Relating Toxicity to Transfection: Using Sphingosine To Maintain Prolonged Expression in Vitro

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ABSTRACT: Cationic reagents are commonly used to facilitate DNA delivery, and transfection experiments are typically initiated in cell culture where the optimal charge ratio is determined. While transfection rates are often enhanced at higher +/− charge ratios, the cellular toxicity associated with the greater amounts of cationic components at elevated charge ratios is often not considered. In addition, the prolonged effects of cationic lipid uptake on cell viability are not evident in a typical 24–48 h transfection experiment. In this study, we compare the transfection efficiency of cationic lipoplexes to effects on viability of cultured cells in both the short and long term (7 days). Our results indicate that, while minimal toxicity is evident 24 h after exposure to DOTAP-based lipoplexes, cell viability continues to decline and ultimately compromises reporter gene expression at longer times. Substitution of a naturally occurring cationic amphiphile, sphingosine, for DOTAP greatly reduces toxicity and allows high expression to be maintained over prolonged periods.

KEYWORDS: transfection, DOTAP, sphingosine, toxicity, viability, cationic lipid

INTRODUCTION

Cationic lipids and polymers are commonly used to facilitate the delivery of nucleic acids into cells both in vitro and in vivo.1−3 Initial attempts to utilize traditional liposomes to deliver nucleic acids were hampered by very poor association between DNA and neutral or anionic liposomes.4−6 However, further studies demonstrated that much higher rates of association and delivery could be achieved when cationic lipids were employed.7,8 Subsequent studies over the past 25 years have explored the ability of thousands of different cationic materials to bind DNA and facilitate intracellular delivery. Although the ultimate goal of such studies is to develop pharmaceutical agents that could be used to deliver therapeutic polynucleotides (e.g., genes, siRNA, miRNA, aptamers), the initial screening to identify promising cationic agents is typically conducted in cell culture. Accordingly, in vitro transfection rates are used to characterize the delivery efficiency of experimental formulations, and the vast majority of published studies rely solely on cells in culture to assess the potential of novel cationic agents to facilitate intracellular delivery. In addition to the cell type employed for such studies, it is well-recognized that the mixing conditions, charge ratio, particle size, zeta potential, and presence of serum can also have dramatic effects on the transfection rates observed in such experiments.9−14

While many studies continue to investigate the potential of new cationic lipids, it has long been recognized that the incorporation of neutral “helper” lipids into lipoplexes greatly enhances delivery efficiency.15−18 Early studies tested the ability of many different neutral lipids to serve as helper lipids, but cholesterol and dioleoylphosphatidylethanolamine (DOPE) have proven to be consistently effective, and they are predominantly utilized in cationic lipid formulations designed for delivery. The mechanism by which helper lipids enhance delivery has been attributed to their ability to expedite the formation of the inverted hexagonal phase: a non-bilayer lipid structure which is thought to promote fusion with the endosomal membrane and thereby facilitate delivery to the cytoplasm.19 The incorporation of helper lipids also affects the charge density of the bilayer, which undoubtedly alters DNA binding and release.13 In addition, studies have shown that cholesterol enables gene delivery vehicles to resist the destabilizing effects of serum.10,13,18 Furthermore, recent work from our laboratory has documented the formation of cholesterol domains at very high levels of cholesterol that enhance transfection rates in vitro and in vivo.20−25

Despite all the progress surrounding the use of cationic agents to facilitate the intracellular delivery of nucleic acids, the toxicity of cationic materials remains a major concern. While toxicity is less of an issue for reagents designed solely for use in cell culture (e.g., to investigate the effect of specific sequences or chemical modifications of the nucleic acid), the ultimate use of delivery technology for therapeutic applications in vivo requires careful monitoring of cellular toxicity. Toxicity is...
important not only for minimizing adverse effects in vivo (e.g., elevated liver enzymes) but also to ensure that the viability of cells which have been successfully transfected is not compromised by vehicle toxicity. However, most investigations of nucleic acid delivery focus predominantly on achieving the highest possible transfection rates, and studies frequently do not consider the toxicity associated with the delivery vehicle. Furthermore, it is possible that the same effects responsible for the observed enhancement in transfection rates (e.g., endosomal escape, membrane permeation, impaired lysosomal function) also compromise cell viability. This study investigates the effects of vehicle toxicity on transfection in human breast cancer cells (MCF-7) and demonstrates that acute toxicity can correlate positively with transfection in a typical cell culture experiment. However, in vivo applications will likely require prolonged expression of the therapeutic gene, and thus we investigate the longer term effects of toxicity on reporter gene expression. Finally, we consider the use of a naturally occurring cationic amphiphile, sphingosine, as a less toxic alternative to traditional transfection reagents.

**MATERIALS AND METHODS**

**Lipoplex Preparation.** Cholesterol, sphingosine, 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP), and 1,2-diarachidoyl-sn-glycero-3-phosphocholine (DAPC), and 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(7-nitro-2-1,3-benzoxadiazol-4-yl) (NBD-DOPE), were purchased from Avanti Polar Lipids (Alabaster, AL). Lipid formulations were prepared as previously described. Lipoplexes were then prepared at different +/− charge ratios by mixing equal volumes of a modified (CMV removed, ROSA26 added, based upon Watcharanurak et al.26) pSelect-LucSh (Invivogen, San Diego, CA) plasmid encoding luciferase with the suspended liposomes as previously described.20 Lipoplexes were then prepared at different +/− charge ratios by mixing equal volumes of a modified (CMV removed, ROSA26 added, based upon Watcharanurak et al.26) pSelect-LucSh (Invivogen, San Diego, CA) plasmid encoding luciferase with the suspended liposomes as previously described.20 These modifications to the plasmid have been shown to prolong expression for weeks to months.20 Lipofectamine 2000 was purchased from Invitrogen (Carlsbad, CA) and used according to the manufacturer’s instructions. Polyethylenimine (PEI, MW 70K) was obtained from Polysciences Inc. (Warrington, PA) and used at an N/P = 7 as described in our previous work.27

**Transfection Protocol.** MCF-7 cells (American Type Culture Collection # HTB-22; human breast adenocarcinoma cells) were cultured at 37 °C, 5% carbon dioxide with 100% humidity in minimum essential medium (MEM), 10% fetal bovine serum (FBS), 50 U/mL penicillin, and 50 μg/mL streptomycin (all media from Cellgro MediaTech Inc., a Corning Acquisition, Manassas, VA) as previously described.25 For acute transfection experiments, cells were seeded at 20,000 cells/well in 96-well plates 24 h prior to treatment. For studies looking at prolonged effects on transfection and toxicity, cells were seeded at 5,000 cells/well in 96-well plates in order to delay overcrowding and treated as above. We chose this number to stay sufficiently dense in the early time points for the viability assay (MTT assay recommends between 5 and 20,000 cells/well), but also to prevent overcrowding at the later time points.
Lipoplexes were preincubated 1:1 v/v in FBS (i.e., 50% FBS to mimic in vivo serum protein conditions) for 30 min prior to dilution in 100% MEM, and then administered to cells for transfection. Formulations were applied to the center of each well and allowed to incubate for 4 h as described previously.25 After 4 h, the plates were spun at 800 rpm for 15 min in order to ensure that all dead cells were sedimented. The transfection medium was then carefully removed so as to not disturb the cell layer, a PBS wash was added to clear any unassociated particles, and then the spin was repeated prior to removal of the PBS wash. The cells were then returned to 10% FBS growth medium. For each day of the long-term experiments, plates were spun and "washed" as described above, and medium was replaced. This approach allowed us to maintain cells in fresh medium for the duration of the experiment and ensure that all dead cells could be accounted for in the daily viability assays. A separate plate of cells was used for each time point, and plates were spun and washed again as described above and then lysed with 30 μL of Promega lysis buffer in the −80 °C freezer according to the manufacturer’s instructions (Promega, Madison, WI). Lysate was assayed for protein content with a Bio-Rad protein assay (Bio-Rad, Hercules, CA) according to the manufacturer’s instructions (Promega, Madison, WI). Lysate was assayed for protein content with a Bio-Rad protein assay (Bio-Rad, Hercules, CA) according to the manufacturer’s instructions (Promega, Madison, WI).

Figure 3. Effect of complex concentrations on transfection and toxicity in MCF-7 cells after exposure to 50% FBS. Increasing quantities of DOTAP:cholesterol 1:4 (A), cholesterol:DOTAP:DAPC 2:3:5 (B), and DOTAP:cholesterol 1:1 (C) lipoplexes (+/− = 4) were used. Similar experiments using complexes prepared with PEI (D) and lipofectamine (E) are shown for comparison. Luciferase expression (closed squares) and viability (closed circles) were determined. Symbols represent the mean ± one standard deviation from replicate samples, n = 6.

The image contains a figure with graphs depicting the effect of complex concentrations on transfection and toxicity in MCF-7 cells after exposure to 50% FBS. The graphs show the transfection efficiency and viability of cells treated with different lipoplex concentrations. The figure includes comparisons with PEI and lipofectamine for reference. The text also describes the experimental procedures, including preincubation, transfection, and viability assays.
The cells were then counted using a BD FACScalibur (BD Bioscience, San Jose, CA) with the excitation set at 488 nm and the emission at 530 nm. The mean fluorescence intensity was calculated by FlowJo software.

**Cell Viability.** The Invitrogen MTT Assay was used for viability measurements of both the short-term and long-term in vitro experiments. The assay was run per the manufacturer’s instructions, but the cell culture medium was removed and cells were “washed” with PBS (with 800 rpm spins to sediment cells each day as described above). Then 10 μL of 12 mM MTT solution diluted in 100 μL of phenol red-free DMEM was added to each well. The cells were then incubated for 4 h at 37 °C before a 10% SDS, 0.01 M HCl solution was added to each well to dissolve the complex. The plates were again incubated for 4 h at 37 °C, and then read by the THERMOMax plate reader (Molecular Devices, Sunnyvale, CA) at 570 nm.

### RESULTS

Figure 1 depicts a typical experiment wherein the +/− charge ratio is increased from 1 to 8 by incorporating more cationic component into formulations while maintaining a constant amount of plasmid DNA in each preparation. We observe a progressive increase in transfection rate at higher charge ratios that is typically observed when such experiments are used to identify promising formulations. However, transfection rates often plateau at very high charge ratios (+/− = 8), and can even be reduced in some formulations (Figure 1). Even formulations that do not offer improved transfection as charge ratios are increased (e.g., DOTAP:cholesterol 1:1) often exhibit a reduction at the highest charge ratio. It might be expected that very high charge ratios result in excess cation that is not incorporated into the complexes, and previous studies have suggested that the free cationic lipids present at high charge ratios actually contribute to the observed transfection.

To assess the effect of free cationic lipids on transfection, we utilized a low concentration of lipoplexes that exhibits minimal transfection, and added progressively greater amounts of free cationic liposomes to the cell culture medium. As shown in Figure 2, the addition of free cationic lipids progressively increased transfection rates with the two formulations that displayed a positive correlation of transfection with charge ratio (i.e., DOTAP:cholesterol 1:4, cholesterol:DOTAP:DAPC 2:3:5).

Figure 4. Effect of time on expression and viability. Cells transfected (+/− = 4; 3.75 μM) with DOTAP:cholesterol 1:4 (A), cholesterol:DOTAP:DAPC 2:3:5 (B), DOTAP:cholesterol 1:1 (C), PEI (D), and lipofectamine (E) complexes were monitored over 7 days. Luciferase expression (closed squares) and viability (closed circles) were determined. Symbols represent the mean ± one standard deviation from replicate samples, n = 6.
Curiously, free cationic lipids had mixed effects on transfection rates when the lipoplex was formulated at low +/− charge ratios (i.e., 1, 2; data not shown).

In attempting to maximize transfection efficiency in cell culture, researchers typically formulate lipoplexes at the optimal charge ratio (in terms of transfection) and treat cells with progressively higher doses of lipoplexes. As shown in Figure 3, transfection increases at higher doses; this is not surprising considering that cells are administered proportionally greater amounts of the reporter gene at the higher doses. However, because the charge ratio is held constant, each successive increase in the amount of plasmid is accompanied by a corresponding increase in cationic lipid, and thus viability is progressively compromised. As might be expected, transfection efficiencies of all three DOTAP formulations are also compromised at very high doses that result in viabilities approaching 60% (Figure 3). For comparison, transfection mediated by PEI and lipofectamine (Figure 4d,e) shows a more dramatic reduction at high levels of cation due to the greater toxicity of these reagents.

The data shown above are somewhat counterintuitive in that larger amounts of cationic lipid can result in higher transfection despite exhibiting greater toxicity. It is only when viability is severely compromised (i.e., ≈60%) that we observe a decrease in transfection. However, it is important to realize that these experiments were all conducted over a very short time frame (≈24 h), and thus additional effects might be observed at longer times. Accordingly, cells in culture treated with lipoplexes in the same manner as the previous experiments were monitored for a week to observe changes in reporter gene expression and viability over time. As shown in Figure 4, both luciferase expression and viability decreased progressively over time in all DOTAP formulations. For comparison, lipoplexes formulated with PEI or lipofectamine caused much greater losses in viability and correspondingly lower transfection rates (Figure 4d,e). It is important to point out that our experimental protocol involved centrifugation with each medium change to ensure that dead/detached cells are not excluded from the analysis. Cells treated with PBS maintained high viability throughout the experiment, and the MTT signal from PBS-treated cells was used to standardize the signal from cells exposed to lipoplexes.

It is well-recognized that different cationic lipids exhibit varying degrees of potency with regard to transfection, but also with regard to toxicity. Most of our previous work has employed DOTAP, which is considered a lipid with relatively low toxicity that has been employed in clinical trials. However, the data in Figures 1−4 indicate that even DOTAP exhibits considerable toxicity under the conditions which provide maximum transfection, especially when the effects of lipoplex exposure on toxicity are monitored over extended periods (Figure 4). Considering the progressive loss of viability over time, it follows that prolonged expression in vivo might best be achieved by employing a less toxic cationic agent. Although any cationic agent would be expected to exhibit some toxicity, especially at high concentrations, previous reports have shown that sphingosine forms complexes with DNA and can facilitate transfection. Considering that sphingosine is a natural degradation product of ceramide that is present in all mammalian cells, it would be expected that this single-tailed cationic amphiphile might be well-tolerated. Accordingly, we substituted sphingosine for DOTAP in the formulations, and monitored the effect of +/− charge ratio on transfection and toxicity. As shown in Figure 5, both transfection and toxicity were comparable to those observed with lipoplexes formulated with DOTAP (compare to Figures 1 and 3). Similarly, increasing the dose of lipoplexes formulated with sphingosine instead of DOTAP resulted in comparable trends in transfection and viability after a 24 h experiment (compare Figures 3 and 6). However, in contrast to the progressive reduction in reporter gene expression and viability over time observed with DOTAP-containing lipoplexes, reporter gene expression and viability were relatively constant after exposure to lipoplexes formulated with sphingosine (Figure 7). Because the dose of lipoplexes clearly plays a role in toxicity (Figures 3, 5), cell viability after treatment with different doses of lipoplexes formulated with DOTAP and sphingosine were directly compared over time (Figure 8). The results clearly demonstrate that although acute toxicities of the formulations (i.e., after 24 h) were comparable, significant...
differences between DOTAP and sphingosine are observed over time. More specifically, the viability of cells transfected by lipoplexes formulated with DOTAP progressively declined with time, whereas those transfected with sphingosine-containing lipoplexes maintained viabilities comparable to that seen after the initial exposure.

It should be noted that, in addition to the differences in the hydrophobic characteristics between the two cationic agents (i.e., two vs one carbon chain), DOTAP is a quaternary amine with a permanent positive charge whereas sphingosine possesses a primary amine with a $pK_a$ of 6.6. Therefore, only $\approx 14\%$ of sphingosine molecules would be expected to be charged at physiological pH (7.4), and this might be expected to result in marked differences in particle size, zeta potential, and serum stability as compared to DOTAP. Not surprisingly, we do observe differences in certain formulations, but there are no consistent differences across all formulations that can explain the stark differences in toxicity over time (Table 1). Similarly, reduced cell uptake of sphingosine formulations could potentially explain their lower toxicity, so experiments were conducted to assess cell uptake via flow cytometry. The results do indicate a consistent reduction in cell uptake of sphingosine as compared to DOTAP formulations, which may contribute to the observed reduction in toxicity (Figure 9). However, one would expect that reduced uptake would result in lower transfection rates with sphingosine, but this effect was not observed. Furthermore, it is important to note that differences in zeta potential, both before and after serum incubation, were negligible, suggesting that the observed reduction in the uptake of sphingosine lipoplexes was not due to reduced electrostatic interactions with cells. Taken together, we feel that it is unlikely that the differences in particle size, zeta potential, serum stability, and cell uptake can explain the clear differences in toxicity over time between DOTAP and sphingosine lipoplexes.
**DISCUSSION**

It is well-known that $+/-$ charge ratio has a significant impact on transfection rates both in cell culture and *in vivo*. This is typically one of the first variables that is optimized when researchers investigate the potential of a gene delivery system, and greater transfection rates are typically observed as $+/-$ is increased (Figure 1). While this effect is often explained as due to an increased charge on the nanoparticle, it can be reasoned that the electrostatic interaction of the nucleic acid with the cation must eventually become saturated, and thus free cation will likely exist at very high charge ratios (e.g., $+/- > 4$). The role of this free cation is not generally appreciated, and previous studies have shown that lipoplexes that are separated from free cationic liposomes possess lower transfection efficiencies. Our data are consistent with these findings and illustrate that transfection rates can be enhanced by the presence of free cationic liposomes (Figure 2). Considering the well-established toxicity of cationic lipids, it is not surprising that increasing doses of lipoplexes compromise cell viability (Figure 3). The fact that transfection rates continue to increase despite reductions in cell viability suggests that the processes that allow for cation-mediated transfection might be related to the mechanism of toxicity (compare Figures 1 and 3), and thus a reduction in transfection is only observed when cell viability is drastically compromised (e.g., $≈ 60\%$). Previous studies with cationic polymers have reported a similar correlation between toxicity and transfection. It is tempting to speculate that the disruption of the endosomal pathway that facilitates intracellular delivery may also be detrimental for cell viability. Indeed, it is well-established that hydrophobic cations can perturb the endosomal/lysosomal pathway, and that the rupture of organelles that promotes delivery can also elicit a toxic inflammatory cascade. In this context, the data in Figure 4 demonstrate that cell viability continues to decline for 7 days, even though the toxic agent (i.e., cationic lipid or PEI) was removed from the medium in the early stages of the experiment (after 4 h). These findings illustrate that exposure to cationic lipids has a prolonged, detrimental effect on cell viability that ultimately compromises transfection when considered over.

![Figure 8](dx.doi.org/10.1021/mp500604r/Mol.Pharmaceutics.2015,12,264-273)
Table 1. Physical Characteristics of Lipoplexes before and after Serum Exposure

| Formulation                  | Size (nm) | Zeta Potential |
|-----------------------------|-----------|----------------|
|                            | MEM       | FBS            |
| 1:1 DOTAP:cholesterol       | 308 ± 17  | 284 ± 12       |
| 1:4 DOTAP:cholesterol       | 189 ± 17  | 196 ± 14       |
| 2:3:5 cholesterol:DOTAP:DAPC| 218 ± 26  | 321 ± 9        |
| 1:1 sphingosine:cholesterol | 478 ± 30  | 455 ± 12       |
| 1:4 sphingosine:cholesterol | 242 ± 23  | 379 ± 20       |
| 2:3:5 cholesterol:sphingosine:DAPC | 229 ± 31 | 363 ± 15       |

The particle size and zeta potential of lipoplexes incubated in MEM media or 50% FBS. Values are the average ± one standard deviation from replicate samples, n = 3.

Figure 9. Effect of cationic lipid and cholesterol content on cell uptake. MCF-7 cells treated with DOTAP-containing (dark bars) or sphingosine-containing (light bars) lipoplexes were assessed for lipoplex uptake after 4 h by flow cytometry. The x-axis shows the ratios of components described in Figures 1 and 5. Symbols represent the mean ± one standard deviation from replicate samples, n = 3.

extended periods. It follows that achieving prolonged expression of a therapeutic gene will require reagents that not only efficiently transfect cells but also possess minimal toxicity from which transected cells can recover.

Considering the well-known toxicity associated with cationic agents, we investigated the potential to utilize sphingosine, a naturally occurring cationic amphiphile that has been shown to bind DNA and facilitate transfection.33–36 Because sphingosine is generated in lysosomes as a breakdown product of ceramide, cells have mechanisms for degrading/eliminating this molecule, thereby minimizing its toxic effects.37,38 Addition of free, DMSO-solubilized sphingosine to cells is known to activate cathepsin D and trigger apoptosis, but this activation is not observed when sphingosine is incorporated into bilayers.47,48 Accordingly, we reasoned that inclusion of sphingosine into lipoplexes would reduce its potential for triggering cell death, and the lower toxicity we observe is consistent with this suggestion. In comparison to DOTAP, lipoplexes formulated with sphingosine exhibited markedly less toxicity at prolonged times, regardless of the neutral lipid content (Figure 8). In addition, the transfection we observe when lipoplexes are formulated with sphingosine is comparable to that with DOTAP, and thus the lower toxicity of sphingosine offers a significant advantage.

The formulations in this study include either DOTAP or sphingosine in different combinations with neutral lipids. Our previous studies have shown that increasing the cholesterol content (from a DOTAP:cholesterol mole ratio of 1:1 to 1:4) results in significantly enhanced transfection in a variety of cell lines.20,23,25,26 Furthermore, we have documented the formation of a cholesterol domain at the higher cholesterol content, and this is believed to be responsible for the increased efficacy both in vitro and in vivo.20–23,25 Biophysical studies employing liposomes have demonstrated that formation of a cholesterol domain can be facilitated by the incorporation of long, saturated acyl chains.49 We recently investigated this strategy for promoting cholesterol domains and established that the incorporation of saturated, long chain phosphatidylcholines (≥20 carbons) enables domain formation at cholesterol contents as low as 10%.25 Consistent with our previous work at higher cholesterol contents, formation of a domain within lipoplexes incorporating saturated, long chain PCs correlated with a marked increase in transfection.25 The precise mechanism by which cholesterol domains enhance transfection has not been fully elucidated, but may involve differences in trafficking, endosomal escape, and/or nuclear delivery. In the current study, lipoplexes formulated with DAPC (20:0 phosphatidylcholine) and 20% cholesterol exhibited transfection rates comparable to the lipoplexes containing 80% cholesterol, and greater than that observed with lipoplexes lacking a cholesterol domain (i.e., DOTAP:cholesterol 1:1). In each of the three formulations possessing different neutral lipid content, substitution of sphingosine for DOTAP did not dramatically alter transfection rates, although distinct differences in toxicity between these two cationic agents were observed (Figure 8). The observed lower toxicity of sphingosine as compared to DOTAP is somewhat unexpected in light of reports that single-tailed cationic amphiphiles are more toxic than those possessing two acyl chains.44

In conclusion, these results illustrate a counterintuitive effect of increasing transfection efficiency at higher charge ratios despite greater toxicity. Transfection efficiency was only compromised when cell viability was decreased to approximately 60%, i.e., at very high charge ratios, at high lipoplex concentrations, or after prolonged experimental times. Studies monitoring cell viability over time demonstrate that cationic lipid toxicity can be minimal on the time frame of a typical overnight transfection experiment, but that the toxic effects of cationic lipids become more evident at later times. We suggest that decreased cell viability may limit prolonged expression of therapeutic genes, and that this may be important for in vivo applications. Furthermore, formulations incorporating sphingosine as the cationic agent offer transfection efficiencies comparable to DOTAP, but with much lower toxicities, at least in cell culture. Future studies will investigate the ability of sphingosine formulations to transfect cells and maintain prolonged expression in vivo.
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**REFERENCES**

(1) Xu, L.; Anchordoquy, T. J. Drug Delivery Trends in Clinical Trials and Translational Medicine: challenges and opportunities in the delivery of nucleic acid-based therapeutics. *J. Pharm. Sci.* 2011, 100, 38–52.

(2) Smyth Templeton, N. Cationic liposomes as in vivo delivery vehicles. *Curr. Med. Chem.* 2003, 10, 1279–1287.

(3) Maurer, N.; Wong, K. F.; Stark, H.; Louie, L.; McIntosh, D.; Wong, T.; Scherrer, P.; Semple, S. C.; Cullis, P. R. Spontaneous entrapment of poly nucleotides upon electrostatic interaction with ethanol-stabilized cationic liposomes. *Biochem. J.* 2001, 380 (5), 2310–2326.

(4) Fraley, R.; Subramani, S.; Berg, P.; Papahadjopoulos, D. Introduction of liposome-encapsulated SV40 DNA into cells. *J. Biol. Chem.* 1980, 255 (21), 10431–10435.

(5) Fraley, R.; Straubinger, R. M.; Rule, G.; Springer, E. L.; Papahadjopoulos, D. Liposome-mediated delivery of deoxyribonucleic acid to cells: enhanced efficiency of delivery related to lipid composition and incubation conditions. *Biochemistry* 1981, 20 (24), 6978–6987.

(6) Straubinger, R. M.; Papahadjopoulos, D. Liposomes as carriers for intracellular delivery of nucleic acids. *Methods Enzymol.* 1983, 101, 512–527.

(7) Feglnér, P. L.; Gadek, T. R.; Holm, M.; Roman, R.; Chan, H. W.; Wenz, M.; Northrop, J. P.; Ringold, G. M.; Danielsen, M. Lipofection: a highly efficient, lipid-mediated DNA-transfection procedure. *Proc. Natl. Acad. Sci. U.S.A.* 1987, 84 (21), 7413–7417.

(8) Fraley, R. T.; Dellaporta, S. L.; Papahadjopoulos, D. Liposome-mediated delivery of tobacco mosaic virus RNA into tobacco protoplasts: A sensitive assay for monitoring liposome-protoplast interactions. *Proc. Natl. Acad. Sci. U.S.A.* 1982, 79 (6), 1859–1863.

(9) Nchinda, G.; Zachauering, O.; Uberla, K. Increased non-viral gene transfer levels in mice by concentration of cationic lipid DNA complexes formed under optimized conditions. *J. Gene Med.* 2003, 5, 712–722.

(10) Zhang, Y.; Garzon-Rodriguez, W.; Manning, M. C.; Anchordoquy, T. J. The use of fluorescence energy transfer to monitor dynamic changes of lipid-DNA interactions during lipoplex formation. *Biochim. Biophys. Acta* 2003, 1614, 182–192.

(11) Ross, P. C.; Hui, S. W. Lipoplex size is a major determinant of in vitro lipofection efficiency. *Gene Ther.* 1999, 6, 651–659.

(12) Zuidam, N. J.; Hirsch-Lerner, D.; Margulies, S.; Barenholz, Y. Lamellarity of cationic liposomes and mode of preparation of lipoplexes affect transfection efficiency. *Biochim. Biophys. Acta* 1999, 1419, 207–220.

(13) Zhang, Y.; Anchordoquy, T. J. The role of lipid charge density in the serum stability of cationic lipid/DNA complexes. *Biochim. Biophys. Acta* 2004, 1663 (1–2), 143–157.

(14) Yang, J. P.; Huang, L. Overcoming the inhibitory effect of serum on lipofection by increasing the charge ratio of cationic liposome to DNA. *Gene Ther.* 1997, 4 (9), 950–960.

(15) Hui, S. W.; Langner, M.; Zhao, Y. L.; Ross, P.; Hurley, E.; Chan, K. The role of helper lipids in cationic liposome-mediated gene transfer. *Biochim. Biophys. Acta* 1996, 1235, 289–295.

(16) Farhood, H.; Serbina, N.; Huang, L. The role of dioleoyl phosphatidylethanolamine in cationic liposome mediated gene transfer. *Biochim. Biophys. Acta* 1995, 1235, 289–295.

(17) Sakurai, F.; Nishioka, T.; Saito, H.; Baba, T.;Okuda, A.; Matsumoto, O.; Taga, T.; Yamashita, F.; Takakura, Y.; Hashida, M. Interaction between DNA-cationic liposome complexes and erythrocytes is an important factor in systemic gene transfer via the intravenous route in mice: the role of the neutral helper lipid. *Gene Ther.* 2001, 8 (9), 677–686.

(18) Crook, K.; Stevenson, B. J.; Dubouchet, M.; Porteous, D. J. Inclusion of cholesterol in DOTAP transfection complexes increases the delivery of DNA to cells in vitro in the presence of serum. *Gene Ther.* 1998, 5 (1), 137–143.

(19) Hafez, I. M.; Maurer, N.; Cullis, P. R. On the mechanism whereby cationic lipids promote intracellular delivery of polynucleic acids. *Gene Ther.* 2001, 8 (15), 1188–1196.

(20) Xu, L.; Anchordoquy, T. J. Cholesterol Domains in Cationic Lipid/DNA Complexes Improve Transfection. *Biochim. Biophys. Acta*, 2008, 1778 (10), 2177–81.

(21) Xu, L.; Anchordoquy, T. J. Effect of cholesterol nanodomains on the targeting of lipid-based gene delivery in cultured cells. *Mol. Pharmacother.* 2010, 7 (4), 1311–1317.

(22) Xu, L.; Betker, J.; Yin, H.; Anchordoquy, T. J. Ligands located within a cholesterol domain enhance gene delivery to the target tissue. *J. Controlled Release* 2012, 160, 57–65.

(23) Xu, L.; Wempe, M. F.; Anchordoquy, T. J. The effect of cholesterol domains on PEGylated liposomal gene delivery in vitro. *Ther. Delivery* 2011, 2, 51–60.

(24) Zhang, Y.; Bradshaw-Pierce, E. L.; Delille, A.; Gustafson, D. L.; Anchordoquy, T. J. In vivo comparative study of lipid/DNA complexes with different in vitro serum stability: effects on biodistribution and tumor accumulation. *J. Pharm. Sci.* 2008, 97 (1), 237–250.

(25) Betker, J. L.; Kullberg, M.; Gomez, J.; Anchordoquy, T. J. Cholesterol domains enhance transfection. *Ther. Delivery* 2013, 4 (4), 453–62.

(26) Watcharanurak, K.; Nishikawa, M.; Takahashi, Y.; Takakura, Y. Controlling the kinetics of interferon transgene expression for improved gene therapy. *J. Drug Targeting* 2012, 20 (9), 764–769.

(27) Molina, M.d.C.; Allison, S. D.; Anchordoquy, T. J. Maintenance of particle size during the freezing step of the lyophilization process is insufficient for preservation of activity: insight from other structural indicators. *J. Pharm. Sci.* 2001, 90 (8), 1445–1455.

(28) Gao, X.; Huang, L. Potentiation of cationic liposome-mediated gene delivery by polyacids. *Biochemistry* 1996, 35, 1027–1036.

(29) Hoiland, H. E.; Shephard, L.; Sullivan, S. M. Formation of stable cationic lipid/DNA complexes for gene transfer. *Proc. Natl. Acad. Sci. U.S.A.* 1996, 93 (14), 7305–9.

(30) Lv, H.; Shang, S.; Wang, B.; Cui, S.; Yan, J. Toxicity of cationic lipids and cationic polymers in gene delivery. *J. Controlled Release* 2006, 114, 100–109.

(31) Yew, N. S.; Scheule, R. K. Toxicity of cationic lipid-DNA complexes. *Adv. Genet.* 2005, 53, 189–214.

(32) Simberg, D.; Weisman, S.; Talmon, Y.; Barenholz, Y. DOTAP (and other cationic lipids): chemistry, biophysics, and transfection. *Crit. Rev. Ther. Drug Carrier Syst.* 2004, 21, 257–317.

(33) Koiv, A.; Mustonen, P.; Kinnunen, P. K. J. Differential scanning calorimetry study on the binding of nucleic acids to dimyristoylphosphatidylcholine-sphingosine liposomes. *Chem. Phys. Lipid.* 1994, 70, 1–10.

(34) Koiv, A.; Kinnunen, P. K. J. Binding of DNA to liposomes containing different derivatives of sphingosine. *Chem. Phys. Lipids* 1994, 72, 77–86.

(35) Paukku, T.; Lauraeus, S.; Huhtaniemi, I.; Kinnunen, P. K. J. Novel cationic liposomes for DNA-transfection with high efficiency and low toxicity. *Chem. Phys. Lipid.* 1997, 87, 23–29.

(36) Baraldo, K.; Leforestier, N.; Bureau, M.; Mignet, N.; Scherman, D. Sphingosine-based liposome as DNA vector for intramuscular gene delivery. *Pharm. Res.* 2002, 19, 1144–1149.

(37) Cuvillier, O. Sphingosine in apoptosis signaling. *Biochim. Biophys. Acta* 2002, 1585, 153–162.
(38) Elrick, M. J.; Fluss, S.; Colombini, M. Sphingosine, a product of ceramide hydrolysis, influences the formation of ceramide channels. *Biophys. J.* 2006, 91, 1749–1756.

(39) Sasaki, H.; Arai, H.; Cocco, M. J.; White, S. H. pH dependence of sphingosine aggregation. *Biophys. J.* 2009, 96, 2727–2733.

(40) van de Wetering, P.; Cherng, J.-Y.; Talma, H.; Crommelin, D. J. A.; Hennink, W. E. 2-(dimethylamino)ethyl methacrylate based (co)polymers as gene transfer agents. *J. Controlled Release* 1998, 53, 145–153.

(41) Kobayashi, T.; Beuchat, M.-H.; Lindsay, M.; Frias, S.; Palmiter, R. D.; Sakuraba, H.; Parton, R. G.; Gruenberg, J. Late endosomal membranes rich in lysobisphosphatidic acid regulate cholesterol transport. *Nat. Cell Biol.* 1999, 1, 113–118.

(42) Roff, C. F.; Goldin, E.; Comly, M. E.; Cooney, A.; Brown, A.; Vanier, M. T.; Miller, S. P. F.; Brady, R. O.; Pentchev, P. G. Type C Niemann-Pick Disease: use of hydrophobic amines to study defective cholesterol transport. *Dev. Neurosci.* 1991, 13, 315–319.

(43) Reiners, J. J., Jr; Kleinman, M.; Kessel, D.; Mathieu, P. A.; Caruso, J. A. Nonesterified cholesterol content of lysosomes modulates susceptibility to oxidant-induced permeabilization. *Free Radical Biol. Med.* 2011, 50, 281–294.

(44) Lonez, C.; Vandenbranden, M.; Ruysschaert, J. M. Cationic lipids activate intracellular signaling pathways. *Adv. Drug Delivery Rev.* 2012, 64, 1749–1758.

(45) Lamkanfi, M.; Dixit, V. M. Modulation of inflammasome pathways by bacterial and viral pathogens. *J. Immunol.* 2011, 187, 597–602.

(46) Wang, F.; Bexiga, M. G.; Anguissola, S.; Boya, P.; Simpson, J. C.; Salvati, A.; Dawson, K. A. Time resolved study of cell death mechanisms induced by amine-modified polystyrene nanoparticles. *Nanoscale* 2013, 5, 10868–10876.

(47) Kagedal, K.; Zhao, M.; Svensson, I.; Brunk, U. T. Sphingosine-induced apoptosis is dependent on lysosomal proteases. *Biochem. J.* 2001, 359, 335–343.

(48) Heinrich, M.; Wickel, M.; Schneider-Brachert, W.; Sandberg, C.; Gehr, J.; Sch wandner, R.; Weber, T.; Brunner, J.; Krönke, M.; Schütze, S. Cathepsin D targeted by acyl sphingomyelinase-derived ceramide. *EMBO J.* 1999, 18, 5252–5263.

(49) Bach, D.; Wachtel, E. Phospholipid/cholesterol model membranes: formation of cholesterol crystallites. *Biochem. Biophys. Acta* 2003, 1610, 187–197.