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Biochimica et Biophysica Acta 1758 (2006) 222–231
BIOCHIMICA ET BIOPHYSICA ACTA
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Preparation of submicron unilamellar liposomes by freeze-drying double emulsions

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Received 4 December 2005; received in revised form 23 January 2006; accepted 23 January 2006
Available online 28 February 2006

Abstract

A novel method is described for the preparation of sterile submicron unilamellar liposomes. The method is based on the lyophilization of double emulsions containing disaccharides as lyoprotectants in both the inner and outer aqueous phase. Using various phospholipids or mixtures of lipids as emulsifiers, the double emulsions can be prepared by a two-step emulsification, including hydrophilic agents in the inner aqueous phase or lipophilic agents in the oil phase. Then, the double emulsions are lyophilized after sterilization by passing them through a 0.22-μm pore filter. Rehydration of the lyophilized products results in liposomes with a relatively high encapsulation efficiency (for calcein, 87%; 5-fluorouracil, 19%; flurbiprofen, 93%) and a size below 200 nm measured by the dynamic light scattering technique (DLS) and the atomic force microscopy (AFM). The liposomes were found to be unilamellar from freeze-fracture electron micrographs and X-ray diffraction patterns. In addition, the liposomes can be reconstituted just before use by rehydration of the lyophilized products which are relatively stable. Thus, this reproducible and simple technique can be used to prepare sterilized, submicron unilamellar liposomes with a relatively high encapsulation efficiency, and excellent stability during long-term storage.

Keywords: Liposome; Preparation; Freeze-drying; Double emulsion; Encapsulation efficiency; Particle size

1. Introduction

Liposomes, vesicles enclosing an aqueous solution with membranes of phospholipids, have been extensively studied as microparticulate carriers for the efficient delivery of therapeutic agents since the first report by Bangham et al. [1,2]. The superiority of liposomes as drug carriers is now widely recognized and great advances in the liposome field have resulted in the development of some approved liposomal products [3]. However, it is still not easy to prepare acceptable aseptic liposomal drugs with desirable properties, high encapsulation efficiency, and long-term stability without drug leakage and loss [4,5].

When liposomes are used as drug carriers, their size is of major importance, since this influences the behavior of liposomes in biological systems [6]. In order to extravasate into the disease site, the liposomes should be smaller than 120 nm. This requires very efficient loading, otherwise, either therapeutic levels of drug cannot be reached or a very large amount of lipids is needed to obtain these levels. Furthermore, inefficient loading will lead to a great loss of the active agent during loading and complicate the preparation procedure by making it difficult to remove the free agent [5].

As liposomes are usually made of phospholipids which are susceptible to light, heat and oxygen, it is usually not acceptable to sterilize the products for parenteral use by autoclaving. Furthermore, when liposomes exist in aqueous solutions, they may be subject to a series of adverse effects such as aggregation, fusion, phospholipid hydrolysis, and drug leakage, which lead to a short-shelf life [4].

Many efforts have been made to improve the properties of liposomal preparations, especially the encapsulation efficiency, vesicle size, and stability, which are still major problems standing in the way of new advances in this field [5]. Up to now, a number of different methods have been successfully

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doi:10.1016/j.bbamem.2006.01.023

developed for the preparation of liposomes. These methods can be classified for convenience into three categories based on the dispersion technology [7]: (1) mechanical dispersion methods, for example, hand shaking, or vortexing, sonication, and the use of a French press; (2) detergent-solubilizing dispersion methods including solubilizing lecithin with sodium cholate or octylglucoside; (3) solvent dispersion methods, such as ethanol injection, ether infusion, and reverse-phase evaporation (REV). Liposomes prepared by the REV method are known to have a higher encapsulation efficiency for water-soluble agents than those prepared by other methods. However, the REV vesicles usually have a diameter larger than 200 nm [8]. Some measures, such as extrusion and sonication, used to control the vesicle size [9–11] are likely to lead to drug loss. A novel liposomal technology involving freeze-drying of a monophase solution, developed in our lab, can produce stable freeze-dried products that can form submicron liposomes with a narrow size distribution upon rehydration [12]. However, this method results in a low encapsulation efficiency unless it is combined with active loading.

The active loading, namely remote loading of the agents into preformed liposomes by the use of special gradients across the liposome membranes, such as pH gradients [13,14] and ammonium salt gradients [15], can result in a rather high encapsulation efficiency; but it limits the liposomal entrapment to only certain ionizable hydrophilic or amphipathic agents. Another way of loading the agents into preformed liposomes involves the dehydration–rehydration of a mixture of liposomes and the materials to be loaded, and the final liposomes formed, so called DRVs, can entrap up to 80% of the starting materials which can be labile molecules, such as proteins, peptides and nucleic acids. However, the DRVs are usually large multilamellar vesicles [16,17].

In order to improve the stability, studies have been carried out and confirmed that liposomes can be effectively stored in the dried state by lyophilization in the presence of carbohydrates as lyoprotectants [18]. Usually, this lyophilization practice needs multiple-step entrapment procedures for preparing drug-containing liposomes.

In this report, a novel procedure is presented for the preparation of sterile unilamellar liposomes with the desirable properties of a relatively high encapsulation efficiency and a mean diameter less than 200 nm. The methodology includes preparation of agent-containing double emulsions with disaccharides as lyoprotectants in both the inner and outer aqueous phase, sterilization by passage through a 0.22-μm pore filter, lyophilization to remove solvents, and rehydration to obtain an aqueous suspension of liposomes. Also, the methodology has the advantage of allowing complete removal of organic solvents and the preparation of stable dry products which can be rehydrated just before use allowing enhanced storage stability. This approach has been validated for the encapsulation of both hydrophilic and lipophilic agents in vesicles to form a product that would be suitable as an effective carrier for the efficient delivery of therapeutic agents.

2. Materials and methods

2.1. Materials

Soybean phosphatidylcholine (PC, Epikuron 200, purity >92%) and soybean phosphatidylserine (PS, Leci-PS90PN, purity >95%) were generous gifts from Degussa (Freising, Germany). Calcein (Cal) was purchased from Sigma (St. Louis, MO, USA). Cholesterol (CH) was of analytical grade from Tianjin Chemical Reagent Co. Inc (Tianjin, China). Flurbiprofen (Flu) was obtained from Southwest Synthetic Pharmaceutical Co., Ltd. (Chongqing, China) and 5-fluorouracil (5-Fu) was a product of Jiangsu Jintan Pharmaceuticals (Jintan, China). The solvents used for the chromatographic mobile phases were of HPLC grade. All other chemicals were of analytical reagent grade.

2.2. Preparation of liposomes by freeze-drying double emulsions (FDE)

The W1/O/W2 double emulsions were prepared by a two-step emulsification procedure at room temperature (25 °C). Several organic solvents (or mixed solvents) which are immiscible with water, such as ether, chloroform, can be used as the oil phase (O), but cyclohexane was selectively used because its melting point is near that of water. Distilled water was used for the aqueous phase (W). Sucrose (Suc) , lactose (Lac), and sucrose together with mannitol (Man) were used, respectively, as lyoprotectants dissolved in both the inner aqueous phase (W1) and the outer aqueous phase (W2) at a concentration of 5% (w/v). Each model agent was dissolved either in the oil phase (O) or in the inner aqueous phase (W1). Concentrations of the drug solutions used in this study were as follows: calcein, 66.65 μg/ml (0.1 mM) in water; 5-fluorouracil, 1.0 mg/ml (7.7 mM) in water; flurbiprofen, 1.2 mg/ml (5.0 mM) in cyclohexane. The phospholipids were dissolved in cyclohexane to give 2.5% (w/v) (ca. 0.033 mol l^{-1}) solutions of lipid mixture solution. The mass ratio of lipids to lyoprotectants in the final aqueous suspensions of liposomes was 1:5.

2.2.1. Formation of W1/O/W2 double emulsions and sterile filtration

Two milliliters of each lipid mixture solution (O) and 1 ml of aqueous solution (W1) were added to a 10-ml ampoule, and this mixture was then emulsified with a homomixer (Ultra Turrax®, model T18, IKA Works, USA) at 22,000 rpm for 30 s to form 3 ml of W1/O type emulsions (primary emulsions). The primary emulsions were immediately sonicated briefly (200 w, ca. 3 min) with a probe-type sonicator (JY92-2D Sonicator, Xinzhi Biotech. Co, Ltd. Ningbo, China) until the mixture became either clear or a uniformly opalescent microemulsion that did not separate for at least 30 min after sonication. The temperature was kept under 25 °C using a water bath during sonication. The microemulsions were then mixed with 4 ml aqueous solution (W2), and emulsified at 18,000 rpm for 30 s to form mostly the type A of W1/O/W2 double emulsions, namely containing only one internal aqueous droplet [19]. The double emulsions were sterilized by passage through a 0.22-μm filter, then immediately transferred to 5-ml freeze-drying vials with a fill volume of 2 ml and at once frozen.

2.2.2. Freeze-drying

The freeze-drying process was as follows: (1) freezing at −50 °C for 8 h; (2) primary drying −50 °C for 48 h; and (3) secondary drying at 25 °C for 24 h. The chamber pressure was maintained at 20 pascals during the drying process. When the freeze-drying process was over, the vials were immediately filled with nitrogen gas, sealed with rubber caps, and stored at 4 °C.

2.2.3. Reconstitution

When needed, aqueous suspensions of liposomes were immediately formed upon rehydration of the lyophilized products with water to the original volume.

2.3. Characterization of the liposomes

2.3.1. Dynamic light scattering technique

The mean diameters of the liposomes in aqueous solution were determined by the dynamic light scattering technique (DLS) with a submicron particle size analyzer (Ls23, Beckman, USA) [20].

2.3.2. Atomic force microscopy

Atomic force microscopy (AFM) measurements were made at room temperature (20 °C), using a commercial atomic force microscope (SPI3800N Probe Station and SPA-400 SPM Unit, Seiko Instruments, Japan). A tapping mode liquid cell was used to obtain images of liposomes in pure water. Cantilevers with tips (SI-DF3, Seiko Instruments, Japan) with a nominal spring constant of 2 N/m were used. The cantilevers were acoustically excited indirectly both through the cell and the liquid at frequencies of 9 kHz, which is just below their resonant frequency in water. Scan rates were 1 Hz with a pixel number of 256×256. Just before the analysis, the samples were rehydrated and aliquots of 20 μl liposome solution were deposited on mica. After 1 min, the mica was gently rinsed with pure water, and the sample chamber was mounted onto the AFM scanner and the measurements were performed and completed within a few minutes to avoid deformation [21].

2.3.3. Freeze-fracture electron microscopy

The freeze-fracture electron micrographs were obtained as described elsewhere [22], and a freeze-fracture device (Balzers BAF 400D) was used for fracturing.

2.3.4. X-ray diffraction

2.3.4.1. Determination of lamellarity. X-ray diffraction data were collected by diffractometer (D-max 2400, Rigaku, Japan). Samples were exposed to a monochromatic beam with a wavelength of 1.5418 Å, which was produced by bombarding copper with electrons. The scanning was performed from 1.00 to 10.00°, in steps of 0.02°/s at room temperature.

Five samples were analyzed and all involved PS/PC (1:9) with sucrose as lyoprotectant and a lipid/sucrose weight ratio of 1:5. Sample 1 was the lyophilized multilamellar vesicles (MLVs). The MLVs were prepared as follows: first, the lipid film was hydrated with a 5% (w/v) sucrose solution to obtain a 1% (w/v) liposome suspension and then this suspension was extruded 10 times through 0.8-μm pores using a 10-ml thermobarrel extruder (Northern Lipids Inc., Vancouver, Canada). Sample 2 was the lyophilized large unilamellar vesicles (LUVs). LUVs were prepared in the same way as sample 1, except that the sizing process was performed using 100-nm membranes. Sample 3 was the powder of FDE. Sample 4 was prepared as follows: first, a replica of sample 3 was hydrated to obtain an aqueous suspension of liposomes, and then lyophilized. Sample 5 was a solid dispersion of lipids in sucrose matrix which was obtained by freeze-drying tert-butyl alcohol/water cosolvent systems containing lipids and water-soluble materials [12] and this was used as a control.

2.3.4.2. Examination of the lyoprotectant phase. Two samples of the powder of FDE composed of PS/PC (1:9) with lyoprotectants of, respectively, sucrose and sucrose together with mannitol (1:1,w/w), were tested by X-ray diffractometry, and scanning was performed from 3.0 to 45.0°, in steps of 0.04°/s at room temperature.

2.3.5. Determination of encapsulation efficiency and entrapped volume

The encapsulation efficiency (EE) for calcein and the entrapped volume (EV) based on calcein were determined according to the method described by Oku et al. [23]. Briefly, the liposome suspension was diluted 50-fold with aqueous solution containing 5% (w/v) lyoprotectants, and 2 ml of the diluted liposome suspension was used to measure the fluorescence intensity of calcein before (F_{tot}) and after (F_{in}) addition of 50 μl of $CoCl_2$ (1.0 mM) on a fluorometer (F-931; Shanghai Spectroscopic Co, Ltd. China) at excitation and emission wavelengths of 400 and 520 nm, respectively. Subsequently, 100 μl 10% (w/v) TritonX-100 was added and the fluorescence was measured again and the resultant fluorescence intensity (F_r) represents the equilibrium concentration of the free calcein. The EE and EV were calculated from:

$$EE(\%) = (F_{in} \cdot r_1 - F_r \cdot r_2)/(F_{tot} - F_r \cdot r_2) \times 100 = EV(\%) \quad (1)$$

where r_1 and r_2 were the dilution factors due to the addition of $CoCl_2$ solution and Triton X-100 solution, in the present case, 1.025, 1.075, respectively.

For 5-fluorouracil, the EE was estimated from the following equation:

$$EE(\%) = (D_{tot} - D_{free})/D_{tot} \times 100 \quad (2)$$

the free drug (D_{free}) was separated by ultrafiltration through a Millipore filter (Amico8010, Millipore Corporation, Bedford, USA) and assayed spectrophotometrically at a wavelength of 265 nm. The total content of drug (D_{tot}) in the preparations was determined after the liposome suspension was diluted with appropriate methanol to disrupt the liposomes completely and release the encapsulated drugs into the solvents.

The EE for flurbiprofen was calculated from the following equation:

$$EE(\%) = D_{en}/D_{tot} \times 100 \quad (3)$$

the amount of flurbiprofen encapsulated in the liposomes (D_{en}) was determined by HPLC (LC-10AT liquid chromatograph, SPD-10A UV detector, Shimadzu, Kyoto, Japan) at a UV detection wave-length of 222 nm after the liposomes were separated from the extra aqueous solution by ultracentrifugation at 20 000×*g* for 15 min and the pelleted vesicles were dissolved in an appropriate amount of methanol. The mobile phase was methanol/water/acetic anhydride (70:30:0.1, v/v/v).

2.4. Detection of solvent residue

Analyses of the solvent residue in the freeze-dried products were carried out on a gas chromatograph (GC-2010 Gas Chromatograph, Shimadzu, Japan) with an FID detector according to reference [24].

2.5. Stability test

The stability of the dried products was assessed by determination of the liposomes, which were reconstituted from dried products protected from light at 4 °C for different periods of time, using the changes in encapsulation efficiency and mean diameters as described above.

3. Results

3.1. Vesicle size

Examination of FDE vesicles composed of different lipids containing different agents by DLS revealed that the mean diameters were all under 200 nm. The mean diameters of FDE vesicles composed of PS/PC (1: 9) were smaller than those of other formulations. Table 1 provides details of the test results.

AFM micrograph of the PS/PC (1:9) FDE liposomes containing calcein with lyoprotectants of sucrose and mannitol revealed that the sizes of the vesicles were under 150 nm and the mean diameters was 92 nm, in good agreement with data obtained by DLS. Representative AFM topography and phase images of the PS/PC (1:9) FDE liposomes are shown in Fig. 1. The AFM topography image and the cross-section given in Fig. 2 suggested that the FDE liposomes were nearly spherical and little deformation had taken place after sample deposition on the mica.

3.2. Freeze-fracture electron micrograph

The FDE liposomes were shown to be unilamellar vesicles from the freeze-fracture electron micrographs presented in Fig. 3.

3.3. X-ray diffraction

It has been shown that solid state lipids obtained from lyophilization (or from film deposition) spontaneously form

Table 1
Effects of lipid composition and lyoprotectants on the size, encapsulation efficiency and trapped volume of FDE liposomes (n = 3)

Lipid composition (1%, w/v)[a]	Sugar (5%, w/v)[a]	Mean diameters (nm)			Encapsulation efficiency (%)			Trapped volume (based on Cal) (μl/mg)
		Cal	5-Fu	Flu	Cal	5-Fu	Flu	
PC	Suc	112	116	112	69.2	16.1	91.4	6.92
	Lac	131	145	142	62.6	14.4	90.3	6.26
	Suc+Man[b]	114	117	118	67.5	15.9	92.7	6.75
PS/PC (1:9)[c]	Suc	98	97	102	78.4	17.9	90.9	7.84
	Lact	110	108	112	73.5	16.4	89.3	7.35
	Suc+Man[b]	96	95	110	76.6	17.1	91.4	7.66
PS/PC/CH (1:4:5)[c]	Suc	147	152	157	87.6	19.6	93.2	8.66
	Lac	183	189	197	80.4	18.1	92.8	8.04
	Suc+Man[b]	145	149	158	86.7	19.4	93.8	8.77

Abbreviations: Cal, calcein; 5-Fu, 5-fluorouracil; Flu, flurbiprofen; CH, cholesterol; Suc, sucrose; Lac, lactose; Man, mannitol.
[a] The concentration in the final aqueous suspension of liposomes.
[b] Sucrose and mannitol were in a mass ratio of (1:1).
[c] Mole ratio of different lipids.

highly structured stacked lamellae [25]. To identify whether there is similar lamellar structure to that of unilamellar or multilamellar vesicles in the lyophilized products, two controls were used: one was the lyophilized MLVs, which contained stacked lamellae, and the other was the lyophilized LUVs, which provided diffraction information on unilamellar vesicles.

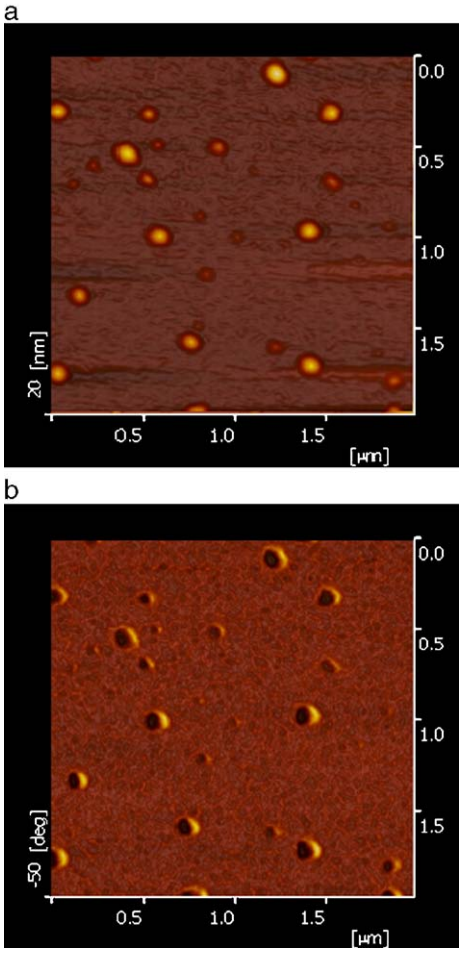


Fig. 1. Representative taping mode AFM topography and phase images of PS/PC (1:9) FDE liposomes, panels a and b, respectively. The AFM measurements were performed immediately after sample deposition on a mica surface in pure water. The liposomes were reconstituted just before AFM tests by rehydration of lyophilized double emulsions containing calcein and lyoprotectants sucrose and mannitol.

The small angle X-ray diffraction patterns are presented in Fig. 4. Characteristically different diffraction patterns are obtained from unilamellar vesicles and from regularly stacked bilayers of multilamellar vesicles. For unilamellar vesicles, where the bilayers have no fixed geometrical relationship to each other, the diffraction pattern displays a very broad peak, which can be used to roughly estimate the bilayer thickness. On the other hand, the regularly stacked bilayers will display a relatively sharp peak [26]. As seen from Fig. 4, the lyophilized MLVs (Sample 1) showed a lamellar repeat spacing of about 58.1 Å (λ = 1.5418 Å, 2θ = 1.52°). The lyophilized LUVs (Sample 2), with an average size of 100 nm, displayed a relatively broad diffraction pattern. Sample 4 had a similar diffraction pattern to that of Sample 2, which means that the FDE liposomes are not multilamellar. Also, the lyophilized products of FDE (Sample 3) exhibited a similar diffraction pattern to that of Sample 2. Thus, the lyophilized products of FDE appear to possess similar single bilayers to those of LUVs. Obviously, Sample 5 exhibited a diffraction pattern different to those of all the other samples, since it had no similar structure to that of MLVs or LUVs [12].

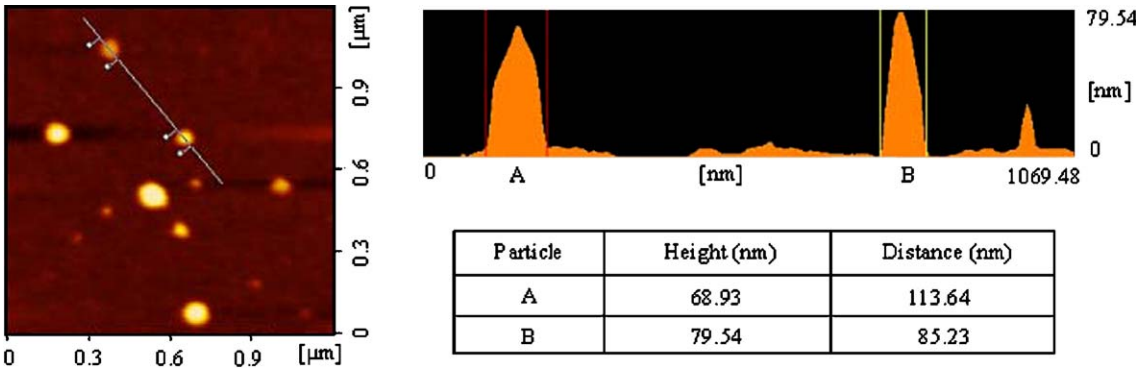
Fig. 5 shows the lyoprotectant of sucrose represented as vitrification in the powder of FDE, while mannitol was in crystalline form.

3.4. Encapsulation efficiency

The encapsulation efficiency of FDE vesicles depends on the lipid composition, lyoprotectants, and the properties of the trapped agents. With sucrose as a lyoprotectant, the FDE liposomes composed of PS/PC/CH (1:8:9) exhibited the highest encapsulation efficiency (Table 1).

3.5. Calculation of the expected diameters

This calculation is based on the assumptions: that the captured aqueous volume of the FDE liposomes equals that of the double emulsions (W1) from which the former are formed; a



Particle	Height (nm)	Distance (nm)
A	68.93	113.64
B	79.54	85.23

Fig. 2. A taping mode AFM topography image including the height cross-section indicated by the straight line. The AFM measurements of PS/PC (1:9) FDE liposomes were performed immediately after deposition on a mica surface in pure water. The liposomes were reconstituted just before AFM tests by rehydration of lyophilized double emulsions containing calcein and lyoprotectants sucrose and mannitol.

phospholipid surface area of 72 $Å^2$ per molecule, condensed to 58 $Å^2$ in the presence of cholesterol; and a uniform population of spherical unilamellar vesicles of diameter:

$$D = 12V/A \quad (4)$$

where V is the total encapsulated volume, and A is the total surface area of a lipid monolayer; the actual thickness of the lipid bilayer and any difference in surface area between inside and outside monolayers have not been taken into account [8]. Calculations based on the entrapped volume of FDE vesicles composed respectively of PC, PS/PC (1:9), and PS/PC/CH (1:8:9) (shown in Table 1) gave values of 145 nm, 165 nm and 207 nm respectively, which are in good agreement with the values obtained by AFM and DLS. The agreement between the calculated and observed diameter values, as well as the freeze-fracture electron micrographs and X-ray diffraction patterns indicate that the FDE liposomes are unilamellar vesicles.

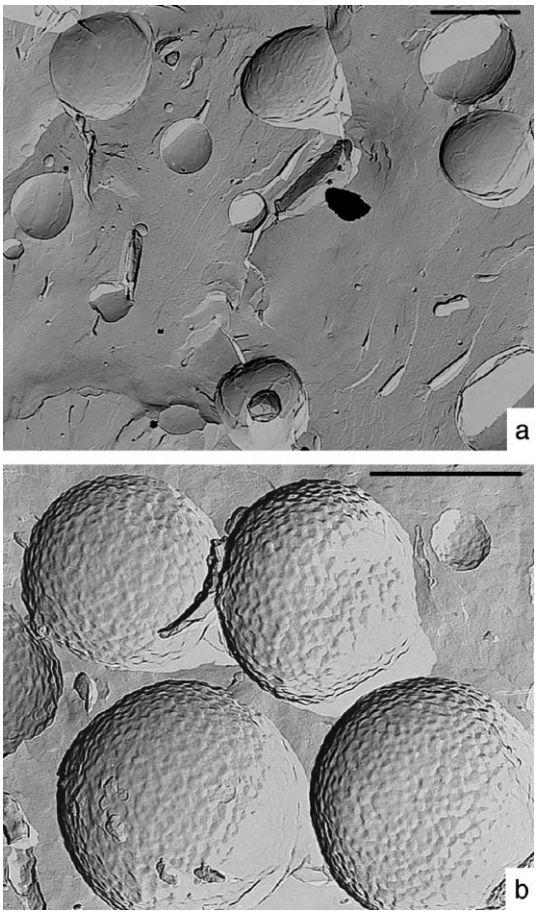


Fig. 3. Typical freeze-fracture electron micrographs of the FDE liposomes. (a) The liposomes were composed of PC with lyoprotectants sucrose and mannitol (1:1, w/w). (b) The liposomes were composed of PS/PC (1: 9) with sucrose as the lyoprotectant. Scale bar indicates 100 nm.

3.6. Solvent residue

No organic solvent residue was found in FDE powder tested by GC. This is an advantage compared with other liposome technologies based on the emulsion method in which organic solvents are usually removed by a rotary evaporation [7].

3.7. Storage stability

Liposomes were formed by rehydration of lyophilized products that were stored protected from the light at 4 °C for 12 months without encapsulation or size change. Only the data of PS/PC (1:9) FDE liposomes containing calcein with lyoprotectants, sucrose and mannitol, are shown in Fig. 6.

4. Discussion

4.1. Formation mechanism of FDE liposomes

The above data have clearly demonstrated that the FDE liposomes are spherical submicron unilamellar vesicles with a relatively high encapsulation efficiency for both hydrophilic and lipophilic agents. A schematic diagram of the processes that might occur during the formation of FDE liposomes is illustrated in Fig. 7. The initial emulsification and sonication of the aqueous phase containing lyoprotectants and the organic phase in the presence of the amphiphatic phospholipid molecules (panel a) will produce submicron water droplets stabilized by a phospholipid monolayer, namely W1/O type primary emulsions (panel b). Such W1/O primary emulsions form into W1/O/W2

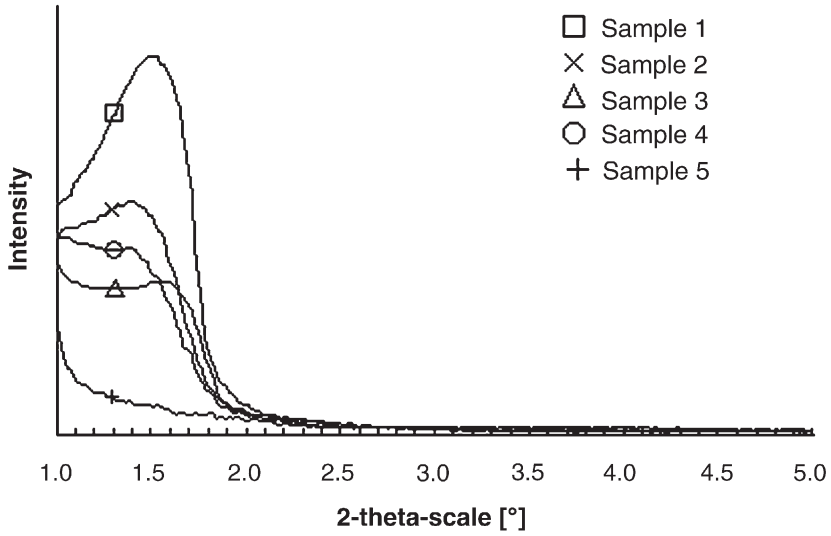


Fig. 4. X-ray diffraction patterns of lyophilized MLVs (sample 1), lyophilized LUVs (sample 2), lyophilized double emulsions (sample 3), lyophilized FDE liposomes (sample 4) and a dispersion of lipids in sucrose matrix (sample 5). Only meaning parts of spectra (2-theta-scale from 1.0 to 5.0°) are shown. All the lyophilized products were composed of PS/PC (1:9) with a mass ratio of sucrose to lipid of 5:1 as lyoprotectant.

double emulsions when they are added to the bulk continuous aqueous phase and emulsified (panel c). Submicron solid spheres surrounded by a phospholipid bilayer with centripetal and acentric polar heads are formed and vitrified when the organic solvents are removed by freeze-drying (panel d). Such submicron structures are confirmed by the X-ray diffraction patterns shown in Fig. 4. In this procedure, carbohydrates act as lyoprotectants as well as physical supports for the submicron solid spherical structures. Upon rehydration with water (panel e), the submicron solid spheres (or dried liposomes) form into an aqueous suspension of liposomes (panel f). The critical point in this procedure is ensuring that the W1/O type primary emulsions do not break up but form into stable W1/O/W2 double emulsions and the submicron solid spherical structures do not collapse, otherwise, the hydrophilic agents will not be encapsulated and the liposome size and size distribution will increase enormously. Therefore, the best possible lyoprotectants are necessary in the freeze-drying process. When the calcein solution was used as only the outer aqueous phase of W1/O/W2 double emulsions, almost none of the calcein was incorporated into the vesicles. This suggests that only the hydrophilic agents previously entrapped in the aqueous phase of W1/O primary emulsions can be incorporated into the final vesicles and further confirmed the existence of the submicron solid spherical structures.

4.2. Influencing factors

It is obvious that a number of variables may be responsible for determining the final products in terms of the FDE vesicle size, structure, and encapsulation efficiency. These include mainly the type of phospholipid and its solubility in the organic solvents, lyoprotectants, and the loaded agents.

4.2.1. Lipids

When charged lipids are used to prepare liposomes, the captured volume per mole lipid is increased due to electrostatic repulsion of different parts of the charged membrane. Also, the charged vesicles will repel each other thereby preventing

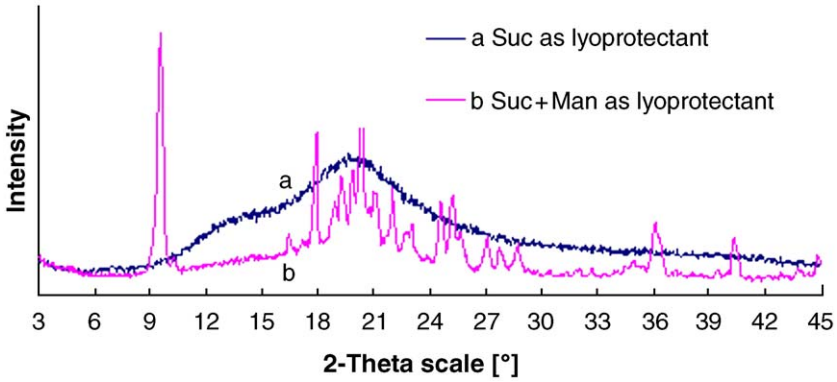


Fig. 5. X-ray diffraction patterns of the samples of the powder of FDE composed of PS/PC (1:9). Diffraction pattern a is the sample with sucrose as lyoprotectant. Diffraction pattern b is the sample with sucrose as lyoprotectant together with mannitol (1:1, w/w).

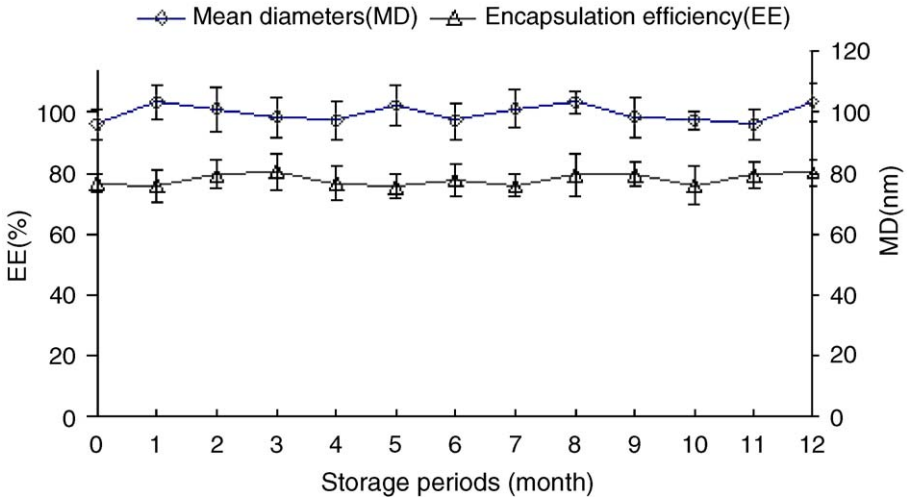


Fig. 6. The storage stability of the lyophilized products composed of PS/PC (1: 9) containing calcein and lyoprotectants sucrose and mannitol. The stability was assessed by determination of the changes in encapsulation efficiency and mean diameters of the liposomes reconstituted by rehydration of lyophilized products protected from light at 4 °C for different periods of time. (n=3).

aggregation and membrane fusion which is often accompanied by leakage and an apparent size increase [27]. Thus, the charged PS/PC (1:9) FDE liposomes are smaller in size than those composed of PC alone, as can be seen in Table 1.

Cholesterol can reduce the fluidity of the membranes at temperatures above the main phase transition temperature (T_m) of the phospholipid [28]. It has been shown that cholesterol 'dries' the lipid/water interface of membranes [29,30], thus enhances close contact and van der Waals interactions between adjacent lipid molecules, thereby contributing to the reduced membrane permeability and prevention of leakage [30–32]. Although it has been shown that cholesterol-containing liposomes exhibit multiple phase transitions when dehydrated, the addition of trehalose will limit the phase separation of the

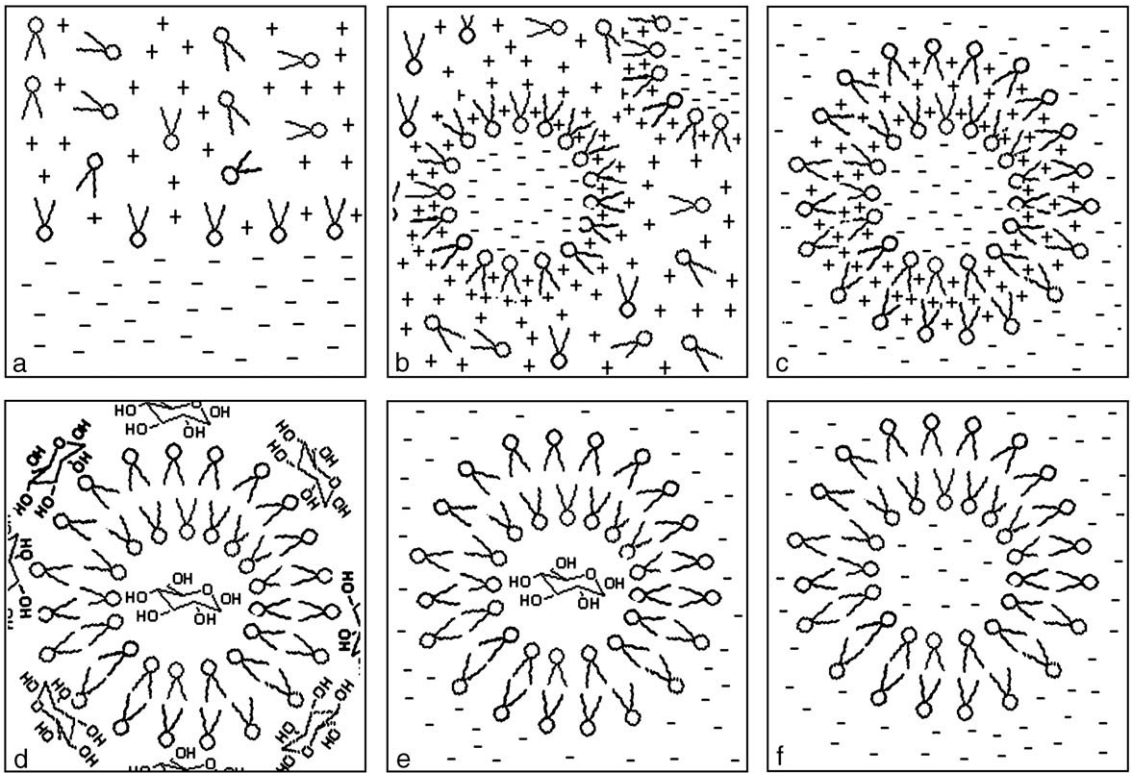


Fig. 7. A schematic diagram of the formation of FDE liposomes. (a) The oil phase containing lipids and the aqueous phase containing lyoprotectants. (b) The formation of W1/O emulsions. (c) The formation of W1/O/W2 double emulsions. (d) The formation of submicron solid spheres surrounded by a phospholipid bilayer by freeze-drying of W1/O/W2 double emulsions, and the lyoprotectants (sugar) act as physical supports for the structure. (e and f) The formation of liposomes upon rehydration with water. "–" and "+" represent water molecules and oil molecules, respectively.

lipidic components and effectively stabilize the membranes upon lyophilization [33]. Other studies have also shown that the fatty acyl composition, cholesterol content and, with the exception of phosphatidylglycerol, acid lipid content do not significantly alter the retention of the aqueous contents by vesicles dehydrated and rehydrated in the presence of trehalose [34]. And it has been reported that different sugars almost equally suppress the rise in T_m upon freeze-drying (at high sugar/phospholipid ratios) and no distinct binding properties of certain sugar molecules to the bilayer have been observed [35,36]. As shown in Table 1, the cholesterol-containing FDE liposomes with lyoprotectants of sucrose or lactose have a higher encapsulation efficiency and larger size, which is consistent with the above conclusions and the results obtained with other methods [8,10]. These effects of cholesterol relate to its molecular structure, weight and properties [37,38].

4.2.2. Agents and processes

The FDE liposomes have higher encapsulation efficiencies for water-soluble calcein and 5-fluorouracil than those prepared by other methods [23,39,40]. However, the encapsulation efficiency of calcein (87%) is much higher than that of 5-fluorouracil (19%).

With the FDE method, the water-soluble agent is first in the dispersed phase (W1) of the W1/O emulsions, then in the inner aqueous phase (W1) of the W1/O/W2 double emulsions, subsequently trapped in the submicron solid spherical structures by freeze-drying, and finally retained in the inner space of liposomes following rehydration. Therefore, theoretically, the initial agent-containing aqueous phase is always separated from the outer aqueous phase by the lecithin-containing oil phase in the double emulsions, which is advantageous for the maintenance of a higher encapsulation efficiency of the water-soluble agent compared with other solvent dispersion methods, such as REV [8]. Later, the organic solvents in the oil phase are removed by freeze-drying which will instantly 'fix' the agent in the inner submicron phase or space surrounded by phospholipids in the solidified matrix and then in the vitrification of lyoprotectants. This is also advantageous for the maintenance of a high encapsulation efficiency. With other liposome methods based on W1/O/W2 emulsions, the organic solvents are usually removed by evaporation at a relatively high temperature [7,41]. Thus, the inner aqueous solution as well as agents may leak out by diffusion through the fluid oil phase. Furthermore, the evaporation process might break down the double emulsions and lead to drug loss.

However, the vesicle membrane fluidity and the physicochemical properties of the drug involved are also major factors affecting the encapsulation efficiency of the liposomes [5,42]. One of the reasons for the rather low encapsulation efficiency of 5-fluorouracil is the fact that 5-fluorouracil could easily and rapidly escape by diffusion through both the oil phase and the lecithin bilayers, since it is neutral, has a low molecular weight and a low partition coefficient between 1-octanol and water (log $P_{octanol/water}$, −0.78). Also, the encapsulation efficiency of drug correlates to some degree with its partition coefficient between 1-octanol and water [5,42,43].

The encapsulation efficiencies of lipophilic agents are usually rather high, since the lipophilic agents are intercalated in the hydrophobic region between the double layers of the phospholipids. These drugs do not leak out due to hydrophobic interaction and, thus, a high encapsulation efficiency is maintained [5,43,44]. This is the case for FDE vesicles which exhibit an encapsulation efficiency of 93% for flurbiprofen.

4.2.3. Lyoprotectants

Disaccharides, such as trehalose, sucrose and lactose, are lyoprotectants known to protect membranes from damage during freeze-drying [45]. Water molecules associated with polar head groups of hydrated phospholipid bilayers may be replaced by sugar molecules which protect liposomes from aggregation and fusion by holding individual vesicles in a vitrified matrix and by reducing the surface tension at the liposome surface during freeze-drying. At the same time, the sugar molecules may prevent disruption of the bilayer by maintaining the phospholipid in a fluid-like state to avoid passage through the gel to the liquid-crystalline phase transition [46–48]. These may also be reasonable explanations for the effects of the lyoprotectants on the size and the encapsulation efficiency of FDE vesicles which have similar or identical structure to that of freeze-dried liposomes.

Mannitol has also been tentatively used as a lyoprotectant for FDE liposomes, but had a much weaker protective effect (data not shown) and no glass-forming properties revealed by X-ray diffraction in Fig. 5. Mannitol encapsulated in liposomes has been reported to localize in the aqueous core and show no evidence of interaction with the lipid head groups [49]. This is also a possible reason for its weaker lyoprotective effects and this is consistent with our results. However, when mannitol together with sucrose (1:1, w/w) was used as a lyoprotectant, the lyoprotective effects were identical with those of sucrose and, in addition, the lyophilized products possessed excellent loosen features and could be very easily and rapidly rehydrated.

4.3. Others

Although no further research has been carried out on the entrapment of FDE liposomes for the agents such as proteins, peptides and nucleic acids, Szoka and Papahadjopoulos have investigated the REV method, which is similar to FDE, and found that alkaline phosphatase could be loaded and retained an appreciable amount of activity (41%) after encapsulation and the encapsulation efficiency is of 43% and 40% for poly(A) and a 25s rRNA, respectively [8]. In fact, during the FDE procedure, the water-soluble agents are always isolated from organic solvents by amphiphilic phospholipid molecules, then freeze-dried, and this may reasonably be used for the entrapment of proteins, peptides and nucleic acids, which are rather susceptible to organic solvents. Since the FDE method has the unique advantages of removing organic solvents by freeze-drying and allowing the preparation of stable dried products, this suggests that the FDE method might also be an important technique for the introduction of various pharmacological and biochemical agents into biological systems both in vitro and in vivo.

In addition, it should also be pointed out that the application of the FDE procedure to remote loading of agents into liposomes by the gradient method is possible and simple [50].

In conclusion, in this investigation, a novel liposome preparation procedure based on double emulsions and lyophilization is described. It can be easily used to produce sterile submicron liposomes with a relatively high encapsulation efficiency for various agents. Because the liposomal drug can be provided as a dry product, many of the problems associated with aqueous liposome preparations can be avoided. Due to its advantages, this approach is valid for the encapsulation of agents in vesicles to form a product that may be suitable for the efficient delivery of therapeutic agents.

Acknowledgements

This work was partly supported by the National Nature Sciences Foundation of China (Grant Number 30472100). We are grateful to Dr. Jinrong Liu for the atomic force micrographs. We thank Aijun Hao,Yong Zhang and all the other members from our laboratory for their help, discussions and suggestions. We would also like to thank Li Zheng from the central laboratory for kind assistance in the experimental work.

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