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Quantitative Silencing of EGFP Reporter Gene by Self-Assembled siRNA Lipoplexes of LinOS and Cholesterol

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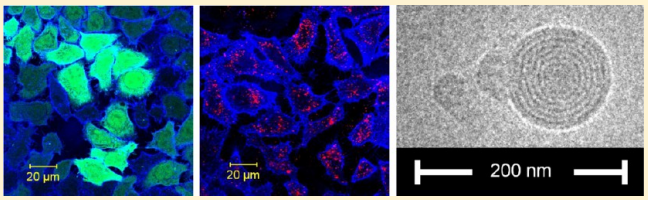
ABSTRACT: Nonviral siRNA vectors prepared by the direct mixing of siRNA and mixtures of an asymmetric N^4,N^9-diacyl spermine conjugate, N^4-linoleoyl-N^9-oleoyl-1,12-diamino-4,9-diazadodecane (LinOS), with either cholesterol or DOPE, at various molar ratios of the neutral lipids, are reported. The effects of varying the lipid formulation and changing the N/P charge ratio on the intracellular delivery of siRNA to HeLa cells and on the siRNA-mediated gene silencing of a stably expressed reporter gene (EGFP) were evaluated. The presence of either cholesterol or DOPE in the mixture resulted in a marked increase in the delivery of the siRNA as well as enhanced EGFP silencing as evaluated by FACS. A LinOS/Chol 1:2 mixture resulted in the highest siRNA delivery and the most efficient EGFP silencing (reduced to 20%) at N/P = 3.0. Lowering the amount of siRNA from 15 pmol to 3.75 pmol, thus increasing the N/P charge ratio to 11.9, resulted in decreasing the amount of delivered siRNA, while the efficiency of gene silencing was comparable to that obtained with 15 pmol (N/P = 3.0) of siRNA. Mixtures of symmetrical N^4,N^9-dioleoyl spermine (DOS) with cholesterol at 1:2 molar ratio showed less siRNA delivery than with LinOS/Chol at N/P = 3.0 (15 pmol of siRNA), and comparable delivery at N/P = 11.9 (3.75 pmol of siRNA). The EGFP silencing was comparable with LinOS and with DOS when mixed with cholesterol 1:2 (lipoplexes prepared with 15 pmol of siRNA), but LinOS mixtures showed better EGFP silencing when the siRNA was reduced to 3.75 pmol. Lipoplex particle size determination by DLS of cholesterol mixtures was 106−118 nm, compared to 194−356 nm for lipoplexes prepared with the spermine conjugates only, and to 685 nm for the LinOS/DOPE 1:1 mixture. Confocal microscopy showed successful siRNA delivery of red tagged siRNA and quantitative EGFP knockdown in HeLa EGFP cells; Z-stack photomicrographs showed that the delivered siRNA is distributed intracellularly. Cryo-TEM of siRNA LinOS/Chol 1:2 lipoplexes shows the formation of multilamellar spheres with a size of ∼100 nm, in good agreement with the particle size measured by DLS. The constant distance between lamellar repeats is ∼6 nm, with the electron-dense layers fitting a monolayer of siRNA. AlamarBlue cell viability assay showed that the lipoplexes resulted in cell viability ≥81%, with LinOS/Chol 1:2 mixtures resulting in cell viabilities of 89% and 94% at siRNA 15 nM and 3.75 nM respectively. These results show that lipoplexes of siRNA and LinOS/Chol mixtures prepared by the direct mixing of the lipid mixture and siRNA, without any preceding preformulation steps, result in enhanced siRNA delivery and EGFP knockdown, with excellent cell viability. Thus, LinOS/Chol 1:2 mixture is a promising candidate as a nontoxic nonviral siRNA vector.



KEYWORDS: *cholesterol, cryo-TEM, lipoplexes, nanoparticles, polyamine, self-assembly, siRNA, spermine, Z-stack*

INTRODUCTION

Small (or short) interfering RNA (siRNA) is a double-stranded RNA (dsRNA), typically 21−25 nucleotides per strand. Sequence-specific post-transcriptional gene silencing by siRNA has many potential therapeutic applications[1] as well as being an important tool in the study of functional genomics. In 1998, Fire, Mello, and co-workers reported the reduction or inhibition (hence genetic "interference") of the expression of a specific gene in *Caenorhabditis elegans* by means of dsRNA that is homologous to 742 nucleotides in the targeted gene,[2] a discovery that was awarded the Nobel Prize in Physiology or Medicine in 2006. In 2001, Elbashir et al. reported that sequence-specific gene silencing with 21 nucleotide siRNA occurs in mammalian cell cultures.[3] The optimum length of siRNA to affect sequence specific gene silencing in mammalian cells is typically less than 30 nucleotides in each strand of the dsRNA. Such a length does not induce interferon synthesis that leads to nonspecific mRNA degradation, but it maintains mRNA sequence-specific degradation.[3] The core complex for mRNA degradation is the RNA induced silencing complex (RISC), a complex of proteins and the siRNA that have a

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complementary sequence to the targeted mRNA. The key proteins in the degradation process belong to the argonaute family of proteins which contain a domain with RNase H (endonuclease) type activity that catalyzes cleavage of the phosphodiester bonds of the targeted mRNA. The assembly of RISC and its subsequent function to mediate sequence-specific mRNA degradation occur in the cytoplasm.[4]

Gene silencing mediated by siRNA requires that the siRNA is protected from various exo- and endonucleases[5] and is delivered intact to the cytoplasm of the target cell.[6] The negative charges of the siRNA phosphate backbone must be masked to facilitate the siRNA−vector complex (lipoplex) binding to the cell membrane, which is then followed by cellular entry of the lipoplex mainly via endocytosis and to a lesser extent by membrane fusion.[7] Thus, a vector is needed to fulfill these requirements. Nonviral vectors used for gene delivery (DNA based) and gene silencing by siRNA or shRNA include lipid-based vectors, polymer-based vectors, e.g., polyethylenimine, carbohydrate-based polymers, e.g., cyclodextrin and chitosan, dendrimers, e.g., polyamidoamine[8] and polypropylenimine, and polypeptides.[9−12] Lipid-based nonviral vectors are widely used for siRNA delivery.[13−15] We have previously designed, synthesized, and characterized fatty acid derivatives of the naturally occurring polyamine spermine, and tested their ability to deliver siRNA to cells in vitro[16−18] and to mediate siRNA dependent gene silencing.[19,20]

In this work, we report the formulations of a new spermine diacyl fatty acid derivative N^4-linoleoyl-N^9-oleoyl-1,12-diamino-4,9-diazadodecane characterized in preparing self-assembled lipoplexes with siRNA either on its own without a helper lipid or in coformulas with cholesterol or DOPE, and without preformulation of liposomes. The prepared lipoplexes were evaluated for their efficiency in delivering siRNA and in mediating gene-silencing and for their effects on cell viability.

MATERIALS AND METHODS

Materials and General Methods. Chemicals were purchased from Sigma-Aldrich (Gillingham, U.K.), and solvents were purchased from Fisher Scientific UK (Loughborough, U.K.). AlamarBlue and cell culture media were purchased from Gibco (Invitrogen Ltd., Paisley, U.K.). HeLa cells stably expressing EGFP were obtained from the Cell Service at Cancer Research UK (CRUK, London Research Institute, Clare Hall Laboratories, South Mimms, London, U.K.). The high resolution (HR) time-of-flight mass spectra were obtained on a Bruker Daltonics micrOTOF mass spectrometer using electrospray ionization (ESI). AllStars negative control siRNA (siNC) and the same tagged with Alexa Fluor 647 (siNC-AF) at the 3′-position were purchased from Qiagen (Crawley, U.K.) as was siRNA against EGFP labeled with Alexa Fluor 647 (siEGFP-AF) at the 3′-position of the sense strand, sequences:

sense strand: 5′-GCAAGCUGACCCUGAAGUUCAUTT-3′
antisense strand: 5′-AUGAACUUCAGGGUCAGCUUGCCG-3′
target DNA sequence: 5′-CGGCAAGCTGACCCTGAAGTTCAT-3′

N^4-Linoleoyl-N^9-oleoyl-1,12-diamino-4,9-diazadodecane (LinOS) and N^4,N^9-dioleoyl-1,12-diamino-4,9-diazadodecane (DOS). We confirmed the authenticity of N^4-linoleoyl-N^9-oleoyl-1,12-diamino-4,9-diazadodecane (LinOS) (HRMS, found $(M + H)^+$ 729.6980, $C_{46}H_{89}N_4O_2$ requires $(M + H)^+$ 729.6986) and N^4,N^9-dioleoyl-1,12-diamino-4,9-diazadodecane (DOS) (HRMS, found $(M + H)^+$ 731.7162, $C_{46}H_{91}N_4O_2$ requires $(M + H)^+$ 731.7137) by the HRMS of homogeneous samples.[20−25]

siRNA Lipoplex Preparation. LinOS, DOS, cholesterol, and DOPE were prepared as ethanolic solutions. For LinOS and DOS mixtures with cholesterol and DOPE, the required volumes of the ethanolic solutions of the single lipids were mixed together. To prepare the lipoplexes, two working liquids A and B were prepared. Liquid A was prepared by adding the required amount of siRNA (siEGFP-AF, siNC-AF, or siNC) to OptiMEM I media, such that the concentration of siRNA was adjusted to 1 pmol/1 μL. Liquid B was prepared by adding the required volume of lipid ethanolic solution to OptiMEM I media, such that the final concentration of LinOS or DOS was 0.75 μg/μL followed by mixing on a vortex mixer for 3 s. Liquid A was added to liquid B, and they were mixed by vortex mixer for 3 s. The lipoplex preparation was then simply allowed to stand for 20 min at 20 °C to allow lipoplex formation by charge neutralization and equilibration. TransIT-TKO was prepared according to the supplier's (Mirus) instructions.

Particle Size and Zeta Potential Measurements. Lipoplexes were prepared by adding siRNA solution (75 μL, 1 μM) in HEPES (pH 7.4, 10 mM) to HEPES (250 μL) containing the specified amount of cationic lipid transfection reagent followed by vortex mixing for 3 s. After 20 min, samples were diluted to a final volume of 3 mL with HEPES buffer and shaken gently for 10 s directly before measurement. Measurements were carried out using Malvern Zetasizer Nano S90 with refractive index 1.59, viscosity 0.89 cP, dielectric constant 79, temperature 25 °C, and equilibrium time 3 min. *Z*-Average diameter (nm) and zeta potential (mV) were recorded as averages of three and six measurements respectively.

Transfection Studies of HeLa Cells Stably Expressing EGFP. Cells were trypsinized at confluency of 80−90% and were seeded at a density of 65,000 cells/well in 24-well plates. They were incubated for 24 h at 37 °C, 5% CO_2, prior to transfection. On the day of transfection, the lipoplex solutions were added to wells containing DMEM (10% FCS) to make the final volume in each well 1 mL and final siRNA concentration 15 nM. The plates were then incubated for 48 h at 37 °C, 5% CO_2. The *N*/*P* charge ratio is calculated as

$$N/P = (\text{no. of moles of cationic lipid} \times 2) / (\text{no. of moles of siRNA} \times \text{no. of bases in one siRNA strand} \times 2)$$

Flow Cytometry (FACS). For analysis of delivery and then reduction of expression of EGFP by flow cytometry (FACS), cells were trypsinized, resuspended in complete DMEM medium without phenol red. Cells were centrifuged (1,000 rpm for 5 min), washed twice by resuspending in PBS containing 0.1% BSA, and then recentrifuged (1,000 rpm for 5 min). The collected cells was then resuspended in PBS and transferred to a flow cytometer tube (Becton Dickinson, U.K.). Cells were analyzed (10,000 or 20,000 events) using a FACSCanto flow cytometer (Becton Dickinson, U.K.), equipped with an argon ion laser at 488 nm for excitation, a long pass (LP) filter at 502 nm and a detector at 530 nm (range ±15 nm) for fluorescence emission, helium/neon laser at 633 nm, and detector for the Alexa Fluor 647 at 660 nm (range ±10 nm). EGFP expression is calculated as

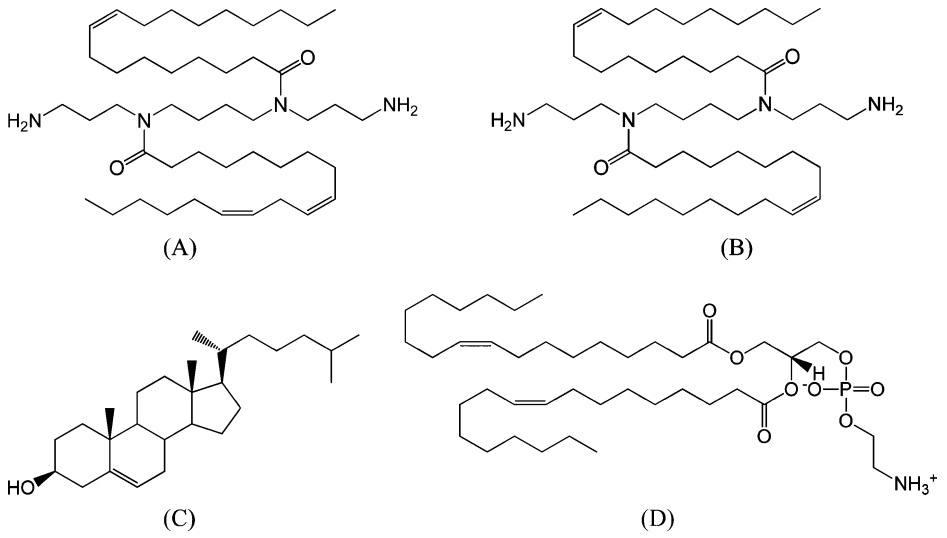

Figure 1. (A) N^4-Linoleoyl-N^9-oleoyl-1,12-diazododecane (LinOS), (B) N^4,N^9-dioleoyl-1,12-diazododecane (DOS), (C) cholesterol, (D) 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE).

$$\%\ \text{EGFP} = \frac{\text{EGFP fluorescence of transfected cells}}{\text{EGFP fluorescence of control cells}} \times 100$$

siRNA delivery was evaluated 48 h post-transfection by means of normalizing the geometric mean fluorescence of the Alexa Fluor 647 of each sample relative to the geometric mean fluorescence of Alexa Fluor 647-siRNA delivered by either of two standards, DOS or TransIT-TKO.

Confocal Microscopy Cell Imaging. Cells were trypsinized at confluency of 80−90%, were seeded at a density of 65,000 cells/well in 24-well plates that have a round-glass coverslip (12 mm diameter), and were incubated for 24 h prior to transfection, which was carried out as described above. After 48 h, the cell culture media were aspirated from each well, and the cells were washed with PBS (3 × 0.5 mL). The cell membrane was then stained with wheat germ agglutinin (WGA) conjugated to Alexa Fluor 555. The concentration of WGA-Alexa Fluor 555 working solution was 5 μg/mL in Hanks balanced salt solution without phenol red. The cells were incubated for 10 min in the dye working solution at 37 °C, 5% CO_2 in the dark. The cells were washed with PBS (3 × 0.5 mL) and then fixed with 4% paraformaldehyde in PBS solution for 20 min at 20 °C in the dark. The coverslips were then removed from each well, left to dry briefly in air, then mounted on glass slides using Mowiol (polyvinyl alcohol from Calbiochem, Nottingham, U.K.) solution as the mounting medium, and left in the dark at 20 °C (18 h) to allow hardening of the mounting medium. The cells were examined using a Carl Zeiss laser scanning microscope LSM 510 meta, with EGFP excitation 488 nm, emission 505−550 nm (band-pass filter), Alexa Fluor 555 excitation 543 nm, emission 560−615 nm (band-pass filter), and Alexa Fluor 647 excitation 633 nm, emission 657−753 nm (meta detector).

Cryo-Transmission Electron Microscopy (Cryo-TEM). siNC lipoplexes were prepared with LinOS/Chol 1:2 (0.75 μg of LinOS per 3.75 pmol of siNC, N/P = 11.9) in 10 mM HEPES buffer. A sample (5 μL) was pipetted onto a previously glow discharged, lacy carbon-coated copper grid (Electron Microscopy Services). The excess was then blotted and the sample plunge frozen into liquid ethane using a Vitrobot plunge freezer (FEI Company). The sample was transferred to a Gatan 626 cryotransfer holder, and the lipoplexes were examined at a temperature of approximately −170 °C in an FEI Tecnai 20 transmission electron microscope operating at 200 kV.

Cell Viability Assay. HeLa cells were trypsinized at confluency of 80−90% and seeded at a density of 6,500 cells/well of 96-well plates. The transfection was carried out using the same protocol as transfecting the 24-well plates, as described above, with the exception of reducing the amount of siNC lipoplexes such that each well typically contains 1.5 pmol of siNC in a final volume of 100 μL/well (15 nM) of DMEM containing 10% FCS. Also, the cell viability of LinOS/Chol (1:2) 3.75 was measured at only 0.375 pmol of siNC/well (3.75 nM). After incubation for 44 h at 37 °C in 5% CO_2, alamarBlue (10 μL) was added to each well. After incubation for 3.5 h at 37 °C in 5% CO_2, the absorbance of each well was measured at 570 and 600 nm using a microplate-reader (VERSAmax), and the amount of reduced alamarBlue at 570 nm was calculated as

$$\text{amount reduced} = A_{570} - (A_{600}R),$$

$$R = \frac{\text{AOx}_{570}}{\text{AOx}_{600}}, \text{ correction factor without cells}$$

where AOx_{570} and AOx_{600} are the absorbance of oxidized alamarBlue at 570 and 600 nm respectively.

Percentage viability is calculated as

$$\%\ \text{viability} = \frac{\text{amount of reduced alamarBlue of sample cells}}{\text{amount of reduced alamarBlue of control cells}} \times 100$$

Statistical Analysis. All data are presented as mean + SD (n = 9). The mean values and SD were determined using MS Office Excel 2003. Statistical significance of differences between data was evaluated by Student's unpaired two tailed t-test. A value of $p < 0.05$ was considered significant, and p values were determined using GraphPad.

RESULTS

We will evaluate the efficiency of both siRNA delivery and gene silencing by siRNA lipoplexes prepared from LinOS (Figure 1A) or DOS (Figure 1B) coformulated with either cholesterol (Figure 1C) or DOPE (Figure 1D) helper lipids. LinOS and DOS are derivatives of the naturally occurring polyamine,

spermine, conjugated to the naturally occurring C18 unsaturated fatty acids: oleic acid (18:1) and/or linoleic acid (18:2). Cholesterol and DOPE are neutral helper lipids widely used in gene therapy as they aid membrane fusion, which may be one of the mechanisms (along with endocytosis) of the functional delivery of lipoplexes.[7]

The lipid dispersions in OptiMEM I media were prepared by addition of ethanolic solutions of the single lipids (Figure 1) or lipid mixtures to OptiMEM I followed by brief mixing on a vortex mixer. This simple procedure avoids the use of sonication or extrusion techniques which are used to prepare single lamellar vesicles and/or reduce the size of the prepared lipid vesicles. Our procedure can be considered as an even more direct method than the ethanol injection vesicle protocol.[26,27]

Figure 2 shows the effect of changing the LinOS/Chol molar ratio on the delivery of siEGFP-AF or siNC-AF in the

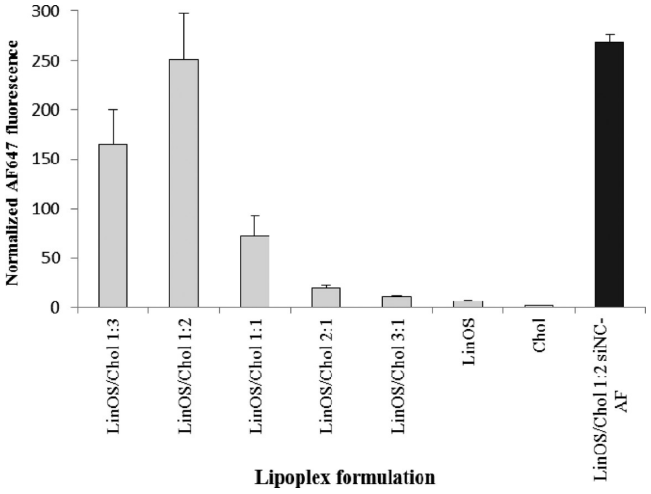

Figure 2. siEGFP-AF delivery to HeLa cells expressed as the normalized values of the geometric mean fluorescence of Alexa Fluor 647 (AF647) 48 h post-transfection of HeLa cells with the lipoplexes prepared with LinOS/Chol and either siEGFP-AF at different LinOS/Chol ratios or siNC-AF at LinOS/Chol 1:2 (per each well, the amounts of LinOS, siEGFP-AF, and siNC-AF were kept constant at 0.75 μg, 15 pmol, and 15 pmol respectively, N/P = 3.0). The LinOS/Chol ratio is the molar ratio. Light gray columns represent lipoplexes prepared with siEGFP-AF; the black column represents lipoplexes of LinOS/Chol 1:2 with siNC-AF.

transfected HeLa cells measured by flow cytometry (FACS). The highest siEGFP-AF delivery was achieved with lipoplexes having a LinOS/Chol ratio of 1:2, as these lipoplexes resulted in normalized Alexa Fluor 647 (AF647) fluorescence of 250. The difference between the value obtained by lipoplexes of LinOS/Chol 1:2 and the closest value of 165 of lipoplexes of LinOS/Chol 1:3 was statistically significant (p = 0.0005). Decreasing the molar ratio of LinOS/Chol from 3:1 to 1:2, i.e., increasing the amount of cholesterol in the mixtures, resulted in an increase in the normalized AF647 fluorescence from 11 to 250 respectively. Coformulation with cholesterol in the lipoplexes of LinOS/Chol 1:2 resulted in a significant increase of normalized AF647 fluorescence when compared with lipoplexes of LinOS only, from 6 with LinOS lipoplexes to 250 with LinOS/Chol 1:2 lipoplexes, which means a ~42-fold increase in siEGFP-AF delivery. The cholesterol data column (Chol) shows that cholesterol alone did not result in any significant siEGFP-AF delivery. Lipoplexes of siNC-AF and LinOS/Chol 1:2 resulted in comparable delivery of siNC-AF when compared with lipoplexes of siEGFP-AF and LinOS/Chol 1:2, normalized AF fluorescence of 268 and 250 respectively (p = 0.28).

Figure 3 shows the effect of changing the LinOS/Chol molar ratio on the percentage expression of EGFP in the transfected

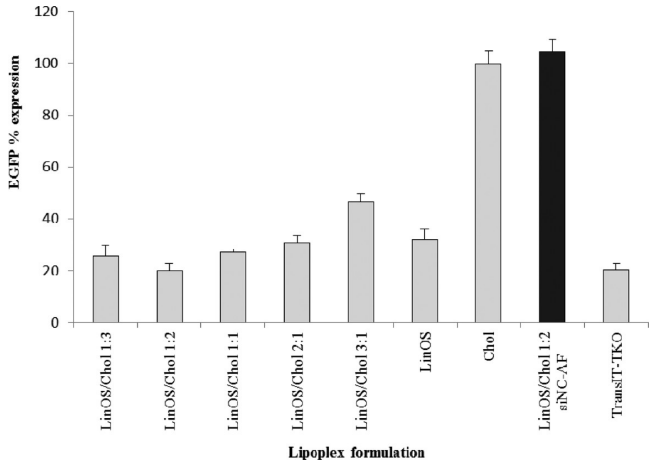

Figure 3. EGFP percentage expression calculated 48 h post-transfection of HeLa cells with the lipoplexes prepared with LinOS/Chol and either siEGFP-AF at different LinOS/Chol ratios or siNC-AF at LinOS/Chol 1:2 (per each well, the amounts of LinOS, siEGFP-AF, and siNC-AF were kept constant at 0.75 μg, 15 pmol, and 15 pmol respectively, N/P = 3.0). Light gray columns represent lipoplexes prepared with siEGFP-AF; the black column represents lipoplexes of LinOS/Chol 1:2 with siNC-AF.

HeLa cells measured by FACS. The LinOS/Chol molar ratio was changed from 3:1 to 1:3. The best lipoplexes were those having a LinOS/Chol ratio of 1:2, as these lipoplexes resulted in a reduction of EGFP percentage expression to 20%, which is statistically significant when compared to the reduction of EGFP obtained by lipoplexes of LinOS/Chol 1:3 (26%, p = 0.0024) and LinOS/Chol 1:1 (27%, p = 0.0001), which were the second best in terms of EGFP expression reduction. Coformulation with cholesterol in the lipoplexes of LinOS/Chol 1:2 resulted in reducing the EGFP percentage expression from 32% for lipoplexes of LinOS only to 20% (p = 0.0001). The cholesterol data column shows that siEGFP-AF only formulated with cholesterol did not have any practically significant effect on EGFP expression (100% ± 5). Lipoplexes of siNC-AF and LinOS/Chol 1:2 did not result in any reduction in EGFP expression (105% ± 5). Transfection of siEGFP-AF using the commercial reagent TransIT-TKO (a proprietary formulation based on a cationic polymer formulation) under the same experimental conditions, siEGFP-AF (15 pmol) formulated with TransIT TKO, resulted in EGFP percentage of expression of 20% (Figure 3). Thus, in terms of transfection efficiency, there was no statistically significant difference between the percentage reductions of EGFP expression due to transfection with lipoplexes of LinOS/Chol 1:2 and TransIT-TKO (p = 1.00).

LinOS/Chol 1:2 lipoplexes with siEGFP-AF resulted in both highest siRNA delivery and most efficient reduction of EGFP (from 100% to 20%, 5-fold). The reduction of EGFP with LinOS/Chol lipoplexes having different LinOS/Chol ratios is affected by the amount of siEGFP-AF delivered. However, it

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can be seen that although siRNA delivery with lipoplexes of LinOS/Chol of molar ratio 1:2 and 3:1 was 250 and 11 respectively (~23-fold), the reduction of EGFP was to 20% and to 46% (~2-fold). Thus, it is difficult to predict the functional biological activity of siRNA based solely on the amount delivered. One explanation is that siRNA lipoplexes might be delivered via different cellular internalization pathways such as clathrin- or caveolin-mediated endocytosis and/or membrane fusion. A recent report showed that the functional delivery of siRNA lipoplexes is not necessarily via endocytic pathways, but rather might be due to another cellular internalization mechanism such as membrane fusion.[7] Thus, although the amounts of siRNA delivered might vary largely, the resultant reduction in EGFP may not correspond exactly with that same large variation.

Figure 4 shows the effect of changing the LinOS/DOPE molar ratio from 1:3 to 3:1 on the delivery of siEGFP-AF or siNC-AF in the transfected HeLa cells. Coformulation with DOPE in the lipoplexes of LinOS/DOPE resulted in a significant increase of normalized AF647 fluorescence when compared with lipoplexes of LinOS only, from 6 with LinOS only to 85 and 78 respectively (p = 0.24) for lipoplexes of LinOS/DOPE 1:3 and 1:1, which gave the highest AF647 normalized fluorescence. Lipoplexes with molar ratio of LinOS/DOPE 1:2 and 2:1 resulted in normalized AF647 fluorescence of 48 and 21 respectively (p = 0.0001). The DOPE data column shows that siEGFP-AF formulation with DOPE only did not result in any practically significant siEGFP-AF delivery. Lipoplexes of siNC-AF and DOPE/Chol 1:1 resulted in normalized AF647 fluorescence of 51.

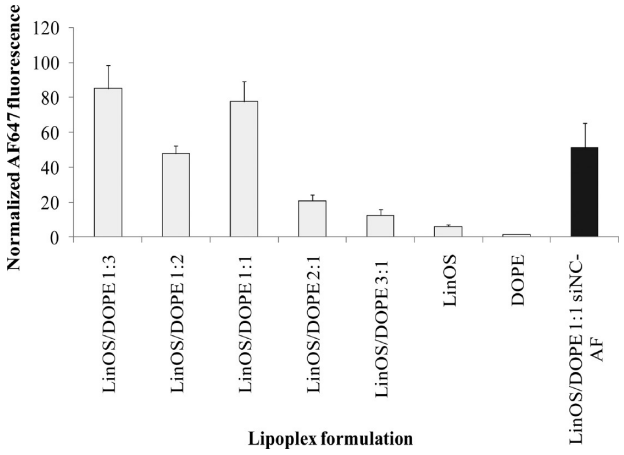

Figure 4. siEGFP-AF delivery to HeLa cells expressed as the normalized values of the geometric mean fluorescence of AF647 48 h post-transfection with the lipoplexes prepared with LinOS/DOPE (LinOS/DOPE) and siEGFP-AF at different LinOS/DOPE ratios and N/P = 3.0 (per each well, the amounts of LinOS and siEGFP-AF were kept constant at 0.75 μg and 15 pmol respectively). Light gray columns represent lipoplexes prepared with siEGFP-AF while the black column represents lipoplexes of LinOS/DOPE 1:1 with siNC-AF.

Figure 5 shows the effect of changing the LinOS/DOPE molar ratio on the percentage expression of EGFP in the transfected HeLa cells. The LinOS/DOPE molar ratio was changed from 1:3 to 3:1. There were very little differences between the percentage expressions of EGFP after transfection with the LinOS/DOPE lipoplexes at all LinOS/DOPE ratios, with LinOS/DOPE 1:1 lipoplexes resulting in EGFP

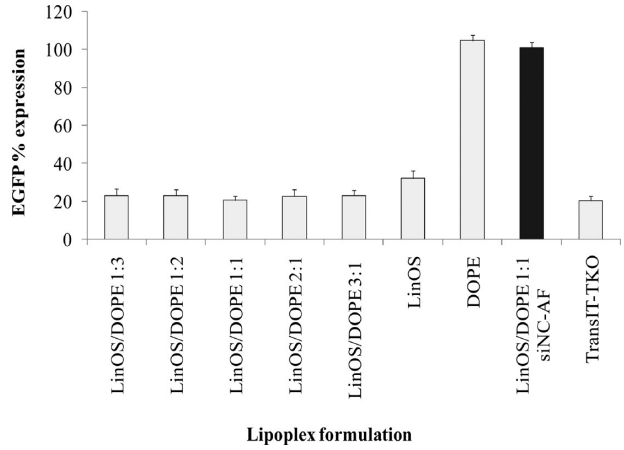

Figure 5. EGFP percentage expression calculated 48 h post-transfection of HeLa cells with the lipoplexes prepared with LinOS/DOPE and siEGFP-AF at different LinOS/DOPE ratios and N/P = 3.0 (per each well, the amounts of LinOS and siEGFP-AF were kept constant at 0.75 μg and 15 pmol respectively). Light gray columns represent lipoplexes prepared with siEGFP-AF while the black column represents lipoplexes of LinOS/DOPE 1:1 with siNC-AF.

percentage expression of 21%, and no statistically significant difference was found between any of the EGFP percentage expressions resulting from the transfection with the LinOS/DOPE lipoplexes. Coformulation with DOPE in the lipoplexes of LinOS/DOPE 1:1 resulted in reducing the EGFP percentage expression from 32% for lipoplexes of LinOS to 21% (p = 0.0001). DOPE column shows that formulating siEGFP-AF with DOPE only did not result in any practically significant effect on EGFP expression (105% ± 3). Lipoplexes of siNC-AF and LinOS/DOPE 1:1 did not result in any reduction in EGFP expression (101% ± 3). There was no statistically significant difference between the percentage expressions of EGFP after transfection with lipoplexes of LinOS/DOPE 1:1 and TransIT-TKO (p = 0.49). Although the amount of delivered siEGFP-AF increased markedly with the addition of DOPE, the differences in the delivered amount did not reflect significant differences in the reduction of EGFP corresponding to the differences in the delivered amount. For example, lipoplexes of LinOS/DOPE 1:1 and 3:1 delivered siEGFP-AF with values of 78 and 12 respectively, and reduced EGFP (from 100%) to 21% and 23% respectively. This, as discussed previously with LinOS/Chol lipoplexes, reflects the possibility of the presence of a specific functional mechanism which results in the required specific gene silencing.[7]

Figure 6 shows the effect of changing the N/P charge ratio from 3.0 to 11.9 by means of reducing the amount of siEGFP-AF (or siNC-AF) from 15 pmol/well of 24-well plates to 3.75 pmol/well, on the normalized AF647 fluorescence in the transfected cells. A comparison between the LinOS/Chol 1:2 lipoplexes and DOS/Chol 1:2 lipoplexes at N/P = 3.0 and 11.9 is also shown. The normalized AF647 fluorescence is significantly higher in the case of lipoplexes prepared with 15 pmol of siEGFP-AF or siNC-AF when compared to the lipoplexes prepared with 3.75 pmol of siEGFP-AF or siNC-AF at N/P = 11.9. siEGFP-AF lipoplexes LinOS/Chol 15 and LinOS/Chol 3.75 resulted in AF647 normalized fluorescence of 250 and 41 respectively and p = 0.0001; siNC-AF lipoplexes LinOS/Chol 15 and LinOS/Chol 3.75 resulted in AF647 normalized fluorescence of 151 and 23 respectively and p = 0.0001. siEGFP-AF lipoplexes of DOS/Chol 15 and DOS/Chol

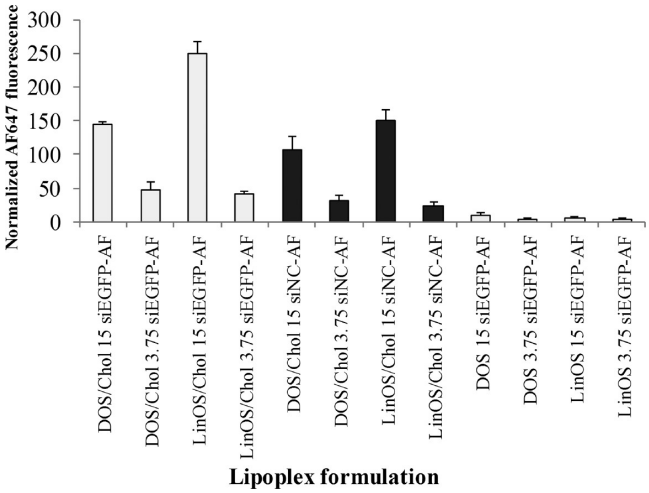

Figure 6. siEGFP-AF delivery to HeLa cells 48 h post-transfection with the lipoplexes prepared with LinOS/Chol 1:2 or DOS/Chol 1:2 at N/P = 3.0 or N/P = 11.9 (per each well, the amounts of LinOS and DOS were kept constant at 0.75 μg). Lipoplex names ending in 15 and 3.75 represent lipoplexes prepared with 15 pmol and 3.75 pmol respectively of siEGFP-AF or siNC-AF. Light gray columns represent lipoplexes prepared with siEGFP-AF while black columns represent lipoplexes prepared with siNC-AF.

3.75 resulted in AF647 normalized fluorescence of 144 and 48 respectively and p = 0.0001; siNC-AF lipoplexes of DOS/Chol 15 and DOS/Chol 3.75 resulted in AF647 normalized fluorescence of 106 and 32 respectively and p = 0.0001. Lipoplexes of LinOS/Chol 15 showed the highest AF647 normalized fluorescence. AF647 normalized fluorescence resulting from transfecting HeLa cells with lipoplexes coformulated with cholesterol were significantly higher than the fluorescence resulting from transfection with DOS or LinOS lipoplexes (DOS 15, DOS 3.75, LinOS 15, and LinOS 3.75, the four columns on the right) with siEGFP-AF.

Figure 7 shows the effect of changing the N/P charge ratio from 3.0 to 11.9, by means of reducing the amount of siEGFP-AF (or siNC-AF) from 15 pmol/well of 24-well plates to 3.75 pmol/well, on the percentage of EGFP expression in the

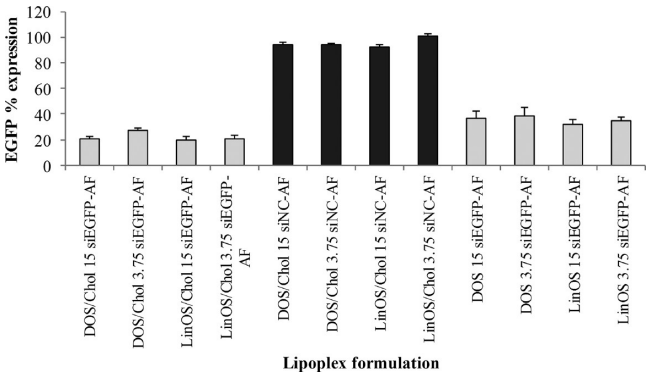

Figure 7. EGFP percentage expression 48 h post-transfection with lipoplexes prepared with LinOS/Chol 1:2 or DOS/Chol 1:2 at N/P = 3.0 or N/P = 11.9 (per well, the amounts of LinOS and DOS were kept constant at 0.75 μg). Lipoplex names followed by 15 and 3.75 represent lipoplexes prepared with 15 pmol and 3.75 pmol respectively of siEGFP-AF or siNC-AF. Light gray columns represent lipoplexes prepared with siEGFP-AF; black columns represent lipoplexes prepared with siNC-AF.

transfected HeLa cells. Also shown in Figure 7 is a comparison between the LinOS/Chol 1:2 lipoplexes and DOS/Chol 1:2 lipoplexes at N/P = 3.0 and 11.9. HeLa cells transfected with lipoplexes of siEGFP-AF and LinOS/Chol 1:2 did not show a significant decrease in the efficiency of transfection, on decreasing the amount of siEGFP-AF from 15 pmol/well to 3.75 pmol/well. Lipoplexes of LinOS/Chol 15 and LinOS/Chol 3.75 resulted in EGFP percentage expression of 20% and 21% respectively (p = 0.42). Lipoplexes of DOS/Chol 1:2 showed a significant change (p < 0.01) of EGFP percentage expression from 21% in the case of DOS/Chol 15 to 28% in the case of DOS/Chol 3.75 lipoplexes (with siEGFP-AF 15 pmol and 3.75 pmol respectively). There were no statistically significant differences between the EGFP percentage expression due to transfection with lipoplexes of siEGFP-AF with either DOS/Chol 15 or LinOS/Chol 15 (21% and 20% respectively, p = 0.42). However, transfection with lipoplexes of LinOS/Chol 3.75 resulted in lower EGFP percentage expression (21%) compared to lipoplexes of DOS/Chol 3.75 (28%), p = 0.0001, and thus we determined experimentally that the lipoplexes of LinOS/Chol 3.75 are superior to the lipoplexes of DOS/Chol 3.75 (Figure 7). Transfection with lipoplexes of siNC-AF (15 pmol or 3.75 pmol) with DOS/Chol 1:2 or LinOS/Chol 1:2 did not result in any significant reduction of EGFP expression. Coformulation of DOS or LinOS with cholesterol reduced the EGFP percentage expression significantly. The EGFP percentage expressions in the transfected HeLa cells for DOS/Chol 15, DOS/Chol 3.75, LinOS/Chol 15, and LinOS/Chol 3.75 were 21%, 28%, 20%, and 21% respectively, compared to DOS 15, DOS 3.75, LinOS 15, and LinOS 3.75 lipoplexes, which resulted in EGFP percentage expressions of 37%, 38%, 32%, and 35% respectively. Thus there are significant improvements (p = 0.0001 for all four respectively) in the gene silencing on mixing with cholesterol. The amount of siEGFP-AF delivered with either DOS/Chol or LinOS/Chol lipoplexes prepared with 15 pmol of siEGFP-AF was higher by ~3-fold and 5-fold respectively (Figure 6) when compared to lipoplexes prepared with 3.75 pmol of siEGFP-AF. The reduction in EGFP expression by the DOS/Chol or LinOS/Chol lipoplexes prepared with either 15 pmol or 3.75 pmol only varied slightly as discussed above (Figure 7).

In Figure 7, the gene silencing of DOS/Chol 1:2 and LinOS/Chol 1:2 with 15 pmol and 3.75 pmol of siEGFP-AF was efficient regardless of the difference in siEGFP-AF delivery (Figure 6), which can be explained on the basis of the actual functional mechanism of siEGFP delivery (e.g., an endocytotic mechanism vs membrane fusion) and which in turn determines the actual functional fraction of the delivered siEGFP-AF.[7] Also, the gene silencing values shown in Figure 7 are close to the maximum reduction in EGFP expression possible after 48 h transfection, based on the ~24 h half-life of EGFP. Thus, quantitative silencing of EGFP reporter gene has been achieved by self-assembled siRNA lipoplexes of LinOS and cholesterol. LinOS/Chol 1:2 mixture resulted in the highest siRNA delivery and the most efficient EGFP silencing (reduced to 20%, i.e., quantitative after ~two half-lives) at N/P = 3.0. The efficiency of EGFP gene silencing was comparable on lowering the amount of siRNA from 15 pmol to 3.75 pmol. The 3.75 pmol of siEGFP-AF payload was chosen specifically to evaluate the effect of increasing the N/P ratio while reducing the payload of siEGFP-AF without changing the amount of cationic lipid. The siEGFP-AF payload was not further reduced below 3.75 pmol because it would have resulted in an exceptionally high N/P

ratio possibly with accompanying negative effects on cell viability.

The cationic lipid/helper lipid ratios which resulted in the best reduction in EGFP expression post-transfection with siEGFP-AF lipoplexes were selected (Figures 3, 5, and 6) for physicochemical analysis (Table 1). Lipoplexes were prepared with either 15 pmol of siNC (N/P = 3.0) or 3.75 pmol of siNC (N/P = 11.9) except for LinOS/DOPE, which was prepared with 15 pmol of siNC only (N/P = 3.0). Self-assembled lipoplexes of siRNA and cholesterol coformulations with DOS or LinOS resulted in particle size (measured by DLS) in the range of 106−127 nm (Table 1). There was a slight increase in particle size in lipoplexes of DOS/Chol 1:2 from 106 to 127 nm upon decreasing the amount of siRNA from 15 pmol to 3.75 pmol (increasing N/P charge ratio from 3.0 to 11.9), p = 0.03. There was no statistically significant difference between the particle sizes of lipoplexes of LinOS/Chol 1:2 with either 15 or 3.75 pmol (p = 0.41). The type of cationic lipid used (DOS or LinOS) in the cholesterol mixtures did not have a significant effect on the resulting particle size at the same N/P charge ratio (same amount of siRNA), where DOS/Chol and LinOS/Chol lipoplexes at N/P = 3.0 (15 pmol of siRNA) resulted in particle size of 106 and 113 respectively (p = 0.47), and DOS/Chol and LinOS/Chol lipoplexes at N/P = 11.9 (3.75 pmol of siRNA) resulted in particle size of 127 and 118 respectively (p = 0.05). Lipoplexes prepared with siRNA and either DOS or LinOS, without any helper lipid, have a particle size in the range of 192−356 nm. There was a statistically significant difference between the DOS and the LinOS lipoplex particle sizes (356 and 294 respectively, p = 0.007) at N/P = 3.0 (15 pmol of siRNA). At N/P = 11.9, the type of cationic lipid did not affect the particle size, with lipoplexes of DOS and LinOS having particle sizes of 192 and 194 respectively (p = 0.72). Increasing the N/P charge ratio to 11.9 (lowering the siRNA amount from 15 to 3.75 pmol) reduced the lipoplex particle size from 356 to 192 nm (DOS, p = 0.0001) and 294 to 194 nm (LinOS, p = 0.0001). Coformulation with cholesterol resulted in significant reduction of the prepared lipoplexes, when comparing the lipoplexes of each cationic lipid with or without cholesterol. The particle sizes of DOS/Chol 15, DOS/Chol 3.75, LinOS/Chol 15, and LinOS/Chol 3.75 (106, 127, 113, and 118 nm respectively) are significantly reduced

Table 1. Effect of Formulation on the Particle Size and ζ-Potential of Lipoplexes of LinOS, DOS, DOS/Chol, LinOS/Chol, and LinOS/DOPE Mixtures

lipoplex formulation	particle size (nm), mean ± SD	PDI, mean ± SD	ζ-potential (+mV), mean ± SD
DOS/Chol 1:2 15 pmol of siNC	106 ± 19	0.35 ± 0.04	58 ± 4
DOS/Chol 1:2 3.75 pmol of siNC	127 ± 8	0.37 ± 0.02	60 ± 5
LinOS/Chol 1:2 15 pmol of siNC	113 ± 13	0.34 ± 0.05	60 ± 4
LinOS/Chol 1:2 3.75 pmol of siNC	118 ± 6	0.35 ± 0.06	56 ± 1
DOS 15 pmol of siNC	356 ± 37	0.48 ± 0.06	56 ± 2
DOS 3.75 pmol of siNC	192 ± 10	0.32 ± 0.05	52 ± 4
LinOS 15 pmol of siNC	294 ± 25	0.41 ± 0.06	53 ± 3
LinOS 3.75 pmol of siNC	194 ± 9	0.33 ± 0.04	60 ± 3
LinOS/DOPE 1:1 15 pmol of siNC	685 ± 83	0.66 ± 0.08	64 ± 4

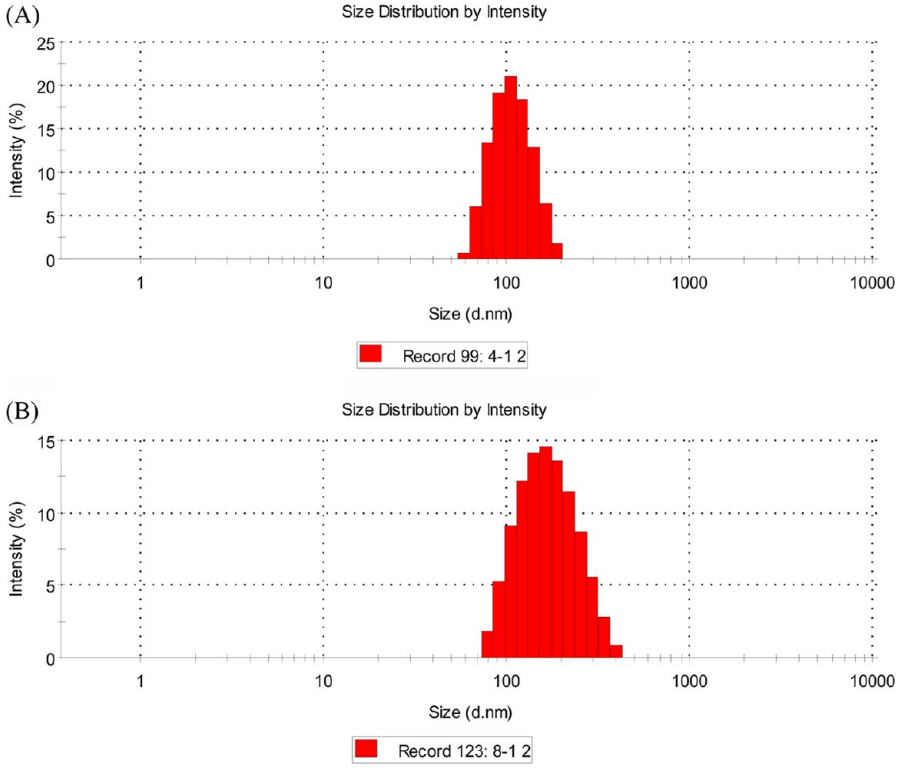

Figure 8. (A) Particle size distribution (DLS) for LinOS/Chol 1:2 lipoplexes prepared with 3.75 pmol of siNC (N/P = 11.9). The hydrodynamic diameter is 117 nm and polydispersity index (PDI) = 0.31 for the shown lipoplexes. (B) Particle size distribution (DLS) for LinOS (only) lipoplexes prepared with 3.75 pmol of siNC (N/P = 11.9). The hydrodynamic diameter is 187 nm and PDI = 0.38 for the shown lipoplexes.

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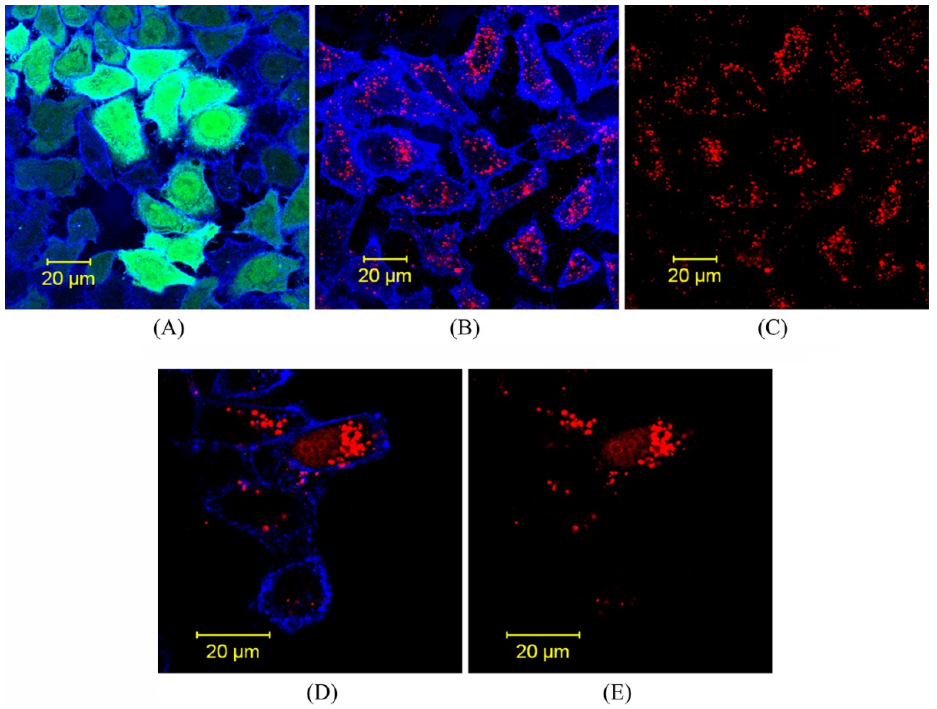

Figure 9. Confocal photomicrographs. EGFP fluorescence (green), cell membrane stained with WGA-Alexa Fluor 555 (blue), and Alexa Fluor 647 (red) which shows tagged siEGFP-AF delivery. (A) Control nontransfected HeLa cells, (B) HeLa cells 48 h post-transfection with lipoplexes of LinOS/Chol 1:2 and siEGFP-AF (3.75 pmol), (C) as panel B with the red channel only turned on for better visualization of the delivered siEGFP-AF, and (D, E) magnified HeLa cells 48 h post-transfection, as in panels B and C respectively.

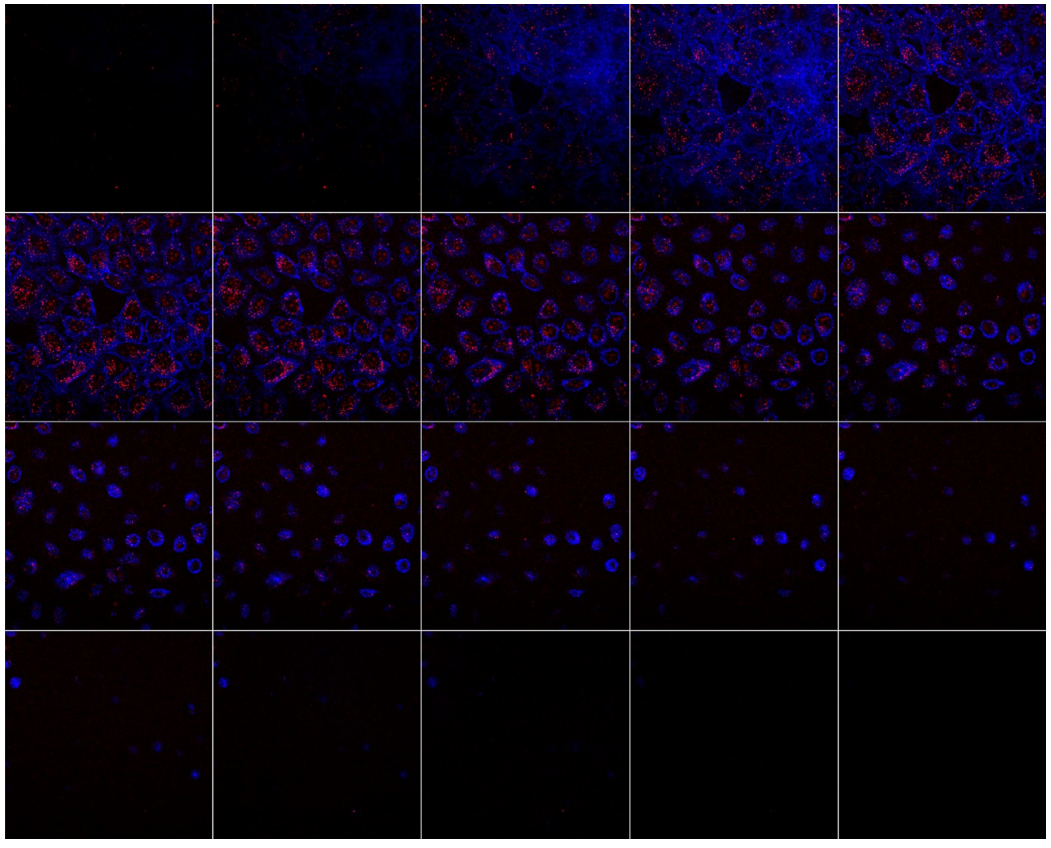

Figure 10. *Z*-Stack confocal photomicrographs. EGFP fluorescence (green), cell membrane stained with WGA-Alexa Fluor 555 (blue), and Alexa Fluor 647 (red) represents tagged siRNA delivery. A *Z*-stack series of photomicrographs representing 20 *Z*-sections in HeLa cells transfected with lipoplexes of LinOS/Chol 1:2 and siEGFP-AF (3.75 pmol).

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compared to DOS 15, DOS 3.75, LinOS 15, and LinOS 3.75 (356 nm, $p = 0.0001$; 192 nm, $p = 0.0001$; 294 nm, $p = 0.0001$; and 194 nm, $p = 0.0001$ respectively). The lipoplexes of LinOS/DOPE 1:1 and 15 pmol of siRNA ($N/P = 3.0$) had the relatively larger particle size of 685 nm compared to the other lipoplex formulations.

The ζ-potentials of the prepared lipoplexes are all positive and lie in the range 53−64 mV (Table 1). There was no significant effect of the coformulation with cholesterol on the ζ-potentials of their prepared lipoplexes compared to the lipoplexes of their cationic lipids without cholesterol. The presence of DOPE caused no or only a very slight increase in the lipoplex ζ-potential (+64 mV) when compared to the other lipoplexes prepared with the same amount of siRNA (15 pmol) and at the same $N/P = 3.0$.

Lipoplex size is an important factor in transfection efficiency though it is not the only determinant factor.[28] Cationic cholesterol derivatized liposomes complexed with siRNA have a size range of 150−500 nm,[29] where selected siRNA lipoplexes were used either to deliver fluorescently tagged scrambled siRNA to different cell lines including HeLa cells or to deliver siRNA silencing GFP in a T293 cell line that stably expresses GFP. Lipoplex size affects the main route of cellular entry where smaller lipoplexes (diameter <300 nm) are likely to enter by clathrin-mediated endocytosis, while larger particles (diameter >500 nm) enter cells by caveoli-mediated endocytosis.[30,31] Also, the entry route that results in functional siRNA mediated gene knockdown might be by fusion with the plasma membrane rather than the endocytosis pathway.[7] The functional delivery of lipoplexes of oligonucleotides in two cell lines, including a HeLa S3 cell line, was recently reported to be by membrane fusion.[32] The authors concluded that lipoplexes internalized in cells by direct membrane fusion improve the functional delivery of oligonucleotide cargoes because they might avoid the endosomal escape step, which is the rate-limiting step for many pDNA and siRNA delivery vectors. However, the lipoplex size used in that study was 869 nm measured in OptiMEM I medium.[32]

Shown in Figure 8 are monomodal populations of LinOS/Chol 1:2 and LinOS (only) lipoplexes. The polydispersity indices (PDI) of the lipoplexes (Table 1) prepared with LinOS or DOS cholesterol mixtures were 0.35−0.37. There was no effect of changing the siRNA amount in the LinOS/Chol or DOS/Chol lipoplexes on the PDI. The PDI of lipoplexes prepared with the cationic lipids only varied from 0.32 to 0.48. Decreasing the amount of siRNA (thus increasing N/P from 3.0 to 11.9) in LinOS or DOS lipoplexes resulted in a decrease in the PDI from 0.48 to 0.32 and 0.41 to 0.33 respectively ($p = 0.02$ and 0.0005). The lipoplexes of LinOS/DOPE 1:1 resulted in a higher PDI of 0.66.

Confocal photomicrographs in Figure 9 show the following: Figure 9A, control nontransfected HeLa cells which stably express EGFP (green) contained within the cell membrane (blue); Figure 9B (63× objective, scan zoom 1.0) and Figure 9C, post-transfection (48 h) with siEGFP-AF, the EGFP expression faded as the gene expression was silenced with the delivery of the siEGFP-AF (red); Figure 9D (63× objective, scan zoom 1.7) and Figure 9E, magnified images which show the reduction of EGFP expression compared to control cells, with the red color of delivered siEGFP-AF. The images in Figure 9 prove that siEGFP-AF was delivered to the EGFP-stably transfected HeLa cells, and also that EGFP gene expression was silenced. This delivery was confirmed by taking a *Z*-stack of images through the thickness of the cells (Figure 10). Confocal microscopy showed successful delivery of red tagged siRNA and quantitative EGFP knockdown in these EGFP HeLa cells.

Z-Stacks are a series of successive optical sections acquired at different positions across the *Z*-axis defining the thickness of the sample perpendicular to the sample's horizontal *XY* plane, and therefore they are useful for visualizing three-dimensional structures. To characterize the intracellular delivery of siEGFP-AF further, *Z*-stack photomicrographs were recorded through a monolayer of transfected HeLa cells (Figure 10). In order to record such a stack, the experiment was set up such that the first optical section was recorded slightly lower than the surface of the cells attached to the coverslip, and then the sections were recorded while slicing through to the opposite surface. This arrangement allows us to identify whether the red color (representing siRNA delivery) is present inside the cell, where there will be no blue color (representing cell membrane) associated with the red color, or the red color is present on/in the cell membrane, in which case the red color will be present simultaneously with the blue color of the cell membrane. It can be seen in the series of *Z*-stack photomicrographs starting from the top left (Figure 10) that the red color appears in the center of the cells, the blue color is present only in the perimeter of the cells, and there is no simultaneous blue color in the middle of the cells where there is red. Thus, we conclude that the majority of the delivered siRNA is present inside the HeLa cells. Minko and co-workers have recently reported the use of *Z*-stack photomicrographs to determine the orientation of the delivered siRNA, where a NuLight DY-547 fluorophore tagged siRNA was delivered to A2780 human ovarian cancer cells by surface neutral, but internally cationic polyamidoamine dendrimers.[8]

Lipoplexes prepared with LinOS/Chol 1:2 and siRNA form spherical multilamellar arrangements (Figure 11) with a size of

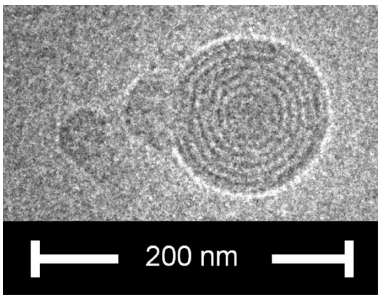

Figure 11. Cryo-TEM image of LinOS/Chol (1:2) siRNA lipoplexes.

∼100 nm, which is in good agreement with the particle size measured by DLS (Table 1) of the same lipoplexes. The constant distance between lamellar repeats is ∼6 nm, with the electron-dense layers fitting a monolayer of siRNA. Recent cryo-TEM images of lipidic aminoglycoside derivatives/siRNA self-assembled lamellar complexes show concentric onionlike structures with the distance between the lamellar repeats being 7 nm.[33] Such siRNA lipoplexes promote efficient siRNA delivery and RNA interference.

Transfection of HeLa cells with lipoplexes of LinOS/Chol and LinOS/DOPE at different molar ratios and 1.5 pmol of siRNA in 96-well plates (Figure 12), $N/P = 3.0$, resulted in cell viabilities of 81−95% of the control cells. The viability resulting from transfection using LinOS (only) lipoplexes was 96% at *N*/

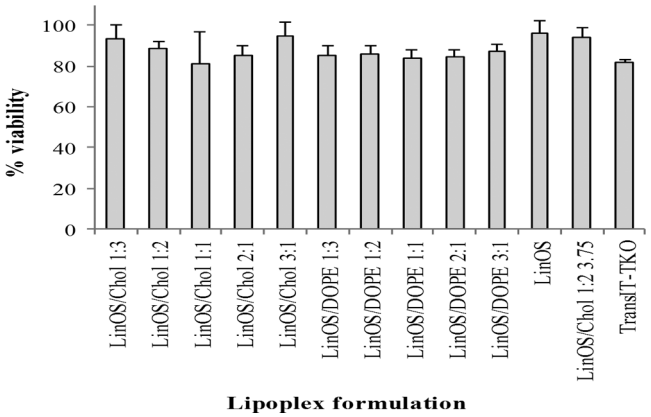

Figure 12. Viability of transfected HeLa cells measured using the alamarBlue assay 48 h post-transfection with lipoplexes prepared with siNC and either LinOS/Chol or LinOS/DOPE at different LinOS/neutral lipid ratios. All were assayed at 1.5 pmol of siNC/well (15 nM), 6,500 cells/well, except LinOS/Chol 1:2 3.75, which had only 0.375 pmol of siNC/well (3.75 nM).

P = 3.0. Transfection with LinOS/Chol 1:2 3.75 resulted in viability of 94%, higher than that of LinOS/Chol 1:2 (89%, p = 0.02). These values are significantly higher than the cell viability on transfection with TransIT-TKO (82%, p = 0.0001 for both). There were small differences in viabilities between LinOS/Chol lipoplexes (81−95%) and LinOS/DOPE lipoplexes (84−87%). Although the amount of lipoplexes chosen in this assay was only one-tenth that used in the 24-well plate assays (for delivery and gene silencing experiments), the siRNA concentration was kept constant in the culture medium in all experiments, i.e., either 15 nM or 3.75 nM.

DISCUSSION

We have evaluated the efficiency of both siRNA delivery and the gene silencing by siRNA lipoplexes prepared from mixtures of LinOS or DOS with either cholesterol or DOPE neutral helper lipids. LinOS and DOS are derivatives of a naturally occurring polyamine, spermine, that has been conjugated to the naturally occurring C18 unsaturated fatty acids: oleic acid (18:1) and/or linoleic acid (18:2). The design of LinOS and DOS is based on the hypothesis that using such natural moieties as the building blocks in the synthetic lipid will result in more benign (less toxic) cationic lipids and that better interactions (mixing) with bilayers both without (cell membrane) and within (endosomal membrane) target cells will increase cellular delivery efficiency and endosome escape.

Cholesterol and DOPE are widely used as helper lipids in DNA liposome and lipoplex preparations,[34] mainly due to their ability to promote nonlamellar lipid arrangements and thus facilitate membrane fusion upon cellular internalization. Herein, we have demonstrated an important increase in both siRNA delivery and the resultant gene silencing efficiency with an increase in the cholesterol content in the lipoplexes (Figures 2 and 3). Cholesterol enhances transfection with DNA lipoplexes by increasing DNase resistance,[35] and cholesterol nanodomains are known to form in lipoplexes having ≥52% of molar cholesterol content.[36] The presence of cholesterol domains in the lipoplexes prepared with ≥60% molar cholesterol content was suggested to result in an increasing resistance to lipoplex aggregation in the presence of 50% serum, and decreased albumin binding to the lipoplexes led to better interaction (fusion) with the cell membrane. Cholesterol and DOPE facilitate the conversion of the lipoplex lamellar phase (L_α) into the nonlamellar inverted hexagonal (H_{II}) and cubic phases, which play an important role in membrane fusion.[37,38] In early and elegant siRNA SNALP delivery studies, MacLachlan and co-workers reported the importance of the saturation of C=C along the lipids chains.[39] They found that, in a series of symmetrical 1,2-dialkyloxy-*N*,*N*-dimethyl-3-aminopropane analogues, as C=C saturation increased, lamellar phase (L_α) to nonlamellar inverted hexagonal (H_{II}) phase transition temperatures increased, an indicator of decreasing fusogenicity, and that less fusogenic particles are more readily internalized by cells, but with lower gene silencing efficiency. They also argued that as electrostatic binding is a precursor to uptake, the pK_a values of the cationic lipid will be important. Their results support an siRNA transfection model in which endosomal release, mediated by fusion with the endosomal membrane, results in cytoplasmic translocation of the siRNA payload.[39] While fully agreeing with their argument, in addition to our two (different) unsaturated acyl chains (18:2 and 18:1), we also have incorporated cholesterol, a known membrane fusogen,[38] in our efficient formulations.

Lipoplexes containing LinOS/DOPE showed enhanced gene silencing (Figure 5) compared to LinOS lipoplex formulations lacking any helper lipid. However, LinOS/DOPE lipoplexes showed less siEGFP-AF delivery (Figure 4) when compared to LinOS/Chol lipoplexes. Lipoplexes containing cholesterol have also been found to be more effective in vivo than those containing DOPE.[40−44] The particle sizes of LinOS/Chol lipoplexes that resulted in the best balance between gene silencing and siEGFP-AF delivery were measured (Table 1). These LinOS/Chol lipoplexes were much smaller than the LinOS/DOPE lipoplexes (Table 1). DOPE containing lipoplexes showed immediate loss of integrity in the presence of serum, which might explain the higher efficiency of cholesterol containing lipoplexes in vivo.[44] Thus, lipoplexes of LinOS/Chol at a 1:2 ratio resulted in the best siEGFP-AF delivery and gene silencing. Further investigation with respect to the effect of decreasing the amount of complexed siRNA from 15 pmol to 3.75 pmol at a LinOS/Chol 1:2 ratio showed the amount of siEGFP-AF delivered was down to 20−33% (5-fold−3-fold). The symmetrical spermine conjugate DOS, which we have demonstrated forms siRNA lipoplexes that efficiently silence EGFP,[20] was chosen to prepare lipoplexes with the DOS/Chol ratio of 1:2, experimentally determined to be the best for LinOS/Chol, to investigate the effect of changing the cationic lipid on the siEGFP-AF delivery and EGFP knockdown. Figure 6 shows that both LinOS and DOS mixtures with cholesterol markedly increased siEGFP-AF delivery, with LinOS/Chol lipoplexes resulting in more enhanced siEGFP-AF delivery. The data in Figure 7 show that the reduction of EGFP expression was essentially the same at both siEGFP-AF concentrations used and for both formulas. Although lipoplexes prepared with 3.75 pmol of siEGFP-AF have a lower amount of siRNA, they therefore have a higher N/P charge ratio, N/P = 11.9 compared with N/P = 3.0 for lipoplexes prepared with 15 pmol of siEGFP-AF, which may play a role in the interactions with cell membranes, hence promoting gene silencing. The differences seen between lipoplexes of LinOS/Chol and DOS/Chol (Figures 6 and 7) can be attributed to the difference between the fatty acids in LinOS and DOS. LinOS contains one oleoyl chain (18:1, one double bond) and one linoleoyl (18:2, two double bonds) while DOS contains two oleoyl chains. The

differences in the hydrophobic volume of these cationic lipids will affect the transfection efficiency of lipoplexes,[45−47] and LinOS lipoplexes were better than DOS in EGFP silencing in HeLa cells (Figure 7). Figures 6 and 7 also show that lipoplexes prepared with scrambled siNC-AF did not result in any significant gene silencing, therefore the reduction in EGFP expression on transfection with siEGFP-AF lipoplexes is due to sequence specific gene silencing, and not due to any off target or lipid related effects, e.g., toxicity. The alamarBlue cell viability assay data (Figure 12) show that the lipoplexes were particularly well tolerated by HeLa cells with viabilities ≥81%. The best viability (94%) for lipoplexes containing cholesterol was achieved using LinOS/Chol 1:2 with 0.375 pmol of siRNA. Such low toxicity (remarkably high cell viability), at the *N*/*P* ratios used, bodes well for future in vivo studies.

The new cationic lipid LinOS was characterized and evaluated for its ability to deliver siRNA to HeLa cells, and for its effect on gene silencing efficiency. LinOS was used to prepare self-assembled lipoplexes with siRNA, either alone or in a coformula with cholesterol or DOPE at various ratios of the cationic lipid/helper lipid. The lipoplexes coformulated with cholesterol resulted in particle size that is smaller than the particle size of lipoplexes coformulated with DOPE. The lipoplexes coformulated with either cholesterol or DOPE were superior to those without cholesterol in terms of efficiency of siRNA delivery, with the lipoplexes having a LinOS/Chol ratio 1:2 resulting in the highest delivery. These lipoplexes resulted in better gene silencing than the lipoplexes of LinOS, and in a comparable manner to the commercial transfecting agent TransIT-TKO in the presence of 10% FCS in the HeLa cell culture media. The prepared lipoplexes resulted in cell viability that is higher than 80% in HeLa cells. These results show that LinOS/Chol mixtures can form self-assembled lipoplexes with siRNA, and are promising nontoxic nonviral vectors for siRNA.

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS USED

DMEM, Dulbecco's modified Eagle's medium; DOS, N^4,N^9-dioleoyl-1,12-diamino-4,9-diazadodecane; EGFP, enhanced green fluorescent protein; FCS, fetal calf serum; HRMS, high-resolution mass spectrometry; LinOS, N^4-linoleoyl-N^9-oleoyl-1,12-diamino-4,9-diazadodecane; WGA, wheat germ agglutinin

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