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ORIGINAL CONTRIBUTION

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Relationship between physicochemical properties and chemical stability of muramyldipeptide derivative B30-MDP in liposomal solutions

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Abstract The muramyldipeptide derivative B30-MDP has immunoadjuvant activity and vesicle-forming ability in aqueous environments. It is therefore important to evaluate the relationship between its physicochemical properties and chemical stability for use as a vaccine adjuvant. We studied the effects of octyl-β-D-glucoside (O.G.) incorporation on the physicochemical properties and chemical stability in aqueous solution at pH 7.4. The changes in particle size and in the membrane fluidity of B30-MDP liposomes, which were induced by the addition of O.G., were measured to confirm the transition from micelle phase to vesicle phase. The degradation of B30-MDP in both liposomal and mixed micellar solutions was measured by reverse-phase high-performance liquid chromatography. This degradation occurred by a pseudo first-order reaction at 313, 323 and 333 K. The shelf-life of the B30-MDP solution supplemented with O.G. was approximately one-seventh of that of B30-MDP alone in the liposomal solution. The changes in the k_{obs} values of B30-MDP correlated well with those in membrane fluidity induced by O.G. incorporation. These results indicate that an increase in membrane fluidity labilizes B30-MDP in liposomal solution.

Key words B30-MDP – Fluidity – Liposome – Octyl-β-D-glucoside – Stability

Introduction

The muramyldipeptide derivative 6-O-(2-tetradecylhexadecanoyl)-N-acetyl-muramyl-L-alanyl-D-isoglutamine (B30-MDP) was synthesized to reduce the toxicity and improve the immunoadjuvant activity of muramyldipeptide [1, 2]. Additionally, in an aqueous environment, B30-MDP has amphipathic properties in vesicle formation, as has a phospholipid [3]. It therefore appeared that B30-MDP would be very useful in the development of improved high immunogenicity vaccines. In practice, Nerome et al. [4] reported that the formation of liposomes with B30-MDP containing influenza hemagglutin and neuraminidase (HANA) antigens enhanced the level and persistence of circulating antibody and cellular immunity in guinea-pigs and mice.

If B30-MDP is to be used in practice as an adjuvant for a liposomal vaccine, the prediction of its chemical stability in liposomal solution is important. It is also necessary to carefully monitor the stability of B30-MDP during liposome manufacture. In view of the generally low stability of antigens, a detergent removal method was used for the manufacturing of B30-MDP liposomal vaccine [5]. Octyl-β-D-glucoside (O.G.) has been chosen as the detergent for this method because O.G. has apparently mild

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affinity for proteins and can be easily removed from a solution containing solubilized antigens and lipids by dialysis [6, 7].

In a previous paper [3], we reported the physicochemical properties of B30-MDP in relation to membrane formation, using polarizing optical microscopy, differential scanning calorimetry and electron spin resonance spectroscopy. The physicochemical characteristics of the gel-to-liquid crystalline phase transition and the fluidity of B30-MDP membrane were clarified.

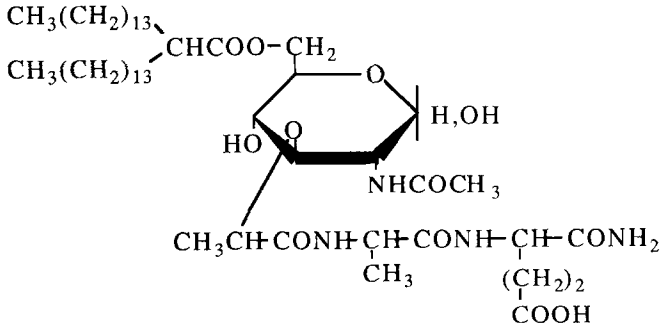
In the present paper, we evaluated the changes in the physicochemical properties of B30-MDP liposome that were induced by the incorporation of O.G., and studied the effect of these changes on the chemical stability of B30-MDP. First, we evaluated the physicochemical effects on the vesicle-forming process from B30-MDP-O.G. mixed micellar solutions. The particle size and membrane fluidity of the liposomal and mixed micellar solution of B30-MDP were measured by the quasi-elastic laser light scattering (QELS) method and the electron spin resonance (ESR) method, and the chemical stability of the liposomal and mixed micellar solutions of B30-MDP was then determined by high-performance liquid chromatography (HPLC). We next evaluated the relationship between the physicochemical properties and the degradation kinetics of B30-MDP.

Experimental

Materials

B30-MDP synthesized by Daiichi Pharmaceutical Co., Ltd. (Tokyo, Japan), was used in the present study. Its chemical structure is shown in Fig. 1. Octyl-β-D-glucoside (O.G.) (97%, Dojindo Laboratories, Kumamoto, Japan) was used without further purification. The spin probe, 5-doxyl-stearic acid (5NS), was obtained from Aldrich Chemical Co., Ltd. (Wl., U.S.A).

Fig. 1 Chemical structure of B30-MDP



Buffer solutions

The 0.05 M phosphate buffers were prepared by dissolving potassium dihydrogen phosphate in water and adjusting the pH to 7.4 with concentrated sodium hydroxide. The ionic strength of the phosphate buffers was adjusted with sodium chloride to remain in the range 0.05 to 0.5.

Particle size

The particle size of the liposomal and mixed micelle solution of B30-MDP was measured by the QELS method with a dynamic light-scattering spectrophotometer (Otsuka Electronics Co., Ltd., model DLS-700 Ar, Japan). The average diameter measured by scattered intensity and the distribution of particle sizes were determined using histograms [8].

ESR method

Spin-labeled B30-MDP solutions were prepared as follows. B30-MDP and 5NS were dissolved separately in chloroform, and these solutions were mixed in a 20-mL round bottomed flask, giving a spin probe content of approximately 0.5% of that of B30-MDP on a molar basis. This solution was evaporated to dryness. An accurately weighed quantity of the lipid film of B30-MDP was hydrated at room temperature with an appropriate buffer solution, and was sonicated with a ultrasonic cleaner (Branson Ultrasonics, model 5200, Japan) for 20 min to make the liposomal or mixed micellar solution.

ESR spectra were recorded as described previously [3]. The experimental temperature was set at 323 K. Order parameter was calculated from measured $T_{||}$ and $T_{\perp}$ in experiments using 5NS as reported by McConnell et al. [9].

Kinetic measurements

The sample solutions were prepared as the same manner as for ESR, but the solution did not contain 5NS. Aliquots of the sample solutions were put into 2 ml ampules in a nitrogen atmosphere and sealed, and were then allowed to stand in constant temperature cabinets, which were set at 313, 323, and 333 K (accuracy $\pm$ 0.3 K).

HPLC Method

A high-performance liquid chromatograph (Jasco, model PU-980, Japan) with a variable-wavelength ultra violet

(UV) spectrophotometer (Jasco, model UV-970, Japan), and an intelligent sampler (Jasco, model 851-AS, Japan) were employed for the quantitative analysis of B30-MDP. Chromatographic separation was carried out using a Shodex ODSpak F-511A column (5 μm; 250 mm × 4.6 mm i.d.) with the mobile phase: methanol-water (19:1) containing 0.1% of perchloric acid. The flow rate was 1.2 ml/min. The column temperature was kept at 313 K by a variable temperature control unit (Jasco, model 860-CO, Japan). The wavelength of the detector was set at 215 nm. The injection volume of the sample was 50 μL. All kinetic measurements were performed in triplicate.

Results and discussion

Effects of O.G. incorporation on size and membrane fluidity of B30-MDP liposome

B30-MDP liposomal vaccine was prepared by the detergent removal method, which utilizes a phase transition from mixed micelle to vesicle. The effect of O.G. on the particle size and membrane fluidity of B30-MDP solutions was evaluated over a range of O.G. concentration from 1 to 100 mmol/dm^3 to confirm the phase transition. The concentration of B30-MDP in the solution was adjusted to 100 μmol/dm^3. Figure 2 shows that the particle size remained constant in a range of O.G. concentrations from 30 to 100 mmol/dm^3 and suddenly increased as the O.G. concentration was decreased to 25 mmol/dm^3, and the particle size of B30-MDP then decreased at an O.G. concentrations below 25 mmol/dm^3. These results indicate that the B30-MDP liposome formed from mixed micelle at O.G. concentration below 25 mmol/dm^3. In addition, the changes in the particle size of B30-MDP liposomes indicated a phase transition. These phenomena were similar to those observed in the phospholipid liposome when the detergent removal method was used [10].

The effect of O.G. on the membrane fluidity of B30-MDP liposome is also shown in Fig. 2. The fluidity of B30-MDP membrane was measured with 5NS the B30-MDP. The results showed that the order parameter, which reflected membrane fluidity [9], did not change between O.G. concentrations of 25 and 100 mmol/dm^3, while the order parameter of B30-MDP membrane increased as the O.G. concentration was decreased below 25 mmol/dm^3. These results indicate that the phase transition of B30-MDP vesicle formation from B30-MDP-O.G. mixed micelle occurred at an O.G. concentration of 25 mmol/dm^3, and was quantitatively evaluated from the standpoint of physicochemical properties such as the changes in particle size and membrane fluidity.

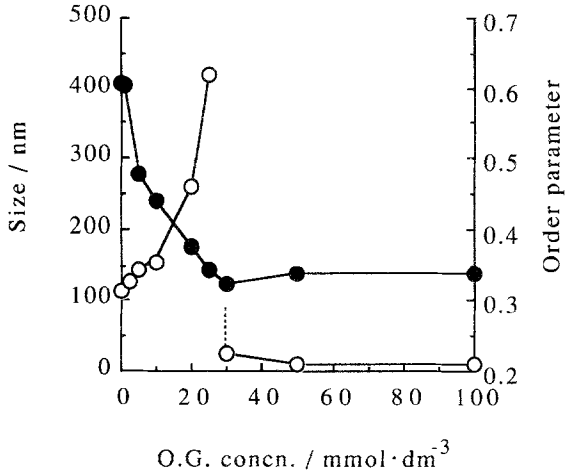


Fig. 2 Effect of O.G. concentration on size and order parameter of B30-MDP solutions (pH 7.4, $\mu = 0.17$). ○, size and ●, order parameter

Chemical stability of B30-MDP in aqueous solutions

The chemical stability of B30-MDP in aqueous solutions was studied to clarify the effect of phase transition on degradation kinetics. The initial concentration of B30-MDP in the sample solutions was adjusted to 100 μmol/dm^3, and the kinetic measurements of B30-MDP solutions were carried out at pH 7.4 because it is necessary to take care to maintain the stability of antigens for practical use as liposomal vaccines. Antigens tend to be generally aggregated and denatured by changes in pH, especially in acidic solution [11–13]. Figure 3 shows that the degradation of B30-MDP in aqueous solutions was performed at various temperatures at a constant pH (pH = 7.4) and a constant ionic strength ($\mu = 0.17$). The semi-logarithmic plots of remaining B30-MDP versus time showed linearity ($r > 0.99$). These data indicate that the degradation of B30-MDP occurred by a pseudo first-order reaction at 313, 323 and 333 K in both liposomal and mixed micellar solutions of B30-MDP. The degradation kinetics of B30-MDP liposome were similar to that observed with the phospholipid liposome [14–16]. In the same articles, it was reported that the degradations of phospholipids in liposome membranes resulted in the formation of lyso-phosphatidylcholine and fatty acid, which led to the conclusion that the degradation of the phospholipids occurred in their hydrophilic portions. It is supposed that the degradation of B30-MDP liposome occurs in the MDP region, where the hydrophilic portion of the B30-MDP membrane is situated. Additionally, if the chemical degradation of B30-MDP occurs only in the monodisperse state, and not in the liposomal state, the degradation kinetics may indicate a pseudo zero-order reaction [17].

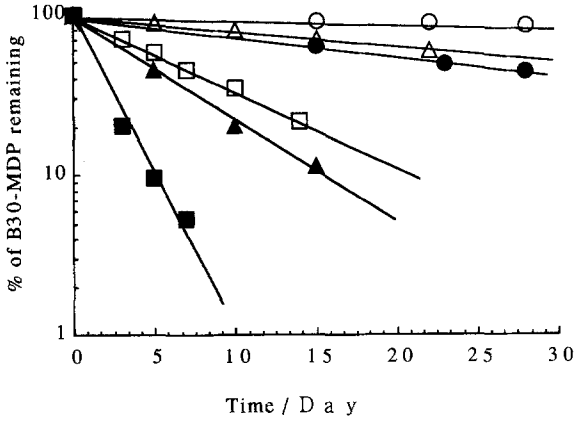


Fig. 3 Plots of the degradation kinetics of B30-MDP at various temperatures (pH 7.4, $\mu = 0.17$). B30-MDP in liposomal solution: ○, 313 K; △, 323 K and □, 333 K. B30-MDP in mixed micellar solution (O.G. concentration: 100 mmol/dm^3): ●, 313 K and ▲, 323 K and ■, 333 K

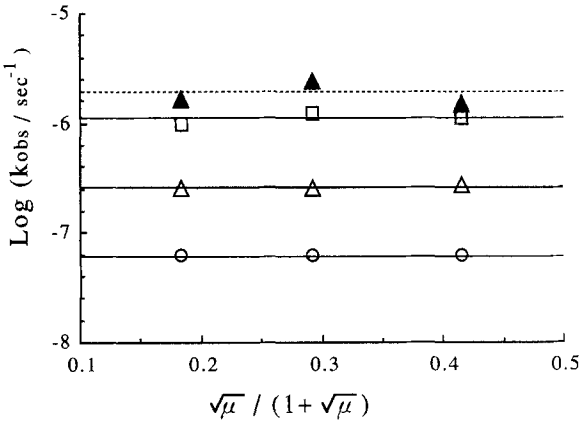


Fig. 4 Effect of ionic strength on degradation rate of B30-MDP at various temperatures at pH 7.4. B30-MDP in liposomal solution: ○, 313 K; △, 323 K and □, 333 K. B30-MDP in mixed micellar solution (O.G. concentration: 100 mmol/dm^3): ●, 313 K and ▲, 323 K and ■, 333 K

The effects of ionic strength on the degradation rate of B30-MDP liposomal and micellar solutions were then evaluated.

Within a restricted range of ionic strength (μ), $\log k_{obs}$ (k_{obs}: observed degradation rate constant) may be expressed by the following equation:

$$\mathrm{Log}\, k_{obs} = \mathrm{Log}\, k\ (\mu = 0) + 2AZ_A Z_B \sqrt{\mu}/(1 + \sqrt{\mu}) \qquad (1)$$

where A is a constant for a given solvent at a given temperature and Z_A and Z_B are the charges on reaction species A and B, respectively [18]. The $\log k_{obs}$ values versus $\sqrt{\mu}/(1 + \sqrt{\mu})$ would be expected to be linear only within the limits of the Debye–Huckel expressions; Carstensen [19] reviewed kinetic ion effects in the pharmaceutical literature and concluded that plots of $\log k_{obs}$ versus $\sqrt{\mu}/(1 + \sqrt{\mu})$ may be linear at ionic strengths below 1.0.

The degradation of B30-MDP at various ionic strengths occurred by a pseudo first-order reaction (data not shown). The plots of $\log k_{obs}$ versus $\sqrt{\mu}/(1 + \sqrt{\mu})$ are shown in Fig. 4. The results showed that the slopes were approximately zero. Thus, an increase in the ionic strength of buffer solutions did not affect the k_{obs} values of B30-MDP in either the liposomal or the mixed micellar solution. It was clear that no kinetic ion effect of B30-MDP was observed in an aqueous environment; it is considered that the k_{obs} value of B30-MDP was closely related to spontaneous hydrolysis or a water-catalyzed reaction [18, 19].

We next evaluated the effect of O.G. incorporation on the degradation of B30-MDP liposome. Figure 3 indicates that the degradation of B30-MDP was accelerated by the addition of O.G. (100 mmol/dm^3). Thus, the effect of temperature and O.G. incorporation on the degradation of B30-MDP in aqueous solution was evaluated by the Arrhenius plots shown in Fig. 5. Here, B30-MDP in 100 mmol/dm^3 O.G. solutions formed an O.G.-B30-MDP mixed micelle as suggested by the results shown in Fig. 2. The degradation of B30-MDP in both the liposomal solution and the mixed micellar solution showed in accordance with the Arrhenius equation, and the degradation rates of B30-MDP, increased by the presence of O.G., did not depend on the changes in the apparent activation energy, but rather on those in the frequency factor, as shown in Fig. 5. From a theoretical point of view, this is consistent with the assumption that the degradation processes of

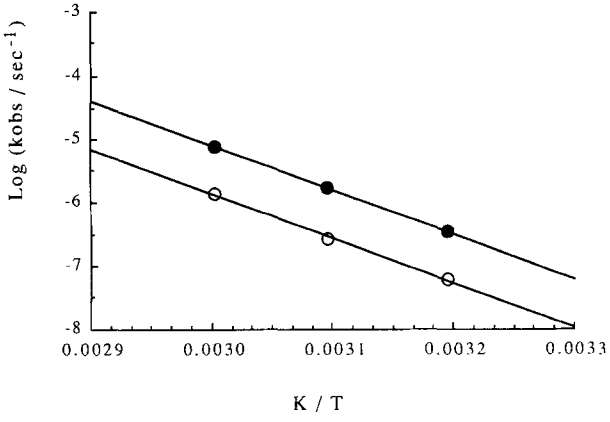


Fig. 5 Effect of temperature on the degradation of B30-MDP by the Arrhenius plots (pH 7.4, $\mu = 0.17$). ○, B30-MDP in liposomal solution and ●, B30-MDP in mixed micellar solution (O.G. concentration: 100 mmol/dm^3)

Table 1 Activation energies (Ea) and frequency factors (A) for the degradation of B30-MDP in aqueous solutions and the calculated shelf lives (t_{90}) at 298 and 288 K.

Formation	Ea(kJ mol^{-1})	A (s^{-1})	t_{90} at 298 K (day)	t_{90} at 288 K (day)
Liposome	134.5	1.58×10^{15}	289	1869
Mixed micelle*	132.6	4.78×10^{15}	45	287

*At an O.G. concentration of 100 mmol/dm^3

B30-MDP in both liposomal and in mixed micellar solutions are the same [20]. It also suggests that the acceleration of B30-MDP degradation occurs independently of the catalytic action of O.G.

On the other hand, it is important to evaluate both the chemical stability of B30-MDP in liposomal solutions and the process of liposome manufacture from the standpoint of producing a B30-MDP vaccine for practical use. The shelf lives (t_{90}) of B30-MDP in liposomal and mixed micellar solutions were calculated using the parameters obtained from the Arrhenius plots in Table 1. The results showed that the t_{90} of B30-MDP in the mixed micellar solution was approximately one-seventh of that seen with B30-MDP alone in the liposomal solution. The minimum shelf-life of B30-MDP as a vaccine adjuvant would in general need to be longer than 1 year, and so B30-MDP liposome must be kept below 288 K. The calculated t_{90} for B30-MDP in mixed micellar solution at 298 K was 45 days, suggesting that the stability of B30-MDP during the process of liposome manufacture is assured. Its stability in liposomal solution has been shown to be much higher than that of other hydrophilic MDP analogs at neutral pH [21]. These results indicate that the degradation of B30-MDP was inhibited by the formation of liposome.

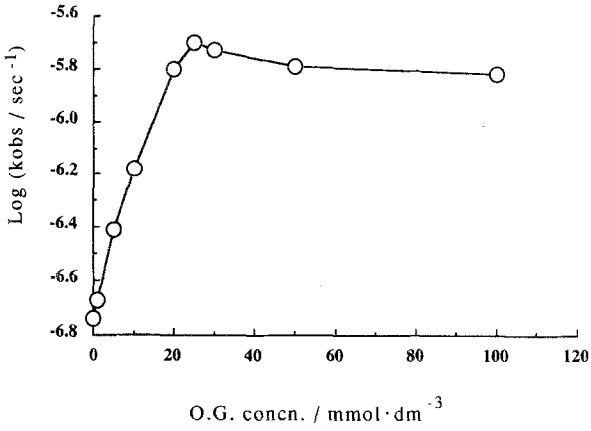


Fig. 6 Effect of O.G. concentration on the observed degradation rate constant of B30-MDP (pH 7.4, $\mu = 0.17$)

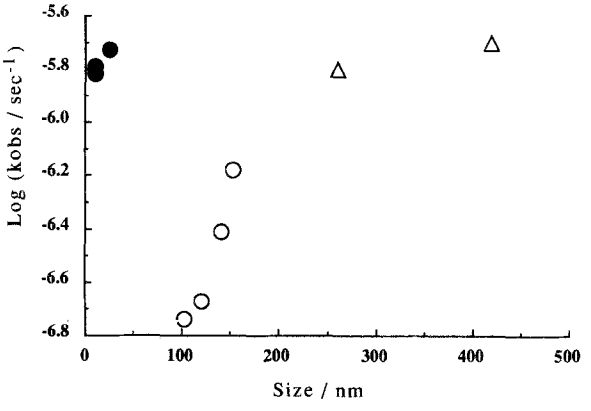


Fig. 7 Correlation between the size of B30-MDP liposomes and the observed degradation rate constants (pH 7.4, $\mu = 0.17$). O.G. concentration: ○, not higher than 15 mmol/dm^3; △, 20 mmol/dm^3 and 25 mmol/dm^3; and ●, 30 mmol/dm^3 or above

Relationship between physicochemical properties and degradation kinetics of B30-MDP

We considered that the chemical stability of B30-MDP was influenced by the presence of O.G., but that the acceleration of its degradation induced by O.G. was a result of its different state of dissolution in the mixed micellar solution, not of the catalysis by O.G. To confirm the distinction between the chemical stability of B30-MDP in liposomal solution and that in micellar solution, we studied the relationship between the physicochemical properties and degradation kinetics of B30-MDP. We first examined the effect of O.G. on the observed degradation rate constant (k_{obs}) of B30-MDP as shown in Fig. 6. The results showed that, while the k_{obs} values of B30-MDP solutions containing O.G. increased with the O.G. concentration, they did not change at concentrations higher than 25 mmol/dm^3. At concentrations above 25 mmol/dm^3, it is thought that B30-MDP formed a mixed micelle, and the k_{obs} values of B30-MDP in the micellar solutions remained constant. This suggests that the degradation of B30-MDP was not affected by the number of micelles of O.G., in contrast with the concept of a "micellar catalyzed reaction" described by Yasuhara et al. [22].

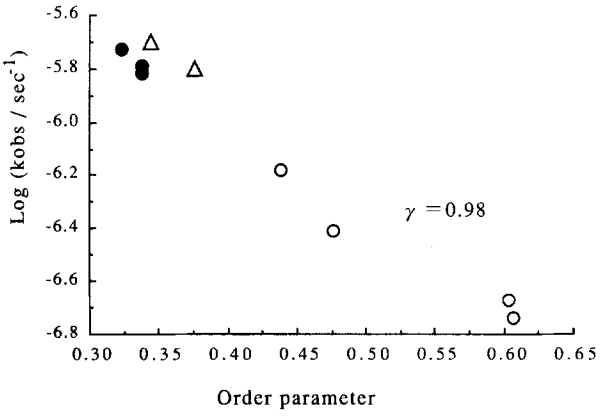


Fig. 8 Correlation between the order parameter of B30-MDP liposomes and the observed degradation rate constants (pH 7.4, $\mu = 0.17$). O.G. concentration: ○, not higher than 15 mmol/dm^3; △, 20 mmol/dm^3 and 25 mmol/dm^3; and ●, 30 mmol/dm^3 or above

It was suspected that the increase in the k_{obs} values of B30-MDP might have corresponded with the progress of B30-MDP liposome solubilization in factors such as the changes in particle size and membrane fluidity shown in Fig. 2. The particle size was therefore compared with the k_{obs} values of B30-MDP in an aqueous environment, as shown in Fig. 7, with results indicating however, that the changes in the particle size of the liposome did not correlate with the changes in the k_{obs} values of B30-MDP. In addition, it was apparent that the relationship between the particle size and the k_{obs} values was of three kinds, and in three phases, according to the increase of O.G. concentration: 1) change in k_{obs} value without change in particle size, 2) no change in k_{obs} value but a change in particle size, and 3) no change in either.

To examine the relationship between the membrane fluidity and chemical stability of B30-MDP, we next compared the order parameter with k_{obs} values (Fig. 8). The changes in the k_{obs} values correlated well with those in membrane fluidity induced by O.G. incorporation, indicating that an increase in membrane fluidity labilizes B30-MDP in liposomal solution. The good correlation found between these parameters suggests that the hydrophilic portion of B30-MDP is the site of degradation reaction, and it was considered that the increase of B30-MDP k_{obs} was dependent on a rise in the frequency of contact with water molecules, resulting from the increase in membrane fluidity.

The present findings suggest that the micelle-vesicle phase transition and the process of solubilization resulting from detergent removal can be monitored by observing changes in particle size and membrane fluidity. The micelle-vesicle phase transition affected the chemical stability of B30-MDP in an aqueous environment, and the changes in the membrane fluidity of B30-MDP liposome correlated with those in its chemical stability. It is supposed that the hydrophilic portion of the B30-MDP molecule probably plays an important role in the physicochemical properties and the chemical stability of the B30-MDP liposome.

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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. Zumbuehl O, Weder HG (1981) Biochim Biophys Acta 640:252–262
6. Baron C, Thompson TE (1975) Biochim Biophys Acta 382:276–285
7. Schneider WJ, Basu SK, McPaul MJ, Goldstein JL, Brown MS (1979) Proc Natl Acad Sci USA 76:5577–5581
8. Eshuis A, Harbers G, Doornink DJ, Mijnlieff PF (1985) Langmuir 1:289–294
9. McConnell HM, Wright KL, McFarland BG (1972) Biochim Biophys Res Commun 47:273–281
10. Ueno M, Akechi Y (1991) Chemistry Lett, 1801–1804
11. Maeda T, Onishi S (1980) FEBS Lett 122:283–287
12. Webster RG, Brown LE, Jackson DC (1983) Virology 126:587–599
13. Yoden S, Kida H, Kuwabara M, Yanagawa R, Webster RG (1986) Virus Res 4:251–261
14. Kensil SR, Dennis EA (1981) Biochemistry 20:6079–6085
15. Grit M, de Smidt JH, Struijke A, Crommelin DJA (1989) Int J Pharmaceu 50:1–6
16. Grit M, Zuidam NJ, Underberg WJ, Crommelin DJA (1993) J Pharm Pharmacol 45:490–495
17. Blaug SM, Wesolowski JW (1959) J Am Pharm Assoc 48:691–694
18. Garret ER, Bojarski JT, Yakatan GJ (1971) J Pharm Sci 60:1145–1154
19. Carstensen JT (1970) J Pharm Sci 59:1140–1143
20. Grit M, Underberg WJ, Crommeliln DJA (1993) J Pharm Sci 83:362–366
21. Powell MF, Foster LC, Becker AR, Lee W (1988) Pharm Res 5:528–532
22. Yasuhara M, Sato F, Kimura T, Muranishi S, Sezaki H (1977) J Pharm Pharmac 29:638–640