Efficient Silencing of EGFP Reporter Gene with siRNA Delivered by Asymmetrical $N^4,N^9$-Diacyl Spermines

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ABSTRACT: It is important to obtain structure-activity relationship (SAR) data across cationic lipids for the self-assembly and non-viral intracellular delivery of siRNA. The aims of this work are to carry out a SAR study on the efficiency of asymmetrical $N^4,N^9$-diacyl spermines in siRNA delivery and EGFP reporter gene silencing, with comparisons to selected mixtures composed of symmetrical $N^4,N^9$-diacyl spermines. Another important aim of these studies is to quantify the changes in cell viability, assayed with alamarBlue, as a function of lipid structure. Therefore, we have designed, synthesized, purified, and assayed novel cationic lipids that are asymmetrical lipopolyamines based on spermine. Flow cytometry and fluorescence microscopy in an EGFP stably transfected HeLa cell line, measuring both delivery of fluorescently tagged siRNAs and silencing the EGFP signal, allowed quantitation of the differences between asymmetrical cationic lipids, mixtures of their symmetrical counterparts, and comparison with commercial non-viral delivery agents. Intracellular delivery of siRNA and gene silencing by siRNA differ with different hydrophobic domains. In these asymmetrical $N^4,N^9$-diacyl spermines, lipids that enhance siRNA uptake do not necessarily enhance siRNA-induced inhibition of gene expression: C18 and longer saturated chains promote uptake, while more unsaturated C18 chains promote gene silencing. These properties are efficiently demonstrated in a new non-toxic cationic lipid siRNA vector, $N^1$-linoleoyl-$N^9$-oleoyl-1,12-diamino-4,9-diazadecane (LinOS) which is also shown to be comparable with or superior to TransIT-TKO and Lipofectamine 2000.

KEYWORDS: Asymmetry, lipoplexes, polyamine, self-assembly, spermine

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INTRODUCTION

RNA interference (RNAi) was first noticed in petunia flowers which showed reduced pigmentation on the introduction of exogenous genes that were meant to increase pigmentation.\(^1\)\(^2\) Fire, Mello, and co-workers reported the reduction or inhibition of gene expression of a specific gene in *Caenorhabditis elegans* by means of dsRNA homologous to the targeted gene.\(^3\) In 2001, gene silencing in mammalian cells mediated by 21 nucleotide dsRNA homologous in sequence to the target gene was reported.\(^4\) Post-transcriptional gene silencing by short or small dsRNA, short interfering RNA (siRNA), typically 21-25 nucleotides in length, is mediated through the RNA-induced silencing complex (RISC) in the cytoplasm. The RISC is comprised of proteins and the anti-sense strand of the siRNA. The key protein that has endoribonuclease activity is one of the Argonaute family of evolutionarily-conserved proteins (e.g. human Argonaute hAgo2)\(^5\) that are essential for development and differentiation, and in most plants and animals defend cells against viral infection. Sequence specific gene silencing is by degradation of mRNA that has a complementary sequence to the anti-sense siRNA strand present in the RISC complex.\(^5\),\(^6\)

siRNA continues to gain popularity in functional genomics studies and as a potential therapeutic that can target many diseases ranging from age-related macular degeneration to different types of cancer and viral infections.\(^7\)-\(^9\) Due to the highly negatively charged nature of the siRNA phosphate backbone, and due to its susceptibility to degradation by exo- and endonucleases,\(^10\) a carrier (vector) is required to ensure successful intracellular siRNA delivery to target cells.\(^11\),\(^12\) Since being introduced as non-viral gene delivery vectors in 1987 by Felgner and co-workers,\(^13\) cationic lipids have been widely studied as non-viral gene and siRNA vectors.\(^14\)-\(^16\)

The majority of phospholipids in mammalian cell membranes are acylated with two different fatty acids at the sn-1 and sn-2 positions of the glycerol 3-phosphate.\(^17\)-\(^21\) We have designed and synthesized various diacyl derivatives of spermine, a naturally occurring polyamine. These diacyl substituents, using either the same or two different fatty acids acylated to either positions \(N^4\) and \(N^6\), or \(N^4\) and \(N^{12}\), of spermine were then characterized and evaluated in vitro as non-viral vectors for the delivery of plasmid DNA (pDNA) and scrambled fluorescently labeled siRNA.\(^22\)-\(^26\) In this work, we report the design and synthesis of seven new \(N^4\) or \(N^6\)-diacylated asymmetrical spermine derivatives, asymmetrical in that they contain two different fatty acid chains, varying in length (from C18 to C24) and/or in their oxidation state. These novel asymmetrical polyamine amides are structurally similar to the naturally occurring diacyl lipids that form lipid cellular membranes.\(^17\)-\(^21\) Using the two positive charges from the terminal primary amino groups, they are rationally designed to neutralize polynucleotides (e.g. siRNA) by titration and without any pre-formulation of liposomes. This titration occurs by the simple mixing of cationic lipids with polyanionic cargoes enabling self-assembly of lipoplex nanoparticles.\(^22\) The
target molecules are also designed to be efficient in coating the siRNA cargo with a protective lipid against serum RNase and therefore to be efficient in siRNA mediated gene silencing. We have reported the use in siRNA delivery of long and very long chains symmetrically substituted on spermine, but prior to our studies, there are no structure-activity relationship (SAR) data on asymmetrical spermine conjugates in gene silencing.

The synthesized spermine conjugates were homogeneous and characterized by NMR, high-resolution mass spectrometry (HRMS), and DLS. Their ζ-potentials were also measured. They were then evaluated as non-viral siRNA delivery vectors and compared with commercially available siRNA delivery systems. To this end, we made use of a HeLa cell line that stably expresses the enhanced green fluorescent protein (EGFP) as a target for gene silencing and measured the efficiency of siRNA delivery and the associated reduction of EGFP expression. In the same system, we also investigated the difference between transfection with lipoplexes of the asymmetrical spermine conjugates (e.g. N⁴-linoleoyl-N⁶-oleoyl spermine), and transfection with lipoplexes of mixtures of their counterpart symmetrical spermine derivatives (e.g. N⁴,N⁶-dilinoleoyl and N⁴,N⁶-dioleoyl). Others have also reported gene silencing studies on a comparable HeLa cell line, on human embryonic kidney 293T cells stably expressing GFP, and on human embryonic retinoblast cells (911 cells) stably expressing luciferase.

MATERIAL AND METHODS

Materials. Chemicals were purchased as follows: N-carbethoxyphthalimide, dicyclohexylcarbodiimide (DCC), 4-dimethylaminopyridine (DMAP), fatty acids, fatty acyl chlorides, hydrazine monohydrate, spermine, and triethylamine (TEA) from Sigma-Aldrich (Gillingham, UK); Mowiol (polyvinyl alcohol) from Calbiochem (Nottingham, UK); TransIT-TKO from Mirus (Cambridge, UK); alamarBlue®, Alexa Fluor® 555-conjugated wheat germ agglutinin (WGA), Lipofectamine 2000, and RiboGreen, from Invitrogen Ltd, (Paisley, Scotland). All solvents were purchased from Fisher Scientific (Loughborough, UK). Anhydrous dichloromethane (DCM) was distilled from calcium hydride. Anhydrous tetrahydrofuran (THF) was distilled from sodium benzophenone ketyl.

Cell culture materials were purchased from Life Technologies (Invitrogen Ltd, Paisley, Scotland) and GIBCO (Paisley, Scotland) except fetal calf serum (FCS, PAA Laboratories, Teddington, UK), G418 (Geneticin, an aminoglycoside antibiotic similar in structure to gentamicin B1) (Sigma, Gillingham, UK), and PBS (Oxoid, Basingstoke, UK). The water used to prepare solutions was from a MilliQ purification system (Millipore, Bedford, MA, USA). HeLa cells were routinely passaged by trypsinization using 0.25% trypsin.
**General Methods.** Glassware used in anhydrous conditions was heated for 16 h at 80 °C, assembled hot, filled with anhydrous nitrogen and allowed to cool to 20 °C before use. Thin layer chromatography (TLC) was performed using aluminium-backed plates coated with Kieselgel 60 F254 (Merck). Ninhydrin (0.2 g) in ethanol (100 mL) was used for detecting polyamines on TLC plates. Column chromatography was performed over flash silica gel 60 (35-75 μm; Prolabo-Merck). NMR spectra were recorded as dilute solution in deuteriochloroform using a Bruker Avance III spectrometer operating at 400.1 MHz spectrometer for $^1$H and at 100.8 MHz for $^{13}$C. Chemical shifts values are recorded in parts per million (ppm) on the δ scale. Coupling constants ($J$) are absolute values (line separations) and recorded in Hz, assigned as s (singlet), m (multiplet), and t (triplet). $^{13}$C NMR assignments were aided by correlation spectroscopies; COSY, HMBC, HMQC spectra were all recorded using automated programmes. The high resolution (HR) time-of-flight (TOF) mass spectra (MS) were obtained on a Bruker Daltonics micrOTOF mass spectrometer using electrospray ionisation (ESI). All lipopolymamines were homogeneous by TLC and showed satisfactory HRMS data (positive ion mode reported as m/z and within 5 ppm).

**siRNAs.** AllStars (scrambled) siRNA negative controls, both without a fluorescent tag (siNC) and tagged with Alexa Fluor® 647 (AF647) at the 3’-position (siNC-AF) were purchased from Qiagen (Crawley, UK) as was siRNA against EGFP labeled with Alexa Fluor® 647 (siEGFP-AF) at the 3’-position of the sense strand. siEGFP-AF sequences are:

- Sense strand: 5’-GCAAGCUGACCCUGAAGUUCAUTT-3’,
- Anti-sense strand: 5’-AUGAACUUCAGGGUCAGCUUGCCG-3’,
- Target DNA sequence: 5’-CGGCAAGCTGACCCTGAAGTTCAT-3’.

**Synthesis of Diacylated Spermines.**

**1,12-Diphthalimido-4,9-diazadodecane.**

To a solution of 1,12-diamino-4,9-diazadodecane (spermine) (2.02 g, 10 mmol) in DCM (30 mL) N-carbethoxyphthalimide (4.38 g, 20 mmol) was added. The solution was stirred at 20 °C for 3 h then evaporated to dryness in vacuo. The residue was purified over silica gel (DCM:MeOH 10:1 then 2:1 v/v) to afford the title compound (2.13 g, 46%). $^1$H NMR, 1.2-1.4 (m, 4H, H6, H7), 1.4-1.7 (m, 6H, H2, H11, 2 x NH), 2.4-2.5 (m, 8H, H3, H5, H8, H10), 3.6 (m, 4H, H1, H12), 7.5-7.7 (m, 8H, aromatic protons). $^{13}$C NMR, 27.1 (C6, C7), 28.2 (C2, C11), 35.5 (C1, C12), 46.3 (C3, C5, C8, C10), 122.7 and 133.6 (aromatic carbons), 168.1 (C=O). HRMS, found (M+H)$^+$ 463.2354, C26H31N4O4 requires (M+H)$^+$ 463.2340. Often, for the synthesis of the following target molecules, the purification of 1,12-diphthalimido-4,9-diazadodecane can be skipped and the crude product taken on directly into the acylation.
**N<sup>4</sup>,N<sup>9</sup>-Dioleoyl-1,12-diamino-4,9-diazadodecane (DOS).**

To a solution of 1,12-diphthalimido-4,9-diazadodecane (2.31 g, 5 mmol) in DCM (30 mL) and TEA (1.4 mL, 10 mmol) oleoyl chloride (3.3 mL, 10 mmol) was added. The solution was stirred for 18 h at 20 °C and then the solvent was evaporated to dryness in vacuo. The residue was reacted with hydrazine monohydrate (2 mL) in a mixture of DCM (15 mL) and THF (15 mL) and heated under reflux for 4 h, cooled to 20 °C, and then the solvent was evaporated in vacuo. The residue was purified over silica gel (DCM:MeOH 10:1 v/v then DCM:MeOH:NH<sub>2</sub>OH 10:5:1 v/v/v) to afford the title compound (1.46 g, 40% over two steps).

**1</sup>H NMR, 0.8 (t, J = 7, 6H, H18'), 1.2-1.3 (m, 40H, H4'-H7', H12'-H17'), 1.4-1.8 (m, 12H, H2, H6, H7, H11, H3'), 1.9-2.1 (m, 8H, H8', H11'), 2.1-2.3 (m, 4H, H2'), 2.6-2.8 (m, 8H, H1, H12, 2 x NH<sub>2</sub>), 3.2-3.5 (m, 8H, H3, H5, H8, H10), 5.3-5.4 (m, 4H, H9', H10').

**13</sup>C NMR, 14.1 (C18'), 22.6-29.7 (C2, C6, C7, C11, C4'-C8', C11'-C17'), 31.9 (C3'), 33.1 (C2'), 38.9-39.2 (C1, C12), 42.5-47.4 (C3, C5, C8, C10), 129.8 (C9', C10'), 172.9 (C1'). HRMS, found (M+H)<sup>+</sup> 731.7162, C<sub>46</sub>H<sub>91</sub>N<sub>4</sub>O<sub>2</sub> requires (M+H)<sup>+</sup> 731.7137.

**N<sup>4</sup>,N<sup>9</sup>-Dierucoyl-1,12-diamino-4,9-diazadodecane (DEruS).**

To a solution of 1,12-diphthalimido-4,9-diazadodecane (0.46 g, 1 mmol) in DCM (30 mL) erucic acid (0.68 g, 2 mmol), DMAP (0.24 g, 2 mmol), and DCC (0.40 g, 2 mmol) were added. The solution was stirred at 20 °C for 18 h under an atmosphere of nitrogen, then filtered and the filtrate was evaporated to dryness in vacuo. The residue was reacted with hydrazine monohydrate (2 mL) in a mixture of DCM (15 mL) and THF (15 mL) and heated under reflux for 4 h, cooled to 20 °C, and then the solvent was evaporated in vacuo and the residue purified over silica gel (DCM/MeOH 10:1 v/v then DCM/MeOH:NH<sub>2</sub>OH 20:10:1 v/v/v) to afford the title compound (0.34 g, 40% over two steps).

**1</sup>H NMR, 0.8 (t, J = 7, 6H, H22'), 1.1-1.3 (m, 60H, H4'-H11', H16'-H21', 2 x NH<sub>2</sub>), 1.4-1.6 (m, 12H, H2, H6, H7, H11, H3'), 1.9 (m, 8H, H12'-H15'), 2.2-2.4 (m, 4H, H2'), 2.5-2.7 (m, 4H, H1, H12), 3.2-3.6 (m, 8H, H3, H5, H8, H10), 5.2 (m, 4H, H9', H10').

**13</sup>C NMR, 14.0 (C22'), 22.6 (C21'), 25.1-32.8 (C2, C6, C7, C11, C4'-C8', C11'-C17'), 31.9 (C3'), 33.1 (C2'), 38.9-39.2 (C1, C12), 42.6-47.4 (C3, C5, C8, C10), 129.8 (C9', C10'), 172.8-173.0 (C1'). HRMS, found (M+H)<sup>+</sup> 843.8364, C<sub>54</sub>H<sub>107</sub>N<sub>4</sub>O<sub>2</sub> requires (M+H)<sup>+</sup> 843.8389; found (M+Na)<sup>+</sup> 865.8194, C<sub>54</sub>H<sub>106</sub>N<sub>4</sub>O<sub>2</sub>Na requires (M+Na)<sup>+</sup> 865.8208.

**N<sup>4</sup>,N<sup>9</sup>-Dilinoleoyl-1,12-diamino-4,9-diazadodecane (DLinS).**

To a solution of 1,12-diphthalimido-4,9-diazadodecane (0.46 g, 1 mmol) in DCM (30 mL) linoleic acid (0.56 g, 2 mmol), DMAP (0.24 g, 2 mmol), and DCC (0.40 g, 2 mmol) were added. The solution was stirred at 20 °C for 18 h under an atmosphere of nitrogen, then filtered and the filtrate was evaporated to dryness in vacuo. The residue was reacted with hydrazine monohydrate (2 mL) in a mixture of DCM (15 mL) and THF (15 mL) and heated under reflux for 4 h, cooled to 20 °C, and then the solvent was evaporated in vacuo and the residue purified over silica gel (DCM/MeOH 10:1 v/v then DCM/MeOH:NH<sub>2</sub>OH 20:10:1 v/v/v) to afford the title compound (0.34 g, 40% over two steps).

**1</sup>H NMR, 0.8 (t, J = 7, 6H, H22'), 1.1-1.3 (m, 60H, H4'-H11', H16'-H21', 2 x NH<sub>2</sub>), 1.4-1.6 (m, 12H, H2, H6, H7, H11, H3'), 1.9 (m, 8H, H12'-H15'), 2.2-2.4 (m, 4H, H2'), 2.5-2.7 (m, 4H, H1, H12), 3.2-3.6 (m, 8H, H3, H5, H8, H10), 5.2 (m, 4H, H9', H10').

**13</sup>C NMR, 14.0 (C22'), 22.6 (C21'), 25.1-32.8 (C2, C6, C7, C11, C4'-C8', C11'-C17'), 33.1 (C2'), 39.2-39.4 (C1, C12), 42.6-47.4 (C3, C5, C8, C10), 129.8 (C9', C10'), 172.8-173.0 (C1'). HRMS, found (M+H)<sup>+</sup> 843.8364, C<sub>54</sub>H<sub>107</sub>N<sub>4</sub>O<sub>2</sub> requires (M+H)<sup>+</sup> 843.8389; found (M+Na)<sup>+</sup> 865.8194, C<sub>54</sub>H<sub>106</sub>N<sub>4</sub>O<sub>2</sub>Na requires (M+Na)<sup>+</sup> 865.8208.

**N<sup>4</sup>,N<sup>9</sup>-Dilinoleoyl-1,12-diamino-4,9-diazadodecane (DLinS).**

To a solution of 1,12-diphthalimido-4,9-diazadodecane (0.46 g, 1 mmol) in DCM (30 mL) linoleic acid (0.56 g, 2 mmol), DMAP (0.24 g, 2 mmol), and DCC (0.40 g, 2 mmol) were added. The solution was stirred at 20 °C for 18 h under an atmosphere of nitrogen, then filtered and the filtrate was evaporated to dryness in vacuo. The residue was reacted with hydrazine monohydrate (2 mL) in a mixture of DCM (15 mL) and THF (15 mL) and heated under reflux for 4 h, cooled to 20 °C, and then the solvent was evaporated in vacuo and the residue purified over silica gel (DCM/MeOH 10:1 v/v then DCM/MeOH:NH<sub>2</sub>OH 20:10:1 v/v/v) to afford the title...
compound (0.29 g, 40% over two steps). $^1$H NMR, 0.8 (t, J = 7, 6H, H18'), 1.2-1.3 (m, 28H, H4'-H7', H15'-H17'), 1.4-1.8 (m, 16H, H2, H6, H7, H11, H3', 2 x NH$_2$), 1.9-2.0 (m, 8H, H8', H14'), 2.0-2.3 (m, 8H, H2'), 2.5-2.7 (m, 8H, H1, H12, H11'), 3.2-3.4 (m, 8H, H3, H5, H8, H10), 5.2-5.3 (m, 8H, H9', H10', H12', H13'). $^{13}$C NMR, 14.0 (C18'), 22.4 (C17'), 25.0-33.0 (C2, C6, C7, C11, C2'-C8', C11', C14'-C16'), 39.0-39.3 (C1, C12), 42.4-47.6 (C3, C5, C8, C10), 127.8-130.0 (C9', C10', C12', C13'), 172.7-173.0 (C1'). HRMS, found (M+H)$^+$ 727.6851, C$_{46}$H$_{87}$N$_4$O$_2$ requires (M+H)$^+$ 727.6824.

**1,12-Diphthalimido-$N^4$-oleyl-4,9-diazadodecane.**

To a solution of 1,12-diphthalimido-4,9-diazadodecane (2.31 g, 5 mmol) in DCM (30 mL) oleic acid (1.6 mL, 5 mmol), DMAP (610 mg, 5 mmol), and DCC (1.03 g, 5 mmol) were added. The solution was stirred at 20 °C for 20 h then filtered and the filtrate was evaporated to dryness in vacuo. The residue was purified over silica gel (DCM:MeOH 20:1 v/v) to afford the title compound (1.34 g, 37%). $^1$H NMR, 0.9 (t, J = 7, 3H, H18'), 1.3 (m, 20H, H4'-H7', H12'-H17'), 1.5-1.6 (m, 8H, H6, H7, H11, H3'), 1.9-2.0 (m, 7H, H2, H8', H11', 1 x NH), 2.1-2.3 (m, 2H, H2'), 2.7-2.8 (m, 4H, H8, H10), 3.2-3.5 (m, 4H, H3, H5), 3.6-3.8 (m, 4H, H1, H12), 5.3 (m, 2H, H9', H10'), 7.7-7.8 (m, 8H, aromatic). $^{13}$C NMR, 14.1 (C18'), 22.6 (C17'), 25.1-31.8 (C2, C6, C7, C11, C3'-C8', C11'-C16'), 33.1 (C2'), 35.3-35.9 (C1, C12), 45.2-50.6 (C3, C5, C8, C10), 123.1-123.3 and 131.8-134.1 (aromatic carbons), 129.7-129.9 (C9', C10'), 168.2-168.4 (C=O phthalimide), 173.0 (C1'). HRMS, found (M+H)$^+$ 727.4801, C$_{44}$H$_{63}$N$_4$O$_5$ requires (M+H)$^+$ 727.4793.

**$N^4$-Linoleoyl-$N^9$-oleyl-1,12-diamino-4,9-diazadodecane (LinOS).**

To a solution of 1,12-diphthalimido-$N^4$-oleyl-4,9-diazadodecane (0.72 g, 1 mmol) and TEA (0.14 mL, 1 mmol) in DCM (30 mL) linoleoyl chloride (0.33 mL, 1 mmol) was added. The solution was stirred at 20 °C for 18 h then the solvent was evaporated to dryness in vacuo. The residue was reacted with hydrazine monohydrate (2 mL) in a mixture of DCM (15 mL) and THF (15 mL) and heated under reflux for 4 h, cooled to 20 °C, and then the solvent was evaporated in vacuo. The residue was purified over silica gel (DCM:MeOH 10:1 v/v then DCM:MeOH:NH$_4$OH 10:5:1 v/v/v) to afford the title compound (0.27 g, 37% over two steps). $^1$H NMR, 0.9 (t, J = 7, 6H, H18', H18''), 1.2-1.4 (m, 34H, H4'-H7', H15'-H17', H4''-H7'', H12''-H17''), 1.4-1.7 (m, 12H, H2, H6, H7, H11, H3', H3''), 1.8-2.0 (m, 12H, H8', H14', H8'', H11'', 2 x NH$_2$), 2.0-2.3 (m, 4H, 2', 2''), 2.6-2.8 (m, 6H, H1, H12, H11'), 3.2-3.4 (m, 8H, H3, H5, H8, H10), 5.3 (m, 6H, H9', H10', H12', H13', H9'', H10''). $^{13}$C NMR, 14.0 (C18', C18''), 22.4 (C17', C17''), 25.0-26.8 (C2, C6, C7, C11, C11'), 27.1-27.2 (8', 14', 8'', 11''), 29.1-29.6 (4'-7', 15'-17', 4''-7'', 12''-17''), 30.9-32.9 (C3', C16', C3'', C16''), 33.0 -36.7 (C2', C2''), 38.9-39.3 (C1, C12), 42.4-47.6 (C3, C5, C8, C10), 127.8-130.0 (C9', C10', C12', C13', C9'', C10''), 172.7-173.0 (C1', C1''). HRMS, found (M+H)$^+$ 729.6980, C$_{46}$H$_{89}$N$_4$O$_2$ requires (M+H)$^+$ 729.6986.
**N^4-Oleoyl-N^9-stearoyl-1,12-diamino-4,9-diazadodecane (OSS).**

To a solution of 1,12-diphthalimido-N^4-oleoyl-4,9-diazadodecane (0.72 g, 1 mmol) and TEA (0.14 mL, 1 mmol) in DCM (30 mL) stearoyl chloride (0.34 mL, 1 mmol) was added. The solution was stirred at 20 °C for 18 h then the solvent evaporated to dryness in vacuo. The residue was reacted with hydrazine monohydrate (2 mL) in a mixture of DCM (15 mL) and THF (15 mL) and heated under reflux for 4 h, cooled to 20 °C, and then the solvent was evaporated in vacuo. The residue was purified over silica gel (DCM:MeOH 10:1 v/v then DCM:MeOH:NH_4OH 10:5:1 v/v/v) to afford the title compound (0.31 g, 43% over two steps).

**1H NMR**, 0.8 (t, *J* = 7, 6H, H18', H18''), 1.2-1.4 (m, 48H, H4'-H7', H12'-H17', H4''-H17''), 1.4-1.8 (m, 16H, H2, H6, H7, H11, H3', H3'', 2 x NH_2), 1.9-2.0 (m, 4H, H8', H11''), 2.1-2.3 (m, 4H, H2', H2''), 2.6-2.8 (m, 4H, H1, H12), 3.2-3.4 (m, 8H, H5, H8, H10), 5.3 (m, 2H, H9', H10'). **13C NMR**, 14.3 (C18', C18''), 22.9 (C17, C17''), 25.4-29.6 (C2, C6, C7, C11, C4'-C8', C11'-C15', C4''-C15''), 31.4, 32.9 (C3', C3''), 32.1 (C16', C16''), 33.3 (C2', C2''), 39.4 (C1, C12), 42.7-47.6 (C3, C5, C8, C10), 130.0 (C9', C10'), 173.1 (C1', C1''). HRMS, found (M+Na)^+ 755.7112, C_{46}H_{92}N_4O_2Na requires (M+Na)^+ 755.7118.

**General Procedure for the synthesis of asymmetrical acyl spermine derivatives using DCC coupling.** The required carboxylic acid (1 mmol), DMAP (122 mg, 1 mmol), and DCC (206 mg, 1 mmol) were added to a solution of 1,12-diphthalimido-N^4-oleoyl-4,9-diazadodecane (726 mg, 1 mmol) in DCM (15 mL). The solution was stirred at 20 °C for 20 h then filtered and the filtrate was evaporated to dryness in vacuo. The residue was reacted with hydrazine monohydrate (2 mL) in a mixture of DCM (10 mL) and THF (10 mL) and heated under reflux for 4 h, cooled to 20 °C, and then the solvent was evaporated in vacuo. The residue was purified over silica gel (DCM:MeOH 10:1 v/v then DCM:MeOH:NH_4OH 100:10:1 v/v/v) to afford the title compound.

**N^4-Arachidonoyl-N^9-oleoyl-1,12-diamino-4,9-diazadodecane (AOS).**

According to the General Procedure, arachidonic acid (304 mg, 1 mmol) was reacted to afford the title compound (301 mg, 40% over two steps). **1H NMR**, 0.9 (m, 6H, J = 7, H20', H18''), 1.2-1.8 (m, 42H, H2, H6, H7, H11, H3', H17'-H19', H3''-H7'', H12''-H17'', 2 x NH_2), 2.0-2.2 (m, 8H, H4', H16', H8', H11''), 2.2-2.4 (m, 4H, 2', 2''), 2.6-2.8 (m, 10H, H1, H12, H7', H10', H13'), 3.2-3.4 (m, 8H, H3, H5, H8, H10), 5.3-5.4 (m, 10H, H5', H6', H8', H9', H11', H12', H14', H15', H9'', H10''). **13C NMR**, 14.1 (C20', C18''), 22.5-22.6 (C19', C17''), 25.3-27.2 (C4', C7', C10', C13', C16', C8', C11'), 29.2-32.7 (C2, C6, C7, C11, C3', C17', C18', C3''-C7', C12''-C16''), 33.1 (C2', C2''), 39.0-39.4 (C1, C12); 42.5-47.3 (C3, C5, C8, C10), 127.5-130.5 (C5', C6', C8', C9', C11', C12', C14', C15', C9'', C10''), 172.6-172.8 (C1', C1''). HRMS, found (M+Na)^+ 753.6982, C_{48}H_{90}N_4O_2 requires (M+Na)^+ 753.6980.

7
**N⁴-Eicosenoyl-N⁹-oleoyl-1,12-diamino-4,9-diazadodecane (EicOS).**

According to the General Procedure, eicosenoic acid (310 mg, 1 mmol) was reacted to afford the title compound (273 mg, 36% over two steps). 

1H NMR, 0.9 (t, J = 7, 6H, H20", H18"), 1.2-1.3 (m, 44H, H4'-H9', H14'-H19', H4''-H7'', H12''-H17''), 1.4-1.8 (m, 12H, H2, H6, H7, H11, H3', H3'"), 2.0 (m, 8H, H10', H13', H8', H11''), 2.2-2.4 (m, 8H, H2', H2'', 2 x NH2), 2.6-2.8 (m, 4H, H1, H12), 3.2-3.45 (m, 8H, H3, H5, H8, H10), 5.3-5.4 (m, 4H, H11', H12', H9'', H10''). 

13C NMR, 14.1 (C20', C18''), 22.7 (C19', C17''), 25.1-32.6 (C2, C6, C7, C11, C3'-C10', C13'-C18', C3''-C8'', C11''-C16''), 33.1 (C2', C2'"), 38.8-39.4 (C1, C12), 42.5-47.5 (C3, C5, C8, C10), 129.8 (C11', C12', C9'', C10''), 172.9-173.4 (C1', C1''), HRMS, found (M+H)+ 759.7450, C₄₈H₉₅N₄O₂ requires (M+H)+ 759.7455.

**N⁴-Erucoyl-N⁹-oleoyl-1,12-diamino-4,9-diazadodecane (EruOS).**

According to the General Procedure, erucic acid (339 mg, 1 mmol) was reacted to afford the title compound (268 mg, 34% over two steps). 

1H NMR, 0.9 (t, J = 7, 6H, H22', H18"), 1.2-1.4 (m, 48H, H4'-H11', H16'-H21', H4''-H7'', H12''-H17''), 1.4-1.7 (m, 16H, H2, H6, H7, H11, H3', H3'", 2 x NH2), 2.0 (m, 8H, H12', H15', H8', H11''), 2.3 (m, 4H, H2', H2'"), 2.8 (m, 4H, H1, H12), 3.2-3.4 (m, 8H, H3, H5, H8, H10), 5.3 (m, 4H, H13', H14', H9'', H10''). 

13C NMR, 14.1 (C22', C18''), 22.6 (C21', C17''), 25.1-27.2 (C2, C6, C7, C11, C12', C15', C8'', C11''), 29.1-32.7 (C3'-C11', C16'-C20', C3''-C7'', C12''-C16''), 33.1 (C2', C2'"), 39.1-39.4 (C1, C12), 42.4-47.3 (C3, C5, C8, C10), 129.7-129.9 (C13', C14', C9'', C10''), 172.8-173.0 (C1', C1''). HRMS, found (M+H)+ 787.7763, C₅₀H₁₀₅N₄O₂ requires (M+H)+ 787.7768.

**N⁴-Lignoceroyl-N⁹-oleoyl-1,12-diamino-4,9-diazadodecane (LigOS).**

According to the General Procedure, lignoceric acid (369 mg, 1 mmol) was reacted to afford the title compound (310 mg, 38% over two steps). 

1H NMR, 0.9 (t, J = 7, 6H, H24', H18"), 1.2-1.4 (m, 64H H4'-H23', H4''-H7'', H12''-H17''), 2 x NH2), 1.4-1.7 (m, 12H, H2, H6, H7, H11, H3', H3'"), 2.0 (m, 4H, H8'', H11''), 2.2-2.3 (m, 4H, H4', H2', H2'"), 2.6-2.8 (m, 4H, H1, H12), 3.2-3.4 (m, 8H, H3, H5, H8, H10), 5.3 (m, 2H, H9'', H10''). 

13C NMR, 14.1 (C24', C18''), 22.6 (C23', C17''), 25.1-27.2 (C2, C6, C7, C11, C8', C11''), 29.2-32.7 (3'-22', 3''-7'', 12''-16''), 33.1 (C2', C2'"), 39.1-39.4 (C1, C12), 42.4-47.3 (C3, C5, C8, C10), 129.7-129.9 (C13', C14', C9'', C10''), 172.8-173.0 (C1', C1''). HRMS, found (M+H)+ 817.8232, C₅₂H₁₀₅N₄O₂ requires (M+H)+ 817.8238.

**N⁴-Nervonoyl-N⁹-oleoyl-1,12-diamino-4,9-diazadodecane (NOS).**

According to the General Procedure, nervonic acid (367 mg, 1 mmol) was reacted to afford the title compound (302 mg, 37% over two steps). 

1H NMR, 0.9 (t, J = 7, 6H, H24'', H18''), 1.2-1.4 (m, 52H H4'-H13', H18'-H23', H4''-H7'', H12''-H17''), 1.4-1.8 (m, 12H, H2, H6, H7, H11, H3', H3'"), 2.0 (m, 8H, H14', H17', H8'', H11''), 2.2-2.4 (m, 4H, H2', H2'"), 2.6-2.8 (m, 4H, H1, H12), 3.2-3.4 (m, 12H, H3, H5, H8, H11, 2 x NH2), 5.3 (m, 4H, H15', H16', H9'', H10''). 

13C NMR, 14.1 (C24', C18''), 22.6 (C23', C17''), 25.5-27.2 (C2, C6, C7, C11, C14', C17', C8'', C11'').
29.1-32.8 (C3'-C13', C18'-C22', C3''-C7'', C12''-C16''), 33.1 (C2', C2''), 39.0-39.5 (C1, C12), 42.5-47.6 (C3, C5, C8, C10), 129.8 (C15', C16', C9'', C10''), 172.7-172.8 (C1', C1''). HRMS, found (M+H)+ 815.8076, C52H103N4O2 requires (M+H)+ 815.8081.

**HeLa cell line stably expressing EGFP (HeLa-EGFP).** The HeLa cell line stably expressing the red-shifted enhanced variant of wild-type GFP (EGFP) used here as a reporter protein was obtained from the Cell Service at Cancer Research UK (CRUK, London Research Institute, Clare Hall Laboratories, South Mimms, London, UK) and was constructed by Dr Yilun Liu. Briefly, the centrin protein (CEN) was subcloned into the expression vector pEGFP-C1 (Clontech, Cowley, UK), downstream of EGFP, under the control of the human CMV promoter. The plasmid was linearized and transfected into HeLa cells. Stably transfected cells were selected with G418 and maintained as a polyclonal cell line, cultivated in DMEM medium supplemented with 10% FCS, penicillin base (50 units/mL), streptomycin (50 μg/mL), and G418 (500 μg/mL) in order to prevent the progressive loss of pDNA occurring during cell cultivation. The percentage of EGFP positive cells was >80% as measured by flow cytometry (FACS) (data not shown) and regularly was verified prior to siRNA transfection experiments.

**Transfection Studies of HeLa Cells Stably Expressing EGFP.** On the day of transfection, cells were trypsinized at a confluency of 80-90% and were seeded at a density of 100,000 cells/well in 24-well plates. The lipoplexes were prepared by mixing the specified amounts of the transfection reagent in OptiMEM serum-free medium (50 μL) with siRNA (15 μL, 1 μM working solution) in OptiMEM serum-free medium. Lipofectamine 2000 (2 μL/well) and TransIT-TKO (4 μL/well) were used in accordance with the manufacturers’ instructions, both were used in the presence of 10% FCS. The solutions were vortex-mixed for 2-3 s, and added to wells containing DMEM (10% FCS and without G418), within 0.5 h of seeding, to make the final volume in each well 1 mL (siRNA final concentration 15 nM). The plates were then incubated for 48 h at 37 °C in 5% CO₂. N/P ratios (defined as the ratio of cationic lipid ammonium ions to RNA phosphate anions) were calculated as:

\[
N/P = \frac{\text{number of moles of cationic lipid}}{\text{number of moles of siRNA} \times \text{total number of phosphates in ds siRNA}} \times 2.0
\]

**Flow Cytometry (FACS).** For analysis of delivery and then reduction of expression of EGFP by flow cytometry (FACS), cells were trypsinized and resuspended in complete DMEM medium without phenol red. Cells were centrifuged (1,000 rpm Falcon 6/300 MSE, London, UK, for 5 min) then washed twice by resuspending in PBS containing 0.1% BSA, and then re-centrifuged (1,000 rpm for 5 min). The collected cells were then resuspended in PBS and transferred to a flow cytometer tube (Becton Dickinson, UK). Cells were then analyzed (10,000
or 20,000 events) using a FACSCanto flow cytometer (Becton Dickinson, UK), 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 green fluorescence emission, and a helium/neon laser at 633 nm for excitation of Alexa Fluor 647 and a detector at 660 nm (range +/- 10 nm). EGFP expression was calculated as:

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

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 Microscopic Cell Imaging.** Cells were trypsinized at a confluency of 80-90%, seeded at a density of 65,000 cells/well in 24-well plates on round glass coverslips (12 mm in diameter) and grown for 24 h prior to transfection which was carried out as described above. At 48 h post-transfection, the cell culture media were aspirated from each well and the cells were washed with PBS (3 x 0.5 mL). The cell membrane was then stained with Alexa Fluor 555-conjugated WGA (5 µg/mL in Hank’s balanced salt solution without phenol red). The cells were incubated for 10 min in the dye working solution at 37 °C in 5% CO₂ in the dark. The cells were then washed with PBS (3 x 0.5 mL) and finally fixed with 4% paraformaldehyde in PBS solution for 20 min at 20 °C in the dark. The coverslips were then removed from the wells, left to dry briefly in air, and then mounted on glass slides using Mowiol (polyvinyl alcohol) solution and left in the dark at 20 °C (18 h) to allow hardening of the mounting media. 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).

**Cell Viability Assay.** HeLa cells were trypsinized at a confluency of 80-90% and seeded at a density of either 6,500 (transfection after 24 h) or 4,000 (transfection after 0.5 h) cells per well of 96-well plates. The transfection was carried out using the same protocol as transfecting the 24-well plates with the exception of reducing the amount of lipoplexes such that each well contains 1.5 pmol siRNA in a final volume of 100 µL/well of DMEM containing 10% FCS. After incubation for 44 h at 37 °C in 5% CO₂, alamarBlue (10 µL) was added to each well. After incubation for 3.5 h at 37 °C in 5% CO₂, the absorbance of each well was measured at 570 nm and 600 nm using a microplate-reader (VERSAmax), and the calculation of the amount of
alamarBlue reduced measured at 570 nm was carried out according to the standard protocol provided by the alamarBlue supplier (Invitrogen). Percentage viability was calculated as:

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

**Particle Size and \(\zeta\)-Potential Measurements.** Lipoplexes were prepared by adding siRNA solution (75 µL, 1 µM in 10 mM HEPES buffer, pH 7.4) to HEPES (250 µL) containing the specified amount of cationic lipid followed by vortex mixing for 2-3 s. Samples were then diluted to a final volume of 3 mL with HEPES buffer and shaken for 10 s. DLS measurements were carried out using a Malvern Zetasizer Nano S90 with refractive index 1.59, viscosity 0.89 cP, dielectric constant 79, at 25 °C with equilibrium time 3 min. Z-Average diameter in nm and \(\zeta\)-potential in mV were recorded as averages of three and six measurements respectively.

**siRNA Binding (RiboGreen Intercalation Assay).** RiboGreen working solution was prepared by diluting RiboGreen stock solution 1 to 400 in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.5 diluted 1 to 20 in RNase free water). RiboGreen working solution (40 µL) was added to each well of a 96-well plate (black bottom) containing free siRNA (1 pmol) or complexed with lipospermines in TE buffer at the lipid/siRNA ratios that showed the best reduction in EGFP expression. Each well contained a final volume of 120 µL. The fluorescence was measured (n = 6) using FLUOstar Optima Microplate Reader (BMG-LABTECH), \(\lambda_{ex} = 480\) nm and \(\lambda_{em} = 520\) nm. The amount of siRNA available to interact with the lipid vector was calculated by subtracting the values of RiboGreen background fluorescence (RiboGreen without siRNA) from each measurement, and expressed as a percentage of the control that contained naked siRNA only, calculated as:

\[
\text{% free siRNA} = \frac{\text{RiboGreen fluorescence of complexes}}{\text{RiboGreen fluorescence of naked siRNA}} \times 100
\]

**Statistical Analysis.** All data are presented as mean + S.D. (n = 9). The mean values and S.D. 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

1,12-Diphthalimido-4,9-diazadodecane

$N^4,N^9$-Dioleoyl-1,12-diamino-4,9-diazadecane (DOS) C18:1 $\Delta^9$ and C18:1 $\Delta^9$

$N^4,N^9$-Dierucoyl-1,12-diamino-4,9-diazadecane (DEruS) C22:1 $\Delta^{13}$ and C22:1 $\Delta^{13}$

$N^4,N^9$-Dilinoleoyl-1,12-diamino-4,9-diazadecane (DLinS) C18:2 $\Delta^{9,12}$ and C18:2 $\Delta^{9,12}$

1,12-Diphthalimido-$N^4$-oleoyl-4,9-diazadodecane

$N^4$-Arachidonoyl-$N^9$-oleoyl-1,12-diamino-4,9-diazadecane (AOS) C20:4 $\Delta^{5,8,11,14}$ and C18:1 $\Delta^9$
$N^4$-Eicosenoyl-$N^9$-oleoyl-1,12-diamino-4,9-diazadodecane (EicOS) C20:1 $\Delta^{11}$ and C18:1 $\Delta^9$

$N^4$-Erucoyl-$N^9$-oleoyl-1,12-diamino-4,9-diazadodecane (EruOS) C22:1 $\Delta^{13}$ and C18:1 $\Delta^9$

$N^4$-Lignoceroyl-$N^9$-oleoyl-1,12-diamino-4,9-diazadodecane (LigOS) C24:0 and C18:1 $\Delta^9$

$N^4$-Linoleoyl-$N^9$-oleoyl-1,12-diamino-4,9-diazadodecane (LinOS) C18:2 $\Delta^{9,12}$ and C18:1 $\Delta^9$

$N^4$-Nervonoyl-$N^9$-oleoyl-1,12-diamino-4,9-diazadodecane (NOS) C24:1 $\Delta^{15}$ and C18:1 $\Delta^9$

$N^4$-Oleoyl-$N^9$-stearoyl-1,12-diamino-4,9-diazadodecane (OSS) C18:1 $\Delta^9$ and C18:0

*Figure 1.* Target asymmetrical and symmetrical $N^4,N^9$-diamides of spermine.
Spermine (1,12-diamino-4,9-diazadecane or N,N'-bis(3-aminopropyl)-1,4-diaminobutane) has two terminal primary amine groups at carbons 1 and 12, and two secondary amine groups with their nitrogen atoms numbered 4 and 9. In order to conjugate two different fatty acids at the $N^4$ and $N^9$ positions, first, the two terminal primary amines were protected with phthalimide, then one fatty acid (oleic, 18:1) was acylated on position $N^4$ using oleic acid/DCC/DMAP to conjugate in a 1:1 stoichiometry with an oleoyl chain (Figure 1). The purified mono-acylated product, 1,12-diphthalimido-$N^4$-oleoyl-4,9-diazadecane, was then conjugated with the second long-chain fatty acid using DCC/DMAP. This method avoids using orthogonal protection of the primary and secondary amino groups with different protecting groups (e.g. phthalimide for the primary amines and then Boc for the secondary amines) and hence we obtained the target products in fewer reactions. Keeping one fatty acid chain constant as oleic acid (18:1), the free secondary amine group of 1,12-diphthalimido-$N^4$-oleoyl-4,9-diazadecane was acylated with: arachidonic (20:4), eicosenoic (20:1), erucic (22:1), lignoceric (24:0), linoleic (18:2), nervonic (24:1), and stearic (18:0) acids. Whilst all the other molecules are named with the oleoyl group at $N^9$, $N^4$-oleoyl-$N^9$-stearoyl-1,12-diamino-4,9-diazadecane (OSS) is named as such to reflect the alphabetical order of oleoyl-stearoyl (IUPAC nomenclature), but this does not alter the relative structures within the diacylated-spermine series. Thus, seven asymmetrical spermine derivatives were synthesised (Figure 1), purified to homogeneity, and then characterized by NMR and HRMS.

![Graph](image.png)

**Figure 2.** siEGFP-AF delivery to HeLa cells expressed as the normalized values of the geometric mean fluorescence of AF647 48 h post-transfection of HeLa cells with the lipoplexes prepared with seven asymmetrical spermine fatty acid conjugates and symmetrical DOS. The amount of siEGFP-AF was kept constant at 15 pmol/well (15 nM).
Figure 2 shows siRNA siEGFP-AF delivery to HeLa cells as evaluated by the normalized geometric mean fluorescence of AF647 covalently bound to siEGFP. The amount of siRNA delivered (at 6 μg cationic lipid/well) as measured by AF fluorescence was 30, 17, and 10 for the C18 fatty acid conjugates OSS, DOS, and LinOS respectively which was related inversely to increases in the number of C=C double bonds in the C18 fatty acids. Increasing the number of unsaturation sites in C24 fatty acids also decreased siRNA delivery (at 6 μg cationic lipid/well, normalized AF fluorescence was 50 and 23 for LigOS and NOS respectively). However, increasing the number of unsaturation sites from one to four in EicOS and AOS respectively, resulted in a decrease in siRNA delivery, from 19 to 10 at 6 μg cationic lipid/well \( (p = 0.0001) \), and from 18 to 14 at 3 μg/well \( (p = 0.0163) \) for EicOS and AOS respectively. Increasing the chain length (EruOS, 22:1 and NOS, 24:1), while keeping the number of C=C double bonds constant, caused an increase in siRNA delivery compared to shorter DOS 18:1 and EicOS 20:1. At a cationic lipid concentration of 6 μg/well, AF647 fluorescence was 17, 19, 35, and 23; and at 3 μg/well: 14, 18, 31, and 24 for DOS, EicOS, EruOS, and NOS respectively. Comparing saturated LigOS (with lignoceroyl chain, 24:0) and OSS (with stearoyl chain 18:0) shows that an increase in the chain length from C18 (OSS) to C24 (LigOS) resulted in an increase in AF647 fluorescence from 30 to 50 respectively \( (p < 0.0001) \).

![Graph showing EGFP expression percentage calculated 48 h post-transfection of HeLa cells with the lipoplexes prepared with seven asymmetrical spermine fatty acid conjugates and symmetrical DOS. The amount of siEGFP-AF was kept constant at 15 pmol/well (15 nM).](attachment:image.png)
Figure 3 shows the reduction of EGFP expression in HeLa cells 48 h post-transfection with lipoplexes of siEGFP and the synthesized asymmetrical fatty acid derivatives of spermine. C18 fatty acids with one or two unsaturation sites resulted in better reduction in EGFP expression. Thus, LinOS (3 µg/well) gave 24% EGFP expression, DOS (6 µg/well) gave 27% EGFP, compared to OSS (with one stearoyl 18:0) which at 3 µg/well gave 38% EGFP and at 6 µg/well gave 38% EGFP. The quantitative FACS data (Figures 2 and 3) are better illustrated by the flow cytometric graphs comprised of individual histograms (Figure 4).

The N/P ratio is defined as the ratio of cationic lipid ammonium ions to RNA phosphate anions. In this case, the numerator is calculated as the number of moles of cationic lipid multiplied by the positive charge carried, 2.0. The value 2.0 reflects the pK\textsubscript{a} of each primary amine (~10.5) and the pH at which the experiments are performed (7.4) using the Henderson-Hasselbalch equation.\textsuperscript{33} The denominator is the total number of phosphate anions which here is calculated from the total number of moles of siRNA multiplied by the total number of phosphates in ds siRNA, where a synthetic 24-mer carries 23 phosphates in each strand following phosphoramidite synthesis. Though the terminal sugars of uncomplexed siRNA will be phosphorylated in cells, lipoplexes of siRNA are prepared before this reaction, at N/P = 23.8 with DOS (6 µg/well), N/P = 21.3 with LigOS (6 µg/well), and N/P = 11.9 with LinOS (3 µg/well) for a fixed amount of siRNA (15 pmol).

Increasing the number of unsaturation sites in C20 fatty acids resulted in better reduction in EGFP expression (down to 37% and 29% at 6 µg/well, p < 0.0001; 52% and 34% at 3 µg/well; p < 0.0001 for EicOS and AOS respectively). There was no significant statistical difference (p > 0.05) between LigOS and NOS with respect to their effects on EGFP expression. Increasing the chain length of the fatty acid spermine derivatives from C18 to C24 while keeping number of C=C double bonds constant generally resulted in higher levels of EGFP across the series of compounds, with EGFP expression reduced to 26%, 38%, 51%, and 54% at 6 µg/well of DOS, EicOS, EruOS, and NOS respectively. There were no differences between EicOS and EruOS at 0.75, 1.5, and 3 µg/well. Increasing the chain length from C18 to C24 in OSS and LigOS respectively resulted in higher levels of EGFP expression at different concentrations of lipids. At 6 µg/well, OSS resulted in EGFP expression of 38% while LigOS resulted in EGFP expression of 56% (p < 0.0001). The efficiency of gene silencing achieved with LinOS (76%), DOS (73%), and OSS (62%) is comparable to that of commercially available reagents TransIT-TKO (4 µL/well, 75%) and Lipofectamine 2000 (2 µL/well, 69%) used in the presence of serum (10% FCS) and in accordance with the manufacturers’ instructions for optimal efficiency.
(a) Control

(b) LigOS

(c) LinOS

(d) TransIT-TKO
Figure 4. Gated FACS analysis of delivery of siEGFP-AF (15 nM, 15 pmol/well) and EGFP expression in HeLa cells (compared to (a) control) 48 h post-transfection with lipoplexes of (b) LigOS (6 µg/well) and (c) LinOS (3 µg/well). Positive controls come with the commercial transfection reagents (d) TransIT-TKO and (e) Lipofectamine 2000. Gate set for a healthy cell population (left column). The AF647 gate (red, middle column) typically shows >95% of parent-gated cells with delivery of siEGFP-AF lipoplexes prepared with asymmetrical spermine fatty acid conjugates. The EGFP gate (green, right column, values measured by geometric mean fluorescence relative to control) shows silencing to ~20% with LinOS lipoplexes, and to ~55% with LigOS lipoplexes even though delivery is essentially quantitative with the latter.

We show representative FACS results (Figure 4) with the typical graphs: forward-scatter/side-scatter (FSC/SSC) dot graph showing the gating of the healthy cell population, the profile with the AF647 gate, and the profile with the EGFP gate. These data are representative in that they are typical and reflect a single delivery and gene silencing experiment (from 1 of 9 wells). The conditions shown are without (as a control) and with siEGFP-AF. A qualitative analysis of the control cells (Figure 4a) shows a healthy and homogeneous population of EGFP fluorescing cells, with weak autofluorescence excluded out of the AF647 gate. Delivery of siEGFP-AF (15 nM, 15 pmol/well) was 6-fold greater with LigOS (6 µg/well) than with LinOS (3 µg/well). However, gene silencing was greater with LinOS lipoplexes than with those prepared with LigOS (see Figure 4 and statistics in Figure 3). Thus, these flow cytometry experiments are also good representatives of cationic lipids showing high delivery with low gene silencing (LigOS), and low delivery with good silencing (LinOS). The commercial transfection reagents TransIT-TKO and Lipofectamine 2000 show very high delivery, but their silencing is no better than that achieved with LinOS (3 µg/well) (Figure 4). Clearly, intracellular delivery and gene silencing are controlled by different processes and therefore functional siRNA delivery is affected by lipoplexes of different cationic lipids to varying extents.34
Figure 5. EGFP expression percentage (grey bars) calculated 48 h post-transfection of HeLa cells with the lipoplexes prepared with asymmetrical spermine fatty acid conjugates and siNC-AF (15 pmol/well, 15 nM). siNC-AF fluorescence shows siRNA delivery (white bars). Cationic lipid amounts used were those that resulted in the best reduction in EGFP expression: AOS (6 µg/well), EicOS (6 µg/well), EruOS (6 µg/well), LigOS (6 µg/well), LinOS (3 µg/well), NOS (6 µg/well), and OSS (6 µg/well).

The EGFP expression and siNC-AF delivery are shown in Figure 5. The amounts of lipids used were those which resulted in the best reduction in EGFP expression with respect to each cationic lipid (see Figure 3). The data show that while the delivery of siNC-AF for each cationic lipid, at the specified concentration, was with comparable efficiency to the delivery results of siEGFP-AF (see Figure 2), there was no significant effect (a maximum of 10% reduction) on EGFP expression percentage. Thus, the reduction in EGFP expression after transfection with siEGFP-AF lipoplexes is mainly due to sequence-specific EGFP knock-down mediated by the delivered siEGFP-AF, and not due to any cationic lipid-related effects, e.g. toxicity.
Figure 6. siEGFP-AF delivery to HeLa cells expressed as the normalized values of the geometric mean fluorescence of AF647 48 h post-transfection of HeLa cells with the mixtures of lipoplexes prepared with DOS/DLinS or DOS/DEruS at different molar ratios (total lipid weight 3 µg/well). The amount of siEGFP-AF was kept constant at 15 pmol/well (15 nM).

Mixtures of cationic lipids have been reported to enhance DNA transfection through synergy. Figure 6 shows siEGFP-AF delivery with lipoplexes of mixtures of DOS/DLinS and DOS/DEruS at different molar ratios of the symmetrical spermine derivatives, as well as comparing their siEGFP-AF delivery with the delivery by the asymmetrical LinOS and EruOS. DOS lipoplexes resulted in AF647 fluorescence of 14 (normalized) and DLinS resulted in delivery of 3. The mixtures of DOS/DLinS of molar ratio 4:1 to 1:4, resulted in AF647 fluorescence of intermediate values between those of DOS and DLinS. When compared to LinOS, the siEGFP-AF delivery with the DOS/DLinS 1:1 lipoplexes resulted in lower AF647 delivery (7 and 12 for DOS/DLinS 1:1 and LinOS respectively, $p < 0.0001$). The mixtures of DOS/DEruS of molar ratio 4:1 to 1:4, resulted in AF647 fluorescence of intermediate values between those of DOS (14) and DEruS (82). There was no statistical significant difference between DOS/DEruS 1:1 and EruOS (33 and 31 respectively, $p = 0.64$). The mixtures mostly behave in a way which reflects the behavior of the individual components (Figure 6). DLinS shows the lowest uptake, consistent with our results (Figure 2) where lipids containing more C=C show lower uptake. DEruS has the highest uptake, also consistent with the results in Figure 2 where the longer and more saturated lipid chains, EruOS, LigOS, and NOS show higher uptake. However, these mixtures of cationic lipids displayed no synergy in siEGFP-AF delivery as found with DNA transfection.

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Figure 7. EGFP expression percentage measured 48 h post-transfection of HeLa cells with the
lipoplexes prepared with mixtures of DOS/DLinS or DOS/DEruS at different molar ratios (total
lipid weight 3 µg/well). The amount of siEGFP-AF was kept constant at 15 pmol/well (15 nM).

Figure 7 shows the reduction in EGFP expression 48 h post-transfection with lipoplexes
of mixtures of DOS/DLinS and DOS/DEruS at different molar ratios of the symmetrical
spermine derivatives, as well as the EGFP expression resulting from transfection with lipoplexes
of the asymmetrical LinOS and EruOS. The mixtures of DOS/DLinS of molar ratio 4:1 to 1:4,
resulted in EGFP expression of intermediate values between those of DOS and DLinS, with
EGFP expression increasing with the increase in DLinS content in the mixture. When compared
to LinOS, the EGFP expression with the DOS/DLinS 1:1 lipoplexes resulted in EGFP expression
of 34% compared to LinOS 24% (3 µg/well, p < 0.0001). The mixtures of DOS/DEruS of molar
ratio 4:1 to 1:4, resulted in EGFP expression of intermediate values between those of DOS and
DEruS. Contrary to our results obtained with DOS/DLinS 1:1 and LinOS, EruOS resulted in
higher EGFP expression (47%) compared to the DOS/DEruS 1:1 mixture (31%, p < 0.0001). The
gene silencing of lipoplexes composed solely of DLinS (55%), and of DEruS (57%) show limited
inhibition of EGFP expression (Figure 7). The mixtures mostly show gene silencing dominated
by DOS rather than reflecting the percentage composition of the individual components. This is
also found in the covalent binding of erucoyl and oleoyl chains in the spermine conjugate EruOS
(gene silencing to 47%), but incorporating two different C18 acyl chains in the asymmetrical
LinOS, linoleoyl and oleoyl, affords the best gene silencing (24%).
**Figure 8.** Confocal photomicrographs of HeLa cells before and after transfection of siEGFP-AF with LinOS (3 µg/well). The cell membrane is stained with Alexa Fluor 555 (blue). Representative pictures are shown. (a) Untransfected HeLa cells stably expressing EGFP (green). (b) Detection of siEGFP-AF (red dots), only 4 h post-transfection. (c) EGFP expression with the green channel on as in (a) and (b), monitored 48 h post-transfection. EGFP expression is largely reduced (cf FACS data, Figure 4b). (d) As (b) with the FACS green and blue channels turned off for clarity.

Figure 8a shows non-transfected (control) HeLa cells expressing EGFP (green) within the cell membrane (blue). Figures 8b and 8d show HeLa cells only 4 h post-transfection with lipoplexes of siEGFP-AF and LinOS (3 µg/well). Figure 8b shows that while siEGFP-AF (red) has been...
delivered, EGFP is still strongly expressed throughout the cell. In Figure 8d, the green channel was turned-off to better visualize the red colour representing siEGFP-conjugated dye AF647 (siEGFP-AF). Note that the lower cell shows higher uptake compared to the upper cell. In Figure 8c, EGFP (green) is barely detectable at 48 h post-transfection, compared to the control untransfected cells shown in Figure 8a. Scattered red dots indicate that siEGFP-AF is still present 48 h post-transfection. Taken together, the photomicrographs shown in Figure 8 demonstrate the apparent efficient uptake of lipoplexes of siEGFP-AF and LinOS (3 µg/well) and the subsequent very dramatic reduction (knock-down) of EGFP expression, at least up to 48 h post-transfection. We note that LinOS (at 3 and even at 6 µg/well, Figure 2) shows the lowest siRNA delivery, but with siEGFP-AF held constant at 15 pmol/well (15 nM), LinOS (3 µg/well) forms the most efficient lipoplexes for gene silencing (Figure 3) even though this high efficacy is not reflected in high delivery.

**Figure 9.** A Z-stack confocal photomicrograph gallery of photomicrographs representing 12 Z-sections in HeLa cells transfected with lipoplexes of LinOS (3 µg/well) and siEGFP-AF (15 pmol). EGFP fluorescence (green), cell membrane stained with WGA-Alexa Fluor® 555 (blue), and Alexa Fluor® 647 (red) representing tagged siRNA delivery.
In order to further highlight the intracellular detection of siEGFP-AF monitored 48 h post-transfection (shown above in Figure 8b and 8d as red dots), where (Figure 8c) there is almost no detectable EGFP expression (with the green channel turned on), a Z-stack gallery was recorded through a monolayer of transfected HeLa cells (Figure 9). Z-Stacks are a series of successive optical sections acquired at different positions along the Z-axis (in 1.0 μm slices). The first optical section was taken slightly lower than the surface of the cells attached to the cover slip, then the sections were recorded while slicing through to the opposite surface. Figure 9 shows that majority of the red colour (representing siRNA delivery) is present inside the cell, the blue colour representing cell membrane is only present at the perimeter of the cells, and occasionally the red colour is present simultaneously with the blue colour indicating Alexa Fluor® 647 (red) (bound to siRNA) within the membrane. As we slice along the Z-axis, there is no blue colour in the middle of the cells where there is red. 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.36

DLS was used to measure the particle sizes (Table 1) of the lipoplexes prepared at the cationic lipid/siRNA ratio that resulted in the largest reduction in EGFP expression. The size range is 145-292 nm (from OSS to AOS) which is in agreement with the size range of lipoplexes previously reported to transfect successfully in cell culture.37 The particle size could determine the main route of cellular entry, with lipoplexes <300 nm likely to enter via clathrin-mediated endocytosis, and lipoplexes >500 nm entering cells via caveoli-mediated endocytosis.37, 38 However, the entry route for functional siRNA delivery might be by fusion with the cell membrane rather than via an endocytic pathway, as demonstrated using selective inhibitors.34 The ζ-potentials of the prepared lipoplexes (Table 1) are all positive (40-59 mV). Such a net positive charge on the lipoplex surface is important in promoting lipoplex-lipoplex repulsion, thus preventing aggregation. The RiboGreen siRNA binding assay results (Table 1) show that the asymmetrical spermine conjugates are able to bind siRNA almost completely, with the fluorescence of RiboGreen dye reduced to values of 1-5% of the control. The two free terminal primary amine groups of the acylated spermine conjugates are practically fully protonated at physiological pH 7.4.

Table 1. Particle diameter, ζ-potential, and siRNA binding assay of fatty acid amides of spermine measured at the cationic lipid/siRNA ratios that showed best knock-down of EGFP (all shown as Mean ± S.D.).

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<table>
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<th>cationic lipid</th>
<th>diameter (nm)</th>
<th>ζ-potential (mV)</th>
<th>% fluorescence of RiboGreen</th>
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<tr>
<td>AOS</td>
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<tr>
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<tr>
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<tr>
<td>LigOS</td>
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<tr>
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<tr>
<td>OSS</td>
<td>145 ± 7</td>
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**Figure 10.** Cell viability (alamarBlue assay) 48 h post-transfection of HeLa cells with the lipoplexes prepared with spermine fatty acid conjugates, using 6,500 cells/well, and transfection with lipoplexes was 24 h after seeding the cells. The amount of siNC was kept constant (1.5 pmol/well, 15 nM) and amounts of lipids were adjusted accordingly to the same cationic lipid/siRNA ratio that resulted in the best reduction in EGFP expression: AOS (0.6 µg/well), EicOS (0.6 µg/well), EruOS (0.6 µg/well), LigOS (0.6 µg/well), LinOS (0.3 µg/well), NOS (0.6 µg/well), and OSS (0.6 µg/well); compared with DOS (0.6 µg/well), Lipofectamine 2000 (Lipof, 0.2 µL/well), and TransIT-TKO (0.4 µL/well).

For pragmatic cell viability assays, we downscaled from the transfection conditions in 24-well (1 mL) format to 96-well (0.1 mL) format, after establishing that 4,000-6,500 cells/well was an optimal range for reproducible results and in order to avoid problems of overconfluency. To keep the ratios and concentrations the same, the weight of cationic lipid and the amount of siRNA were each reduced to one tenth of their original values. The two cell viability assay
conditions we employed reflect the experimental conditions used in gene silencing (0.5 h between seeding and transfection) and in confocal microscopy where the time after seeding was 24 h to allow the cells to attach well and distribute evenly on the cover slips. In comparison, when using FACS to quantify gene silencing, there was no requirement for a long time between seeding and transfection as there was no requirement for stronger attachment to cover slips.

The weight of lipid chosen in Figure 10 is based on the ratio of lipid/siEGFP-AF that resulted in the best reduction in EGFP expression as seen in Figure 3. The percentage cell viabilities, shown in Figure 10, indicate that transfection of HeLa cells with lipoplexes of siRNA and the synthesized asymmetrical spermine derivatives resulted in cell viabilities above 74%, with LinOS (0.3 μg/well) resulting in 88% viability and DOS 85% viability (0.6 μg/well) which is comparable to TransIT-TKO (81%) and better than Lipofectamine 2000 (62%).

![Graph showing cell viability after transfection](image)

**Figure 11.** Cell viability (alamarBlue assay) 48 h post-transfection of HeLa cells with the lipoplexes prepared with spermine cationic lipids using 4,000 cells/well and transfection 0.5 h after seeding. The amount of siRNA (siNC) was kept constant at 1.5 pmol/well (15 nM) and amounts of cationic lipids were adjusted accordingly.

Figure 11 shows the cell viabilities after transfection of HeLa cells using a lower number of cells (4,000 cell/well in 96-well format) and carrying out the transfection within 0.5 h of seeding the cells, aiming to make the transfection conditions harsher for the cells, such that individual differences between each of the cationic lipids at their different concentrations can be compared. Figure 11 also shows that increasing the amounts of lipids per well from 0.075 to 0.6 μg resulted in a decrease in cell viability. The viabilities resulting from transfection with lipoplexes of LinOS and DOS (the cationic lipids that showed the best reduction in EGFP expression as shown in Figure 3) were reduced from 96% to 84% to 65% to 52% (LinOS) and
from 95% to 77% to 50% to 33% (DOS) at 0.075, 0.15, 0.3, and 0.6 μg/well respectively. The lowest cell viabilities were due to transfection with EruOS lipoplexes (from 94%, 53%, 26%, and 14% at 0.075, 0.15, 0.3, and 0.6 μg/well of EruOS respectively).

**DISCUSSION**

We have designed, synthesized, purified, and tested in a variety of physico-chemical and cell biological assays asymmetrical lipopolyamines based on spermine, possessing two primary amines. These are novel divalent cationic lipids. Assessment using both flow cytometry and fluorescence microscopy in human HeLa cells, measuring both delivery of the fluorescently tagged siRNA and silencing the EGFP signal in a stably transfected cell line, allowed quantitation of the differences between asymmetrical cationic lipids, selected mixtures of their symmetrical counterparts, and comparison with commercially available non-viral delivery agents. In this study, we report the design of a series of novel spermine based cationic lipids, making variations in the hydrophobic domain by changing the type of fatty acids conjugated to the parent spermine molecule. Koynova et al. reported the impact of changing the hydrophobic moiety of cationic lipids on DNA transfection efficiency. Data from 20 cationic phosphatidylcholine (PC) derivatives show that the chain saturation state and chain length, representing hydrophobic volume and cationic lipid hydrophilic-lipophilic balance (HLB), are major factors that determine DNA transfection efficiency, with variations in efficiency by more than two orders of magnitude, and also that lipid chain asymmetry has a strong impact on DNA transfection efficiency while keeping the HLB of cationic lipids constant.

The human cervical epithelial adenocarcinoma HeLa cell line is a well-described model of cancer cells and hence is a relevant tool for studies on siRNA- or shRNA-mediated silencing. In this paper, as a first step towards assessing the efficiency of our compounds at delivering siRNA into cells, we chose HeLa cells stably expressing a reporter gene coding for the fluorescent protein EGFP, under the control of a constitutive promoter, a strategy used in a number of other studies. The advantages of using a stably transfected cell line are that this simple experimental system allows expression of the protein, translated from the reporter gene at a steady-state level, and a fast assessment on the effect of EGFP-directed siRNA delivery by flow cytometry (FACS) and fluorescence microscopy. Stable transfection of the target gene of interest (with expression vector pEGFP-C1 pDNA in this study) only requires the cells to be kept under selective pressure with an antibiotic (aminoglycoside G418 in our system) to prevent loss of pDNA during cell cultivation. The loss of genome-integrated exogenous pDNA with cell division during in vitro cultivation is a known phenomenon and it can only be reduced/prevented by keeping the cells under some pressure, with a non-toxic antibiotic (G418) concentration.
Furthermore, as in our experimental system cell populations studied are asynchronous, any potential effects of the cell cycle on the reporter gene are averaged and the related protein levels will vary with the efficiency in silencing of the delivered siRNA.

Whilst these studies are in HeLa cells stably expressing EGFP to test the efficiency of the lipoplexes of asymmetrical lipopolyamines to deliver siRNA and then achieve gene silencing, they are comparable with recently reported siRNA delivery in C6 glioma cells stably expressing GFP, and antisense studies in human cervical cancer HeLa705 cells containing an aberrant luciferase gene. Likewise, this in vitro evaluation strategy has been used in HeLa-EGFP cells and HeLa expressing either EGFP or luciferase. It is likely that any correlation with in vivo performance will follow results obtained at high serum levels. siRNA for intravenous administration must be stable in full (human) serum. Our use of 10% FCS is a step towards this.

Non-viral DNA vectors prepared from cationic lipids that are composed of asymmetrical fatty acid chains have been reported to have higher in vitro and/or in vivo efficiencies in comparison with their symmetrical counterparts. Several hypotheses have been put forward to explain the superior DNA transfection efficiency of asymmetrical cationic lipids. Ali et al. reported that fatty chain asymmetry in membrane lipids affects the physical environment of liquid-crystalline bilayers. PCs showing a relatively large difference in chain length (larger asymmetry, e.g. 18:0/8:0 or 18:0/10:0 PCs) resulted in 20-25% greater in-plane elasticity compared to PCs with less asymmetry (16:0-18:1 PCs) and to symmetrical PCs (di18:1). Heyes et al. suggested that the difference in length between the fatty chains allows for better overlapping within biological lipid bilayers following the work of Balasubramaniam et al. who proposed that asymmetrical lipids have increased fusogenicity.

The effect on DNA transfection efficiency of changing the saturation state in one chain was reported by Koynova et al. Comparing two asymmetric cationic phospholipid derivatives, oleoyldecanoylethyl-PC (C18:1/C10:0) and stearoyldecanoylethyl-PC (C18:0/C10:0), resulted in the former compound with the oleoyl chain showing 50-fold more DNA transfection efficiency than the latter with the saturated stearoyl chain. This DNA transfection efficiency result was attributed to the enhanced fusibility of the (C18:1/C10:0) lipid with cellular lipid membranes, and to the facilitated release of DNA due to phase reorganization, lamellar to non-lamellar transitions, and extensive phase coexistence as a result of lipid mixing at physiological temperatures. The processes of siRNA lipoplex delivery (uptake) and gene silencing are different from each other and different from DNA transfection. Our gene silencing of EGFP expression results show that siRNA lipoplexes self-assembled with lipopolyamines possessing one or two centres of unsaturation in the acyl chains (e.g. DOS, LinOS) lead to more gene silencing compared to similar lipoplexes containing saturated chains of the same length (e.g. stearoyl in OSS). siRNA uptake was highest with the symmetrical DEruS.
Synergistic effects of mixtures of cationic lipids have also been reported to enhance DNA transfection. However, mixtures of \(N\)-[1-(2,3-dimyristoyloxy)propyl]-\(N\),\(N\),\(N\)-trimethylammonium (DMTAP) and \(N\)-[1-(2,3-dioleoyloxy)propyl]-\(N\),\(N\),\(N\)-trimethylammonium (DOTAP) show intermediate values of DNA transfection efficiencies compared to using pure parent lipids as a linear function of lipid mixture composition.\(^{35}\) Enhanced DNA transfection following the use of lipid mixtures is possibly due to the change of the hydrophobic volumes of the lipids, related to the average hydrophobicity of the mixture and/or the heterogeneous distribution of the hydrophobic domains of the mixtures. Also, the presence of more than one lipid results in an increase in the degrees of freedom of lipoplex-membrane interactions eventually leading to better fusogenicity.\(^{35}\) siRNA lipoplex formation and delivery do not necessarily follow the same constraints as those of DNA lipoplexes due to the significant structural differences between DNA and siRNA regarding molecular weight and rigidity. Thus, the aforementioned hypotheses regarding DNA self-assembled lipoplexes should be applied cautiously to siRNA lipoplexes.

The use of mixtures e.g. DOS/DLinS and DOS/DEruS, at different molar ratios of the symmetrical spermine derivatives, afforded similar or lower siEGFP-AF delivery in comparison with their related asymmetrical cationic lipids (LinOS and EruOS). Reduction in EGFP expression 48 h after transfection with lipoplexes prepared from mixtures of these symmetrical cationic lipids was poorer than that achieved with the related asymmetrical cationic lipid LinOS, but better than that achieved with EruOS. In the alamarBlue assay, cell viability of LinOS lipoplexes was 88%. LinOS is an excellent, efficient, non-toxic cationic lipid in HeLa cells, comparable or superior to commercially available reagents TransIT-TKO and Lipofectamine 2000.

The amount of siEGFP-AF delivered to HeLa cells was lower with more C=C double bonds in the lipid moiety. Increasing the chain length, while keeping the number of C=C constant, caused an increase in siRNA delivery. C18 fatty acids with one or two unsaturation sites resulted in better reduction in EGFP expression and increasing the number of unsaturation sites in C20 fatty acids also resulted in better reduction in EGFP expression in HeLa cells 48 h post-transfection. Increasing the chain length of the fatty acid spermine derivatives from C18 to C24 while keeping number of C=C double bonds constant was generally less efficient for gene silencing. We have shown that reduction in EGFP expression after transfection with siEGFP-AF lipoplexes is due to sequence-specific EGFP knock-down mediated by the delivered siEGFP-AF, and not due to any cationic lipid-related effects, e.g. toxicity. We conclude that the effects of different hydrophobic domains are not equal on intracellular delivery of siRNA and on gene silencing by siRNA. Control of these processes can be achieved with different \(N^4,N^9\)-diacyl spermines where gene silencing is efficiently demonstrated using lipoplexes of LinOS.
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ABBREVIATIONS USED

EGFP, enhanced green fluorescent protein; FCS, fetal calf serum; HRMS, high-resolution mass spectrometry; WGA, wheat germ agglutinin.

REFERENCES


