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Varying the Chain Length in N^4,N^9-Diacyl Spermines: Non-Viral Lipopolyamine Vectors for Efficient Plasmid DNA Formulation

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Abstract: The aims of this work are to study the effect of varying the chain length in synthesized N^4,N^9-diacyl spermines on DNA condensation and then to compare their transfection efficiencies in cell lines. The five novel N^4,N^9-diacyl lipopolyamines: N^4,N^9-[didecanoyl, dilauroyl, dimyristoyl, dimyristoleoyl, and dipalmitoyl]-1,12-diamino-4,9-diazadodecane were synthesized from the naturally occurring polyamine spermine. The abilities of these novel compounds to condense DNA and to form nanoparticles were studied using ethidium bromide fluorescence quenching and nanoparticle characterization techniques. Transfection efficiency was studied in FEK4 primary skin cells and in an immortalized cancer cell line (HtTA), and compared with a saturated (distearoyl) analogue and also with the non-liposomal transfection formulation Lipogen, N^4,N^9-dioleoyl-1,12-diamino-4,9-diazadodecane. By incorporating two aliphatic chains and changing their length in a stepwise manner, we show efficient circular plasmid DNA (pEGFP) formulation and transfection of primary skin and cancer cell lines. Two C14 chains (both saturated or both *cis*-monounsaturated) were efficient transfecting agents, even in the presence of serum, but they were too toxic. N^4,N^9-Dioleoyl spermine efficiently condenses pDNA and achieves the highest transfection levels with the highest cell viability among the studied lipopolyamines in cultured cells even in the presence of serum.

Keywords: Gene delivery; lipopolyamine; NVGT; primary skin cancer cells; transfection

Introduction

Progress is being made toward gene therapy realizing its potential and becoming an efficient medicine for the treatment of diseases such as inherited blindness, inflammation, cancer, for neuronal delivery,[1] or for vaccination.[2,3] However, the significant problems still associated with viral gene therapy, especially immunogenicity, mammalian toxicity, and the limited payload of DNA, ensure that the goal of intracellular protein levels at therapeutic concentrations moves even more toward utilizing non-viral gene therapy (NVGT).[4,5] Within the broad term NVGT, except for naked (free) DNA being trapped inside cells during mitosis, the (prodrug) DNA must be formulated for delivery. Our NVGT focus is on lipopolyamines composed of two long-carbon chains (or a steroid) covalently bound to a polyamine e.g.

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spermine (1,12-diamino-4,9-diazadodecane).[6,7] Other NVGT research groups are investigating a variety of alternative approaches, including naked DNA, gene gun (bound to gold particles), electroporation, polycation-mediated DNA delivery, and the use of a wide variety of cationic lipids (lipoplexes),[8−15] and cationic polymers (polyplexes),[16,17] in the search for an "intelligent" material which can overcome the key obstacles still found in poly-nucleic acid delivery,[18,19] for reviews see refs 1, 20−24.

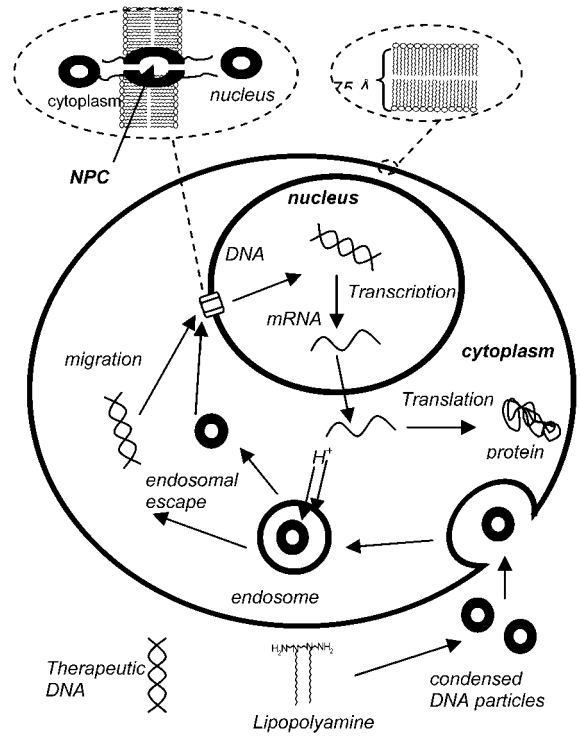

Figure 1. The process of NVGT by endocytosis and the barriers that must be crossed by toroidal condensed DNA nanoparticles, illustrated for lipopolyamines in the formulation of lipoplexes leading to DNA delivery and ultimately to the goal of intracellular protein synthesis.

The essential requirements for gene delivery are the transport of DNA through the cell membrane and ultimately to the nucleus. The design of an efficient formula for the delivery of genetic material requires a detailed understanding of the barriers that hinder this process (Figure 1). Efficient NVGT formulations should be able to deliver safely the required DNA across the various cellular barriers to the nucleus.[25] These barriers include complex formation between the DNA and the lipopolyamine leading to DNA nanoparticle (lipoplex) formation by electrostatic charge neutralization and overall packing as condensed DNA, and then transport across

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cell-membranes, either by adsorptive endocytosis or mediated by cations, both routes leading to internalization of the lipoplex.[26]

Our aims are to design and develop efficient, non-toxic, non-viral vectors for in vitro and possible in vivo applications, using our novel spermine conjugates, based on change to the type, length, position, and number of the hydrophobic anchors.[6,7] These cationic lipids (lipospermines) probably assist in the self-assembling of polycationic scaffolds, as well as facilitating absorptive endocytosis and/or fusion with cell membranes. These lipospermines form spontaneous complexes with negatively charged poly nucleic acids to condense DNA (using pEGFP as the reporter gene), leading to formation of nanoparticles after removal of small counterions from both cationic carriers and DNA (a thermodynamically favored step which drives and stabilizes complex formation). Thus, these formed nanoparticles are suitable for gene delivery. The complexes are monitored using ethidium bromide (EthBr) fluorescence quenching,[27] transfection efficiency, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cytotoxicity assays.[28] We have therefore designed a series of novel shorter chain lipopolyamines in order to prepare lipoplex formulations (without any prepreparation of liposomes) of pDNA for non-viral gene delivery and transfection of target cells as they are designed to form nanoparticles which will efficiently enter cells by endocytosis (Figure 1). These vectors are designed to have simplicity of use based upon DNA condensation by anion titration.

Herein we report investigations of the effects on DNA formulation with variation in the length of the two fatty chains in the lipospermine moiety. We make sequential changes to chain length from C10 (decanoyl) to C18 (oleoyl), through C12 (lauroyl), C14 (myristoyl and myristoleoyl) and C16 (palmitoyl). In our experiments we have chosen to deliver a 4.7 kbp plasmid encoding for enhanced green fluorescent protein (pEGFP), with a molecular weight of about 3.1 MDa (given an average of 330 Da per nucleotide, 660 Da per bp,[29] carrying 9,400 negative charges. We report the synthesis and characterization of the nanoparticles, transfection results, DNase protection and toxicity with the five new synthesized lipospermine formulations in both primary and cancer cell lines, and compare our results with those obtained with a saturated analogue containing two C18 chains, N^4,N^9-distearoyl spermine [6,7] and the non-liposomal lipospermine Lipogen (N^4,N^9-dioleoyl spermine) formulation.[6,7]

Materials and Methods

Materials. Chemicals, including spermine, acyl chlorides, reagents, solvents, and buffers, were routinely purchased from Sigma-Aldrich (Gillingham, U.K.) except where indicated, and cell culture materials were from Life Technologies (Paisley, Scotland).

Synthesis of N^4,N^9-Substituted Spermine Derivatives. Spermine was used as the starting material for the synthetic process, outlined in Figure 2. The tetra-amine (203 mg, 1.0 mmol) in methanol (10 mL) was protected on both the primary amino functional groups by reaction with ethyl trifluoroacetate (0.3 mL, 2.2 equiv) in methanol (10 mL), under anhydrous nitrogen, and the reaction mixture was stirred for 18 h at 20 °C. The solvent was evaporated to dryness in vacuo to form N^1,N^{12}-ditrifluoroacetyl-1,12-diamino-4,9-diazadodecane as a yellow oily residue. This residue was taken into the next step without purification. Fatty acyl chloride (decanoyl, lauroyl, myristoyl, myristoleoyl, oleoyl, palmitoyl, stearoyl) (2.2 equiv) and triethylamine (2.5 equiv) were added to the diprotected spermine solution in CH_2Cl_2 and methanol (1:1, v/v). The solution was stirred for 72 h at 20 °C and then evaporated to dryness in vacuo. The residue was dissolved in CH_2Cl_2, and the solution was filtered and evaporated to dryness in vacuo to form N^4,N^9-(didecanoyl, dilauroyl, dimyristoyl, dimyristoleoyl, dioleoyl,[30] dipalmitoyl, or distearoyl)-N^1,N^{12}-ditrifluoroacetyl-1,12-diamino-4,9-diazadodecane, as typified by N^1,N^{12}-ditrifluoroacetyl-N^4,N^9-dimyristoleoyl-1,12-diamino-4,9-diazadodecane (706 mg, 87%, R_f = 0.9 CH_2Cl_2:MeOH, 5:1, v/v) which showed MS, FAB$^+$ found 811.2 (100%, *m/z* [M + H]$^+$), $C_{42}H_{73}F_6N_4O_4$ requires 811.6.

For the removal of the ditrifluoroacetyl protecting groups, the tetra-amide (e.g. 700 mg, 1 mmol) was dissolved in methanol (20 mL), and the pH of the solution was increased by saturating with ammonia gas, when it was left for 18 h at 20 °C and then evaporated to dryness in vacuo to give an oily residue (e.g. 554 mg, 97%) that was purified over silica gel (CH_2Cl_2:MeOH 10:1, v/v, then CH_2Cl_2−MeOH−concentrated aqueous NH_3 10:5:1, v/v/v) to afford the seven desired lipopolyamine conjugates as their free bases. All the synthesized lipopolyamines were homogeneous on silica gel thin-layer chromatography (CH_2Cl_2−MeOH−concentrated aqueous NH_3 25:10:1, v/v/v) and were characterized by ^{1}H nuclear magnetic resonance (NMR) at 400 MHz and ^{13}C NMR spectroscopy at 100 MHz. NMR assignments follow from correlation spectroscopies and all lipopolyamines showed satisfactory high-resolution mass spectrometric (HRMS) data (reported in Da and within 5 ppm), as typified by N^4,N^9-didecanoyl spermine **1** found (*m/z* [M + H]$^+$) 511.4929, $C_{30}H_{63}N_4O_2$ requires 511.4951 (Δ ppm 4.3);

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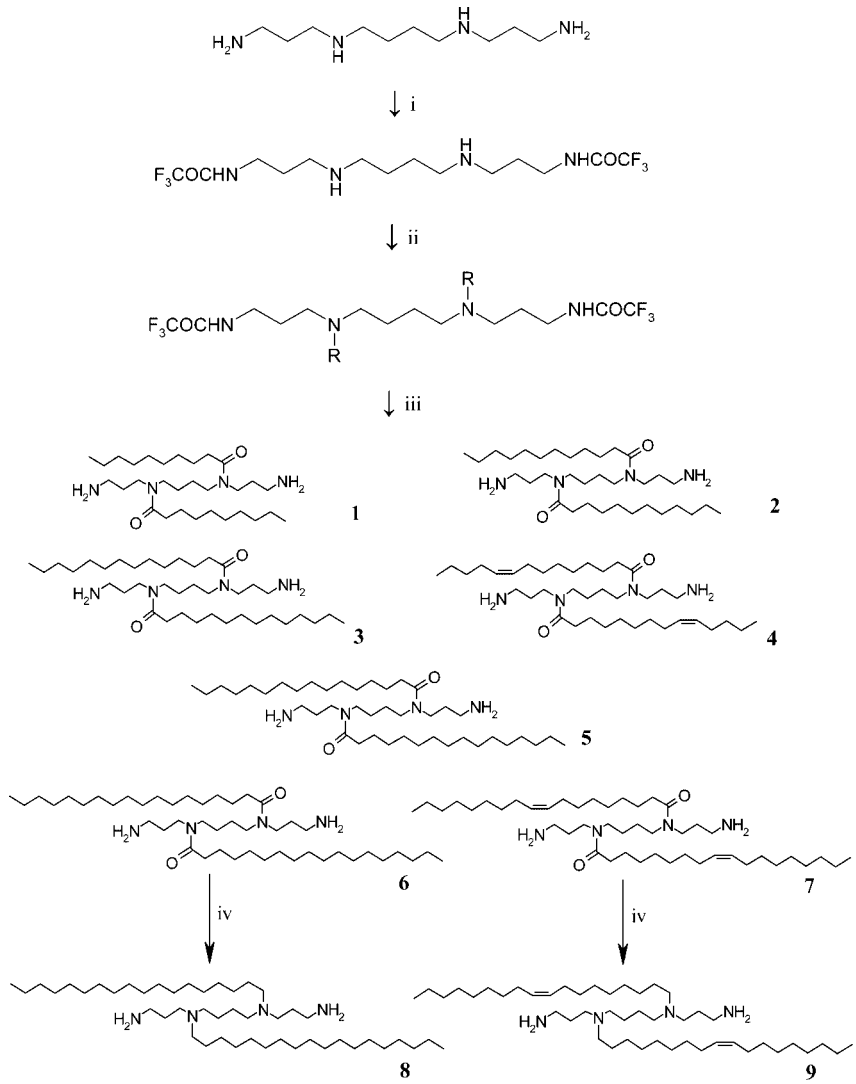

Figure 2. Synthetic scheme of spermine based cationic lipids: N^4,N^9-didecanoyl spermine **1**, N^4,N^9-dilauroyl spermine **2**, N^4,N^9-dimyristoyl spermine **3**, N^4,N^9-dimyristoleoyl spermine **4**, N^4,N^9-dipalmitoyl spermine **5**, N^4,N^9-distearoyl spermine **6,** N^4,N^9-dioleoyl spermine **7**, N^4,N^9-distearyl spermine **8,** and N^4,N^9-dioleyl spermine **9**. Reagents: (i) ethyl trifluoroacetate, 18 h at 20 °C; (ii) fatty acyl chloride, R = (decanoyl **1**, lauroyl **2**, myristoyl **3**, myristoleoyl **4**, palmitoyl **5**, stearoyl **6**, and oleoyl **7**), triethylamine, CH_2Cl_2 and methanol (1:1, v/v), 72 h at 20 °C; iii) methanol saturated with ammonia gas, 18 h at 20 °C; (iv) $LiAlH_4$, anhydrous THF, 24 h at reflux.

N^4,N^9-dilauroyl spermine **2** found (*m/z* $[M + H]^+$) 567.5561, $C_{34}H_{71}N_4O_2$ requires 567.5577 (Δ ppm 2.8); N^4,N^9-dimyristoyl spermine **3** found (*m/z* $[M + H]^+$) 623.6170, $C_{38}H_{79}N_4O_2$ requires 623.6203 (Δ ppm 5.3); N^4,N^9-dimyristoleoyl spermine **4** found 619.5872 (*m/z* $[M + H]^+$), $C_{38}H_{75}N_4O_2$ requires 619.5890 (Δ ppm 2.9); N^4,N^9-dipalmitoyl spermine **5** found (*m/z* $[M + H]^+$) 679.6796, $C_{42}H_{87}N_4O_2$ requires 679.6829 (Δ ppm 4.9); N^4,N^9-distearoyl spermine **6** found (*m/z* $[M + H]^+$) 735.7438, $C_{46}H_{95}N_4O_2$ requires 735.7455 (Δ ppm 2.3); N^4,N^9-dioleoyl spermine **7** found (*m/z* $[M + H]^+$) 731.7115, $C_{46}H_{91}N_4O_2$ requires 731.7142 (Δ ppm 3.7). To a solution of **6** or **7** (1 mmol) in anhydrous THF (40 mL), an excess of lithium aluminum hydride (0.11 g, 3 mmol) was added and heated under reflux for 24 h, then cooled to 20 °C and cautiously quenched. The residue was purified over silica gel (CH_2Cl_2:MeOH 10:1 v/v then CH_2Cl_2:MeOH:NH_4OH 10:5:1 v/v/v) to afford the target dialkylated tetra-amines (Figure 2): N^4,N^9-distearyl spermine **8** found (*m/z* $[M + H]^+$) 707 7880, $C_{46}H_{99}N_4$ requires 707.7864 (Δ ppm 2.3); N^4,N^9-dioleyl spermine **9** found (*m/z* $[M + H]^+$) 703.7511, $C_{46}H_{95}N_4$ requires 703.7484 (Δ ppm 3.8).

Amplification and Purification of Plasmid DNA (pEGFP). DNA plasmid[29] encoding enhanced green fluorescent protein (pEGFP), purchased from Clontech, was transformed into *Escherichia coli* JM 109 bacterial strain (Promega). The transformed cells were grown in larger quantities of Luria−Bertani (LB) broth supplemented with 125 mg/L ampicillin. pEGFP plasmid was produced in large-scale using HiSpeed plasmid purification Maxi kit (Qiagen) according to the manufacturers protocol. DNA yields and purity were determined spectroscopically (OD_{260}/OD_{280} = 1.80 to 1.90 OD, optical density) and by agarose gel (1%) analysis.

DNA Condensation (Ethidium Bromide Fluorescence Quenching Assay). Each concentration of the DNA stock solutions (approximately 1 μg/μL, 1 mL) was determined spectroscopically (Milton Roy Spectronic 601 spectrometer, 1 cm path length, 3 mL cuvette),[29] and 6 μg (approximately 6 μL) of DNA was diluted to 3 mL with buffer (20 mM NaCl, 2 mM HEPES, 10 μM EDTA, pH 7.4) in a glass cuvette stirred with a microflea. Immediately prior to analysis, EthBr solution (3 μL, 0.5 mg/mL) was added to the stirring solution and allowed to equilibrate for 10 min. Separately each lipopolyamine, aliquots (5 μL), according to the ammonium/phosphate (*N*/*P*, +/−) charge ratio required, were then added to the stirring solution and the fluorescence measured after 1 min equilibration using a Perkin-Elmer LS 50B luminescent spectrometer (λ_{ex} = 260 nm and λ_{em} = 600 nm with slit width 5 nm) while stirring using an electronic stirrer (Rank Bros. Ltd.).[27] The total lipopolyamine solution added to the DNA solution did not exceed 5% of the total volume of the solution, so no correction was made for sample dilution. The fluorescence was expressed as the percentage of the maximum fluorescence when EthBr was bound to the DNA in the absence of competition for binding and was corrected for background fluorescence of free EthBr in solution.

Gel Electrophoresis. Each sample of plasmid DNA (0.5 μg), either free or complexed with different lipopolyamine concentrations, was analyzed by gel electrophoresis for about 1 h under 75 V/cm, through an agarose gel (1%) containing EthBr (1 μg/mL) in Tris-acetate−EDTA 1 × (40 mM Tris-acetate and 1 mM EDTA) buffer. The (unbound) free DNA in the agarose gel was visualized under UV using GeneGenius (Syngene, Cambridge, U.K.).

Lipoplex Particle Size. The average particle size for the lipoplexes formed (at their optimum charge ratio of transfection), after mixing with a vortex mixer, was determined using a NanoSight LM10 (NanoSight Ltd., Salisbury, U.K.). All measurements were carried out on lipoplexes with 1 μg/mL plasmid DNA in HEPES buffer at pH 7.4 in a sample volume of 0.2 mL. Results were analyzed with the Nanoparticle Tracking Analysis (NTA) software.

ζ-Potential Measurements. The ζ-potential measurements for the lipoplexes formed (at their optimum charge ratio of transfection), after mixing with a vortex mixer, were determined using a DelsaNano Zeta Potential (Beckman Coulter, High Wycombe, U.K.). All measurements were carried out on lipoplexes with 3 μg/mL pDNA in HEPES buffer at pH 7.4 in a sample volume of 2 mL.

DNase I Protection Assay. Briefly, in a typical assay, pEGFP plasmid (1 μg) was complexed with varying amounts of the representative cationic lipid using the indicated lipopolyamine:DNA *N*/*P* charge ratios in a total volume of 30 μL in HEPES buffer, pH 7.4, and incubated at 20 °C for 30 min on a rotary shaker. Subsequently, the complexes were treated with DNase I (10 μL, at a final concentration of 1 μg/mL) in the presence of 20 mM $MgCl_2$ and incubated for 20 min at 37 °C. The reactions were then halted by adding EDTA (to a final concentration of 50 mM) and incubated at 60 °C for 10 min in a water bath. The aqueous layer was washed with phenol:chloroform:isoamyl alcohol (50 μL, 25:24:1 v/v/v) and centrifuged at 10,000 rpm for 5 min. The aqueous supernatants were separated, loaded (15 μL) on a 1% agarose gel (pre-stained with EthBr 1.0 μg/mL) and electrophoresed at 100 V for 1 h.

Cell Culture and Transfection Experiments. Two cell lines were used in the transfection experiments, a human primary skin fibroblast cells FEK4[31] derived from a foreskin explant and a human cervix carcinoma, HeLa derivative and transformed cell line (HtTA). The HtTA cells being stably transfected with a tetracycline-controlled transactivator (tTA) consisting of the tet repressor fused with the activating domain of virion protein 16 of the herpes simplex virus (HSV). Cells were cultured in Earle's minimal essential medium (EMEM) supplemented with fetal calf serum (FCS) 15% in the case of FEK4 and 10% in the case of HtTA cells, penicillin and streptomycin (50 IU/mL each), glutamine (2 mM), and sodium bicarbonate (0.2%).

Transfection Experiments in the Absence or Presence of Serum. For the transfection (gene delivery) and the resultant gene activity (transfection efficiency), FEK4 and HtTA cells were seeded at 50,000 cells/well in 12-well plates in EMEM (2 mL) containing FCS for 24 h to reach a plate confluency of 50−60% on the day of transfection. Then the media were replaced by 0.4 mL of Opti-MEM (serum free media, Gibco BRL) for 4 h at 37 °C in 5% CO_2. The lipoplex was prepared by mixing pEGFP (1 μg in 50 μL) with the cationic lipopolyamine in Opti-MEM (typically 10 μg in 50 μL) according to the *N*/*P* charge ratio at 20 °C for 30 min and then incubated with the cells (final volume of 0.5 mL) for 4 h at 37 °C in 5% CO_2 in Opti-MEM (in the absence of serum). Then the cells were washed and cultured for a further 44 h in full growth medium at 37 °C in 5% CO_2 before the assay.

Or for transfection in the presence of serum, as in the above procedure, but on the day of transfection, the media were replaced by fresh EMEM (0.4 mL) containing FCS. The lipoplex was prepared as above and then incubated with the cells for 4 h at 37 °C in 5% CO_2 in full growth medium (in the presence of serum). Then the cells were washed and cultured for a further 44 h in full growth medium at 37 °C in 5% CO_2 before the assay.

The transfection efficiency was calculated based on the percentage of the cells that expressed enhanced green fluorescent protein (EGFP positive cells) in the established range (M_1) with correction for the background fluorescence of the control sample, λ_{ex} = 495 nm and λ_{em} = 518 nm, using a fluorescence activated cell sorting (FACS) machine (Becton Dickinson FACS Vantage dual laser instrument, argon ion laser 488 nm). Therefore, at the end of the 44 h of growth, cells were washed twice with PBS (1 mL/well)

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to remove FCS and dead cells. Only a subset of the data obtained from healthy cells (the major population) was analyzed through a gate setting. Typically, fluorescence parameters for 10,000 events were acquired at green fluorescence (FL1) and data were expressed as histograms. In the histogram of events at different fluorescence intensities of the control group, the fluorescence intensity range (M_1) was set as a constant range (<5% overlap) throughout the experiments ($n = 9$).

In Vitro Cytotoxicity (MTT) Assay. FEK4 and HtTA cells were seeded in 96-well plates at 8,000 cells/well and incubated for 24 h at 37 °C in 5% CO_2. Lipoplexes e.g. N^4,N^9-dioleoyl spermine complexed with pEGFP were added in the same way as in the transfection protocol. After incubation for 44 h, the media were replaced with fresh media (90 μL) and sterile filtered MTT solution (10 μL, 5 mg/mL) to reach a final concentration of 0.5 mg/mL. Then the plates were incubated for a further 4 h at 37 °C in an atmosphere of 5% v/v CO_2. After incubation, the media and the unreacted dye were aspirated and the formed blue formazan crystals were dissolved in 200 μL/well of dimethyl sulfoxide (DMSO). The color produced was measured using a plate-reader (VERSAmax) at $\lambda = 570$ nm. The percent viability relative to control wells containing cells without added DNA or lipopolyamine is calculated by (test absorbance/control absorbance) × 100.[32]

Results and Discussion

Synthesis of Lipospermines. The tetra-amine spermine was used as the starting material for the synthesis of the seven desired lipopolyamines: N^4,N^9-didecanoyl spermine **1**, N^4,N^9-dilauroyl spermine **2**, N^4,N^9-dimyristoyl spermine **3**, N^4,N^9-dimyristoleoyl spermine **4**, N^4,N^9-dipalmitoyl spermine **5**,[33] N^4,N^9-distearoyl spermine **6** and N^4,N^9-dioleoyl spermine **7** (Figure 2). The tetra-amine was protected on both the primary amino functional groups with ethyl trifluoroacetate (2.2 equiv) in methanol. Each corresponding fatty acyl chloride (e.g. myristoyl, myristoleoyl, oleoyl, stearoyl, etc.) was used as the acylating agent together with triethylamine in CH_2Cl_2 and methanol (1:1). Deprotection in methanol saturated with ammonia gas and flash column chromatography afforded the seven target lipospermines, homogeneous on silica gel thin-layer chromatography e.g. N^4,N^9-dioleoyl spermine **7** $R_f = 0.44$ (CH_2Cl_2−MeOH−concentrated aqueous NH_3 25:10:1 v/v/v). After purification to homogeneity, the structures of the target compounds were established by ^{1}H and ^{13}C NMR spectroscopy and confirmed by HRMS. The compounds were fully characterized by ^{1}H (at 400 MHz) and ^{13}C NMR (at 100 MHz) spectroscopy; the characteristic ^{1}H NMR signals included δ 0.8−0.9 ppm methyl protons, 1.2−1.3 ppm many methylene chain protons, and 5.3−5.4 ppm for the alkene protons (where required), while the characteristic ^{13}C NMR signals included δ 13.9−14.1 ppm methyls, 22.3−39.1 ppm methylenes, 45.1−49.2 ppm (C3 and C5), 129.5−129.7 *cis*-alkenes C9′ and C10′ (where required), and 172.7−174.5 ppm the amide carbonyl groups. A solution of the diamide **6** or **7**, in anhydrous THF, was reduced with an excess of lithium aluminum hydride and purified over silica gel (CH_2Cl_2:MeOH 10:1 v/v then CH_2Cl_2:MeOH:NH_4OH 10:5:1 v/v/v) to afford the two target tetra-amines N^4,N^9-distearyl spermine **8** and N^4,N^9-dioleyl spermine **9** (Figure 2).[34]

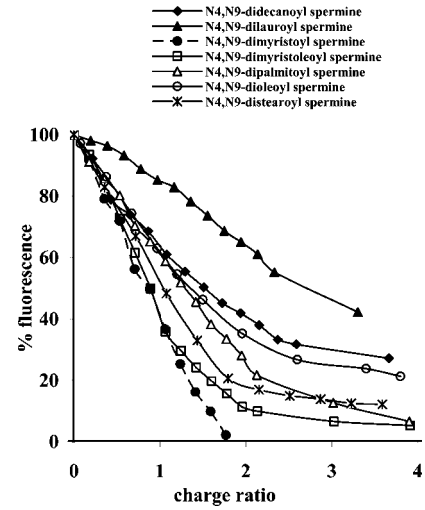

Figure 3. Plot of EthBr fluorescence quenching assay of pEGFP complexed with different lipospermines.

DNA Condensation. In Figure 3, we show the DNA condensation ability of the synthesized lipopolyamines in an EthBr fluorescence quenching assay. These results show that N^4,N^9-dimyristoyl spermine **3** and N^4,N^9-dimyristoleoyl spermine **4** have the best DNA condensing ability, more than 90% EthBr fluorescence quenching at *N*/*P* charge ratio 2, while N^4,N^9-dipalmitoyl **5** and N^4,N^9-distearoyl **6** show such a result at $N/P = 3$, then N^4,N^9-didecanoyl spermine **1** and N^4,N^9-dioleoyl spermine **7** show 75% fluorescence quenching at $N/P = 4$ and last N^4,N^9-dilauroyl spermine **2** is only able to achieve about 60% fluorescence quenching at the same *N*/*P* charge ratio.

The gel electrophoresis results (data not shown) show that the majority of spermine conjugates were able to prevent the migration of pEGFP DNA efficiently, as a result of neutralization of DNA phosphate negative charges by the lipopolyamine ammonium positive charges at their optimized respective charge ratios (*N*/*P*) of transfection. Thus, by completely inhibiting the electrophoretic mobility of plasmid DNA, we conclude that they are charge neutralized, experi-

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Table 1. Particle Size (Mean ± S.D.) and ζ-Potential of pEGFP Complexes with the Studied N^4,N^9-Diacyl Lipopolyamines

lipospermine	charge ratio (*N*/*P*)	lipoplex diameter (nm)	ζ-potential (+) mV
N^4,N^9-didecanoyl spermine **1**	10	150 (6)	4.77
N^4,N^9-dilauroyl spermine **2**	20	170 (12)	14.32
N^4,N^9-dimyristoyl spermine **3**	10	150 (13)	6.78
N^4,N^9-dimyristoleoyl spermine **4**	12	130 (9)	8.21
N^4,N^9-dipalmitoyl spermine **5**	20	160 (10)	9.24
N^4,N^9-disteraroyl spermine **6**	15	220 (21)	nd
N^4,N^9-dioleoyl spermine **7**	2.5	150 (12)	2.17

mental evidence which supports the pEGFP DNA condensation data that we obtained from the EthBr fluorescence quenching assay (Figure 3). Of course, it is not essential that the DNA is fully condensed, provided that nanoparticles are formed, if entry is via the clathrin coated pit, in comparison with relying upon entry during mitosis as in the use of naked DNA.

Lipoplex Particle Size and Zeta-Potential Measurements. The particle size and ζ-potential characterization measurements were carried out on the lipoplexes at their optimum *N*/*P* charge ratio of transfection (see Table 1). Particle size characterization by laser diffraction showed that the nanoscale particle size of the formed complexes ranged from 130 nm (N^4,N^9-dimyristoleoyl spermine **4**) to 170 nm (N^4,N^9-dilauroyl spermine **2**) and 220 nm (N^4,N^9-distearoyl spermine **6**) with an average particle size of 161 nm (Table 1).

ζ-Potential is an important parameter helping to predict the stability of the formulation as well as the ability of the positively charged nanoparticles to interact with cell membranes. ζ-Potential depends on several factors, including pH, ionic charge, ion size, and concentration of ions in solution. The formed nanoparticles are considered to be stable when they have pronounced ζ-potential values, either positive or negative, but the tendency to aggregate is higher when the ζ-potential is close to zero.[7] The surface charge, as determined by ζ-potential measurements on the lipoplexes at their optimum *N*/*P* charge ratio of transfection, show that all values are positive (Table 1), and they ranged from +2.17 mV (N^4,N^9-dioleoyl spermine **7**) to +14.32 mV (N^4,N^9-dilauroyl spermine **2**); the measured ζ-potential for naked DNA is −1.02 mV.

DNase I Protection. The DNase I protection assay was carried out using plasmid DNA complexed with different *N*/*P* charge ratios of the lipopolyamines. Reasonably intense undigested DNA bands were detected in the gel as a control, and there was no band in the lane for uncondensed DNA which was fully digested by DNase I as negative control (Figure 4). We performed the DNase protection assay using different *N*/*P* charge ratios of our lipopolyamines e.g. for N^4,N^9-dioleoyl spermine **7** the intense band of undigested DNA appeared at *N*/*P* charge ratio 3 (Figure 4) which means that the lipoplex formed from condensed pEGFP DNA with

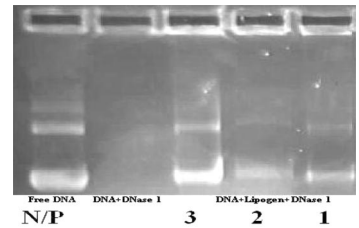

Figure 4. Typical 1% agarose gel of fluorescent EthBr intercalated in pEGFP, assay of DNase I protection on complexation (lipoplex formation) with N^4,N^9-dioleoyl spermine **7**.

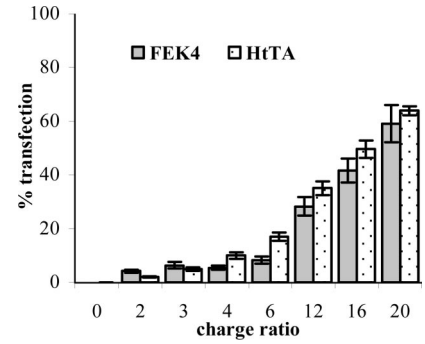

Figure 5. Lipofection of the primary skin cell line FEK4 and the cancer cell line HtTA transfected with pEGFP (1 μg) complexed with N^4,N^9-dilauroyl spermine **2** at different *N*/*P* charge ratios.

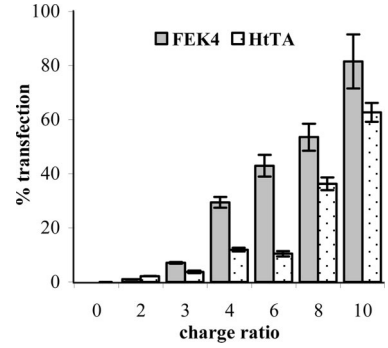

Figure 6. Lipofection of the primary skin cell line FEK4 and the cancer cell line HtTA transfected with pEGFP (1 μg) complexed with N^4,N^9-dimyristoyl spermine **3** at different *N*/*P* charge ratios.

this lipopolyamine is effectively protected from DNase I enzyme, and this may contribute to the lipoplex serum stability.

Transfection Experiments and In Vitro Cytotoxicity. The transduction of EGFP into a primary skin cell line FEK4 and a cancer cell line (HeLa-derived HtTA) was investigated. The optimum concentrations (and corresponding *N*/*P* charge ratios) for transfection were experimentally determined by using ascending *N*/*P* charge ratios of lipopolyamines. From our typical results with these new lipopolyamines we conclude that the transfection efficiency (Figures 5, 6, and 7) and also the toxicity (decrease in cell viability) (Figures 8 and 9) increase as a function of *N*/*P* charge ratio.

That our lipopolyamines are pDNA delivery vectors that can achieve high transfection efficiency is immediately seen

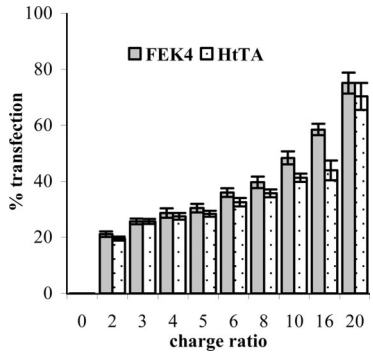

Figure 7. Lipofection of the primary skin cell line FEK4 and the cancer cell line HtTA transfected with pEGFP (1 μg) complexed with N^4,N^9-dipalmitoyl spermine **5** at different *N*/*P* charge ratios.

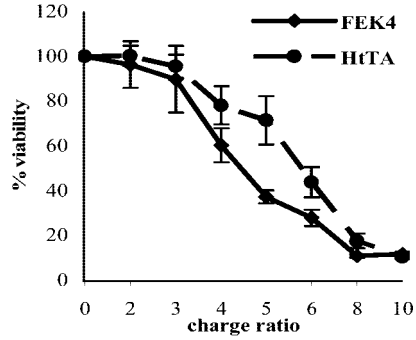

Figure 8. Cytotoxicity effect of pEGFP (1 μg) complexed with N^4,N^9-didecanoyl spermine **1** at different *N*/*P* ratios on the primary skin cell line FEK4 and the cancer cell line HtTA.

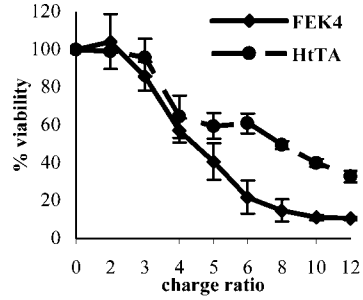

Figure 9. Cytotoxicity effect of pEGFP (1 μg) complexed with N^4,N^9-dimyristoleoyl spermine **4** at different *N*/*P* ratios on the primary skin cell line FEK4 and the cancer cell line HtTA.

from our representative flow cytometric FACS analysis (gated in the control for live cells) with N^4,N^9-dimyristoleoyl spermine **4** in both cell lines (Figure 10). After 4 h transfection and then 44 h growing, at the end of those 48 h, cells were washed twice with PBS (1 mL/well) to remove FCS and dead cells. Typically, 10,000 events were collected. Recordings were made at green fluorescence (FL1), and data were expressed as histograms. Only a subset of the data obtained from healthy cells (the major population) was analyzed through a gate setting. In the histogram of events at different fluorescence intensity control group, the fluorescence intensity range (M_1) was set as a constant range

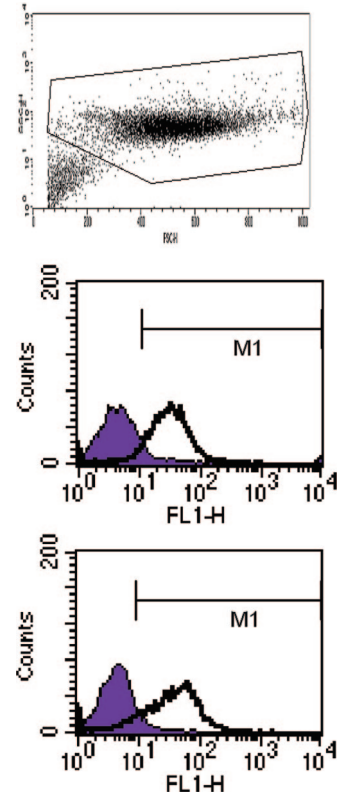

Figure 10. FACS analysis showing the live population gated (above), and of FEK4 (middle) and of HtTA (below) after 48 h transfection of pEFGP complexed with N^4,N^9-dimyristoleoyl spermine **4**: ■ untransduced cells, □ EGFP-positive cells.

throughout the experiments. The transfection efficiency was calculated based on the percentage of the cells that expressed EGFP (positive cells) in the established range (M_1) with correction for the background fluorescence of the control sample with $\lambda_{ex} = 495$ nm and $\lambda_{em} = 518$ nm. Further transfection efficiency studies (carried out in triplicate on 3 separate experiments, $n = 9$) showed that for N^4,N^9-dioleoyl spermine **7** there was no significant difference between the transfection efficiency in the presence (FEK4 82.9% $\pm$ 4.3, HtTA 75.4% $\pm$ 5.5) or absence (FEK4 85.3% $\pm$ 1.4, HtTA 78.0% $\pm$ 1.7) of serum. Encouraged by these positive results, all our subsequent transfection experiments are performed in the presence of serum.

The required lipopolyamine amounts and the corresponding *N*/*P* charge ratios to deliver pEGFP (1 μg in 50 μL, in a total final volume of 0.5 mL) for optimum high levels of transfection were found to be N^4,N^9-didecanoyl spermine (7.7 μg, *N*/*P* = 10.0), N^4,N^9-dilauroyl spermine (17.2 μg, *N*/*P* = 20.0), N^4,N^9-dimyristoyl spermine (9.4 μg, *N*/*P* = 10.0), N^4,N^9-dimyristoleoyl spermine (11.2 μg, *N*/*P* = 12.0), N^4,N^9-dipalmitoyl spermine (20.5 μg, *N*/*P* = 20.0), N^4,N^9-dioleoyl spermine (2.8 μg, *N*/*P* = 2.5) and N^4,N^9-distearoyl spermine (16.7 μg, *N*/*P* = 15). The transfection results (histograms in Figures 11 and 12) indicate that there is no significant difference in the transfection ability of the different lipospermines (at their optimum pDNA delivery *N*/*P* charge ratio)

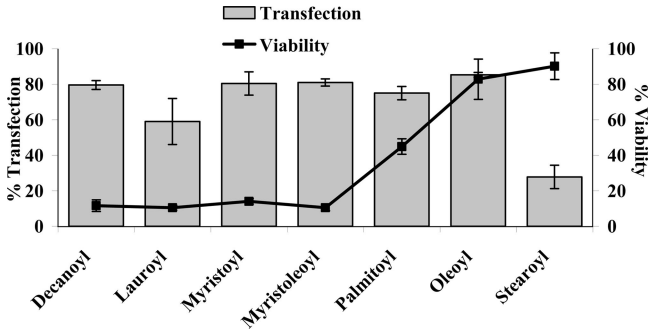

Figure 11. Lipofection and cytotoxicity effects of pEGFP (1 μg) complexed with decanoyl (N^4,N^9-didecanoyl spermine **1**) (7.7 μg), lauroyl (N^4,N^9-dilauroyl spermine **2**) (17.2 μg), myristoyl (N^4,N^9-dimyristoyl spermine **3**) (9.4 μg), myristoleoyl (N^4,N^9-dimyristoleoyl spermine 8**4**) (11.2 μg), palmitoyl (N^4,N^9-dipalmitoyl spermine **5**) (20.5 μg), oleoyl (N^4,N^9-dioleoyl spermine **7**) (2.8 μg), and stearoyl (N^4,N^9-distearoyl spermine **6**) (16.7 μg) on the primary skin cell line FEK4. The data represent 3 different experiments (3 replicates each), and the error bars represent the SD.

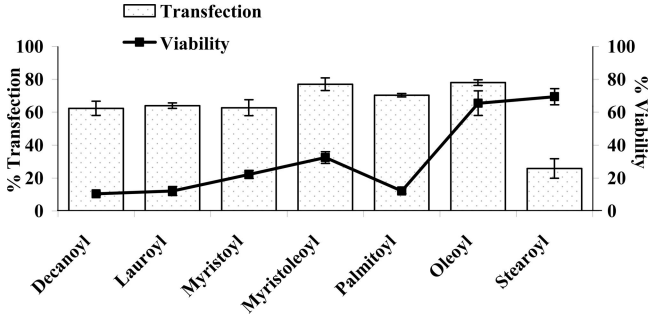

Figure 12. Lipofection and cytotoxicity effects of pEGFP (1 μg) complexed with decanoyl (N^4,N^9-didecanoyl spermine **1**) (7.7 μg), lauroyl (N^4,N^9-dilauroyl spermine **2**) (17.2 μg), myristoyl (N^4,N^9-dimyristoyl spermine **3**) (9.4 μg), myristoleoyl (N^4,N^9-dimyristoleoyl spermine **4**) (11.2 μg), palmitoyl (N^4,N^9-dipalmitoyl spermine **5**) (20.5 μg), oleoyl (N^4,N^9-dioleoyl spermine **7**) (2.8 μg), and stearoyl (N^4,N^9-distearoyl spermine **6**) (16.7 μg) on the HtTA cancer cell line. The data represent 3 different experiments (3 replicates each), and the error bars represent the SD.

on both cell lines, even in the presence of serum, except with N^4,N^9-distearoyl spermine **6**. However, the cell viability (MTT assay) results (solid lines on Figures 11 and 12) indicate that N^4,N^9-dioleoyl spermine typically shows 80% and 70% cell viability for FEK4 and HtTA cells, respectively. The shorter chain lipospermines, especially di-C14, both saturated and *cis*-monounsaturated, while efficient at cell transfection, are toxic to both cell lines (viability less than 40%, and often under 20%). It is often proposed that toxicity increases with increasing *n*-alkyl chain length, e.g. going from C2 to C8 [35−37] and similarly to C10,[38] as such cationic surfactants are known to increase membrane permeability, although this is not an immutable rule, as was found for C2 to C12 in the 8-alkylberberine chloride series where upon elongating the aliphatic chain, toxicity decreased gradually.[39] We are not proposing that our results contradict this, rather that our dioleoyl spermine is used at a significantly low *N*/*P* charge ratio, and the shorter chain analogues at higher *N*/*P* ratios.

In this study, we have investigated effects resulting from changes in the length of the symmetrical diacyl fatty chain formulations of lipospermines on DNA condensation and cellular delivery. The results from pEGFP condensation, investigated by EthBr fluorescence quenching assay, revealed that five of our synthetic lipopolyamines were able to condense DNA to less than 20% EthBr fluorescence, where DNA is defined as condensed.[40] However, N^4,N^9-dilauroyl spermine had not quenched the EthBr fluorescence to 20% by *N*/*P* charge ratio 3.5 (Figure 3). Particle size of the final nanoscale formulation is also an important factor in improving gene delivery.[41,42] Particle size results (Table 1) showed an average particle size of 161 nm. On the relationship between particle size and transfection efficiency, all our lipoplexes are in the range 130−220 nm, and they transfected target cell lines efficiently. If internalization is via a clathrin coated pit, there will be an upper-size limit of 250−300 nm,[43] although larger sizes of nanoparticles still do achieve transfection, presumably by a different mechanism.[44] Nanoparticles have relatively higher intracellular uptake than

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microparticles.[45] Also, on the nanoscale, smaller-size polyplexes are more able to enter cells and thereby increase the efficiency of transfection.[46]

The lipid moiety in our cationic lipids interacts with the phospholipid bilayer of the cell membrane, and that facilitates cell entry, either in crossing the membrane bilayer and/or in helping to weaken the endosomal bilayer and thereby aid escape into the cytosol. DNA, either as a nanoparticle or now free (uncomplexed) from the condensing lipopolyamine, must now traffic to the nucleus and cross the nuclear membrane which is thought to occur through the NPC or by direct association with the chromatin during mitosis. After nuclear entry, the payload DNA should successfully be able to give the desired protein through transcription and translation (Figure 1). The first key step in this gene formulation is DNA condensation into nanoparticles by masking the negative charges of the phosphate backbone. This titration with a lipopolyamine causes alleviation of charge repulsion between remote phosphates along the DNA helix leading to collapse into a more compact structure that facilitates cell entry. The packing of the lipid chains make a significant contribution to this DNA compaction (Figure 3).

We have previously reported the importance of the substituents in the lipid moiety conjugated to the cationic polyamine to achieve improvements in DNA condensation efficiency for non-liposomal formulations where the lipid moiety must be considered in shape (volume) and substituent pattern, as well as the polyamine moiety and its pK_a values.[6,7,47−50] The design and synthesis of novel cationic lipids based on the tetra-amine spermine, as non-liposomal formulations, where the lipid moiety is a long carbon chain, were largely instigated by Remy et al.[51] and Behr et al.[52] with their design and preparation of the highly efficient lipopolyamine dioctadecylamidoglycylspermine (DOGS, Transfectam). We have previously reported that N^4,N^9-dioleoyl spermine is significantly less toxic and more efficient than both Lipofectin and Lipofectamine in the primary skin cell line FEK4, and we also cited the cytotoxic effects of these liposomal formulations.[6] It is generally agreed that the length and type of the aliphatic chains incorporated into cationic lipids significantly affect their transfection efficiency, but this needs experimental verification. Thus, a series of vectors differing in their hydrophobic domains have been prepared. Behr and co-workers made a series of lipopolyamines and found that their gene delivery efficiency was independent of chain saturation (oleoyl vs stearoyl lipopolyamines).[51] Further, when comparing saturated chains, McGregor et al. found C18 chains to be optimal in a series of gemini surfactants according to the order C18 > C16 > C14,[53] which was also reported by Scherman and co-workers with a series of linear polyamine-based vectors.[54] Results obtained with DMRIE[55] (1,2-dimyristyloxypropyl-3-dimethyl-hydroxyethylammonium bromide, an analogue of DOTMA), alkyl acyl carnitine esters[56] and bis-ether lipids related to DOTAP[57] have shown that a comparison of vectors based solely on the lengths of the two saturated aliphatic chains led to the order C14 > C16 > C18 (in terms of transgene expression). Early liposomal studies therefore led to the proposal that a shorter chain length may facilitate intermembrane mixing, an important factor in endosomal escape.[55] A common moiety is the use of *cis*-monounsaturated alkyl chains e.g. the oleoyl group (C18). This leads to higher transfection levels than the corresponding saturated e.g. stearoyl (C18) derivatives, a result possibly related to the issues of hydrophobic moiety hydration or packing.[55,56]

The remarkably different results obtained between N^4,N^9-distearoyl spermine **6** and N^4,N^9-dioleoyl spermine **7**, the former with poor transfection and the latter with high transfection, but both showing good cell viability at practical concentrations for pDNA delivery with N^4,N^9-dioleoyl sper-

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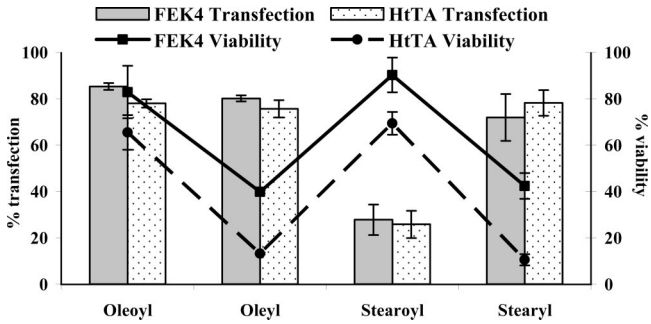

Figure 13. Lipofection and cytotoxicity effects of pEGFP (1 μg) complexed with oleoyl (N^4,N^9-dioleoyl spermine **7**) (2.8 μg), oleyl (N^4,N^9-dioleyl spermine **9**) (4.3 μg), stearoyl (N^4,N^9-distearoyl spermine **6**) (16.7 μg) and stearyl (N^4,N^9-distearyl spermine **8**) (6.7 μg) on the primary skin cell line FEK4 and the HeLa derived cancer cell line HtTA.

mine **7**, made us investigate further these vectors. We designed and prepared alkyl analogues with four positive charges at physiological pH from amide reduction to the tetraamines. Therefore, the corresponding N^4,N^9-distearyl spermine **8** and N^4,N^9-dioleyl spermine **9** were compared with the two di-C18 acyl lipospermines, the saturated N^4,N^9-distearoyl spermine **6** and the mono-*cis*-unsaturated N^4,N^9-dioleoyl spermine **7**. The results (Figure 13, histograms) show that there is not a large difference in the transfection efficiency, neither gain nor loss, on doubling the number of positive charges in the conjugate derived from N^4,N^9-dioleoyl spermine, while there is a great gain in the transfection efficiency for the conjugate compared to N^4,N^9-distearoyl spermine **6**. Thus, N^4,N^9-distearoyl spermine **6** shows transfection efficiencies of 28% and 26% (FEK4 and HtTA respectively), N^4,N^9-distearyl spermine **8** 72% and 87%, N^4,N^9-dioleoyl spermine **7** 85% and 78%, and N^4,N^9-dioleyl spermine **9** 80% and 76%, but the cell viabilities drop from around 65−90% to 10−42%. Therefore, the two alkyl analogues are much more toxic (Figure 13, lines) to both cell lines.

By incorporating two aliphatic chains and then stepwise changing their length, we have shown that our synthesized lipospermines, except distearoyl, afford transfection results in a similar range (essentially equally efficient), but at different *N*/*P* ratios of which N^4,N^9-dioleoyl spermine has the lowest (*N*/*P* = 2.5) while the others ranged over *N*/*P* ratios 10−20. This may give a lead to their higher toxicity, often more than 60% compared with the ∼25% observed for N^4,N^9-dioleoyl spermine which, with its two unsaturated chains, is also much more efficient than the saturated analogue N^4,N^9-distearoyl spermine. N^4,N^9-Dioleoyl spermine is an excellent vector for NVGT.

Abbreviations Used

DOGS, dioctadecylamidoglycylspermine; EGFP, enhanced green fluorescent protein; EMEM, Earle's minimal essential medium; EthBr, ethidium bromide; FCS, fetal calf serum; HRMS, high-resolution mass spectrometry; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NPC, nuclear pore complex; NVGT, non-viral gene therapy; pEGFP, plasmid enhanced green fluorescent protein.

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