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The Effect of PS Content on the Ability of Natural Membranes to Fuse with Positively Charged Liposomes and Lipoplexes

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Abstract. Supramolecular aggregates containing cationic lipids have been widely used as transfection mediators due to their ability to interact with negatively charged DNA molecules and biological membranes. First steps of the process leading to transfection are partly electrostatic, partly hydrophobic interactions of liposomes/lipoplexes with cell and/or endosomal membrane. Negatively charged compounds of biological membranes, namely glycolipids, glycoproteins and phosphatidylserine (PS), are responsible for such events as adsorption, hemifusion, fusion, poration and destabilization of natural membranes upon contact with cationic liposomes/lipoplexes. The present communication describes the dependence of interaction of cationic liposomes with natural and artificial membranes on the negative charge of the target membrane, charges which in most cases were generated by charging the PS content or its exposure. The model for the target membranes were liposomes of variable content of PS or PG (phosphatidylglycerol) and erythrocyte membranes in which the PS and other anionic compound content/exposure was modified in several ways. Membranes of increased anionic phospholipid content displayed increased fusion with DOTAP (1,2-dioleoyl-3-trimethylammoniumpropane) liposomes, while erythrocyte membranes partly depleted of glycocalix, its sialic acid, in particular, showed a decreased fusion ability. The role of the anionic component is also supported by the fact that erythrocyte membrane inside-out vesicles fused easily with cationic liposomes. The data obtained on erythrocyte ghosts of normal and disrupted asymmetry, in particular, those obtained in the presence of Ca^{2+}, indicate the role of lipid flip-flop movement catalyzed by scramblase. The ATP-depletion of erythrocytes also induced an increased sensitivity to hemoglobin leakage upon interactions with DOTAP liposomes. Calcein leakage from anionic liposomes incubated with DOTAP liposomes was also dependent on surface charge of the target membranes. In all experiments with the asymmetric membranes the fusion level markedly increased with an increase of temperature, which supports the role of membrane lipid mobility. The decrease in positive charge by binding of plasmid DNA and the increase in ionic strength decreased the ability of DOTAP liposomes/lipoplexes to fuse with erythrocyte ghosts. Lower pH promotes fusion between erythrocyte ghosts and DOTAP liposomes and lipoplexes. The obtained results indicate that electrostatic interactions together with increased mobility of membrane lipids and susceptibility to form structures of negative curvature play a major role in the fusion of DOTAP liposomes with natural and artificial membranes.

Key words: Cationic liposomes — Lipid asymmetry — Phosphatidylserine — Fusion — Gene therapy

Introduction

Gene therapy has been postulated as a promising strategy for the treatment of cancer, infectious viral diseases and hereditary disorders [15, 25, 35, 36, 52, 66]. Among other viral and non-viral systems for DNA delivery (for examples, *see* references [1, 10, 14, 30, 47]), cationic liposomes seem to be effective and safe carriers. Cationic lipids are synthetic amphiphiles composed of a hydrophobic part (usually two hydrocarbon chains) and a positively charged headgroup. Most of them tend to form liposomes when dispersed in an aqueous phase. Due to their positive charge they spontaneously interact with DNA and

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form larger aggregates [33, 34, 39, 43, 53, 57, 68]. The amphiphilic properties of cationic lipid molecules together with positive charge and defined phase behavior of liposomes or lipoplexes composed of them, make possible interactions with negatively charged membranes such as adsorption, fusion, poration and destabilization [50, 51, 62]. Some cationic lipids are able to penetrate natural membranes and localize in the inner leaflet, forming invaginations and even endosome-like vesicles [22].

Cationic lipids have been proven to be effective as transfection reagents both in vitro and in vivo. However, the mechanism of lipofection is still not well understood. The initial event occurring between cationic liposomes or lipoplexes and negatively charged plasma membrane is adsorption. Electrostatic and hydrophobic interactions may lead to hemifusion, fusion, poration or, alternatively, receptor-mediated endocytosis may occur [18, 67]. All these events are possible ways of DNA entry into the cytoplasm. It is known that the fusion process between membranes of opposite charge might be important for transfection efficiency on the stage of interaction with plasma membrane and endosomal membrane [2, 20, 38, 44, 46, 55, 58, 63]. It was postulated that cationic lipids could induce flip-flop of PS from the cytosolic face of endosomal membrane, resulting in its destabilization and release of DNA [7, 23, 64, 65]. DNA dissociation from lipoplexes was observed to coincide with their fusion with endosomal membrane [37]. Endosome destabilizing activity of some cationic lipids, as demonstrated experimentally [60], is the property responsible for high efficiency of promoting endosomal escape [40].

The cell surface possesses negative charge mainly due to the presence of glycoproteins and glycolipids. Anionic phospholipids, such as PS, are also components of the plasma membrane that can add negative charge to the cell surface, but an efficient mechanism restricts aminophospholipids to the inner leaflet of the plasma membrane bilayer [12]. At least three enzymatic activities are responsible for the natural lipid asymmetry of plasma membrane: MgATP-dependent aminophospholipid translocase (flippase), MgATP-dependent cholinephospholipid translocase (floppase) and Ca^{2+}-dependent scramblase, which facilitates bi-directional migration across the bilayer of all phospholipids. Activation of scramblase and inhibition of the aminophospholipid translocase cause a collapse of lipid asymmetry and exposure of PS on the cell surface [17, 61]. It has been shown that lipid bilayers of opposite charge fuse easily in liposome systems [42, 43, 58]. Liposomal fusion can result from neutralization of charge on lipid species, as induced by changes in pH or by the addition of neutralizing multivalent ions [8, 16, 21]. It is also known that fusion between natural membranes and cationic liposomes occurs due to the negative surface charge of plasma membranes [4, 24]. Some lipids used in mixtures with cationic lipids as helper lipids promote fusion with anionic membranes. The property of these lipids, mainly phosphatidylethanolamines, and liposomes/lipoplexes composed of them, results from a preference for an inverted hexagonal phase, which upon interaction with negatively charged membranes may lead to the creation of structures, which mediate the fusion process [26, 29].

Particularly, fusion of cationic liposomes with erythrocyte membranes has been well documented [29, 54], but it has not been studied what might be the role of PS exposed on the outer leaflet of erythrocyte membrane in fusion with cationic liposomes. PS as a component of the inner leaflet is an important factor affecting interactions between plasma membrane and membrane skeleton. Disturbance of these interactions leads to a change in mechanical properties of erythrocyte membrane [32]. It has been known for a long time that ATP-depleted erythrocytes undergo vesiculation and this phenomenon may be connected to a loss of interactions between inner leaflet PS and membrane skeleton [31]. Moreover, adsorption of positively charged and sometimes labile lipid aggregates, such as cationic liposomes/lipoplexes on the erythrocyte membrane surface may lead to aggregation of integral glycoproteins and affect the membrane skeleton/membrane interactions, similarly as it occurs in the presence of divalent cations and cationic polypeptides of surfactant activity [9].

Here we study the role of electrostatic interactions in the fusion between cationic liposomes or lipoplexes containing various proportions of plasmid DNA and natural or artificial membranes of negative charge. The role of membrane lipid mobility in this process was shown by comparing the level of fusion at temperatures of 30, 37 and 45°C. As a model served negatively charged liposomes containing a varying proportion of anionic phospholipid and erythrocyte membranes of which PS content, PS exposure and surface charge could be changed by various treatments.

Materials and Methods

Chemicals

DOTAP (1,2-dioleoyl-3-trimethylammoniumpropane) was purchased from Northern Lipids (Vancouver, British Columbia, Canada). Egg-PC (egg phosphatidylcholine), egg-PS (egg phosphatidylserine) and PG (phosphatidylglycerol) were obtained from Lipid Products (South Nutfield, UK). NBD-PE (N-(7-nitrobenz-2-oxa-1, 3-dia-zol-4-yl)-1,2-dihexadecanoyl-*sn*-3-phosphoetanolamine) and Rh-PE (N-(lissamine™ rhodamine B sulfonyl)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine) were purchased from Molecular Probes (Eugene, OR).

Preparation of Liposomes and Lipoplexes

Chloroform solutions of lipids were dried under a stream of nitrogen followed by the removal of solvent under high vacuum for 2 hours. Lipids were hydrated in a buffer containing 20 mM Tris, 150 mM NaCl, pH 7.4. The obtained suspension of MLV's was extruded 10 times through 400-nm- (in the case of anionic liposomes) or 100-nm (in the case of DOTAP liposomes) pore-size polycarbonate filters.

Plasmid DNA pEGFP-C1 4.7 kb (Clontech) was isolated with a Midi Prep isolation kit (Eppendorf) according to the manufacturer's instructions. Lipoplexes were prepared by addition of DNA solution to liposome suspension at a +/− charge ratio of 4/1, 6/1, 8/1 or 12/1, vortexing, and a 1-hour incubation at room temperature.

Size distributions of liposomes and lipoplexes were determined by PCS on a Zeta-Sizer 5000, Malvern Instruments.

Preparation of Erythrocyte Membranes

Blood drawn from sheep was prevented from coagulation by mixing with sterilized anticoagulant solution (75 mM sodium citrate, 38 mM citric acid, 125 mM glucose) solution and used within 3 days.

The procedure was carried out at 4°C. Erythrocytes were collected and washed three times with a buffer containing 20 mM Tris-HCl, 150 mM NaCl and PMSF (10 μg/ml) pH 7.4. To prepare erythrocyte ghosts of disturbed asymmetry ("normal ghosts" and ghosts in which scrambling was promoted by the presence of Ca^{2+}), cells were lysed and washed four times with 35 vol of buffer containing (in mM) 5 Tris, 10 NaCl, PMSF (10 μg/ml) pH 7.4 or 5 Tris, 10 NaCl, PMSF (10 μg/ml), 0.5 MgATP, 1 $MgCl_2$, and 2 $CaCl_2$, pH 7.4. Ghosts of normal asymmetry were prepared by erythrocyte lysis in a buffer containing (in mM) 5 Tris, 10 NaCl, PMSF (10 μg /ml), 0.5 MgATP and 1 $MgCl_2$, pH 7.4 (according to [11, 32]). After addition of a small volume of 3 M NaCl to obtain a 150 mM concentration of NaCl, the ghost suspension was incubated at 37°C for 40 min for membrane resealing. The ghost suspensions prepared in the presence of MgATP were washed two times with 20 mM Tris, 150 mM NaCl, PMSF (10 μg/ml), pH 7.4.

In some cases ghosts were prepared by using hypotonic buffer composed of 5 mM MES, 10 mM NaCl, PMSF (10 μg/ml), pH 7.1 and isotonic buffer composed of 20 mM MES, 150 mM NaCl, PMSF (10 μg/ml), pH 7.1.

Inside-out vesicles were prepared by incubation of erythrocyte ghosts in a low ionic strength solution (0.1 mM EDTA, PMSF 10 μg/ml, pH 7.4) at 37°C for 30 minutes. The membranes were separated by centrifugation at 40,000 × *g* for 30 min at 4°C. Finally, inside-out vesicles were washed and re-suspended in buffer containing 20 mM Tris, 150 mM NaCl, 10 μg/ml PMSF, pH 7.4. Peripheral protein-free membranes were also prepared after treatment of ghosts with 0.1 M NaOH for 30 min on ice. The membranes were separated from supernatant by centrifugation at 40,000 × *g* for 30 minutes and washed twice with a buffer composed of 20 mM Tris, 150 mM NaCl, 10 μg/ml PMSF, pH 7.4.

PS-enriched erythrocyte ghosts were prepared (according to Manno et al. [32]) by the addition of 0.5, 0.2 or 0.05 mg of egg PS in a small volume of chloroform solution to 2 ml of ghost suspension (1.5 mg of protein/ml) and vortexing. PS-loaded membranes were collected by centrifugation using a sucrose cushion and washed two times with a buffer containing 20 mM Tris, 150 mM NaCl, 10 μg /ml PMSF, pH 7.4.

To partially remove glycoproteins extending on the surface of "normal ghosts", we prepared ghosts from erythrocytes treated for 1 h with 0.25% trypsin, 2 mM $CaCl_2$ at 37°C. After that, erythrocytes were washed in isotonic buffer and the normal procedure of ghost preparation was applied. Alternatively, erythrocytes were treated with neuraminidase: 20 ml of 10% suspension of erythrocytes in isotonic MES buffer, pH 5.0, was incubated with 0.125 U/ml of neuraminidase for 1 h at 37°C. As determined with the phenol method [13], the total sugar content of these membranes was reduced approximately by a factor of two.

Protein content was determined by the modified method of Lowry [45]. Phospholipid concentration was determined by a phosphorus assay [48].

Lipid Mixing–Fusion Assays

Fusion between membranes of opposite charge was monitored as lipid mixing by measuring the decrease in energy transfer after dilution of two fluorescent probes, NBD-PE and Rh-PE, in unlabeled membranes [56]. Cationic liposomes or lipoplexes containing 1% mol of both probes were added to unlabeled membranes (at a ratio of 25 nmol of lipid of labelled liposomes or lipoplexes to 100 nmol of unlabeled liposomes, and in the case of natural membranes an equivalent of 100 nmol of phosphate: 120 μg of ghost protein or 80 μg of inside-out vesicle protein) and incubated at an appropriate temperature for 15 min in a total volume of 1 ml. The samples were kept on ice until measurements of fluorescence were performed. Fluorescence was measured as emission of NBD-PE at 536 nm, using an excitation wavelength of 463 nm on a Kontron SFM 25 spectrofluorimeter. The degree of lipid mixing was calculated as:

$$\Delta F/\Delta F_{\max}(\%) = 100\ [(F-F_0)/(F_{\max}-F_0)]$$

where F_0 is initial fluorescence and $F_{\max}$ is maximal fluorescence measured after addition of 20 μl of 10% SDS to a sample.

All experiments were performed 4–5 times, usually in triplicate, and the data represent mean ± SD (standard deviation).

Hemolysis

We carried out experiments comparing hemoglobin leakage from fresh and ATP-depleted erythrocytes. ATP-depleted erythrocytes were prepared by overnight incubation of washed fresh erythrocytes in test buffer containing 10 mM Tris, 150 mM NaCl and 10 μg/ml PMSF, pH 7.4 at 4°C.

Washed erythrocytes, 650×10^6 cells, were incubated with different concentrations of DOTAP liposomes. The total volume of each sample was 0.5 ml. After a 30-min incubation, the samples were centrifuged at 1,500 × *g* for 5 min and hemoglobin concentration in supernatants was determined spectrophotometrically at 410 nm. The degree of hemolysis was determined by comparing to 100% hemolysis achieved by lysis in water.

To asses erythrocyte membrane osmotic resistance, freshly prepared and ATP-depleted erythrocytes (280×10^6 cells) after centrifugation were re-suspended in 1 ml of 10 mM Tris, 2 mM $MgCl_2$, Im M EGTA pH 7.4 containing variable concentrations of NaCl (0–150 mM), incubated for 30 min at room temperature and centrifuged for 5 min at 800 × *g*. Hemoglobin content in supernatants was estimated by measuring absorbance at 410 nm.

Calcein Leakage from Anionic Liposomes upon Contact with DOTAP Liposomes

Dry lipid films composed of PC and PS were hydrated in buffer (20 mM Tris, 150 mM NaCl, pH 7.4) containing 30 mM calcein. After extrusion through 400 nm polycarbonate filter, liposomes were separated from external calcein solution by gel filtration using a Sephadex G 50 column equilibrated with 20 mM Tris, 150 mM NaCl, pH 7.4 buffer. A fixed amount of the obtained liposome

suspension (100 nmol of lipid) was incubated with increasing concentrations of DOTAP liposomes for 30 min at 37°C (total sample volume, 1 ml) and 50 μl of each sample was added to 1.95 ml of buffer in a spectrofluorimeter cuvette. The fluorescence measurements were performed using excitation wavelength 490 nm and emission wavelength 513 nm. The level of calcein leakage was estimated using the following equation:

$$\text{leakage}(\%) = 100\ [(F-F_0)/(F_{\max}-F_0)],$$

where F_0 is fluorescence of a sample which was not incubated with DOTAP liposomes, $F_{\max}$ is maximal fluorescence after addition of 10 μl of 10% SDS to each sample.

Results

Effect of Anionic Compound Content in the Target Membranes on Lipid Mixing Efficiency During Fusion with DOTAP Liposomes

In Fig. 1*A* and 1*B* lipid mixing during fusion between anionic liposomes, 400 nm in diameter, containing increasing amounts of PS or PG, and 100 nm DOTAP liposomes is compared. No fusion was observed when anionic liposomes were composed of PS/PC or PG/PC at a molar ratio of 1/9 (only a small increase in fluorescence occurred at a temperature of 60°C, not shown). As expected, with the increasing content of anionic lipid in PS/PC or PG/PC liposomes, the level of lipid mixing occurring during fusion with DOTAP liposomes increased, reaching a value of 75–90% for anionic lipid content of 40–50% (*see* Fig. 1*A* and *B*).

To answer the question of what the effect is of PS content in the erythrocyte membrane, the erythrocyte ghosts were enriched in PS by incubation with small volumes of a chloroform solution of PS according to Manno et al. [32] (*see* Materials and Methods). The results presented in Fig. 1*C* indicate that the enrichment in PS of the purified erythrocyte membranes markedly increased their ability to fuse with DOTAP liposomes. For example, at a temperature of 30°C almost a four-fold increase in lipid mixing efficiency was observed for erythrocyte ghosts, which had been incubated with 0.17 mg PS/mg ghost protein in 2 ml of ghost suspension compared to "normal ghosts" treated the same way.

Inside-out vesicles are small vesicles (about 50–150 nm in diameter) arising from erythrocyte ghosts after dissociation of the membrane skeleton. They expose the inner face of cell membrane still rich in PS and PE, even though during preparation some scrambling occurs. Much higher levels of fusion between DOTAP liposomes and these membranes (compared to "normal ghosts") (Fig. 1*D*) can be observed. It may be a result of high PS and PE content in the inner membrane monolayer and the lack of membrane skeleton that could promote close contact between interacting lipid membranes, leading to fusion and fast and effective lipid mixing. Peripheral protein-free membranes obtained by treatment of erythrocyte ghosts with 0.1 M NaOH also fused with DOTAP liposomes with very high effectiveness (Fig. 1*D*).

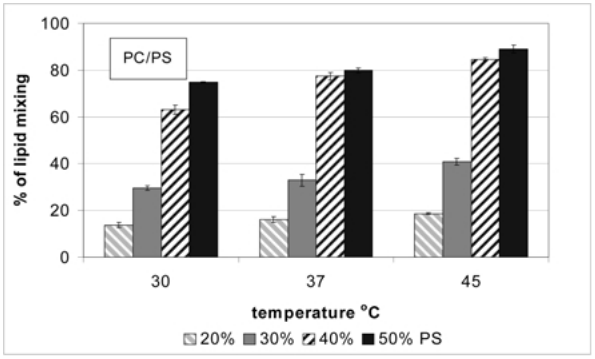
To test the effect of glycocalix presence at the cell surface we prepared ghosts from erythrocytes pretreated with trypsin and neuraminidase. As shown in Fig. 1*E*, the ghosts prepared from erythrocytes treated with trypsin, in contrast to "normal ghosts", did not fuse with DOTAP liposomes during a 15-minute incubation at 30°C. Ghosts prepared from erythrocytes treated with neuraminidase are also characterized by a lower ability to fuse with DOTAP liposomes (Fig. 1*F*). Decreased ability of fusion of membranes partly depleted of glycocalix implies that glycocalix, as a source of the membrane negative surface charge, strongly promotes close contact with positively charged liposomes.

It should be noted that the effect of temperature on fusion efficiency was rather complicated. In the case of artificial membranes (liposomes), the effect of temperature was low (usually not higher than 10%), indicating a rather small role of increased lipid mobility on fusion efficiency. On the other hand, the effect of increased temperature on the fusion efficiency between cationic liposomes and erythrocyte membranes was quite substantial (2 to 4 fold increase), indicating a possible role in increased mobility of lipid components of the initially asymmetrical membrane.

Comparison of Lipid Mixing Levels During Fusion of DOTAP Liposomes with Ghosts of Natural and Disrupted Lipid Asymmetry

The presence of MgATP and Mg^{2+} during preparation of ghosts from fresh erythrocytes results in membranes of sustained natural asymmetry and no increased exposure of PS on the outer leaflet of the membranes was observed by fluorescent annexin V assay (*not shown*). An absence of MgATP and Mg^{2+} or addition of Ca^{2+} during preparation is known to disturb natural asymmetry of ghosts. MgATP is necessary for flippase activity, which is responsible for maintaining lipid asymmetry. Ca^{2+}, reported as a flippase inhibitor, activates scramblase and an ATP–dependent Ca^{2+}-pump, which causes faster ATP depletion and, as a result, PS exposure [11, 32].

As presented in Fig. 2*A*, fusion with DOTAP liposomes is much more effective for ghosts prepared in the absence of MgATP and Mg^{2+} ("normal ghosts") or in the presence of MgATP, Mg^{2+} and Ca^{2+} than for ghosts prepared in the presence of MgATP and Mg^{2+}. The effect is visible at the temperatures of 30, 37 and 45°C. The results suggest that enrichment of the surface of the membrane in PS or conditions promoting exposure of PS, promote fusion with cationic liposomes.



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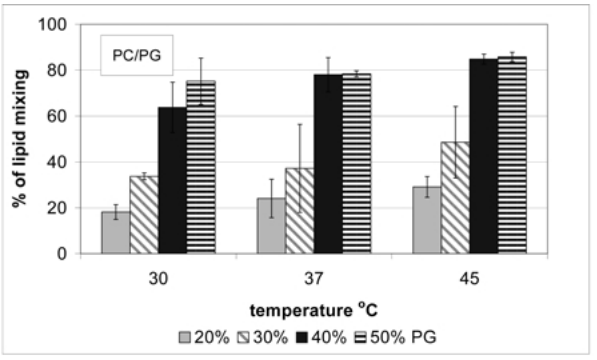

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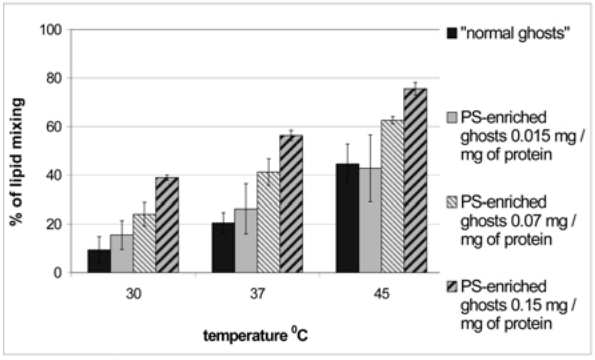

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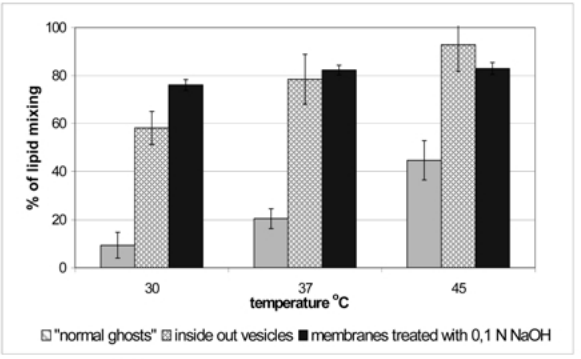

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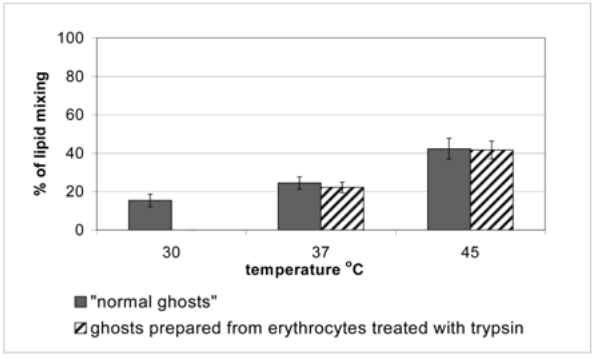

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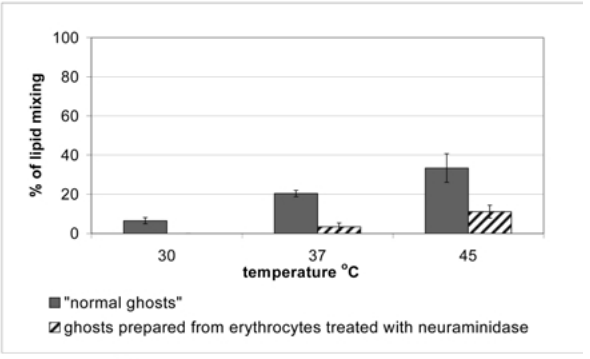
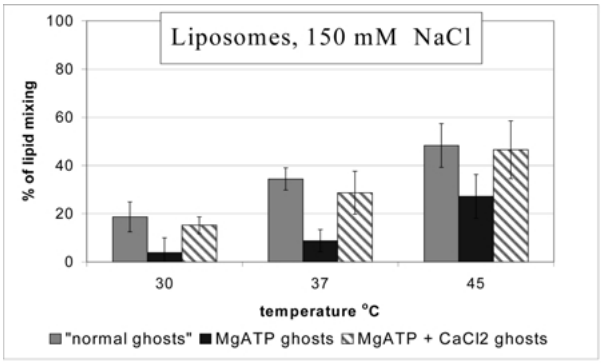


F

Fig. 1. The level of fusion between DOTAP liposomes and liposomal or natural membranes depends on surface charge of negatively charged membranes. (*A*) The effect of PS content in liposomes composed of PC/PS on efficiency of fusion with DOTAP liposomes. (*B*) The effect of PG content in liposomes composed of PC/PG on efficiency of fusion with DOTAP liposomes. (*C*) Enrichment of ghosts with PS increases their ability to fuse with DOTAP liposomes. (*D*) Inside-out vesicles and peripheral protein-free erythrocyte membranes fuse easily with DOTAP liposomes. (*E*) Ghosts prepared from erythrocytes pre-treated with trypsin do not fuse with DOTAP liposomes when incubated at 30°C. (*F*) Ghosts prepared from erythrocytes pre-treated with neuraminidase reveal lower ability for fusion with DOTAP liposomes. The liposomes and erythrocyte membranes were prepared as described in Materials and Methods. DOTAP liposomes, 100 nm in diameter, labelled with 1% NBD-PE and 1% Rh-PE at a concentration of 25 μM of lipid were incubated with 100 μM PC/PS or PC/PG liposomes, 400 nm in diameter (*A*, *B*), 120 μg of protein of erythrocyte ghosts (*C*, *D*, *E*, *F*) or 80 μg of protein of inside-out vesicles or 0.1 M NaOH treated erythrocyte membranes (*D*) for 15 min. in a total volume of 1 ml (buffer composed of 20 mM Tris, 150 mM NaCl, PMSF 10 μg/ml, pH 7.4) at an indicated temperature. After incubation, samples were kept on ice. The measurements were performed as described in Materials and Methods.



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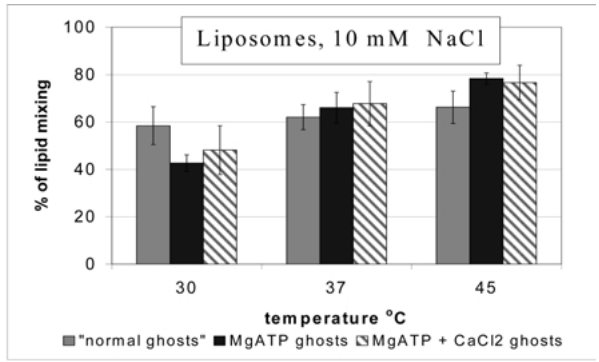

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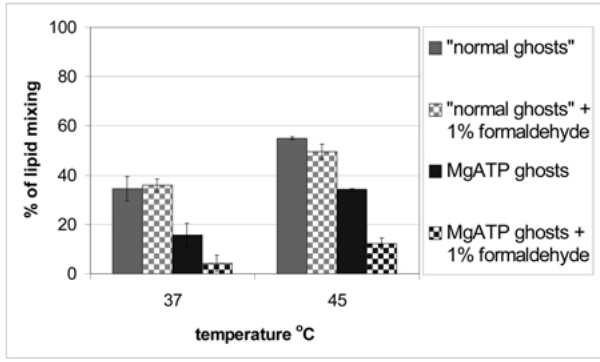


C

Fig. 2. Fusion of erythrocyte ghosts of normal and disturbed asymmetry with DOTAP liposomes. (*A*) Results obtained in buffer containing 150 mM NaCl. (*B*) Results obtained in buffer containing 10 mM NaCl. (*C*) The effect of 1% formaldehyde treatment on efficiency of fusion between ghost membranes and DOTAP liposomes (buffer containing 150 mM NaCl). Ghosts (120 μg of protein/ml) were incubated with 25 μM of Rh-PE and NBD-PE-labelled DOTAP liposomes, as described in the legend to Fig. 1 and Materials and Methods.

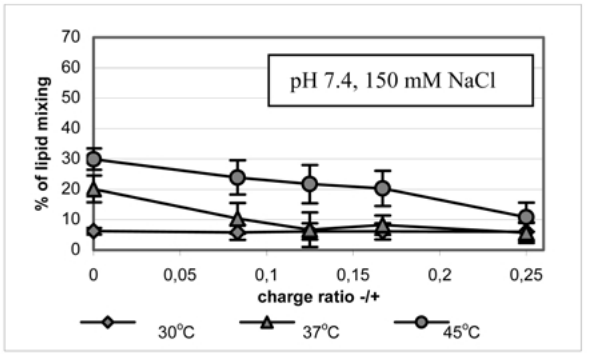
When the membranes of erythrocyte ghosts were incubated and re-suspended for a fusion experiment in a buffer containing 10 mM NaCl, the levels of lipid mixing were much higher (at the temperatures of 30, 37 and 45 °C) than the levels of fusion observed in a buffer containing 150 mM NaCl (compare Fig. 2*A* and *B*). At 10 mM NaCl no distinct differences were observed for membranes prepared in the presence of MgATP and in conditions promoting PS exposure. These results suggest that electrostatic interactions between charged components of contacting membranes are the main forces influencing the process of fusion.

Treatment with 1% formaldehyde (as a component of test buffer containing 150 mM NaCl) during a 15-minute incubation of MgATP ghosts with DOTAP liposomes at 37 and 45°C results in a decrease in the level of lipid mixing compared to the level obtained in the absence of formaldehyde. On the other hand, for "normal ghosts" no effect of formaldehyde treatment is observed (Fig. 2*C*). This result may suggest that fixed MgATP ghosts could be more resistant to flip-flop of PS.

Fusion between Erythrocyte Membranes and Lipoplexes Composed of 100 nm DOTAP Liposomes and Plasmid DNA (pEGFP)

Lipoplexes composed of DOTAP liposomes and plasmid DNA at different charge ratio are less susceptible to fusion with erythrocyte ghosts than initial liposomes (Fig. 3*A*). The correlation between −/+ charge ratio and efficiency of fusion between lipoplexes and erythrocyte membranes is clearly seen: the higher the charge ratio, the lower the ability to fuse with erythrocyte ghosts. It is well known that in systems containing phosphatidylethanolamines low pH promotes fusion between lipid membranes. Also phosphatidylserine is expected to prefer negative curvature at lower pH, close to pK_a values [19]. We observed higher efficiencies of fusion between erythrocyte ghosts and DOTAP liposomes when experiments were carried out in conditions of low pH (pH 5.2) than in conditions of neutral pH (compare Fig. 3*A* and *B*).

Lipoplexes composed of DOTAP/pEGFP at charge ratio of +/− 8/1 (400 nm as determined by



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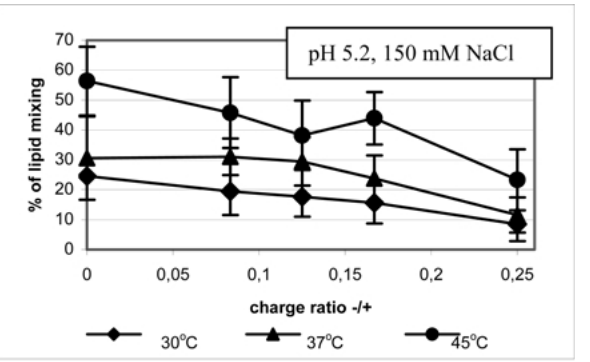

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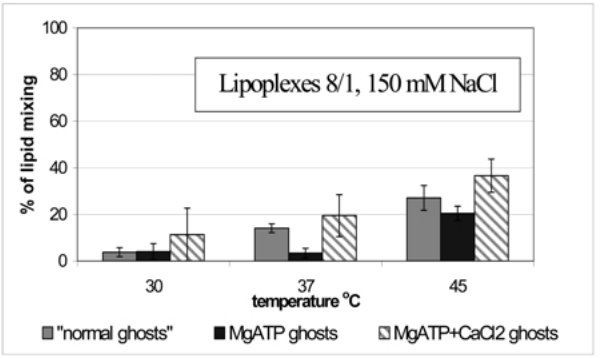

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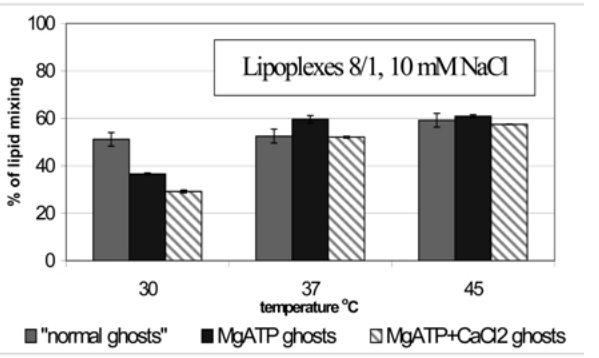

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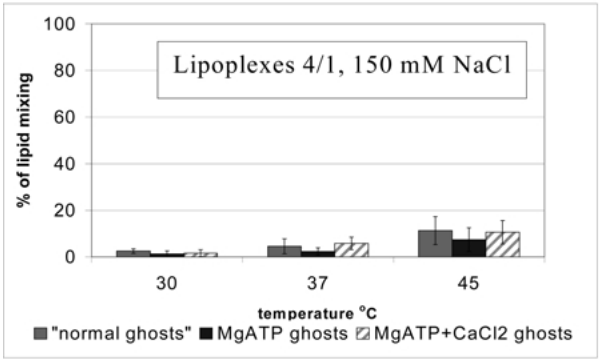
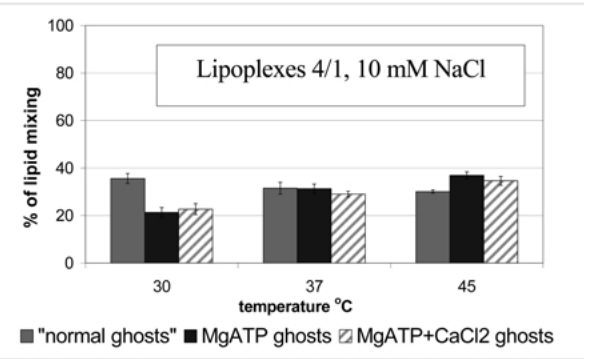


E



F

Fig. 3. Lipoplexes prepared from DOTAP liposomes and plasmid DNA fuse with erythrocyte ghosts with lower efficiencies than free liposomes. (*A*) "Normal ghosts" in fusion with DOTAP liposomes and lipoplexes prepared at different charge ratios in conditions of neutral pH 7.4 and 150 mM NaCl. (*B*) "Normal ghosts" in fusion with DOTAP liposomes and lipoplexes prepared at different charge ratios in conditions of acidic pH 5.2 and 150 mM NaCl. (*C*) Efficiencies of fusion between lipoplexes composed of DOTAP and plasmid DNA (pEGFP) at charge ratio of +/− 8/1 and erythrocyte ghosts in buffer containing 150 mM NaCl. (*D*) Efficiencies of fusion between lipoplexes composed of DOTAP and plasmid DNA (pEGFP) at charge ratio of +/− 8/1 and erythrocyte ghosts in buffer containing 10 mM NaCl. (*E*) Efficiencies of fusion between lipoplexes composed of DOTAP and plasmid DNA (pEGFP) at charge ratio of +/− 4/1 and erythrocyte ghosts in buffer containing 150 mM NaCl. (*F*) Efficiencies of fusion between lipoplexes composed of DOTAP and plasmid DNA (pEGFP) at charge ratio of +/− 4/1 and erythrocyte ghosts in buffer containing 10 mM NaCl. Lipoplexes were prepared by mixing a suspension of 100 nm DOTAP liposomes with water solution of plasmid DNA (pEGFP) at a charge ratio of +/− 12/1, 8/1, 6/1 or 4/1, and a 1-hour incubation at room temperature. The size of formed aggregates 12/1 and 8/1 determined by the PCS method was approximately 400 nm. Lipoplexes 6/1 were larger with mean diameter of about 800 nm. The diameter of lipoplexes 4/1 was too large to be measured by PCS. The measurements of fusion were performed as described in Materials and Methods.

PCS) fused still quite easily with erythrocyte ghosts in a buffer containing 150 mM NaCl, but even at 60°C (*not shown*) the levels of fusion never reached a value higher than 50%. Similarly to the characteristics of fusion between DOTAP liposomes and erythrocyte ghosts, higher efficiencies of fusion were observed for

membranes prepared in conditions promoting lipid scrambling. The effect of decrease in ionic strength is also visible, however, it is not so clear as for the fusion between DOTAP liposomes and ghosts (*compare* Fig. 3*B* and *C*), and a 100% value of lipid mixing was never obtained, even at a temperature of 60°C (*not shown*).

Lipoplexes prepared at a charge ratio of +/− 4/1 (the diameter was too large to be measured by PCS) fused with erythrocyte membranes very poorly. Only at a temperature of 60°C (*not shown*) more effective lipid mixing occurred. At decreased ionic strength (10 mM NaCl) increased lipid mixing effectiveness could be observed, but only up to a value of about 40% (see Fig. 3*D* and *E*).

It seems clear that lower net positive charge of the lipoplexes compared with the same concentration of free DOTAP liposomes due to charge neutralization, and also high stability of aggregates formed from strongly interacting plasmid DNA and cationic liposomes, are the reasons of the decreased fusion activity of lipoplexes compared to DOTAP liposomes.

Interactions of DOTAP Liposomes with Erythrocytes or Anionic Liposomes Lead to Leakage of Their Content

The results presented below indicate that stability of erythrocyte membrane depends on ATP content. Normal and ATP-depleted erythrocytes (650×10^6 cells) were incubated for 30 minutes with increasing concentrations of DOTAP liposomes, and the hemolysis level was estimated after centrifugation by hemoglobin present in supernatants, as determined spectrophotometrically. As it can be seen in Fig. 4*A*, for both populations the hemolytic activity of DOTAP liposomes is very low at 37°C. The shape of the curves representing plots of the degree of hemolysis against DOTAP concentration, at 37°C, resembles the shape of a saturation curve with a maximal level of hemolysis oscillating around a value of 1%. Essentially no difference between normal and ATP-depleted erythrocytes was observed at 37°C. Much higher hemolysis was observed when erythrocytes were incubated with DOTAP liposomes at a temperature of 45°C. The curves representing dependence of hemolysis on DOTAP concentration at 45°C have the shape of straight lines. Comparing both populations of erythrocytes, the slope of the curve is 3 times higher for ATP-depleted erythrocytes (8.83 versus 2.9).

In Fig. 4*C* the effect of DOTAP liposomes on release of calcein in PS/PC liposomes is shown. Liposomes containing 20% PS released their content up to the level of about 20% after incubation with DOTAP liposomes at a concentration of 200 μM, whereas liposomes composed of PS/PC 1:1 were more intensively disturbed — the level of release reached a value of about 60% (Fig. 4*C*). Leakage is probably accompanied by fusion and rupture of vesicles upon electrostatic interactions with DOTAP membranes.

In conclusion, the effect of DOTAP liposomes on erythrocyte and on liposomal membrane rich in PS is different. The leakage of liposomal membrane is much higher than that observed for intact erythrocyte membrane.

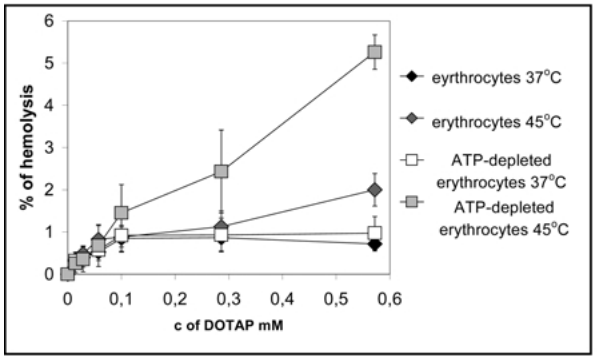
Discussion

Fusion between cationic liposomes/lipoplexes and negatively charged liposomes or natural membranes has been extensively studied [1, 23, 27, 28, 41, 42, 58, 59]. Liposomal fusion can be induced by changing the physical parameters of the system, such as temperature, curvature of the target membrane, and/or surface tension. Induction of fusion is also possible via charge neutralization resulting from electrostatic interactions of oppositely charged membranes or from binding of multivalent ions [8, 21].

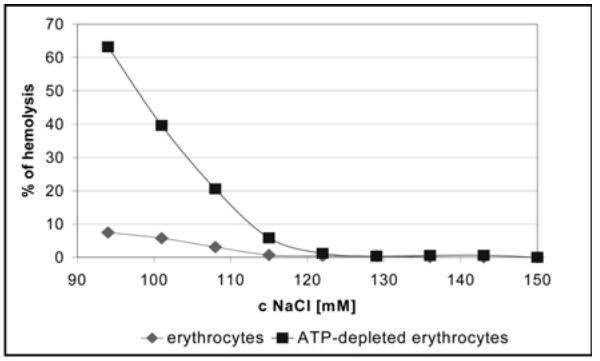
The results presented above indicate a crucial role of electrostatic interactions in the fusion of cationic (DOTAP) liposomes with artificial (liposome) and natural (erythrocyte) membranes. The novelty of this study comprises the notion that ATP-dependent lipid asymmetry of erythrocyte ghosts causes some restraint of fusion upon contact with cationic liposomes, and that enhancement of fusion efficiency can be achieved by enrichment of the outer leaflet of the membrane in PS via disturbing this natural lipid asymmetry. In addition it was found that increase in lipid mobility markedly enhanced the fusion process, in particular in the case of erythrocyte membranes (i.e., membranes initially asymmetric) treated in various ways, *see* e.g., Figures 1*C*, *E*, 2*A*, 3*A*, *C*, 4*A*, *B*.

The observation in the liposomal system that the level of lipid mixing due to fusion with DOTAP liposomes markedly depends on PS or PG content in anionic liposomes composed of PC/PS or PC/PG mixtures is consistent with a number of studies exploring fusion between cationic liposomes composed of various synthetic lipids with negatively charged liposomes [4, 41, 43]. The efficiency of lipid mixing slightly depends on the temperature of incubation, indicating a rather marginal role of increased mobility of lipid molecules in liposomal membranes, which are essentially symmetric (there are no differences in lipid composition of inner and outer monolayer).

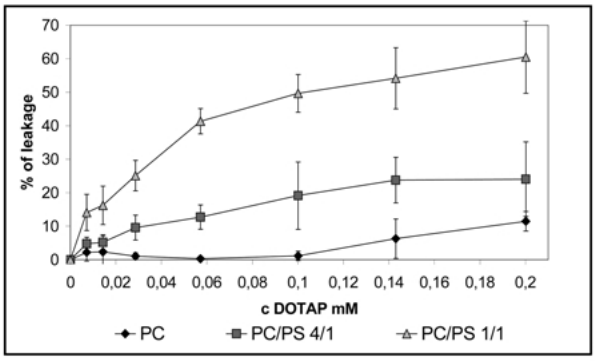
A correlation between the presence of PS in erythrocyte membranes and the possibility of effective fusion with cationic liposomes was found. The ghosts in which the outer membrane monolayer was enriched with PS did fuse with higher efficiencies than the "normal ghosts". As we expected, the ghosts



A



B



C

Fig. 4. Interactions of DOTAP liposomes with erythrocytes or anionic liposomes lead to leakage of initially encapsulated solute (hemoglobin or calcein). (*A*) Hemolytic activity of DOTAP liposomes against normal and ATP-depleted erythrocytes. (*B*) Hemolysis of normal and ATP-depleted erythrocytes induced by osmotic pressure. (*C*) Calcein leakage from anionic liposomes upon interactions with DOTAP liposomes. Experiments were performed as described in Materials and Methods.

prepared in the presence of Mg^{2+} and MgATP (to prevent scrambling) fused with lower efficiencies than "normal ghosts". A similar effect was observed in the case of the effect of phospholipid asymmetry on fusion between large anionic liposomes induced by Ca^{2+} [16]. In another study by Bailey & Cullis [3], fusion was restrained by asymmetric distribution of highly fusogenic synthetic amino lipids restricted to the inner leaflet of lipid vesicles by pH gradient. From a number of studies [5, 49] it is known that erythrocytes exposing PS are more sensitive to fusion induced by Ca^{2+}. We also observed that a 1% formaldehyde treatment of MgATP ghosts additionally decreased efficiency of fusion with DOTAP liposomes at the temperatures of 37 and 45°C, whereas there was no effect of formaldehyde in the case of "normal ghosts". MgATP ghosts fixed by formaldehyde could be more resistant to scrambling and therefore to lipid mixing induced by DOTAP liposomes due to chemical modification of aminophospholipids, which could be more strictly immobilized and restricted to the inner leaflet of the membrane.

It should be noted that lipid composition of the surface of the target membrane resulting from scrambling and disturbing of normal lipid asymmetry (obtained as a consequence of absence of Mg^{2+} and MgATP or activation of scramblase) may affect not only surface charge of the membrane but also those physical properties of the membrane that are under control of membrane skeleton and Mg^{2+}, such as stability and integrity, as some of the interactions of membrane skeleton with membrane bilayer seem to depend on PS presence in the inner monolayer [32].

In contrast to liposomal membranes, temperature-induced lipid mobility strongly affects the fusion ability, probably due to disruption of lipid asymmetry in the bilayer. It should be noted that no fusion was detected after 15-minute incubation of a mixture of liposomes and ghosts at 21°C. Temperature-induced fusion could also be a result of induction of nonlamellar structures resulting from the presence of PE (the natural content in the outer leaflet equals to about 20% of its total amount, as reviewed by Devaux [12], and is elevated by lipid scrambling). The high levels of fusion of DOTAP liposomes and inside-

out vesicles or peripheral protein-free membranes obtained by treatment of erythrocyte ghosts with 0.1 M NaOH, observed here, support the postulated hypothesis of a role of PS in enhancement of fusion and are also in agreement with the known fact that phosphatidylethanolamines facilitate fusion [4]. Also, the observation of elevated fusion between erythrocyte ghosts and DOTAP liposomes at pH of 5.2 (compared to experiments carried out in conditions of neutral pH 7.4), could be explained by the presence of PE on the surface of the membrane.

We also carried out experiments which addressed the question of whether lipoplexes composed of DOTAP and plasmid DNA (pEGFP) can effectively fuse with erythrocyte membranes. Lower pH promoted fusion of lipoplexes with erythrocyte ghosts but the ability of fusion was, as for neutral pH, strongly dependent on the charge ratio. From other studies it is known that electrostatic interactions and fusion of oppositely charged membranes, if they occur, should promote DNA release from lipoplexes [58]. As we observed a lower ability to fuse for lipoplexes than for initial liposomes, even in conditions of mildly acidic pH, we conclude that dissociation of plasmid DNA from DOTAP lipoplexes could still be difficult, even if the possibility of fusion existed and was enhanced by applying conditions of low pH or high temperature. Decreased NaCl concentration also gave increased values of lipid mixing but the efficiency of fusion was never 100%, even after incubation at 60°C. The effect of decreased ionic strength on fusion between erythrocyte ghosts and DOTAP liposomes or lipoplexes is consistent with other studies [28, 41, 43] and indicates the role of charge neutralization in the fusion of negatively charged membranes with cationic liposomes. It should be noted that the effect of temperature on fusion efficiency with DOTAP liposomes/lipoplexes in the case of experiments carried out at a 10 mM NaCl concentration was rather small, indicating a smaller role of temperature-dependent lipid mobility than that in the case of the physiological ionic strength. It should be noted, however, that since erythrocyte membranes were equilibrated in a buffer containing 10 mM NaCl before the fusion experiment, their changed properties such as elevated permeability and perturbed interactions between membrane skeleton and the inner face of the membrane bilayer could be partly responsible for their lower resistance to fusion.

It should be mentioned that processes of membrane fusion and destabilization occurring upon contact of oppositely charged lipid vesicles are often accompanied by leakage of their contents [8, 42]. Here we observed that interactions of DOTAP liposomes with erythrocytes or anionic liposomes lead to leakage of their contents. Substantial leakage of hemoglobin after incubation of ATP-depleted and intact erythrocytes with DOTAP liposomes was observed only at 45°C. The leakage was larger in the case of ATP-depleted erythrocytes, indicating the role of the loss of lipid asymmetry in the susceptibility to hemolysis. The osmotic fragility of these cells was also lower. Similar implications are proposed in a study by Baumann & Sowers [6] where the authors report that subpopulation of osmotically sensitive membranes is less resistant to electrofusion. In the liposomal system we observed increased leakage of calcein initially encapsulated in liposomes upon contact with DOTAP liposomes for vesicles of larger surface charge.

Recent studies on mechanisms of transfection mediated by cationic liposomes support the view that although the main way of DNA entry into cytoplasm is endocytosis, the fusion process accompanying destabilization of endosomes is important for transfection efficiency [37, 38, 63]. The present study demonstrates that in the case of DOTAP liposomes, lipid asymmetry of natural membranes is a factor which prevents fusion. Efficiency of fusion is mainly dependent on surface charge of target membranes and cationic liposomes/lipoplexes but could be increased by acidic pH or temperature, indicating involvement of nonlamellar structures.

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