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# Efficient Gene Delivery into Cells by a Surprisingly Small Three-Armed Peptide Ligand

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## Materials and Methods

**General Remarks:** Solvents were dried and distilled before use. Millipore water was obtained with a Micropure from *TKA*. All reactions were carried out in oven dried glassware. Lyophilization was carried out with an Alpha 1-4 2D plus freeze drying apparatus from *Christ*. Analytical TLC was carried out on $SiO_2$ aluminium foils ALUGRAM SIL G/$UV_{254}$ from *Macherey-Nagel*. Reversed phase column chromatography was done with an *Armen Instrument* Spot Flash Liquid Chromatography MPLC apparatus with *RediSep* C-18 Reversed-Phase columns. The IR spectra were recorded on a FT-IR 430 spectrometer from *Jasco* with a *Pike* MIRacle ATR sampling accessory. Bands are quoted in $cm^{-1}$ and the following abbreviations are used: w, weak; m, medium; s, strong; br., broad. $^{1}H$- and $^{13}C$-NMR spectra were recorded on a DRX 500 MHz spectrometer from *Bruker* at ambient temperature. The chemical shifts are reported in parts per million (ppm) relative to the deuterated solvent $CDCl_3$ or DMSO-$d_6$. The following abbreviations are used for peak multiplicities: s, singlet; d, doublet, m, multiplet; br, broad. HR-ESI-mass spectra were received by using a *Bruker* BioTOF III. Melting points were obtained in open glass capillary tubes using an apparatus from *Büchi* and are quoted uncorrected. Determination of pH values was carried out with a pH-Meter 766 Calimatic from *Knick*. UV spectra were obtained with a *Jasco* V-660 spectrometer, fluorescence spectra with a *Varian* Cary Eclipse spectrometer. Isothermal Titration Calorimetry (ITC) experiments were conducted on a *Microcal* VP-ITC microcalorimeter. Origin 7.0 software, supplied by the manufacturer, was used for data acquisition and analysis. Microwave assisted SPPS was carried out with a *CEM* Discover. AFM imaging was carried out with an Innova Scanning Prope Microscope from *Veeco* and *Olympus* N-type silicon cantilevers AC-160TS in tapping mode on freshly cleaved mica surface from *Plano GmbH*. The analysis was done utilizing the software Gwyddion (Vers. 2.19). Dynamic Light Scattering (DLS) experiments were performed using a Zetasizer-Nano ZS from *Malvern* equipped with a 4 mW He-Ne laser (633 nm wavelength) at a fixed detector angle of 173° with an avalanche photodiode detector.

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**Cell lines and DNA:** The human embryonic kidney cell line HEK293T (ATCC-No. CRL-1573), the human cervix carcinoma cell line HeLa (ATCC-No.CCL-2), and the murine fibroblast cell line NIH/3T3 (ATCC-No. CRL-1658) were obtained from the American Type Culture Collection and maintained as recommended in complete Dulbecco`s Modified Eagle Medium (DMEM) supplemented with 10 % fetal bovine serum, 1 % Glutamine and 1 % Antibiotic-Antimycotic (*Invitrogen*) at 37° C in a humidified atmosphere of 5 % $CO_2$. Calf thymus DNA (ctDNA) was obtained from *Aldrich*, dissolved in sodium cacodylate buffer (0.05 M, Ph 7), cooled to 4° C for 20 h, sonicated (8 × 4 sec) and filtered through a 0.45 µm PTFE filter.

**Transfection and Microscopy:** Per well, $1 \times 10^4$ cells were seeded in 96 well cell culture plates (*Greiner bio-one*) in a total medium volume of 200 µl 24 h before transfection. The cationic transfection reagent polyethylenimine (PEI, pH 6.8, *Sigma-Aldrich*) was used at a concentration of 0.12 mM or 0.24 mM in PBS (*Invitrogen*) with 2 µg of plasmid DNA per well. Transfection with **1** and **2** was carried out in a concentration range of 0.12, 0.18, 0.24 and 0.30 mM with 2 µg plasmid DNA in a total volume of 30 µl in PBS buffer. **2** was also tested at 0.36 mM. Transfection efficiency was analyzed 24 and 48 h after transfection with an inverted fluorescence microscope (Axiovert 200M, *Carl Zeiss*) with a 10X air objective. Images were processed and analyzed using MetaMorph 6.3r6 (*Molecular Devices*) and Adobe Photoshop CS2 (*Adobe Systems*).

**Endosomal Escape Assay:** To facilitate endosomal escape, cells were incubated with complete medium containing 25 µM chloroquine 30 min before transfection. Transfection was carried out with 0.24 mM **1**, **2**, or PEI and 2 µg plasmid-DNA.

**Alamar Blue Cell Viability Assay:** HEK293T cells were grown and transfected as described above. Before transfection, 24 h and 48 h after transfection cells were incubated with Alamar Blue dye (*Invitrogen*, 10 % v/v) for 3 h at 37° C at 5 % $CO_2$. Fluorescence was measured at 590 nm using a multimode reader (GloMax-Multi+DetectionSystem, *Promega*).

**Plasmid DNA Labeling and Co-localization Experiments:** Plasmid DNA was labeled with the PromoFluor-500 Nick Translation Labeling Kit (*PromoKine*) according to the manufacturer's

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instructions. Briefly, following nick translation of the pF143-GFP vector with PromorFluor-500-dUTPs for 2 h at 15° C, cleanup of the mixture was performed using a DNA purification Kit (*Macharey-Nagel*) as advised. For transfection, Hela cells were seeded in 8-well plates, grown for 24 h and transfected with 2 µg of Lamp1-RFP Plasmid-DNA mixed with 1 mM PEI. After an additional incubation of 24 h, cells were transfected again with 0.2 µg of labeled DNA mixed with 0.24 mM PEI, **1**, **2**, or with the labeled DNA alone and examined under a confocal fluorescence microscope (SP5 LCSM, *Leica*) 2 and 4 h after transfection. Images were processed using LAS AF software (*Leica*) and *Adobe* Photoshop CS2.

**UV/Vis Titrations:** All measurements were carried out in aqueous sodium cacodylate buffer (0.01 M, pH 7.00 ± 0.01) in quartz UV microcuvettes (1 cm) equipped with a stopper at 25° C. The pH was adjusted with aqueous HCl or NaOH. To a solution of **1** or **2** (800 µL, $2 \times 10^{-5}$ M) a stock solution of ctDNA ($7.5 \times 10^{-4}$ M) was added in aliquots (5 – 100 µL; total: 700 µL).

**Ethidium Bromide Displacement Assay:** Spectra were recorded at 25° C in aqueous sodium cacodylate buffer (0.01 M, pH 7.00 ± 0.01) in quartz fluorescence microcuvettes (1 cm) equipped with a stopper. To a solution of ethidium bromide (900 µL, 0.75 µM, 1 eq) ctDNA or pF143-GFP plasmid (3.00 µM, 4 eq) was added and incubated for 15 min. The fluorescence emission was then measured from 560 to 650 nm utilizing an excitation wavelength of 520 nm. To this mixture a stock solution (50 µM) of **1**, **2** or **3** was added in aliquots (1–32 µl). After each addition the cuvette was gently shaken and the mixture was incubated for 1 min to ensure that the equilibria were established (no change was observed with longer incubation time). An excerpt of the fluorescence emission at 600 nm was corrected for ethidium bromide´s own emission and plotted against [EB]/[**1**], [EB]/[**2**], and [EB]/[**3**], respectively. An exponential decay first order function was fitted using Origin 7.0. The reciprocal x-value at half of the maximum fluorescence emission is the $IC_{50}$ value representing the equivalents of **1**, **2** or **3** that are necessary to displace half of the ethidium bromide from the EB/DNA complex.

**Isothermal Titration Calorimetry:** All measurements were carried out in sodium cacodylate buffer (0.01 M, pH 7.00 ± 0.01) at 25° C. All solutions were ultrasonicated and degassed in vacuum prior to the experiments. Aliquots of **1** (0.3 mM, 30 × 5 µL), **2** (0.6 mM, 35 × 5 µL), or **3** (0.06 mM, 25 × 5 µL)

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were injected from a 297 µl rotating syringe (307 rpm) into the calorimeter reaction cell containing 1.45 ml of a ctDNA or pF143-GFP plasmid solution (0.1 mM). Blank experiments were conducted to determine the heats of dilution of **1**, **2**, **3** and the nucleic acids. These were subtracted from the heats measured in the titration experiments. Data was analyzed using Origin 7.0 software according to a single set of sites binding mode.

**AFM sample preparation:** Samples were prepared by diluting an aqueous ctDNA stock solution (0.5 mg/ml, untreated) with water to 5 µg/ml (16 µM phosphates). To this solution $MgCl_2$ (263 µM) and **1** (5–25 µM) or **2** (8–50 µM) were added. For each measurement 10 µl of the mixture were dropped onto a freshly cleaved mica surface and dried by spin coating (20 rps for 1 min and 100 rps for 2 min).

**DLS experiments:** All measurements were carried out in sodium cacodylate buffer (0.01 M, pH 7.00 ± 0.01) at 25° C in UV-transparent microcuvettes (1 cm) equipped with a stopper. Mixtures of ctDNA (50 µM) and **1** (5–20 µM) or **2** (5–50 µM) were prepared and filtered prior to measuring via 0.20 µm nylon filters. The autocorrelation functions of the backscattered light fluctuations were analyzed with the DTS 6.20 software from *Malvern* providing the hydrodynamic diameter (Z-average), polydispersity and size distribution (NNLS analysis).

**Solid-Phase Peptide Synthesis of (5):** The reaction was carried out in a flask equipped with a glass-frit on a *Heidolph* Rotamax 120 shaker. SASRIN-resin (300 mg, 1.3 mmol/g) was swollen in DCM/DMF (7/3, 10 ml) for 1 h. To a solution of Fmoc-Phe-OH (471 mg, 1.17 mmol, 3 eq) in DCM/DMF (7/3, 10 ml) DIC (182 µl, 1.17 mmol, 3 eq) was added dropwise at 0° C under argon atmosphere and stirred for 20 min at room temperature. This solution, HOBt (179 mg, 1.17 mmol, 3 eq) and catalytic amounts of DMAP was added to the resin and shaken for 20 h under argon atmosphere. After washing (3 × 10 ml DMF) the coupling and washing steps were repeated two more times. Afterwards the resin was treated with acetic anhydride (110 µl, 1.17 mmol, 3 eq) in 3 % NMM/DMF (10 ml) for 20 h. After another washing step with DMF (3 × 10 ml) Fmoc-deprotection was achieved by shaking the resin in 20 % piperidin/DMF (10 ml) for 20 min twice followed by washing with DMF (6 × 10 ml). Fmoc-Lys(Boc)-OH (547 mg, 1.17 mmol, 3 eq) and *N*-Boc-guanidinocarbonyl pyrrole **4**

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(347 mg, 1.17 mmol, 3 eq) were coupled with PyBOP (609 mg, 1.17 mmol, 3 eq) in 3 % NMM/DMF (10 ml) under argon atmosphere for 20 h. The resin was washed with DMF (3 × 10 ml) and DCM (6 × 10 ml) and dried under reduced pressure for one hour. In order to cleave the product the resin was shaken five times for one hour with 1 % TFA, 2.5 % TIS and 2.5 % water in DCM. The resin was washed three times with the cleavage mixture and the combined filtrates were concentrated at high vacuum at room temperature. Water (50 ml) was added and the mixture was freezedried in vacuum. The resulting solid was purified by MPLC on C18 reversed phase silica gel (water to 50 % methanol/water within 30 min) to give **5** as a white solid (74 mg, 28 %). mp > 177° C (decomposition); IR (ATR): $\tilde{\nu}$ [$cm^{-1}$] = 3145 [br. w], 2948 [s], 1683 [s], 1623 [s], 1521 [s], 1247 [s], 1199 [s], 1143 [s], 971 [s], 833 [s], 771 [s] $cm^{-1}$. $^{1}$H-NMR (500 MHz, $CDCl_3$): δ [ppm] = 1.36 (s, 9H, Boc-C$H_3$), 1.38-1.47 (m, 8H, Lys-C$H_2$), 1.59 (s, 9H, Boc-C$H_3$), 1.68-1.93 (m, 2H, Lys-C$H_2$), 2.95-3.08 (m, 2H, Lys-C$H_2$), 3.12-3.30 (m, 2H Phe-C$H_2$), 4.75 (s, 1H, amide-N*H*); 4.89-5.01 (m, 1H, Phe-C*H*), 5.69-5.80 (m, 1H, Lys-C*H*), 6.56-6.65 (m, 2H, Phe-C$H_{ar}$, pyrrole-C*H*), 6.80-6.88 (m, 2H, Phe-C$H_{ar}$), 7.01-7.13 (m, 4H, amide-N*H*, 2 × Phe-C$H_{ar}$, pyrrole-C*H*), 8.35-8.44 (m, 1H, amide-N*H*), 9.73 (br. s, 1H, guanidine-N*H*), 10.41 (br. s, 1H, guanidine-N*H*), 11.65 (s, 1H, pyrrole-N*H*), 11.82 (s, 1H, $CO_2H$), 12.01 (s, 1H, guanidine-N*H*). $^{13}$C-NMR (125 MHz, $CDCl_3$): δ [ppm] = 22.8 (Lys-$CH_2$), 28.0 (Boc-$CH_3$), 28.5 (Boc-$CH_3$), 29.9 (Lys-$CH_2$), 35.0 (Lys-$CH_2$), 39.0 (Phe-$CH_2$), 40.5 (Lys-$CH_2$), 52.0 (Lys-*C*H), 55.0 (Phe-*C*H), 78.9 (Boc-$C_q$), 84.9 (Boc-$C_q$), 109.8 (pyrrole-*C*H), 119.6 (pyrrole-*C*H), 126.5 (Phe-$CH_{ar}$), 127.2 (Phe-$CH_{ar}$), 129.2 (Phe-$CH_{ar}$), 131.63 (pyrrole-$C_q$), 136.6 (Phe-$C_q$), 155.3 ($C_q$), 156.0 ($C_q$), 157.7 ($C_q$), 159.1 ($C_q$), 161.7 ($C_q$), 171.1 ($C_q$), 178.8 ($C_q$). HR-MS (neg. ESI) m/z calculated for $C_{32}H_{44}N_7O_9$ $[M - H^+]^-$ 670.3206, found 670.3342.

**Tripodal Ligand (1):** To a solution of 71 mg **5** (0.11 mmol, 3 eq) and 41 mg HATU (0.11 mmol, 3 eq) in DMF (5 ml) 150 µl DIPEA were added and the mixture was stirred for 1 h under argon atmosphere. After the addition of 6 mg of the aromatic triamine scaffold **6** (0.04 mmol, 1 eq) the solution was stirred for another 36 h under argon atmosphere. Water (10 ml) was added and the resulting suspension extracted with ethyl acetate (3 × 25 ml). The combined organic layers were dried

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with $Na_2SO_4$ and the solvent was removed in vacuum. The resulting solid was purified by MPLC on normal phase silica gel (cyclohexane to ethyl acetate within 30 min), dissolved in 50 % TFA/DCM (10 ml) and stirred for 16 h under argon atmosphere. After removal of the solvent in vacuum the crude product was purified by MPLC on C18 reversed phase silica gel (water to methanol within 30 min, 0.1 % TFA), re-dissolved in 1 M aqueous HCl and freeze dried. The last step was repeated twice and after an additional lyophilization in pure water **1** was obtained as a white, voluminous hydrochloric salt (16 mg, 26 %). mp > 250° C (decomposition); IR (ATR): $\tilde{\nu}$ [$cm^{-1}$] = 3152 [br. m], 2905 [w], 2362 [s], 2336 [s], 1701 [s], 1685 [s], 1657 [s], 1541 [s], 1517 [s], 1289 [m], 828 [w], 685 [m]. $^1$H-NMR (TFA-salt), (500 MHz, DMSO-$d_6$): δ [ppm] = 0.94-1.10 (m, 6H, Lys-C$H_2$), 1.17-1.62 (m, 12H, Lys-C$H_2$), 2.63-3.11 (m, 12 H, 3 × Lys-C$H_2$, 3 × Phe-C$H_2$); 4.07-4.60 (m, 12H, 3 × Lys-C*H*, 3 × Ar-C$H_2$, 3 × Phe-C*H*), 6.83-6.91 (m, 3H, pyrrole-C*H*), 6.97 (s, 3H, C$H_{ar}$), 7.01 (s, 3H, C$H_{ar}$), 7.06-7.27 (m, 18H, 3 × pyrrole-C*H*, 15 × Phe-C$H_{ar}$), 7.70 (br, s, 9H, Lys-N$H_3$), 8.30-8.67 (m, 12H, 3 × amide-N*H*, 3 × pyrrole-N*H*, 6 × *g*uanidine-N*H*), 11.38 (s, 3H pyrrole-N*H*), 12.48 (s, 3H, guanidine-N*H*). $^{13}$C-NMR (TFA-salt), (125 MHz, DMSO-$d_6$): δ [ppm] = 22.2 (Lys-*C*$H_2$), 26.6 (Lys-*C*$H_2$), 31.4 (Lys-*C*$H_2$), 37.8 (Phe-*C*$H_2$), 38.7 (Lys-*C*$H_2$), 42.2 (*C*$H_2$), 52.6 (Lys-*C*H), 53.9 (Phe-*C*H), 113.7 (pyrrole-*C*H), 115.0 (pyrrole-*C*H), 125.0 (*C*$H_{ar}$), 126.3 (Phe-*C*$H_{ar}$), 127.9 (Phe-*C*$H_{ar}$), 129.2 (*C*$H_{ar}$), 129.4 (*C*$H_{ar}$), 137.9 (Phe-$C_q$), 139.3 ($C_q$), 139.5 ($C_q$), 158.4 ($C_q$), 158.7 ($C_q$), 158.9 ($C_q$), 171.0 ($C_q$), 171.4 ($C_q$). HR-MS (pos. ESI) m/z calculated for $C_{75}H_{97}N_{24}O_{12}$ $[M + H^+]^+$ = 1525.7712, found 1525.7583.

**Microwave Assisted Solid-Phase peptide Synthesis of Divalent Ligand (3):** The reaction was carried out in a fritted, microwave-transparent 25 ml polyethylene column with a CEM Discover microwave apparatus. Fmoc-Rink-Amide (125 mg, 0.94 mmol/g) was swollen in DMF (3 ml) for 1 h. Fmoc removal was achieved by irradiating the resin in 20 % piperidine/DMF (3 ml) for 1 min and 5 min at 20 W and a maximum temperature of 60° C followed by washing with DMF (6 × 3 ml). The first residue Fmoc-Gly-OH (210 mg, 0.70 mmol, 6 eq) was attached to the resin by microwave irradiation for 20 min at 20 W and a maximum temperature of 60° C under argon atmosphere with PyBOP (367 mg, 0.70 mmol, 6 eq) in 5 % DIPEA/DMF (3 ml) and consequent washing with DMF (3 × 3 ml). Coupling

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and washing steps were repeated. The resin was then treated with acetic anhydride (110 µl, 1.18 mmol, 10 eq) in 5 % DIPEA/DMF (3 ml) under coupling conditions. After Fmoc deprotection the aromatic template **7** (440 mg, 0.70 mmol, 6 eq), Fmoc-Lys(Boc)-OH (330 mg, 0.70 mmol, 6 eq), again Fmoc-Lys(Boc)-OH (330 mg, 0.70 mmol, 6 eq), Fmoc-Arg(Pbf)-OH (457 mg, 0.70 mmol, 6 eq) and *N*-Boc-guanidinocarbonyl pyrrole **4** (280 mg, 0.70 mmol, 6 eq) were coupled with PyBOP (367 mg, 0.70 mmol, 6 eq) as described above, also repeating coupling and washing steps. The resin was washed with DCM (3 × 3 ml), methanol (3 × 3 ml), and DCM (3 × 3 ml) and dried under reduced pressure for one hour. To cleave the product, the resin was transferred to a flask equipped with a frit onto a *Heidolph* Rotamax 120 shaker. There it was shaken under argon atmosphere in a mixture containing 95 % TFA, 2.5 % water, and 2.5 % TIS for four hours and washed twice with the cleavage mixture. The filtrates were combined and concentrated in high vacuum at room temperature. Diethyl ether (40 ml) was added and the resulting suspension was centrifuged. The supernatant solvent was decanted and the solid was washed with diethyl ether and centrifuged again. After decanting, the raw product was dissolved in little methanol, water (30 ml) was added, and the mixture was freeze-dried in vacuum. The resulting solid was purified by MPLC on C8 reversed-phase silica gel (10 % to 25 % methanol/water in 45 min, 0.1 % TFA) to obtain **3** as voluminous white solid (24 mg, 8 %). mp > 250° C (decomposition); IR (ATR): $\tilde{\nu}$ [$cm^{-1}$] = 3273 [br. w], 1636 [br. s], 1541 [br. s], 1457 [w], 1280 [br. m], 1195 [br. m]. $^{1}$H-NMR (TFA-salt), (500 MHz, DMSO-$d_6$): δ [ppm] = 1.22-1.39 (m, 8H, 4 × Lys-C$H_2$), 1.42-1.84 (m, 24H, 4 × Arg-C$H_2$, 8 × Lys-C$H_2$), 2.69-2.78 (m, 8H, 4 × Lys-C$H_2$), 3.12 (td, 4H, $^{3}J_1$ = 6.87 Hz, $^{3}J_2$ = 13.80 Hz, 2 × Arg-C$H_2$), 3.78-3.92 (ddd, $^{3}J_1$ = $^{3}J_2$ = 16.65 Hz, $^{3}J_3$ = 6.56 Hz, Gly-C$H_2$), 4.09-4.31 (m, 6H, 2 × C$H$, 2 × C$H_2$), 4.36 (dd, $^{3}J_1$ = 15.41 Hz, $^{3}J_2$ = 5.01 Hz, 2H, 2 × C$H$), 4.47 (dd, $^{3}J_1$ = 14.11 Hz, $^{3}J_2$ = 6.50 Hz, 2H, 2 × C$H$), 6.91 (d, $^{3}J$ = 3.59 Hz, 2H, 2 × Pyr-C$H_{ar}$), 7.12 (d, $^{3}J$ = 4.37 Hz, 2H, 2 × Pyr-C$H_{ar}$),7.14 (br. s, 8H, 2 × Arg-N$H_4^+$), 7.17 (s, 2H, N$H_2$), 7.28 (s, 2H, 2 × N$H$), 7.51 (s, 1H, C$H_{ar}$), 7.64 (s, 2H, 2 × C$H_{ar}$), 7.75 (br. s, 12H, 4 × Lys-N$H_3^+$), 8.04 (d, $^{3}J$ = 6.42 Hz, 2H, 2 × N$H$), 8.17 (d, $^{3}J$ = 7.10 Hz, 2H, 2 × N$H$), 8.20-8.79 (m, 13H, 5 × N$H$, 2 × GCP-N$H_4^+$), 11.51 (s, 2H, 2 × GCP-N$H$), 12.52 (s, 2H, 2 × Pyr-N$H$). $^{13}$C-NMR (TFA-salt), (125 MHz, DMSO-$d_6$): δ [ppm] = 22.24 (C$H_2$), 25.18 (C$H_2$), 26.44 (C$H_2$),

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26.52 ($CH_2$), 28.83 ($CH_2$), 30.87 ($CH_2$), 31.27 ($CH_2$), 38.54 ($CH_2$), 38.57 ($CH_2$), 40.34 ($CH_2$), 41.88 ($CH_2$), 42.41 ($CH_2$), 44.00 (*C*H), 52.51 (*C*H), 52.68 (*C*H), 113.67 ($CH_{ar}$), 115.65 ($CH_{ar}$), 124.37 ($CH_{ar}$), 125.79 ($C_q$), 128.89 ($CH_{ar}$), 134.15 ($C_q$), 139.42 ($C_q$), 156.85 ($C_q$), 159.11 ($C_q$), 166.35 ($C_q$), 171.31 ($C_q$), 171.47 ($C_q$), 171.57 ($C_q$),171.60 ($C_q$). HR-MS (pos. ESI) m/z calculated for $C_{61}H_{102}N_{28}O_{122}$ [M + 2H+]$^{2+}$ = 709.4111, found 709.4035.

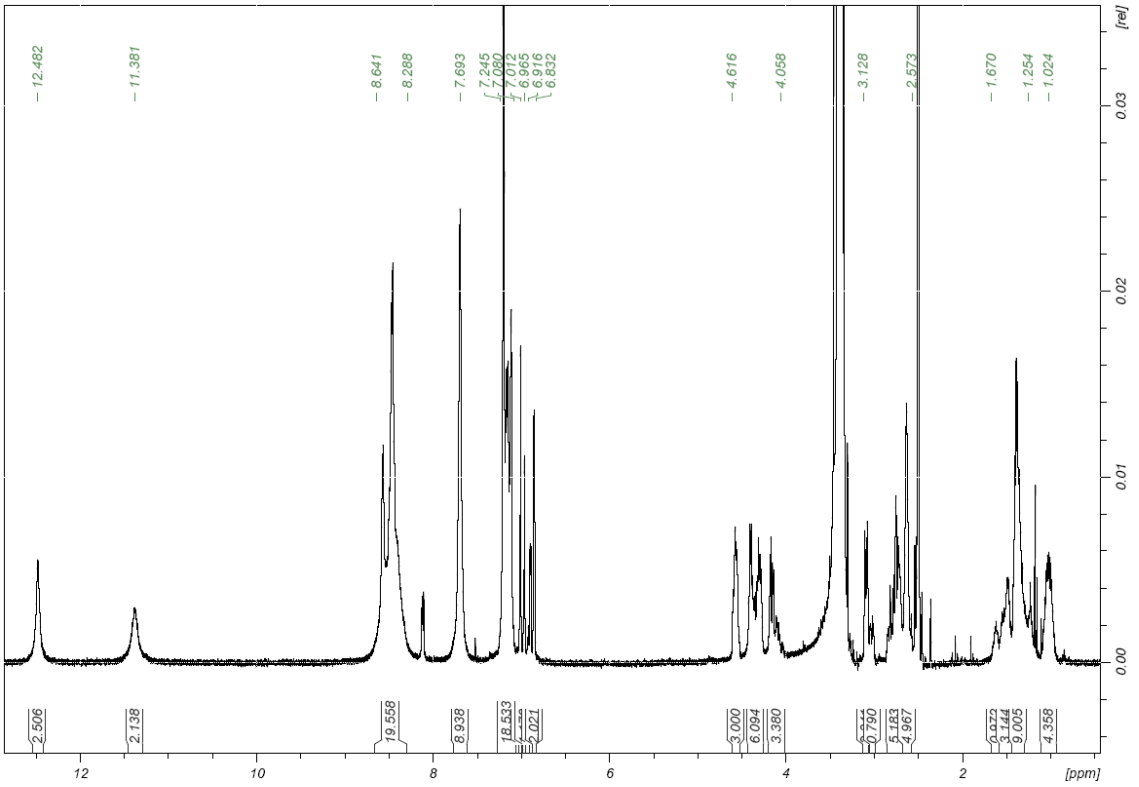


**Fig. S1.** $^{1}$H NMR of **1** in DMSO-$d_6$ recorded at 500 MHz.

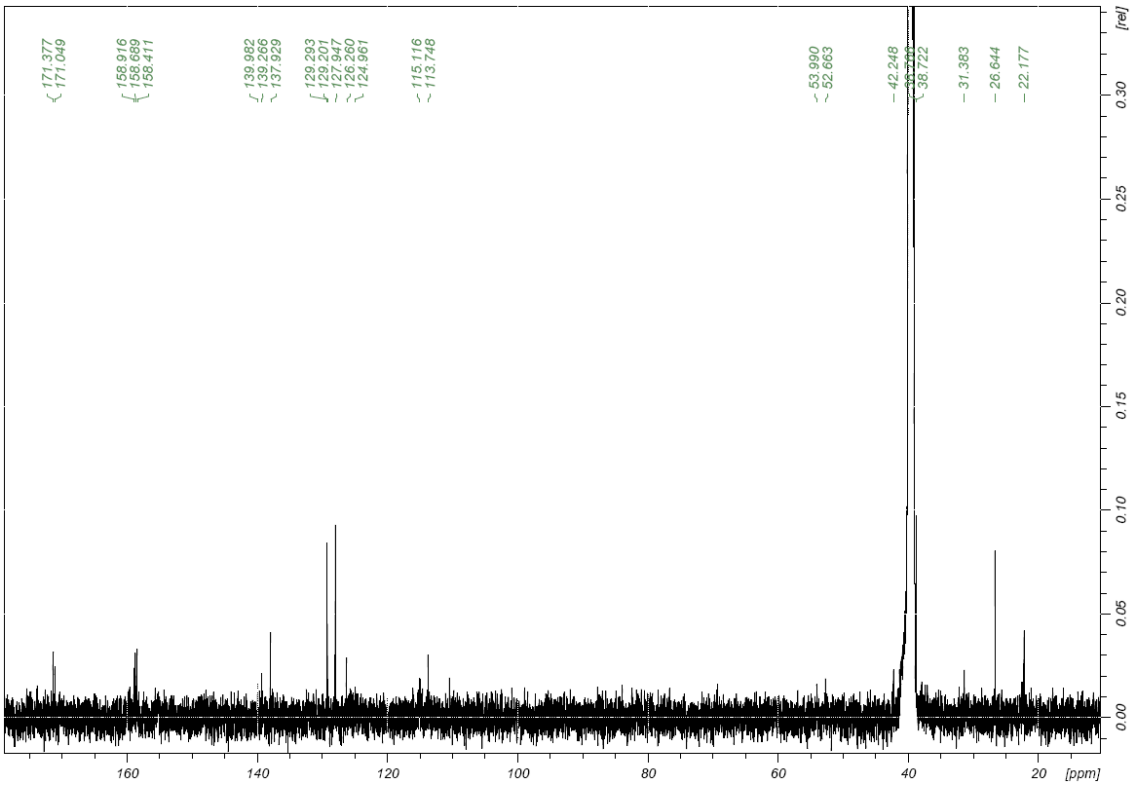


**Fig. S2a.** $^{13}$C NMR of **1** in DMSO-$d_6$ recorded at 125 MHz.

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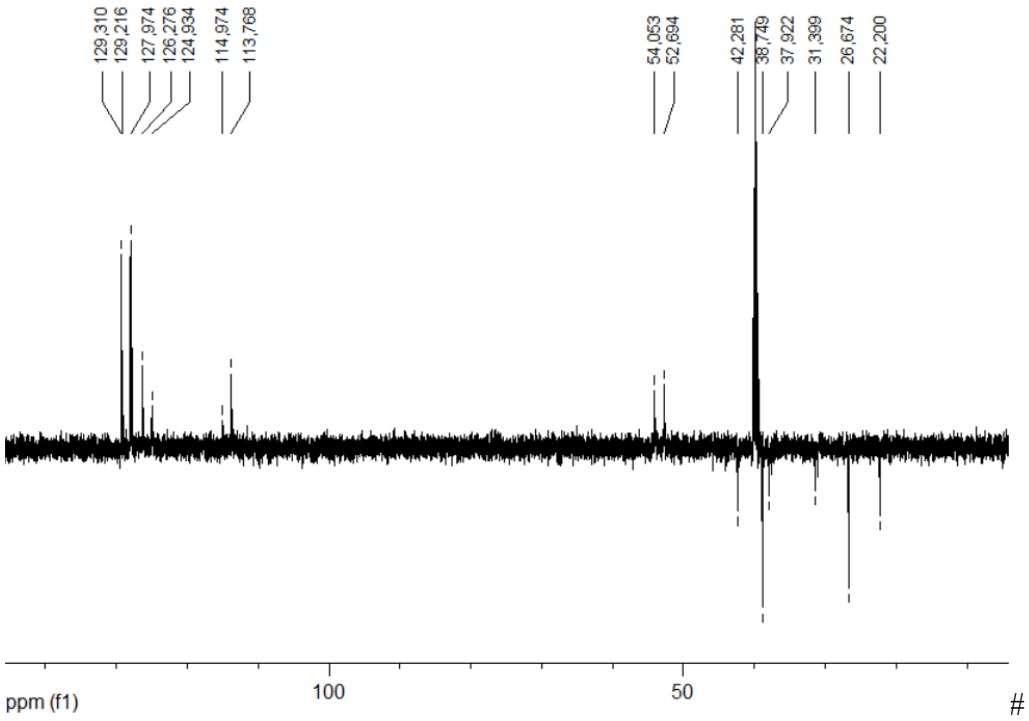


**Fig. S2b.** DEPT135 NMR of **1** in DMSO-$d_6$ recorded at 125 MHz.

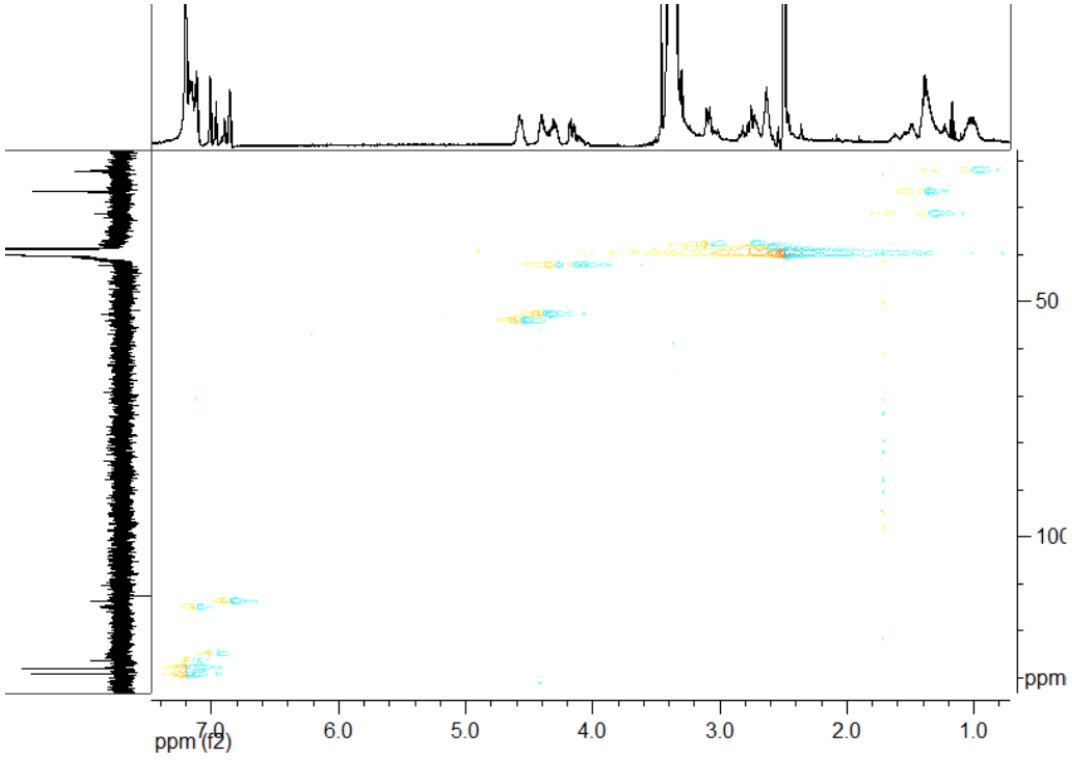


**Fig. S2c.** HSQC NMR of **1** in DMSO-$d_6$ recorded at 125 MHz.

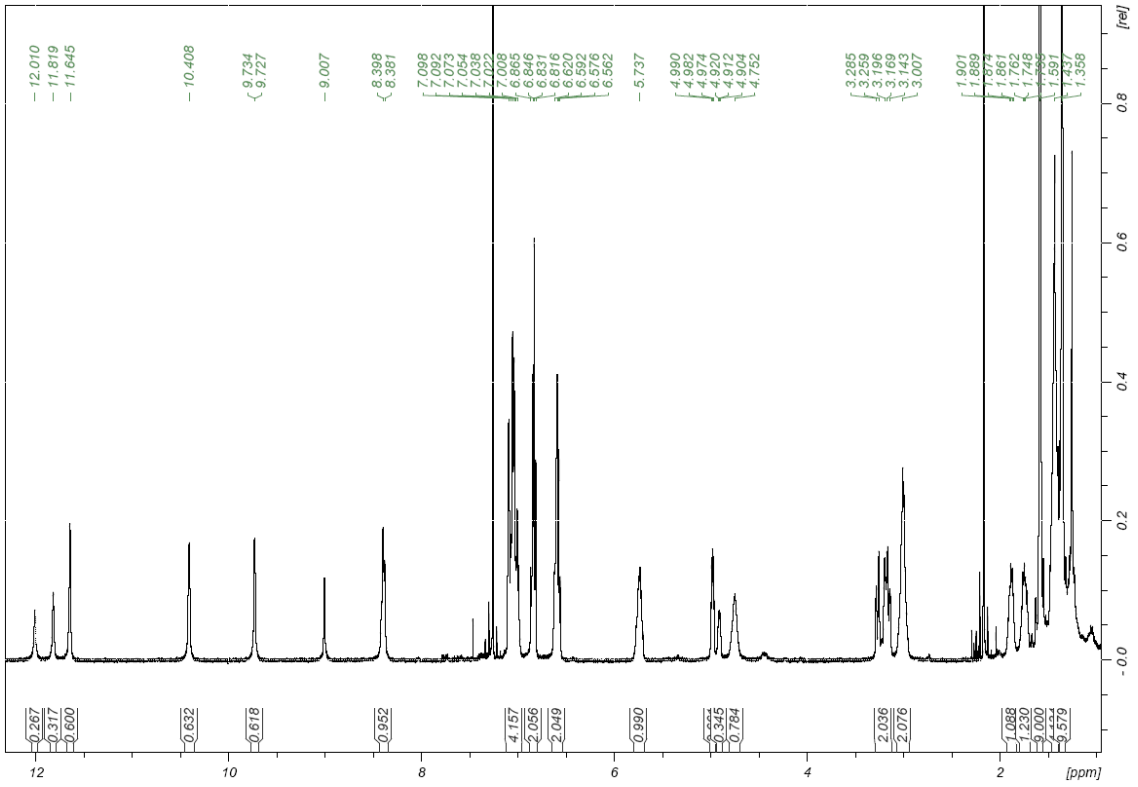


**Fig. S3.** $^{1}$H NMR of **5** in $CDCl_3$ recorded at 500 MHz.

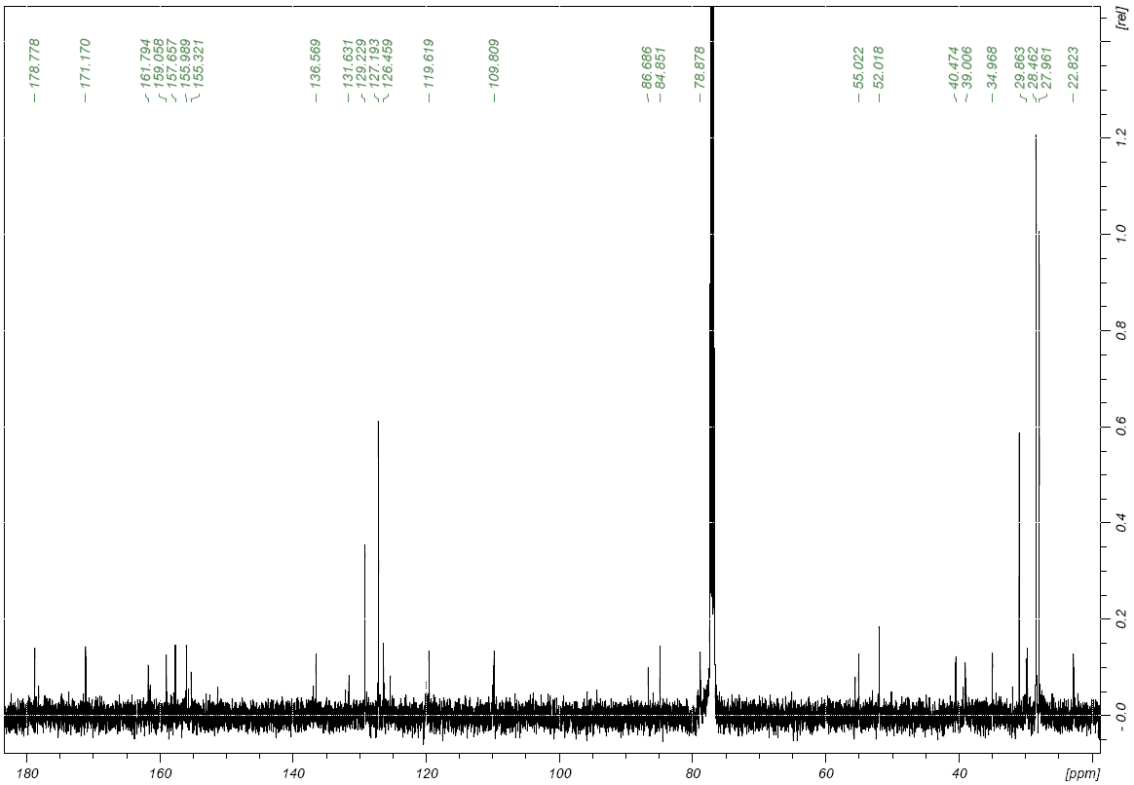


**Fig. S4.** $^{13}$C NMR of **5** in $CDCl_3$ recorded at 125 MHz.

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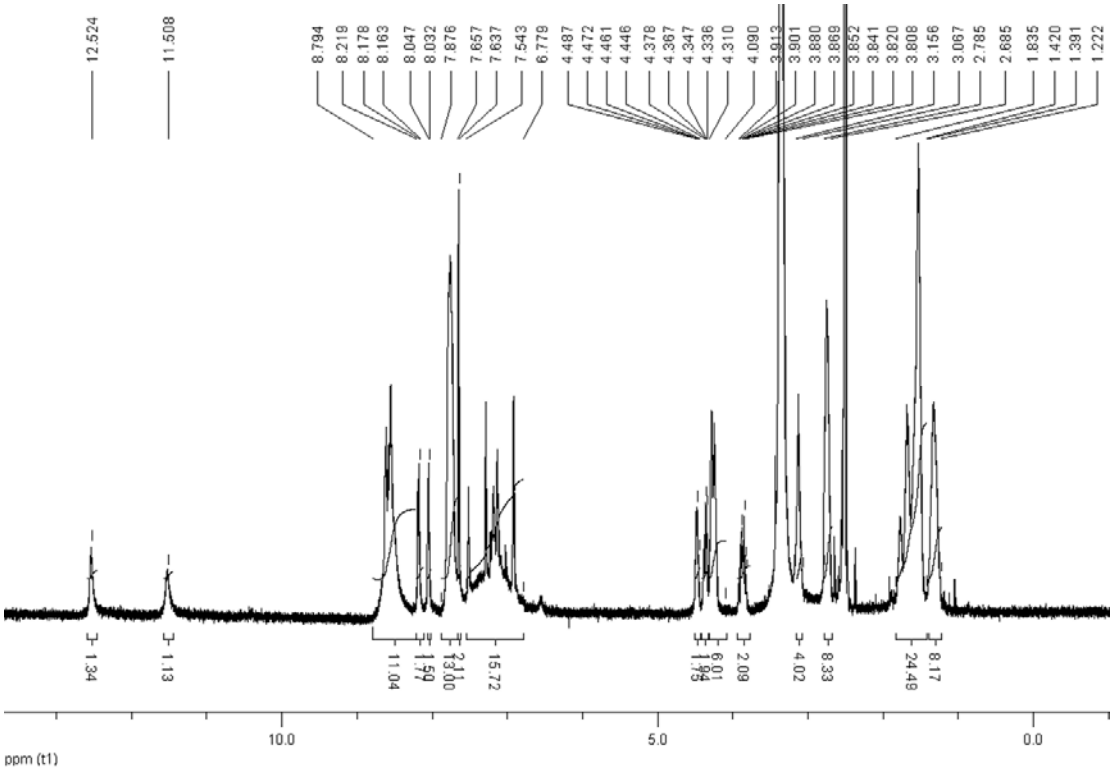


**Fig. S5.** $^{1}$H NMR of **3** in DMSO-$d_6$ recorded at 500 MHz.

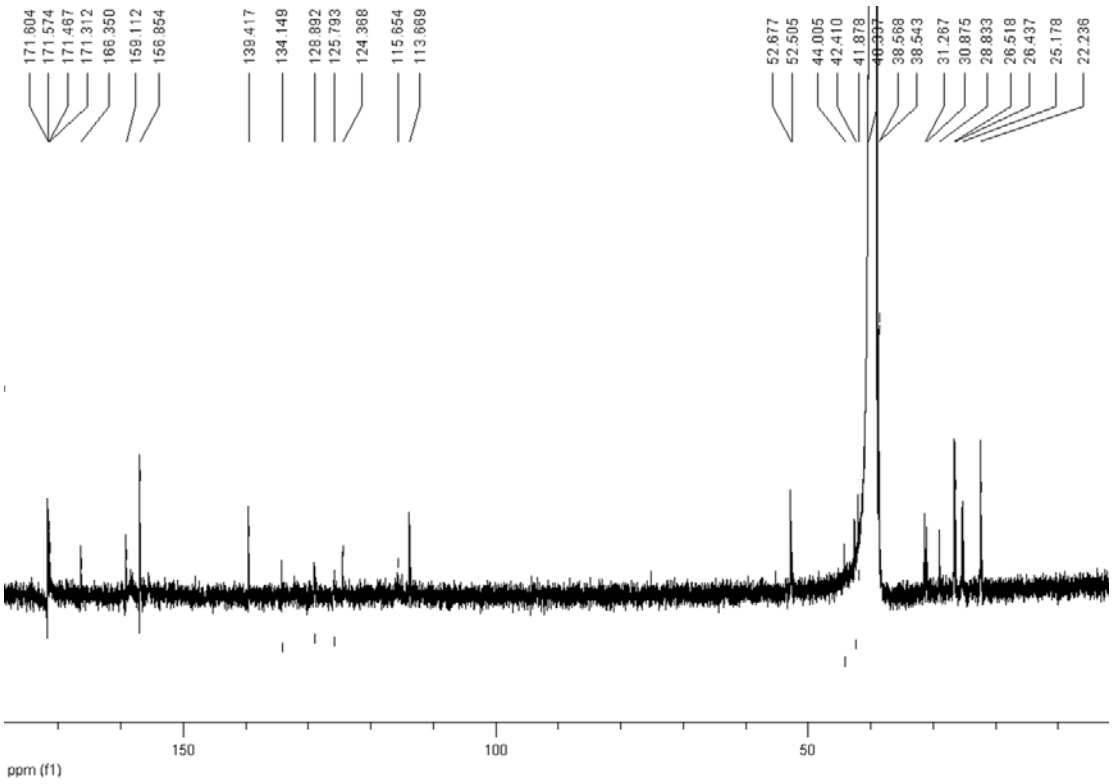


**Fig. S6.** $^{13}$C NMR of **3** in DMSO-$d_6$ recorded at 125 MHz.

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**Table S1.** Supplementary results for AFM and DLS for **1**

| $r^a$ | N/P | AFM[b] [nm] | DLS Size Distribution by Volume[c] [nm] | DLS Size Distribution by Numbers[c] [nm] | DLS Z-average [nm] / PDI |
|---|---|---|---|---|---|
| 0 | 0 | H[d] = 0.5, 1.0, 1.5<br>W[d] = 31.0 ± 5.4<br>L[d] = 460 ± 175 | 39.4 (98%) | 22.3 (100%) | 73.4 / 0.608 |
| 0.1 | 0.6 | - | 50.26 (41%) / 451.2 (59%) | 45.9 (99%) / 307.4 (1%) | 278.0 / 0.333 |
| 0.3 | 1.8 | H = 1.5 ± 0.4<br>D = 500 ± 100 | 397.6 (91%) | 211.1 (100%) | 284.0 / 0.303 |
| 0.4 | 2.4 | $H_1$ = 14.3 ± 4.2<br>$W_1$ = 700 ± 350;<br>$H_2$ = 1.3 ± 0.2<br>$D_2$ = 4000-9000 | 322.2 (2%) / 1465 (98%) | 269.1 (56%) / 1174 (44%) | 1243 / 0.293 |
| 0.5 | 3.0 | H = 17.5 ± 5.2<br>D = 350 ± 100 | -[e] | -[e] | -[e] |
| 1.5 | 9.0 | H = 17.0 ± 6.0<br>D = 150 ± 25 | - | - | - |

a) r = [compound] / [ctDNA]; b) Distances were measured at half height; c) Hydrodynamic diameter and percentage of total volume/numbers; d) H = height, W = width, L = length, D = diameter; e) Precipitation.

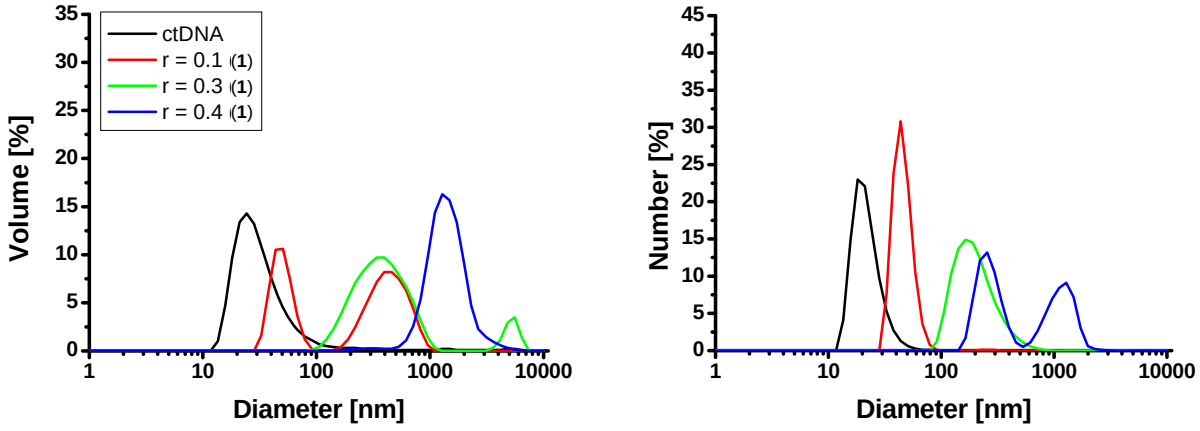


**Fig. S7.** Volume- (left) and number-weighted (right) size distribution of complexes between ctDNA and **1** obtained from DLS measurements at pH 7.0 in 0.01 M sodium cacodylate buffer; [ctDNA] = 50 µM; r = [compound] / [ctDNA].

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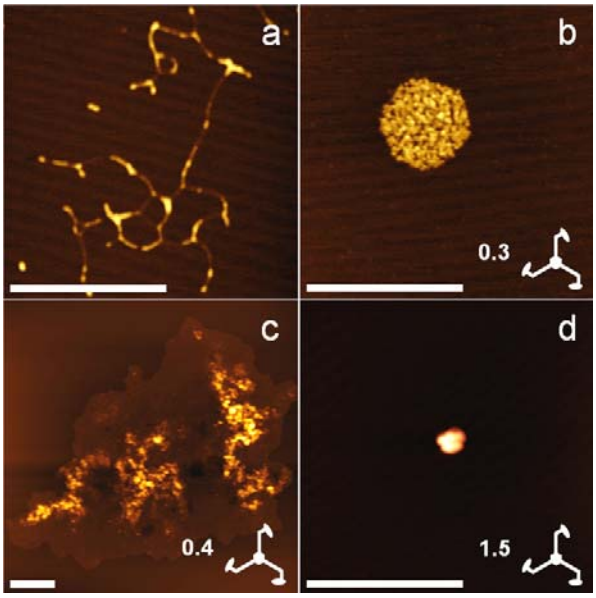


**Fig. S8.** AFM images obtained on mica surface with the microscope operating in tapping mode. Addition of **1** leads to condensation of ctDNA. a) [ctDNA] = 16 µM, [$MgCl_2$] = 263 µM; b) [**1**] = 5 µM (0.3 eq); c) [**1**] = 6.5 µM (0.4 eq); d) [**1**] = 25 µM (1.5 eq). White scale bar always equals 1 µm.

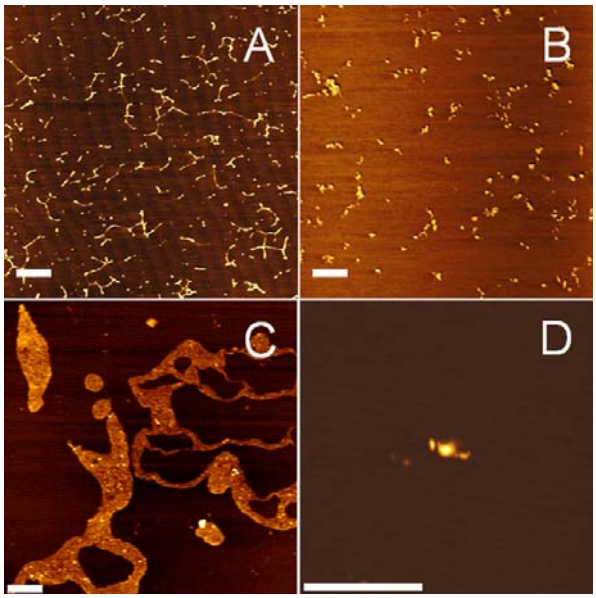


**Fig. S9.** AFM images obtained on mica surface with the microscope operating in tapping mode. Addition of **2** leads to condensation of ctDNA. A) [ctDNA] = 16 µM, [$MgCl_2$] = 263 µM, B) [**2**] = 8 µM (0.5 eq), C) [**2**] = 16 µM (1.0 eq), D) [**2**] = 50 µM (3.1 eq). White scale bar always equals 1 µm.

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**Table S2.** Supplementary results for AFM and DLS for **2**

| $r^a$ | N/P | AFM[b] [nm] | DLS Size Distribution by Volume[c] [nm] | DLS Size Distribution by Numbers[c] [nm] | DLS Z-average [nm] / PDI |
|---|---|---|---|---|---|
| 0 | 0 | H[d] = 0.5, 1.0, 1.5<br>W[d] = 31.0 ± 5.4<br>L[d] = 460 ± 175 | 39.4 (98%) | 22.3 (100%) | 73.4 / 0.608 |
| 0.1 | 0.4 | - | 31.5 (96%) | 20.00 (100%) | 77.0 / 0.479 |
| 0.5 | 2.0 | H = 0.5, 1.0, 1.5<br>D[d] = 150 ± 30 | 26.8 (76%) /<br>128.7 (24%) | 24.2 (100%) | 113.5 / 0.247 |
| 1.0 | 4.0 | H = 1.1 ± 0.3 | 1529 (98%) | 1414 (100%) | 2222 / 0.392 |
| 1.5 | 6.0 | -[e] | -[e] | -[e] | -[e] |
| 3.1 | 12.4 | H = 16.2 ± 4.9<br>D = 90 ± 30 | - | - | - |

a) r = [compound] / [ctDNA]; b) Distances were measured at half height; c) Hydrodynamic diameter and percentage of total volume/numbers; d) H = hight, W = width, L = length, D = diameter; e) Precipitation.

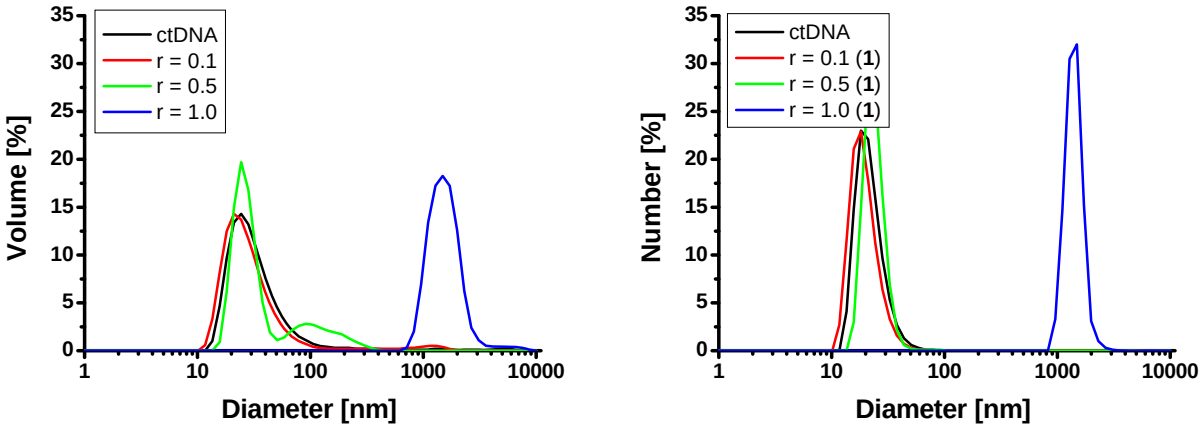


**Fig. S10.** Volume- (left) and number-weighted (right) size distribution of complexes between ctDNA and **2** obtained from DLS measurements at pH 7.0 in 0.01 M sodium cacodylate buffer; [ctDNA] = 50 µM; r = [compound] / [ctDNA].

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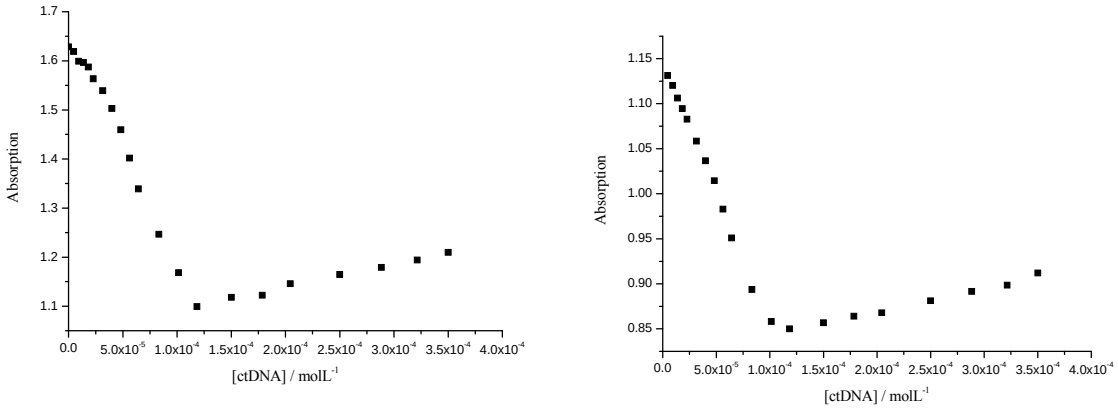


**Fig. S11.** UV titration: To a solution of **1** (20 µM, left) or **2** (30 µM, right) in aqueous sodium cacodylate buffer (0.01 M) at pH 7 aliquots of ctDNA were added. The graphs represent excerpts at 300 nm. Absorption is corrected for dilution.

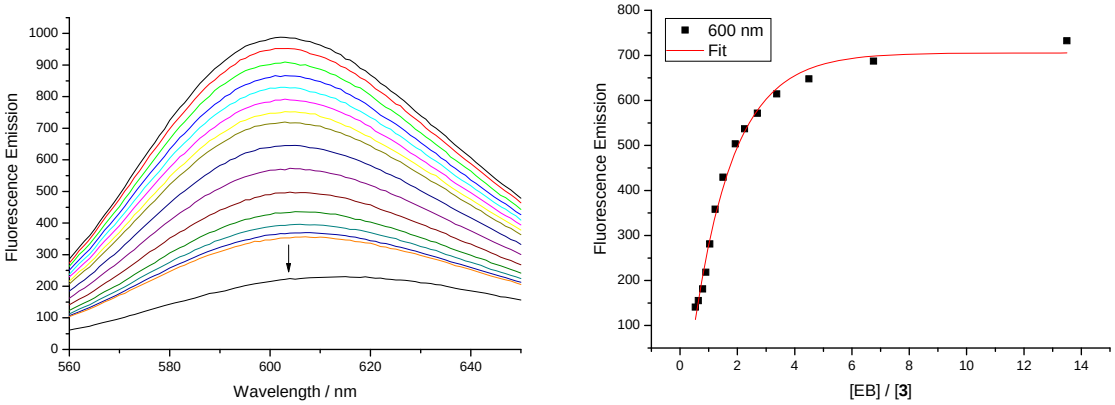


**Fig. S12.** Ethidium bromide (EB) displacement titration in 0.01 M sodium cacodylate buffer at pH 7: [ctDNA] = 3.00 µM (1.00 eq), [EB] = 0.75 µM (0.25 eq) (left); **1** was added in aliquots; Excerpt at 600 nm corrected for EB´s own emission, fitted with exponential decay first order function (right).

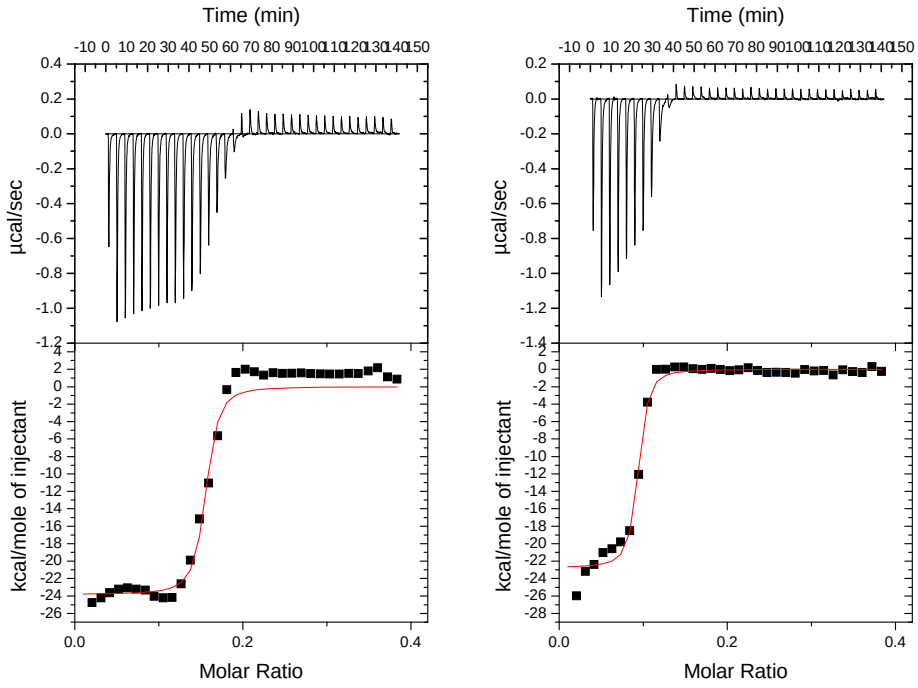


**Fig. S13.** ITC titration in sodium cacodylate buffer (0.01 M) at pH 7: **1** (0.3 mM) was added to ctDNA (0.1 mM, left) or pF143-GFP (0.1 mM, right).

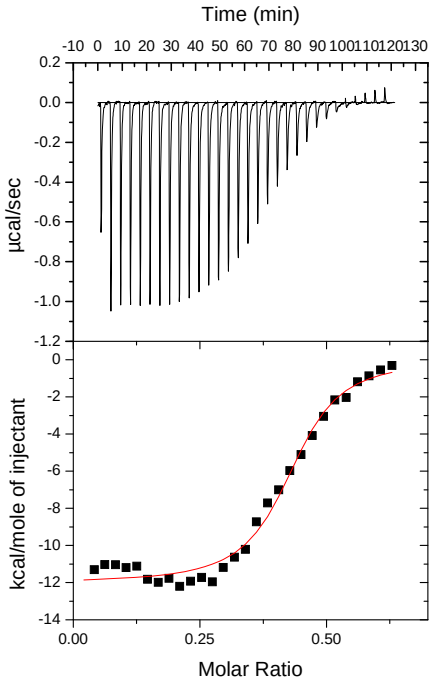


**Fig. S14.** ITC titration in sodium cacodylate buffer (0.01 M) at pH 7: **2** (0.6 mM) was added to ctDNA (0.1 mM).

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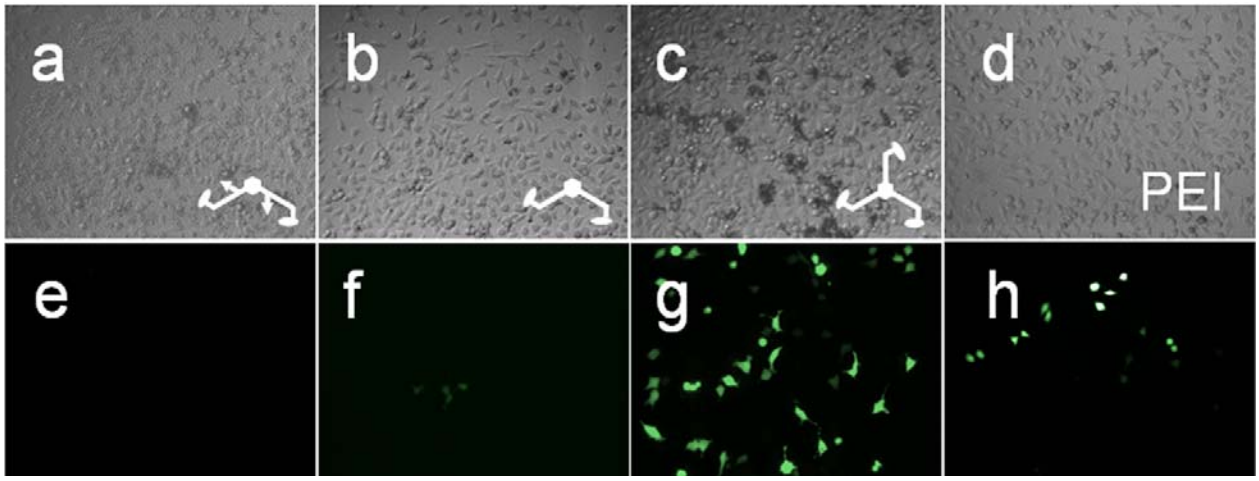


**Fig. S15.** Brightfield (a–d) and fluorescence images (e–h) of HeLa cells 48 h after transfection with 2 µg pF143-GFP plasmid and either 0.24 mM **3** (N/P = 64; a, e), **2** (N/P = 32; b, f), **1** (N/P = 48; c, g), or PEI (N/P = 4640; 0.18 mM, d, h).

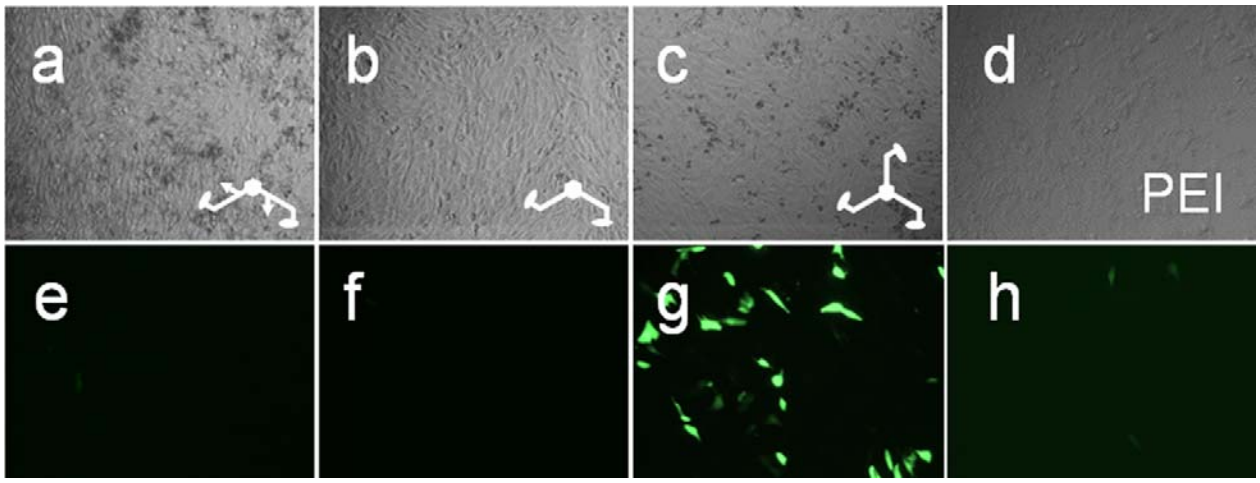


**Fig. S16.** Brightfield (a–d) and fluorescence images (e–h) of NIH cells 48 h after transfection with 2 µg pF143-GFP plasmid and either 0.24 mM **3** (N/P = 64; a, e), **2** (N/P = 32; b, f), **1** (N/P = 48; c, g), or PEI (N/P = 4640; 0.18 mM, d, h).

**Table S3.** Transfection efficiency for HeLa and NIH cell lines

| Compound | Transfection Efficiency [%] | |
|---|---|---|
| | HeLa | NIH |
| **1** | 15.6 ± 1.7 | 14.5 ± 2.6 |
| **2** | 1.4 ± 0.2 | 0 |
| **3** | 0 | 0.3 ± 0.4 |
| PEI | 4.2 ± 0.7 | 0.4 ± 0.5 |

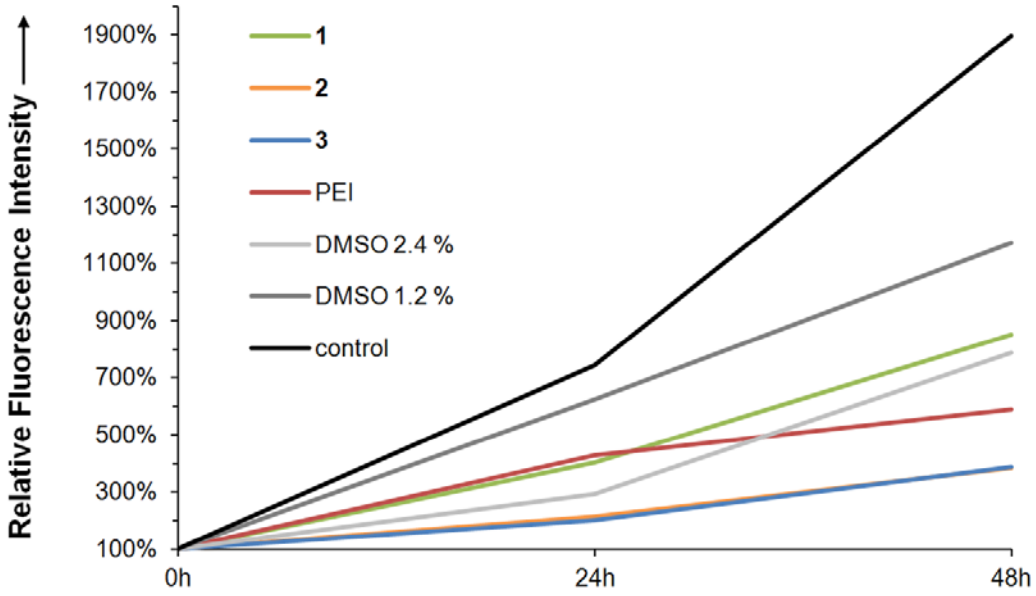


**Fig. S17.** Vital HEK293T cells metabolically convert the dye Alamar Blue to the red-fluorescent resorufin. The graph illustrates the fluorescence level 24 h and 48 h after transfection with **1**, **2**, **3** (containing 2.4 % DMSO), PEI, or DMSO (1.2 % and 2.4 %). The fluorescence intensity is normalized to the fluorescence level before transfection as 100 %. Vital cells feature high relative fluorescence intensities and vice versa. Although **1** contains 2.4 % DMSO it is less cytotoxic than PEI (without DMSO).

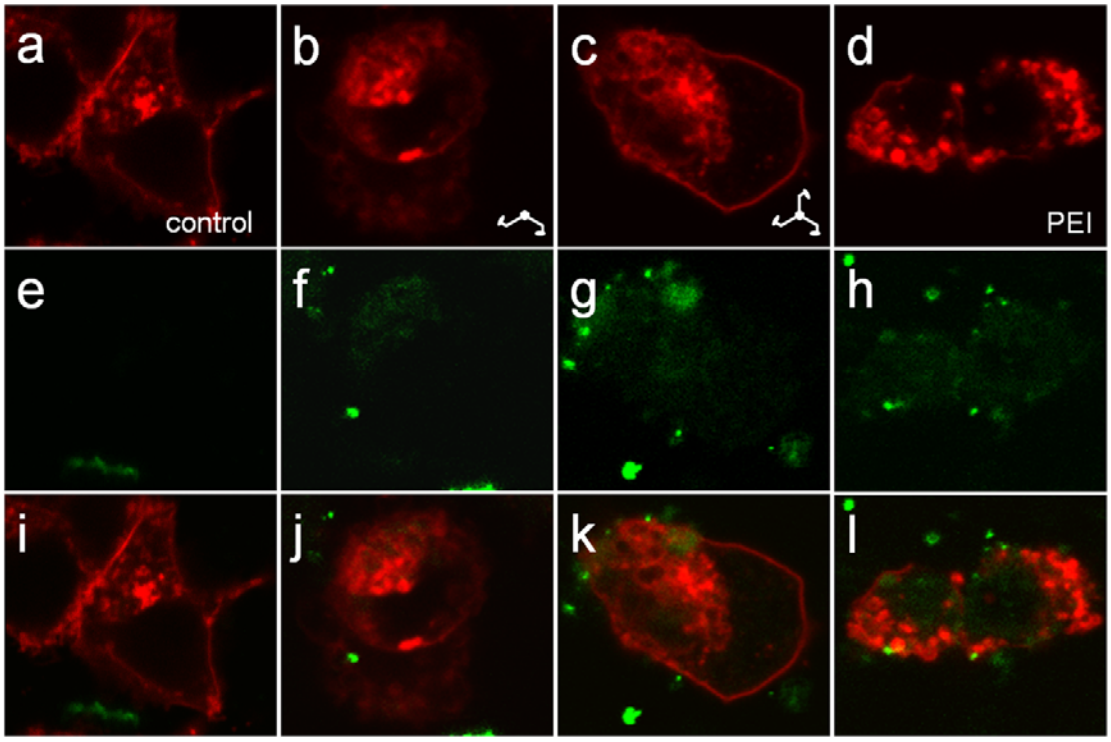


**Fig. S18.** Confocal microscopy images from HEK293T live-cell co-localization experiments with pure DNA (control; a, e, i), **2** (b, f, j), **1** (c, g, k), and PEI (d, h, l) after 2 h; a-d) Lamp1-RFP plasmid (2 µg) transfected with PEI (1 mM) for membrane and endosome staining; e-h) Fluorescence labeled ds-DNA (0.2 µg) transfected 24 h later with **1**, **2** and PEI (0.24 mM); i-l) merger of pictures above.

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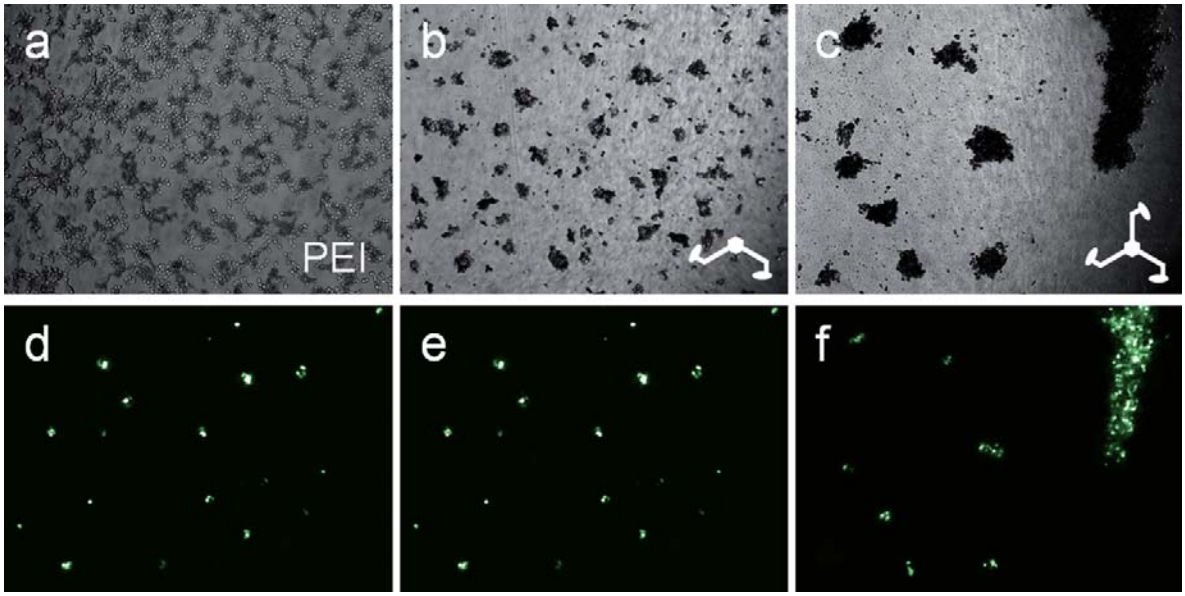


**Fig. S19.** Brightfield (a–c) and fluorescence images (d–f) of HEK293T. Cells were treated with 25 µM chloroquine 30 min before transfection with 2 µg pF143-GFP plasmid and either PEI (a, d), **2** (b, e), or **1** (c, f) (all 0.24 mM). Pictures were taken 16 h afterwards.