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Transfection with fluorinated lipoplexes based on fluorinated analogues of DOTMA, DMRIE and DPPES

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Abstract

Fluorinated double-chain (poly)cationic lipids (one or both of these chains being ended by a highly fluorinated tail) which are close analogues of DOTMA, DMRIE or DPPES were designed as synthetic vectors for gene delivery. For *N*/*P* ratios (*N* = number of amine functions of the lipid; *P* = number of DNA phosphates) from 0.8 to 5, these fluorinated cationic lipids condensed DNA, with or without the use of DOPE, to form fluorinated lipoplexes. No specific cell toxicity was evidenced for these new fluorinated lipoplexes. The efficiency of some of the fluorinated lipoplexes to transfect lung epithelial A549 cells was comparable to that of the first generation of fluorinated lipoplexes made from fluorinated analogues of DOGS (Transfectam) [Bioconjug. Chem. 12 (2001) 114]. These results, combined with the higher in vivo transfection potential found for fluorinated lipoplexes than for conventional lipoplexes or PEI polyplexes [J. Gene Med. 3 (2001) 109], confirm that fluorinated lipoplexes are very promising gene transfer systems.
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1. Introduction

Synthetic nonviral gene (or DNA) transfer systems into cells have become very promising novel forms of molecular medicine. Although at present, the expression levels obtained with synthetic gene transfer vectors based on (poly)cationic lipids, liposomes, or polymers (e.g. lipoplexes or polyplexes, respectively [1–5]) are transient and lower than with viral vectors, these systems present several advantages including low-cost and large-scale production, safety, and capacity to deliver large gene fragments. However, there is still a need for the development of lipoplexes or polyplexes with improved and original properties.

Fluorinated lipoplexes, that is, lipoplexes formulated with highly fluorinated lipids, are such promising transfection systems. Indeed, highly fluorinated analogues of DOGS (Transfectam®) [6,7] and highly fluorinated helper lipids, analogues of dioleoylphosphatidylethanolamine (DOPE) [8], were recently shown to lead to lipoplexes that exhibited a higher in vitro and in vivo transfection potential than conventional lipoplexes [6–8] or even than PEI polyplexes [8]. The remarkable transfection potency of the fluorinated lipoplexes (resulting from the formulation of DNA with fluorinated cationic lipids) was attributed to the unique lipophobic and hydrophobic character of fluorinated chains, thus preventing DNA from interactions with lipophilic and hydrophilic biocompounds, and from degradation [7].

We report herein on the synthesis of a series of new highly fluorinated lipo(poly)amines (see structures in Fig. 1) and on their abilities of compacting and transferring DNA into cells. These new compounds are close analogues of DOTMA [9], DMRIE [10] and DPPES [11] which are also very frequently used as synthetic gene transfer agents. The present study was aimed at extending the library of fluorinated cationic lipids that enable gene transfer. It was also aimed at examining the transfection efficiency of the fluorinated lipoplexes formulated with these new fluorinated cationic lipids as compared with the first generation of fluorinated DOGS analogues and with conventional cationic lipids such as DOGS (see structures in Fig. 1).

The framework of the fluorinated analogues of DOTMA (e.g. DF4C11-TMA) and DMRIE (e.g. DF4C11-DMHEA) consists of a hydrophobic part, which includes two

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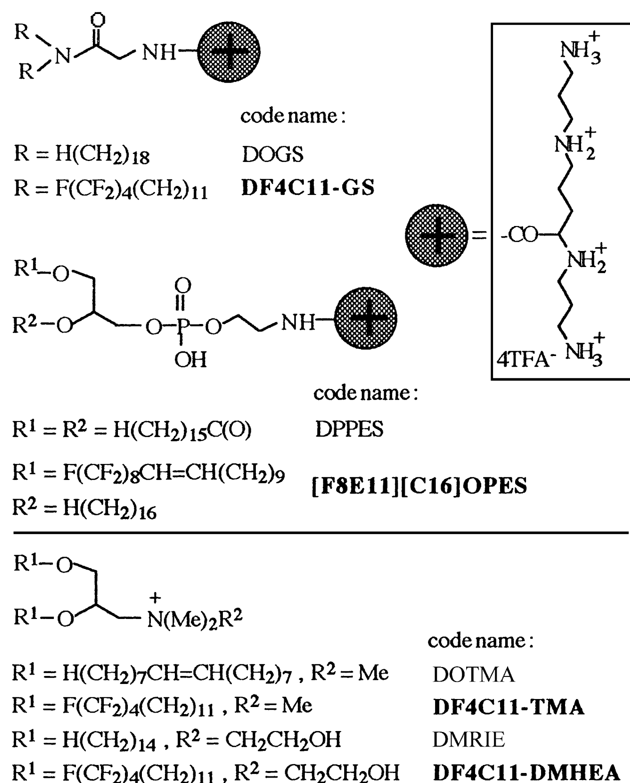


Fig. 1. Chemical structure of the fluorinated and reference cationic lipids used or mentioned in this study: DF4C11-TMA, DF4C11-DMHEA, [F8E11][C16]OPES and DF4C11-GS [6] are fluorinated analogues of DOTMA [9], DMRIE [10], DPPES [11] and DOGS [2], respectively.

$F(CF_2)_4(CH_2)_{11}$ (F4C11) chains. These chains were shown in the DOGS series to lead to the most efficient gene transfer element (i.e. DF4C11-GS) [6]. [F8E11][C16]OPES is a fluorinated analogue of DPPES, and contains a mixed fluorocarbon/hydrocarbon hydrophobic framework. This compound derives from the fluorinated [F8E11][C16]OPE phosphoethanolamine which was found, in vitro and in vivo, to be a more efficient helper lipid than DOPE [8]. As the use of DOPE as co-lipid was found to enhance the transfection activity of most (poly)cationic lipids (including the fluorinated analogues of DOGS [6]), we report also on the transfection efficacy of DF4C11-TMA, DF4C11-DMHEA and [F8E11][C16]OPES used in conjunction with DOPE. The lipoplexes formulated with these new fluorinated cationic lipids were further compared with the most efficient formulations made from the fluorinated DOGS analogue, that is, DF4C11-GS, and with DOGS [6].

2. Materials and methods

2.1. General experimental and analytical conditions

Most of the reactions were performed in anhydrous solvents under dry and oxygen-free nitrogen. Anhydrous solvents were prepared by standard methods. The purifications by column chromatography were carried out using silica gel 60 (Merck, 70–230 mesh) and chloroform ($CHCl_3$), dichloromethane (CH_2Cl_2), methanol (MeOH) or mixtures thereof as indicated. Unless noted otherwise, the ratios describing the composition of solvent mixtures represent relative volume. Advancing of the reaction was followed by thin layer chromatography (TLC) on silica plates F_{254} (Merck). The following developing systems were used: UV light, $KMnO_4$, H_2SO_4/EtOH, Dragendorff reagent (Sigma), ninhydrin reagent (Sigma).

11-(*F*-butyl)undecyl toluene-4-sulfonate and tetra-*N*,*N*′,*N*″,*N*‴-(*tert*-butyloxycarbonyl)-spermine-5-carboxylic acid were synthesised according to Refs. [12] and [6], respectively. *Rac*-3-[11-(*F*-octyl)undec-10-enyl]-2-(hexadecyl)glycero-1-phosphoethanolamine, [F8E11][C16]OPE, was from our laboratory [8]. DOPE was purchased from Sigma. All other organic chemicals were from Aldrich or Fluka.

^{1}H, ^{13}C and ^{19}F NMR spectra were recorded at 200, 50.3, and 188.3 MHz, respectively, on a Bruker AC-200. Chemical shifts were measured relative to $CHCl_3$ (δ 7.27 ppm) or CH_3OD (δ 3.35 ppm) for ^{1}H, relative to $CDCl_3$ (δ 76.9 ppm) for ^{13}C and expressed indirectly in relation to TMS, and relative to CCl_3F as internal reference for ^{19}F. The following abbreviations are used to describe the signal multiplicities: s (singlet), d (doublet), t (triplet), q (quadruplet), and m (multiplet). Chemical shifts are expressed in parts per million and listed as follows: shift in parts per million (multiplicity, coupling, integration and attribution). Electrospray ionisation mass spectrometry (ESI-MS) in positive mode was performed on a Finnigan MAT LCQ equipped with an atmospheric pressure ionisation (API) source. Elemental analyses were carried out by the Service Central de Microanalyses du CNRS.

2.2. Synthesis of the fluorinated cationic lipids

2.2.1. N-[1-(2,3-di-11-(F-butyl)undecyloxy)propyl]-N,N,N-trimethylammonium iodide, DF4C11-TMA

A suspension of NaH (360 mg, 15.0 mmol) and 0.6 g of 3-dimethylamino-1,2-propanediol in 50 ml of toluene was stirred during 30 min at 50 °C. Then, 6.0 g (11 mmol) of 11-(*F*-butyl)undecyl toluene-4-sulfonate was added and the resulting mixture was heated at reflux during 24 h. After hydrolysis, the aqueous phase was extracted with $CHCl_3$. The organic phase was then washed with water, dried over Na_2SO_4 and evaporated. Flash chromatography of the residue over a silica gel column (180 g, $CHCl_3$ then $CHCl_3$/MeOH: 99/1: v/v) afforded 2.1 g (2.4 mmol, 58%) of *N*-[1-(2,3-di-11-(*F*-butyl)undecyloxy)propyl]-*N*,*N*-dimethylamine as a colourless oil. [TLC ($CHCl_3$/MeOH 99/1: 9/1: v/v; ninhydrin): Rf = 0.35. ^{1}H NMR ($CDCl_3$): δ 1.18–1.42 (s large, 28H, $CF_2(CH_2)_2(C\underline{H}_2)_7$); 1.42–1.65 (m, 8H, $CF_2CH_2C\underline{H}_2$, $C\underline{H}_2CH_2O$); 1.99 (tt, $^3J = 7.5$ Hz, $^3J_{HF} = 19.1$ Hz, 4H, CF_2CH_2); 2.24 (s, 6H, CH_3); 2.34–2.42 (m, 2H, CH_2N); 3.34–3.64 (m, 7H, CH_2O, CHO). ^{13}C NMR

($CDCl_3$): δ 20.0 (t, $^3J_{CF}$ = 4 Hz, $CF_2CH_2\underline{C}H_2$); 26.2 ($\underline{C}H_2(CH_2)_2O$); 29.0, 29.1, 29.3, 29.4, 29.5, 29.6 ($CF_2(CH_2)_2(\underline{C}H_2)_6$); 30.2 ($\underline{C}H_2CH_2O$); 30.7 (t, $^2J_{CF}$ = 22 Hz, $CF_2\underline{C}H_2$); 46.0 (CH_3); 61.0 (CH_2N); 70.1 ($O\underline{C}H_2CH$); 71.5, 72.0 (CH_2O); 77.2 (CH). ^{19}F NMR ($CDCl_3$): δ − 81.4 (3F, CF_3); − 115.2 (2F, CF_2CH_2); − 125.0 (2F,$CF_3CF_2C\underline{F}_2$); − 126.5 (2F, $CF_3C\underline{F}_2$)]. Then, 130 mg (0.15 mmol) of this dimethylamine derivative in 3 ml of MeI was stirred overnight at room temperature. After evaporation of excess MeI and triturating with diethylether, 150 mg of DF4C11-TMA (0.15 mmol, 100%) as a white powder was obtained. ^{1}H NMR ($CDCl_3$): δ 1.15–1.47 (s large, 28H, $CF_2(CH_2)_2(C\underline{H}_2)_7$); 1.47–1.70 (m, 8H, $CF_2CH_2C\underline{H}_2$, $C\underline{H}_2CH_2O$); 2.05 (tt, ^3J = 7.5 Hz, $^3J_{HF}$ = 19.1 Hz, 4H, CF_2CH_2); 3.34–3.73, 3.95–4.16 (m, 15H, $C\underline{H}_2N$, $C\underline{H}_2O$, $C\underline{H}O$, CH_3). ^{13}C NMR ($CDCl_3$): δ 20.1 (t, $^3J_{CF}$ = 4 Hz, $CF_2CH_2\underline{C}H_2$); 26.1, 26.2 ($\underline{C}H_2(CH_2)_2O$); 29.1, 29.2, 29.4, 29.5 ($CF_2(CH_2)_2(\underline{C}H_2)_6$); 30.0 ($\underline{C}H_2CH_2O$); 30.8 (t, $^2J_{CF}$ = 22 Hz, $CF_2\underline{C}H_2$); 55.2 (CH_3); 68.1, 68.3, 69.3, 72.1, 73.5 (CH_2O, CH_2N); 73.5 (CH). ^{19}F NMR($CDCl_3$): identical to that of *N*-[1-(2,3-di-11-(*F*-butyl)undecyloxy)propyl]-*N*,*N*-dimethylamine. ESI-MS: m/z = 878.8, in agreement with the calculated mass for $[M]^+ = C_{36}H_{58}F_{18}NO_2$. Anal. calc. for ($C_{36}H_{58}F_{18}INO_2$) C, 42.99; H, 5.81; N, 1.39. Found: C, 43.28; H, 6.01; N, 1.53.

2.2.2. Synthesis of N-[1-(2,3-di-11-(F-butyl)undecyloxy)-propyl]-N-[2-hydroxyethyl]-N,N-dimethylammonium bromide, DF4C11-DMHEA

A mixture of 1.3 g (1.5 mmol) of *N*-[1-(2,3-di-11-(*F*-butyl)undecyloxy)propyl]-*N*,*N*-dimethylamine, 0.5 ml NEt_3 (3.6 mmol), 4 ml of 2-bromoethanol (56 mmol) in 50 ml of THF was stirred during 12 h at room temperature, then during 4 h at reflux. After filtration and evaporation, the residue was dissolved in CH_2Cl_2. The organic phase was extracted with water, dried over Na_2SO_4. Flash chromatography of the residue obtained after evaporation over a silica gel column (60 g, CH_2Cl_2 then CH_2Cl_2/MeOH: 95/5: v/v) led to 1.2 g (1.2 mmol, 80%) of DF4C11-DMHEA as a white powder.

TLC (CH_2Cl_2/MeOH: 9/1: v/v; H_2SO_4, Dragendorff): Rf = 0.40. ^{1}H NMR ($CDCl_3/CD_3OD$): δ 1.12–1.44 (s large, 28H, $CF_2(CH_2)_2(C\underline{H}_2)_7$); 1.44–1.70 (m, 8H, $CF_2CH_2C\underline{H}_2$, $C\underline{H}_2CH_2O$); 1.99 (tt, ^3J = 7.5 Hz, $^3J_{HF}$ = 19.1 Hz, 4H, CF_2CH_2); 3.18 (s, 6H, CH_3); 3.30–3.73, 3.84–4.05 (m, 13H, CH_2N, CH_2O, CHO). ^{13}C NMR ($CDCl_3/CD_3OD$): δ 20.6 (t, $^3J_{CF}$ = 4 Hz, $CF_2CH_2\underline{C}H_2$); 26.6, 26.7 ($\underline{C}H_2(CH_2)_2O$); 29.6, 29.7, 29.8, 29.9, 30.0 ($CF_2(CH_2)_2(\underline{C}H_2)_6$); 30.6 ($\underline{C}H_2CH_2O$); 31.3 (t, $^2J_{CF}$ = 2 Hz, $CF_2\underline{C}H_2$); 53.4, 53.5 (CH_3); 56.5, 67.8, 69.5, 69.9, 72.5 (CH_2O, CH_2N); 73.8 (CH). ^{19}F NMR ($CDCl_3/CD_3OD$): identical to that of *N*-[1-(2,3-di-11-(*F*-butyl)undecyloxy)propyl]-*N*,*N*-dimethylamine. ESI-MS: m/z = 908.8, in agreement with the calculated mass for $[M]^+ = C_{37}H_{60}F_{18}NO_3$. Anal. calc. for ($C_{37}H_{60}BrF_{18}NO_3$) C, 44.95; H, 6.12; N, 1.42. Found: C, 45.65; H, 6.25; N, 1.49.

2.2.3. Synthesis of N′-(rac-1-[11-(F-octyl)undec-10-enyl]-2-(hexadecyl)glycero-3-phosphoethanoyl)-sperminecarboxamide, [F8E11][C16]OPES (as its tetra trifluoroacetate ammonium salt)

A solution of 60 mg (0.09 mmol) tetrabutyloxycarbonyl-spermine carboxylic acid [6], 19 mg (0.095 mmol) of *N*-hydroxysuccinimide (HOSu) and 11 mg (0.095 mmol) of dicyclohexylcarbodiimide (DCC) in 2 ml of CH_2Cl_2 was stirred at room temperature during 12 h. The mixture was cooled to 0 °C and filtrated. Then, 100 mg (0.083 mmol) of [F811][C16]OPE (*F*-PE) and 40 μl (0.30 mmol) of NEt_3 were added and the resulting mixture was stirred at room temperature during 12 h. The organic phase was washed with water, dried over Na_2SO_4. Flash chromatography of the residue obtained after evaporation over a silica gel column (10 g, CH_2Cl_2/MeOH 99/1 to 9/1: v/v) led to 70 mg (0.043 mmol, 53%) of *N′*-(*rac*-1-[11-(*F*-octyl)undec-10-enyl]-2-(hexadecyl)glycero-3-phosphoethanoyl)-tetrabutoxycarbonylspermine-carboxamide, [F8E11][C16]OPES(Boc) as a colourless oil. TLC (CH_2Cl_2/MeOH: 9/1: v/v; ninhydrin): Rf = 0.48. ^{1}H NMR ($CDCl_3$): δ 0.89 (t, ^3J = 6.1 Hz, 3H, CH_3); 1.08–1.38 (s large, 38H, $CH_3(C\underline{H}_2)_{13}$, $(C\underline{H}_2)_6(CH_2)_2O$); 1.38–1.88 (m, 48H, $C\underline{H}_2CH_2O$, CH_3 (Boc), $C\underline{H}_2CH_2N(H)Boc$, $CHC\underline{H}_2C\underline{H}_2$); 2.11–2.29 (m, 2H, $CH{=}CHC\underline{H}_2$); 2.87–3.35 (m, 10H, CH_2NBoc, $CH_2N(H)Boc$); 3.35–3.71 (m, 11H, $C\underline{H}_2N(H)C(O)$, CH, $C\underline{H}_2OC\underline{H}_2$, $C\underline{H}_2OC\underline{H}$); 3.71–4.12 (m, 4H, $CHC\underline{H}_2OP$, $POC\underline{H}_2CH_2N$); 5.46–5.73 (m, 1H, $CF_2C\underline{H}{=}CH$); 6.27–6.52 (m, 1H, $CF_2CH{=}C\underline{H}$). ^{13}C NMR ($CDCl_3$): δ 14.0 (CH_3); 22.7 ($CH_3\underline{C}H_2$); 26.2 ($\underline{C}H_2CH_2CH_2O$); 28.4 ($CH_3$ (Boc)); 28.0, 29.1, 29.4, 29.6, 29.8, 30.2 ($\underline{C}H_2CH_2O$, $CH_3(CH_2)_2(\underline{C}H_2)_{10}$, $CH{=}CHCH_2(\underline{C}H_2)_5$); 31.9, 32.0 ($CH_3CH_2\underline{C}H_2$, $=CH\underline{C}H_2$); 40.2 ($OCH_2\underline{C}H_2N$); 64.1 (d, $^2J_{CP}$ = 5 Hz, $PO\underline{C}H_2CH_2N$); 65.2 (d, $^2J_{CP}$ = 5 Hz, $CH\underline{C}H_2OP$); 70.6, 71.7 ($\underline{C}H_2OCH_2$, $\underline{C}H_2OCH$); 77.7 (d, $^3J_{CP}$ = 8 Hz, $\underline{C}HO$); 78.6, 79.5, 80.8 ($\underline{C}(CH_3)_3$); 116.7 (t, $^2J_{CF}$ = 23 Hz, $CF_2\underline{C}H{=}CH$); 143.0 (t, $^3J_{CF}$ = 9 Hz, $CF_2CH{=}\underline{C}H$ *trans*); 156.5 (C(O)); 171.5 ($N\underline{C}(O)CH$). The spermine CH_2 and CH resonances appear as large signals between 37 and 47 ppm. ^{19}F NMR ($CDCl_3$): δ − 81.2 (3F, CF_3); − 106.8 (0.2F, CF_2CH *cis*); − 111.6 (1.8F, CF_2CH *trans*); − 121.9, − 122.4, − 123.2, − 123.9 (2F, 4F, 2F, 2F, $CF_3CF_2(CF_2)_5$); − 126.6 (2F, CF_3CF_2). ^{31}P {^{1}H} NMR ($CDCl_3$): δ − 2.45 (s).

The Boc-deprotection was quantitatively achieved by dissolving and keeping [F8E11][C16]OPES(Boc) in a large excess of TFA at room temperature for 1 h. The excess TFA was removed by co-evaporation in the presence of cyclohexane. Then, the product was triturated in CH_2Cl_2 to give the desired [F8E11][C16]OPES lipospermine, as its tetra-TFA ammonium salt.

^{1}H NMR (CD_3OD): δ 0.89 (t, ^3J = 6.1 Hz, 3H, CH_3); 1.17–1.71 (s large, 42H, $CH_3(C\underline{H}_2)_{14}$, $(C\underline{H}_2)_7CH_2O$); 1.71–2.38 (m, 10 H, $=CHC\underline{H}_2$, $CHC\underline{H}_2C\underline{H}_2$, $C\underline{H}_2CH_2N^+$); 2.89–3.27 (m, 10H, CH_2N^+); 3.40–3.72 (m, 9H, $C\underline{H}_2O$, $C\underline{H}O$, $C\underline{H}_2N(H)C(O)$); 3.71–4.12 (m, 4H, $CHC\underline{H}_2OP$, $POC\underline{H}_2CH_2$, $C(O)C\underline{H}$); 5.46–5.73 (m, 1H, $CF_2C\underline{H}{=}CH$); 6.27–

6.52 (m, 1H, $CF_2CH{=}C\underline{H}$). ^{13}C NMR (CD_3OD): δ 14.4 (CH_3); 22.7, 28.5 ($CHC\underline{H}_2C\underline{H}_2$); 23.7 ($CH_3\underline{C}H_2$); 27.3 ($\underline{C}H_2CH_2CH_2O$); 25.4 ($\underline{C}H_2CH_2N^+$); 29.2, 30.1, 30.2, 30.3, 30.4, 30.5, 30.6, 30.7, 30.8 ($\underline{C}H_2CH_2O$, $CH_3(CH_2)_2(\underline{C}H_2)_{10}$, $CH{=}CHCH_2(\underline{C}H_2)_5$); 33.0, 33.1 ($CH_3CH_2\underline{C}H_2$, $=CHCH_2$); 37.8, 37.9, 45.0, 45.7 (CH_2N^+); 41.5 (d, $^3J_{CP}=6$ Hz, $\underline{C}H_2N(H)C(O)$); 61.5 ($C(O)\underline{C}H$); 64.9, 66.5 ($CHC\underline{H}_2OP$, $PO\underline{C}H_2CH_2$); 71.6, 71.7, 72.7 ($\underline{C}H_2O\underline{C}H_2$, $\underline{C}H_2OCH$); 79.0 (d, $^3J_{CP}=8$ Hz, $\underline{C}HCH_2OP$); 117.3 (t, $^2J_{CF}=23$ Hz, $CF_2\underline{C}H{=}CH$); 145.4 (t, $^3J_{CF}=9$ Hz, $CF_2CH{=}\underline{C}H$); 168.4 ($C(O)N(H)$). ^{19}F NMR (CD_3OD): δ −76.0 (13.5F, $CF_3C(O)O$); −81.3 (3F, CF_3); −106.8 (0.2F, CF_2CH *cis*); −111.7 (1.8F, CF_2CH *trans*); −121.9, −122.5, −123.4, −123.9 (2F, 4F, 2F, 2F, $CF_3CF_2(C\underline{F}_2)_5$); −126.7 (2F, $CF_3C\underline{F}_2$). ^{31}P {^{1}H} NMR (CD_3OD): δ −2.32 (s). ESI-MS: m/z = 1237.2, in agreement with the calculated mass for $[M+Na]^+ = C_{49}H_{90}F_{17}N_5O_7P + Na^+$.

2.3. Preparation of DNA complexes

The plasmid pTG11033 (pCMV-intronHMG-luciferase-SV40pA), used for the preparation of the DNA complexes and for transfection assays, was produced by Transgène (Strasbourg, France). pTG11033 is a plasmid of 9572 bp. The endotoxin content of the plasmid preparation was checked using a Limulus Amebocyte Lysat kit (Biogenic, France). This value was below 1 endotoxin unit/mg of plasmid, hence below the 5 e.u./mg of DNA recommended for in vivo protocols. The quantities of compound used were calculated according to the desired DNA concentration of 0.1 mg/ml, the *N*/*P* ratio, the molar weight and the number of positive charges in the selected cationic lipid (CL). The *N*/*P* ratio of 5, for example, corresponds to the molar amount of CL necessary to have a ratio of five amino group nitrogens (for 1 mol CL) per one phosphate in the DNA (330 Da mean Mw), as described elsewhere [1,13,14]. The CL/DNA complex is formulated by adding a desired volume of the CL preparation at a concentration of 10 mg/ml (in 20 mM HEPES buffer, pH 7.5) to the desired volume of DNA solution to reach desired DNA concentration (e.g. 0.1 mg/ml). Thus, for the preparation of the [F8E11][C16]OPES/DNA complex at *N*/*P* ratio 5 and 0.1 mg/ml DNA, 50 μl of a [F8E11][C16]OPES solution (10 mg/ml in EtOH) was transferred to a borosilicate glass tube (16 × 100 mm). The solvent was evaporated in a Rotavap evaporation system (45 °C, 30 pm, 0.2 bar, 40 min). Fifty microliters of HEPES 20 mM, pH 7.5 was added to the film obtained. The preparation was vortexed for 12 h. Then, 47.9 μl of this preparation was added to 952.1 μl DNA solution [100 μl DNA (1 mg/ml) diluted with 852.1 μl HEPES buffer]. This preparation was vortexed for 10 s and was used within 1 h for the particle size measurements and the in vitro transfection experiments.

When the composition further comprises DOPE, the desired volume of 10 mg/ml chloroform solution (to get 1:1 CL/DOPE, mol/mol) was added to the volume of CL solution (10 mg/ml in EtOH) and then the mixture was transferred to a borosilicate glass tube. The following steps were as described above.

2.4. Measurement of the size of the lipoplexes

The sample was diluted with HEPES 20 mM in the measurement tube and homogenised and the average sizes were measured by photon correlation spectroscopy using a Coulter N4Plus particle size analyser, as described elsewhere [5,6]. The formulations and analyses were reproduced twice.

2.5. Agarose gel electrophoresis

Each sample was analysed and plasmid integrity in each sample was confirmed by electrophoresis after decomplexing the lipoplexes with sodium dodecyl sulfate, following the procedures described elsewhere [5,6].

2.6. In vitro transfection of A549 cells

The same transfection protocol as that described for the fluorinated analogues of DOGS was used [6]. Briefly, 24 h before transfection, A549 cells (epithelial cells derived from human pulmonary carcinoma) were grown in Dulbecco's modified Eagle culture medium (DMEM) (GIBCO-BRL, Life Technologies, Cergy Pontoise, France), containing 10% foetal calf serum, FCS (Sigma, Saint Quentin Fallavier, France), in 96-well plates (2×10^4 cells per well), in a wet (37 °C) and 5% CO_2/95% air atmosphere. Volume of DNA/CL:DOPE (1:*x*, *x* = 0, 1 mol) lipoplex (10, 5 and 1 μl) was diluted to 100 μl in DMEM supplemented with 10% FCS to obtain various amounts of DNA (1, 0.5 and 0.1 μg, respectively) in the preparation. The culture medium was removed and replaced by 100 μl of DMEM supplemented with 10% FCS and containing the desired amount of DNA. After 4 and 24 h, 50 and 100 μl of DMEM supplemented with 30% and 10% FCS, respectively, were added. After transfection (48 h), the culture medium was discarded and the cells were washed twice with 100 μl of PBS and then lysed with 50 μl of lysis buffer (Promega, Charbonnières, France). The lysates were frozen at −40 °C awaiting analysis of luciferase activity. These measurements were done for 10 s on 10 μl of the lysis mixture in a Berthold LB96P luminometer in dynamic mode, using the "Luciferase" determination system (Promega) in 96-well plates. The total protein concentration per well was determined using conventional techniques (BCA test, Pierce, Montluçon, France). For cells grown in the absence of lipoplexes, a well contains around 30–50 μg of proteins. The percentage of cell viability of the lipoplexes was calculated as the ratio of the total protein amount per well of the transfected cells relative to that measured for untreated cells × 100%. The given means ± S.E.M. were calculated from two or three repetitions in two independent experiments.

2.7. Statistical analyses

Statistical tests were performed with STATGRAPHICS Plus5.0® software. Analysis of variance (ANOVA) was run on the logarithmic transformation of transfection levels [Log10(femtograms luciferase/milligram protein)] and on the cell viability to fit normal distributions of the data.

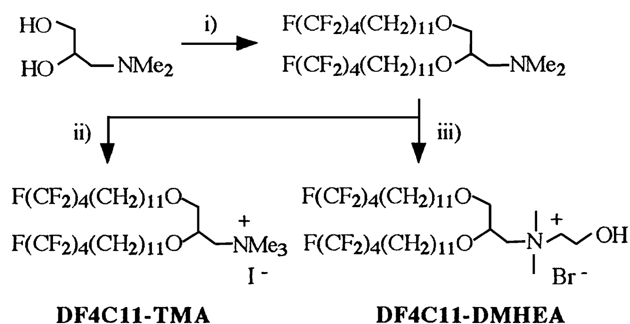
For the fluorinated DF4C11-TMA, DF4C11-DMHEA and [F8E11][C16]OPES lipids, three factors, that is, DOPE (added or not), DNA amount/well (0.1, 0.5 and 1 μg/well) and *N*/*P* ratio (5, 2.5, 1.25 and 0.8), were analysed as source of the variation of cell viability percentages and of logarithmic transformation of the transfection levels. When two- or three-way interactions ($p < 0.05$) between factors were found to be statistically significant, multiple comparison procedure [Tukey's Honestly Significant Difference (HSD)] was used to isolate which factor levels differed from the others.

To compare the transfection efficiencies and the effects on cell viability of these new fluorinated lipids to those of DF4C11-GS and DOGS, a multiple-sample comparison procedure was run. The method being used to discriminate among the means was HSD procedure.

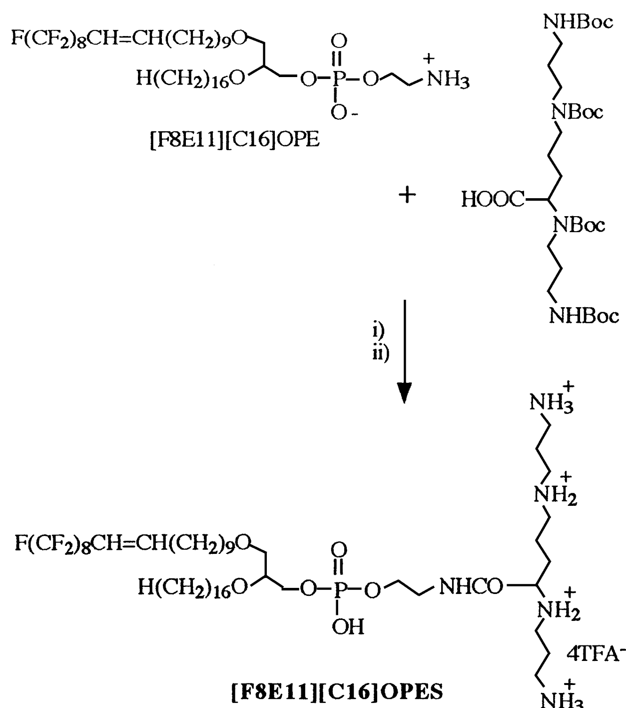
3. Results and discussion

3.1. Synthesis

The syntheses of the fluorinated (poly)cationic lipids DF4C11-TMA, DF4C11-DMHEA and [F8E11][C16]OPES were performed using the procedures described for their respective hydrocarbon DOTMA [9], DMRIE [10] and DPPES [11] analogues and are shown in Schemes 1 and 2. Their specificity lies in the use of perfluoroalkylated derivatives which are not commercially available. The first step of the synthesis of DF4C11-TMA and DF4C11-DMHEA was common to both derivatives (Scheme 1). It consisted into the *O*-alkylation of the dianion of 3-dimethylamino-1,2-propanediol using 11-(*F*-butyl)undecyl tosylate [9]. Quaternarisation of the amine function using a large



Scheme 1. Synthetic route to DF4C11-TMA and DF4C11-DMHEA. (i) NaH/toluene, then $F(CF_2)_4(CH_2)_{11}OTs$; (ii) excess MeI; (iii) excess $BrCH_2CH_2OH$, NEt_3, THF.



Scheme 2. Synthetic route to the fluorinated [F8E11][C16]OPES lipospermine. (i) DCC/HOSu, CH_2Cl_2/THF; (ii) excess TFA/CH_2Cl_2.

excess of methyl iodide or 2-bromoethanol afforded DF4C11-TMA or DF4C11-DMHEA, respectively.

The synthesis of the fluorinated lipospermines [F8E11][C16]OPES (isolated as its tetra-TFA ammonium salt, as attested by ^{19}F NMR) was achieved by coupling the fluorinated glycerophosphoethanolamine [F8E11][C16]OPE [8] with tetra-*tert*-butoxycarbonylspermine-5-carboxylic acid [6] in the presence of DCC/HOSu as coupling agent, followed by the quantitative Boc-deprotection with TFA [6,11]. The characterisation and purification of this highly hygroscopic tetra-TFA ammonium salt was best performed on the Boc-protected derivative.

3.2. Lipoplex formation and characterisation

The capability of the various fluorinated lipids (F-lipids) to condense DNA and to form lipoplexes was analysed with or without an equimolar amount of DOPE. These studies were performed with pTG11033 plasmid, also used for the in vitro transfection assays (see below). The procedure applied for the lipoplex preparation relies on the dilution from a micellar or liposomal F-lipid or F-lipid/DOPE 1:1 mol dispersion in buffer, using *N*/*P* ratios of 5, 2.5, 1.25 and 0.8. The sizes of the lipoplexes were analysed by light scattering spectroscopy (LSS). Table 1 collects the mean particle sizes measured for the different lipoplexes.

For an excess of cationic charges, such as *N*/*P* ratios of 2.5 and 5, all the fluorinated aminolipids condensed DNA into cationic lipoplexes with mean particle sizes in the 170–220 nm range, except for DF4C11-TMA for which precip-

Table 1
Mean size of the lipoplexes formed between plasmid pTG11033 and DF4C11-TMA, DF4C11-DMHEA, or [F8E11][C16]OPES, as determined by light scattering spectroscopy

Cationic lipid	N/P ratio[a]	Lipoplexes	
		Mean diameter in nanometers (S.D.)	
		Without DOPE	With DOPE
DF4C11-TMA	5	p[b]	300 (110)
	2.5	p[b]	400 (150)
	1.25	340 (110)	p[b]
	0.8	300 (90)	460 (170)
DF4C11-DMHEA	5	160 (large)	300 (110)
	2.5	220 (large)	325 (110)
	1.25	p[b]	p[b]
	0.8	380 (130)	450 (150)
[F8E11][C16]OPES	5	170 (60)	200 (75)
	2.5	180 (60)	p[b]
	1.25	p[b]	p[b]
	0.8	370 (130)	350 (130)
DF4C11-GS	5	125 (40)	280 (80)
	2.5	p[b]	p[b]
	1.25	p[b]	p[b]
	0.8	340 (120)	340 (120)

The data concerning the DF4C11-GS formulations were taken from Ref. [6].
[a] N = lipid cation equivalents; P = plasmid phosphate equivalents.
[b] p = precipitate.

itates were observed. Co-formulation of the N/P 2.5 and 5 lipoplexes with an equimolar amount of DOPE resulted in a lipoplex size increase (200–400 nm), although for the [F8E11][C16]OPES/DOPE N/P 2.5 complexes, a precipitate formed.

For N/P 1.25, and with or without the addition of DOPE, one observed often precipitates. For N/P 0.8, the fluorinated lipo(poly)amines condensed DNA into "anionic" lipoplexes of a mean size in the 300- to 460-nm range. The addition of an equimolar amount of DOPE had no significant effect on lipoplex size (320–550 nm).

Altogether, these results in terms of impact of N/P ratio on lipoplex stability, that is, formation of precipitates for N/P 1.25 corresponding to "neutrally" charged lipoplexes and "stable" dispersions for N/P ratios corresponding to negatively and positively charged lipoplexes, are close to those reported for other lipopolyamines [15] and for the fluorinated analogues of DOGS [6] (see data for DF4C11-GS in Table 1).

These formulations were also analysed by gel electrophoresis (results not shown) which indicated the presence of "free" plasmid and of complexed plasmid accessible to ethidium bromide intercalation for the DF4C11-TMA and DF4C11-DMHEA formulations, even for the highest N/P ratio investigated (N/P 5). When DOPE was used as co-lipid of DF4C11-TMA and DF4C11-DMHEA, only the N/P 0.8 and 1.25 formulations displayed "free" and accessible plasmid. By contrast, the DNA was totally protected from ethidium bromide interaction for the [F8E11][C16]OPES lipoplexes, with or without DOPE, except for N/P 0.8.

3.3. In vitro transfection

The transfection potential of DF4C11-TMA, DF4C11-DMHEA and [F8E11][C16]OPES, without and with an equimolar amount of DOPE, was assayed in vitro on A549 cells. These assays were performed by incubating the lipoplexes with the cells in the presence of 10% foetal calf serum for 24 h. All N/P 0.8, 1.25, 2.5 and 5 lipoplex formulations, even those that precipitated, were tested. Their transfection efficiency [expressed in femtograms (fg) of luciferase/milligram (mg) of protein] was evaluated for a DNA amount of 0.1, 0.5 and 1 μg/well as compared to naked DNA and to reference lipoplexes based on DF4C11-GS or DOGS (without and with an equimolar amount of DOPE). Cells treated with naked DNA under equivalent conditions showed expression levels of about 10^{2-3} fg of luciferase/mg of protein. The cell viability of the lipoplexes was also checked by determining the total protein amount per well of the transfected cells relative to that measured for untreated cells (for which the total protein amount per well is in a 30- to 60-μg/well range).

Fig. 2 presents the transfection results for the fluorinated lipoplexes formulated without and with an equimolar amount of DOPE obtained for a DNA amount of 0.1, 0.5 and 1 μg/well. As for most of the conventional (poly)cationic lipids tested so far, the transfection efficiency and the cell tolerance of the fluorinated lipoplexes were related to the concentration of the lipoplexes (expressed as DNA amount/well) and the lipoplex formulation (DOPE added or not, N/P ratio).

The variance analysis of the logarithmic transformation of the transfection levels obtained with the fluorinated lipoplexes showed that three factors (DOPE added or not, DNA amount/well and N/P ratio of the lipoplexes) had significant effects on their variability ($p < 0.001$; data not shown; see also discussion below). The two factors contributing the most to the variance were the concentration of the lipoplexes (DNA) and the use of DOPE for the formulation of the lipoplexes. Furthermore, the variance analysis showed also a high two-way interaction between the DOPE and N/P ratio factors ($p < 0.001$; data not shown), indicating that the effect of DOPE on the transfection level depended on the N/P ratio used for the formulation of the lipoplexes.

Concerning the toxicity of the lipoplexes, the variance analysis of cell survival showed in the case of DF4C11-DMHEA and DF4C11-TMA that the factors having the highest impact on variance were the DNA concentration and DOPE factors ($p < 0.001$; data not shown). Two two-way interactions were also found, one between the DOPE and DNA concentration factors, and another one between the DOPE and N/P ratio factors, indicating that the effect of DOPE on cell survival of the lipoplexes depended on the DNA concentration and on the N/P ratio of these lipoplexes. For [F8E11][C16]OPES, the DNA concentration and N/P ratio factors had the main contribution to the variability

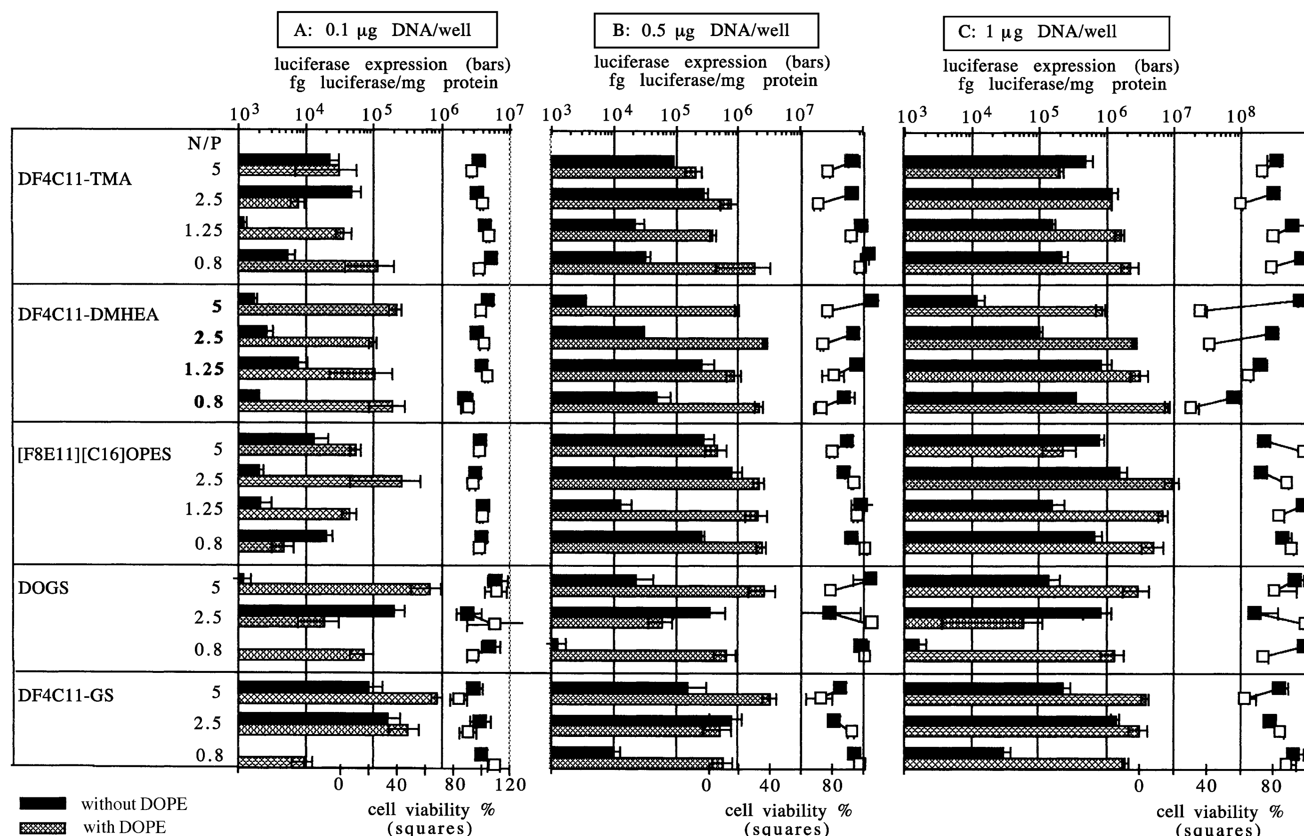


Fig. 2. Luciferase expression (bars) and cell viability (squares) of the fluorinated lipoplexes made of pTG11033 and DF4C11-TMA, DF4C11-DMHEA or [F8E11][C16]OPES without and with an equimolar amount of DOPE, as compared to DOGS or DF4C11-GS controls in A549 cells for a lipoplex concentration of 0.1, 0.5 and 1 μg/well (panels A, B and C, respectively). Lipofection was performed in the presence of 10% foetal calf serum. Means ± S.E.M. are given.

of cell survival ($p<0.001$) while DOPE had no impact ($p>0.05$; data not shown).

Analysing means of transfection values with respect to the concentration of the lipoplexes, it appears, as illustrated in Fig. 3, panels A1–3, that the transfection efficiency of the fluorinated lipoplexes significantly increased on raising the DNA concentration from 0.1 to 0.5 and to 1 μg/well ($p<0.05$). An optimum of gene expression was attained for a DNA amount of 0.5 μg/well ($p>0.05$ between 0.5 and 1 μg/well). It is further noteworthy that these fluorinated lipoplexes allowed luciferase expression levels often compatible with cell growth (cell viability percentages between 77 ± 1% and 92 ± 1% for 0.5 μg DNA/well which declined to 59 ± 1% to 80 ± 1% for 1 μg DNA/well; see Fig. 4, panels A1–3).

Co-formulation of the lipoplexes with DOPE increased significantly the transfection efficiency of the three new fluorinated lipids tested ($p<0.001$; see Fig. 3, panels B1–3) as for most of the conventional (poly)cationic lipids tested so far, highlighting again the unique properties of DOPE as helper lipid. The difference of means showed that the helper effect of DOPE was the highest for DF4C11-DMHEA (Fig. 3, panel B3) and the lowest for DF4C11-TMA (Fig. 3, panel B2). Co-formulation of the lipoplexes with DOPE decreased however significantly cell viability in the case of DF4C11-TMA and DF4C11-DMHEA ($p<0.05$; Fig. 4, panels B2 and 3) but had no significant effect in the case of [F8E11][C16]OPES lipoplexes ($p>0.05$; Fig. 4, panel B1). The concomitant rise of transfection efficiency and toxicity when co-formulating the DF4C11-DMHEA and DF4C11-TMA lipoplexes with DOPE is likely related to an increase of the lipoplex cellular uptake.

Analysing means of transfection values with respect to the charge of the lipoplexes (*N*/*P* ratio), no systematic trend could be evidenced with increasing the *N*/*P* ratio from 0.8 to 5 (Fig. 3, panels C1–3). One can, however, notice that the [F8E11][C16]OPES- and DF4C11-TMA-based lipoplexes behave very similarly, the highest mean levels of transfection being attained for the *N*/*P* ratio of 0.8 and 2.5. Surprisingly in the case of DF4C11-DMHEA, transfection decreased with increasing the *N*/*P* ratio from 0.8–2.5 to 5 (Fig. 3, panel C3). For the formulations based on the [F8E11][C16]OPES- and DF4C11-TMA lipids, their cell viability was lower for the cationic *N*/*P* 2.5 and 5 lipoplexes than for the "negative" (*N*/*P* 0.8) and "neutral" (*N*/*P* 1.25) ones (Fig. 4, panels C1 and 2). By contrast, for DF4C11-

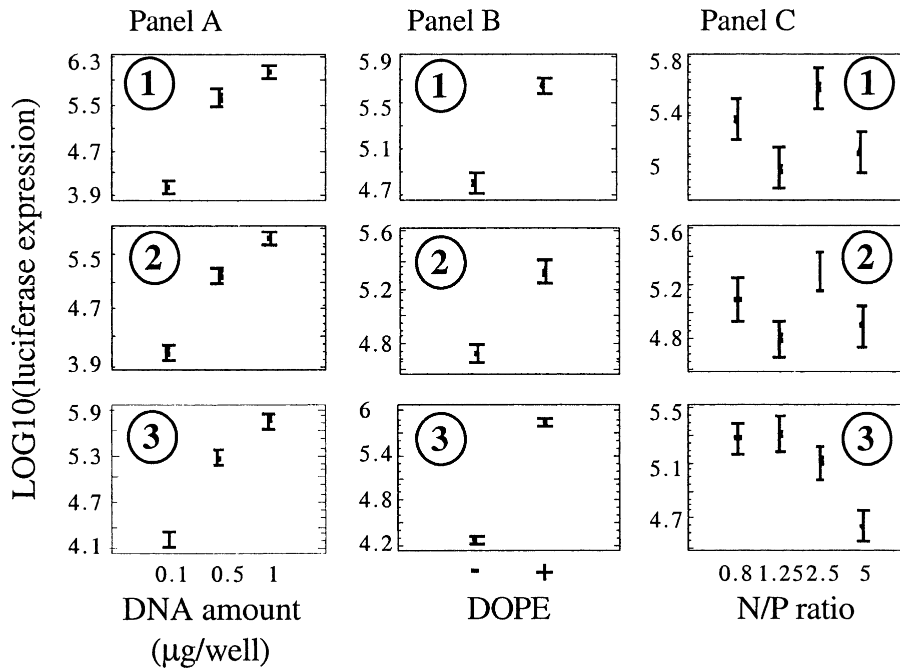


Fig. 3. Means and 95.0% Tukey HSD intervals of the logarithmic transformation of transfection levels [Log10(femtograms luciferase/milligram protein)] for the fluorinated lipoplexes based on [F8E11][C16]OPES (line 1), DF4C11-TMA (line 2), or DF4C11-DMHEA (line 3) with respect to the lipoplex concentration (panel A), the presence (+) or not (−) of DOPE (panel B), or to the lipoplex *N*/*P* ratio (panel C).

DMHEA lipid, the most toxic formulations were those corresponding to the "negative" (*N*/*P* 0.8) ones (Fig. 4, panel C3).

It is further noticeable that several *N*/*P* 0.8 or 1.25 lipoplexes investigated displayed comparable luciferase expression levels ($p>0.05$) than the more cationic *N*/*P* 2.5 and 5 ones (see Fig. 2 and Fig. 3, panels C1–3), while luciferase expression optimum is rather reported for *N*/*P* ratios ≥ 3 in the literature. One should mention that agarose gel electrophoresis showed for most of the *N*/*P* 0.8 or 1.25

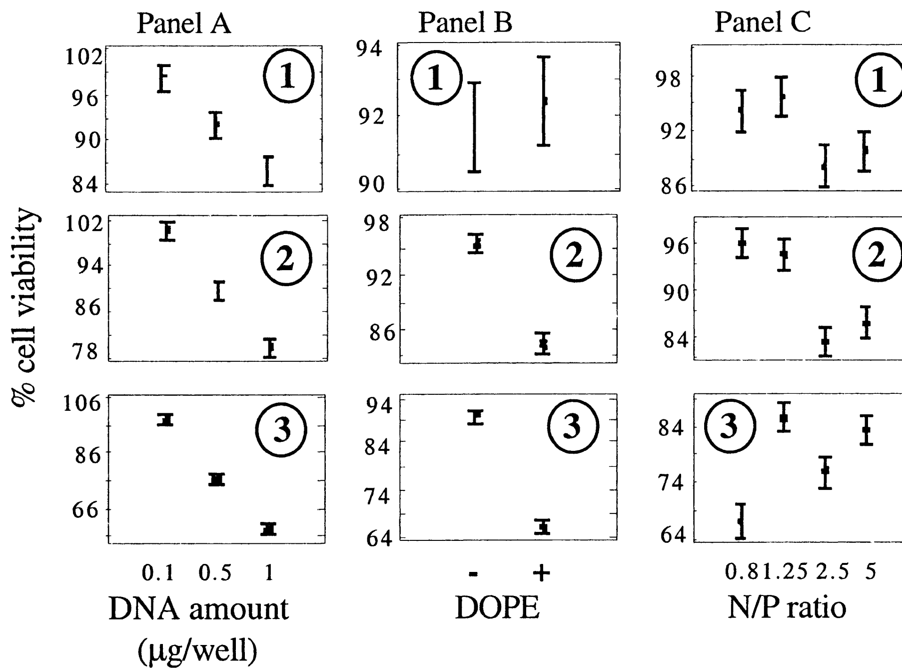


Fig. 4. Means and 95.0% Tukey HSD intervals of the cell viability for the fluorinated lipoplexes based on [F8E11][C16]OPES (line 1), DF4C11-TMA line (2), or DF4C11-DMHEA (line 3) with respect to the lipoplex concentration (panel A), the presence (+) or not (−) of DOPE (panel B), or to the lipoplex *N*/*P* ratio (panel C).

DF4C11-TMA-, DF4C11-DMHEA- or [F8E11][C16] OPES-based lipoplexes the presence of "free" DNA and of complexed DNA accessible to ethidium bromide. These results indicate that fully complexed and protected DNA is not a prerequisite for optimal in vitro transfection. Optimal lipofection with partially complexed DNA ($N/P < 1$ lipoplexes) was also found for DOGS:DOPE and DF4C11-GS:DOPE controls (see Fig. 2) and reported for DMRIE [10] and DMRIE analogues [16].

That an optimum of transfection can be obtained with "negatively" charged lipoplexes indicates most likely that the charge of the DNA complex plays an important role not only for cell entry, but also for intracytoplasmic DNA delivery and/or traffic to the nucleus. It is generally assumed that the synthetic lipofection systems provide non-specific gene transfer into cells which involves electrostatic interactions of the cationized DNA particles with the anionic proteoglycans expressed on the cell surface followed by endocytosis [17,18]. If the cell entry process is favoured when increasing the cationic charge of the lipoplexes, the intracytoplasmic DNA delivery and its traffic to the nucleus seem to be favoured when the charge of the lipoplexes once inside the cell is lowered to negative values, in agreement with intracytoplasmic and/or intranuclear microinjection experiments of partially ($N/P < 1$) or fully ($N/P > 1$) complexed DNA [19,20].

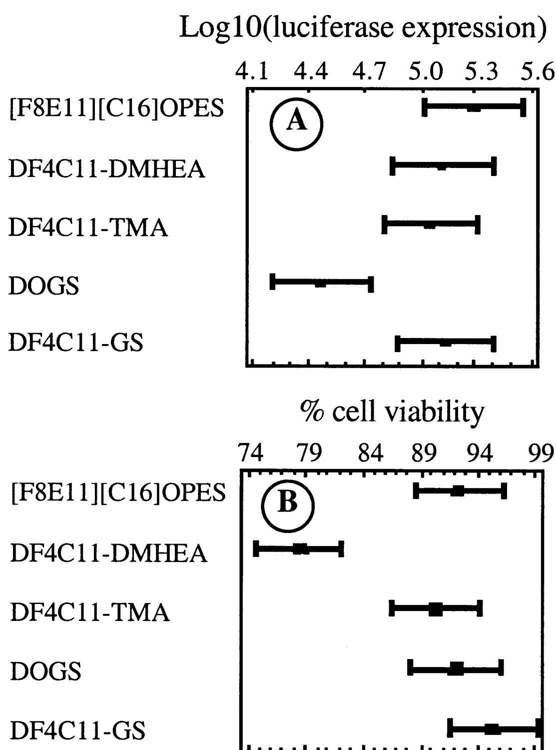


Fig. 5. Means and 95.0% Tukey HSD intervals of the logarithmic transformation of transfection levels [Log10(femtograms luciferase/milligram protein); panel A] and of the cell viability (panel B) for the fluorinated DF4C11-TMA-, DF4C11-DMHEA- or [F8E11][C16]OPES-based lipoplexes and for control formulations made with DF4C11-GS or DOGS, irrespectively to the DNA concentration, the presence or not of DOPE, or the lipoplex N/P ratio.

The aim of this study was also to evaluate the transfection potential of the lipoplexes formulated with these new fluorinated cationic lipids as compared with the most efficient fluorinated cationic lipid reported so far for transfecting A549 cells, that is, DF4C11-GS [6]. This fluorinated reference lipid led to lipoplexes that were more efficient than those resulting from its conventional DOGS analogue (see results shown in Fig. 2, and Fig. 5A), both lipids being, in our in vitro transfection protocol, among the most efficient cationic lipids for transfecting A549 cells [6].

Multiple-sample comparisons were run on the logarithmic transformations of the transfection levels and on cell viability percentages for each of the three new lipids tested and for the DF4C11-GS and DOGS controls to determine which means, hence which lipids, were significantly different from which others. Fig. 5 summarises the calculated means corresponding to the transfection and cell survival data for their respective lipoplexes. As illustrated in Fig. 5A, the new fluorinated lipids showed transfection level means, and consequently transfection efficiencies, that are (i) comparable to those of DF4C11-GS ($p > 0.05$) and (ii) significantly higher ($p < 0.05$) than those of the conventional DOGS lipid. If the [F8E11][C16]OPES-, DF4C11-TMA-, DF4C11-GS- and DOGS-based lipoplexes gave comparable mean cell viabilities ($p > 0.05$), these formulations display significantly higher cell viabilities than the DF4C11-DMHEA-based lipoplexes ($p < 0.05$) (Fig. 5B).

4. Conclusion

All these results together combined with the higher in vitro and in vivo transfection potential found for fluorinated lipoplexes as compared with that of conventional lipoplexes [6–8] or even of PEI polyplexes [8] confirm that fluorinated lipoplexes are appealing and very promising candidates for gene transfer purposes, highlighting the diversity of lipids leading to efficient transfection. Although the in vitro results obtained here with the fluorinated analogues of DOTMA, DMRIE and DPPES using a standard transfection protocol lead to no improvement over the first generation of fluorinated lipids (e.g. the fluorinated analogues of DOGS), they are nevertheless very encouraging. Moreover, these in vitro findings do not prejudge all of their in vitro and in vivo behaviours which remain to be investigated. In addition, these results confirm also that lipophobicity and restricted miscibility of the lipoplex lipids with the endogenous lipids does not preclude efficient gene transfer and expression. This study underscores also the determinant impact of the unique lipophobic and hydrophobic character of the fluorinated lipids on the transfection potency of the fluorinated lipoplexes they form with DNA, preventing DNA from interactions with lipophilic and hydrophilic biocompounds, and from degradation [7].

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