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pH-dependent release property of dioleoylphosphatidyl ethanolamine liposomes

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Abstract−pH-sensitive liposomes were prepared by a detergent removal method. Dioleoylphosphatidylethanolamine (DOPE) and cholesteryl hemisuccinate (CHEMS) were combined for the preparation of the liposomes so that the molar ratios of DOPE to CHEMS are 9/1, 8/2, 6/4 and 5/5. On transmission electron micrographs, hundreds of nm sized - multilamella vesicles were observed. The degrees of fluorescence quenching were approximately 70-80%, indicating that closed vesicles were formed. According to the results of the pH-dependent release experiment with the liposome composed of DOPE/CHEMS (5/5), no significant release was observed in the pH region ranging from 6 to 8. At pH of 5, an appreciable amount of calcein was released. The patterns of pH-dependent releases from liposomes composed of DOPE/CHEMS (6/4) and DOPE/CHEMS (8/2) were almost the same as those from liposomes composed of DOPE/CHEMS (5/5). With the liposomes composed of DOPE/CHEMS (9/1), unlike the other liposomes described above, almost 90% release was observed at pH 6. In this case, since the amount of a complementary molecule, CHEMS, is relatively small, the liposomes would be subjective to destabilization even at a small change in the degree of deionization of the carboxylic group. This may explain why the liposome of DOPE/CHEMS (9/1) exhibits a significant release at a relatively high pH, pH 6.0.

Key words: Liposome, Dioleoylphosphatidylethanolamine, Cholesteryl Hemisuccinate, pH-sensitive Release

INTRODUCTION

Since liposomes have been proposed as a carrier of drug, much attention has been paid to the design of stimuli-sensitive liposomes. Specific functions of the liposomes are induced by direct interaction [1,2], pH [3,4] and temperature [5-10]. The sensitivity of liposomes to stimuli can be controlled by either altering the composition of membrane or modifying the surface. Recently, temperature-sensitive releases of liposomes were proposed by coating liposomes with hydrophobically modified poly(*N*-isopropylacrylamide) (poly(NIPAM)) [7-10]. The polymer maintains an expanded form below its lower critical solution temperature (LCST) of around 32 °C, and attains a contracted form beyond the temperature. Release of the materials entrapped in the inner aqueous phase of liposomes is caused by the interaction of the polymer and lipid membranes. On the other hand, pH-sensitive liposomes were prepared by combining dioleoylphosphatidylethanolamine (DOPE) and the copolymer of NIPAM and methacrylic acid [11]. Since the molecule of DOPE is conical and its packing parameter is greater than 1, the phospholipids tend to form a non-bilayer structure in a physiological condition. In order to form a bilayer, it needs complementary molecules which fill the space among the head groups of DOPE. The hydrophobically modified copolymers of NIPAM and methacrylic acid were used as pH-sensitive complementary molecules. It is a weakly acidic amphiphile and could stabilize DOPE bilayers at neutral pH. Among pH-sensitive complementary molecules, cholesteryl hemisuccinate (CHEMS) has been the most frequently employed [12-16]. It is also a weakly acidic amphiphilic molecule and could stabilize DOPE bilayers at physiological pH.

Until now, few data have been obtained about the correlation between the composition of pH-sensitive liposome and the pH where the liposomes start to release. In this study, compositionally different kinds of pH-sensitive liposomes were prepared by using DOPE and CHEMS. The effects of the molar ratio on the pH at which the liposome starts to release were investigated. In parallel, pH-dependent changes in the size of the liposomes were observed to explain the effects of molar ratio on the pH where the liposome starts to release.

EXPERIMENTAL

1. Materials

Dioleoylphosphatidylethanolamine (DOPE) was purchased from Fluka. Deoxycholate, cholesteryl hemisuccinate (CHEMS) and calcein, a fluorescence marker for release tests, were purchased from Sigma Chemical Co. All other reagents were of analytical grade.

2. Preparation of DOPE/CHEMS Liposome by Detergent Removal Method

DOPE solution in chloroform (10 mg/ml) and CHEMS solution in chloroform (10 mg/ml) were put into a 50 ml round bottom flask so that the molar ratios of DOPE to CHEMS were from 5 : 5 to 9 : 1, and the total amount of lipid was 20 mg. The solvent was evaporated in a rotary evaporator at a reduced pressure to obtain a mixed dry film of DOPE and CHEMS. The dry films were dispersed into 2 ml of HEPES (pH 7.4) containing calcein (50 mM) and deoxy cholate (DOC, 0.09%), and they were sonicated for 20 min with a bath-type sonicator (Ultrasonic processor, VCX 500, SONIX, USA). The liposomal suspensions were allowed to stand at room temperature for 4-5 h. DOC and unentrapped calcein was removed by a gel permeation chromatography by using a Sephadex G-50 column

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(1.5 cm×50 cm). The final concentrations of lipid were adjusted to 2 mg/ml.

3. Transmission Electron Microscopy

The suspensions of liposomes were negatively stained with freshly prepared phosphotungstic acid solution (2%, pH 6.8). The stained liposomal suspension was transferred onto a formvar/carbon coated grid (200 mesh) and it was air-dried at room temperature. Electron microphotographs were taken on an electron microscope (LEO-912AB OMEGA, LEO, Germany) with magnification of 50,000.

4. % Quenching of Fluorescence

The % quenching of liposome-entrapped calcein was determined by the formula, % quenching=$(1-F_i/F_f)\times 100$, where F_i is the initial fluorescence after removing free calcein, and F_f is the total fluorescence after DOC is added to the suspension of calcein-containing liposomes so that final DOC concentration is 0.2%. The fluorescence intensities were measured at room temperature.

5. pH-dependent Calcein Release

Calcein-containing liposome suspensions of 0.15 ml, 2 mg lipid/ml, in HEPES (pH 7.4), were injected into a fluorescence cell containing 2.6 ml of HEPES, preadjusted to pH ranging from 5 to 8. The change in fluorescence was monitored at 524 nm with excitation at 494 nm. The percent release of calcein was determined as follows.

$$\% \text{ release}=(F_t-F_i)/(F_f-F_i)\times 100$$

where F_t is the intensity of fluorescence at a given pH and F_i is the initial intensity at pH 7.4. F_f is the total fluorescence after adding DOC so that the final concentration is 0.2%. Since the intensity of the fluorescence strongly depends on pH, F_t was corrected by using calibration curves.

6. Dynamic Light Scattering

The pH-dependent changes in the size were measured with a particle size analyzer (ZetaPlus 90, Brookhaven Instrument Co., USA). The content of liposome in the suspension was adjusted to 0.03%. pH was varied from 5-8 with 1 mM NaOH and 1 mM HCl solutions.

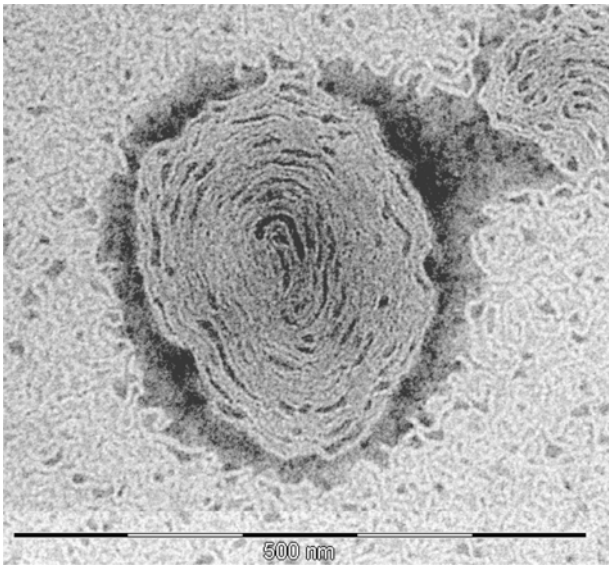

Fig. 1. Transmission electron microphotograph of liposomes composed of DOPE/CHEMES (9 : 1). Magnification is ×50,000.

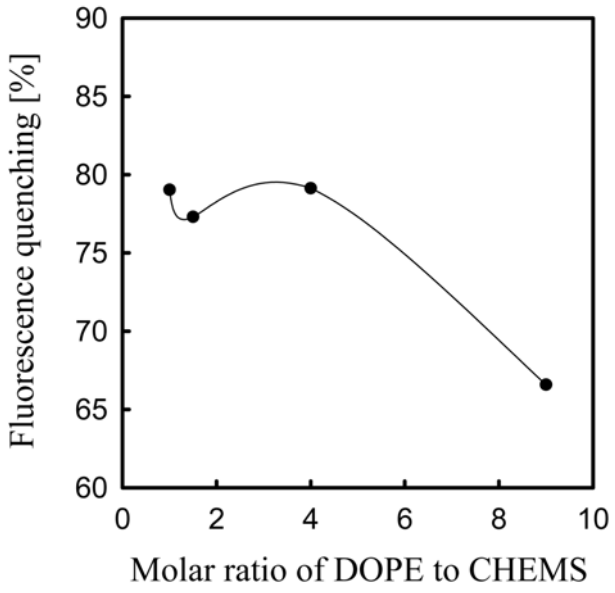

Fig. 2. Fluorescence quenching of calcein entrapped in DOPE/CHEMS liposomes.

RESULTS AND DISCUSSION

1. Transmission Electron Microscopy

Fig. 1 shows the electron microphotograph of liposomes, where the DOPE to CHEMES ratio is 9 : 1. Multi-lamella vesicles were observed together with non-vesicular multilayers. According to the results of quenching experiments, the values ensured that most of the particles in suspensions were enclosed vesicles. In fact, the negative staining technique involves a dry process. Therefore, dehydration of the vesicles may alter the vesicular structure. This may account for the non-vesicular bilayer structure.

2. % Quenching of Fluorescence

Fig. 2 shows the degree of quenching of fluorescence. Calcein, a fluorescence marker, is known to be self-quenched at a higher concentration. Thus, higher quenching means that the concentration of calcein is high enough to suppress its fluorescence. This phenomenon occurs when the concentrated calcein solution is enclosed by vesicles. When the ratios of DOPE to CHEMS were 5/5, 6/4, 8/2, the quenching % was approximately 80%. The value is close to the quenching of phosphatidylcholine liposomes, which are readily formed without a complementary molecule such as CHEMS. It indicates that DOPE/CHEMS at the ratios of 5/5, 6/4, 8/2 form tightly closed vesicles. On the other hand, when a small amount of the complementary molecule was included, such as DOPE/CHEMS molar ratio of 9/1, the degree of quenching decreased to 67%, a relatively low value. It means that the liposomal membranes were unstable and it was somewhat destabilized during the gel permeation chromatography process, or the liposomal membrane was so loose that an appreciable amount of entrapped calcein released. Therefore, it seems that the amount of CHEMS should be more than 8 : 2 to obtain stable liposomes. This result is in good agreement with previous research [15].

3. pH-dependent Calcein Release

Since the liposomes were prepared at pH 7.4 and the release ex-

periment was performed at various pHs, which is different from pH 7.4, the fluorescence intensity at the various pHs should be converted to the intensity at pH 7.4. Fig. 3 shows the calibration curve of calcein fluorescence at various pHs, where the degree of release was observed. The slope decreased with decreasing pH. It means that the intensity of fluorescence is suppressed at acidic pHs. Using these curves, the fluorescence intensities need to be corrected.

Fig. 3 shows the pH-dependent calcein release from liposome composed of DOPE/CHEMS (5/5). In the pH region ranging from 6 to 8, no significant release was observed in 60 sec. At pH 5, an appreciable amount of calcein was released. Since the pK value of carboxylic acid of CHEMS is 5.8, the carboxylic acid would be ionized above a pH, such as pH 6, 7 and 8, so that the head group is large enough to stabilize DOPE bilayers. On the other hand, when the pHs were below the pK value, such as pH 5, the carboxylic acid would be deionized and thus the size of head group of CHEMS decrease. Accordingly, the DOPE bilayer would be destabilized into non-bilayer structures, giving rise to a higher release. The patterns of

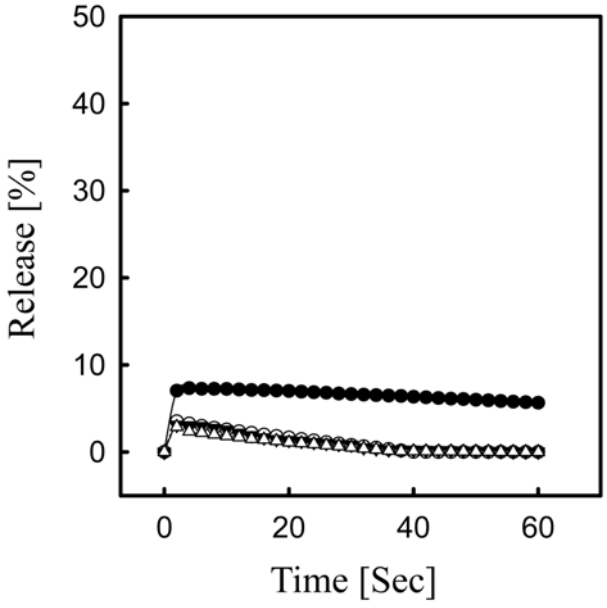

Fig. 3. pH-dependent calcein release from liposomes composed of DOPE/CHEMS (5/5). pH 5 (●), pH 6 (○), pH 7 (▼), pH 8 (△).

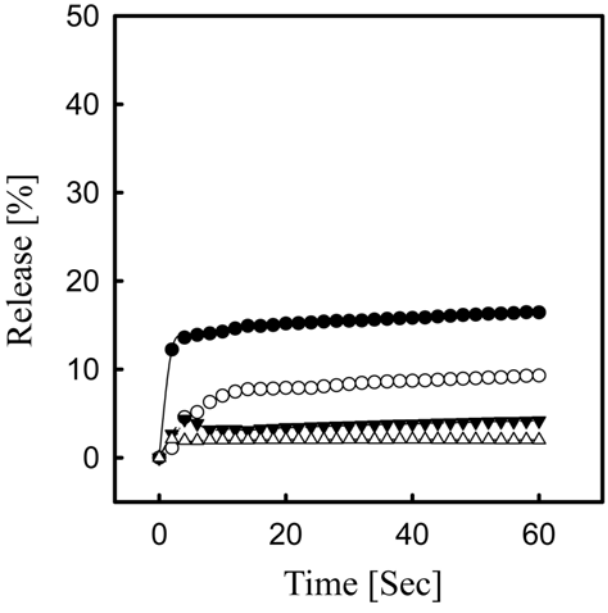

Fig. 4. pH-dependent calcein release from liposome composed of DOPE/CHEMS (6/4). pH 5 (●), pH 6 (○), pH 7 (▼), pH 8 (△).

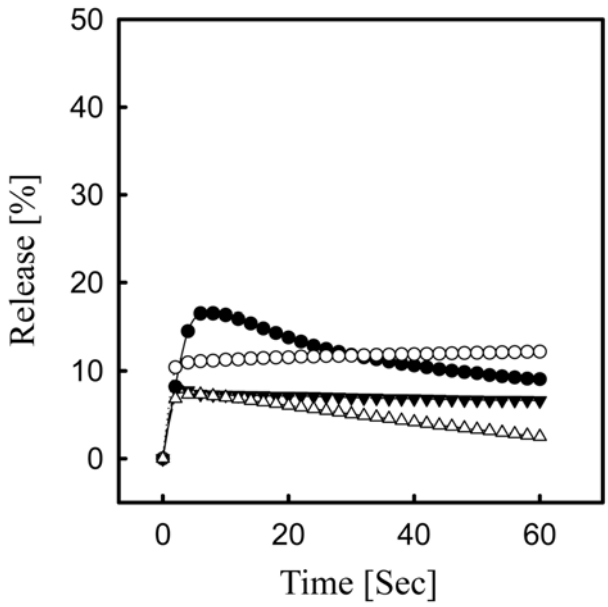

Fig. 5. pH-dependent calcein release from liposome composed of DOPE/CHEMS (8/2). pH 5 (●), pH 6 (○), pH 7 (▼), pH 8 (△).

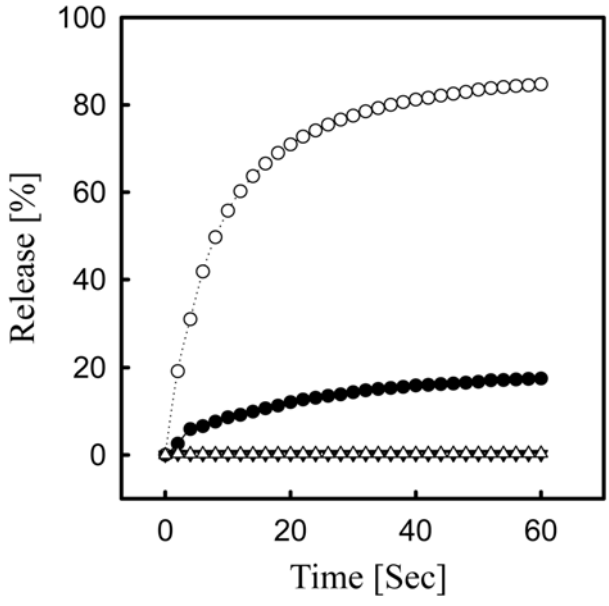

Fig. 6. pH-Dependent calcein release from liposome composed of DOPE/CHEMS (9/1). pH 5 (●), pH 6 (○), pH 7 (▼), pH 8 (△).

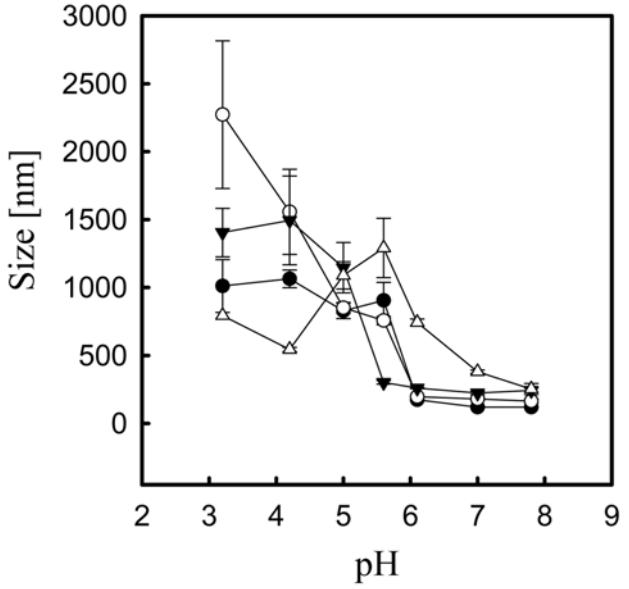

Fig. 7. Change in the size of DOPE/CHEMS liposome with pH. DOPE/CHEMS (5 : 5 ▼, 6 : 4 ○, 8/2 ●, 9 : 1 △).

pH-dependent releases from liposomes composed of DOPE/CHEMS (6/4) and DOPE/CHEMS (8/2) were almost the same as those from liposomes composed of DOPE/CHEMS (5/5) (Fig. 4, Fig. 5). The degree of the release, however, started to be appreciable at pH 6, and some higher releases were observed with the liposomes of DOPE/CHEMS (6/4) and DOPE/CHEMS (8/2).

Fig. 6 shows the pH-dependent calcein release from liposome composed of DOPE/CHEMS (9/1). Unlike the other liposomes described above, a significant amount of the calcein release at pH 6, and almost 90% release was observed at pH 6. In this case, the amount of a complementary molecule, CHEMS, is relative small. Therefore, the liposomes would be subjective to destabilization even at the small change in the degree of deionization of carboxylic group. This may explain why the liposome of DOPE/CHEMS (9/1) exhibits a significant release at a relatively high pH, pH 6.

4. Dynamic Light Scattering

Fig. 7 shows the change in the size of liposome with pH. In case of DOPE/CHEMS liposome of 5 : 5, 6 : 4, 8 : 2, the size was almost constant with respect to pH when pH decreased from 8.0 to 6.0 and the value was around 250 nm. When pH decreased to 5.0, the size started to increase and it reached values greater than 1,000 nm in more acidic conditions. This is possibly because the deionization of CHEMS in the acidic condition would result in the destabilization of DOPE liposomal membrane. From our previous report, the DOPE liposomes are known to destabilize into hexagonal phase when the complementary molecules lost their function of the stabilization [17,18]. Thus the larger size would be due to hexagonal phases. In addition, the change of the closed vesicle to non vesicular hexagonal phase could account for the pH-sensitive release described in section 3.3. Obviously, pH, where calcein starts to release significantly, is in accordance with the value, where the size starts to increase.

On the other hand, in case of liposome of DOPE/CHEMS (9 : 1), the size increased markedly at pH 6.0. In this case, the amount of a complementary molecule, CHEMS, is relative small. Therefore, the liposomes would be subject to destabilization even at a small change in the degree of deionization of carboxylic group. This may explain why the liposome of DOPE/CHEMS (9/1) exhibits an outstanding increase in size at a relatively high pH, pH 6.

CONCLUSION

The pH-sensitive DOPE liposome could be formed with aid of CHEMS. The lower critical ratio of DOPE/CHEMS for the formation of liposomes was around 9 : 1. The liposomes composed of DOPE/CHEMS (5 : 5, 6 : 4, and 8 : 2) were stable at pH 6, pH 7, pH 8 in terms of the release and the size. And they became unstable at pH 5. On the other hand, the liposomes composed of DOPE/CHEMS (9 : 1) were stable at pH 7 and pH 8, and they became unstable at pH 6. That is, the pH where liposomes start to release is closely related to the ratio of DOPE/CHEMS.

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REFERENCES

1. E. O. Lee, J. G. Kim and J.-D. Kim, *J. Biochem.*, **112**, 671 (1992).
2. A. Huang, Y. S. Tsao, S. J. Kennel and L. Huang, *Biochim. Biophys. Acta*, **716**, 140 (1983).
3. M.-J. Choi, H.-S. Han and H. Kim, *J. Biochem.*, **112**, 694 (1992).
4. M. Maeda, A. Kumano and D. A. Tirrell, *J. Am. Chem. Soc.*, **110**, 7455 (1988).
5. M. B. Yatvin, J. N. Weinstein, W. H. Dennis and R. Blumenthal, *Science*, **202**, 1290 (1978).
6. J. N. Weinstein, R. L. Magin, R. L. Cysyl and D. S. Zaharko, *Cancer Res.*, **40**, 1388 (1980).
7. H. Hayashi, K. Kono and T. Takagishi, *Biochim. Biophys. Acta*, **1280**, 127 (1996).
8. J.-C. Kim, S. K. Bae and J.-D. Kim, *J. Biochem.*, **121**, 15 (1997).
9. K. Kono, R. Nakai, K. Morimoto and T. Takagishi, *Biochim. Biophys. Acta*, **1416**, 239 (1999).
10. K. Kono, A. Henmi, H. Yamashita, H. Hayashi and T. Takagishi, *J. of Controlled Release*, **59**, 63 (1999).
11. M. Zignani, D. C. Drummond, O. Meyer, K. Hong and J.-C. Leroux, *Biochim. Biophys. Acta*, **1463**, 383 (2000).
12. R. M. Straubinger, N. Düzgünes and D. Papahadjopoulos, *FEBS Lett.*, **179**, 148 (1984).
13. D. Liu and L. Huang, *Biochim. Biophys. Acta*, **1022**, 348 (1990).
14. D. C. Litzinger and L. Huang, *Biochim. Biophys. Acta*, **1113**, 201 (1992).
15. H. Ellens, J. Bentz and F. C. Szoka, *Biochemistry*, **23**, 1532 (1984).
16. P. R. Cullis and B. De Kruijff, *Biochim. Biophys. Acta*, **559**, 399 (1979).
17. J.-C. Kim and J.-D. Kim, *Colloid Surf. B: Biointerfaces*, **24**, 45 (2002).
18. J.-C. Kim, M.-S. Kim and J.-D. Kim, *Korean J. Chem. Eng.*, **16**, 28 (1999).