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Spectroscopic investigation of the molecular state of nystatin encapsulated in liposomes

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Abstract

The stability and spectral properties of nystatin-encapsulating liposomes, composed of various combinations of dipalmitoyl phosphatidylcholine (DPPC), cholesterol (CH) and distearoyl-*N*-(monomethoxy poly(ethylene glycol)succinyl) phosphatidylethanolamine (DSPE-PEG), were studied in order to elucidate the molecular state and localization of nystatin encapsulated in liposomes. Localization of nystatin at the surface region of the liposomal membrane was investigated by PEG/dextran two-phase partition and measurement of the fluorescence quenching of nystatin by *p*-xylene-bis-pyridinium bromide (DPX). In DPPC/DSPE-PEG liposomes and DPPC/CH/DSPE-PEG liposomes, containing 151 and 160 μg nystatin per mg lipid, respectively, nystatin appeared to be present at the surface region of the liposomal membranes. Self-quenching of nystatin fluorescence was observed in DPPC/CH and DPPC/CH/DSPE-PEG liposomes even at low encapsulated amounts, suggesting the localization of nystatin in CH-incorporating membranes. In CH-free liposomes, nystatin molecules were at first delocalized in the membranes and then self-associated at a higher level of encapsulation. Absorption and circular dichroism (CD) spectra were also measured to examine the monomeric and aggregated states of nystatin in liposomes. High encapsulation efficacy was observed in DPPC and DPPC/DSPE-PEG liposomes, but the highest stability and retention of nystatin in liposomes were observed in DPPC/CH/DSPE-PEG liposomes, evaluated in terms of the nystatin and calcein release from nystatin-encapsulating liposomes in vitro. From the results, possible encapsulation mechanisms of nystatin in liposomes narrowed down to the following three points; interaction with lipid membrane, adsorption on the liposomal surface and complex formation with DSPE-PEG.

Keywords: Nystatin; Liposome; Polyethylene glycol; Fluorescence; Circular dichroism spectra

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 first-line therapy for invasive aspergillosis. Nystatin, which has a similar structure to AmB, has a broader spectrum of action than AmB and is used 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 reduced toxicity and good activity in mice was established (Mehta et al., 1987a,b; Wallace et al., 1997) by using dimyristoylphosphatidylcholine (DMPC)/dimyristoylphosphatidylglycerol (DMPG). Concerning the optimal lipid formulation of AmB- or nystatin-encapsulating liposomes for intravenous administration, we have already demonstrated that the incorporation of an amphipathic polyethyleneglycol derivative (distearoyl-*N*-(monomethoxy poly(ethylene glycol)-succinyl) phosphatidylethanolamine, DSPE-PEG), significantly increased the encapsulation efficacy of AmB (Moribe et al., 1998, 1999b) or nystatin (Moribe et al., 1999a) in cholesterol (CH)-incorporating liposomes. AmB-encapsulating PEG liposomes and PEG immunoliposomes showed a long circulation time in blood and a high therapeutic efficiency against invasive pulmonary aspergillosis in mice (Otsubo et al., 1998). However, enhanced encapsulation of nystatin tends to occur in sterol-free liposomes, which is different from the case of AmB. Furthermore, nystatin does not readily form a complex with DSPE-PEG, compared with AmB. The mechanism of the interaction between nystatin and sterol-free lipid membranes has been studied by many researchers (Petersen et al., 1987; Castanho et al., 1992; Coutinho and Prieto, 1995; Milhaud et al., 1997), but the mechanism of encapsulation and the molecular state of nystatin in liposomes have not been fully established.

In the present study, the stability and molecular states of nystatin in four kinds of liposomes, dipalmitoyl phosphatidylcholine (DPPC), DPPC/cholesterol (CH), DPPC/DSPE-PEG and DPPC/CH/DSPE-PEG liposomes, were examined in order to elucidate the encapsulation mechanism of nystatin in liposomes. The localization of nystatin on the outer surface of the liposomal membranes was investigated by PEG/dextran two-phase partition and measurement of the fluorescence quenching of nystatin in liposomes. The molecular states of nystatin in these liposomes were studied by measuring fluorescence self-quenching, anisotropy of nystatin and absorption and circular dichroism (CD) spectra. Finally, stability of liposomes and location of nystatin in nystatin-encapsulating liposomes were also studied in terms of calcein and nystatin release from nystatin-encapsulating liposomes in vitro.

2. Materials and methods

2.1. Materials

Nystatin and cholesterol (CH) were purchased from Wako Pure Chemicals (Osaka, Japan) and used without further purification. ^{3}H-CH was obtained from New England Nuclear Japan (Tokyo, Japan). Dipalmitoylphosphatidylcholine (DPPC), distearoylphosphatidyl ethanolamine (DSPE) and monomethoxy polyethylene glycol succinimidyl succinate (PEG-OSu) with an average molecular weight of 2000 Da (2 kDa) were kindly provided by Nippon Oil and Fats (Tokyo, Japan). *p*-Xylene-bis-pyridinium bromide (DPX) was obtained from Molecular Probes Inc. (Eugene, OR). Other chemicals used were of reagent grade. DSPE-PEG was synthesized as reported previously (Maruyama et al., 1992).

2.2. Preparation of liposomes

Lipid compositions used were DPPC, DPPC/DSPE-PEG (2/0.13 molar ratio) and DPPC/CH/DSPE-PEG (2/1/0.19 molar ratio). Liposomes were prepared as previously described (Moribe et al., 1999a). Briefly, nystatin and DSPE-PEG 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 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 109–135 nm, as measured by dynamic light scattering (ELS 800, Otsuka Electronics Co., Tokyo). The extruded nystatin-encapsulating liposomes were also centrifuged at $2 \times 10^5 \times g$ for 15 min to separate non-entrapped nystatin and to concentrate the liposomes. The resulting particle sizes of these liposomes were almost same those of before centrifugation as shown in Table 1. 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). Initial weight ratio of nystatin to lipid was 0.75/5 mg (initial molar ratio of nystatin to lipid in CH-free and CH-incorporated liposomes were 0.119 and 0.100, respectively) and the encapsulated amounts of nystatin in DPPC, DPPC/CH, DPPC/DSPE-PEG and DPPC/CH/DSPE-PEG liposomes were 150, 13, 151 and 84 μg mg^{-1} lipid, respectively.

2.3. PEG/dextran two-phase partition

The PEG/dextran two-phase system was prepared as described (Tilcock et al., 1993; Moribe et al., 1997). Briefly, a 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 nystatin-encapsulating liposomes was done as follows. ^{3}H-CH-labeled liposomes (100 μl) 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.

Table 1
Particle size of nystatin-encapsulated liposomes[a] (nm)

Lipid composition	−Nystatin	+Nystatin			
	After extrusion/centrifugation	After extrusion	After extrusion/centrifugation	Before partition[b]	After partition[c]
DPPC	109 (4.8)	122 (1.1)	135 (8.1)	115 (5.5)	113 (2.8)
DPPC/CH	109 (3.0)	116 (1.8)	116 (3.2)	116 (2.9)	111 (4.6)
DPPC/DSPE-PEG	112 (2.5)	113 (0.9)	119 (1.8)	117 (2.6)	113 (7.0)
DPPC/CH/DSPE-PEG	119 (3.7)	122 (1.6)	118 (5.1)	129 (3.9)	126 (5.2)

[a] Particle size was measured three times and shown as the average. S.D. is shown in the parenthesis.
[b] Particle size of the liposomes were measured after mixing with PEG/dextan two-phase system.
[c] Particle size of the liposomes were measured at 30 min after the mixing with PEG/dextran two-phase system.

2.4. Quenching of nystatin fluorescence by DPX

DPX has been used as a hydrophilic quencher to evaluate the fusion of liposomes or the leakage of drugs (Ladokhin et al., 1995; Blackwood et al., 1996). Since DPX does not permeate through the liposomal membranes, fluorescence quenching of nystatin encapsulated in liposomes by addition of DPX reflects the amount of nystatin at the liposomal surface. The prepared liposomes were diluted to a final concentration of 3 μM nystatin with 9% sucrose solution and the desired concentration of DPX was added (0–53 mM). Fluorescence measurements were done on a Shimadzu RF-5000 spectrofluorometer (Kyoto, Japan) with excitation and emission wavelengths of 318 and 425 nm, respectively. After measurement of the fluorescence intensity at 30°C, relative fluorescence intensity was standardized in terms of F_0/F and the Stern–Volmer constant was calculated by use of the following equation:

$$\frac{F_0}{F} = K_{sv}[\mathrm{DPX}] + 1,$$

where F_0 or F is the fluorescence intensity of nystatin in the absence or presence of DPX, [DPX] is the DPX concentration and K_{sv} is the Stern–Volmer constant. The degree of nystatin quenching by DPX was estimated in terms of the Stern–Volmer constant, the order of which would reflect the extent to which nystatin molecules are located on the surface of the liposomal membranes.

2.5. Fluorescence measurements

Fluorescence intensity of nystatin in liposomes were measured as described above. The prepared liposomes were diluted to the desired concentration of nystatin (3 μM) with 9% sucrose solution.

Fluorescence anisotropy (r) of nystatin was calculated by use of the following equation:

$$r = \frac{(I\mathrm{a} - I\mathrm{b}(G))}{(I\mathrm{a} + 2I\mathrm{b}(G))},$$

where Ia and Ib are the intensities of light emitted with its polarization plane parallel and perpendicular to that of the exciting beam, respectively, and G is an instrument anisotropy factor. The obtained data were compared with those of Castanho et al. (1992).

2.6. Circular dichroism (CD) spectra

Circular dichroism (CD) spectra were recorded with a JASCO J-700 spectropolarimeter (Tokyo, Japan). The nystatin 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; response, 0.25; scan speed, 100 nm min^{-1}; accumulation, five times. All the data were expressed in terms of $\Delta\varepsilon$ ($\mathrm{dm}^3\ \mathrm{mol}^{-1}\ \mathrm{cm}^{-1}$), which is the differential molar dichroic absorption coefficient.

2.7. Calcein and nystatin release profile of nystatin-encapsulating liposomes in vitro

Stability and location of nystatin in nystatin-encapsulating liposomes were estimated in terms of the extent of calcein and nystatin release in vitro.

Calcein has been used as a fluorescent aqueous marker to investigate the membrane permeability (Allen and Cleland, 1980; New, 1990; Katsu, 1999) and the release profiles from liposomes reflects the stability. Calcein and nystatin-encapsulated liposomes were prepared as described above by hydration with 50 mM calcein/150 mM sucrose/10 mM Hepes buffer (pH 7.4). The prepared liposomes were centrifuged at $2 \times 10^5 \times g$ for 15 min and washed with phosphate-buffered saline (PBS, pH 7.4) to remove the untrapped calcein. Lipid concentration of the prepared liposomes was adjusted to 10 mg ml^{-1} by 9% sucrose solution. A 200 μl aliquot of the sample was added to 800 μl of ddY mouse plasma and the mixture was incubated at 37°C. After the desired period of incubation, a 50 μl aliquot of the sample was collected, diluted with 950 μl of PBS. A 50 μl aliquot of the sample was collected again to measure 100% release of calcein by adding a 50 μl of 10% Triton X-100 solution. The rest of the sample was centrifuged at $2 \times 10^5 \times g$ for 30 min to ob-

tain the supernatant. The amount of calcein released was determined by measuring the fluorescence intensity at 515 nm (excitation at 470 nm) after the lipid concentration was adjusted to 5 μg ml^{-1}.

Nystatin release from nystatin-encapsulating liposomes in vitro was also measured to estimate the molecular localization of nystatin in liposomes. Prepared nystatin-encapsulating liposomes were diluted with 9% sucrose solution to adjust the nystatin concentration to 1 mg $^{-1}$ml. A 0.4 ml aliquot of the sample was added to 1.6 ml of ddY mouse plasma and the mixture was 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 30 min to obtain the supernatant. A 0.2 ml aliquot of the supernatant was mixed with 1.8 ml of 99% methanol and the mixture was centrifuged at $2000 \times g$ for 30 min. The amount of nystatin released was calculated from the fluorescence intensity of the supernatant as described above.

Data were expressed as the mean of triplicate measurements.

3. Results and discussion

3.1. Surface state of liposomal membranes in the presence of nystatin

Nystatin has a similar structure to AmB, but its encapsulation characteristics are different. We have already demonstrated that enhanced encapsulation of nystatin can be achieved in sterol-free and DSPE-PEG-incorporating liposomes (Moribe et al., 1999a). In that case, nystatin molecules may be adsorbed or may interact with DSPE-PEG at the liposomal surface. Therefore, PEG/dextran two-phase partition and measurement of the fluorescence quenching of nystatin by *p*-xylene-bis-pyridinium bromide (DPX) were performed to evaluate the surface state of the liposomal membranes in the presence of nystatin.

First, we employed the PEG/dextran two-phase partition method (Table 2). Lipid composition of 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 (Tilcock et al., 1993). Thus, if nystatin-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, Woodle et al., 1992; Moribe et al., 1997). 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 examine the change of the surface state of PEG liposomes caused by encapsulation of nystatin. Four liposomal formulations, DPPC, DPPC/CH (2/1, mol/mol), 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 nystatin, were prepared with average diameters in the range 109–135 nm (Table 1). 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 and at the interface (Table 2). Particle size of these liposomes was not changed before and after the partitioning experiments (Table 1). DPPC liposomes were found mostly at the interface (87.5%), but were partly partitioned to the lower phase (27%) after encapsulation of nystatin. Because of the low encapsulation of nystatin, partitioning behavior of DPPC/CH liposomes was almost same in the presence or absence of nystatin. Enhanced partitioning of DPPC/DSPE-PEG2K and DPPC/CH/DSPE-PEG2K liposomes into the upper phase was observed, but these liposomes were also

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

Lipid composition	Initial molar ratio	Encapsulated amount (μg mg^{-1} lipid)	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/nystatin	2/0.27	150	2.3	1.1	71.0	5.5	27.0	4.4
DPPC/CH	2/1		1.4	0	87.9	1.2	10.7	1.2
DPPC/CH/nystatin	2/1/0.38	13	0.7	0.4	78.6	2.6	20.7	2.3
DPPC/DSPE-PEG 2K	2/0.13		97.3	2.3	1.5	2.5	1.2	2.2
DPPC/DSPE-PEG 2K/nystatin	2/0.13/0.27	151	70.8	8.2	27.8	8.2	1.4	0
DPPC/CH/DSPE-PEG 2K	2/1/0.19		94.4	1.6	3.7	1.5	2.0	0.1
DPPC/CH/DSPE-PEG 2K/nystatin	2/1/0.19/0.38	84	83.1	6.9	9.4	9.3	7.5	2.4
DPPC/CH/DSPE-PEG 2K/nystatin	2/1/0.19/0.76	160	70.0	7.5	28.7	9.4	1.3	1.9

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

Table 3
Fluorescence quenching of nystatin in liposomes by DPX[a]

Lipid composition	Encapsulated amount (μg mg^{-1} lipid)	Stern–Volmer constant[b] K_{sv} (M^{-1})	Correlation coefficient r
DPPC	150	3.8	0.995
DPPC/CH	13	5.0	0.970
DPPC/DSPE-PEG	151	8.2	0.995
DPPC/CH/DSPE-PEG	84	2.7	0.963
DPPC/CH/DSPE-PEG	160	7.5	0.956

[a] Nystatin concentration was 3 μM.
[b] Stern–Volmer plot, $F_0/F = K_{sv}[DPX]+1$.

partly partitioned to the interface or lower phase after encapsulation of nystatin. At this lipid composition, the surface state of DPPC/CH/DSPE-PEG liposomes encapsulating nystatin at 84 μg mg^{-1} lipid did not change significantly. However, partitioning to the interface was observed in the case of higher encapsulation (160 μg mg^{-1} lipid). These results suggested that nystatin molecules were partly localized at the surface region of the liposomal membrane when the encapsulated amount of nystatin was high.

To estimate the surface state of nystatin-encapsulating liposomes quantitatively, fluorescence quenching of nystatin encapsulated in liposomes by DPX was investigated, and the results are summarized in Table 3. DPX has been used as a hydrophilic quencher to evaluate the fusion of liposomes or the leakage of drugs (Ladokhin et al., 1995; Blackwood et al., 1996). Since DPX can not permeate the liposomal membranes, fluorescence quenching of nystatin encapsulated in liposomes by addition of DPX reflects the amount of nystatin at the surface region of the liposomal membranes. The degree of nystatin quenching by DPX was estimated in terms of the Stern–Volmer constant, the order of which would reflect the extent to which nystatin molecules are located on the surface. As shown in Table 3, K_{sv} value of DPPC/CH liposomes was extraordinarily high in spite of the low encapsulated amounts (13 μg mg^{-1} lipid), suggesting that nystatin molecules may be localized on the surface of the liposomal membranes. Incorporation of DSPE-PEG enhanced the K_{sv} value from 3.8 to 8.2 (cp. DPPC and DPPC/DSPE-PEG liposomes) at almost same encapsulated amounts. In the case of DPPC/CH/DSPE-PEG liposomes, a low K_{sv} value (2.7) was observed at low encapsulation of nystatin (84 μg mg^{-1} lipid), whereas a high value 7.5 was seen at a high level of nystatin encapsulation (160 μg mg^{-1} lipid). These results correspond well to those shown in Table 2 and indicate that localization of nystatin molecules in the surface region of DPPC/DSPE-PEG and DPPC/CH/DSPE-PEG liposomes depends on the encapsulated amounts.

3.2. Molecular state of nystatin in liposomes

We have already demonstrated the encapsulation characteristics of nystatin in liposomes (Moribe et al., 1999a). Compared with AmB, nystatin does not readily form a complex with DSPE-PEG and the enhanced encapsulation of nystatin would be attributed to the CH-free liposomal membranes, not to the complex formation with DSPE-PEG (Moribe et al., 1999a,b). Investigation of the molecular localization and states of nystatin in liposomal membranes contributes to clear the mechanism of encapsulation of nystatin in liposomes. Because of the intrinsic fluorescence property of nystatin, the molecular states of nystatin in four kinds of liposomes were estimated by measurements of fluorescence and CD spectra of nystatin in these liposomes.

Fig. 1 shows the change of the fluorescence intensity of nystatin as a function of the encapsu-

lated amount in liposomes. When the nystatin concentration was fixed at 3 μM, the fluorescence intensity decreased with increasing encapsulated amount of nystatin. Fluorescence intensity of nystatin in DPPC/CH and DPPC/CH/DSPE-PEG liposomes at low encapsulated amounts showed almost same value, though the encapsulated amounts of nystatin in DPPC/CH liposomes were very small. Sterol-free, DPPC and DPPC/DSPE-PEG liposomes also showed similar changes of the fluorescence intensity and the changes were larger than that of DPPC/CH/DSPE-PEG liposomes. These results indicate that the fluorescence intensity of nystatin in liposomes is influenced by CH and not by DSPE-PEG.

To interpret the quenching of the nystatin fluorescence in these liposomes two possibilities may be considered, one is self-association or aggregate formation of nystatin in liposomal membranes (Castanho et al., 1995; Milhaud et al., 1997) and the other is molecular interaction with CH (Castanho et al., 1992). As shown in Table 1, we cannot observe the particle size increase by encapsulation of nystatin in every lipid composition. Since nystatin is not encapsulated in DPPC/CH liposomes so much and nystatin fluorescence in DPPC and DPPC/DSPE-PEG liposomes decreased with increasing encapsulated amount of nystatin to the same level as that of DPPC/CH/DSPE-PEG liposomes, the lower fluorescence intensity of nystatin in CPPC/CH and DPPC/CH/DSPE-PEG liposomes can be attributed to the former mechanism.

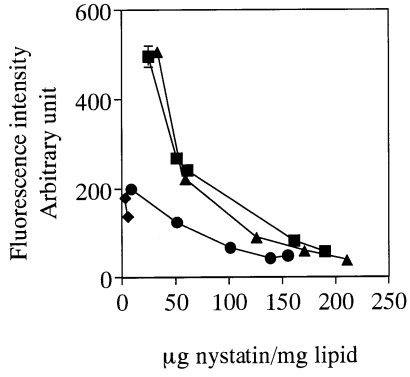

Fig. 1. Change of fluorescence intensity with increasing content of nystatin in liposomes at 30°C. (■) DPPC liposomes; (◆) DPPC/CH liposomes; (▲) DPPC/DSPE-PEG liposomes; (●) DPPC/CH/DSPE-PEG liposomes. Nystatin concentration was adjusted to 3 μM in sucrose solution. The excitation and emission wavelengths of nystatin were 318 and 425 nm, respectively. Values are mean ± S.D. and $n = 3$.

The change of the fluorescence intensity of nystatin has also been investigated by using dimyristoylphosphatidylcholine vesicles at low nystatin-to-lipid molar ratio (< 0.02, Petersen et al., 1987). The quenching behavior of nystatin they reported was not dynamic quenching, but static quenching. Interestingly, the quenching behavior is similar to that of DPPC/CH/DSPE-PEG liposomes, but not to that of sterol-free DPPC or DPPC/DSPE-PEG liposomes. The apparently contradictory results can be attributed to the difference of the encapsulated amount of nystatin. Restriction of the reorientational motion of nystatin monomer, as they mentioned, would also contribute to the quenching behavior of DPPC/CH/DSPE-PEG liposomes.

These results indicate that the localization of nystatin in CH-incorporating membranes could be evaluated on the basis of self-quenching of nystatin fluorescence, and that nystatin molecules were at first delocalized and then self-associated at a higher level of encapsulation in CH-free membranes.

The effect of the encapsulated amount of nystatin on the fluorescence anisotropy in nystatin-encapsulating liposomes is shown in Fig. 2. Decreased anisotropy represents increased nystatin mobility (Castanho et al., 1992). When the temperature of the measurements was 30°C, the fluorescence anisotropy of nystatin in the four kinds of liposomes was almost the same, showing a slight decrease with encapsulated amount of nystatin within the range 0.2–0.25. The decreased anisotropy can be attributed to quenching of the polarization, as a result of fluorescence intensity quenching (Petersen et al., 1987), but the degree of quenching was very little in the four kinds of liposomes at these high encapsulated amounts. Fluorescence anisotropy of nystatin in aqueous solution has also been reported by Castanho et al. (1992). They found that the anisotropy of nystatin in monomeric and aggregated states was 0.05 and 0.28, respectively. From the results, we speculate

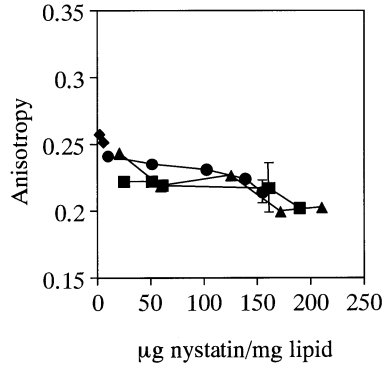

Fig. 2. Change of anisotropy with increasing content of nystatin in liposomes at 30°C. (■) DPPC liposomes; (◆) DPPC/CH liposomes; (▲) DPPC/DSPE-PEG liposomes; (●) DPPC/CH/DSPE-PEG liposomes. Nystatin concentration was adjusted to 3 μM in sucrose solution. The excitation and emission wavelengths were 318 and 425 nm, respectively. The anisotropy was calculated from the fluorescence of nystatin as described in Section 2. Values are mean ± S.D. and $n = 3$.

that the molecular motion of nystatin is restricated by encapsulation in liposomes, though the nystatin encapsulated in the three kinds of liposomes has more freedom of movement than nystatin in the aggregated state in water.

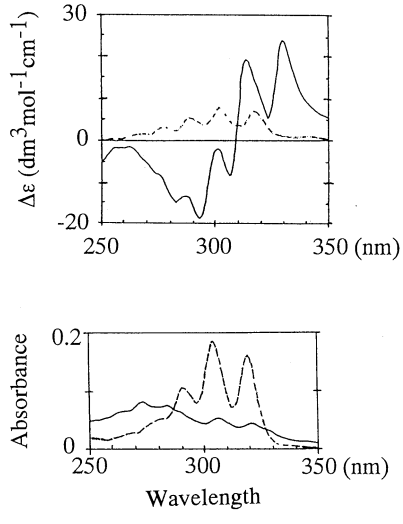

Fig. 3. Circular dichroism and absorption spectra of nystatin in methanol and in 9% sucrose solution. Nystatin concentration of the sample was adjusted to 3 μM in methanol (dashed line) or in 9% sucrose solution (solid line).

Next, we examined the absorption and CD spectra of nystatin in liposomes to evaluate the molecular state of nystatin. Because the CD spectrum of nystatin in phosphate-buffered solution is poorly reproducible, little work has been done in this area (Bolard et al., 1991; Milhaud et al., 1997). Absorption and CD spectra of nystatin in 9% sucrose solution and methanol are shown in Fig. 3. The concentration of nystatin for the CD spectral measurements was fixed at 3 μM, and the absorption and CD spectra of nystatin in water and in sucrose solution were identical (data not shown). In the absorption spectra, three nystatin concentration-dependent peaks at 318, 305 and 290 nm were seen in methanol solution. These can be assigned to the monomeric state of nystatin. In sucrose solution, in addition to the monomeric peaks at 319 and 306 nm, we observed a broad peak at 274 nm, due to the self-associated or aggregated state of nystatin (Petersen et al., 1987; Milhaud et al., 1997; Milhaud and Michels, 1999). In the CD spectra, two positive bands at 330 and 314 nm and three negative bands at 307, 293 and 283 nm in sucrose solution and four positive bands at 318, 302, 289 and 278 nm in methanol solution were observed. Milhaud et al., reported three types of CD spectra of nystatin in phosphate-buffered solution, depending on the concentrations of nystatin and phosphate buffer (Milhaud et al., 1997). The first was a totally negative CD spectrum at concentrations of nystatin lower than 15 μM in 10 mM phosphate buffer solution. Three negative bands at 323, 307 and 296 nm and a positive band at 276 nm were observed at higher concentrations, while three positive bands at 321, 305 and 293 nm and three negative bands at 330, 315 and 276 nm were observed at concentrations above 250 μM in 100 mM phosphate buffer solution (this was assigned to the superaggregated state of nystatin). Though the CD spectra observed by us in sucrose solution were different from those of Milhaud et al., the difference can be attributed to the difference of the solution, i.e. electrolyte phosphate buffer and nonelectrolyte sucrose solution or water.

Absorption and CD spectra of nystatin in liposomes are shown in Fig. 4. In the absorption spectra, the positions of the spectral bands were almost the same (323, 307 and 293 nm) at every

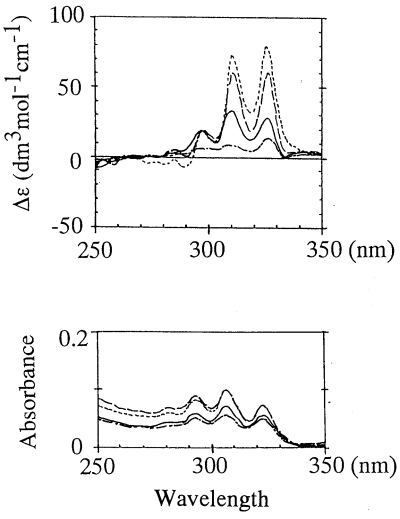

Fig. 4. Circular dichroism and absorption spectra of nystatin-encapsulating liopsomes in 9% sucrose solution. DDPC liposomes (dashed line), DPPC/CH liposomes (dashed and dotted line), DPPC/DSPE-PEG liposomes (dotted line), DPPC/CH/DSPE-PEG liposomes (solid line). Encapsulated amounts of nystatin in DPPC, DPPC/CH, DPPC/DSPE-PEG and DPPC/CH/DSPE-PEG liposomes were 150, 13, 151 and 84 μg mg^{-1} lipid, respectively. Nystatin concentration of the samples was adjusted to 3 μM.

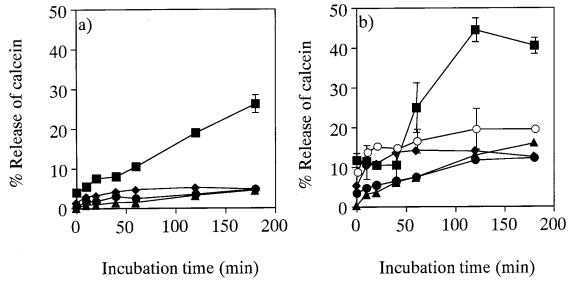

Fig. 5. Calcein release profiles from liposomes in the absence (a); or presence (b) of nystatin after incubation with ddY mouse plasma at 37°C. (■) DPPC liposomes; (◆) DPPC/CH liposomes; (▲) DPPC/DSPE-PEG liposomes; (●) DPPC/CH/DSPE-PEG liposomes. Encapsulated amounts of nystatin in DPPC, DPPC/CH, DPPC/DSPE-PEG and DPPC/CH/DSPE-PEG liposomes were 150, 13, 151 and 84 μg mg^{-1} lipid, respectively. Open circles represent DPPC/CH/DSPE-PEG liposomes with nystatin encapsulation of 160 μg mg^{-1} lipid. Liposomes were prepared as described in Section 2 by hydration with 50 mM calcein/150 mM sucrose/10 mM Hepes buffer (pH 7.4). The amount of calcein released was determined by measuring the fluorescence intensity at 515 nm (excitation at 470 nm) after the lipid concentration was adjusted to 5 μg ml^{-1}. Values are mean ± S.D. and $n = 3$.

lipid composition. Broad and lower absorption spectra were observed in DDPC/CH and DPPC/CH/DSPE-PEG liposomes, which can be attributed to the lower encapsulation of nystatin (13 and 84 μg mg^{-1} lipid). In the CD spectra, three positive bands at 324, 309 and 297 nm were observed at every lipid composition, but the values of $\Delta\varepsilon$ were different. However, the CD spectrum of DPPC/CH/DSPE-PEG liposomes was not changed at high encapsulation of nystatin (160 μg mg^{-1} lipid, data not shown), indicating that the lower absorbance and dichroic absorption coefficient reflect restriction of nystatin mobility, as shown in Figs. 1 and 2. Wavelengths of positive CD bands of nystatin-encapsulating liposomes at 324 or 309 nm were intermediate between those of nystatin in methanol (318 or 302 nm) and in sucrose solution (330 or 314 nm), respectively. Compared with the results reported by Milhaud et al., the CD spectral bands of nystatin in these liposomes were almost the same as those at concentrations above 250 μM in 100 mM phosphate buffer solution, i.e. the superaggregated state of nystatin, except for the negative broad band at 276 nm (Milhaud et al., 1997).

From the results in Figs. 3 and 4, two kinds of molecular states of nystatin, i.e. monomeric and super aggregated, were present in all kinds of liposomes. But further investigation of the molecular states of nystatin in various solutions is required to allow quantitative evaluation of the states in liposomes.

3.3. Liposomal stability and location of nystatin in nystatin-encapsulating liposomes in vitro

Finally, liposomal stability and location of nystatin in nystatin-encapsulating liposomes were studied in terms of calcein and nystatin release from nystatin-encapsulating liposomes. Membrane stability of four kinds of liposomes on incubation with mouse plasma at 37°C was estimated by calcein release and the results are shown in Fig. 5a. DPPC liposomes showed the lowest stability in these lipid compositions. Increased release of calcein by encapsulation of nystatin is thought to reflect the destabilization of bilayer membrane by the incorporation of nystatin in the

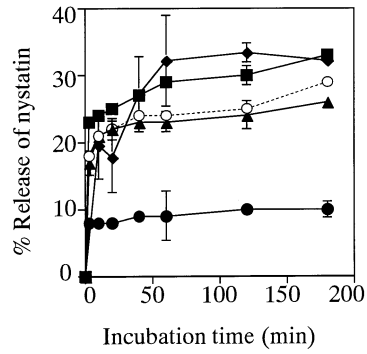

Fig. 6. Nystatin release profiles from nystatin-encapsulating liposomes after incubation with ddY mouse plasma at 37°C. (■) DPPC liposomes; (◆) DPPC/CH liposomes; (▲) DPPC/DSPE-PEG liposomes; (●) DPPC/CH/DSPE-PEG liposomes. Encapsulated amounts of nystatin in DPPC, DPPC/CH, DPPC/DSPE-PEG and DPPC/CH/DSPE-PEG liposomes were 150, 13, 151 and 84 μg mg^{-1} lipid, respectively. Open circles represent DPPC/CH/DSPE-PEG liposomes with nystatin encapsulation of 160 μg mg^{-1} lipid. Nystatin concentration of the samples was adjusted to 1 mg ml^{-1}. Values are mean ± S.D. and $n = 3$.

membrane core. That was observed in every lipid composition and the highest stability of nystatin-encapsulated liposomes was observed in DPPC/DSPE-PEG (151 μg mg^{-1} lipid) and DPPC/CH/DSPE-PEG liposomes (84 μg mg^{-1} lipid) as shown in Fig. 5b. But the higher encapsulation of nystatin in DPPC/CH/DSPE-PEG liposomes (160 μg mg^{-1} lipid) reduced the stability of the membrane.

Fig. 6 shows the nystatin relaease profile from nystatin-containing liposomes in vitro. Burst release of nystatin was observed within 5 min after the start of incubation, and thereafter, nystatin release became very slow at every lipid composition. As shown in Fig. 5b, stability of the liposomal membrane was almost, same, except that of DPPC liposomes, but the release profiles of nystatin were different. Higher level of nystatin release was observed in DPPC/CH and DPPC/DSPE-PEG liposomes, compared with that of calcein release. When the encapsulated amount of nystatin in DPPC/CH/DSPE-PEG liposomes was 84 μg mg^{-1} lipid, the lowest level of nystatin release was observed. When the encapsulated amount increased at 160 μg mg^{-1} lipid, however, higher release of nystatin, the amount of which was almost same to that of DPPC/DSPE-PEG liposomes (151 μg mg^{-1} lipid), was observed. These results suggested that encapsulation of nystatin induced the localization on the surface region of the liposomal membranes, especially in DPPC/DSPE-PEG liposomes. On the contrary, a high release of nystatin from DPPC liposomes with a low level of nystatin on the liposomal surface, as shown in Tables 2 and 3 reflects the low stability of liposomal membrane as shown in Fig. 5b.

From the results, molecular states of nystatin in CH-incorporating liposomes were speculated as follows. Maximum encapsulated amount of nystatin in DPPC/CH liposome was determined as 13 μg mg^{-1} lipid. Assuming that encapsulated amount of nystatin in DPPC/CH/DSPE-PEG liposome is the sum of that of DPPC/CH liposome and amount of nystatin interacted with DSPE-PEG, the calculated maximum encapsulated amount in DPPC/CH/DSPE-PEG-liposome is 78 μg mg^{-1} lipid. When the encapsulated amount of nystatin in DPPC/CH/DSPE-PEG liposome is 84 μg mg^{-1} lipid, which is almost same to the calculated value, the lowest amounts of release profiles of calcein and nystatin were observed as shown in Fig. 5b and Fig. 6. But the amounts of release increased with the higher encapsulation of nystatin (160 μg mg^{-1} lipid), indicating that nystatin would be located both inside and on the liposomal membrane, when the encapsulated amounts exceeded the calculated value.

On the contrary, it is difficult to determine the maximum encapsulated amounts of nystatin in DPPC or DPPC/DSPE-PEG liposomes, because of the enhanced encapsulation of nystatin in sterol-free membranes. Aggregate and micelle formation would occur with the high encapsulation.

In conclusion, possible encapsulation mechanisms of nystatin in liposomes narrowed down to the following three points; interaction with lipid membrane, adsorption on the liposomal surface and complex formation with DSPE-PEG. As for the interaction with lipid membrane, nystatin molecules are self-associated and incorporated in lipid membrane by hydrophobic interaction (Castanho et al., 1992; Castanho et al., 1995; Milhaud

and Michels, 1999; Milhaud et al., 1997). Mechanisms of localization of nystatin in the surface region of the liposomal membrane include adsorption or complex formation with DSPE-PEG. We have already demonstrated nystatin binding to pre-formed liposomes (Moribe et al., 1999a). Saturable binding was observed in DPPC and DPPC/DSPE-PEG liposomes and linearly increasing binding in DPPC/CH/DSPE-PEG liposomes. Since small amounts of nystatin molecules are located at the surface of DPPC liposomes, as shown in Table 3, adsorption of nystatin on the liposomal membrane may occur independently of lipid composition. Incorporation of DSPE-PEG, which forms complex with nystatin, also contributes to the adsorption of nystatin on the membrane surface and the resulting high encapsulation as shown in Table 3. In terms of the stability and retention of nystatin in liposomes, DPPC/CH/DSPE-PEG with relatively low level of encapsulated amounts of nystatin would be the optimum lipid composition.

These encapsulation characteristics and the mechanism of encapsulation are apparently different from those of AmB (Moribe et al., 1998, 1999a). For example, AmB is more easily interacted with DSPE-PEG and the complex formation enhances the encapsulation in liposomes. On the contrary, nystatin is more easily incorporated in CH-free liposomal membrane in the presence or absence of DSPE-PEG. These physicochemical properties may influence on the different toxicity and therapeutic efficacy of both drugs in practical uses.

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