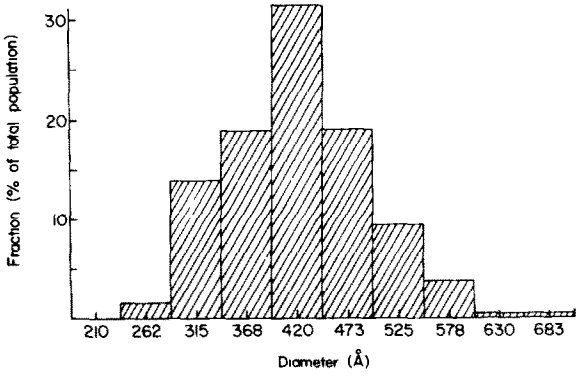

Fig.2. Size distribution of FUV after 4 treatments by the French press based on electron micrographs counting 800 vesicles (see text for details).

The vesicle preparation obtained by the French press is strongly dependent on the pressure; reducing the pressure to 15 000 p.s.i. gave lower yield of small vesicles. We suggest that the new type liposomes, prepared by repeated subjection to French pressure cell at 20 000 p.s.i. be denoted FUV (French press unilamellar vesicles).

Another parameter used for the characterization of these membraneous structures was the ratio between the number of PC molecules on the outer surface of the aggregates and the number of those molecules which do not face the outer bulk solution ($I \equiv N_{out}/N_{in}$). In micellar structures all the PC molecules face the outer solution. On the other hand in multibilayers only a small fraction does, thus for these liposomes I is much smaller than 1. For unilamellar vesicles, $1 < I < 2.2$ and the value of this

ratio decreases with increasing size of the vesicles [20–22]. It is especially sensitive to size changes in relatively small vesicles and since discrimination between populations of such vesicles by various techniques is not easy, determination of the I is probably one of the most adequate criteria for size evaluation of small vesicles.

The value of I can be experimentally measured by recording the proton magnetic resonance (PMR) spectrum of a dispersion after the addition of various lanthanide cations. These cations shift the choline head group signal of those PC molecules with which it can interact (peaks a_0 in fig.3A_1 and B_1) while the signal of the choline head groups of PC molecules in inner surfaces (peaks a_i) remain unchanged in their chemical shift [23]. The spectra of the liposomes prepared by the French press, before and after the addition of $PrCl_3$, are presented in fig.3B and $3B_1$, respectively. The ratio of intensities of peak a_0 to a_i is equal to 1.5, as compared to a value of ~2.2 observed from a sample of SUV obtained by further sonication of the preparate made by the French press (fig.$3A_1$). Identical spectra were also obtained 2 days after the addition of the $PrCl_3$, both in terms of the chemical shift difference and intensity ratios of a_0 to a_i. This again indicated the stability and integrity of the formed membranes and show that these vesicular aggregates are impermeable to Pr^{3+}.

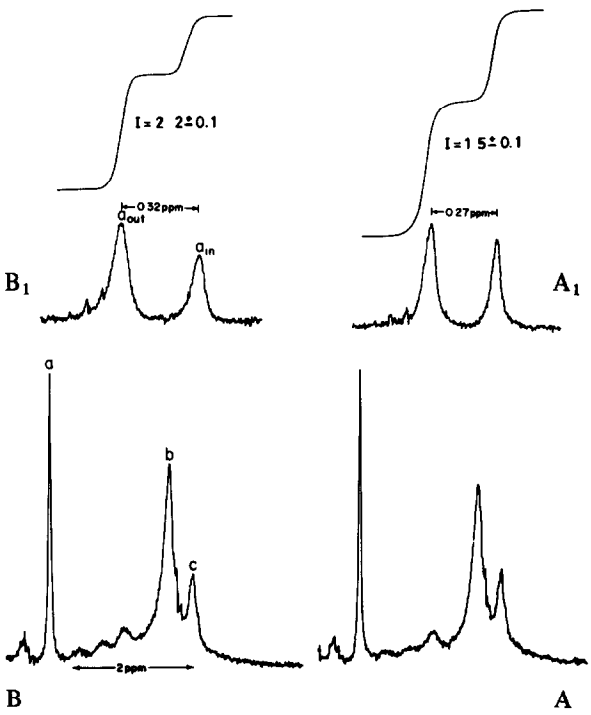

Fig.3. PMR spectra of vesicles prepared from egg PC (100 mM in D_2O) by (A) French Press and (B) further sonication (10 min heat system 350 W sonication at 0°C) of the sample prepared by the French press. Signals (a) are of the choline head groups, peaks (b) are due to the bulk methylene signals and (c) are of the terminal methyl groups. Spectra A_1 and B_1 are of the choline head groups and are obtained after the addition of $PrCl_3$ (30 mM) to the dispersions from which spectra A and B were recorded, respectively. The upper trace is an integration of the areas underneath the two peaks a_0 and a_i.

These measurements also provide an estimate for the average size of these vesicles, which according to theoretical considerations must be ~400 Å diam. [24]. The sonicated dispersion, for which I = 2.2, of ~220 Å diam. [21] namely the liposomes prepared by the French press have an average diameter almost twice that of sonicated vesicles. This value is in accordance with 315–525 Å diam. obtained from the electron micrographs.

One may predict that if the radius of a liposome is a major factor in determining the 'fusibility', then FUV will probably have a lower tendency to fuse with cells than SUV. Still they might have a high tendency to fuse with cells, since the packing of the PC molecules within them is quite disrupted as compared to LUV. This conclusion is based on the quite narrow lines observed for the methylene protons in the PMR spectrum (peak b has a linewidth of 25 Hz in FUV and 20 Hz in SUV).

In the context of the possible use of liposomes for drug application this may certainly be regarded as an advantage. There are more advantages to this method of preparation:

(i) Ease of preparation;
(ii) Reproducibility;
(iii) Its being nondestructive in terms of degradation, oxidation and protein denaturation;
(iv) High yield even at high lipid concentration;
(v) The possibility of preparing large volume of vesicle preparations.

These properties along with the much larger entrapped volume than that of SUV and the ease of preparation of large volumes of high concentration FUV make these vesicles a promising tool for drug

entrapment and vesicle–cell interaction studies. Preliminary experiments showed that other lipids can also be included in the FUV without altering their size. More specifically, liposomes prepared by this method from egg PC and cholesterol at various molar ratios ($\leqslant$ 48 mol % cholesterol) all showed a value of $I \simeq 1.5$ (~400 Å diam.). The potential of this method of preparation for reconstitution of membranes is presently under investigation.

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