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Preparation of novel double liposomes using the glass-filter method

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Abstract

The glass-filter method, a newly developed preparative method for liposomes, was applied for preparation of novel double liposomes. Double liposomes were prepared by filtering a suspension of liposomes prepared using a G4 filter (pore size: 10–16 μm) into a G3 filter (pore size: 40–100 μm) coated with a similar lipid layer. The morphological structure of the double liposomes was confirmed using scanning electron microscopy by the freeze-fracture method to be multivesicular vesicles consisting of small liposomes enveloped in larger liposomes. The diameter of liposomes prepared using the G4 filter was 0.8–2 μm and that of liposomes prepared using the G3 filter or double liposomes was 5–10 μm. These results suggested that the particle size of liposomes is dependent on the pore size of the glass-filter. The encapsulation efficiencies of double liposomes for brilliant blue FCF (BB) and erythrosine (ER) were higher than those of liposomes prepared by the standard Bangham method. Double liposomes showed suppressed release of BB or ER compared with normal liposomes. In particular, no release of BB was observed from the double liposomes prepared with stearylamine. These findings implied that the outer lipid layer protects the inner liposomes. The glass-filter method is the only method that we can get the double liposomes in a short period, and double liposomes prepared by this novel method had adequate size and good stability in vitro.

Keywords: Double liposomes; Preparation; Glass-filter method; Drug loading; Stability

1. Introduction

Liposomes have been utilized as effective drug carriers, a vehicle for gene delivery and biomembrane models (Desormeaux and Bergeron, 1998; Liu and Huang, 2002; Chatterjee and Agarwal, 1988). Liposomes are generally prepared an ultrasonication or by the method of Bangham, and liposomes can be classified as (1) multilamellar vesicles (MLV; 0.2–10 μm), (2) small unilamellar vesicles (SUV; 25–50 nm) or (3) large unilamellar vesicles (LUV; 100 nm–1 μm). However, for these liposomes, the volume of the inner phase is small and the loading volume of water-soluble drugs is low. Therefore, a method for producing reverse-phase evaporation vesicles (REV), which are LUV

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with a large inner phase, was developed (Szoka and Papahadjopoulos, 1978). Further, a technique for producing a novel type of liposomes, designated here as 'double liposomes', was also developed. Double liposomes consist of smaller liposomes encapsulated in a lipid layer, i.e. multivesicular vesicles (Kim et al., 1983; Talsma et al., 1987; Walker et al., 1997). The outer lipid layer protects the inner liposomes against degradation by several enzymes, and therefore they are thought to be more effective as drug carriers than normal liposomes. In this study, a glass-filter method that can prepare liposomes in a short time (Machida et al., 1992) was used instead of the Bangham method (Bangham et al., 1965). In addition, the physicochemical characteristics, drug loading and stability of novel double liposomes prepared by the glass-filter method were investigated.

2. Materials and methods

2.1. Materials

Hydrogenated soybean phosphatidylcholine (H-soyaPC) was kindly supplied by NOF Co. Ltd (Tokyo, Japan). Stearylamine (SA) and phosphatidylserine (PS) were purchased from Sigma Chemical Company (St. Louis, USA). Brilliant blue FCF (BB) and erythrosine (ER) were from Tokyo Kasei Kogyo Co., Ltd (Tokyo, Japan) and Wako Pure Chemical Industries, Ltd (Osaka, Japan), respectively. The other chemicals and solvents were of reagent grade and were used without further purification.

2.2. Preparation

Twenty-six micromole H-soyaPC was dissolved alone or with 2.6 μM SA or 2.6 μM PS as lipids with electrical charges in chloroform. BB or ER in ethanol (1 mM) was poured into the lipid solution. The mixture was infiltrated into a G4 glass-filter (pore size: 10–16 μm) and chloroform was evaporated with a gentle stream of nitrogen gas at room temperature. The lipid layer formed on the glass-filter was hydrated with 1 ml of phosphate buffered saline (pH 7.4) for 10 min and the glass-filter was soaked in a water-bath and was sonicated for 30 min at 60 °C. Then, 3 ml of buffer solution was passed through the filter repeatedly by alternately pressing syringes connected to both sides of the filter to form the liposomes. Double liposomes were prepared by filtering a suspension of liposomes prepared using a G4 filter into a G3 filter (pore size: 40–100 μm) coated with a similar lipid layer.

The structure of the double liposomes was observed using a Nikon biological micrograph. The mean diameter was determined by the microscopic method. The detailed morphological structure of the double liposomes was confirmed using scanning electron microscopy by the freeze-fracture method.

2.3. Drug loading

Four ml of PBS was added to 1 ml of suspension of liposomes containing BB or ER, the suspension was centrifuged (1800 rpm, 10 min) and the supernatant was removed. This process was repeated twice. To destroy the liposomes, 1 ml of 10% Triton X-100 was added to the pellet. The solution was analyzed spectrophotometrically at 630 or 524 nm for BB or ER, respectively. Encapsulation efficiency (%) was calculated by the following formula:

$$\text{Encapsulation efficiency}(\%) = \text{Elemental drug in pellet}/\text{Elemental drug added}$$

2.4. Stability

Four milliliter of PBS was added to 1 ml of suspension of liposomes containing BB or ER, and the suspension was centrifuged (1800 rpm, 10 min). The supernatant was removed, and 4 ml of Japanese Pharmacopoeia XIII (JP XIII) first fluid (pH 1.2) was added to the pellet. After incubation with stirring at 50 strokes/min for 1 h at 37 °C, the samples were centrifuged, and then the supernatant (4 ml) was taken as the sample solution. Twenty milliliter of JP XIII second fluid (pH 6.8) was poured into the pellet, and the suspension was

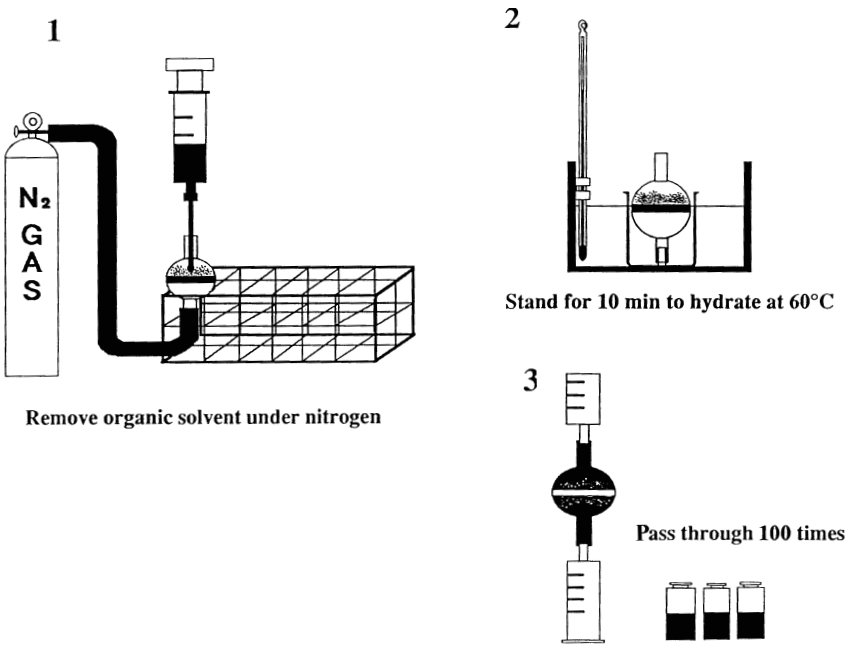

Fig. 1. Preparation of liposomes by the glass-filter method. (1) Remove organic solvent under N_2 gas. (2) Stand for 10 min to hydrate at 60 °C. (3) Pass through 100 times.

incubated with stirring at 100 strokes/min for 1 h at 37 °C. At selected time intervals, 5 ml of the suspension was taken, and the sample solution was analyzed spectrophotometrically.

3. Results and discussion

3.1. Preparation

MLV, liposomes using phosphatidylcholine with a high transformation temperature or the addition of cholesterol results in stability against gastric juice, bile acid and lipase (Rowland and Woodley, 1981). In addition, addition of tocopherol (Hunt and Tsang, 1981) as an antioxidant and of collagen (Pajean and Herbage, 1993) as a membrane stabilizer were reported to lead to membrane stabilization. In this study, H-soyaPC was used as a lipid because (i) the transformation temperature of H-soyaPC is high (53 °C), (ii) hydrogenation prevents membrane lability, and (iii) it is inexpensive. Further, the addition of cholesterol was not a prerequisite for the preparation; i.e. highly stable liposomes could be prepared using only one kind of lipid. These suggested that H-soyaPC had a brilliant future as a lipid for liposome. Fig. 1 delineates a schematic diagram of method for preparation of liposomes. Hydration was performed at 60 °C, which is above the transformation temperature of H-soyaPC as determined by differential scanning calorimetry.

Fig. 2 shows photomicrographs of liposomes prepared using the glass-filter method. The particle sizes of normal liposomes and double liposomes are summarized in Table 1 and Table 2, respectively. The diameter of liposomes prepared using the G4 filter was confirmed to be 0.8–2 μm, while liposomes prepared using the G3 filter and double liposomes were 5–10 μm in diameter. These results suggested that the particle size of liposomes was dependent on the pore size of the glass-filter. As outer liposomes were prepared using a G3 glass-filter, the majority of the size of double liposomes was distributed around the mean particle size (data not shown). Thus, deviatory small values were precluded from the size measurement because they were thought to be non-doubled inner liposomes.

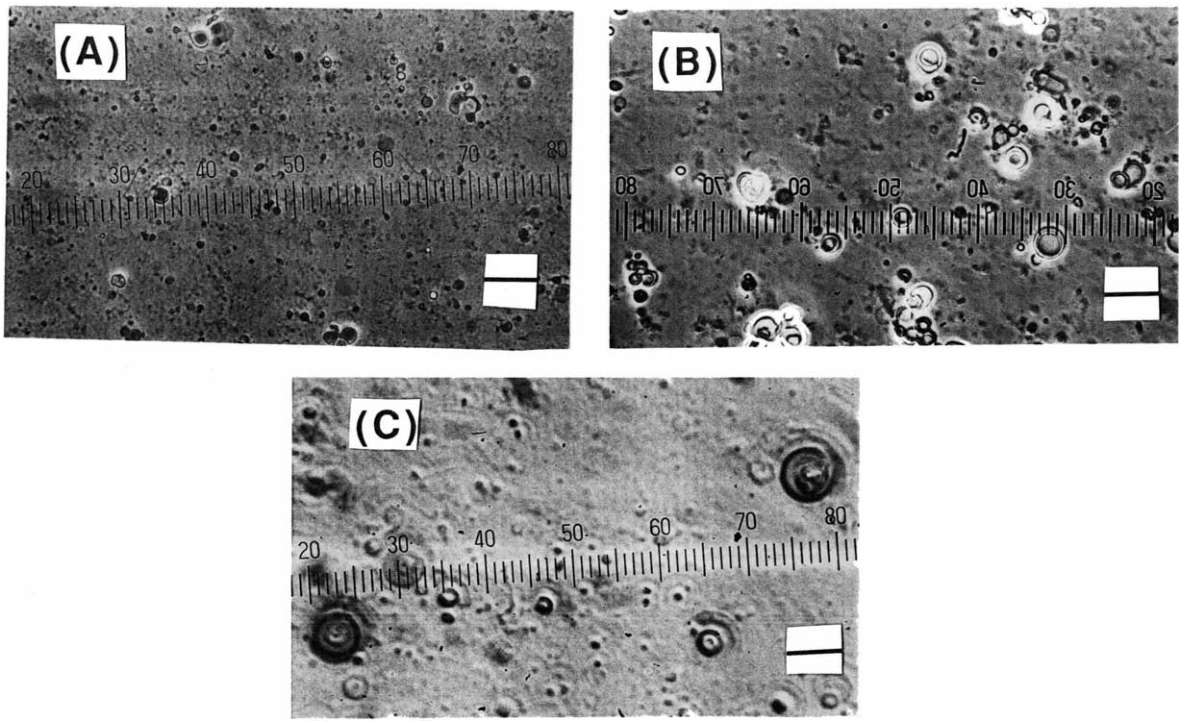

Fig. 2. Photomicrographs of liposomes prepared using the glass-filter method. (A) Liposomes prepared using G4 glass-filters. (B) Liposomes prepared using G3 glass-filters. (C) Double liposomes. Bars, 10 μm.

It was necessary to pressurize the G4 filter using N_2 cylinder because the use of the G4 filter went beyond the bounds of compression using the syringe. Liposomes with diameters in the nanometer range may be prepared using filters with smaller pores if high pressure apparatus can be developed. Fig. 3 shows the morphological structure of double liposomes. As shown in Fig. 3, the double liposomes were confirmed to be multivesicular vesicles, in which small liposomes were enveloped by an outer lipid bilayer. A few photographs were also confirmed to be as 'doubled' structures. Two small liposomes were encapsulated in a large liposome as indicated in this figure. An outer liposome observed encapsulated from 1 to 3 liposomes, further studies are needed to evaluate the number of the inner liposomes.

3.2. Encapsulation efficiency

The encapsulation efficiencies of normal liposomes are shown in Table 1. Those of double liposomes are shown in Table 2. The encapsulation efficiencies of BB and ER were higher than those of liposomes prepared by Bangham method. It was possible to add BB and ER directly to the lipid solution in this glass-filter method, and therefore the encapsulation efficiencies were increased; encapsulation efficiency was approximately 10% when BB was added to the aqueous phase, but 20–50% when BB was added to the lipid solution. Similarly, the encapsulation efficiencies of ER were increased to 40–70%. Addition of the drug to the lipid solution was considered to lead to an increase of local drug concentration compared with when the drug was added to the aqueous phase. The drug was also considered to be eluted from the lipid layer into the inner aqueous phase even after the end of liposome formation. The encapsulation efficiencies of ER were higher than those of BB. ER is water-soluble and its structure includes iodic affixture of fluorescein. The compatibility of the lipid layer and ER may have been enhanced because the polarity of ER was lower

Table 1
Encapsulation efficiencies of model drugs and particle size of normal liposomes

Drug	Lipid composition (glass-filter or molar ratio)	Total lipid concentration (μM)	Concentration of drug (μM)	Encapsulation efficiency (%)	Mean particle size (μM)
Brilliant blue FCF (BB)	H-soyaPC (G4)[a]	26	1	19.2	1.6
	H-soyaPC	26	1	28.9	5.4
			2	24.2	6.6
			4	25.0	5.6
		52	1	41.2	7.5
			2	50.8	8.6
			4	45.6	10.5
	H-soyaPC/SA (10:1)	52	2	27.4	5.8
	H-soyaPC/PS (10:1)	52	2	40.1	6.2
Erythrosin (ER)	H-soyaPC (G4)[a]		1	41.8	1.2
	H-soyaPC		1	40.0	4.6
			2	46.2	5.4
			4	38.0	6.9
			1	68.6	6.6
			2	59.7	8.8
			4	49.7	9.7
	H-soyaPC/SA (10:1)		2	62.0	6.6
	H-soyaPC/PS (10:1)		2	25.0	6.8

[a] Prepared using G4 glass-filter. G3 glass-filter was used for the others.

Table 2
Encapsulation efficiencies of model drugs and particle size of double liposomes

Drug	Lipid composition (Glass-filter)	Total lipid concentration (μM)	Concentration of drug (μM)	Encapsulation efficiency (%)	Mean particle size (μm)
BB	H-soyaPC (G4)	26	1	28.0	
ER	H-soyaPC (G3)	26	1	58.0	5.4
BB	H-soyaPC (G4)	26	2	26.0	
ER	H-soyaPC (G3)	26	2	57.6	8.0
BB	H-soyaPC (G4)	52	1	46.8	
ER	H-soyaPC (G3)	52	1	56.4	10.3
BB	H-soyaPC (G4)	52	2	46.0	
ER	H-soyaPC (G3)	52	2	60.0	9.8
BB	H-soyaPC (G4)	52	2	50.2	
ER	H-soyaPC (G3)	52	2	28.0	8.4
BB	H-soyaPC/SA (G4)	52	2	30.8	
ER	H-soyaPC/PS (G3)	52	2	68.7	7.3
BB	H-soyaPC/SA (G4)	52	2	50.6	
ER	H-soyaPC/PS (G3)	52	2	45.2	7.2

than that of BB due to the structure of ER. Moreover, we added electrically charged lipids into the lipid layer based on the previous report that the repulsion by electrical charge prevented the aggregation of liposomes and that the loading volume was increased due to expansion of the

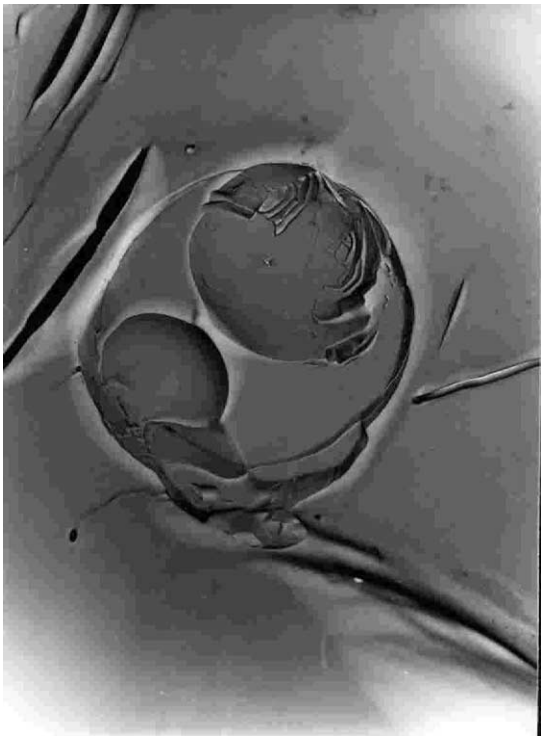

Fig. 3. Freeze-fracture electron micrographs of double liposomes. Bar, 1 μm.

aqueous phase in liposomes (Benita et al., 1984). It was confirmed that the encapsulation efficiencies of BB were increased in the following order: no addition (±) > PS (−) > SA (+). In the case of ER, the encapsulation efficiencies were in the following order: SA (+) > no addition (±) > PS (−). It was not obvious whether the electrical characteristics of the drug itself or addition of the drug into the lipid layer was responsible for the differences in the encapsulation efficiencies. Further studies are needed to clarify the reasons for the characteristics of drug loading.

3.3. Stability

The release patterns of BB or ER from the double liposomes are shown in Fig. 4. No release of BB or ER was observed in JP XIII first fluid (data not shown); however, rapid release was observed within 30 min from the shift into JP XIII second fluid and drugs were released slowly within the following 2 h. It was possible that drug molecules present on the surface of the lipid layer or leakage into the spaces between inner and outer liposomes at the preparation of double liposomes were responsible for the initial release. As shown in Fig. 4, double liposomes showed suppressed release of BB or ER compared with normal liposomes. In particular, no release of BB from the double liposomes with SA was observed. These findings implied that the outer lipid layer protects the inner liposomes. Further, no leakage from the liposomes occurred when double liposomes were prepared using glass-filters.

In conclusion, despite the good solubility of BB and ER in water, the encapsulation efficiencies could be increased by the glass-filter method.

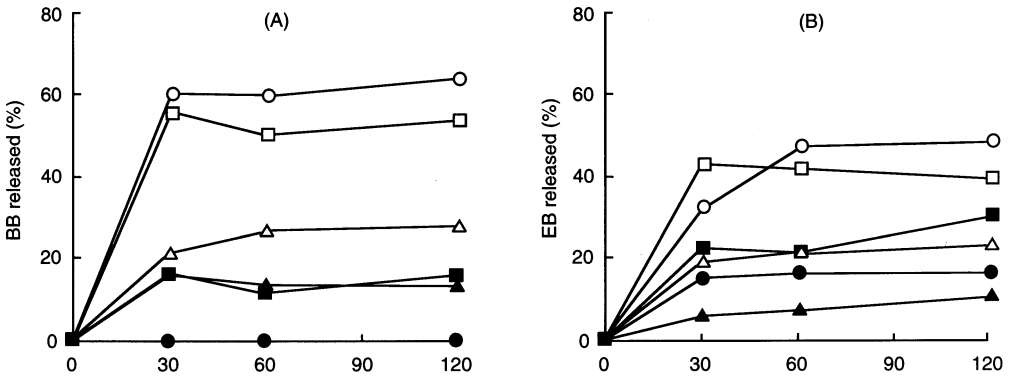

Fig. 4. Release profiles of model drug from liposomes in the JP XIII 2nd fluid after treatment with the JP XIII 1st fluid for 1 h. Open symbols represent the release pattern from normal liposomes, closed symbols represent that from double liposomes. Squares, circles and triangles indicate H-Soya PC alone, that with addition of SA and addition of PS, respectively.

Further, double liposomes showed suppressed release of BB or ER compared with normal liposomes. Double liposomes prepared by the glass-filter method may be a good candidate for drug carriers for oral administration.

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