# Supplemental Material to

## Efficient and Synergetic DNA Delivery with Pyridinium Amphiphiles - Gold Nanoparticle Composite Systems Having Different Packing Parameters

**Adrian Kizewski[1], Marc A. Ilies[2,3]***

[1]College of Science and Technology, Temple University, 1803 N Broad Street, Philadelphia, PA-19122

[2]Department of Pharmaceutical Sciences and Moulder Center for Drug Discovery Research, Temple University School of Pharmacy, 3307 N Broad Street, Philadelphia, PA-19140

[3]Temple Materials Institute, 1803 N Broad Street, Philadelphia, PA-19122

* To whom correspondence should be addressed: Dr. Marc A. Ilies, Department of Pharmaceutical Sciences, Temple University School of Pharmacy, 3307 N Broad Street, Philadelphia, PA-19140, Tel 215-707-1749, Fax 215-707-5620, E-mail: mailies@temple.edu

**Materials.** Sodium citrate dihydrate, carbon tetrabromide, triphenylphosphine, Dowex 1X8-200 were from Sigma-Aldrich, potassium thioacetate was from Alfa Aesar, $HAuCl_4 \cdot 3\ H_2O$ was from LabChem Inc (Pittsburg, PA), $HS\text{-}(CH_2)_{11}O(CH_2CH_2O)_3H$ (TEG-SH) was from Nanoscience Instruments (Phoenix AZ), 12-amino-1-dodecanol was from TCI America (Portland, OR), solvents (HPLC quality), acids and bases were from Fisher Scientific (Pittsburg, PA) or EMD Chemicals (Gibbstown, NJ), deuterated solvents were from Acros (NJ). Trimethylpyrylium hexafluorophosphate was prepared following literature procedure [1]. DOPE were from Avanti Polar Lipids (Alabaster, AL) and was used as received. Tris Acetate EDTA (TAE) buffer, Lambda DNA/*Hin*d III markers, Blue juice – Blue/Orange Loading dye were from Promega (Madison, WI). DNA plasmid - gWiz™ Luc plasmid encoding the firefly luciferase gene was from Aldevron (Fargo, ND). The GelStar Nucleic acid gel stain was from Lonza (Rockland, ME). Agarose (ultrapure) was from Invitrogen (Carlsbad, CA).

**Techniques**: The purity and the structure identity of the intermediary and final products were assessed by a combination of techniques that includes thin-layer chromatography (TLC), high performance liquid chromatography (HPLC), $^1$H- and $^{13}$C-NMR, and elemental analysis.

Melting points were determined on a Thermolyne heating stage microscope (Dubuque, IA), equipped with an Olympus 5X objective and are uncorrected. The NMR spectra were recorded in DMSO-$d_6$, at ≈300 K with a Bruker Avance III spectrometer operating at 400 MHz for $^1$H-NMR, at 100 MHz for $^{13}$C-NMR and at 376 MHz for $^{19}$F-NMR. Chemical shifts are reported as δ values, using TMS as internal standard for proton spectra and the solvent resonance for carbon spectra. Coupling constants (*J*) are reported in Hz. Peak shapes were denoted as follows: s, singlet; d, doublet; t, triplet; q, quadruplet; cv, cvintuplet; sext, sextuplet; hept, heptuplet; m, multiplet; bs, broad singlet. Attributions were done by means of chemical shifts, peak integration, COSY, HMQC and HMBC experiments, and model spectra. HPLC-DAD-MS analysis (ESI) was done on an Agilent 1100 HPLC system equipped with a G1315A DAD and a 6130 quadrupole mass spectrometer. Preparative liquid chromatography was done on a Gilson GX-281 automated LC system equipped with two 333/334 pumps, a Phenomenex Gemini 5 μm C18 110 Å 50 x 30 mm column and a UV/VIS-155 detector. Elemental analyses were done by combustion, for C, H, N, with an a Perkin Elmer 2400 Series II CHNS analyzer and the results were found ± 0.4% within the theoretical values. Combustion and HPLC confirmed the purity of all compounds to be over 95%. Thin layer chromatography (TLC) was carried out on $SiO_2$-precoated aluminum plates (silica

gel with F254 indicator; layer thickness 200 μm; pore size 60 Å, from Sigma-Aldrich), eluted with MeOH/$CHCl_3$ 10/90 (v/v) unless specified otherwise.

**Synthesis of gold nanoparticles** (adapted after [2, 3])

All glassware was cleaned with a solution of aqua regia (3 parts HCl 37% and 1 part $HNO_3$ 68%), washed three times with deionized $H_2O$, and then dried. A stock solution of 0.01 % by weight $HAuCl_4$ was prepared by dissolving 50 mg $HAuCl_4 \cdot 3\ H_2O$ in 500 mL of distilled $H_2O$. A stock solution of 1% by weight sodium citrate was also prepared by dissolving 1 g of sodium citrate dihydrate in 100 mL distilled $H_2O$. Both solutions were filtered through a 0.2 μm filter and stored in the dark.

In a 100 mL round bottom flask equipped with a magnetic stir bar 25 mL of 0.01% $HAuCl_4$ stock solution was diluted to 50 mL with distilled water, heated under stirring to boiling and subsequently treated with 0.71 mL of sodium citrate 1% stock solution. The solution developed a deep blue color initially, which changed to a deep red color in about 1 min. Boiling and stirring was continued for 10 minutes, after which heating was discontinued, and the solution was allowed to cool under stirring to room temperature. The resulting colloidal solution of gold nanoparticles was filtered through a 0.2 μm filter to remove any large aggregates, treated with 500 μL of 0.1 M NaOH to raise the pH to 10 and stored in the dark at room temperature for future use.

**Physicochemical characterization of gold nanoparticles**

The resulting gold nanoparticles were characterized by their aspect, surface plasmon spectrum, dynamic light scattering, zeta potential measurements and transmission electron microscopy (TEM) (Figure 1b-1f).

The plasmonic spectrum was recorded using a Spectramax M2 spectrophotometer in the range 400 – 850 nm. Dynamic light scattering and zeta potential measurements were performed on a Zetasizer Nano ZS (Malvern Instruments, Malvern, UK) using a disposable Malvern DTS 1060 measurement cell. The readings were all made at 25°C at normal resolution, using the instrument's automated feature. A refractive index of 0.200 and a max absorbance of 3.320 were used for gold nanoparticles size measurements. The volume results were considered in all cases,

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

Transmission electron microscopy (TEM) was performed on a JEOL JEM1400 EM with an operating voltage of 80 KeV. In all cases, 2 μL of the suspensions were placed on carbon-coated, type-A 300 mesh copper TEM grids (Ted Pella, Inc., Redding, CA), the excess water was wicked away, and the grids were air-dried for 10-15 min. Images were captured using a Gatan Outer SCAN 1000 CCD Camera. Image capture, processing, and analysis were performed with Gatan Digital Micrograph software. See main text for all results and discussion.

**Synthesis of the 1-(2-mercaptoethyl)-2,4,6-trimethylpyridinium hexafluorophosphate 2**

An amount of 1.5 g (5.6 mmol) of trimethylpyrylium hexafluorophosphate was suspended in 10 mL dichloromethane in a 100 mL round bottom flask. The suspension was stirred for 5 min and was treated with a solution obtained by dissolving 0.453 g (5.87 mmol) aminothiol in 20 mL of dichloromethane, under stirring. Triethylamine (50 μL, 0.35 mmol) was added as a catalyst and the yellow-green homogeneous solution was refluxed for 15 min under inert gas. Acetic acid (100 μL, 1.6 mmol) was added to facilitate the pyridinium ring closure and the solution was refluxed for 1 h, when the initial yellow-green color of the solution faded. The solution was cooled to 25 $^{o}$C, 0.5 mL of 25% aqueous ammonium hydroxide was added, and the solution was brought to reflux 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 ether (3 x 20 mL) to remove the pyridine and excess aminothiol. The ether layer was decanted away and the residue was partitioned between 15 mL 5% aqueous $HPF_6$ and 30 mL DCM in a separatory funnel. The organic layer was removed, the aqueous layer was extracted four more times, using 15 mL of DCM each time. The organic extracts were combined, back-extracted with brine and dried on anhydrous sodium sulfate. Evaporation of the solvent yielded 1.61 g of crude product. Flash chromatography on $SiO_2$ using MeOH/$CH_2Cl_2$ gradients yielding 1.22 g pure pyridinium thiol **2** (66.9% yield).

1-(2-mercaptoethyl)-2,4,6-trimethylpyridinium hexafluorophosphate **2** white crystals, mp = 134-136 $^{o}$C, Yield 68%; $^{1}$H-NMR (dmso-d$^{6}$), δ, ppm: 7.74 (s, 2H, H-3 $Py^{+}$ ), 4.55 (t, $J$ = 7.9 Hz, 2H, $Py^{+}$-C$\underline{H_2}$), 3.06 (t, $J$ = 8.6 Hz, H1, SH), 2.96 (q, $^{2}J_1$ = 16.0 Hz, $^{3}J_2$ = 8.3 Hz, 2H, $Py^{+}$-$CH_2$-C$\underline{H_2}$-

SH), 2.82 (s, 6H, $2CH_3\alpha Py^+$), 2.49 (t, 3H, $CH_3\gamma$ $Py^+$); $^{13}$C-NMR (dmso-d$^6$), δ, ppm: 157.5 (C-4 $Py^+$), 154.4 (2C, C-2,6 $Py^+$), 128.0 (2C, C-3,5 $Py^+$), 53.7 ($Py^+$-$\underline{C}H_2$), 21.0 ($CH_3\gamma$ $Py^+$), 20.8 ($Py^+$-$CH_2$-$\underline{C}H_2$-SH), 20.6 (2C, $2CH_3\alpha Py^+$); $^{19}$F-NMR (dmso-d$^6$), δ, ppm: -70.1 (d, $J$ = 711.3 Hz, $PF_6^-$) Elemental analysis and LC-MS: Found: C, 36.95; H, 5.06; N 4.31%; M+, 182.1. $C_{10}H_{16}F_6NPS$ requires C, 36.70; H, 4.93; N 4.28%; M+, 182.3.

***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 deionized 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 pyridinium salt **2** (as hexafluorophosphate) were weighed and dissolved in MeOH (about4 mL). The MeOH 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 iPrOH and dried under vacuum.

1-(2-mercaptoethyl)-2,4,6-trimethylpyridinium chloride **3** white sticky glue, Yield 86%; $^1$H-NMR (dmso-d$^6$), δ, ppm: 7.74 (s, 2H, H-3 $Py^+$ ), 4.55 (t, $J$ = 7.9 Hz, 2H, $Py^+$-$C\underline{H_2}$), 3.06 (t, $J$ = 8.6 Hz, H1, SH), 2.96 (q, $^2J_1$ = 16.0 Hz, $^3J_2$ = 8.3 Hz, 2H, $Py^+$-$CH_2$-$C\underline{H_2}$-SH), 2.82 (s, 6H, $2CH_3\alpha Py^+$), 2.49 (t, 3H, $CH_3\gamma$ $Py^+$); $^{13}$C-NMR (dmso-d$^6$), δ, ppm: 157.5 (C-4 $Py^+$). 154.4 (2C, C-2,6 $Py^+$), 128.0 (2C, C-3,5 $Py^+$), 53.7 ($Py^+$-$\underline{C}H_2$), 21.0 ($CH_3\gamma$ $Py^+$), 20.8 ($Py^+$-$CH_2$-$\underline{C}H_2$-SH), 20.6 (2C, $2CH_3\alpha Py^+$); $^{19}$F-NMR (dmso-d$^6$), δ, ppm: (No Signal) Elemental analysis and LC-MS: Found: C, 55.35; H, 7.66; N 6.45%; M+, 182.1. $C_{10}H_{16}ClNS$ requires C, 55.16; H, 7.41; N 6.43%; M+, 182.3.

**Synthesis of the 1,6-bis(2,4,6-trimethylpyridinium)-3,4-dithiahexane (di-hexafluorophosphate 4 and dichloride 5)**

In a 50 mL round bottom flask 0.5 g (1.5 mmol) of 1-(2-mercaptoethyl)-2,4,6-trimethylpyridinium hexafluorophosphate **2** was dissolved into 15 mL DCM. Ammonia in methanol (7N, 0.1 mL, 40 mmol) was added to this solution and air was passed through the solution via a capillary until TLC showed no thiol left (about 1h). The solvent was evaporated to dryness and the residue was subjected to flash chromatography on $SiO_2$ using $MeOH/CH_2Cl_2$ gradients followed by crystallization from methanol to yield 0.43 g pure pyridinium disulfide **4** (86% yield). Counterion exchange was performed similarly to thiol **3** on 0.15 g disulfide **4**, yielding 0.095 g of **5** as clear viscous oil (95%).

1,6-Bis(2,4,6-trimethylpyridinium)-3,4-dithiahexane di-hexafluorophosphate **4**: white crystals, mp = 186-189 $^{o}$C, Yield 86%; $^{1}$H-NMR (dmso-d$^{6}$), δ, ppm: 7.78 (s, 4H, H-3Py$^{+}$ ), 4.71 (t, $J$ = 8.1 Hz, 4H, 2Py$^{+}$-C$\underline{H_2}$), 3.35 (m, 4H, 2Py$^{+}$-CH$_2$-C$\underline{H_2}$-S), 2.86 (s, 12H, 2CH$_3$αPy$^{+}$), 2.50 (bs, 6H, 2CH$_3$γPy$^{+}$); $^{13}$C-NMR (dmso-d$^{6}$), δ, ppm: 157.9 (2C, C-4Py$^{+}$), 154.5 (4C, C-2,6Py$^{+}$), 128.2 (4C, C-3,5Py$^{+}$), 51.0 (2C, Py$^{+}$-$\underline{C}$H$_2$), 39.7 (2C, CH3γPy$^{+}$), 32.7 (2C, Py$^{+}$-CH$_2$-$\underline{C}$H$_2$-S), 20.9 (2C, CH$_3$γ Py$^{+}$), 20.4 (4C, CH$_3$αPy$^{+}$); $^{19}$F-NMR (dmso-d$^{6}$), δ, ppm: -70.14 (d, $J$ = 711.2 Hz, $PF_6^-$); Elemental analysis and LC-MS: Found: C, 36.85; H, 4.66; N 4.35%; $M^{2+}/2$, 181.1. $C_{20}H_{30}F_{12}N_2P_2S_2$ requires C, 36.81; H, 4.63; N 4.29%; $M^{2+}/2$, 181.3.

1,6-Bis(2,4,6-trimethylpyridinium)-3,4-dithiahexane di-chloride **5**: clear viscous oil, Yield 95%; $^{1}$H-NMR (dmso-d$^{6}$), δ, ppm: 7.78 (s, 4H, H-3Py$^{+}$ ), 4.71 (t, $J$ = 8.1 Hz, 4H, 2Py$^{+}$-C$\underline{H_2}$), 3.35 (m, 4H, 2Py$^{+}$-CH$_2$-C$\underline{H_2}$-S), 2.86 (s, 12H, 2CH$_3$αPy$^{+}$), 2.50 (bs, 6H, 2CH$_3$γPy$^{+}$); $^{13}$C-NMR (dmso-d$^{6}$), δ, ppm: 157.9 (2C, C-4Py$^{+}$), 154.5 (4C, C-2,6Py$^{+}$), 128.2 (4C, C-3,5Py$^{+}$), 51.0 (2C, Py$^{+}$-$\underline{C}$H$_2$), 39.7 (2C, CH3γPy$^{+}$), 32.7 (2C, Py$^{+}$-CH$_2$-$\underline{C}$H$_2$-S), 20.9 (2C, CH$_3$γ Py$^{+}$), 20.4 (4C, CH$_3$αPy$^{+}$); $^{19}$F-NMR (dmso-d$^{6}$), δ, ppm: no signal; Elemental analysis and LC-MS: Found: C, 55.35; H, 6.66; N 6.05%; $M^{2+}/2$, 181.1. $C_{20}H_{30}Cl_2N_2S_2$ requires C, 55.41; H, 6.98; N 6.46%; $M^{2+}/2$, 181.3.

**Synthesis of 1-(12-mercaptododecyl)-2,4,6-trimethylpyridinium chloride 10 and 1,26-Bis(2,4,6-trimethylpyridinium)-13,14-dithiatetraeicosan chloride 11** (see Scheme 1)

**A) Synthesis of 1-(12-hydroxydodecyl)-2,4,6-trimethylpyridinium hexafluorophosphate 6**

In a 50 mL round bottom flask 1.02 g (5 mmol) of 12-amino-1-dodecanol was suspended in 40 mL DCM and was treated under stirring with a solution obtained by dissolving 1.5 g (5.6 mmol) of trimethylpyrylium hexafluorophosphate **1** in 20 mL DCM. To the homogeneous orange solution triethylamine was added (100 µL, 0.7 mmol) as catalyst and the yellow solution thus obtained was refluxed for 15 min. Acetic acid (3 mL, 16.6 mmol) was added to facilitate the pyridinium ring closure and the solution was subsequently refluxed for 1 h, when the color lightens. After cooling at room temperature 1 mL of 25% aqueous ammonium hydroxide was added, and the solution was brought to reflux 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 ether (3 x 50 mL) to remove the pyridine and excess aminododecanol. The ether layer was decanted away and the residue was absorbed onto 5 g $SiO_2$. Flash chromatography on $SiO_2$ using MeOH/$CH_2Cl_2$ gradients yielded 2.2 g (92%) of pure pyridinium dodecanol **6**.

1-(12-hydroxydodecyl)-2,4,6-trimethylpyridinium hexafluorophosphate **6**: clear viscous oil, Yield 92%; $^1$H-NMR ($CDCl_3$), δ, ppm: 7.47 (s, 2H, H-3 $Py^+$), 4.37 (t, $J$ = 8.5 Hz, 2H, $Py^+$-$\underline{CH_2}$), 3.63 (t, $J$ = 6.6 Hz, 2H, $Py^+$-$(CH_2)_{11}$-$\underline{CH_2}$-OH), 2.78 (s, 6H, $2CH_3\alpha Py^+$), 2.51 (s, 3H, $CH_3\gamma Py^+$), 2.01 (bs, 1H, OH), 1.75 (cv, $J$ = 8.0 Hz, 2H, $Py^+$-$CH_2$-$\underline{CH_2}$), 1.56 (cv, $J$ = 6.9 Hz, 2H, $\underline{CH_2}$-$CH_2$-OH), 1.48 (cv, $J$ = 7.5 Hz, 2H, $2Py^+$-$CH_2$-$CH_2$-$\underline{CH_2}$), 1.43-1.20 (m, 14H, $\underline{CH_2}$ from alkyl chain); $^{13}$C-NMR ($CDCl_3$), δ, ppm: 157.9 (C-4 $Py^+$), 153.7 (2C, C-2,6 $Py^+$), 128.9 (2C, C-3,5 $Py^+$), 63.0 ($CH_2$-OH) 52.4 ($Py^+$-$\underline{C}H_2$), 32.6, 29.5, 29.4, 29.3, 29.3, 28.9, 28.7, 26.6, 25.7 (all $CH_2$ from alkyl chain), 21.4 ($CH_3\gamma Py^+$), 20.7 (2C, $2CH_3\alpha Py^+$); $^{19}$F-NMR ($CDCl_3$), δ, ppm: -73.4 (d, $J$ = 711.8 Hz, $PF_6^-$); LC-MS: Found: $M^+$ 306.3. $C_{20}H_{36}NO^+$ requires $M^+$, 306.3.

**B) Synthesis of 1-(12-bomododecyl)-2,4,6-trimethylpyridinium hexafluorophosphate 7**

The pyridinium dodecanol **6** (1.96 g, 4.35 mmol) was dissolved in 40 mL dry DCM together with 2.17 g (6.53 mmol) of dry carbon tetrabromide and was treated dropwise, under stirring, with a solution obtained by dissolving 1.71 g (6.53 mmol) of triphenylphosphine in 5 mL of dry DCM. The addition of the $PPh_3$ solution was continued until TLC (5/95 MeOH/DCM) showed complete conversion of alcohol to bromide. The solvent was evaporated to dryness and the crude product was absorbed onto 5 g $SiO_2$. Flash chromatography on $SiO_2$ using MeOH/$CH_2Cl_2$ gradients yielded 1.9 g (85%) of pure 12-bromododecylpyridinium **7**.

**1-(12-bomododecyl)-2,4,6-trimethylpyridinium hexafluorophosphate 7**: yellowish amorphous compound; Yield 85%; $^1$H-NMR ($CDCl_3$), δ, ppm: 7.47 (s, 2H, H-3 $Py^+$), 4.36 (t, $J$ = 8.6 Hz, 2H, $Py^+$-$\underline{CH_2}$), 3.42 (t, $J$ = 6.8 Hz, 2H, $Py^+$-$(CH_2)_{11}$-$\underline{CH_2}$-Br), 2.77 (s, 6H, $2CH_{3\alpha}Py^+$), 2.50 (s, 3H, $CH_{3\gamma}Py^+$), 1.85 (cv, $J$ = 7.2 Hz, 2H, $\underline{CH_2}$-$CH_2$-Br), 1.75 (cv, $J$ = 7.8 Hz, 2H, $Py^+$-$CH_2$-$\underline{CH_2}$), 1.47 (cv, $J$ = 7.6 Hz, 2H, $Py^+$-$CH_2$-$CH_2$-$\underline{CH_2}$), 1.39-1.20 (m, 14H, $\underline{CH_2}$ from alkyl chain); $^{13}$C-NMR ($CDCl_3$), δ, ppm: 157.9 (C-4$Py^+$), 153.7 (2C, C-2,6$Py^+$), 128.9 (2C, C-3,5$Py^+$), 63.0 ($CH_2$-OH) 52.4 ($Py^+$-$\underline{C}H_2$), 34.2 ($\underline{C}H_2$-Br), 32.8 ($\underline{C}H_2$-$CH_2$-Br), 29.4, 29.35, 29.3, 28.9, 28.73, 28.7, 28.1 26.6 (all $\underline{C}H_2$ from alkyl chain), 21.4 ($CH3_{\gamma}Py^+$), 20.8 (2C, $2CH_{3\alpha}Py^+$); $^{19}$F-NMR ($CDCl_3$), δ, ppm: -73.5 (d, $J$ = 712.6 Hz, $PF_6^-$); LC-MS: Found: $M^+$, 368.1. $C_{20}H_{35}BrN^+$ requires $M^+$, 368.2.

**C) Synthesis of 1-(12-acetylthiododecyl)-2,4,6-trimethylpyridinium hexafluorophosphate 8 and chloride 9**

Potassium thioacetate 0.214 g (1.87 mmol) was suspended in 20 mL anhydrous acetone and treated with a solution obtained by dissolving 0.321 grams (0.624 mmol) of pyridinium dodecylbromide **7** in 5 mL dry acetone. The suspension was refluxed for 1 h (TLC and NMR control), after which the solvent was evaporated to dryness and the crude product was subjected to flash chromatography on $SiO_2$ using MeOH/$CH_2Cl_2$ gradients (0/100 to 10/90 v/v). The pure fractions collected were grouped and the solvent was evaporated, yielding 110 mg pyridinium thioacetate **8** as hexafluorophosphate. An additional 47 mg product were obtained after grouping the impure fractions and running the flash chromatography one more time, thus raising the overall

yield to 49.4%. Reaction was redone using the same procedure on 0.5 g bromide **7** yielding an additional 257 mg thioacetate **8** (52%).

**1-(12-Acetylthiododecyl)-2,4,6-trimethylpyridinium hexafluorophosphate 8**: Pale-yellow amorphous compound; Yield: 52%; $^{1}$H-NMR ($CDCl_3$), δ, ppm: 7.43 (s, 2H, H-3 $Py^+$), 4.44 (m, 2H, $Py^+$-$\underline{CH_2}$), 2.88 (t, *J* = 7.4 Hz, 2H, $Py^+$-$(CH_2)_{11}$-$\underline{CH_2}$-SCO), 2.83 (s, 6H, $2CH_{3\alpha}Py^+$), 2.55 (s, 3H, $CH_{3\gamma}Py^+$), 2.34 (s, 3H, SCO-CH3), 1.78 (m, 2H, $Py^+$-$CH_2$-$\underline{CH_2}$), 1.56 (cv, *J* = 7.3 Hz, 2H, $\underline{CH_2}$-$CH_2$-SCO-$CH_3$), 1.51 (m, 2H, $Py^+$-$CH_2$-$CH_2$-$\underline{CH_2}$), 1.45-1.20 (m, 14H, $\underline{CH_2}$ from alkyl chain). LC-MS: Found: $M^+$, 364.4. $C_{22}H_{38}NOS^+$ requires M+, 364.3.

Ion Exchange ($PF_6^-$ to $Cl^-$): Pyridinium dodecylthioacetate hexafluorophosphate **8** (250 mg, 0.5 mmol) was dissolved in about 15 mL of methanol and the counterion was exchanged to chloride using Dowex 1X8-200 ion exchange resin following the same protocol as in the case of compound **3**. Evaporation of methanol yielded chloride **9** in quantitative yield, which was purified via preparative liquid chromatography using MeCN/$H_2O$ gradients (from 5/95 to 95/5), to generate 173 mg of pure product (88%).

**1-(12-Acetylthiododecyl)-2,4,6-trimethylpyridinium chloride 9**: pale yellow amorphous compound; Yield: 88%; $^{1}$H-NMR ($CDCl_3$), δ, ppm: 7.44 (s, 2H, H-3 $Py^+$), 4.55 (t, *J* = 8.5 Hz, 2H, $Py^+$-$\underline{CH_2}$), 2.88 (s, 6H, $2CH_{3\alpha}Py^+$), 2.86 (t, *J* = 7.4 Hz, 2H, $Py^+$-$(CH_2)_{11}$-$\underline{CH_2}$-SCO), 2.52 (s, 3H, $CH_{3\gamma}Py^+$), 2.32 (s, 3H, SCO-CH3), 1.74 (cv, *J* = 7.8 Hz, 2H, $Py^+$-$CH_2$-$\underline{CH_2}$), 1.56 (cv, *J* = 7.2 Hz, 2H, $\underline{CH_2}$-$CH_2$-SCO-$CH_3$), 1.49 (cv, *J* = 7.6 Hz, 2H, $Py^+$-$CH_2$-$CH_2$-$\underline{CH_2}$), 1.40-1.20 (m, 14H, $\underline{CH_2}$ from alkyl chain); $^{13}$C-NMR ($CDCl_3$), δ, ppm: 196.1 (S$\underline{C}$O-$CH_3$), 157.2 (C-4$Py^+$), 154.3 (2C, C-2,6Py+) , 128.6 (2C, C-3,5$Py^+$), 52.8 ($Py^+$-$\underline{C}H_2$), 30.7 ($\underline{C}H_3$-COS), 29.5, 29.45, 29.4, 29.35, 29.1, 29.09, 29.06, 28.9, 28.8, 26.7 (all $\underline{C}H_2$ from alkyl chain), 21.5 ($CH_{3\gamma}Py^+$), 21.3 (2C, $2CH_{3\alpha}Py^+$); $^{19}$F-NMR ($CDCl_3$), δ, ppm: (no signal); LC-MS: Found: $M^+$, 364.4. $C_{22}H_{38}NOS^+$ requires M+, 364.3.

**D) Synthesis of 1-(thiododecyl)-2,4,6-trimethylpyridinium chloride 10 and disulfide 11**

In a 25 mL round bottom flask 60 mg (0.15 mmol) pyridinium dodecylthioacetate **9** was dissolved into 2 mL of degassed MeOH and treated with 100 µL of aqueous HCl 37 %. The mixture was refluxed under nitrogen for 1 h, then the solvent was evaporated to dryness and the residue was subjected to reverse phase liquid chromatography using MeCN(0.1% HCOOH)/$H_2O$ (0.1% HCOOH) gradients (from 5/95 to 95/5), to generate 40.3 mg of pure pyridinium thiol **10** (75%).

**1-(12-Mercaptododecyl)-2,4,6-trimethylpyridinium chloride 10**: $^1$H-NMR ($CDCl_3$), δ, ppm: 7.48 (s, 2H, H-3 $Py^+$), 4.60 (t, *J* = 8.5 Hz, 2H, $Py^+$-$\underline{CH_2}$), 2.91 (s, 6H, $2CH_{3\alpha}Py^+$), 2.53 (s, 3H, $CH_{3\gamma}Py^+$), 2.52 (q, *J* = 7.4 Hz, 2H, $\underline{CH_2}$-SH), 1.76 (cv, *J* = 7.8 Hz, 2H, $Py^+$-$CH_2$-$\underline{CH_2}$), 1.60 (cv, *J* = 7.1 Hz, 2H, $\underline{CH_2}$-$CH_2$-SH), 1.51 (cv, *J* = 7.6 Hz, 2H, $Py^+$-$CH_2$-$CH_2$-$\underline{CH_2}$), 1.88-1.44 (m, 14H, $\underline{CH_2}$ from alkyl chain), 1.33 (t, *J* = 7.7 Hz, 1H, SH); $^{13}$C-NMR ($CDCl_3$), δ, ppm: 157.4 (C-4$Py^+$), 154.2 (2C, C-2,6Py+) , 128.9 (2C, C-3,5$Py^+$), 53.4 ($Py^+$-$\underline{C}H_2$), 34.0 ($\underline{C}H_2$-$CH_2$-SH), 29.5, 29.4, 29.39, 29.34, 29.14, 29.12, 29.1, 29.0, 28.4 (all $\underline{C}H_2$ from alkyl chain), 24.7 ($\underline{C}H_2$-SH), 21.8 (2C, $2CH_{3\alpha}Py^+$), 21.7 ($CH_{3\gamma}Py^+$); $^{19}$F-NMR ($CDCl_3$), δ, ppm: no signal; Elemental analysis-LC-MS: Found: C, 67.31; H, 10.06; N 4.05%; $M^+$, 322.2. $C_{20}H_{36}ClNS$ requires C, 67.09; H, 10.14; N 3.91%; $M^+$, 322.3.

In a 25 mL round bottom flask 40 mg (0.1 mmol) pyridinium dodecylthioacetate **9** was dissolved into 2 mL of degassed MeOH and treated with 50 mg $K_2CO_3$. The mixture was stirred overnight at room temperature, then the solvent was evaporated to dryness and the residue was subjected to reverse phase liquid chromatography using MeCN(0.1% HCOOH)/$H_2O$ (0.1% HCOOH) gradients (from 5/95 to 95/5), to generate 30.4 mg of pure disulfide **11** (85%).

**1,26-Bis(2,4,6-trimethylpyridinium)-13,14-dithiatetraeicosan chloride 11**: $^1$H-NMR ($CDCl_3$), δ, ppm: 7.49 (s, 2H, H-3 $Py^+$), 4.53 (t, *J* = 8.5 Hz, 2H, $Py^+$-$\underline{CH_2}$), 2.86 (s, 6H, $2CH_{3\alpha}Py^+$), 2.59 (t, *J* = 7.4 Hz, 2H, $Py^+$-$(CH_2)_{11}$-$\underline{CH_2}$-S), 2.45 (s, 3H, $CH_{3\gamma}Py^+$), 1.66 (cv, *J* = 7.9 Hz, 2H, $Py^+$-$CH_2$-$\underline{CH_2}$), 1.58 (cv, *J* = 7.3 Hz, 2H, $\underline{CH_2}$-$CH_2$-S), 1.42 (cv, *J* = 7.5 Hz, 2H, $2Py^+$-$CH_2$-$CH_2$-$\underline{CH_2}$), 1.35-1.05 (m, 14H, $\underline{CH_2}$ from alkyl chain); $^{13}$C-NMR ($CDCl_3$), δ, ppm: 156.8 (C-4$Py^+$), 153.4 (2C, C-2,6Py+) , 128.2 (2C, C-3,5$Py^+$), 52.5 ($Py^+$-$\underline{C}H_2$), 38.5 ($\underline{C}H_3$-COS), 28.9, 28.7, 28.69, 28.62, 28.60,

28.42, 28.38, 28.32, 28.2, 27.7 (all $\underline{C}H_2$ from alkyl chain), 26.0, 20.92 ($CH_{3\gamma}Py^+$), 20.86 (2C, $2CH_{3\alpha}Py^+$); $^{19}$F-NMR ($CDCl_3$), δ, ppm: (no signal); Elemental analysis-LC-MS: Found: C, 67.44; H, 10.15; N 4.07%; $M^{2+}/2$, 321.5. $C_{40}H_{70}Cl_2N_2S_2$ requires C, 67.28; H, 9.88; N 3.92%; $M^{2+}/2$, 321.2.

**Surface modification of Au nanoparticles with TEG-SH and Pyridinium thiol 10**

Stock solutions of thiols **2**, **3**, **10**, disulfides **4**, **5**, **11** and TEG-SH **12** were prepared in nitrogen-degassed absolute EtOH, each at a concentration of 0.02 M, as recommended in literature [4, 5].

All attempts to change the surface ligands from citrate directly to pyridinium thiols through incubation of the citrate-decorated Au NPs with dilute EtOH solutions of thiols **2**, **3**, **10**, or disulfides **4**, **5**, **11** failed (flocculation of gold colloid occurred in all cases). We decided to co-decorate the Au surface with TEG-SH **12** in order to stabilize the Au NPs during the charge reversal process that occurs during ligand exchange. The TEG-SH **12** alone can stabilize the AuNPs in solution, as proved by following experiment.

**1. Colloidal Surface Modification of Au NPs with TEG-SH 12:**

In a polyalomer centrifuge tube 4 mL colloidal solution of Au NPs prepared as indicated above was centrifuged at 15500 g (15000 rpm) for 15 minutes at room temperature. The supernatant was discarded and the red Au NPs pellet was treated directly with 60 µL of TEG-SH **12** ethanol stock (2 x $10^{-2}$ M), mixed and diluted with 1.5 mL DI water. The suspension was sonicated for 5 min, to ensure thorough ligand exchange. The color remained similar to that of the initial colloidal Au NPs stock, indicating a nanosystems size similar to the starting one. The colloidal solution was centrifuged at 15500 g (15000 rpm) for 15 minutes at room temperature to pellet the Au NPs, the supernatant was removed, and the functionalized Au NPs nanoparticles were resuspended in 1.5 mL DI water. The physicochemical properties of the TEG-SH- functionalized Au NPs (size, zeta potential, surface plasmon spectrum) were assessed as presented above. The average size of the Au NP increased from 21 nm to 25 nm while the average zeta potential remained relatively constant (initial value – 8.4 mV, final value – 7.9 mV). There was only one

major peak in the surface plasmon spectra, with a maximum at 527 nanometers, similar to the initial value. Lack of aggregation was confirmed by the surface Plasmon spectra which did not show any peaks above 600 nm wavelength.

**2. Colloidal Surface Modification of Au NPs with Pyridinium thiol 10 and TEG-SH 12:**

In a polyalomer centrifuge tube 4 mL colloidal solution of Au NPs prepared as indicated above was centrifuged at 15500 g (15000 rpm) for 15 minutes at room temperature. The supernatant was discarded and the red Au NPs pellet was treated directly with 60 μL of TEG-SH **12** ethanol stock (2 x $10^{-2}$ M) and 20 μL of Pyridinium thiol **10** (2 x $10^{-2}$ M) at the same time, mixed and diluted with 1.0 mL DI water. Sonication was performed for 5 minutes, to ensure a homogenous solution and was stored overnight at room temperature. The next day the color of colloidal solution was unchanged indicating a stable nanosystems with NP size similar to the starting one. The physicochemical properties of the crude pyridinium/TEG co-decorated Au NPs are presented in Figure 1 (bright red color, average size 27.0 nm, average zeta potential + 23.3 mV, one major peak in surface plasmon centered at 519 nanometers). There were no signs of aggregation based on the surface Plasmon spectra (no peak over 600 nm).

The solution was transferred into a polyalomer centrifuge tube, and centrifuged at 15500 x g (15000 rpm) for 15 minutes at room temperature. After centrifugation was completed the supernatant (transparent in color) was removed, and the light red pellet was resuspended in 1.5 mL DI water. The physicochemical properties of the pyridinium/TEG co-decorated Au NPs are presented in Figure 1 (bright red color, average size 27.8 nm, average zeta potential + 29.1 mV, one major peak in surface plasmon centered at 520 nanometers). There were no signs of aggregation based on the surface Plasmon spectra (no peak over 600 nm). Assuming an equal affinity of the two thiols for the Au surface, and complete removal of the weak citrate ligand by the strong thiol chemisorption, we considered the surface derivatized with TEG-SH **12**/PySH **10** at a ratio of 3/1. Calculated total positive charge /mL co-decorated Au NP: 1.33 x $10^{15}$.

Mention must be made that when the ratio of TEG-SH **12**/PySH **10** was less than 3/1 (e. g. 2/1 or 1/1) the final nanosystems was not stable in time. The PySH **10** can be substituted with disulfide **11** but longer incubation times (48 h) are required for generating a homogeneous

nanosystem The shorter thiols **2**, **3** and disulfides **4** and **5** were not able to generate a stable nanosystems even in the presence of TEG-SH **12**.

**Lipoplex generation from cationic liposomes and DNA with or without pyridinium Au NPs**

***Liposomal preparation and characterization***

***Liposome Preparation:*** Stock solutions (3 mM) of the cationic lipid **Ole** were prepared from powder in glass vials using $CHCl_3$/MeOH (2/1) as solvent (organic stock). For DOPE a solution of the same concentration (3 mM) was made in $CHCl_3$. All solutions were swirled, purged with nitrogen, and capped securely; when not in use they were stored in the -20 $^oC$ freezer.

Two preparations were made for **Ole** lipid – lipid alone, and lipid mixed with an equimolar amount of DOPE. Thus 200 μL of the **Ole** organic stock solution was transferred into two glass vials (total cationic lipid in each vial was 600 nmol). For the **Ole**/DOPE 1/1 formulation 200 μL of DOPE stock was added into the second vial. The volume was adjusted to 600 μL with $CHCl_3$/MeOH (2/1). The organic solvent was evaporated to dryness in the SpeedVac for 1 h, and then the samples were further dried under vacuum in a desiccator for another 1 h. The dry lipid films were hydrated with 1 mL of deionized 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) as indicated above.

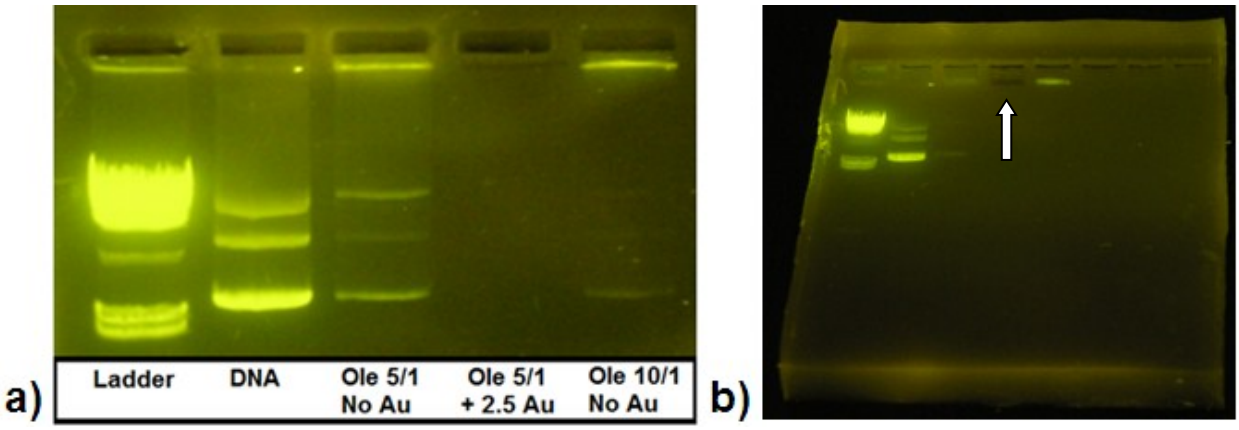
***Lipoplex preparation and characterization***

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. A

secondary Ole liposomal stock solution (0.1 mM) was used in this experiment, generated through dilution with DI water of the concentrated liposomal stock solution (0.6 mM) previously prepared.

In three eppendorff tubes, 5 μL of diluted DNA stock were treated with either 40 μL **Ole** liposomes, 40 μL **Ole** liposomes (0.1 mM) and 910 μL pyridinium-decorated Au NPs (PyAuNPs), and with 80 μL **Ole** liposomes (0.1 mM) (lipid/DNA ratios of 5/1, 5(lipid)+2.5(PyAuNPs)/1, 10/1). 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. From the **Ole**/DNA 5/1 lipoplex 10 μL were removed for gel electrophoresis, while from **Ole**/DNA 10/1 lipoplex 15 μL were removed for the same purpose. The remaining lipoplex solution was used in both cases for size and zeta potential measurements. In the case of mixed **Ole**/PyAuNPs/DNA 5(lipid)+2.5(PyAuNPs)/1 formulation the suspension was centrifuged 5 min at 1000 rpm when all lipoplexes were pelleted at the bottom of the vial. An amount of 880 μL supernatant were removed and the pellet was resuspended in remaining 75 μL aqueous solution. Out of this lipoplex suspension 15 μL were used for gel electrophoresis experiment and the remaining lipoplex solution was used for size and zeta potential experiments.

***Gel electrophoresis of lipoplexes*** In the gel electrophoresis experiment an amount of 10 μL of **Ole**/DNA 5/1 lipoplex , 15 μL mixed **Ole**/PyAuNPs/DNA 5(lipid)+2.5(PyAuNPs)/1, 15 μL **Ole**/DNA 10/1 formulation was aliquoted out in three small eppendorf vials. The volume of first vial was adjusted to 15 μL with DI water. 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 at normal incidence (perpendicular, see below Supplemental Figure 1a and Figure 2) and at an angle of 45 o in order to allow better observance of mixed Ole/PyAuNPs/DNA lipoplexes (see below Supplemental Figure 1b).




Supplemental Figure 1. Agarose gel electrophoretic mobility of lipoplexes generated from Ole cationic lipid and gWizLuc plasmid DNA in the absence and in the presence of pyridinium-decorated Au NPs: frontal aspect of gel (a) and gel photographed at 45$^{o}$ angle (b), revealing the Ole/PyAuNP/DNA complexes (arrow) of red-violet color. A DNA ladder generated from Lambda DNA/*Hin*d III and the initial gWizLuc plasmid were used as references.

***Lipoplex characterization*** The remaining lipoplex preparation from each formulation 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, as indicated above.

***General procedure for transfection and cytotoxicity experiments***

The lipoplexes were tested for their ability to transfect the NCI-H23 lung cancer cell line that was proved to have a good sensitivity to transfection by pyridinium-based synthetic transfection systems.[6-8] The cells were maintained in 10% fetal bovine serum (FBS) enriched RPMI 1640 medium (CellGro, Houston, TX) at 37 $^{o}$C in a humidified atmosphere of 95% air/5% $CO_2$. Twenty-four hours prior to transfection cells were transferred to 96-well microtiter plates (Cellstar 655180, Greiner Bio-One) at a density of 2000 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.

***Preparation of lipoplexes*** was done similarly to previous experiment with the exception that more concentrated solutions of liposomes (0.6 mM instead of 0.1 mM) and PyAuNPs (total positive charge /mL co-decorated Au NP: $2.66 \times 10^{15}$ instead of $1.33 \times 10^{15}$) were used in order to allow the encapsulation and delivery of 0.016 μg DNA per well containing 2000 cells in a reasonably small volume.

In the first experiment (no co-lipid used in liposomal formulations), 5 μL of a 0.5 mg/mL gWiz™ Luc plasmid DNA solution were diluted to a final volume of 50 μL with DI water. An amount of 3 μL of the diluted DNA solution were aliquoted out in 4 small eppendorff vials and were treated with either 4 μL **Ole** liposomes (0.6 mM), 4 μL **Ole** liposomes (0.6 mM) and 273 μL PyAuNPs, 4 μL **Ole** liposomes (0.6 mM) and 576 μL PyAuNPs and with 80 μL **Ole** liposomes (0.1 mM) (lipid/DNA ratios of 5/1, 5(lipid)+2.5(PyAuNPs)/1, 5(lipid)+5(PyAuNPs)/1, 10/1). 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. For the **Ole**/DNA 5/1 and 10/1 lipoplexes the volume was adjusted to 32 μL with DI water. The mixed **Ole**/PyAuNPs/DNA 5(lipid)+2.5(PyAuNPs)/1 and **Ole**/PyAuNPs/DNA 5(lipid)+5(PyAuNPs)/1 formulation were centrifuged 5 min at 1000 rpm when all lipoplexes were pelleted at the bottom of the vial and an amount of 248 μL supernatant were removed from the first formulation and 551 μL from the second one and the pellet was resuspended in remaining 32 μL aqueous solution.

For transfection/cytotoxicity experiments 27 μL of the lipoplex formulations were diluted with 873 μL Optimem® (Invitrogen) and the 100 μL of Optimem-based lipoplex suspension were placed in each well containing 2000 cells (4 wells/each formulation, two plates). The two 96-well plates were returned to the incubator for 30 min, then the lipoplex formulation was removed and cells in each well were washed with 200 μL of PBS. After removal of PBS each well received 100 μL media, 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.

In the second experiment co-lipids used in liposomal formulations: an **Ole**/DOPE 1/1 formulation (0.6 mM) was prepared as described above. The commercial reagent Lipofectamine® (Invitrogen) was used as control, following the manufacturer's specifications. A diluted stock of approximate the same lipid concentration as **Ole**/DOPE 1/1 was generated by diluting 2 μL of commercial reagent was to a final volume of 20 μL.

In the actual experiment 5 μL of a 0.5 mg/mL gWiz™ Luc plasmid DNA solution were diluted to a final volume of 50 μL with DI water. An amount of 3 μL of the diluted DNA solution were aliquoted out in 10 small eppendorff vials and were treated with either 0.8 μL **Ole**/DOPE liposomes, 0.8 μL **Ole**/DOPE liposomes + 109 μL PyAuNPs (added simultaneously), 0.8 μL **Ole**/DOPE liposomes + 109 μL PyAuNPs (first added **Ole**/DOPE 1/1, vial tapped 1 min, then added the PyAuNPs), 0.8 μL **Ole**/DOPE liposomes + 109 μL PyAuNPs (first added PyAuNPs, vial tapped 1 min, then added the **Ole**/DOPE 1/1), 0.8 μL **Ole**/DOPE liposomes + 218 μL PyAuNPs (added simultaneously), 2.4 μL **Ole**/DOPE liposomes, 109 μL PyAuNPs, 218 μL PyAuNPs, 3.67 μL diluted Lipofectamine®, and 1 μL water (as negative control), which corresponds to charge ratios cationic species/DNA of: lipid/DNA 1/1, lipid+1(PyAuNPs)/1 (simultaneous mixing), lipid+1(PyAuNPs)/1 (sequential mixing, lipid first), lipid+1(PyAuNPs)/1 (sequential mixing, lipid second), lipid+1(PyAuNPs)/1 (simultaneous mixing), lipid/DNA 3/1, 1(PyAuNPs)/1, 2(PyAuNPs)/1, Lipofectamine®/DNA 3/1, negative control (only DNA). 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 lipoplex volume was adjusted to 32 μL with DI water or through centrifugation, removal of supernatant and resuspension in the case of gold-containing lipoplexes, as described in the first transfection experiment.

For transfection/cytotoxicity experiments 27 μL of the lipoplex formulations were diluted with 873 μL Optimem® (Invitrogen) and the 100 μL of Optimem-based lipoplex suspension were placed in each well containing 2000 cells (4 wells/each formulation, two plates). The two 96-well plates were returned to the incubator for 30 min, then the lipoplex formulation was removed and cells in each well were washed with 200 μL of PBS. After removal of PBS each well received 100 μL media, 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.

***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 15 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 [9] was performed along with the luciferase and BCA assays. Forty-eight hours post-transfection, 10 μL of WST-1 tetrazolium dye solution (Roche, Mannheim, Germany) was added to each well (still containing the regular media). A blank was prepared by mixing 100 μL of media and 10 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.

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