

HIGHLY FUSOGENIC CATIONIC LIPOSOMES TRANSIENTLY PERMEABILIZE THE PLASMA MEMBRANE OF HeLa CELLS

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Abstract: Cationic liposomes can efficiently carry nucleic acids into mammalian cells. This property is tightly connected with their ability to fuse with negatively charged natural membranes (i.e. the plasma membrane and endosomal membrane). We used FRET to monitor and compare the efficiency of lipid mixing of two liposomal preparations – one of short-chained diC14-amidine and one of long-chained unsaturated DOTAP – with the plasma membrane of HeLa cells. The diC14-amidine liposomes displayed a much higher susceptibility to lipid mixing with the target membranes. They disrupted the membrane integrity of the HeLa cells, as detected using the propidium iodide permeabilization test. Morphological changes were transient and essentially did not affect the viability of the HeLa cells. The diC14-amidine liposomes were much more effective at either inducing lipid mixing or facilitating transfection.

Key words: Cationic liposomes, Fusion, Transfection, Plasma membrane integrity

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Abbreviations used: DiC14-amidine – N-t-butyl-N'-tetradecylaminopropionamide; DMPC – dimyristoylphosphatidylcholine; DOTAP – 1,2-dioleoyl-3-trimethylammonium-propane; FRET – fluorescence resonance energy transfer; MEM α – minimum essential medium alpha; NBD-PE – N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-1,2-dihexadecanoyl-*sn*-3-phosphoethanolamine; PBS – phosphate buffered saline; PC – phosphatidylcholine; PMSF – phenylmethylsulphonyl fluoride; PS – phosphatidylserine; Rh-PE – N-(lissamineTM rhodamine B sulfonyl)-1,2-dihexadecanoyl-*sn*-glycero-3-phospho-ethanolamine; SDS – sodium dodecyl sulphate

INTRODUCTION

Liposomes composed of cationic lipids are widely used as transfection vectors. Due to their positive charge, they interact with nucleic acids and negatively charged cellular membranes. Adsorption on the cell surface is a prerequisite to further events such as the hemifusion, fusion, poration or engulfment processes. Endocytosis is the main mode of lipoplex entry into the cell [1-6]. However, fusion with the endosomal membrane followed by its disruption is thought to be a key event enabling lipoplex cargo release from the endosomal compartment [2, 7-11].

Short-chained lipids are more fusogenic than long-chained ones, and liposomes composed of cationic lipids of different acyl chain lengths fuse more easily than those made up of lipids with identical acyl chains [12]. DiC14-amidinium, a short-chained cationic lipid, has been reported to possess a high level of transfection activity even in the absence of helper lipids *in vitro* and *in vivo* [13, 14]. It was also recently successfully used as an immunoadjuvant [15].

In this study, we compared the properties of diC14-amidinium and the commonly used, commercially available cationic lipid DOTAP (long-chained with one double bond in the middle of each of its two alkyl chains) in terms of their ability to fuse (measured as lipid mixing) with and transfect HeLa cells in culture. Our results indicate that diC14amidinium liposomes have a high potential to transiently alter the organisation of natural membranes.

MATERIALS AND METHODS

Chemicals

DOTAP (1,2-dioleoyl-3-trimethylammoniumpropane) was purchased from Northern Lipids (Vancouver, Canada). NBD-PE (N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-1,2-dihexadecanoyl-*sn*-3-phosphoethanolamine) and Rh-PE (N-(lissamineTM rhodamine B sulfonyl)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine) were purchased from Molecular Probes (Eugene, OR, USA). TRITC-phalloidin and PMSF were obtained from Sigma (St. Luis, MI, USA). DiC14-amidinium (N-t-butyl-N'-tetradecylaminopropionamidinium) was synthesized as described earlier [20]. Egg-PC and DMPC were purchased from Lipid Products (South Nutfield, UK). Human epithelial carcinoma HeLa cells were obtained from the Institute of Immunology and Experimental Therapy, (Wrocław, Poland).

Cell culture

Cells were grown at 37°C, under 5% CO₂ to 80% confluence in MEM α medium with 10% heat-inactivated foetal bovine serum containing 2 mM glutamine and 100 U/ml penicillin, 100 μ g/ml streptomycin and 100 μ g/ml neomycin (Gibco, Paisley, UK). They were passaged every three days using 0.25% trypsin/0.05% EDTA, and seeding at a 1:6 dilution.

Preparation of cationic liposomes and lipoplexes

Chloroform solutions of the two cationic lipids were dried under a stream of nitrogen, and kept under a high vacuum for 2 h to completely remove the solvent. The lipids were hydrated in a buffer of 20 mM Hepes and 150 mM NaCl, pH 7.4. The liposomal suspension was sonicated for 60 s and subsequently extruded twice through a 200-nm pore-size polycarbonate filter in an extruder (PPH Marker, Wrocław, Poland) at a temperature of 50°C. The liposomes prepared for the lipid-mixing assay contained the fluorescent probes Rh-PE and NBD-PE, each at a concentration of 1 mol%. The size of the liposomes was determined by PCS in a ZetaSizer 5000 (Malvern Instruments, UK). The sonicated DOTAP liposomes were about 100 nm in diameter and were stable, while the diC14amidine liposomes tended to increase their diameter at room temperature in the presence of 150 mM NaCl; after 2 h, their average size had increased to 300 nm. The liposome/lipoplex suspensions were finally sterilized by passage through a 200-nm filter in one way only in a sterile vial.

Plasmid DNA pEGFP-C1 4.7 kb (Clontech) was isolated using a Midi Prep isolation kit (Eppendorf) according to the manufacturer's instructions. Lipoplexes were prepared by adding a DNA solution to a liposome suspension at a +/- charge ratio of 8:1, vortexing, and incubating for 1 h at room temperature. The average size of the DOTAP lipoplexes was ~400 nm while the diC14-amidine-based lipoplexes had a diameter of about 500 nm.

Preparation of erythrocyte membranes

Blood drawn from sheep was prevented from coagulating by mixing it with a sterilized anticoagulant solution (75 mM sodium citrate, 38 mM citric acid, 125 mM glucose). The blood was used within 3 days. Isolation of ghosts was carried out at 4°C, and they were resealed as described previously [17, 18] in conditions maintaining normal lipid asymmetry. The phospholipid concentration was determined using a phosphorus assay [19].

Lipid mixing assay and microscopic observations

Trypsinized HeLa cells were washed and resuspended in a buffer of 20 mM Hepes, 140 mM NaCl, 3.5 mM KCl, 1 mM MgCl₂, 1 mM CaCl₂ and 100 µg/ml glucose, pH 7.4, and counted using a Bürcker's chamber. Lipid mixing, as the indicator of fusion between membranes of opposite charge, was monitored by measuring the fluorescence energy transfer between two fluorescent probes, NBD-PE and Rh-PE, in unlabeled membranes [20, 21]. Briefly, liposomes containing 1 mol% of both probes were added at a concentration of 15 µM (lipid) to a cell suspension (1x10⁶ cells/ml) in the test buffer, and were incubated for 1 h. Erythrocyte ghosts (100 nmol of phospholipids) in 20 mM Hepes, 150 mM NaCl, 10 µg/ml PMSF, pH 7.4, were mixed with liposomes (25 nmol lipid) in a total volume of 1 ml. Fluorescence measurements and calculations were carried out according to [20].

Microscopic observations were performed using an Olympus B211 fluorescent microscope to detect the lipid-mixing between the NBD-PE/Rh-PE-labeled

cationic liposomes and HeLa cells. Cells were seeded in a 24-well plate at 7×10^5 cells per well and left in the incubator for 24 hours. The cells were rinsed twice with PBS, covered with medium without serum and subsequently pre-incubated for 1 h at 37°C, and then liposomes were added to a final concentration of 25 μ M (lipid). After 1 h incubation (37°C) in the presence of liposomes, the cells were rinsed with PBS, covered with PBS, and observed directly under blue light to bring about NBD-PE fluorescence in the case of the fluorescent probes having been diluted within the cellular membranes.

To evaluate the effect of incubation of cells with cationic liposomes on plasma membrane integrity, the cells were incubated for 1 h with liposomes as described above, rinsed twice with PBS, and treated with 0.5 μ g/ml propidium iodide for 10 min. They were subsequently observed using a fluorescent microscope. To see whether the permeabilization of the plasma membrane affected the viability of the cells, the above test was performed on cells seeded at density of 50000 per well of a 24-well plate after 1 h incubation with diC14-amidine liposomes or lipoplexes cultured for 24 or 48 h.

Transfection protocol

The cells, which had been seeded in 24-well plates at a density of 7×10^5 cells per well on the day before the experiment, were rinsed twice with PBS, covered with 1 ml of medium without serum, and pre-incubated for 1 h at 37°C. Then, lipoplexes composed of DOTAP or diC14-amidine cationic liposomes and plasmid DNA pEGFP (encoding “enhanced green fluorescent protein”, 4.7 kb) at a +/- charge ratio of 8:1 were added. After 1 h incubation, the cells were rinsed twice with PBS, covered with 1 ml of medium containing 10% FBS, and left in a cell culture incubator for 48 h. To evaluate the transfection efficiency, the number of cells emitting green fluorescence seen in the observation field was divided by the total number of cells. The counting procedure was repeated at least 5 times for each well.

RESULTS AND DISCUSSION

Erythrocyte ghosts and HeLa cells were chosen as model systems to compare the lipid-mixing activity of diC14-amidine and DOTAP liposomes. The data presented in Fig. 1A and B indicates diC14-amidine liposomes have higher lipid-mixing efficiencies than those prepared from DOTAP, which concurs with the results of others [13, 22]. They also show a higher efficiency at interacting with HeLa cells, also demonstrated previously by others [23]. Our experiments were performed at different temperatures to evaluate how the increase in lipid fluidity (a set of highly temperature-dependent features of the lipid bilayer) affects the lipid-mixing process (Fig. 1A). For both preparations of liposomes, the efficiency of lipid mixing was increased at higher temperatures. For the two temperature values tested (30 and 37°C), lipid mixing was much more efficient for diC14-amidine than for the DOTAP liposomes (Fig. 1A). The lipid-mixing efficiency after incubation at 30°C was 6 times higher for diC14-amidine than

for DOTAP liposomes (about 60% versus 10% lipid mixing, respectively). The lipid-mixing efficiency is dependent on lipid fluidity; therefore, a temperature increase induces higher lipid-mixing efficiencies, in particular in the case of DOTAP liposomes. As the diC14-amidine-induced increase in lipid-mixing efficiency is not that dramatic (30% at 37°C compared to an almost two-fold increase for DOTAP liposomes), it may be suggested that diC14-amidine monomers first induce an increase in membrane fluidity facilitating fusion events. This view would concur with postulated idea of penetration of diC14amidine into the inner lipid layer and electrostatic interaction with PS [24]. Experiments carried out at 45°C (not shown) also support these conclusions: in the case of erythrocyte ghosts the temperature-dependent increase in lipid-mixing capacity upon incubation with DOTAP liposomes is high, while the increase of this parameter upon treatment with diC14amidine liposomes is moderate. In the case of trypsinized HeLa cells, both liposome preparations induced a moderate increase in lipid mixing when experiments were carried out at 45°C (Fig. 1B, not shown for the highest temperature).

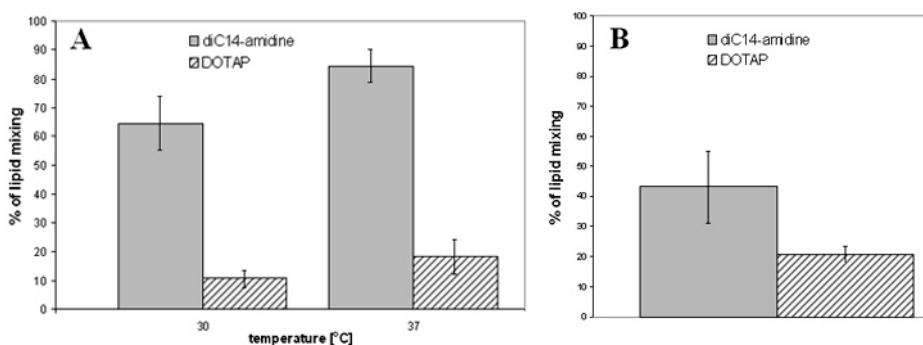


Fig. 1. Efficiencies of lipid mixing obtained upon interactions of diC14-amidine or DOTAP liposomes with erythrocyte ghosts or trypsinized HeLa cells. A. Liposomes at a concentration of 25 μ M (lipid) were incubated with erythrocyte ghosts (100 nmol P_i/ml) at the indicated temperatures for 15 min. B. Trypsinized HeLa cells (10^6 in total volume of 1ml) were incubated with liposomes at a concentration of 15 μ M (lipid) at 37°C. The values represent the mean \pm S.D. (n = 6). For details see Materials and Methods.

When HeLa cells were incubated with diC14-amidine liposomes labelled with the fluorescent probes NBD-PE and Rh-PE, the cellular membranes observed under blue light became green-stained, indicating lipid mixing with diC14-amidine liposomes. In Fig. 2A, the yellow-orange spots adsorbed on the cell surface (arrows) represent unfused liposomes, but they could also be intracellular endocytosed objects (arrowheads). Bright green-stained objects possibly representing fused liposomes or endovesicles can be also seen (Fig. 2A, the cell indicated by a circle). It is likely that lipid mixing mainly occurred with

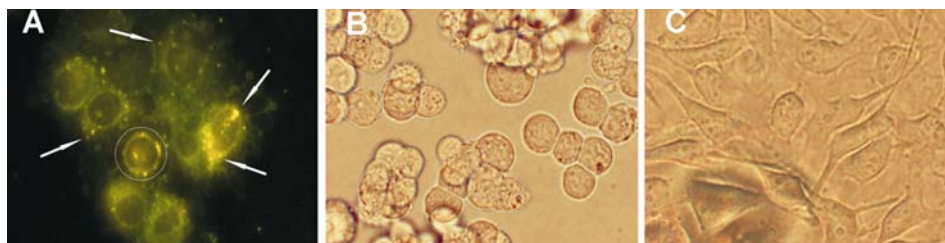


Fig. 2. HeLa cells treated with diC14-amidine liposomes display high lipid-mixing effect and undergo morphological changes. A - Fluorescent image of cells which were incubated with diC14-amidine liposomes labelled with NBD-PE and Rh-PE. Yellow-orange spots represent liposomes adsorbed on the cell surface (arrows). Green fluorescence (plasma membranes) indicates dilution of fluorescent probes. B - Differential interference contrast image of the same cells and C - Untreated cells. Magnification 500x.

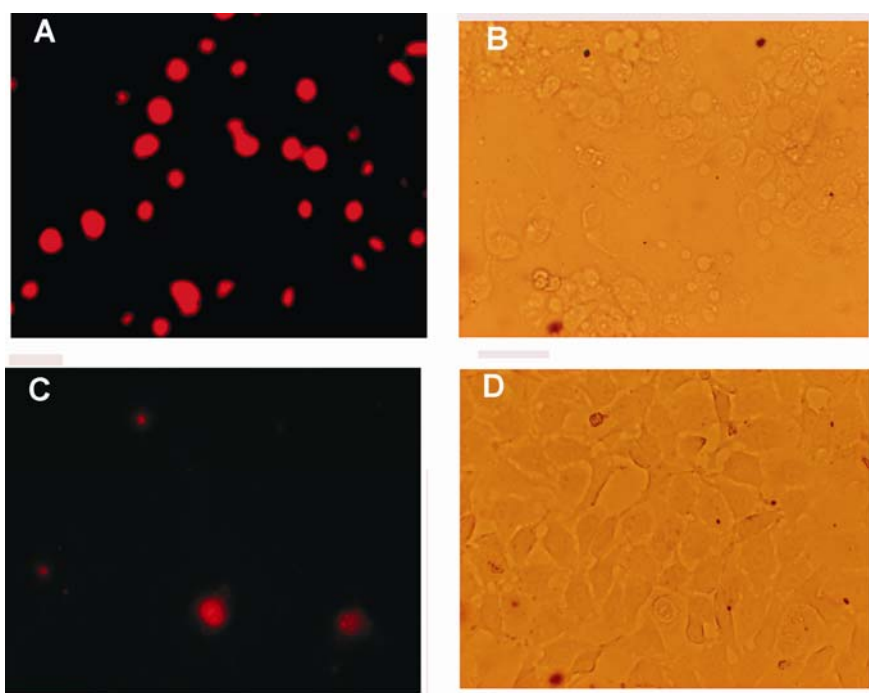


Fig. 3. HeLa cells incubated with DiC14-amidine liposomes become permeable to propidium iodide. A - Cells incubated with diC14-amidine liposomes – fluorescent image. B - Cells incubated with diC14-amidine liposomes – differential interference contrast image. C - Cells incubated with DOTAP liposomes – fluorescent image. D - Cells incubated with DOTAP liposomes – differential interference contrast image. For details see Materials and Methods.

the plasma membrane, since trypsinization should reduce the cellular uptake of liposomes via adsorptive endocytosis [25, 26].

HeLa cells treated with diC14-amidine liposomes in a standard cell culture medium MEM α containing 1 mM CaCl₂ undergo characteristic morphological changes resembling a necrotic morphology (Fig. 2B, compare with untreated cells, Fig. 2C). Moreover, the plasma membranes of HeLa cells incubated for 1 h with diC14-amidine liposomes became permeable to propidium iodide, which was rather rarely observed after incubation with DOTAP liposomes (Fig. 3A and C). DiC14-amidine was shown by others to possess haemolytic activity, which is consistent with the above observation [26]. Both effects (i.e. morphological changes and membrane permeabilising activity) induced by diC14-amidine are related. The disturbance in membrane integrity may induce an elevation of the Ca²⁺ level in the cytosol, which was shown by others to cause actin filament disassembly [27]. Observation of the morphology of the actin cytoskeleton (with TRITC-phalloidin) showed neither the changes related to the membrane morphology nor visible changes in actin filament density (data not shown). Independently of the kind of mechanism(s) underlying these changes (which should certainly be the subject of further studies), they are transient, as most (>80%) of the cells survived that treatment. Their permeability to propidium iodide was largely decreased to ~10% 24 h after the removal of the liposomal suspension from the culture medium (Fig. 4). Moreover, a similar, even greater effect was observed when HeLa cells were treated for 1 h with diC14-amidine lipoplexes with DNA at a charge ratio of 8:1 (Fig. 4).

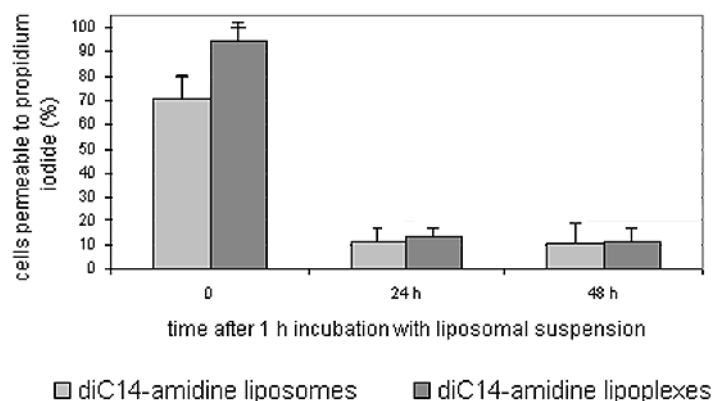


Fig. 4. The permeabilisation of HeLa cells induced by diC14-amidine liposomes and lipoplexes is transient. Cells were incubated with diC14-amidine liposomes or lipoplexes prepared at a +/- charge ratio of 8:1 for 1 h at 37°C, and washed and treated with a solution of propidium iodide in PBS. Alternatively, the cells after 1 h incubation with a solution of liposomes/lipoplexes were rinsed twice with PBS, covered with culture medium and further incubated for 24 or 48 h. After that, they were treated with propidium iodide. Then, the cells were washed with PBS and analyzed by fluorescence microscopy. The data represent the mean \pm S.D. (n = 4).

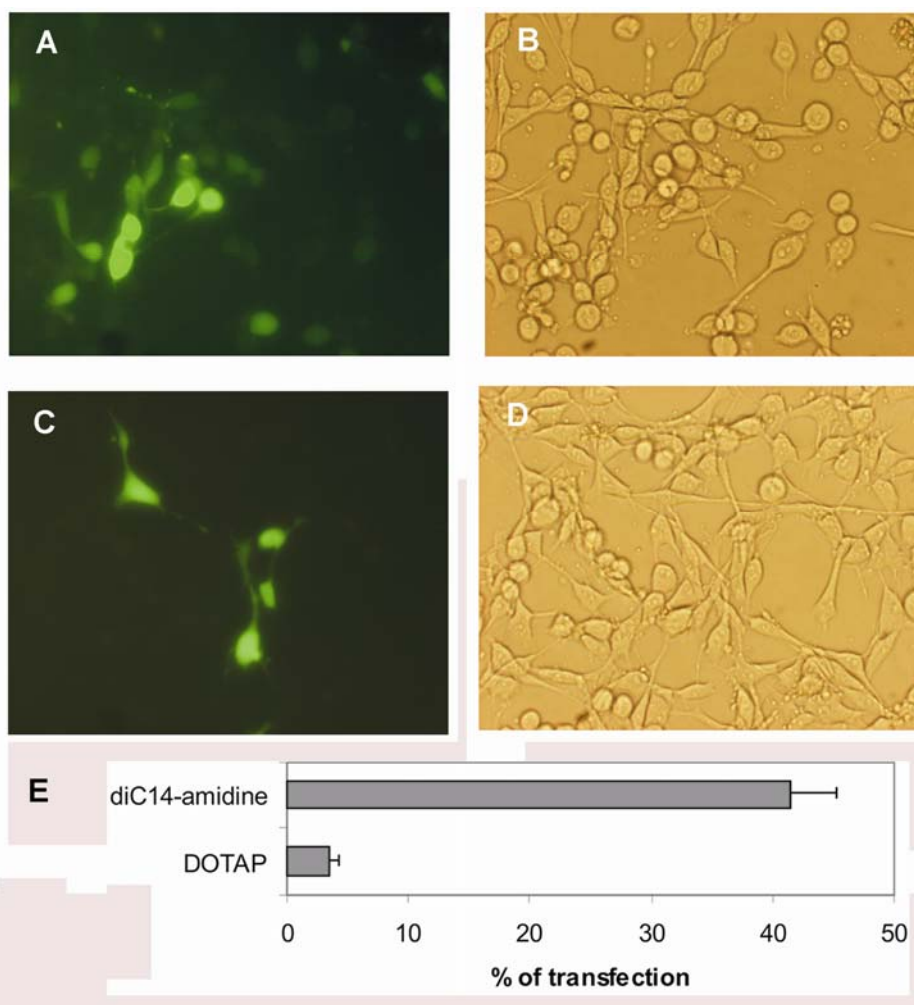


Fig. 5. A comparison of the transfection efficiencies obtained for diC14-amidine and DOTAP lipoplexes used as carriers of plasmid DNA encoding enhanced green fluorescence protein pEGFP. Lipoplexes were prepared at a \pm charge ratio of 8:1, and incubated with HeLa cells for 1 h at 37°C in the absence of serum. A, B - Fluorescent (A) and phase contrast (B) images of HeLa cells transfected with diC14-amidine lipoplexes. C, D - Fluorescent (C) and phase contrast (D) images of cells transfected with DOTAP lipoplexes. Magnification 133x. E - Transfection efficiencies observed for diC14-amidine and DOTAP lipoplexes expressed as % of cells emitting green fluorescence. The values represent the mean \pm S.D.

Keeping these observations in mind, we performed transfection experiments with lipoplexes prepared from diC14-amidine or DOTAP liposomes and plasmid DNA encoding green fluorescent protein (GFP) in the same experimental conditions. The transfection efficiency of the diC14-amidine lipoplexes was

markedly higher than that observed for DOTAP lipoplexes (Fig. 5A, C, E), exceeding 40%, which is comparable to other highly efficient preparations, e.g. Metafectene [28]. The transient increase in membrane permeability after treatment with diC14-amidine liposomes might be involved in the highly effective transfection process compared to that mediated by DOTAP lipoplexes. The experimental data of other researchers suggests that an intracellular Ca^{2+} increase may be responsible for the enhanced level of transfection obtained using cationic lipoplexes [29]. The results presented here may indicate that both properties – the high lipid-mixing efficiency upon interaction with the natural membranes and their transfection activity – are related. Short-chained diC14-amidine is clearly an agent capable of affecting the integrity of natural membranes more efficiently than DOTAP. This effect could be a result of fusion-associated lipid mixing within the cytosolic monolayer of the plasma membrane due to the electrostatic interactions between the positively charged diC14-amidine and negatively charged PS, as was suggested for the erythrocyte membrane [24]. These interactions may lead to the formation of nonlamellar structures which could both promote fusion and affect membrane integrity. The morphology of the cells transfected using diC14-amidine liposomes appeared unchanged in most cases, suggesting that the necrotic-like changes observed after incubation with liposomes were transient and the processes leading to them were not lethal.

In summary, we found a correlation between the ability of cationic liposomes to alter HeLa cell membrane organization, their lipid-mixing efficiency and the efficiency of transfection. The differences between the effects of the two cationic lipid liposomes on cellular membranes are apparently due to the alkyl chain length and unsaturation. It was suggested [11, 30] that the transfection activity of various types of lipoplexes was moderately dependent on the uptake but mainly on the subsequent stages of transfection, i.e. escape from the endosome. There is also data that at least in the case of some cell lines (e.g. monocytic leukaemia THP1 cells), a lack of correlation between the fusion and transfection efficiencies was observed [31]. Therefore, further studies should be carried out on the cell type specificity and the detailed mechanism(s) of transient permeabilisation and the resealing (recovery) of the plasma membrane barrier properties.

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