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Encapsulation characteristics of nystatin in liposomes: effects of cholesterol and polyethylene glycol derivatives

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Abstract

In this study, we characterized the encapsulation of amphipathic nystatin into liposomes with or without cholesterol (CH) and a polyethylene glycol derivative, distearoyl-*N*-(monomethoxy poly(ethylene glycol)succinyl)phosphatidylethanolamine (DSPE–PEG). The highest encapsulation efficacy of nystatin into liposomes (151 μg nystatin/mg lipid) was obtained with a cholesterol-free lipid composition containing 6 mol% of DSPE–PEG. The encapsulation efficacy was decreased by the incorporation of CH and improved by the incorporation of DSPE–PEG. In liposomes composed of dipalmitoylphosphatidylcholine (DPPC)/CH (2:1, mol/mol), the highest encapsulation efficacy of nystatin liposomes (84 μg/mg lipid) was achieved by the addition of DSPE–PEG and hydration with 9% sucrose solution, as compared with 13 μg/mg lipid without DSPE–PEG. The encapsulated amount increased with increasing amount of DSPE–PEG used and plateaued at 6 mol% of DSPE–PEG. The optimum molecular weight of PEG in DSPE–PEG was 2000 and a larger molecular weight resulted in lower encapsulation. The incorporation of CH affected the self-association of nystatin with lipid membranes, which was detected by fluorescence measurement. The molecular interaction between an amino group in nystatin and a phosphate group in DSPE–PEG plays an important role in efficient encapsulation of nystatin. Finally, the encapsulation characteristics of nystatin were compared with those of amphotericin B (AmB). Nystatin more readily associated with CH-free lipid membranes, but, AmB more readily interacted with DSPE–PEG. The results indicated that the differences in the molecular association of AmB or nystatin with lipids or DSPE–PEG are reflected in the encapsulation characteristics in liposomes.

Keywords: Nystatin; Liposome; Polyethylene glycol; Fluorescence; ^{31}P-NMR

1. Introduction

The polyene macrolide antibiotics are used in the treatment of fungal infections, which have recently increased in incidence due to the use of immunosuppressive drugs and the increasing frequency of AIDS (Hiemenz and Walsh, 1996; Pons

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et al., 1997). Amphotericin B (AmB) has been used as the first-line therapy for invasive aspergillosis. Because the usefulness of parenteral administration of AmB as an AmB deoxycholate formulation (Fungizone®) is limited by severe side effects such as renal toxicity, the use of AmB encapsulated in liposomes or other lipid carriers has recently been developed. Commercial preparations so far introduced include AmBisome®, ABELECT® (AmB-lipid complex), and Amphocil™ (AmB colloidal dispersion), which is suitable for intravenous therapy (Janknegt et al., 1992; Gates and Pinney, 1993; Van Etten et al., 1995; Hiemenz and Walsh, 1996). Nystatin, which has a similar structure to AmB, is derived from *Streptomyces noursei* and has a broader spectrum of action than AmB. Nystatin has been used also for the treatment of cutaneous, vaginal, and oral candidases by oral (Schafer et al., 1996), pleural (Oehling et al., 1975), inhalation (Sinclair et al., 1978) and topical administration (Than et al., 1980). However, toxicity problems such as thrombophlebitis, fever, chills, and nausea, have prevented parenteral application of nystatin (Newcomer et al., 1955). Since nystatin has different biological properties from those of AmB and sometimes acts on AmB-resistant fungi, the intravenous administration of nystatin would be desirable. Recently, a liposomal formulation of nystatin with good activity in mice was established (Mehta et al., 1987a,b; Wallace et al., 1997). Dimyristoylphosphatidylcholine (DMPC) and dimyristoylphosphatidylglycerol (DMPG) in a molar ratio of 7:3 were used in the formulation, but the suitability and the physicochemical properties of such formulations are not well understood.

Recently, we have found that the incorporation of an amphipathic polyethyleneglycol derivative (distearoyl-*N*-(monomethoxy poly(ethylene glycol)succinyl)phosphatidylethanolamine, DSPE–PEG), which was added to provide prolonged circulation characteristics, also increased significantly the encapsulation efficiency of AmB into liposomes (Moribe et al., 1998). These liposomes showed a long circulation time in blood and a high therapeutic efficiency against invasive pulmonary aspergillosis in mice (Otsubo et al., 1998).

In the present study, the encapsulation characteristics of nystatin in liposomes were examined and compared with those of AmB. It was found that nystatin associated more readily with sterol-free lipid membranes, while AmB interacted more readily with DSPE–PEG.

2. Materials and methods

2.1. Materials

Nystatin and cholesterol (CH) were purchased from Wako Pure Chemicals (Osaka, Japan) and used without further purification. Dipalmitoylphosphatidylcholine (DPPC), distearoylphosphatidylethanolamine (DSPE), distearoylphosphatidic acid (DSPA), monomethoxy polyethylene glycol succinimidyl succinate (PEG–OSu) with an average molecular weight of 1000 (1K), 2000 (2K) or 5000 (5K) Da, 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 as reported previously (Maruyama et al., 1992). The structures of some of these compounds, as well as the nystatin derivatives described below, are shown in Fig. 1.

2.2. Synthesis of nystatin derivatives

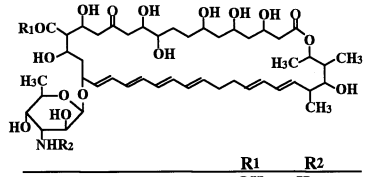
Nystatin derivatives, *N*-acetylnystatin and nystatin methylester, were synthesized as reported (Mechlinski and Schaffner, 1972). *N*-Acetylnystatin: nystatin (1 g) was dissolved with stirring in 10 ml of DMSO at room temperature and diluted with 10 ml of absolute methanol. The mixture, containing partially precipitated antibiotic, was cooled on ice and 0.12 g of acetic anhydride was added stepwise during 10 min with stirring. After a further 10 min, the product was isolated by precipitation in 100 ml of anhydrous ethyl ether, followed by centrifugation and washing with ethyl ether. Nystatin methylester: nystatin (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, which was

prepared in the usual way from *N*-methyl-*N*-nitroso-ptoluenesulfonamide (Diazald (R)) for 1 min. 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^2; chloroform/methanol/0.025 M borate buffer, pH 8.3 (2/2/1 v/v, lower phase)).

2.3. Preparation of liposomes

The lipid composition of nystatin–PEG-liposomes was DPPC/CH (2/1 molar ratio) with 6 mol% of DSPE–PEG 2K (average molecular weight 2000). Nystatin 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 mixture was evaporated to make a lipid film. The lipid film was hydrated with 9% sucrose by vortex mixing, and the mixture was frozen and thawed four times. Liposomes were extruded through Nuclepore polycarbonate membranes of 0.4, 0.2 and 0.1 μm pore size (Nuclepore Co., CA), resulting in an average particle size of 120 nm, as measured by dynamic light scattering (ELS 800, Otsuka Electronics Co., Tokyo). The extruded nystatin–PEG-liposomes were also centrifuged at $2 \times 10^5 \times g$ for 15 min to separate non-entrapped nystatin and to concentrate the liposomes. These 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 nystatin concentration was measured spectrophotometrically at 318 nm in methanol. The encapsulated amount of nystatin was usually expressed as the weight ratio of nystatin to lipid (equivalent amounts of DPPC and CH).

Nystatin and nystatin derivatives



	R1	R2
nystatin	-OH	-H
N-acetylnystatin	-OH	-COCH3
nystatin methylester	-OCH3	-H

DSPE-PEG and PEG derivatives

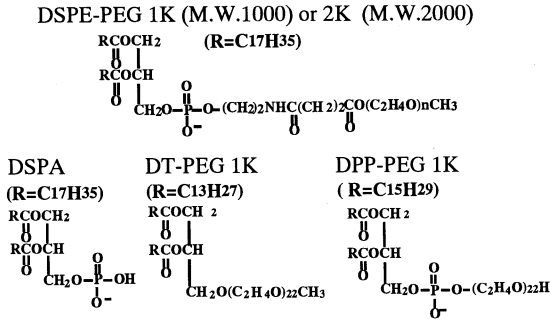


Fig. 1. Chemical structures of nystatin and PEG derivatives.

2.4. Fluorescence measurement of nystatin binding

Self-association of nystatin with liposomal membranes was investigated by fluorescence measurement as reported (Coutinho and Prieto, 1995). Nystatin-free liposomes were prepared as described in Section 2. Lipid concentration was adjusted to 5 mM as a stock solution. Appropriate volumes of nystatin stock solution dissolved in methanol, liposome stock solution and 9% sucrose solution were mixed to make the desired samples. Final nystatin concentration was 10 μM and the final concentration (v/v) of methanol in solutions was below 1%. Nystatin binding to the liposomal membranes was detected in terms of the increase of the fluorescence intensity. The prepared samples were incubated at 30°C for 1 h in the dark, and fluorescence measurement was preformed with excitation and emission wavelengths of 318 and 425 nm, respectively.

2.5. Preparation of nystatin–DSPE–PEG solution

A solution of 20 mg of nystatin in methanol was mixed with a solution of 20 mg of DSPE–PEG in chloroform. After evaporation of the

Table 1
Encapsulation of nystatin in liposomes

Lipid composition	Molar ratio (mol/mol)	Solvent	Nystatin encapsulation[a] (μg nystatin/mg lipid)	S.D. (±)
DPPC	2	9% Sucrose	149.5	2.1
DPPC/DSPE–PEG 2K	2:0.12	9% Sucrose	151.0	11.6
DPPC/CH	2:1	9% Sucrose	12.8	2.7
	2:1	Saline	10.2	4.4
DPPC/CH/DSPE–PEG 2K	2:1:0.19	9% Sucrose	83.9	6.1
	2:1:0.19	Saline	25.9	5.7
	2:1:0.19	10 mM Citric acid (pH 3)	13.5	0.9
	2:1:0.19	10 mM Tris–HCl (pH 9)	21.5	0.6
DPPC/CH	1:1	9% Sucrose	10.0	3.1
DPPC/CH/DSPE–PEG 2K	1:1:0.12	9% Sucrose	33.3	13.8

[a] Initial weight ratio of nystatin to total lipid was 0.15 (w/w).

organic solvent, 1 ml of 9% sucrose solution was added to the mixture and the free nystatin was removed by centrifugation at $3000 \times g$ for 15 min as a precipitate. The remaining mixture was extruded through a 400 nm filter. Nystatin and lipid concentrations were determined as described above.

2.6. ^{31}P-NMR measurement

Nystatin–DSPE–PEG solutions were prepared as described above, except for hydration with D_2O. Free nystatin was removed by passing the solution through a 0.4 μm membrane filter. Lipid concentration was adjusted to 20 mg/ml. 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.

3. Results

3.1. Encapsulation behavior of nystatin in liposomes

The amount of nystatin encapsulated in liposomes is summarized in Table 1. In CH-free lipid compositions such as DPPC and DPPC/DSPE–PEG, the highest encapsulation efficacy (149.5 and 151 μg nystatin/mg lipid, respectively) were obtained by using 9% sucrose solution for hydration. The encapsulation efficacy decreased with the incorporation of CH, but was improved by the incorporation of DSPE–PEG. In DPPC/CH liposomes with a 2/1 molar ratio, high efficacy of nystatin encapsulation (83.9 μg/mg lipid) was obtained by the addition of 6 mol% of DSPE–PEG to the liposomes and by using 9% sucrose solution for hydration. Hydration with an electrolyte solution such as saline in DSPE–PEG-free liposomes or DSPE–PEG-containing liposomes resulted in a low nystatin encapsulation (< 25.9 μg/mg lipid). Since nystatin is insoluble in an electrolytic solution as well as a sugar solution, we speculated that some interaction between nystatin and DSPE–PEG might play an important role in the improvement of nystatin encapsulation. On the other hand, no improvement of the nystatin encapsulation was observed in DPPC/CH/DSPE–PEG (1/1/0.12, m/m), suggesting that the presence of CH disfavors the nystatin encapsulation and that the interaction between nystatin and DSPE–PEG, which may contribute to the nystatin encapsulation, is not strong.

Table 2
Effect of DSPE–PEG on the encapsulation of nystatin in liposomes hydrated with 9% sucrose solution

Lipid composition	Molar ratio (mol/mol)	DSPE–PEG (mol%)	Nystatin encapsulation (μg nystatin/mg lipid)	S.D. (±)
DPPC/CH/DSPE–PEG 1K	2:1:0.19	6	74.1[a]	2
DPPC/CH/DSPE–PEG 2K	2:1:0.19	6	83.9[a]	6
DPPC/CH/DSPE–PEG 3K	2:1:0.19	6	62.9[a]	13
DPPC/CH/DSPE–PEG 5K	2:1:0.19	6	17.4[a]	15
DPPC/CH/DSPE–PEG 2K	2:1:0.09	3	128.3[b]	14
DPPC/CH/DSPE–PEG 2K	2:1:0.19	6	186.6[b]	21
DPPC/CH/DSPE–PEG 2K	2:1:0.30	9	185.4[b]	31
DPPC/CH/DSPE–PEG 2K	2:1:0.41	12	190.1[b]	40

[a] Initial weight ratio of nystatin to total lipid was 0.15 (w/w).
[b] Initial weight ratio of nystatin to total lipid was 0.30 (w/w).

The effects of the molecular weight and the incorporated amount of DSPE–PEG on the encapsulation of nystatin in CH-containing liposomes are summarized in Table 2. When the incorporated amount of DSPE–PEG was fixed at 6 mol%, high encapsulation of nystatin was observed in DSPE–PEG 1K and 2K-containing liposomes (74.1 and 83.9 μg nystatin/mg lipid, respectively), whereas DSPE–PEG-containing liposomes with longer PEG chains showed decreased encapsulation. The amount of 6 mol% incorporation of DSPE–PEG into liposomes seemed reasonable from the viewpoint of inhibition of micelle formation and achieving a long-circulation property in vivo. The encapsulated amount of nystatin was also influenced by the amount of DSPE–PEG incorporated. It increased as the amount of DSPE–PEG 2K was increased, reaching a plateau at 6 mol% DSPE–PEG. Compared with the results for AmB, a lower incorporation of DSPE–PEG was enough for saturation of the encapsulation of nystatin (Moribe et al., 1998).

In the above experiments, the initial weight ratio of nystatin was fixed at 0.15 or 0.30 to investigate the relative encapsulation efficacy. Encapsulation efficacy was also affected by the initial amount of nystatin used for the preparation. As shown in Fig. 2, the encapsulated amount of nystatin increased with the initial weight ratio of nystatin to lipid. Of the three types of liposomes, DPPC/DSPE–PEG-liposomes showed the highest encapsulation efficacy at high initial weight ratio. However, a higher nystatin to lipid ratio resulted in lower stability and yield, so the weight ratio used in Tables 1 and 2 was considered to be a good compromise.

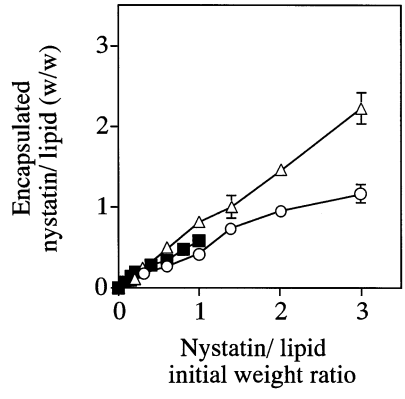


Fig. 2. Effect of the initial weight ratio of nystatin to lipid on nystatin encapsulation in liposomes. ■, DPPC-liposomes, △, DPPC/DSPE–PEG-liposomes; ○, DPPC/CH/DSPE–PEG-liposomes.

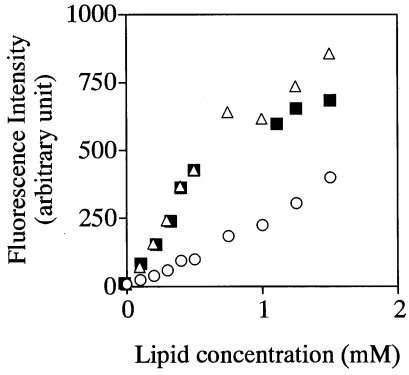


Fig. 3. Self-association of nystatin with liposomal membranes estimated in terms of the enhancement of fluorescence intensity at 30°C. ■, DPPC-liposomes; △, DPPC/DSPE–PEG-liposomes; ○, DPPC/CH/DSPE–PEG-liposomes. Nystatin was dissolved in methanol. Liposomes were prepared as described in Section 2 without nystatin. The lipid concentration was adjusted to 5 mM as a stock solution. Nystatin concentration in each sample was fixed at 10 μM. Nystatin in a constant volume and liposomes were added to each sample from the stock solutions and each mixture was incubated at 30°C for 1 h. The final concentration of methanol in the solution was below 1% (v/v).

3.2. Self-association of nystatin with liposomal membranes

Since nystatin self-associates with lipid membrane (Coutinho and Prieto, 1995; Milhaud et al., 1997), high encapsulation of nystatin in sterol-free liposomes may reflect the preferred nystatin interaction with sterol-free lipid membranes. To confirm this, the binding affinity of nystatin to the liposomal membranes was studied by fluorescence measurement in DPPC-, DPPC/DSPE–PEG- and DPPC/CHIDSPE–PEG-liposomes. As shown in Fig. 3, the fluorescence intensity of nystatin was enhanced with increasing lipid concentration in these liposomes. CH-free liposomes (DPPC, DPPC/DSPE–PEG) showed a similar increase of fluorescence, reaching a plateau at high lipid concentration. On the other hand, the fluorescence intensity in DPPC/CH/DSPE–PEG-liposomes increased linearly about half as fast as in the sterol-free liposomes. These results indicate that the incorporation of CH inhibits the association of nystatin with liposomal membranes, even in the presence of DSPE–PEG.

3.3. Molecular interaction between nystatin and DSPE–PEG

The encapsulation efficacy of nystatin into DPPC/CH/DSPE–PEG-liposomes was influenced by the solution used for hydration, the incorporated amount of DSPE–PEG and the molecular weight of PEG, suggesting that molecular interactions occur between nystatin and DSPE–PEG. The nystatin–DSPE–PEG solution was prepared as described in Section 2, and the interaction between the components was investigated.

As the interaction mode, two possibilities were considered. One is complex formation between nystatin and DSPE–PEG via the interaction between certain functional groups. The other is solubilization of nystatin by DSPE–PEG. In this case, nystatin molecules would be incorporated in the acyl chains and may form pore structures in an aggregated state. The sample formed a pale yellow solution, which was stable at 4°C for at least a month. Fig. 4 shows the nystatin/DSPE–PEG molar ratio of the prepared nystatin/DSPE–PEG solution. The nystatin interaction with DSPE–PEG increased depending on the initial molar ratio and reached $\sim 1/1$ at a 5/1 initial molar ratio. Since no further increase of the ratio was observed at higher initial molar ratio, the molar ratio of nystatin/DSPE–PEG interaction was estimated as 1/1.

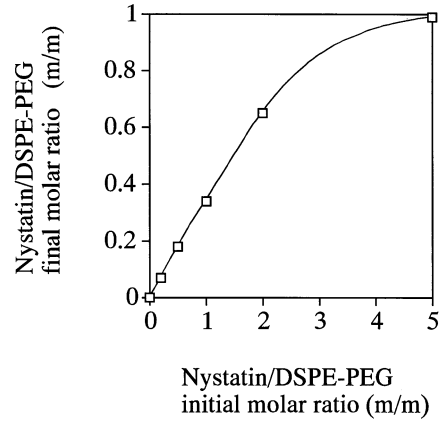


Fig. 4. Effect of initial molar ratio on the interaction between nystatin and DSPE–PEG.

Table 3
Effect of nystatin and DSPE–PEG derivatives on encapsulation[a]

Nystatin derivative	DSPE–PEG derivative	Nystatin encapsulation[b] (μg nystatin/mg lipid)	S.D. (±)
Nystatin	DT–PEG 1K	22.4	4
Nystatin	DSPA	71.2	6
Nystatin	DPP–PEG 1K	75.1	16
Nystatin	DSPE–PEG 1K	83.9	6
Nystatin	DSPE–PEG 2K	83.9	6
Nystatin methyl ester	DSPE–PEG 2K	80.2	16
Acetylnystatin	DSPE–PEG 2K	1.8	1

[a] DPPC/CH/DSPE–PEG 2K (2/1/0.19, mol/mol).
[b] Initial weight ratio of nystatin to total lipid was 0.15 (w/w).

Next, the mechanism of encapsulation of nystatin into PEG-liposomes was examined by using PEG and nystatin derivatives (Fig. 1). The results are summarized in Table 3. When α-monomethoxy-ω-(1,2ditetradecanoyloxyglyceryl) polyoxyethylene, which has only a PEG moiety (DT-PEG 1K), was used, the encapsulated amount of nystatin was 22.4 μg/mg lipid. With DSPA, which has a phosphate group, nystatin encapsulation increased (71.2 μg/mg lipid) and with α-(dipalmitoylphosphatidyl)-ω-hydroxy polyoxyethylene (DPP–PEG 1K), while DSPE–PEG 1K, which have both the phosphate group and the PEG moiety, also gave high values (75.1 and 83.9 μg/mg lipid, respectively). Thus, a phosphate group, which has a negative charge, is required for high encapsulation of nystatin, and the PEG moiety is not so important in terms of enhanced encapsulation of nystatin.

The encapsulated amount of nystatin methylester, a carboxyl groupmodified derivative of nystatin, was almost the same as that of nystatin, while that of acetylnystatin, an amino group-modified derivative of nystatin, was almost zero. These results indicated that the amino group may play an important role in the encapsulation of nystatin by interacting with the phosphate group in DSPE–PEG.

Participation of the phosphate group in the interaction was confirmed by the ^{31}P-NMR spectrum (Fig. 5). In the spectrum of DSPE–PEG, the phosphate signal was observed at 2.05 ppm. In the nystatin–DSPE–PEG solution, the signal was partially shifted to the higher magnetic field (−9.08 ppm), which is consistent with an amino group/phosphate group interaction.

4. Discussion

Encapsulation of nystatin in liposomes was characterized with regard to the effects of CH and

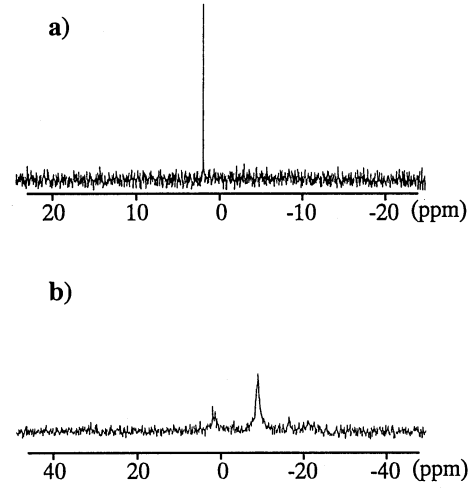


Fig. 5. ^{31}P-NMR spectra of nystatin–DSPE–PEG solutions. (a) DSPE–PEG, (b) nystatin–DSPE–PEG. Samples were prepared by hydration with D_2O and filtered through a 0.4 μm membrane filter. ^{31}P-NMR spectra were recorded at 162 MHz at 300 K.

DSPE–PEG, and compared with that of AmB.

The preferential association of nystatin with sterol-free lipid membranes has been reported (Coutinho and Prieto, 1995; Milhaud et al., 1997) and was confirmed in this study with DPPC- and DPPC/CH/DSPE–PEG-liposomes (Fig. 3). These phenomena influence nystatin encapsulation into liposomes. As shown in Table 1 and Fig. 2, the highest encapsulation efficacy was observed in DPPC/DSPE–PEG-liposomes. Since DPPC-liposomes also showed high encapsulation efficacy at low initial nystatin to lipid ratio, self-association of nystatin with the sterol-free lipids may occur during liposome preparation. In DPPC/DSPE–PEG-liposomes, the encapsulated amount of nystatin continued to increase even at high initial nystatin to lipid ratio (Fig. 2). The difference of encapsulation efficacy between DPPC– and DPPC/DSPE–PEG-liposomes reflects the interaction between nystatin and DSPE–PEG, but an encapsulated amount ~ 1 mg nystatin/mg lipid (almost equal to 1/1 molar ratio) was too large to allow formation of liposomal membranes with normal physicochemical properties. Therefore, though high encapsulation efficacy was obtained at high initial nystatin to lipid ratio, the need for stability and good membrane properties means that a low initial nystatin to lipid ratio is more suitable for the preparation of nystatin-encapsulated liposomes.

The decrease of nystatin encapsulation by incorporation of CH was improved by the incorporation of DSPE–PEG, when the DPPC/CH molar ratio was 2/1 (Table 1). Although the improvement reflects the molecular interaction between nystatin and DSPE–PEG in the liposome preparation, no enhanced encapsulation of nystatin was observed in DPPC/CH- (1/1, m/m) liposomes even in the presence of DSPE–PEG. These results indicate that the incorporation of CH disfavors nystatin encapsulation, and that the encapsulation amount depends mainly on the CH content.

Incorporation of CH decreased the encapsulation efficacy, but incorporation of CH and DSPE–PEG in nystatin-encapsulating liposomes was required for the long-circulating property in blood to achieve high therapeutic efficacy in practical use. Even in DPPC/CH/DSPE–PEG-liposomes, the encapsulation efficacy (83.9 1 μg/mg lipid) was higher than that of DMPC/DMPG-liposomes (maximum incorporation: 60 μg/mg lipid), which have been used for experimental therapy (Mehta et al., 1987a,b; Wallace et al., 1997). Therefore, we mainly investigated the encapsulation characteristics of nystatin with CH- and DSPE–PEG-incorporated liposomes.

Increased DSPE–PEG incorporation resulted in increased encapsulation of nystatin, but this plateaued at above 6 mol% incorporation (Table 2). Enhanced encapsulation was also observed in PEG-liposomes when the molecular weight of PEG was 1000 or 2000 but decreased with a larger molecular weight of PEG. Comparing the encapsulation characteristics of nystatin with those of AmB, nystatin associated more readily with sterol-free lipid membranes. For example, the encapsulated amounts of AmB and nystatin in DPPC-liposomes were 62 and 149.5 μg/mg lipid, respectively. On the other hand, AmB was more readily interacted with DSPE–PEG, and even in DPPC/CH (1/1, m/m) liposomes, enhanced encapsulation of AmB was observed (61 μg/mg lipid). These results indicated that the difference of the association of AmB and nystatin with lipids or DSPE–PEG is reflected in the encapsulation characteristics of the liposomes.

The molecular interaction between nystatin and DSPE–PEG is different from that of AmB and DSPE–PEG. Complex formation involving interactions of the amino and polyene moieties of AmB with the phosphate and PEG moieties of DSPE–PEG, respectively, has already been reported (Moribe et al., 1998). In this case, in addition to the complex formation between AmB and DSPE–PEG by interaction between the functional groups, solubilization of AmB by DSPE–PEG may also occur. On the other hand, molecular interaction between nystatin and DSPE–PEG involves only the amino group of nystatin and phosphate group of DSPE–PEG (Table 3). Therefore, the molecular interaction between nystatin and DSPE–PEG may promote solubilization of nystatin molecules into the lipid membrane.

Multilamellar vesicles (MLV) containing nystatin, which are composed of DMPC/DMPG, have been used for experimental therapy and showed good antifungal activity in mice (Mehta et al., 1987a,b; Wallace et al., 1997). However, there are some problems with the intravenous administration of these liposomal formulations. Actually, the liposomal formulation of nystatin is a injectable dosage with relatively high encapsulation efficacy, however, is cleared rapidly from blood circulation and accumulated in the reticuloendothelial system (RES) or the lung. Therefore, the liposomal formulation of nystatin, which has a long-circulation property in blood as shown in this study, is of better than that of MLV for the therapeutic experiments of systemic fungal infection.

The lipid composition including the encapsulation efficacy of nystatin is also an important factor to determine the therapeutic efficacy. Increased incorporation of CH in liposomes contributes to the stability in blood, but reduces the encapsulation efficacy of nystatin. Increased incorporation of DSPE–PEG contributes to the long-circulation property in blood and enhanced encapsulation efficacy of nystatin, but in this case, micelle formation and the corresponding toxicity problem may occur. So, we think that PEG-liposome, which is composed of DPPC/CH/DSPE–PEG with the molar ratio of 2/1/0.19, is the optimum liposomal formulation for the therapeutic experiment. Further fundamental studies, e.g., on the stability and mechanisms of nystatin encapsulation in PEG-liposomes, would be required to find optimum experimental conditions for the therapeutic uses. Because of the intrinsic fluorescence of nystatin, detailed structural information about the localization of nystatin in PEG-liposomes should be straightforward to obtain.

In conclusion, we have characterized nystatin encapsulation in liposomes in relation to the incorporation of CH and DSPE–PEG. High encapsulation efficacy was obtained in CH-free PEG-liposomes. In CH-containing liposomes, enhanced encapsulation was obtained by incorporation of DSPE–PEG of low molecular weight. Molecular interaction between nystatin and DSPE–PEG appears to involve the interaction of the amino group of nystatin with the phosphate group of DSPE-PEG, which may result in solubilization of nystatin in the liposomal membrane. These phenomena are different from those in the case of AmB, and the association of AmB or nystatin with lipids or DSPE–PEG plays an important role in determining the encapsulation characteristics.

These results may help in the development of effective pharmaceutical formulations for injectable hydrophobic drugs with reduced side effects.

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