Volume 305, number 3, 185–188 FEBS 11223 July 1992


# Interaction between oleic acid-containing pH-sensitive and plain liposomes

## Fluorescent spectroscopy studies

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Received 7 May 1992

The energy transfer method has been applied to study the interaction between pH-sensitive liposomes (phosphatidyl ethanolamine/oleic acid/cholesterol, 4:2:4 molar ratio) and plain liposomes (phosphatidyl choline/phosphatidyl ethanolamine/cholesterol, 4:2:3 molar ratio). It was shown that a slow fusion process occurs between two types of liposomes. Also, the transfer of oleic acid from pH-sensitive liposomes to plain liposomes takes place. This transfer results in the increased permeability of both pH-sensitive and plain liposomes, facilitating the release of liposome-entrapped fluorescent dye. The data obtained were used for a possible explanation of the mechanism of intracytoplasmic drug delivery by pH-sensitive oleic acid-containing liposomes.

pH-sensitive liposome; Membrane fusion; Oleic acid; Energy transfer method

## 1. INTRODUCTION

It has been shown that the use of pH-sensitive liposomes (in particular those containing oleic acid) increases the cytoplasmic delivery of liposome-entrapped compounds [1,2]. pH-Sensitive liposomes are known to release their contents when the pH value decreases. A decreased pH is characteristic of the internal compartment of endosomes formed in the process of the capture of liposome by cells. This fact may explain the release of the liposomal content into the inner endosome compartment. The penetration of the compound through the endosomal membrane into the cytoplasm still remains unexplained.

Connor and Huang [3] consider several possible routes for the cytoplasmic delivery of the liposomal content. According to the first of these, the decrease in pH value induces the fusion of a liposome with the endosome membrane and the subsequent release of liposomal content into the cytoplasm. The second possibility implies the primary release of the substance delivered from liposomes and then its diffusion from the endosome following the concentration gradient. The third way is endosome membrane permeabilization upon interaction with pH-sensitive liposomes.

The data of [4] demonstrating the active fusion process between pH-sensitive liposomes and inner mytochondrial membranes seem to confirm the possibility of membrane fusion between liposome and endosome. Huang and Liu [4] used cholesterol-free liposomes. At the same time it is known that cholesterol strongly influences lipid membrane properties [5–7], and being incorporated into pH-sensitive liposomes it increases their stability and encapsulation efficacy at neutral pH values [8].

In the present study we have investigated the interaction between cholesterol- and oleic acid-containing pH-sensitive liposomes and pH-insensitive liposomes, the phospholipid composition of which imitates that for the endosomal membrane to some extent.

*Abbreviations:* PE, egg phosphatidyl ethanolamine; PC, egg phosphatidyl choline; Chol, cholesterol, OA, oleic acid; SRh, sulforhodamine B; [$^{14}$C]PE, [$^{14}$C]dioleoyl PE; Rh-PE, *N*-(lysamine rhodamine B sulfamine)-PE; NBD-PE, *N*-(7-nitro-1,2,3-benzoxadiazol-4-yl)-PE; pH-Lip, pH-sensitive liposomes; P-Lip, plain liposomes; FT, liposomes, prepared by freeze-thawing method; REV, liposomes prepared by reverse phase evaporation; PBS, phosphate-buffered saline; TLC, thin layer chromatography.

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## 2. MATERIALS AND METHODS

### 2.1. *Materials*

PE was purified according to [9]. PC was a product of Kharkov Plant of Bacterial Preparations (Ukraine). TLC in chloroform/methanol/water (65:25:4) with the development in iodine vapour proved PC homogenicity. Chol, OA, calcein and SRh were the products of Sigma

Co. [$^{14}$C]PE, and [$^{3}$H]OA were obtained from Amersham. Rh-PE and NBD-PE were from Molecular Probes. All components of buffer solutions were of ASA grade. Distilled and deionized water was used in all experiments. Diethyl ether was kept for 48 h over the water layer to remove products of peroxidation oxidation [10].

2.2. *Preparation of pH-Lip*

Liposomes were prepared by freeze–thawing method [11]. To prepare the lipid film, the mixture of PE/OA/Chol (4:2:4 molar ratio) in chloroform was dried under reduced pressure using a rotor evaporator (Rotadest). Traces of chloroform were removed by freeze-drying. When necessary [$^{14}$C]PE and [$^{3}$H]OA (up to 0.2 and 1.0 $\mu$Ci/mg liposomal lipid, respectively) or NBD-PE and Rh-PE (upto 1% of total lipid) were added to the lipid mixture. The lipid film was hydrated with PBS (140 mM NaCl, 10 mM Na phosphate, 1 mM EDTA and 0.04% Na azide), pH 8.0, or with a self-quenching 60 mM calcein solution in 10 mM Na phosphate buffer, pH 8.0 and incubated overnight at 4°C under argon. The suspension obtained was sonicated at 0°C and 30 W on a Labsonic ultrasonicator (Lab-Line Instruments) until transparency. During the sonification, the pH was adjusted to 8.0 with 1 M NaOH. To make FT, liposomes obtained were frozen in liquid nitrogen three times and thawed at room temperature, and then passed through a stack of two 0.2 $\mu$m polycarbonate filters (Nuclepore Corp.) until a preparation was obtained that was not sedimentable in the ultracentrifuge. The lipid concentration was 2 mg/ml. According to the size measurements (Autosizer II, Malvern) the mean diameter of liposomes was 0.22 $\mu$m. For calcein-containing FT, non-entrapped fluorescent dye was removed by gel filtration (Sepharose CL-4B).

2.3. *Preparation of P-Lip*

Liposomes were prepared as FT (see above) from PC, PE and Chol (4:2:3 molar ratio). The same lipid composition was also used to prepare REV [12]. In the latter case the lipid mixture was dissolved in diethyl ether at 10 mg/ml concentration. The ether solution was supplemented with PBS, pH 7.5, or with self-quenching 40 mM SRh in 10 mM Na phosphate buffer up to an ether-to-water volume ratio 3:1. The mixture was sonicated until a stable suspension was obtained. REV P-Lip were formed upon ether removal under reduced pressure, passed twice through a 0.4 $\mu$m polycarbonate filter, sedimented in the ultracentrifuge, washed and resuspended. Non-entrapped fluorescent dye was removed by gel-filtration. SRh-containing liposomes were used within 8 h after preparation.

2.4. *OA redistribution between pH-Lip and P-Lip*

100 $\mu$l of FT pH-Lip (total lipid concentration 80 $\mu$M) labeled with [$^{14}$C]PE and [$^{3}$H]OA, were supplemented with 100 $\mu$l of REV P-Lip (ca. 400 $\mu$M total lipid), and the mixture was incubated for 5 to 60 min at 20°C. REV P-Lip were precipitated by centrifugation for 10 min at 30,000 × g, and the efficacy of precipitation and OA exchange was estimated following the $^{14}$C and $^{3}$H radioactivity in both precipitate and supernatant. Normally, 85–95% of [$^{14}$C]PE activity remained in the supernatant, whereas the main part of the [$^{3}$H]OA activity was gradually transferred to the precipitate. The kinetics of OA redistribution between FT pH-Lip and REV P-Lip was studied following [$^{3}$H]OA radioactivity in the supernatant and precipitate samples. Each time point was measured in duplicate on a Liquid Scintillation Counter LKB 1215-11.

2.5. *Interactions between pH-Lip and P-Lip*

FT pH-Lip labeled with Rh-PE and NBD-PE, were added up to 2.5 $\mu$M concentration (as total lipid) to 22.5 $\mu$M REV P-Lip or FT P-Lip. The interaction (fusion) was registered following the increase in sample fluorescence at 530 nm (exitation wavelength 450 nm). The fluorescence intensity value at zero time was considered as characteristic of the sample without any fusion. The fluorescence intensity value corresponding to complete lipid mixing was obtained upon the addition of 1% Triton X-100 to the system.

2.6. *Permeability of pH-Lip in the presence of P-Lip*

FT or REV P-Lip (400 $\mu$M as total lipid) were supplemented with FT pH-Lip (up to 20 $\mu$M) containing 60 mM calcein. Calcein release was monitored following the fluorescence intensity increase at 520 nm (excitation at 490 nm). The measurements were performed on a F-4010 Hitachi spectrofluorimeter. The initial fluorescence intensity corresponded to zero release; the fluorescence intensity upon the addition of Trition X-100 corresponded to complete release. The fluorescence intensity of the initial sample was ca. 1/10 of the fluorescence upon Triton X-100 addition.

2.7. *Permeability of P-Lip in the presence of pH-Lip or OA*

SRh-containing FT P-Lip (24 $\mu$M total lipid, 40 mM SRh) were supplemented with FT pH-Lip up to 48 or 480 $\mu$M or with OA up to 9 or 90 $\mu$M. Dye release was registered following the increase in fluorescence intensity at 590 nm (exitation at 560 nm). The initial fluorescence of the sample was 10- to 30-fold less than that of the sample with liposomes completely destroyed with Triton X-100.

## 3. RESULTS AND DISCUSSION

P-Lip were used composed of PC, PE and Chol in a 4:2:3 molar ratio. This lipid composition to some extent imitates lipid composition of endoplasmic membranes of many eukariotic cells [13], if one substitutes all the membrane lipids except PE for PC.

The interaction between pH-Lip and P-Lip was studied using the energy transfer method, which allows for the following of the energy transfer between two fluorophores incorporated into pH-Lip membranes. If the fusion takes place upon the mixing of labeled liposomes with unlabeled ones, the dilution of fluorescent-labeled lipids in the bilayer proceeds, which in turn changes their fluorescence spectra [14,15].

NBD-PE and Rh-PE (1% mol each) were incorporated into membranes of FT pH-Lip as fluorescence donor and acceptor, respectively [15]. Fluorescent-labeled liposomes were supplemented with a 10-fold excess of non-labeled P-Lip (no difference was found between FT and REV P-LP), and the fusion process was registered following the increase in NBD (energy donor) fluorescence intensity. The data in Fig. 1 demonstrate that slow fusion proceeds in the system used. A pH decrease from 6.0 to 5.1 accelerates fusion, confirming pH-sensitivity of the liposomes used [16,17]. In the control system (Rh-PE- and NBD-PE-containing PC-Chol

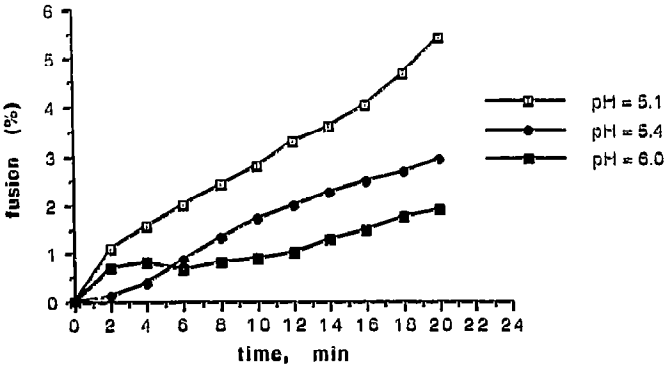


Fig. 1. Time-depent fusion of FT pH-Lip (2.5 $\mu$M) with FT P-Lip (22.5 $\mu$M) at different pH values. pH-Lip contains 1% mol of both NBD-PE and Rh-PE.

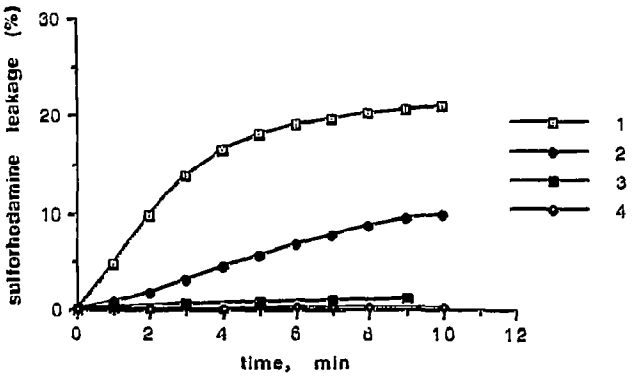


Fig. 2. The release of SRh from FT P-Lip (24 $\mu$M) during their incubation with FT pH-Lip, pH-Lip concentration (as total lipid): 480 $\mu$M, pH 5.8, curve 1, 48 $\mu$M, pH 5.8, curve 2; 480 $\mu$M, pH 7.5, curve 3; PBS, pH 5.8, curve 4.

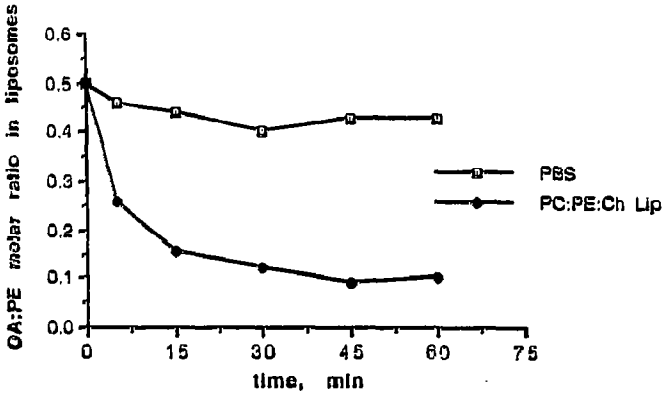


Fig. 3. The loss of OA by FT pH-Lip (80 $\mu$M) during the incubation in PBS (curve 1) and in the presence of 400 $\mu$M REV P-Lip (curve 2). pH 5.8 See section 2 for details.

liposomes with P-Lip) no fluorescence intensity increase was observed under the same conditions.

The addition of FT pH-Lip causes an increase in the FT P-Lip permeability. Fluorescent dye included into P-Lip at a self-quenching concentration, dilutes upon its stimulated release, which results in an increase in fluorescence (Fig. 2). Higher pH-Lip concentrations and lower pH values stimulate P-Lip destabilization. The most pronounced effect can be observed at 480 $\mu$M pH-Lip concentration and pH 5.8 (which is characteristic of the endosome internal compartment). The effect noticeably decreases at 48 $\mu$M pH-Lip. At pH 7.5, even 480 $\mu$M pH-Lip practically do not affect SRh-loaded P-Lip. The addition of the control PC-Chol liposomes to the dye- loaded P-Lip did not cause an increase in fluorescence, i.e. did not affect P-Lip.

pH-Lip used in the present study contained OA as an important component required for imparting pH-sensitivity to liposomes [16,17]. The data in Fig. 3 directly point to the OA transfer from FT pH-Lip to REV P-Lip, which can be involved in the P-Lip permeability increase. In the process of co-incubation of P-Lip and pH-Lip the OA/PE ratio in pH/Lip decreases, and on the plateau region (reached after about 15 min upon pH-Lip and P-Lip mixing) it was only 0.1, whereas initially the ratio was as high as 0.5 (The total quantity of lipids and OA in all samples was determined according to the corresponding radioactivity label.)

To prove the involvement of OA in P-Lip membrane destabilization we have studied the interaction of free OA with FT P-Lip. Fig. 4 presents the data on the rate of SRh release from P-Lip in the presence of free OA. Free OA destabilizes P-Lip, the effect being even higher than that provoked by the equivalent quantity of OA, associated with the liposomal membrane. A pH decrease accelerates P-Lip destabilization by both free and liposomal OA. To cause the destabilization, quite a small concentration of OA is needed, since its increase from 9 to 90 mM only slightly influences the process.

The loss of OA by pH-Lip also affects their own permeability. Fig. 5 shows the stability of 60 mM calcein-containing FT pH-Lip in the presence of P-Lip at different pH values. The incubation of pH-Lip in the presence of either FT or REV P-Lip provokes an increase in pH-Lip permeability. The most probable explanation for this phenomenon can be OA transfer from pH-Lip to P-Lip.

Assuming similar processes can take place during liposome-to-endosome interaction, the data obtained suggest a possible hypothetical mechanism for the penetration of the matter entrapped into pH-sensitive oleic acid-containing liposomes through the endosome membrane: 1. During the process of pH-Lip capture by the cell, OA from pH-Lip is redistributed between liposome and endosome membranes; 2. Because of a pH decrease inside the endosome and OA loss, pH-Lip are destroyed; 3. Simultaneously the endosomal membrane is destabilized by the incorporated OA; 4. The intraliposomal content leaves the endosome through the destabilized membrane into the cytoplasm following the

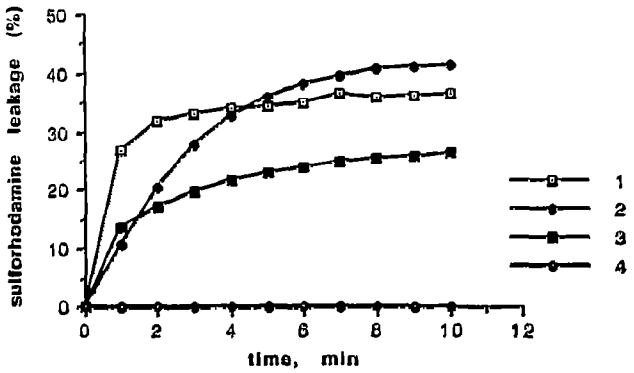


Fig. 4. The release of SRh from FT P-Lip (24 $\mu$M) during the incubation in the presence of OA. OA concentration: 90 $\mu$M, pH 5.8 (curve 1); 9 $\mu$M, pH 5.8 (curve 2); 90 $\mu$M, pH 7.5 (curve 3); PBS, pH 5.8 (curve 4).

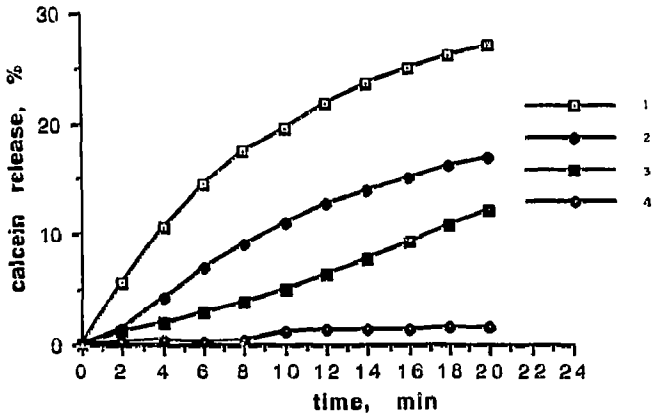


Fig. 5. The release of calcein from FT pH-Lip (20 $\mu$M) in the presence of FT P-Lip (400 $\mu$M): pH 6.3, curve 1; pH 6.9, curve 2; and in PBS: pH 6.3, curve 3; pH 6.9, curve 4.

concentration gradient. Further experiments should reveal the finer details of the mechanism proposed.

## REFERENCES

[1] Collins, D., Norley, S.T., Rouse, B. and Huang, L. in: Liposomes as Drug Carriers (G. Gregoriadis, Ed.), Wiley, New York, 1988, pp. 761–770.
[2] Nayar, R., Fidler, I.J. and Schroit, A.J . in: Liposomes as Drug Carriers (Gregoriadis, Ed.), Wiley, New York, 1988, pp. 771–782.
[3] Connor, J. and Huang, L. in: Cell Fusion (A.E. Sowers, Ed.), Plenum Press, New York, 1987, pp. 285–299.
[4] Huang, L. and Liu, S.S. (1984) Biophys. J. 45, N2(2), 75a.
[5] Christiansson, A., Kuypers, F.A., Roelofsen, B., Wirtz, K.W.A. and Op-Den-Kamp, J.A.F. (1984) Chem. Phys. Lipids 35, 247–258.
[6] Chauhan, A., Chauhan, V.P.S. and Brockerhoff, H. (1986) Biochemistry 25, 1569–1573.
[7] Bittman, R. and Blau, L. (1986) Biochim. Biophys. Acta 863, 115–120.
[8] Straubinger, R.M., Duzgunes, N. and Papahadjopoulos, D. (1985) FEBS Lett. 179, 148–154.
[9] Bergelson, L.D., Dyatlovitskaya, E.V., Molotkovsky, Yu.G., Batrakov, C.G., Barsukova, L.I. and Prokazova, N.V. in: Preparativnaya Biokhimia Lipidov (Preparative Biochemistry of Lipids, Russ.) Nauka, Moscow, 1981, pp. 141–142.
[10] Ellens, H., Bents, J. and Szoka, F.C. (1984) Biochemistry 23, 1532–1538.
[11] Pick, U. (1981) Arch. Biochem. Biophys. 212, 186–194.
[12] Szoka, F. and Papahadjopoulos, D. (1978) Proc. Natl. Acad. Sci. USA 75, 4194–4198.
[13] Ivkov, V.G. and Berestovsky, G.I. in: Lipidny Bisloy Biologicheskikh Membran (Lipid Bilayer of Biological Membranes, Russ.) Nauka, Moscow, 1982, p. 224.
[14] Struck, D.K., Hoekstra, D. and Pagano, R.E. (1981) Biochemistry 20, 4093–4099.
[15] Duzgunes, N., Allen, T.M., Fedor, J. and Papahadjopoulos, D. (1987) Biochemistry 26, 8435–8442.
[16] Wang, Ch.-Y., Hughes, K.W. and Huang, L. (1986) Plant Physiol. 82, 179–184.
[17] Wang, Ch.-Y. and Huang, L. (1987) Proc. Natl. Acad. Sci. USA 84, 7851–7855.