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Introduction of a Disulfide Bond into a Cationic Lipid Enhances Transgene Expression of Plasmid DNA

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We have introduced a convenient method of synthesis for disulfide-containing cationic lipids. The lipid, 1,2-dioleoyl-sn-glycero-3-succinyl-2-hydroxyethyl disulfide ornithine conjugate (DOGSDSO), was synthesized and used to prepare liposomes in combination with DOPE. The rationale behind the selection of the disulfide bond was to produce a lipid which could be selectively destabilized within the cytosol of the cell. The disulfide bond of DOGSDSO was shown to be cleaved by reductive media leading to destabilization of the liposome/DNA complex, thus increasing the release of pDNA compared to a non-disulfide-containing analog. The introduction of a disulfide bond increases the transfection activity using model animal cell lines. The transfection activity and toxicity of DOTAP, DOGSDSO and its analog in three cell lines were compared. The amount of transgene (e.g. luciferase) produced with the use of DOGSDSO/DOPE was greater than that of DOTAP/DOPE and up to 50 times more than that of its non-disulfide analog. The results indicate disulfide-containing cationic liposomes may act as excellent vectors for gene transfection. © 1998 Academic Press

Key Words: **disulfide; plasmid DNA; gene delivery; liposome.**

Cationic liposomes are promising non-viral systems for use in gene delivery. Although the biochemical and biophysical mechanisms of cationic liposomes assisted gene transfection and expression are not thoroughly understood, the barriers involved in the transfection process *in vitro* generally include the following events (1): *A.* Formation of the liposome/DNA complex; *B.* Entry of complex into the cell; *C.* Escape of DNA from the endosome; *D.* Dissociation of DNA from the liposome; *E.* Entry of DNA into the nucleus; and *F.* DNA transcription. Some of the barriers can be approached by rationale design of the delivery systems {A-E} while others {F} will relate to the innate properties of the plasmid itself (e.g., promoters, enhancers etc.).

A conflict is encountered overcoming the first and fourth barriers. Cationic liposomes are required not only to complex DNA in the test tube so that the DNA is protected from nucleases and enabling them to enter the cell, but also to enable them to easily release for expression. To address this conflict in requirements, a new lipid, DOGSDSO, was synthesized. This lipid contains a reversible disulfide bond between the polar head group of the lipid and its lipophilic tail. After forming the lipid/DNA complex and transporting the plasmid DNA into cells, DOGSDSO was designed to take advantage of the relatively high intracellular concentration of reductive substances cleaving the disulfide bond, thus releasing the DNA. The higher cytoplasm concentration of DNA should increase transgene expression. A new method is described here for the synthesis of cationic lipids containing a disulfide bond (e.g., DOGSDSO). To test the importance of the disulfide bond a second lipid containing an alkyl connection (DOGSHDO) was synthesized and compared to DOGSDSO. In all liposome preparations the cationic lipid was mixed with an equal molar ration of DOPE.

MATERIALS AND METHODS

Chemicals. DOTAP, DOPE, and 1,2-dioleoyl-sn-glycero-3-succinate were purchased from Avanti Polar Lipids (Alabaster, AL). Calcein-AM was purchased from Molecular Probes, Inc. (Eugene, OR). Other organic reagents were bought from Aldrich Chemicals (Milwaukee, WI).

Plasmid DNA. All supplies were purchased from Promega (Madison, WI). Plasmid DNA was obtained from *E. coli* (strain JM-109) which had been transfected with a pGL3 luciferase producing plasmid. Plasmid DNA was isolated using a Megawizard DNA purification kit. The concentration and purity of pDNA were determined spectrophotometrically.

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Abbreviations: DOTAP: 1,2-dioleoyl-3-trimethylammonium-propane; DOPE: L-dioleoyl phosphatidylethanolamine; DOGSDSO: 1′,2′-dioleoyl-sn-glycero-3′-succinyl-2-hydroxyethyl disulfide ornithine conjugate; DOGSHDO: 1′,2′-dioleyl-sn-glycero-3′-succinyl-1,6-hexanediol ornithine conjugate.

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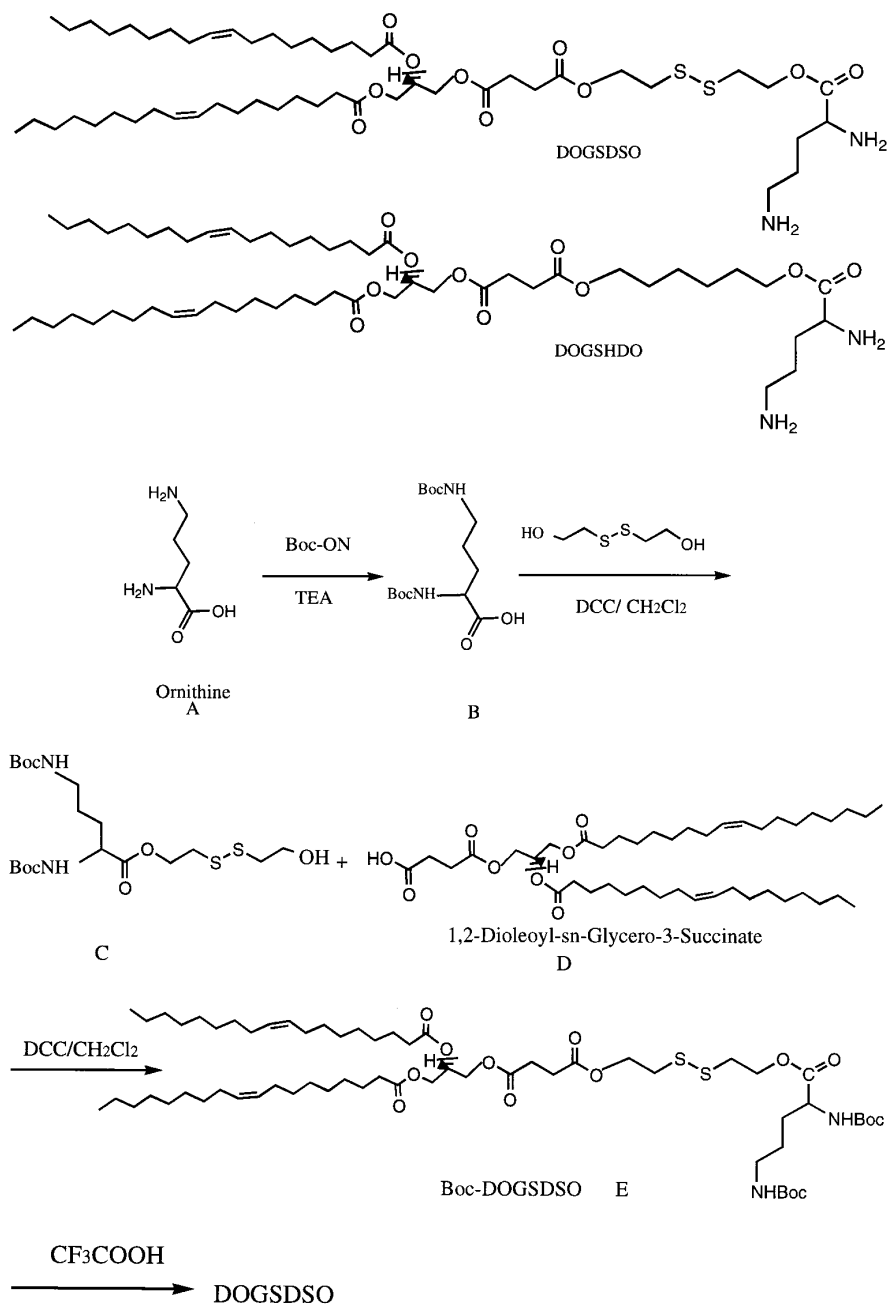

FIG. 1. Scheme for the synthesis of DOGSDSO. Boc-ON, 2-(*tert*-butoxycarbonyloxyimino)-2-phenylacetonitrile; TEA, triethylamine; DCC, 1,3-dicyclohexylcarbodiimide.

Synthesis of DOGSDSO (Fig.1). Amino groups of L-ornithine were protected with Boc-ON in wet 1,4-dioxane to produce B(2). B was esterified with 5 equivalents of 2-hydroxyl disulfide to form C (DCC, 1.1 equiv., RT, in CH_2Cl_2) (3). C and D (1:1) were condensed to generate Boc-DOGSDSO (DCC, 1.1 equiv., RT, in CH_2Cl_2). Thin layer chromatography (TLD) was used to follow all the reactions. A normal separation method of silica gel chromatography was used to purify B, C, D and E. The molecular weight of E was determined by ionization spray positive mode mass spectrometry, MW=1171(calculated 1171.7 for $C_{62}H_{110}N_2O_{14}S_2$). ^1H NMR ($CDCl_3$, 300 HZ): 0.90 [t, 2×(CH_3)], 1.20−1.69[m, 26×(CH_2)], 1.44[s, 2×(CH_3)$_3$], 2.01 [q, 4×(C=C − CH_2)], 2.31 [m, 2×(CH_2C=O)], 2.71 [s, O=CCH_2CH_2C=O], 2.91 [t, CH_2SSCH_2], 3.12 [q, CH_2N], 4.18 [m, a-H], 4.36 [2×(CH=CH)], 4.62 [broad s, NH], 5.05 − 5.12[broad s, NH], 5.22 − 5.40 [m, OCH_2CHOCH_2O]. Before using the lipid to prepare liposomes, E was deprotected (CF_3COOH, 0°C, 15min) (4), and excessive trifluoroacetic acid was dried under high vacuum to yield DOGSDSO. The overall yield was 45%-50% based on the amount of starting amino acid. Because DOGSDSO was unstable on silica during chromatography, its purity was checked by TLC, and it was used directly to prepare liposomes. A similar synthetic method and conditions were used to synthesize the non-sulfide analog DOGSHDO with similar chemical yields.

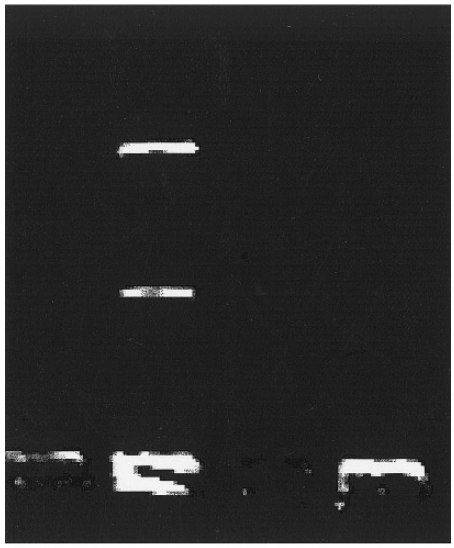

FIG. 2. Electrophoretic analysis of binding of 1 μg of pDNA complexed with 16 μg liposomes, and the freeing of plasmid DNA from the complexes in reductive environment. A. DOGSDSO/DOPE lipid/ pDNA; B. DOGSDSO/DOPE/pDNA + 10 mM DTT; C. DOGSHDO/ DOPE/ pDNA ; D: DOGSHDO/DOPE/pDNA +10 mM DTT

Liposome preparation. DOTAP, DOGSDSO, and DOGSHDO were dissolved and mixed with a neutral lipid DOPE (1:1 molar ratio) (5) in chloroform respectively. The solution was evaporated in a round-bottom flask using a rotary evaporator at room temperature. The lipid film was dried by nitrogen for an additional ten minutes to insure all solvent was evaporated. The lipid film was then suspended in sterile water to make a concentration of 1 mg/ml based on the amount of DOTAP, DOGSDSO or DOGSHDO. The resultant mixtures were shaken for 30 minutes, followed by sonication by using Sonic Dismembrator (Fisher Scientific) for 5 minutes at 5 watts to form homogenized liposomes. The particle size distribution of the liposomes was measured using a NICOMP 380 ZLS instrument (Santa Babara, CA) with the volume-weight distribution parameter. The average diameter of all liposomes was between 220-350 nm regardless of the cationic lipid used. The liposomes were stored at 4°C until use. The liposomes demonstrated similar transfection activity when used for gene transfection for up to two months when stored under these conditions (data not shown).

Dissociation of DNA from the lipid/DNA complex in reductive media. One microgram of DNA was complexed with 16 μg DOGSDSO/DOPE or DOGSHDO/DOPE in PBS (1 mL) (pH=7.4). After the initial mixing, the complexes were incubated for 30 minutes at room temperature. DL-Dithiothreitol (DTT) in PBS (pH=7.4) was then added to complexes to form a final concentration of 10 mM. The mixtures were incubated for 12 hours at 37°C. Released plasmid DNA from the complex was visualized by SYBR staining of a 0.7% TBE agarose gel.

Cell culture and transfection. SKnSH cells, HEK 293 cells, and COS-1 cells (obtained through ATCC) were maintained in RPMI 1640, DMEM, and DMEM (high glucose) respectively, and supplemented with 10 % fetal bovine serum and antibiotics (penicillin 100 μg/ml and streptomycin 100 μg/ml). All cells were maintained in 5% CO_2 and at 37°C in humidified air. Cell lines were cultured and seeded in 24 well plates (2×10^5 cells/well) and grown to 60-80 % confluence in 1 ml of media with serum. pDNA/lipid complexes were made and incubated at room temperature in serum free media for 30 minutes and used for transfection. Before transfection, serum containing media was changed to serum free media and the transfection mixtures were added to the cells. After 4.5 hours, the media was changed to growth media containing serum, and the cells were grown for another 48 hours. Firefly luciferase activity was measured using a luciferase assay. Following transfection and incubation, cells were rinsed twice with phosphate buffer saline. 100 μl of luciferase lysis buffer (0.1 M potassium phosphate buffer pH 7.8, 2 mM EDTA, 1% Triton X-100, 1 mM DTT) was added to the cells. The cells were lysed by shaking at room temperature for 15 minutes. Luciferase activity was quantified by using a Monolight 2010 Luminometer. 100 μl of luciferase assay buffer (30 mM Tricine, 3mM ATP, 15 mM $MgSO_4$, 10mM DDT, pH=7.8) and 20 μl of cell lysate were added to a 100 μl injection of 1mM D-luciferin, pH=6.3 (Molecular Probes, Eugene,

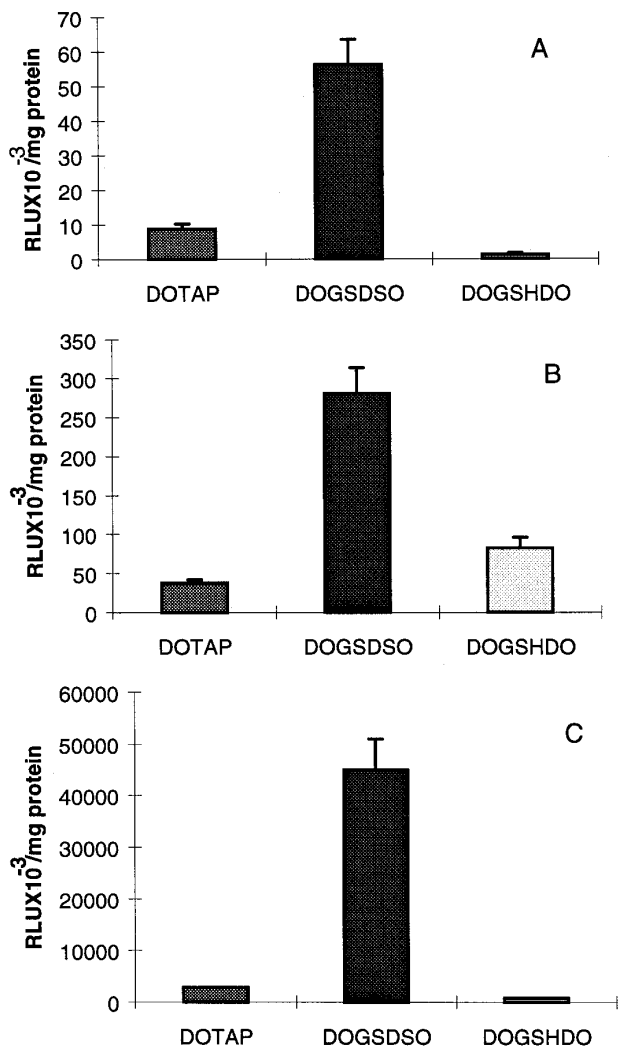

FIG. 3. Transfection activity of DOTAP/DOPE, DOGSDSO/ DOPE and DOGSHDO/DOPE. A. HEK 293 cells; the amount of each liposome was 48 μg/ml. B. SKnSH cells, the amount of liposome was 30 μg/ml. C. COS-1 cells the amount of liposome was 12 μg/ml. The amount of DNA used was 3 μg/ml for all types of cells. Data is shown as mean $\pm$ s.d. (n=4).

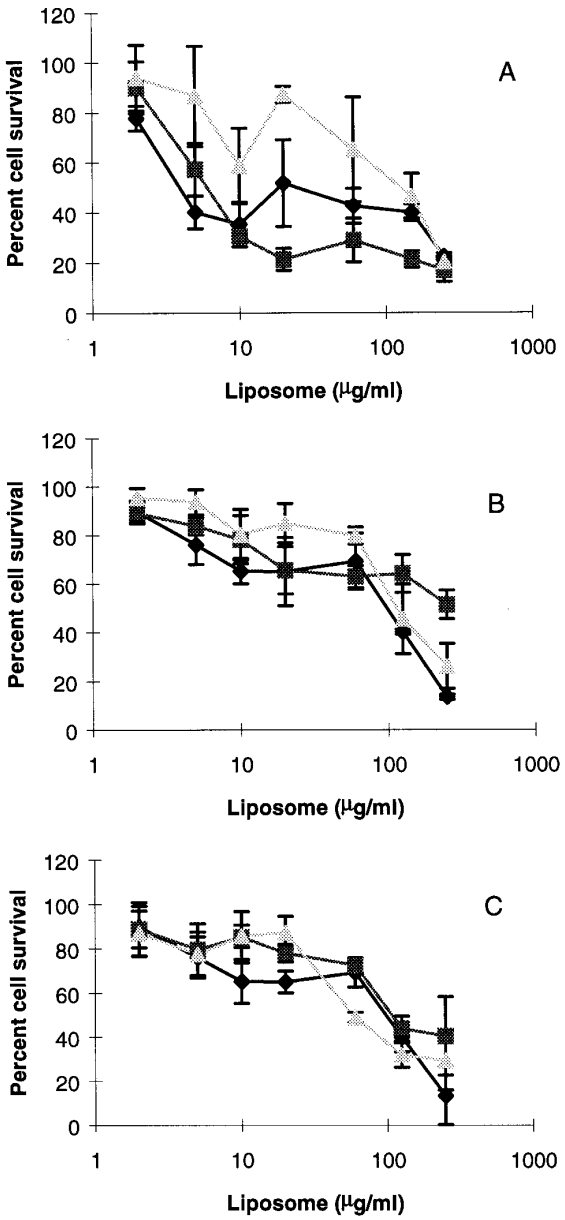

FIG. 4. Toxicity assays. After three cell lines were treated with liposomes for 48 hours, cells were incubated with 100 μl calcein-AM (1 μg/ml) at room temperature for 30 min, then released calcein fluorescence were measured. Cell survival was calculated as percentage cell survival using the equation stated in methods. A. HEK 293 cells. B. SKnSH cells. C. COS-1 cells. ♦: DOTAP/DOPE: ■: DOGSDSO/DOPE; ▲: DOGSHDO/DOPE. Data is shown as mean ±s.d. (n=3).

OR). The light emission over a 10 second reaction period was integrated. The total protein concentration in the cell lysate was measured by the BCA protein assay reagent (Pierce, Rockford, IL). Luciferase activity in cultured cells was expressed as relative light units (RLU) per mg of protein. Experiments were done in quadruplicate with each experiment repeated at least twice, and within in a series.

Toxicity assay. The cellular toxicity of DOGSDSO/DOPE, DOGSHDO/DOPE and DOTAP/DOPE in the three cell lines was determined by using a calcein release assay (6). Before the assay, cells were plated in a 96 well plate (1×10^5/well) and grown to 60-80% confluence. Cells were treated with media containing increasing amounts of liposome and incubated for an additional 48 hours. After specific treatments, cells were washed three times with PBS and incubated with 100 μl of calcein-AM (1μg/ml in PBS) for 30 minutes at room temperature. Calcein fluorescence intensity was measured at the excitation and emission wavelengths of 485 nm and 538 nm respectively, on a Perkin Elmer LS 50B Spectrophotometer. Cell survival was calculated as a percentage using the following equation:

$$\text{Percentage of Cell Survival} = \frac{\text{Measured Signal} - \text{Minimum Signal}}{\text{Maximum Signal} - \text{Minimum Signal}} \times 100$$

The maximum signal was the fluorescence signal obtained from cells not treated by liposomes. The minimum signal was the background signal.

RESULTS AND DISCUSSION

The described procedure for the lipid synthesis is a new method to synthesize disulfide bond-containing cationic lipids. Compounds containing disulfide bonds are able to participate in disulfide exchange reactions over a broad range of conditions from acid to basic pH and in a wide variety of buffer constitutes and physiological conditions (7). Because of their special chemical properties, disulfide conjugate techniques have been widely used in drug delivery to achieve high delivery efficiencies (8-11). The most common method used in bioconjugates involves cross-linking or modification reactions using disulfide exchange processes to form disulfide linkage with sulfhydryl-containing molecules (7-11). However, this method is not suitable for the syntheses of most cationic lipids due to their specific chemical structures. We synthesized DOGSDSO by using a convenient method without the use of a cross-linking strategy.

Lipid/DNA complexes were treated with either media containing 10 mM DTT or DTT free-media for 12 hours at 37°C to evaluate the dissociation of DNA from complexes in reductive media. Only DNA complexed by DOGSDSO/DOPE liposomes and treated with DTT was released (Fig. 2). DNA complexed by DOGSHDO/DOPE liposome was not released in either environment. Without treatment of DTT, no DNA was released from the DOGSDSO/DOPE/DNA complex after incubatation for 12 hours at 37°C. This fact indicates that the dissociation of DNA from lipid complexes is difficult and may act as a barrier for gene transfection. When exposed to the high intracellular reductive substances, the disulfide bond of DOGSDSO may be reduced and DNA can be released. It is postulated that higher plasmid DNA concentrations within the cytoplasm will result in greater production of the transgene.

We tested the transfection activity of DOTAP/DOPE, DOGSDSO/DOPE, and DOGSHDO/DOPE liposomes in three cell lines. In initial experiments, the optimum

concentration of DOGSDSO/DOPE was determined and used for these studies. When other concentrations of the lipid were used in transfection, a similar trend was always seen but with different magnitudes of transgene expression. In these preliminary studies we evaluated the importance of the disulfide bond in the lipid structure as compared to its structural analog, DOGSHDO. DOTAP/DOPE was evaluated as a second control liposome composed of a commonly used cationic lipid. The goals of the experiment were in the evaluation of the disulfide linkage and not in producing a more effective gene delivery lipid. The RLU/mg protein represents the transgene expressed after transfection. These values ranged from 7×10^4 to 6×10^7 (Fig. 3) which is indicative of the cell lines used. DOGSDSO/DOPE demonstrated greater transgene expression than DOTAP/DOPE in all cell lines tested. In the cell lines tested there was a significant difference ($p < 0.01$) between treatment with DOGSDSO/DOPE and the other two formulations while there was no significant difference between DOTAP/DOPE and DOGSHDO/DOPE treatments. Compared to its analog, DOGSHDO, which has very similar structure except the disulfide bond, DOGSDSO has a greater than 50-fold effect on transfection activity in 293 cells and COS-1 cells and a 4 fold increase in SKnSH cells.

Using a calcein release assay we also tested cell viability of three cell lines to the liposome preparations. In two of the cell lines (293 cells and SKnSH cells) there was a significant difference ($p<0.01$) between all three treatments while in the COS cells treatment with DOGSDSO/DOPE was only significant different from treatment with DOTAP/DOPE liposomes. The toxicity results suggest DOGSDSO/DOPE liposomes did not improve transfection activity solely through its difference in toxic effects. It is highly possible that DOGSDSO/DOPE liposomes improve transfection activity by the specific mechanism of releasing the DNA in the reductive environment of the cytoplasm. At this time we can not exclude other mechanisms such as increase cellular uptake or endosomal escape as other viable explanations.

We tested cell viability of three cell lines to the liposome preparations using a calcein release assay. DOGSDSO/DOPE liposomes have a relatively low toxicity in SKnSH cells and COS-1 cells compared to the other two liposomes, but it demonstrated greater toxicity to 293. The toxicity results suggested DOGSDSO/DOPE did not improve transfection activity solely through its less toxic effects. It is highly possible that DOGSDSO/DOPE liposomes improve transfection activity by the specific mechanism of releasing the DNA in the reductive environment of the cytoplasm. At this time we can not exclude other mechanisms such as increase cellular uptake or endosomal escape as other viable explanations.

In summary, we have reported a method for the synthesis of disulfide bond-containing cationic lipids. Liposomes made of DOGSDSO/DOPE produced greater amounts of a reporter enzyme as compared to either DOTAP/DOPE liposomes or its analog DOGSHDO/DOPE liposomes. This report is one of the first to address the release of plasmid DNA as a potential barrier in non-viral gene delivery through rational design of the delivery system. In future studies, we will continue this work to verify that the DNA is being released within the cytoplasm, and that the greater transgene effect produced is due to higher cytoplasm concentrations of DNA. It should be pointed out that the experiments reported were done in mitotic cells in which the nuclear membrane is continuously being reformed. Therefore, plasmid DNA may have easier entrance to the transcripitional machinery of the cell as compared to post-mitotic cells.

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