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Molecular localization and state of amphotericin B in PEG liposomes

Kunikazu Moribe, Kazuo Maruyama *, Motoharu Iwatsuru

Faculty of Pharmaceutical Sciences, Teikyo University, Sagamiko, Kanagawa 199-0195, Japan

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Abstract

We investigated the molecular localization and state of amphotericin B (AmB) encapsulated in polyethylene glycol (PEG)-coated liposomes. AmB-encapsulating PEG-liposomes composed of dipalmitoylphosphatidylcholine (DPPC), cholesterol (CH) and distearoyl-*N*-(monomethoxy poly(ethylene glycol)succinyl) phosphatidylethanolamine (DSPE-PEG, average MW of the PEG chain 2000) were prepared by hydration with 9% sucrose solution and extrusion. The amount of AmB encapsulated in the liposomes increased with incorporation of DSPE-PEG and decreased with that of CH. The molecular localization and state of AmB were investigated by PEG/dextran two-phase partition, potassium permeability measurement, fluorescence quenching measurement and circular dichroism (CD) spectroscopy. The results suggest that there are two types of AmB localization in PEG-liposomes, one of which corresponds to the complex of AmB with DSPE-PEG on the membrane surface, while the other corresponds to the pore form of AmB in the hydrophobic core of the liposomal membrane. AmB in PEG liposomes was present in both aggregated and monomeric states.

Keywords: Amphotericin B; Liposome; Polyethyleneglycol; Partition; Fluorescence; Circular dichroism spectra

1. Introduction

A polyene macrolide antibiotics amphotericinB (AmB) is used potently for the treatment of systemic fungal infections in spite of its toxicity. Recently, carrier systems, such as AmB-lipid complex and a liposomal formulation of AmB, have been developed to improve the therapeutic index (Alder-Moore and Proffitt, 1993; Hiemenz and Walsh, 1998; Van Etten et al., 1993, 1995a,b). We have reported a novel polyethylene glycol (PEG)-coated liposomal AmB formulation with a high encapsulation rate of AmB and a long-circulating activity, which were achieved by the complex formation of AmB with distearoyl-*N*-(monomethoxy poly(ethylene glycol)succinyl) phosphatidiylethanolamine (DSPE-PEG) (Moribe et al., 1997a, 1998). This method is very simple and easy, and was extended to the preparation of PEG immunoliposomes conjugated with monoclonal antibodies at the distal terminus of the PEG

* Corresponding author. Tel.: +81-426-853724; fax: +81-426-853432.

E-mail address: maruyama@pharm.teikyo-u.ac.jp (K. Maruyama)

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chain, which exhibited high targeting affinity to the lung and high therapeutic efficiency against murine invasive pulmonary aspergillosis (Otsubo et al., 1998).

The molecular interaction between AmB and liposomes as a model membrane system has been studied by many researchers. Pore formation by assembled AmB molecules in a lipid bilayer has been investigated by fluorescence (Bolard et al., 1991; Hartsel et al., 1991), ESR (Hartsel et al., 1988) and CD (Bolard et al., 1991 Hartsel et al., 1988) 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 (Bolard et al., 1991; Hartsel et al., 1993). NMR and CD studies have also cast light on the structure of AmB aggregates and an AmB-lipid complex (Balakrishnan and Easwaran, 1993a,b). However, these investigations only provide information about the interaction of AmB with preformed liposomes and do not explain the encapsulation mechanism of AmB in liposomes.

In the case of AmB-encapsulating PEG liposomes, the encapsulated amount of AmB depends on the lipid composition. Though the incorporation of CH in AmB-encapsulating liposomes contributes to the membrane stability and the long-circulation behavior, the effect of CH on the encapsulation process itself is unknown. The mechanism of encapsulation of AmB in PEG liposomes has been suggested to involve complex formation between AmB and DSPE-PEG (Moribe et al., 1998). However, the location of AmB molecules in the liposomal membrane in the presence or absence of CH and DSPE-PEG also remains unclear.

In this study, we examined the molecular localization and state of AmB in the liposomal membranes. AmB-encapsulating liposomes composed of DPPC, DPPC/CH, DPPC/DSPE-PEG and DPPC/CH/DSPE-PEG were prepared to investigate the encapsulation efficiency and the mechanism of encapsulation of AmB into liposomal membranes. The effects of CH, kind of lipid and initial added amount of AmB on the encapsulation of AmB were also investigated, and the membrane surface state of the liposomes was examined by PEG-dextran two-phase partition measurement. The location of AmB in the liposomal membrane was confirmed by fluorescence quenching and potassium permeability measurements. We proposed that AmB exist in two forms: as a complex form between AmB and DSPE-PEG on the surface of the liposomal membrane and as an AmB pore in the hydrophobic core of the liposomal membrane. Circular dichroism measurements indicated that AmB is present in these liposomes in both monomeric and aggregated states. In terms of the molecular states of AmB, the results are in good agreement with those of AmB incorporated into a block copolymer systems (Yu et al., 1998).

2. Materials and methods

2.1. Materials

AmB was kindly donated by Bristol–Myers Squibb Pharmaceutical Research (Tokyo, Japan). Cholesterol (CH) and ^{3}H-CH were purchased from Wako Pure Chemicals (Osaka, Japan) and New England Nuclear Japan (Tokyo, Japan), respectively. Dipalmitoylphosphatidylcholine (DPPC), distearoylphosphatidyl-ethanolamine (DSPE), monomethoxy polyethylene glycol succinimidyl succinate (PEG-OSu) with an average molecular weight of PEG chain 2000 (2K) and other lipids were kindly provided by Nippon Oil and Fats (Tokyo, Japan). 1,6-Diphenyl-1,3,5-hexatriene (DPH) and 1-[4-(trimethylamino)phenyl]-6-phenylhexa-1,3,5-triene (tmaDPH) were purchased from Nacalai Tesque (Kyoto, Japan) and Dojin (Kumamoto, Japan), respectively. 8-Hydroxypyrene-1,3,6-trisulfonic acid (pyranine), a fluorescent probe used as a pH indicator, was obtained from Molecular Probes, (OR). PEG with an average molecular weight of 8000 and dextran T-500 were from Sigma (St Louis, MO) and Pharmacia Biotech (Tokyo, Japan), respectively. Other chemicals used were of reagent grade. DSPE-PEG was synthesized according to the reported method (Maruyama et al., 1992).

2.2. Preparation of liposomes

AmB-encapsulated liposomes composed of DPPC and DPPC/CH (2/1 molar ratio), and DPPC and DPPC/CH (2/1 molar ratio) with 6 mol% of DSPE-PEG 2K (average molecular weight 2000) were prepared as described (Moribe et al., 1998). In the case of DPPC and DPPC/CH liposomes, AmB dissolved in methanol was mixed with DPPC and CH in chloroform. In the case of DPPC/DSPE-PEG and DPPC/CH/DSPE-PEG liposomes, AmB and DSPE-PEG were dissolved in methanol and chloroform, respectively, and mixed, followed by the addition of DPPC and CH in chloroform. The initial added amount of AmB was 0.75 mg AmB/5 mg lipid (DPPC + CH). Each mixture was evaporated to make a lipid film. The lipid film was hydrated by vortex mixing in 9% sucrose and was frozen and thawed four times. Liposomes were extruded through a Nuclepore polycarbonate membrane of 0.4, 0.2 and 0.1 mm pore size (Extruder, Lipex Biomembrane, Canada and Nuclepore, CA) resulting in an average particle size of 100–130 nm, as measured by dynamic light scattering (ELS 800, Otsuka Electronics, Tokyo). When the extrusion of the sample was clogged by precipitate, it was performed little by little after dilution of the samples. Most of the free AmB was removed by this procedure. The extruded AmB-encapsulating liposomes were also centrifuged at 2×10^5 *g* for 15 min to separate non entrapped drug interacting with DSPE-PEG in the supernatant and to concentrate the liposomes. The prepared liposomes are large unilamellar vesicles, and PEG chains are located on both sides of the liposomal membrane. The phospholipid concentration was determined by phosphate assay, and the AmB concentration was measured spectrophotometrically at 405 nm in methanol. The encapsulated amount of AmB was usually expressed as the weight ratio of AmB to lipid.

2.3. PEG/Dextran two-phase partition

The PEG/dextran two-phase system was prepared as described (Moribe et al., 1997b). Briefly, 5% (w/w) PEG8000 and 5% (w/w) dextran T-500 two-phase system in 1:1 volume ratio was prepared in 0.01 M sodium phosphate (pH 7.0) containing 0.15 M sodium chloride (non charge-sensitive system) by mixing the appropriate weights of the following stock solutions: 22% (w/w) dextran T-500, 30% (w/w) PEG 8000, 0.44 M sodium phosphate (pH 7.0), 0.6 M sodium chloride and distilled water (Tilcock et al., 1993). The two phases were equilibrated at 25°C, separated and stored at 4°C until used.Phase-partitioning of AmB-encapsulating liposomes was done as follows. One hundred microliters of ^{3}H-CH-labeled liposomes was added to a mixture of PEG 8000 (top phase) and dextran T-500 (bottom phase, 0.75 ml of each) equilibrated at 25°C in an 80×10 mm glass tube. The phases were mixed for 1 min by repeated inversion, then a 50 μl aliquot was taken for total radioactivity counting. The mixture was left at 25°C for a further 30 min, then 25 μl of each phase (PEG 8000/top phase, dextran T-500/bottom phase) was sampled for counting and the distribution of liposomes between the phases was determined. The amount of liposomes at the interface was calculated by subtracting the sum of the radioactivity in the PEG 8000 and dextran T-500 phases from the total radioactivity.

2.4. Potassium permeability measurement

Pore formation of AmB molecules in the PEG-liposomal membrane was confirmed by potassium permeability measurement, using a modification of the method of Bolard et al. (1991). In brief, PEG liposomes were prepared by hydration with sucrose-containing potassium buffer (130 mM sucrose; 50 mM K_2SO_4; 10 mM KH_2PO_4; 9 mM pyranine pH adjusted to 7.1 with KOH) and extrusion through a polycarbonate filter of 400 nm pore-size. The initial weight ratio of AmB to lipid was 0.38 mg/5 mg in order to avoid the quenching of pyranine fluorescence by AmB. After preparation of the liposomes, external pyranine was removed by gel-chromatography with Bio-Gel A-1.5 m column against pyranine-free buffer. The fluorescence intensity of pyranine encapsulated in AmB-PEG liposomes was used as a pH indicator within the range of 6.4–7.8 (Bolard et al., 1991). After dilution of the sample to 0.1

mM concentration, 2 ml was placed in a quartz cuvette. A pH gradient was created by addition of 10 μl of 1 N H_2SO_4. Change of the fluorescence intensity was measured at 510 nm with excitation at 450 nm. Potassium leakage from the bilayer membrane occurs by K^+/H^+ exchange when AmB and carbonyl cyanide p-(trifluoromethoxy) phenylhydrazone (FCCP, Sigma), used as an uncoupler to increase H^+ permeability, are incorporated in the membrane. So a change of the fluorescence intensity would occur upon addition of FCCP, if AmB molecules were incorporated in the core of the liposomal membranes. In this study, 5 μl of FCCP (0.2 μM in DMSO) was added to the samples at 1 min and complete uncoupling was achieved by addition of 10 μl of valinomycin (0.3 mM in DMSO) at 10 min after addition of H_2SO_4.

2.5. Fluorescence quenching

DPH has been used to estimate the membrane property in the hydrophobic part of the membrane (Lentz, 1989). Because of being anchored by its cationic moiety, which may interact with phosphate groups of phospholipids, tmaDPH has also been used to estimate the membrane property at the membrane/water interface (Illinger et al., 1995). So, the quenching of DPH or tmaDPH fluorescence in the presence of AmB in liposomal membrane would reflect the localization of the AmB molecules close to the fluorescent probes. Because of the different localization of DPH or tmaDPH in the liposomal membranes, we used both DPH and tmaDPH to estimate the location of the AmB molecules in liposomes. DPH or tmaDPH dissolved in chloroform or chloroform/methanol (1/1, v/v), respectively, was mixed with lipids to make a lipid film. The molar ratio of AmB to lipid was 1:150. AmB-incorporating liposomes containing DPH or tmaDPH were prepared as described above and the lipid concentration was adjusted to 0.1 mM by the sucrose solution. Fluorescence intensity measurement was done at an excitation wavelength of 318 nm and an emission wavelength of 425 nm. Relative fluorescence intensity was standardized in terms of F_0/F and the Stern–Volmer constant was calculated by use of the following equation:

$$F_0/F = k[C] + 1$$

where F_0 or F is the fluorescence intensity in the absence or presence of AmB; $[C]$ is AmB concentration and k is the Stern–Volmer constant. The degree of DPH or tmaDPH quenching by AmB was estimated by the Stern–Volmer constant, and the order of which would reflects the degree of AmB molecules located in the core or close to the hydrophilic part of the membranes.

2.6. Circular dichroism spectra

Circular dichroism (CD) spectra were recorded with a JASCO J-700 spectropolarimeter (Tokyo, Japan). The AmB concentration of the sample was adjusted to 5 μM and CD spectra were measured under the following conditions: band-width, 1.0 nm; step-resolution, 0.1 nm; sensitivity, 50 mdeg; response, 0.25; scan-speed, 100 nm/min; accumulation, five times. All the data were expressed in terms of $\Delta\varepsilon$ (M^{-1} cm^{-1}) which is the differential molar dichroic absorption coefficient.

3. Results and discussion

3.1. Encapsulation characteristics of AmB in liposomes

As we have reported previously, the co-incorporation of DSPE-PEG2K in liposomes resulted in greatly enhanced encapsulation of AmB, suggesting that complex formation occurs between AmB and DSPE-PEG2K molecules. This finding was confirmed in the present study (Table 1). However, the amount of AmB encapsulated was also affected by the lipid composition. We first examined the effect of CH as a lipid component on the encapsulation of AmB. In DPPC liposomes without CH and DSPE-PEG2K, the encapsulated amount of AmB was 62 μg/mg lipid. Since AmB itself is not soluble in water, pore structures assembled from AmB molecules, as already reported by many researchers (Hartsel et al., 1988, 1991; Bolard et al., 1991), may be formed in the lipid bilayer. However, the presence of CH molecules in the DPPC-liposomal membranes

Table 1
Effect of cholesterol on the encapsulation of AmB in liposomes hydrated with 9% sucrose solution[a]

Lipid composition	Molar ratio (mol/mol)	AmB encapsulation (μg AmB/mg lipid)	SD ±
DPPC	2	62	14.0
DPPC/DSPE-PEG2K	2:0.12	149	12.7
DPPC/CH	2:1	14	2.3
DPPC/CH/DSPE-PEG2K	2:1:0.19	111	5.7
DPPC/CH	1:1	9	3.0
DPPC/CH/DSPE-PEG2K	1:1:0.12	61	15.0

[a] Initial weight ratio of AmB to total lipid is 0.15 w/w.

drastically decreased the AmB encapsulation. A similar effect of CH was also observed in DPPC/DSPE-PEG2K liposomes, though, 61 μg of AmB/mg lipid was still encapsulated in DPPC/CH/DSPE-PEG liposomes at 1:1 molar ratio of CH. The DPPC/CH molar ratio did not change during or after liposome preparation, as found previously by lipid and CH quantitative analysis (Moribe et al., 1997a, 1998). The absence of complex formation between AmB and CH molecules in liposomal membrane has been confirmed by other researchers (Bolard et al., 1991). These results indicate that the presence of CH inhibited AmB encapsulation, whereas DSPE-PEG enhanced it, even in the presence of a substantial amount of CH. Because the initial ratio of AmB to lipid was fixed at 0.15 w/w, the encapsulated amount of 149 μg/mg lipid in DPPC/DSPE-PEG liposomes is maximum. So the effect of initial weight ratio of AmB to lipid on the encapsulation was studied (Fig. 1). In DPPC liposomes, AmB encapsulation was maximally 62 μg/mg lipid. The presence of DSPE-PEG in DPPC/CH liposomes increased the encapsulated amount of AmB with increasing initial weight ratio, reaching a plateau. In the case of DPPC/DSPE-PEG liposomes, the encapsulated amount of AmB was proportional to the initial ratio within the initial weight ratio of 0.45. The highest encapsulation efficacy was observed in DPPC/DSPE-PEG liposomes. Encapsulation efficacy in DPPC/CH/DSPE-PEG liposomes was high below an initial weight ratio of 0.1, but it decreased at higher ratios, suggesting inhibition of the encapsulation by CH.

Next, the effect of CH and DSPE-PEG on the encapsulation was examined. Fig. 2 shows the encapsulation amount of AmB in DPPC/CH liposomes and DPPC/CH/DSPE-PEG liposomes. The encapsulated amount in DPPC/CH/DSPE-PEG liposomes was higher than that in DPPC/CH liposomes at every CH content. In DPPC/CH liposomes, the encapsulated amount of AmB was drastically decreased even at low levels of CH incorporation, whereas it was almost unaffected in DPPC/CH/DSPE-PEG liposomes until the CH incorporation reached 30 mol%, then it decreased with further incorporation of CH. These results indicated that incorporation of DSPE-PEG contributes to high encapsulation of AmB even in the presence of CH. In the case of DPPC/CH liposomes, since most of the AmB did not interact with lipids during liposome preparation, the non encapsulated AmB was removed by filtration with a polycarbonate filter on extrusion. In the case of DPPC/CH/DSPE-PEG liposomes, however, there

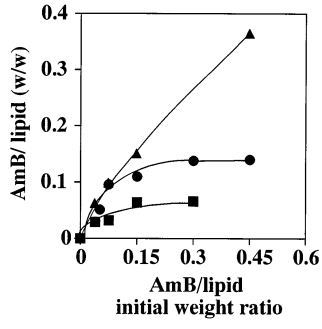

Fig. 1. Effect of initial amount of AmB on the encapsulation into liposomes. (■) DPPC liposomes; (▲) DPPC/DSPE-PEG liposomes; (●) DPPC/CH/DSPE-PEG liposomes.

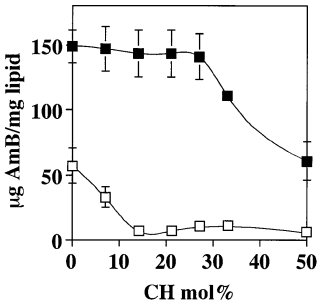

Fig. 2. Effect of CH on the AmB encapsulation into liposomes. (□) DPPC/CH liposomes; (■) DPPC/CH/DSPE-PEG liposomes.

is a possibility that micelle or aggregate formation may account for the decreased encapsulation of AmB at high levels of CH incorporation. In any case, incorporation of CH tends to block the encapsulation of AmB into liposomes, and non encapsulated AmB remains as aggregates or micellar solution.

3.2. Surface state of AmB-encapsulating liposomes

To investigate whether AmB is located on the outer surface of the liposomal membranes, we employed PEG/dextran two-phase partition (Tilcock et al., 1993). The kinetics of the partitioning of liposomes in aqueous two-phase partition systems is dependent upon the vesicle size, net charge and hydrophilicity or lipophilicity of the membrane surface. Thus, if AmB-encapsulating and non encapsulating liposomes, prepared by the same procedure, show different partitioning behavior in the same phase system, their surface properties must differ. Since partitioning behavior provides an index of surface properties as mentioned above, this method can be used analytically.

The surface potential of PEG-liposomes is negative, though the zeta potential is almost neutral under physiological conditions (150 mM NaCl, Moribe et al., 1997b). Taking these electrical surface properties into consideration, a non charge-sensitive phase system consisting of 5% (w/w) PEG8000 and 5% (w/w) dextran T-500, 0.01 M sodium phosphate, 0.15 M sodium chloride (pH 7.0) was used to estimate the alteration of the surface state of PEG-liposomes caused by encapsulation of AmB.

Three liposomal formulations, DPPC, DPPC/DSPE-PEG2K (2/0.13, mol/mol), and DPPC/CH/DSPE-PEG2K (2/1/0.19, mol/mol) labeled with ^{3}H-CH, with or without AmB, were prepared with average diameters in the range of 100–130 nm. Labeling with ^{3}H-CH in DPPC and DPPC/DSPE-PEG2K liposomes did not affect the partitioning behavior of these liposomes, compared with DPPC/CH and DPPC/CH/DSPE-PEG2K liposomes, respectively (data not shown). Partitioning behavior of these liposomes measured at 30 min after mixing was expressed in terms of the percentages of total liposomes in the two phases

Table 2
Partitioning of AmB-encapsulating liposomes in the PEG/dextran two-phase system[a]

Lipid composition	Initial molar ratio	% Liposomes added[b]					
		Upper phase	S.D.	Interface	S.D.	Lower phase	S.D.
DPPC	2	7.7	0	87.5	0.5	4.9	0.5
DPPC/AmB	2/0.27	15.1	0.5	77.5	1.3	7.4	1.8
DPPC/DSPE-PEG 2K	2/0.13	97.3	2.3	1.5	2.5	1.2	2.2
DPPC/DSPE-PEG 2K/AmB	2/0.13/0.27	90.3	2.9	7.6	2.1	2.1	0.8
DPPC/CH/DSPE-PEG 2K	2/1/0.19	94.4	1.6	3.7	1.5	2	0.1
DPPC/CH/DSPE-PEG 2K/AmB	2/1/0.19/0.38	88.3	2.5	9.8	2.2	1.9	0.2

[a] Phase system composed of 5% (w/w) PEG8000 and 5% (w/w) dextran T-500 in 0.01 M sodium phosphate (pH 7.0) containing 0.15 M NaCl.

[b] Values are mean ($\pm$S.D.) $n = 3$.

and at the interface, as shown in Table 2. DPPC liposomes were found mostly at the interface (87.5%) and encapsulation of AmB had little effect on the partitioning behavior. Enhanced partitioning of DPPC/DSPE-PEG2K and DPPC/CH/DSPE-PEG2K liposomes into the upper phase was observed, and this partitioning was almost unaffected by encapsulation of AmB. We have already reported that AmB release caused by incubation with mouse serum was about 10% at 2 h and that AmB encapsulation did not influence the high level of blood residence, compared with that of PEG liposomes (Moribe et al., 1998). Thus, we consider that encapsulation of AmB into liposomes did not markedly influence the outer surface properties of the liposomal membrane and that most of the AmB molecules were located in the core or on the inner side of the liposomal membrane.

3.3. Molecular localization and state of AmB in PEG liposomes

The mechanism of AmB encapsulation appears to be different in the presence or absence of DSPE-PEG in liposomes. To confirm the molecular location and state of AmB in AmB-encapsulating liposomes, potassium permeability, fluorescence quenching and CD spectral measurements were carried out.

Pore formation by AmB molecules in the core of the liposomal membrane was confirmed by potassium permeability measurement. Fig. 3 shows the change of fluorescence of pyranine encapsulated in AmB-containing DPPC and PEG liposomes upon addition of H_2SO_4. Because of the low encapsulation efficacy of AmB in DPPC liposomes, the change of the fluorescence intensity is relatively small. After addition of H_2SO_4, slight decreases of the fluorescence were observed in AmB-DPPC and AmB-DPPC/CH/DSPE-PEG liposomes. The changes of fluorescence were almost the same, and were decreased by addition of FCCP and valinomycin, indicating that pore structure was formed by AmB molecules in both liposomal membranes, allowing H^+/K^+ exchange. But in AmB-DPPC/DSPE-PEG liposomes, addition of H_2SO_4 caused a progressive

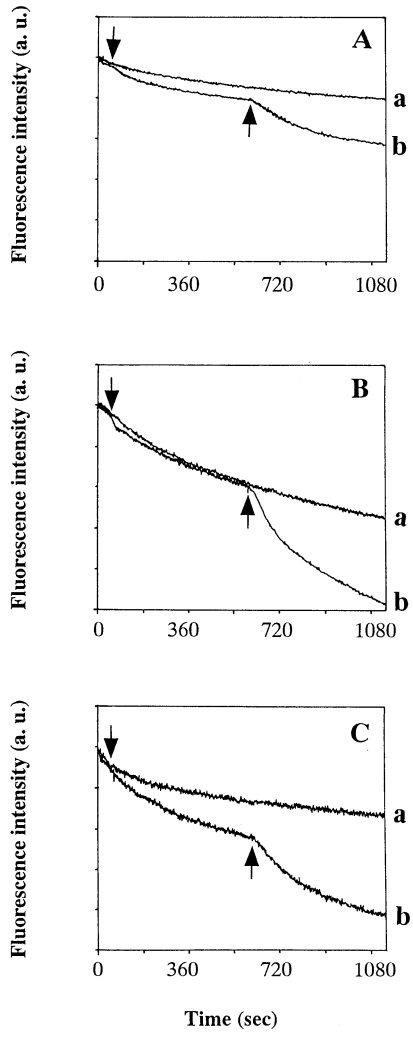

Fig. 3. Fluorescence change of pyranine encapsulated in AmB-containing liposomes in the presence of a pH gradient and of a protonophore FCCP, as a function of time. (A) DPPC liposomes; (B) DPPC/DSPE-PEG2K liposomes; and (C) DPPC/CH/DSPE-PEG2K liposomes. (a) Change of pyranine fluorescence after addition of H_2SO_4 alone; and (b) change of pyranine fluorescence on addition of FCCP at 1 min (first arrow) and valinomycin at 10 min (second arrow) after a pH gradient was formed. Other experimental procedures were described in Section 2.

decrease of the fluorescence, which was not affected by the addition of FCCP. In this case, the membrane properties of AmB-DPPC/DSPE-PEG liposomes were maintained, because the fluorescence intensity was drastically decreased by addi-

tion of valinomycin. Generally, lack of CH in the liposomal membrane reduces the membrane stability. Though the mechanism of these phenomena is not clear, incorporation of AmB in the sterol-free PEG-liposomal membrane may allow H^+ permeation simply in response to a pH gradient.

The molecular location of AmB in liposomal membrane was also investigated in terms of the fluorescence quenching of DPH and tmaDPH. DPH and tmaDPH have been used to estimate the membrane property in the core (Lentz, 1989) and the membrane/water interface (Illinger et al., 1995), respectively. Fluorescence quenching of DPH or tmaDPH in AmB-DPPC, AmB-DPPC/DSPE-PEG and AmB-DPPC/DSPE-PEG liposomes shows a Stern–Volmer type quenching behavior. AmB concentration was calculated from the encapsulated amount of AmB at constant lipid concentration. Stern–Volmer constants of liposomes are given in Table 3. Fluorescence quenching of DPH by AmB was in the order of DPPC > DPPC/DSPE-PEG > DPPC/CH/DSPE-PEG liposomes. Since DPH was located at the center of the membrane bilayer, the quenching behavior reflects the AmB encapsulation in the core of the membrane. Fluorescence quenching of tmaDPH by AmB was in the order of DPPC > DPPC/DSPE-PEG = DPPC/CH/DSPE-PEG liposomes, and Stern–Volmer constants of tmaDPH-containing liposomes were relatively low compared with those of DPH-containing liposomes. These results indicated that AmB is located further from the hydrophilic groups.

Table 3
Stern–Volmer constants of AmB-encapsulating liposomes[a]

Lipid composition	Stern–Volmer constant (μM^{-1})	Correlation factor (r)
DPH		
(In methanol)	0.028	0.991
DPPC	4.223	0.963
DPPC/DSPE-PEG 2K	0.771	0.993
PPPC/CH/DSPE-PEG 2K	2.668	0.991
TmaDPH		
(In methanol)	0.001	0.961
DPPC	0.965	0.979
DPPC/DSPE-PEG 2K	0.452	0.991
DPPC/CH/DSPE-PEG 2K	0.430	0.976

[a] Liposome sample was prepared by using sucrose solution with 0.1 mM lipid concentration.

We have previously reported that enhanced encapsulation of AmB was induced by complex formation between AmB and DSPE-PEG. We speculated that AmB molecules were located on the surface of the liposomal membrane, interacting with DSPE-PEG. But, from the results described here, it is clear that AmB molecules were also located in the core of the membrane, in addition to on the surface of the membrane. Considering the difference of the encapsulation efficacy between DPPC/CH and DPPC/CH/DSPE-PEG liposomes, encapsulation of AmB in the core of the membrane was also induced by DSPE-PEG.

Finally, the molecular state of AmB in liposomes was examined by CD spectral measurement. Positive and negative peaks at 325–330 nm and 343–348 nm were assigned to aggregated states of AmB (Gaboriau et al., 1997). Since CD spectra with three positive peaks at higher wavelength (378, 397 and 412 nm) were almost same to that of AmB in water at low concentrations ($< 5 \times 10^{-8}$ M) (Bolard et al., 1980) or in methanol (data not shown) or ethanol (Rinnert and Thirion, 1977), these were assigned to the monomeric state of AmB. CD spectra of AmB aqueous solution showed a positive peak at 330–340 nm and negative peaks at higher wavelength (data not shown), being apparently different from that of AmB-encapsulating liposomes. As shown in Fig. 4, monomeric and aggregated states of AmB were observed in all liposomes. Molecular states of AmB in these two kinds of states are in good agreement with those of AmB incorporated into a block copolymer systems (Yu et al., 1998). The positive peak shifts from 330 to 320 nm in these liposomes may reflect the fact that heating during the process of liposome preparation induces a superaggregated state (Gaboriau et al., 1997). In DPPC/DSPE-PEG liposomes, increase

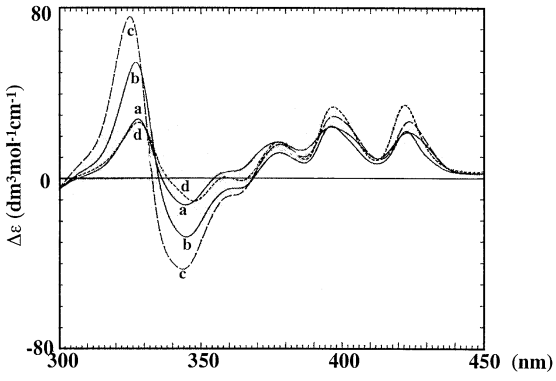

Fig. 4. Circular dichroism (CD) spectra of AmB-encapsulating liposomes. (a) DPPC liposomes (60); (b) DPPC/DSPE-PEG2K liposomes (149); (c) DPPC/DSPE-PEG2K liposomes (290); and (d)DPPC/CH/DSPE-PEG2K liposomes (111). The encapsulated amount of AmB (μg AmB/mg lipid) in liposomes is shown in parenthesis.

of the encapsulated amount of AmB contributes to the increase of the aggregated state of AmB. These results indicated that in every lipid composition of liposomes, AmB molecules existed in both monomeric and aggregated states. For further investigation of the membrane structures of liposomes containing AmB, X-ray diffraction or electron microscopy would be useful, but there are difficulties in the preparation of samples.

In conclusion, we proposed that the structure of AmB-encapsulating liposomes is as follows. In AmB-DPPC liposomes, AmB is located in the core of the membrane. However, the encapsulated amount of AmB in DPPC liposomes is low. In DPPC/DSPE-PEG liposomes, AmB is located in the core and on the surface of the membrane. DPPC/DSPE-PEG liposomes showed the highest levels of AmB encapsulation, reflecting the complex formation between AmB and DSPE-PEG. In DPPC/CH/DSPE-PEG liposomes, AmB is also located in the core and on the surface of the membrane, as in the case of DPPC/DSPE-PEG liposomes. From the results of fluorescence quenching measurement, the lower encapsulation efficacy in DPPC/CH/DSPE-PEG liposomes, compared with that of DPPC/DSPE-PEG liposomes, reflects the AmB localization in the core of the membrane. Complex formation between AmB and DSPE-PEG plays an important role in the high encapsulation of AmB. From the viewpoint of the stability of the liposomes and encapsulation of AmB, DPPC/CH/DSPE-PEG liposomes appear to be the best carrier for AmB for therapeutic purposes.

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