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Research Paper

N^4,N^9-Dioleoyl Spermine Is a Novel Nonviral Lipopolyamine Vector for Plasmid DNA Formulation

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Purpose. To study the effect of synthesized N^4,N^9-dioleoyl spermine on DNA condensation and then measure its transfection efficiency in cell culture.
Methods. The lipopolyamine was synthesized from the naturally occurring polyamine spermine. The ability of this novel compound to condense DNA was studied using ethidium bromide fluorescence quenching and light scattering assays. Transfection efficiency was studied in primary skin cells (FEK4) and in an immortalized cancer cell line (HtTA), and compared with the commercially available transfection formulations Lipofectin and Lipofectamine.
Results. The synthesized N^4,N^9-dioleoyl spermine formula is efficient at condensing calf thymus and circular plasmid DNA and effectively transfects both primary skin cells and cancer cell lines at low charge ratios of (+/− ammonium/phosphate) 2.5.
Conclusions. N^4,N^9-Dioleoyl spermine condenses DNA and achieves high transfection levels in cultured cells.

KEY WORDS: FEK4; gene delivery; lipopolyamine; N^4,N^9-dioleoyl spermine; transfection.

INTRODUCTION

It is widely believed that gene therapy will become an efficient medicine for the treatment of diseases such as cancer, cystic fibrosis and for vaccination. 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 mechanism of gene delivery to the nucleus. Different strategies have been used for the delivery of genetic material into target cells, classified as viral or non-viral delivery systems (1–3). Viral delivery systems depend on the development of genetically-modified viruses to utilize their capability of efficiently delivering DNA into cells without their pathogenic characteristics (3). Although high efficiency is achieved by viral vectors, there are concerns about their use which include: a limit in the size of the DNA delivered (the "payload"), endogenous viral recombination, unexpected anti-vector immune response, and oncogene activation (4–7). Since the design and formulation of Lipofectin by Felgner and co-workers, reported in 1987 (8), the focus on nonviral vectors for DNA delivery has shown a remarkable increase worldwide (9–15).

Efficient nonviral formulation should be able to deliver safely the required DNA across the various cellular barriers to the nucleus. These barriers that hinder the delivery of DNA to its physical site of action (the nucleus) have been summarized in Fig. 1. They include complex formation between the DNA and cationic lipid or polymer that leads to condensation of DNA into nanoparticles. Cell-membrane entry is thought to be mediated by cationic substances, which interact with the DNA payload, and can then cause adsorptive endocytosis and internalization of the complex. Also, the lipid moiety in cationic lipids interacts with the phospholipid bilayer of the cell membrane that facilitates cell entry. The internalized material is fused with early endosome. That leads to sorting to the late endosomal compartment, at this stage the DNA complex should escape the endosomal vesicle before the later stage of the lysosome where the DNA will be degraded. After endosomal escape, the DNA (either complexed or dissociated from the condensing agent) should find its way to the nucleus and cross the nuclear membrane which is thought to occur through the nuclear pore complex (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 the processes of transcription and translation.

For drug formulators, it is difficult to deliver a drug molecule of 3.3 kDa molecular weight carrying 10 negative charges, but in the case of the (prodrug) DNA, a 5 kbp

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ABBREVIATIONS: DCC, dicyclohexylcarbodiimide; DOGS, dioctadecylamidoglycylspermine; DOSPER, 1,3-dioleoyloxy-2-(6-carboxyspermine; EGFP, enhanced green fluorescent protein; EMEM, Earle's minimal essential medium; EthBr, ethidium bromide; FCS, fetal calf serum; HOBt, hydroxybenzotriazole; NPC, nuclear pore complex; PEI, polyethylenimine; PLL, poly-L-lysine.

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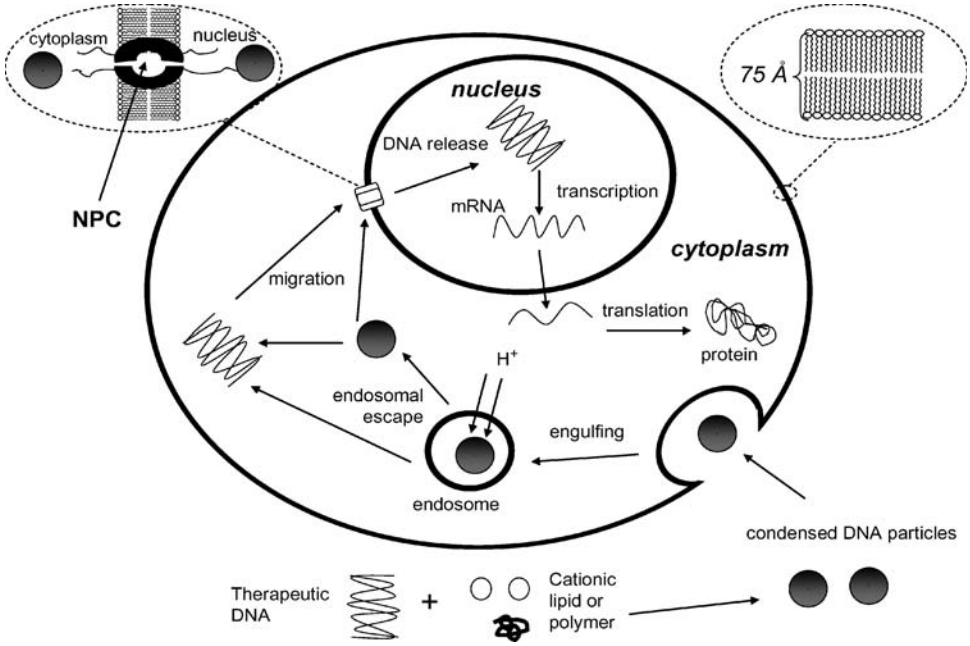

Fig. 1. Steps in the process of nonviral gene therapy by endocytosis showing the barriers for DNA nanoparticles, from the formation of the DNA-polycation complex (condensed DNA particles) to protein synthesis.

plasmid has a molecular weight of about 3.3 megadaltons and carries 10,000 negative charges. So the first key step in gene formulation is DNA condensation into a nanoparticle form through masking the negative charges of the phosphate backbone which causes alleviation of charge repulsion between remote phosphates on the DNA helix leading to collapse into a more compact structure (2,16). The importance of DNA condensation is attributed to the correlation of the transfection efficiency with the formation of DNA nanoparticles that are essential for the delivery of DNA through the cell membrane (16–20).

Cationic lipids are considered to be the major gene carriers among the non-viral delivery systems. They have the ability to condense DNA into particles that can be readily endocytosed by cultured cells, and facilitate endosomal escape leading to efficient delivery to the nucleus (21). They can be classified as liposomal and non-liposomal nonviral delivery vectors. Liposomal delivery vectors usually contain two types of lipids, a cationic lipid (positively charged amphiphile) for DNA condensation and cellular membrane interaction, and a neutral helper lipid (phospholipid), most use dioleoylphosphatidyl-ethanolamine (DOPE) (Fig. 2) to increase transfection efficiency as it has a membrane fusion promoting ability (8,22,23). Nonliposomal cationic-lipid delivery vectors combine both the characteristics of cationic and helper lipids.

The synthesis of the lipopolyamine dioctadecylamidoglycylspermine (DOGS) by Behr and co-workers (24) as a promising transfecting agent, encouraged several laboratories to focus on the synthesis of novel cationic lipids based on the naturally occurring polyamine spermine, for example, RPR120535 (25) and 1,3-dioleoyloxy-2-(6-carboxyspermine) DOSPER (26) (Fig. 2). The design of a novel lipopolyamine formula for DNA condensation and cellular delivery relies on previous and continuing studies of the structure-activity relationships of DNA binding and condensation by polyamines (16,27–30). Although lipopolyamines are less efficient in comparison with viral vectors, their promising lower toxicity than viral vectors ensures a continuous effort to design novel lipopolyamines with improved transfection efficiency. In this study, we synthesized and formulated a novel lipospermine in which the tetra-amine spermine (the cationic moiety) and dioleoyl chains (the lipophilic moiety) that is reported to improve the transfection efficiency by fusion with cellular membrane (31). These unsaturated chains are linked by amide bonds at the secondary amino groups of spermine to form N^4,N^9-dioleoyl spermine (commercially available as LipoGen) (32). These amide linkers have the advantages of being both biodegradable and less toxic than the ether bonds in DOTMA (33,34). The ability of this synthetic lipopolyamine to condense DNA was studied using ethidium bromide (EthBr) fluorescence quenching and light scattering assays. Transfection efficiency was studied in an immortalized cancer cell line (HtTA), and in primary skin cells (FEK4) for the first time. The difficulties found in efficiently transfecting primary cell lines were largely overcome with this nonliposomal formulation comprising a vector with two covalently bound oleoyl chains. The results are compared with two commercially available (liposomal) transfection formulations, Lipofectin® and Lipofectamine that incorporate such oleoyl or oleyl (C18) chains.

MATERIALS AND METHODS

Materials

Chemicals, including polyamines spermine, polyethylenimine (PEI), and poly-L-lysine (PLL), reagents, solvents, buffers, and DNA were routinely purchased from Sigma-Aldrich, UK, except where indicated. Lipofectin and Lipo-

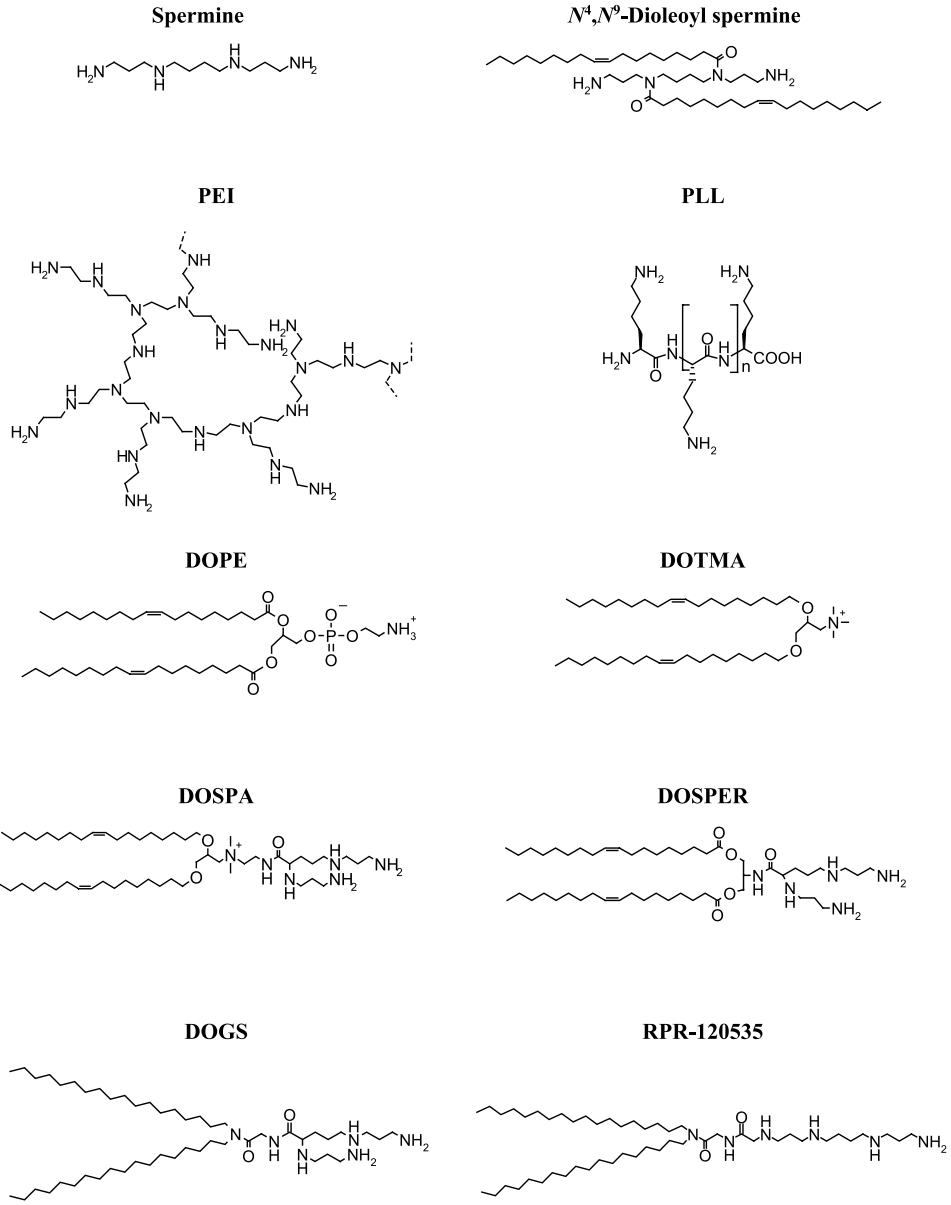

Fig. 2. Chemical structures of spermine, PEI, PLL, DOPE, DOTMA, and spermine based lipopolyamines.

fectamine reagents from Invitrogen (Life Technologies Gibco BRL) and cell cultures materials from Life Technologies (Paisley, Scotland).

Synthesis of N^4,N^9-Dioleoyl Spermine

Spermine was used as the starting material for the synthesis process (35), outlined in Fig. 3. Spermine was protected on the primary amino functional groups with ethyl trifluoroacetate (2.2 eq) in methanol and the reaction mixture was stirred for 18 h at 25°C. The solvent was evaporated to dryness *in vacuo* to form N^1,N^{12}-ditrifluoroacetyl-1,12-diamino-4,9-diazadodecane. Dicyclohexylcarbodiimide (DCC, 2.5 eq), 1-hydroxybenzotriazole (HOBt, 0.2 eq) and oleic acid (2.2 eq) were added to the diprotected spermine solution in CH_2Cl_2 and methanol (1:1). The solution was stirred for 18 h at 25°C. The solvent was evaporated to dryness *in vacuo*. The residue was dissolved in CH_2Cl_2 and the solution filtered and evaporated to dryness *in vacuo* to form N^4,N^9-dioleoyl-N^1,N^{12}-ditrifluoroacetyl-1,12-diamino-4,9-diazadodecane. For the removal of the di-trifluoroacetyl groups, the tetra-amide was dissolved in methanol and the pH of the solution was increased by saturating with ammonia gas, then it was left (18 h) and evaporated to dryness *in vacuo* to give a residue which was purified over silica gel (CH_2Cl_2-MeOH 5:3 v/v, then CH_2Cl_2-MeOH-conc. aq. NH_3 25:10:1 v/v/v) to afford N^4,N^9-dioleoyl spermine R_f 0.3 (CH_2Cl_2-MeOH-conc. aq. NH_3 25:10:1 v/v/v).

Amplification and Purification of Plasmid DNA (pEGFP)

DNA plasmid encoding enhanced green fluorescent protein (pEGFP) purchased from Clontech was transformed into *Escherichia coli* JM 109 bacterial strain (Promega). The

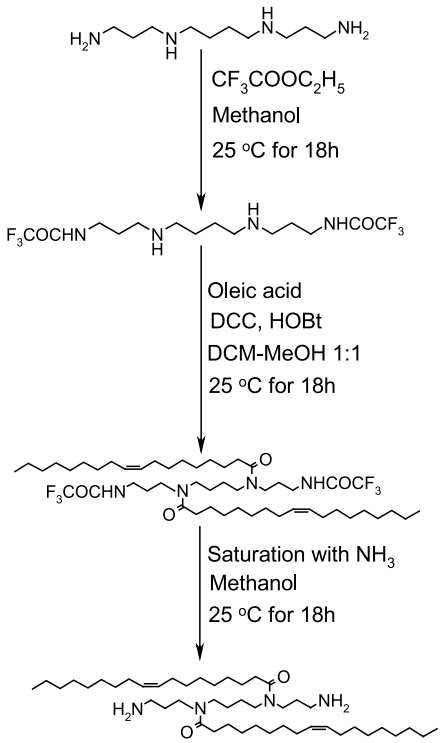

Fig. 3. Synthesis of N^4,N^9-dioleoyl spermine.

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% agarose) analysis.

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) (2) 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 micro-flea. 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 for each polyamine or lipopolyamine (spermine, poly-L-lysine (average molecular weight 9600 Da, PLL 9.6k), polyethylenimine (average molecular weight 2000 Da, PEI 2K), N^4,N^9-dioleoyl spermine, Lipofectin, and Lipofectamine) aliquots (5 μl, according to the ammonium/phosphate (+/−) charge ratio required) were then added to the stirring solution and the fluorescence measured after 1 min equilibration using Perkin-Elmer LS 50B luminescent spectrometer (λ_{excit} = 260 nm and λ_{emiss} = 600 nm with slit width 5 nm) while stirring using an electronic stirrer (Rank Bros. Ltd.) (36). The total polyamine 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.

Light Scattering Assay

DNA (60 μg, 60 μl of 1 mg/ml solution) was diluted to 3 ml with HEPES buffer (2 mM HEPES, 20 mM NaCl, 10 μM EDTA, pH 7.4) in a cuvette with a micro-flea, and the concentration determined spectroscopically (Milton Roy Spectronic 601 spectrometer, 1 cm path length, 3 ml cuvette). Then aliquots (5 μl, according to the ammonium/phosphate (+/−) charge ratio) of the tested polyamines were then added to the stirring solution and the absorbance (light scattering) at 320 nm was measured after 1 min stirring to allow the mixture to reach equilibrium. The increase in absorbance due to the scattered light was expressed as the percentage of relative maximum apparent absorbance (% rel. max. app. abs.) due to light scattering of the bound polyamine with DNA.

Cell Culture and Transfection Experiments

Two cell lines were used in the transfection experiment, a human primary skin fibroblast cells FEK4 (37) 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 foetal 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%).

For the transfection (gene delivery) and the resultant gene activity (transfection efficiency), FEK4 and HtTA cells were seeded at 1×10^5 cell/well in 6 well plates in 4 ml EMEM media with FCS for 24 h to reach a plate confluency of 50–60% on the day of transfection. The complex was prepared by mixing 2 μg of pEGFP with the cationic liposomes or lipopolyamine in Opti-MEM (serum free media, Gibco BRL) according to the charge ratio at room temperature for 30 min and then incubated with the cells for 4 h at 37°C in 5% CO_2. Then the cells were washed and cultured for further 44 h in growth medium at 37°C in 5% CO_2 before the assay.

Levels of enhanced green fluorescent protein (EGFP positive cells) in the transfected cells were detected and corrected for background fluorescence of the control cells using a fluorescence activated cell sorting (FACS) machine (Becton Dickinson FACS Vantage dual Laser Instrument, argon ion laser 488 nm). The transfection efficiency was calculated based on the percentage of the cells that expressed EGFP (positive cells) in the total number of cells.

Cytotoxicity (MTT) Assay of the Formed Lipoplexes

FEK4 and HtTA cells were seeded in 96 well plates at 8000 cell/well and incubated for 24 h at 37°C in 5% CO_2. N^4,N^9-Dioleoyl spermine complexed with pEGFP was added in the same way as the transfection protocol. After incubation for 44 h, the media was replaced with 90 μl of fresh media and

10 μl of sterile filtered MTT solution (Sigma-Aldrich, UK) (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 produced color was measured using a plate-reader (VERSAmax) at wavelength 570 nm. The % viability related to control wells containing cells without DNA and/or polymer and is calculated by (test absorbance/control absorbance) × 100 (38). The same protocol was applied in case of the commercially available reagents Lipofectin and Lipofectamine.

RESULTS

Synthesis of N^4, N^9-Dioleoyl Spermine

The synthesized N^4,N^9-dioleoyl spermine (Fig. 3) was homogenous on silica gel thin-layer chromatography and was fully characterized by ^{1}H-NMR (at 400 MHz) and ^{13}C-NMR and high-resolution accurate mass spectroscopy.

Ethidium Bromide Fluorescence Quenching Assay

To study the ability of N^4,N^9-dioleoyl spermine to condense calf thymus DNA and pEGFP as well as compared this effect with the effect of different known polycations spermine, PLL (39) and PEI (40) (Fig. 2) to condense DNA. Figure 4 shows the ability of the studied polyamines to displace EthBr from DNA. The binding ability was in the order PEI 2K > N^4,N^9-dioleoyl spermine > PLL 9.6K > spermine according to the charge ratio. The charge ratio was calculated according to the ammonium/phosphate (+/−) ratio for: spermine, 202.35 g/mole with four nitrogen atoms that can be protonated; PLL 9.6K, one positive charge/lysine monomer; N^4,N^9-dioleoyl spermine, with two positive charges. In the case of PEI 2K the charge ratio was calculated as 25% of the amino groups in the polymer that can be protonated, assuming that 43.1 g/mol is the repeating unit of PEI that contains one nitrogen atom (41,42). It seems to be that there is no agreement in the literature for the calculation of the PEI/DNA ratio. Although it is calculated as PEI nitrogen/DNA phosphate (40) not as charge ratio, it is reported that every third atom of PEI polymer is a protonatable nitrogen (40), but one in five of the protonatable nitrogens in PEI are protonated at pH 7 (43). However, Wagner (11) reported that one of every seven nitrogens within PEI polymer is protonated at pH 7. The polycations (PEI 2K and PLL 9.6K) are known for their ability to condense DNA efficiently at a relatively low charge ratio compared to spermine. On the other hand, the results revealed that N^4,N^9-dioleoyl spermine is able to condense DNA at a lower charge ratio than PLL 9.6K and spermine (Fig. 4), and produces a 50% fluorescence decrease at charge ratio 0.52. Also, Fig. 5 shows DNA condensation ability of N^4,N^9-dioleoyl spermine in comparison with the commercially available, cationic lipid, liposomal formulations Lipofectin and Lipofectamine. All three cationic lipid formulations have the ability to condense completely DNA through the displacement of EthBr leading to fluorescence quenching. At lower charge ratios N^4,N^9-dioleoyl spermine has better ability to suppress the fluorescence than Lipofectin and Lipofectamine. On studying the effect of N^4,N^9-dioleoyl spermine on the type of DNA (calf thymus DNA, and plasmid pEGFP), it was found that there is no significant variation in the condensation ability of the studied lipopolyamine on the type of DNA.

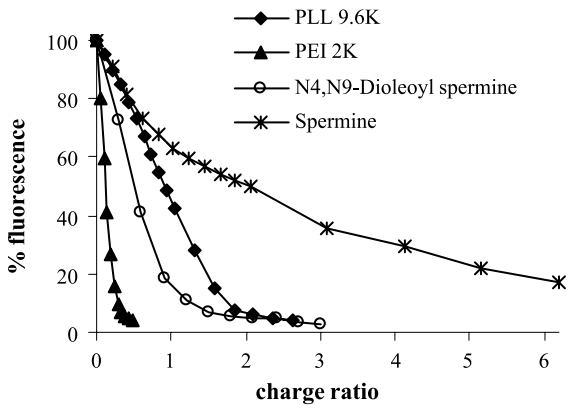

Fig. 4. Plot of EthBr displacement assay of calf thymus DNA complexed with PLL 9.6K, PEI 2K, N^4,N^9-dioleoyl spermine, and spermine.

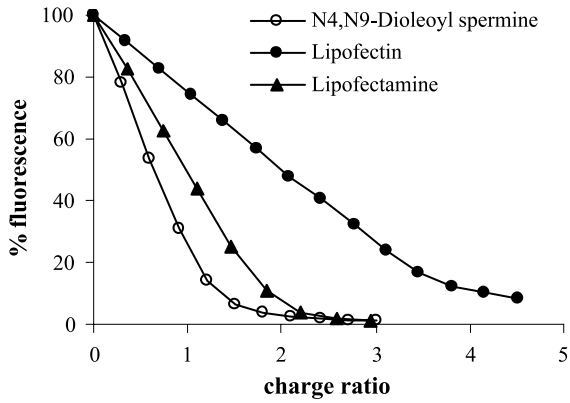

Fig. 5. Plot of EthBr displacement assay of calf thymus DNA complexed with N^4,N^9-dioleoyl spermine, Lipofectin, and Lipofectamine.

Light Scattering Assay

This experiment has been carried out to investigate the condensation of DNA by polyamines and the formation of particles (44). The apparent UV absorbance at 320 nm (where there is no DNA absorbance above 300 nm) was measured (28,45,46) showing light scattering. The results from Fig. 6 indicated the formation of particles upon interaction of spermine, PLL 9.6K and PEI 2K. In addition, Fig. 7 shows that the light scattering due to particle formation increases with the increase in the displaced EthBr and reaches the maximum at approximately the same charge ratio at which there is a maximum EthBr displacement, although the concentration of DNA used in light scattering experiments is ten times the concentration used in fluorescence quenching experiments which is related to the lack of sensitivity of light scattering experiment in comparison with fluorescence assay. Also, from light scattering results, there is a decrease in the % relative maximal apparent absorbance (% rel. max. app. abs.) after reaching the maximum absor-

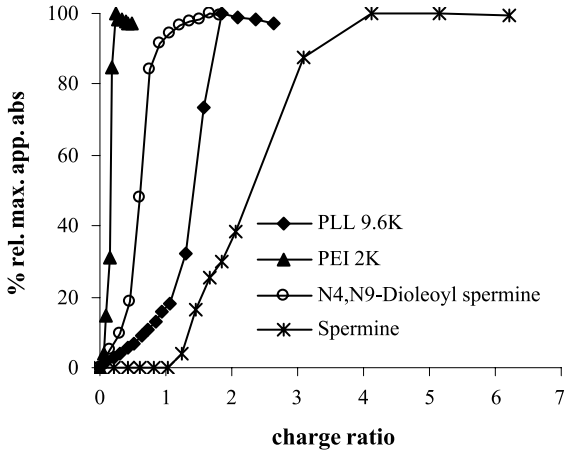

Fig. 6. Light scattering assay (% relative maximum apparent absorbance at λ = 320 nm) of calf thymus DNA complexed with PLL 9.6K, PEI 2K, N^4,N^9-dioleoyl spermine and spermine.

bance, which could be attributed to the formation of polyamine-DNA aggregates as reported by Gosule and Schellman (47).

Transfection Experiments

The transfection efficiency of pEGFP into FEK4 primary cell line and the cancer HtTA cells was studied using N^4,N^9-dioleoyl spermine and the commercially available reagents (Lipofectin and Lipofectamine). The transfection results of pEGFP into FEK4 indicated higher transfection ability of N^4,N^9-dioleoyl spermine (75%) and Lipofectamine (66%) formulations over Lipofectin (18%); there is no significant difference in the transfection activity between N^4,N^9-dioleoyl spermine and Lipofectamine (Fig. 8). On the other hand, both N^4,N^9-dioleoyl spermine and Lipofectamine formulations show a similar transfection activity in HtTA cells (about 70%), higher than Lipofectin (58%). N^4,N^9-Dioleoyl spermine transfects the cells best at charge ratio (+/−) 2.5 (5.54 μg/ml), Lipofectin at charge ratio 0.6 (5.0 μg/ml), while Lipofectamine transfect both cell lines at charge ratio 3.7 (10.0 μg/ml).

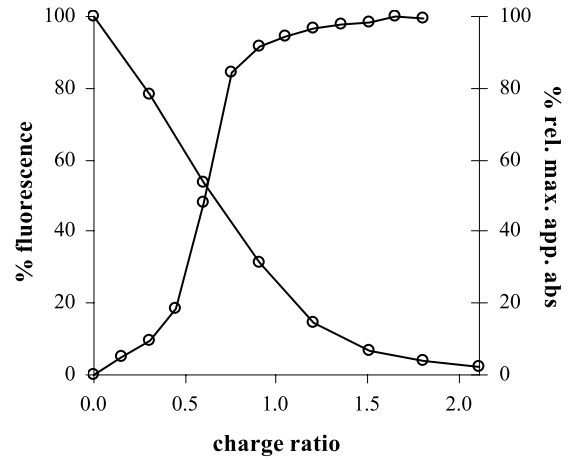

Fig. 7. Comparison of EthBr displacement and light scattering assays of calf thymus DNA with N^4,N^9-dioleoyl spermine.

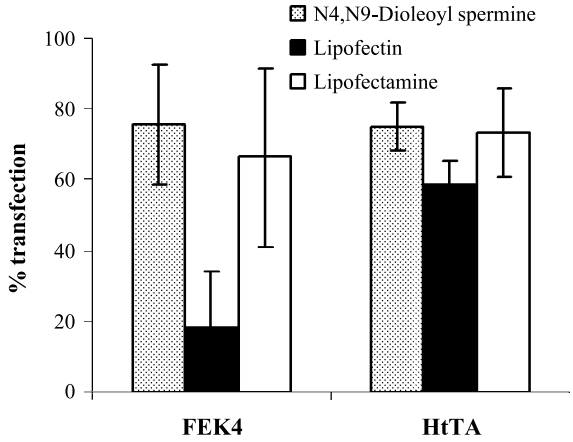

Fig. 8. Lipofection of FEK4 and HtTA cells transfected with pEGFP complexed with N^4,N^9-dioleoyl spermine, Lipofectin or Lipofectamine (at their respective N/P ratios for best transfection). The data represent 3 different experiments (3 replicates each) and the error bars represent the standard deviation.

In Vitro Cytotoxicity

The cytotoxicity of N^4,N^9-dioleoyl spermine was studied in FEK4 and HtTA cells using MTT assay (48) (Fig. 9). The IC_{50} (the concentration at which cell growth is inhibited by 50%) (42) values for the free polycation in FEK4 and HtTA

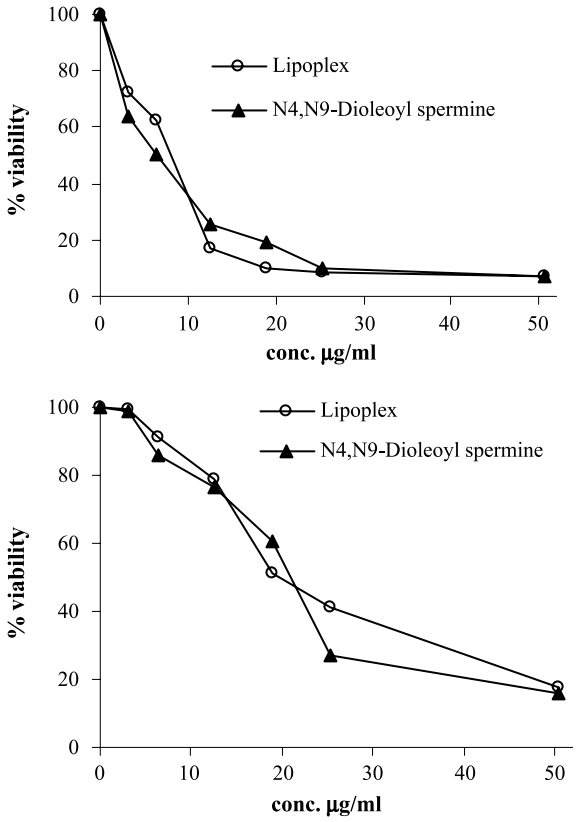

Fig. 9. Viability of HtTA cells (above) and FEK4 primary skin cells (below) after application of different concentrations of N^4,N^9-dioleoyl spermine either free or complexed with pEGFP.

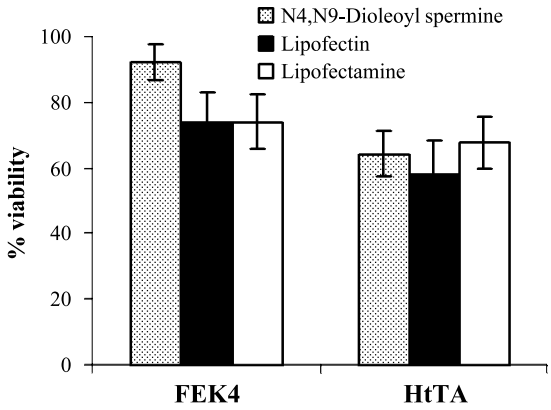

Fig. 10. Cytotoxicity effect of pEGFP (2 μg/ml) complexed with either N^4,N^9-dioleoyl spermine (5.54 μg/ml), Lipofectin (5 μg/ml) or Lipofectamine (10 μg/ml) in FEK4 and HtTA cells.

cells were 20.47 and 6.31 μg/ml respectively, and for the lipoplex were 19.82 and 8.35 μg/ml respectively. The results indicate that there is no significant difference in the toxic effect (IC_{50}) of the free polycation over the lipoplex in the case of HtTA cells and FEK4 cells for the studied lipopolyamines. N^4,N^9-Dioleoyl spermine show a significant difference in the % viability comparing to either Lipofectin and Lipofectamine in the primary skin cell line FEK4, but there is no difference in the case of the cancer cell line HtTA (Fig. 10). These results also revealed that N^4,N^9-dioleoyl spermine toxicity is higher (lower concentrations) in the case of HtTA cells more than with the primary cell line FEK4 which could be attributed to the ease of transfection of immortalized cancer cell lines over primary cell lines.

DISCUSSION

Non-viral delivery systems can be defined to include the use of plasmid DNA alone (so-called naked DNA) (49,50) as well as DNA complexed to synthetic carriers such as cationic lipids (51–54) or polymers (55). The use of an efficient carrier for nucleic acid delivery is considered to be a determinant factor for the successful application of gene therapy (56). This carrier is responsible for the complex process of gene delivery to the nucleus (57).

Ethidium bromide (EthBr) (2,7-diamino-10-ethyl-9-phenylphenanthridinium bromide, Fig. 11) is a cationic dye that displays a marked increase in the fluorescence upon binding with DNA and RNA through the intercalation between the EthBr phenanthridinium-moiety and adjacent base-pairs of DNA sequences (36,58). Within the prerequisites for delivery of DNA across intact cytoplasmic membrane are condensation and masking the negative charge of the phosphate backbone. Condensation of DNA occurs when about 90% of the charge on DNA is neutralized (16,47).

The ability of the cationic lipid N^4,N^9-dioleoyl spermine to compact DNA more efficiently than both spermine and the powerful condensing agent PLL a cationic polymer (9), (Fig. 4) shows the importance of the lipid moiety we have bound to the cationic polyamine in order to achieve improvements in its ability to condense DNA, cellular entry, and lowering the toxicity of the polyamine conjugate (11,59,60).

The formation of N^4,N^9-dioleoyl spermine-DNA lipoplex at lower charge ratio decreases the toxicity of the DNA delivering lipopolyamine. As the mammalian cell membrane is a semipermeable membrane formed of phospholipids bilayer that allows the transport of macromolecules by endocytosis, neutralization of the negative charges on the DNA by polycations will improve the delivery of DNA through the cell membrane because of the presence of negative charges on both DNA and cell membrane. Also, the positively charged lipid complex will mediate transfection by fusion with cell membranes (60,61). It was found that both the number of positive charges and their distribution on the surface of the molecule have profound effects on DNA condensation (28,62,63).

In addition, in a study on the transfection activity of cholesterol carbamate cationic lipids (28), Blagbrough and co-workers reported that the carbamate with a spermine polyamine moiety has the highest transfection activity with its ability to condense DNA efficiently. Our findings are in agreement with the literature, the cationic liposomal formulation Lipofectin, with its cationic moiety DOTMA containing one positively charged quaternary ammonium group, has lower ability to displace the EthBr from DNA than Lipofectamine formulation that contains DOSPA (Fig. 2) with its four positively charged nitrogens (15) and N^4,N^9-dioleoyl spermine with its two positively charged nitrogens. The higher ability of N^4,N^9-dioleoyl spermine (two positive charges) over Lipofectamine (four positive charges) in DNA condensation, though N^4,N^9-dioleoyl spermine has a lower number of positive charges/molecule, may be attributed to the distribution of the positive charges on the molecule allowing a higher affinity of the vector for DNA and leading to the efficient displacement of EthBr. Another variable is the liposomal formulation of Lipofectamine compared to the non-liposomal formulation of N^4,N^9-dioleoyl spermine.

The helper lipid DOPE which is the second component of the cationic liposomes is used to increase the transfection activity of the cationic liposome through its ability to destabilize lipid bilayers leading to endosomal destabilization with subsequent increase in the total cellular uptake of the delivered DNA (23,64). Lipospermines (Fig. 2) with their cationic headgroup sometimes form micelles (in the absence of DNA) rather than the bilayer produced by the small cationic quaternary ammonium headgroup of DOTMA (Lipofectin formulation) (43,65). N^4,N^9-Dioleoyl spermine combines in its structure the two oleoyl chains that have the characteristics of the fusogenic lipid DOPE and the cationic polyamine spermine (Fig. 2). Thus, DNA is condensed by the

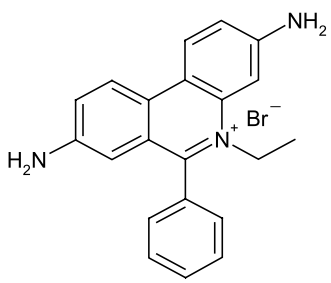

Fig. 11. Structure of ethidium bromide.

two primary amines and coated by the dioleoyl lipophilic moiety. The DNA condensation results of N^4,N^9-dioleoyl spermine (Fig. 5) show a higher efficiency than either liposomal formulation. The transfection results reveal higher transfection ability (levels of expression) of N^4,N^9-dioleoyl spermine compared to Lipofectin (Fig. 8) in FEK4 and HtTA cells. These results indicate the roles of both number and distribution of positive charges along the polyamine backbone on the ability of the compound to condense DNA. On the other hand, there is no significant difference in the transfection activity between N^4,N^9-dioleoyl spermine and Lipofectamine in both FEK4 and HtTA cell lines, which indicates the importance of lipid coating over the DNA molecule on both the condensation and cellular delivery of DNA in case of the liposomal and non liposomal formulations (65). N^4,N^9-Dioleoyl spermine achieved high levels of transfection in both a cancer cell line and a primary skin cell line (73%), which indicates the ability of this vector to deliver DNA. Cell viability results revealed improved FEK4 viability more than the cancer cells HtTA (Fig. 9). Also, N^4,N^9-dioleoyl spermine showed a significant improvement in primary FEK4 cell viability over the liposomal formulatios (Fig. 10), but no significant difference in HtTA cells. Previous studies on Lipofectin and Lipofectamine on different cell lines showed the cytotoxic effects of these liposomal formulations (66–70). In conclusion, a lipopolyamine vector has been developed for DNA delivery. This new non-liposomal formulation has the ability to transfect primary skin cells more efficiently than the commercially available liposomal Lipofectin formulation.

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