Effect of Phosphatidylcholine and Cholesterol on pH-Sensitive Liposomes

Norio HAZEMOTO,*,[1] Masanobu HARADA, Shigeki SUZUKI, Fusao KAIHO, Makoto HAGA and Yuriko KATO

Faculty of Pharmaceutical Sciences, Science University of Tokyo, Ichigaya-Funagawara-machi, Shinjuku-ku, Tokyo 162, Japan. Received October 20, 1992

We previously reported that liposomes composed of phosphatidylethanolamine (PE) and fatty acid exhibited pH-dependent leakage, aggregation and fusion (N. Hazemoto, M. Harada, N. Komatubara, M. Haga and Y. Kato, *Chem. Pharm. Bull.*, 38, 748 (1990)). In this study, we have examined the effects of phosphatidylcholine (PC) and cholesterol (Chol) on the pH-sensitivity of liposomes. Contents-leakage from liposomes was always accompanied by a change in light-scattering, suggesting that aggregation or fusion of liposomes causes the leakage. The pH-sensitivity was observed only when liposomes contained less than 32 mol% of PC. The leakage *vs.* pH curves shifted to the more acidic regions as the PC content of the liposomes increased, but the maximum leakage (%) did not change. The effect of cholesterol on the pH-sensitivity depended on the PC/PE ratio of the liposomes. Addition of cholesterol to PC/PE/oleic acid (OA) liposomes system induced two effects, that is, aggregation of liposomes *via* the reduction in PC content and the stabilization of the liposomal membrane. It was shown that pH-sensitivity can be controlled by addition of the appropriate amount of PC and/or Chol to liposomal lipids.

Keywords pH-sensitive liposome; phosphatidylcholine; cholesterol; destabilization

In recent years, pH-sensitive liposomes have attracted attention as effective carriers for the cytoplasmic delivery of biologically active molecules such as DNA[2a] and toxins.[2b] Their potential stems from their ability to fuse with other lipid membranes and release entrapped contents. Conventional pH-insensitive liposomes, once endocytosed by a cell, are taken into the lysosomal compartment and degraded, resulting in low activity. In contrast, pH-sensitive liposomes are internalized in acidic endosomes, which lead to destabilization of the liposomal membrane and fusion with the endosomal membrane under acidic conditions, with the concomitant release of the entrapped contents into the cytoplasm.[3,4]

pH-Sensitive liposomes are usually composed of phosphatidylethanolamine (PE) and a protonatable amphiphile.[3,5,6] Liposomes do not exhibit pH-sensitivity when PE is replaced with phosphatidylcholine (PC). However, very little effort has been made to study the pH-sensitivity in PE/PC mixed systems, and so we prepared liposomes containing PC and cholesterol (Chol) in addition to PE and oleic acid (OA), and examined whether they still retained pH-sensitivity. We found that pH-sensitivity was observed up to about 32 mol% of PC. In this range, the threshold pH shifted to more acidic regions with increasing PC content. The effect of Chol on the pH-sensitivity of PE/PC/OA mixed systems was further studied. Addition of Chol to these systems induced the aggregation of liposomes *via* a reduction in PC content and a slight stabilization of the liposomal membrane.

Materials and Methods

Materials Transphosphatidylated phosphatidylethanolamine (PE) from egg phosphatidylcholine was purchased from Avanti Polar Lipids, Inc. (Bermingham, AL., U.S.A.). Phosphatidylcholine (PC), cholesterol (Chol) and oleic acid (OA) were obtained from Sigma Chemical Co. (St. Louis, MO., U.S.A.). Aminonaphthalene-3,6,8-trisulfonic acid (ANTS) and *N,N'-p*-xylylenebispyridinium bromide (DPX) were purchased from Molecular Probes, Inc (Eugene, OR., U.S.A.). All lipids were stored as chloroform stock solutions at −20 °C. The purity of the lipids was confirmed chromatographically on silica gel plates.

Preparation of Liposomes Liposomes of different compositions were prepared by a reverse-phase evaporation method according to Szoka and Papahadjopoulos.[7] Lipid (10 μmol) was dissolved in 1 ml of ether and combined with 0.34 ml of an aqueous solution containing 12.5 mM ANTS, 45 mM DPX, 50 mM NaCl and 5 mM Tris–HCl buffer, pH 9.0 and sonicated for 5 min in an ultrasonic bath. The resulting emulsion was evaporated in a rotary evaporator at 25 °C under reduced pressure (400 mmHg) to remove ether. After collapse of the gel, evaporation was continued under high vacuum (150 mmHg). The liposomes were extruded through a polycarbonate membrane with a 0.2 μm pore diameter (Nuclepore, Pleasanton, CA) and separated from free ANTS/DPX on a 15 × 170 mm Sepharose 4B column equilibrated with a buffer consisting of 145 mM NaCl, 5 mM Tris, pH 9.0, and 0.2 mM EDTA at a flow rate of 0.5 ml/min. Lipid concentrations were determined by phosphate analysis.[8]

Measurements of Contents-Leakage and Light-Scattering Fluorescent ANTS was encapsulated together with the quencher DPX in liposomes as described above. Leakage of ANTS from liposomes was followed by the increase in fluorescence due to the reduction in DPX quenching.[9] ANTS fluorescence at 530 nm was measured with an excitation wavelength of 360 nm. Maximum fluorescence after solubilization of the liposomes with 5% Triton X-100 was taken as 100% leakage. Light-scattering change was monitored at 450 nm with a Hitachi fluorescence spectrophotometer. The pH of the liposome suspension was measured by a Horiba pH electrode.

Results and Discussion

Effects of PC on pH-Sensitivity To investigate the effect of PC on the pH-sensitivity, we prepared various liposomes consisting of PE, OA and PC. The mol% of OA was first set at 20%, and only the molar ratio of PC to PE was varied. The time course of aqueous contents-leakage is shown in Fig. 1a when the pH of the medium was lowered from 9.0 to 5.5. The rate of contents-leakage decreased with increasing PC content. When the PC/PE ratio was greater than 0.5, the leakage rate was greatly reduced. Light-scattering changes of these liposome solutions were also measured when the pH of the medium was lowered to 5.5 (Fig. 1b). There was a parallel relationship between the increase in aqueous contents-leakage and the increase in light-scattering intensity. It is well known that the light-scattering change is a good index of liposome aggregation.[10] The contents-leakage observed, therefore, must involve aggregation of the liposomes. A considerable

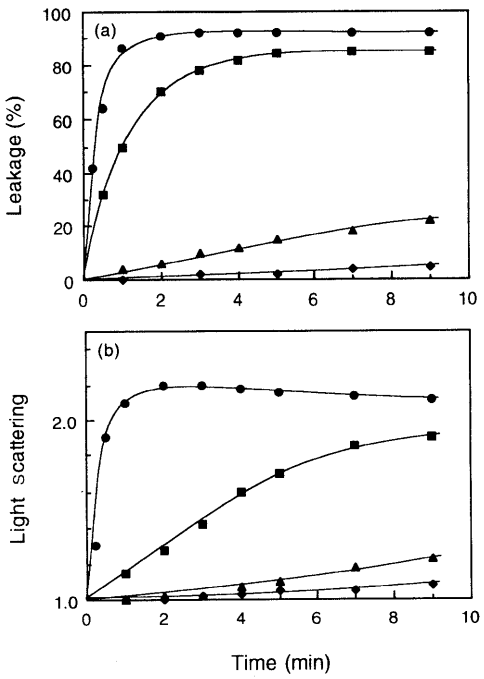

Fig. 1. Time Courses of Contents-Leakage (a) and Light Scattering Change (b) of Liposomes Consisting of PE, OA and PC

At time zero, the pH of liposome suspensions (50 μM lipid) was reduced from 9.0 to 5.5. Light scattering changes were measured at 450 nm and relative increase was calculated. The mol% of OA was kept at 20% and the molar ratio of PC to PE was varied. ●, PC/PE = 1/9; ■, PC/PE = 1/3; ▲, PC/PE = 1/2; ◆, PC/PE = 1/1.

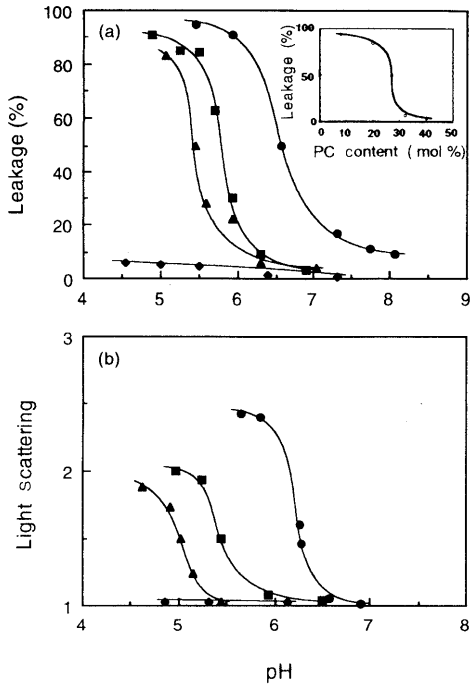

Fig. 2. pH-Dependence of Contents-Leakage (a) and Light Scattering Change (b) of Liposomes

The leakage (%) and light scattering change at 15 min after injection of an acid solution is plotted against the pH of the liposome suspension. The description of other symbols is the same as in Fig. 1. Inset, the leakage (%) at pH 5.5 is plotted against PC content.

number of other studies have suggested that contents leakage is involved in the aggregation and/or fusion of the liposomal membrane.[9,11] The pH-dependence of contents-leakage from the liposomes and the corresponding light-scattering change are shown in Figs. 2a and 2b. The contents-leakage was observed at ratios PC/PE = 1/9, 1/3 and 1/2, but practically no pH-sensitivity was observed at a ratio PC/PE = 1 over the pH range examined. The leakage *vs.* pH curves shifted to more acidic regions with increasing PC content. The light-scattering behavior of these liposome solutions corresponded to the contents-leakage, indicating that the aggregation or fusion of the liposomes plays an important role in the contents-leakage process. It is likely that PC has some effect in stabilizing the lipid membrane of PE and OA or preventing aggregation of the liposomes. Consequently, more acidic conditions are necessary to induce contents-leakage from PC-rich liposomes.

The effect of PC content on the leakage at pH 5.5 is shown in the inset in Fig. 2a. The pH-sensitivity of liposomes was very susceptible to slight variations in the PC content around 27%. There are two possible explanations for this effect of PC on the pH-sensitivity of liposomes. One is the decrease in the pK_a of OA with the change of electrical field in the vicinity of the choline group by addition of PC. The other is that PC-containing liposomes are hard to aggregate because of the repulsive hydration force between bilayers[12] or to form a non-bilayer structure.[13] The latter is likely since the properties of the liposomal membrane changed critically around a PC content of 27% and further increase in PC content made the liposomes pH-insensitive. NMR studies of a dioleoylphosphatidylethanolamine (DOPE) and dioleoylphosphatidylcholine mixture system by Tilcock *et al.*[14] showed that a concentration of DOPC greater than 20 mol% stabilized the bilayer structure. In our system, it seems that liposomal membranes containing over 27 mol% PC adopt a bilayer structure even if the fatty acid is protonated by addition of acid.

Effects of Cholesterol on pH-Sensitivity and Lipid Concentration on Contents-Leakage The time course of contents-leakage from the liposomes containing cholesterol and the corresponding light-scattering change are shown in Figs. 3a and 3b. Addition of cholesterol to the liposomes (PC/PE = 1/9 and 1/3) decreased the leakage rate, on the other hand, in the liposomes (PC/PE = 1/2 and 1) the rate was slightly increased. These findings indicate that the effect of cholesterol on the contents-leakage depends on the PC/PE ratio of the liposomal membrane.

The pH-dependence of leakage from the liposomes containing 40% cholesterol in addition to PE, PC and OA is shown in Fig. 4a and the corresponding light-scattering change in Fig. 4b. Contents leaked from the cholesterol-containing liposomes (PC/PE/OA/Chol = 0.48/4.32/1.2/4 μmol) at a more acidic pH compared with those containing no cholesterol (*cf.* PC/PE/OA = 0.8/7.2/2 in Fig. 2a). In the liposomes (PC/PE/OA/Chol = 2.4/2.4/1.2/4 μmol), however, addition of cholesterol significantly increased liposome aggregation (Fig. 4b) and the contents-leakage below pH 5.5. This effect of cholesterol may be explained in terms of PC content. The PC mol% of the liposomes (PC/PE/OA = 4/4/2) was 40, suggesting that the liposomes are pH-insensitive (*cf.* inset of Fig. 2a). Addition of cholesterol to these liposomes decreases the PC content to 24%, inducing more aggregation and leakage. We found that cholesterol itself has no effect on the aggregation of

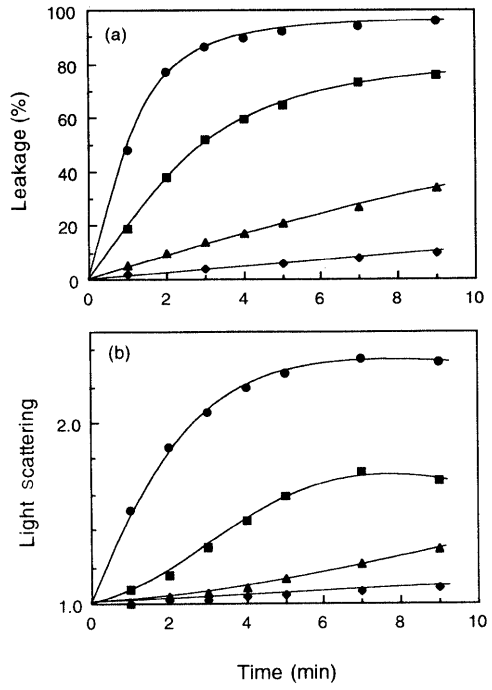

Fig. 3. Time Courses of Contents-Leakage (a) and Light Scattering Change (b) of Liposomes Consisting of PE, OA, PC and Chol

Cholesterol (40%) was added to the lipid composition in Fig. 1. PC/PE ratio: ●, 1/9; ■, 1/3; ▲, 1/2; ◆, 1/1.

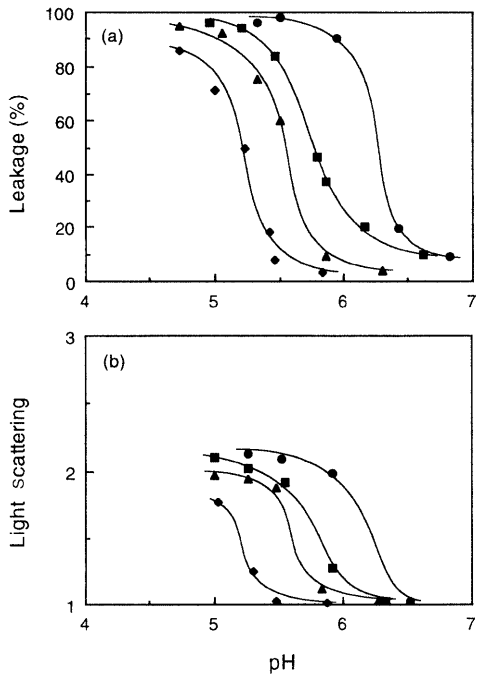

Fig. 4. pH-Dependence of Contents-Leakage (a) and Light Scattering Change (b) of Liposomes Containing Cholesterol (40%)

PC/PE ratio: ●, 1/9; ■, 1/3; ▲, 1/2; ◆, 1/1.

liposomes. The contents-leakage and light-scattering of the liposomes containing various amounts of cholesterol are shown in Figs. 5a and 5b. The contents-leakage *vs.* pH curves shifted slightly to acidic regions, but the light-scattering was unchanged with increasing cholesterol-content. This indicates that cholesterol stabilizes liposomal membrane slightly but it does not affect aggregation of

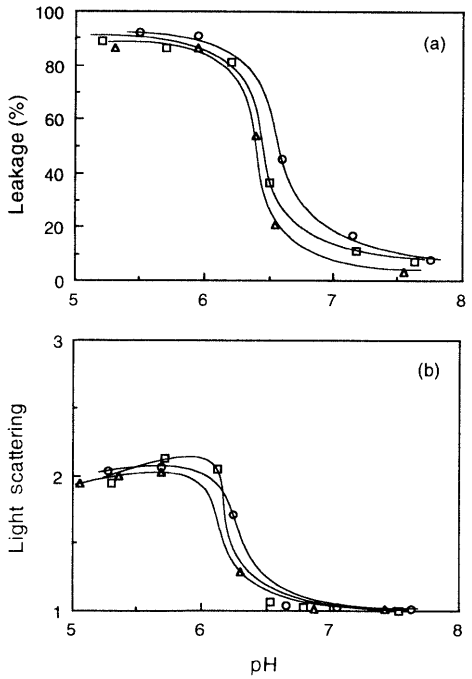

Fig. 5. Effect of Cholesterol on the pH-Sensitivity of Liposomes

(a) contents-leakage, (b) light scattering change, ○, PE/OA=8/2; □, PE/OA/Chol=6.4/1.6/2.0; △, PE/OA/Chol=4.8/1.2/4.0.

the liposomes. It is well known that cholesterol stabilizes the lamellar structure of the lipid membrane.[15] Thus, addition of cholesterol to a PC/PE/OA liposome system may induce two effects, namely, aggregation of liposomes *via* the reduction in PC content, which is seen at a high PC content (PC/PE/OA=4/4/2) and stabilization of the liposomal membrane, which is seen at a low PC content (PC/PE/OA=0.8/7.2/2). A similar result was obtained by Tilcock *et al.*[14] They showed that a DOPE/DOPC mixture system, containing 40% cholesterol, tended to adopt a hexagonal configuration (H_{II}) at 40 °C. Liu and Huang[16] studied the role of cholesterol in the stability of pH-sensitive LUV (large unilamellar vesicles) using calcein as a fluorescence marker, and showed that the pH value at which half-maximum calcein release took place remained at pH 7, and the maximum contents-leakage decreased linearly with increasing cholesterol content. The results shown in Figs. 2a and 4a, however, clearly indicate that the maximum contents-leakage is nearly 100%, and is unaffected by cholesterol content. This discrepancy cannot be explained convincingly at present.

We examined the effect of lipid concentration on the contents-leakage of liposomes, since contact between individual liposomes is necessary for destabilization of the liposomal membrane. The rate of leakage was increased with increasing lipid concentration both in liposomes consisting of PC/PE/OA=2/6/2 and PC/PE/OA/Chol=1.2/3.6/1.2/4. This finding suggests that liposome–liposome interaction is necessary to induce contents-leakage *via* aggregation. Similar results have been obtained in PE liposomes containing various fatty acids.[6]

The present results show that the pH-sensitivity can be controlled by the addition of appropriate amounts of PC or Chol to liposomal lipids. This may provide a useful technique for designing pH-sensitive liposomes able to

deliver their contents to the cytoplasm of cells *via* the endocytic process.

References and Notes

1) Present address: *Faculty of Pharmaceutical Sciences, Nagoya City University, Tanabe-dori, Mizuho-ku, Nagoya 467, Japan.*
2) *a*) C. Y. Wang and L. Huang, *Proc. Natl. Acad. Sci. U.S.A.*, **84**, 7851 (1987); *b*) D. Collins and L. Huang, *Cancer Res.*, **47**, 735 (1987).
3) R. M. Straubinger, N. Düzgünes and D. Papahadjopoulos, *FEBS Lett.*, **179**, 148 (1985).
4) J. Conner and L. Huang, *J. Cell Biol.*, **101**, 582 (1985).
5) R. Leventis, T. Diacova and J. R. Silvius, *Biochemistry*, **26**, 3267 (1987).
6) N. Hazemoto, M. Harada, N. Komatsuba, M. Haga and Y. Kato, *Chem. Pharm. Bull.*, **38**, 748 (1990).
7) F. C. Szoka and D. Papahadjopoulos, *Proc. Natl. Acad. Sci. U.S.A.*, **75**, 4194 (1978).
8) G. Bartlett, *J. Biol. Chem.*, **234**, 466 (1959).
9) H. Ellence, J. Bwenz and F. C. Szoka, *Biochemistry*, **23**, 1532 (1984).
10) N. Düzgünes, R. M. Straubinger, P. A. Baldwin, D. S. Friend and D. Papahadjopoulos, *Biochemistry*, **24**, 3091 (1985); S. Ohki, N. Duzgunes and K. Leonards, *ibid.*, **21**, 2127 (1982).
11) J. Conner, M. D. Yatbin and L. Huang, *Proc. Natl. Acad. Sci. U.S.A.*, **81**, 1715 (1984).
12) D. LeNeveu, R. P. Rand and V. A. Parsegian, *Nature* (London), **259**, 601 (1976); L. J. Lis, M. McAlister, N. Fuller, R. P. Rand and V. A. Parsegian, *Biophys. J.*, **37**, 657 (1982).
13) P. R. Cullis and B. D. Kruijef, *Biochem. Biophys. Acta*, **507**, 207 (1978).
14) C. P. S. Tilcock, M. B. Balley, S. B. Farren and P. R. Cullis, *Biochemistry*, **21**, 4596 (1982).
15) J. Senior and G. Gregoriadis, *Life Sci.*, **30**, 2123 (1982).
16) D. Liu and L. Huang, *Biochem. Biophys. Acta*, **981**, 254 (1989).