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RESEARCH PAPER

Characterization of Amphotericin B Liposome Formulations

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ABSTRACT

Liposomes composed of hydrogenated soya phosphatidylcholine (Emulmetik 950®)/cholesterol/charged lipids [dicetyl phosphate (−) or stearylamine (+)] were developed. The hydrogenated soya phosphatidylcholine/cholesterol/charged lipid liposomes at molar ratios of 1:1:0, 7:2:0, 7:2:1 (−), and 7:2:1 (+), with and without the entrapped amphotericin B (0.05 mg AmB/mg lipid), were prepared by a chloroform-film method with sonication. The charges of liposomes were characterized by a Zeta–Meter. The negative liposomes with and without the entrapped AmB showed higher surface charge density than other formulations. The size distribution of liposomes determined by standard error of the mean (SEM) was in the range of 0.115 to 0.364 μm. The smallest size was observed in the negative liposomes with the entrapped drug [7:2:1 (−) AmB]. The lamellarity of more than 15 layers was observed by transmission electron microscope (TEM) in the neutral liposomes with the entrapped drug [7:2 AmB]. The transition temperature and enthalpy of transition (ΔH) were determined by differential scanning calorimetry (DSC). Positive liposomes with the entrapped and unentrapped AmB demonstrated higher ΔH of the first peak than other formulations, indicating higher rigidity of liposomal membrane. The AmB contents in liposomes were determined by high-performance liquid chromatography (HPLC) with ultraviolet (UV) detection at 382 nm. The percentages of entrapment of AmB in all formulations were above 85%. The positive liposome [7:2:1 (+)

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AmB] formulation, which gave the highest thermal stability, was selected for further skin absorption evaluation.

Key Words: Amphotericin B; Liposomes; Hydrogenated soya phosphatidylcholine; Cholesterol; Dicetyl phosphate; Stearylamine.

INTRODUCTION

Liposomes have been initially used as models for studying biological membranes and later considered more frequently as drug carriers.[1–9] Liposomes are widely used as carriers for a variety of drugs, including their application to topical delivery.[10] The topical liposome products have been exploited by the cosmetic industries since 1987, due to their moisturizing properties. In addition, liposomes not only enhance the drug penetration into the skin with the slow release of drug, but also decrease the clearance of drug by minimizing its absorption into the bloodstream.

Amphotericin B (AmB) is the drug of choice for systemic fungal infection.[11] Liposomal AmB products are currently available for systemic, eye and lung applications.[11–17] However, there is none for topical application. Owing to the bulky structure of AmB, it cannot be absorbed through the skin.[12] Since liposomes can enhance the skin absorption,[18] the entrapment of AmB in liposomes might be advantageous. Because of its lipophilic property, AmB will be incorporated in the lipid bilayers. This will result in a maximum entrapment of AmB in liposomes. Thus, AmB entrapped in liposomes is a considered approach for topical application.

The objective of this study was to develop liposome formulations for AmB from various lipid compositions, containing neutral, positive, or negative charged lipid. The best formulation, evaluated from charges, zeta potential, size and size distribution, lamellarity, transition temperature, enthalpy of transition, and the percentages of entrapment of AmB in liposomes, was selected for further skin absorption study.

MATERIALS AND METHODS

Materials

AmB, cholesterol (CHL), dicetyl phosphate (DCP) and stearylamine (SA) were obtained from from Sigma Co. (St. Louis, MO). Fungizone® (FGZ), consisting of 50 mg AmB, 41 mg sodium desoxycholate, and 20.2 mg sodium phosphate was kindly donated by Bristol–Myers Squibb Ltd. (Bangkok, Thailand). Hydrogenated soya phosphatidylcholine (Emulmetik 950®) (HSC) was a gift from JJ-Degussa (T) Ltd., Bangkok. Acetonitrile and methanol were of high-performance liquid chromatography (HPLC) grade, whereas ethylenediaminetetraacetic acid disodium salt dihydrate, chloroform, potassium dihydrogen orthophosphate, sodium hydroxide pellets, and dimethyl sulfoxide were of analytical reagent grade.

Preparation of Liposomes

Liposome dispersion samples were prepared by a conventional chloroform film method with sonication. The lipids used were HSC, CHL, and charged lipids (SA for positively charged lipid and DCP for negatively charged lipid). Four different liposome formulations composed of HSC/CHL=1:1 and 7:2; HSC/CHL/SA=7:2:1(+) and HSC/CHL/DCP=7:2:1(−) at molar ratios, with and without the entrapped AmB (in the form of FGZ) were prepared (Table 1). The concentration of AmB was 0.05 mg per mg of total lipid. For each lot, an amount of 40 mL of each liposome dispersion sample was prepared.

The lipid mixture and AmB were dissolved in 10 mL of chloroform and 30 mL of methanol respectively, and the two solutions were then mixed. For liposome formulations without AmB, the lipid mixture was dissolved in 40 mL of chloroform. The solution was vacuum-desiccated (400 mbar, 65°C, 45 rpm) for 60 min. A thin film layer formed was further dried by vacuum-desiccator for 30 min and then flushed with nitrogen gas for 1 min. The thin film was resuspended in 40 mL of 0.1 M phosphate buffer (pH 7.4), weighed, and swelled by swirling in a water bath (80°C, 190 rpm) for 30 min. The dispersion was cooled to room temperature and the weight of the dispersion was adjusted to the weight before swelling. The resulting liposomes were sonicated with a microtip probe sonicator (Vibracell, Sonics & Materials, Inc., Danbury, CT) for 30 min and were then flushed with nitrogen gas for 1 min, to remove dissolved oxygen. The weight of liposomes was adjusted to the initial weight prior to sonication. The liposome dispersion samples were kept at 4°C and protected from light. Prior to use, they were filtered through Whatman filter paper No. 42 (pore size: 2.5 μm).

Table 1. Compositions of eight different liposome formulations.[a]

	Formulations							
Compositions	1:1	7:2	7:2:1 (+)	7:2:1 (−)	1:1 AmB	7:2 AmB	7:2:1(+) AmB	7:2:1(−) AmB
HSC	0.133	0.175	0.167	0.160	0.133	0.175	0.167	0.160
CHL	0.067	0.025	0.024	0.023	0.067	0.025	0.024	0.023
SA	–	–	0.009	–	–	–	0.009	–
DCP	–	–	–	0.017	–	–	–	0.017
AmB	–	–	–	–	0.010	0.010	0.010	0.010
Molar ratio	1:1	7:2	7:2:1	7:2:1	1:1	7:2	7:2:1	7:2:1
Expected charges	None	None	(+)	(−)	None	None	(+)	(−)

[a]The content of each compound was expressed as gram per 40 mL of liposome dispersion samples.

The freeze-dried liposome powder was prepared using a freeze dryer (Model Lioalfa 10, Telstar, Barcelona, Spain). Vials containing 5 mL of each liposome sample were put into a freeze dryer, with the following prefreeze conditions: 25°C for 1.5 h, −32°C for 1.5 h, and −36°C for 1 h. When the condenser was at −42°C, the primary drying vacuum pump was maximum at 0.35 mbar and minimum at 0.019 mbar for 10 h. The duration of secondary drying with shelf heat at 25°C was 3 h.

Physical Properties Determination of Liposomes

The charges of liposomes were characterized by an electrophoresis type GT-2 cell, which had the molybdenum cylinder anode (+) and platinum rod cathode (−) (Zeta–Meter System 3.0, Zeta–Meter, Inc., Long Island City, NY). Liposome dispersion samples (0.5 mL) dispersed in 15 mL of deionized water were filled into an electrophoresis cell. The ''voltage setting'' switch was set on ''75'' and ''ocular micrometer'' on ''full.'' The liposomes were tracked and monitored 20–30 times as when they moved along the tracking line. If they moved to the left (anode), they were negatively charged, and to the right (cathode), they were positively charged. The experiments were performed in triplicate.

The particle size and size distribution of liposomes were measured by a scanning electron microscope (SEM 840A JEOL, JEOL Ltd., Tokyo 196–8558, Japan) at magnification ranging from 10,000 to 30,000. The freeze-dried liposome powder was picked up by a toothpick and gently spread on the adhesive copper tape attached to the brass stubs. The sample surface on the stubs was covered with gold (Gold coater JFC-1100E Ion Sputtering Device, JEOL Ltd.), and the stubs were examined after coating. The mean and standard deviation of particle size were calculated from 100 particles.

The lamellarity of liposomes was determined by a transmission electron microscope (TEM 1200S JEOL). A formvar-coated grid was inserted horizontally into a drop of liposome on the parafilm for 3 min. This grid was pulled out, blotted side of grid with filter paper, and left dry for 3 min. This grid was then turned with its face down floating on uranyl acetate for 10 min and protected from light. This grid was then picked up, blotted side with filter paper and left dry for 3 min, and then examined with TEM. For particle size and lamellarity observations, the photographs were taken and the contact prints were developed. The determination of liposome size was performed by comparing the apparent size of particles with a bar in the photograph.

The transition temperature (T_c) and enthalpy of transition (ΔH) were determined from the thermogram, generated by a differential scanning calorimeter (Perkin Elmer DSC7, Perkin Elmer Ltd., Norwalk, CT). An amount of 2 to 5 mg of liposomes was put on the 40 μL-aluminium pan with cover. The pan was sealed and put in the sample holder. The reference holder was the 40-μL empty pan with cover. The temperature ranging from 30° to 250°C was used with scan rate of 5°C/min. The FGZ, HSC, CHL, SA, and DCP were also run, using similar DSC conditions. This study was performed in six replicates.

Determination of the Amount of AmB Entrapped in Liposomes

The content of AmB in FGZ and the percentages of entrapment of AmB in all liposome formulations were determined by HPLC. The filtrate obtained from the filtration of liposome formulations through the Whatman filter paper No. 42 was used to determine the total amount of AmB, following 1000-fold dilution with methanol. Another 1-mL filtrate containing 0.25 mg/mL of AmB was centrifuged at 50,000 *g* (4°C)

for 1 h in a Beckman Avanti™ 30 centrifuge (Palo Alto, CA). A 900-μL supernatant was removed and diluted to 10 mL with methanol. This solution was further diluted (10 times) with methanol, to determine the amount of unentrapped AmB. The pellets collected from the centrifuge tubes were resuspended to 50 mL with methanol. This solution was then diluted (10 times) with methanol, to determine the amount of entrapped AmB in liposomes. The loading of AmB in liposomes was also calculated. The experiments were performed in six replicates.

HPLC Assay for the Determination of AmB

Qualitative and quantitative analyses of AmB in FGZ and in liposome formulations were performed by an HPLC (HP1100, Vectra XM series 4, Hewlett Packard, Waldbronn, Germany). An ODS-Hypersil C_{18} (250 × 4 mm i.d., 5-μm particle size) HPLC column was used. The mobile phase was a mixture of acetonitrile/2.5 mM disodium edetate in water (45:55, v/v). High-performance liquid chromatography was performed isocratically at ambient temperature and a flow rate of 1 mL/min with ultraviolet (UV) detection at 382 nm. Under this condition, the retention time of AmB was 4.7 min. Every sample was filtered through a 0.45-μm membrane filter, prior to injection onto the HPLC column (injection volume, 50 μL). Linearity of HPLC was determined from five working standard solutions of AmB in DMSO/methanol (1:999, v/v), over the concentration range 0.02–1.50 μg/mL. The correlation (r^2), intercept, and slope of standard curves were calculated. The peak areas of samples were calculated and compared with those obtained from the standard curve, to determine the amount of AmB. This experiment was performed in duplicate and each replicate sample was injected for three times.

RESULTS

Charges and Zeta Potential of Liposomes

The charges observed from the direction of particle movement and zeta potential values of eight liposome formulations are presented in Table 2. The 1:1, 7:2,

Table 2. Physical appearances and pH[a] charges and zeta potential values of eight liposome formulations.

Formulations	Appearances		pH[b]	Charges	Zeta potential[c] (mV)
	Freshly prepared	Stored at 4°C for 1 d			
1:1	Translucent, white dispersion	Translucent, white dispersion with sediment (1 mm height)	7.517 + 0.006	Negative	39.6 ± 6.9
7:2	Translucent, white dispersion	Translucent, white dispersion with a thin layer sediment	7.517 + 0.006	Negative	36.3 ± 4.8
7:2:1(+)	Translucent, white dispersion	Translucent, white dispersion	7.537 + 0.006	Positive	32.6 ± 2.9
7:2:1(−)	Translucent, white dispersion	Translucent, white dispersion	7.483 + 0.006	Negative	68.9 ± 1.1
1:1AmB	Translucent, yellow dispersion	Translucent, yellow dispersion with sediment (0.5 mm height)	7.54 + 0.01	Negative	57.1 ± 0.3
7:2AmB	Translucent, yellow dispersion	Translucent, yellow dispersion with sediment (2 mm height)	7.54 + 0.02	Negative	59.4 ± 1.2
7:2:1(+)AmB	Translucent, yellow dispersion	Half translucent, yellow dispersion with sediment (10 mm height)	7.52 + 0.03	Positive	47.4 ± 1.2
7:2:1(−)AmB	Translucent, yellow dispersion	Translucent, yellow dispersion with a thin layer sediment	7.49 + 0.01	Negative	62.9 ± 5.3

[a]In comparison, the pH of 0.1 M phosphate buffer solution used as dispersion medium for liposome formulations was 7.49 + 0.01 (n = 3).
[b]Experimental data represent the mean + SD of three determinations.
[c]Experimental data represent the mean ± SD of three determinations.

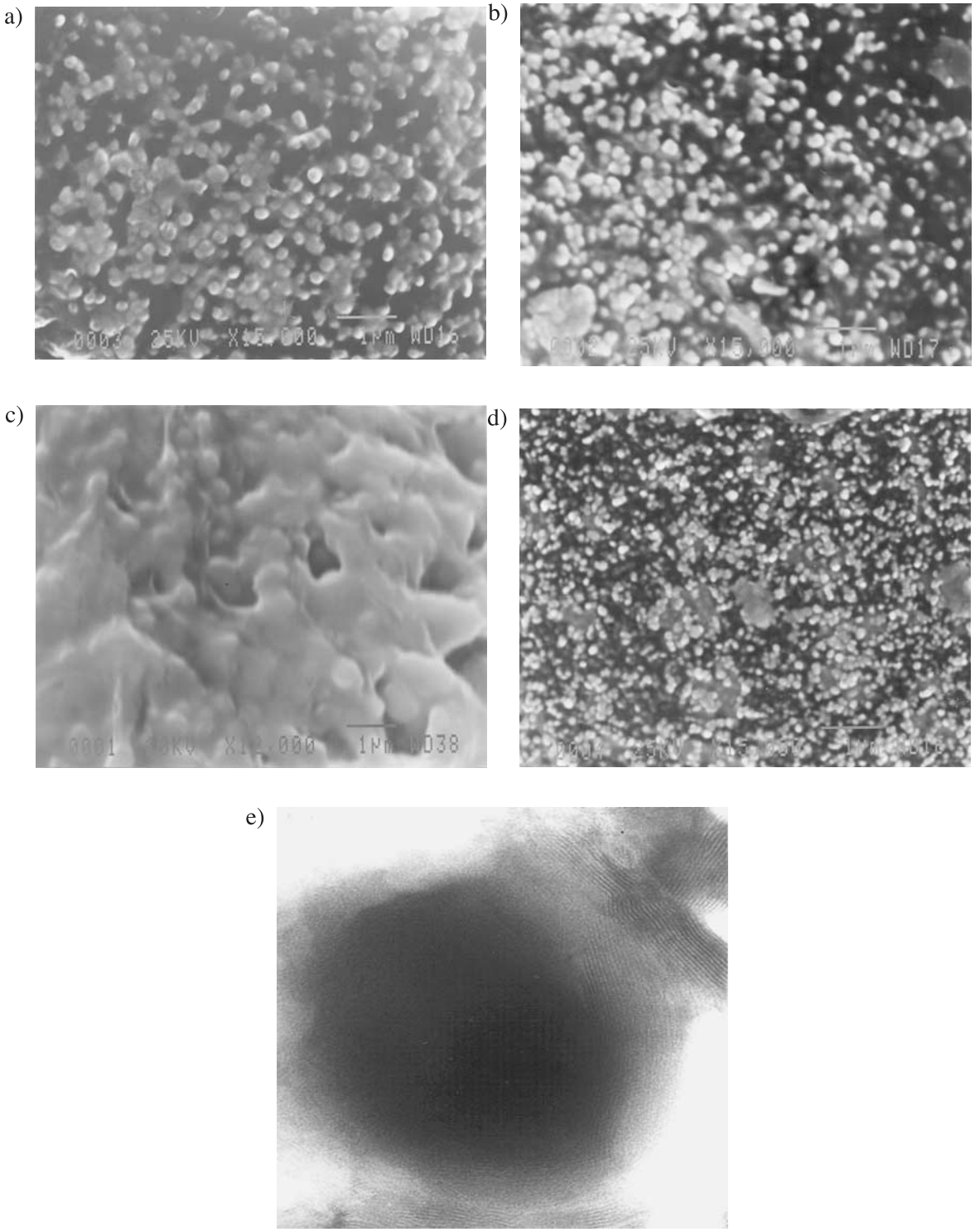

Figure 1. Scanning electron micrographs of the (a) 1:1AmB liposomes (15000×, 0.154 μm); (b) 7:2AmB liposomes (15000×, 0.154 μm); (c) 7:2:1(+)AmB liposomes (12000×, 0.364 μm); (d) 7:2:1(−)AmB liposomes (15000×, 0.115 μm). (e) The lamellarity of the 7:2 AmB liposome formulation.

Table 3. Transition temperature (T_c) and enthalpy of transition (ΔH) of various compounds and liposome formulations.[a]

Compounds or liposomes	Transition temperature (°C)		Enthalpy of transition (J/g)	
	1st Peak	2nd Peak	1st Peak	2nd Peak
FGZ	93.1±1.8	164.7±0.1	224.6±17.7	45.8±1.1
HSC	61.82±0.05	75.9±0.7	1.654±0.006	33.2±3.3
CHL	148.0±0.7	–	68.1±0.6	–
SA	46.0±1.6	–	277.7±1.3	–
DCP	69.0±1.4	–	204.7±4.7	–
1:1	152.0±9.3	215.6±4.0	145.1±46.7	24.2±1.5
7:2	146.9±4.2	214.3±9.8	119.6±37.8	65.9±17.9
7:2:1(+)	131.0±6.3	215.4±3.1	484.4±40.9	67.6±32.4
7:2:1(−)	143.0±7.4	214.5±7.1	177.8±11.8	63.0±16.6
1:1AmB	134.7±1.8	213.4±3.2	172.5±8.8	33.4±0.5
7:2AmB	140.3±2.8	214.3±4.2	249.4±37.1	43.2±12.0
7:2:1(+)AmB	108.8±2.3	206.0±8.9	299.9±54.8	88.2±13.3
7:2:1(−)AmB	85.0±12.9	200.7±9.2	183.9±7.7	152.4±4.8

[a]Experimental data represent the mean ± SD of six determinations.

7:2:1(−), 1:1AmB, 7:2AmB, and 7:2:1(−)AmB liposome formulations showed negative charges (Table 2). The 7:2:1(+) and 7:2:1(+)AmB liposomes were positively charged with some particles showed negative charges (Table 2).

Liposome Size and Lamellarity

Figure 1 shows the micrographs of liposome formulations with the entrapped AmB. The mode of liposome size was used instead of mean, since the liposomes showed abnormal distribution. The size distribution of all liposome formulations, with and without the entrapped AmB, was in the range of 0.115 to 0.364 μm. The smallest size of 0.115 μm was observed in the negative liposomes with the entrapped drug [7:2:1(−)AmB]. The lamellarity of more than 15 layers was observed in the neutral 7:2 liposomes with the entrapped AmB.

Transition Temperature and Enthalpy of Transition

From DSC curves, the lyophilized liposomal powder of eight formulations exhibited two peaks between 30° and 250°C. The T_c and ΔH of FGZ, HSC, CHL, SA, DCP, and eight liposome formulations are summarized in Table 3. The DSC curves of CHL, SA, and DCP exhibited single endothermic peaks at 148°, 46° and 69°C, respectively. The DSC curve of HSC showed two endothermic peaks, at 62° and 76°C.

Determination of the Percentages of Entrapment of AmB in Liposomes

The standard curve of AmB in DMSO/methanol (1:999, v/v) was demonstrated to be linear (r^2=0.9999)

Table 4. Percentages of entrapment of AmB, free AmB, and the amount of AmB per total lipid in liposome formulations.[a]

Formulations	Form of drug	% Entrapment	Loading of AmB (μg/mg lipid)[b]
1:1AmB	Total drug	100.0	–
	Entrapped drug	88.6±6.4	32.9±1.1
	Free drug	3.1±3.3	–
7:2AmB	Total drug	100.0	–
	Entrapped drug	85.8±3.7	36.9±2.2
	Free drug	0.2±0.1	–
7:2:1(+)AmB	Total drug	100.0	–
	Entrapped drug	90.1±2.5	35.1±4.8
	Free drug	0.19±0.05	–
7:2:1(−)AmB	Total drug	100.0	–
	Entrapped drug	86.3±3.4	34.3±8.0
	Free drug	1.9±1.0	–

[a]Experimental data represent the mean±SD of six determinations.
[b]Total lipid in 1 mL of liposome dispersion samples was 5 mg.

over the concentration range 0.02–1.50 μg/mL. The regression equation was as follows:

$$y = 354.34x - 8.01$$

where y=the peak area of AmB (mAU*s) and x=the concentration of AmB (μg/mL). The coefficients of variation calculated during replicate assays varied between 0.4% and 5.5%. The content of AmB determined by HPLC was 42±4% (n=3) of the total weight of FGZ product, close to the labeled amount (45%). The percentages of AmB entrapped in liposomes, the free drug, and the loading of AmB in liposome formulations are shown in Table 4.

DISCUSSION

The negative charges present on the surface of liposomes even without DCP (1:1, 7:2, 1:1AmB, 7:2AmB) may be due to free fatty acid resulting from the partial decomposition of lipids at high temperature (80°C) during preparation of liposomes.[19] However, the 7:2:1(−) and 7:2:1(−)AmB formulations demonstrated higher negatively charged density than other liposome formulations (Table 2), since these liposomes contained negatively charged lipid (DCP). It should be noted that differences in zeta potential might also be due to size differences. The entrapped AmB liposomes had a tendency of higher zeta potential than the unentrapped AmB liposomes (Table 2). Thus, low coagulation should be expected in the entrapped AmB liposomes. However, the entrapped AmB liposomes showed greater coagulation and sedimentation than the unentrapped AmB liposomes (Table 2). The sodium ion in FGZ may accelerate coagulation, by reducing the electrical double layers of particles.[20]

Liposomes without the entrapped AmB demonstrated wider size distribution than the liposomes with the entrapped AmB. The entrapment of AmB in liposomes showed smaller and more uniform size than the liposomes without the entrapped AmB.

The liposome formulations with the presence of AmB were better than those without AmB, because the transition temperature of AmB in the form of FGZ (93–165°C) was between those of HSC (76°C) and CHL (148°C) (Table 3). Thus, the lipid bilayers can be formed easier in the hydration process from the lipids with AmB than the lipids alone.

The ΔH of the second peak of 1:1 and 1:1AmB formulations cannot be obtained accurately, since decomposition occurred. The first small peak of DSC curve of HSC was the pretransition and the second peak was the main transition.[21] Two endothermic peaks were observed in all liposome formulations, with and without the entrapped AmB (Table 3). The first peak demonstrated the transition of the gel phase to the liquid phase of lipid mixture, while the second peak expressed the physical interaction between lipid compositions. The neutral liposomes (1:1AmB, 7:2AmB) and positively charged liposomes [7:2:1(+)AmB] showed higher T_c of the first peak of about 44° and 16°C respectively, than the free drug (FGZ) (Table 3). In contrast, the negatively charged liposomes [7:2:1(−)AmB] demonstrated lower T_c of the first peak (±8°C) than the free drug (Table 3). This may indicate higher stability of AmB in the neutral and positive liposomes than in the negative liposomes. All liposome formulations with and without the entrapped AmB showed lower ΔH values of the first peak than the free drug, except for the positively charged liposomes ([7:2:1(+)] and [7:2:1(+)AmB]), which exhibited higher ΔH of about 260 and 75 J/g respectively, than the free drug (Table 3).

The T_c and ΔH values of the charged liposome formulations were different from the uncharged ones (Table 3). The high ΔH of the first peak of 7:2:1(+) formulations demonstrated a strong binding between the positively charged lipid (SA) and the negatively charged free fatty acid of HSC in the lipid bilayers. However, the 7:2:1(+)AmB formulations exhibited lower T_c and ΔH. This might be due to the effect of zwitter ion of AmB, which may enhance the aggregation of AmB with the positive charges in the lipid bilayers, thereby causing the heterogeneity (lowering of ΔH) of lipid bilayers. The lowering of T_c may also cause the enhancement of phase separation.[22]

The low T_c of the first peak and high ΔH of the second peak of the 7:2:1(−)AmB formulations were observed (Table 3). The zwitter ion property of AmB, the positive charge from sodium salt in FGZ, and the negatively charged lipid (DCP) in liposomes may enhance the aggregation, which in turn will result in phase separation and low T_c value. The high ΔH of the second peak in this liposome system might be due to the interaction between the positive sodium ion and the negative charge of DCP.

The percentages of entrapment of AmB in the 1:1AmB, 7:2AmB, 7:2:1(+)AmB, and 7:2:1(−)AmB liposome formulations were above 85% (Table 4). This result was broadly comparable with that reported previously.[23] The larger size of the 7:2:1(+)AmB liposomes may exhibit higher volume of entrapment. The percentages of entrapment obtained from the free drug concentration in supernatant were not used, since

they appeared not to be reliable (high SD) (Table 4). The loading of AmB in liposomes varied from 33 to 37 μg of AmB per mg of lipid. This value is important for the calculation of doses of AmB in liposome formulations.

In conclusion, the liposomes composed of HSC/CHL/charged lipids at molar ratios of 1:1:0, 7:2:0, 7:2:1(+), and 7:2:1(−) with the entrapped AmB were demonstrated as multilamellar vesicles, with particle sizes ranging from 0.115 to 0.364 μm. The 7:2:1(+)AmB formulation seemed to demonstrate higher stability than other formulations, since it showed the highest ΔH. The percentages of entrapment of AmB in all liposome formulations were more than 85%. The best formulation might be the positively charged 7:2:1(+)AmB liposomes, which exhibited the highest thermal stability. This formulation will be further evaluated for topical use by skin absorption study.

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