



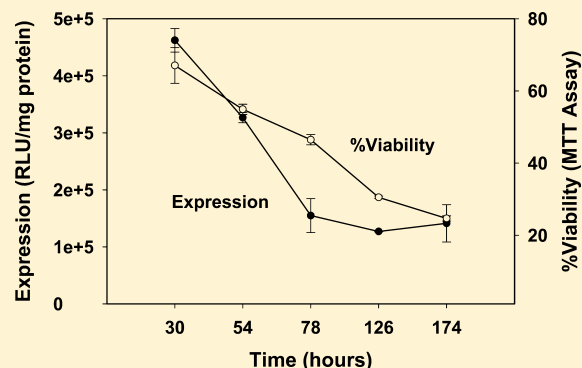
Relating Toxicity to Transfection: Using Sphingosine To Maintain Prolonged Expression *in Vitro*

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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

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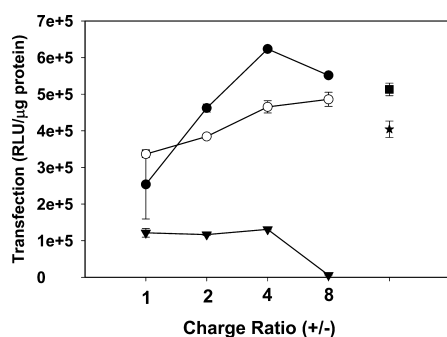


Figure 1. Effect of charge ratio on transfection by DOTAP-containing lipoplexes. Progressively increasing quantities of cationic lipid were used to prepare lipoplexes, which were subsequently assessed for transfection efficiency in MCF-7 cells after exposure to 50% FBS. Luciferase expression after treatment of cells with increasing charge ratios of DOTAP:cholesterol 1:4 (closed circles), cholesterol:DOTAP:DAPC 2:3:5 (open circles), and DOTAP:cholesterol 1:1 (closed triangles) lipoplexes are depicted. Transfection rates of lipofectamine (star) and PEI (N/P = 7; closed square) formulations are shown for comparison. Symbols represent the mean \pm one standard deviation from replicate samples, $n = 6$.

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.²⁵ 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.²⁶ 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

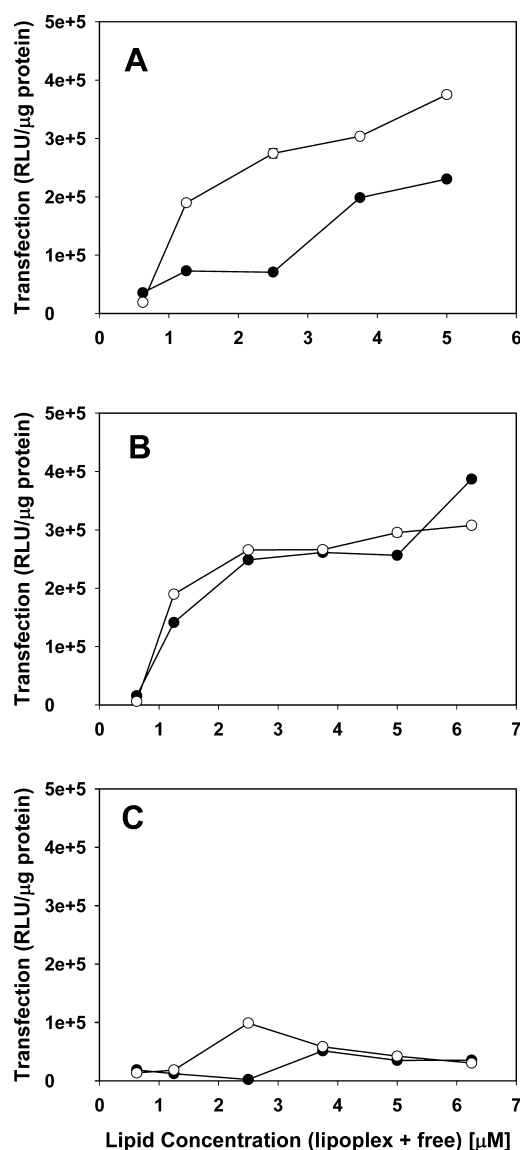


Figure 2. Effect of free cationic liposomes on transfection in MCF-7 cells after exposure to 50% FBS. Increasing quantities of cationic liposomes were added to DOTAP-containing lipoplexes prepared at +/- = 4 (open circles) or +/- = 8 (closed circles). Luciferase expression of DOTAP:cholesterol 1:4 (A), cholesterol:DOTAP:DAPC 2:3:5 (B), and DOTAP:cholesterol 1:1 (C) lipoplexes are depicted. Symbols represent the mean \pm one standard deviation from replicate samples, $n = 6$.

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.

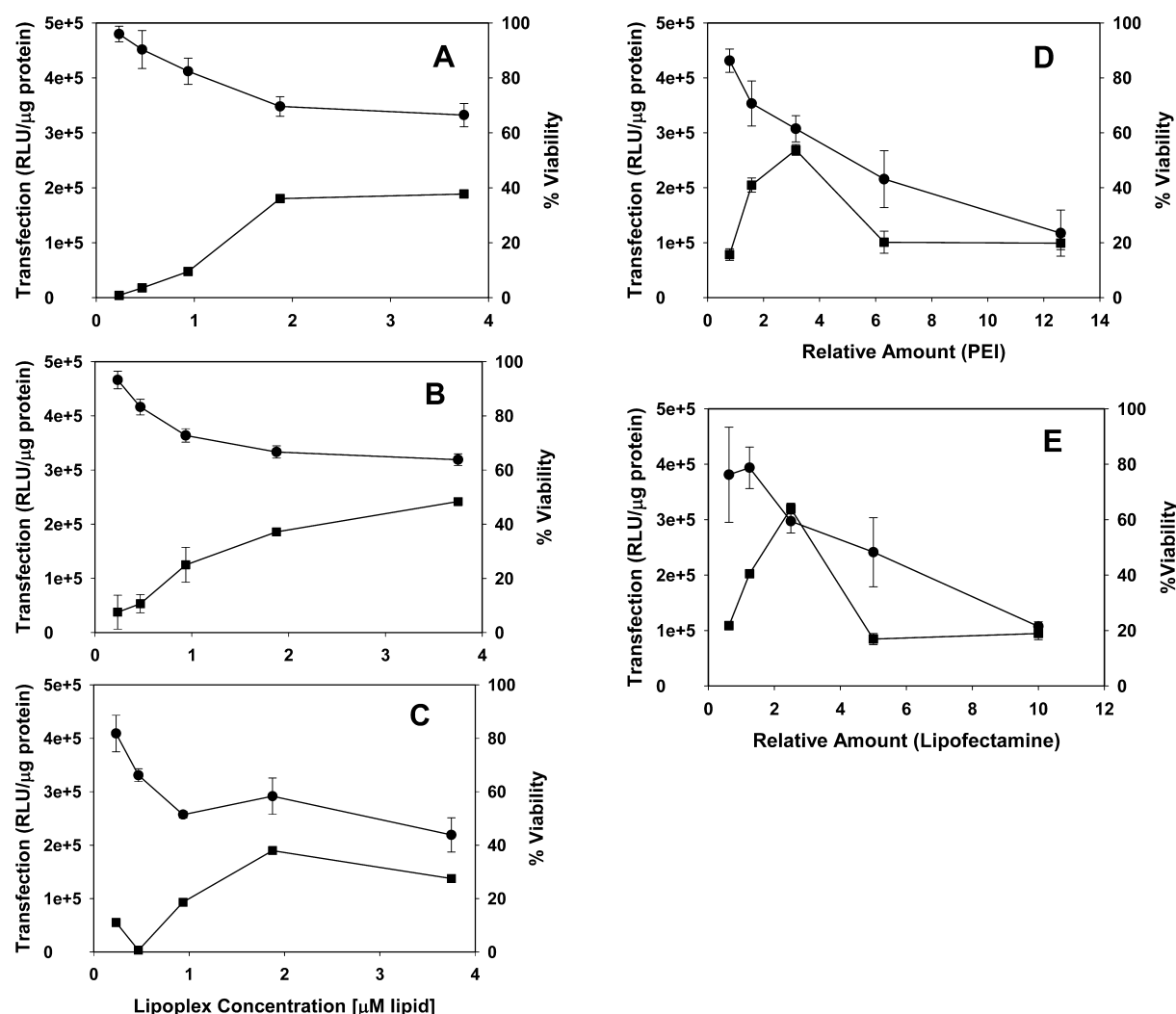


Figure 3. Effect of complex concentrations on transfection and toxicity in MCF-7 cells after exposure to 50% FBS. Increasing quantities of DOTAP:cholesterol 1:4 (A), cholesterol:DOTAP:DAPC 2:3:5 (B), and DOTAP:cholesterol 1:1 (C) lipoplexes (\pm 4) were used. Similar experiments using complexes prepared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

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 μ L 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) on a 96-well

THERMOMax plate reader (Molecular Devices, Sunnyvale, CA). Luminescence was quantified using a Monolight luminometer according to the manufacturer’s instructions (BD Biosciences, San Jose, CA).

Particle Sizing and Zeta Potential Measurements.

Sizes of the lipoplexes were determined by dynamic light scattering with a Nicomp 380 ZLS (PSS Nicomp, Santa Barbara, CA) as previously described.²⁵ Zeta potential of the lipoplexes was determined by a Malvern ZetaSizer Nano Series Nano-ZS (Malvern Instruments, Worcestershire, U.K.). Samples were preincubated 1:1 v/v in MEM or FBS for 30 min prior to making the size and zeta potential measurements. Incubated samples were then diluted 1:100 in PBS prior to measurement to reduce light scattering. A Bio-Rad protein assay was used to measure the amount of serum protein binding as previously described.²⁵

Determination of Cell Uptake by Flow Cytometry.

MCF7 cells were treated with lipoplexes incorporating 1 mol % NBD-DOPE as described above, and cells were then washed twice with PBS and collected by centrifugation at 800 rpm for 15 min. The pellets were fixed with a 1% formalin PBS solution and then quenched with 0.1% Trypan blue as previously

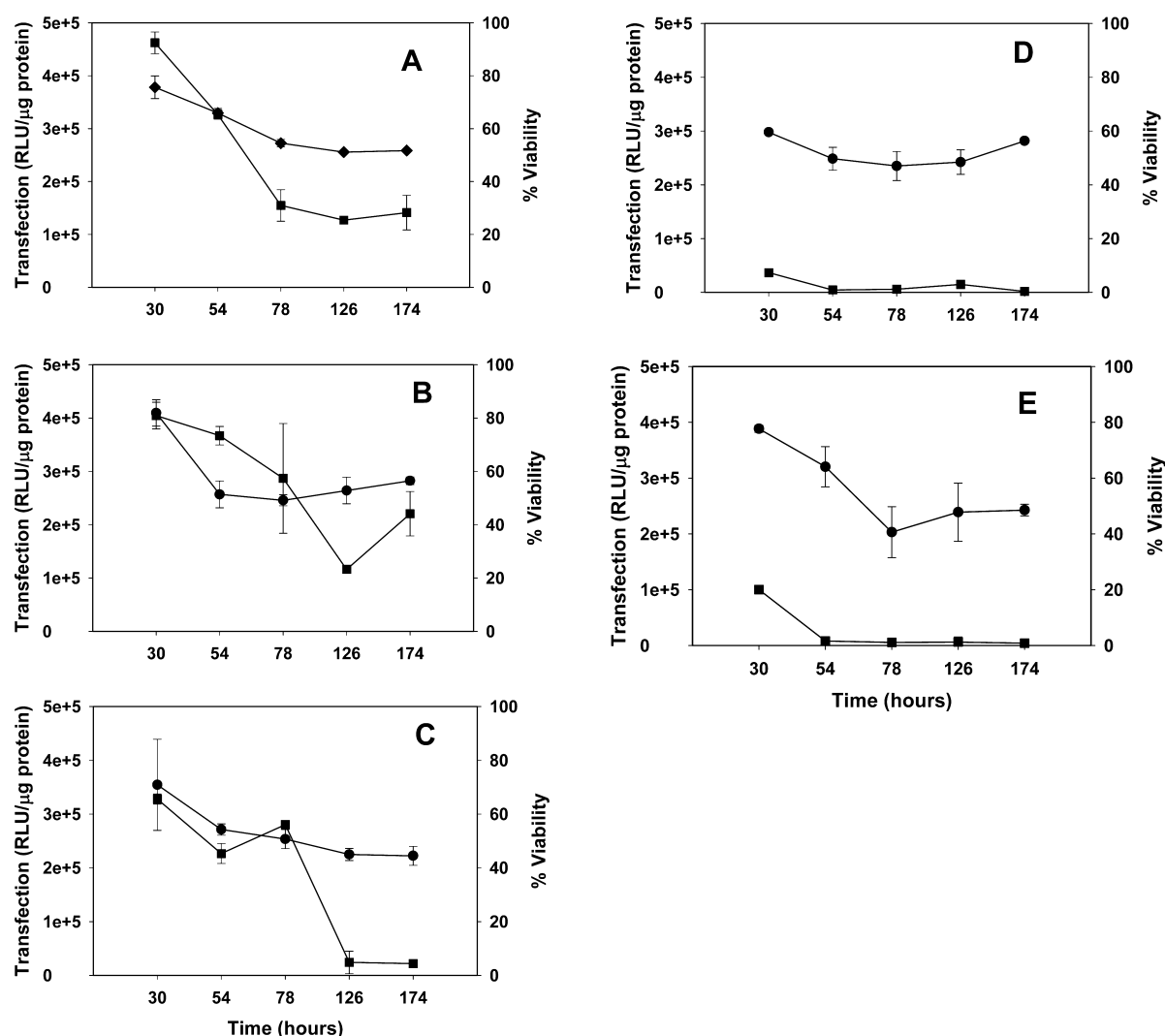


Figure 4. Effect of time on expression and viability. Cells transfected ($\pm = 4$; $3.75 \mu\text{M}$) with DOTAP:cholesterol 1:4 (A), cholesterol:DOTAP:DAPC 2:3:5 (B), DOTAP:cholesterol 1:1 (C), PEI (D), and lipofectamine (E) complexes were monitored over 7 days. Luciferase expression (closed squares) and viability (closed circles) were determined. Symbols represent the mean \pm one standard deviation from replicate samples, $n = 6$.

described.²¹ 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 $10 \mu\text{L}$ of 12 mM MTT solution diluted in $100 \mu\text{L}$ 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 \pm 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 ($\pm = 8$), 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.^{28,29} 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).

Curiously, free cationic lipids had mixed effects on transfection rates when the lipoplex was formulated at low \pm 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., $\approx 60\%$) 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.^{30,31} Most of our previous work has employed DOTAP, which is considered a lipid with relatively low toxicity that has been employed in clinical trials.³² 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.^{33–36} Considering that sphingosine is a natural degradation product of ceramide that is present in all mammalian cells,^{37,38} 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 \pm charge ratio on transfection and toxicity. As shown in Figure 5, both transfection and toxicity were comparable to those observed with lipoplexes formulated

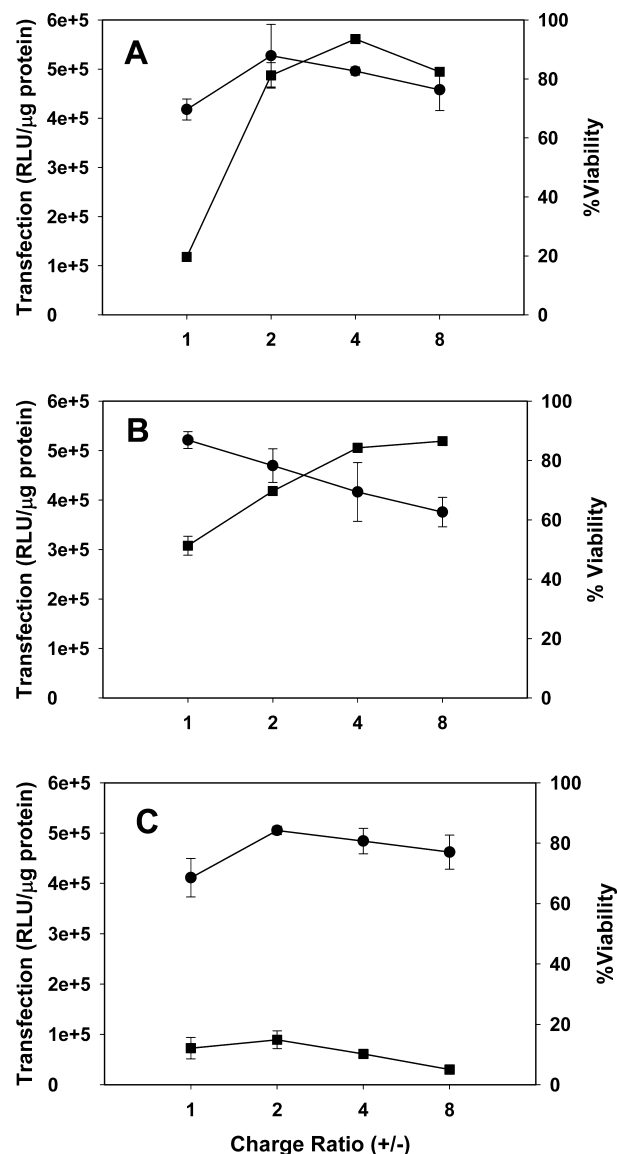


Figure 5. Effect of charge ratio of sphingosine-containing lipoplexes on transfection and toxicity in MCF-7 cells after exposure to 50% FBS. Progressively increasing quantities of cationic lipid were used to prepare lipoplexes, which were subsequently assessed for transfection (closed squares) and viability (closed circles). Treatment with different charge ratios of sphingosine:cholesterol 1:4 (A), cholesterol:sphingosine:DAPC 2:3:5 (B), and sphingosine:cholesterol 1:1 (C) lipoplexes are depicted. Symbols represent the mean \pm one standard deviation from replicate samples, $n = 6$.

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

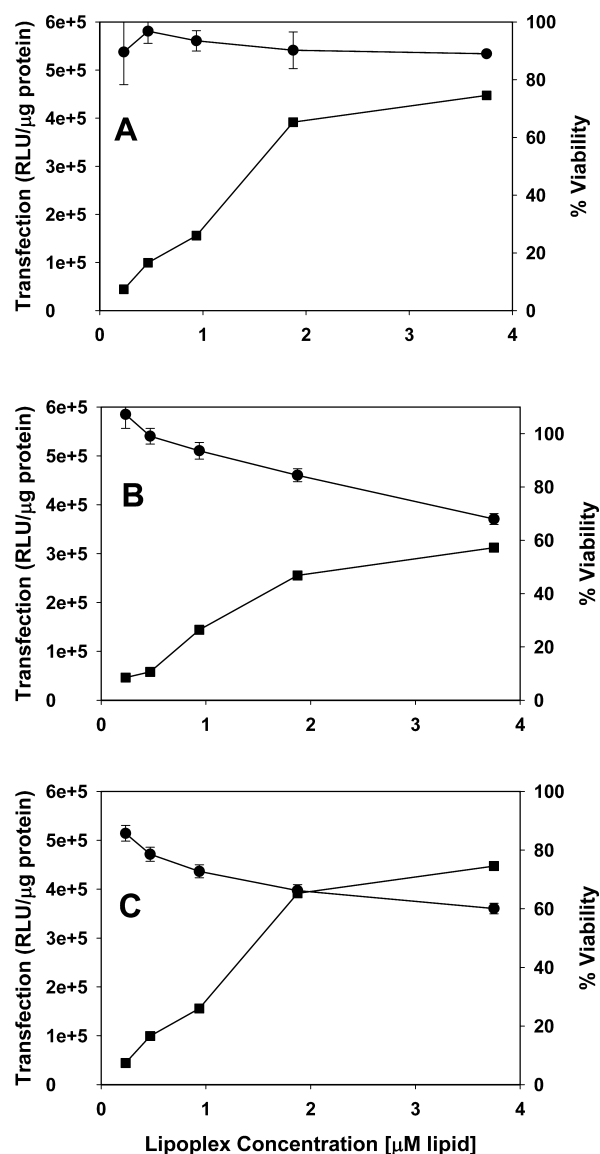


Figure 6. Effect of lipoplex concentration on transfection and toxicity in MCF-7 cells after exposure to 50% FBS. Increasing quantities of sphingosine:cholesterol 1:4 (A), cholesterol:sphingosine:DAPC 2:3:5 (B), and sphingosine:cholesterol 1:1 (C) lipoplexes (+/- = 4) were used. Luciferase expression (closed squares) and viability (closed circles) were determined. Symbols represent the mean \pm one standard deviation from replicate samples, $n = 6$.

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 6.6.³⁹ 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

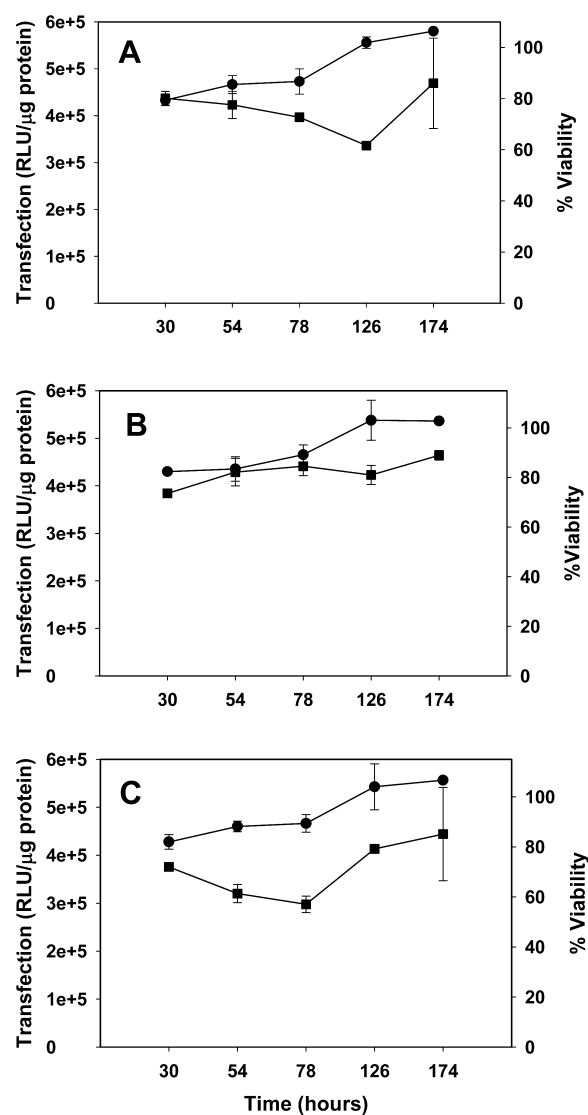


Figure 7. Effect of time on expression and viability. Cells transfected with sphingosine:cholesterol 1:4 (A), cholesterol:sphingosine:DAPC 2:3:5 (B), and sphingosine:cholesterol 1:1 (C) lipoplexes (+/- = 4) were monitored over 7 days. Luciferase expression (closed squares) and viability (closed circles) were determined. Symbols represent the mean \pm one standard deviation from replicate samples, $n = 6$.

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.

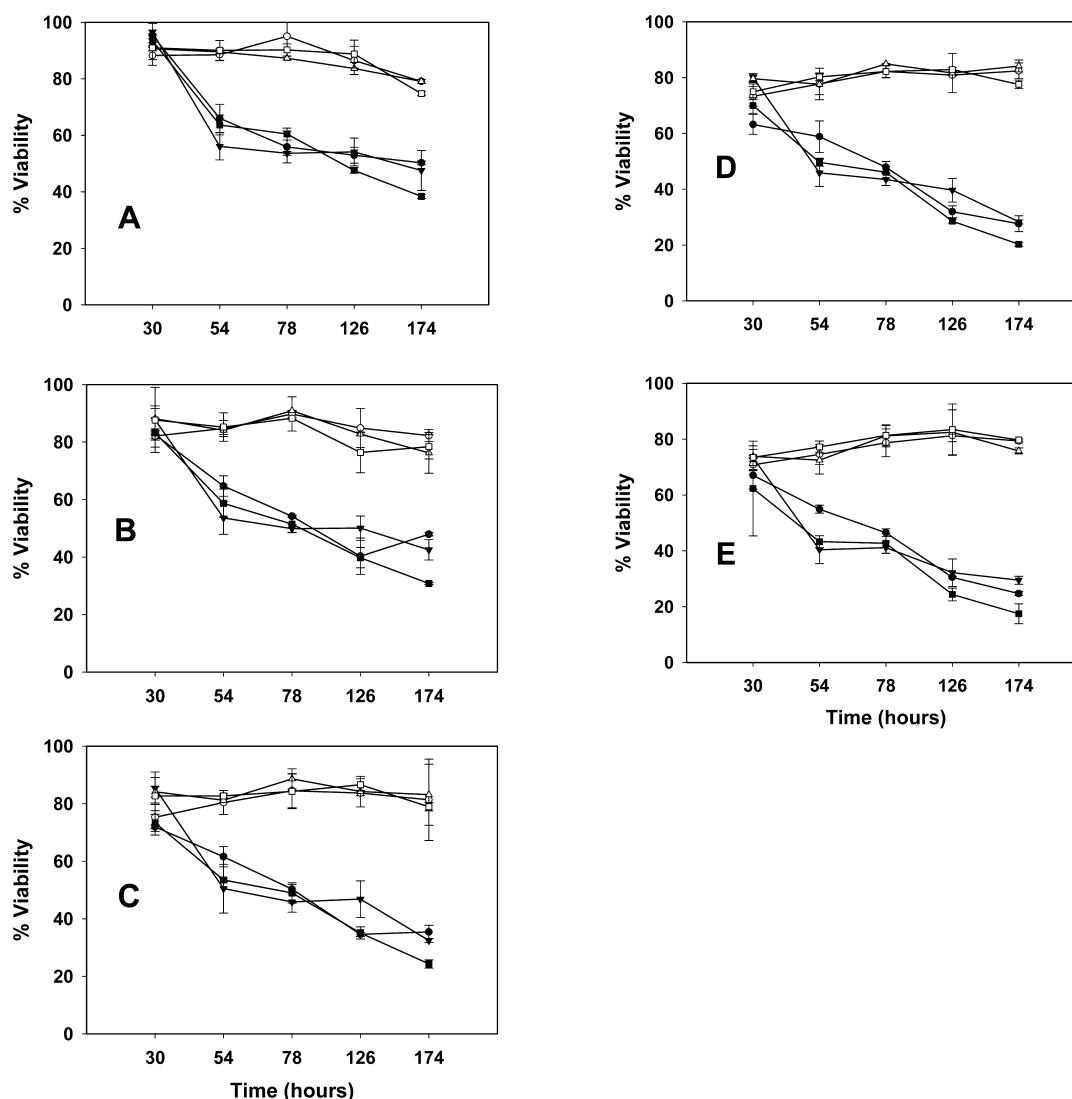


Figure 8. Effect of time and lipoplex concentration on viability. Cells transfected with lipoplex concentrations of 0.23 (A), 0.47 (B), 0.94 (C), 1.88 (D), and 3.75 μ M (E) cationic lipid at \pm = 4 were assessed for viability over 7 days. Lipoplexes formulated with sphingosine (open symbols) maintained higher viability than those formulated with DOTAP (closed symbols). Lipoplexes formulated at ratios of cationic lipid to cholesterol of 1:4 (circles), 2:3:5 (triangles), and 1:1 (squares) were compared. Symbols represent the mean \pm one standard deviation from replicate samples, n = 6.

DISCUSSION

It is well-known that \pm charge ratio has a significant impact on transfection rates both in cell culture and *in vivo*.^{10,12,13} Accordingly, 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 \pm 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., \pm > 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.^{28,29} 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., \approx 60%). Previous studies with cationic polymers have reported a similar correlation between toxicity and transfection.⁴⁰ 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,^{41–43} and that the rupture of organelles that promotes delivery can also elicit a toxic inflammatory cascade.^{44–46} 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

Table 1. Physical Characteristics of Lipoplexes before and after Serum Exposure^a

	size (nm)	
	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
	zeta potential	
	MEM	FBS
1:1 DOTAP:cholesterol	46 ± 3	-45 ± 1
1:4 DOTAP:cholesterol	55 ± 3	-47 ± 4
2:3:5 cholesterol:DOTAP:DAPC	53 ± 2	-51 ± 1
1:1 sphingosine:cholesterol	34 ± 3	-47 ± 1
1:4 sphingosine:cholesterol	43 ± 2	-41 ± 1
2:3:5 cholesterol:sphingosine:DAPC	48 ± 4	-41 ± 1

^aThe particle size and zeta potential of lipoplexes incubated in MEM media or 50% FBS. Values are the average ± one standard deviation of triplicate samples.

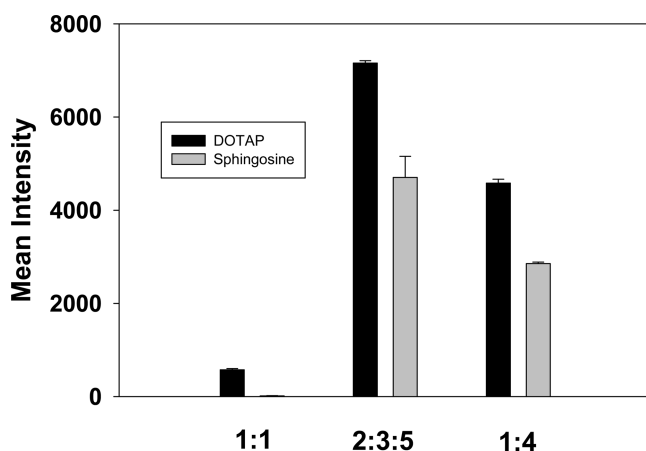


Figure 9. Effect of cationic lipid and cholesterol content on cell uptake. MCF-7 cells treated with DOTAP-containing (dark bars) or sphingosine-containing (light bars) lipoplexes were assessed for lipoplex uptake after 4 h by flow cytometry. The *x*-axis shows the ratios of components described in Figures 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 transfected 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.^{10,13,20,21,23} 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 lipids with 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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Notes

The authors declare no competing financial interest.

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