Electronic Supplementary Material (ESI) for ChemComm.

Guanidiniocarbonyl Pyrrole (GCP) Conjugated PAMAM-G2, a Highly Efficient Vector for Gene Delivery: The Importance of DNA Condensation**

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1. Materials and Methods

General Remarks:

Solvents were dried and distilled before use. All other reagents were used as obtained from Aldrich, Fluka or Alfa Aesar unless otherwise specified. All reactions were carried out in oven dried glassware. Reactions were monitored by TLC on silica gel plates (*Machery-Nagel* POLYGRAM SIL G/UV254). 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}. The ^{1}H NMR spectra were recorded at 300 MHz or 500 MHz and the ^{13}C NMR spectra at 150 MHz from *Bruker* at ambient temperature. The chemical shifts are relative to the signals of the used solvent: DMSO-d_6 (δ ^{1}H = 2.50 and δ ^{13}C = 39.52) and $CDCl_3$ (δ ^{1}H = 7.26 and δ ^{13}C =77.16). The apparent coupling constants are given in Hertz. The description of the fine structure means: s = singlet, bs = broad singlet, d = doublet, t = triplet, m = multiplet. 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*. Fluorescence spectra were obtained with a *Varian* Cary Eclipse spectrometer. 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-Nelaser (633 nm wavelength) at a fixed detector angle of 173° with an avalanche photodiode detector. Isothermal Titration Calorimetry (ITC) experiments were conducted on a *Microcal* VPITC microcalorimeter. Origin 7.0 software, supplied by the manufacturer, was used for data acquisition and analysis.

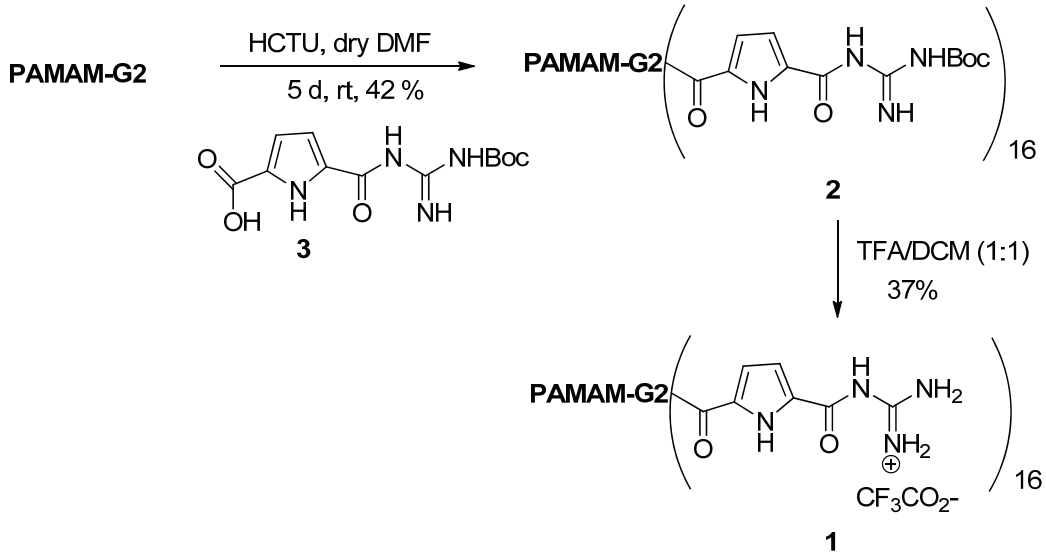
Cell lines and DNA: The human embryonic kidney cell line HEK293T (ATCC-No. CRL-1573), the human cervix carcinoma cell line HeLa (ATCC-No.CCL-2), were obtained from the American Type Culture Collection and maintained as recommended in complete Dulbecco`s Modified Eagle Medium (DMEM) supplemented with 10 % fetal bovine serum, 1 % Glutamine and 1 % Antibiotic-Antimycotic (*Invitrogen*) at 37° C in a humidified atmosphere of 5 % CO2. 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, 1×10^4 cells were seeded in 96 well cell culture plates (*Greiner bio-one*) in a total medium volume of 100 μl 24 h before transfection. The cationic transfection reagent polyethylenimine (PEI, pH 6.8, *Sigma-Aldrich*) was used at a concentration of 0.15 mM in PBS (*Invitrogen*) with 2 μg of plasmid DNA per well. Transfection with **1,** PAMAM-G2 and also PEI was carried out in a concentration range of 0.6, 1, and 10 μM with 2 μg plasmid DNA in a total volume of 30 μl in PBS buffer. PAMAM-G2 was also tested at 0.2 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 in a concentration range of 0.6, 1, and 10 μM with **1**, PAMAM-G2, PEI, also at 200 μM of PAMAM-G2 and at 150 μM of PEI in the presence of 2 μg plasmid-DNA.

Alamar Blue Cell Viability Assay: HeLa cells were grown and transfected as described above. After, 24h and 48h 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*).

2. Synthetic steps



3. Experimental procedures and characterization

Synthesis of intermediate 2:

To a solution of guanidinio pyrrole carboxylic acid **3** (182 mg, 0.46 mmol) and HCTU (191 mg, 0.461 mmol) in dry DMF (10 mL), DIPEA (0.1 mL, 0.552 mmol) was added and the solution was stirred at room temperature for 45 min. First, PAMAM-G2 (60 mg, 0.208 mmol) was dissolved in 300 μL DMF, then solution was added to the reaction mixture and it was stirred for 5 days at room temperature. After removing DMF from the reaction mixture, ethyl acetate (50 mL) was added. A light brown solid was precipitated out. The solid product was filtered and washed with ethyl acetate and ether. Finally it was dried under vacuum to give desired compound as a light brown solid (61 mg, 42 %). We proceeded next step without further purification. Mp: 232-237 °C; ^{1}H NMR (300MHz, DMSO-d_6): δ 11.29 (bs, 15H, N*H*), 9.30 (bs, 12H, N*H*), 8.57 (bs, 12H, N*H*), 8.40 (bs, 9H, N*H*), 8.20 (bs, 20H, N*H*), 6.80-6.73 (m, 32H, C*H*), 3.63-3.57 (m, 8H, CH_2), 3.29-3.20 (m, 124H, CH_2), 3.14-3.04 (m, 41H, CH_2), 3.00-2.95 (m, 21H, CH_2), 2.54-2.50 (m, 5H, CH_2), 2.46-2.31 (m, 29H, CH_2), 1.45-1.43 (s, 144H, CH_3); ^{13}C NMR (150 MHz, DMSO-d_6): δ 162.29, 161.37, 159.87, 158.38, 158.21, 132.41, 129.37, 128.33, 125.37, 120.97, 115.13, 113.73, 111.79, 109.22, 80.92, 53.59, 51.67, 49.24, 46.33, 45.75, 41.83, 40.03, 39.91, 39.77, 39.64, 39.50, 39.36, 39.22, 39.08, 38.47, 38.34, 35.76, 27.73, 27.60; FT-IR (KBr, cm^{-1}): 3094, 1725, 1627, 1541, 1468, 1144; MS is not measured due to solubility problem.

Synthesis of the hybrid compound (1):

To a solution of the Boc-protectecd tetra guanidiniocarbonyl pyrrole (50 mg, 0.0064 mmol) in CH_2Cl_2 (4 mL), TFA (4 mL) was added and the reaction mixture was stirred at room temperature for 6 h. After removal of CH_2Cl_2 and TFA under reduced pressure, the residual light brown gum was diluted with ethyl acetate (50 mL). A light brown solid was precipitated out. The resulting precipitate was filtered and washed thoroughly with ethyl acetate. Finally it was purified by MPLC on C8 reversed-phase silica gel (45 % to 55 % methanol/water in 65 min, 0.1 % TFA) to obtain **1** as a slightly brown powder (23 mg, 38 %). Mp: 184-188 °C; ^{1}H NMR (300MHz, DMSO-d_6): δ 12.25 (bs, 13H, N*H*), 11.53 (bs, 11H, N*H*), 8.71 (bs, 20H, N*H*), 8.56 (m, 29H, N*H*), 8.37 (m, 17H, N*H*), 7.15 (bs, 16H, C*H*), 6.84-6.83 (m, 16H, C*H*), 3.43-3.19 (m, 181H, CH_2), 2.59-2.55 (m, 47H, CH_2); ^{13}C NMR (150 MHz, DMSO-d_6): δ 159.80, 159.38, 159.15, 158.71, 155.26, 132.60, 125.48, 117.67, 115.55, 115.37, 115.22, 112.34, 51.16, 48.94, 48.63, 38.43, 38.32, 36.45, 34.33, 33.61, 30.68, 28.82; FT-IR (KBr, cm^{-1}): 3312, 1654, 1638, 1181, 1123; MALDI-TOF-MS (pos.) m/z calculated for $C_{254}H_{384}N_{122}O_{60}$ $[M + H]^+$ 6107.54, found 6130.50 $[M + Na]^+$.

4. Spectra

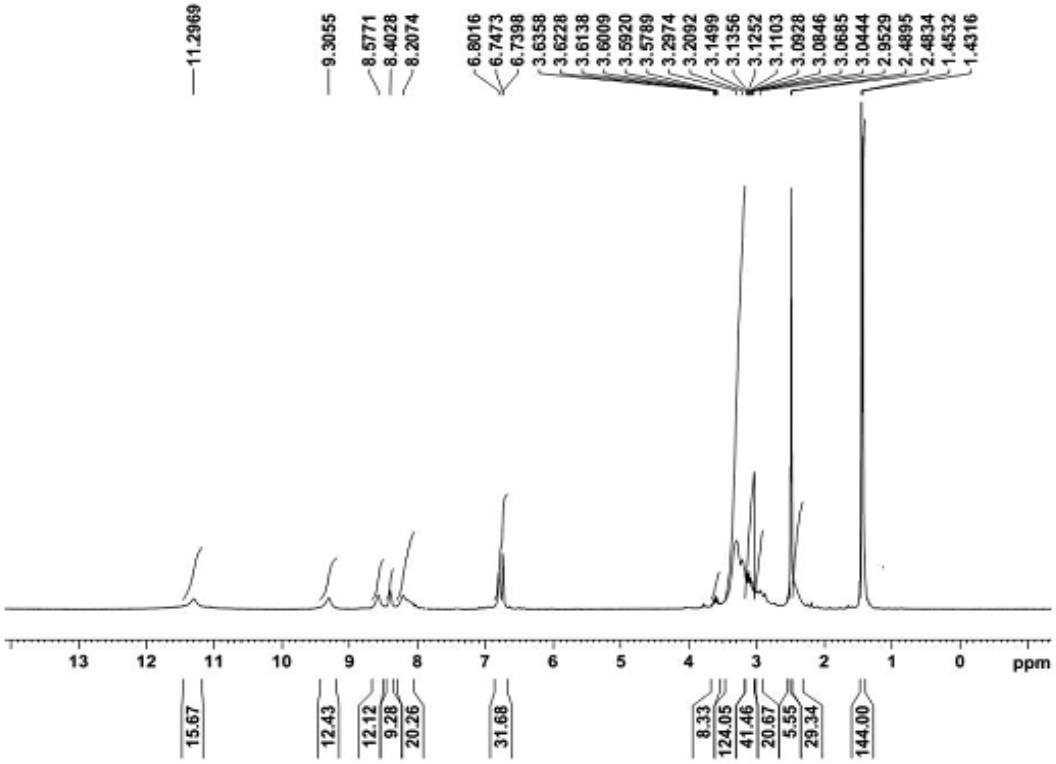


Figure S1: ^{1}H NMR spectrum of intermediate **2** in DMSO-d_6, 300 MHz.

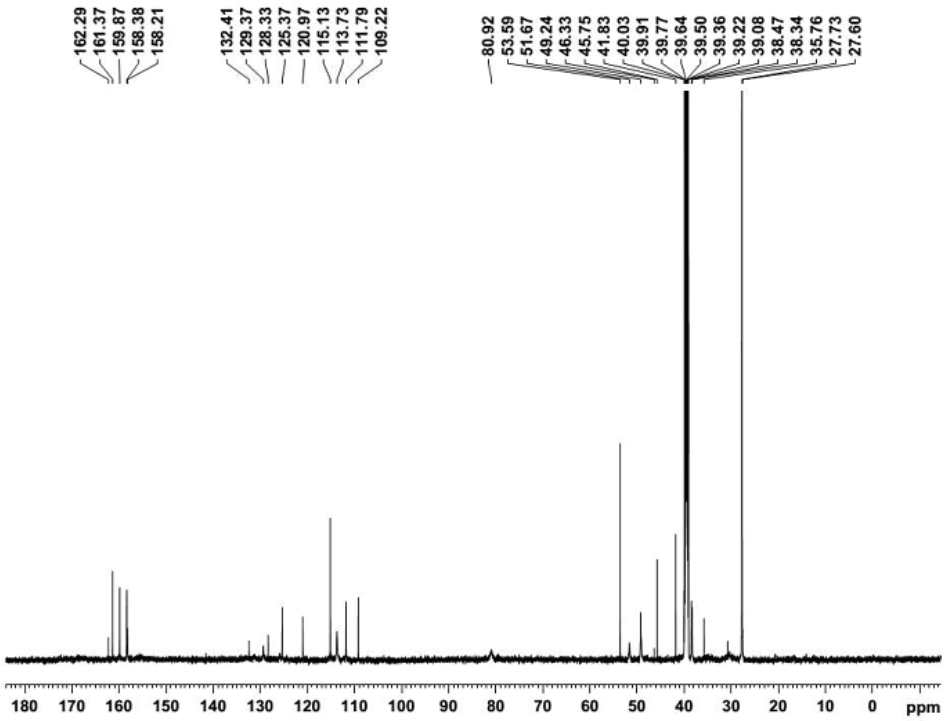


Figure S2: ^{13}C NMR spectrum of intermediate **2** in DMSO-d_6, 150 MHz.

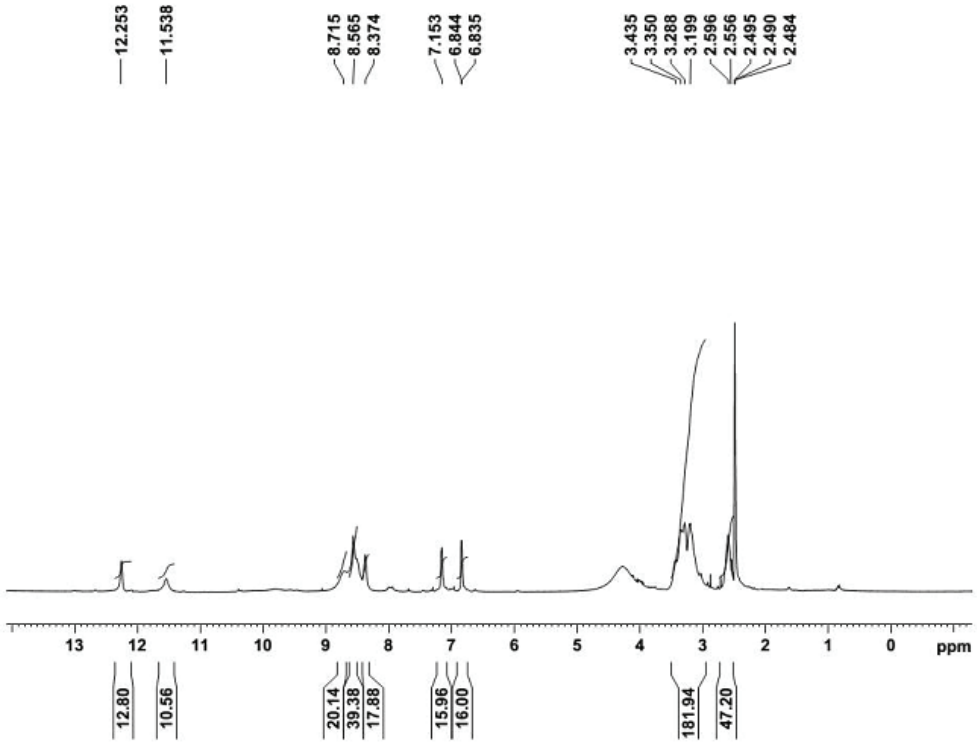


Figure S3: ^{1}H NMR spectrum of **1** in DMSO-d_6, 300 MHz.

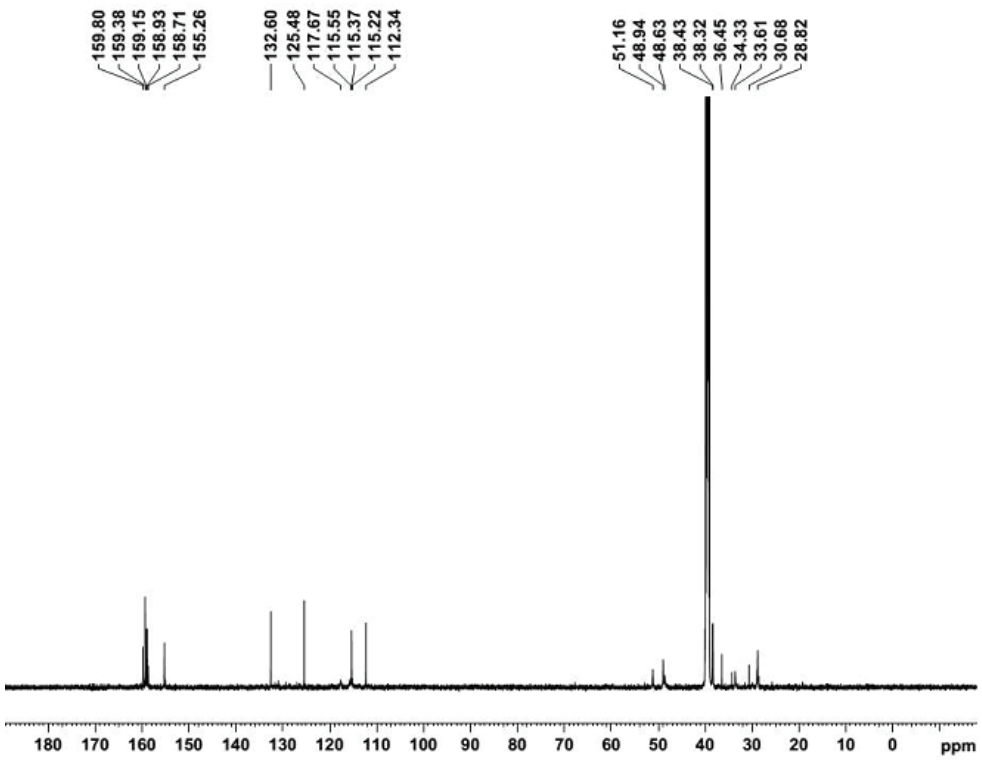


Figure S4: ^{13}C NMR spectrum of **1** in DMSO-d_6, 150 MHz.

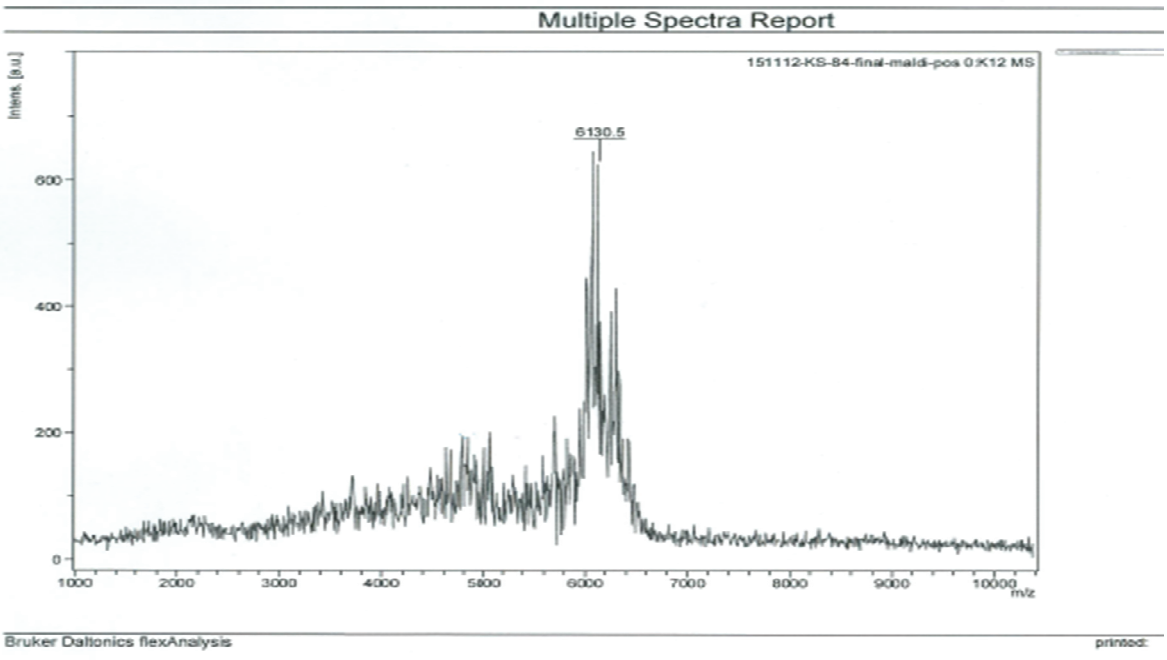
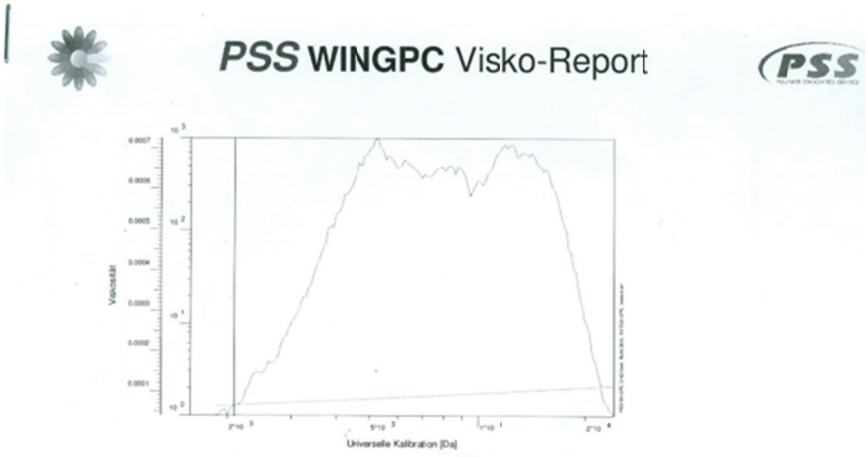


Figure S5: MALDI-TOF-Mass spectrum of **1**.

5. GPC analysis

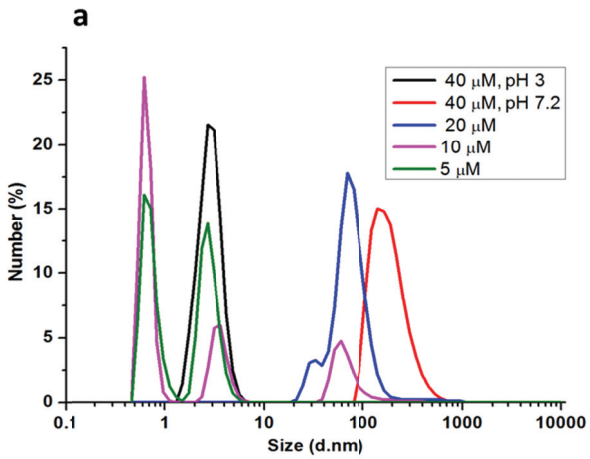


Probe:	Vial 2: KS-84 - 1	Methode:	C:\PSS WinGPC Messungen\GPC-3
Basislinie von:	19,97 ml bis 22,96 ml	Integration von:	19,97 ml bis 22,96 ml
Kalibration:	PMMA02122015-3.CAL	Kalibriertyp:	Viscometry
MHK-A (Kal.):	0,614	MHK-K (Kal.):	0,0292
Int.Stand.-K:	25,37 ml	Int.Stand.-M:	0,00 ml
Pumpe:	Jasco PU-2080	Flußrate:	1,00 ml/min
Eluent:	DMAc	Injektvolumen:	200 µl
Säulen:	PSS Gram lin 10µm		
Detektor 1:	ETA-2020 iP	Versatz:	0,000 ml
Detektor 2:	ETA-2020 dP	Versatz	0,000 ml
Detektor 3:	ETA-2020 RI	Versatz:	0,033 ml
Operateur:	Supervisor	Probenkonzentration:	4,0000 g/l

Detektor	Mn (D)	Mw (D)	D	Mp (D)	Fläche (V*ml)
ETA-2020 RI	5.601	8.483	1,51	5.180	0,0661

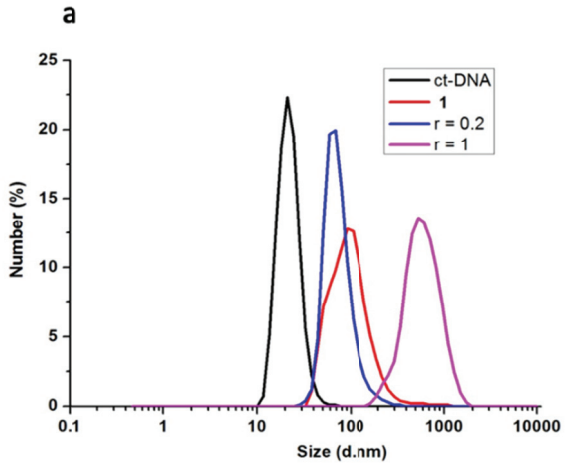
6. DLS experiments

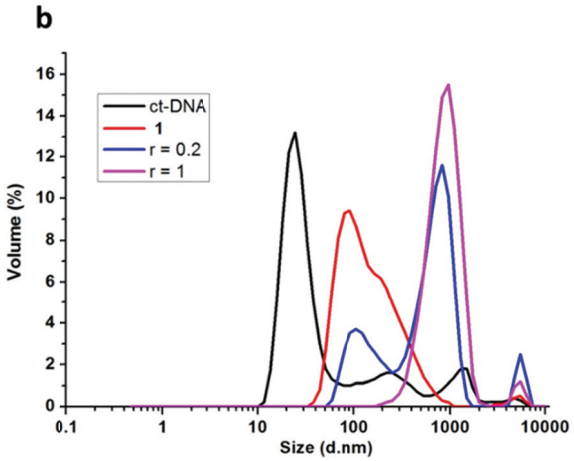
A 40 µM cacodylate buffer solution pH 7.0 (I = 0.01 M) of ctDNA, incubated with different ratios of **1** (r = 0.2, 1) and PAMAM-G2 (r = 0.5, 1), respectively. All measurements were carried out at 25° C in UV-transparent microcuvettes (1 cm) equipped with a stopper. The solution was filtered prior to measure *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).



Sample	Size (d.nm)	% Number	Pdi
1, 40 μM, pH 3	2.9	100	0.601
1, 40 μM, pH 7.3	192.2	100	0.965
1, 20 μM, pH 7.3	78.63, 31.53	89.8, 1.8	0.586
1, 10 μM, pH 7.3	0.65, 3.58, 78.39	60, 20, 20	0.189
1, 5 μM, pH 7.3	0.71, 2.81	49.9, 50.1	0.103

Figure S6: (a) Concentration and pH dependent number-weighted size distribution of **1**.

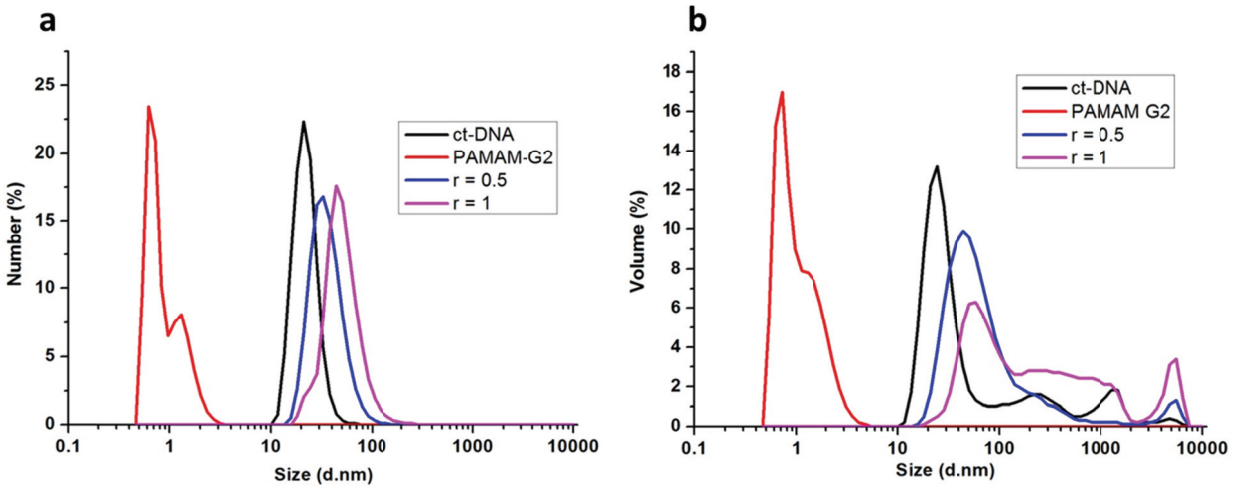




Sample	Size (d.nm)	% Number	Pdi
Ct-DNA	22.41	100	0.451
1	109.7	100	0.652
r = 0.2	76.36	100	0.220
r = 1	627.3	100	0.231

Sample	Size (d.nm)	% Volume	Pdi
Ct-DNA	28.05, 239.5, 1268	72.1, 16.6, 9.6	0.451
1	172.0, 4906	98.4, 1.6	0.652
r = 0.2	729.9, 135.1, 5590	69.0, 26.0, 5.0	0.220
r = 1	888.4, 5352	97.2, 2.8	0.231

Figure S7: (a) Number-weighted size distribution complexes; (b) Volume-weighted size distribution of complexes between ctDNA and **1** obtained from DLS measurements at pH 7.0 in 0.01 M sodium cacodylate buffer; [ctDNA] = [**1**] = 40 μM; r = [compound] / [ctDNA].



Sample	Size (d.nm)	% Number	Pdi
Ct-DNA	22.41	100	0.451
PAMAM-G2	0.70, 1.37	66.3, 33.7	0.598
r = 0.5	37.03	100	0.389
r = 1	53.23	100	0.527

Sample	Size (d.nm)	% Volume	Pdi
Ct-DNA	22.41	100	0.451
PAMAM-G2	1.12	100	0.598
r = 0.5	89.37, 1404, 4839	94.9, 0.8, 4.2	0.389
r = 1	75.18, 341.0, 1043	47.9, 25.3, 15.0	0.527

Figure S8: (a) Number-weighted size distribution of complexes; (b) Volume-weighted size distribution of complexes between ctDNA and PAMAM-G2 obtained from DLS measurements at pH 7.0 in 0.01 M sodium cacodylate buffer; [ctDNA] = [PAMAM-G2] = 40 µM; r = [compound] / [ctDNA].

7. Zeta potential

We exactly used the same ligand/DNA ratio at which the transfection studies were performed.

Ligand 1	Zeta potential (mV)	PAMAM-G2	Zeta potential (mV)
0.6 µM (N/P = 28)	10.9	0.6 µM (N/P = 9)	11.5
1 µM (N/P = 47)	28.2	1 µM (N/P = 16)	16.5
10 µM (N/P = 470)	33.8	10 µM (N/P = 160)	32.6

The overall positively charged ligand-DNA aggregates were formed.

8. 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 (400 µL, 1 µM, 1 eq), ctDNA (4.00 µM, 4 eq) was added and incubated for 15 min. The fluorescence emission was then measured from 540 to 670 nm utilizing an excitation wavelength of 520 nm. To this mixture a stock solution (5 µM) of **1**, and 1 µM of PAMAM-G2 was added in aliquots (1–13 µl) of **1** and 0.5-8 µL of PAMAM-G2. 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**], and [EB]/[PAMAM-G2], 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**, PAMAM-G2 that are necessary to displace half of the ethidium bromide from the EB/DNA complex.

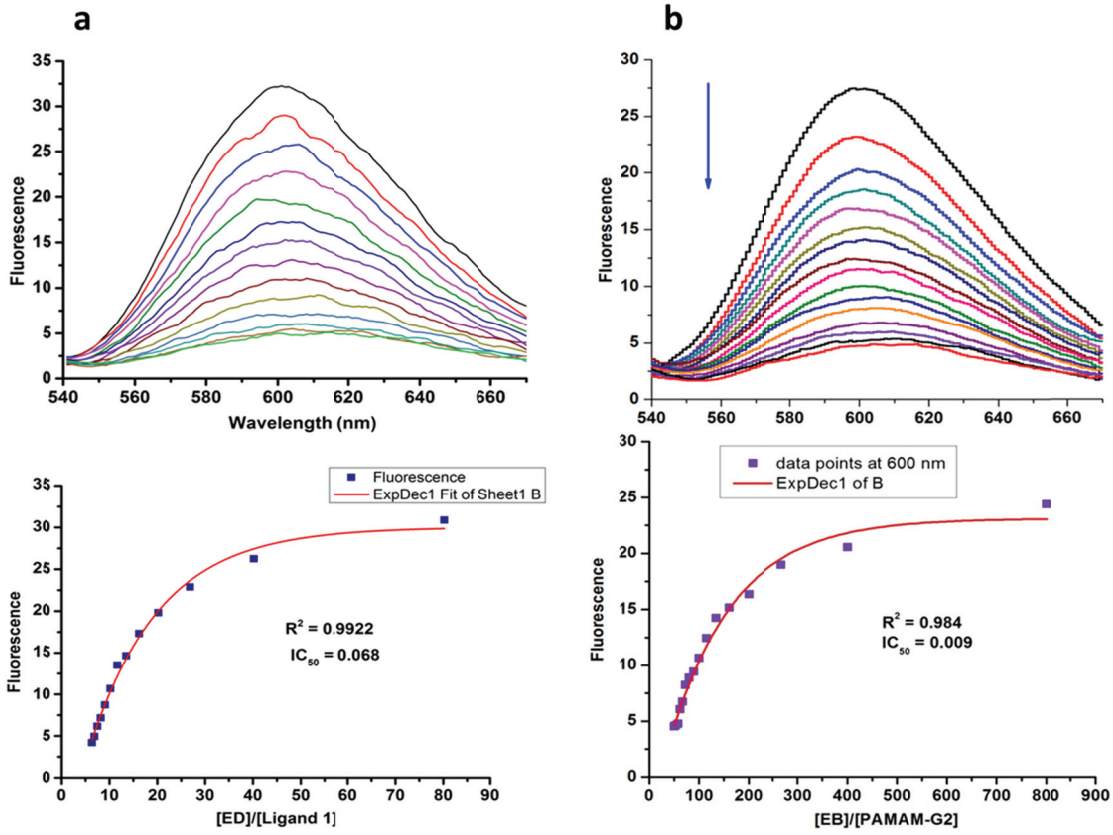


Figure S9. (a) Ethidium bromide (EB) displacement titration with 5 µM stock solution of **1** and the data is fitted with exponential decay first order function (Left), with IC_{50} value is 0.068 nM; (b) (EB) displacement titration with 1 µM stock solution of PAMAM-G2 and the data is fitted with exponential decay first order function (Right), with IC_{50} value is 0.009 nM.

***PAMAM-G2** has 7.5 times stronger binding affinity than ligand **1**.*

9. 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** (0.15 mM, 30 x 1 µL), and PAMAM-G2 (0.15 mM, 40 x **1** µL) were injected from a 297 µL rotating syringe (307 rpm) into the calorimeter reaction cell containing 1.45 mL of a ctDNA solution (0.02 mM). Data was analyzed using Origin 7.0 software according to a sequential binding sites model.

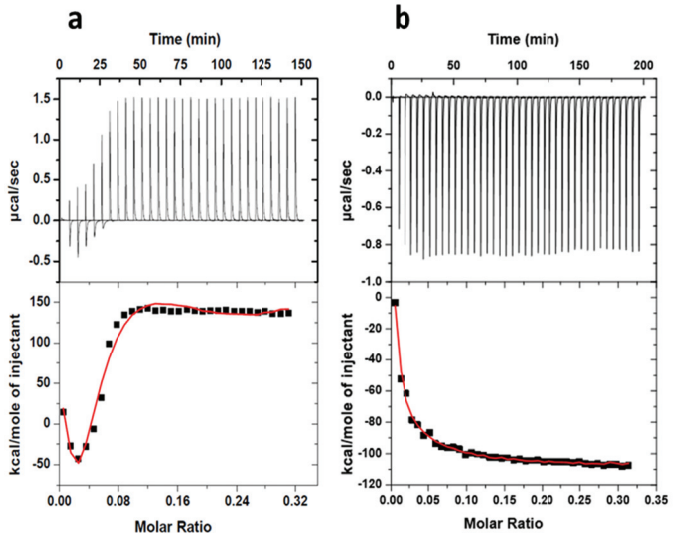


Table1: Thermodynamic parameters of ITC results

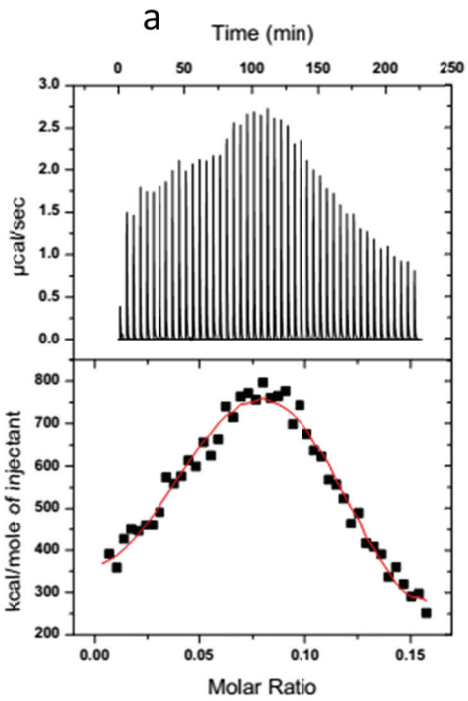
Hybrid compound **1**				**PAMAM-G2**			
K_a (x 10^5 M^{-1})	ΔH [kcal mol^{-1}]	ΔS [kcal mol^{-1}]	No.[a]	K_a (x 10^5 M^{-1})	ΔH [kcal mol^{-1}]	ΔS [kcal mol^{-1}]	No.[a]
K1 = 0.394	ΔH1 = 112.8 ±3.36	ΔS1 = 0.399 ±0.03	5	K1 = 0.335	ΔH1 = 92.63 ±0.67	ΔS1 = 0.331±0.002	3
K2 = 2.10	ΔH2 = -6633 ±144	ΔS2 = -22.2 ±0.89		K2 = 89.6	ΔH2 = -356.5 ±8.7	ΔS2 = -1.16 ±0.02	
K3 = 13.0	ΔH3 = 17640 ±289	ΔS3 = 59.2 ±0.75		K3 = 1.11	ΔH3 = 87.05 ±0.36	ΔS3 = 0.315 ±0.003	
K4 = 0.933	ΔH4 = -87970 ±486	ΔS4 = -295 ± 8.35					
K5 = 0.738	ΔH5 = 31,8000 ±2180	ΔS5 = 1070 ±105.6					

[a] indicates number of sequential binding sites; Experiment is carried out at 25 °C.

Figure S10: ITC titration in sodium cacodylate buffer (0.01 M) at pH 7. (a) **1** (0.15 mM) was added to ct-DNA (0.02 mM); (b) PMAM-G2 (0.15 mM) was added to ct-DNA (0.02 mM). **Table1** represents thermodynamic parameters of ITC results.

Reverse titration

Reverse titration for compound **1** was performed in which aliquots of ctDNA (0.15 mM, 45 x 1 µL), were injected into the calorimeter reaction cell containing 1.45 mL of compound **1** solution (0.02 mM).



Hybrid compound **1**			
K_a (x 10^5 M^{-1})	ΔH [kcal mol $^{-1}$]	ΔS [kcal mol $^{-1}$]	No.[a]
K1 = 0.221	ΔH1 = 1191 ±155	ΔS1 = 4.02 ±0.03	6
K2 = 0.249	ΔH2 = 8398 ±237	ΔS2 = 28.2 ±0.21	
K3 = 1.74	ΔH3 = 160900 ±580	ΔS3 = 540 ±6.5	
K4 = 0.673	ΔH4 = 72990 ±3600	ΔS4 = 245 ± 3.5	
K5 = 1.58	ΔH5 = -9767000 ±1330	ΔS5 = -32800 ±1256	
K6 = 23.3	ΔH6 = 10640000 ±1070	ΔS6 = 35700 ±2250	

[a] indicates number of sequential binding sites

Figure S11: Reverse ITC titration in sodium cacodylate buffer (0.01 M) at pH 7. (a) Positive heat exchanges were observed when aliquots of ctDNA (0.15 mM) were injected into **1** (0.02 mM). The data were fitted in sequential binding sites model with number 6.

10. AFM sample preparation

A 20 µM cacodylate buffer solution pH 7.0 (I = 0.01 M) of ctDNA (containing 263 µM magnesium chloride), incubated with different ratios of **1** (r = 0.2, 1) and PAMAM-G2 (r = 1). For each measurement 10 µL of the mixture was dropped onto a freshly cleaved mica surface and dried by spin coating (50 rps for 2 min) at room temperature and analyzed by tapping-mode AFM. r = [ligand]/[ctDNA].

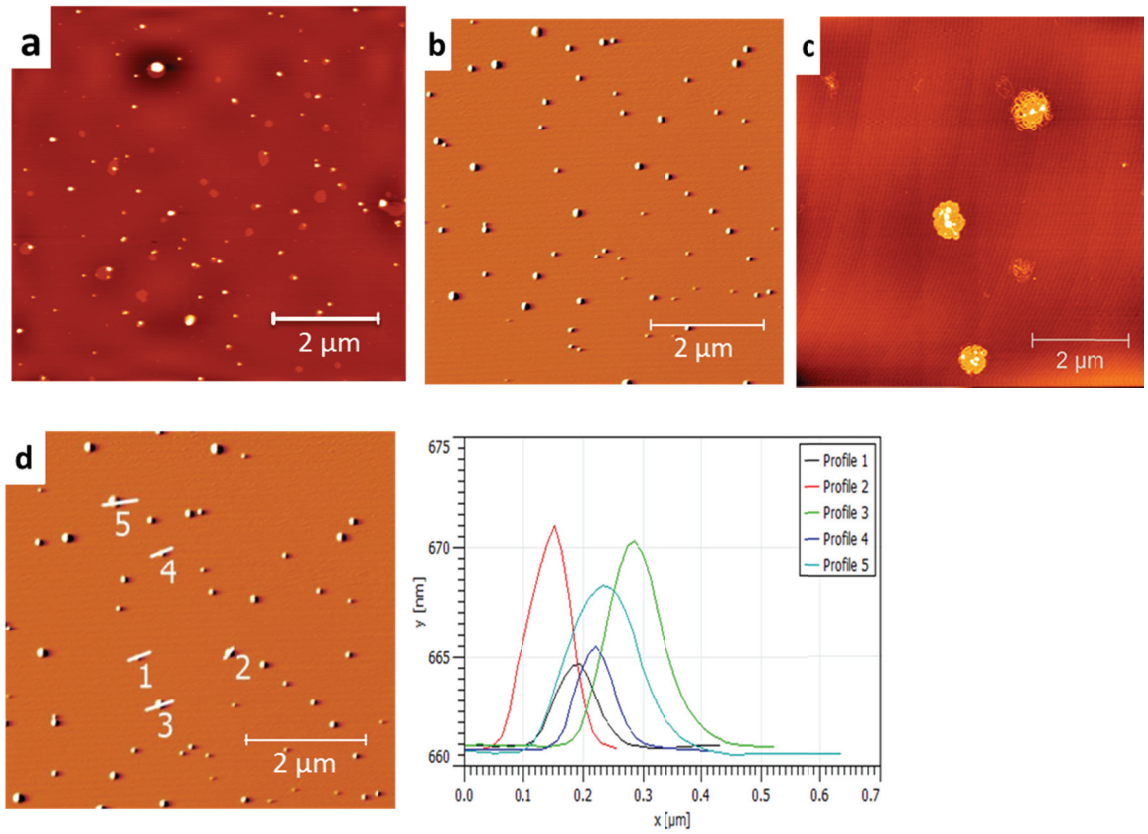


Figure S12: AFM images of (a) compound **1,** r = 0.2; (b) compound **1,** r = 1; (c) PAMAM-G2, r = 1, Z scale 4 nm; (d) height image of compound **1**, r = 1; r = [ligand]/[ctDNA].

*Addition of 0.2 equivalents of **1** led to the formation of weak condensed circular aggregates of ctDNA (Figure S12: a). With further increasing the ratios r = 1, very tight and compact polyplexes were formed (Figure S12: b). PAMAM-G2 weakly condensed ctDNA at r = 1 (Figure S12: c).*

11.Circular dichroism (CD) study

All measurements were carried out in aqueous sodium cacodylate buffer **(**0.01 M, pH 7.00 ± 0.01) in quartz UV microcuvettes (1 mm) equipped with a stopper at 20° C. To a solution of ctDNA **(**200 µL, 100 µM), a stock solution of **1** (1 mM) and PAMAM-G2 (1 mM) was added in aliquots. CD spectra were collected with a Jasco J-810 spectrometer.

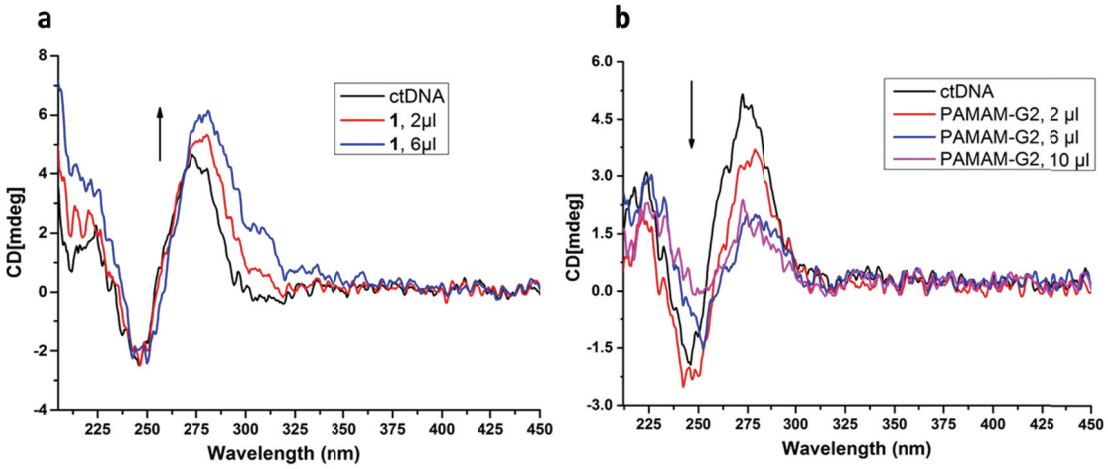


Figure S13: (a) Positive band at 275 nm is increasing upon addition of compound **1**; (b) Positive band at 275 nm is decreasing upon addition of PAMAM-G2.

12.Transfection results on Hela cells in the presence of Chloroquine

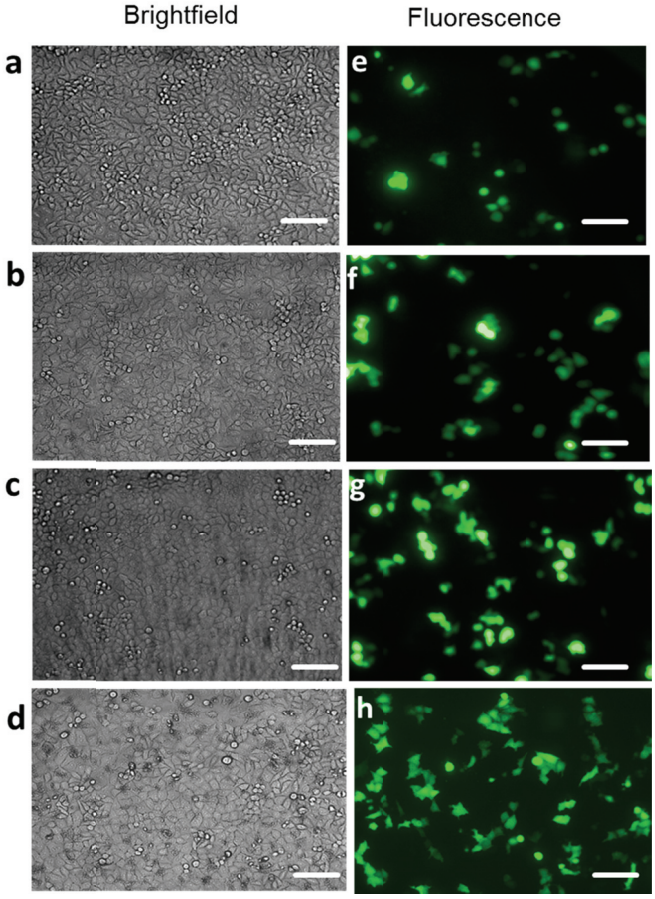


Figure S14. Transfection of 2 µg pF143-GFP plasmid when using **1** at different concentrations and PEI (0.15 µM) in the presence of 25 µM chloroquine. Brightfield (a–d) and fluorescence (e–h) images of HeLa cells 48 h after transfection with 0.6 µM (N/P = 28; a,e), 1 µM (N/P = 47; b,f), 10 µM (N/P = 470; c,g), and 150 µM PEI (N/P = 3866; d,h). Both PAMAM-G2 and PEI is unable to transfect in the concentration ranges (0.6 µM to 10 µM). Scale bar = 50 µm.

13. Transfection results of PAMAM-G2 at 200 µM on HeLa cells

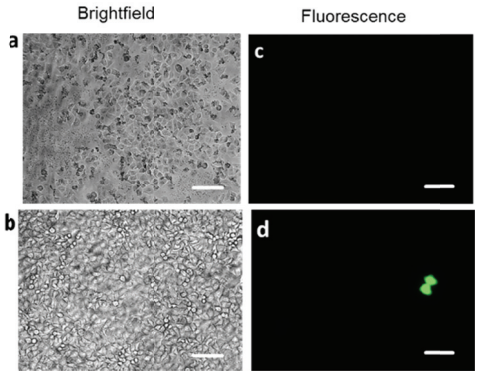


Figure S15. Transfection of 2 µg pF143-GFP plasmid when using PAMAM-G2 at 200 µM (N/P = 1888) in the presence of 25 µM chloroquine. Brightfield (a–b) and fluorescence (c–d) images of HeLa cells 48 h after transfection in the absence (a,c), and presence (b,d), of chloroquine. Scale bar = 50 µm.

PAMAM-G2 weakly condensed ctDNA. It is the main influence of its zero transfection efficiency.

14. Transfection results in Hek cells in the presence and absence of chloroquine

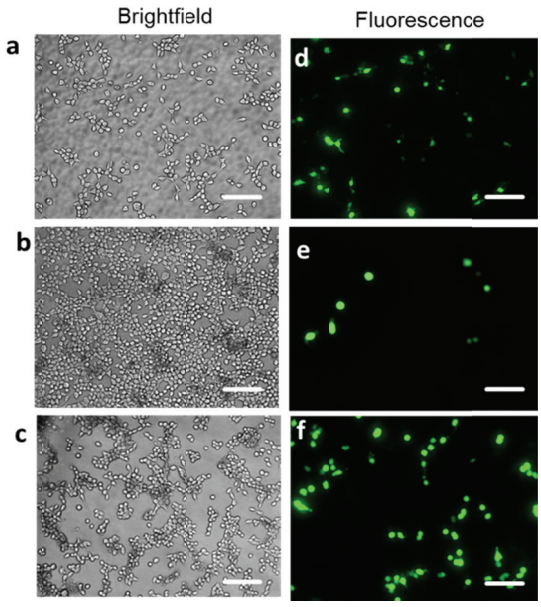


Figure S16. Transfection of 2 µg pF143-GFP plasmid when using **1**, PAMAM-G2 and PEI in the absence of chloroquine. Brightfield (a–c) and fluorescence (d–f) images of Hek cells 48 h after transfection with 10 µM **1** (N/P = 470; a,d), 200 µM PAMAM-G2 (N/P = 1888; b,e) and 150 µM PEI (N/P = 3866; c,f). Scale bar = 50 µm.

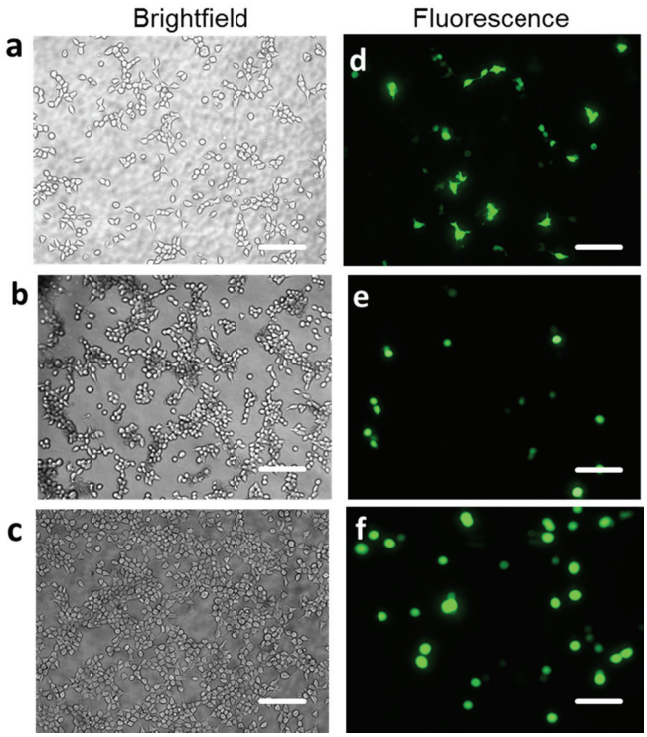


Figure S17. Transfection of 2 µg pF143-GFP plasmid when using **1**, PAMAM-G2 and PEI in the presence of 25 µM chloroquine. Brightfield (a–c) and fluorescence (d–f) images of Hek cells 48 h after transfection with 10 µM **1** (N/P = 470; a,d), 200 µM PAMAM-G2 (N/P = 1888; b,e) and 150 µM PEI (N/P = 3866; c,f). Scale bar = 50 µm.

15. UV-Vis spectroscopy

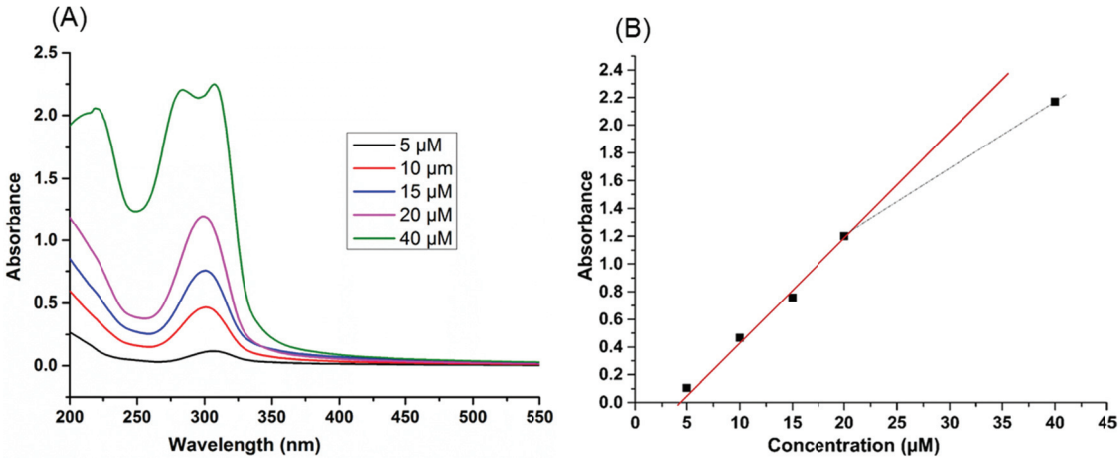


Figure S18: (a) Absorbance of compound **1** at various concentration. (b) Deviation of Lambert-Beer law at 40 µM.