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A Simple Method for the Preparation of Liposomes for Pharmaceutical Applications: Characterization of the Liposomes

STEPHEN PERRETT, MICHAEL GOLDING AND W. PATRICK WILLIAMS

Biomolecular Sciences Division, King's College London, Campden Hill, London W8 7AH, UK

Abstract—A new method for the preparation of liposomes is described that avoids the use of pharmaceutically unacceptable solvents and energy-expensive procedures such as sonication. The method is based on the initial formation of a proliposome mixture containing lipid, ethanol and water, which is converted to lipsomes by a simple dilution step. Measurements using 6-carboxyfluorescein as a marker indicate that water-soluble drugs can be trapped with extremely high efficiency (65–80% depending on lipid composition). The structural organization of the proliposome mixture and the final liposomes were characterized using electron microscopy and ^{31}P-NMR.

The potential of liposomes as drug-delivery systems has been widely recognized (Gregoriadis 1976; Szoka & Papahadjopoulos 1980; Mayer et al 1986). Interest has tended to concentrate on the use of liposomes for drug-targeting in systemic applications. Problems associated with the development of efficient targeting procedures have meant, however, that the use of liposomes in such applications has been largely confined to laboratory studies. It is becoming increasingly clear that liposomes also have considerable potential for use in a wide range of non-systemic applications. This field has received relatively little attention. The main reasons for this are the high cost of pure lipids required for the production of high entrapment vesicles and the lack of a simple practical method for the routine or large-scale production of such vesicles. The requirements in terms of lipid purity for such applications are less stringent than for systemic applications. In the longer term, therefore, it is the availability of a simple, efficient technique for the preparation of liposomes which can be scaled up reliably and economically that is likely to be the limiting factor rather than the cost of the starting materials.

Liposomes are normally characterized in terms of their size and the number of lipid bilayers surrounding their central aqueous compartment. The main categories are multilamellar vesicles (Bangham et al 1965), small unilamellar vesicles (Huang 1969; Johnson & Bangham 1969; Batzri & Korn 1973), large unilamellar vesicles (Deamer & Bangham 1976; Szoka & Papahadjopoulos 1978; Olson et al 1979) and cell-size vesicles (Kim & Martin 1981). Of these, multilamellar vesicles are likely to be of the most general use. The conditions required for their preparation also tend to be much less demanding.

A variety of procedures for the formation of multilamellar vesicles of different types has been described in the literature (Bangham et al 1965; Kirby & Gregoriadis 1984; Gruner et al 1985; Kim et al 1985; Payne et al 1986; Pigeon et al 1987). All of these methods, however, suffer from one or more drawbacks in terms of suitability for bulk manufacture for pharmaceutical applications. These vary from a requirement for pharmaceutically unacceptable solvents, which could give rise to undesirable solvent residues, to the involvement of energy-expensive steps such as sonication which cannot readily be scaled up. The percentage entrapment achievable by some of the methods is also inherently very low. In this study, we describe a new method for the preparation of multilamellar vesicles, involving the production of an initial proliposome preparation which is converted to a liposome dispersion by a simple dilution step that avoids these problems.

Correspondence to: W. P. Williams, Biomolecular Sciences Division, King's College London, Campden Hill, London W8 7AH, UK.

Materials and Methods

Liposome preparation

All lipids used were purchased from Lipid Products (Redhill, UK). Egg-yolk phosphatidylcholine (Grade 1) was supplied in chloroform:methanol. Lipid mixtures consisting of 42 μmol phosphatidylcholine (PC) and 12 μmol cholesterol (CHL), either alone or together with 6 μmol phosphatidic acid (PA) or 6 μmol stearylamine (ST) were supplied in a premixed form in 1 mL chloroform:methanol dispensed into individual ampoules. Lipid dissolved in chloroform:methanol was used in the present experiments for the sake of convenience. When the avoidance of chloroform residues is of prime importance, ethanolic lipid solutions or dry lipid can be used as alternative starting materials.

Liposomes were prepared using a method involving the production of an initial proliposome mixture which was subsequently converted to a standard lipsome dispersion by dilution in buffer. Typically, the proliposome mixture was prepared by transferring 100 mg of PC, or the contents of three ampoules of mixed lipids corresponding to a similar weight of PC, to a small stoppered tube. The lipid was thoroughly dried under nitrogen. It was then dissolved in warm ethanol (80 mg) and 25 mM Tris-HCl, pH 7·4 (200 mg) was added to yield a (100:80:200 w/w/w) lipid:ethanol:water mixture. This mixture was heated to 60°C for a few minutes and then allowed to cool to room temperature (20°C) yielding a proliposome mixture. The proliposome mixture was finally converted to a liposome suspension by

the dropwise addition of 25 mM Tris-HCl, pH 7·4 to a final volume of 10 mL. The suspension was vortex-mixed throughout this last stage.

Entrapment studies

The entrapment efficiency of the liposomes was examined using 6-carboxyfluorescein as a marker. The marker was normally added in the buffer used to form the proliposome mixture. Its concentration in the proliposome mixture was adjusted so as to yield a concentration of 2 μg mL^{-1} in the final diluted liposome preparations. Entrapment efficiences were determined by centrifuging the liposomes at 100 000 *g* at 20°C for 1 h to pellet the liposomes, and assaying the supernatant spectrophotometrically at 490 nm for non-trapped marker. The trapped fraction was then calculated by difference.

Three-phase diagrams

Three-phase diagrams of lipid/ethanol/water systems were constructed by determining the positions of the boundaries between the clear isotropic, the precipitated bilayer, and liposomal regions of the diagram. The position of the boundary between the former two regions was determined by adding increasing amounts of buffer to different concentrations of PC in ethanol and noting the point at which precipitation of lipid first took place. The boundary between the liposomal region and the hydrated bilayer region was estimated from measurements of the entrapment of 6-carboxyfluorescein in mixtures containing different ratios of PC and ethanol as detailed above. The proliposome compositions giving rise to maximum entrapment were taken to mark the approximate boundary between the two regions.

Inulin inaccessible space

Liposomes were prepared as described above. [^{3}H]Inulin (0·05 μCi) was added to 10 mL samples of the liposomes. One set of samples was retained for counting and the others centrifuged at 100 000 *g* at 20°C for 1 h to pellet the liposomes. Portions (1·0 mL) were taken both from the supernatant of the centrifuged samples and the original liposome suspensions. Nine mL of scintillation fluid cocktail T (BDH Chemicals, Poole, UK) was then added to each sample and the samples counted for 5 min using a Beckman LS6800 scintillation counter. The inulin inaccessible space per unit volume of the liposomes (V) was calculated from the formula:

$$V = \frac{N_1 - N_2}{N_1} \quad (1)$$

where N_1 and N_2 are the number of counts min^{-1}, corrected for differences in quenching efficiency, for the supernatant and the liposome suspension, respectively. The value of the inulin inaccessible space per mole of lipid (V_i) was then calculated from the value of V and the weight of lipid per unit volume of liposome suspension.

Electron microscopy

Small samples (10–20 μL) of the liposome pellet were loaded into small copper holders and rapidly frozen in a liquid nitrogen jet freezer (Polaron, UK). The samples were freeze-fractured and replicated using a Polaron freeze-fracture device. Samples for negative staining were prepared by mixing one drop of the concentrated liposome suspension with a similar volume of 2% ammonium molybdate. A drop of this mixture was then transferred to a thin carbon film supported on an electronmicroscope grid. Excess liquid was removed by adsorption onto a filter-paper. All samples were examined in a Jeol 100CX II electron microscope.

^{31}P-NMR measurements

Samples for ^{31}P-NMR measurements contained 0·5–1·0 g lipid. Spectra were measured at a scan frequency of 101·256 MHz with 80% phosphoric acid as a standard, using a Bruker WM-250 spectrometer. Samples were not spun during the measurement and signals were averaged over 800–2000 scans to increase the signal-to-noise ratio.

Size measurements

The average diameter and size distribution of the liposomes were measured using a Malvern 4700 Laser Scattering Device.

Partition coefficient measurements

The partition coefficient of ethanol between lipid and water, defined as K = (g ethanol per g lipid)/(g ethanol per g water), was estimated as follows. 100 mg of PC was first dried in a rotary evaporator. The dried lipid film was then dispersed in a 2% ethanol solution. The resulting lipsomes were precipitated by centrifugation at 100 000 *g* at 20°C for 1 h. The concentration of ethanol in the supernatant fraction was measured using a Sigma ethanol determination kit and the concentration of ethanol in the lipid phase was then calculated by difference. The directly measured values of the partition coefficient were corrected for the presence of non-solvent water associated with the lipid headgroups using the relationship:

$$K = K' + f \quad (2)$$

derived by Katz & Diamond (1974a). K′ is the measured partition coefficient, K the true partition coefficient, and f the equivalent weight of water in g (g lipid)$^{-1}$ that completely excludes solutes.

Results

Three-phase diagram

The organization of lipid/ethanol/water mixtures can be conveniently summarized in terms of three-phase diagrams of the type presented in Fig. 1 for the pure PC system. The

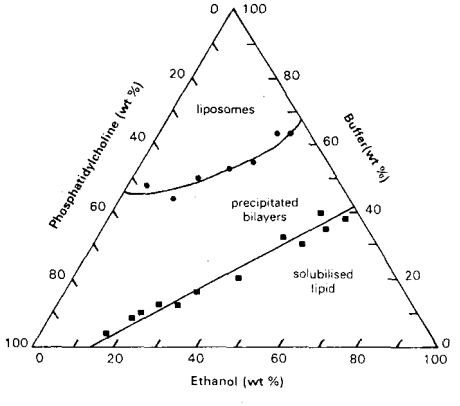


FIG. 1. Three-phase diagram for PC:ethanol:water (buffer) mixtures at 20°C. See text for details.

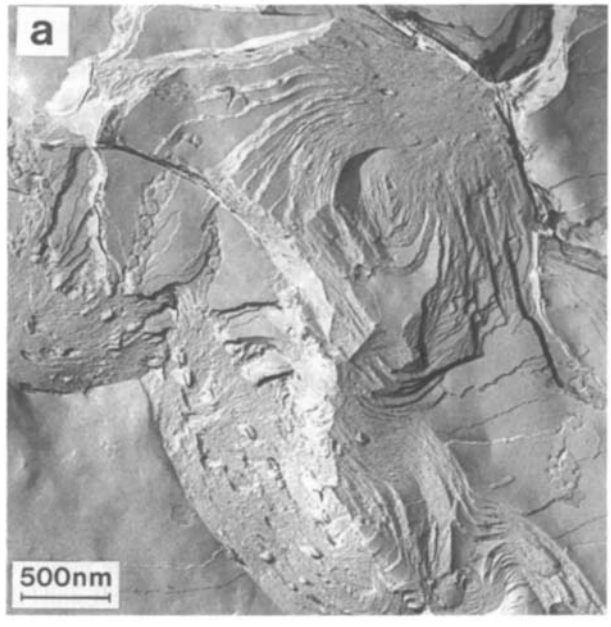
diagram is divided into three main regions corresponding to a clear solution of lipid in aqueous ethanol, suspensions of stacked hydrated lipid bilayers in excess aqueous ethanol, and liposomes. The boundaries between these regions were determined as described in the methods section. Mixtures lying in either of the two former regions constitute proliposome mixtures. Addition of excess buffer to such mixtures leads to the spontaneous formation of liposomes.

The precise positions of the boundaries between the different regions are difficult to determine as the regions themselves are not completely homogenous. Dissolved lipid and hydrated lipid co-exist in the neighbourhood of the border between the clear solution and hydrated bilayer regions. Similarly, precipitated hydrated bilayers and liposomes will co-exist near the boundary between these two latter regions. Our estimate of the position of the boundary between the solubilized lipid and precipitated bilayer regions is in good agreement with that previously reported by Tinker & Saunders (1968) for the boundary between the turbid and isotropic solution regions in this system. The position of the boundary between the hydrated bilayer and liposomal regions has not been previously explored.

Structural studies

The structure of the proliposome mixture varies with the relative amounts of lipid, ethanol and water (buffer) present in the mixture, the nature of the lipid used and temperature. Precipitated mixtures containing relatively low concentrations of water normally consist of extensive highly stacked lipid bilayers of the type shown in the freeze-fracture electronmicrograph presented in Fig. 2a. Mixtures containing higher concentrations of water, particularly those close to the border of the liposomal region, often contain tightly packed vesicles that have formed during the cooling phase of proliposome preparation. This is illustrated in Fig. 2b.

The structural organization of proliposome mixtures used for the preparation of PC liposomes was also investigated using ^{31}P-NMR. A series of ^{31}P-NMR scans for a range of PC proliposome mixtures containing lipid and ethanol in the ratio 100:80 (w/w) together with varying proportions of buffer is presented in Fig. 3. Proliposome mixtures containing less than 25 wt% water were clear liquids at 20°C and were characterized by sharp peaks reflecting isotropic averaging of the rapidly rotating lipid molecules. Tinker & Saunders (1968), using light scattering techniques, have shown that the lipid present in solutions of this type is in the form of small micelles consisting of 12–14 molecules of PC rather than as free monomers.



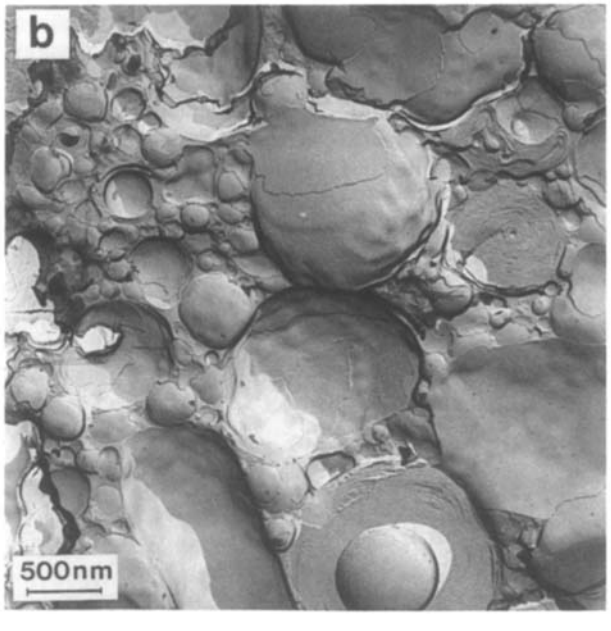


FIG. 2. Typical freeze-fracture electronmicrographs of proliposome mixtures containing phosphatidylcholine:ethanol:water (buffer) in the ratio (a) 100:80:100 and (b) 100:80:200 (w/w/w).

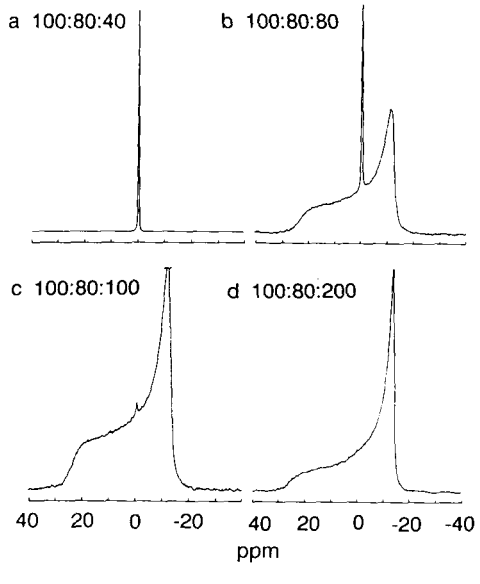


FIG. 3. Typical ^{31}P-NMR scans obtained for mixtures containing different proportions of phosphatidylcholine:ethanol:water (buffer). Measurements were made at 21°C.

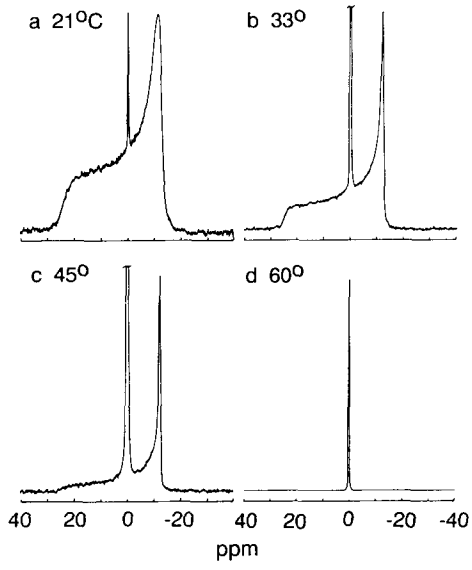


FIG. 4. ^{31}P-NMR scans for a proliposome mixture containing phosphatidylcholine:ethanol:water (buffer) in the ratio 100:80:85 (w/w/w) measured at different temperatures.

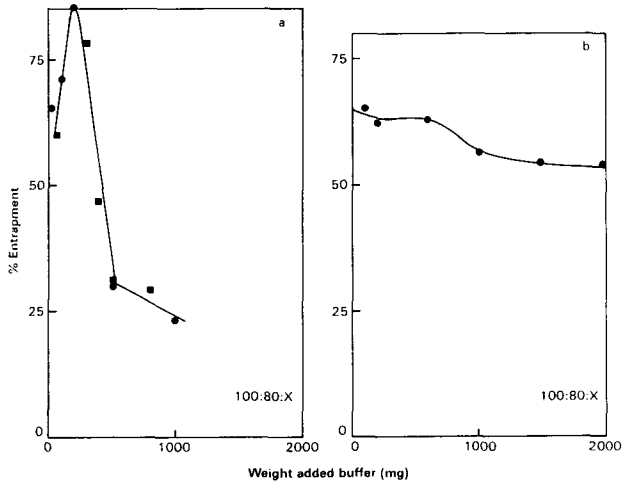


FIG. 5. Plots of the percentage entrapment of 6-carboxyfluorescein in (a) phosphatidylcholine liposomes and (b) phosphatidylcholine/cholesterol/phosphatidic acid liposomes prepared by the dilution of proliposome mixtures to a final concentration of 10 mg mL^{-1}, as a function of the weight of buffer (X) incorporated in the original proliposome mixtures. Different symbols refer to separate experiments.

As the amount of water present was increased and the lipid precipitated out of solution, a broad peak with a clear shoulder typical of the presence of lipid bilayers (Cullis & de Kruijff 1979), appeared and grew at the expense of the isotropic peak. Above 36 wt% water, no isotropic peak occurred. Further additions of water appear to lead to a relative lowering of the shoulder of the bilayer signal. The reason for this decrease is not known. It is possibly associated with the reorganization of the precipitated lipid into a concentrated liposome suspension.

The physical state of the proliposome mixtures is strongly dependent on temperature. The solubility of lipid in aqueous ethanol increases markedly with increasing temperature as illustrated by the increasing contributions of the isotropic component to the series of ^{31}P-NMR scans presented in Fig. 4. This strong temperature dependence is an important feature of the system as mixtures at the boundary of the proliposome region often convert from their original proliposome form to liposomes on cooling.

Choice of proliposome mixture composition

Typical plots showing the entrapment efficiency of the marker dye by PC and PC/CHL/PA liposomes are presented in Fig. 5. In these particular experiments, the proliposome mixtures contained 100 mg lipid and 80 mg ethanol together with varying amounts of aqueous phase. The entrapment efficiency of the liposomes, formed following dilution of the proliposome mixtures to a final volume of 10 mL, was plotted as a function of the weight of aqueous phase, present in the original proliposome mixtures.

In the case of the PC liposomes (Fig. 5a), maximum entrapment occurred when the liposomes were prepared from a proliposome mixture with a lipid:ethanol:water ratio of 100:80:200 (w/w/w). Liposomes prepared from proliposome mixtures of this composition routinely yielded percentage entrapments of 75–85%. Mixtures containing lower proportions of aqueous phase showed slightly reduced entrapment but a very much more dramatic decrease in entrapment efficiency occurred if the proportion of aqueous phase was increased above this optimal value. This sharp cut-off value, as discussed above, reflects the complete conversion of the initial proliposome mixture into a suspension of highly concentrated liposomes. Once the mixture has reached this critical point, inclusion of more aqueous phase in the proliposome mixture simply leads to the dilution of an existing liposome suspension and thus to a reduction in final trapping efficiency.

The corresponding plot for the entrapment efficiency of PC/CHL/PA liposomes (Fig. 5b), shows a much less marked dependence on the amount of aqueous phase incorporated in the original proliposome mixture. The absence of a clear cut-off value of the type seen for PC liposomes reflects the presence of the negatively charged PA. Similar results (not shown) were obtained using lipid mixtures containing positively charged stearylamine. Addition of water to lipids dissolved in ethanol leads to a precipitation of the lipid in the form of hydrated bilayers. In the case of charged lipids, mutual electrostatic repulsion between these bilayers results in extensive rearrangement during the heating stage of the proliposome preparation. This, in turn, leads to the trapping of larger volumes of buffer, and accompanying marker, within the liposomes formed from such mixtures.

The choice of proliposome composition is clearly depen-

Table 1. Percentage entrapment of 6-carboxy fluorescein in liposomes prepared from proliposome mixtures.

Lipid	Molar ratio	Lipid:ethanol:water (w/w/w)	Entrapment*
PC	—	100:80:200	77·7±5·7
PC/CHL	42:12	100:80:200	69·0±2·9
PC/CHL/PA	42:12:6	100:80:500	64·4±4·3
PC/CHL/ST	42:12:6	100:80:500	78·8±3·3

* Results of 6 independent measurements ± s.d.

dent on the composition of the lipid used in liposome preparation and must be optimized for different applications. A representative set of entrapment efficiences obtainable for the marker 6-carboxyfluorescein for the lipid mixtures used in this study is presented in Table 1. No detectable leakage of the marker was observed over a period of a weeks storage at 4°C.

Two-stage dilution method

The requirement that the drug, or marker, be incorporated into the proliposome could impose undesirable limitations on liposome loading levels in cases where the drug is heat-sensitive or ethanol insoluble. In practice, this can often be overcome by adopting a two-stage dilution procedure. The first dilution step involves the addition of the drug dissolved in just sufficient buffer to convert the proliposome mixture to a concentrated liposome suspension. The final liposome preparation can then be made up by a second dilution step using drug-free buffer.

Typical results obtained for PC and PC/CHL/PA liposomes using the marker 6-carboxyfluorescein are presented in Fig. 6. An initial proliposome mixture was prepared consisting of lipid:ethanol:water in the ratio 100:80:40 (w/w/w). No marker was included at this stage. This mixture was then converted to liposomes in two stages as described above. As in the previous entrapment experiments, the proliposome mixture contained 100 mg of lipid and was diluted to a final volume of 10 mL. The entrapment efficiency of the resulting liposomes was measured and expressed as a function of the total amount of aqueous phase present in the mixture after the first dilution step.

In the case of PC (Fig. 6a), the resulting liposomes show a similar pattern of entrapment efficiency to that obtained on incorporating the marker directly into the proliposome mixture (Fig. 5a). In both cases maximum entrapment is seen at a lipid:ethanol:water ratio of about 100:80:200 (w/w/w).

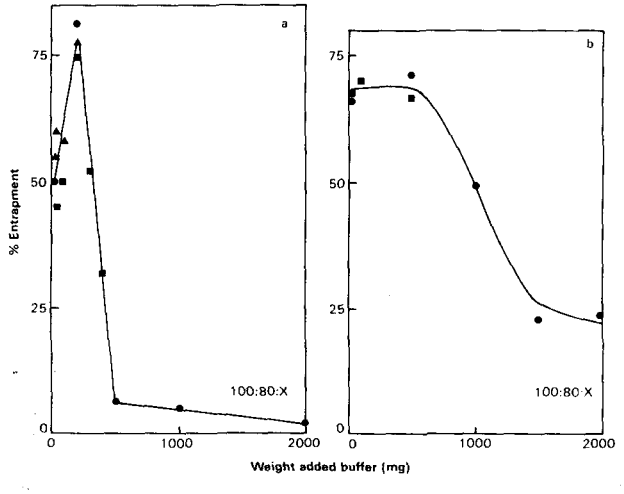


FIG. 6. Plots of the percentage entrapment of 6-carboxyfluorescein in (a) phosphatidylcholine liposomes and (b) phosphatidylcholine/cholesterol/phosphatidic acid liposomes prepared by a two-stage dilution of proliposome mixtures to a final concentration of 10 mg mL^{-1}, as a function of the weight of buffer (X) added in the first stage of the two-stage dilution method described in the text. Different symbols refer to separate experiments.

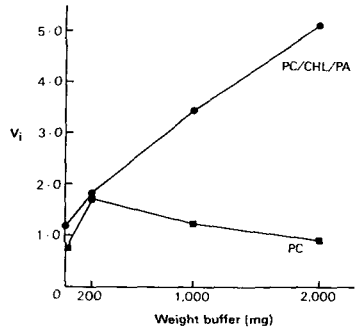


FIG. 7. Plot of the inulin impermeable space of phosphatidylcholine and phosphatidylcholine/cholesterol/phosphatidic acid liposomes prepared from proliposome mixtures containing lipid and ethanol in the ratio of 100:80 (w/w) as a function of the weight of buffer (X) in the original proliposome mixtures.

The corresponding results for the PC/CHL/PA system (Fig. 6b), show little or no difference to those obtained for the conventional method of proliposome preparation (Fig. 5b) for small additions of aqueous phase. There is, however, a marked difference if the weight of aqueous phase added in the first dilution stage exceeds 500 mg (corresponding to a lipid:ethanol:water ratio of 100:80:500 w/w/w). Under these conditions, a sharp decrease in percentage entrapment occurs similar to that seen for the PC system. This decrease, as discussed below, reflects the fact that the PC/CHL/PA system takes up much lower amounts of water before conversion to liposomes at room temperature than at elevated temperatures.

Inulin inaccessible space measurements

Measurements of the inulin inaccessible space of PC and PC/CHL/PA liposomes prepared from proliposome mixtures of different composition are presented in Fig. 7. To facilitate comparison, the measurements were performed using the same proliposome compositions as employed for the entrapment experiments. The measurements clearly demonstrate the greater capacity of the PC/CHL/PA proliposome mixtures to take up water. This capacity only exists at the elevated temperatures involved in proliposome preparation. It is the absence of such uptake at room temperature that accounts for the decrease in entrapment of 6-carboxyfluorescein in PC/CHL/PA proliposome mixtures subjected to two-stage dilution (cf. Figs 5b, 6b).

The final internal volume of liposomes formed from PC proliposome mixtures, in contrast, is only weakly dependent on the proportion of water originally present in the proliposome mixture. In the absence of charged lipids, there is little or no tendency for the proliposome mixtures to take up more water at higher temperatures. This accounts for the similarity of the entrapment efficiency plots presented in Figs 5a and 6a.

Characterization of final liposomes

A typical freeze-fracture electronmicrograph of a suspension of PC liposomes formed by the dilution of a proliposome mixture containing lipid:ethanol:water in the ratio 100:80:20 (w/w/w) is presented in Fig. 8a. The diameter of most of the liposomes appears to lie in the range 0·1–2·0 μm.

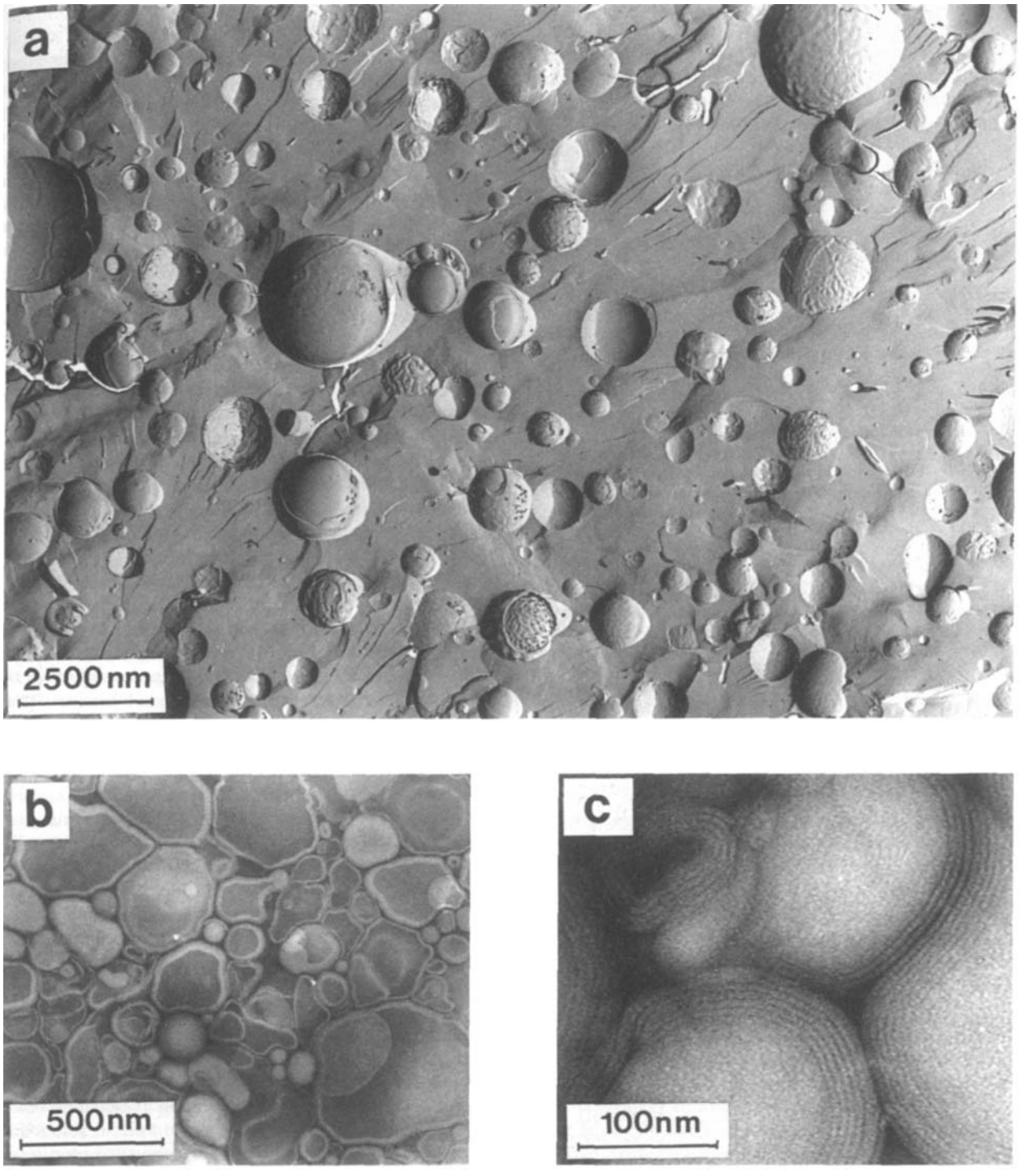


FIG. 8. Typical freeze-fracture electronmicrograph (a) and negative-stain electronmicrograph (b, c) of phosphatidylcholine liposomes prepared by the proliposome method.

Cross-fractures indicate that they are normally multi- or oligolamellar. This is confirmed by the electronmicrographs of negatively stained samples presented in Fig. 8b, c. The roughness seen in the exposed fracture-faces of some of the liposomes is probably due to post-fracture freeze-etching associated with the loss of the small amounts of residual ethanol associated with the liposomes (see below).

A more accurate assessment of the size of the liposomes was made using laser scattering. Histograms illustrating the size distribution of liposomes prepared from PC and PC/CHL/PA mixtures are presented in Fig. 9. The PC liposomes show a bimodal distribution with most of the liposomes having diameters of between about 0·3 and 0·5 μm and a smaller population of larger liposomes with diameters

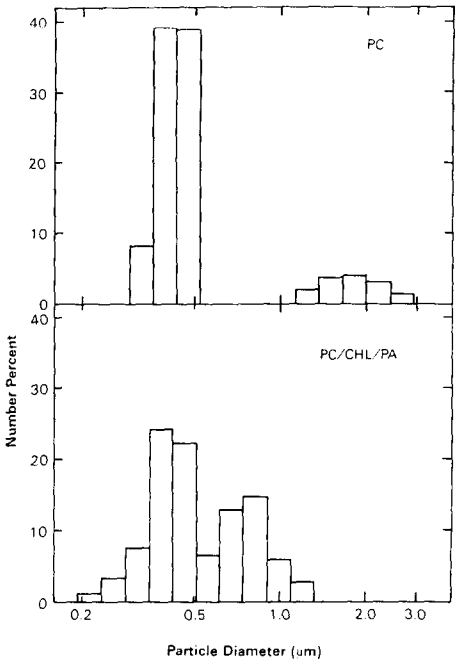


FIG. 9. Histograms showing the size distributions of phosphatidylcholine and phosphatidylcholine/cholesterol/phosphatidic acid liposomes prepared from proliposome mixtures containing lipid/ethanol/water (buffer) in the ratio 100:80:200 and 100:80:500 (w/w/w), respectively, plotted in terms of number percentage.

centred around 2 μm. The diameters of the PC/CHL/PA liposomes, in contrast, showed a single broad peak spanning a range from about 0·2 μm to just over 1 μm. Little variation in size was seen between batches of liposomes of given composition. Measurements (not shown) of liposomes prepared from proliposomes of different composition, however, revealed a considerable variation of liposome size with proliposome composition. In general, the further the proliposome mixture from the border between the liposome and proliposome regions of the three-phase diagram the smaller the liposomes. The inclusion of charged lipids in the proliposome mixture also tended to reduce the size of liposomes.

Residual solvent

The partition coefficient of ethanol between the aqueous and lipid phases was estimated as described in the methods section. Three independent measurements yielded values of 0·366, 0·334 and 0·327, respectively. These measured partition coefficient values were converted to true partition coefficient values by the addition of a factor f, corresponding to the equivalent weight of non-solvent water in the system as described in the methods section. Using a value of f = 0·18 (Katz & Diamond 1974b) for the partition of non-electrolytes into dimyristoyl PC liposomes, the average value of K for the three experiments was 0·522 at 20°C. Katz & Diamond (1974b) obtained a rather lower value of 0·441 at 25°C for the partition coefficient of ethanol in their study but this could well reflect a difference between the saturated lipid used in their experiments and the egg-yolk PC used here. The amount of ethanol directly associated with the lipid bilayers, as discussed below, is in any case extremely low.

Discussion

The preparation of multilamellar vesicles was first described by Bangham et al (1965). Their method was based on the formation of a dry lipid film which was subsequently converted to liposomes by mechanical agitation and/or sonication in the presence of aqueous media. Gruner et al (1985) and Pigeon et al (1987) have reported alternative methods for forming multilamellar vesicles based on a reverse-phase-evaporation procedure originally developed by Szoka & Papahadjopoulos (1978) for the formation of large unilamellar vesicles. These methods involve the emulsification of lipids in ether and the subsequent removal of organic solvent by evaporation. Kim et al (1985) have reported a different method of preparation based on the formation of chloroform-ether spherules in water. Again the final stage in the preparation involves a controlled evaporation of organic solvent.

Kirby & Gregoriadis (1984) have used a rather different approach in which they first prepare small unilamellar or multilamellar vesicles by conventional sonication methods and then dehydrate them by freeze-drying before rehydration in the presence of medium containing the material to be entrapped. Membrane fusion during the rehydration step leads to the formation of oligolamellar and multilamellar liposomes with high entrapment efficiencies. Payne et al (1986) have adopted yet another approach involving the initial production of a dry preparation formed by the evaporation of solutions of lipid in organic solvents in the presence of sorbitol or sodium chloride as a passive carrier. The liposome preparations are then formed by reconstitution in aqueous media.

Most of these methods involve the use of solvents that could give rise to pharmaceutically unacceptable residues. The elimination of such residues is likely to be particularly difficult and expensive under conditions of bulk manufacture. Many of the methods also involve sonication steps which are again difficult to scale-up. The proliposome approach that is described in this paper avoids these difficulties.

The method is based on the simple idea that mixtures of membrane lipids, ethanol and water can be used to form a concentrated proliposome preparation which can be converted to a stable liposome dispersion simply by dilution with excess aqueous phase. In general, the organization of lipid/ethanol/water mixtures can be described in terms of three-phase diagrams of the type shown in Fig. 1. Diagrams of this type divide into three principal areas; lipid in solution in aqueous ethanol, hydrated bilayers suspended in aqueous ethanol and a liposomal region. Addition of excess aqueous phase to lipid mixtures in either of the former regions (the proliposome regions) lead to the spontaneous formation of liposomal suspensions.

The precise form of the three-phase diagram will vary with the lipid and with temperature. The lipid in the proliposome mixture may be present in solution, in the form of stacked bilayer sheets (Fig. 2a), or as mixtures of sheets and vesicles (Fig. 2b). Given the micellar properties of lipids, the stacked sheets will themselves also almost certainly be part of larger closed structures. The relative proportions of sheet and vesicular structures depends mainly on the position of the

mixture in the three-phase diagram but could be modified by further processing such as extrusion.

The essential feature of the proliposome mixture is its ability to rearrange on dilution to form a stable liposomal suspension. Water-soluble drugs, or markers, included in the proliposome mixture are trapped within the liposomes formed by this technique with very high efficiency (Fig. 5, Table 1). The rearrangement occurring on dilution means that, if desired, the drug can be included in the diluting buffer rather than in the original proliposome. Under these conditions, high drug entrapment can still be achieved by use of a two-stage dilution procedure involving the initial addition of drug in a small volume of buffer that is insufficient to take the overall composition of the mixture out of the proliposome region followed by dilution in the usual way (Fig. 6).

The liposomes produced by the proliposome technique are multilamellar vesicles with diameters centred around 0·5 μm (Figs 8, 9). Liposomes formed from proliposome mixtures containing charged lipids tend to have appreciably larger entrapped volumes per mole of lipid than those formed from neutral lipids (Fig. 7). The average diameter of liposomes formed from mixtures containing charged lipids was found to be smaller than that of liposomes formed from neutral lipids (Fig. 9). This suggests that this increased entrapped volume reflects an increase in the total number of liposomes formed, and hence a decrease in the average number of bilayers per liposome, when charged lipid is present. This would be consistent with the increased electrostatic repulsion between bilayer surfaces when charged lipids are present.

The main difference between multilamellar vesicles formed by the present method and those formed by most other solvent-based techniques is the absence of toxic solvent residues. Relatively high concentrations of ethanol are present in the proliposome mixture but this can be advantageous as it allows the storage of the proliposome mixture in a sterile form suitable for the subsequent in-situ formation of liposomes. The liposome dispersions formed in this study contained 10 mg mL^{-1} PC and 8 mg mL^{-1} ethanol. Using our value of 0·522 for the partition coefficient of ethanol, this corresponds to an ethanol content in the lipid phase of 4·15 μg (mg lipid)$^{-1}$. This means that only 0·52% of the ethanol in the system is directly associated with the lipid bilayers. The presence of ethanol at these low levels has little or no effect on the permeability of water-soluble markers such as 6-carboxyfluorescein.

In conclusion, the liposomes produced by the proliposome technique described in this paper, are characterized by extremely high entrapment efficiencies, which by utilization of the two-stage dilution procedure are suitable for use with a wide range of drugs of differing water and alcohol solubilities. The method itself has a number of important advantages over existing methods of production of multilamellar vesicles for pharmaceutical purposes. It is simple and avoids the use of sonication steps or pharmaceutically unacceptable solvents, and it is eminently suitable for scaling-up for production purposes. The proliposome material is also suitable for extended storage for subsequent in-situ formation of liposomes.

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