er rate constants, k_2, were obtained from the slope of a plot of k_{obs} versus amine with more than five concentrations of more than three runs and were reproducible to within ±3%.

Product analysis. p-Nitrophenylacetyl chloride was reacted with excess p-chloroaniline with stirring for more than 15 half-lives at −15.0 °C in acetonitrile, and the products were isolated by evaporating the solvent under reduced pressure. The physical constants after recrystallization were:

p-NO_2-$C_6H_4CH_2$-C(=O)NHC_6H_4-p-Cl: mp 158-160 °C; δ_H ($CDCl_3$), 6.6-7.4 (Ph-H, 8H, m), 3.6 (NH, 1H, s), 4.3 (CH_2, 2H, s). ν_{max}, (KBr), 3400 (N-H). 1760 (C=O). m/z, 290 (M^+).

Acknowledgment. We thank Korea Science and Engineering Foundation and Inha University for support of this work.

References

1. (a) Bender, M. L. *Chem. Rev.* **1960**, *53*, 60. (b) Jencks, W. P. *Chem. Soc. Rev.* **1981**, *10*, 354.
2. Williams, A. *Chem. Soc. Rev.* **1994**, *23*, 93.
3. Palling, D. J.; Jencks, W. P. *J. Am. Chem. Soc.* **1984**, *106*, 4869.
4. Bond, P. M.; Castro, E. A.; Moodie, R. B. *J. Chem. Soc. Perkin Trans. 2* **1976**, 68.
5. (a) Litvinenko, L. M.; Shpanko, I. V.; Korostylev, A. P. *Zh, Org. Khim.* **1981**, *17*, 972. (b) Shpanko, I. V. *Mendeleev Commun.* **1991**, 119. (c) Rogne, O. *J. Chem. Soc. Perkin Trans. 2* **1975**, 1486. (d) Lee, I.; Shim, C. S.; Chung, S. Y.; Kim, H. Y.; Lee, H. W. *J. Chem. Soc., Perkin Trans. 2* **1988**, 1919.
6. Song, B. D.; Jencks, W. P. *J. Am. Chem. Soc.* **1989**, *111*, 8479.
7. Jedrzejczak, M.; Motie, R. E.; Satchell, D. P. N.; Satchell, R. S.; Wassef, W. N. *J. Chem. Soc., Perkin Trans. 2* **1994**, 1471.
8. Yew, K. H.; Koh, H. J.; Lee, H. W.; Lee, I. *J. Chem. Soc., Perkin Trans. 2* **1995**, 2263.
9. Koh, H. J.; Lee, H. C.; Lee, H. W.; Lee, I. *Bull. Korean Chem. Soc.* **1996**, *17*, 712.
10. Kim, T. H.; Huh, C.; Lee, B.-S.; Lee, I. *J. Chem. Soc., Perkin Trans. 2* **1995**, 2257.
11. Oh, H. K.; Woo, S. Y.; Shin, C. H.; Lee, I. *J. Org. Chem.* **1997**, *62*, 5780.
12. Oh, H. K.; Lee, J. Y.; Yun, J. H.; Park, Y. S.; Lee, I. *Int. J. Chem. Kinet.*, In press.
13. Lee, I. *Chem. Soc. Rev.* **1995**, *24*, 223.
14. Lee, I.; Koh, H. J.; Lee, B. C. *J. Phys. Org. Chem.* **1994**, *7*, 50.
15. Lee, I. *Adv. Phys. Org. Chem.* **1992**, *27*, 57.
16. Dewar, M. J. S.; Daugherty, R. C. *The PMO Theory of Organic Chemistry*; Plenum, New York, 1975; p 212.
17. (a) le Noble, W. J.; Asano, T. *J. Am. Chem. Soc.* **1975**, *97*, 1778. (b) le Noble, W. J.; Miller, A. R. *J. Org Chem.* **1979**, *44*, 889. (c) Lee, I.; Rhyu, K. W.; Lee, H. W.; Shim, C. S.; Oh, H. K. *J. Phys. Org. Chem.* **1990**, *3*, 751.
18. Lee, I.; Koh, H. J.; Lee, H. W. *J. Chem. Soc., Perkin Trans. 2* **1993**, 1575.
19. (a) Lee, I.; Shin, C. S.; Kim, H. Y.; Lee, H. W. *J. Chem. Soc., Perkin Trans. 2* **1988**, 1919. (b) Bordwell, F. G.; Boutan, P. J. *J. Am. Chem. Soc.* **1956**, *78*, 87.
20. Lee, I.; Koh, H. J.; Lee, B.-S.; Lee, H. W.; Choi, J. H. *Bull. Korean Chem. Soc.* **1995**, *11*, 435.
21. Guggenheim, E. A. *Phil. Mag.* **1926**, *2*, 538.

New pH-Sensitive Liposomes Using Bis(6-hemisuccinyloxyhexyl) Fumarate

Ji Young Jin and Youn-Sik Lee*

Faculty of Chemical Engineering and Technology, Institute of Cardiovascular Research, Chonbuk National University, Chonju 561-756, Korea

Received January 26, 1998

In order to develop pH-sensitive liposomes that are stable in plasma, liposomes containing membrane-spanning bipolar amphiphiles as protonatable components were studied. Sonicated small unilamellar liposomes composed of dioleoylphosphatidylethanolamine (DOPE), dioleoylphosphatidylcholine (DOPC) and bis(6-hemisuccinyloxyhexyl) fumarate (BHF) in a 3 : 1 : 1 molar ratio are stable at neutral pH, but destabilized at weakly acidic pH with 50% leakage of entrapped materials at about pH 5.5. The liposomes are relatively stable in plasma such that only a few percent entrapped calcein was released in 50% plasma within 1.5 h incubation at 37 °C, while about 10% entrapped calcein was released from sonicated liposomes composed of DOPE , DOPC, and oleic acid (OA) in a 3 : 1 : 1 molar ratio under the identical conditions. The aqueous contents mixing and lipid components mixing experiments suggest that the protonation of BHF may induce fusion between the liposomes, followed by the release of the entrapped materials.

Introduction

Immunoliposomes are taken up by cells through the endocytic pathway, and many drugs, especially the macromolecular drugs, are hydrolyzed in the lysosome, thus resulting in poor biological activities of the delivered drug.[1] The pH-sensitive liposomes have been designed to circumvent the lysosomal catabolic degradation by delivering

*To whom correspondence should be addressed.

entrapped drugs at a pre-lysosomal site. It is believed that a liposome-endosome fusion occurs with the release of entrapped drugs into the cytoplasm when the pH-sensitive liposomes are exposed to endosomes of pH 5.0-6.5.

One approach to the pH-sensitive liposome systems is to construct liposomes by combining phosphatidylethanolamine (PE) with amphiphiles containing a titratable group which include fatty acids,[2,3] palmitoyl homocysteine,[4] N-succinyldioleoylphosphatidylethanolamine (COPE),[5] cholesterol hemisuccinate (CHEMS)[6] and diacylsuccinylglycerol (DASG).[7,8] Unsaturated PE such as dioleoylphosphatidylethanolamine (DOPE) prefers to adopt an inverted hexagonal (H_{II}) phase rather than the bilayer sheet under the physiological conditions.[9] The titratable amphiphiles stabilize the PE bilayers at neutral pH and allow the intra- and/or inter-liposomal bilayers to be in contact with one another at acidic pH. Then the adhered PE bilayers become destabilized, followed by mixing and/or releasing of the encapsulated aqueous contents.[6,10-12] However, the stability of the pH-sensitive liposomes is relatively poor as compared to phosphatidylcholine (PC)-based liposomes. For example, the pH-sensitive liposomes composed of unsaturated PE and oleic acid (OA) rapidly aggregate and become leaky in plasma.[13] In order to overcome this problem, several different liposome systems such as liposomes composed of unsaturated PE and double chained-protonatable components instead,[5,7,8] partially polymerized liposomes,[14] and PC liposomes bearing a poly(ethylene glycol) derivative with carboxyl groups,[15] have been designed.

Recently, we reported a new type of pH-sensitive liposomes containing a membrane-spanning bipolar amphiphile such as bis(6-hemisuccinyloxyhexyl) muconate (BHM) as a protonatable component (Figure 1).[16,17] Liposomes composed of DOPE, dioleoylphosphatidylcholine (DOPC) and BHM in a 3 : 3 : 1 molar ratio were stable at neutral pH, but leaky at acidic pH. However, BHM molecules in the liposomal membranes were readily polymerized *via* 1,2-polymerization process on the expose of light (254 nm) due to the muconyl group in the molecule.[17] The polymerization may alter the liposomal properties such as fusion, lipid mixing, and leakage. Thus we synthesized bis(6-hemisuccinyloxyhexyl) fumarate (BHF) instead, as a protonatable component, which is much less sensitive to light. In this paper, we report some important properties of new pH-sensitive liposomes containing the bipolar amphiphiles.

Experimental

Materials. Calcein, 1,6-hexanediol, fumaryl chloride, succinic anhydride and tert-butyldimethylsily chloride were purchased from Sigma Chemical Co. Thin layer chromatography (TLC) plate and silica gel (200-400 mesh ASTM) were purchased from E. Merk Co. N-(7-nitro-2,1,3-benoxadiazol-4-yl) dioleoylphosphatidylethanolamine (NBD-DOPE) and N-(lissamine rhodamine B sulfonyl) dioleoylphosphatidylethanolamine (Rh-DOPE), DOPE, and DOPC were purchased from Avanti Polar Lipids (Birmingham, AL). 8-Aminonaphthalene-1,3,6-trisulfonic acid disodium salt (ANTS) and p-xylenebispyridinium bromide (DPX) were purchased from Molecular Probes, Inc.

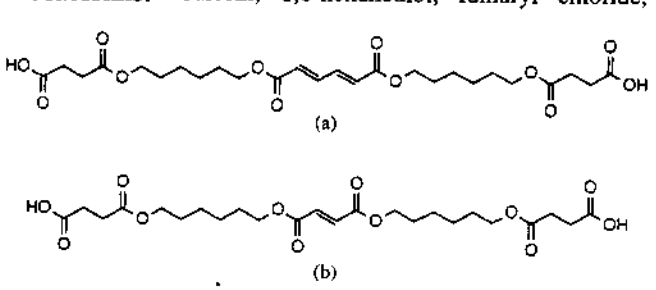

Figure 1. Chemical structures of (a) bis(6-hemisuccinyloxyhexyl) muconate (BHM) and (b) bis(6-hemisuccinyloxyhexyl) fumarate (BHF).

Synthesis of bis(6-hemisuccinyloxyhexyl) fumarate (BHF). BHF was synthesized by a modification of the procedure of Jin *et al.*[17] Briefly, bis(6-hydroxyhexyl) fumarate was obtained by reaction between fumaryl chloride and 6-tert-butyldimethylsilyloxyhexan-1-ol which was prepared from 1,6-hexanediol and tert-butyldimethylsilyl chloride, followed by desilylation with an aqueous HCl solution. Finally, a reaction of bis(6-hydroxyhexyl) fumarate with succinic anhydride in toluene yielded BHF as a white solid. The overall yield of BHF was about 30% based on fumaryl chloride. Analysis. Calcd. $C_{12}H_{36}O_{12}$: C, 55.81; H; 7.02. Found: C, 55.99; H, 6.99. mp 76 °C. ^{1}H NMR ($CDCl_3$): δ 1.6-1.8 (m, 16H, CH_2), 2.5 (t, 4H, CH_2-COO), 2.6 (t, 4H, CH_2-COO), 4.1 (t, 4H, CH_2-OOC), 4.2 (t, 4H, CH_2-OOC), 6.8 (s, 2H, -CH=CH-). UV: λ_{max}=220 nm, methanol, ε=11,500.

Measurements of pH-sensitivity. Various combinations of phospholipids such as DOPE and DOPC with BHF were dried from chloroform solution with a rotary evaporator. The dried lipid films were vacuum desiccated for 1 h and suspended in an aqueous calcein solution (50 mM adjusted to 300 mOsm/L with NaCl, pH 8.0). The samples were then incubated at 4 °C overnight and then sonicated with a tip type sonicator (Misonic Inc.) for 10 min, followed by a 10 min resting period. The samples were then sonicated for an additional 10 min. The suspension was chromatographed on Bio-Gel A 0.5-m column equilibrated with PBS (phosphate buffered saline, 300 mOms/L, pH 7.4) to remove unentrapped calcein. The lipid concentration of each sample was adjusted by measuring the UV absorbance of BHF in methanol at 220 nm. The mean diameter of the liposomes composed of DOPE, DOPC and BHF in a 3 : 1 : 1 molar ratio was estimated to be 100±20 nm by laser light scattering (Shimadzu Sald-2001). Liposomes (4.4×10^{-4} M) were incubated in a PBS solution of various pH at 37 °C for 1 h, and then adjusted to pH 7.4 by adding an appropriate amount of 0.1 mM NaOH solution. A fluorometer (Simoaminoco Luminescence Spectrometer, Series 2) was used to measure the fluorescence intensity of calcein. The excitation and emission wavelengths were 490 nm and 520 nm, respectively. Percent release was calculated from the following formula:

$$\% \text{ Release} = [(F_t - F_0)/(F_\infty - F_0)] \times 100$$

where F_0=fluorescence intensity of liposomes at pH 7.4, F_t= fluorescence intensity after acid incubation at different pH and F_∞=fluorescence intensity after lysis of liposomes with Triton X-100.

Measurements of plasma stability. Calcein-containing liposomes (1.7×10^{-4} M) were prepared by the procedure as described above to determine the stability of lipo-

somes in human plasma. The liposome suspension and the same volume of human plasma were prewarmed to 37 °C and mixed together. 2 mL of the mixture were taken for the fluorescence measurement at different incubation time-points. Percent release of liposomal calcein was calculated as above, except F_0=fluorescence intensity of liposomes in PBS at time zero, F_t=fluorescence intensity at different time-points.

Aqueous content mixing assay. The extent of aqueous content mixing was measured for DOPE/DOPC/BHF (3/1/1) liposomes by the ANTS/DPX method.[12] With the ANTS/DPX fusion assay, mixing of aqueous contents of ANTS and DPX-containing liposomes was registered at pH 5.0 and 6.0 as a decrease in ANTS fluorescence due to quenching of ANTS fluorescence by DPX. All solutions were buffered with 10 mM Tris-HCl at pH 7.4. In one population of liposomes, 12.5 mM ANTS was entrapped, and 45 mM DPX was entrapped in a second liposome population. The fluorescence scale was calibrated with the fluorescence of a 1/1 mixture of ANTS/DPX liposomes in Tris-HCl buffer taken as 100% fluorescence (0% fusion). The 0% fluorescence level was set to the intensity of the liposomes containing the coencapsulated ANTS/DPX. Excitation was at 360 nm and emission at 545 nm.

Lipid mixing assay. DOPE/DOPC/BHF (3/1/1) containing 1 mol% each of the fluorescent lipid analogues NBD-PE and Rh-PE were mixed with DOPE/DOPC/BHF (3/1/1) liposomes devoid of the fluorescent lipids. The ratio of fluorescent to nonfluorescent liposomes was 1/9. Lipid mixing was registered as an increase in fluorescence from the NBD probe, which is due to a decreased energy transfer between NBD-PE and Rh-PE as the two probes are diluted. The 100% fluorescence level was set with DOPE/DOPC/BHF (3/1/1) liposomes containing 0.1 mol% each of NBD-PE and Rh-PE at the same total lipid concentration (4.6 mM). Excitation was set at 450 nm, and emisson was measured at 530 nm.

Results and Discussion

BHF did not form monolayer vesicles by itself due to the difference in the outer and inner radius curvature of the vesicle.[16-19] Any combination of DOPE with BHF at pH 7.4 did not form vesicles, either. Various combinations of DOPE, DOPC and BHF were tested in order to determine a proper ratio of the components in preparing the pH-sensitive liposomes. A mixture of DOPE, DOPC and BHF in a 3 : 1 : 1 molar ratio gave the best results in terms of the stability and pH-sensitivity of liposomes. For example, a mixture of DOPE, DOPC and BHF in a 4 : 1 : 1 molar ratio did not encapsulate the calcein solution, indicating that the mixture does not form any stable liposomes. The pH-sensitivity of liposomes composed of DOPE, DOPC and BHF decreased as the ratio changes from 3/1/1 to 3/3/1 (data not shown). This is a somewhat different result from that of the DOPE/DOPC/BHM mixtures where the best pH-sensitivity with enough stability was observed from DOPE/DOPC/BHM (3/3/1) liposomes. Furthermore, hydration of the mixtures appeared to be easier when the mixtures contained BHF rather than BHM. The calcein-containing liposomes composed of DOPE, DOPC and BHF in a 3 : 1 : 1 molar ratio in PBS of pH 7.4 were stored at 4 °C, and 2 mL of the mixture were taken for the fluorescence measurement at different incubation time-points. Less than 10% of the encapsulated material were released in the buffer solution for two months. This result indicates that the liposome suspension can be prepared in a large quantity and used for a long-term research.

pH-sensitivity. DOPE/DOPC/BHF (3/1/1) liposomes are stable at neutral pH, but leaky at acidic pH as reported for DOPE/DOPC/BHM (3/3/1) liposomes (Figure 2). The release of entrapped calcein in the liposomes increases as the pH decreases with 50% leakage at about pH 5.5, indicating that the liposomes are pH-sensitve. The liposomes of pH 4.8 and 5.8 had increased fluorescence intensity that reached a plateau within 40 and 50 min, respectively (Figure 3). This result indicates that the leakage rates of the

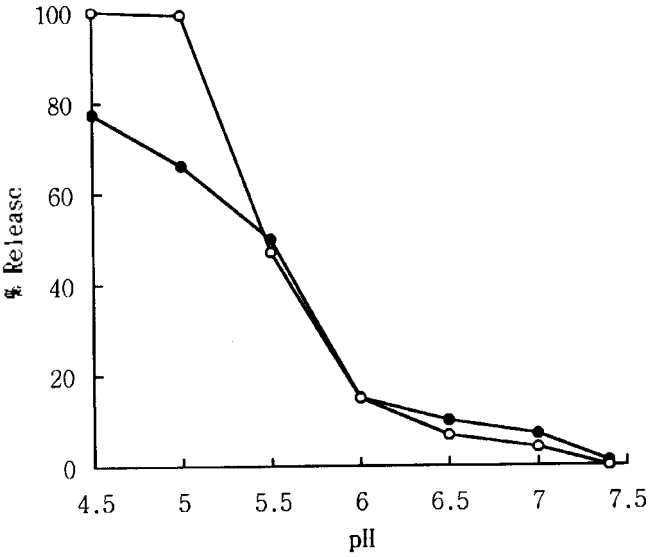

Figure 2. pH-dependent calcein release from DOPE/DOPC/BHF (3/1/1) (●), DOPE/DOPC/BHM (3/3/1) (○) liposomes in PBS at 37 °C. The percentage values varied by less than ±5% between three runs.

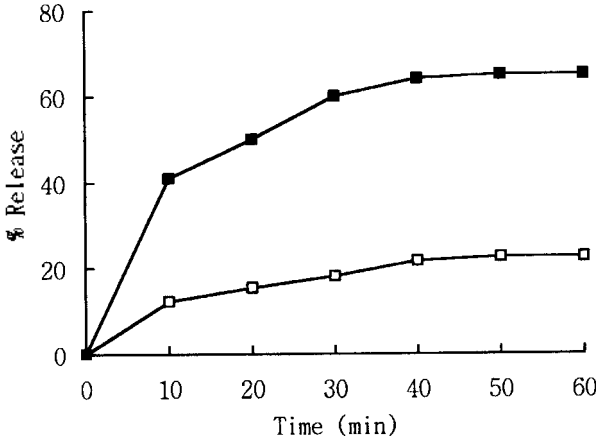

Figure 3. Time courses of leakage of entrapped calcein from DOPE/DOPC/BHF (3/1/1) liposomes in PBS of pH 4.8 (■) and 5.8 (□) at 37 °C. Each time point was measured in duplicate and mean values were taken for the plot.

liposomes are pH-dependent and the 1h-incubation at 37 °C is long enough for measurements of the fluorescence intensity of the released calcein.

To find the role of the BHF in the liposomes, DOPE/DOPC (3/1) liposomes devoid of BHF were prepared and tested for the pH-sensitivity at 37 °C. But there was no significant change in the fluorescence intensity as the pH of the medium decreased. This result indicates the leakage of the entrapped calcein from the liposomes is initiated by the protonation of BHF since the pKa of BHF should lie in the region of 4.2-5.6.[6]

Plasma stability. Leakage of entrapped calcein from DOPE/DOPC/BHF (3/1/1) and DOPE/DOPC/OA (3/1/1) liposomes incubated in 50% human plasma at 37 °C was recorded by measurements of calcein fluorescence (Figure 4). Only a few percent leakage from the liposomes containing BHF was observed while about 10% of the entrapped calcein was released from the liposomes containing OA within 1.5 h. This result indicates that the bipolar BHF molecules more stabilize the pH-sensitive liposomes in plasma than the fatty acid molecules. In order to remove the membrane-spanning bipolar amphiphiles embedded in the liposome membrane, one has to get rid of water molecules associated with the inner hydrophilic headgroup and push it through the hydrophobic membrane core. This process would be energetically very unfavorable. However, if all the bipolar molecules assume a U-shaped conformation rather than the extended transmembrane arrangement, they will be also readily removed from the liposomal membranes. It has not been determined yet whether the fumaryl-based amphiphile actually is fully extended or U-shaped (bent) in the liposomal membranes. Even though the bipolar amphiphiles are randomly arranged in the membranes, *i.e.*, partly extended and partly U-shaped, liposomes composed of the bipolar molecules will be more stable in plasma as compared to liposomes composed of the conventional protonatable components instead. Futhermore, the bipolar amphiphile can be regarded as an integral protein. Thus it is believed that the enhanced stability of DOPE/DOPC/BHF (3/1/1) liposomes in plamsa is at least partly due to the presence of BHF in the bilayer membranes.

Aqueous content mixing. Most of pH-sensitive liposomes reported so far undergo fusion or a phase transition to the H_{II} phase at weakly acidic pH, followed by the leakage of the entrapped materials.[6,10-12] We therefore examined the effects of pH on properties of the DOPE/DOPC/BHF (3/1/1) liposomes in order to determine if these or other mechanisms are responsible for the release of entrapped calcein. Liposome fusion cannot occur without mixing of the aqueous contents in liposomes. The fusion process between liposomes can be easily assayed by measuring the decrease in the fluorescence of ANTS when the ANTS-entrapped liposomes are mixed with another DPX-entrapped liposomes. The fluorescence intensity of ANTS decreased by about 10 and 20% within 40 min at pH 6.0 and 5.0, respectively (Figure 5). The percentages of fusion are not great compared to those of the acid-induced leakage shown in Figure 2 at the similar pH. However, this is understandable because the percentage of ANTS quenched by DPX at a given time depends upon both the fusion kinetics and the leakage kinetics of the fused products even though the number of fused liposomes increases with time.[12] In fact, vesicles composed of double-chained anionic bipolar molecules did fuse with one another when the amphiphiles became uncharged at low pH.[22] Thus this experimental result suggests that the bilayer destabilization and leakage may occur subsequent to the acid-induced fusion between the liposomes.

Lipid mixing. Lipid components should be mixed if the liposomes undergo fusion.[11] The extent of lipid mixing between liposomes can be easily estimated by measuring the increase in the fluorescence of NBD-PE when liposomes containing both Rh-PE and NBD-PE are diluted with liposomes devoid of the fluorophores. If the lipid components are mixing, the fluorophores will be also diluted, and the efficiency in quenching of the NBD-PE fluorescence by Rh-PE will be lowered accordingly, resulting in increase in the NBD-PE fluorescence. The lipid mixing was tested as described in the experimental section by using the procedure of Struck *et al.*[23] The extent of lipid mixing

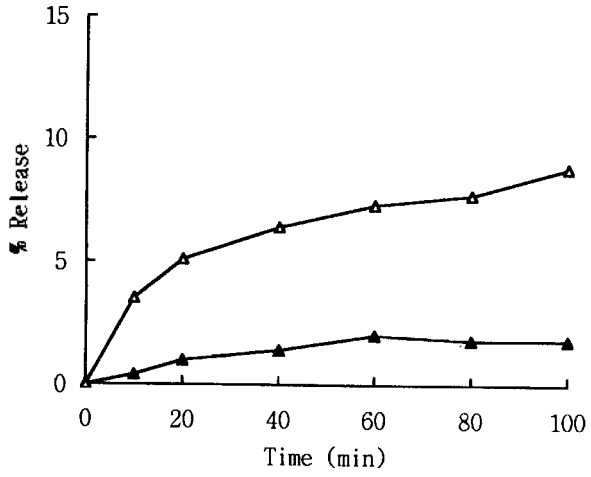

Figure 4. Release of entrapped calcein from DOPE/DOPC/BHF (3/1/1) (▲) and DOPE/DOPC/OA (3/1/1) (△) liposomes in 50% human plasma at 37 °C. The percentage values varied by less than ±3% between three runs.

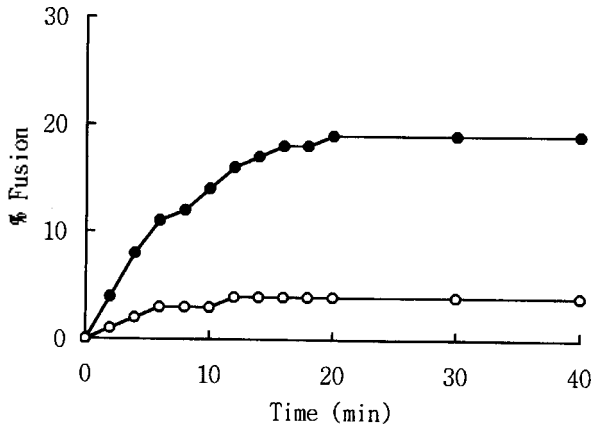

Figure 5. Time courses of aqueous contents mixing entrapped in DOPE/DOPC/BHF (3/1/1) liposomes at pH 5.0 (●) and 6.0 (○). Each time point was measured in duplicate and mean values were taken for the plot.

increased from 0 to 22, 55, and 74% as the pH of the medium decreased from 7.4 to 6.7, 6.2 and 5.0, respectively. This result indicates that the lipid components in DOPE/DOPC/BHF (3/1/1) liposomes are mixed at weakly acidic conditions. Based on the results obtained from the aqueous contents mixing and lipid components mixing experiments, the liposome-liposome fusion is probably responsible for the leakage of the entrapped materials from the DOPE/DOPC/BHF (3/1/1) liposomes at acidic pH.

Summary

Liposomes composed of DOPE, DOPC and BHF in a 3 : 1 : 1 molar ratio are stable at neutral pH, but become leaky at weakly acidic conditions. The pH-sensitive liposomes are more stable than DOPE/DOPC/OA (3/1/1) liposomes in plasma. The enhanced stability of the liposomes is partly due to the presence of BHF, a membrane-spanning component. Based on the results obtained from the aqueous contents mixing and lipid components mixing experiments, the leakage of entrapped calcein from the pH-sensitive liposomes is probably induced by the liposome-liposome fusion that occurs on the protonation of BHF at acidic conditions.

Acknowledgment. The authors wish to acknowledge the financial support of the Korea Research Foundation made in the program year of 1996 (Grant No. 01-D-0266). We also thank Hee-Jun Kim and Eun-Joeng Kim for the technical assistance in the fluorescence measurements.

References

1. Litzinger, D. C.; Haung, L. *Biochim. Biophys. Acta* **1992**, *1113*, 201.
2. Haung, L.; Liu, S. S. *Biophys. J.* **1984**, *45*, 72.
3. Conner, J.; Haung, L. *Cancer Res.* **1986**, *46*, 3431.
4. Conner, J.; Yatvin, M. B.; Haung, L. *Proc. Natl. Acad. Sci. USA* **1984**, *81*, 1715.
5. Nayar, R.; Schroit, A. J. *Biochemistry* **1985**, *24*, 5967.
6. Ellen, H.; Bentz, J.; Szoka, F. C. *Biochemistry* **1984**, *23*, 1532.
7. Collins, D.; Litzinger, D. C.; Haung, L. *Biochim. Biophys. Acta* **1990**, *1025*, 234.
8. Tari, A. M.; Fuller, N.; Boni, L. T.; Collins, D.; Rand, P.; Haung, L. *Biochim. Biophys. Acta* **1994**, *1192*, 253.
9. Cullis, O. R.; De Kruiff, B. *Biochim. Biophys. Acta* **1979**, *559*, 399.
10. Ellens, H.; Siegel, D. P.; Alford, D.; Yeagle, P. L.; Boni, L.; Lis, L. J.; Quinn, P. J.; Bentz, J. *Biochemistry* **1989**, *28*, 3692.
11. Duzgunes, N.; Allen, T. M.; Fedor, J.; Papahadjoulos, D. *Biochemistry* **1987**, *26*, 8435.
12. Ellen, H.; Bentz, J.; Szoka, F. C. *Biochemistry* **1985**, *24*, 3099.
13. Conner, J.; Norley, N.; Haung, L. *Biochim. Biophys. Acta* **1986**, *884*, 474.
14. Choi, M-J.; Han, H-S.; Kim, H. *J. Biochem.* **1992**, *112*, 694.
15. Kono, K.; Zenitan, K-I.; Takagishi, T. *Biochim. Biophys. Acta* **1994**, *1193*, 1.
16. Jeong, M. H.; Lee, Y-S.; Jin, J. Y.; Ko, S. B. *Bull. Korean Chem. Soc.* **1996**, *17*, 875.
17. Jin, J. Y.; Ko, S. B.; Jeong, M. H.; Choi, K. H.; Lee, Y-S. *J. of Kor. Ind. & Eng. Chem.* **1997**, *8*, 59.
18. Okahata, Y.; Kunitake, T. *J. Am. Chem. Soc.* **1979**, *101*, 5231.
19. Bader, H.; Ringsdorf, H. *J. Polym. Sci., Polym. Chem. Ed.* **1982**, *20*, 1623.
20. Bader, H.; Ringsdorf, H. *Faraday Discuss. Chem. Soc.* **1986**, *81*, 329.
21. Moss, R. A.; Fujita, T.; Okumura, Y. *Langmuir* **1991**, *7*, 2415.
22. Fuhrhop, J-H.; David, H-H.; Mathieu, J.; Liman, U.; Winter, H-J.; Boekema, E. *J. Am. Chem. Soc.* **1986**, *108*, 1785.
23. Struck, D.; Hoekstra, D.; Pagano, R. E. *Biochemistry* **1981**, *20*, 4093.