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Efficient Escape from Endosomes Determines the Superior Efficiency of Multicomponent Lipoplexes

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Designer multicomponent lipoplexes have recently emerged as especially promising transfection candidates, since they are from 10 to 100 times more efficient than binary complexes usually employed for gene delivery purposes. Here, we show, for the first time, that after internalization binary complexes of lower transfection potency remain in compact perinuclear endosomes, while multicomponent systems have intrinsic endosomal rupture properties that allow plasmid DNA to escape from endosomes with extremely high efficiency. Endosomal rupture results in an extraordinarily homogeneous distribution of unbound plasmid DNA throughout the cytoplasm and in the nucleus.

Synthetic cationic liposomes, which form complexes (lipoplexes) with polyanionic DNA, are presently the most widely used nonviral gene carriers.[1] Among the formulations reported up to date, designer multicomponent (MC) lipoplexes, incorporating from three to six lipid species simultaneously, have emerged as especially promising transfection candidates, since they are from 10 to 100 times more efficient than binary complexes usually employed for gene delivery and gene therapy purposes.[2−4] A persuasive example of the superiority of MC lipoplexes with respect to binary ones has been recently provided by the four-component system incorporating the cationic lipids 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) and (3β-[*N*-(*N*′,*N*′-dimethylaminoethane)-carbamoyl])-cholesterol (DC-Chol) and the neutral helper lipids dioleoylphosphatidylethanolamine (DOPE) and dioleoylphosphocholine (DOPC) (1:1:1:1 molar ratio). Such a MC system was found to be 100× more effective as a DNA transfection agent (in fibroblasts NIH 3T3, ovarian CHO, and cancer A17 cells[2−4]) than an equimolar DOTAP−DOPC binary formulation despite their similar physical properties and virtually identical lipoplex organization.[5] Here, we show, for the first time, that the superior efficiency of MC lipoplexes does correlate with their distinctive capability to escape from endosomes and release DNA both into the cytoplasm and in the nucleus. Our results may be potentially important for the development of new strategies toward the rational design of lipoplexes by enhancing their endosomal destabilization ability.

DOTAP, DC-Chol, DOPC, DOPE, and the fluorescently labeled NBD−DOPC and NBD−DOPE were purchased from Avanti Polar Lipids (Alabaster, AL) and used without further purification. The Cy3-labeled 2.7 kbp plasmid DNA was purchased from Mirus Bio Corporation. Liposome dispersions were routinely prepared (final lipid concentration 1 mg/mL). Chinese hamster ovary (CHO-K1) cells were cultured and maintained in a humidified, 5% CO_2 atmosphere at 37 °C in Dulbecco's modified Eagle's medium (Gibco, Paisley, U.K.) 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 μg of plasmid with 10 μL of sonicated lipid dispersions. Such volumes were dictated by liposome concentration to obtain positively charged lipoplexes (cationic lipid/DNA charge ratio, ρ, ∼4).[5] 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.

Confocal images of CHO-K1 cells 4 h after incubation with DOTAP−DOPC/DNA lipoplexes are shown in Figure 1 (panels A and B). Transfection by two-component lipoplexes resulted in a distribution of small complexes homogeneous in size largely associated with the cell periphery. This observation correlates with the results of previous dynamic light scattering measurements,[5] showing that DOTAP−DOPC/DNA lipoplexes are small size complexes (average size ∼200 nm). Lipoplex−cell interaction at the plasma membrane often resulted in direct detection of naked DNA outside the cells (not reported). This finding indicates that destabilization of DOTAP−DOPC/DNA lipoplexes at the cell surface occurred, thus leading to extracellular release of DNA. Interestingly, this observation was in good agreement with the results of previous synchrotron small-angle X-ray scattering (SAXS) and electrophoresis investigations,[4,5] showing that DOTAP−DOPC/DNA lipoplexes are extremely unstable against disintegration by cellular lipids and rapidly release DNA. Following internalization, the plasmid has to

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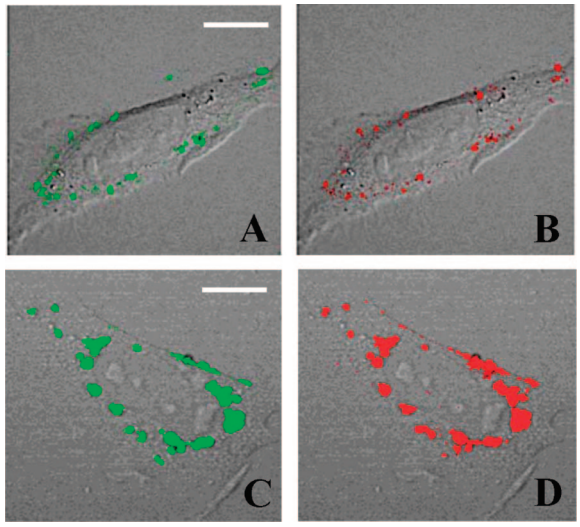

Figure 1. Confocal microscopy of CHO-K1 cells 4 h (panels A and B) and 48 h after treatment with DOTAP−DOPC/DNA lipoplexes that contained NBD-labeled lipids (green) and Cy3-labeled plasmid DNA (red). Within 4 h, DOTAP−DOPC/DNA lipoplexes resulted in a distribution of complexes largely associated with the plasma membrane, while at 48 h a specific perinuclear localization was observed (panels C and D). Scale bars are 10 μm.

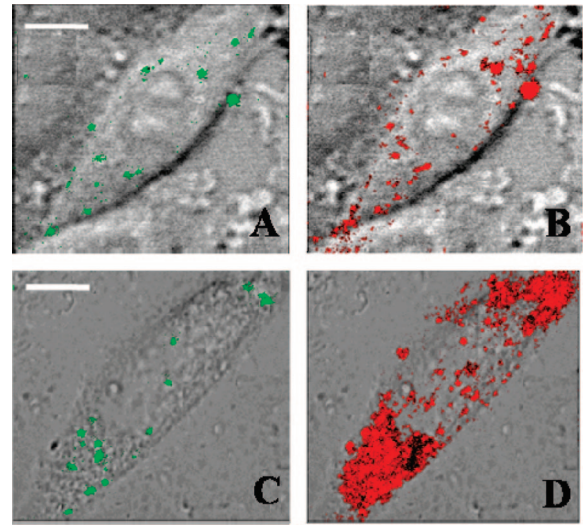

Figure 2. Confocal microscopy of CHO-K1 cells 4 h (panels A and B) and 48 h (panels C and D) after treatment with DOTAP−DC-Chol−DOPC−DOPE/DNA. Within 4 h, lipoplexes were distributed throughout the cytoplasm and at the cell periphery, while at 48 complexes largely released DNA into both cytoplasm and nucleus. Intracellular localization of free plasmid DNA can be better visualized by the three-dimensional reconstruction reported in the Supporting Information. Scale bars are 10 μm.

escape into the cytoplasm to avoid degradation at the lysosomal level.[6−8] Thus, prompt release from an endosomal compartment is supposed to constitute one of the critical steps in determining the efficiency of transfection.[7] After 48 h, DOTAP−DOPC/DNA lipoplexes showed a distinct perinuclear accumulation (Figure 1, panels C and D). These data indicate that the binary lipoplexes were processed along the endocytic pathway,[7−10] leading to their localization in late endosomal/lysosomal compartments. In addition, CHO-K1 cells incubated with DOTAP−DOPC/DNA lipoplexes appeared almost devoid of cytoplasmic plasmid DNA, suggesting that such binary formulation is defective in facilitating endosomal escape of nucleic acids, resulting in entrapment of plasmid DNA in endosomes.[10]

By contrast, the distribution of fluorescent compounds in CHO-K1 cells incubated with DOTAP−DC-Chol−DOPC−DOPE/DNA complexes (Figure 2) was completely different from that observed with binary ones. After 4 h, DOTAP−DC-Chol−DOPC−DOPE/DNA lipoplexes were mainly distributed throughout the cytoplasm and to some extent at the cell periphery (Figure 2, panels A and B).

This means that, over the same time scale, MC lipoplexes are more easily internalized than binary ones. In addition, no extracellular DNA release was observed. This observation confirmed our expectation that, at the early stages of internalization, MC lipoplexes are more stable[4,5] and offer a more efficient DNA protection than DOTAP−DOPC/DNA ones. Bearing in mind the remarkable difference in transfection activity between DOTAP−DOPC/DNA and DOTAP−DC-Chol−DOPC−DOPE/DNA lipoplexes,[2−4] these observations reinforce that fast complex dissociation is a critical attribute of poor transfection[5] and that efficient protection of DNA by cationic lipids resulting in successful intracellular delivery of DNA is of crucial importance for efficient transfection.[6−8]

After 48 h, complexes (Figure 2, panels C and D) 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 (Figure 2, panel D; a three-dimensional reconstruction is reported in the Supporting Information). It may be reasonable to judge that the spreading red regions of Figure 2 (panel D) represent the distribution of plasmid DNA exiting from the endosomal or lysosomal stage into the cytoplasm.[10,11] General observations in our experiments with MC lipoplexes were the following: (i) no significant indication of transfer of fluorescent lipids to the plasma membrane; (ii) a much higher red fluorescence intensity in the cytoplasm; (iii) minor, if any, perinuclear accumulation. All of these findings likely indicate that MC lipoplexes must have entered the cells through endocytosis[12] and that a large fusion between lipoplexes and endosomal membranes resulting in plasmid DNA release occurred.[12] To our knowledge, such an extraordinarily homogeneous distribution of unbound plasmid DNA throughout the cytoplasm has not been reported so far. According to the literature,[7,10−13] the most reliable explanation for such an observation is that MC lipoplexes were slightly processed along the endocytic pathway and were mostly able to avoid lysosomal degradation.

Confocal images therefore provided mechanistic insights into the origin of the observed enhanced transfection properties of multicomponent mixtures of lipids which are otherwise either essentially transfection incompetent or poorly transfecting when used in binary combinations. Here, we claim that the superior activity of MC lipoplexes does strictly correlate with their distinctive capability to escape from endosomes and release DNA.

We also observe that DNA fluorescence was also detectable in the nucleoplasm (Supporting Information). Incidentally, the latter observation is noteworthy, since it is not often that plasmid fluorescence in the nuclei has been observed.[11] These results are fully in line with the notion that disruption of the endosomal membrane barrier allows plasmid DNA to diffuse readily to enter the nucleus.[7−13]

Despite the fact that the mechanisms involved in dissociation of DNA from lipoplexes are still poorly understood, some generalities have been proposed: (i) the unbinding of DNA from lipoplexes involves neutralization of cationic lipid by cellular anionic lipids;[13] (ii) phase evolution of lipoplex lipids upon interaction with cellular lipids appears to be decisive for

transfection success;[14,15] (iii) fusion of lipoplexes with endosomal membranes should facilitate DNA release from endosomes into the cytoplasm, and thus promote DNA expression.[11] The neutralization of cationic lipid carriers by anionic cellular lipids, required for DNA release, involves some local contact and the adhesion−condensation mechanism[16] predicts that such interaction critically depends on the membrane charge density. Since the MC and binary lipoplexes employed in the present study exhibit virtually identical membrane charge densities as well as cationic lipid/DNA charge ratios,[4,5] such physical parameters cannot be taken into account to provide an explanation for the impressively different cytosolic plasmid delivery. Second, after mixing with anionic membrane-mimicking vesicles, used to simulate lipoplex−endosomal membrane interaction, MC and binary lipoplexes exhibited a practically identical phase behavior.[4,5,17] As a result, neither cationic/anionic charge neutralization nor the structural changes of lipoplexes upon interaction with anionic cellular lipids could be used to explain the superior DNA release efficiency of MC lipoplexes. On the other hand, since mixing of lipoplex with cellular lipids has been interpreted as fusion, the ability of MC lipoplexes to perturb the endosomal bilayer facilitating the plasmid release may be connected to their higher surface activity and fusogenicity.[14] This phenomenon is perhaps related to nonideal mixing, domain formation, and/or higher probability for packing defects in the multicomponent systems relative to single-component bilayers or binary mixtures.[5,15]

Aside from clarifying the mechanism of endosomal escape, we have provided relevant insights toward clarifying the superior activity of MC lipoplexes. While binary complexes of lower transfection potency remain in compact perinuclear endosomes, MC systems have intrinsic endosomal rupture properties that allow plasmid DNA to escape from endosomes with extremely high efficiency. In an effort to increase the efficiency of the endosomal escape step, our results may be important for the rational design of novel lipoplex formulations and transfection kits for use in nonviral gene therapy.

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Supporting Information Available: Three-dimensional (3D) reconstruction of the plasmid DNA distribution visible in Figure 2 (panel D). This 3D rendering ensures that free plasmid DNA was localized intracellularly both in the cytoplasm and in the nucleus. This material is available free of charge via the Internet at http://pubs.acs.org.

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