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Cation-dependent fusogenicity of an *N*-acyl phosphatidylethanolamine

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Abstract

N-acyl phosphatidylethanolamines (NAPEs) are natural lipid components of many organisms. *N*-acylation of unsaturated phosphatidylethanolamines with a saturated fatty acid converts them from non-lamellar organizing lipids into lamellar organizing, acidic lipids which can interact with cations and potentially return to non-lamellar structures. These special properties make NAPEs candidates for fusogens. We tested the fusogenicity of one of the NAPEs, *N*-dodecanoyl-dioleoylphosphatidylethanolamine (N-C12-DOPE) mixed with dioleoylphosphatidylcholine (DOPC) in liposomes. Binding and fusion to erythrocyte ghosts in the presence of 3 mM Ca^{2+} required at least 60 mol% of N-C12-DOPE. Fusion was not observed when phosphatidylglycerol or phosphatidylserine was substituted for *N*-C12-DOPE, indicating specificity for properties of this lipid. Binding of *N*-C12-DOPE/DOPC (70:30) liposomes required 1 mM Ca^{2+} while 1.25 mM Ca^{2+} and Mg^{2+} were sufficient for lipid mixing and delivery of encapsulated dextrans to erythrocyte ghosts. These liposomes also bound and possibly mixed lipid with nucleated U-937 cells in a Ca^{2+}- and endocytosis-dependent manner. Low pH-dependent fusion with ghosts was observed in the absence of any divalent cation, indicating that fusion with U-937 cells could result after endocytosis into the acidic endosomes. The possible mechanisms for N-C12-DOPE mediated binding and fusion and the potential application of these liposomes as delivery vehicles for therapeutic agents are discussed. © 1998 Elsevier Science B.V.

Keywords: Fusion; Binding; *N*-acyl phosphatidylethanolamine; Liposome

1. Introduction

The vast majority of phospholipids that form biological membranes contain two long acyl chains. However, a small percentage of phospholipids with more than two long chains also exists in some biological membranes. The functions of one class, the *N*-acyl phosphatidylethanolamines (NAPEs), are as yet poorly understood. These lipids have been suggested as precursors of *N*-acyl ethanolamines (NAEs), a group of lipids that exhibit a wide range of biological functions [for a review, see [1]], including anti-inflammatory effects [2,3]. The presence of NAPEs in many types of cells under normal physiological con-

Abbreviations: PE, phosphatidylethanolamine; PC, phosphatidylcholine; PG, phosphatidylglycerol; PS, phosphatidylserine; DO-, dioleoyl-; NAPE, *N*-acyl phosphatidylethanolamine; NAE, *N*-acyl ethanolamine; N-C12-DOPE, *N*-dodecanoyl-dioleoylphosphatidylethanolamine; NBD-PE, *N*-(7-nitro-2,1,3,-benzoxadiazol-4-yl)phosphatidylethanolamine (egg); Rh-PE, *N*-(lissamine rhodamine B sulfonyl)phosphatidylethanolamine (egg); TMR, tetramethylrhodamine B; TMR-70kD dextran, tetramethylrhodamine B conjugated 70kD dextran; $C_{12}E_8$, octaethylene glycol monododecyl ether; RET, resonance energy transfer; LUV, large unilamellar vesicle; TES, *N*-[*tris*(hydroxymethyl)methyl]-2-aminoethanesulfonic acid; ^{1}H NMR, proton nuclear magnetic resonance; H_{II}, hexagonal II; TLC, thin layer chromatography; BSA, bovine serum albumin

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ditions suggests that they may also possess other important membrane functions.

Studies on the structure and phase behavior of NAPEs in aqueous dispersion [4–6] show that *N*-acylation of certain unsaturated phosphatidylethanolamines (PEs), a group of non-lamellar phase lipids, with a saturated fatty acid can convert them into acidic lamellar phase lipids. Incorporation of NAPEs into PC liposomes has also been reported to decrease the membrane permeability of the vesicles [7] and to stabilize them in the presence of serum [8]. The bilayer stabilizing effect of these lipids has been suggested to result from the insertion of longer *N*-acyl chains, i.e. *N*-acyl chains $\geq$ C10, into the hydrophobic core of the membrane, causing immobility and a higher degree of hydration of the headgroups [5,6].

One of these lamellar NAPEs has been shown to interact with Ca^{2+} to return to non-lamellar hexagonal II (H_{II}) structures [4]. Such non-lamellar structures have been proposed as intermediates for membrane fusion processes [9–11]. Hence these lipids may be able to impart fusogenicity to membranes, which can be modulated by Ca^{2+} and *N*-acyl chain structure. Because NAPEs are also naturally occurring lipids, it was of interest to investigate their potential as components of liposomes that can fuse and deliver reagents into cells.

We chose to study *N*-dodecanoyl-dioleoylphosphatidylethanolamine (N-C12-DOPE), an intermediate *N*-acyl chain length that may be long enough to be buried inside the liposomal membrane and, in the presence of a target membrane, bears the potential to be released to mediate hydrophobic binding and/or fusion. We report here the Ca^{2+}- and proton-dependent fusogenic properties of liposomes containing this lipid using model liposome-cell fusion systems.

2. Materials and methods

2.1. Materials

DOPC, brain PS, dioleoylphosphatidylethanolamine (DOPE), dioleoylphosphatidylglycerol (DOPG) were purchased from Avanti Polar Lipids (Alabaster, AL). *N*-(7-nitro-2,1,3,-benzoxadiazol-4-yl)phosphatidylethanolamine (egg) (NBD-PE), *N*-(lissamine rhodamine B sulfonyl)phosphatidylethanolamine (egg) (Rh-PE), tetramethylrhodamine B conjugated 70kD dextran (TMR-70D) were purchased from Molecular Probes (Eugene, OR). Octaethylene glycol monododecyl ether ($C_{12}E_8$) and triethylamine were purchased from Fluka (Roukonkona, NY). *N*-[*tris* (hydroxymethyl)methyl]-2-aminoethanesulfonic acid (TES) was purchased from Calbiochem (La Jolla, CA). Lauric anhydride was purchased from Aldrich (Milwaukee, WI). Human prothrombin, factor V/Va, and factor Xa were purchased from Enzyme Research Laboratories (South Bend, IN). Sarcosine–Pro–Arg–*p*-nitroanilide was purchased from Sigma (St. Louis, MO). U-937 cells were purchased from American Type Culture Collection (Rockville, MD).

2.2. NAPE synthesis and characterization

Briefly, DOPE dissolved in $CHCl_3$ (500 mg, 0.67 mmol) was stirred for 24 h at room temperature with lauric anhydride (513 mg, 1.34 mmol) and triethylamine (726 mg, 7.2 mmol). Thin layer chromatograph (TLC) analysis showed the disappearance of all starting materials. The solvent was evaporated and the residue was purified by flash column chromatography on a silica gel (230–400 mesh, Aldrich, Milwaukee, WI) using the solvent gradients of $CHCl_3$/MeOH, 100:0, 98:2, 95:5, 90:10, and 80:20. The desired compound was pooled and concentrated under vacuum. The obtained product was dissolved in a minimum amount of $CHCl_3$ and the solution was passed through an Acrodisc CR syringe filter (0.2 μm, Fisher Scientific, Malvern, PA) to remove the silica gel. After removal of chloroform, the product was lyophilized from cyclohexane and characterized by proton nuclear magnetic resonance (^{1}H NMR, Bruker Instrument, Manning, MA, 300 MHz, $CDCl_3$) and TLC (data not shown). The final yield of N-C12-DOPE was 626 mg, quantitatively.

After the initial experiments, larger quantities of N-C12-DOPE were purchased from Avanti Polar Lipids. No significant difference was observed between the two stocks of N-C12-DOPE, as characterized by ^{1}H NMR and fluorescence fusion assays (data not shown). Fig. 1 shows the chemical structure of N-C12-DOPE.

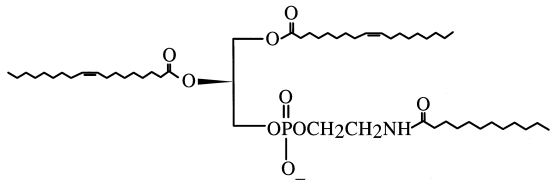


Fig. 1. Chemical structure of N-C12-DOPE.

2.3. Liposome preparation

NBD/Rh labeled large unilamellar vesicles (LUVs) were prepared as described before [12]. Briefly, the lipid mixture in chloroform was dried under a nitrogen stream to a thin film, which was then left under vacuum overnight to remove residual solvent. The lipid film was hydrated with TES buffered saline (10 mM TES, 0.1 mM EDTA, 154 mM NaCl, pH 7.4). Brief vortexing was applied to ensure complete hydration. After ten cycles of freeze/thaw in liquid nitrogen/room-temperature water bath, the sample was extruded ten times through 0.1 μm polycarbonate membrane filter (Poretics, Livermore, CA). The liposomes were stored at 4°C.

For dextran encapsulated LUVs, no fluorescent lipid label was used. The dry lipid film was hydrated with 50 mg/ml TMR-70D in 10 mM TES buffered saline. After freeze/thaw and extrusion, the LUVs were separated from unencapsulated dextran by passing through a 45 × 1.3 cm Biogel-A50 gel filtration column (Bio-Rad Laboratories, Richmond, CA). LUVs eluted at the void volume. The liposomes were stored at 4°C and used within one week after preparation.

The phospholipid concentration of each liposome preparation was determined by phosphate assay [13]. The approximately 0.1 μm size of the liposomes was confirmed (data not shown) on a Nicomp submicron particle sizer (Nicomp Instruments, Goleta, CA) using quasi-elastic light scattering.

2.4. Preparation of resealed and unsealed human erythrocyte ghosts

Resealed ghosts are referred to as erythrocyte ghosts unless otherwise specified. Resealed erythrocyte ghosts were prepared as described before [14,15]. Briefly, fresh human blood was washed several times with cold 10 mM TES buffered saline to remove plasma and white cells. Then 2 ml of washed erythrocytes (50% hematocrit) were pre-swelled in cold hypotonic solution containing 8 ml H_2O and 9.6 ml 10 mM TES buffered saline, and then pelleted at 850 × *g* for 5 min. The pellet was resuspended in 40 ml cold lysis buffer (10 mM Tris, 0.1% BSA, 2 mM $MgCl_2$, and 0.1 mM EGTA) and incubated on ice for at least 2 min. After addition of 4.5 ml 10 × resealing buffer (1.22 M NaCl, 30 mM KCl, 0.15 M Na_2HPO_4, 50 mM KH_2PO_4, and 2 mM $MgCl_2$), the sample was incubated at 37°C for 40 min. The resealed ghosts were pelleted at 1750 × *g* for 10 min and washed several times with 10 mM TES buffered saline until no hemoglobin could be observed in the supernatant. The ghosts were stored at 4°C and used within one week.

The unsealed ghosts were prepared as described before [16]. Briefly, erythrocytes were washed several times with 10 mM TES buffered saline to remove plasma and white cells. The pellet was then resuspended in cold 5 mM Na_2HPO_4 and spun at 14 000 × *g* for 15 min. The pellet was then washed with the same buffer until the supernatant was clear. The unsealed ghosts were stored in the same buffer at 4°C and used within a week.

2.5. Prothrombinase assay

The phospholipid asymmetry of the erythrocyte membranes was measured by prothrombinase assay which detects the presence of PS in the outer monolayer of the membrane. The assay was performed as described before [17] with some modifications. Briefly, 4×10^5 cells were incubated at 37°C for 3 min in Tris buffered saline (50 mM Tris–HCl, 120 mM NaCl, pH 7.4) containing 6 mM $CaCl_2$, 0.33 unit ml^{-1} factor V/Va, 0.33 unit ml^{-1} factor Xa, and 1.3 unit ml^{-1} prothrombinase. The total reaction volume was 1 ml. After 15 mM EDTA was added to stop the reaction, the cells were pelleted by centrifugation. About 900 μl supernatant was mixed with 100 μl chromogenic substrate sarcosine–Pro–Arg–*p*-nitroanilide (500 μM) and OD_{405} was measured kinetically. The rates (ΔOD/min) of intact erythrocytes and unsealed erythrocyte ghosts were taken as 0 and 100% accessible PS, respectively. Using this

scale, the accessible PS on the resealed ghosts was found to be about 16%.

2.6. Fluorescent binding and lipid mixing assays

For each assay, either 100 nmol liposomes and 5×10^8 erythrocyte ghosts or 10 nmol liposome and 1×10^7 U-937 cells were mixed. The total volume was brought to 100 μl with 10 mM TES buffered saline. For assays at low pH, an equal volume of 50 mM citrate buffered saline (50 mM citrate, 90 mM NaCl, 0.1 mM EDTA, pH 4.7) and liposome-ghost mixture were mixed to yield a final pH of 4.9 and a final volume of 106 μl. The mixture was incubated at 37°C for indicated periods of time with or without divalent cations. The mixture was then centrifuged for 5 min at $3500 \times g$ to pellet ghosts or $300 \times g$ to pellet U-937 cells. The unbound liposomes were removed with the supernatant, leaving approximately 10% of the unbound liposomes with the ghost pellet (see below for correction) and $< 5\%$ with the U-937 pellet (no correction made). The pellet was resuspended in 100 μl TES buffered saline and transferred to a cuvette containing the same buffer at room temperature to measure lipid mixing and percent bound. The total volume in the cuvette was 2 ml. All the data presented in this paper are averages of three identical experiments unless otherwise specified.

Lipid mixing between NBD/Rh labeled liposomes and unlabeled ghosts or U-937 cells was measured in 10 mM TES buffered saline by the NBD/Rh resonance energy transfer (RET) assay [18]. The NBD fluorescence was recorded at room temperature on a PTI Alphascan fluorometer (South Brunswick, NJ) in a cuvette with continuous stirring. The excitation wavelength was 450 nm, with a 450 ± 20 nm bandpass filter (Melles Griot, Irvine, CA) to further refine the light beam. The emission wavelength was 530 nm with a > 500 nm highpass filter (Schott Glass Technologies, Duryea, PA). The NBD fluorescence of a freshly prepared liposome-cell mixture, i.e. without incubation and spin, was also measured with and without 0.2% $C_{12}E_8$ to give the 0 and 100% lipid mixing, respectively.

The fluorescence of Rh in the presence of 0.2% $C_{12}E_8$ was used to measure liposome binding. The excitation wavelength was 560 nm with a 550 ± 20 nm bandpass filter (Melles Griot, Irvine, CA) to further refine the light beam. The emission wavelength was 600 nm with a > 570 highpass filter (Schott Glass Technologies, Duryea, PA). The fluorescence level of cells alone in the presence of 0.2% $C_{12}E_8$ was taken as 0% binding. The Rh fluorescence of a freshly prepared liposome-cell mixture (no sedimentation) was also measured in the presence of 0.2% $C_{12}E_8$ to yield 100% binding.

The extents of lipid mixing and binding were calculated as follows:

$$\%\ \text{lipid mixing} = 100 \times \frac{\left[(N_S - N_C) \times R_{0D}/R_{SD}\right] + N_C - N_0}{\left[(N_{SD} - N_{CD}) \times R_{0D}/R_{SD}\right] + N_{CD} - N_0},$$

$$\%\ \text{binding} = 100 \times \frac{R_{SD} - R_{CD}}{R_{0D} - R_{CD}},$$

where N_S – NBD fluorescence of sample; N_C – NBD fluorescence of cells alone; N_0 – NBD fluorescence of freshly prepared liposome-cell mixture; N_{SD} – NBD fluorescence of sample in the presence of detergent; N_{CD} – NBD fluorescence of cells alone in the presence of detergent; R_{0D} – Rh fluorescence of freshly prepared liposome-cell mixture in the presence of detergent; R_{SD} – Rh fluorescence of sample in the presence of detergent; and R_{CD} – Rh fluorescence of cells alone in the presence of detergent.

Binding percentages for ghosts were corrected for the residual unbound fluorescence by essentially assuming 10% of the supernatant remained in each pellet. Therefore:

$$\%\ \text{binding(corr.)} = 1.11 \times (\%\text{measured}) - 11.1.$$

Similar corrections were small to negligible for % lipid mixing of any samples with significant binding ($> 25\%$).

2.7. Fluorescence microscopy

25 nmol N-C12-DOPE/DOPC (70 : 30) liposomes, encapsulating TMR-70kD dextran or labeled with NBD/Rh, and 5×10^8 erythrocyte ghosts were incubated at 37°C for 30 min with, or without 1.25 mM Ca^{2+}/Mg^{2+}. At the end of the incubation period, ice cold 10 mM TES buffered saline with or without Ca^{2+}/Mg^{2+} was added to each sample to bring the volume to 1 ml. The sample was centrifuged for

5 min at 3500 × *g* and the pellet was washed once with the same buffer to remove unbound liposomes. The pellet was then resuspended in the same buffer and examined under an Olympus BH-2 fluorescence microscope (Olympus, Lake Success, NY), equipped with a 545 nm excitation filter (Olympus) and a > 580 nm dichroic mirror (Olympus).

3. Results

The fusogenic nature of N-C12-DOPE was tested using erythrocyte membranes as a model system for biological membranes. These cells are incapable of endocytosis, so that any observed fusion can be attributed to direct fusion with the plasma membrane. Since the high concentration of hemoglobin inside intact erythrocytes interferes with fluorescent fusion assays, erythrocyte ghosts were used. The phospholipid asymmetry of intact erythrocyte membranes [19] is largely maintained in these ghost membranes, as determined by prothrombinase assay (see Section 2).

The ability of N-C12-DOPE to mediate liposome binding and membrane fusion in the presence of cations was tested by labeling the liposomes with 0.75 mol% NBD-PE and 0.75 mol% Rh-PE. These liposomes and erythrocyte ghosts were typically incubated at 37°C for 1 h or less with various concentrations of Ca^{2+} and/or Mg^{2+}. After incubation, unbound liposomes were removed by centrifugation. Lipid mixing was measured by dequenching of NBD fluorescence resulting from dilution of probes into the much larger ghost membrane and subsequent decrease of RET between the probes. Binding was estimated by measuring the total Rh fluorescence associated with the detergent solubilized pellet of ghosts and comparing to the maximal possible binding. Lipid mixing was expressed as the percentage of maximal fusion among the bound liposomes (see Section 2 for details).

Because Ca^{2+} has been reported to affect the lamellar-to-nonlamellar phase transition of one of the NAPEs [4], we initially studied the binding and lipid mixing of N-C12-DOPE/DOPC (70:30) liposomes with erythrocyte ghosts in buffer with 3 mM Ca^{2+}. Fig. 2 shows the time course of these processes at 37°C. Over 80% binding and about 20% lipid mixing were observed under these conditions. Both binding and lipid mixing were quite rapid, reaching their maximal extents in 5–10 min. Therefore, it appeared that N-C12-DOPE could be a fusogen under some conditions.

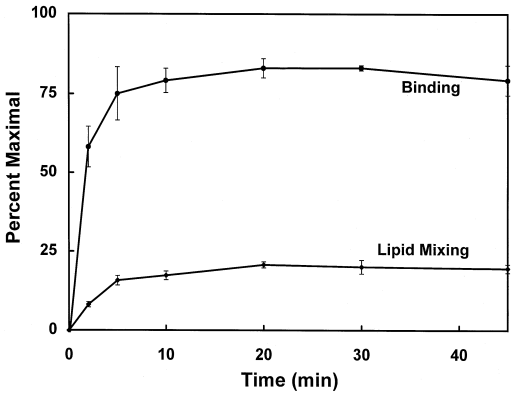


Fig. 2. The kinetics of binding and lipid mixing between N-C12-DOPE/DOPC (70:30) liposomes and erythrocyte ghosts at 37°C, measured by NBD/Rh RET assay. The liposomes and ghosts were incubated at 37°C for the indicated time periods in the presence of 3 mM Ca^{2+}. The NBD and Rh fluorescence were measured after the unbound liposomes were removed by centrifugation. Lipid mixing and binding are expressed as percentages of detergent values as described in Section 2.

3.1. Divalent cation-dependence of binding and lipid mixing with erythrocyte ghosts

The divalent cation dependence of these events was then investigated. Fig. 3(A) and (B) show this dependence of binding and lipid mixing, respectively, after 1 h incubation at 37°C. While both Ca^{2+} and Mg^{2+} mediated binding, Ca^{2+} was more effective than Mg^{2+}. The two cations together induced even more binding. For instance, the presence of both Ca^{2+} and Mg^{2+} at 1 mM each resulted in nearly 100% binding, compared to about 55% with 1 mM Ca^{2+} alone and 14% with 1 mM Mg^{2+} alone. These data suggested that the effects of Ca^{2+} and Mg^{2+} may be somewhat synergistic in this case.

Lipid mixing was observed at Ca^{2+} concentrations above 1 mM (Fig. 3(B)). The extent of lipid mixing increased with Ca^{2+} concentration. By contrast, the highest extent of lipid mixing that was induced by Mg^{2+} alone was about 5% of complete mixing. At a 1:1 Ca^{2+}/Mg^{2+} ratio, the lipid mixing extents were

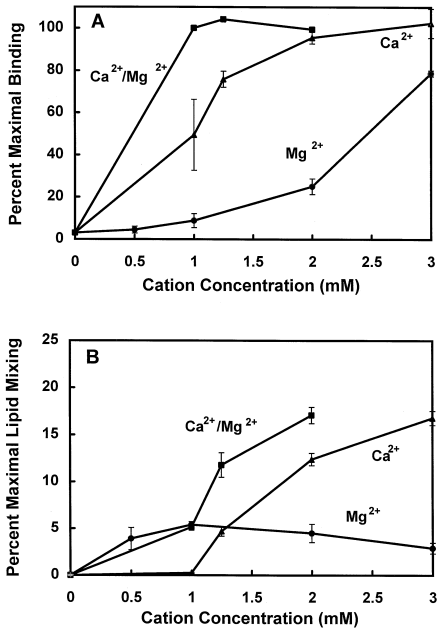


Fig. 3. Divalent cation-dependence of binding (A) and lipid mixing (B) between N-C12-DOPE/DOPC (70:30) liposomes and erythrocyte ghosts, measured by NBD/Rh RET assay. The NBD/Rh labeled liposomes and unlabeled ghosts were incubated at 37°C for 1 h in the presence of indicated concentrations of cation(s). When both Ca^{2+} and Mg^{2+} were used, each of them was present at the labeled concentration.

apparently additive. Clearly, divalent cations were required for lipid mixing under these conditions.

3.2. N-C12-DOPE-dependence of binding and lipid mixing

In order to determine whether the observed cation-dependent binding and lipid mixing were also dependent on *N*-C12-DOPE, liposomes containing various percentages of this lipid were used. Their interactions with erythrocyte ghosts after 1 h incubation at 37°C in the presence of 3 mM Ca^{2+} are shown in Fig. 4. Essentially, no lipid mixing and less than 5% binding were observed with liposomes containing 40 mol% or less of N-C12-DOPE. On the other hand, liposomes containing 60 mol% or more N-C12-DOPE achieved more than 94% binding. The extents of lipid mixing of liposomes containing 60 and 80 mol% N-C12-DOPE were 7.5 and 20%, respectively. These results demonstrate that N-C12-DOPE is required to render the liposomes fusogenic and that it must be present in a substantial amount.

3.3. Comparison with other negatively charged liposomes

It is well known that Ca^{2+} can mediate both binding and fusion between liposomes containing negatively charged acidic phospholipids, such as PS [20,21]. Thus, it was of interest to determine whether the observed Ca^{2+}-dependent binding and lipid mixing required any special properties of N-C12-DOPE.

Using the NBD/Rh RET assay, we compared binding and lipid mixing of N-C12-DOPE/DOPC (80:20) liposomes to other negatively charged liposomes, DOPG/DOPC (80:20) and brain PS/DOPC (80:20) liposomes (Fig. 5). All these liposomes bear the same negative surface charge density. The liposomes were incubated with erythrocyte ghosts at 37°C for 1 h in the presence of 3 mM Ca^{2+}. Although nearly 64% of PS-containing liposomes and about 20% of DOPG-containing liposomes bound to the ghosts, less than 5% lipid mixing was observed with either of these liposomes. By contrast, under the same conditions about 84% of the N-C12-DOPE

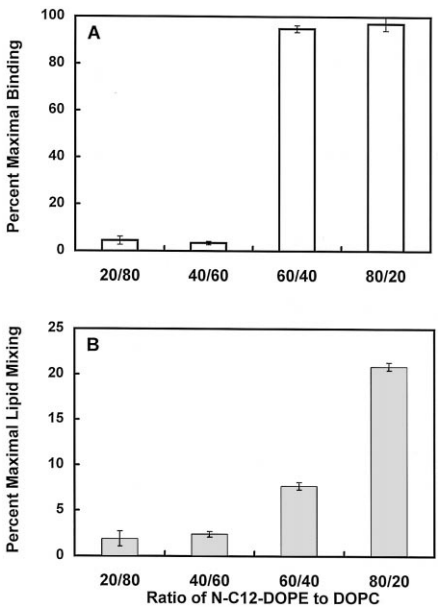


Fig. 4. N-C12-DOPE-dependence of binding (A) and lipid mixing (B), measured by NBD/Rh assay. The NBD/Rh labeled N-C12-DOPE/DOPC liposomes and unlabeled ghosts were incubated at 37°C for 1 h in the presence of 3 mM Ca^{2+}.

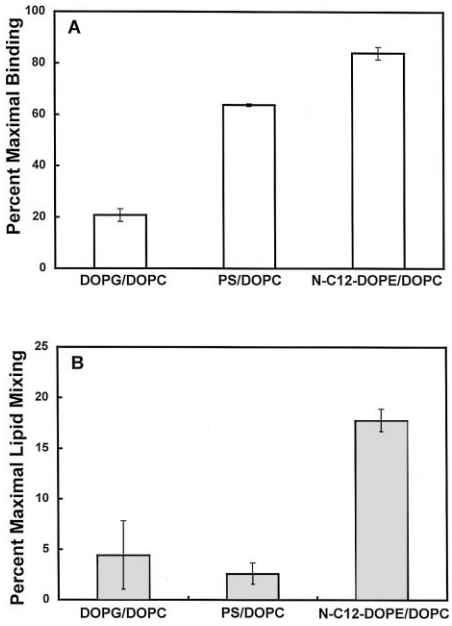


Fig. 5. Comparison of DOPG/DOPC (80:20), brain PS/DOPC (80:20), and N-C12-DOPE/DOPC (80:20) liposomes in terms of binding (A) and lipid mixing (B) with erythrocyte ghosts, measured by NBD/Rh RET assay. The liposomes and ghosts were incubated at 37°C for 1 h in the presence of 3 mM Ca^{2+}.

containing liposomes associated with ghosts, and about 17% lipid mixing was detected. As discussed later, this suggests that indeed some unique properties of N-C12-DOPE may play an important role in both binding and lipid mixing processes.

3.4. Microscopic observation of lipid mixing

Confirmation of the observed binding and lipid mixing events was sought microscopically using the NBD/Rh labeled N-C12-DOPE/DOPC (80:20) liposomes. The liposomes and ghosts were incubated at 37°C with, or without 1.25 mM Ca^{2+}/Mg^{2+}. After 30 min incubation, the samples were washed to remove unbound liposomes and examined under the fluorescence microscope. Binding of liposomes should result in punctate fluorescence associated with ghost membranes. Upon fusion, lipid labels would be diluted into the much larger ghost membrane, resulting in diffuse fluorescence. Fig. 6(top panel) shows that, in the absence of Ca^{2+}/Mg^{2+}, only a small percentage of liposomes bound to the ghosts and almost no fusion occurred. Ghosts that have undergone lipid mixing were observed only in the presence of 1.25 mM Ca^{2+}/Mg^{2+} (Fig. 6, bottom). In these cells, a diffuse, ringed pattern of fluorescence was observed, as expected for membrane labeling. Therefore, the results are qualitatively similar to those obtained with the fluorometer measurements, suggesting cation-dependent lipid mixing.

3.5. Divalent cation-dependent content delivery into erythrocyte ghosts

True fusion must result in mixing of the aqueous contents of liposomes and erythrocyte ghosts. Thus, we used N-C12-DOPE/DOPC (70:30) liposomes with encapsulated tetramethylrhodamine-conjugated 70kD dextran (TMR-70D) to assess contents mixing with the ghosts.

Ghosts and dextran encapsulating liposomes were treated as described in the above section and examined under the fluorescence microscope. As discussed above, binding would be represented by punctate fluorescence, whereas contents delivery would be indicated by diffuse fluorescence resulting from dilution of dextrans inside the ghosts. Fig. 7(B) (upper right) shows the sample that had been incubated at 37°C without Ca^{2+}/Mg^{2+}. While there was some ghost-associated punctate fluorescence, indicative of binding, apparently little fusion had occurred, as judged by the absence of ghosts with diffuse fluorescence. By contrast, ghosts with punctate fluorescence as well as those with diffuse fluorescence can be observed in the upper (A) and lower (C, higher magnification) left panels, where the sample had been incubated with 1.25 mM Ca^{2+}/Mg^{2+} at 37°C. Furthermore, the total amount of fluorescence associated with the ghosts also appeared much higher than without Ca^{2+}/Mg^{2+}. Fusion may also be indicated by the somewhat larger appearance of the ghosts containing dextrans, although this may also be related to out-of-focus fluorescence. Whereas some leakage of TMR-dextran from liposomes was observed under these conditions (data not shown), no significant uptake of free dextrans by the ghosts was detected when they were incubated with comparable concentration of free dextrans under the same conditions (D, lower right). From this, it is concluded that true fusion, i.e. delivery of contents, had occurred.

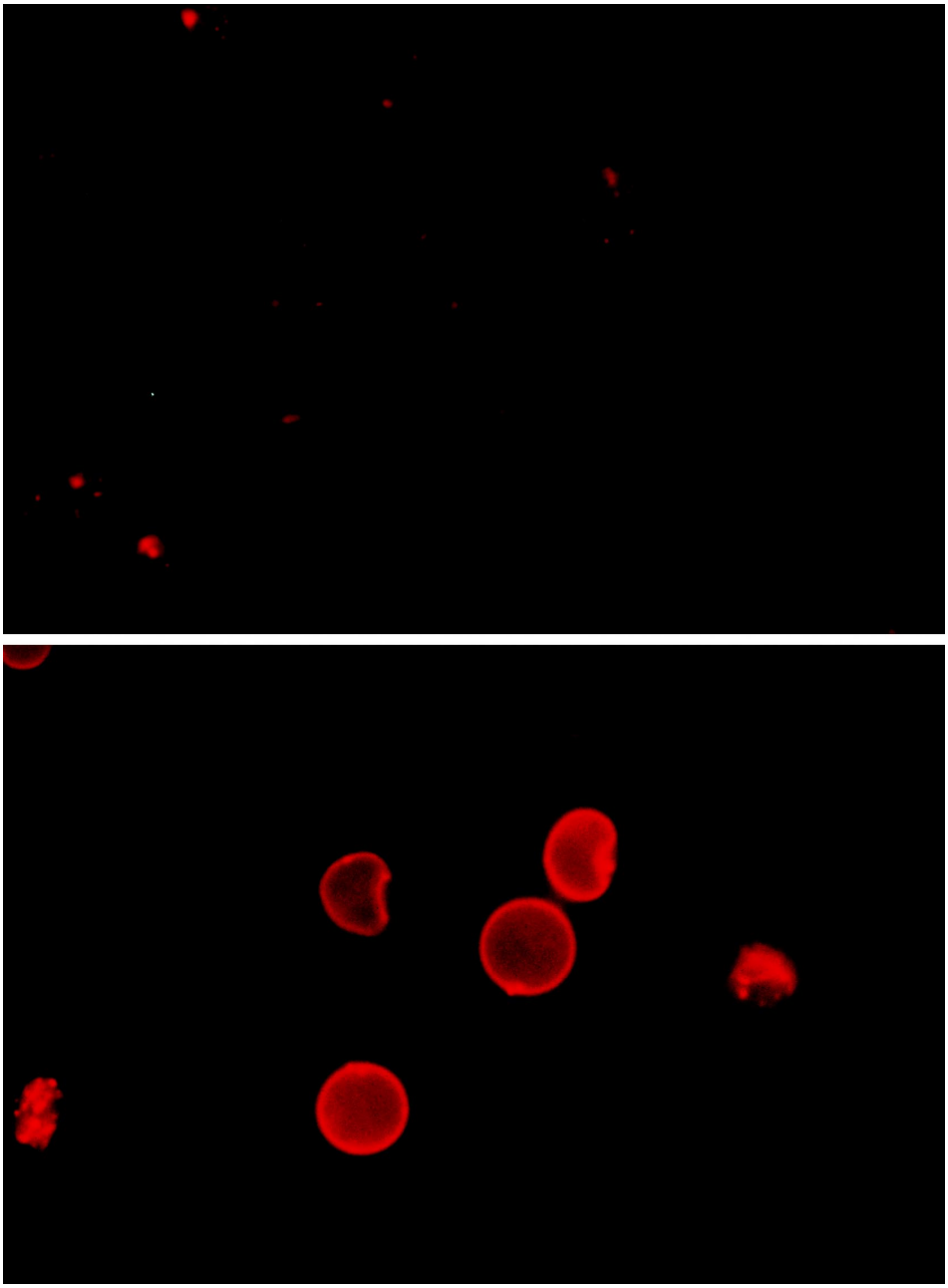
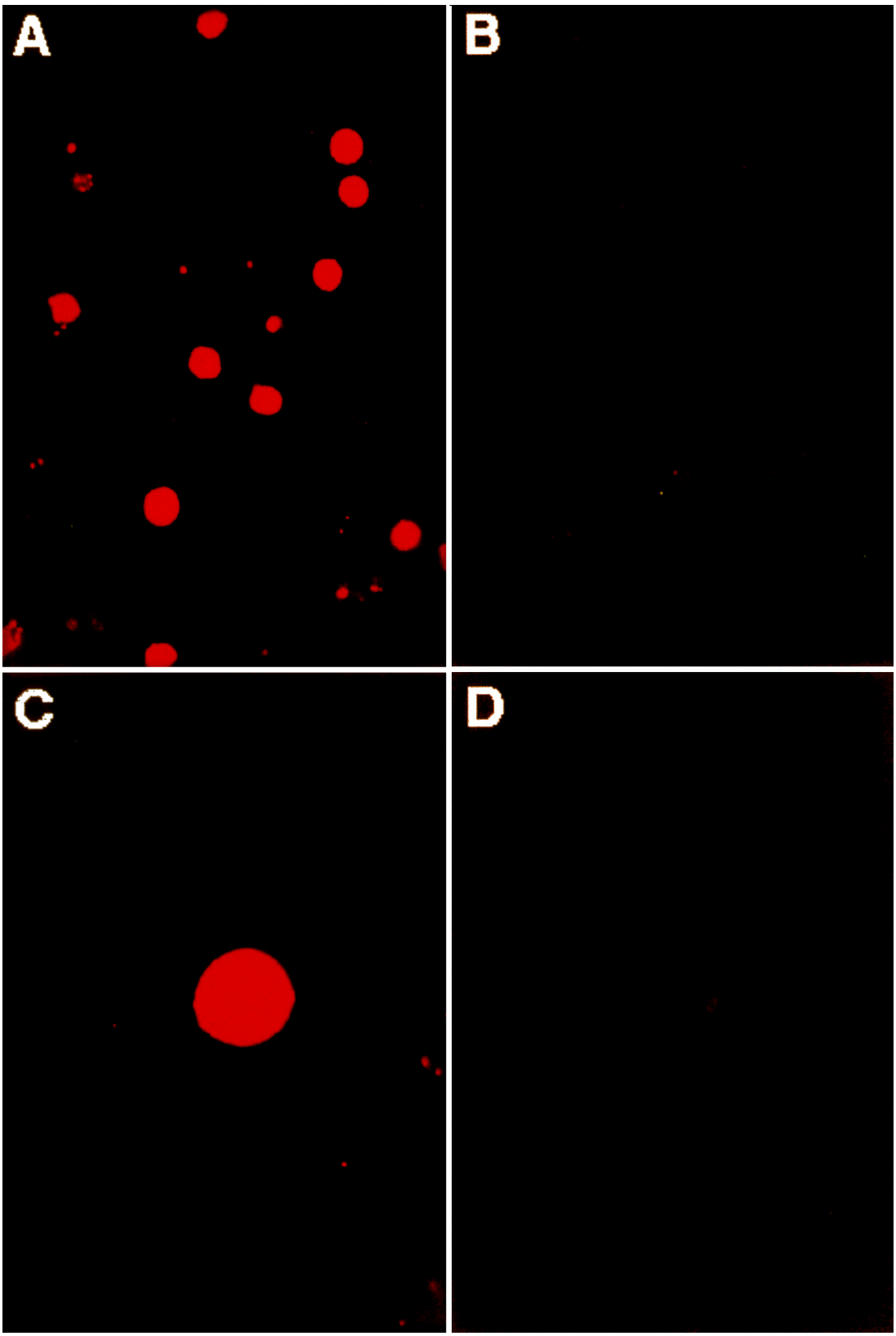


Fig. 6. Fluorescence micrographs of lipid mixing between N-C12-DOPE/DOPC (70:30) liposomes and erythrocyte ghosts. The NBD/Rh labeled liposomes were incubated with ghosts for 30 min without (upper panel) or with (lower panel) 1.25 mM Ca^{2+}/Mg^{2+}. After the incubation, the ghosts were washed to remove unbound liposomes. The total magnification is 1000× for each micrograph.


A
B
C
D

Fluorescence microscopy was also used to estimate the efficiency of these events in terms of the percentage of ghosts involved, as apposed to the percentage of liposomes described earlier. Percentages of ghosts that engaged in liposome binding, lipid mixing, and content delivery were estimated by comparing the numbers of total ghosts, ghosts with any fluorescence, and those with diffuse fluorescence in each sample. The results are summarized in Table 1. For both labels, significant portions of the ghosts either had associated liposomes or fused with liposomes, suggesting that N-C12-DOPE mediated binding and fusion are not specific for any small subpopulation of the ghosts. However, the percentage of ghosts showing lipid mixing is much higher than the percentage of ghosts showing contents delivery. Possible explanations for this result are discussed later.

3.6. Partially Ca^{2+}-independent binding and lipid mixing with nucleated cells

To test the ability of N-C12-DOPE/DOPC (70:30) liposomes to bind and fuse with nucleated cells, we used a human histiocytic lymphoma cell line (U-937 cells) as the target membrane. Binding and lipid mixing between NBD/Rh labeled liposomes and unlabeled cells were measured after 1 h incubation at 37°C with, or without 3 mM Ca^{2+}. As shown in Fig. 8, about 30% binding was observed and 60% lipid mixing was detected among the bound liposomes in the presence of Ca^{2+}. However, even in the absence of Ca^{2+}, 35% binding and 30% lipid mixing were observed, indicating that a different mechanism of binding and lipid mixing may have been used. Since U-937 are endocytic cells, lipid mixing may have occurred in response to uptake of the liposomes into the increasingly acidic endosomes.

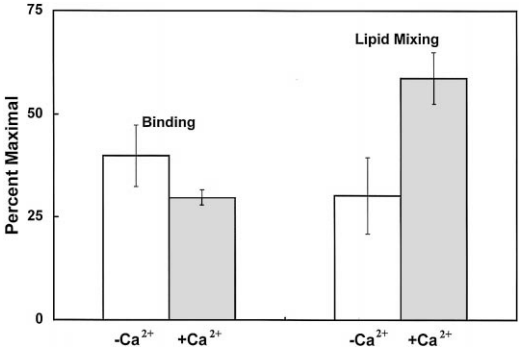


Fig. 8. Binding and lipid mixing between N-C12-DOPE/DOPC (70:30) liposomes and U-937 cells, measured by NBD/Rh RET assay. The liposomes and U-937 cells were incubated at 37°C for 1 h with, or without 3 mM Ca^{2+}.

3.7. pH-Dependence of binding and lipid mixing to erythrocyte ghosts

If N-C12-DOPE/DOPC (70:30) liposomes are endocytosed by nucleated cells, the acidic environment inside the endosomes may neutralize the negative surface charges of the liposomes, leading to binding and fusion with the luminal side of the endosomal membrane. Thus, the pH-dependence of N-C12-DOPE mediated binding and fusion was studied.

Using erythrocyte ghosts as model target membranes, we compared the extent of binding and lipid mixing of N-C12-DOPE/DOPC (70:30) liposomes at pH 7.4 and pH 4.9 (Fig. 9) after 1 h incubation at 37°C. No divalent cation was present in this set of experiments. Essentially, neither binding nor lipid mixing was observed at neutral pH. However, about 78% binding and nearly 10% lipid mixing were detected at low pH. This suggests that both binding

Table 1
Percentages of erythrocyte ghosts with N-C12-DOPE/DOPC (70:30) liposomes, bound or fused [a]

Probes	Ca^{2+}/Mg^{2+}	Fields counted	Total ghosts counted [b]	% Fused ghosts [c]	% Bound ghosts [d]
TMR-70kD	+	12	550	13.4 ± 6.7	44.9 ± 12.6
TMR-70kD	−	12	504	0.0 ± 0.0	60.7 ± 21.3
NBD/Rh	+	3	225	53.3 ± 18.8	71.7 ± 12.6
NBD/Rh	−	3	244	1.1 ± 1.2	100.2 ± 9.2

[a] The liposomes and ghosts were incubated at 37°C for 30 min with, or without 1.25 mM Ca^{2+}/Mg^{2+}.
[b] Counted under light microscope.
[c] Number of ghosts with diffuse fluorescence × 100/number of total ghosts.
[d] Number of fluorescent ghosts × 100/number of total ghosts.

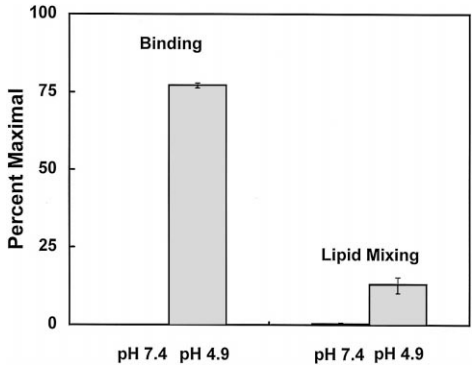


Fig. 9. pH-Dependence of binding and lipid mixing between N-C12-DOPE/DOPC (70:30) liposomes and erythrocyte ghosts at 37°C, measured by NBD/Rh RET assay. The liposomes and ghosts were incubated at indicated pH for 1 h in the absence of any divalent cation.

and lipid mixing between N-C12-DOPE/DOPC (70:30) liposomes and erythrocyte ghosts are pH-dependent, and that low pH is capable of triggering both events. No significant change of NBD and Rh fluorescence was observed when liposomes were incubated at pH 4.9 alone (data not shown). Thus, N-C12-DOPE appears to act as a pH-dependent as well as a Ca^{2+}-dependent fusogen.

4. Discussion

The data we have presented demonstrate the fusogenic nature of one of the *N*-acyl-PEs. Incorporation of these lipids into liposomes or their generation in cellular membranes could render such membranes fusogenic in the presence of physiologically relevant cations. Fusion of other pure lipid liposomes with cells has been observed, but these studies have generally utilized positively charged liposomes [22] or those dependent on the low pH of the endosome [23–25]. By contrast, liposomes containing *N*-acyl phospholipids may fuse on the basis of properties distinct from the other liposomes and may have different physiological properties.

We can only speculate on the mechanism of these fusion events at present. Fusion of any membrane with a cellular membrane requires a mechanism to overcome the steric hindrance of cellular proteins and carbohydrates as well as any electrostatic repulsion. Ultimately upon very close approach of membrane surfaces, hydration forces may also become important. In the cases studied, the electrostatic repulsion between the negatively charged N-C12-DOPE and the predominantly negatively charged cell-surface carbohydrates and proteins is probably overcome by binding of the divalent cations or protonation at low pH. The divalent cations may also help to bridge between the liposomal surface and charged groups at the exterior of the cellular glycocalyx.

The mechanism by which the steric hindrance of the glycocalyx is overcome is more difficult to rationalize. It is possible that thermal motions are sufficient to allow occasional productive contact between cellular and liposomal membranes to occur. However, the fact that the PS-containing liposomes in this study bound quite well to the ghosts but did not fuse is consistent with special requirements for bridging the gap presented by the glycocalyx and/or overcoming the non-fusogenic nature of the plasma membrane lipids. Several properties unique to the *N*-acyl-PEs (as opposed to PS and PG) may satisfy such requirements.

It is likely that the *N*-acyl-PEs, in the presence of divalent cations, would have a strong tendency for negative spontaneous curvature [26] due to insertion of the third acyl chain into the bilayer [4,6,27,28]. This would lead to inverted hexagonal II phase in multilamellar systems, which has been demonstrated for *N*-palmitoyl–DOPE [4] and another multichain phospholipid, cardiolipin [29,30]. *N*-oleoyl–DOPE apparently forms an inverted hexagonal phase even in the absence of divalent cations [5]. One way in which this property can promote fusion is to facilitate the formation of fusion intermediates that may require similar negative spontaneous curvature [31,32]. The curvature preference may also lead to prefusion hydrophobic exposure via acyl chain packing mismatches.

Data for DPPE-based *N*-acyl lipids suggest that acyl chains that are less than 10 carbons do not insert well into the bilayer [6]. No data on the DOPE derivatives exist, but the 12 carbon DOPE derivative studied here is only slightly longer, and it may be capable of extension away from the bilayer, especially in the presence of Ca^{2+}. Extension into a second, target membrane would relieve the strong curvature stress likely to be induced by Ca^{2+} in the original membrane. However, it is likely that only a

small portion of the *N*-acyl chains would be stabilized in this manner, only at points of membrane–membrane contact, thereby enhancing binding between the membranes without nullifying the fusogenic impetus of the curvature stress. In some respects, this putative process could possess similarities to influenza hemagglutinin-mediated fusion [for reviews, see [33]].

Once the glycocalyx is penetrated and liposomal and cell-membrane lipids can approach closely, hydration may be an important factor. The observation that Ca^{2+} is a stronger mediator of fusion than Mg^{2+} is consistent with a requirement for partial dehydration of the liposome surface, which would be better promoted by Ca^{2+} [34–36].

Binding and possible fusion of N-C12-DOPE/DOPC liposomes to nucleated cells were also observed. Surprisingly, neither of them was completely Ca^{2+}-dependent, despite the overall negative charge of the plasma membrane. It has been proposed [37–39] that some nucleated cells may possess specific receptors for liposomes containing negatively charged lipids, such as PS. Thus, it is possible that binding of N-C12-DOPE/DOPC liposomes to U-937 cells is partially mediated by specific high affinity receptors in addition to cations. Endocytosis following receptor binding could lead to pH-dependent fusion with the endosomal membrane or degradation in the lysosomes. Although we have not yet obtained results that can distinguish between these two possibilities, observed binding and fusion of N-C12-DOPE/DOPC liposomes with erythrocyte ghosts at pH 4.9 is consistent with fusion of liposomes with the endosomal membrane [40] before exposure to lysosomal enzymes.

N-C12-DOPE/DOPC liposomes were also capable of delivering encapsulated dextrans into erythrocyte ghosts via cation-mediated binding and fusion. After 30 min incubation at 37°C, more than 13% of the ghosts acquired dextrans (Table 1). The actual percentage may be even higher because some of the ghosts may have only fused with a few liposomes and their fluorescence would be too low to be observed microscopically. Leakage of encapsulated dextrans under these conditions (data not shown) could be another cause of underestimation of delivery. The fact that over 50% of ghosts show lipid mixing with NBD/Rh labeled liposomes under the same experimental conditions (Table 1) also supports this hypothesis, although the percentage of fused ghosts may also be overestimated using lipid probes due to potential artifactual probe transfer and lipid mixing in the absence of contents mixing.

The number of maximum liposome binding sites on an approximately 5 μm diameter spherical ghost can be estimated using the projection of a spherical 0.1 μm diameter liposome. About 10 000 liposomes could fit on the surface of one ghost. Taking the area occupied by each phospholipid as 0.7 nm^2, each 100 nm diameter liposome contains approximately 45 000 phospholipid molecules. The highest lipid/ghost ratio used here, i.e. 100 nmol/5×10^8, gives about 2600 liposomes per ghost, far below that necessary to cover the entire ghost surface. Thus, the delivery efficiency may be improved by increasing liposome/ghost ratio (data not shown), although at very high liposome/cell ratio, lipid toxicity could be a potential problem. Conversely, the percent of lipid mixing could potentially be increased by decreasing the liposome/ghost ratio.

Liposomes have been extensively studied as delivery systems for various drugs and nucleic acids [41–43]. Encapsulation of drugs and nucleic acids into liposomes can protect them from enzyme degradation, reduce their toxicity, and provide the potential of specific targeting. Delivery through fusion between liposomal and cellular membranes is highly desirable due to its potential high efficiency, targeting ability and wide application range. Since not all cells are endocytic nor is it always desirable to utilize the endocytic pathway, the ability to deliver in the absence of endocytosis may be important in many situations. Here, we have shown delivery of encapsulated dextrans into non-endocytic erythrocyte ghosts using N-C12-DOPE/DOPC liposomes. These data suggest that NAPEs may constitute an important component for liposomes that can be used as delivery vehicles for therapeutic agents.

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