Quantitative Evaluation of DNA Dissociation from Liposome Carriers and DNA Escape from Endosomes During Lipid-Mediated Gene Delivery

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Abstract

Nonviral vectors are highly attractive for gene therapy from a clinical point of view, and cationic lipid nanoparticles in particular have generated considerable interest. However, despite considerable recent advances, problems associated with low transfection efficiencies remain to be resolved to fully meet the potential of these vectors. The trafficking of plasmid DNA (pDNA) from the extracellular space up to the nucleus is prevented by several barriers, including liposome/pDNA dissociation within the endosome and pDNA escape into the cytosol. The aim of this work was to develop and optimize a tool that could offer simultaneous quantitative information both on the intracellular dissociation of oligonucleotides from lipid nanoparticles, and on the DNA escape from endocytic compartments. The ability to follow in real time both of these processes simultaneously (in a quantitative manner) is expected to be of high value in the rationalization and conception of new lipid nanoparticle vectors for gene delivery for therapeutic purposes. To this effect, a combination of Förster resonance energy transfer (FRET) and colocalization microscopy was employed. We show that it is possible to distinguish between liposome/pDNA dissociation and depletion of DNA within endosomes, providing resolution for the detection of intermediate species between endocytic particles with intact lipoplexes and endosomes devoid of DNA because of DNA escape or degradation. We demonstrate that after endocytosis, exceptionally few endocytic particles are found to exhibit simultaneously DNA/lipid colocalization and low FRET (DNA/lipid dissociation). These results clearly point to an extremely short-lived state for free plasmid within endosomes, which either escapes at once to the cytosol or is degraded within the endocytic compartment (because of exposure of DNA). It is possible that this limitation greatly contributes to reduction in probability of successful gene delivery through cationic lipid particles.

Introduction

PLASMID DNA (pDNA) COMPARED WITH VIRAL VECTORS has several advantages as a gene delivery system in preventive and therapeutic vaccination, since it is easy to manipulate and can be produced and purified in large amounts. The ideal vaccine should incorporate features such as safety (meeting concerns about integration, tolerance, and autoimmunity) and appropriated humoral and cellular elicitation (long-lived protection), and should be easy to administer, inexpensive to manufacture, and stable in storing procedures (the supercoiled pDNA isoform used in vaccines is temperature stable, eliminating the need for expensive cold chains) (Donnelly *et al.*, 2003; Srivastava and Liu, 2003).

Although significant improvements have been made in the delivery of pDNA to cells, there are still several barriers to gene expression during the delivery process. (1) Enzymatic degradation of the pDNA by extracellular and cytosolic nucleases: First, the plasmid has to be sufficiently protected to survive the nucleases present in the extracellular medium and reach intact to the cell neighborhood (Escoffre *et al.*, 2010). (2) Low efficiency of plasmid uptake: To reach intact to the nucleus, the pDNA must be endocytosed, and overcome the different diffusional barriers and the degradation by nucleases (present in lysosomes and cytosol) (Lechardeur *et al.*, 1999; Pollard *et al.*, 2001). (3) Degradation in the endosome or inability to escape from it: Following internalization, the clathrin-coated vesicles

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discard their coats and fuse with the early endosomes that have acidic pH (6.0–6.2) that mature into late endosomes (pH 5.5–6.0), which will eventually fuse to lysosomes (pH $\approx$ 5.0). The pDNA should be able to escape to the cytoplasm to reach the nucleus before the fusion to lysosome, where occurs the degradation of pDNA by the action of acid hydrolases (Cooper, 2013). (4) Low efficiency in the transcription/translation process because of problems in the nuclear uptake and mRNA export (Lechardeur *et al.*, 1999; Pollard *et al.*, 2001).

The nuclear envelope is the subsequent barrier that the DNA has to overcome, and the entry is thought to occur through the nuclear pores (around 10 nm in diameter), by the nuclear pore complex, which allows an active, energy-dependent transport, or a passive transport of macromolecules in a similar way to protein import and export (Lechardeur and Lukacs, 2006) or during cell division, when the disassembly of the nuclear envelope occurs (Miller and Dean, 2009). For transcription to be effective, the pDNA must dissociate from the condensed complexes before entering the nucleus. In the end, the elements of the gene expression system in the plasmid will influence the transfection efficiency (Luo and Saltzman, 2000).

Before reaching the nucleus, the supercoiled DNA can be converted to the linear and open circular isoforms, which are not protected against exo- and endonucleases (Houk *et al.*, 2001; Schleef and Schmidt, 2004). In order to improve DNA delivery and transfection efficiency (by increasing the number of DNA molecules that reach intact to the nucleus and are correctly expressed), these barriers should be overcome too.

There are several ways to deliver DNA to cells, one of the most widely used DNA delivery systems consists of a plasmid with an expression cassette complexed with a liposome (lipoplex) (Lechardeur and Lukacs, 2002). Liposomes are nonimmunogenic because of their lack of protein components and can be tailored to yield the desired size, surface charge, and morphology (Schwendener *et al.*, 2010). The effectiveness of liposomes for transfection depends on the number of lipid layers, electric charge, composition, and preparation method (Petrovsky and Aguilar, 2004). For transfection, cationic liposomes are generally employed. These are composed of a mixture of cationic (e.g., DOTAP) and a zwitterionic fusogenic lipid (e.g., DOPE) (Ho *et al.*, 2010).

When the pDNA is condensed/complexed with cationic liposomes, it is converted into an orderly collapsed state (Bloomfield, 1997), allowing the reduction of its size/volume and improving stability (Knight and Adami, 2003). The positively charged molecular structure of the cationic lipid binds the negatively charged DNA, and the hydrophobic moiety of the liposome participates in the formation of the condensed lipoplexes (Wetzer *et al.*, 2001). It is the compacted state of the DNA within the lipoplex that is responsible for the high efficacy of the liposomes in protecting the pDNA from the attack of the nucleases and, by reducing its size, allows it to pass through small openings (Bloomfield, 1997). In addition, the complex must be dismantled in order for the plasmid to become transcriptionally active (Houk *et al.*, 2001). The zwitterionic fusogenic lipid component helps in the endosomal escape and reduces the cytotoxicity of cationic lipids (Yoshioka *et al.*, 2009).

Although the mechanisms involved in dissociation of DNA from lipoplexes are still poorly understood, it is well

known that, once in the endocytic pathway, the plasmid may become degraded when reaching the lysosomes. Taking this into consideration, it is crucial that the plasmid acquires cytosolic access at an earlier stage, meaning an escape from (early) endosomal compartments. This is likely to be initiated by interaction of lipoplex lipid components with the endosomal membrane, possibly culminating in membrane fusion and destabilization of endosomal membrane (Caracciolo *et al.*, 2009).

The exchange of lipids between the lipoplex and the endosomal membrane is driven by electrostatic interaction between the cationic lipids of the lipoplex and the anionic lipids found on the endosome. The diffusion of anionic lipids into the lipoplex leads to charge neutralization and consequently to the release of pDNA from the lipoplex. On the other hand, fusion with the lipoplex membrane is likely to destabilize endosomes, providing an escape route for pDNA. Importantly, the incorporation of a fusogenic lipid as DOPE in lipoplexes greatly enhances gene expression as well (Lam, 2012).

Förster resonance energy transfer (FRET) has been exploited as a tool to monitor dissociation of DNA from the cationic liposome, because of fusion with anionic liposomes (Zelphati and Szoka, 1996). Through the use two labeled lipids within the cationic liposome acting as FRET donor and acceptor, FRET has also been used to monitor the fusion of liposomes with endocytic membranes, which leads to FRET cancelation (Wang and MacDonald, 2004; El-Sayed *et al.*, 2008, 2009; Akita *et al.*, 2009). However, these studies probe only the fusion of lipoplex and endosome membrane, failing to distinguish between the two crucial events required for cytosol access of DNA (DNA/lipid dissociation and DNA escape from the endosome compartment).

The simultaneous quantitative monitoring of pDNA dissociation from lipid nanoparticles and endosomal escape is challenging. In this context, the aim of this work was to present a methodology able to offer simultaneous quantitative information on both the intracellular dissociation of oligonucleotides from lipid nanoparticles, and on the DNA escape from endocytic compartments. To do so, we employ a combination of colocalization and FRET microscopy. The sensitivities of the two methods for different spatial scales (1–10 nm for FRET and 250–400 nm for confocal microscopy) allow us to detect simultaneously the dissociation of DNA from liposomes within endocytic particles through FRET and DNA shedding from these organelles through colocalization studies. We show that it is possible to distinguish between liposome/pDNA dissociation and depletion of DNA within endosomes, providing resolution for the detection of intermediate species between endocytic particles with intact lipoplexes and endosomes devoid of DNA because of DNA escape or degradation.

Materials and Methods

Preparation of labeled large unilamellar vesicles

The large unilamellar vesicles (LUVs) used in this study were prepared using a mixture of the cationic lipid 1,2-dioleoyl-3-trimethyl-ammonium-propane (DOTAP), and the zwitterionic phospholipid 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE). 1,2-Dioleoyl-*sn*-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl)

(DOPE-Rho) was included as a liposome marker. Lipid suspensions were prepared in chloroform from the stock solutions in a 75:25:1 (DOTAP:DOPE:DOPE-Rho) ratio. All the lipids were obtained from Avanti Polar Lipids.

The lipid solutions were protected from light, dried under an N₂ flux, and left in vacuum overnight. In the next day, the lipid film was resuspended in 1 ml of phosphate buffered saline (PBS) to a final lipid concentration of 1 mM, and 5 freeze-thaw cycles were performed to homogenize the liposome composition. To obtain unilamellar vesicles and homogenize the size of the liposomes, this solution was extruded at room temperature with an Avanti Mini-Extruder using 100-nm-pore-size polycarbonate membranes.

Plasmid DNA purification and labeling

Plasmid pVAX1GFP (3697 bp) is based on the commercially available plasmid pVAX1LacZ (6050 bp; Invitrogen) in which the *lacZ* reporter gene was replaced by the *eGFP* (green fluorescent protein) gene (Azzoni *et al.*, 2007). This vector also contains the human cytomegalovirus immediate early promoter (CMV promoter), the bovine growth hormone polyadenylation (BGH PolyA) sequence, a kanamycin resistance gene for selection in *Escherichia coli*, and a pMB1 origin (pUC-derived).

Plasmid DNA was replicated in *E. coli* (DH5 α) and purified using an in-house procedure based in hydrophobic interaction chromatography and size-exclusion chromatography (Diogo *et al.*, 2005). Its concentration was determined by a Nanovue Plus Spectrophotometer (GE Healthcare), and agarose gel electrophoresis was performed to assess its quality and relative amount of supercoiled DNA compared with circular and linear forms.

pDNA was labeled using Label IT Tracker Intracellular Nucleic Acid Localization Kit (Mirus) with Cy5 fluorescent label reagent. The labeling procedures were made in accordance with the manufacturer's protocol, consisting of 1 hr incubation of the pDNA with the Cy5 fluorophore and buffer, followed by an ethanol precipitation to remove the unbound Cy5. The labeled pDNA was pelleted and washed with 70% (v/v) ethanol by centrifugation, and resuspended in sterile water.

Chinese hamster ovary cell culture

Chinese hamster ovary (CHO) cells were grown in 10 ml Dulbecco's modified Eagle's medium (DMEM) (Gibco; high glucose, with pyruvate and glutamine and without HCO₃ and phenol red) supplemented with 10% (v/v) fetal bovine serum (FBS) (Gibco; heat inactivated) and 1% penicillin and streptomycin (PenStrep, from Gibco), in 75 cm² culture flasks (5% CO₂ at 37°C). Cells were grown up to 80% confluence and passed to a new T-75 flask or seeded in 8-well μ -Slide confocal microscope plates (from Ibidi). These μ -Slides were previously treated with 200 μ l of polylysine for 2 hr and washed twice with PBS (from Gibco) and once with complete DMEM.

The cell seeding procedure consisted of initial washing of the cells in the T-75 flask with 10 ml of PBS. In order to release the adherent cells from the T-75 flask surface, 4 ml of 0.05% trypsin-EDTA solution (from Gibco) was added, followed by 5 min incubation at 37°C. After this incubation period, 6 ml of DMEM supplemented with 10% FBS and

1% PenStrep was added to the T-75. Cells were transferred to a 15 ml falcon tube and centrifuged in a conventional bench-top centrifuge (5 min, 1500 g). The cell pellet was resuspended in 10 ml DMEM supplemented with 10% FBS and 1% PenStrep and cells were counted using a hemocytometer. The volume containing the desired amount of cells (3×10^4 cells per well) was transferred to the 8-well μ -Slides, and DMEM supplemented with 10% FBS and 1% PenStrep was added up to a final volume of 200 μ l.

Cell transfection with lipoplexes

CHO cells were seeded in 8-well μ -Slides 48 hr before transfection to allow complete adherence and a confluence of 80–90% on the day of transfection. Plasmid DNA (0.5 μ g) and liposomes (0.5 μ g) were resuspended in DMEM without FBS, antibiotics, and phenol red and incubated together for 20 min at room temperature (final volume of 50 μ l) to allow the formation of the lipoplexes. Cells in each well were prepared for transfection by removing the old medium and washing with 200 μ l of PBS to remove dead cells and toxins. The lipoplexes were added to each well, and 150 μ l of DMEM (without FBS, antibiotics, and phenol red) was added to a final volume of 200 μ l. The cells were incubated for 2, 4, or 24 hr (37°C, 5% CO₂). After 2 hr incubation, the medium was changed in all the 8-well μ -Slides to a complete medium (DMEM supplemented with 10% FBS and 1% PenStrep, without phenol red). Controls for DNA labeling (without rhodamine-DOPE labeling), liposome labeling (without Cy5 labeling), and plasma membrane labeling were performed.

Plasma membrane labeling

After transfection, cells in all wells (except in one control condition) were labeled with N-[6-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]hexanoyl]-sphingosine-1-phosphocholine (C6-NBD-SM) (Avanti Polar Lipids), previously diluted in PBS to a final concentration of 1 μ M. CHO cells were incubated with 200 μ l of the labeling solution, after the medium had been removed, for 15 min at 37°C and 5% CO₂. After incubation, cells were washed two times with 200 μ l of PBS and one time with 200 μ l of complete medium (DMEM supplemented with 10% FBS and 1% PenStrep, without phenol red).

Immobilization of lipoplexes for calibration of DNA densities

The ratio of fluorescence signals from the labeled lipid and labeled DNA was calibrated to provide information on the density of DNA molecules within the surface of the lipoplexes during internalization (Kucerka *et al.*, 2005). For this calibration, biotinylated lipoplexes obtained with different DNA concentrations were immobilized in an avidin-coated surface for confocal data acquisition. Biotinylation of the liposomes was achieved through the use of biotinylated phospholipid 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-N-(cap biotinyl) (DOPE-Cap-biotin) (Avanti Polar Lipids) (Sarmiento *et al.*, 2012). Lipid suspensions were prepared in chloroform from the stock solutions in a 75:23.99:1:0.01 (DOTAP:DOPE:DOPE-Rho:DOPE-Cap-biotin) proportion. The labeled pDNA was mixed with the liposomes as already

described, and these lipoplexes were immobilized in 8-well μ -Slides coated with avidin from egg white (Sigma Chemical). Avidin coating was carried out through a 30 min incubation of the slides with an avidin solution at 0.5 mg/ml (room temperature). Slides were washed three times with MilliQ water to remove excess avidin.

Zeta-Sizer measurements

The average diameter of the liposomes was determined at 25°C using a Zeta-Sizer (Zeta-Sizer Nano Series; Malvern Instruments). The liposomes were measured alone and also complexed with DNA, diluted in PBS to a final concentration of 0.05 μ g/ml. The DNA and liposomes were incubated for 30 min in a 1:1 ratio.

Confocal fluorescence microscopy

All measurements were performed on a Leica TCS SP5 inverted confocal laser scanning microscope (Leica Microsystems CMS GmbH). The excitation lines provided by the argon laser were focused into the sample by an apochromatic water immersion objective (63 $\times$, NA 1.2; Zeiss Jena Germany). The out-of-focus signals were blocked by using a 111.4- μ m-diameter pinhole in front of the image plane. The emission was detected through the spectrophotometric detection system of this microscope.

Four different fluorophores were detected by confocal microscopy: Cy5 (pDNA), Rho-DOPE (liposomes), C6-NBD-SM (plasma membrane), and GFP (when the *GFP* gene is expressed). C6-NBD-SM was employed only for plasma membrane staining for short incubation times, when GFP expression was absent. GFP or C6-NBD-SM imaging was achieved through excitation with an Ar 458 nm laser and detection at 500 $\pm$ 30 nm. Rho-DOPE fluorescence data acquisition was carried out with excitation with an Ar 514 nm laser and detection at 630 $\pm$ 10 nm. Cy5 (DNA) signal was measured using a HeNe 633 nm laser, and detection at 680 $\pm$ 20 nm. For the colocalization studies, images of the samples were acquired in the three channels (NBD/GFP, rhodamine-DOPE, and Cy5). These images were merged using ImageJ (Wayne Rasband, NIH).

Time-resolved fluorescence spectroscopy

Fluorescence decay measurements were carried out making use of the time-correlated single-photon timing technique, as described elsewhere (Loura *et al.*, 2000). Rho-DOPE was excited at 560 nm, and fluorescence emission was acquired at 600 nm. The emission wavelength was selected by a Jobin Yvon HR320 monochromator (Horiba Jobin Yvon Inc.). Quartz cuvettes with dimensions 0.5 $\times$ 0.5 cm² from Hellma Analytics were used. Blank decays were acquired and photon counts were negligible. Data analysis was performed with the TRFA software (Scientific Software Technologies Center) based on the Levenberg–Marquardt algorithm. The goodness of the fit was judged from the experimental χ^2 , weighted residuals and autocorrelation plot. In every analysis, χ^2 was less than 1.3, and both residuals and autocorrelation were randomly distributed around zero.

The FRET efficiency can be described by the following equation (Lackowicz, 2006):

$$E = 1 - \frac{\tau_{DA}}{\tau_D} \quad \text{Eq.1}$$

where τ represents the fluorescence lifetime from the donor in the absence (D-unquenched) and in the presence of the acceptor (DA-quenched) (Lackowicz, 2006).

Intensity-based FRET measurements

For quantitative FRET measurements, the three-cube method was employed (Chen *et al.*, 2006). In this methodology, images are acquired using three different conditions or channels: (1) the donor channel (IDD, donor excitation and emission), (2) the FRET channel (IDA, donor excitation, acceptor emission), and (3) the acceptor channel (IAA, acceptor excitation and emission). Channel conditions were described previously in the section Confocal Fluorescence Microscopy.

Since fluorescence spectral overlap is generally observed between donors and acceptors in a FRET experiment, controls must be carried out to quantify correction factors, which can be applied to the uncorrected images in order to recover the isolated signals from the donor (I_{dd}), the acceptor (F_A), and sensitized emission or acceptor fluorescence after donor excitation (F_c). These so-called crosstalk parameters were obtained experimentally from samples with only donor (Rho-DOPE) or only acceptors (Cy5-DNA) as described elsewhere (Chen *et al.*, 2006). The donor fluorescence lost because of FRET equals F_c/G , where G is a correction factor that must be obtained from samples with known FRET efficiency. In this way, the total donor fluorescence (if no FRET was present) is $F_D = I_{dd} + F_c/G$. The ratio of donor fluorescence (corrected for FRET) and acceptor fluorescence is

$$\frac{F_D}{F_A} = \frac{I_{dd} + \frac{F_c}{G}}{F_A} \quad \text{Eq.2}$$

This ratio is independent of FRET efficiency and is proportional to the ratio of donor and acceptor concentrations, giving information on colocalization of the molecules, independently of direct interaction.

On the other hand, FRET efficiencies (E values) provide information on the interaction between donor and acceptor molecules, and are determined from (Chen *et al.*, 2006):

$$E = \frac{F_c/G}{I_{dd} + (F_c/G)} \quad \text{Eq.3}$$

In the case of pDNA interaction with cationic liposomes, the F_D/F_A ratio and the E values provide complimentary information, in the sense that they allow us to follow both the concentration of pDNA in the lipoplex and the extent of adhesion of DNA to the lipoplex surface during trafficking. The analysis of the images obtained by confocal microscopy measurements was carried out using ImageJ (Wayne Rasband, NIH) and homemade software in a Matlab environment (Mathworks).

The images from the samples with both donor and acceptor were corrected for the background fluorescence. Rho-DOPE and Cy5 were chosen for labeling the liposomes and the DNA, respectively (Figs. 1A and 2A), since they are a good FRET pair and emit fluorescence at longer wavelengths. In this way, negligible bleedthrough was observed by the C6-NBD-SM (plasma membrane marker) and from GFP to the channels used for calculation of the F_D/F_A ratios and the E values. Generally, after 24 hr transfection, the images could be acquired without plasma membrane labeling, since GFP expression levels were sufficient

FIG. 1. Plasmid DNA density ($\mu\text{g}/\text{cm}^2$) and its influence on Cy5 fluorescence intensity values. (A) Liposome immobilization (biotin and avidin interaction). (B) Fluorescence lifetime values of the donor in the absence and presence of the acceptor. (C and D) Relationship between ratios of fluorescence intensity from rhodamine-DOPE and pDNA-Cy5 (F_D/F_A) and pDNA-Cy5 density ($\mu\text{g}/\text{cm}^2$). (E and F) Rhodamine-DOPE (donor) to pDNA-Cy5 (acceptor) FRET efficiency and pDNA-Cy5 density ($\mu\text{g}/\text{cm}^2$). Frequency is the normalized frequency of events (normalized number of particles detected) for each ratio of F_D/F_A (C) and FRET efficiency (E). In (D) and (F), results are expressed as means $\pm$ SEM ($n=10-35$). Analysis of confocal imaging data was carried out using ImageJ (Wayne Rasband, NIH) and homemade software in a Matlab environment (Mathworks). Lines are guides to the eye. FRET, Förster resonance energy transfer; pDNA, plasmid DNA. Color images available online at www.liebertpub.com/hgth

FIG. 2. Colocalization of liposome markers and DNA. (A) Lipoplex scheme: *green*, liposome label Rho-DOPE; *red*, pDNA label Cy5. (B) 3D reconstruction of a cell 4 hr after transfection showing an internalized lipoplex and another adsorbed to the plasma membrane. (C) Confocal microscopy of CHO cells 2, 4, and 24 hr after transfection: *blue*, plasma membrane label C6-SM-NBD-SM at 2 and 4 hr, and GFP at 24 hr. CHO, Chinese hamster ovary. Color images available online at www.liebertpub.com/hgtb

for imaging. Fluorescent particles corresponding to lipoplexes, unloaded liposomes, or free DNA were selected, and average I_{dd} , F_C , and F_A values were determined for each particle.

Results and Discussion

Liposome characterization using Zetasizer

The size of the liposomes was determined using dynamic light scattering. The sizing results obtained show that the mean diameter of the liposomes complexed with the pDNA was higher than the diameter of the liposomes alone (Table 1). The carrier size is a critical parameter in determining circulation half-life of liposomes. Based on their size and number of bilayers, the type of liposome used in this work is LUVs with an average diameter higher than 100 nm (Laouini *et al.*, 2012). As mentioned previously, the method used for the formation of

TABLE 1. THE PARTICLE SIZE (z-AVERAGE) IN NANOMETERS AND POLYDISPERSITY INDEX VALUES OF LIPOSOMES ALONE OR COMPLEXED WITH DNA (LIPOPLEXES) IN A MASS PROPORTION (1:1) IN PHOSPHATE BUFFERED SALINE

	Particle size (nm)	Polydispersity index
Liposomes	160.0 ± 1.2	0.32 ± 0.08
Liposomes/pDNA	240.0 ± 3.2	0.46 ± 0.06

pDNA, plasmid DNA.
Errors are standard deviation values ($n = 2$).

these LUVs had involved a process of freezing and thawing, followed by extrusion, whereby LUV's size reflects the diameter of the filter pore to which it was forced to pass. In this case, extrusion through filters with 100 nm pores typically yields large LUVs with a mean diameter of 120–140 nm (Lasic, 1997). As the addition of pDNA was done after liposome formation and not during the hydration step, it was already expected that DNA should complexate with the exterior of the liposome (Fig. 2A), increasing its diameter.

The polydispersity index (PDI) of the liposomes alone was found to be relatively lower than that of liposomes complexed with pDNA. The large PDI of the liposomes/pDNA complexes suggested a heterogeneous liposome population in terms of size.

The analysis of the correlograms given by the Zetasizer software indicated the presence of several liposome aggregates, but in a very low percentage, which does not seem to interfere in the transfection process (data not shown).

FRET and colocalization studies on immobilized liposomes

In this study, we aimed to characterize the process of DNA dissociation from internalized lipoplexes using two different approaches: FRET and colocalization microscopy. While FRET is sensitive to direct interaction between DNA-Cy5 (acceptor) and rhodamine-DOPE (donor) within the lipoplex (distances below 10 nm) (Lackowicz, 2006), colocalization studies provide information on the spatial overlap of the Lipid and DNA within the confocal microscopy lateral resolution (typically 250–400 nm) (Pawley, 2006). Since the latter distances are on the order of sizes of endocytic particles, changes in colocalization of DNA and lipid signal will reflect the escape of pDNA from endosomes into the cytosol or pDNA endocytic degradation, while changes in FRET efficiencies reflect the detachment of pDNA from the liposome membrane. In this way we can monitor both events for individual lipoplexes and characterize the evolution overtime.

As a proof of principle that our approach is able to efficiently follow pDNA detachment from the liposome, controls were executed on immobilized biotinylated liposomes (Fig. 1A), as described in the Materials and Methods section.

As previously described, because of the presence of FRET, in order to determine the ratio of fluorescence signals from the liposome marker (rhodamine-DOPE) and the labeled pDNA (DNA-Cy5) (F_D/F_A), a correction must be made to the donor fluorescence lost as a result of FRET (Eq. 2). The donor fluorescence lost as a result of FRET can be determined from the sensitized emission of the acceptor (acceptor fluorescence as a consequence of FRET by multiplication with a calibration factor G ; see the Materials and Methods section for details). The value of G was obtained by comparing microscopy data from immobilized liposomes with the real FRET efficiencies measured from fluorescence lifetimes of rhodamine-DOPE in the presence of different concentrations of pDNA-Cy5 (Fig. 1B). On the other hand, The F_A value is proportional to acceptor concentration, regardless of FRET, and no corrections are necessary for this value.

DOPE-RHO decays were performed and the amplitude-weighted average lifetime (τ) was quantified (Eq. 1) by using 2–3 fluorescence lifetimes and the obtained values were $\tau_{\text{Only donor}} = 2.37$ ns; $\tau_{0.33 \mu\text{g/cm}^2} = 0.65$ ns; $\tau_{0.07 \mu\text{g/cm}^2} = 1.83$ ns;

$\tau_{0.03 \mu\text{g/cm}^2} = 1.87$ ns (Fig. 1B). The corresponding FRET efficiencies (Eq. 3) are $E_{0.33 \mu\text{g/cm}^2} = 73\%$; $E_{0.07 \mu\text{g/cm}^2} = 23\%$; $E_{0.03 \mu\text{g/cm}^2} = 23\%$. From these FRET efficiencies and by comparison with fluorescence microscopy data on the same liposomes, a value of $G = 0.93$ was determined and this was used throughout this work to determine the F_D/F_A ratios.

The ratio F_D/F_A provides us with a quantitative measure of the degree on colocalization of the DNA with the lipoplex. This was confirmed on immobilized liposomes in the presence of different concentrations of pDNA-Cy5 (Fig. 1C and D). To determine DNA densities in the surface of liposomes, we assumed that 100% of the DNA is incorporated in lipoplexes and that the lipid surface molecular area was 72.4 Å, which is typical of a phospholipid with monounsaturated acyl-chains in the fluid phase (Kucerka *et al.*, 2005). The experimental conditions were settled in a fixed lipid mass of 0.5 μg and a variable pDNA mass, corresponding to DNA densities on liposome surface of 0.33, 0.17, 0.07, and 0.03 $\mu\text{g/cm}^2$, respectively (Fig. 1).

The histograms obtained for the different concentrations of labeled DNA clearly show that some heterogeneity in DNA content is observed for each pDNA-Cy5 concentration. At lower concentrations of DNA, a fraction of the liposomes exhibit very high F_D/F_A values, probably reflecting the presence of liposomes almost fully devoid of DNA (Fig. 1C). As expected, a significant decrease in F_D/F_A (enrichment with DNA) is observed on the liposomes incubated with higher concentrations of DNA (Fig. 1D), confirming that this methodology is able to monitor depletion of pDNA from liposomes.

Similar results are obtained for FRET efficiencies (E) in the same liposomes. For the smaller pDNA-Cy5 density considered (0.03 $\mu\text{g/cm}^2$), a fraction of liposomes show E values close to zero, once more reflecting the presence of a liposome population almost unloaded of DNA (Fig. 1F). FRET efficiency values are also shown to be dependent on DNA densities, with higher values for higher DNA concentrations, as expected (Fig. 1E).

In this way, we confirmed that the above-described methodologies are able to efficiently monitor release of DNA from lipoplexes, and can be applied to monitor these events in living cells.

Optimization of transfection

The concentration of the different markers (liposome, DNA, and plasma membrane) as well as the quantity of lipoplexes delivered to the cells were optimized to ensure that cell viability was not affected, while achieving ideal acquisition conditions for microscopy. A 1:100 molar fraction of rhodamine-DOPE:total lipid was chosen for the lipoplex mixture.

Several DNA:liposome proportions were also tested, and the 0.5 μg DNA:0.5 μg liposome was considered the ideal, since higher quantities had a negative effect on the cell viability. This mixture has a ratio of 72 pDNA molecules per liposome, which is likely to correspond to a saturation of liposome surface with DNA. The DNA-Cy5 surface density in this case was determined to be approximately 0.33 $\mu\text{g/cm}^2$, corresponding to the highest DNA concentration used in the control experiments with immobilized liposomes (Fig. 2). This concentration of DNA-Cy5 elicited a robust FRET

efficiency ($E \approx 0.72$), which was essential for sensitivity of the assay for DNA unloading from the lipoplex.

Different C6-NBD-SM concentrations were tested for plasma membrane labeling and $1 \mu\text{M}$ was considered sufficient, since at higher concentrations, C6-NBD-SM negatively affected the cell viability after a short period of time.

Both particles exhibiting very low or very high (saturating) levels of fluorescence, in comparison with intact lipoplexes, were not considered in the analysis. Particles with very low signals are likely to correspond to degraded DNA or fusion of lipoplexes with intracellular membranes, while particles with very strong fluorescence signals corresponded to very large clusters of lipoplexes, which are probably formed in the endocytic pathway.

Monitoring of pDNA trafficking through fluorescence colocalization and FRET

As already discussed, given the comparable dimensions of endocytic vesicles and the resolution of confocal microscopy, coincidence detection of the fluorescence signals of a liposome marker and of the DNA is expected to offer information on either release of DNA from endosomes where liposomes remain trapped after internalization or endocytic degradation of the oligonucleotide. In order to obtain information on this process, fluorescence colocalization analysis, using different labels for the pDNA, liposomes, and plasma membrane, was performed before and after expression of GFP (Fig. 2).

The images were acquired 2, 4, and 24 hr posttransfection. After only 2 hr posttransfection, it was possible to identify liposomes inside CHO cells, mainly near the plasma membrane, confirming that the uptake of lipoplexes by the cells was very fast. Several endosomes containing non-dissociated internalized lipoplexes were visible (Fig. 2C, 2h), and also some intact liposomes without DNA. A 3D reconstruction of a CHO cell 4 hr after initiation of transfection shows a lipoplex already internalized and another adsorbed to the plasma membrane (Fig. 2B).

In the 4 hr posttransfection stage, there were still non-dissociated internalized lipoplexes (Fig. 2C, 4h). In some cases, liposomes within endosomes that had no DNA fluorescence appear to have already fused with endocytic membranes, as the fluorescence from the lipid marker becomes more diffuse in many cases, possibly reflecting homotypic fusion of early endosomes into larger vesicles. The obtained results corroborate the results achieved by Akita *et al.* (2004), which showed that the efficacy of pDNA trafficking as well as transfection may occur within 3 hr.

Transcription of the transfected gene can be analyzed within 24 hr after introduction of the DNA, and a significant fraction of cells presented GFP expression at this point (Fig. 2C, 24 hr). For this to happen, the pDNA had to dissociate from the liposome and escape from the endosomes, in order to enter the nucleus and become transcriptionally active. The remaining cells in this image (Fig. 2C, 24h) present nonlocalized red fluorescence because of the degradation of DNA and release of Cy5. In these red cells, although significant Cy5 label is present, the transfection was not efficient since no GFP expression was detected, possibly because of DNA degradation.

The quantitative analysis of the microscopy data during trafficking of lipoplexes is shown in Fig. 3. The population

of intact lipoplexes (0 hr, green) was measured with immobilized liposomes and is included in all graphs for comparison with the detected populations at the different time points. At 2 hr after transfection (Fig. 3A), the DNA load of the bulk population of internalized lipoplex is comparable to the intact lipoplexes, displaying $E \approx 0.5$ –1 and $F_D/F_A \approx 0.5$ –4. As already mentioned, a minor fraction of the internalized lipoplexes is already disengaging from the DNA load at this time point, displaying $E < 0.5$. However, although DNA disengagement is observed for these lipoplexes, both instances of DNA colocalization ($F_D/F_A \approx 3.5$) and noncolocalization within endocytic particles ($F_D/F_A \approx 8$) are still observed, corresponding to two different stages of lipoplex trafficking, before and after either endosomal escape of pDNA or pDNA degradation.

An internalized particle with high DNA-Cy5 fluorescence and absent rhodamine-DOPE fluorescence ($F_D/F_A \approx 0$), corresponding to cytosolic free DNA, is also already detected (Fig. 3A), confirming efficient escape of DNA from the endocytic pathway very briefly after incubation with lipoplexes. It should be noted that the lifetime of dissociated pDNA in the cytosol is expected to range from 5 min to 2 hr (Lechardeur *et al.*, 1999; Bureau *et al.*, 2004), and as such their detection is more unlikely. Interestingly, with the exception of one particle, the population of noninternalized lipoplexes 2 hr after the onset transfection remains virtually identical to the initial population (Fig. 3B), reflecting the stability of the lipoplexes in the extracellular environment. Four hours after transfection, the population of liposomes displaying low FRET efficiency ($E < 0.25$) increases significantly, as a result of continuous disengagement of DNA from internalized lipoplexes (Fig. 3C). Moreover, almost all of these disengaging lipoplexes exhibit low colocalization with DNA ($F_D/F_A > 4$). At 24 hr after the onset of transfection, the number of endosomes exhibiting both DNA disengagement and low DNA colocalization increases even further, while events of endocytic pDNA colocalization with disengaged liposomes remain very rare (Fig. 3D).

At this stage, the number of internalized lipoplexes is expected to be significantly higher than the number of analyzed particles (endosomes and lysosomes), as a result of fusion of endocytic compartments. Surprisingly, even after 24 hr, a nonnegligible pool of intact internalized lipoplexes is still found within CHO cells, suggesting that a pool of endocytic particles has not still fully mature. Significant fusion of endocytic membranes is expected for mature endosomes and lysosomes, and aggregation of lipoplexes is observed as an increase in FRET efficiencies even in the presence of lower quantities of DNA within endosomes ($F_D/F_A > 4$), as a result of the proximity to the lipoplex membrane of DNA-Cy5 from other aggregating lipoplexes. Additionally, at this point, several events of fully dissociated DNA ($F_D/F_A \approx 0$) are once more observed, both on the nucleus and the cytosol.

In the control experiments with immobilized lipoplexes, the low FRET efficiencies ($E < 0.25$) observed for several internalized lipoplexes are only observed for DNA concentrations 4–5 times lower than the one present in intact lipoplexes, and this value was used as a threshold in the quantification of endosomes with unloaded lipoplexes. To quantify the fraction of these “unloaded” lipoplexes, which are still colocalizing with pDNA in the endosome, a

FIG. 3. Trafficking of lipoplexes in CHO cells. Intracellular lipoplexes measured at 2 (A and B), 4 (C), and 24 (D) hr after transfection. Mean $\pm$ SEM values for F_D/F_A ratios (E) and E (F) are also shown. These values are the result of 3 independent measurements with 20–100 lipoplexes analyzed in each one. Color images available online at www.liebertpub.com/hgtb

FIG. 4. Lipoplexes dissociation along the trafficking process. The percentage of measured endocytic particles showing dissociation of DNA from lipoplexes ($E < 0.25$) but still loaded with pDNA-Cy5 ($F_D/F_A = 0.5-4$) is shown in *black*, while the percentage of endocytic particles showing dissociation and shedding of pDNA-Cy5 ($F_D/F_A > 4$) is shown in *gray*. These values are the result of 3 independent measurements with 20–100 lipoplexes analyzed in each one.

threshold of $F_D/F_A = 4$ was considered, since before internalization and for the pDNA concentration used here, values for this ratio are virtually always below this number because of a saturation of the liposome surface with the pDNA. For immobilized intact lipoplexes, this ratio increases consistently with decreasing concentrations of pDNA (Fig. 1), and after DNA release from the endosome or degradation in the endocytic compartment, F_D/F_A is expected to raise above the threshold value of 4. The quantification of the population of lipoplexes unloaded of DNA and colocalizing or not with the fluorescence signal of DNA-Cy5 is shown on Fig. 4.

Although the fraction of endocytic particles showing dissociation of DNA seems to reach a steady state at 4 hr, the number of lipoplexes releasing DNA is expected to be much higher at 24 hr, since significant fusion of endocytic compartments has occurred at this point. On the other hand, the rarity of the observations of internalized lipoplexes displaying both disengagement from the DNA (low FRET) and colocalization with the DNA fluorescence signal ($F_D/F_A \approx 0.5-4$) clearly indicates that while lipoplex dissociation is in most cases a slow process, once DNA disengagement is achieved, DNA escape from the endosome or DNA degradation within the endocytic compartment is much faster.

Conclusions

Nonviral vectors are highly attractive for gene therapy from a clinical point of view, and cationic lipid nanoparticles in particular have generated considerable interest. However, despite considerable recent advances, problems associated with low transfection efficiencies remain to be resolved to fully meet the potential of these vectors. For this reason, a quantitative description of the intracellular trafficking of plasmids delivered by these carriers is required to fully understand the factors governing the efficacy of gene expression. Taking this into consideration, a strategy of dual-monitoring of both the direct

interaction of pDNA with the conjugated liposome and of the colocalization of both within the same organelle was employed in living cells incubated with lipoplexes. Direct interaction between pDNA and liposome was assessed for each individual lipoplex through FRET microscopy, while colocalization of pDNA and loaded liposomes was evaluated through standard confocal microscopy. The latter is expected to be sensitive to endocytic escape or degradation of pDNA.

In the first hours of the experiment, shedding of DNA is observed only for internalized lipoplexes, since almost all lipoplexes that are not endocytosed remain intact. This confirms that while allowing for intracellular dissociation of pDNA, the lipid formulation used for the preparation of these lipoplexes provides efficient protection from nuclease attack and helps in cell uptake and escape from the endosome, optimizing the delivery route/method and maximizing the quantity of pDNA with potential to reach the nucleus.

After 2 hr from the onset of the transfection procedure, several lipoplexes had already been endocytosed, and the presence of free pDNA within the cytosol was identified. Results show that the dissociation of pDNA from the liposomes as measured by FRET is a slow process. Although the number of endocytic particles showing dissociation of pDNA seems to reach a steady state at 4 hr, the number of lipoplexes releasing DNA is expected to be much higher at 24 hr, since significant fusion of endocytic compartments has taken place. Surprisingly, 24 hr posttransfection, a small fraction of endosomes still contain intact lipoplexes, although the large majority of endosomes display significant depletion of DNA.

Most importantly, we demonstrate that after endocytosis, exceptionally few endocytic particles are found to exhibit simultaneously DNA/lipid colocalization and low FRET (DNA/lipid dissociation). These results clearly point to an extremely short-lived state for free plasmid within endosomes, which either escapes at once to the cytosol or becomes susceptible to degradation, as a result of the increased exposure of its free form. It is possible that this limitation greatly contributes to reduction in probability of successful gene delivery.

The quantitative dual-monitoring of FRET between pDNA and liposome as well as of the colocalization of both on endocytic particles is shown here to be useful in the characterization of trafficking of lipoplexes in living cells. This tool has the potential to be of great value in the identification of intracellular barriers for nuclear insertion of pDNA, and further contributes to the rationalization and conception of new lipid nanoparticle vectors for gene delivery for therapeutic purposes.

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Author Disclosure Statement

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References

- Akita, H., Ito, R., Khalil, I.A., *et al.* (2004). Quantitative three-dimensional analysis of the intracellular trafficking of plasmid DNA transfected by a nonviral gene delivery system using confocal laser scanning microscopy. *Mol. Ther.* 9, 443–451.
- Akita, H., Kudo, A., Minoura, A., *et al.* (2009). Multi-layered nanoparticles for penetrating the endosome and nuclear membrane via a step-wise membrane fusion process. *Biomaterials* 30, 2940–2949.
- Azzoni, A.R., Ribeiro, S.C., Monteiro, G.A., and Prazeres, D.M. (2007). The impact of polyadenylation signals on plasmid nuclease-resistance and transgene expression. *J. Gene Med.* 9, 392–402.
- Bloomfield, V.A. (1997). DNA condensation by multivalent cations. *Biopolymers* 44, 269–282.
- Bureau, M.F., Naimi, S., Torero Ibad, R., *et al.* (2004). Intramuscular plasmid DNA electrotransfer: biodistribution and degradation. *Biochim. Biophys. Acta* 1676, 138–148.
- Caracciolo, G., Caminiti, R., Digman, M.A., *et al.* (2009). Efficient escape from endosomes determines the superior efficiency of multicomponent lipoplexes. *J. Phys. Chem. B* 113, 4995–4997.
- Chen, H., Puhl, H.L., 3rd, Koushik, S.V., *et al.* (2006). Measurement of FRET efficiency and ratio of donor to acceptor concentration in living cells. *Biophys. J.* 91, L39–L41.
- Cooper, G. (2013). *The Cell: A Molecular Approach*. (Sinauer Associates Inc., New York).
- Diogo, M.M., Queiroz, J.A., and Prazeres, D.M. (2005). Chromatography of plasmid DNA. *J. Chromatogr. A* 1069, 3–22.
- Donnelly, J., Berry, K., and Ulmer, J.B. (2003). Technical and regulatory hurdles for DNA vaccines. *Int. J. Parasitol.* 33, 457–467.
- El-Sayed, A., Khalil, I.A., Kogure, K., *et al.* (2008). Octaarginine- and octalysine-modified nanoparticles have different modes of endosomal escape. *J. Biol. Chem.* 283, 23450–23461.
- El-Sayed, A., Masuda, T., Khalil, I., *et al.* (2009). Enhanced gene expression by a novel stearylated INF7 peptide derivative through fusion independent endosomal escape. *J. Control. Release* 138, 160–167.
- Escoffre, J.M., Teissie, J., and Rols, M.P. (2010). Gene transfer: how can the biological barriers be overcome? *J. Membr. Biol.* 236, 61–74.
- Ho, E.A., Ramsay, E., Ginj, M., *et al.* (2010). Characterization of cationic liposome formulations designed to exhibit extended plasma residence times and tumor vasculature targeting properties. *J. Pharm. Sci.* 99, 2839–2853.
- Houk, B.E., Martin, R., Hochhaus, G., and Hughes, J.A. (2001). Pharmacokinetics of plasmid DNA in the rat. *Pharm. Res.* 18, 67–74.
- Knight, J.D., and Adami, R.C. (2003). Stabilization of DNA utilizing divalent cations and alcohol. *Int. J. Pharm.* 264, 15–24.
- Kucerka, N., Tristram-Nagle, S., and Nagle, J.F. (2005). Structure of fully hydrated fluid phase lipid bilayers with monounsaturated chains. *J. Membr. Biol.* 208, 193–202.
- Lackowicz, J.R. (2006). *Principles of Fluorescence Spectroscopy*. (Springer, Berlin).
- Lam, W. (2012). Endosomal escape pathways for non-viral nucleic acid delivery systems. In *Molecular Regulation of Endocytosis*. B. Ceresa, ed. (Intech, Rijeka) pp. 429–456.
- Laouini, A., Jaafar-Maalej, C., Limayem-Blouza, I., *et al.* (2012). Preparation, characterization and applications of liposomes: state of the art. *J. Colloid Sci. Biotechnol.* 1, 147–168.
- Lasic, D.D. (1997). *Liposomes in Gene Delivery*. (CRC Press, Boca Raton, FL).
- Lechardeur, D., and Lukacs, G.L. (2002). Intracellular barriers to non-viral gene transfer. *Curr. Gene Ther.* 2, 183–194.
- Lechardeur, D., and Lukacs, G.L. (2006). Nucleocytoplasmic transport of plasmid DNA: a perilous journey from the cytoplasm to the nucleus. *Hum. Gene Ther.* 17, 882–889.
- Lechardeur, D., Sohn, K.J., Haardt, M., *et al.* (1999). Metabolic instability of plasmid DNA in the cytosol: a potential barrier to gene transfer. *Gene Ther.* 6, 482–497.
- Loura, L.M., Fedorov, A., and Prieto, M. (2000). Partition of membrane probes in a gel/fluid two-component lipid system: a fluorescence resonance energy transfer study. *Biochim. Biophys. Acta* 1467, 101–112.
- Luo, D., and Saltzman, W.M. (2000). Synthetic DNA delivery systems. *Nat. Biotechnol.* 18, 33–37.
- Miller, A.M., and Dean, D.A. (2009). Tissue-specific and transcription factor-mediated nuclear entry of DNA. *Adv. Drug Deliv. Rev.* 61, 603–613.
- Pawley, J. (2006). *Handbook of Biological Confocal Microscopy*. (Springer, Berlin).
- Petrovsky, N., and Aguilar, J.C. (2004). Vaccine adjuvants: current state and future trends. *Immunol. Cell Biol.* 82, 488–496.
- Pollard, H., Tomanianitz, G., Amos, J.L., *et al.* (2001). Ca^{2+} -sensitive cytosolic nucleases prevent efficient delivery to the nucleus of injected plasmids. *J. Gene Med.* 3, 153–164.
- Sarmiento, M.J., Prieto, M., and Fernandes, F. (2012). Reorganization of lipid domain distribution in giant unilamellar vesicles upon immobilization with different membrane tethers. *Biochim. Biophys. Acta* 1818, 2605–2615.
- Schleef, M., and Schmidt, T. (2004). Animal-free production of ccc-supercoiled plasmids for research and clinical applications. *J. Gene Med.* 6 Suppl 1, S45–S53.
- Schwendener, R.A., Ludwig, B., Cerny, A., and Engler, O. (2010). Liposome-based vaccines. *Methods Mol. Biol.* 605, 163–175.
- Srivastava, I.K., and Liu, M.A. (2003). Gene vaccines. *Ann. Intern. Med.* 138, 550–559.
- Wang, L., and MacDonald, R.C. (2004). New strategy for transfection: mixtures of medium-chain and long-chain cationic lipids synergistically enhance transfection. *Gene Ther.* 11, 1358–1362.
- Wetzer, B., Byk, G., Frederic, M., *et al.* (2001). Reducible cationic lipids for gene transfer. *Biochem. J.* 356, 747–756.
- Yoshioka, T., Yoshida, S., Kurosaki, T., *et al.* (2009). Cationic liposomes-mediated plasmid DNA delivery in murine hepatitis induced by carbon tetrachloride. *J. Liposome Res.* 19, 141–147.
- Zelphati, O., and Szoka, F.C., Jr. (1996). Mechanism of oligonucleotide release from cationic liposomes. *Proc. Natl. Acad. Sci. USA* 93, 11493–11498.

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