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Preparation and characterization of semi-solid phospholipid dispersions and dilutions thereof

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Abstract

Highly concentrated, viscous to semi-solid phospholipid dispersions with phosphatidylcholine (PC) contents up to 600 mg/g or 780 mM were obtained by high-pressure homogenization. Dilution of these pastes with excess buffer led to 'classical' liposome dispersions. The dilution technique determined the homogeneity of the liposome dispersions. Handshaking yielded heterogeneous dispersions, which according to cryo-electron microscopy contained large multivesicular vesicles (MVVs) as well as small unilamellar vesicles (SUVs). By using a ball mill for dilution, however, the phospholipid pastes could be completely transferred into uniform SUVs with mean diameters of about 20–40 nm. The absence of bigger particles could be demonstrated both by a membrane filtration test through 0.2 μm pore filters and photon correlation spectroscopy. Lipid paste formation and subsequent dilution into liposomes led to high encapsulation efficiencies of the hydrophilic model compound 5,6-carboxyfluorescein. For true SUV dispersions, encapsulation efficiencies rose with increasing lipid contents up to a maximum of over 45% at original lipid contents of 600 mg/g. According to geometrical considerations, the packing of SUVs reaches densest sphere packing at this lipid content. In conclusion, semi-solid, vesicular PC pastes can be diluted by ball milling into homogeneous SUV dispersions with high encapsulation efficiency for hydrophilic compounds.

Keywords: Electron microscopy; Encapsulation efficiency; High-pressure homogenization; Liposome; Phosphatidylcholine; Small unilamellar vesicle

Abbreviations: FF-TEM, freeze-fracture transmission electron microscopy; MLV, multilamellar vesicle; MVV, multivesicular vesicle; NS-TEM, negative-staining transmission electron microscopy; PC, phosphatidylcholine; SPC, soy phosphatidylcholine; SUV, small unilamellar vesicle.

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1. Introduction

In recent years liposomes have gained great interest mostly as carriers for drugs, diagnostics and vaccines. Despite the enormous effort in research and development on liposomes as drug carriers, only a small number of liposomal products have arrived on the market so far. This may be due to various reasons. To our understanding, one of the problems which almost all preparation techniques for liposomal drug carriers have in common is the poor loading of water-soluble compounds into liposomes; with hydrophilic substances, usually only a minor proportion of the active compound ends up inside the liposomes. This proportion becomes particularly small when small liposomes are prepared. Small liposome sizes on the other hand are mandatory for many applications. Upon intravenous (i.v.) administration, for example, small liposomes have been found to circulate longer in the blood stream than large liposomes (Juliano and Stamp, 1975; Abra and Hunt, 1981; Allen and Everest, 1983). The unencapsulated portion of the active compound usually has to be removed from the preparation by, for example, size exclusion chromatography, ion-exchange or dialysis techniques. From the standpoint of industrial liposome production, all these purification methods are time consuming, costly, cannot be scaled up easily and are difficult to handle under aseptic precautions. Furthermore, with most hydrophilic low-molecular-weight active compounds, after removal of the unentrapped material leakage of the active compound out of the liposomes is observed. The permeability of liposome membranes is widely dependent on the physico-chemical properties of the bilayer and entrapped molecule, and on the temperature. For glucose, permeability coefficients ranging from 10^{-12} to 10^{-10} cm/s have been reported (Bresseleers et al., 1984), which would translate to release half-times in the magnitude of several hours to several days for 100 nm unilamellar vesicles. For another model compound, 5(6)-carboxyfluorescein, release half-times ranging from 13 to 190 days have been found with multilamellar liposomes (MLVs) of different lipid compositions (Hernandez-Caselles et al., 1990). The enormous variability of leakage values of this compound in the literature may, besides the above stated reasons, also be due to different permeabilities of different purity grades of the dye used (Domingo et al., 1989). Among the therapeutically used compounds whose entrapment into liposomes has been tried, the 8-aminoquinoline derivatives, for example, showed leakage half-times of several days (Alving and Swartz, 1984). Thus, leakage rates often are shelf-life limiting for liposomal preparations or do not allow storage of (unstabilized) liposomes at all. Various techniques have been developed to overcome this problem, for example, remote loading of preformed liposomes shortly before application through a pH gradient (Madden et al., 1990). Transmembrane ammonium sulfate gradients have been described to yield efficient and stable entrapment of anthracyclines (Haran et al., 1993). This concept, however, is restricted to certain amphipatic weak bases. Another promising and generally applicable approach is to improve encapsulation efficiency, if possible to such an extent that removal of unentrapped drug may be omitted. There are two principal ways to improve encapsulation efficiency: (1) to make fewer but bigger liposomes instead of many small ones; and/or (2) to make more liposomes per preparation volume, i.e. to increase the lipid concentration. With regard to an increase of liposome size: at least when the intended way of application is i.v. injection, it is generally accepted that relatively small liposome sizes are stringent to avoid rapid clearance from the blood. Ideally, liposome size should not exceed 200 nm. So far, all techniques yielding liposomes well below 200 nm, however, seem to be hampered by low encapsulation efficiencies. Sachse and coworkers (Schneider et al., 1994) recently described a continuous high-pressure filter extrusion process yielding liposome sizes of 100–200 nm and entrapping hydrophilic contrast agents quite efficiently, so that removal of unentrapped contrast agent appeared avoidable. With this high-pressure extrusion technique, relatively high lipid concentrations were achieved. With regard to an increase of the phospholipid concentration: theoretically, a correlation between increasing lipid concentration and improved en-

trapment of hydrophilic compounds is obvious, so that the greater the amount of lipid present, the larger the membrane area, i.e. the more liposomes are formed per unit volume. This causes an increase of the proportion of the aqueous volume entrapped in liposomes compared to the total aqueous volume. As long as the active compound is truly water-soluble and does not bind to the membrane and as long as steric hindrance effects are negligible, the encapsulation efficiency should be determined merely by the ratio of internal to total aqueous volume. However, no systematic study on liposome preparations with lipid concentrations exceeding 300 mM has been undertaken so far to our knowledge. This is probably due to experimental difficulties. It is almost impossible to obtain homogeneously hydrated lipid samples of the above concentration range by the usual thin film technique within reasonable time periods because the dispersions are highly viscous. Furthermore, it must be taken into account that liposome formation with these lipid concentrations may be disturbed. Two experiences from earlier studies encouraged us to work with 'highly concentrated' lipid dispersions: we noticed that high-pressure homogenization can achieve 'forced hydration' of phospholipids and that certain high-pressure homogenizers, such as the APV Micron Lab 40, can process quite heterogeneous and viscous material without congestion problems.

The aim of the present study, therefore, was to investigate the preparation of highly concentrated phospholipid dispersions at concentrations of 250 mg/g or 300 mM and above by high-pressure homogenization in terms of formation of liposomes and encapsulation of hydrophilic markers.

2. Materials and methods

2.1. Preparation and dilution of lipid pastes

The preparation of lipid dispersions was performed by analogy with the one-step technique described previously (Brandl et al., 1990, 1993). In brief: dry powdered lipids were mixed with aqueous medium, e.g. 5,6-carboxyfluorescein solution and either allowed to swell under magnetic stirring or directly fed into a laboratory-scale high-pressure homogenizer (APV Micron Lab 40, APV Homogeniser, Lübeck, Germany). In case of binary lipid mixtures, a film or spongy cake was formed by dissolution of the lipids in ethanol and subsequent rotary evaporation or freeze-drying. Lipids used were a phosphatidylcholine (PC)-rich fraction of egg lecithin with at least 80% PC (Lipoid E 80, Lipoid, Ludwigshafen, Germany) or PC fractions from soy beans ($\geq$95–98% PC), natural or in fully hydrogenated form (Lipoid SPC or SPC-3, Lipoid, Ludwigshafen, Germany, or Phospholipon 100 or 100H, Nattermann Phospholipid, Cologne, Germany, respectively). Cholesterol (Croda, Nettetal, Germany) was recrystallized twice from methanol. The buffer was isotonic phosphate buffered saline with a pH of 7.4. The lipid/buffer or lipid/marker solution mixtures (batch sizes of 30–40 g) were homogenized at pressures between 15 and 160 MPa up to 15 times. If not stated otherwise, standard conditions of 70 MPa and 10 homogenization cycles were employed. The device used for resuspension of the concentrated lipid dispersions was a Retsch oscillating ball mill equipped with Eppendorf tubes and 2 mm glass balls. 5,6-Carboxyfluorescein (CF, Eastman Fine Chemicals, Rochester, NY, USA) was dissolved upon addition of NaOH and purified by column chromatography using Sephadex LH20 gel (Pharmacia, Freiburg, Germany). The solution was adjusted to pH 7.4 before use and diluted to 2 mM with phosphate-buffered saline.

2.2. Marker and lipid assays

Liposomes were separated from unentrapped marker by size exclusion chromatography using Sephadex G25 medium (Pharmacia, Freiburg, Germany). In order to obtain clear solutions, the liposomes were transformed into mixed micelles by addition of cholate in excess (Gallensalzmischung für die Mikrobiologie, Merck, Darmstadt, Germany). Free and liposomal marker were quantified fluorimetrically (excitation at 490 nm, emission at 518 nm) against a calibration curve. Encapsulation efficiency was calculated as the percentage of liposomal marker compared to total

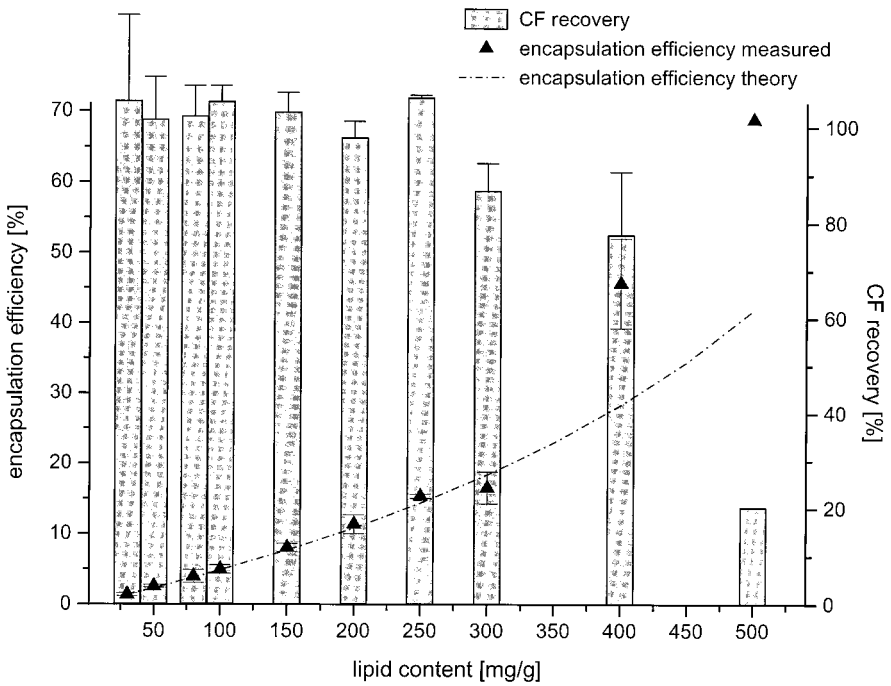

Fig. 1. Encapsulation efficiency and marker recovery of CF upon entrapment in SPC liposomes, prepared by 5-fold homogenization at 70 MPa and subsequent dilution and gel chromatography in relation to lipid content. Results of three consecutive experiments given as mean ± S.D., except for data point at 500 mg/g (single value).

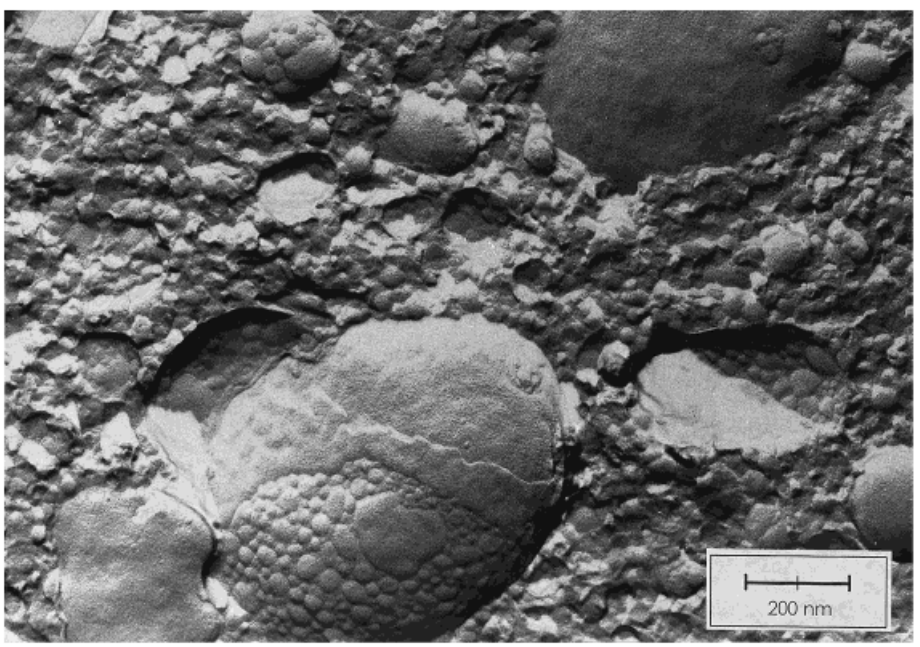

Fig. 2. Liposome paste, SPC content 450 mg/g, prepared by 10-fold high-pressure homogenization at 70 MPa; freeze-fracture electron micrograph.

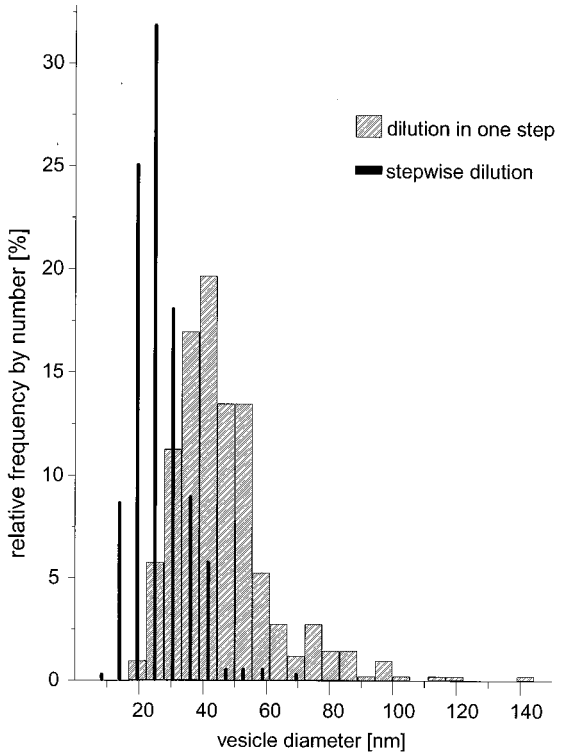

Fig. 3. Results of NS-TEM particle size analysis of vesicle dispersions obtained by various dilution techniques from homogenized 400 mg/g SPC paste.

(free and liposomal) marker. Recovery was calculated as the percentage of free and liposomal marker compared to the theoretical marker content. Theoretical encapsulation efficiencies of the marker into liposomes were calculated and plotted using Origin software (Microcal, Northampton, MA, USA). The calculation is based on purely geometrical considerations:

$$EE = \frac{v_{in} \cdot \frac{n}{x}}{v_{tot} - v_{li} \cdot n} \cdot 100\% = \frac{\frac{4}{3}(r_{out} - d_{bil})^3 \pi \cdot \frac{c \cdot N_A}{x}}{v_{tot} - v_{li} \cdot c \cdot N_A} \cdot 100\%$$

where: EE is encapsulation efficiency [%]; v_{in} is the aqueous volume inside one vesicle [nm^3]; n is the number of PC molecules per unit volume (1 l) [l^{-1}]; x is the number of PC molecules per vesicle; v_{tot} is the unit volume of 1 l equal to 10^{24} nm^3 [nm^3]; v_{li} is the lipid volume of one vesicle [nm^3]; r_{out} is the outer radius of the vesicle [nm]; d_{bil} is the thickness of the bilayer [nm]; c is the lipid concentration [mol/l]; and N_A is the Avogrado number.

The following assumptions were made using data from the literature (Lasic, 1993): small unilamellar liposomes with 21 nm external diameter and a bilayer thickness of 3.7 nm contain 2377 PC molecules per vesicle with a lipid volume of 1.223 nm^3 per PC molecule.

Phospholipids in liposome preparations were quantified by using the Stewart assay as described in New (1990). The optical density of the phospholipid/ferrothiocyanate complex in chloroform was read at a wavelength of 485 nm and compared with a calibration curve.

2.3. Electron microscopy and particle size analysis

Freeze-fracture transmission electron microscopy (FF-TEM) was carried out as described earlier (Brandl et al., 1997). In brief: small amounts of the lipid paste were shock-frozen in liquid ethane. The sample was fractured using a precooled freeze-fracture unit (BAF 301, Baltec, Balzers, Liechtenstein) at 173 K and between 3.5×10^{-6} and 1.4×10^{-5} Pa. The fractured surfaces were etched for 30 s at 173 K and vapor was deposited unidirectionally with platinum/carbon (2 nm) and carbon (30 nm) at an angle of 40–45°. The obtained replica was floated off and rinsed with ethanol/water or ethanol/chloroform mixtures. Micrographs were taken on a Philips EM400 transmission electron microscope operated at an acceleration voltage of 80 kV.

For negative-staining electron microscopy, specimens were prepared according to standard protocols: carbon-coated copper grids (Science Service, Munich, Germany) were hydrophilized by glow discharge. A drop of the liposome dispersion was placed on the grid, allowed to adsorb, and the surplus was removed by blotting paper. A drop of uranyl acetate solution, 2% (by mass,

Serva, Heidelberg, Germany) was added and the surplus was removed again. Grids were allowed to dry at ambient conditions and inspected with a Philips EM400 transmission electron microscope operated at an acceleration voltage of 80 kV. For particle size analysis, large-format prints of the micrographs at final magnifications of 100000-fold or higher were used to count at least 400 particles semi-automatically. Diameters were digitalized using a Summasketch II pad (Summagraphics, Seymour, CT, USA) and Trackon software (Ritzmann, Teningen, Germany). For calculation and plotting, Statgraphics software (STSC, Rockville, MD, USA) and Origin were used.

Cryo-electron microscopy was performed as follows: a small drop of the liposome dispersion was placed on a perforated carbon film grid (Science Service, Munich, Germany), and the surplus was removed with blotting paper. The resulting thin film was immediately shock-frozen by plunging into liquid ethane at 90 K using a Zeiss cryo-unit (Carl Zeiss, Oberkochen, Germany). Specimens were transferred with a cryo-transfer unit to a Zeiss CEM 902 equipped with a cold stage (Carl Zeiss, Oberkochen, Germany) and visualized at a constant temperature of 100 K. All images were zero-loss filtered, i.e. taken at a loss of energy of $\Delta E = 0$ eV. Micrographs were taken using the plate camera under minimum dose focussing conditions, i.e. approximately at a dose of 10^{-2} C/cm^2.

Alternatively, particle size distributions of the liposome dispersions were measured by dynamic laser light scattering using a Malvern Zetamaster (Malvern Instruments, Malvern, Worcestershire, UK).

For detection of large particles, a membrane filtration test was performed. Aliquots of the preparation were filtered through membrane filters of 0.2 μm pore size using a syringe and either polycarbonate membranes (Nucleopore, Tübingen, Germany) placed in a reusable filter holder or disposable Minisart NML units (Sartorius, Göttingen, Germany) containing a cellulose acetate membrane. Marker and phospholipid content were assayed before and after filtration as above.

3. Results and discussion

In order to study the influence of phospholipid concentration on encapsulation efficiency of hydrophilic compounds in small unilamellar vesicles (SUVs), a series of lipid dispersions was prepared with increasing soy phosphatidylcholine (SPC) contents from 30 mg/g or 40 mM to 500 mg/g or 650 mM. Coarse PC dispersions were obtained by mixing the lipid with CF solution on a magnetic stirrer. When lipid contents of 250 mg/g or more were used, lumps of lipid were observed which would not disappear even after several hours of magnetic stirring. Despite this macroscopic inhomogeneity, the coarse dispersions were fed into an APV Micron Lab 40 high-pressure homogenizer. After one or two homogenization cycles at 70 MPa, all dispersions appeared fully homogeneous. The dispersions showed increasing viscosities with rising lipid concentrations. Above lipid contents of 300 mg/g, they had a semi-solid or paste-like consistency. For chromatographic separation on a size exclusion gel column, the pastes were diluted. Surprisingly, all pastes up to lipid contents of 500 mg/g could be easily diluted by adding buffer and shaking. The diluted dispersions were macroscopically homogeneous. Resuspension of the more concentrated semi-solid preparations, however, required extended mixing by handshaking or on a vortex mixer for several minutes until macroscopically visible aggregates disappeared. Encapsulation efficiencies and recovery of carboxyfluorescein from SPC dispersions of increasing lipid content, prepared by 5-fold homogenization at 70 MPa and subsequent dilution, were determined. The results are given in Fig. 1 in relation to the PC contents of the original dispersions (before dilution). For comparison, the theoretical encapsulation efficiencies into SUVs were calculated as stated above and plotted as well. The encapsulation efficiency values (EEs) in general were found to be relatively small, below 20% for the usual lipid concentrations up to 200 mg/g. This is what one would expect for SUVs. Up to 300 mg/g, EEs were rising moderately with increasing lipid content, whilst beyond this limit they rose much more steeply, reaching maximum values of almost 70% encapsulation at 500 mg/g lipid content. At the

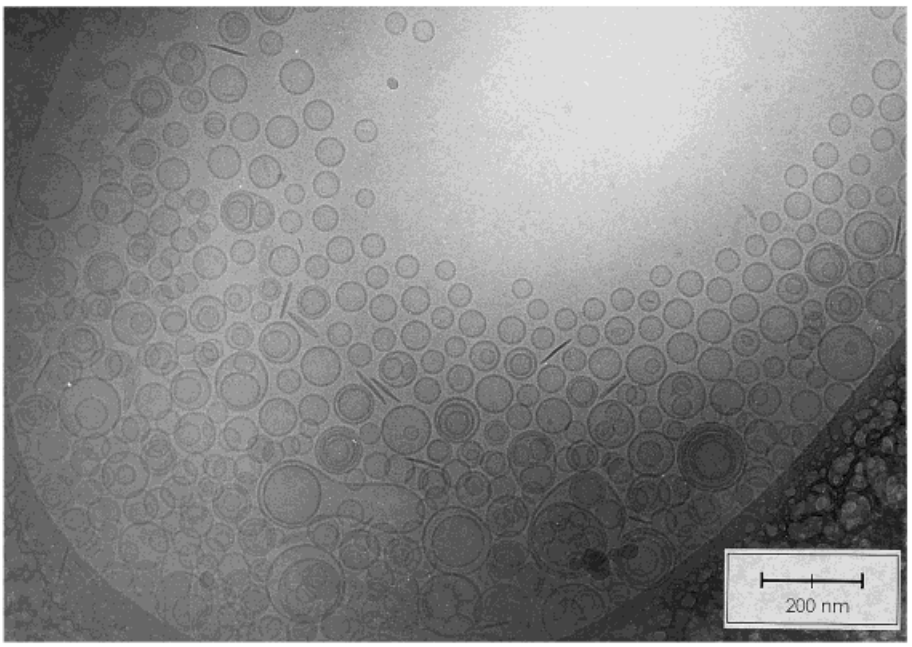

Fig. 4. Cryo-electron micrograph of liposome dispersion obtained by dilution by handshaking from HSPC/CH (1:1, mol/mol) paste. Paste of total lipid content 300 mg/g prepared by 10-fold high-pressure homogenization at 70 MPa.

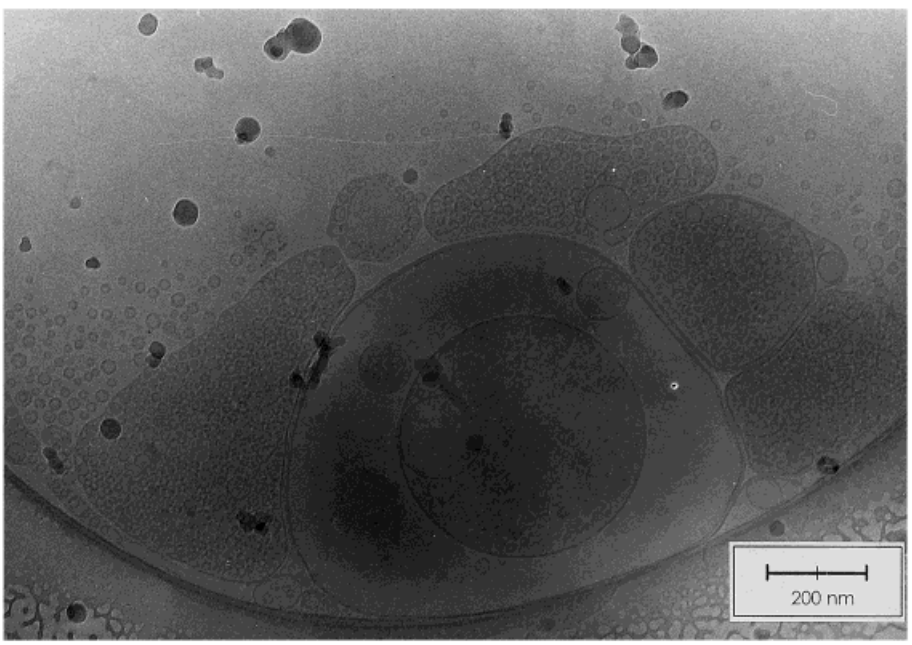

Fig. 5. Cryo-electron micrograph of liposome dispersion obtained by dilution by handshaking from 300 mg/g SPC paste. Paste prepared by 10-fold high-pressure homogenization at 140 MPa.

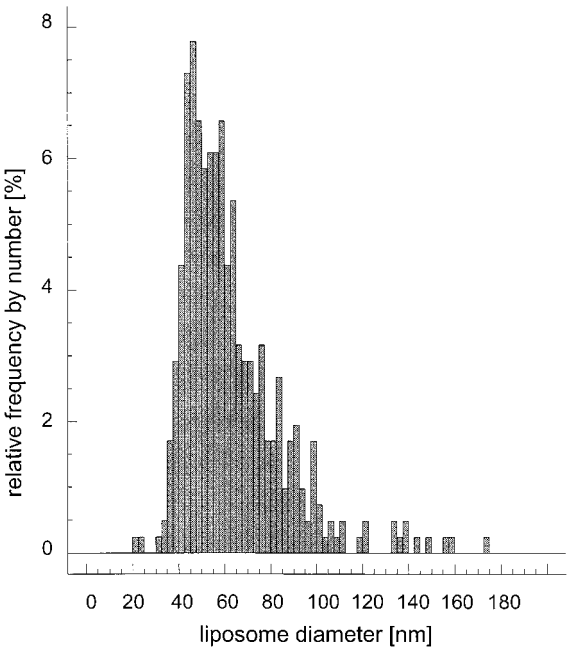

Fig. 6. Result of cryo-TEM particle size analysis of the sample displayed in Fig. 4.

same time, the experimental values were in very good agreement with the theoretically predicted encapsulation efficiencies up to the mentioned border of 300 mg/g lipid. Apparently, up to this limit the assumptions of the prediction, namely small, homogeneously loaded, unilamellar vesicles of 21 nm diameter, are well fulfilled. Above lipid concentrations of 300 mg/g, however, the measured encapsulation efficiencies were markedly higher than expected for SUVs from the theory. At the same time, the recovery of marker after homogenization, dilution and column chromatographic separation was close to 100% up to 300 mg/g lipid, but decreased more and more at higher lipid concentrations. For 500 mg/g lipid pastes, a recovery of 20% was determined in one case. In subsequent runs, no liposomes could be recovered from the column at all. Most of the fluorescent mass was observed staying on top of the column, which explains the low recovery value or inability to detect liposomes. Apparently, the more concentrated dispersions after dilution contained marker/lipid aggregates which were too coarse to pass the sephadex gel bed.

In order to better understand the structures, especially in these highly concentrated lipid dispersions and their dilutions, various transmission electron micrographic techniques were employed. The aim was to analyze the morphology of the original as well as the resuspended dispersions. In Fig. 2, a typical freeze-fracture electron micrograph of such a lipid paste is given. The micrograph is a replica obtained from a 450 mg/g SPC dispersion prepared by 10-fold homogenization at 70 MPa. The picture shows densely packed, round, very small fracture faces of vesicles. Apparently, this lipid paste contains many small unilamellar vesicles in the size range well below 100 nm. Some larger vesicles with diameters of up to 1 μm are seen as well with these pastes. They can be referred to as multivesicular vesicles (MVVs) rather than multilamellar vesicles (Talsma et al., 1987). The multivesicular morphology becomes visible when the uppermost membrane layer of the large vesicles is broken off during the preparation of the specimen and the

Table 1
Influence of resuspension technique on entrapment, recovery and filterability

Resuspension technique	EE (%)	CF recovery (%)	Lipid loss on filtration (%)	CF loss on filtration (%)
Dilution in one step by handshaking/vortexing	44.2 ± 1.1	77.2 ± 6.4	ND	ND
Dilution in steps by handshaking/vortexing	27.7 ± 0.4	94.9 ± 3.9	ND	ND
Dilution in steps by ball milling for 8 min	35.7 ± 1.0	ND	0.4 ± 0.3	5.5 ± 8.0
Dilution in steps by ball milling for 16 min	32.3 ± 1.7	ND	0.1 ± 0.0	6.0 ± 5.5
Dilution in steps by ball milling for 32 min	37.1 ± 0.7	ND	0.4 ± 0.1	23.7 ± 17.0

EE, encapsulation efficiency; ND, not determined. Results of three consecutive experiments given as mean $\pm$ S.D.

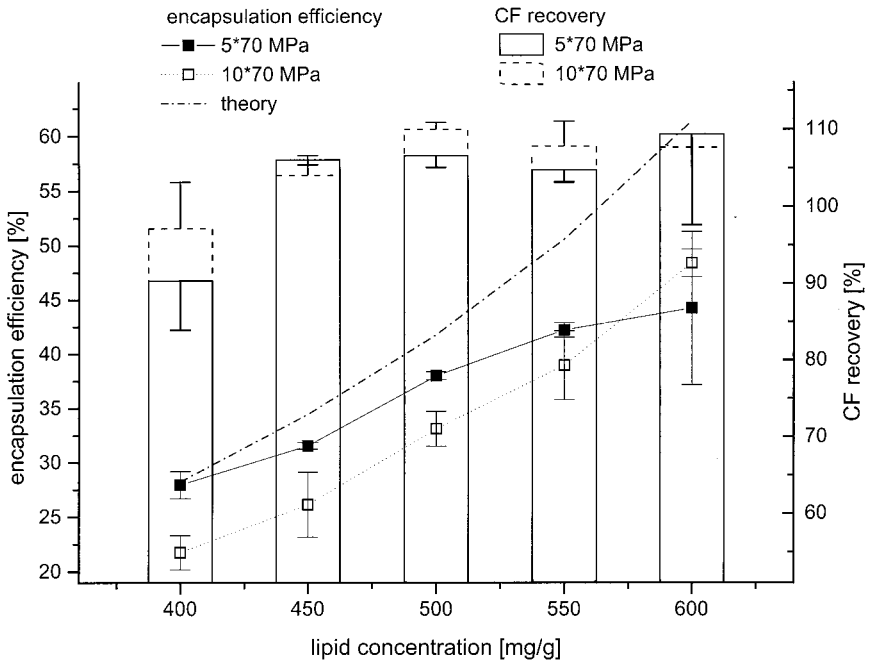

Fig. 7. Encapsulation efficiency of CF and recovery upon entrapment in E 80 liposomes, prepared by 5- (5×70 MPa) or 10-fold (10×70 MPa) homogenization at 70 MPa, and subsequent dilution and gel chromatography in relation to lipid content. Results of three consecutive experiments given as mean $\pm$ S.D.

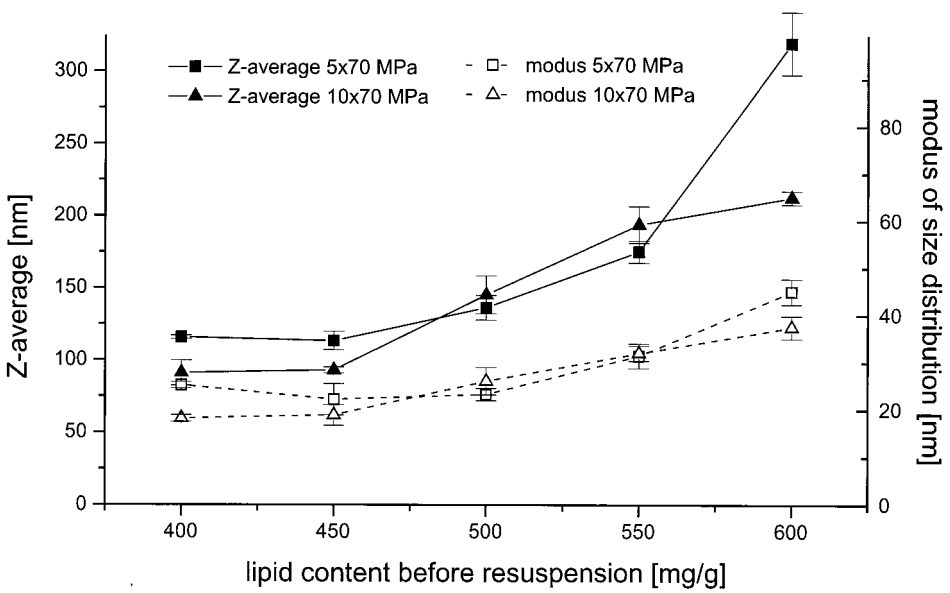

Fig. 8. PCS particle sizes (Z-averages and mode values of the distributions by number) of liposome dispersions obtained upon dilution of E 80 pastes prepared by 5- (5×70 MPa) or 10-fold (10×70 MPa) homogenization at 70 MPa, in relation to lipid content before dilution. All values are given as mean $\pm$ S.D. of three consecutive measurements.

structure underneath is exposed. Due to the paste-like appearance and the vesicular morphology, we are using the expression 'vesicular phospholipid gels' for this sort of preparation. Further results of FF-TEM evaluation of the morphology of semi-solid phospholipid pastes have recently been published elsewhere (Brandl et al., 1997).

The second question is: what is the morphology and size of the particles which form when the pastes are diluted? This question was addressed both by negative-staining and cryo-electron microscopy. Negative-staining transmission electron (NS-TEM) micrographs have primarily been used for particle size analysis. In Fig. 3, the particle size distribution is given for a vesicle preparation obtained by dilution by handshaking in one step from a 400 mg/g SPC paste (micrograph not shown). The paste had been prepared by 10-fold homogenization at 70 MPa. The obtained particle size distribution has its mode at 40 nm and ranges up to diameters of more than 100 nm. As NS-TEM does not allow to gain insight into the inner structure of the particles, cryo-electron microscopy was employed to study the morphology of the diluted dispersions in more detail. Two examples for typical structures seen in cryo-electron micrographs are given in Figs. 4 and 5. The dispersions were obtained by dilution of pastes containing 300 mg/g lipid. The sample displayed in Fig. 4 consisted of an equimolar mixture of hydrogenated soy PC (HSPC) and cholesterol (CH), homogenized 10-fold at 70 MPa. The sample shown in Fig. 5 was just SPC homogenized 10-fold at 140 MPa. Both figures have in common that the particles observed upon dilution of the liposome gels were vesicles. The vesicles in Fig. 4 were quite small and mostly unilamellar. Some needle-like structures on the images were assumed to represent cholesterol crystals. A vesicle size distribution, as obtained by measuring vesicle diameters on cryo-electron micrographs such as that in Fig. 4, is shown in Fig. 6. As the vesicles tend to arrange themselves in the holes of the grid within the water film so that smaller vesicles are preferentially found in the center of the hole and bigger vesicles rather towards the edges, it was important to choose representative micrographs for size analysis. The size distribution indicated small and relatively homogeneously distributed diameters. More than 95% of the vesicles were within the size range 30–110 nm, with the mode of the distribution occurring at 45 nm. In contrast, the appearance of the preparation displayed in Fig. 5 was quite different. Here, besides a small fraction of SUVs, some large vesicles in the micrometer range were seen. With some of these larger vesicles, their inner structures could be clearly discerned: they were apparently filled with SUVs. These vesicle-filled vesicles resemble the multivesicular vesicles (MVVs) already seen in the freeze-fracture micrographs of some of the pastes before dilution.

It is not clear so far why MVVs were sometimes observed and sometimes not. However, even if not observed on cryo-electron micrographs, these bigger particles cannot be excluded because it is difficult to trap particles like the observed MVVs within the frozen water film. From the frequency at which they occur in cryo-micrographs, it is not possible to discover the proportion in which they were present in the sample. From the standpoint of someone who wants to produce SUVs, these MVVs are unfavorable. An observation made when occasionally modifying the resuspension procedure seemed helpful in this respect: instead of adding excess buffer in one step, a 400 mg/g SPC paste homogenized 10-fold at 70 MPa was diluted step-wise by adding small portions of buffer and mixed at each step by vortexing. Particle sizes were again determined by NS-TEM and compared to those of the same paste that had been diluted in one step (Fig. 3). The size distribution was shifted to smaller sizes and was narrower than that obtained with the sample diluted in one step. Now more than 99% of the vesicles had sizes below 50 nm, with the mode of the distribution at 25 nm. No particles above 100 nm were seen on the grids. It has to be mentioned, however, that NS-TEM is not ideal for quantifying the number of particles in the micrometer range. During specimen preparation, the big particles might here also be drained off by touching the grid with blotting paper or the outermost membranes might be ruptured due to osmotic effects.

In further experiments, different resuspension techniques should be studied more systematically

and the range of analytical techniques should be extended with respect to the detection of large particles. The use of an oscillating ball mill for resuspension of the pastes was tried. For determination of large MVVs after resuspension, a filtration through 0.2 μm polycarbonate membrane filters was done. The CF and PC losses during this filtration step were quantified fluorimetrically and by the Stewart assay, respectively. The results are given in Table 1 together with those of the above experiments.

The dilution process in one step yielded the highest encapsulation efficiencies, whilst the stepwise resuspension process, no matter whether by handshaking or ball milling, led to somewhat lower EE values. This effect might be attributed to the reduction of large multivesicular vesicles in the latter preparations. MVVs have a larger internal volume and thus would entrap relatively high amounts of CF. After ball milling, the remainder of larger particles, as determined both from PC loss and loss of entrapped CF during filtration through 0.2 μm polycarbonate membranes, was quite low. In the literature, PC recovery values after filtration through 0.2 μm filters have been reported (Elorza et al., 1993) to be as low as 50% for heterogeneous reverse-phase evaporation vesicles. For small vesicles prepared by a microfluidizer, a loss of active ingredient up to 33% and of lipid up to 18% upon membrane filtration has been reported recently (Sorgi and Huang, 1996). Here, after ball milling some loss of entrapped marker (up to 30%) but only minimal loss in PC (below 0.5%) was measured after filtration of samples. The higher loss of marker as compared to PC might be due to removal of large marker-filled vesicles by filtration. With polycarbonate membranes it cannot be ruled out that, to some extent, extrusion of larger vesicles through the pores of the filter might occur, which would lead to an underestimation of the big particles. For subsequent experiments, cellulose acetate membrane filters were therefore used, as they do not have straight pores like the polycarbonate membranes and thus should preferentially lead to removal rather than extrusion of vesicles.

For further experiments, resuspension by stepwise dilution and ball milling were chosen. Concentrated liposome gels were made from E 80 with increasing lipid concentrations from 400 to 600 mg/g by 5- or 10-fold homogenization at 70 MPa. Encapsulation efficiencies were determined as well as recovery of CF after dilution and gel chromatography. Mean particle sizes were estimated by PCS analysis after dilution and samples were filtered through 0.2 μm filters and analyzed for loss of entrapped CF. The results of encapsulation and recovery of marker are given in Fig. 7. Encapsulation efficiencies were found to be quite high. They were continuously rising, from about 25% at 400 mg/g to 45% at 600 mg/g lipid, with a tendency to higher values after 5-fold homogenization compared to those after 10-fold homogenization, except for the 600 mg/g dispersion. All experimental EE values were slightly below the theoretically predicted values, and the divergence of experimental data from theory increased with rising lipid contents. This indicates that the formation and loading of vesicles at these high lipid concentrations shows more and more aberration from theory. The recovery of CF after dilution and column chromatography in all cases was close to, or actually slightly above, 100%. This was taken as proof that with the chosen resuspension procedure, large lumps which cannot pass the gel column did not occur any more. The estimated mean particle sizes are not only given as Z-averages but also as peaks of the distribution by number (mode) for comparison with above electron microscopic data (Fig. 8). The Z-averages of the resuspended dispersions were well reproducible and ranged between 100 and 200 nm with a tendency to increasing values with increasing lipid content. In contrast, both polydispersity index and fit error showed a great variability among consecutive measurements of the same sample, ranging from 0.005 to 0.5 and 0.002 to 0.4, respectively. It remained unclear, in which cases the software of the zetamaster decided for the good fit and poor polydispersity index and vice versa. The same measurements expressed as the mode of the number distribution yielded values between 20 and 40 nm, again with a tendency to larger diameters with increasing lipid contents. The values after 5-fold and 10-fold homogenization did not differ much, except for the 600 mg/g preparation

which had a higher Z-average of 300 nm and a mode of 45 nm after five homogenization cycles. In our experience, such mode values are typical for SUV dispersions. The amount of entrapped CF before and after filtration through 0.2 μm filters did not differ significantly for all lipid concentrations studied here (up to 550 mg/g, data not shown). This was taken as a further hint that large MVVs were almost absent in the resuspended liposome dispersions.

4. Conclusions

The results reported here indicate that by high-pressure homogenization the preparation of liposomes can be extended to dispersions of quite high lipid concentrations of up to 600 mg/g. The obtained semi-solid dispersions contain densely packed vesicular structures, as seen on FF-TEM micrographs. Depending on the lipid content and hydration conditions, these vesicles can vary in size and morphology from small and unilamellar to very large (around 1 μm) multivesicular vesicles. Mixing of these pastes with excess buffer yields 'classical' liquid liposome dispersions. Depending on the mixing procedure, the obtained liposomes show different morphology and particle size. Upon dilution by handshaking, a fraction of large multivesicular vesicles was seen on cryo-electron micrographs, as well as uniform small unilamellar vesicles. By resuspending the pastes step-wise using a ball mill, the occurrence of larger particles (in the micrometer range) could be widely suppressed, as indicated by PCS and filtration experiments. By this technique, apparently the outermost membrane of the MVVs could be disrupted and the SUVs contained in their interior released. The driving force for the current experiments was to achieve an improvement of encapsulation efficiency of hydrophilic compounds into small unilamellar liposomes. With lipid concentrations usually used for liposome preparation, the encapsulation efficiencies for SUVs range from about 1 to 20%. For more heterogeneous preparations containing larger vesicles, such as reverse-phase evaporation vesicles or dehydration–rehydration vesicles, higher EEs of 50% and above have been reported (Szoka and Papahadjopoulos, 1978; Kirby and Gregoriadis, 1984). Gregoriadis further described a way to reduce vesicle size and improve size homogeneity of DRVs by subsequent microfluidization without major loss of entrapped drug (Gregoriadis et al., 1990). With the 'liposome gel resuspension' technique described here, high encapsulation efficiencies of close to 50% as well as small and uniform vesicle sizes could be achieved at the same time.

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