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Brief Article

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Cholesterol-Dependent Macropinocytosis and Endosomal Escape Control the Transfection Efficiency of Lipoplexes in CHO Living Cells

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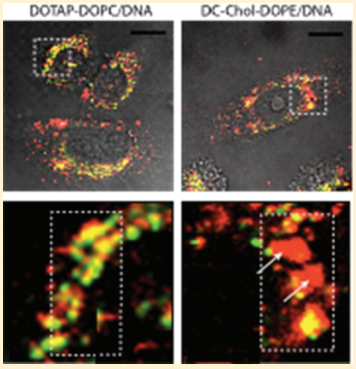
Supporting Information

ABSTRACT: Here we investigate the cellular uptake mechanism and final intracellular fate of two cationic liposome formulations characterized by similar physicochemical properties but very different lipid composition and efficiency for intracellular delivery of DNA. The first formulation is made of cationic lipid 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) and the zwitterionic helper dioleoylphosphocholine (DOPC), while the second one is made of the cationic 3β-[*N*-(*N*,*N*-dimethylaminoethane)-carbamoyl] cholesterol (DC-Chol) and the zwitterionic lipid dioleoylphosphatidylethanolamine (DOPE). Combining pharmacological and imaging approaches we show that both DOTAP-DOPC/DNA and DC-Chol-DOPE/DNA lipoplexes are taken up in Chinese hamster ovary (CHO) living cells mainly through fluid-phase macropinocytosis. Our results also indicate that lipoplex macropinocytosis is a cholesterol-sensitive uptake mechanism. On the other side, both clathrin-mediated and caveolae-mediated endocytosis play a minor role, if any, in the cell uptake. Colocalization of fluorescently tagged lipoplexes and Lysosensor, a primary lysosome marker, reveals that poorly efficient DOTAP-DOPC/DNA lipoplexes are largely degraded in the lysosomes, while efficient DC-Chol-DOPE/DNA systems can efficiently escape from endosomal compartments.



KEYWORDS: *cationic liposomes, lipoplexes, cell transfection, endocytosis, macropinocytosis, endosomal escape, lysosome degradation*

INTRODUCTION

Gene delivery is an extremely promising therapeutic approach for a vast array of medical disorders including cancer, genetic and acquired diseases. Among nonviral carriers, cationic liposome/DNA complexes (lipoplexes) have advantages in terms of simplicity of use and ease of large-scale production.[1−3] Furthermore, they are much less immunogenic than their viral counterpart and potentially not limited in DNA packaging capacity. However, the use of lipoplexes has not yet been translated into clinical applications because of unsatisfactorily low transfection efficiency (TE). Such poor TE depends on the lack of knowledge of the mechanisms whereby lipoplexes are able to overcome transfection barriers to then induce transgene expression.[4,5] The first barrier to transfection is the cell uptake. Upon arrival near the cell, lipolexes must associate with the plasma membrane, either through electrostatic interactions or by ligand−receptor binding. Despite that some lipoplex formulations can enter cells via a temperature-independent mechanism,[6,7] it is well-accepted that both nonspecifically and ligand-bound complexes enter cells principally via endocytic processes. Lipoplex endocytosis is divided into (i) clathrin-mediated endocytosis, (ii) caveolae-mediated endocytosis, and (iii) fluid-phase macropinocytosis.[8] This broad classification does not account for phagocytosis and clathrin- and caveolae-independent endocytosis. However, phagocytosis occurs only in specialized cells such as macrophages, neutrophiles or monocytes that have not been used in the present investigation, while clathrin- and caveolae- independent endocytosis is still largely unknown. Thus, we restrict to these forms of endocytic internalization that are the most studied pathways for nonviral carriers.[9−11] Lipoplexes taken up by different endocytic pathways are processed by the cells in different ways. This could determine their intracellular trafficking and, in turn, their gene transfer efficiency. Studies aimed at investigating the mechanism of internalization and the final fate of lipoplexes are therefore highly auspicious.

Herein, we investigate the cellular uptake and the final intracellular fate of two lipoplex formulations by a combination of pharmacological and imaging approaches. The first formulation was the widely used delivery system made of the cationic lipid 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) and the zwitterionic lipid dioleoylphosphocholine (DOPC). The second one was the binary system made of the cationic 3β-[*N*-(*N*,*N*-dimethylaminoethane)-carbamoyl] cholesterol (DC-Chol) and the zwitterionic helper lipid dioleoylphosphatidylethanolamine (DOPE). These formulations were chosen because they exhibit virtually identical

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physical−chemical properties such as size, zeta-potential, DNA-protection ability and structure at the nanoscale.[12−14] Despite the similarity in the physical−chemical properties, the marked diversity in lipid composition results in a different biological activity with DC-Chol-DOPE/DNA lipoplexes being almost one order of magnitude more efficient than DOTAP-DOPC/DNA complexes. The widely used DOTAP-DOPC liposome formulation was chosen due to its liquid-crystalline phase of lipid bilayer that could promote favorable interaction with fluid-phase domains present in the plasma membrane. On the other hand, DC-Chol-DOPE formulation was chosen because it does contain DC-Chol, a cationic derivative of cholesterol, which is supposed to interact favorably with cholesterol-rich domains (i.e., caveolae and rafts) in the plasma membrane.

Here we show that both the lipoplex formulations are taken up in Chinese hamster ovary (CHO) cells mainly through fluid-phase macropinocytosis, while both clathrin-mediated and caveolae-mediated endocytosis play a minor role, if any, in the process. Combining pharmacological and imaging results we suggest that lipoplex macropinocytosis is a cholesterol-sensitive uptake mechanism. Investigating the ultimate intracellular fate of lipoplexes, we find that poorly efficient DOTAP-DOPC/DNA lipoplexes are largely degraded in the lysosomes, while DC-Chol-DOPE/DNA lipoplexes successfully escape the endosome pathway.

EXPERIMENTAL SECTION

Liposomes Preparation. Cationic 1,2-dioleoyl-3-trimethylammonium propane (DOTAP), [3β[*N*-[(*N*′,*N*′-dimethylamino)ethyl]carbamoyl]]cholesterol (DC-Chol), zwitterionic dioleoylphosphatidylethanolamine (DOPE), and dioleoylphosphocholine (DOPC) were purchased from Avanti Polar Lipids (Alabaster, AL) and used without further purification. Briefly, binary mixtures of DOTAP-DOPC and DC-Chol-DOPE CLs, at molar fractions of neutral lipid in the bilayer Φ = neutral lipid/total lipid (mol/mol) = 0.5, were dissolved in chloroform, and the solvent was evaporated under vacuum for at least 24 h. The obtained lipid films were hydrated with the appropriate amount of a Tris-HCl buffer solution (10^{-2} M, pH 7.4) to achieve the desired final concentration (1 mg/mL). The suspension was sonicated to clarity using a bath sonicator (20 kHz, 100 W, 10 min). The obtained liposome solutions were stored at 30 °C for 24 h to achieve full hydration.[15] Mean diameter and zeta-potential were measured at 25 °C by a Malvern NanoZetaSizer spectrometer (experimental details and results are given in Table S1 in the Supporting Information).

Lipoplex Praparation. For transfection experiments, plasmid DNA (pGL3 which codifies for firefly luciferase) (Promega, Madison, WI) was used. The vector pGL3-control employed in this study was transformed into *Escherichia coli* strain DH5α and grown in Luria−Bertani medium supplemented with ampicillin. The plasmid DNA was purified using a Maxiprep kit (Qiagen, Crawley, U.K.), and the concentration was determined spectrophotometrically at 260 nm.

For laser scanning confocal microscopy experiments the Cy3-labeled 2.7 kbp plasmid DNA was purchased from Mirus Bio Corporation (Madison, WI). Self-assembled lipoplexes were obtained by mixing adequate quantities of the DNA solutions to suitable volumes of liposome dispersions. All samples were prepared at a fixed cationic lipid/DNA charge ratio (mol/mol), i.e., ρ = cationic lipid (by mole)/DNA (base) = 3. Such a value was chosen because it corresponds to the middle of a typical plateau region observed for optimal transfection conditions.[3,7] The size and zeta potential of DOTAP-DOPC/DNA and DC-Chol-DOPE/DNA lipoplexes are 202 and 190 nm, and 48.9 and 50.2 mV, respectively (data not reported).

Luciferase Transfection Studies with Endocytosis Inhibitors. Different endocytosis inhibitors were purchased from Sigma-Aldrich (St. Louis, MO). Chinese hamster ovary (CHO) cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with Glutamax I (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) (Invitrogen) at 37 °C and 5% CO_2 atmosphere, splitting cells every 2−4 days to maintain monolayer coverage. Twenty-four hours before transfection, 150 000 cells were seeded per well into 24 well culture plates, in order to reach 70−80% confluence during transfection. CHO cells were pretreated with chlorpromazine (60 μM), wortmannin (10 μM), genistein (200 μM), and methyl-β-cyclodextrin (500 μM) in Optimem for 30 min prior to addition of lipoplexes to the cells. Inhibitor concentrations were chosen according to previous works.[16,17] Levels of proteins produced by treated cells were the same as those of untreated cells. This guaranteed that treating cells with inhibitors, at least at the concentrations used in the present study, did not result in any appreciable cell death. Lipoplexes were prepared in Optimem (Invitrogen) by mixing for each well of 24 well plates 0.5 μg of plasmid with 5 μL of sonicated lipid dispersion. These complexes were left for 20 min at room temperature before adding them to the cells. During transfection the cells were incubated in the presence of inhibitors (4 h at 37 °C). Afterward, the cells were washed 3× with PBS before they were incubated in 1 mL of growth medium for 48 h. Finally, cells were washed in PBS and harvested in 200 μL of lysis buffer (Promega, Madison, WI). A total of 20 μL of cell suspension was diluted in 100 μL of luciferase reaction buffer (Promega), and the luminescence was measured 10 s using a luminometer (Berthold, Bad Wildbad, Germany). Results were expressed as relative light units per mg of cell proteins as determined by BioRad Protein Assay Dye Reagent (Bio-Rad, Hercules, CA). Each experiment was performed in quadruplicate and repeated three times.

Cell Culture, Transfection, and Colocalization Assays. CHO-K1 were purchased from American Type Culture Collection (CCL-61 ATCC) and were grown in Ham's F12K medium supplemented with 10% of fetal bovine serum at 37 °C and in 5% CO_2. For transfection experiments, lipoplexes were prepared in PBS (Invitrogen) by mixing 1 μL of Cy3-labeled DNA with 10 μL of sonicated lipid dispersions. These complexes were left for 20 min at room temperature before adding them to the cells. In order to identify the endocytic vesicles involved in the internalization of lipoplexes, we performed colocalization assays in living cells. CHO-K1 cells were coincubated with lipoplexes and different endocytic fluorescent markers: 1 mg/mL 70 kDa dextran−FITC conjugate at 37 °C for 30 min to label macropinosomes, 50 mM Lysosensor for 30 min to label lysosomes, 2 μg/mL transferrin−Alexa488 conjugate for 30 min to label recycling and sorting endosomes. Transfection of Caveolin-E^{1}GFP[18] was carried out using lipofectamine reagent (Invitrogen) according to the manufacturer's instruction. Colocalization of green and red signals was analyzed by means of the Pearson's coefficient (Rr) using the "colocalization finder" plugin of the ImageJ software (NIH Image; http://rsbweb.nih.gov/ij/).[19]

Laser Scanning Confocal Microscopy Experiments. Laser scanning confocal microscopy (LSCM) experiments were

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performed with the Olympus Fluoview 1000 (Olympus, Melville, NY) confocal microscope interfaced with a 405 nm diode laser, a 488 nm Argon laser, and 543 nm helium−neon laser. Glass bottom Petri dishes containing transfected cells were mounted in a temperature-controlled chamber at 37 °C and 5% CO_2 and viewed with a 60× 1.25 numerical aperture water immersion objective. The following collection ranges were adopted: 500−540 nm (EGFP, Alexa488−transferrin and FITC−dextran 70 kDa), 555−655 nm (Cy3), and 460−530 (Lysosensor). Images were collected in sequential mode to eliminate emission cross talk between the various dyes.

RESULTS AND DISCUSSION

The goal of the present work was to analyze the uptake mechanism and the intracellular routing of two distinct lipoplex formulations in CHO cells. It is well accepted that the first interaction between complexes and cell surface proteoglycans is electrostatic, while the uptake mechanism can be severely affected by some physical features of lipoplexes such as surface charge and size. To exclude effects due to charge and size, DOTAP-DOPC/DNA and DC-Chol-DOPE/DNA lipoplexes were chosen. Indeed, despite very different lipid compositions, these formulations exhibit virtually identical size and zeta-potential.[12−14] To define the mechanisms involved in the internalization and intracellular fate of lipoplexes, CHO cells were pretreated with chemical drugs that specifically affect distinct uptake routes and intracellular pathways under well-established conditions. The inhibitors used in the present study and the respective pathways they target are listed in Table 1.

Table 1. Inhibitors Used and Targeted Endocytosis Pathways

inhibitor	endocytosis pathways
genistein	caveolae-mediated
chlorpromazine	clathrin-mediated
wortmannin	macropinocytosis
methyl-β-cyclodextrin	cholesterol dependence

Figure 1 shows the relative transfection efficiency (RTE) of DOTAP-DOPC/DNA and DC-Chol-DOPE/DNA lipoplexes in the presence of inhibitors defined as percentages of those without inhibitors. Genistein, reported to block caveolae-mediated uptake processes, did not substantially affect the activity of lipoplexes (RTE ≈ 90%). Chlorpromazine is an amphiphilic drug that prevents the recycling of clathrin proteins from endosomes back to the cell membrane, thus inhibiting the formation of new clathrin-coated pits. Pretreatment of the cells with chlorpromazine resulted in a slight decrease of TE (85% < RTE < 95%). Fluid-phase macropinocytosis is a triggered process used by the cell to internalize large amounts of fluid and membrane. Macropinocytosis is characterized by the formation of large, irregular primary endocytic vescicles, called macropinosomes, after closure of ruffling membrane domains. Macropinosomes are dynamic structures that frequently move inward toward the center of the cell. Treatment of cells with wortmannin, a powerful inhibitor of macropinocytosis, did remarkably affect TE of DOTAP-DOPC/DNA systems (RTE ≈ 10%). On the other hand, a minor effect, if any, was observed in the case of DC-Chol-DOPE lipoplexes, (RTE ≈ 90%). Lastly, cholesterol depletion from the plasma membrane by methyl-β-cyclodextrin resulted in a strong reduction of TE for both formulations suggesting a key role of cholesterol in the lipoplex uptake and subsequent trafficking. RTE of DOTAP-DOPC/DNA lipoplexes was about 50%, while inhibition of DC-Chol-DOPE/DNA lipoplexes was almost complete (RTE ≈ 5%). In summary, the pharmacological interference study showed that (i) major involvement of clathrin-mediated and caveolae-mediated endocytosis can be excluded; (ii) fluid-phase macropinocytosisis is the major pathway responsible for DOTAP-DOPC-mediated cell transfection; (iii) cholesterol depletion has a dramatic effect on the efficiency of DC-Chol-DOPE/DNA lipoplexes.

We are aware that some drugs may induce unpredictable side effects that inhibit the various uptake mechanisms. Thus, to rule out any possible side effect of these treatments on gene expression per se, LSCM on CHO untreated cells was performed. LSCM allowed us to visualize the intracellular trafficking of fluorescently labeled lipoplexes (red) in living cells in the presence of various endocytic markers (green). Colocalization of red and green signals gave rise to visible yellow/orange punctate structures. To perform a quantitative analysis of confocal images, the degree of colocalization of green and red fluorescence signals was calculated by means of the Pearson's coefficient (Experimental Section) (Table 2).

First, a transfection with caveolin-E[1]GFP was used to label caveolae. At 1 h of treatment with lipolexes (Figure 2, panel A), no clear colocalization of red and green fluorescent signals was found, indicating that DOTAP-DOPC/DNA and DC-Chol-DOPE/DNA lipoplexes were not taken up, at least significantly, through this pathway. Correlation analysis of confocal images confirmed this suggestion (average Pearson's coefficients are

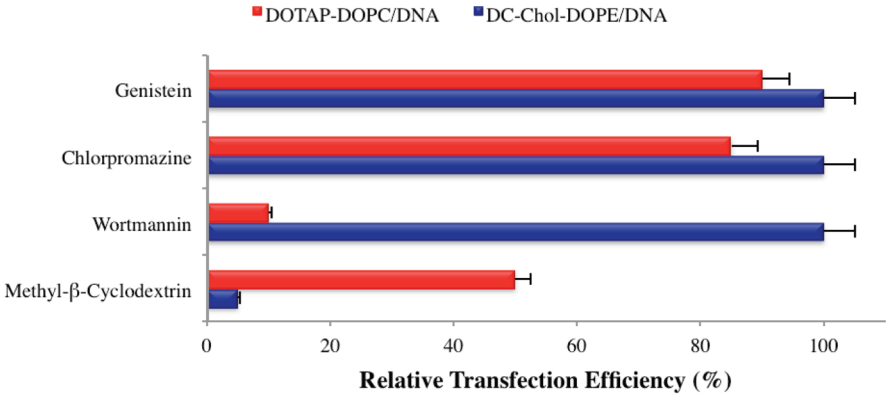

Figure 1. Relative transfection efficiency of DOTAP-DOPC/DNA and DC-Chol-DOPE/DNA lipoplexes in the presence of inhibitors.

Table 2. Colocalization with Endocytic Markers[a]

	caveolin	transferrin	dextran
DOTAP-DOPC/DNA	−0.04 ± 0.06 (7)	0.08 ± 0.06 (10)	0.41 ± 0.09 (10)
DC-Chol-DOPE/DNA	−0.01 ± 0.05 (7)	0.06 ± 0.05 (10)	0.39 ± 0.08 (10)

[a]Pearson's coefficients for colocalization of lipoplexes with endocytic markers.

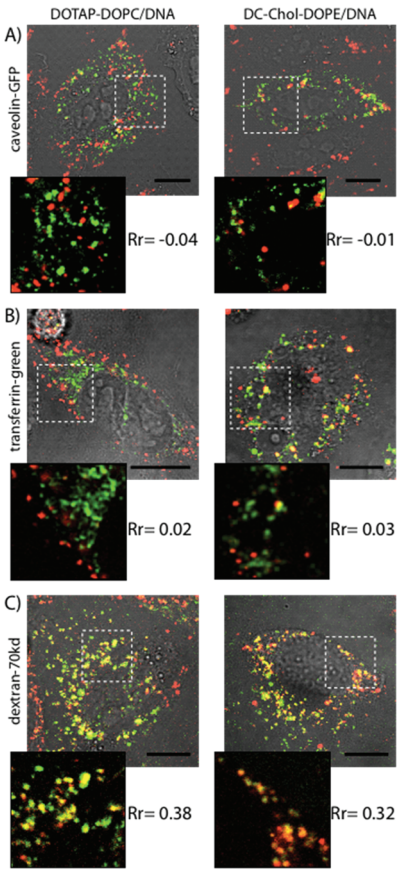

Figure 2. Colocalization of DOTAP-DOPC/DNA and DC-Chol-DOPE/DNA signals (red) with endocytic markers (green). No colocalization was observed with caveolin-E[1]GFP (caveolar pathway) (A) and Alexa488−transferrin (clathrin pathway) (B) while high correlation (yellow vesicles) was observed with 70 kDa dextran (macropinocytosis) (C). The Pearson's coefficient (Rr) is shown for each measurement. Scale bar =10 μm.

reported in Table 2). This observation was consistent with the results from the pharmalogical investigation reported in Figure 1 showing that, when caveolae-mediated uptake processes are blocked by genistein, the TE of lipoplexes remains practically unchanged.

To investigate if lipoplexes enter CHO cells via the clathrin-coated vesicle-mediated endocytosis pathway, Alexa488-labeled transferrin, marker for clathrin dependent endocytosis, was used. At t = 30 min (Figure 2, panel B), the two fluorescence signals were not colocalized (Table 2). A slight increase of the degree of colocalization of lipoplexes with the clathrin pathway is usually observed with time (not shown). This, however, may be due to macropinosomes slowly reaching the clathrin-positive endosomial checkpoint. Once again, this result is in very good agreement with the results of the pharmacological interference reported in Figure 1 that led us to exclude major involvement of clathrin-dependent internalization for both these formulations.

Lastly, a fluorescently labeled 70 kDa dextran, a fluid-phase uptake marker, was used for macropinocytosis visualization. Since macropinocytosis is a fluid phase uptake, we would expect it to be a slower process. Thus, to further support our findings, measurements were performed after incubating the lipoplexes with cells for varying time periods of 15 min, 30 min, 1 h, and 3 h. At t = 3 h we observe that both the lipoplex formulations considerably colocalized with dextran in living CHO cells (Figure 2, panel C). The quantitative colocalization analysis of the signals of lipoplexes and macropinosomes did not show appreciable differences between DOTAP-DOPC/DNA and DC-Chol-DOPE/DNA lipoplexes (Table 2). This result supports the conclusion that both the lipoplex formulations were predominatly taken up by the macropinocytosis pathway.[20] This result is in good agreement with the results of the inhibitory study confirming that fluid phase macropinocytosis is largely responsible for the transfection of DOTAP-DOPC/DNA lipoplexes. On the other hand, TE of DC-Chol-DOPE/DNA lipoplexes was not affected by wortmannin, while it was suppressed by methyl-β-cyclodextrin. However, this apparent contradiction can be resolved as soon as one recognizes that lipoplex macropinocytosis can implicate different molecular mechanisms involving either fluid-phase or cholesterol-rich domains simultaneously. While there is general consensus about the active role of fluid phase domains in macropinocytosis, the concept that this endocytic pathway is also dependent on cholesterol is now emerging. The precise mechanism is presently unknown, but cholesterol depletion might lead to a redistribution of other lipids required for the formation of ruffles with the result that formation of macropinocytic vesicles cannot occur.[21,22]

It has been recently shown that shape coupling between lipoplex and membrane lipids regulates lipoplex−microdomain interaction, thus determining the success of internalization.[23] The plasma membrane itself is no longer considered a uniform structure but rather a patchwork of microdomains that can compartmentalize signaling. Some of us have shown that the propensity of lipoplexes to interact with cellular lipids is regulated by the shape coupling between lipoplex and membrane lipids.[3] According to such suggestions,[3,23] we propose that DC-Chol-rich lipoplexes are internalized by macropinocytosis after interaction with lipid rafts where sphingolipids and cholesterol pack tightly, excluding most phospholipids with unsaturated hydrocarbon tails. This idea is in good agreement with pharmacological results showing that cholesterol depletion has a major effect on DC-Chol-DOPE/DNA lipoplexes. For the same reason, interaction of cholesterol-like (DC-Chol) and cone-shaped (DOPE) lipids with cylinder shaped lipids (that are abundant in fluid phase membrane domains) is inefficient. This suggestion could help toward explaining why DC-Chol-DOPE/DNA lipoplexes are not deeply affected by treatment of cells with wortmannin. The overall scenario is completely symmetrical when DOTAP-DOPC/DNA lipoplexes are considered. Indeed, this formulation is made of cylinderlike lipids that could favorably interact with fluid-phase domains that are richer in unsaturated lipids of similar shape. This same shape coupling argument is supported by the TE results showing complete inhibition after treatment of cells with wortmannin. On the other hand, DOTAP-DOPC/DNA lipoplexes can only partially interact with cholesterol-rich "lipid rafts", due to high packing competition between lipoplex

and cellular lipids.[3,22] This unfavorable interaction may result in a low incorporation efficiency and might explain why their final efficiency is much less affected by cholesterol depletion. In summary, the fluorescence colocalization investigation and pharmacological inhibitor study support the following conclusions: (i) fluid phase macropinocytosis is the major pathway responsible for lipoplex internalization in CHO living cells and (ii) it is a cholesterol-dependent mechanism; (iii) macropinocytosis is affected by a regulatory mechanism that couples the lipid composition of lipoplexes and that of the cell surface.

In our measurements, 3 h of treatment is enough to observe the fluorescent signal of lipoplexes inside the cell. To account for differences in TE the ultimate intracellular fate of lipoplexes was finally investigated. To this end, we used Lysosensor, which accumulates in acidic cell organelles, and is primarily a lysosome marker (Figure 3). Thus, the DNA signal colocalized

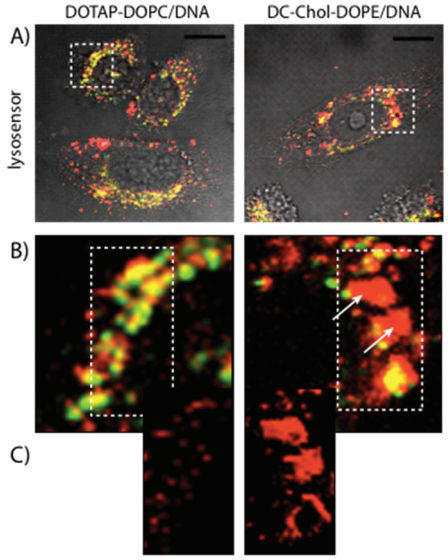

Figure 3. Colocalization of DOTAP-DOPC/DNA and DC-Chol-DOPE/DNA signals (red) with Lysosensor (lysosome marker, green), after 3 h of lipoplex treatment (A). This analysis reveals that DOTAP-DOPC/DNA is completely delivered to the lysosomal compartment, while DC-Chol-DOPE/DNA is able to avoid lysosomal entrapment (B, C). Scale bar = 10 μm.

with Lysosensor signal (yellow clusters) was interpreted as DNA in lysosomes. After 3 h of incubation with DOTAP-DOPC/DNA lipoplexes a marked colocalization of red and green fluorescence in large perinuclear vesicular structures was observed. This observation suggests that DOTAP-DOPC/DNA lipoplexes were largely degraded in the lysosomes and provides a reasonable explanation for their low TE. Strickingly, when CHO cells were transfected with DC-Chol-DOPE/DNA lipoplexes a much lower degree of colocalization with Lysosensor was found. At the same time, large (>1 μm) patches of red fluorescence appear indicating that DC-Chol-DOPE/DNA lipoplexes largely escaped the endosome pathway before reaching lysosomes (Figure 3C reports the difference between the red and yellow signals). Accordingly, if the escape efficiency is calculated as the intensity of the red signal divided by the total intensity of the red and yellow signals,[24] the red patches yield efficiency values close to 1. Confocal images therefore suggest that efficient escape from endosomes explains the superior efficiency of DC-Chol-DOPE/DNA lipoplexes with respect to the poorly transfecting DOTAP-DOPC/DNA complexes. This ability of DC-Chol-DOPE/DNA lipoplexes to escape the endosomal compartments may be due to their phase behavior upon interaction with cellular lipids. Recent evidence strongly suggests that the phase evolution of lipoplex lipids plays a prominent role in destabilizing the endosomal membrane. Particularly relevant in this regard is the presence of nonbilayer structures, which may promote membrane destabilization and/or disrupt membrane integrity. In this regard, DOPE, due to its cone-like molecular geometry,[25,26] has intrinsic ability to promote lamellar-to-hexagonal phase transitions at low pH. Lowering of the pH in endosomal compartments causes local phase separation into DOPE-enriched domains.[25] This, in turn, allows the formation of a hexagonal phase of DOPE at low pH. The formation of a nonbilayer phase at the level of the lipoplex interacting with the endosomal membrane[27] is crucial for bringing about sufficient perturbation and/or destabilization of the endosomal membrane and efficient release of DNA from the lipoplexes, which is then ejected into the cytoplasm. Several strategies have been explored to favor endosomal escape and intracellular trafficking to the nucleus.[28,29] Most of them are based on the drop in pH nanocarriers experience when they move from the extracellular environment (pH $\approx$ 7.4) to the lumen of early endosomes (pH $\approx$ 6), up to their final intracellular destination (late endosomes and lysosomes, pH $\approx$ 5). Among these strategies, pH-responsive lipids with enhanced fusogenic character at low pH can greatly help toward the destabilization of the endosomal membrane.[30] As a consequence of the incorporation of such pH-responsive helper lipids, the transfection efficiency was found to increase by a factor of up to 100 compared to the use of other common colipids.[30] Another possibility is the inclusion of pH-sensitive components such as histidine and imidazole[31] in the cationic lipids. The imidazole ring of histidine is a weak base with the intrinsic ability to become positively charged at pH < 6. This can induce membrane fusion and/or membrane permeation in an acidic medium. The accumulation of histidine residues inside acidic vesicles can induce a proton sponge effect, which increases their osmolarity and promotes their swelling. Both these phenomena can induce membrane fusion and/or membrane permeation in an acidic medium with the result that the gene payload can efficiently escape the endosomal compartments.[31]

To further support our conclusions, cells were transfected with DC-Chol-DOPC/DNA lipoplexes. Since DC-Chol-DOPC/DNA lipoplexes do contain the same molar fraction of DC-Cholesterol as that of DC-Chol-DOPE/DNA ones, these experiments were useful to confirm our conclusions about the role of cholesterol-dependent macropinocytosis in lipoplex uptake. On the other hand, the replacement of DOPE with DOPC in DC-Chol-containing lipoplexes allowed us to discriminate whether the high endosomal escape of DC-Chol-DOPE/DNA lipoplexes was effectively due to the peculiar fusogenic properties of DOPE. After 3 h of incubation with CHO cells, DC-Chol-DOPC/DNA lipoplexes did not significantly colocalize with caveolin-E[1]GFP (Figure S1 in the Supporting Information, panel A; Rr = 0.02). Likewise, the fluorescence signals of lipoplexes and Alexa488-labeled transferrin did not give rise to any appreciable colocalization (Figure S1 in the Supporting Information, panel B; Rr = −0.08). On the other hand, DC-Chol-DOPC/DNA lipoplexes largely colocalized with dextran (Figure S1 in the Supporting Information, panel C; Rr = 0.35). These results led us to

conclude that, when CHO cells are transfected with DC-Chol-DOPC/DNA lipoplexes, (i) major involvement of caveolae- and clathrin-dependent internalization can be excluded; (ii) macropinocytosis is the major pathway responsible of internalization. Pharmacological cholesterol depletion from the plasma membrane by methyl-β-cyclodextrin strongly affected the biological activity of DC-Chol-DOPC/DNA lipoplexes, with inhibition being nearly complete (RTE < 0.1%, results not reported for space consideration). In summary, these results strongly supported our conclusion that cholesterol-dependent macropinocytosis is likely to be the preferential entry route of lipoplexes in CHO living cells. Lastly, fluorescently tagged DC-Chol-DOPC/DNA lipoplexes were found to considerably colocalize with Lysosensor (Figure S2 in the Supporting Information, Rr = 0.58). Furthermore, broad patches of red fluorescence, as those observed in cells treated with DC-Chol-DOPE/DNA lipoplexes, were not detected. Even though a precise quantification of the fluorescence signals is beyond the scope of the present work, the observation that DC-Chol-DOPC/DNA lipoplexes are less able to avoid lysosomal degration with respect to their DOPE-containing counterpart corroborates our suggestion about the role played by DOPE in promoting the escape of the gene payload from the endosomal compartments.

In conclusion, we have shown that both DOTAP-DOPC/DNA and DC-Chol-DOPE/DNA lipoplex formulations are taken up in CHO living cells mainly through fluid-phase macropinocytosis, while both clathrin-mediated and caveolae-mediated endocytosis play a minor role, if any, in the cell uptake. Combining pharmacological and imaging results we also speculate that lipoplex macropinocytosis is a cholesterol-sensitive uptake mechanism. We claim that the superior activity of DC-Chol-DOPE/DNA lipoplexes does strictly correlate with their distinctive capability to escape from endosomes and release DNA. This result may have deep biological impact to predict the intracellular trafficking of lipoplexes and should be carefully considered for the rational design of efficient lipid gene vectors. The existence of a correlation between cholesterol-dependent macropinocytosis and efficient endosomal escape is currently under investigation in our laboratory.

■ ASSOCIATED CONTENT

Supporting Information

Figures depicting colocalization of DC-Chol-DOPC/DNA, tables of experimental data, and additional experimental details. This material is available free of charge via the Internet at http://pubs.acs.org.

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■ REFERENCES

(1) Felgner, P. L.; Ringold, G. M. Cationic liposome-mediated transfection. *Nature* **1989**, *337*, 387−388.

(2) Ramezani, M.; Khoshhamdam, M.; Dehshari, A.; Malaekeh-Nikouei, B. The influence of size, lipid composition and bilayer fluidity of cationic liposomes on the transfection efficiency of nanolipoplexes. *Colloids Surf. B: Biointerfaces* **2009**, *72*, 1−5.

(3) Pozzi, D.; Caracciolo, G.; Caminiti, R.; Candeloro De Sanctis, S.; Amenitsch, H.; Marchini, C.; Montani, M.; Amici, A. Toward the Rational Design of Lipid Gene Vectors: Shape Coupling between Lipoplex and Anionic Cellular Lipids Controls the Phase Evolution of Lipoplexes and the Efficiency of DNA Release. *ACS Appl. Mater. Interfaces* **2009**, *10*, 2237−224.

(4) Elouahabi, A.; Ruysschaert, J. M. Formation and intracellular trafficking of lipoplexes and polyplexes. *Mol. Ther.* **2005**, *11*, 11336−11347.

(5) Medina-Kauwe, L. K.; Xie, J.; Hamm-Alvarez, S. Intracellular trafficking of nonviral vectors. *Gene Ther.* **2005**, *12*, 1734−1751.

(6) Resina, S.; Prevot, P.; Thierry, A. R. Physico-Chemical Characteristics of Lipoplexes Influence Cell Uptake Mechanisms and Transfection Efficacy. *PloS One* **2009**, *4*, e6058.

(7) Marchini, C.; Pozzi, D.; Montani, M.; Alfonsi, C.; Amici, A.; Candeloro De Sanctis, S.; Digman, M. A.; Sanchez, S.; Gratton, E.; Amenitsch, H.; Fabbretti, A.; Gualerzi, C. O.; Caracciolo, G. Role of temperature-independent lipoplex−cell membrane interactions in the efficiency boost of multicomponent lipoplexes. *Cancer Gene Ther.* **2011**, *18* (8), 543−552.

(8) Gonçalves, C.; Mennesson, E.; Fuchs, R.; Gorvel, J.-P.; Midoux, P.; Pichon, C. Macropinocytosis of Polyplexes and Recycling of Plasmid via the Clathrin-Dependent Pathway Impair the Transfection Efficiency of Human Hepatocarcinoma Cells. *Mol. Ther.* **2004**, *10*, 373−385.

(9) Zuhorn, I. S.; Hoekstra, D. On the mechanism of cationic amphiphile-mediated transfection. To fuse or not to fuse: is that the question? *J. Membr. Biol.* **2002**, *189*, 167−179.

(10) Hoekstra, D.; Rejman, J.; Wasungu, L.; Shi, F.; Zuhorn, I. S. Gene delivery by cationic lipids: in and out of an endosome. *Biochem. Soc. Trans.* **2007**, *35*, 68−71.

(11) Rejman, J.; Oberle, V.; Zuhorn, I. S.; Hoekstra, D. Size-dependent internalization of particles via the pathways of clathrin- and caveolae-mediated endocytosis. *Biochem. J.* **2004**, *377*, 159−169.

(12) Marchini, C.; Montani, M.; Amici, A.; Pozzi, D.; Caminiti, R.; Caracciolo, G. Surface area of lipid membranes regulates the DNA-binding capacity of cationic liposomes. *Appl. Phys. Lett.* **2009**, *94*, 033903.

(13) Caracciolo, G.; Pozzi, D.; Caminiti, R.; Marchini, C.; Montani, M.; Amici, A.; Amenitsch, H. Transfection efficiency boost by designer multicomponent lipoplexes. *Biochim. Biophys. Acta* **2007**, *1768*, 2280−2292.

(14) Caracciolo, G.; Marchini, C.; Pozzi, D.; Caminiti, R.; Amenitsch, H.; Montani, M.; Amici, A. Structural stability against disintegration by anionic lipids rationalizes the efficiency of cationic liposome/DNA complexes. *Langmuir* **2007**, *23*, 4498−4508.

(15) Pozzi, D.; Amenitsch, H.; Caminiti, R.; Caracciolo, G. How lipid hydration and temperature affect the structure of DC-Chol-DOPE/DNA lipoplexes. *Chem. Phys. Lett.* **2006**, *422*, 439−445.

(16) Rejman, J.; Bragonzi, A.; Conese, M. Role of clathrin- and caveolae mediated endocytosis in gene transfer mediated bylipo- and polyplexes. *Mol. Ther.* **2005**, *12*, 468−474.

(17) Zuhorn, I. S.; Kalicharan, R.; Hoekstra, D. Lipoplex-mediated Transfection of Mammalian Cells Occurs through the Cholesterol-dependent Clathrin-mediated Pathway of Endocytosis. *J. Biol. Chem.* **2002**, *277*, 18021−18028.

(18) Serresi, M.; Bizzarri, R.; Cardarelli, F.; Beltram, F. Real-time measurement of endosomal acidification by a novel genetically encoded biosensor. *Anal. Bioanal. Chem.* **2009**, *393*, 1123−1133.

(19) Zinchuk, V.; Zinchuk, O.; Okada, T. Quantitative colocalization analysis of multicolor confocal immunofluorescence microscopy

images: pushing pixels to explore biological phenomena. *Acta Histochem. Cytochem.* **2007**, *40*, 101−111.
(20) Zhang, X.-X.; Allen, P. G.; Grinstaff, M. Macropinocytosis is the Major Pathway Responsible for DNA Transfection in CHO Cells by a Charge-Reversal Amphiphile. *Mol. Pharmaceutics* **2011**, *8*, 758−766.
(21) Grimmer, S.; van Deurs, B.; Sandvig, K. Membrane ruffling and macropinocytosis in A431 cells require cholesterol. *J. Cell Sci.* **2002**, *115*, 2953−2962.
(22) Grimmer, S.; Iversen, T.-G.; van Deurs, B.; Sandvig, K. Endosome to Golgi Transport of Ricin Is Regulated by Cholesterol. *Mol. Biol. Cell* **2000**, *11*, 4205−4216.
(23) Marchini, C.; Pozzi, D.; Montani, M.; Alfonsi, C.; Amici, A.; Amenitsch, H.; Candeloro De Sanctis, S.; Caracciolo, G. Tailoring Lipoplex Composition to the Lipid Composition of Plasma Membrane: A Trojan Horse for Cell Entry? *Langmuir* **2010**, *26*, 13867−13873.
(24) Akita, H.; Kogure, K.; Moriguchi, R.; Nakamura, Y.; Higashi, T.; Nakamura, T.; Serada, S.; Fujimoto, M.; Naka, T.; Futaki, S.; Harashima, H. Nanoparticles for ex vivo siRNA delivery to dendritic cells for cancer vaccines: Programmed endosomal escape and dissociation. *J. Controlled Release* **2010**, *143*, 311−317.
(25) Zuhorn, I. S.; Hoekstra, D. On the Mechanism of Cationic Amphiphile-mediated Transfection. To Fuse or not to Fuse: Is that the Question? *J. Membr. Biol.* **2002**, *189*, 167−179.
(26) Smisterova, J.; Wagenaar, A.; Stuart, M. C. A.; Polushkin, E.; Ten Brinke, G.; Hulst, R.; Engberts, J. B. F. N.; Hoekstra, D. Molecular shape of the cationic lipid controls the structure of cationic lipid/dioleylphosphatidylethanolamine-DNA complexes and the efficiency of delivery. *J. Biol. Chem.* **2001**, *276*, 47615−47622.
(27) Koynova, R.; Wang, L.; Tarahovsky, Y.; MacDonald, R. C. Lipid Phase Control of DNA delivery. *Bioconjugate Chem.* **2005**, *16*, 1335−1339.
(28) Midoux, P.; Breuzard, G.; Gomez, J. P.; Pichon, C. Polymer-based gene delivery: a current review on the uptake and intracellular trafficking of polyplexes. *Curr. Gene Ther.* **2008**, *8*, 335−352.
(29) Guo, X.; Huang, L. Recent advances in nonviral vectors for gene delivery. *Acc. Chem. Res.* **2011**. DOI: 10.1021/ar200151m.
(30) Mével, M.; Neveu, C.; Gonçalves, C.; Yaouanc, J. J.; Pichon, C.; Jaffrès, P. A.; Midoux, P. Novel neutral imidazole-lipophosphoramides for transfection assays. *Chem. Commun.* **2008**, *27*, 3124−3126.
(31) Midoux, P.; Pichon, C.; Yaouanc, J. J.; Jaffrès, P. A. Chemical vectors for gene delivery: a current review on polymers, peptides and lipids containing histidine or imidazole as nucleic acids carriers. *Br. J. Pharmacol.* **2009**, *157*, 166−178.