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Macropinocytosis Is the Major Pathway Responsible for DNA Transfection in CHO Cells by a Charge-Reversal Amphiphile

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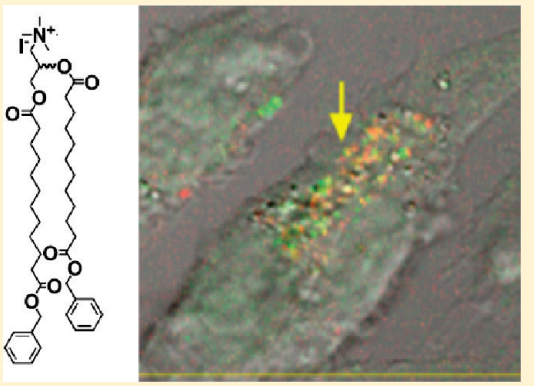
ABSTRACT: The cellular uptake of a functional charge-reversal amphiphile:DNA lipoplex is described. First, pharmacological inhibitors were applied to block different endocytosis pathways. By examining the resulting transfection activities, it was found that endocytosis was the pathway leading to transfection in Chinese hamster ovary (CHO) cells. When the specific pathway of macropinocytosis was inhibited, β-galactosidase expression was significantly depleted (90%); meanwhile the inhibition of clathrin-mediated pathway only brought a 30% decrease in expression; and the inhibition of caveolae-mediated pathway did not affect expression. Furthermore, a transfection kinetics study revealed that the cellular uptake responsible for gene expression was a slower process compared to clathrin-mediated endocytosis, consistent with fluid-phase uptake compared to receptor-mediated uptake. Next, a fluorescence colocalization study was used to visualize the DNA lipoplex uptake pathways. The colocalization of the DNA lipoplex and Cascade Blue, a fluid-phase uptake marker, was observed. Meanwhile, the colocalization of the DNA lipoplex and transferrin, a clathrin-mediated endocytosis marker, was also seen. However, no colocalization was observed with the endosome/lysosome marker Lysotracker. Our results indicate that macropinocytosis, not the commonly seen clathrin-mediated endocytosis for cationic lipids, is the major pathway leading to gene transfection in CHO cells for this charge-reversal amphiphile.



KEYWORDS: gene delivery, transfection, charge-reversal, amphiphile, macropinocytosis, lipid, DNA

INTRODUCTION

Gene therapy is of interest for treating, preventing, or controlling a myriad of diseases, and activities are ongoing at both the research and clinical levels. Traditionally, DNA delivery has been classified into two major categories based on the type of delivery vector: viral-mediated[1−5] and nonviral (i.e., synthetic)[6−10] mediated. Viral vectors (infection) are by far the most effective and efficient means of DNA delivery, but their use is tempered by some safety and immunogenicity concerns. Alternatively, nonviral vectors have the advantages of ease of production, better stability and low immunogenicity, but have the drawback of low transfection efficiency. Consequently, significant research is being conducted on increasing transfection efficiency through the synthesis of new small molecule and polymer amphiphiles and understanding the intracellular steps in the transfection pathway.

To overcome the low transfection efficiencies of synthetic lipid-based vectors, investigators have developed and evaluated helper lipids as well as functional or stimulus-responsive vectors. In the first approach, zwitterionic helper lipids such as dioleoylphosphatidylethanolamine (DOPE)[11] and its derivatives[12] have been used to facilitate fusion of the bilayer with the membrane of the endosome, to increase the transfection efficacy.[12,13] The second strategy entails the use of functional or stimulus-responsive vectors. Many of these functional vectors, which are able to respond to a stimulus that changes their chemical or physical properties, were designed based on the specific steps in the transfection pathway to facilitate DNA delivery. The transfection pathway usually starts with cellular entry of the DNA−vector complex (i.e., lipoplex) through endocytosis to endosomal escape, and finally to nuclear targeting and DNA expression. As a result, a wide variety of synthetic vectors have been prepared that are responsive to pH,[14−17] reducing conditions,[18−21] enzymes,[22−24] or temperature.[25,26] For example, vectors containing imidazoles or histidines[27] have been studied since they can induce membrane fusion and act like a proton sponge in an acidic environment, such as that found in the endosome, to promote DNA release into cytoplasm.

Our effort to address this challenge has yielded a charge-reversal amphiphile **1**.[28] The net charge of this cationic lipid is reversed from +1 to −1 upon hydrolysis of the terminal ester (Figure 1). This change in amphiphile structure will lead to a loss of DNA binding once inside the cell to facilitate transfection. This charge-reversal amphiphile transfects DNA in several cell lines at high efficiency.[28] Herein, we report the cellular uptake, intracellular trafficking and transfection mechanism of this gene delivery vector by a combination of pharmacological and imaging approaches. We show that the lipoplex formed by DNA and amphiphile **1** was taken up in Chinese hamster ovary (CHO) cells mainly through macropinocytosis.

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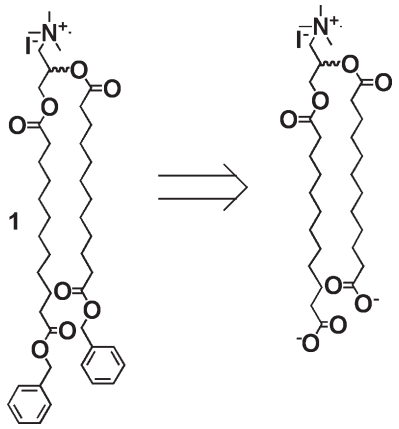

Figure 1. Structure of charge-reversal amphiphile **1** and proposed mechanism of charge switching from +1 to −1.

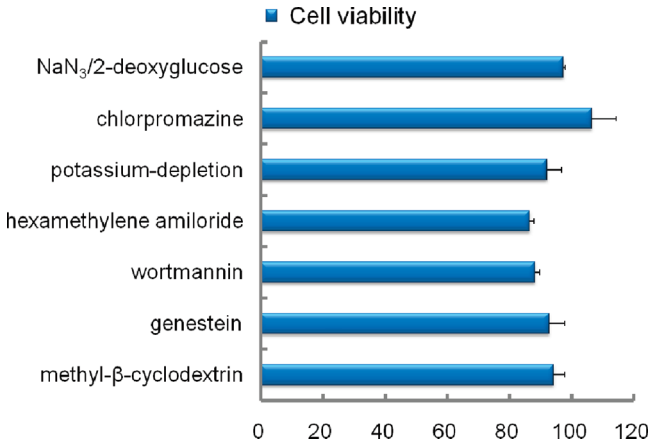

Figure 2. Cell viability after inhibitor treatments.

MATERIALS AND METHODS

Reagents and Cells. All the chemicals used in the pharmacological inhibition studies were purchased from Sigma-Aldrich (St. Louis, MO) without further purification. Amphiphile **1** was synthesized as described earlier and readily forms liposomes.[28] Lipoplexes were prepared following our published procedure[28] at the charge ratio of 5/1. The size and zeta potential of the liposomes and lipoplexes are 374 and 277 nm, and 50.2 and 27.7 mV, respectively.

The pGeneGrip rhodamine-labeled β-galactosidase or GFP-encoding DNA and CellScrub buffer was purchased from Gene Therapy Systems (San Diego CA). Alexa Fluor 647-conjugated dextran (M_w = 10,000; Ex/Em: 647/670), Cascade Blue hydrazide (Ex/Em: 402/420), Alexa Fluor 633-conjugated transferrin (Ex/Em: 632/647), Lysotracker Blue DND-22 (Ex/Em: 373/422) and BODIPY FL (Ex/Em: 505/511) labeled C_{12}−HPC were purchased from Invitrogen (Calsbard CA).

Chinese hamster ovary cells were purchased from American Type Culture Collection (ATCC) (Manassas, VA). The cells were maintained in F-12K medium (ATCC), supplemented with 100 units/mL of penicillin, 100 μg/mL of streptomycin, 2 mM glutamine (ATCC) and 10% heat-inactivated fetal bovine serum (Longza, Allendale, NJ). The cells were cultured in 6-well Costar tissue culture plates in a 5% CO_2 humidified incubator at 37 °C.

Transfection and Viability Assays. Generally, 24 h prior to transfection, the cells were seeded in a 24-well plate at a density of 1×10^5/well so that, by the time of transfection, the cells reached 60−70% confluence. During transfection, cell culture medium was removed and 20 μL of serum-free medium containing lipoplexes, which were composed 0.5 μg of psv-β-galactosidase control vector (Promega, Madison WI) and amphiphile **1** at a 1:5 charge ratio, was added. After incubation for 3 h, 300 μL of fresh serum-containing medium was added and the cells were incubated for another 48 h. Cell viability was measured using CellTiter 96 AQueous Non-Radioactive Cell Proliferation Assay (MTS) (Promega). β-Galactosidase Enzyme Assay System (Promega) was used to obtain the amount of β-galactosidase expression. Total protein was analyzed using Coomassie Protein Assay Reagent (Thermo Fisher Scientific, Waltham MA). Transfection efficiency was calculated as mU β-gal/mg total protein.

Inhibitor Treatment. The pharmalogical inhibitors were used at the following concentrations: sodium azide 0.01%, 2-deoxyglucose 20 mM, chloropromazine 10 μM, hexamethylene amiloride 20 μM, wortmannin 10 μM, genestein 50 μM, and methyl-β-cyclodextrin 5 mM. These concentrations were identified through a series of cell proliferation/cytotoxicity assays with various inhibitor concentrations. The cell viability assay results of the final selected inhibitor concentrations are shown in Figure 2.

The cells were pretreated with the inhibitors for 1 h at 37 °C and transfected with the lipoplexes in the presence of inhibitors for 3 h at 37 °C. Then the medium was removed and cells were washed 3 times with PBS and cultured in fresh growth medium in the absence of inhibitors for 48 h prior to β-galactosidase expression assay. In the case of K^+ depletion, cells were washed with K^+-free buffer (140 mM NaCl, 20 mM HEPES, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mg/mL glucose, pH7.4) once, hypotonic buffer (K^+-free buffer diluted 1:1 with water) once and finally K^+-free buffer 3 times. Then cells were transfected with lipoplex in K^+-free buffer for 1 h at 37 °C. As a control, cells were washed and transfected in a similar buffer but containing 10 mM KCl. The buffer was then removed, and fresh growth medium was added.

Flow Cytometry. Twenty-four hours before transfection, 1.5×10^6 cells were seeded into a 6-well plate. Prior to transfection, cells were incubated in the presence of inhibitors for 1 h as described above. Then, lipoplexes containing 5 μg of rhodamine-labeled β-gal in 1 mL of serum-free medium were added. After transfection for 3 h, the cells were washed twice with PBS before they were incubated for 20 min in CellScrub buffer to remove membrane-attached lipoplexes. Subsequently, the cells were washed twice with PBS, trypsinized, washed again with PBS, and resuspended in PBS containing 0.05% BSA. The analysis was carried out on a LSRII flow cytometer (Becton-Dickson, Germany) using FACSDiva (Becton-Dickson, Germany) software with a 561 nm excitation laser and a 610/20 filter. 10,000 cells were measured in each sample. Transfection in the absence of inhibitors and cells only was used as controls.

Transfection Kinetics Study. Cells were transfected using the lipoplex for 15 min, 30 min, 1 h or 3 h as described above. After the indicated time period, cells were washed with 10 unit/mL heparin in PBS twice, and then incubated in growth medium for 48 h prior to β-galactosidase expression assay.

Laser Scanning Confocal Microscopy. 48 h before transfection, 1.5×10^5 cells were seeded on a collagen-coated glass bottom 35 mm dish (MatTeck, Ashland MA). Cells were then transfected by lipoplex with 4 μg of rhodamine-labeled GFP-encoding DNA in 1 mL of serum-free medium supplemented

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Table 1. Inhibitors Used and Targeted Endocytosis Pathways

NaN_3/2-deoxyglucose	general endocytosis inhibitor
chlorpromazine	clathrin-mediated
potassium depletion	
hexamethylene amiloride	macropinocytosis
wortmannin	
genistein	caveolae-mediated
methyl-β-cyclodextrin	cholesterol dependence

with 2 mM HEPES. After 3 h, 2 mL of fresh growth medium supplemented with 2 mM HEPES was added.

Cells were imaged using an Olympus Fluo-View 1000 laser scanning confocal microscope in sequential mode to eliminate emission cross-talk between the various dyes. XYZ and XYT acquisition was controlled using the Olympus FV10 software, version 1.8b. Three dimensional *Z*-stacks of confocal slices were acquired using the FV10v1.8b software, with the sampling step size set to half the axial resolution of the lens for ideal sampling.

Live cells were maintained on the microscope in an F12 based imaging media consisting of F12 and 20 mM HEPES, pH7.4, supplemented with 10% serum, glutamine, serum and antibiotics. Laser and detector settings were set to minimize phototoxicity as judged by the continued cell movements, and lack of blebbing and rounding up.

Transfection in the Presence of Ca^{2+} or Chloroquine. The transfection procedure was the same as described above except that 4 mM $CaCl_2$ or 50 μM chloroquine was present in the transfection medium in the 3 h period. In the former case, 1 mM $CaCl_2$ was also present in the growth media in the 48 h period prior to β-galactosidase expression assay.

RESULTS AND DISCUSSION

Endocytosis has been recognized as the most common cellular uptake pathway for gene delivery carriers.[29−31] To date, five major pathways have been distinguished,[32−36] and their characteristics are described briefly as follows: (1) Phagocytosis, which occurs only in specialized cells such as macrophages, neutrophiles or monocytes. Since Chinese hamster ovary (CHO) cells were used for all transfection experiments in this study, this is not a relevant pathway for this study. (2) Macropinocytosis, which is caused by membrane ruffles that fuse with the plasma membrane and form large endocytotic vesicles (up to 1−5 μm) known as macropinosomes. They either fuse with lysosomes or are recycled back to the cell surface. (3) Clathrin-mediated endocytosis, which is a process whereby ligand-bound receptors are internalized via clathrin-coated vesicles and subsequently transformed to early endosomes. The ligands are transferred into late endosomes for potential degradation in lysosomes, whereas the receptors are recycled and transported back to the plasma membrane. (4) Caveolae-mediated endocytosis, which is characterized by the evolution of caveolae-derivatives of the subdomains of sphingolipid and cholesterol-rich cell membrane fractions. After internalization, these ligands either transfer into caveosomes and then translocate to the endoplasmic reticulum or the Golgi complex, or they enter the endosomal pathway. (5) Clathrin- and caveolae- independent endocytosis which includes those pathways not usually classified by the above criteria. Many of their regulatory mechanisms are actively investigated and still unknown.

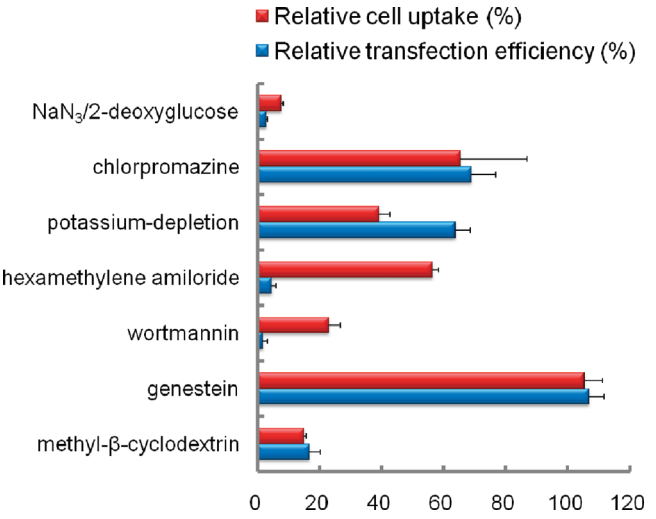

Figure 3. Relative cell uptake and transfection efficiencies in the presence of inhibitors were expressed as percentages compared to those without inhibitors.

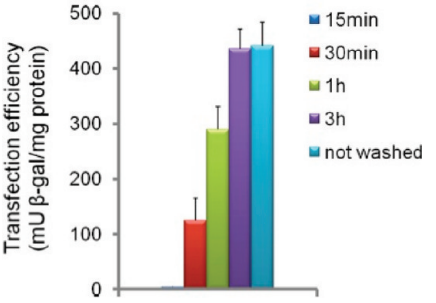

Figure 4. Kinetics of transfections by **1**. Cells were washed after internalization of different time periods, and transfection efficiency was compared to unwashed cells.

Pharmacological inhibitors have been widely used to study endocytic pathways.[37−41] The advantages of using this method are that cells are affected as a population and delayed side effects or the need for compensatory mechanisms are minimized, since cells are usually exposed to inhibitors for a short time period.[40] The inhibitors used in this study and the respective pathways they target are listed in Table 1.

First, optimal inhibitor concentrations were determined by cell proliferation assays which involved treating the cells with various inhibitor concentrations for 4 h and then recording data 48 h after inhibitor treatment. Inhibitor concentrations that showed at least 85% cell viability compared to nontreated cells were used for the cellular uptake and transfection studies. For cellular uptake experiments, generally, cells were preincubated in the presence of inhibitors for 1 h. After transfection with rhodamine-labeled DNA lipoplexes for 3 h, during which inhibitors were also present, transfection medium was removed and cells were washed and analyzed by flow cytometry. The cellular uptake data were expressed as the percentage of DNA uptaken compared to no inhibitor-treated cells. For transfection experiments, β-galatosidase expressions were determined 48 h after cells were transfected for 3 h. The transfection efficiencies were reported as the percentage of β-galatosidase expression compared to no inhibitor-treated cells.

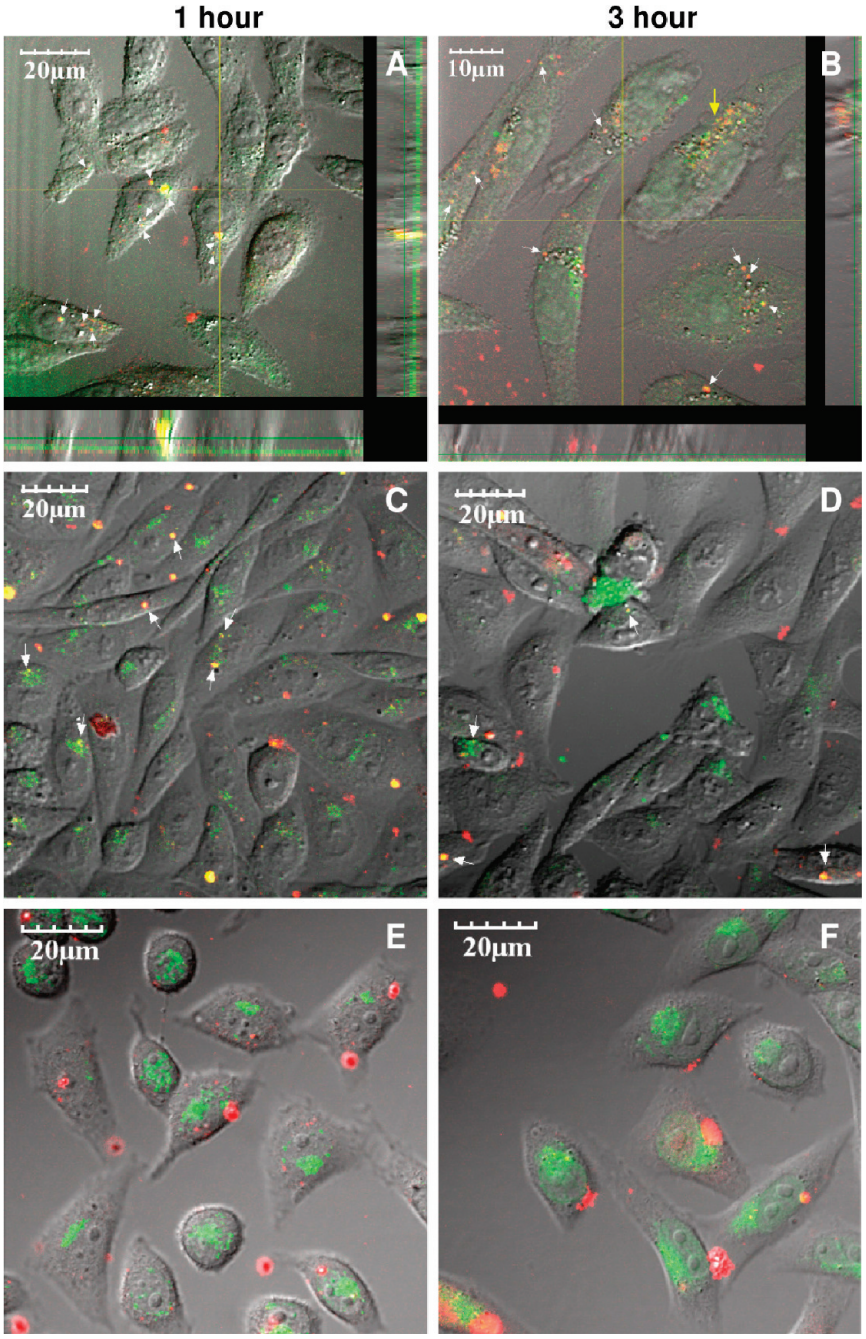

Figure 5. Rhodamine-labeled DNA transfected by amphiphile **1** at 1 h and 3 h in the presence of Cascade Blue (A, B), transferrin (C, D) or Lysotracker (E, F). Red: Rhodamine-labeled GFP-encoding DNA; Green: Cascade Blue (A, B); Alexa Fluor 633-conjugated transferrin (C, D); Lysotracker Blue (E, F). The arrows point out some of the representative examples of colocalization. Note: Pseudocolor was used to enhance contrast and facilitate observation.

The results in Figure 3 suggest that endocytosis is the dominant pathway of cellular uptake, since treatment with NaN_3/2-deoxyglucose almost completely diminished uptake of lipoplexes (>90%). When the clathrin-mediated pathway was blocked (by chlorpromazine or potassium depletion), uptake of lipoplexes decreased about 35% and 60% respectively. Meanwhile, when macropinocytosis was blocked (by hexamethylene amiloride or wortamannin), 44−78% uptake was inhibited. Furthermore, treating with genestein did not affect uptake, suggesting that the caveolae-mediated pathway probably did not play a significant role. The addition of methyl-β-cyclodextrin afforded a reduction in uptake by 85%, confirming the role of cholesterol in the process.

As for transfection efficiencies, endocytosis is the pathway also responsible for transfection as seen after NaN_3/2-deoxyglucose treatment. When the clathrin-mediated pathway was blocked, transfection efficiency only decreased by about 30−35%. In comparison, ≥95% was inhibited when macropinocytosis was blocked. Similar to the uptake study, treating with genestein did not affect transfection. Additionally, the 80% inhibition observed

when treated with methyl-β-cyclodextrin confirmed that membrane cholesterol was also needed for transfection.

The pharmacological interference study showed that macropinocytosis is the major pathway responsible for transfection, while the clathrin-mediated pathway is the minor one. The likely reason why there was more inhibition (~25%) on cellular uptake after potassium-depletion than chlorpromazine treatment is probably that, in the potassium-depletion experiment, cells were transfected for only 1 h instead of 3 h as in all other experiments. As macropinocytosis is a slower process, fewer DNA lipoplexes were taken up by cells in the 1 h period. Wortamannin blocks macropinocytosis by inhibiting phosphoinositide 3-kinase (PI3K), which is one of the key enzymes involved in phosphoinositide metabolism. It has been reported that it can block internalization of known ligands of the clathrin- and caveolae-mediated pathways.[42] In comparison, selective inhibitors of Na^+/H^+ exchange, such as hexamethylene amiloride, may have fewer side effects and should be considered as the first choice for macropinocytosis inhibitors.[40] Thus, the inhibition of cellular uptake by wortamannin can be attributed to its pleiotropic effects on multiple pathways. Both genestein and methyl-β-cyclodextrin inhibitions involve cholesterol, but through different mechanisms. Genestein is a specific inhibitor of tyrosine-specific protein kinases.[43,44] Methyl-β-cyclodextrin can deplete cholesterol from cell membranes rapidly within 1 h by forming soluble inclusion complexes with cholesterol.[45] Even though methyl-β-cyclodextrin is commonly considered a blocker of caveolae-mediated pathway, it can also interfere with the other endocytosis pathways.[41,46−48]

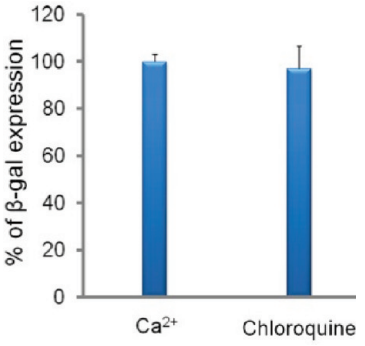

Figure 6. Transfection in the presence of Ca^{2+} or chloroquine compared to untreated cells.

With regard to other studies on the endocytic process of cationic lipid-mediated DNA transfection, the results by the groups of Hoekstra and Conese[39] are particularly relevant. Hoekstra and colleagues demonstrated through a series of potassium depletion, cholesterol dependencies, and transferrin colocalization studies that endocytosis of cationic SAINT/DOPE lipoplexes occurs through the cholesterol-dependent clathrin-mediated endocytosis pathway in the COS-7, CHO, and HepG2 cells.[46] Through pharmacological inhibitor and fluorescence colocalization studies by the Conese group,[39] it was found that, in A549 and HeLa cells, clathrin-mediated endocytosis was also responsible for the cellular uptake of DOTAP lipoplexes, while caveolae-mediated endocytosis was mainly responsible for the uptake of polyethylenimine (PEI) 25K polyplexes. The kinetics of transfection showed that DOTAP lipoplex uptake was a faster process than that of PEI. Since macropinocytosis is a fluid phase uptake, we would expect it to be a slower process. Thus, to further support our findings, transfections were performed after incubating the lipoplexes with cells for varying time periods of 15 min, 30 min, 1 h and 3 h. After the given time, cells were washed with heparin-containing (10 units/mL) PBS to remove membrane bound lipoplexes and a β-galactosidase assay was performed after 48 h. The results (Figure 4) show that minimal gene expression was obtained after a 15 min internalization period. For the 30 min and 1 h incubation times, some expression was observed. After 3 h of exposure, the expression level was maximized and comparable to when the cells were exposed to the lipoplex for 48 h (i.e., unwashed samples). These observations are consistent with our earlier inhibition study results (Figure 3). However, the results serve as a contrast to the clathrin-dependent uptake mechanism of DOTAP-mediated transfection, where 15 min of incubation internalization afforded significant gene expression, with the caveat that these studies have been performed in different cell lines.[39]

Next, laser scanning confocal microscopy was used to directly visualize the intracellular trafficking of the lipoplexes. Rhodamine-labeled GFP-encoded DNA was transfected by amphiphile **1** in the presence of various endocytosis markers. Live cell images taken at 1 and 3 h are shown in Figure 5. Cascade Blue hydrazide

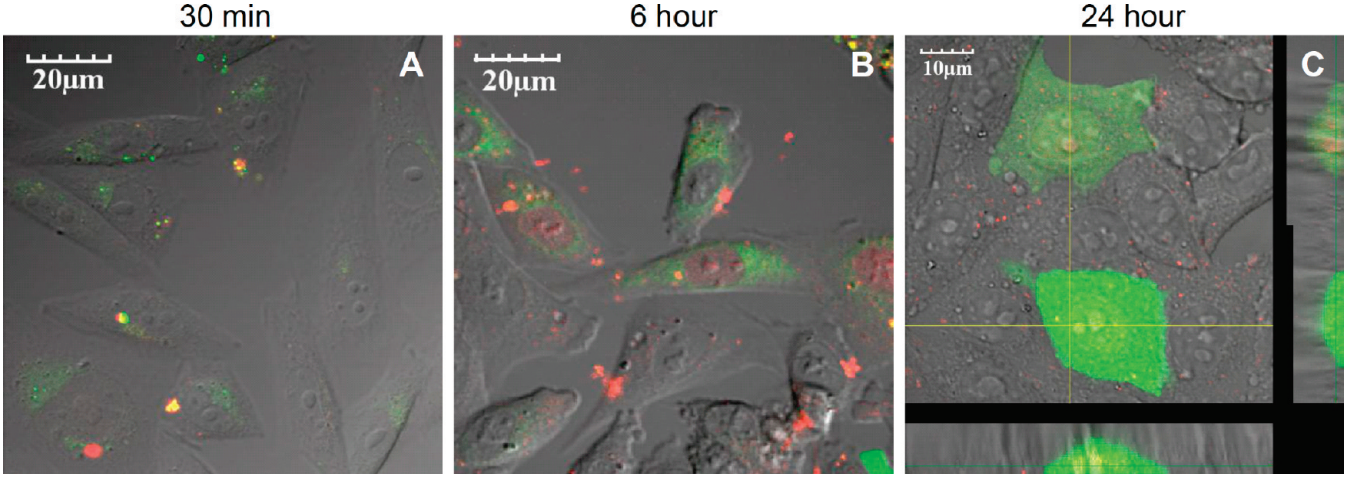

Figure 7. Confocal images of cells at different time points after transfection: (A) 30 min; (B) 6 h; (C) 24 h. Red: rhodamine-labeled GFP-encoding DNA. Green: BODIPY-FL labeled C_{12}-HPC (A and B) or GFP expression (C).

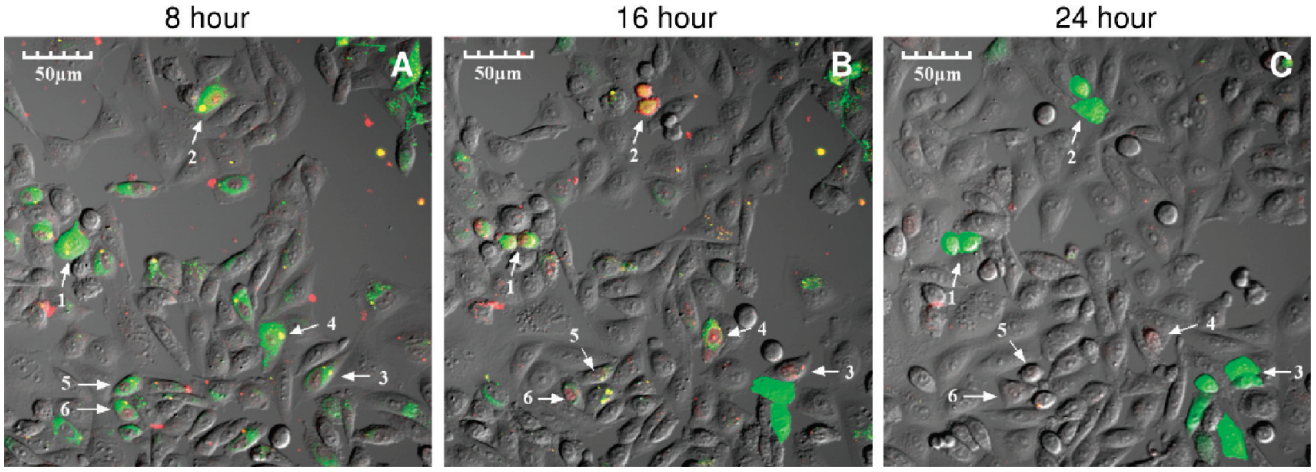

Figure 8. Confocal images of the same area of cells at different time points after transfection: (A) 8 h; (B) 16 h; (C) 24 h. Red: rhodamine-labeled GFP-encoding DNA. Green: BODIPY-FL labeled C_{12}-HPC or GFP expression.

(Invitrogen), a small-molecule fluid-phase uptake marker, was used for macropinocytosis visualization. When cells were coincubated with Cascade Blue, the colocalization of DNA and Cascade Blue was observed (Figures 5A and 5B). Consistent with uptake through constitutive macropinocytosis, not all pinosomes, defined by the presence of Cascade Blue, contained DNA. At 3 h, colocalization of DNA and Cascade Blue was seen in a significant number of vesicles in the cell pointed out by the yellow arrow in Figure 5B. This result supports the conclusion that the lipoplexes were taken up by the macropinocytosis pathway. When cells were coincubated with Alexa Fluor 633-conjugated transferrin (Invitrogen), a common clathrin-mediated endocytosis marker, some colocalization was found, indicating that some lipoplexes were also taken up through this pathway. This observation was consistent with the results from the inhibitor study, confirming that clathrin-mediated endocytosis is partially responsible for the transfection. Next we used Lysotracker Blue DND-22 (Invitrogen), which accumulates in acidic cell organelles, and is primarily a lysosome maker. No colocalization was observed when Lysotracker was incubated with the lipoplex, indicating that the lipoplex did not reach the lysosome, or it escaped the endosome pathway before reaching lysosomes, or it greatly changed the pH of lysosome. Since the amphiphile lacks a mechanism to increase the pH, the last possibility can be excluded. Altogether these observations support the macropinocytosis pathway for cellular uptake for the amphiphile **1**:DNA lipoplex.

To examine whether chloroquine (CQ) or $CaCl_2$ alters gene transfer efficiency, transfection experiments were performed in the presence of these two agents. CQ is a lysosomotropic agent known to improve transfection efficiency[13,49,50] by facilitating DNA release from the endosome by destabilizing the endosome,[51] and/or by inhibiting enzymatic degradation in lysosomes.[52,53] Ca^{2+} has also been shown to increase transfection efficiency by disrupting the endosome/lysosome membrane, thus facilitating DNA to escape lysosomal degradation.[54,55] As shown in Figure 6, neither CQ nor Ca^{2+} had an effect on transfection efficiency. There are three possible reasons for these findings: (1) the lipoplex did not go through the endosome pathway; (2) the lipoplex had its own mechanism to escape lysosomal degradation; and (3) escape of the lipoplex from the endosome/lysosome is not a limiting step in the transfection process, which means that, despite the effects of CQ or Ca^{2+}, the outcome of protein expression is not affected. The first reason can be excluded, since the above data indicates that the endosomal pathway is involved. Although undetermined at this stage which of the last two reasons is accurate, this observation does not contradict our findings obtained from the pharmacological interference and confocal microscopy studies.

In addition to the colocalization study described earlier, to further investigate the intracellular trafficking and transfection kinetics, lipoplexes were prepared from amphiphile **1** with 2 mol % BODIPY FL (Ex/Em: 505/511) labeled C_{12}-HPC and a rhodamine-labeled GFP-encoding DNA. The lipoplexes were then added to cells, and confocal images of live cells were taken at varying time points. As seen in Figure 7A, after 30 min of exposure, the fluorescent signal for DNA and the lipoplex was observed inside the cell cytoplasm. The DNA and amphiphiles existed as punctate structures, presumably endocytic vesicles, where the two fluorescent signals are colocalized. This observation was consistent with the results shown in Figure 4: gene transfer was achieved after 30 min of lipoplex internalization. After 6 h (Figure 7B), the fluorescently labeled DNA was present in the nucleus in a more diffused form, suggesting that it was no longer in a condensed state as in the lipoplex. Meanwhile, the signal for the amphiphile was observed throughout the cytoplasm but not in the nucleus. This indicates that the lipoplexes disassembled and the DNA had dissociated from the lipoplexes before entering the nucleus, such that the fluorescent amphiphiles did not enter the nucleus, with the caveat that we can only detect the BODIPY lipid and it is representative of both lipids used in the study. It is believed that plasmid DNA can enter the nucleus during cell division as the nuclear envelope breaks down temporarily, since dividing cells can exhibit higher transfectability than nonmitotic cells.[56−58] Moreover, direct injection of DMRIE/DOPE lipid/DNA complexes into the cytoplasm or even into the nucleus of *Xenopus* oocytes resulted in negligible levels of gene expression,[59] indicating that DNA may need to dissociate from the lipoplexes for subsequent activity. Figure 6C shows two GFP-expressing cells after 24 h. The fluorescence was observed from both lipids and GFP. Since the fluorescence of GFP was throughout the whole cell and its intensity was much greater than that of the amphiphiles, the fluorescence from the lipid was difficult to observe in these cells. It seemed that DNA

accumulated in round-shaped structures, most probably cell nucleoli. However, by examining the intensity profiles of the rhodamine and GFP fluorescence, it was found that the rhodamine signal in the nucleoli was not significantly higher than that in the adjacent area; instead it was the GFP signal that was much lower in the cell nucleoli (data not shown). This was also observed in an earlier study by S. Subramanian and F. Srienc, who showed that when CHO cells were transfected, GFP was uniformly distributed throughout the cytoplasm and the nucleus except for the nucleoli (which appeared to exclude GFP).[60]

When confocal images were taken at later time points after transfection, additional information was obtained. As shown in Figure 8A at 8 h, some cells were already expressing GFP, such as the cell labeled 1. In other cells, such as cells labeled 2−6, DNA was seen in the nucleus and lipids were seen throughout the cytoplasm. Once again, no lipids were seen in the nucleus. At 16 h (Figure 8B), cells 1 and 2 had divided and no GFP expression was observed. It is important to note that the disappearance of DNA and the lipid fluorescence signals does not necessarily mean that they were transported out of cells; it is possible that this is a result of photobleaching given the continuous imaging of the same area (photobleaching was observed in controls under the same conditions). At 24 h (Figure 8C), the daughter cells of cell 1 were still expressing GFP, while cells 2 and 3 also showed GFP expression after division.

CONCLUSION

In summary, the cellular uptake pathway and intracellular trafficking of a charge-reversal amphiphile:DNA lipoplex were examined. By applying pharmacological interferences, we found that endocytosis is the dominant pathway for the cellular uptake of the DNA lipoplexes. When clathrin-mediated endocytosis was inhibited, transfection decreased by 30−35%. When macropinocytosis was inhibited, 95% of the transfection efficiency was inhibited. Inhibition of caveolae-mediated endocytosis appeared to have no effect on transfection. The results from the time-course study supported the macropinocytosis pathways, as this process is relatively slow compared to the receptor-mediated endocytosis. Using laser scanning confocal microscopy, we found that DNA was colocalized with Cascade Blue (a fluid-phase uptake marker) and transferrin (a clathrin-mediated pathway marker) but not with Lysotracker (an early lysosome marker). This indicated that the DNA lipoplexes were taken up by both macropinocytosis and clathrin-mediated endocytosis. Because macropinosomes are thought to be inherently leaky vesicles compared with other types of endosomes,[61] this pathway may afford greater DNA release into the cytoplasm and less lysosomal degradation. Recently, Izumisawa et al. reported cell line-dependent internalization pathways of a decaarginine-PEG-lipid (R10B) delivered DNA.[62] The R10B-lipoplex was internalized primarily through clathrin-mediated endocytosis in PC-3 and KB cells, and macropinosytosis in HeLa cells. The highest transfection efficiency by the R10B-lipoplex was observed in HeLa cells.

Further cellular trafficking studies by confocal microscopy indicated that the DNA dissociated from lipoplexes before entering cell nuclei and that the lipids did not enter through the nuclear envelope. Additionally, DNA entering the nucleus seemed not to guarantee protein expression.

Cellular uptake and endosome escape have been two of the major hurdles in gene transfer, and various methods have been used to overcome them. Many cationic lipids have been found to enter the cells by clathrin-mediated endocytosis, such as DOTAP,[39] lipopolylysine/DOPE[63] and SAINT-2/DOPE.[46] In the present study, we showed that macropinocytosis was the major pathway leading to transfection by a charge-reversal amphiphile in CHO cells. This finding illustrates the importance of macropinocytosis in cellular trafficking of cationic lipoplexes and may provide an alternative strategy in the rational design of additional transfection agents.

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