pH-Sensitive Liposomes Composed of Phosphatidylethanolamine and Fatty Acid

Norio HAZEMOTO,* Masanobu HARADA, Nobuko KOMATSUBARA, Makoto HAGA and Yuriko KATO

Faculty of Pharmaceutical Sciences, Science University of Tokyo, Ichigaya-Funagawara-machi, Shinjuku-ku, Tokyo 162, Japan.
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pH-induced destabilization, aggregation and fusion of liposomes composed of phosphatidylethanolamine (PE) and various fatty acid were studied. Destabilization was examined as a fluorescent change caused by leakage of co-encapsulated aminonaphthalene-3,6,8-trisulfonic acid (ANTS) and *N,N-p*-xylylenebispyridinium bromide (DPX). Fusion was monitored by two different methods, that is, intermixing assay of internal aqueous contents of liposomes, and lipid dilution assay of liposomes labeled with fluorescent phospholipids.

Contents leakage from liposomes was observed by lowering the pH, and pH where the leakage began depended on fatty acid used. Fifty percent leakage of contents from PE liposomes containing α-hydroxypalmitic acid or α-hydroxystearic acid was observed at pH 5.5, that from liposomes containing stearic acid or palmitic acid was observed at pH 6.5—6.7, and that from ricinoleic acid at pH 7.2. Aggregation and fusion of the respective liposomes also occurred at a similar pH region. These results were interpreted by the notion that the protonation of the fatty acid triggers a series of pH-sensitive events. The liposomes developed in this study may be useful as a drug carrier which could release the contents in response to pH changes in their environment.

Keywords pH-sensitive liposome; fatty acid; phosphatidylethanolamine; fusion; destabilization

pH-sensitive liposomes, which are stable at neutral pH but become destabilized at weakly acid pH, have attracted interest for their potential to release their content in a specific environment. Liposomes composed of palmitoylhomocystein and phosphatidylcholine (PC) were first designed to target areas of the body, such as primary tumors and metastases, where pH is less than physiological.[1] Recently pH-sensitive liposomes have also been proposed to deliver encapsulated macromolecules to the cytoplasm of cell, since these liposomes can fuse with the membrane of acidic intracellular vacuoles, resulting in the release of the contents of the liposome into the cytoplasm.[2,3] pH-sensitive liposomes are generally composed of pH-sensitive substances such as double chain amphiphiles,[4] oleic acid (OA),[3] cholesterolhemisuccinate,[5] 2,3-seco-5α-cholestan-2,3-dioic acid[6] or pH-sensitive polymer[7] in addition to PC and phosphatidylethanolamine (PE) as a main component. Liposomes composed of fatty acid may be suitable as a drug carrier because of their biocompatibility and simplicity of preparation.

In this paper, we examined pH-dependent leakage, aggregation and fusion of PE liposomes containing various fatty acids, *e.g.* stearic acid, palmitic acid, oleic acid, linoleic acid, recinoleic acid, α-hydroxypalmitic acid and α-hydroxystearic acid. Results demonstrated that the behaviors of these liposomes under various pH conditions are dependent upon the nature of the fatty acid that constitutes the liposomal membrane.

Materials and Methods

Materials PE prepared by transphosphatidylation of egg PC, *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)-PE (NBD-PE) and *N*-(lissamine Rhodamine B sulfonyl)-PE (Rh-PE) were purchased from Avanti Polar Lipids, Inc. (Birmingham, AL). OA and linoleic acid (LA) were obtained from Sigma Chemical Co. (St. Louis, MO). Stearic acid (SA), palmitic acid (PA) and dipicolinic acid (DPA) were from Wako Pure Chemical Ind., Ltd. (Osaka). α-Hydroxypalmitic acid (HOPA) and α-hydroxystearic acid (HOSA) were from Larodan Fine Chemicals (Malmo, Sweden). Ricinoleic acid (RA) and terbium trichloride (Tb) were from Nacalai Tesque (Kyoto). Aminonaphthalene-3,6,8-trisulfonic acid (ANTS) and *N,N-p*-xylylenebispyridinium bromide (DPX) were purchased from Molecular Probes, Inc. (Eugene, OR). All lipids were stored as chloroform stock solutions at −20 °C until used. The purity of lipids was confirmed chromatographically on silica gel plates.

Liposome Preparation Liposomes were prepared essentially by the method of Bangham *et al.*[8] Briefly, PE (7 μmol) and fatty acid (3 μmol) dissolved in chloroform were placed in a round-bottomed flask and dried to give a thin lipid film by evaporating. The lipid was suspended in 1 ml aqueous solution to be encapsulated and vortexed vigorously for 5 min. The resulting multilamellar vesicles were extruded under N_2 pressure at room temperature through polycarbonate membrane of 0.4 μm pore diameter (Nuclepore, Pleasanton CA). Liposomes were separated from a free fluorescent marker by gel filtration on a Sepharose 4B column (15 × 170 mm) equilibrated in 2 mM Tris, 148 mM NaCl, 0.2 mM ethylenediaminetetraacetic acid (EDTA), pH 9.0 (280 mOsm) at elution speed 0.5 ml/min. The size distribution of the liposomes was measured in an Autosizer, model 700 (Mulvern, England). The diameter was 343 ± 101 nm (mean ± S.D.) for PE/SA liposomes, 378 ± 130 nm for PE/PA liposomes, 292 ± 93 nm for PE/OA liposomes, 253 ± 82 nm for PE/LA liposomes, 322 ± 107 nm for PE/HOPA liposomes, 417 ± 136 nm for PE/HOSA liposomes, and 362 ± 123 nm for PE/RA liposomes. Lipid concentrations were determined by phosphate analysis.[9]

Measurements of Contents Leakage Release of contents from liposome was measured by encapsulating 12.5 mM ANTS/45 mM DPX/68 mM NaCl in 2 mM Tris–HCl buffer, pH 9.0 and following the increase in ANTS fluoresence upon dilution of contents into the medium. A full level of leakage (100% leakage) was determined by lysing the liposomes with 5% Triton X-100 (final concentration, 0.05%). ANTS fluoresence at 530 nm was measured in a Hitachi F-300 fluorescence spectrophotometer with an excitation wavelength of 360 nm.

Fusion Assays Membrane fusion was monitored by the Tb/DPA fluorescence assay for the intermixing of internal aqueous contents of liposomes.[10] Tb solution comprised of 5 mM $TbCl_3$/50 mM sodium citrate/48 mM NaCl was entrapped in one population of liposomes. DPA solution comprised of 50 mM DPA/73 mM NaCl was entrapped in a second liposome population. The two populations were suspended in 2 mM Tris–HCl buffer (pH 9.0) containing 148 mM NaCl/0.2 mM EDTA at equal concentrations of lipid (0.05 mol/ml). Intermixing of aqueous contents entrapped within the two liposome populations result in an increase in fluorescence signal due to formation of a Tb/DPA complex. Leakage of the Tb/DPA complex from liposomes leads to a decrease in fluorescence signal as a result of its dilution and interaction with EDTA. Fluorescence measurements were made with an excitation wavelength of 276 nm and emission wavelength of 545 nm, using a Toshiba cutoff filter to eliminate the contribution of light scattering to the signal.

Fusion was also monitored by the dilution assay of fluorescent lipid probes based on resonance energy transfer.[11] NBD-PE and Rh-PE were incorporated into one population of liposomes at 1 mol% each. The labeled liposomes were mixed with liposomes containing no fluorescent lipids at a ratio of 1 : 9. When such liposomes are fused, the two probes

are diluted with the other lipids. This dilution reduces the surface density of the fluorescent probe resulting in a decreased efficiency of resonance energy transfer, which is measured experimentally. NBD fluorescence measurements were made by using an excitation wavelength of 470 nm and an emission wavelength of 530 nm.

Determination of Light Scattering and Turbidity Aggregation of the liposomes was followed by an increase in turbidity at 450 nm in a Hitachi 220A spectrophotometer. Light-scattering change was monitored at 450 nm with a Hitachi fluorescence spectrophotometer.

Other Methods The pH of the liposome suspension was lowered by injecting 200 mM acetate solution (300 mOsm). Final pH of the suspension was measured by a Horiba pH electrode.

Results

pH Dependence of the Contents Leakage from Various Types of Liposomes We evaluated the pH sensitivity of liposomes by monitoring the release of pH-insensitive fluorophore ANTS from the liposomes into the medium. The pH of the medium was lowered from pH 9.0 to appropriate pH by adding acetate solution to the liposome suspensions. Figure 1 shows the time course of contents leakage from liposomes composed of PE and fatty acid such as SA, HOPA or RA at various pH. At pH 8.0 and 7.0, the leakage of contents was observed only in PE/RA liposomes. At pH 6.0, the leakage was observed in both PE/SA and PE/RA liposomes. PE/HOPA liposomes leaked contents only under a more acidic condition, pH 5.0. These results imply that the fatty acid constituting PE liposomes play an important role in the pH sensitivity of the contents leakage.

We also examined the pH-dependent leakage of liposomes containing other fatty acids. In Fig. 2, the extent of content leakage from liposomes was plotted as a function of pH. Values of the % leakage at 15 min after injection of an acid solution were plotted. A substantial contents leakage from liposomes was observed upon lowering the pH from 9.0 to acidic region. Leakage from PE/RA liposomes was induced by addition of less acid, and 50% of the contents leaked out at pH 7.6. PE liposomes containing SA, PA, OA or LA leaked 50% of the contents in the pH region of 6.5—6.9. PE/HOPA and PE/HOSA liposomes did not leak in this pH region, but 50% leakage was observed at pH 5.3 and 5.5, respectively. Liposomes consisting of pure PE showed broad response to pH change compared with other liposomes, and 50% leakage was observed at pH 6.8. These effects of the fatty acid could be described as follows: fatty acid stabilize the lipid bilayer above a certain pH, while below this pH, liposomes containing fatty acid are in a more unstable state than those made of pure PE. Then, the pH response curve of liposomes containing fatty acid show a drastic change in the pH region.

pH Dependence of Light Scattering and Turbidity The effect of pH on liposomal aggregation was examined. As shown in Fig. 3, light scattering of liposome suspensions increased by lowering the pH from a basic condition, and then gradually decreased in more acidic pH. The increase of light scattering was observed below about pH 7.2 in PE/SA and PE/OA liposomes, pH 6.2 in PE/HOPA and pH 8.2 in PE/RA liposomes. The pH dependence of light scattering change was similar to that of contents leakage in corresponding liposomes.

Figure 4 shows the time course of turbidity change induced by lowering the pH from 9.0 to 6.0. Turbidity increased rapidly in liposomes containing SA or OA. In

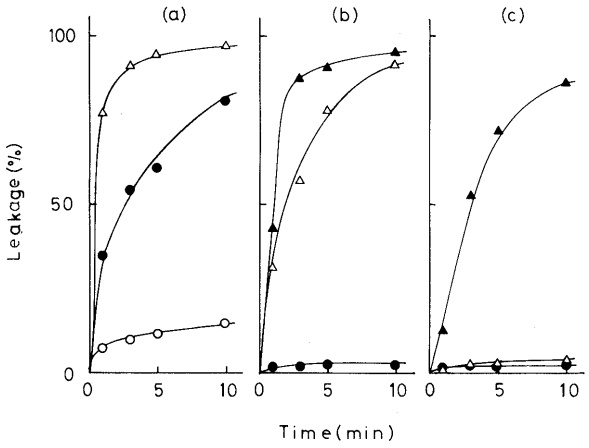

Fig. 1. Time Course of pH-Induced Contents Leakage from Liposomes

Leakage was followed by the dequenching of ANTS/DPX initially encapsulated in the liposomes combining PE with (a) RA, (b) SA, or (c) HOPA. At time zero, the pH of liposome suspensions (50 μM lipid) was reduced from 9.0 to the value: ○, pH 8.0; ●, pH 7.0; △, pH 6.0; ▲, pH 5.0.

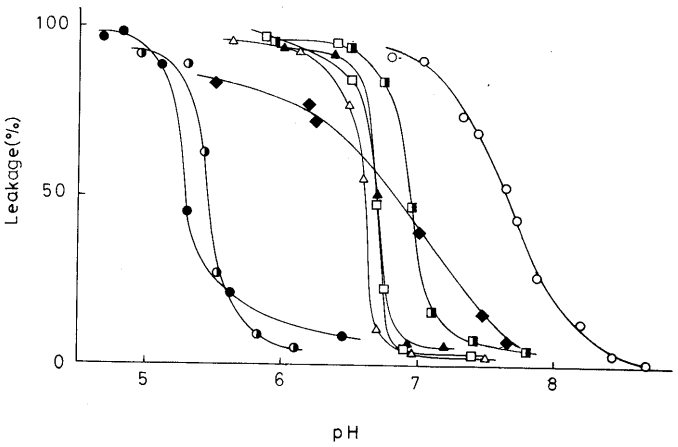

Fig. 2. pH-Dependence of the Contents Leakage from Liposomes

The leakage (%) from liposomes combining PE (◆) with ○, RA; ◑, HOSA; ●, HOPA; △, SA; ▲, PA; □, OA; ◨, LA was measured as in Fig. 1. Other details are described in the text.

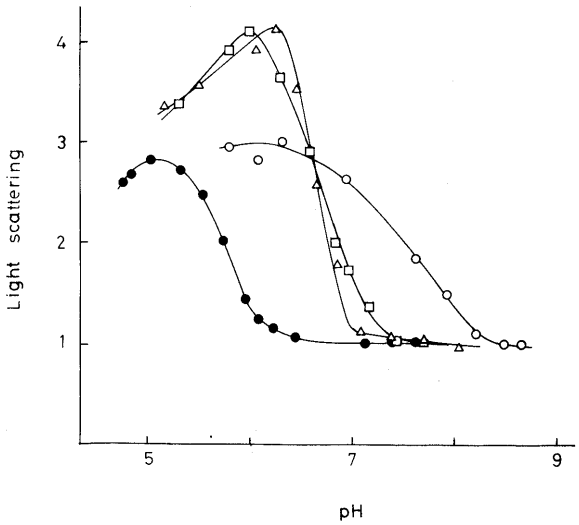

Fig. 3. pH-Dependence of Light Scattering Changes of Liposomal Suspensions

Light scattering changes at 5 min after acid injection into the suspension (50 μM lipid) of liposomes combining PE with RA (○), HOPA (●), SA (△) or OA (□) were measured at 450 nm and relative increase in light scattering was calculated.

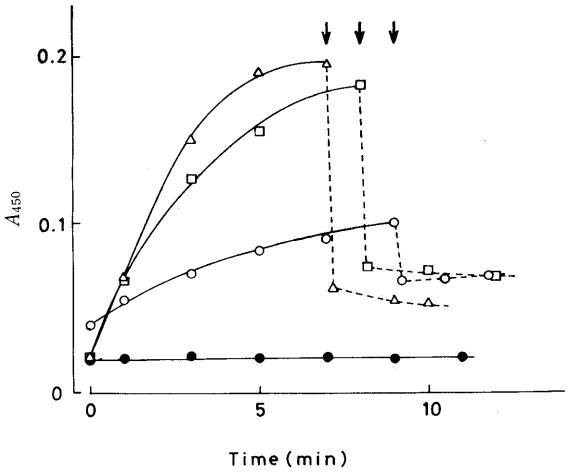

Fig. 4. Time Course of pH-Induced Turbidity Changes of Liposomal Suspensions

Turbidity change at 450 nm was monitored after acidification of the liposome suspensions (50 μM lipid) from pH 9.0 to 6.0. The pH was returned to 9.0 at times indicated by the arrows by the addition of NaOH solution. Liposomes were composed of PE and fatty acid: ○, RA; ●, HOPA; △, SA; □, OA.

PE/HOPA liposomes, turbidity did not change at pH 6, but changed at pH 5. The dashed lines show subsequent turbidity change after the pH was returned to pH 9. The turbidity level recovered to some extent but not to the same level. This suggests that reversible aggregation occurred, and that a partially irreversible event such as fusion took place.[12]

pH-Induced Fusion We first examined the effects of pH on the intermixing of aqueous contents between various types of liposomes, using the Tb/DPA assay for contents mixing. The time course of content mixing in some liposomes is shown in Fig. 5. In each liposome, an increase in fluorescence was observed after the addition of acetic acid, which was ascribed to the formation of Tb/DPA complex, initially encapsulated in separate populations of liposomes. PE/SA and PE/OA liposomes showed a rapid mixing of contents when the pH was reduced to below about 7.0. The subsequent decay of the signal indicates that dilution and dissociation of the Tb/DPA complex occurred, following the destabilization of the liposome membrane.[3,10] Apparent maximum was observed at pH 5.9. In PE/RA and PE/HOPA liposomes, slower mixing of contents was observed when the pH was reduced to below 7.7 or 6.8. Liposomes containing other fatty acid, *e.g.* PA or LA exhibited a similar pH-dependent mixing of contents with PE/SA liposomes. The difference of the overall kinetics by lipid compositions may reflect a different mechanism in the fusion process.

In order to examine pH-induced fusion of the liposomes, we also tried a lipid dilution assay, which is not subject to the complicating effects of contents leakage. As shown in Fig. 6, lipid mixing as well as contents mixing took place when these liposomes were exposed to more acidic conditions. The rate of lipid mixing in PE/HOPA and PE/HOSA liposomes was slower than that in PE/SA and PE/OA liposomes. The threshold pH of lipid mixing was about 5.5 in PE/HOPA liposomes, 6.5 in PE/OA, 6.7 in PE/SA and 8.2 in PE/RA. These results indicate that PE liposomes containing fatty acid have the ability to cause fusion at appropriate pH, depending on the lipid composition of liposome.

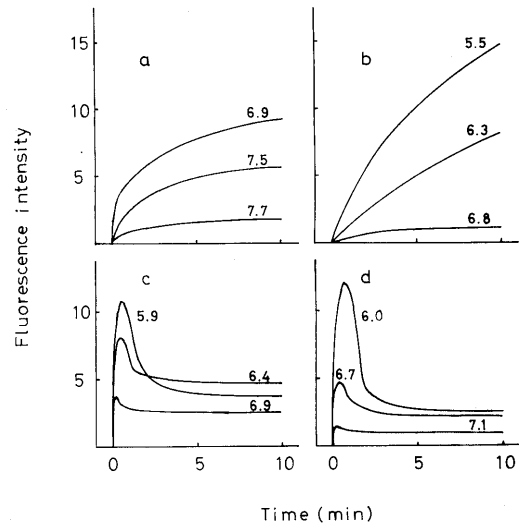

Fig. 5. Time Courses of Contents Mixing in Various Liposmes

Contents mixing for liposomes combining PE with (a) RA, (b) HOPA, (c) SA, or (d) OA was monitored by a Tb/DPA assay. At time zero, the pH of liposome suspensions was changed to the indicated values. Other details of the assays were as described in Materials and Methods.

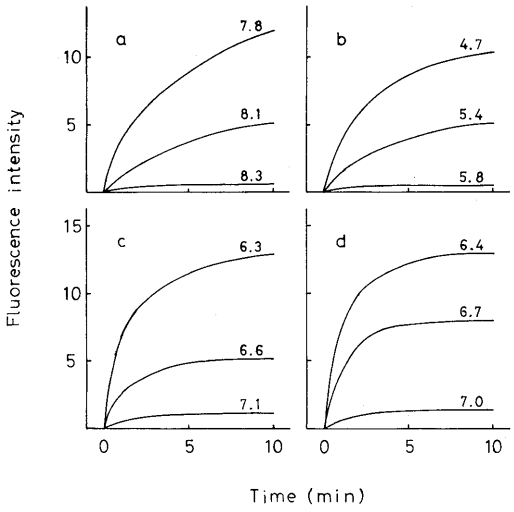

Fig. 6. Time Courses of Lipid Mixing in Various Liposomes

Lipid mixing for liposomes combining PE with (a) RA, (b) HOPA, (c) SA, or (d) OA was monitored by a fluorescent probe dilution assay. At time zero, the pH of liposome suspensions was changed to the indicated values. Other details were as described in Materials and Methods.

Discussion

The results presented in this study demonstrate that liposomes composed of PE and fatty acid can cause pH-dependent leakage, aggregation and fusion. As shown in Fig. 2, contents leakage in each type of liposome exhibited a different pattern of pH-dependence. In addition, the leakage patterns were fairly close in the pH range where scattering change (Fig. 3) and fusion (Figs. 5 and 6) were observed. As described in other pH-sensitive liposome systems,[13] leakage of the encapsulated contents requires liposome–liposome interaction. This is because the destabilization process involves the formation of the intermembranous intermediate (IMI)[14] or isotropic phase. In our pH-sensitive liposome system, it is also conceivable that contents leakage was caused by the formation of a non-

bilayer structure in the process of aggregation and fusion.

PE has a much less interfacial hydration than PC and has the propensity to form nonbilayer, as described in many reports.[15] The presence of dissociated fatty acid at higher pH values would increase the hydration of the membrane surface and the repulsive hydration force between bilayers of liposome. On the other hand, protonation of titratable groups at low pH would reduce the interfacial hydration and repulsive force. It would become easy for liposomes to come into contact, and would cause the formation of a nonbilayer structure which bring about the leakage of contents. From this viewpoint, it appears that whether the carboxyl group dissociates or not is a primary determinant of the stabilization of PE liposomes. We have no data concerning the pK_a of fatty acids under the same conditions as our experiment, but it is well known that pK_a of α-hydroxy fatty acid is generally less than that of normal fatty acid. Therefore, threshold pH of destabilization in liposomes composed of HOPA or HOSA would shift to a more acidic pH region. Recinoleic acid, however, has hydroxyl group at 12 position and it might have little effect on pK_a of the carboxyl group. Recinoleic acid may have a property to destabilize the lipid bilayer.

pH-sensitive liposomes have attracted attention as effective carriers for the cytoplasmic delivery of biologically active molecules.[2] Their potential is largely due to their ability to fuse with other lipid membrane and to release contents in low pH environments. Negatively charged liposomes are internalized mainly through an endocytic pathway.[2,16] The efficiency of delivery of contents is expected to be greater if the liposome undergoes fusion with endocytic vesicles (roughly pH 5.0—6.5). Then, it will be of interest to compare the interactions of cells with the variety of liposomes prepared in this study.

In conclusion, our results demonstrate that pH-sensitive liposomes allowed to fuse and release contents at desired pH could be prepared by altering the lipid composition. Such liposomes may be useful as a drug delivery system which could release contents in response to a local pH condition.

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