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RESEARCH PAPER

Physicochemical Properties of Amphotericin B Liposomes Prepared by Reverse-Phase Evaporation Method

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ABSTRACT

The physicochemical properties of phosphatidylcholine-cholesterol liposomes containing amphotericin B and prepared by reverse-phase evaporation method were studied. Uniformly dispersed liposomal suspensions were obtained by employing 3:1 ratio (by volume) of diethyl ether to normal saline, 5 min sonication time at 7°C, and evaporation of diethyl ether at 25°C. Microscopic examination showed that the prepared liposomes were spheroids with unilamellar, oligolamellar, or multilamellar structure. The liposomes containing amphotericin B 2.0 mol% of total lipid led to the highest percentage of drug entrapment. Liposomes with maximum entrapment efficiency were obtained from using 250 μmol of total lipid. The liposomal amphotericin B possessing the highest drug entrapment efficiency (approximately 95%) with particle size range of 1307–1451 nm was the one composed of 1:1 molar ratio of phosphatidylcholine to cholesterol.

Key Words: Liposomes; Amphotericin B; Reverse-phase evaporation method; Physiochemical properties; Oligolamellar vesicles.

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INTRODUCTION

Amphotericin B finds its clinical uses in treatment of AIDS-related systemic fungal infections, neutropenic cancer, infections of the CNS, lung, peritoneum, genito-urinary system, eyes, and skin. However, the treatment has become increasingly complex due to its low selectivity, high toxicity, and the development of resistance to the drug. The most promising approach for improving the therapeutic efficacy as well as minimizing toxicity of amphotericin B has recently been pointed out to be the entrapment of drug within liposome vesicles since they are easily prepared, biocompatible, and non-toxic.[1] It was also shown that incorporation of amphotericin B into certain type of liposomes could markedly reduce the drug toxicity without loss of antifungal activity.[2,3] The lower level of toxicity had permitted larger doses to be given, and consequently, the liposomal formulations were more effective than free amphotericin B.[4,5] The exceptional therapeutic values of amphotericin B liposomal drug delivery systems are now well clarified.[6–8]

Although in recent years there have been intense developments of various liposomal preparations as well as concurrent evaluations, both in vitro and in vivo, on their advantageous therapeutic efficacy and toxicity,[9] almost all of them were prepared by somewhat conventional procedures such as chloroform-film method. Studies on formulation factors of liposomes entrapping especially the hydrophobic drugs, which were manufactured by some other processes, and their potential for large-scale production, were rarely reported in the literature. The reverse-phase evaporation method developed by Szoka and Papahadjopoulos[10,11] appears to be one method, among the liposome manufacturing processes, that could commercially be scaled up to large volume with advantages for encapsulating drugs, proteins, nucleic acid, and other bioactive agents. The vesicles formed are large, unilamellar in nature with the mean diameter between 200 and 500 nm, and the entrapment efficiency as high as 60%. Besides, the mechanism of vesicle formation in this process has been proposed to be different from that of the chloroform-film method. The vesicles are formed via the collapse of the ''inverted micelles'' in w/o emulsion into a viscous gel-like state when the organic phase is removed by evaporation, which in turn becomes an aqueous liposomal dispersion. It is thus of interest to examine the effect of lipid composition on the entrapment of hydrophobic drug such as amphotericin B into liposomes using this process.

The objective of the present study was to investigate the effects of some formulation factors, i.e., amount of drug added, total lipid content, and lipid composition ratio, on the physicochemical properties (entrapment efficiency, particle-size distribution, and microscopic appearance) of prepared amphotericin B liposomes.

MATERIALS

Amphotericin B USP (AmB) was purchased from Aldamex, Riken, Switzerland. Phosphatidylcholine (PC) (Epikuron® 200) was obtained from Lucas Meyer, Hamburg, Germany. Cholesterol NF (Chol) and dl-α-tocopherol acetate (Vit E) were obtained from E. Merck, Darmstadt, Germany. Diethyl ether ACS reagent and absolute methanol AR grade were supplied by J. T. Baker, Phillipsburg, NJ, USA.

METHODS

Preparation of AmB Liposomes

The AmB liposomes were prepared by the reverse-phase evaporation method as previously described[10] with minor modifications. Amphotericin B 50 mg was dissolved and adjusted to volume with methanol in a 250-mL volumetric flask to a concentration of 0.2 mg/mL. Aliquots of this solution were transferred to a 250-mL round-bottom flask with ground-glass quickfit neck to yield various contents of AmB (0.5, 0.7, 0.9, 1.0, 1.5, 2.0, 3.0, 5.0, and 7.0 mol% of total lipid). Various contents of total lipid at 150, 200, 300, 350, 400, and 450 μmol with molar ratios of PC to Chol at 10:0, 9:1, 8:2, 7:3, 6:4, and 5:5, were dissolved in each of these aliquots of AmB solution, followed by addition of Vit E at 0.6 mol% of total lipid. The mixture was warmed at 40°C under reduced pressure in a rotary evaporator (Eyela, Tokyo Rikakikai, Japan) until a dry, uniform, thin film on the inner wall of the flask was obtained.

The film was further dissolved in 45 mL of diethyl ether, after which 15 mL of 0.9% sodium chloride solution was introduced into the lipid solution by rapid injection through a spinal

needle. The flask was closed with a glass stopper immediately and placed in an ultrasonic bath (Cole Parmer, Vernon Hills, IL, USA) filled with a weak detergent solution. This mixture was sonicated at 7°C for 5 min so that the w/o emulsion was formed. The emulsion was then transferred to a rotary evaporator to slowly evaporate the solvent out under reduced pressure at 25°C until a viscous gel was formed, which subsequently became a homogeneously aqueous suspension. The evaporation process was further continued for at least 30 min at 30–35°C to ensure complete removal of all traces of organic solvent. This liposomal suspension was finally dispersed in 20 mL normal saline solution and subjected to further purification.

Purification of the Resultant Liposomes

The unencapsulated materials as well as residual organic solvent were removed by dialysis against 0.9% sodium chloride solution overnight using cellulose membrane tubing (Spetra/Por, Spectrum Medical Industries, New Brunswick, NJ, USA) with molecular-weight cutoff at 12,000, which was previously stored in normal saline solution before use. The system was maintained at 30°C by means of a circulating water bath. The dialyzing solution was continuously stirred with a magnetic stirrer and changed six times during dialysis for 24 hr. The system was protected from light by wrapping with aluminum foil throughout.

Study of Physicochemical Properties of Prepared AmB Liposomes

Scanning Electron Microscopy (SEM)

The morphology of the prepared vesicles was examined using SEM (Jeol Model SEM-5300, Tokyo, Japan) with accelerating voltage of 10 kV. Samples were fixed with 2% osmium tetroxide before coating with gold,[12] and photographed at 2000× and 7500× magnifications.

Transmission Electron Microscopy (TEM)

The microscopic appearance of the prepared vesicles was examined using negative-staining TEM (Jeol Model JEM-200CX, Tokyo, Japan) with accelerating voltage of 30 kV. Samples on 300-mesh copper grids were stained with 1% phosphotungstic acid, air dried,[13] and photographed at 75,000 × magnification.

Particle-Size Measurement

A submicron particle analyzer (Coulter Model N4MD, Coulter Corporate Communications, Hialeah, FL, USA) was used to determine the particle size of prepared AmB liposomes by measuring the rate of fluctuations in laser light intensity scattered by particles as they diffused through the liposomal suspension at 25°C. The intensity of scattered light was detected by a photomultiplier at an angle of 90° relative to the laser beam. The concentration of the liposomes in the dispersion that was analyzed was approximately 0.5 mg/mL. The particle count/sec was within the range of 2.00×10^5–6.00×10^5. The reproducibility was expressed in terms of the standard deviation of three runs. The solvent used was normal saline and the assumed refractive index was 1.333.

Drug-Entrapment Efficiency

One milliliter of AmB liposomal suspension each of which before and after dialysis, was diluted and adjusted to volume with methanol in a 25-mL volumetric flask, and the amount of drug was determined spectrophotometrically at 405.2 nm (Model GBC 918, GBC Scientific Equipment, Sydney, Australia). The percentage entrapment efficiency of drug was calculated by

$$\% \text{ Entrapment efficiency} = \frac{\text{Content of AmB in post-dialyzed liposomes}}{\text{Content of AmB in pre-dialyzed liposomes}} \times 100 \quad (1)$$

RESULTS AND DISCUSSION

Microscopic Appearance

The AmB liposomes prepared in this study visually appeared as white to yellowish suspensions. Figure 1 is the SEM photomicrograph showing that

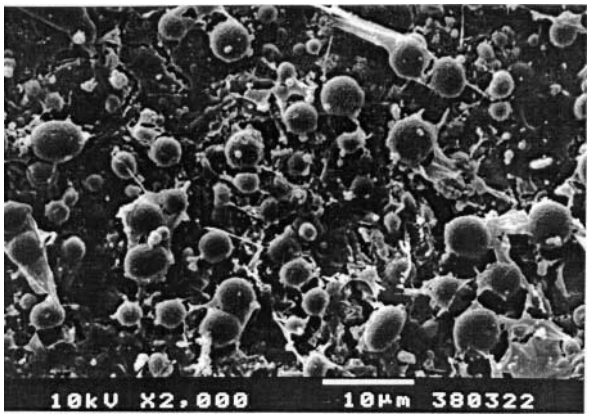

Figure 1. Scanning electron photomicrograph of amphotericin-B liposomes prepared by reverse-phase evaporation method.

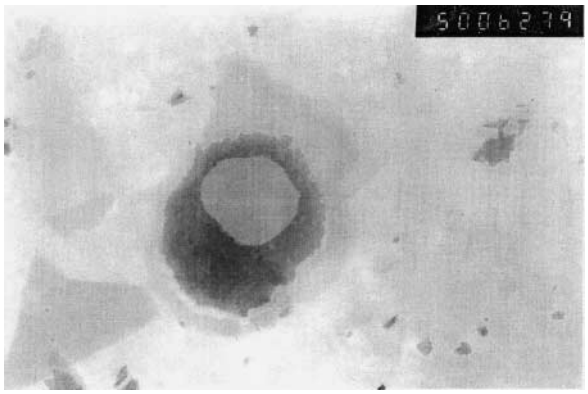

Figure 2. Transmission electron photomicrograph of amphotericin-B liposomes prepared by reverse-phase evaporation method. Magnification: 75,000×; 75 mm = 1 µm.

the vesicles are spherical in nature. The TEM photomicrograph in Fig. 2 also illustrates the unilamellar or oligolamellar structure of prepared liposomes, which are the typical characteristics of vesicles prepared by reverse-phase evaporation method.[14]

Particle-Size Distribution and Drug Entrapment

Effect of AmB Content

The average particle size of prepared AmB liposomes determined by laser-beam scattering was within the range of approximately 750–1500 nm, depending on their lipid compositions. When the amount of AmB added to the formulation exceeded 3 mol% of total lipid, no uniformly dispersed liposomal suspension was obtained because of precipitation of AmB. This may be due to the limited solubility in diethyl ether of AmB (0.01 mg/mL), which could not be further entrapped in the liposomes.

The effect of the amount of AmB—up to 2.0 mol% of the amount of total lipid (400 µmol PC)—on the entrapment efficiency of AmB liposomes is shown in Table 1. The percentages of drug entrapped in five formulations were significantly different ($p < 0.05$, using analysis of variance, or ANOVA). The most efficiently entrapping formulation was the one containing the highest amount of AmB at 2.0 mol% of total lipid followed by the next adjacent 1.0 and 0.9 mol% of AmB in an descending order ($p < 0.05$, Least Significant Difference test, or LSD). However, those of the last three formulations (0.9, 0.7, and 0.5 mol%) were not signifi-

Table 1. Effect of AmB content on entrapment efficiency of AmB liposomes using 400 µmol PC.

AmB content (mol% (mg))	Entrapment efficiency			
	Batch no.[a]			
	1	2	3	Mean (SD) (%)[b]
2.0 (7.40)	94.61	93.71	94.58	94.30 (0.51)
1.0 (3.70)	91.33	92.27	91.71	91.77 (0.47)
0.9 (3.33)	88.70	90.89	88.82	89.47 (1.23)
0.7 (2.59)	88.83	89.30	87.95	88.69 (0.69)
0.5 (1.85)	87.91	88.39	87.51	87.93 (0.44)

[a]Average of two determinations for each batch.
[b]Average of three batches.

Table 2. Effect of total lipid content on particle size and entrapment efficiency of AmB liposomes using 0.65 mol% (2.40 mg) AmB with 0.6 mol% α-tocopherol.

Total lipid content (μmol)	Particle size Mean (SD) (nm)[a]	Entrapment efficiency Mean (SD) (%)[a]
150	827.5 (33.0)	87.84 (0.66)
200	865.7 (58.2)	88.56 (2.04)
250	928.7 (28.2)	92.63 (0.95)
300	889.5 (16.1)	91.36 (0.58)
350	863.7 (116.4)	89.77 (1.02)
400	793.8 (23.1)	88.78 (0.69)
450	750.1 (24.0)	87.84 (0.78)

[a]Average of three batches.

cantly different. The increase in entrapment efficiency with the increasing amount of AmB added during preparation of the liposomes could be attributed to the high concentration of drug in the organic phase during the reverse of emulsion phase, resulting in a larger amount of AmB being intercalated in the bilayers.

Effect of Total Lipid Content

The average particle sizes and percentage drug-entrapment efficiencies of AmB liposomes prepared at various contents of total lipid are shown in Table 2. The mean diameters of vesicles were between 750 and 928 nm, with no significant difference among all formulations ($p > 0.05$, Kruskal-Wallis test). This result indicates that the amount of total lipid used in preparation of AmB liposomes by this method did not significantly affect the liposome size. Nevertheless, it should be noted that vesicle size tended to increase as the percentage of entrapped drug increased, which was probably due to the presence of more AmB molecules residing in the bilayers. Moreover, the average particle size of the liposomes without AmB using 450 μmol PC was 717.8 ± 101.1 nm ($n = 5$), which was not significantly different from those with entrapped drug ($p > 0.05$, t-test).

The entrapment efficiencies of all formulations were found to be within the range of 87.84%–92.63%, the maximum of which occured at 250–300 μmol of total lipid content with statistically significant difference from other compositions ($p < 0.05$, ANOVA). The decline in entrapment efficiency as the total amount of lipid being increased may be attributed to other factors such as viscosity of the emulsion during reverse-phase evaporation process.[15]

Table 3. Effect of PC-to-Chol ratio on particle size and entrapment efficiency of AmB liposomes using 0.65 mol% (2.40 mg) AmB with 250 μmol total lipid content and 0.6 mol% α-tocopherol.

PC:Chol Ratio	Particle size Mean (SD) (nm)[a]	Entrapment efficiency Mean (SD) (%)[a]
10:0	928.7 (28.2)	92.63 (0.95)
9:1	921.9 (29.6)	90.39 (1.73)
8:2	1,029.0 (55.6)	91.97 (0.57)
7:3	1,075.5 (73.3)	92.56 (1.34)
6:4	1,232.9 (169.7)	94.00 (1.22)
5:5	1,317.3 (154.8)	95.72 (1.64)

[a]Average of three batches.

Effect of PC-to-Chol Ratio

The average particle sizes and percentage drug entrapment efficiencies of AmB liposomes prepared at various amount ratios of PC to Chol are shown in Table 3, which reveals that incorporation of more Chol would yield significantly larger vesicles ($p < 0.05$, Kruskal-Wallis test). It was reported that Chol enhanced the rigidity of bilayer membrane above the phase-transition temperature of the constituent phospholipids, resulting in an increased elastic modulus, which inhibited curving of the bilayer.[16,17]

The content of Chol is one of the important parameters in the design of liposomal preparations. Cholesterol does not itself form the bilayer structure but it can be incorporated into the phospholipid bilayers. Since it is amphipathic, Chol could be inserted into the membrane with its hydroxyl group oriented toward the aqueous surface and the aliphatic

chain aligned parallel to the acyl chains in the center of the bilayer. When the bilayers are in the fluid crystal state, above the phase-transition temperature of the structural phospholipid, the presence of the rigid steroid nucleus of Chol alongside the carbons of the phospholipid chain has the effect of reducing the freedom of motion of these carbons. Furthermore, Chol forms a weak complex with phospholipids in the bilayer phase via H-bonding with the carbonyl oxygen of neighboring phospholipids. The limited freedom of acyl chains causes the membranes to condense, with a reduction in area, into a closer packing with decreased fluidity.[18,19] Therefore, the permeability of the liposomal bilayers to the entrapped drug will decrease.[20]

The entrapment efficiencies of all liposomal formulations were found to be within the range of 90.39%–95.72%, which were significantly enhanced with the increasing Chol content ($p < 0.05$, ANOVA), except in the case of one without Chol. It was, however, observed that Chol separated out from the bilayers when PC-to-Chol ratio was higher than 5:5. Since AmB is the hydrophobic drug that would be bound inside the hydrophobic region of the bilayer, the enhancing effect of Chol on the entrapment efficiency may be attributed to the rigidifying effect in the fluid crystal state, facilitating the complete formation of the vesicles with the bilayer-bound drug during the phase-reversal process.

CONCLUSION

Amphotericin B liposomes prepared by reverse-phase evaporation method possessed the oligolamellar structure with average particle size of 750–1500 nm. The entrapment efficiency was increased with the increasing amount of the drug added at constant phospholipid content. The maximum entrapment efficiency occurred at 250–300 μmol of total lipid content. The optimal conditions that which provided the highest drug entrapment efficiency was 250 μmol of total lipid at 1:1 molar ratio of PC to Chol and the drug content at 2.0 mol% of total lipid.

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