Supplemental Material

Tuning the self-assembling of pyridinium cationic lipids for efficient gene delivery into neuronal cells

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Summary:

1. General procedure for the synthesis of pyridinium cationic lipids

A. Preparation of 1-(3,4-dihydroxyphenylethyl)-2,4,6-trimethylpyridinium hexafluorophosphate **3** (adapted after [38])

An amount of 2.5 g (13.18 mmol) 3-hydroxytyramine hydrochloride was dissolved in the minimum amount of deionized water (1-2 mL) and subsequently diluted with 12.5 mL ethanol. The homogeneous solution was treated with 1.85 mL (1.33 g, 13.12 mmol) triethylamine, dropwise, in order to generate the dopamine free base, and added dropwise over a stirred suspension of 5.71 g (15.82 mmol) 2,4,6-trimethylpyrylium hexafluorophosphate [ATB Science of Synthesis] in 12.5 mL ethanol in 100 mL round bottom flask. The reaction mixture turns orange and all pyrylium salt dissolves; it was subsequently treated with another batch of triethylamine (1.85 mL, 1.33 g, 13.12 mmol), refluxed for 15 min, and then treated with 4 mL (4 g, 66.66 mmol) acetic acid. After another 3 h reflux (TLC control MeOH/DCM 2/8 v/v) it was treated with 4 mL aqueous NH_4OH 25%, and refluxed again for 5 min in order to convert any unreacted pyrylium salt into the corresponding pyridine. The solvent was evaporated to dryness and the residue was washed with ethyl ether (50 mL) in order to dissolve the pyridine and any the other amines present in solution. The ether was decanted and discarded, and the resulting precipitate was filtered and washed two more times with ethyl ether (2 x 50 mL). After drying, it was suspended into 10 mL of deionized water, stirred for 5 minutes, filtered, and washed with 2 x 5 mL deionized water. The crude product was triturated with 25 mL HPF_6 5%, filtered, and recrystallized from 75 mL methanol, yielding 2.61 g. Concentration of mother liquor yielded another 0.95 g product (3.56 g overall, 67%), mp 217-220 ^oC (lit [38] mp 218-220 ^oC).

B. General procedure for the preparation of pyridinium lipids **4**

In a 25 mL round bottom flask 0.6 g (1.5 mmol) 1-(3,4-dihydroxyphenylethyl)-2,4,6-trimethylpyridinium hexafluorophosphate **3** were dissolved into 10 mL dry dimethylformamide (DMF). Alkyl bromide (3 mmol) was subsequently added, followed by 0.42 g (3 mmol) anhydrous K_2CO_3. The flask was capped with a septum and the stirred suspension was degassed with a stream of dry nitrogen for 1h. The suspension was heated under stirring at 70 ^oC for 48 hours, after which it was quenched by pouring the reaction mixture into 100 mL cold water. The oily mixture was extracted with CH_2Cl_2 (5 x 30 mL), and the water was discarded. The combined DCM extracts were washed with 50 mL aqueous HPF_6 5%, 50 mL aqueous saturated $NaHCO_3$,

and were dried on anhydrous Na_2SO_4; evaporation of the solvent yielded the crude product. Flash chromatography ($CHCl_3$/MeOH, using gradient elution 97.5/2.5 → 70/30 v/v) followed by crystallization from hexane/ethyl acetate 7/3 v/v solvent mixture [65] and/or ethanol yielded the pure compound.

1-(3,4-didecyloxyphenylethyl)-2,4,6-trimethylpyridinium hexafluorophosphate **4Dec** (yield 48%) ^{1}H-NMR ($CDCl_3$), δ, ppm: 7.39 (s, 2H: Hβ-pyridinium), 6.75 (d, *J* = 8.1 Hz, 1H: H5 Ph), 6.52 (d, *J* = 1.9 Hz, 1H: H2 Ph), 6.47 (dd, *J* = 1.9, 8.1 Hz, 1H: H6 Ph), 4.61 (t, *J* = 7.0 Hz, 2H: CH_2-Py^+), 3.93 (t, *J* = 6.6 Hz, 2H: CH_2-O), 3.88 (t, *J* = 6.6 Hz, 2H: CH_2-O), 3.00 (t, *J* = 7.0 Hz, 2H: CH_2-Ph), 2.60 (s, 6H: CH_3 α-Py^+), 2.48 (s, 3H: CH_3 γ-Py^+), 1.77 (m, 4H: 2 $\underline{CH_2}CH_2O$), 1.44 (m, 4H: 2 $\underline{CH_2}CH_2CH_2O$), 1.26 (m, 24H: 12 CH_2 from fatty chains), 0.88 (t, *J* = 6.6 Hz, 6H: 2CH_3 from fatty chains); ^{13}C-NMR (DMSO-d_6), δ, ppm: 158.3 (Cγ-pyridinium), 154.7 (2Cα-pyridinium), 150.1 (C-3, Ph), 149.2 (C-4, Ph), 129.1 (3C, 2Cβ-pyridinium + C-1, Ph), 128.3 (C-2, Ph), 121.3 (C-6, Ph), 114.8 (C-5, Ph), 69.9 (O-CH_2), 69.8 (O-CH_2), 53.9 ($\underline{C}H_2$-Py^+), 34.8 ($\underline{C}H_2$-Ph), 32.3 (2C), 29.99 (3C), 29.95 (2C), 29.83 (3C), 29.7 (2C), 26.4 (2C), 23.0 (2C) (all from fatty chains), 21.7 (CH_3 γ-pyridinium), 21.4 (2CH_3 α-pyridinium), 14.4 (2C: 2 CH_3 from fatty chains); ^{19}F-NMR (DMSO-d^6), *δ*, ppm: - 75.3 (*J* = 712 Hz, PF_6^-); HPLC: 96.2%; Anal ($C_{36}H_{60}NO_2^+$ PF_6^-) C, H, N.

1-(3,4-didodecyloxyphenylethyl)-2,4,6-trimethylpyridinium hexafluorophosphate **4Lau**: Yield 46%; ^{1}H-NMR ($CDCl_3$), δ, ppm: 7.38 (s, 2H: Hβ-pyridinium), 6.75 (d, *J* = 8.1 Hz, 1H: H5 Ph), 6.52 (d, *J* = 1.9 Hz, 1H: H2 Ph), 6.47 (dd, *J* = 1.9, 8.1 Hz, 1H: H6 Ph), 4.61 (t, *J* = 6.8 Hz, 2H: CH_2-Py^+), 3.93 (t, *J* = 6.6 Hz, 2H: CH_2-O), 3.88 (t, *J* = 6.6 Hz, 2H: CH_2-O), 3.00 (t, *J* = 6.8 Hz, 2H: CH_2-Ph), 2.60 (s, 6H: CH_3 α-Py^+), 2.48 (s, 3H: CH_3 γ-Py^+), 1.77 (m, 4H: 2 $\underline{CH_2}CH_2O$), 1.44 (m, 4H: 2 $\underline{CH_2}CH_2CH_2O$), 1.26 (m, 32H: 16 CH_2 from fatty chains), 0.88 (t, *J* = 6.6 Hz, 6H: 2CH_3 from fatty chains); ^{13}C-NMR (DMSO-d_6), δ, ppm: 158.3 (Cγ-pyridinium), 154.8 (2Cα-pyridinium), 150.1 (C-3, Ph), 149.3 (C-4, Ph), 129.1 (3C, 2Cβ-pyridinium + C-1, Ph), 128.3 (C-2, Ph), 121.3 (C-6, Ph), 114.8 (C-5, Ph), 69.9 (O-CH_2), 69.8 (O-CH_2), 54.0 ($\underline{C}H_2$-Py^+), 34.8 ($\underline{C}H_2$-Ph), 32.3 (2C), 30.08 (6C), 30.0 (6C), 29.8 (2C), 29.7 (2C), 26.4 (2C), 23.0 (2C) (all from fatty chains), 21.8 (CH_3 γ-pyridinium), 21.4 (2CH_3 α-pyridinium), 14.4 (2C: 2 CH_3 from fatty

chains); ^{19}F-NMR (DMSO-d^6), δ, ppm: - 73.8 (J = 712 Hz, PF_6^-); HPLC: 96.7%; Anal ($C_{40}H_{68}NO_2^+$ PF_6^-) C, H, N.

1-(3,4-ditetradecyloxyphenylethyl)-2,4,6-trimethylpyridinium hexafluorophosphate **4Myr**: Yield 50%; ^{1}H-NMR ($CDCl_3$), δ, ppm: 7.38 (s, 2H: Hβ-pyridinium), 6.75 (d, J = 8.1 Hz, 1H: H5 Ph), 6.52 (d, J = 1.9 Hz, 1H: H2 Ph), 6.47 (dd, J = 1.9, 8.1 Hz, 1H: H6 Ph), 4.61 (t, J = 7.0 Hz, 2H: CH_2-Py$^+$), 3.93 (t, J = 6.6 Hz, 2H: CH_2-O), 3.87 (t, J = 6.6 Hz, 2H: CH_2-O), 3.00 (t, J = 7.0 Hz, 2H: CH_2-Ph), 2.59 (s, 6H: CH_3 α-Py$^+$), 2.48 (s, 3H: CH_3 γ-Py$^+$), 1.77 (m, 4H: 2 $\underline{CH_2}CH_2O$), 1.44 (m, 4H: 2 $\underline{CH_2}CH_2CH_2O$), 1.26 (m, 40H: 20 CH_2 from fatty chains), 0.88 (t, J = 6.6 Hz, 6H: 2CH_3 from fatty chains); ^{13}C-NMR (DMSO-d_6), δ, ppm: 158.2 (Cγ-pyridinium), 154.7 (2Cα-pyridinium), 150.1 (C-3, Ph), 149.2 (C-4, Ph), 129.1 (3C, 2Cβ-pyridinium + C-1, Ph), 128.3 (C-2, Ph), 121.3 (C-6, Ph), 114.7 (C-5, Ph), 69.84 (O-CH_2), 69.76 (O-CH_2), 53.9 ($\underline{C}H_2$-Py$^+$), 34.7 ($\underline{C}H_2$-Ph), 32.2 (2C), 30.04 (6C), 30.0 (6C), 29.8 (2C), 29.7 (4C), 26.4 (2C), 23.0 (2C) (all from fatty chains), 21.7 (CH_3 γ-pyridinium), 21.3 (2CH_3 α-pyridinium), 14.4 (2C: 2 CH_3 from fatty chains); ^{19}F-NMR (DMSO-d^6), δ, ppm: - 73.8 (J = 712 Hz, PF_6^-); HPLC: 97.1 %; Anal ($C_{44}H_{76}NO_2^+$ PF_6^-) C, H, N.

1-(3,4-dihexadecyloxyphenylethyl)-2,4,6-trimethylpyridinium hexafluorophosphate **4Pal**: Yield 48%; ^{1}H-NMR ($CDCl_3$), δ, ppm: 7.38 (s, 2H: Hβ-pyridinium), 6.78 (d, J = 8.1 Hz, 1H: H5 Ph), 6.52 (d, J = 1.9 Hz, 1H: H2 Ph), 6.47 (dd, J = 1.9, 8.1 Hz, 1H: H6 Ph), 4.61 (t, J = 7.0 Hz, 2H: CH_2-Py$^+$), 3.93 (t, J = 6.6 Hz, 2H: CH_2-O), 3.87 (t, J = 6.6 Hz, 2H: CH_2-O), 3.00 (t, J = 7.0 Hz, 2H: CH_2-Ph), 2.59 (s, 6H: CH_3 α-Py$^+$), 2.48 (s, 3H: CH_3 γ-Py$^+$), 1.77 (m, 4H: 2 $\underline{CH_2}CH_2O$), 1.43 (m, 4H: 2 $\underline{CH_2}CH_2CH_2O$), 1.26 (m, 48H: 24 CH_2 from fatty chains), 0.87 (t, J = 6.6 Hz, 6H: 2CH_3 from fatty chains); ^{13}C-NMR (DMSO-d_6), δ, ppm: 158.2 (Cγ-pyridinium), 154.7 (2Cα-pyridinium), 150.1 (C-3, Ph), 149.2 (C-4, Ph), 129.1 (3C, 2Cβ-pyridinium + C-1, Ph), 128.3 (C-2, Ph), 121.3 (C-6, Ph), 114.7 (C-5, Ph), 69.9 (O-CH_2), 69.8 (O-CH_2), 53.9 ($\underline{C}H_2$-Py$^+$), 34.7 ($\underline{C}H_2$-Ph), 32.3 (2C), 30.1 (10C), 30.0 (6C), 29.8 (2C), 29.7 (4C), 26.4 (2C), 23.0 (2C) (all from fatty chains), 21.7 (CH_3 γ-pyridinium), 21.3 (2CH_3 α-pyridinium), 14.4 (2C: 2 CH_3 from fatty chains); ^{19}F-NMR (DMSO-d^6), δ, ppm: - 73.5 (J = 712 Hz, PF_6^-); HPLC: 96.0%; Anal ($C_{48}H_{84}NO_2^+$ PF_6^-) C, H, N.

1-(3,4-dioctadecyloxyphenylethyl)-2,4,6-trimethylpyridinium hexafluorophosphate **4Ste**: Yield 44%; ^{1}H-NMR ($CDCl_3$), δ, ppm: 7.39 (s, 2H: Hβ-pyridinium), 6.75 (d, *J* = 8.1 Hz, 1H: H5 Ph), 6.52 (d, *J* = 1.9 Hz, 1H: H2 Ph), 6.47 (dd, *J* = 1.9, 8.1 Hz, 1H: H6 Ph), 4.62 (t, *J* = 7.0 Hz, 2H: CH_2-Py^+), 3.93 (t, *J* = 6.6 Hz, 2H: CH_2-O), 3.88 (t, *J* = 6.5 Hz, 2H: CH_2-O), 3.00 (t, *J* = 7.0 Hz, 2H: CH_2-Ph), 2.60 (s, 6H: CH_3 α-Py^+), 2.49 (s, 3H: CH_3 γ-Py^+), 1.77 (m, 4H: 2 $\underline{CH_2}CH_2O$), 1.44 (m, 4H: 2 $\underline{CH_2}CH_2CH_2O$), 1.26 (m, 56H: 28 CH_2 from fatty chains), 0.88 (t, *J* = 6.8 Hz, 6H: $2CH_3$ from fatty chains); ^{13}C-NMR (DMSO-d_6), δ, ppm: 158.2 (Cγ-pyridinium), 154.8 (2Cα-pyridinium), 150.1 (C-3, Ph), 149.3 (C-4, Ph), 129.1 (3C, 2Cβ-pyridinium + C-1, Ph), 128.3 (C-2, Ph), 121.3 (C-6, Ph), 114.7 (C-5, Ph), 69.9 (O-CH_2), 69.8 (O-CH_2), 53.9 ($\underline{C}H_2$-Py^+), 34.8 ($\underline{C}H_2$-Ph), 32.3 (2C), 30.1 (12C), 30.0 (8C), 29.8 (2C), 29.7 (4C), 26.4 (2C), 23.0 (2C) (all from fatty chains), 21.7 (CH_3 γ-pyridinium), 21.4 ($2CH_3$ α-pyridinium), 14.4 (2C: 2 CH_3 from fatty chains); ^{19}F-NMR (DMSO-d^6), *δ*, ppm: -74.0 (*J* = 712 Hz, PF_6^-); HPLC: 96.1%; Anal ($C_{52}H_{92}NO_2^+$ PF_6^-) C, H, N.

1-(3,4-dioleyloxyphenylethyl)-2,4,6-trimethylpyridinium hexafluorophosphate **4Ole** (yield 42%); ^{1}H-NMR ($CDCl_3$), δ, ppm: 7.39 (s, 2H: Hβ-pyridinium), 6.75 (d, *J* = 8.0 Hz, 1H: H5 Ph), 6.53 (s, 1H: H2 Ph), 6.47 (d, *J* = 8.0 Hz, 1H: H6 Ph), 5.34 (m, 4H, 2 CH=CH), 4.62 (t, *J* = 7.0 Hz, 2H: CH_2-Py^+), 3.93 (t, *J* = 6.6 Hz, 2H: CH_2-O), 3.88 (t, *J* = 6.5 Hz, 2H: CH_2-O), 3.00 (t, *J* = 7.0 Hz, 2H: CH_2-Ph), 2.60 (s, 6H: CH_3 α-Py^+), 2.49 (s, 3H: CH_3 γ-Py^+), 2.00 (m, 8H, 4 CH_2-CH=CH), 1.77 (m, 4H: 2 $\underline{CH_2}CH_2O$), 1.44 (m, 4H: 2 $\underline{CH_2}CH_2CH_2O$), 1.31 (m, 24H: 12 CH2 from fatty chains), 1.26 (m, 16H: 8 CH_2 from fatty chains), 0.88 (t, *J* = 6.8 Hz, 6H: $2CH_3$ from fatty chains); ^{13}C-NMR (DMSO-d_6), δ, ppm: 158.2 (Cγ-pyridinium), 154.7 (2Cα-pyridinium), 150.1 (C-3, Ph), 149.2 (C-4, Ph), 130.2 (2C, CH=CH), 130.1 (2C, CH=CH), 129.1 (3C, 2Cβ-pyridinium + C-1, Ph), 128.3 (C-2, Ph), 121.3 (C-6, Ph), 114.7 (C-5, Ph), 69.9 (O-CH_2), 69.8 (O-CH_2), 53.9 ($\underline{C}H_2$-Py^+), 34.8 ($\underline{C}H_2$-Ph), 32.2 (2C), 30.2 (2C), 30.1 (3C), 29.9 (3C), 29.8 (2C), 29.7 (2C), 29.6 (4C), 27.6 (4C), 26.39 (2C), 26.37 (2C), 23.0 (2C) (all from fatty chains), 21.7 (CH_3 γ-pyridinium), 21.4 ($2CH_3$ α-pyridinium), 14.4 (2C: 2 CH_3 from fatty chains); ^{19}F-NMR (DMSO-d^6), *δ*, ppm: - 73.4 (*J* = 712 Hz, PF_6^-); HPLC: 98.6%; Anal ($C_{52}H_{88}NO_2^+$ PF_6^-) C, H, N.

Ion Exchange Procedure. An amount of 70 g of Dowex 1X8-200 ion exchange resin (chloride form) were suspended in 200 mL deionized water in an Erlenmeyer flask and kept at room temperature for three days to fully inflate. It was transferred into a glass column and washed with deionized water (200 mL), with 5% aqueous HCl (1 L), then again with deionised water until neutral pH was reached. After a final wash with MeOH, the resin was retrieved from the column and stored in an Erlenmeyer flask under MeOH.

Prior to counterion exchange, about 10 g resin was transferred into a small column and washed with 200 mL MeOH. Separately, an amount of 50 mg of pure lipid **4** (as hexafluorophosphate) were weighed and dissolved in MeOH (1-4 mL, depending on the solubility of the material). The methanol solution was transferred on top of the ion exchange column and the compound was eluted with methanol (flow rate 1-3 mL/min). Sample was cycled through the column repeatedly (10 times minimum) until ^{19}F-NMR analysis of a small aliquot taken from the eluent showed no fluorine peak corresponding to PF_6^-. Evaporation of solvent yielded the product, which was recrystallized from ethyl acetate and dried under vacuum; all compounds were found >96% pure by HPLC.

1-(3,4-didecyloxyphenylethyl)-2,4,6-trimethylpyridinium chloride **5Dec** (yield 65%); ^{1}H-NMR ($CDCl_3$), δ, ppm: 7.39 (s, 2H: Hβ-pyridinium), 6.75 (d, *J* = 8.1 Hz, 1H: H5 Ph), 6.52 (d, *J* = 1.9 Hz, 1H: H2 Ph), 6.47 (dd, *J* = 1.9, 8.1 Hz, 1H: H6 Ph), 4.61 (t, *J* = 7.0 Hz, 2H: CH_2-Py^+), 3.93 (t, *J* = 6.6 Hz, 2H: CH_2-O), 3.88 (t, *J* = 6.6 Hz, 2H: CH_2-O), 3.00 (t, *J* = 7.0 Hz, 2H: CH_2-Ph), 2.60 (s, 6H: CH_3 α-Py^+), 2.48 (s, 3H: CH_3 γ-Py^+), 1.77 (m, 4H: 2 $\underline{CH_2}CH_2O$), 1.44 (m, 4H: 2 $\underline{CH_2}CH_2CH_2O$), 1.26 (m, 24H: 12 CH_2 from fatty chains), 0.88 (t, *J* = 6.6 Hz, 6H: 2CH_3 from fatty chains); ^{19}F-NMR (DMSO-d^6), δ, ppm: no signal; HPLC: 97.6%; Anal ($C_{36}H_{60}NO_2^+$ Cl^-) C, H, N.

1-(3,4-didodecyloxyphenylethyl)-2,4,6-trimethylpyridinium chloride **5Lau** (yield 78.6%); ^{1}H-NMR ($CDCl_3$), δ, ppm: 7.38 (s, 2H: Hβ-pyridinium), 6.75 (d, *J* = 8.1 Hz, 1H: H5 Ph), 6.52 (d, *J* = 1.9 Hz, 1H: H2 Ph), 6.47 (dd, *J* = 1.9, 8.1 Hz, 1H: H6 Ph), 4.61 (t, *J* = 6.8 Hz, 2H: CH_2-Py^+), 3.93 (t, *J* = 6.6 Hz, 2H: CH_2-O), 3.88 (t, *J* = 6.6 Hz, 2H: CH_2-O), 3.00 (t, *J* = 6.8 Hz, 2H: CH_2-Ph), 2.60 (s, 6H: CH_3 α-Py^+), 2.48 (s, 3H: CH_3 γ-Py^+), 1.77 (m, 4H: 2 $\underline{CH_2}CH_2O$), 1.44 (m, 4H: 2

$\underline{CH_2}CH_2CH_2O$), 1.26 (m, 32H: 16 CH_2 from fatty chains), 0.88 (t, *J* = 6.6 Hz, 6H: $2CH_3$ from fatty chains); ^{19}F-NMR (DMSO-d^6), *δ*, ppm: no signal; HPLC: 96.0 %; Anal ($C_{40}H_{68}NO_2^+$ Cl^-) C, H, N.

1-(3,4-ditetradecyloxyphenylethyl)-2,4,6-trimethylpyridinium chloride **5Myr** (yield 92.3%); ^{1}H-NMR ($CDCl_3$), δ, ppm: 7.38 (s, 2H: Hβ-pyridinium), 6.75 (d, *J* = 8.1 Hz, 1H: H5 Ph), 6.52 (d, *J* = 1.9 Hz, 1H: H2 Ph), 6.47 (dd, *J* = 1.9, 8.1 Hz, 1H: H6 Ph), 4.61 (t, *J* = 7.0 Hz, 2H: CH_2-Py$^+$), 3.93 (t, *J* = 6.6 Hz, 2H: CH_2-O), 3.87 (t, *J* = 6.6 Hz, 2H: CH_2-O), 3.00 (t, *J* = 7.0 Hz, 2H: CH_2-Ph), 2.59 (s, 6H: CH_3 α-Py$^+$), 2.48 (s, 3H: CH_3 γ-Py$^+$), 1.77 (m, 4H: 2 $\underline{CH_2}CH_2O$), 1.44 (m, 4H: 2 $\underline{CH_2}CH_2CH_2O$), 1.26 (m, 40H: 20 CH_2 from fatty chains), 0.88 (t, *J* = 6.6 Hz, 6H: $2CH_3$ from fatty chains); ^{19}F-NMR (DMSO-d^6), *δ*, ppm: no signal; HPLC: 97.1 %; Anal ($C_{44}H_{76}NO_2^+$ Cl^-) C, H, N.

1-(3,4-dihexadecyloxyphenylethyl)-2,4,6-trimethylpyridinium chloride **5Pal** (yield 74.8%); ^{1}H-NMR ($CDCl_3$), δ, ppm: 7.38 (s, 2H: Hβ-pyridinium), 6.78 (d, *J* = 8.1 Hz, 1H: H5 Ph), 6.52 (d, *J* = 1.9 Hz, 1H: H2 Ph), 6.47 (dd, *J* = 1.9, 8.1 Hz, 1H: H6 Ph), 4.61 (t, *J* = 7.0 Hz, 2H: CH_2-Py$^+$), 3.93 (t, *J* = 6.6 Hz, 2H: CH_2-O), 3.87 (t, *J* = 6.6 Hz, 2H: CH_2-O), 3.00 (t, *J* = 7.0 Hz, 2H: CH_2-Ph), 2.59 (s, 6H: CH_3 α-Py$^+$), 2.48 (s, 3H: CH_3 γ-Py$^+$), 1.77 (m, 4H: 2 $\underline{CH_2}CH_2O$), 1.43 (m, 4H: 2 $\underline{CH_2}CH_2CH_2O$), 1.26 (m, 48H: 24 CH_2 from fatty chains), 0.87 (t, *J* = 6.6 Hz, 6H: $2CH_3$ from fatty chains); ^{19}F-NMR (DMSO-d^6), *δ*, ppm: no signal; HPLC: 96.3%; Anal ($C_{48}H_{84}NO_2^+$ Cl^-) C, H, N.

1-(3,4-dioctadecyloxyphenylethyl)-2,4,6-trimethylpyridinium chloride **5Ste** (yield 66.3%); ^{1}H-NMR ($CDCl_3$), δ, ppm: 7.39 (s, 2H: Hβ-pyridinium), 6.75 (d, *J* = 8.1 Hz, 1H: H5 Ph), 6.52 (d, *J* = 1.9 Hz, 1H: H2 Ph), 6.47 (dd, *J* = 1.9, 8.1 Hz, 1H: H6 Ph), 4.62 (t, *J* = 7.0 Hz, 2H: CH_2-Py$^+$), 3.93 (t, *J* = 6.6 Hz, 2H: CH_2-O), 3.88 (t, *J* = 6.5 Hz, 2H: CH_2-O), 3.00 (t, *J* = 7.0 Hz, 2H: CH_2-Ph), 2.60 (s, 6H: CH_3 α-Py$^+$), 2.49 (s, 3H: CH_3 γ-Py$^+$), 1.77 (m, 4H: 2 $\underline{CH_2}CH_2O$), 1.44 (m, 4H: 2 $\underline{CH_2}CH_2CH_2O$), 1.26 (m, 56H: 28 CH_2 from fatty chains), 0.88 (t, *J* = 6.8 Hz, 6H: $2CH_3$ from fatty chains); ^{19}F-NMR (DMSO-d^6), *δ*, ppm: no signal; HPLC: 97.0%; Anal ($C_{52}H_{92}NO_2^+$ Cl^-) C, H, N.

1-(3,4-dioleyloxyphenylethyl)-2,4,6-trimethylpyridinium chloride **5Ole** (yield 74.9%); ^{1}H-NMR ($CDCl_3$), δ, ppm: 7.39 (s, 2H: Hβ-pyridinium), 6.75 (d, *J* = 8.0 Hz, 1H: H5 Ph), 6.53 (s, 1H: H2 Ph), 6.47 (d, *J* = 8.0 Hz, 1H: H6 Ph), 5.34 (m, 4H, 2 CH=CH), 4.62 (t, *J* = 7.0 Hz, 2H: CH_2-Py^+), 3.93 (t, *J* = 6.6 Hz, 2H: CH_2-O), 3.88 (t, *J* = 6.5 Hz, 2H: CH_2-O), 3.00 (t, *J* = 7.0 Hz, 2H: CH_2-Ph), 2.60 (s, 6H: CH_3 α-Py^+), 2.49 (s, 3H: CH_3 γ-Py^+), 2.00 (m, 8H, 4 CH_2-CH=CH), 1.77 (m, 4H: 2 $\underline{CH_2}CH_2O$), 1.44 (m, 4H: 2 $\underline{CH_2}CH_2CH_2O$), 1.31 (m, 24H: 12 CH2 from fatty chains), 1.26 (m, 16H: 8 CH_2 from fatty chains), 0.88 (t, *J* = 6.8 Hz, 6H: 2CH_3 from fatty chains); ^{19}F-NMR (DMSO-d^6), *δ*, ppm: no signal; HPLC: 97.3%; Anal ($C_{52}H_{88}NO_2^+$ Cl^-) C, H, N.

2. Liposomal preparation and characterization

Liposome Preparation: Stock solutions (3 mM) of the cationic lipids were prepared from powder in glass vials using $CHCl_3$/MeOH (2/1) as solvent (organic stock). For DOTAP, DOPE and cholesterol, solutions of the same concentration (3 mM) were made in $CHCl_3$. All solutions were swirled, purged with nitrogen, and capped securely; when not in use they were stored in the -20 ^{o}C freezer.

Three preparations were made for each lipid – lipid alone, lipid mixed with an equimolar amount of cholesterol, and lipid mixed with an equimolar amount of DOPE. Thus 200 μL of the corresponding organic stock was transferred into a glass vial (total cationic lipid in each vial was 600 nmol). A control of DOTAP/Chol 1/1 was also made. The samples were diluted with $CHCl_3$/MeOH (2/1) to a final volume of 800 μL. The organic solvent was evaporated to dryness in the SpeedVac for 1 h, and then the samples were further dried under vacuum in a dessicator for another 1 h. The dry lipid films were hydrated with 1 mL of deionised water yielding a 0.6 mM cationic lipid suspension. The vials were purged with sterile nitrogen passed through a 0.22 μm filter, sonicated at room temperature for 1 min, and then left overnight to hydrate.

The next day, each vial was freeze-thawed 10 times (-70°C/65°C) and subsequently sonicated twice for 15 minutes at 65°C with a 15 minute pause between cycles yielding homogeneous liposomal formulations.

Liposome Characterization A volume of 500 μL of each liposomal preparation was introduced into a disposable Malvern DTS 1060 measurement cell. The size and zeta potential of the liposomes were measured using a Zetasizer Nano (Malvern Instruments). The readings

were all made at 25°C at normal resolution, using the instrument's automated feature. For the size measurements, the volume results were used in all cases, and the results were reported as the average of 10-20 runs. Zeta potentials were measured in millivolts (mV).

Lipoplex preparation and characterization

Solutions of plasmid DNA (gWiz™ Luc plasmid, Aldevon), and ladder Lambda DNA/*Hin*d III (Promega), both 0.05 μg/μL, were prepared in sterile conditions, using nuclease-free water.

Diluted stock solutions (0.2 mM, 300 μL each) of the cationic liposome formulations were made from 100 μL the original stock solutions (0.6 mM) and 200 μL nuclease-free water. In six eppendorff tubes, 20 μL of diluted DNA stock were treated with 16 μL, 32 μL, 48 μL, 64 μL, and 128 μL of diluted liposomal preparation (lipid/DNA ratios of 1/1, 2/1, 3/1, 4/1 and 8/1). The vials were tapped gently for 1 min to ensure proper mixing, and then allowed to rest at room temperature for 30 min for proper lipoplex compaction. The volume of all lipoplex suspensions was adjusted with nuclease-free water to 150 μL. This lipoplex stock solution was used for both gel electrophoresis and size/zeta potential measurements.

Gel electrophoresis of lipoplexes In the gel electrophoresis experiment an amount of 15 μL of each lipoplex formulation was aliquoted out in small eppendorf vials and each vial subsequently received 3 μL of Blue/Orange Loading dye (Promega). A DNA standard was made by mixing 2 μL of diluted DNA stock with 13 μL nuclease free water and 3 μL of of Blue/Orange Loading dye. The same procedure was used to make a ladder reference standard using the Lambda DNA/*Hin*d III marker. The final volume in all vials was 18 μL. The lipoplex/dye mixtures were loaded into a 1% Agarose gel in 1X TAE buffer, pre-stained with GelStar® (Lonza) nucleic acid stain (10 μL in 50 mL gel suspension). Gel electrophoresis was carried out at 75 mV for 75 min. DNA bands were visualized with a Mighty Bright transilluminator (Hoefer), and the gel was photographed with an Olympus C-5060 digital camera.

Lipoplex characterization The remaining 135 μL from each lipoplex preparation was diluted to a final volume of 500 μL with nuclease-free water and transferred into a disposable Malvern DTS 1060 measurement cell. The size and zeta potential of the lipoplexes were measured using a Zetasizer Nano (Malvern Instruments) at 25°C at normal resolution. Volume

results were used for size data, and results were reported as the average of 10-20 runs. Zeta potentials were measured in millivolts (mV).

3. General procedure for transfection and cytotoxicity experiments

Preparation of lipoplexes was done similarly to previous experiment, using a cationic lipid/DNA charge ratio of 3/1. In a typical experiment, for each cationic liposomal formulation to be tested an amount of 3 μL of a 0.5 mg/mL gWiz™ Luc plasmid DNA solution was aliquoted out in a sterile eppendorf vial and was treated with 24 μL of the liposomal formulation (0.6 mM, prepared as indicated above). The vials were tapped gently to ensure proper mixing, and then allowed to rest at room temperature for 30 min for complete lipoplex compaction. Nuclease-free water (92 μL) was used to dilute lipoplex preparation. This lipoplex stock solution was used for transfection, cytotoxicity, size, and zeta potential measurements.

Transfection and viability experiments From the lipoplex stock solution, an amount of 100 μL was aliquoted out for each cationic lipid formulation to be tested, and was diluted with 800 μL Optimem.

The lipoplexes were tested for their ability to transfect six cancer cell lines – two lung carcinomas (NCI-H23, A549), one breast carcinomas (MCF-7), two prostate carcinoma (DU-145, PC3) and a colon carcinoma (Caco-2). The cells were maintained in 10% fetal bovine serum (FBS) enriched medium at 37 ^{o}C in a humidified atmosphere of 95% air/5% CO_2. The following media were used: RPMI 1640 (CellGro, Houston, TX) for NCI-H23, MCF-7, and PC3 cells, Eagle's minimum essential medium (ATCC) for DU-145 and CaCo-2 cells, Ham's F12K medium, Kaighn modification (CellGro) for the A549 cells. Twenty-four hours prior to transfection cells were transferred to 96-well microtiter plates (Cellstar 655180, Greiner Bio-One) at a density of 20,000 cells/well. Each well received 100 μL of appropriate medium, and the plate was incubated in the same conditions as above. All experiments were done in quadruplicate. Two plates were made for each experiment, one for transfection, and another one for cytotoxicity. The error bars in figures represent one standard deviation from the average value.

Immediately before transfection the medium was removed, and the cells from each well were briefly washed with 200 μL sterile PBS. After removal of the PBS solution each well received 100 μL of lipoplex stock solution, and the plates were returned to the incubator for 2

hours. An additional 100 µL of medium was added to each well at this time, and the plates were incubated for further 48 hours, after which the transfection efficacy was determined using the first cell plate and the associated cytotoxicity was assessed using the second cell plate, transfected in similar conditions as the first one.

The pulse transfection experiment was done similarly, except that after 2h incubation time with cells the lipoplexes were removed, cells were washed with sterile PBS and then each well received 200 µL of medium. Cell plates were incubated for further 48 hours, after which the transfection efficacy was determined using the first cell plate and the associated cytotoxicity was assessed using the second cell plate, transfected in similar conditions as the first one.

Transfection efficiency: luciferase and protein content assay

Forty-eight hours after transfection, the medium was aspirated and the wells were washed briefly with 200 µL PBS. After removal of PBS the cells were lysed by adding 100 µL 1X reporter lysis buffer (Promega) to each well and incubating the plate at 37 ^{o}C for 10 minutes. The cell lysate was collected and used for luciferase and protein assays.

For the luciferase assay, 20 µL of cell lysate was transferred to a test tube and assessed directly by means of BD Monolight 3010 luminometer (BD Biosciences, San Jose, CA) using a luciferase assay kit (E4030) from Promega.

The protein content was quantified using a bicinchoninic acid (BCA) assay (Thermo Scientific, Rockford, IL). The BCA assay was prepared as specified in its manufacturer's instructions; 40 µL of cell lysate were treated with 1 mL of BCA reagent in an acryl cuvette and the solution was incubated for 1 hour at 37^{o}C. The light absorption of the solution was then read at 562 nm by means of a Hach DR/4000U UV-VIS Spectrometer (Loveland, CO), and the protein content was estimated by comparison to bovine serum albumin standards. The luciferase activity was normalized by the protein content and expressed as relative luminescence units/µg of protein (RLU/µg protein).

Viability assay In order to quantify the relative cytotoxicity of the non-viral cationic vectors, a WST-1 standard viability method [56] was performed along with the luciferase and BCA assays. Forty-eight hours post-transfection, 20 µL of WST-1 tetrazolium dye solution (Roche, Mannheim, Germany) was added to each well (still containing the serum and the liposomal preparation). A blank was prepared by mixing 100 µL Optimem, 100 µL of serum and 20 µL of tetrazolium dye solution, and the plate was incubated at 37 ^{o}C in the CO_2 incubator.

After 3 hours the colorimetric measurement was performed at 450 nm (with a reference wavelength of 650 nm that was subtracted) by means of a SpectramaxM2 microplate reader (Molecular Devices, Sunnyvale, CA). The value corresponding to the blank was deducted from the value corresponding to each well. Viability was expressed as percentage of the control, represented by cells that underwent the same treatments but without cationic lipoplexes.

Lipoplex characterization The remaining 19 μL from each lipoplex preparation was diluted to a final volume of 500 μL with nuclease-free water and transferred into a disposable Malvern DTS 1060 measurement cell. The size and zeta potential of the lipoplexes were measured using a Zetasizer Nano (Malvern Instruments) at 25°C at normal resolution. Volume results were used for size data, and results were reported as the average of 10-20 runs. Zeta potentials were measured in millivolts (mV).

4. General procedure for transfection experiments in the presence of variable amounts of serum

The preparation and characterization of the liposomes and lipoplexes were done as indicated above, using a lipid/DNA charge ratio of 3/1. Besides DOTAP/Chol 1/1 control, Lipofectamine transfection reagent (Invitrogen, Carlsbad, CA) was used as a positive control, following the manufacturer's protocol. In this case, 3.75 μL of reagent was diluted to 24 μL nuclease-free water and added over 3 μL plasmid DNA stock solution (0.5 mg/mL) pre-diluted to 50 μL water. The vial was tapped gently to ensure proper mixing, and then allowed to rest at room temperature for 30 min for complete lipoplex compaction. Nuclease-free water (50 μL) was used to dilute lipoplex preparation. This lipoplex stock solution was used for transfection, size, and zeta potential measurements.

From all lipoplex stock solutions, an amount of 100 μL was aliquoted out for each cationic lipid formulation to be tested, including the two positive controls, and was diluted with 800 μL of media containing the amount of serum indicated in each case (0%, 5%, 10%, 20%, and 40%). The lipoplexes were tested for their ability to transfect the NCI-H23 cell line, following the same protocol and experimental conditions as indicated above. Immediately before transfection the medium was removed, and the cells from each well were briefly washed with 200 μL sterile PBS. After removal of the PBS solution each well received 100 μL of lipoplex stock solution (containing in this case variable amounts of serum), and the plates were returned

to the incubator for 2 hours. An additional 100 μL of 10% serum medium was added to each well at this time, and the plates were incubated for further 48 hours, after which the transfection efficacy was determined via luminometry and was corrected for protein content in the standard way (indicated above).

5. Neuronal culture, transfection and viability imaging

Neurons were dissociated from 1–2-day-old Sprague–Dawley rats as previously described [60]. In brief, newborn rats were euthanized by decapitation and the brains removed and immersed in ice-cold Hanks balanced salt solution (HBSS; Mediatech, Manassas, VA). Animal protocols were reviewed and approved by the Institutional Animal Care and Use Committee. The nucleus accumbens was identified, removed, and minced; the cells were subjected to enzymatic and mechanical dissociation. A similar procedure was applied for cortical neurons. Cells were plated on 25 mm glass coverslips in Neurobasal-A medium (Invitrogen, Carlsbad, CA) containing 1% GlutaMax (Invitrogen), 2% antibiotic–antimycotic (Mediatech) and 10% fetal bovine serum. Cultures were maintained at 37 °C in a humidified atmosphere with 5% CO2. Cytosine β-arabinofuranoside (1 μM) was added to inhibit glial cell proliferation [66]. Cultures were used for transfection and viability (calcium imaging) after 3 days.

Transfection was performed as indicated above, using lipoplexes generated from **5Ole**/DOPE (1:1 molar ratio) and gWiz™ GFP plasmid (Aldevron, Fargo, ND) at a cationic lipid/DNA charge ratio of 3/1. Before transfection the old media was removed and cells were washed with warm PBS. Lipoplexes were diluted with Neurobasal-A medium and incubated with neurons. After 2h incubation time at 37 °C the transfection media was removed, cells were washed twice with PBS and incubated with Neurobasal-A medium for 24 h.

Viability was assessed via measurements of intracellular Ca^{2+} concentration, $[Ca^{2+}]_I$, performed as previously described [60, 67, 68]. Briefly, cells were incubated with 5 μM fura-2AM (Invitrogen, Carlsbad, CA) in HBSS at room temperature for 45 min, washed three times with dye-free HBSS, and then incubated for another 45 min. Coverslips were mounted in an open bath chamber (RP-40LP, Warner Instruments, Hamden, CT) on the stage of an inverted microscope Nikon Eclipse TiE (Optical Apparatus Co., Ardmore, PA). The microscope was equipped with a Perfect Focus System and a Photometrics CoolSnap HQ2 CCD camera (Roper Scientific, Optical Apparatus Co.). Fura-2 AM fluorescence (emission = 510 nm), following

alternate excitation at 340 and 380 nm, was acquired at a frequency of 0.25 Hz. Images were acquired and analyzed using NIS-Elements AR 3.1 software (Nikon/Optical Apparatus Co.). The ratio of the fluorescence signals (340/380 nm) was converted to Ca^{2+} concentration [61]. Cultured cells with neurites at least three times longer than the cell bodies, which responded to 20 mM KCl solution by an increase in $[Ca^{2+}]^i$ were considered neurons [60].