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Biochimica et Biophysica Acta 1280 (1996) 107–114

BBA Biochimica et Biophysica Acta

Non-phospholipid fusogenic liposomes

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Received 27 March 1995; revised 8 November 1995; accepted 22 November 1995

Abstract

We have demonstrated the capacity of non-phospholipid liposomes composed primarily of dioxyethylene acyl ethers and cholesterol to fuse with membranes composed primarily of phospholipid. Phase-contrast microscopy, freeze-fracture electron microscopy and a macromolecular probe indicate that these non-phospholipid liposomes can fuse with the plasma membranes of erythrocytes and fibroblasts. Furthermore, fluorescence probe experiments have demonstrated fusion between phosphatidylcholine liposomes and non-phospholipid liposomes. Mixing of internal contents was shown by a terbium/dipicolinate assay. Mixing of membrane lipid components was demonstrated by measuring (i) fluorescence resonance energy transfer between *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)phosphatidylethanolamine and *N*-(lissamine rhodamine B sulfonyl)phosphatidylethanolamine, after phosphatidylcholine liposomes were mixed with non-phospholipid liposomes, and (ii) reduced concentration quenching of rhodaminephosphatidylethanolamine and octadecylrhodamine incorporated into phosphatidylcholine liposomes after mixing with the non-phospholipid liposomes. The degree of apparent fusion reported by the different probe techniques ranged from 25% to 64%.

Keywords: Fusogenic lipid; Liposome–cell fusion

1. Introduction

For many reasons, there has been increasing interest recently in non-phospholipid liposomes, NPL, made of non-phospholipid, 'membrane mimetic' [1] lipids (see [2–5] for reviews). NPL are attractive because of ease of manufacture, stability and versatility, and are being increasingly applied to experimental pharmaceutical studies [5–12]. This paper concerns the fusogenic properties of NPL made of dioxyethylene acyl ethers and cholesterol.

A number of different approaches have been used to create phospholipid liposomes that have the capacity to fuse with cellular membranes. In some fusogenic liposomes fusogenicity derives from membrane-associated proteins [13,14] or peptides [15,16]. In others it depends on specific interactions between the liposomes and target membrane receptors. Liposomes containing negatively charged phospholipids becoming fusogenic in the presence of calcium [17,18]. pH-sensitive liposomes fuse with endosomal membranes at low pH [16,19].

Here we demonstrate the fusogenic properties of NPL containing dioxyethylene acyl ethers, single-tailed, non-phospholipid amphiphiles as principal membrane constituents. These liposomes can fuse with phosphatidylcholine liposomes, PL, at neutral pH, as demonstrated by fluorescence assays monitoring the mixing of both liposomal aqueous compartments and membrane lipids. These NPL also fuse with living cells, as demonstrated by phase-contrast microscopy, by freeze-fracture electron microscopy showing altered lateral distribution of intramembranous particles [20–27] representing plasma membrane transmembrane proteins, and by transfer of a macromolecular probe.

Abbreviations: CL, calcein; DPA, dipicolinic acid; HRP, horseradish peroxidase; PL, phosphatidylcholine liposome; NPL, non-phospholipid liposomes; (N-NBD-PE), *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)phosphatidylethanolamine; (N-Rh-PE), *N*-(lissamine rhodamine B sulfonyl)phosphatidylethanolamine; R18, octadecyl rhodamine B hydrochloride; RET, resonance energy transfer; Tes, *N*-tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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SSDI 0005-2736(95)00286-3

2. Materials and methods

2.1. Chemicals

2,2′-Azino-bis(ethylbenzthiazole)-6-sulfonic acid, cholesterol, dipicolinic acid (DPA; pyridine-2,6-dicarboxylic acid), dioxyethylene hexadecyl and octadecyl ethers, egg yolk phosphatidylcholine, L-histidine, glyceryl octadecyl ether, horseradish peroxidase (HRP), H_2O_2, oleic acid, $TbCl_3$, Tes and Tris-HCl were obtained from Sigma (St. Louis, MO). *N*-(Lissamine rhodamine B sulfonyl)phosphatidylethanolamine (N-Rh-PE) and *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)phosphatidylethanolamine (N-NBD-PE) were bought from Avanti Polar Lipids. Octadecyl rhodamine B hydrochloride (R18) was obtained from Molecular Probes. Triton X-100 and calcein (CL) came from Merck.

2.2. Vesicle preparation

For fluorescence studies, NPL composed of dioxyethylene acyl ether and cholesterol (3:1, molar ratio) were prepared by extruding the molten lipid phase vigorously at (50°C) through a 0.46 mm ID needle into medium A (20 mM NaCl, 2 mM L-histidine and 2 mM Tes buffer at pH 7.4). The final lipid concentration was 12.5–62.5 mM. The vesicles were sonicated for 15 min with a 50% active cycle, using a Vibra Cell instrument (Bioblock, France) at 0°C, to 150–300 nm diameter, systematically controlled in all assays by photon correlation spectroscopy using a NS4 submicron particle analyzer of Coultronics. Negative staining electron microscopy, using phosphotungstic acid and a magnification of $\times 100\,000$ showed that most of the vesicles were unilamellar.

Egg yolk phosphatidylcholine liposomes (PL) were prepared from a (2:1, v/v) chloroform/methanol solution by evaporation under N_2, swelling the lipid in medium A and sonicating at 0°C as to reach a vesicle diameter in the 150–250 nm range, measured by photon correlation spectroscopy. The final lipid concentration was 10 mM. These liposomes were unilamellar according to negative staining electron microscopy.

2.3. Fluorescence measurements

Continuous monitoring of fluorescence used a recording Aminco-Bowman (series 2) spectrophotofluorometer, with a magnetically stirred sample chamber and temperature maintained using a thermostated circulating water bath.

2.4. Leakage from the intraliposomal water compartment

The release of water-soluble liposomal contents after mixing PL with NPL was evaluated using the increased fluorescence of CL, initially encapsulated within PL at the highly self-quenching concentration of 100 mM [28–31]. After removing extraliposomal CL by filtration on Sephadex G-25, the CL-laden vesicles were mixed with an excess of NPL and CL fluorescence monitored with time (excitation at 490 nm; emission at 515 nm). To calculate percent CL leakage, the fluorescence of CL-free PL was taken as zero and the fluorescence intensity after lysis of PL with Triton X-100 (0.5%, v/v) as 100%.

2.5. Mixing of liposomal water compartments

We monitored the interaction between Tb^{3+} and dipicolinic acid [30,32]. Mixing these reagents creates the $[Tb(DPA)^{3-}]_3$ complex, with a 10^4 enhancement of terbium fluorescence [29,30,32–35]. To measure mixing of internal contents, NPL were prepared in presence of 50 mM DPA, and PL in presence of 2.5 mM $TbCl_3$. Unencapsulated material was removed on Sephadex G-25 using an elution buffer composed of medium A containing 1 mM EDTA (pH 7.4). The EDTA blocks formation of $[Tb(DPA)^{3-}]_3$ in the extraliposomal compartment. After mixing one ml aliquots of the two liposome populations (final lipid concentration varied from 0.30 to 1 μmol/ml according to liposome preparation), terbium fluorescence was recorded at 25°C (excitation at 276 nm; emission at 545 nm). In control experiments, DPA-laden PL were substituted for the DPA-containing NPL. To generate calibration curves, aliquots of the $TbCl_3$-containing PL vesicles were filtered through Sephadex G-25 to remove EDTA, 20 mM free DPA added and sodium cholate (0.5%, w/v) mixed in to release vesicle contents. The fluorescence after this procedure was taken as 100%. The extent of apparent fusion F_t was calculated according to:

$$F_t = [(I_t - I_0)/(I_d - I_0)] \times 100$$

where I_0 is the fluorescence intensity at time = 0, I_t is the fluorescence intensity at time = t, and I_d is the fluorescence following detergent disruption of liposomes.

2.6. Lipid mixing — two-probe assay

This method relies on resonant energy transfer (RET) between two fluorescent phosphatidylethanolamine derivatives, N-NBD-PE and N-Rh-PE incorporated into PL bilayers. (This 'probe dilution' method is insensitive to vesicle aggregation [35]). The efficiency of RET from the energy donor, N-NBD-PE, to the energy acceptor, N-Rh-PE, depends on the concentration of the probes in the bilayer surface [31,35–39]. Any fusion of labeled PL with NPL will result in diffusional redistribution of membrane lipids (including the marker fluorophores), increased distance between the fluorophores and thereby decreased RET efficiency. This will enhance the fluorescence of the donor (N-NBD-PE) and diminish that of the of acceptor (N-Rh-PE). Labeled PL (egg yolk phosphatidylcholine/N-Rh-PE/N-NBD-PE; 99:0.5:0.5, molar ratio) were prepared as above, except that hydration was in 20

mM Tris, 10 mM NaCl at pH 7.4 (medium B). The labeled PL were mixed with NPL (1:1, lipid molar ratio) to a total lipid concentration of 100 μM. For fluorescence measurements the excitation and emission monochromators were set at 465 and 530 nm, respectively. The sample volume was 2 ml and fluorescence was monitored at 25°C. The residual fluorescence of the PL, in absence of NPL was taken as zero. The fluorescence after dilution of the fluorophore by disruption of the liposomes with Triton X-100 and correcting for the effect of detergent on the quantum yield of N-NBD-PE, was taken as 100% [39]. The correction factor (1.8–2.0) was determined by incorporating N-NBD-PE into the PL at 0.1% and measuring the fluorescence intensity before and after addition of Triton X-100 [39]. Fusion was quantitated as in [38], taking into account the ratio of labeled to unlabeled vesicles

2.7. Lipid mixing — single-probe assay

When N-Rh-PE [40] or R18 [31,38,41], are incorporated at a sufficiently high concentrations into PL membranes, fluorescence emission is 'self-quenched', probably by RET between N-Rh-PE molecules or between R18 molecules. Self-quenching efficiency depends on the concentration of the fluorophore in the lipid bilayer and is directly proportional to the ratio of fluorophore to total lipids. The decrease of self-quenching following addition of unlabeled NPL provides an index of fusion.

PL containing 5 mol% N-Rh-PE or R18 in medium B were mixed with NPL (1:4, molar ratio) in the same medium at a final lipid concentration of 100 μM. Under these conditions the fluorophore probe concentration is < 1 nmol/ml and the fluorescence intensity varies linearly with probe concentration [38,41]. The fluorescence emission measurements were carried out in a 2 ml volume, at 25°C, with continuous stirring. The excitation and emission monochromators were set at 560 and 590 nm, respectively.

2.8. Cells

Erythrocytes, isolated from freshly drawn, heparinized blood were pelleted at 3500 rpm and washed thrice with 5 volumes of phosphate-buffered saline. 3T3 fibroblasts, propagated conventionally, were studied at confluence.

2.9. Phase-contrast microscopy

Interaction of NPL with control and NPL-treated cells in phosphate-buffered saline was studied using vesicles composed of dioxyethylene hexadecyl ether/cholesterol (molar ratio 3 : 1), dioxyethylene octadecyl ether/cholesterol (molar ratio 3 : 1) and glyceryl octadecyl ether/cholesterol (molar ratio 3 : 1), both with and without 0.025 mol% oleic acid (to provide a negative surface charge). Preliminary studies showed no difference in cellular effects due to oleic acid. However, the oleic acid-containing vesicles were less prone to aggregate at high vesicle concentrations and over long durations of time. The interaction of NPL with control and NPL-treated cells was monitored at 25°C and 37°C for 15–120 min at vesicle concentrations ranging about 10^{12} particles per ml to about 10^{6} particles per ml.

2.10. Electron microscopy

Dioxyethylene cetyl ether/cholesterol/oleate vesicles (about 10^{6} vesicles/cell) were reacted for 18 h at room temperature with either erythrocytes or fibroblasts and the cells then examined by freeze-fracture electron microscopy essentially as in [24]

2.11. Transfer of horseradish peroxidase to erythrocytes

For encapsulation of HRP, this molecule was included in the aqueous phase during formation of NPL composed of dioxyethylene cetyl ether/cholesterol [5,6]. Empty, otherwise identical vesicles were prepared in parallel. Free HRP was removed by five centrifugal washings over a 10% dextran density barrier. The last wash was saved. A 1:10 dilution of HRP-laden NPL (about 10^{13} vesicles per ml) was incubated with $2.3 \cdot 10^{6}$ erythrocytes at 37°C for 1 h. Control erythrocytes included: (i) cells washed at zero time, (ii) cells incubated with a 1:10 dilution of the final wash solution and (iii) cells incubated with vesicles laden with final wash. At the end of the incubation, the red cells were separated centrifugally from the NPL. No erythrocyte lysis occurred. The cells were then disrupted in octyl glucoside (100 mg/ml). This was followed by a standard assay involving addition of 100 μl substrate, 2,2′-azino-bis(ethylbenzthiazole)-6-sulfonic acid (10 mg/ml) and 100 μl H_2O_2 (0.3%). After 10 min the color at 405 nm was read against a standard curve.

3. Results

3.1. Interaction of NPL with PL

3.1.1. Mixing of PL and NPL aqueous compartments

Mixing of the aqueous compartments of PL and NPL is demonstrated in Fig. 1. Whereas addition of DPA-laden PL to Tb^{3+}-laden PL does not cause significant changes in fluorescence (Fig. 1, curve A), addition DPA-laden NPL to Tb^{3+}-laden PL markedly enhances fluorescence intensity (Fig. 1; curve B). This is due to the formation of $[Tb(DPA)^{3-}]_3$ complexes [31]. Since EDTA, in the suspension medium, prevents formation of extraliposomal $[Tb(DPA)^{3-}]_3$ complexes, the increase of fluorescence can be ascribed to the formation of a common aqueous compartment following fusion of Tb^{3+}-laden PL and DPA-laden NPL. The data give an apparent initial fusion rate of

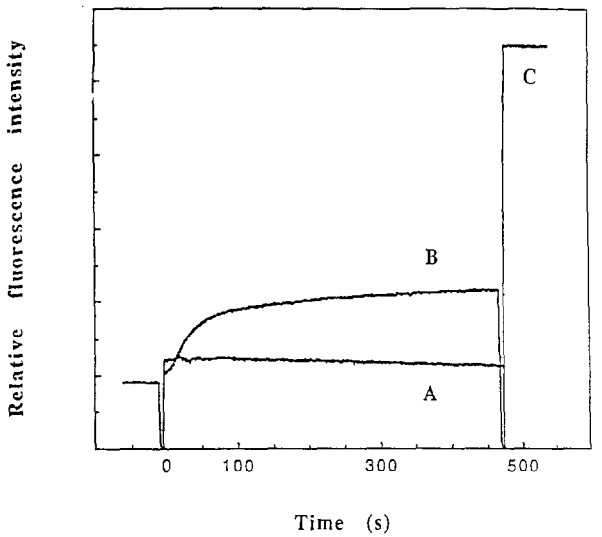

Fig. 1. Fluorescence intensity of Tb^{3+}-laden PL after mixing with DPA-laden NPL or DPA-laden PL. At zero time Tb^{3+}-laden PL were mixed with DPA-laden PL (A) or DPA-laden NPL (B), (1:1, v/v) at the final lipid concentration of 0.3 and 0.33 μmol/ml, respectively, in presence of 1 mM EDTA. Fluorescence intensity was monitored continuously at 545 nm and 25°C. Curve C was obtained after detergent solubilization of liposomes, in absence of EDTA, as explained in Section 2. The small increase in apparent fluorescence at zero time, probably reflects the increased vesicle concentration after addition of PL or NPL.

about 0.3% maximum/sec and maximal fusion of 30% in 10 min. (Table 1, column 2).

3.1.2. Leakage

To better evaluate the extent of fusion, it was important to determine whether small molecules might pass between intraliposomal and external aqueous compartments during the fusion process. For this, we loaded PL with 100 mM CL, at which concentration this fluorophore is concentration-quenched, and measured the increase of fluorescence occurring after addition of NPL (Fig. 2). We found maximum leakage of 28% at 25°C, with a plateau after 2–3 min, and an initial rate of 2.5% maximum/sec.

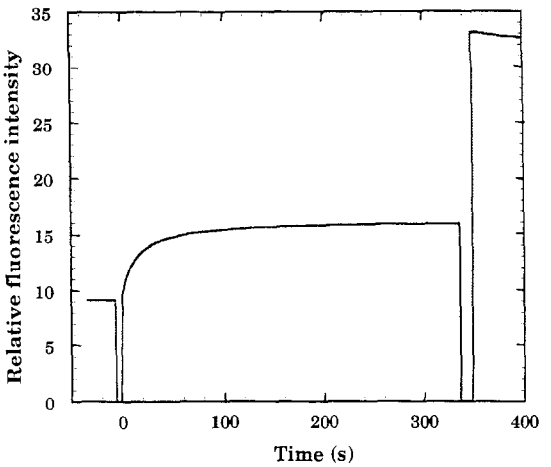

Fig. 2. CL leakage from PL mixed with NPL. At zero time excess of NPL were mixed with CL-laden PL. The final lipid concentration was 0.77 μm/ml. Fluorescence intensity was monitored continuously at 515 nm and 25°C. 100% fluorescence intensity was obtained after lysis with Triton X-100 (0.5%, v/v).

3.1.3. Mixing of PL and NPL membranes — two-probe studies

In the two-probe lipid intermixing experiments, we monitored the relative fluorescence intensities of N-NBD-PE, the RET donor, and N-Rh-PE, the acceptor, at their emission wavelengths, after addition of NPL to the PL suspension. Fig. 3 shows the time-dependent increase in the fluorescence of N-NBD-PE following combination of N-NBD-PE-labeled PL with NPL at a lipid ratio of 1:1 and a total lipid concentration of 100 μM. Mixing of N-NBD-

Table 1
NPL-induced fusion estimated from mixing of water-soluble liposome contents and mixing of membrane lipid

Time (min)	Percent fusion			
	ICM [a]	Membrane lipid mixing		
		Two-probe [b]	Single-probe	
			N-Rh-PE [a]	R18 [c]
0	0	0	0	0
1	18.6	42.6	24.6	11.3
3		50.8		15.1
4	24.4			
5	25.6	55.2	33.0	17.3
10	27.8	60.4	35.0	20.4
15	30.0	63.4	39.0	23.5
20		64.2	40.0	25.0

[a] Two assays.
[b] Four assays.
[c] Five assays.

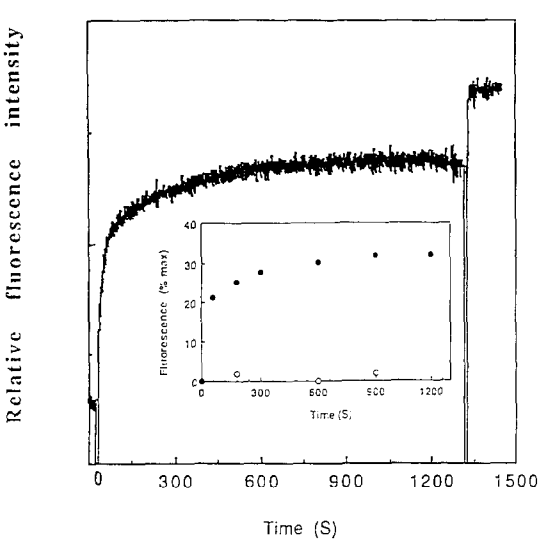

Fig. 3. NBD fluorescence intensity after mixing of PL containing N-NBD-PE and N-Rh-PE (99:0.5:0.5, molar ratio) with NPL (lipid molar ratio 1.0) at a final lipid concentration of 100 μM (25°C). The inset depicts percent maximum fluorescence computed from kinetic curves. (●) Labeled PL/NPL; (o) labeled/unlabeled PL.

PE-labeled PL with unlabeled PL caused no change in fluorescence (Fig. 3, inset). Since the fluorescent probes have been shown not to exchange between phospholipid vesicles [36], the increase in the fluorescence intensity of N-NBD-PE can be attributed to its increased separation from N-Rh-PE following interaction of the labeled PL with NPL. We found a maximum fluorescence increase of $32 \pm 4\%$ which corresponded to about 64% fusion (Table 1, column 3).

3.1.4. Mixing of PL and NPL membranes — single-probe studies

In these experiments we monitored the fluorescence of N-Rh-PE-labeled PL, or of R18-labeled PL, after mixing them at a vesicle lipid molar ratio of 1:4 with NPL. In both cases, addition of NPL caused an increase of probe fluorescence maximal after 15–20 min. The maximum fluorescence increases were 31% and 20% with N-Rh-PE and R18, which corresponded to fusion values of 40% and 25%, respectively (Table 1, columns 4 and 5).

3.2. Interaction of NPL with cells

3.2.1. Erythrocytes — phase-contrast microscopy

Addition to freshly washed erythrocytes of PL, or of any of the NPL formulations tested, did not lead to any release of hemoglobin, suggesting no leakage of macromolecules. However, NPL made with dioxyethylene hexadecyl or octadecyl ethers caused slight to massive crenation (Fig. 4a) and, depending on concentration, conversion of the erythrocytes into bizarre, grape-like clusters, with attached or budded-off, hemoglobin-filled tubules (Fig. 4b). The erythrocyte deformation process occurred within 15 min. at room temperature and was detected at vesicle concentrations as low as 10^2 per cell for the hexadecyl ether and 10^3 vesicles per cell for the octadecyl ether (1 : 10 000 and 1 : 1000 dilutions, respectively). In contrast, PL or vesicles made with glyceryl octadecyl ether did not cause red cell deformation even at high concentrations (data not shown). Budded-off tubules could be centrifugally isolated and osmotically lysed to release hemoglobin.

3.2.2. Erythrocytes — electron microscopy

The nature of the red cell deformation process caused by dioxyethylene acyl ethers has been elucidated by freeze-fracture electron microscopy. As shown in Fig. 5, the interaction of dioxyethylene cetyl ether/cholesterol/oleate NPL with erythrocytes markedly disturbs the normal distribution of intramembranous particles representing transmembrane proteins. Instead of the well established statistical distribution of particles without particle-free domains [42], the fracture faces of the NPL-treated cells exhibit huge bald areas that contain few free intramembranous particles. Most of the intramembranous particles have aggregated into clusters. The bald, particle-free areas range in area from 1 to 3 μm^2, equivalent to 2–6 times the surface area of an NPL.

3.2.3. Fibroblasts electron microscopy

To determine whether fusion of dioxyethylene acyl ether vesicles might also occur with cultured fibroblasts, we repeated the erythrocyte experiments with 3T3 cells at a concentration of about 10^{11} vesicles per ml. Comparison of treated with untreated cells (Fig. 6) shows a pattern analogous to that observed with erythrocytes. The normally randomly distributed particles are aggregated and much of the surface area is occupied by particle-free, bald patches.

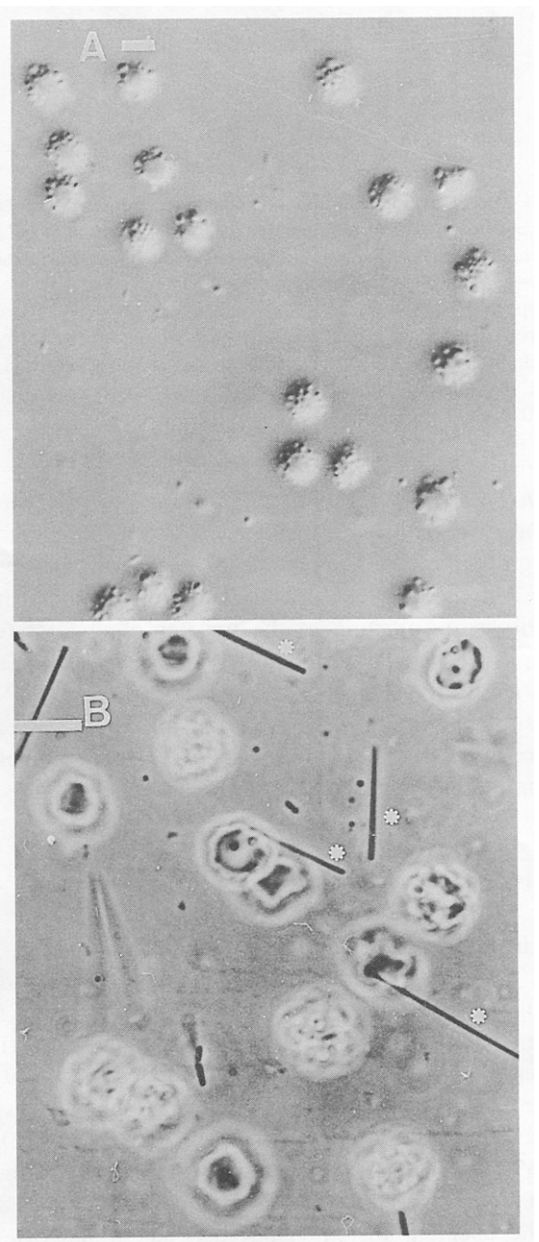

Fig. 4. Phase-contrast visualization of the effects of fusogenic NPL on erythrocytes. (A) Crenation with globular surface protrusions; NPL dilution 1 : 100; incubation 1 h; original magnification ×225. (B) Hemoglobin-filled tubules (stars) pinched off severely deformed erythrocytes; incubation overnight; NPL dilution 1 : 100; original magnification ×500. Bar corresponds to 10 μm.

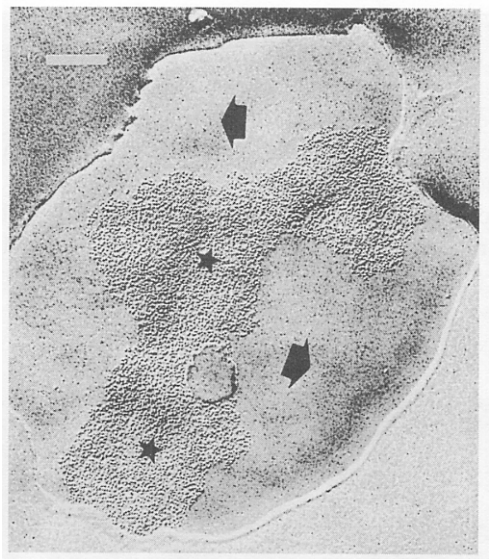

Fig. 5. Freeze-fracture electron micrograph of the effects of NPL on erythrocytes. In normal erythrocytes the fracture face is populated by numerous intramembranous particles — representing transmembrane proteins — that are distributed randomly. The fracture face of the NPL-treated erythrocytes shown here shows only rare solitary particles. The rest are clumped together in a massive aggregate (stars), separated by large particle-free areas (arrows). We think this is due to a localized lateral phase segregation induced by NPL lipids. Original magnification ×16000. Bar corresponds to 0.5 μm.

3.2.4. Transfer of horseradish peroxidase to erythrocytes

Table 2 shows the results of these experiments. Although erythrocyte hemoglobin gives a backgroud peroxidase activity, the NPL treated cells exhibit much higher activity corresponding to the transfer of about 6.6% of encapsulated HRP, or about 10^6 molecules/cell. Control cells gave only background activity, suggesting HRP entry takes place during the period of membrane alteration demonstrated by light and electron microscopy, rather than by simple binding of HRP/NPL vesicles.

4. Discussion

Kinetic studies, using Tb^{3+}-laden PL and DPA-laden NPL give an apparent initial rate of aqueous compartment mixing of 0.3% max/sec at 25°C with an apparent maximum of 30% (Fig. 1; Table 1). This compares to literature values of 0.15% max/sec [30], 0.3% max/sec [29] and 4% max/sec [18] for the phosphatidyl serine liposome/Ca^{2+} system at 25°C (maximum > 30%), and > 16% max/sec [31] for the cardiolipin liposome/influenza virus system at 37°C system (maximum > 80%).

The apparent fusion values depend on the extent of leakage, during fusion, of small molecules out of the intraliposomal compartments. Such leakage is well documented [30], but has not been found to correlate well quantitatively with fusion. Leakage may be slow and small relative to fusion or large and extensive [30]. We evaluated leakage by the release of a CL, a small water-soluble fluorescent molecule (M_r 622) and a suitably sized probe for Tb^{3+}, DPA, $[Tb(DPA)^{3-}]_3$ and EDTA. The initial CL efflux rate, 2.3% of maximum at 25°C is considerably

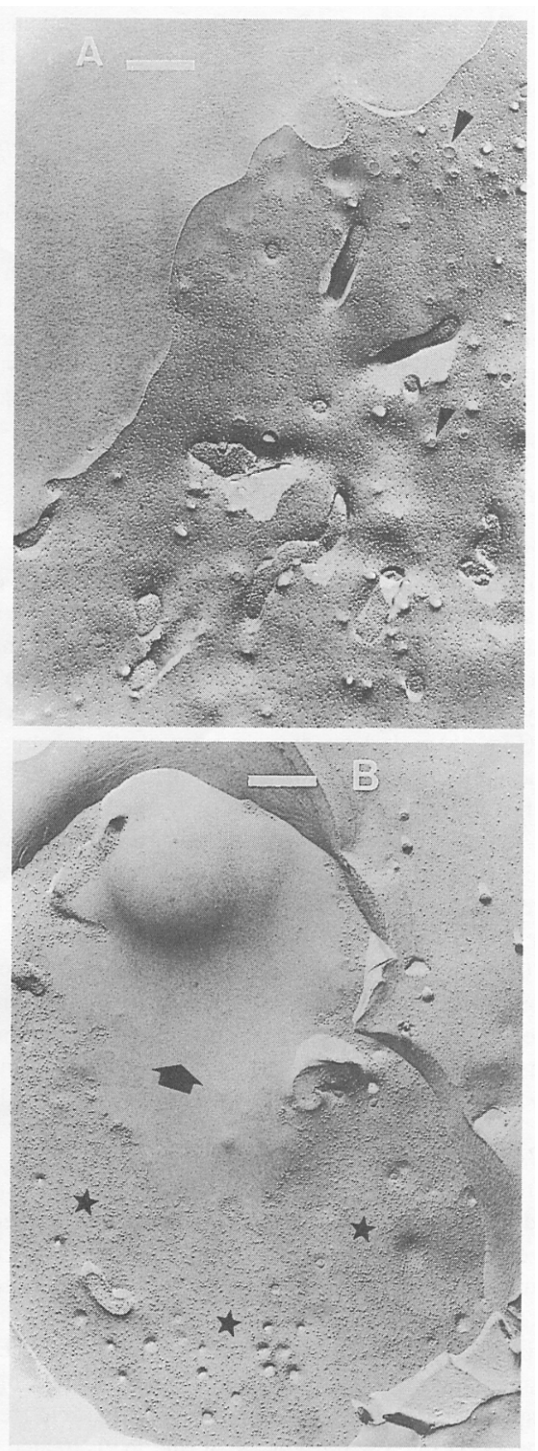

Fig. 6. Freeze-fracture electron micrographs of the effects of NPL on fibroblasts. (A) In normal fibroblasts the intramembranous particles are distributed randomly. Note 'stumps' of fractured microvilli (arrow). (B) NPL-treated fibroblast. Note particle aggregation (stars) and particle-free area (arrows). Original magnification ×18000. Bar corresponds to 0.5 μm.

Table 2
Transfer of HRP from fusogenic dioxyethylene hexadecyl ether/cholesterol NPL to erythrocytes

Conditions	HRP ($\mu g/10^6$ cells)
Erythrocytes + blank NPL	10 [a]
Erythrocytes + final wash	10 [a]
Erythrocytes + HRP-laden NPL	172

[a] These values represent the peroxidase activity of erythrocyte hemoglobin.

larger than the initial rate of aqueous compartment mixing, 0.3% of maximum. Although efflux of DPA is likely to be slower than that of CL because of its lower initial concentration (50 mM vs. 100 mM), one must assume fairly rapid efflux and therefore a decrease in the rate and extent of $[Tb(DPA)^{3-}]_3$ formation and fluorescence enhancement. Influx of EDTA and efflux of Tb^{3+} and $[Tb(DPA)^{3-}]_3$ would further decrease fluorescence enhancement. Without taking into account the effects of leakage, the maximum enhancement of Tb^{3+} fluorescence suggests about 30% mixing of aqueous compartments after 10 min at 25°C. Equating maximum CL leakage (28%) at 25°C with possible DPA leakage would give 42% fusion. These data fall within the range of results obtained by others with CL or carboxyfluorescein [28–31]. However, even this figure is likely to be low, because the efflux of Tb^{3+} and $[Tb(DPA)^{3-}]_3$ into an EDTA-containing medium are also likely reduce the apparent fusion values.

The two-probe RET experiments gave a maximum fluorescence increase of 34 ± 4 after 10–15 min. With liposomes possessing identical surface areas and a 1:1 vesicle ratio, complete intermixing of membrane lipids should give a 50% fluorescence increase at infinite dilution [38,39]. Accordingly, the fluorescence increase observed after the interaction of PL and NPL would correspond to about 64% fusion after 15 min (Table 1, column 3). The decrease in the fluorescence of the acceptor, N-Rh-PE, suggests a maximal fusion value of 63% (data not shown).

Since NPL contain 25 mol% cholesterol and since RET from N-NBD-PE to R18 is increased in the presence of cholesterol [40], the fusion value of 63–64% may underestimate the actual degree of fusion. It is not, however, inconsistent with our data on leakage and mixing of internal contents and compares reasonably well with values obtained for the phosphatidylserine liposome/Ca^{2+} system and the cardiolipin liposome/influenza virus system (Table 3).

The maximum fluorescence increases in the single-probe experiments were 31% and 20% with N-Rh-PE and R18, respectively. With the 1:4 lipid molar ratio used, the fluorescence increment should correspond to 80% of the signal at infinite dilution, giving a fusion value of 40% and 25% for the N-Rh-PE and R18 respectively (Table 1, columns 4 and 5). However, as stated earlier, cholesterol markedly increases self-quenching of R18 and N-Rh-PE in PL [40]. At 25°C, 50 mol% cholesterol and 5 mol% R18 or N-Rh-PE, for example, R18 fluorescence quenching is about 16% that found in cholesterol-free PL. Because the NPL contain 25% cholesterol, it is possible that the values obtained in the single probe experiments may substantially underestimate the degree of fusion.

The use of fluorophores to monitor membrane fusion has heretofore been limited to systems containing only phospholipid-based membranes and we cannot now be certain how these systems apply quantitatively to the interaction of NPL with PL. It is clear, however, that the interaction of dioxyethylene cetyl ether/cholesterol NPL with PL causes transient leakage of small, water-soluble probes from the PL and, concomitantly, continuity between the aqueous compartments of the two types of liposome. In addition, both the single- and double-probe RET experiments suggest that the interaction of the NPL with PL leads to considerable intermingling of the respective membrane lipids.

The work on erythrocytes and fibroblasts indicates that dioxyethylene acyl ether-containing NPL can fuse not only with artificial phospholipid bilayers but also with the

Table 3
RET in different fusogenic systems: negatively charged liposomes/Ca^{2+}, negatively charged liposomes/virus and PL/NPL

Labeled material	Unlabeled material	Molar ratio	Ca^{2+} (mM)	Temp. (°C)	% Fluoresc. max.	% Fusion	Initial rate (%max./s)	Reference
CL/DOP	CL/DOPC	1:1	11	25	60		2.5	[34,39]
PS	PS	1:1	2				38	[39]
			4				70	
			5				83	
			6				88	
PS	PS	1:4	2	37	20	25	0.6	[38]
			3				2.7	
			4		60	85	5.1	
			5				6.1	
CL	Influenza	1:1		37	40	80	8.0	[31]
PS	Influenza	1:1		37	30	60	1.4	[38]
PL	NPL	1:1		25	32.2	64	0.58	

plasma membranes — which are phospholipid-based — of at least two cell types. The electron microscopic data are consistent with considerable lipid intermixing, but also suggest that the injection of foreign lipid into the target cell membranes leads to interference with the normal lateral distribution of trans-membrane proteins. Interestingly, there is no evidence of macromolecule (hemoglobin) leakage under fusion conditions that lead to considerable leakage of CL although the data show significant transfer of HRP from NPL to erythrocytes.

We suspect that the fusogenic capacity of the dioxyethylene acyl ether/cholesterol NPL derives from the -O-$(CH_2CH_2O)_2$ head group, depending on chain length, and that it involves a lamellar → hexagonal (H_{II}) transition. Physicochemical studies on a series of polyoxyethylene acyl ethers [43] have shown that these molecules can form hexagonal phases. Moreover, plasmenylethanolamines, differing from phosphatidylethanolamines only by replacement of the ester linkage in the proximal portion of the *sn* − 1 constituent by a single vinyl ether (-CH_2-O-CH=CH- versus -CH_2-CO-CH_2-CH_2-), have been shown stimulate membrane fusion [44].

Acknowledgements

This work was supported by grants from "l'Agence Nationale de Recherche sur le SIDA" (ANRS), and from "l'Association pour la Recherche sur le cancer" (ARC) and by Micro Vesicular Systems. M. El Baraka is a recipient of a CNRS postdoctoral scholarship. We thank Dr. V. Speth, University of Freiburg, for the phase-contrast and electron micrographs.

References

[1] Fendler, J. (1982) Membrane Mimetic Chemistry, 522 pp., Wiley, New York.
[2] Wallach, D.F.H. and Philippot, J.R. (1993) in Liposome Technology, Vol. 1, 2nd Edn. (Gregoriadis, G., ed.), pp. 141–156, CRC Press, Boca Raton, FL.
[3] Philippot, J.R., Milhaud, P. Puyal, C. and Wallach, D.F.H. (1994), in Liposomes as Tools in Basic Research and Industry (Philippot, J.R. and Schuber, F., eds.), pp. 44–54, CRC Press, Boca Raton, FL.
[4] Wallach, D.F.H. and Mathur, R. (1994) in Liposomes as Tools in Basic Research and Industry (Philippot, J.R. and Schuber, F., eds.), pp. 241–251, CRC Press, Boca Raton, FL.
[5] Varanelli, C., Kumar, S. and Wallach, D.H.F. (1994) in Liposomes as Tools in Basic Research and Industry (Philippot, J.R. and Schuber, F., eds.), pp. 253–263, CRC Press, Boca Raton, FL.
[6] Azmin, M.N., Florence, A.T., Handjani-Vila, R.M., Stuart, J.F., Vandenberghe, G. and Whittaker, J.S. (1985) J. Pharm. Pharmacol. 37, 237–242.
[7] Azmin, M.N., Florence, A.T., Handjani-Vila, R.M., Stuart, J.F., Vandenberghe, G. and Whittaker, J.S., (1985) J. Microencapsulation 3, 95–100.
[8] Rogerson, A., Cummings, J., Wilmott, N. and Florence A.T., (1987) J. Microencapsulation 4, 321–328.
[9] Rogerson, A., Cummings, J., Wilmott, N. and Florence, A.T. (1988) J. Pharm. Pharmacol. 40, 337–342.
[10] Kerr, D.J., Rogerson, A., Morrison, G.J., Florence, A.T. and Kaye, S.B. (1988) Br. J. Cancer 58, 432–436.
[11] Moser, P., Marchand-Avrier, M., Labrude, P. and Vigneron, C. (1990) Pharm. Acta Helv. 65, 82–92.
[12] Parthasarathi, G., Udupa, N., Umadevi, P. and Pillai, G.K., (1994), J. Drug Target 2, 173–182.
[13] Sechoy, O., Philippot, J.R. and Bienvenüe, A. (1989) Exp. Cell Res. 185, 122–131.
[14] Bron, R., Ortiz, A. and Wilschut, J. (1994) Biochemistry 33, 9110–9117.
[15] Puyal, C., Milhaud, P., Bienvenüe, A. and Philippot, J.R. (1995) Eur. J. Biochem. 228, 697–703.
[16] Kato, T., Lee, S., Ono, S., Agawa, Y, Aoyagi, H., Ohno, M. and Nishino, N. (1991) Biochim. Biophys. Acta 1026, 186–197.
[17] Wilschut, J. and Papahadjopoulos, D. (1979) Nature 281, 690–692.
[18] Hoekstra, D. (1982) Biochim. Biophys. Acta 692, 171–175.
[19] Collins, D., (1994) in Liposomes as Tools in Basic Research and Industry (Philippot, J.R and Schuber, F., eds.), pp. 201–214, CRC Press, Boca Raton, FL.
[20] Speth, V., Wallach, D.F.H., Weidekamm, E. and Knüfermann, H. (1972) Biochim. Biophys. Acta 255, 386–394.
[21] Wunderlich, F., Speth, V., Batz, W. and Kleinig, H. (1973) Biochim. Biophys. Acta 298, 39–49.
[22] Speth, V. and Wunderlich, F. (1973) Biochim. Biophys. Acta 291, 621–628.
[23] Bhakdi, S., Speth, V., Knüfermann, H., Wallach, D.F.H. and Fischer, H. (1974) Biochim. Biophys. Acta 356, 300–308.
[24] Wunderlich, F., Batz, W., Speth, V. and Wallach, D.F.H. (1974) J. Cell Biol. 61, 633–640.
[25] Wunderlich F., Wallach, D.F.H., Speth, V. and Fischer, H. (1974), Biochim. Biophys. Acta 373, 34–43.
[26] Boggs, J.M., Clement, I.R. and Moscarello, M.A. (1980) Biochim. Biophys. Acta 601, 134–151.
[27] Robinson, J.M., Roos, D.S., Davidson, R.L. and Karnovsky, M.J. (1979) J. Cell Sci. 40, 63–75.
[28] Vidal, M., Bienvenüe, A., Sainte-Marie, J. and Philippot, J.R. (1984) Eur. J. Biochem. 138, 399–405.
[29] Wilschut, J., Düzgünes, N., Fraley, R. and Papahadjopoulos, D. (1980) Biochemistry 19, 6011–6021.
[30] Düzgünes, N. and Wilschut, J. (1993) Methods Enzymol. 220, 3–14.
[31] Stegmann, T., Hoekstra, D., Scherphof, G. and Wilschut, J. (1985) Biochemistry 24, 3107–3113.
[32] Barela, T.D. and Sherry, D., (1976) Anal. Biochem., 71, 351–357.
[33] Rosenberg, J., Düzgünes, N. and Kayalar, C. (1983) Biochim. Biophys. Acta 735, 173–180.
[34] Wilschut, J., Nir, S., Scholma, J. and Hoekstra, D. (1985) Biochemistry 24, 4630–4636.
[35] Düzgünes, N., Allen, T.M., Fedor, J. and Papahadjopoulos, D. (1987) Biochemistry 26, 8435–8442.
[36] Kok, J.W., Beest, M., Scherphof, G. and Hoekstra, D. (1990) Eur. J. Cell Biol. 53, 173–184.
[37] Struck, D.K., Hoekstra, D. and Pagano, R.E. (1981) Biochemistry 20, 4093–4099.
[38] Hoekstra, D., De Boer, T., Klappe, K. and Wilschut, J. (1984) Biochemistry 23, 5675–5681.
[39] Hoekstra, D. and Düzgünes, N. (1993) Methods Enzymol. 220, 15–32.
[40] McDonald, R.I. (1990) J. Biol. Chem. 265, 13533–13539.
[41] Hoekstra, D. and Klappe, K. (1993) Methods Enzymol. 220, 261–276.
[42] Steck, T.L., Weinstein, R.S., Straus, J.H. and Wallach, D.F.H. (1970) Science 168, 255–257.
[43] Mitchell, J., Tiddy, G.J.T., Waring, L., Bostock, T. and McDonald, M.P. (1983) J. Chem. Soc. Faraday Trans I 79, 975–1000.
[44] Glaser, P.E. and Gross, R.W. (1994) Biochemistry 33, 5805–5812.