Encapsulation of Drugs by Lyophilized, Empty Dipalmitoylphosphatidylcholine Liposomes

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This study was concerned with whether drug-entrapping liposomes can be prepared by using lyophilized, empty dipalmitoylphosphatidylcholine (DPPC)–liposomes. It was found that drug-entrapping liposomes could be prepared by warming a mixture of lyophilized empty DPPC–liposomes and aqueous drug solution over the solid–liquid crystal phase-transition temperature (T_c), indicating that warming ($> T_c$) resealed the defect in the bilayer membranes after incorporation of the drug into the empty liposomes. The pharmaceutical potential of this new method was examined, and the results proved that drug-containing liposomes could be prepared readily or immediately before use, and with reproducibly high efficiency (40—70%) of entrapment.

Keywords liposome; freeze-drying; entrapment; preservation; dipalmitoylphosphatidylcholine (DPPC); structural defect; annealing; rehydration

Many attempts to use liposomes as a drug carrier have recently been made in the pharmaceutical and clinical fields.[1-17] However, it has been difficult to find an approach suitable for practical use in therapy. One major problem is to achieve high efficiency of drug-entrapment by liposomes and another is to preserve drug-containing liposomes stably for a long period. To overcome the latter problem, freezing and freeze-drying of liposomes have been studied,[3,7,9-17] and it was reported that a cryoprotectant was useful to stabilize drug-containing liposomes during freezing and freeze-drying.[13-15] On the other hand, nucleation of ice during the freezing process not only concentrates liposomes and free drug but also makes the liposomes unstable, resulting in their aggregation and fusion. Methods for producing drug-containing liposomes by freeze-thawing[18] or freeze-drying[9,10] of an aqueous mixture of empty SUV (small unilamellar vesicles) and a drug are known, but the mechanism of incorporation of drugs into the empty liposomes is not necessarily clear. In this paper, the stability of dipalmitoylphosphatidylcholine (DPPC)–liposomes to freezing and freeze-drying is described and a new method to entrap drugs in DPPC–liposomes is demonstrated and discussed.

Experimental

Materials The following materials were used; DL-dipalmitoylphosphatidylcholine (DL-DPPC; types I and I-S; from Sigma Chemical Co.), dicetylphosphate (DCP; from Sigma Chemical Co.), dipalmitoylphosphatidylglycerol (PG; from Sigma Chemical Co.), dipalmitoylphosphatidic acid (PA; from Sigma Chemical Co.), L-dipalmitoylphosphatidylcholine (L-DPPC; from Avanti Phospholipids, Inc. and Nippon Oil & Fats Co., Ltd.), stearylamine (SA; from Tokyo Kasei Co.), cholesterol (Chol; from Nakarai Chemical Co.), egg phosphatidylcholine (egg PC; from Avanti Phospholipids, Inc.), 5-fluorouracil (5-FU; from Daikin Industrial Co.). Bovine insulin, FITC-dextran (FD-10S), human serum albumin (HSA) and bovine serum albumin (BSA) were purchased from Sigma Chemical Co. Cephalothin, cephalexin and latamoxef were obtained from Shionogi & Co., Ltd. These materials were used without further purification.

Retention of FITC-Dextran Entrapped in Liposomes after Freeze-Drying and Rehydration Large vesicle (LV) and small vesicles (SV) entrapping FITC-dextran (5 mg/ml) alone or with trehalose (50 mg/ml; TR) or mannitol (50 mg/ml; MA) were freeze-dried with the water containing TR (0.02 mg/ml; a), TR (50 mg/ml) or MA (50 mg/ml), respectively. The lipid concentration of the liposome suspensions subjected to freeze-drying was 5 mg/ml. The freeze-dried liposomes were rehydrated with a large amount of the same vehicle as that of the initial liposome suspension, and the retention of the entrapped solute was evaluated. LV were prepared according to the conventional hydration method. SV were prepared by extrusion of LV through a 0.1 μm Nuclepore membrane. Particle size was measured by a dynamic light scattering method (Coulter N4) and retention was evaluated by measurement of fluorescence.

Preparation and Lyophilization of Empty Liposomes Lipid mixture in chloroform solution was dried to leave a thin film on the wall of a round-bottomed flask by removal of the organic solvent using a rotary evaporator. The lipid film was hydrated with water and agitated at 50 °C. When egg PC (100 mg) was used, the lipid film was hydrated with aqueous solution containing mannitol (the weight ratio to total lipid was 1—1.5) and vortexed at room temperature. The obtained lipid suspension (5 ml) was then frozen in a dry ice–acetone bath and dried by a freeze dryer (Tokyo Rika Kikai Co., Ltd.; EYELA FD-80 freeze dryer). In some experiments, SUV were lyophilized; they were prepared by sonication (Ohtake sonifier, tip-type) of the turbid suspension described above followed by collection of the optically clear supernatant after ultracentrifugation (85000 *g* for 30 min). The lyophilized liposomes were stocked at −20 °C unless otherwise mentioned.

Rehydration of lyophilized liposomes was done under various conditions of lipid and drug concentrations, temperature and incubation time. These conditions are indicated in each table. Drug-containing liposomes were separated from free drug and washed three times by ultracentrifugation (100000 *g* × 60 min).

Encapsulation Efficiency and Captured Volume of Liposomes Drug-containing liposomes were disrupted by addition of Triton X-100 and the total amount of the released drug was determined, usually by high performance liquid chromatography (HPLC) (Shimadzu LC-6A, SPD-6A, RF-535). The concentration of 5-FU was determined under the following HPLC conditions: Nucleosil $_{10}C_{18}$, 0.01 M KH_2PO_4, 265 nm. The concentration of latamoxef was determined under the following HPLC conditions: μ-Bondapak C_{18}, 0.05 M ammonium acetate/methyl alcohol = 11/1, 275 nm. The concentrations of FITC-dextran and calcein were determined by measurement of fluorescence (Hitachi F-3000). The concentration of insulin was determined according to the method of Terabe *et al.*[19] The encapsulation efficiency and the captured volume were calculated by means of the following equations.

$$\text{encapsulation efficiency (\%)} = \frac{\text{total amount of drug entrapped in liposomes}}{\text{total amount of drug applied}} \times 100 \quad (1)$$

$$\text{captured volume} = \frac{\text{\% encapsulation} \times \text{volume (}\mu\text{l) of drug solution applied}}{100 \times \text{total amount (mg) of lipid applied}} \quad (2)$$

If the drug is not adsorbed or partitioned in the lipid bilayers, the captured volume calculated from the above equation signifies the volume of aqueous compartment of the liposomes.

Drug Entrapment Efficiency by Freeze-Dried Empty Liposomes Stored at Various Temperatures The liposomes prepared by the conventional vortexing method were extruded through a 0.2 μm Nuclepore membrane. Next, 0.3 ml portions of the extruded liposome suspension (17 mg lipid/ml) were pipetted into vials (BVK-30) and freeze-dried under vacuum (1×10^{-1} mbar, −20 °C). After 24 h, the vials were filled with nitrogen gas

and sealed with rubber stoppers. The samples were stored at different temperature (3, 25 and 40 °C) and rehydrated with 0.2 ml portions of aqueous solution of FITC-dextran (2.6 mg/ml) or latamoxef (5.3 mg/ml). Entrapment efficiency was represented as the mean with standard deviation in parentheses ($N=4$).

Results

Retention of FITC-dextran entrapped in liposomes after freeze-drying and rehydration is shown in Table I. The results from liposomes of different sizes prepared with egg PC or DPPC indicated that SV displayed higher retention than LV, and egg PC liposomes exhibited higher retention than DPPC liposomes. Table I also shows that TR is an effective cryoprotectant against freeze-drying, as reported previously.[14,15]

In contrast, when MA was used as a cryoprotectant or when sufficient TR was not used, the release of a large amount of the contents was observed as well as an increase in particle size of SV, indicating fusion between liposomes (enlargement of liposome size was observed under an optical microscope). In the case of DPPC–LV almost all the contents were released even in the presence of TR. Such instability of liposomes appeared to be related to disintegration and reintegration of the lipid bilayer membranes during the freeze-drying and rehydration processes. In general, ice formation disrupts bilayer membranes of liposomes. From a comparison with the results for egg PC liposomes, DPPC–liposomes were assumed to be rehydrated with more incomplete annealing or restoration of the membrane. Such membrane damage was expected to be repaired by warming above the phase transition temperature (T_c), thereby resulting in an improvement of retention. The next experiments were done to confirm this. Empty multilamellar vesicles (MLV) and SUV were prepared from DL-DPPC according to the conventional vortexing method and sonication method, respectively, then freeze-dried. Table II shows the incorporation of drugs into empty DPPC–liposomes by incubation of an aqueous mixture of freeze-dried empty liposomes and a drug.

Table II demonstrates that rehydration of freeze-dried empty liposomes by aqueous solution containing drugs without heating or addition of drugs to the aqueous suspension of freeze-dried empty liposomes after preheating ($>T_c$) resulted in negligible entrapment of drugs by the liposomes. In contrast, heating above T_c after rehydration of freeze-dried empty liposomes with drug solution (operation DT shown in Table II) produced considerable entrapment. These results implied that freeze-drying of DPPC–liposomes produced defects large enough to allow insulin or 5-FU to permeate through the bilayer membrane, the integrity of which was then reinstated by heating above T_c after rehydration. The operation DT shown in Table II could be a novel and simple way to prepare drug-containing liposomes. Thus, we evaluated the possibility of pharmaceutical application of this new method in regard to various lipid components and several drugs. Table III shows that this method was widely applicable to various lipids and drugs, and that high efficiency of entrapment could be achieved under suitable conditions. Further, the manipulation was very simple. The reproducibility of the entrapment efficiency by this method within a day or during storage was also tested. The results in Table IV showed a slight decrease in the potential of freeze-dried empty liposomes to incorporate drugs during long-term preservation.

TABLE I. Retention of FITC-Dextran Entrapped in Liposomes after Freeze-Drying and Rehydration

Liposome		Before freeze-drying		After rehydration	
		Size (nm)	Cryop.	Size (nm)	Retention (%)
PA/DPPC	LV	≥3000	TR	≥3000	6.1
(5/100)	LV	≥3000	MA	≥3000	5.7
	SV	100—200	TR	100—200	48.7
	SV	100—200	MA	≥3000	38.3
	SV	100—200	—	≥3000	11.6
PA/egg PC	LV	≥3000	TR	≥3000	37.4
(5/100)	LV	≥3000	MA	≥3000	11.1
	SV	100—200	TR	100—200	88.4
	SV	100—200	MA	≥3000	44.3

Cryop., cryoprotectant.

TABLE II. Entrapment by Rehydration of Lyophilized Empty DPPC–Liposomes with Aqueous Solution Containing a Drug

Operation	Entrapment (%)	Captured volume (μl/mg lipid)
a. Insulin		
DR	3.0	0.2
TD	1.6	0.1
DT	60.5	4.0
b. 5-FU		
DR	0.1	0.04
TD	0.1	0.04
DT	36.7	14.3

Lyophilized empty DL-DPPC MLVs (a) and SUVs (b) were rehydrated with 0.01 N HCl aqueous solution (7 μl/mg lipid) of insulin (10 mg/ml) and aqueous solution (40 μl/mg lipid) of 5-FU (5 mg/ml) respectively. DR: The mixture of freeze-dried empty liposomes (FDEL) and aqueous drug solution was kept at room temperature. TD: After the mixture of FDEL and water was heated at 50 C, a drug was added at room temperature. DT: The mixture of FDEL and aqueous drug solution was heated at 50 C for 10 min (for a) or 5 min (for b).

TABLE III. Entrapment of Various Drugs by Rehydration of Lyophilized Empty Liposomes

Lipid composition of liposomes	Drug	Volume of solution for rehydration (μl/mg lipid)	Warming ($>T_c$)	Entrapment (%)
DL-DPPC	Cephalothin	14	50 °C × 1 min	34.5
	Cephalexin	38	50 °C × 2 min	37.3
	Latamoxef	40	50 °C × 1 min	25.0
	Insulin	13	50 °C × 5 min	71.6
	5-FU	40	50 °C × 5 min	41.8
	5-FU	13	50 °C × 1 min	36.6
	BSA	15	50 °C × 1 min	66.7
DL-DPPC/SA (9/1)	Latamoxef	13	50 °C × 1 min	42.3
	5-FU	13	50 °C × 1 min	39.9
L-DPPC/SA (100/1)	5-FU	40	50 °C × 5 min	44.0
DL-DPPC/DCP (9/1)	Latamoxef	13	50 °C × 1 min	28.7
	5-FU	13	50 °C × 1 min	33.3
L-DPPC/DCP (100/3)	5-FU	40	50 °C × 5 min	39.6
L-DPPC/PG (10/3)	5-FU	40	50 °C × 5 min	49.3
L-DPPC/PA (100/1)	5-FU	40	50 °C × 5 min	45.2
DL-DPPC/Chol (1/1)	5-FU	40	50 °C × 5 min	23.7
Egg PC	HSA	40	Room temp.	37.3

5-Fu, 5 mg/ml; BSA, 5 mg/ml; HSA, 5 mg/ml; cephalothin, 4 mg/ml; cephalexin, 5 mg/ml; latamoxef, 5 mg/ml; insulin, 6.5 mg/ml.

TABLE IV. Drug Entrapment Efficiency by Freeze-Dried Empty Liposomes Stored at Various Temperatures

Temperature (°C)	Liposomes, PA/DPPC (1/10)[a]				Liposomes, SA/DPPC (1/10)[b]			
	Initial	1 d	1 week	4 weeks	Initial	1 d	1 week	4 weeks
3	51.0 (0.3)	53.5 (7.2)	40.8 (4.5)	39.7 (3.3)	40.3 (3.1)	51.7 (2.7)	46.3 (3.0)	38.3 (1.4)
25	51.0 (0.3)	51.9 (1.6)	40.2 (2.3)	39.1 (2.6)	40.3 (3.1)	45.1 (2.6)	40.1 (10.8)	32.7 (3.3)
40	51.0 (0.3)	50.7 (2.3)	51.3 (6.7)	21.8 (9.0)	40.3 (3.1)	42.5 (3.8)	33.1 (6.1)	26.2 (2.4)

a) Test compound, FITC-dextran. *b*) Test compound, latamoxef.

However, rather high potential still remained after 4-week storage, except at 40 °C. The preservation conditions adopted here did not induce considerable change in liposome size (those measured with a Coulter counter TA were 3—4 μm for PA/DPPC (1/10) liposomes and 2—3 μm for SA/DPPC (1/10) liposomes).

Discussion

Among methods to prepare drug-containing liposomes, vortexing and the reverse phase evaporation (REV) method are used widely, but there are also other methods, *e.g.* an aqueous mixture of empty liposomes and drugs is subjected to dehydration by evaporation,[6] freeze-thawing or freeze-drying.[9,10,18] These methods have the common point that both solutes and liposomes are concentrated locally by dehydration or ice-nucleation, leading to high efficiency of drug entrapment. The mechanism by which drugs are incorporated into empty liposomes in these methods may involve damage to the bilayer membrane resulting from growth of the ice phase,[20,21] increase in osmotic pressure due to concentration of salt or solute, phase transition (solid–liquid crystal), or fusion of liposomes during the process of freezing, drying, or thawing. Which process is the major one has not yet been established.

The results in Table I indicate that egg PC liposomes freeze-dried with TR display higher retention of entrapped drugs, suggesting that TR is an effective cryoprotectant, as reported before.[14,15] However, DPPC liposomes (especially LV) demonstrate exceedingly low retention even in the presence of TR. The reason for this low retention might be lack of annealing of freeze-drying-induced damage in the bilayer membranes upon rehydration. In contrast, in the case of egg PC liposomes, the damage might be readily annealed during rehydration because hydrated egg PC membrane is in a liquid state at room temperature. A similar phenomenon is also observed during the freeze-thawing process. Table V shows the incorporation of calcein into liposomes by freeze-thawing an aqueous mixture of empty egg PC or DPPC MLV and calcein. The difference in entrapment efficiency between the two kinds of liposomes might be ascribed to whether the phase of the lipid bilayer membranes is solid or liquid crystal after thawing. The entrapment of calcein by egg PC liposomes is speculated to be primarily a result of its penetration through defects in the bilayer membrane preformed by ice nucleation during the freeze-thawing process and to be secondarily completed by resealing of the defects during the thawing process. The resealing process might proceed easily since phase transition of egg PC bilayer membrane from solid to liquid crystal occurs before ice-melting during the thawing process. In contrast, the damage to the DPPC membrane is not annealed during the thawing process because the T_c is higher than room temperature, thereby resulting in negligible entrapment.

TABLE V. Incorporation of Calcein into Empty Liposomes (MLV) by Freeze-Thawing

Liposome	Entrapment (%)
DPPC/PA (10/0.5)	0.0
Egg PC/PA (10/0.5)	2.2

Empty liposomes (MLV) were prepared according to the conventional vortexing method. The mixture of calcein (2 mM) and liposomes (3.8 mg/ml) was subjected to freeze-thawing twice.

On the basis of these considerations, a simple new method was proposed, by which drug-containing DPPC liposomes can be readily prepared from freeze-dried empty liposomes. It was assumed that the inclusion of a resealing process of freeze-dried DPPC–liposomes during rehydration with drug solution by heating above the T_c might complete the drug entrapment. The validity of this assumption was supported by the results shown in Table II, and the proposed method was proved to be feasible. The steps involved in this method are as follows: freeze-drying of liposome ruptures the membrane; freeze-dried, membrane-damaged DPPC–liposomes are resuspended without annealing; mixing of freeze-dried empty DPPC–liposomes and aqueous drug solution results in incorporation of drug through the defects of the membrane; after the rehydration, the defects are resealed by warming ($> T_c$). In the case of freeze-dried egg PC liposomes, the incorporation and the resealing might occur simultaneously during the hydration at room temperature.

The applicability of the above-proposed method to various lipid compositions and several drugs was tested and the results were satisfactory. High entrapment efficiency could be reproducibly obtained under adequate conditions (Tables III and IV). The principal advantages of the new method may be summarized as follows: (i) the manipulation is simple; (ii) drug-containing liposomes can be prepared immediately before use; (iii) high entrapment efficiency can be reproducibly obtained; (iv) this method can incorporate the process of microbe removal by the membrane extrusion of empty liposomes before freeze-drying. However, there is still much room for improvement in this method for pharmaceutical application. The influence of rehydration conditions such as the kind of salt or additive, salt concentration, *etc.* on liposome characteristics is being clarified and will be the subject of a subsequent paper.

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