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ORIGINAL ARTICLE

Role of temperature-independent lipoplex–cell membrane interactions in the efficiency boost of multicomponent lipoplexes

C Marchini¹, D Pozzi², M Montani¹, C Alfonsi¹, A Amici¹, S Candeloro De Sanctis², MA Digman³, S Sanchez³, E Gratton³, H Amenitsch⁴, A Fabbretti¹, CO Gualerzi¹ and G Caracciolo²

¹Department of Bioscience and Biotechnology, University of Camerino, Camerino, Italy; ²First Faculty of Medicine, Department of Chemistry, Sapienza University of Rome, Rome, Italy; ³Laboratory for Fluorescence Dynamics, Department of Biomedical Engineering, University of California, Irvine, CA, USA and ⁴Institute of Biophysics and Nanosystems Research, Austrian Academy of Sciences, Graz, Austria

Multicomponent lipoplexes have recently emerged as especially promising transfection candidates, as they are from 10 to 100 times more efficient than binary complexes usually employed for gene delivery purposes. Previously, we investigated a number of chemical–physical properties of DNA–lipid complexes that were proposed to affect transfection efficiency (TE) of lipoplexes, such as nanoscale structure, size, surface potential, DNA-protection ability and DNA release from complexes upon interaction with cellular lipids. Although some minor differences between multicomponent and binary lipoplexes were found, they did not correlate clearly with efficiency. Instead, here we show that a marked difference between the cell internalization mechanism of binary and multicomponent lipoplexes does exist. Multicomponent lipoplexes significantly transfect cells at 4 °C, when endocytosis does not take place suggesting that they can enter cells via a temperature-independent mechanism. Confocal fluorescence microscopy experiments showed the existence of a correlation between endosomal escape and TE. Multicomponent lipoplexes exhibited a distinctive ability of endosomal escape and release DNA into the nucleus, whereas, poorly efficient binary lipoplexes exhibited minor, if any, endosomal rupture ability and remained confined in perinuclear late endosomes. Stopped-flow mixing measurements showed that the fusion rates of multicomponent cationic liposomes with anionic vesicles, used as model systems of cell membranes, were definitely shorter than those of binary liposomes. As either lipoplex uptake and endosomal escape involve fusion between lipoplex and cellular membranes, we suggest that a mechanism of lipoplex–cellular membrane interaction, driven by lipid mixing between cationic and anionic cellular lipids, does explain the TE boost of multicomponent lipoplexes.

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Introduction

Research efforts are currently focused on studying diseases of genetic origin by introducing the defective gene(s) in malfunctioning target cells.¹ As circulation of free DNA is hindered by nuclease degradation, designing effective carrier vectors that compact and protect oligonucleotides is an urgent task. Initial efforts are concentrated on using viral carriers, as these vectors exhibited high efficiency at delivering nucleic acids to numerous cell lines. Major drawbacks associated with viral vector systems, including toxicity, immunogenicity

and limitations with respect to scale-up procedures, encouraged the investigation of non-viral scaffolds.^{2–4} Among these, cationic liposome (CL)/DNA complexes (lipoplexes), because of their least immunogenic nature, robust manufacture, ability to deliver large pieces of DNA and ease in handling and preparation techniques, are finding increasing uses in non-viral gene therapy.^{5–8} While non-viral gene therapy provides easier scale-up, better pharmaceutical control and potentially better safety, a major obstacle limiting this approach is poor transfection efficiency (TE). Over the last two decades, efforts to improve lipoplex transfection have been largely empirical. However, it is now well recognized that progress in enhancing lipofection efficacy requires understanding of transfection mechanisms. Therefore, ongoing research in development of nonviral gene delivery systems is toward identification of rate-limiting steps.^{9–11} The cell presents multiple barriers to DNA/vector complexes *en route* to the nucleus, which must be overcome to deliver exogenous

Correspondence: Dr G Caracciolo, First Faculty of Medicine, Department of Chemistry, Sapienza University of Rome, Piazzale A. Moro 5, 00185 Rome, Italy.

E-mail: g.caracciolo@caspur.it

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DNA into the cell nucleus of the host cell to allow its expression.

Vectors must be internalized, move through the cytoplasm, release DNA into the nucleus where DNA transcription occurs. Very early steps in the transfection process involve binding of the vector to the cell surface and its uptake. Although endocytosis is generally considered to be the main entering pathway for lipoplexes, mechanisms other than endocytosis have been hypothesized to be responsible for the functional DNA delivery.^{12,13} An exchange mechanism must thus take place between lipoplexes and plasma membranes that would lead to destabilization of the lipoplex structure.

To avoid degradation in lysosomes, the plasmid has to escape into the cytosol before reaching this organelle. Lysosomal degradation dictates a time limit for the escape of lipoplexes from the endosomes into the cytoplasm. Thus, prompt release from the endosomal compartment presumably constitutes a critical step in determining the TE. However, little insight is available about endosomal membrane destabilization and the concomitant dissociation and release of plasmids.

To better understand the mechanisms of cellular transfection and hence the phenomena responsible for efficiency differences between transfection reagents, we investigated the mechanisms of uptake and intracellular trafficking of three lipoplex formulations. These were chosen because they exhibited the most striking difference in TE.^{14–16} 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)-carbonyl]-cholesterol (DC-Chol) and the zwitterionic helper lipid dioleoylphosphatidylethanolamine (DOPE). The third lipoplex formulation was the multicomponent (MC) system encapsulating the four lipid species simultaneously. When NIH 3T3, Chinese hamster ovary (CHO) and cancer A17 cells were treated with these three formulations, MC lipoplexes were up to $100 \times$ more effective than DOTAP-DOPC/DNA ones and from 3 to 10 times more efficient than DC-Chol-DOPE/DNA systems, depending on the cell line used.^{14–17} In previous studies,^{14–17} we investigated some recognized physical-chemical characteristics^{18–27} that might account for the superior TE of the most efficient lipoplexes, such as their lipid composition, nanostructure, size, surface potential, propensity to be disintegrated by anionic lipids and ability to release DNA. However, as no clear correlation was found, we believe that parameters other than these physical-chemical properties of lipoplexes can modulate their transfection behavior.

In this study, we investigated the internalization mechanisms of these formulations. The relative contribution of temperature-dependent (TD) and temperature-independent (TI) lipoplex uptake on TE was evaluated by performing experiments at either 37 or 4 °C. A clear correlation between the internalization mechanism of lipoplexes and their TE was found.

Materials and methods

Liposome preparation

DOTAP, DC-Chol, DOPE, DOPC and the fluorescently labeled NBD-DOPC and NBD-DOPE were purchased from Avanti Polar Lipids (Alabaster, AL) and used without further purification. DOTAP-DOPC, DC-Chol-DOPE and MC CLs made of DOTAP, DC-Chol, DOPE and DOPC (1:1:1:1 molar ratio) were prepared according to standard protocols.¹⁷ In brief, each binary mixture, at a molar fraction of neutral lipid in the bilayer $\Phi = (\text{neutral lipid}/\text{total lipid}) (\text{mol/mol}) = 0.5$, was 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 Tris-HCl buffer solution (10^{-2} M, pH 7.4) to achieve the desired final concentration (1 mg ml^{-1}). The same experimental protocol was used to prepare negatively charged liposomes mimicking membrane (MM) made of anionic lipids dioleoylphosphatidylglycerol and dioleoylphosphatidic acid and zwitterionic DOPC and DOPE (1:1:1:1 molar ratio).

Lipoplexes preparation

For transfection experiments, plasmid DNA (pGL3 which codifies for firefly luciferase; Promega, Madison, WI) was employed. For confocal fluorescence microscopy experiments, Cy3-labeled 2.7-kbp plasmid DNA (Mirus Bio Corporation, Madison, WI) was used. By mixing adequate amounts of the DNA solutions to suitable volumes of liposome dispersions, self-assembled DOTAP-DOPC, DC-Chol-DOPC-DOPE and DOTAP-DC-Chol-DOPC-DOPE (MC) lipoplexes were obtained. All samples were prepared at a fixed cationic lipid/DNA charge ratio (mol/mol), that is, $\rho = (\text{cationic lipid (by mole)}/\text{DNA base}) = 3.5$. This ratio was chosen because it corresponds to the lowest amount of cationic lipid required to maximize TE (Supplementary Figure 1). Lipofectamine (Invitrogen, Carlsbad, CA) was used as control following the manufacturer's protocol.

Transfection efficiency experiments

Cell lines were cultured in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 1% penicillin-streptomycin (Invitrogen) and 10% fetal bovine serum (Invitrogen) at 37 °C and 5% CO₂ atmosphere, splitting the cells every 2–4 days to maintain monolayer coverage. For luminescence analysis, mouse fibroblast NIH 3T3 and ovarian CHO cells were transfected with pGL3 control plasmid (Promega). In order to investigate the temperature-dependence of lipoplex uptake, NIH 3T3 and CHO cells were transfected with lipoplexes at either 37 or 4 °C. The day before transfection, cells were seeded in 24-well plates (150 000 cells per well) using medium without antibiotics. Cells were incubated until they were 75–80% confluent, which generally took 18–24 h. For TE experiments, lipoplexes were prepared in OptiMem (Invitrogen) by mixing for each well of 24-well plates 0.05 μg of plasmid with 5 μl of sonicated lipid dispersions ($\sim 1 \text{ mg ml}^{-1}$). Complexes were left for 20 min at room temperature before adding them to the cells. On the day

of transfection, the growth medium was replaced with 400 μ l of Optimum and the cells were incubated for 30 min at either 37 or 4 °C (keeping the well plates containing cells on ice), before adding 100 μ l of lipoplexes in Optimum. Cells were incubated at either 37 or 4 °C for an additional 4 h to permit transient transfection. Finally, to avoid internalization of complexes that could remain bound to the cell surface after medium replacement, the cells were extensively washed 3 \times with PBS at the corresponding temperatures (preheated at 37 °C or pre-cooled at 4 °C) before Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum at 37 °C was added. After 48 h of incubation at 37 °C for both conditions, the cells were analyzed for luciferase expression using Luciferase Assay System from Promega. Briefly, cells were washed in PBS and harvested in 200 μ l 1 \times reporter lysis buffer (Promega). Of the cell suspension, 20 μ l was diluted in 100 μ l luciferase reaction buffer (Promega) and the luminescence was measured 10 s using a Berthold AutoLumat luminometer LB-953 (Berthold, Bad Wildbad, Germany). Results were expressed as relative light units per mg of cell proteins as determined by Bio-Rad Protein Assay Dye Reagent (Bio-Rad, Hercules, CA). Each condition was performed in quadruplicate and repeated three times. Lipofectamine (Invitrogen) commercially available CLs were used as control following the manufacturer's protocol.

Confocal fluorescence microscopy experiments

Chinese hamster ovary (CHO-K1) cells were cultured and maintained in a humidified, 5% CO₂ atmosphere at 37 °C in Dulbecco's modified Eagle's medium (Gibco, Paisley, UK) supplemented with 10% fetal bovine serum and nonessential amino acids, and splitting the cells every 2–4 days to maintain monolayer coverage. For transfection experiments, lipoplexes were prepared in PBS (Invitrogen) by mixing 0.5 μ g of Cy3-labeled plasmid DNA with 10 μ l of sonicated lipid dispersions. These complexes were left for 20 min at room temperature before adding them to the cells. Confocal fluorescence microscopy experiments were performed with the Olympus Fluoview 1000 (Olympus, Melville, NY) confocal microscope.

Cytotoxicity assay

Toxicity studies, for comparison of cationic lipoplexes, were performed using the 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium bromide (Invitrogen) cell viability assay. Cells (2×10^4) per well were plated in 96-well plates and incubated with lipoplexes as previously described. After 24 h, 10 μ l of 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium bromide solution (5 mg ml⁻¹) was added to each well to achieve a final concentration of 0.5 mg 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium bromide ml⁻¹ and incubated for 2–4 h (CO₂, 37 °C), to allow the production of the dark-blue formazan crystal. Cells were lysed with lysis buffer (20% SDS in 50% dimethyl formamide) and further incubated for 5–6 h to dissolve the formazan crystals. The resulting purple solution was analyzed spectrophotometrically at 580 nm. Different controls were studied (cells alone, Lipofectamine).

The toxicity experiments were performed as two independent experiments in quadruplicate.

Stopped-flow experiments

The light scattering experiments were carried out using a stopped-flow apparatus Kintek SF2004 (Kintek Corporation, Austin, TX). The excitation wavelength was set to 436 nm and the emitted signal was recorded at 90° to the incident beam without any filter. Experiments were carried out by rapidly mixing equal volumes (20 μ l each) of reactants contained in the two syringes of the stopped-flow apparatus. The first syringe was filled with MM anionic vesicles in 10 mM Tris-HCl, pH 7.4, whereas the second syringe was filled with DOTAP-DOPC, DC-Chol-DOPC-DOPE or MC CLs in 10 mM Tris-HCl, pH 7.4. All experiments were performed at 10, 20, 30 and 37 °C. In a single experiment, 1000 data points were acquired.

Results

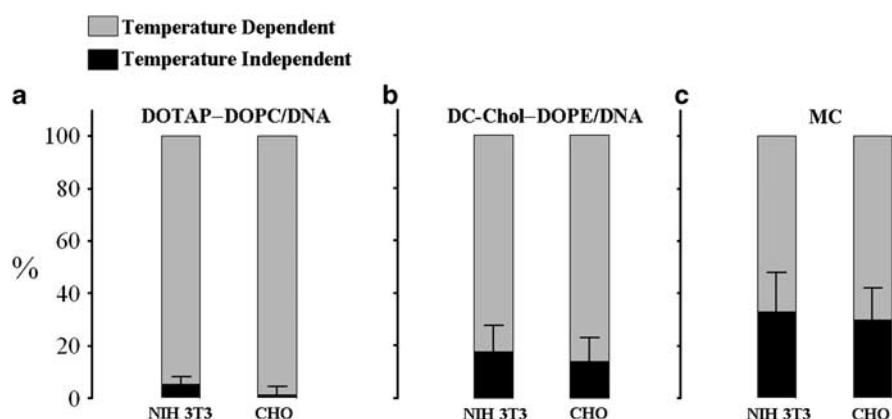
Uptake mechanism

Endocytosis, which is a vesicle-mediated process, is considered the main route used by lipoplexes. Generally, experiments aimed at clarifying the various endocytic pathways operating in eukaryotic cells are performed by using specific endocytic inhibitors, such as filipin, chlorpromazine, wortmannin, genistein and so on.^{13,28,29} However, evaluating the contribution of endocytic routes is not the purpose of our study and, as a consequence, inhibitors were not used here. On the other side, endocytosis is well known to be a TD process and thus does not take place at 4 °C, while some transport mechanisms through plasma membrane are supposed to be TI.^{22,30–34} For evaluating the contribution of the TD or TI lipoplex uptake on TE, experiments at either 37 or 4 °C were performed. When NIH 3T3 cells were transfected at 4 °C, all the lipoplex formulations were able to transfect cells (Table 1). The first obvious consequence of such an observation is that a mechanism other than endocytosis is responsible, at least in part, for lipoplex-mediated internalization. However, TE was found to decrease (Table 1) with decreasing factor TE(37 °C)/TE(4 °C) varying from ~ 20 for DOTAP-DOPC/DNA to ~ 5.6 for DC-Chol-DOPE/DNA lipoplexes and to ~ 2.4 for MC lipoplexes (Table 1). It is noteworthy to observe that similar results were obtained with CHO cells (Table 1). According to the literature, TE was modeled as the sum of two distinct contributions: a TI term, (TE)_{TI} = TE(4 °C), which is due to lipoplexes entered via a mechanism other than endocytosis and a second TD term, (TE)_{TD} = TE(37 °C) – (TE)_{TI}, which is due by lipoplexes internalized by endocytosis. Estimation of the percentage of (TE)_{TD} and (TE)_{TI} is shown in Figure 1. When DOTAP-DOPC formulation was used to deliver plasmid DNA to cells, TD mechanism was dominant accounting for the largest percentage of TE ($100 \times ((TE)_{TD}/TE) \sim 90$ to 95%), suggesting that lipoplexes internalized by endocytosis contributed the most to TE (Figure 1a). DC-Chol-DOPE/DNA lipoplexes exhibited a decrease in the involvement

Table 1 Transfection efficiency (TE) in RLU per mg of cellular proteins of DOTAP–DOPC/DNA, DC-Chol–DOPE/DNA and MC lipoplexes at 37 and 4 °C in mouse fibroblast NIH 3T3 and ovarian CHO cells

Cell line	DOTAP–DOPC/DNA		DC-Chol–DOPE/DNA		MC/DNA	
	TE (RLU per mg protein)		TE (RLU per mg protein)		TE (RLU per mg protein)	
	37 °C	4 °C	37 °C	4 °C	37 °C	4 °C
NIH 3T3	$(2.0 \pm 0.7) \times 10^7$	$(1.1 \pm 0.5) \times 10^6$	$(2.4 \pm 0.6) \times 10^8$	$(4.3 \pm 0.4) \times 10^7$	$(8.0 \pm 0.7) \times 10^8$	$(3.3 \pm 0.8) \times 10^8$
CHO	$(1.2 \pm 0.5) \times 10^8$	$(2.9 \pm 0.9) \times 10^6$	$(8.0 \pm 0.3) \times 10^8$	$(1.0 \pm 0.2) \times 10^8$	$(1.3 \pm 0.5) \times 10^9$	$(5.0 \pm 0.4) \times 10^8$

Abbreviations: CHO, Chinese hamster ovary; DC-Chol, 3β-[N-(N,N-dimethylaminoethane)-carbamoyl]-cholesterol; DOPC, dioleoylphosphocholine; DOTAP, 1,2-dioleoyl-3-trimethylammonium-propane; MC, multicomponent; RLU, relative light units.

**Figure 1** Estimation of the percentage of $(TE)_{TD}$ (black bars) and $(TE)_{TI}$ (gray bars) for DOTAP–DOPC/DNA (a) and MC (b) lipoplexes in NIH 3T3 and CHO cell lines. Error bars were calculated by error propagation.

of TD mechanism to TE ($100 \times ((TE)_{TD}/TE) \sim 80$ to 85%) (Figure 1b). On the other hand, when cells were treated with MC lipoplexes, the contribution of the TI term was much more significant, the relative percentages of $(TE)_{TD}$ and $(TE)_{TI}$ being roughly comparable (Figure 1c). In summary, these findings suggest that (i) in addition to endocytosis, lipoplexes enter cells via a TI mechanism; (ii) internalization mechanism correlates with TE: specifically, the higher the TE of lipoplexes, the higher the contribution of the TI term. TE data were used to compare efficiencies of the lipoplex formulations. To better compare the relevance of $(TE)_{TD}$ and $(TE)_{TI}$, we defined the following ratios:

- R = ratio between measured transfection efficiencies of MC and DC-Chol–DOPE/DNA lipoplexes with respect to that of DOTAP–DOPC/DNA lipoplexes.
- R_{TI} = ratio between measured $(TE)_{TI}$ of MC and DC-Chol–DOPE/DNA lipoplexes with respect to that of DOTAP–DOPC/DNA lipoplexes.
- R_{TD} = ratio between calculated $(TE)_{TD}$ of MC and DC-Chol–DOPE/DNA lipoplexes with respect to that of DOTAP–DOPC/DNA lipoplexes.

This is a very natural choice because DOTAP–DOPC/DNA lipoplexes exhibited the lowest TE as well as the lowest involvement of TI mechanism to TE. Table 2

Table 2 TE of MC and DC-Chol–DOPE/DNA lipoplexes with respect to that of DOTAP–DOPC/DNA lipoplexes

	NIH 3T3			CHO		
	R	R_{TD}	R_{TI}	R	R_{TD}	R_{TI}
MC	39.2	24.2	313.0	6.4	3.9	173.6
DC-Chol–DOPE/DNA	11.9	10.3	40.0	3.76	3.30	34.24

Abbreviations: CHO, Chinese hamster ovary; DC-Chol, 3β-[N-(N,N-dimethylaminoethane)-carbamoyl]-cholesterol; DOPC, dioleoylphosphocholine; DOTAP, 1,2-dioleoyl-3-trimethylammonium-propane; MC, multicomponent.

R is the ratio between the measured transfection efficiencies (TE); R_{TD} is the ratio between calculated $(TE)_{TD}$; R_{TI} is the ratio between measured $(TE)_{TI}$ of MC and DOTAP–DOPC/DNA lipoplexes.

shows R , R_{TI} and R_{TD} for NIH 3T3 and CHO cell lines. While R and R_{TD} of both MC and DC-Chol–DOPE/DNA lipoplexes were found to be roughly the same ($R \sim R_{TD}$), R_{TI} was about one order of magnitude larger than both of them. Interestingly, we also observe that R_{TI} of MC lipoplexes was from ~ 5 (in CHO cells) to ~ 8 (in NIH 3T3 cells) times larger than that of DC-Chol–DOPE/DNA lipoplexes.

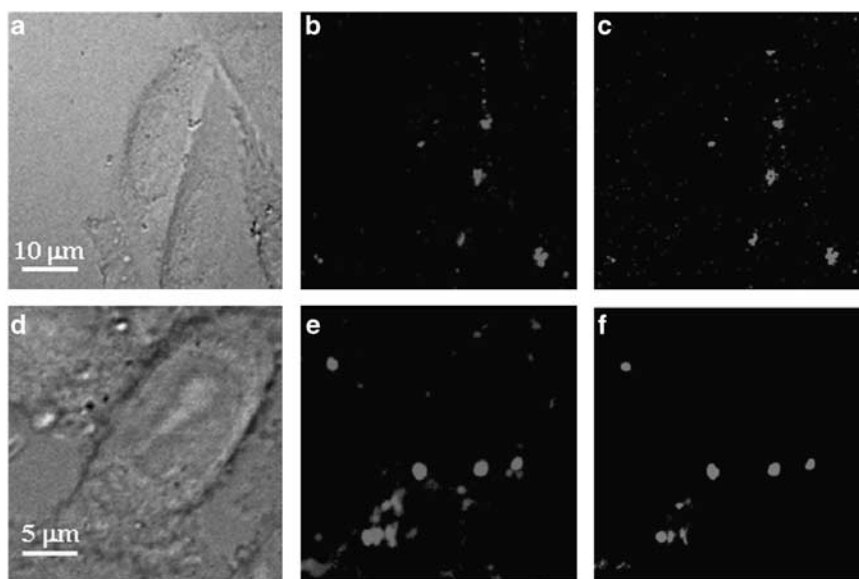


Figure 2 Confocal microscopy of CHO-K1 cells 4 h after treatment with DOTAP-DOPC/DNA (a–c) and MC (d–f) lipoplexes. Lipoplex formulations contained NBD-labeled lipids (green) and Cy3-labeled plasmid DNA (red).

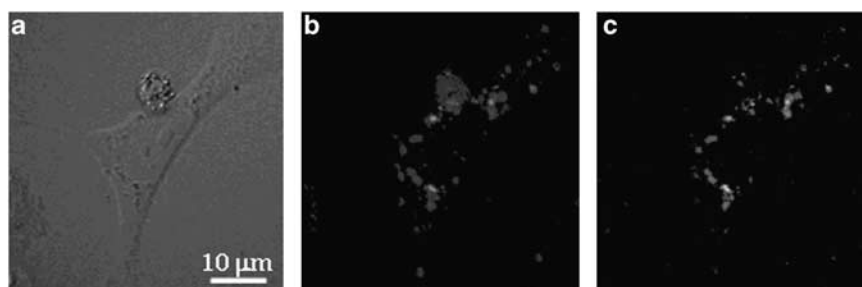


Figure 3 Confocal microscopy of CHO-K1 cells 4 h after treatment with DOTAP-DOPC/DNA lipoplexes (a–c). Lipoplex formulations contained NBD-labeled lipids (green) and Cy3-labeled plasmid DNA (red).

Intracellular trafficking

Figure 2a shows confocal images of CHO-K1 cells treated with DOTAP-DOPC/DNA lipoplexes. After 4 h incubation, transfection by two-component lipoplexes resulted in a distribution of small complexes homogeneous in size associated with the cell periphery, as demonstrated by colocalization of fluorescent plasmid DNA (red, Figure 2b) and lipid (green, Figure 2c). By contrast, the distribution of fluorescent compounds in CHO-K1 cells incubated with MC lipoplexes (Figure 2d) was completely different from the binary formulations. After 4 h, MC lipoplexes were mainly distributed throughout the cytoplasm and around the cell nucleus (Figure 2e and f). Only a minor fraction was localized at the cell periphery. This means that, over the same time scale, MC lipoplexes were more easily internalized than binary ones. DC-Chol-DOPE/DNA lipoplexes exhibited an intermediate behavior in that they were found either at the plasma membrane or in the cytoplasm (not reported). However, we also observed that DOTAP-DOPC/DNA lipoplexes–cell interaction at the plasma membrane often resulted in detection of both complexes and naked DNA outside the

cells (Figure 3). This finding suggests that lipoplex–cell interaction results in most complexes being left and disintegrated outside the cell. This observation was in agreement with the previous synchrotron SAXS and electrophoresis investigations^{14,15,35} showing that DOTAP-DOPC/DNA lipoplexes are extremely unstable against disintegration by cellular lipids and rapidly release plasmid DNA. As a consequence, a considerable amount of aggregated DNA (Figure 3b) was left on or adjacent to the cell surface when lipoplex structure was destabilized by interaction with plasma membranes. On the opposite, when MC lipoplexes were used, no appreciable extracellular DNA release was observed. The latter observation confirmed our expectation that, at the early stages of internalization, MC lipoplexes offer a more efficient DNA protection than DOTAP-DOPC/DNA ones.^{14,15,35}

After 48 h, DOTAP-DOPC/DNA lipoplexes showed a distinct perinuclear accumulation (Figure 4a–c) with all DNA encapsulated within complexes. These data indicate that binary lipoplexes were processed along the endocytic pathway,^{13,24,36,37} leading to their localization in late endosomal/lysosomal compartments. After 48 h, MC

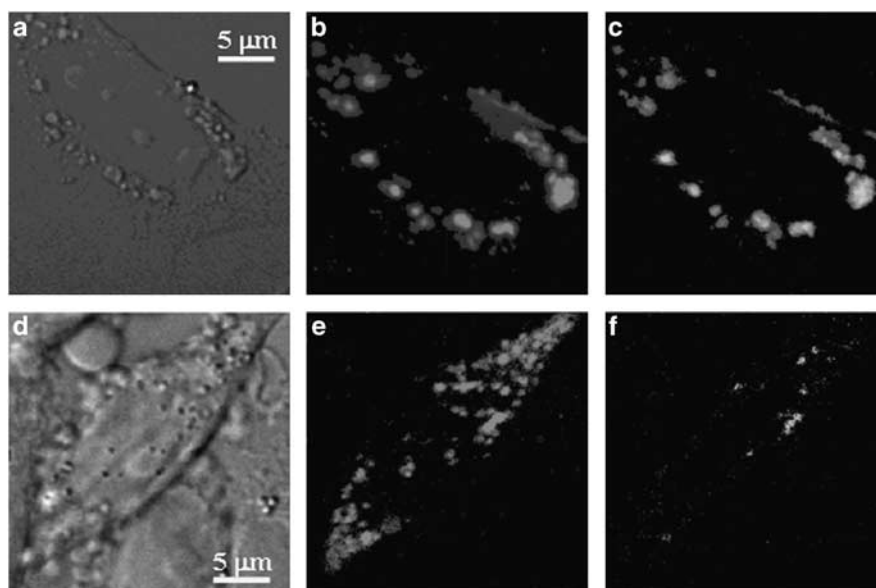


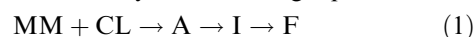
Figure 4 Confocal microscopy of CHO-K1 cells 48 h after treatment with DOTAP-DOPC/DNA (**a–c**) and MC (**d–f**) lipoplexes. Lipoplex formulations contained NBD-labeled lipids (green) and Cy3-labeled plasmid DNA (red).

complexes (Figure 4d–f) smaller than those observed with the binary formulation were found intracellularly. While fluorescence from lipids was clearly localized, DNA had visibly spread into the cytoplasm. It may be reasonable to judge that the spreading red regions in Figure 4e represent the distribution of plasmid DNA exiting from the endosomal or lysosomal stage into the cytoplasm. Minor, if any, perinuclear accumulation was observed. Our findings indicate that escape from endosomes is not a rate-limiting step when cells are treated with MC lipoplexes. DNA fluorescence was also detected in the nucleoplasm. The latter observation is noteworthy, as it is not often that plasmid fluorescence in the nuclei has been observed. To better investigate the intracellular distribution of plasmid DNA, scans at different focus depths were taken (120 images with 0.25 μm as the slice size) for a large number of cells. A representative three-dimensional reconstruction of a cell incubated for 48 h with MC lipoplexes is reported in Supplementary Figure 2. Free plasmid was abundantly found in the nucleus. As both cell internalization and endosomal escape may require fusion between lipoplex and cell membranes, we asked whether the superior ability of MC lipoplexes to fuse with cell membranes is due to their peculiar fusogenicity. To test such suggestion stopped-flow experiments were performed.

Lipid mixing

The ability of lipoplex formulations to fuse with cell membranes can be evaluated by stopped-flow mixing that is a very powerful technique to determine the relevant time constants for aggregation and fusion rates.^{38,39} As model system of cell membranes we used anionic MM liposomes made of anionic and zwitterionic lipid species common in cellular membranes. Figure 5a shows the time dependence of the scattered light intensity upon stopped-

flow mixing of MM anionic vesicles and CLs at 10 °C. This is the lowest accessible temperature in the stopped-flow apparatus close to the temperature where endocytosis does not occur (4 °C). For the sake of clarity, results are reported for DOTAP-DOPC and MC cationic vesicles. For both the temperatures, DC-Chol-DOPE CLs exhibited an intermediate behavior. The scattered intensity increases to a maximum, then it decreases up to a plateau is reached. There may be multiple steps between aggregation and the final fusion product, but, for simplicity and lack of specific information, three discrete steps can be found: aggregation, liposome destabilization and fusion. According to previous studies,³⁸ the overall process can be described by the following equation:



Where MM represents membrane-mimicking anionic liposome, CL is a CL, A is an aggregate arising from MM and CL interaction, I is an intermediate state that leads to the final fusion product, F. As the scattering intensity of a particle is proportional to the square of its molecular mass (and is independent of the shape of the molecule), the association of two particles to a single larger particle results in an increase of the intensity of light scattering. According to this model, the initial increase in scattered intensity was fitted by a single exponential

$$y = 1 - a_1 \exp(-k_1 t), \quad (2)$$

where k_1 is the time constant of initial aggregation. Estimates of the aggregation rate, $\tau_1 = 1/k_1$, could be obtained from the time constant of the exponential law fitting the data and are listed in Table 3. As evident, in this time regime, the kinetics of formation of MM-CL aggregates does not depend significantly on the CL formulation with the aggregation times being roughly

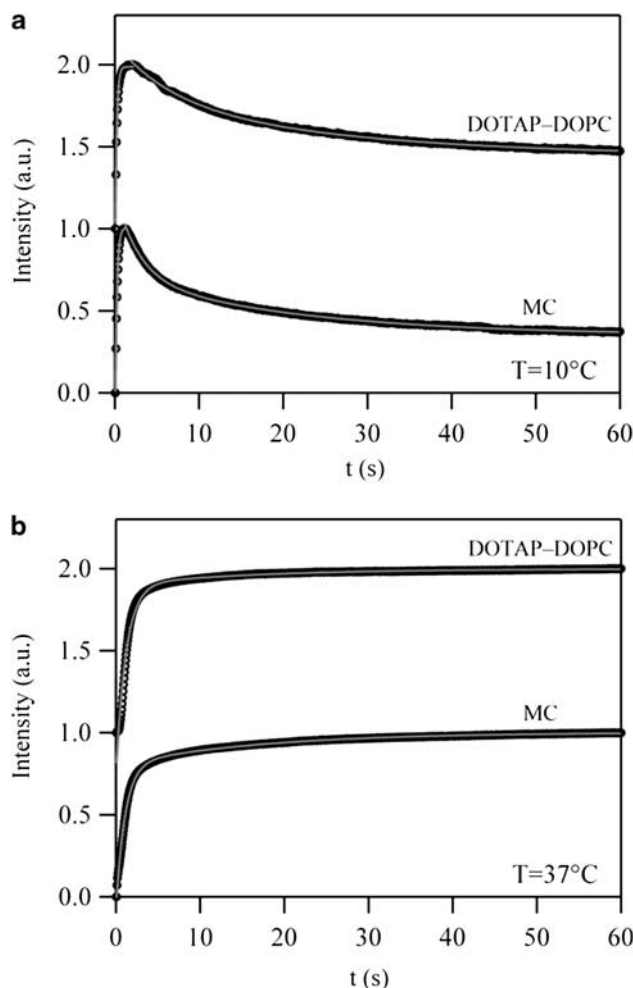


Figure 5 Changes in light scattering as a function of time after MM anionic and cationic liposomes were mixed at 10 °C (a) and 37 °C (b). Red solid lines are the best fits to the data obtained by using Equations 2 and 3 (10 °C) and Equation 5 (37 °C).

the same ($\tau_1 \sim 200$ ms). Initial changes in light scattering subsequent to mixing MM vesicles and CLs is proportional to the square of the initial liposome concentration and is driven by electrostatic attraction. Thus, at a fix liposome concentration, the membrane charge density is the major determinant of this rate. These findings are therefore in agreement with the results of previous zeta-potential measurements showing that DOTAP-DOPC and MC cationic vesicles have very close membrane charge density.

A second kinetic phase, however, manifests a reduction in intensity (Figure 5a) related to a fast and a slow process identified as rearrangement of the complex^{38,39} to a more compact intermediated structure (I) that finally results in the final fusion product, F. Kinetic traces were best fitted by a double exponential

$$y = 1 - a_2 \exp(-k_2 t) - a_3 \exp(-k_3 t) \quad (3)$$

The rates of bi-exponential fits derived from the assembly kinetics of MM and CLs are summarized in Table 3. As

Table 3 Time constants for aggregation, prefusion and final fusion events

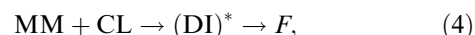
	Temperature	τ_1 (s)	τ_2 (s)	τ_3 (s)
DOTAP-DOPC	10 °C	0.19 ± 0.02	6.1 ± 0.1	24.4 ± 0.3
MC		0.21 ± 0.02	2.7 ± 0.1	21.3 ± 0.1
DOTAP-DOPC	37 °C	ND	1.2 ± 0.1	17.0 ± 0.1
MC		ND	1.1 ± 0.1	16.2 ± 0.1

Abbreviations: DOPC, dioleoylphosphocholine; DOTAP, 1,2-dioleoyl-3-trimethylammonium-propane; MC, multicomponent; ND, not determined.

Constants were derived from Equations 2 and 3 (10 °C) and Equation 5 (37 °C).

evident, the time constants ($\tau_2 = 1/k_2$ and $\tau_3 = 1/k_3$) derived for MC CLs are much lower than those associated with DOTAP-DOPC CLs. This finding indicates that, at 10 °C, MM anionic liposomes fuse with MC CLs much faster than they do with DOTAP-DOPC vesicles.

The rates of aggregation, destabilization and final fusion are extremely susceptible to exogenous factors such as temperature. Figure 5b shows kinetic traces collected at 37 °C. While both a rapid and a slow intervesicle lipid mixing were observed, there was no lag phase separating them, so three distinct processes could not be detected. This means that the fast component (with time constant k_1) was more temperature dependent than the other two components (with time constants k_2 and k_3 respectively). As a result, the rate of formation of dimer aggregates may be too fast to be distinguished from the second step. In this case, a more simple model can be used³⁹:



where (DI)* indicates the combination of the undistinguishable aggregation and destabilization steps. The kinetic traces were therefore fitted by the following double exponential model, where

$$y = 1 - a_2 \exp(-k_2 t) - a_3 \exp(-k_3 t) \quad (5)$$

the relevant time constants for aggregation-destabilization (k_2) and final fusion (k_3) are listed in Table 3. As evident, two major conclusions can be drawn: (i) first, for a given liposome formulation, the overall rate of fusion is faster with increasing temperature; (ii) second and most importantly, at each temperature, MC cationic membranes fuse with anionic MM liposomes faster than DOTAP-DOPC membranes do.

Cytotoxicity

Data on the cytotoxicity of lipoplexes used in this study are reported in Supplementary Material. As shown in Supplementary Figure 3, MC and DC-Chol-DOPE/DNA lipoplexes have the highest percentage of viable cells compared with untreated control, which corresponds to the lowest toxicity, followed by DOTAP-DOPC/DNA lipoplexes and Lipofectamine (commercial kit used as a control). No significant cytotoxicity was observed in cells treated with the employed lipoplex formulations.

Discussion

Designer MC lipoplexes have recently emerged as especially promising transfection candidates, as they are up to 100 times more efficient than binary complexes, usually employed for gene delivery purposes.^{14,15} In previous studies, we investigated a number of properties of DNA–lipid complexes that might explain the superior TE of MC lipoplexes with respect to binary ones, such as structure on the nanoscale, size, surface potential and DNA-protection ability. Although some differences were found, they did not correlate clearly with efficiency. Thus, factors other than the investigated physical–chemical parameters of lipoplexes could have a central role in transfection. At present, little is known about the mechanisms of cellular transfection, while such knowledge is urgently needed for improving gene delivery.³⁶ First, we investigated the importance of the uptake mechanism on TE. To distinguish between TD and TI mechanisms, we performed TE experiments at 4 °C where endocytosis is strongly reduced. We observed that all lipoplex formulations are able to transfect NIH 3T3 and CHO cell lines at 4 °C (Table 1). These results imply that a mechanism other than endocytosis is responsible for lipoplex internalization. We determined that the percentage of TD, TE, (TE)_{TD}, was largely dominant (more than 95%) when cells were treated with DOTAP–DOPC/DNA lipoplexes. On the contrary, Figure 1 shows that TD and TI TE of MC lipoplexes were similar. Data analysis reported in Table 2 made it clear that the superior TE of MC lipoplexes was largely due to the TI contribution, (TE)_{TI}. Taken together, these data indicate a possible relation between uptake mechanism and TE of lipoplexes. Poorly efficient DOTAP–DOPC/DNA lipoplexes almost exclusively use a TD transport (endocytosis-like), whereas highly efficient MC lipoplexes use either a TD or a TI uptake mechanism. Our finding that a second internalization route has a relevant role in efficient lipofection is in very good agreement with the conclusions of the recent study by Lu *et al.*,⁴⁰ who found that a not-endocytic pathway is responsible for the cellular uptake of short interfering RNA/cationic lipid complexes. Even more remarkably, we underline that a clear correlation between TE and the mechanism of internalization was found: the higher the TI contribution the higher the TE.

Several lipoplex internalization mechanisms that may contribute to TE have been postulated to exist in mammalian cells. Among them, direct plasma membrane fusion was the first mechanism proposed. The former suggestion was essentially based on the ability of CLs to mix with negatively charged lipid membranes^{41,42} and with erythrocyte membranes.⁴³ Such mechanism should resemble the nucleic acid delivery performed by viruses, which penetrate through the plasma membrane by spontaneous merger of membranes promoted by fusion proteins. Likewise, insertion of a fusion protein inside liposomes was shown to promote membrane fusion facilitating intracellular penetration of liposome content.³² In lipid fusion, contacting lipid bilayers undergo phase change, which results in the mixing of bilayers with

each other. Therefore, intermembrane lipid mixing is usually taken as an indication of membrane fusion. As lipid mixing is known to occur also at very low temperature,^{44–46} we hypothesized that lipid exchange between lipoplex and cellular membranes largely controls the uptake mechanism at 4 °C and might also contribute to the intracellular transport of plasmid DNA at higher temperature. To test this suggestion, stopped-flow measurements were performed. Stopped-flow is a powerful technique that permits accurate characterization of interaction between liposomes. We have applied stopped-flow mixing in an attempt to semi-quantitatively compare liposome aggregation and lipid-mixing driven fusion rates, without application of a complex kinetic analysis of the data. Our conclusions are that the aggregation rates correlate with lipid composition of lipoplex bilayer. Specifically, results reported in Table 3 showed that interaction between anionic MM and cationic MC membranes is much faster than that between MM and DOTAP–DOPC ones. These data support our conclusion that the major relevance of the TI contribution to TE observed in MC lipoplexes can be due to their high fusogenicity.

Once in the cell, plasmid DNA must be able to escape endosomal trafficking. If DNA is not released from endosomes, it is shuttled to the lysosomes, where it is degraded by the abundant nucleases and transfection may fail.^{13,36,37} Improving endosomal escape remains a significant challenge in lipoplex design. Confocal imaging experiments (Figure 4) have demonstrated that the superior transfection efficacy of MC lipoplexes does correlate with their efficient endosomal escape. Numerous research groups are currently trying to overcome this rate-limiting step to transfection through incorporation of some lysosomotropic agents, such as chloroquine and/or fusogenic peptides, which are practically inactive at neutral pH, but become membrane lytic at pH ~ 5 (typical of endosomes).⁴⁸ It is therefore remarkable that highly efficient MC lipoplexes exhibited a distinctive ability of escape from endosomes with no lytic or fusogenic agent added. We also observed that DNA fluorescence was detectable in the nucleoplasm of the cells treated with MC lipoplexes (Supplementary Figure 2). These results are fully in line with the notion that disruption of the endosomal membrane barrier allows plasmid DNA to diffuse readily and to enter the nucleus. On the contrary, poorly efficient binary lipoplexes, with virtually identical physical–chemical properties than MC ones^{14–17} exhibited minor, if any, endosomal rupture ability and remained confined in perinuclear late endosomes (Figure 4). This finding confirms that inability to escape from endosomal compartments is a primary cause of low TE.

Endosomal escape is a well-established but still poorly understood concept. However, there is general consensus that destabilization of endocytic vesicles should occur via direct fusion between the lipoplex and the endosomal membrane.⁴⁷ Membrane fusion proceeds by means of defined transition states, including intermediates in which the proximal leaflets of the fusing membranes are merged, whereas the distal leaflets are separate (fusion stalk), followed by the opening of small aqueous ‘fusion pores’.⁵⁰ In the case of lipofection, the cationic amphiphiles may

indeed transfer from binding to nucleic acid to associating with the anionic lipids of the inner monolayer of endosomes. Upon lipoplex–endosomes membrane fusion, anionic lipids laterally diffuse in the lipoplex membrane surface neutralizing the cationic lipids in a flip-flop process and consequently release DNA through pores formed in the mixed membranes.⁴⁹ The described fusion-dependent mechanism of DNA release requires a robust lipid mixing to be efficient. Recent publication on the correlation between lipoplex properties and uptake mechanisms points out that lipid composition controls the rate of intermembrane fusion and has an important role in determining fusogenicity.³⁴ In principle, the superior capacity of MC lipoplexes to fuse with anionic cellular membranes may be due to the presence of DOPE, a cone-like lipid that is well known for its fusogenicity. However, MC lipoplexes contain a percentage of DOPE in their bilayer (25%) that is twofold lower than that of DC-Chol–DOPE/DNA lipoplexes (50%), while the latter complexes exhibited a much lower TI contribution to TE (Table 2). This observation suggests that factors other than lipid composition (that is, DOPE content) has a role in determining the fusogenicity of lipoplexes.

In a recent publication,⁵¹ some of us showed that decrease in free energy due to lipid mixing is the driving force responsible for the fusion-induced formation of mixed membranes. Such a decrease in free energy is strongly influenced by the local density of each lipid species and is maximum when an ideal lipid mixing occurs. Furthermore, it can be demonstrated that, given the lipid composition of endosomal membranes, the higher the number of lipid components the vector is composed of, the higher the decrease in free energy. As a result, large lipid mixing promoted by highly fusogenic MC lipoplexes^{14,15,52,53} is expected to result in facilitated formation of the prefusion state, which finally leads to the formation of membrane pores, and to the observed DNA release (Figure 4).

In summary, we have provided the following body of evidence: (i) lipoplexes enter cells not only via endocytosis-like internalization but also by a TI mechanism, (ii) poorly efficient binary lipoplexes predominantly use TD transport, whereas highly efficient MC lipoplexes use either TD and TI uptake mechanism, (iii) MC lipoplexes exhibited a distinctive ability of escape from endosome, whereas poorly efficient binary lipoplexes, with virtually identical physical–chemical properties than MC ones, do not. As both TI lipoplex uptake and endosomal escape are likely to involve fusion between lipoplex and cellular membranes,^{12,13,36} here we propose that a novel mechanism of lipoplex–cellular membrane interaction, most likely controlled by lipid mixing, can account for the TE boost of MC. We therefore believe that the possibility of a fusion mechanism promoted by robust lipid mixing should be considered when developing lipoplexes designed for *in vivo* or *ex vivo* nucleic acid transfer.

Conflict of interest

The authors declare no conflict of interest.

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