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ORIGINAL CONTRIBUTION

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Effect of cholesterol or phospholipids incorporation on vesicle formation of muramyldipeptide derivative B30-MDP

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Summary The muramyldipeptide derivative B30-MDP has immunoadjuvant activity and vesicle-forming ability in aqueous solutions. To assist in the clinical application of B30-MDP to liposomal vaccine, we investigated the physicochemical properties including membrane fluidity, surface charge and particle size of B30-MDP vesicles containing cholesterol, dipalmitoylphosphatidylcholine (DPPC) or dipalmitoylphosphatidylglycerol (DPPG).

The membrane fluidity of B30-MDP/cholesterol vesicles was slightly influenced by cholesterol concentration and temperature. The membrane fluidity of B30-MDP/phospholipid vesicle was dependent on temperature. ESR spectra clearly showed the good miscibility of cholesterol with B30-MDP and the occurrence of phase separation between B30-MDP and phospholipid.

The surface charge and particle size of B30-MDP/cholesterol vesicles were hardly influenced by cholesterol concentration in the membrane because the membrane surface was covered with the hydrophilic region of B30-MDP. The effect of this hydrophilic region of B30-MDP on the surface charge and particle size of B30-MDP/phospholipid vesicle was greater than that of phospholipid.

This study showed that the membrane structure of B30-MDP/cholesterol vesicle differed from that of B30-MDP/phospholipid vesicle. Further, the hydrophilic region of B30-MDP is considered to play an important role in the physicochemical properties and formation of the vesicle.

Key words Cholesterol – membrane fluidity – muramyldipeptide – phospholipid – vesicle

Introduction

The muramyldipeptide derivative 6-O-(2-tetradecylhexadecanoyl)-N-acetylmuramyl-L-alanyl-D-isoglutamine (B30-MDP) was synthesized to reduce the toxicity and improve the immunoadjuvant activity of muramyldipeptide [1, 2]. B30-MDP is an amphipathic compound which has double alkyl chains consisting of 14 carbons as the lipophilic region and an MDP containing a carboxylic group as the hydrophilic region. B30-MDP therefore forms vesicles by itself in aqueous solutions [3]. These characteristics suggested that B30-MDP would be useful in the development of improved high-immunogenicity vaccines. Experimentally, Nerome et al. [4] reported that the formation of liposomes with B30-MDP containing influenza hemagglutin and neuraminidase antigens enhanced the level and persistence of circulating antibody and

cellular immunity in guinea-pigs and mice. However, clinical use of B30-MDP as an adjuvant for liposomal vaccines requires the evaluation of its physicochemical properties, including membrane fluidity, surface charge and vesicle size [5–9].

We previously reported the physicochemical properties of B30-MDP membrane obtained using polarizing optical microscopy, differential scanning calorimetry and electron spin resonance (ESR), and described the relationship between its physicochemical properties and chemical stability of B30-MDP in vesicle formation [10]. In the present study, we evaluated the effect of cholesterol or phospholipids incorporation on the physicochemical properties and vesicle formation of B30-MDP using the ESR [11], zeta-potential [12] and quasi-elastic laser light scattering (QELS) methods [13].

Materials and methods

Chemicals

Muramyldipeptide derivative, [6-O-(2-tetradecylhexadecanoyl)-N-acetylmuramyl-L-alanyl-D-isoglutamine: B30-MDP], synthesized by Daiichi Pharmaceutical Co., Ltd. (purity not less than 99%, Tokyo, Japan), was used [1]. Dipalmitoylphosphatidylcholine (DPPC) and dipalmitoylphosphatidylglycerol (DPPG) (purity not less than 99%; Nichiyu Liposome Co., Ltd., Tokyo, Japan) were used without further purification. Cholesterol was obtained from Sigma Chemical Co. (purity not less than 99%, MO, U.S.A.). The spin labeled reagents 5-doxyl-stearic acid (5NS), 12-doxyl-stearic acid (12NS), and 2,2,6,6,-tetramethylpiperidine-N-oxyl (TEMPO) were obtained from Aldrich Chemical Co., Ltd. (Wisconsin., U.S.A.). Octyl-β-D-glucoside (OG) (purity not less than 97%; Dojindo Laboratories, Kumamoto, Japan) was used without further purification. All other chemicals used were of the highest grade commercially available.

Preparation of vesicle samples

Vesicle samples for ESR and zeta potential measurement were prepared by the Bangham method [14]. Briefly, B30-MDP, cholesterol and phospholipids were dissolved separately in chloroform and the solutions were mixed in a 20-ml round-bottomed flask. Cholesterol content ranged from 0% to 75% (molar basis) and that of each phospholipid from 0% to 100% (molar basis) against B30-MDP. In addition, the content of spin probes 5NS and 12NS was set at 0.5% (molar basis) of the total, where necessary. The sample solutions were evaporated to dryness and the films were hydrated at a temperature above 323 K with phosphate-buffered saline (PBS) and sonicated with an ultrasonic cleaner (Model 5200; Branson Ultrasonics Japan) for 30 min to make multi-lamella vesicles (average diameter 300–400 nm). The prepared vesicles were then filtrated using a membrane filter (Millipore filter, pore size 0.45 μm) and PBS was added to bring the total component concentration to 10 mmol/dm^3. For vesicles for experiments using TEMPO, TEMPO solution (10 mmol/dm^3 in PBS) was added and adjusted to a final concentration of 30 μmol/dm^3. Total component concentration was adjusted to 10 mmol/dm^3.

Vesicle samples for particle size measurement were prepared by the detergent removal method [15]. The lipid film was solubilized with PBS containing OG 10% (w/v). Total component concentration was adjusted to 100 mmol/dm^3. The sample solutions were packed into seamless cellulose tubing and dialyzed against several changes of 2000 volumes of PBS with stirring several times to remove OG.

Electron spin resonance (ESR) measurement

ESR spectra were recorded at the X-band as described previously [3]. ESR measurement was performed at 298, 310.5 and 323 K. The outer hyperfine splitting constant, Amax, was obtained from the separation 2Amax between the low-field maximum and the high-field minimum peaks in experiments using 5NS and 12NS, and TEMPO parameter was calculated from the measured high field nitroxide hyperfine line as described by McConnell et al. [11].

Zeta potential measurement

The electrophoretic mobilities of the vesicles in PBS were measured with a electrophoretic light scattering spectrophotometer (model ELS-800 He-Ne laser 10 mW; Otsuka Electronics Co., Ltd., Japan) at 298 K. The zeta potential was then obtained by the Smoluchowski equation [12].

Particle size measurement

The particle size of the vesicles was measured by the QELS method with a dynamic light scattering spectrophotometer (model DLS-700 Ar laser 75 mW; Otsuka Electronics Co., Ltd., Japan) at 293 K. Average diameter was measured by scattered intensity, and the distribution of particle size was determined using histograms [13].

Results and discussion

ESR measurement

ESR measurements were performed to determine the membrane fluidity of B30-MDP with cholesterol, DPPC or DPPG. Membrane fluidity was expressed by the Amax and TEMPO parameters [11]: an increase in fluidity was signified by a decrease in Amax or an increase in TEMPO parameter. The average diameter of the vesicles used in ESR measurements was 300–400 nm.

Changes in membrane fluidity measured with 5NS

The fluidity of the vesicle membranes was measured with 5NS, in which the nitroxide monitoring group is positioned near the membrane surface. Figure 1 shows the Amax of 5NS in B30-MDP membranes with cholesterol, DPPC or DPPG at 298, 310.5 and 323 K.

In the case of B30-MDP/cholesterol membrane, fluidity of the membrane was slightly decreased with increasing concentration of cholesterol at 298, 310.5 and 323 K. When DPPC was added to B30-MDP membrane, fluidity of the membrane was unchanged with increasing concentration of DPPC at 298 K. At 310.5 K, the fluidity of B30-MDP/DPPC membrane was higher than that of both DPPC and B30-MDP lone membrane. At 323 K, the fluidity of B30-MDP/DPPC membrane was increased at the DPPC concentration of 25% and above. DPPC lone membrane has a gel-to-sub gel phase transition (so-called pretransition) temperature of about 308 K and a sub gel-to-liquid crystalline phase transition (so-called main transition) temperature of about 315 K [16, 17]. Changes in B30-MDP/DPPC membrane fluidity were clearly related to that in phase of DPPC lone membrane, namely gel, sub gel and liquid crystalline phase. The behavior of the B30-MDP/DPPC membrane was closely similar to that of B30-MDP/DPPG membrane.

Miscibility of B30-MDP/cholesterol and B30-MDP/phospholipid membrane was evaluated from ESR spectra of 5NS. Figure 2 shows typical ESR spectra of 5NS in B30-MDP membranes with cholesterol or DPPG at 323 K. When homogeneous distribution occurs in a binary system membrane, the ESR spectra obtained from the spin probe in the membrane indicates the unchanged form, not peak width, at various proportions of B30-MDP in the system. However, if phase separation of the membrane occurs, the ESR spectra is observed by the composite of the two individual signals, and two separated signals appear in the lower and higher magnetic field of the spectra. This is because the distribution of spin probe differs between the two component parts in the membrane.

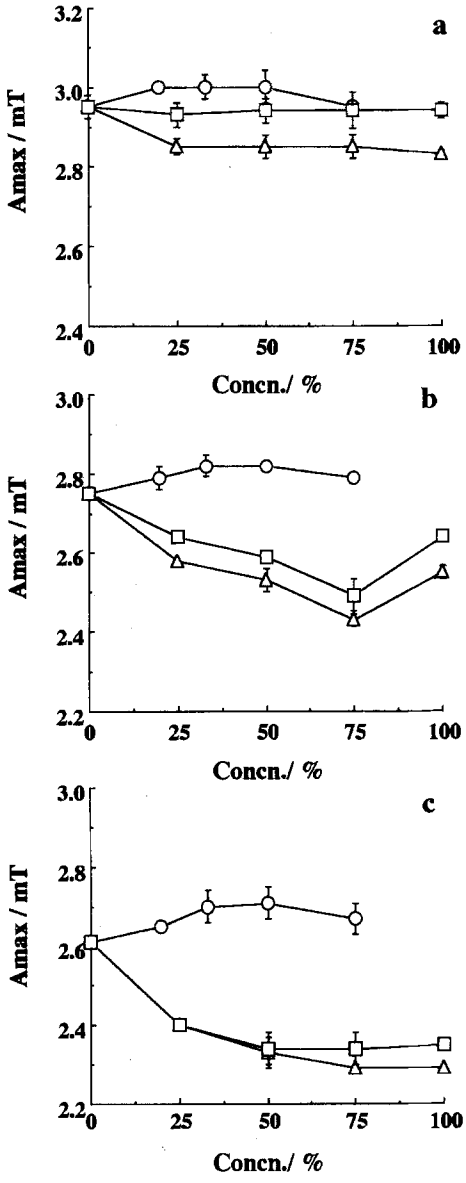

Fig. 1 Relationship between concentration of cholesterol, DPPC or DPPG and Amax value for 5NS in the membranes at various temperatures. ○: cholesterol, □: DPPC, △: DPPG, a: 298 K, b: 310.5 K, c: 323 K Vertical bars denote SD for a series of three separate determinations

The ESR spectra B30-MDP/cholesterol membranes were similar to that of the B30-MDP alone membrane (Fig. 2a–d). These results indicate that cholesterol had good miscibility with B30-MDP in membrane formation, and was probably distributed homogeneously in the B30-MDP membrane.

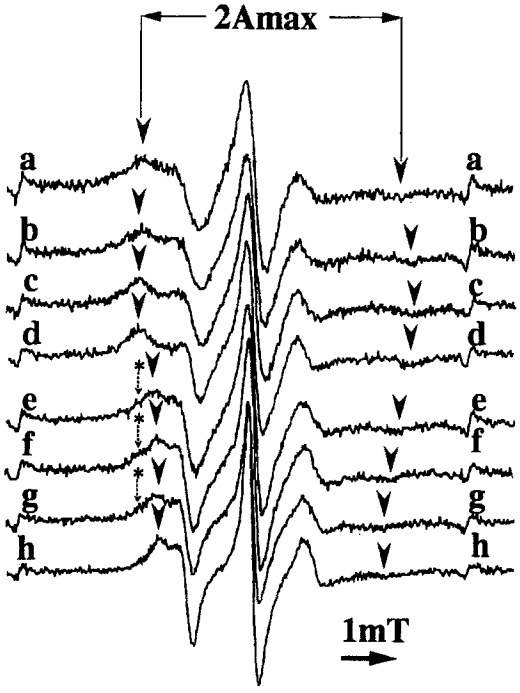

Fig. 2 Typical ESR spectra of 5NS in B30-MDP/cholesterol or B30-MDP/DPPG vesicles at 323 K. a: B30-MDP lone vesicle, b: 33% of cholesterol, c: 50% of cholesterol, d: 75% of cholesterol, e: 25% of DPPG, f: 50% of DPPG, g: 75% of DPPG, h: DPPG lone vesicle. The solid arrows indicate the low-field maximum and the high-field minimum peaks. The dotted arrows with star symbols indicate the low-field maximum peak corresponding to B30-MDP

On the other hand, the anisotropy of the spectra decreased with increasing concentration of DPPG in the membrane, and the appearance of two separated signals in the lower magnetic field was observed in the ESR spectra of B30-MDP/DPPG membranes (Fig. 2e–g). In addition, although the two separated signals should have been observed at in higher magnetic field in the spectra, this was not measurable owing to low sensitivity. The Amax values of B30-MDP/DPPG membrane decreased with increasing DPPG concentration, whereas the positions of the peaks indicated by star symbols did not move with increasing DPPG concentration but were the same as that of the peak of the lower magnetic field obtained from B30-MDP lone membrane. It was considered that the peaks indicated by star symbols originated from B30-MDP cluster formation in the B30-MDP/DPPG membrane, and thus the phase separation between B30-MDP and DPPG occurred in the membrane. The change in the ESR spectra caused by the addition of DPPC to B30-MDP membrane was similar to that by DPPG (data not shown). It was considered that phase separation between B30-MDP and DPPC also occurred in the B30-MDP/DPPC membrane.

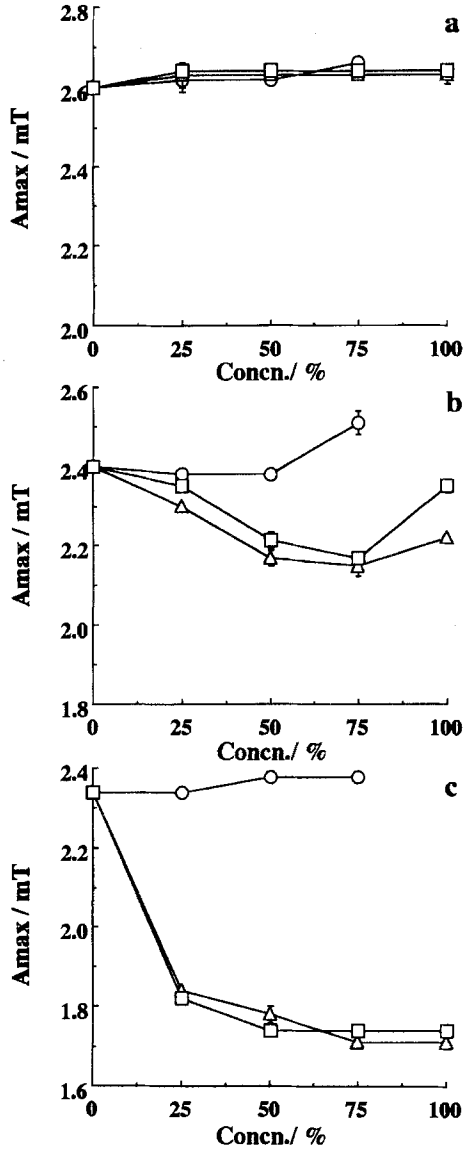

Fig. 3 Relationship between concentration of cholesterol, DPPC or DPPG and Amax value for 12NS in the membrane at various temperatures. ○: cholesterol, □: DPPC, △: DPPG, a: 298 K, b: 310.5 K, c: 323 K Vertical bars denote SD for a series of three separate determinations

Changes in membrane fluidity measured with 12NS

The fluidity of the membranes was measured with 12NS, in which the nitroxide monitoring group is positioned near the central region of the alkyl chain of the lipid bilayer. Amax of 12NS in B30-MDP membrane with cholesterol, DPPC or DPPG at 298, 310.5 and 323 K were shown in Fig. 3. The results indicated that the membrane fluidity of the central region agreed with the results mentioned for 5NS above. In particular, membrane fluidity was clearly changed by the addition of phospholipids at 323 K.

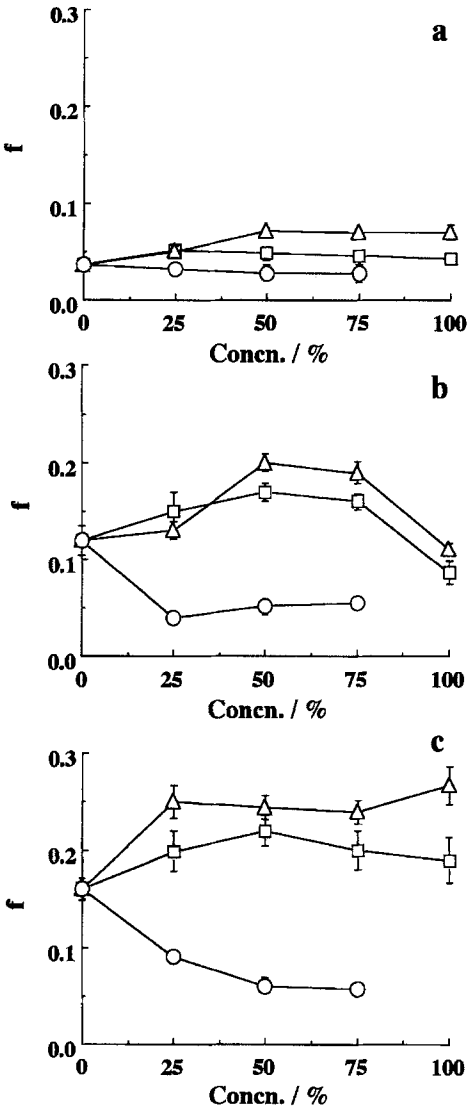

Fig. 4 Relationship between concentration of cholesterol, DPPC or DPPG and TEMPO parameter (f) in the membrane at various temperatures. ○: cholesterol, □: DPPC, △: DPPG, a: 298 K, b: 310.5 K, c: 323 K Vertical bars denote SD for a series of three separate determinations

Changes in membrane fluidity measured with TEMPO

TEMPO penetrates from outer aqueous phase to the membrane, the fluidity of which can be expressed as the TEMPO parameter. Results for B30-MDP with cholesterol, DPPC or DPPG at 298, 310.5 and 323 K by this parameter were shown in Fig. 4. Cholesterol enhanced the rigidity of B30-MDP membranes. It was considered that cholesterol was well miscible with B30-MDP and that the lateral diffusion rates of B30-MDP were reduced by hydrophobic incorporation with cholesterol. The changes in fluidity of B30-MDP membrane with phospholipids agreed with the results with 5NS and 12NS. In particular, it was likely that the fluidity of B30-MDP/phospholipid membrane at 310.5 K was more disordered than that of B30-MDP or phospholipid lone membrane.

Effect of cholesterol incorporation on the membrane fluidity of B30-MDP

In a previous paper, we reported that B30-MDP membrane occurs in a liquid crystalline phase at temperatures above 278 K, and that membrane fluidity at the liquid crystalline phase is initially almost as low as that of phospholipids at gel phase [3]. Further, we assumed that the low fluidity of B30-MDP membrane at the liquid crystalline phase was caused by the stabilizing effect of hydrogen-bonding on the hydrophilic region of B30-MDP [3]. Accordingly, the fluidity of B30-MDP/cholesterol membrane was slightly decreased by increasing the concentration of cholesterol (Figs. 1–4), although it is well known that cholesterol clearly reduces the lateral diffusion rates of phospholipids at the liquid crystalline phase [16–18]. It was considered that the decrease of membrane fluidity by increasing the concentration of cholesterol was due to a cooperative phenomenon of the stabilizing effect of hydrogen-bonding on the hydrophilic region of B30-MDP and the hydrophobic interaction of cholesterol. On the other hand, the homogeneous distribution of cholesterol in B30-MDP membrane shown in the ESR spectra (Figs. 2b–d) suggest that, if the stabilizing effect of the bulky hydrophilic region in B30-MDP is destroyed by the presence of a large amount of cholesterol in the membrane, the fluidity of B30-MDP/cholesterol membrane is reduced by the hydrophobic interaction of cholesterol.

Effect of phospholipids incorporation on the membrane fluidity of B30-MDP

The effects of DPPC and DPPG incorporation on the fluidity of B30-MDP were closely similar. The changes in B30-MDP/phospholipid membrane fluidity did not relate to the difference between DPPC and DPPG in surface charge or structure, but rather depended on experimental temperature (Figs. 1, 3, 4). Thus, B30-MDP/phospholipid membrane fluidity related to the phase of phospholipid lone membrane, namely gel, sub gel and liquid crystalline phase. The ESR spectra of 5NS in B30-MDP/phospholipid membranes indicated that phase separation occurred between B30-MDP and phospholipid (Fig. 2e–g). These data agreed with our previous report in which phase

separation between B30-MDP and phospholipid was determined using differential scanning calorimeter [3].

At the liquid crystalline phase temperature range of the phospholipids lone membrane, the fluidity of B30-MDP/phospholipid membrane was increased at the phospholipid concentration of 25% or more (Figs. 1c, 3c, 4c). Regarding the addition of phospholipid to B30-MDP membrane, it was considered that membrane fluidity was increased with decreasing a network of stabilizing effect of MDP region [3]. Because the stabilizing effect of the MDP region on membrane fluidity was retained, phase separation occurred between B30-MDP and phospholipid in the membrane, and the cluster of B30-MDP did not affect the fluidity of the whole membrane.

Figures 1b, 3b and 4b indicate the presence of maximum fluidity value versus phospholipid concentration was observed at the sub gel phase temperature range of the phospholipids lone membrane. B30-MDP membrane was disordered by the addition of phospholipid, and phospholipid membrane was disordered by the addition of B30-MDP. These results suggest that the stabilizing effect of MDP region at the membrane surface was disordered by the presence of phospholipid, and that the hydrophobic interaction of phospholipid at sub gel phase was disturbed by the presence of B30-MDP. We consider that, if the stabilizing effect of the surface structure in B30-MDP lone membrane is lost, the flexibility of the alkyl chain of B30-MDP at liquid crystalline phase will be higher than that of phospholipid at sub gel phase.

At the gel phase temperature range of the phospholipid lone membrane, the fluidity of B30-MDP/phospholipid membrane was unchanged with increasing phospholipid concentration. The fluidity of B30-MDP lone membrane and phospholipid lone membrane was closely similar. The fluidity of B30-MDP membrane was stabilized by the effect of hydrogen-bonding on the hydrophilic region of B30-MDP, and that of phospholipid membrane is stabilized by the hydrophobic interaction of alkyl chains of phospholipid at gel phase. Accordingly, it was considered that the alkyl chain flexibility of the B30-MDP/phospholipid membrane was stabilized by the hydrophobic interaction of phospholipid at gel phase, while the stabilizing effect of the MDP region on membrane surface fluidity was disturbed by the presence of phospholipid.

Zeta potential and particle size measurements

Zeta potential measurement

Zeta potential measurement provides direct information about the B30-MDP vesicle surface charge and allows estimation of how the B30-MDP vesicle surface is effect of these on the zeta potential of B30-MDP membrane at 298 K. That of B30-MDP lone vesicle was about −60 mV. This result indicates that zeta potential is influenced by the surface distribution of the dissociated carboxylic group in the MDP region of B30-MDP in the vesicle. The addition of cholesterol to B30-MDP vesicle caused almost no increase in zeta potential. The surface charge of a neutral phospholipid: DPPC lone vesicle was about 0 mV, while that of an acidic phospholipid: DPPG lone membrane was about −40 mV (Fig. 5). The maximum error in this measurement was about 0.9 mV. Although the surface charge of both phospholipids lone membranes greatly differed from that of the B30-MDP lone membrane, the magnitude of changes in the surface charge of B30-MDP/phospholipid vesicles did not increase in a phospholipid concentration-dependent manner. In addition, the surface charge of 75% cholesterol-containing membrane was lower than that of 75% DPPG-containing membrane (Fig. 5).

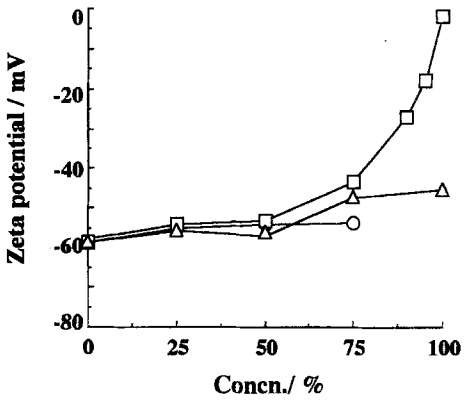

Fig. 5 Relationship between concentration of cholesterol, DPPC or DPPG and zeta potential on the membrane surface at 298 K. ○: cholesterol, □: DPPC, △: DPPG

The results of ESR measurements indicated that cholesterol has good miscibility with B30-MDP in membrane formation and reduce the lateral diffusion rates of the alkyl chain order of B30-MDP in the membrane. It was therefore considered that the carboxylic group in the MDP region approaches the outer region of the B30-MDP/cholesterol vesicle surface while cholesterol was located in the alkyl chain region of B30-MDP in the membrane. Thus, the membrane surface of B30-MDP/cholesterol vesicle was covered by the MDP region and the surface charge of the membrane was influenced by the carboxylic group in the MDP region.

In the case of B30-MDP/phospholipid membrane, the charge density arising of the carboxylic group in the MDP region in the membrane was decreased by the presence of phospholipid in the membrane surface; however,

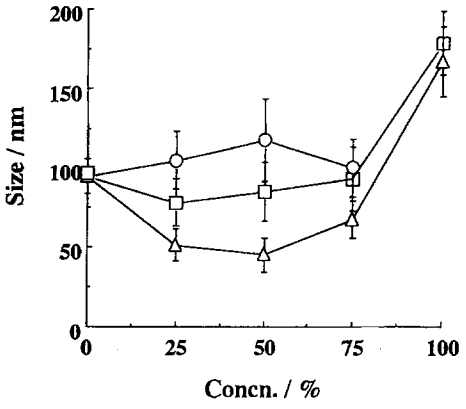

Fig. 6 Relationship between concentration of cholesterol, DPPC or DPPG and particle size of the vesicles at 293 K. ○: cholesterol, □: DPPC, △: DPPG Vertical bars denote SD for a series of three separate determinations

changes in the surface charge of B30-MDP/phospholipid membrane was observed when phospholipid at 75% or above was added to the membrane. The MDP region is bigger than the hydrophilic region of phospholipid. It was speculated that the carboxylic group in the bulky MDP region approaches the outer region of the B30-MDP/phospholipid vesicle surface, and the effect of B30-MDP on the zeta potential is greater than that of phospholipid. In addition, the effect of phase separation between B30-MDP and phospholipid in the membrane on the surface charge was not clarified.

Particle size measurement

In view of the low stability of antigens against high temperature and organic solvents, a detergent removal method was used to manufacture B30-MDP liposomal vaccine [15, 19, 20]. The effect of the addition of cholesterol, DPPC or DPPG on the particle size of B30-MDP vesicles prepared by the detergent removal method was shown in Fig. 6.

The size of B30-MDP/cholesterol vesicles slightly increased with increasing cholesterol concentration in the vesicle. This effect of cholesterol incorporation (Fig. 6) can be explained by the experimental fact that cholesterol slightly enhances the rigidity of bilayer membranes at room temperature, as described above, and slightly inhibits the curving of the bilayers [21].

Regarding B30-MDP/DPPC vesicles, particle size was hardly changed by increases in DPPC concentration below 75%, but increased at concentrations of 75% and above. Thus, the particle size of DPPC vesicles was larger than that of B30-MDP/DPPC vesicles. When DPPG was added to B30-MDP, particle size first decreased with increasing DPPG concentration and then gradually increased. The size of the B30-MDP/DPPG vesicles was smaller than that of both the B30-MDP and DPPG alone vesicles (Fig. 6).

Jiskoot et al. [22] reported that the incorporation of charged compounds reduces particle size according to the increase in electrostatic repulsion force at the vesicle surface. They noted that the curvature of the vesicle is stabilized by the electrostatic repulsion force, because the charged hydrophilic regions are further separated from each other at a high degree of curvature.

The particle size of the DPPC vesicles was therefore larger than that of B30-MDP/DPPC vesicles, while B30-MDP/DPPG vesicles were smaller than both the B30-MDP and DPPG lone vesicles.

The results of ESR measurements indicated that the low fluidity of B30-MDP membrane at the liquid crystalline phase was caused by the stabilizing effect of hydrogen-bonding on the hydrophilic region of B30-MDP [3], and that phase separation occurred in the B30-MDP/phospholipid membrane. Furthermore, the effect of the carboxylic group in the MDP region of B30-MDP on the B30-MDP/phospholipid membrane affected the surface charge and particle size of the membrane. It was apparent that the hydrophilic region of B30-MDP played an important role in B30-MDP/phospholipid vesicle formation.

In addition, the average diameter of B30-MDP/cholesterol and B30-MDP/phospholipid vesicles ranged from 50 to 130 nm. A relationship was seen between particle size and the amount of cholesterol or phospholipid added; we therefore presumed that the particle size of B30-MDP vesicles in the detergent removal method can be controlled not only by the dilution rate [23–25] but also by incorporation with phospholipids or cholesterol.

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References

1. Kusumoto S, Inage M, Shiba T, Azuma I, Yamamura Y (1978) Tetrahedron Lett 49:4899–4902
2. Adam A, Lederer E (1984) Med Res Rev 4:111–152
3. Ando S, Tsuge H, Miwa A (1994) Colloid Polym Sci 272:1281–1288
4. Nerome K, Yoshioka Y, Ishida M, Oka T, Kataoka T, Inoue A, Oya A (1990) Vaccine 8:503–509
5. Davis D, Gregoriadis G (1987) Immunology 61:229–234
6. Bakouche Q, David F, Gerlier D (1987) Eur J Immunol 17:1839–1842

7. Kinsky SC, Yasuda T, Tadakuma T (1982) J Biol Res Mod 8:308–310
8. Latif NA, Bachhawat BK (1987) Immunol Lett 15:45–51
9. Francis MJ, Fry CM, Rowlands DJ, Brown F, Bittle JL, Houghten RA, Lerner RA (1985) J Gen Virol 66: 2347–2354
10. Ando S, Tsuge H, Miwa A (1995) Colloid Polym Sci 273:399–404
11. McConnell HM, Wright KL, McFarland BG (1972) Biochim Biophys Res Commun 47:273–281
12. Tatulian SA (1983) Biochim Biophys Acta 736:189–195
13. Eshuis A, Harbers G, Doornink DJ, Mijnlieff PF (1985) Langmuir 1:289–294
14. Bangham AD, Standish MM, Watkins JC (1965) J Mol Biol 13:238–244
15. Zumbuehl O, Weder HG (1981) Biochim Biophys Acta 640:252–262
16. Mabrey S (1981) In: Knight CG (ed) Liposomes: From physical structure to therapeutic applications. Elsevier, Amsterdam, pp 128–131
17. Lasic DD (1993) In: Lasic DD (ed) Liposomes from Physics to Applications. Elsevier, Amsterdam, pp 33–34
18. Shimshich EJ, McConnell HM (1973) Biochim Biophys Res Commun 53:446–451
19. Webster RG, Brown LE, Jackson DC (1983) Virology 126:587–599
20. Yoden S, Kida H, Kuwabara M, Yanagawa R, Webster RG (1986) Virus Res 4:251–261
21. Poznanski MJ, Juliano RL (1984) Pharmacol Rev 36:277–336
22. Jiskoot W, Teerlink T, Beuvery EC, Crommelin DJA (1986) Pharm Weekbl Sci 8:259–265
23. Schwender RA, Asanger M, Weder HG (1981) Biochim Biophys Res Commun 100:1055–1062
24. Zumbuehl O, Weder HG (1981) Biochim Biophys Acta 640:252–262
25. Ueno M, Tanford C, Reynolds JA (1984) Biochemistry 23:3070–3076