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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 co-localization study was used to visualize the DNA lipoplex uptake pathways. The co-localization of the DNA lipoplex and Cascade Blue[®], a fluid-phase uptake marker, was observed. Meanwhile, the co-localization of the DNA lipoplex and transferrin, a clathrin-mediated endocytosis marker, was also seen. However, no co-localization 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 both at the research and clinical level. Traditionally, DNA delivery has been classified into two major categories based on the type of delivery vector: *viral*-mediated¹⁻⁵ and *non-viral* (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, non-viral 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 stimuli-responsive vectors. In the first approach, zwitterionic helper lipids such as dioleoylphosphatidylethanolamine (DOPE)¹¹ and its derivatives¹² 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 stimuli-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 histidines²⁷ 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 efficiency²⁸. 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.

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 procedure²⁸ 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 pGeneGripTM rhodamine-labeled β -galactosidase or GFP-encoding DNA and CellScrubTM buffer was purchased from Gene Therapy Systems (San Diego CA). Alexa Fluor[®] 647-conjugated dextran (Mw=10,000; Ex/Em: 647/670), Cascade Blue[®] hydrazide (Ex/Em: 402/420), Alexa Fluor[®] 633-conjugated transferrin (Ex/Em: 632/647), LyotrackerTM Blue DND-22 (Ex/Em: 373/422) and BODIPY[®] FL (Ex/Em: 505/511) labeled C₁₂-HPC were purchased from Invitrogen (Calsbad 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 unit/mL of penicillin, 100 μ g/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% CO₂ humidified incubator at 37 °C.

Transfection and Viability Assays

Generally, 24 hours 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 media was removed and 20 μ L of serum-free media containing lipoplexes, which were composed 0.5 μ g psv- β -galactosidase control vector (Promega, Madison WI) and amphiphile **1** at a 1:5 charge ratio, was added. After incubation for 3 hours, 300 μ L of fresh serum-containing media was added and the cells were incubated for another 48 hours. Cell viability was measured using Cell Titer 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.

Inhibitors Treatment

The pharmacological inhibitors were used at the following concentrations: sodium azide 0.01%, 2-deoxyglucose 20 mM, chlorpromazine 10 μ M, hexamethylene amiloride 20 μ M, wortmannin 10 μ M, genestein 50 μ M, 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 hour at 37 °C and transfected with the lipoplexes in the presence of inhibitors for 3 hours at 37 °C. Then the media was removed and cells were washed 3 times with PBS and cultured in fresh growth media in the absence of inhibitors for 48 hours 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 CaCl₂, 1 mM MgCl₂, 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 hour 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 media was added.

Flow Cytometry

24 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 hour as described above. Then, lipoplexes containing 5 μ g rhodamine-labeled β -gal in 1 mL serum-free medium were added. After transfection for 3 hours, the cells were washed twice with PBS before they were incubated or 20 minutes in CellScrub[™] buffer to remove membrane-attached lipoplexes. Subsequently, the cells were washed twice with PBS, trypsinized, washed again with PBS, and re-suspended 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 were used as controls.

Transfection Kinetics Study

Cells were transfected using the lipoplex for 15 minutes, 30 minutes, 1 hour or 3 hours 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 hours prior to β -galactosidase expression assay.

Laser Scanning Confocal Microscopy

48 hours before transfection, 1.5×10^5 cells were seeded on a collagen-coated glass bottom 35 mm dish (MatTek, Ashland MA). Cells were then transfected by lipoplex with 4 μ g rhodamine-labeled GFP-encoding DNA in 1 mL of serum-free media supplemented with 2 mM HEPES. After 3 hours, 2 mL of fresh growth media 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 photo-toxicity 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 μ M chloroquine was present in the transfection media in the 3 hour period. In the former case, 1 mM CaCl_2 was also present in the growth media in the 48 hour 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 distinguished³²⁻³⁶ and their characteristics are described briefly as follows: (1) Phagocytosis, which occurs only in specialized cells such as macrophages, neutrophils 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 μ m) 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 pathway not usually classified by the above criteria. Many of their regulatory mechanisms are actively investigated and still unknown.

Pharmacological inhibitors have been widely used to study endocytic pathways³⁷⁻⁴¹. 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 period⁴⁰. 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 hours and then recording data 48 hours after inhibitor treatment. Inhibitor concentrations that showed at least 85% cell viability compared to non-treated cells were used for the cellular uptake and transfection studies. For cellular uptake experiments, generally, cells were pre-incubated in the presence of inhibitors for 1 hour. After transfection with rhodamine-labeled DNA lipoplexes for 3 hours, during which inhibitors were also present, transfection media was removed and cells were washed and analyzed by flow cytometry. The cellular uptake data were expressed as the percentage of DNA uptake compared to no inhibitor-treated cells. For transfection experiments, β -galactosidase expressions were determined 48 hours after cells were transfected for 3 hours. The transfection efficiencies were reported as the percentage of β -galactosidase expression compared to no inhibitor-treated cells.

The results in Figure 3 suggest that, endocytosis is the dominant pathway of cellular uptake, since treatment with $\text{NaN}_3/2$ -deoxyglucose almost completely diminished lipoplexes uptake (>90%). When clathrin-mediated pathway was blocked (by chlorpromazine or potassium depletion), lipoplexes uptake decreased about 35% and 60% respectively. Meanwhile, when macropinocytosis was blocked (by hexamethylene amiloride or wortmannin), 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 $\text{NaN}_3/2$ -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 clathrin-mediated pathway is the minor one. The likely reason 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 hour instead of 3 hours as in all other experiments. As macropinocytosis is a slower process, less DNA lipoplexes were taken up by cells in the 1 hour period. Wortmannin 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 inhibitors⁴⁰. Thus, the inhibition of cellular uptake by wortmannin 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 hour 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}

With regards to other studies on the endocytic process of cationic lipid-mediated DNA transfection, the results by the groups of Hoekstra and Conese³⁹ are particularly relevant.

Hoekstra and colleagues demonstrated through a series of potassium depletion, cholesterol dependencies, and transferrin co-localization 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 co-localization 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 minutes, 30 minutes, 1 hour and 3 hours. After the given time, cells were washed with heparin-containing (10 unit/mL) PBS to remove membrane bounded lipoplexes and a β -galactosidase assay was performed after 48 hours. The results (Figure 4) show that minimal gene expression was obtained after a 15 minutes internalization period. For the 30 minutes and 1-hour incubation times, some expression was observed. After 3 hours of exposure, the expression level was maximized and comparable to when the cells were exposed to the lipoplex for 48 hours (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 minutes 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 hour and 3 hours are shown in Figure 5. Cascade Blue[®] hydrazide (Invitrogen), a small-molecule fluid-phase uptake marker, was used for macropinocytosis visualization. When cells were co-incubated with Cascade Blue[®], the co-localization of DNA and Cascade Blue[®] was observed (Figure 5A and 5B). Consistent with uptake through constitutive macropinocytosis, not all pinosomes, defined by the presence of Cascade Blue[®], contained DNA. At 3 hours, co-localization 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 co-incubated with Alexa Fluor[®] 633-conjugated transferrin (Invitrogen), a common clathrin-mediated endocytosis marker, some co-localization was found, indicating 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 co-localization 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. All together these observations support the macropinocytosis pathway for cellular uptake for the amphiphile **1**:DNA lipoplex.

To examine whether chloroquine (CQ) or CaCl₂ alter 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}. Ca²⁺ 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 co-localization study described earlier, to further investigate the intracellular trafficking and transfection kinetics, lipoplexes were prepared from amphiphile **1** with 2mol% 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 minutes 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 co-localized. This observation was consistent with the results shown in Figure 4: gene transfer was achieved after 30 minutes of lipoplex internalization. After 6 hours (Figure 7B), the fluorescently labeled DNA were 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 non-mitotic 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 expression⁵⁹, indicating that DNA may need to dissociate from the lipoplexes for subsequent activity. Figure 6C shows two GFP-expressing cells after 24 hours. 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 hours, 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 hours (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 same conditions). At 24 hours (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 co-localized 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 DOTAP³⁹, lipopolylysine/DOPE⁶³ 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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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

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.

Figure 5.

Rhodamine-labeled DNA transfected by amphiphile **1** at 1hr and 3hr 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 co-localization. Note: pseudo-color was used to enhance contrast and facilitate observation.

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

Figure 7. Confocal images of cells at different time points after transfection: A. 30min; B. 6 hour; 24 hour. Red: rhodamine-labeled GFP-encoding DNA; Green: BODIPY-FL labeled C₁₂-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 hours; B. 16 hours; C. 24 hours. Red: rhodamine-labeled GFP-encoding DNA; Green: BODIPY-FL labeled C₁₂-HPC or GFP expression.

Table 1
Inhibitors used and targeted endocytosis pathways

NaN ₃ /2-deoxyglucose	general endocytosis inhibitor
chlorpromazine potassium depletion	clathrin-mediated
hexamethylene amiloride wortmannin	macropinocytosis
genistein	caveolae-mediated
methyl- β -cyclodextrin	cholesterol dependence