Enhanced Encapsulation of Amphotericin B into Liposomes by Complex Formation with Polyethylene Glycol Derivatives

Kunikazu Moribe,[1] Eitaro Tanaka,[1] Kazuo Maruyama,[1,2] and Motoharu Iwatsuru[1]

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Purpose. A highly efficient method was developed for the encapsulation of amphotericin B (AmB) in liposomes, and the mechanism involved was characterized.

Methods. AmB was encapsulated in dipalmitoylphosphatidylcholine/cholesterol (DPPC/CH, 2:1) liposomes after complex formation with distearoyl-N-(monomethoxy poly(ethylene glycol)succinyl) phosphatidylethanolamine (DSPE-PEG). Hydration of lipids was done with 9% sucrose solution.

Results. The encapsulated amount of AmB was 111 μg/mg lipid, which was much higher than that obtained by the same method without DSPE-PEG (14 μg/mg lipid). The amount encapsulated increased with amount of DSPE-PEG used and with PEG molecular weight. Encapsulation efficacy was also influenced by the type of PEG derivatives used and by the modification of AmB, suggesting the involvement of complex formation between AmB and DSPE-PEG. Absorption and ^{31}P-NMR spectral analyses indicated that interactions between the amino and phosphate groups and between the polyene and PEG moieties in AmB and DSPE-PEG, respectively, play an important role in the complex formation.

Conclusions. Complex formation of AmB with DSPE-PEG allows the highly efficient encapsulation of the drug in liposomes. This simple technique should be applicable to other hydrophobic drugs.

KEY WORDS: amphotericin B; liposome; polyethylene glycol; ^{31}P-NMR.

INTRODUCTION

The polyene macrolide antibiotic amphotericin B (AmB) has been used in the treatment of invasive fungal infections in immunosuppressed patients. However, the usefulness of parenteral administration of AmB as an AmB-deoxycholate formulation (Fungizone®) is limited by severe side effects. In an attempt to overcome the toxicity problem, the use of AmB encapsulated in liposomes or other lipid carriers has recently been tried (1). Commercial preparations so far introduced include AmBisome®, ABLC (AmB-lipid complex), and Amphocil®, which have different structural and pharmacokinetic characteristics (2). AmBisome®, which consists of small unilamellar liposomes containing distearoylphosphatidylglycerol, shows a relatively prolonged circulation time in blood, contributing to the higher therapeutic efficacy than that of Fungizone® (3).

However, some problems remain. For example, liposomal AmB is taken up by the reticuloendothelial system *in vivo* and it is difficult to incorporate AmB into liposomes in large amounts using conventional methods. To overcome those problems, long-circulating liposomes composed of hydrogenated soybean phosphatidylcholine and cholesterol (CH) with an amphipathic polyethyleneglycol derivative (distearoyl-N-(monomethoxy poly(ethylene glycol) succinyl) phosphatidylethanolamine, DSPE-PEG) in the presence or absence of distearoylphosphatidylglycerol (DSPG) have been developed. Van Etten *et al.* reported that DSPE-PEG-incorporating AmB liposomes showed prolonged circulation and higher therapeutic efficiency, compared with AmBisome and AmB liposomes without DSPE-PEG (4). Recently, we have found that the presence of DSPE-PEG, which was added to acquire the prolonged circulation characteristics, also significantly increased the encapsulation efficiency of AmB into liposomes. These liposomes showed a long circulation time in blood and a high therapeutic efficiency against invasive pulmonary aspergillosis in mice (5–7). Furthermore, AmB-encapsulating PEG-immunoliposomes, which were conjugated with monoclonal antibodies at the distal terminus of the PEG chain, exhibited high targeting affinity to the lung and high therapeutic efficiency against murine invasive pulmonary aspergillosis (6,7).

In the present study, we characterized the factors affecting the encapsulation efficiency of AmB into PEG-liposomes and we investigated the mechanism of the encapsulation. Encapsulation mechanisms of AmB into conventional liposomes as a model membrane system have been studied by many researchers. Pore formation of AmB in a lipid bilayer has been studied by fluorescence (8), ESR (9) and CD (8) spectral techniques, and it was proposed that the association of AmB with sterols, especially ergosterol, plays an important role in generating the transmembrane pore structure (8,10). NMR and CD studies also cast light on the structure of AmB aggregates and an AmB-lipid complex (11,12). Furthermore, recent theoretical studies by computer simulation techniques revealed an 8-membered AmB channel structure (13) and the complexation of AmB with sterols (14).

The presence of DSPE-PEG increased the encapsulation rate of AmB into liposomes, but the mechanism appeared to be different from that of the formation of conventional liposomes. The encapsulation efficacy of AmB into PEG-liposomes was influenced by the solution used for hydration and by the amount of DSPE-PEG used (5–7), suggesting the involvement of complex formation between AmB and DSPE-PEG. This was confirmed by the chloroform/water partition method, and by absorption and ^{31}P-NMR spectral analyses.

[1] Faculty of Pharmaceutical Sciences, Teikyo University, Sagamiko, Kanagawa 199-0195, Japan.

[2] To whom correspondence should be addressed. (e-mail: maruyama@pharm.teikyo-u.ac.jp)

ABBREVIATIONS: AmB, amphotericin B; DSPE-PEG, distearoyl-N-(monomethoxy poly(ethylene glycol)succinyl) phosphatidylethanolamine; CH, cholesterol; DSPG, distearoylphosphatidylglycerol; ESR, electron spin resonance; CD, circular dichroism; NMR, nuclear magnetic resonance; DPPC, dipalmitoylphosphatidylcholine; DSPE, distearoyl-phosphatidylethanolamine; DSPA, distearoylphosphatidic acid; PEG-OSu, monomethoxy polyethylene glycol succinimidyl succinate; DT-PEG, α-monomethoxy-ω-(1,2-ditetradecanoyloxyglyceryl) polyoxyethylene; DPP-PEG, α-(dipalmitoylphosphatidyl)-ω-hydroxy-polyoxyethylene; Ac-AmB, N-acetylamphotericin B; AME, amphotericin B methylester; DMSO, dimethyl sulfoxide; Diazald(R), N-methyl-N-nitroso-p-toluenesulfonamide; TLC, thin layer chromatography; EDTA, ethylenediamine tetraacetic acid; CHE, cholesteryl hexadecylether; DDW, double distilled water.

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MATERIALS AND METHODS

Materials

AmB was kindly donated by Bristol-Myers Squibb Pharmaceutical Research (Tokyo, Japan.). CH and ^{3}H-cholesteryl hexadecylether (^{3}H-CHE) were purchased from Wako Pure Chemicals (Osaka, Japan) and New England Nuclear Japan (Tokyo, Japan), respectively. Dipalmitoylphosphatidylcholine (DPPC), distearoyphosphatidylethanolamine (DSPE), distearoylphosphatidic acid (DSPA), monomethoxy polyethylene glycol succinimidyl succinate (PEG-OSu) with an average molecular weight of 1000 (1K), 2000 (2K) or 5000 (5K) daltons, and other PEG derivatives were kindly provided by Nippon Oil and Fats (Tokyo, Japan). Other chemicals used were of reagent grade. DSPE-PEG was synthesized according to the reported method (15). The structures of some of these compounds, as well as the AmB derivatives described below are shown in Fig. 1.

Synthesis of AmB Derivatives

AmB derivatives, N-acetyl-amphotericin B (Ac-AmB) and amphotericin B methylester (AME), were synthesized as reported by Mechlinski and Schaffner (16). Ac-AmB: AmB (1 g) was dissolved with stirring in 10 ml of DMSO at room temperature and diluted with 10 ml of absolute methanol. The mixture, containing partially precipitated antibiotic, was cooled on ice and 0.12 g of acetic anhydride was added stepwise during 10 minutes with stirring. After a further 10 minutes, the product was isolated by precipitation in 100 ml of anhydrous ethyl ether, followed by centrifugation and washing with ethyl ether. AME: AmB (1 g) was dissolved in 10 ml of DMSO at room temperature and diluted with 1 ml of absolute methanol. The solution was cooled on ice to 6°C, then treated with 7 ml of diazomethane reagent (prepared in the usual way from Diazald (R) (N-methyl-N-nitroso-p-toluenesulfonamide)) for 1 minute. After the esterification was completed, the product was isolated by precipitation in 200 ml of anhydrous ethyl ether followed by centrifugation and washing with ethyl ether. Completion of both reactions was checked by TLC. (Silica gel G plate 20 × 20 cm; chloroform/methanol/0.025 M borate buffer, pH 8.3 (2/2/1 v/v, lower phase)). The purity was above 95%, as estimated by NMR measurement.

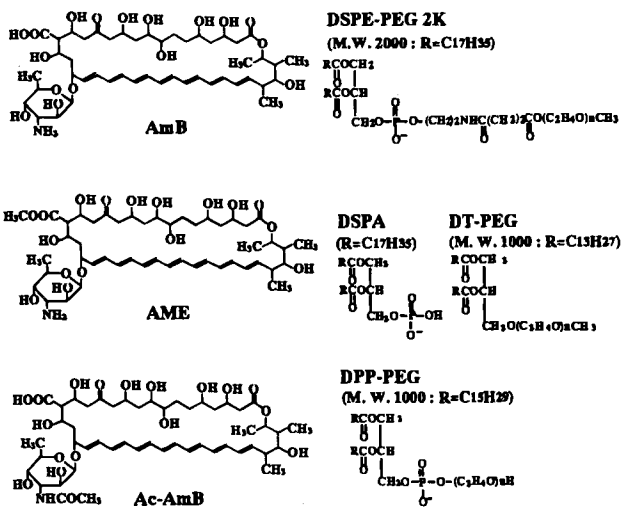

Fig. 1. Chemical structures of AmB and PEG derivatives.

Preparation of Liposomes

The lipid composition of AmB-PEG-liposomes was DPPC/CH (2/1 molar ratio) with 6 mol% of DSPE-PEG 2K (average molecular weight 2000). AmB and DSPE-PEG (2/1 molar ratio) were dissolved in methanol and chloroform, respectively, and the solutions were mixed. Then a solution of DPPC and CH in chloroform was added and the whole was evaporated to make a lipid film. The lipid film was hydrated by vortex mixing in 9% sucrose, and the mixture was frozen and thawed 4 times. Liposomes were extruded through a Nuclepore polycarbonate membrane of 0.4, 0.2 and 0.1 μm pore size (Nuclepore Co., CA, USA) resulting in an average particle size of 130 nm, as measured by dynamic light scattering (ELS 800, Otsuka Electronics Co., Tokyo). Most of the free AmB was removed by this procedure. The extruded AmB-PEG-liposomes were also centrifuged at $2 \times 10^5 \times g$ for 15 min to separate non-entrapped drug interacting with DSPE-PEG and to concentrate the liposomes. Prepared liposomes are large unilamellar vesicles in which PEG chains are located on both sides of the liposomal membrane. The phospholipid concentration was determined by phosphate assay, and the AmB concentrations were measured spectrophotometrically at 405 nm in methanol. The encapsulated amount of AmB was usually expressed as the weight ratio of AmB to lipid (equivalent amount of DPPC and CH).

Stability of AmB-PEG-Liposomes *In Vitro*

Stability of AmB-PEG-liposomes *in vitro* was estimated by the extent of AmB release from liposomes. Prepared AmB-PEG-liposomes were diluted with 9% sucrose solution to adjust the AmB concentration to 0.2 mg/ml. A 0.1 ml aliquot of the sample was added to 1 ml of ddY mouse plasma and incubated at 37°C. After the desired period of incubation, a 0.1 ml aliquot of the sample was collected, diluted with 0.9 ml of phosphate-buffered saline and centrifuged at $2 \times 10^5 \times g$ for 15 min to obtain the supernatant. The supernatant was homogenized with 1 ml of 99% methanol and 4 ml of 10 mM phosphate buffer (pH 7.4) containing 2 mg/ml of 1-amino-4-nitronaphthalene (Aldrich, Milwaukee, WI, USA) as an internal standard. The amount of AmB released was measured by HPLC (5). Released AmB was extracted on BOND-ELUT® (Varian, Harbor City, CA, USA) from the supernatant, eluted by 1 ml of acetonitrile/2.5 mM EDTA (60/40, v/v), and was measured by HPLC (Nihon Bunko Ltd., Tokyo, Japan.). Wakosil 5C18 column (4.0 mm × 30 cm, Wako Pure Chemical Industries Ltd.) was used and the mobile phase was 40% acetonitrile in 10 mM acetate buffer (pH 4.0), with a flow rate of 1.0 ml/min. AmB and the internal standard had retention times of 8.0 and 11.0 min, respectively. The column effluent was monitored for absorbance at 385 nm.

Stability of AmB-PEG-Liposomes *In Vivo*

Bare-liposome, which was composed of DPPC/CH (2/1, m/m) and AmB-PEG-liposomes labeled with ^{3}H-CHE were injected into 6-week-old male ddY mice via the tail vein with 10 mg lipid/kg body in 0.2 ml of sucrose. At desired times

after the injection, blood was collected by eye puncture from the anesthetized mice. The biodistribution of liposomes and AmB were determined by means of radioactivity and HPLC measurements, respectively, as described above.

Chloroform/Water Two-phase Partition

Intermolecular interaction between AmB and DSPE-PEG in aqueous solution was easily observed by using a chloroform/water partition system. The principle of this method is based on the solubility difference of the two drugs. DSPE-PEG is soluble in chloroform and forms micellar structure in water. But AmB is poorly soluble in these solvents and can exist in them only as aggregated states. In the presence of DSPE-PEG, AmB can exist in the water phase by solubilization or interacting with DSPE-PEG. In the chloroform/water system, however, most of the DSPE-PEG is partitioned to the organic phase, depending on the solubility difference against both solvents. Therefore, if AmB exists in the water phase, upon addition of chloroform the AmB separates out at the interface between the chloroform and water phases as a precipitate. AmB-DSPE-PEG aqueous solution was prepared as follows: A solution of 20 mg of AmB in methanol was mixed with a solution of 20 mg of DSPE-PEG in chloroform. After evaporation of the organic solvent, 1 ml of 9% sucrose solution was added to the mixture and the free AmB was removed by centrifugation at 3,000 $\times g$ for 15 min as a precipitate. One ml of chloroform was added to the aqueous solution dropwise to observe the change of state.

Absorption Spectra of AmB and DSPE-PEG

The interaction of AmB and DSPE-PEG was studied by absorption spectroscopy, which is modified method as reported by Mazerski *et al.* (17). AmB was dissolved in DMSO at a concentration of 10^{-3} M. A DSPE-PEG stock solution was prepared in methanol at a concentration of 10^{-3} M. Appropriate amounts of AmB and DSPE-PEG solutions were added to 9% sucrose solution. Spectroscopic measurement was done 1 h after mixing, and in all experiments, the concentration of AmB was 2×10^{-6} M.

^{31}P-NMR Measurement

PEG-liposomes and AmB-PEG-liposomes were prepared as described above, except for hydration with D_2O. Free AmB was removed by passing through a 0.45 μm cellulose acetate filter (Toyo Roshi Kaisha, Ltd., Japan) to obtain a concentrated and not viscous sample. Lipid concentration was adjusted to 20 mg/ml. To prepare the AmB-DSPE-PEG complex, AmB dissolved in methanol was mixed with DSPE-PEG dissolved in chloroform. After evaporation of the organic solvent, D_2O was added to the mixture and the AmB not associated with liposome was removed by filtration with a 0.45 μm filter. The sample was transferred into a 10 mm bore NMR tube and the ^{31}P-NMR spectrum was recorded on a Bruker AVANCE-400 spectrometer.

RESULTS AND DISCUSSION

Encapsulation of AmB in PEG-Liposomes

The biodistribution and therapeutic efficiency of AmB-PEG-liposomes have been reported, but this is the first study of the efficacy and mechanism of the encapsulation of AmB in PEG-liposome. The amount of AmB encapsulated in liposomes (DPPC/CH = 2/1, m/m) is summarized in Table 1. High efficiency of AmB encapsulation (111 μg AmB/mg lipid; 1:10 molar ratio) was obtained by the addition of DSPE-PEG (6 mol% of total lipid) to the liposomes and by using isotonic sugar solution, such as 9% sucrose solution, for hydration. DSPE-PEG-non-incorporating liposome encapsulated only a small amount of AmB (14 μg AmB/mg lipid), and in this case, AmB may exist in the liposomal membrane in the form of pore structure, as reported by several researchers (8–13). In this experiment, the AmB: DSPE-PEG molar ratio was fixed at 2:1, because the encapsulated amount of AmB did not increase any further when more AmB was used (data not shown). Since a higher AmB:DSPE-PEG ratio also resulted in lower recovery, the molar ratio used in this study was considered to be a good compromise.

The effect of the solution used for hydration on the encapsulation was also studied by using several kinds of solutions. A relatively high encapsulated amount of AmB in the PEG-liposomes was obtained with isotonic sugar solutions. However, hydration by electrolytic solution, such as saline and PBS, decreased the encapsulation efficacy. Hydration with double distilled water (DDW) also enhanced the AmB encapsulation, suggested that ionic strength of the solvents affected the encapsulation of AmB. Since, AmB is not only soluble in an electrolytic solution but a sugar solution, we speculated that some of the interaction between AmB and DSPE-PEG might play an important role on the encapsulation.

The effects of the molecular weight and the incorporated amount of DSPE-PEG on the encapsulation of AmB are summarized in Table 2. When the incorporated amount of DSPE-PEG was fixed at 6 mol%, the encapsulated amount of AmB in DSPE-PEG 1K-incorporating liposomes showed a relatively low value (69 μg AmB/mg lipid), whereas DSPE-PEG-incorporating liposomes with longer PEG chains showed higher encapsulation of AmB (109–122 μg AmB/mg lipid). The amount of 6 mol% incorporation of DSPE-PEG into liposomes seemed reasonable from the viewpoint of inhibition of micelle formation and the long-circulation property *in vivo*. In the case of DSPE-PEG 2K-incorporating liposomes, the thickness of the PEG molecular water layer was estimated by Shimada *et al.* as 2.0 nm (18), which is close to the molecular size of AmB reported

Table 1. Encapsulation of AmB into Liposomes

Lipid composition[a]	Solvent	Encapsulation efficacy (AmB/lipid)	
		μg/mg[b]	mol/10^3 mol
DPPC/CH	sucrose	14 (2.3)	17
DPPC/CH/DSPE-PEG2K	sucrose	111 (5.7)	101
DPPC/CH/DSPE-PEG2K	saline	10 (2.3)	
DPPC/CH/DSPE-PEG2K	PBS	9 (2.4)	
DPPC/CH/DSPE-PEG2K	glucose	87 (13.6)	80
DPPC/CH/DSPE-PEG2K	lactose	96 (7.7)	88
DPPC/CH/DSPE-PEG2K	trehalose	113 (6.9)	104
DPPC/CH/DSPE-PEG2K	DDW	102 (5.6)	93

[a] Incorporated amount of DSPE-PEG2K was 6 mol%.
[b] Values are mean (± SD). n = 3–5.

Table 2. Effect of DSPE-PEG on the Encapsulation of AmB into Liposomes Hydrated with 9% Sucrose Solution

Lipid composition DSPE-PEG	mol%	Encapsulation efficacy[a] μg AmB/mg lipid
DPPC/CH/ DSPE-PEG1K	6	69 (7.5)
DPPC/CH/ DSPE-PEG2K	6	111 (5.7)
DPPC/CH/ DSPE-PEG3K	6	109 (9.9)
DPPC/CH/ DSPE-PEG5K	6	122 (9.2)
DPPC/CH/ DSPE-PEG2K	3	78 (11.0)
DPPC/CH/ DSPE-PEG2K	6	111 (5.7)
DPPC/CH/ DSPE-PEG2K	10	199 (5.7)
DPPC/CH/ DSPE-PEG2K	12	251 (25.3)
DPPC/CH/ DSPE-PEG2K	15	250 (26.4)

[a] Values are mean (± SD). n = 3–5.

by P. Ganis *et al.* (19). Thus, the molecular weight of DSPE-PEG, which corresponded to the thickness of the PEG molecular water layer, affected the encapsulation of the AmB, indicating that AmB molecule may be interacting with the PEG moiety in PEG-liposome. Encapsulated amount of AmB was also influenced by the amount of PEG incorporated. It increased as the amount of DSPE-PEG 2K was increased, reaching a plateau at 20 mol% DSPE-PEG. Micelle formation may occur at levels of DSPE-PEG above 15 mol% (20).

On the basis of these results, AmB-PEG-liposomes composed of DPPC/CH/DSPE-PEG 2K (2/1/0.19, m/m) were used for the study of the stability of AmB-PEG-liposomes *in vitro* and *in vivo*.

Stability of AmB-PEG-Liposomes *In Vitro* and *In Vivo*

Figure 2 shows the AmB release profile from AmB-PEG-liposomes after incubation with mouse plasma at 37°C. Burst release of AmB (7.5% of the total) was observed within 5 min after the start of incubation, and thereafter, AmB release became very slow (additional release of AmB was 2.5% within 2 h after the burst release). These results indicated that most of the AmB was stably existed in the liposomes. Stability of AmB in AmB-PEG-liposomes was also studied *in vivo* (Fig. 3). Suitable ^{3}H-CHE levels and AmB concentrations were chosen to allow

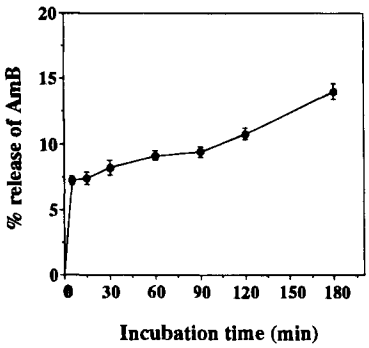

Fig. 2. AmB release from AmB-PEG-liposomes after incubation with ddY mouse plasma. Diluted AmB-PEG-liposome (0.2 mg AmB/ml) sample (0.1 ml) was added to 1 ml of ddY mouse plasma and the mixture was incubated at 37°C.

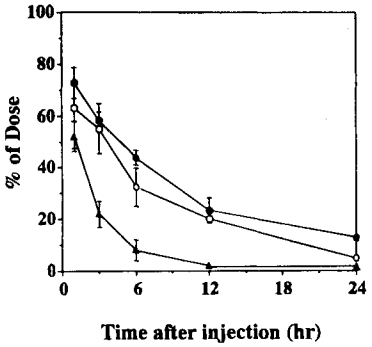

Fig. 3. Blood clearance of liposome and liposome-associated AmB after i.v. injection of AmB-PEG-liposome into ddY mice. (●) liposomes (^{3}H-CHE), (○) liposome-associated AmB, (▲) bare liposomes (^{3}H-CHE). Injected dose of AmB-PEG-liposomes was 2 mg AmB/kg. Data are expressed as percent of injected dose of ^{3}H-CHE, mean ± SD (n = 3) or as percent of injected dose of AmB, mean ± SD (n = 3).

convenient estimation of the blood clearance of AmB-PEG-liposomes and AmB associated with the liposomes. AmB-PEG-liposomes showed a prolonged circulation time, comparing with that of bare-liposomes. This result was also similar to that of PEG-liposomes not containing AmB (data not shown). The AmB levels of AmB-PEG-liposomes as a percentage of the injected dose in blood were equivalent to the lipid level of AmB-PEG-liposomes, suggesting that the AmB was stably associated with PEG-liposomes in blood and that the presence of AmB in the liposomes did not influence the long-circulating property of the PEG-liposomes.

Mechanism of Encapsulation

The encapsulation efficacy of AmB into PEG-liposomes was influenced by the solution used for hydration, the incorporated amount of PEG and the molecular weight of PEG (7,8), suggesting that some of the molecular interactions occur between AmB and DSPE-PEG. To confirm this, a solution of AmB and DSPE-PEG was prepared as described in Methods, and the interaction between the components was investigated. The sample formed a yellow solution, which was stable at 4°C for at least a month. Next, chloroform was added to the mixed sample and AmB-PEG-liposomes. All the AmB separated as a precipitate at the interface between the chloroform and water phases, indicating that AmB was interacting with DSPE-PEG in the water phase.

As the interaction mode, two possibilities were proposed. One is the complex formation between AmB and DSPE-PEG by interaction between some functional groups. Another is a solubilization of AmB by DSPE-PEG. In this interaction mode, AmB molecules are incorporated in the acyl chains and may form pore structures as an aggregated state.

Molecular interaction between AmB and DSPE-PEG was also studied by absorption spectra of AmB in the presence of DSPE-PEG in sucrose solution system, using a modification of the method employed for investigating the complex formation between AmB and sterols (17). AmB absorption spectra was derived from the polyene structure. AmB shows three absorption peaks (365, 386, and 409 nm) assigned to monomeric form and broad peak (330–350 nm) assigned to polymeric aggregate

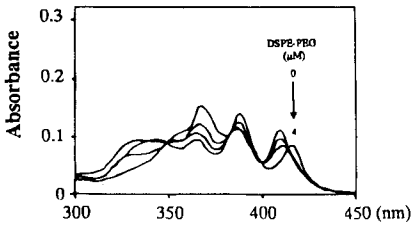

Fig. 4. Absorption spectra of AmB in the presence of DSPE-PEG. A modification of the method reported by Mazerski *et al.* (22) was used. AmB concentration in sucrose solution was fixed at 2 μM. DSPE-PEG concentration was 0, 1, 2 or 4 μM.

form. AmB concentration was adjusted to 2×10^{-6} M to reduce the effect of the aggregate. The absorption peak at 409 nm decreased when the amount of DSPE-PEG increased and the peak position was ultimately shifted to 414 nm as shown in Fig. 4. These results indicated that the polyene moiety of AmB contributes to the interaction leading to the complex formation.

Next, the mechanism of encapsulation of AmB into PEG-liposomes was examined by using PEG and AmB derivatives (Fig. 1). The results are summarized in Table 3. When α-monomethoxy-ω-(1,2-ditetradecanoyloxyglyceryl) polyoxyethylene (DT-PEG1K) or DSPA which has only a PEG moiety or phosphate group, respectively, was used, AmB was not encapsulated. With α-(dipalmitoylphosphatidyl)-ω-hydroxy polyoxyethylene (DPP-PEG1K) and DSPE-PEG1K, which both have a phosphate group and a PEG moiety, the encapsulated amounts of AmB were 35 and 69 μg/mg lipid, respectively. Thus both the phosphate group and the PEG moiety in DSPE-PEG is required for the high encapsulation of AmB, and the PEG moiety may interact with the polyene moiety of AmB (see Fig. 4). The encapsulated amount of AME, an amino group-modified derivative of AmB, was almost the same as that of AmB, while that of Ac-AmB was only about the half as much, suggesting that the amino group may play an important role in the encapsulation of AmB. Presumably it interacts with the phosphate group in DSPE-PEG.

Participation of the phosphate group in the complex formation was confirmed by the ^{31}P-NMR spectra (Fig. 5). In the spectrum of DSPE-PEG, the phosphate signal was observed at 2.05 ppm. The signal was broadened in the case of PEG-liposomes, and two peaks, which were assigned to DSPE-PEG

Table 3. Effect of AmB and DSPE-PEG Derivatives on the Encapsulation[a]

AmB derivative	PEG derivative	Encapsulation efficacy[b] μg AmB/mg lipid
AmB	DT-PEG 1K	9 (4.5)
AmB	DSPA	20 (3.6)
AmB	DPP-PEG 1K	35 (4.0)
AmB	DSPE-PEG 1K	69 (7.5)
AmB	DSPE-PEG 2K	111 (5.7)
AME	DSPE-PEG 2K	113 (11.9)
Ac-AmB	DSPE-PEG 2K	50 (10.0)

[a] Lipid composition: DPPC/CH(2/1)/PEG-derivative (6 mol%).
[b] Values are mean (± SD). n = 3–5.

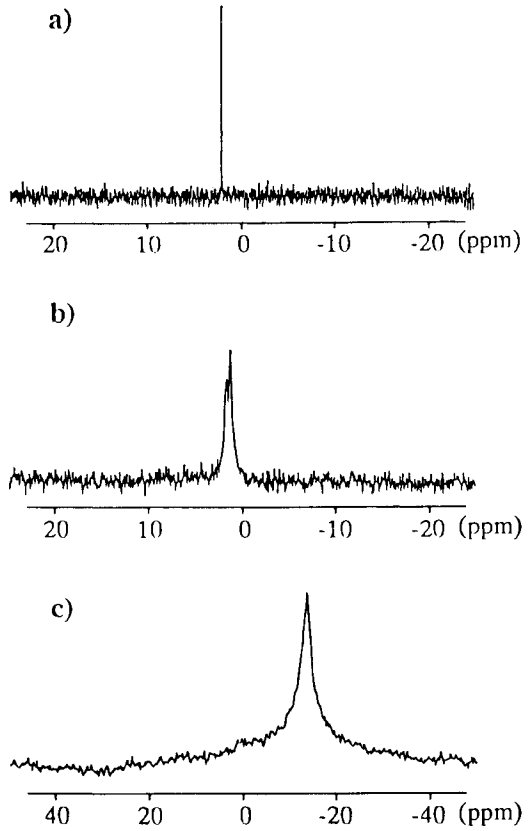

Fig. 5. ^{31}P-NMR spectra of AmB-PEG-liposomes. a) DSPE-PEG, b) PEG-liposomes, c) AmB-PEG-liposomes. Liposomes were prepared by hydration with D_2O and filtered through a 0.45 μm filter. ^{31}P-NMR spectra were recorded at 162 MHz at 300 K.

(1.73 ppm) and DPPC (1.22 ppm), were observed. In the case of AmB-PEG-liposomes, however, the signal appears at −13.8 ppm. This is consistent with an amino group/phosphate group interaction.

Thus, we considered that complex formation between AmB and DSPE-PEG was occurred in the process of AmB-PEG-liposome preparation, which involved interactions between the amino group and phosphate group and between the polyene and PEG moieties of AmB and DSPE-PEG, respectively. It remains to be seen whether AmB pore formation occurs in the lipid membrane of PEG-liposomes, which corresponds to the solubilization of AmB by DSPE-PEG. Since we used DPPC and CH as a basic lipid composition in this experiment, the effect of CH on the encapsulation of AmB should be taken into consideration. Incorporation of CH apparently reduced the encapsulation of AmB; for example, an increase of CH content (DPPC/CH molar ratio changed from 2/1 to 1/1) reduced the amount of encapsulated AmB from 111 to 60 μg AmB/mg lipid. The constancy of the DPPC/CH molar ratio before and after the liposome preparation was confirmed by lipid and CH concentration measurement. We also confirmed that no AmB-CH complex was formed in this experiment. Taking these results into consideration, further study by using liposomes with other lipid composition, is required to confirm the location of AmB in the liposomal membrane.

In conclusion, we have achieved the highly efficient encapsulation of AmB in DPPC/CH liposomes by using DSPE-PEG,

and we have established that the mechanism of the AmB encapsulation involves complex formation between AmB and DSPE-PEG. The complex formation appears to involve interactions of the amino and polyene moieties of AmB with the phosphate and PEG moieties of DSPE-PEG, respectively. This simple and efficient encapsulation method using DSPE-PEG should also be applicable to the encapsulation of other polyene antibiotics, such as nystatin, as well as to other hydrophobic drugs with appropriate functional groups. It may provide an effective pharmaceutical formulation for injectable hydrophobic drugs with reduced side effects.

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