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Supporting Information:

Tuning of Endosomal Escape and Gene Expression by Functional Groups, Molecular Weight and Transfection Medium: A Structure-Activity Relationship Study

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List of Tables.

List of Figures.

ADDITIONAL METHODS

Materials.

All chemicals were used as received unless stated otherwise. BocAEAm was prepared according to a previously described procedure.[1, 2] The chain transfer agents 2-(Butylthiocarbonothioylthio) propanoic acid (PABTC) and 2-(Butylthiocarbonothioylthio)propanoic acid-*N*-hydroxysuccinimide (NHS-PABTC) were prepared following previously reported procedures.[3] Dimethylamino propyl acrylamide (DMAPAm) was obtained from ABCR (Germany). Dimethylamino ethyl acrylamide (DMAEAm) was obtained from ABCR and purified by column chromatography (silica, ethyl acetate). Sodium hydrogen carbonate ($NaHCO_3$) and sodium chloride (NaCl) was obtained from Fisher (Germany). Acryloyl chloride (97%), 1,3-bis-(*tert*-butoxycarbonyl)-2-methyl-2-thiopseudourea (98%), ethylene diamine and 1,3-diaminopropane (≥99%) were obtained from Sigma (Germany). Trimethylamine (≥99.5%) was obtained from Carl Roth (Germany). Di-tert-butyl dicarbonate and trifluoroacetic acid (TFA) were obtained from TCI (Germany). 2,2'-Azobis(2,4-dimethylvaleronitrile) (V65B) was obtained from FUJIFILM Wako Chemicals (Germany). HPLC grade dimethylformamide (DMF) (HiPerSolv CHROMANORM) was obtained from VWR (Germany). Anhydrous *N,N*-dimethylacetamide (99.8%, DMAc) was obtained from Sigma. 1,4-dioxane (>99.5%) was obtained from Carl Roth and purified over inhibitor remover beads (for hydroquinone and monomethyl ether hydroquinone) at 4 °C. Dichloromethane (DCM) was obtained from a solvent purification system (SPS) on site, tetrahydrofuran (THF), methanol, hexane and ethyl acetate were distilled on site. DY-635 amine and DY-635 NHS ester were obtained from Dyomics (Germany). 2-(1H-Benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) was obtained from Iris Biotech (Germany). 4-methylmorpholine (99%) was obtained from Alfa Aesar (Germany). For biological studies, following substances were ordered from suppliers in brackets: cell culture media and

supplements (Biochrom, Merck Millipore, Germany), Opti-MEM™ reduced serum medium (Gibco, Thermo Fisher, Germany), fetal calf serum (FCS, Capricorn Scientific, Germany), alamarBlue™ solution, YOYO™-1 iodide (Life Technologies, Thermo Fisher, Germany), trypsin-EDTA-solution, Triton X-100, 0.4% trypan blue solution and Hanks' balanced salt solution, calcein, L-α-phosphatidylcholine (PC), L-α-phosphatidylethanolamine, dioleyl (PE, Sigma-Aldrich), Bis(monomyristoyl-glycero)phosphate (BMP, Avanti Polar Lipids, US), 1% ethidium bromide solution (EtBr, Carl Roth), heparin sodium salt from porcine intestinal mucosa (Alfa Aesar) and linear poly(ethyleneimine) (LPEI, M_w = 25 kg mol^{-1}) and branched PEI (BPEI, M_w = 10 kg mol^{-1}, Polysciences, Germany). Plasmid DNA (pDNA) encoding the enhancend green fluorescent protein (EGFP) for transfection studies was isolated with the Giga Plasmid Kit (Qiagen, Germany) from *E. coli* containing pEGFP-N1 (4.7 kb, Clontech, USA). For all other studies, like pDNA binding or uptake, the ready-to-use plasmid pCMV-GFP (PlasmidFactory, Germany) was used.

Instruments.

Nuclear magnetic resonance (NMR) spectroscopy. ^{1}H NMR (300 MHz) and DEPT ^{13}C (75 MHz) spectra were recorded on a Bruker AC 300 MHz spectrometer at 300 K. The delay time (d1) was set at 1 s for ^{1}H NMR and 2 s for DEPT ^{13}C. Chemical shifts (δ) are reported in ppm.

Size exclusion chromatography (SEC). SEC was conducted on one of two instruments. Dimethylacetamide (DMAc)-SEC was conducted using an Agilent 1200 series instrument equipped with differential refractive index (DRI) and UV/vis (DAD) detector. The liquid chromatography system used 1 × PSS GRAM 30 Å column (300 × 0.8 mm, 10 μm particle size) and 1 × PSS GRAM 1000 Å column (300 × 0.8 mm, 10 μm particle size). The DMAc eluent contained 0.21 wt.% LiCl as additive. Samples were run at 1 mL min^{-1} at 40 °C. Analyte samples

were filtered through a polytetrafluoroethylene (PTFE) membrane with 0.45 μm pore size prior to injection. Poly(methyl methacrylate) (PMMA) narrow standards (PSS) were used to calibrate the SEC system. Aq.-SEC was conducted using a Jasco instrument equipped with DRI and UV (DAD) detector. The liquid chromatography system used 2 × PSS NOVEMA-MAX column (300 × 0.8 mm, 10 μm particle size). The aqueous eluent contained 0.1% TFA + 0.1 mol L^{-1} NaCl as additive. Samples were run at 1 mL min^{-1} at 30 °C. Analyte samples were filtered through a nylon membrane with 0.45 μm pore size prior to injection. Poly(2-vinylpyridine) (P2VP) narrow standards (Polymer Source Inc. Dorval, Quebec, Canada) were used to calibrate the SEC system. Experimental $M_{n,SEC}$ and $Đ$ (M_w/M_n) values of synthesized polymers were determined using PSS WinGPC UniChrom GPC software.

Titration. Titration of the polymers was conducted using a Metrohm OMNIS integrated titration system. For a typical measurement, the polymer was dissolved in 125 mM NaCl (in ultrapure water) (1.0 mg mL^{-1}), which was acidified with addition of 1 M hydrochloric acid (pH ~ 2). The polymers were titrated (with dynamic flow rate adjustment) against 0.1 M NaOH solution up to a pH value of 12. The PGPAm polymers were titrated against 0.5 M NaOH. The pK_a values were estimated using the Henderson-Hasselbalch equation (5) from equivalence points determined by the OMNIS titration software.

$$\mathrm{pH}=\mathrm{p}K_a+\log\frac{[\mathrm{A}^-]}{[\mathrm{HA}]} \qquad (5)$$

Flow cytometry. Flow cytometry was conducted on either the Cytomics FC 500 or the CytoFlex S by Beckman Coulter. With both instruments 10^4 cells were analyzed regarding their forward and sideward scattering (FSC, SSC) and their fluorescence at λ_{Ex} = 488 with a 525 nm bandpass filter, since all employed stains (YOYO-1, EGFP, calcein) were green fluorescent.

Microplate reader. Fluorescence intensity measurements for EBA, HRA, alamarBlue™, LDH and BMP assays as well as absorption measurements for hemolysis and aggregation assays were performed on the Infinite M200 PRO microplate reader (Tecan, Germany) with λ_{Ex} / λ_{Em} used as indicated in the respective method sections and gain set to optimal.

Monomer Synthesis.

Synthesis of 1,3-Di-Boc-guanidinopropyl acrylamide, GPAm^{diBoc}. 1,3-Bis(*tert*-butoxycarbonyl)-2-methyl-2-thiopseudourea (10.34 g, 3.56×10^{-2} moles) in dry DCM (85 mL) was added dropwise *via* a pressure-equalizing dropping funnel to a solution of 1,3-diaminopropane (7.99 g, 9.0 mL, 1.08×10^{-1} moles) in DCM (85 mL) in a 250 mL round-bottomed flask equipped with a magnetic stirring bar. Following complete addition, the reaction was left to stir at room temperature overnight. A white precipitate formed. Dry DCM (40 mL) was added, and the solution was filtered to remove the precipitate. The solution as washed with deionized H_2O (3 × 200 mL) and brine (2 × 200 mL), the organic layer was dried over $MgSO_4$ and the DCM was removed under vacuum to yield crude 2-[1,3- Bis(tert-butoxycarbonyl)guanidine]ethylamine as a colourless, slightly turbid oil (12.40 g, 3.92×10^{-2} moles). The crude product was dissolved in dry DCM (200 mL) and transferred to a 500 mL two-necked round-bottomed-flask equipped with a magnetic stirring bar. Et_3N (6.6 mL, 4.74×10^{-2} moles) was added, the flask was fitted with a pressure-equalising dropping funnel, sealed, purged with argon, and cooled in an ice bath. Acryloyl chloride (2.84 g, 3.20 mL, 3.94×10^{-2} moles) in dry DCM (40 mL) was added dropwise and the reaction was left to stir at room temperature overnight, to give a clear pale-yellow solution. Dry DCM (100 mL) was added, then saturated $NaHCO_3$ (400 mL) was added and the aqueous layer was extracted with DCM (3 × 3400 mL). The organic layers were dried over $MgSO_4$ and the solvent was removed under vacuum to give a viscous yellow oil, which was subjected to flash column chromatography

(silica, hexane/ethyl acetate) to afford 2-[1,3-Bis(*tert*-butoxycarbonyl)guanidine]propyl acrylamide as a white solid. ^{1}H NMR (300 MHz, 300 K, $CDCl_3$, δ): 11.44 (s, 1 H, –NH(–N=C)–N***H***–), 8.47 (t, 6.1 Hz, 1 H, –CH_2–N***H***–(C=N–)NH–), 7.91 (t, 1 H, –CH_2–N***H***–(C=O)–), 6.30 (1 H, –(C=O)–C***H***=CH2), 6.28 (d, 1 H, –(C=O)–CH=C***H*****$_2$**), 5.55 – 5.59 (dd, 1 H, –(C=O)CH=C***H*****$_2$**), 3.46 – 3.52 (m, 2 H, –C***H*****$_2$**–NH–(C=N–)NH–), 3.30 – 3.36 (m, 2 H, –C***H*****$_2$**–NH–(C=O)–), 1.69 (m, 2 H, –CH_2–C***H*****$_2$**–CH_2–), 1.49 (2 × s, 18 H, –O–C((C***H*****$_3$**)$_3$). ^{13}C NMR (75 MHz, 300 K, CDCl3, δ): 131.8 (CH_2=***C***H–(C=O)–), 125.4 (***C***H_2=C–), 37.1 (–***C***H_2–NH–(C=O)–), 34.8 (–***C***H_2–NH–(C=N–)NH–), 29.7 (–CH_2–***C***H_2–CH_2–), 28.3 (–O–C((***C***H_3)$_3$), 28.0 (–O–C((***C***H_3)$_3$). MS: $[M + H]^+$ 371.22 (calculated), 371.23 (found).

Synthesis of N-t-butoxycarbonyl-N'-acryloyl-1,2-diaminoethane (AEAm^{Boc}). Ethylene diamine (30.02 g, 5.00×10^{-1} moles) was dissolved in dry DCM (280 mL) in a 500 mL round-bottomed-flask equipped with a magnetic stirring bar. The flask was fitted with a pressure-equalising dropping funnel, sealed, purged with argon, and cooled in an ice bath. Di-tert-butyl dicarbonate (27.6 g, 1.25×10^{-1} moles) in dry DCM (120 mL) added dropwise with stirring over 1 h. The reaction was allowed to reach room temperature and left to stir overnight. The resulting solution was filtered to remove precipitate, and concentrated under vacuum. Deionized H_2O (350 mL) added, and the solution was filtered to remove the resulting precipitate (*N*,*N*'-(bis-*tert*-butoxycarbonyl)-1,2-diaminoethane). The aqueous solution was saturated with NaCl, and extracted with EtOAc (3 x 300 mL), the organic layers combined, dried over $MgSO_4$ and concentrated under vacuum to yield *N-tert*-butoxycarbonyl-1,2-diaminoethane as a clear oil (14.39 g, 8.985×10^{-2} moles), which was dissolved in dry DCM (300 mL) and transferred to a 500 mL two-necked round-bottomed-flask equipped with a magnetic stirring bar. Et_3N (15.0 mL, 1.08×10^{-1} moles) was added, the flask was fitted with a pressure-equalizing dropping funnel, sealed,

purged with argon, and cooled in an ice bath. Acryloyl chloride (7.30 mL, 8.96×10^{-2} moles) in dry DCM (100 mL) was added dropwise and the reaction was left to stir at room temperature overnight. The solution was concentrated under vacuum, dissolved in deionized H_2O (300 mL), and extracted with $CHCl_3$ (3 × 300 mL). The organic layers were combined, dried over MgSO4 and concentrated under vacuum to give a white solid, which was subjected to flash column chromatography (silica, hexane/ethyl acetate) to afford *N-tert*-butoxycarbonyl-*N*'-acryloyl-1,2- as a white solid. ^{1}H NMR (300 MHz, 300 K, $CDCl_3$, δ): 6.65 (br, 1 H, –N***H***–(C=O)CH=CH_2), 6.22 – 6.28 (dd, 17.1 Hz, 1.4 Hz, 1 H, –NH–(C=O)CH=C***H₂***), 6.05 – 6.14 (dd, 17.1 Hz, 10.1 Hz, 1 H, –NH–(C=O)C***H***=CH_2), 5.60 – 6.64 (dd, 10.1 Hz, 1.6 Hz, 1 H, –NH–(C=O)CH=C***H₂***), 5.11 (br, 1 H, –N***H***–(C=O)O–), 3.40 – 3.45 (m, 2 H, –C***H₂***–NH–(C=O)CH=CH_2), 3.27 – 3.32 (m, 2 H, –C***H₂***–NH–(C=O)O–), 1.42 (s, 9 H, –O–C((C***H₃***)$_3$). ^{13}C NMR (75 MHz, 300 K, CDCl3, δ): 130.9 (CH_2=***C***H–(C=O)–), 126.3 (***C***H_2=C–), 41.0 (–***C***H_2–NH–(C=O)CH=CH_2), 40.1 (–C***H₂***–NH–(C=O)O–), 28.3 (–O–C((***C***H_3)$_3$). MS: $[M + H]^+$ 237.13 (calculated), 237.12 (found).

Calculations for RAFT Polymerization.

Monomer conversion (*p*) was calculated from ^{1}H NMR data by comparing the integrals of vinyl peaks (5.5-6.3 ppm) against an external reference (1,3,5-trioxane, 5.14 ppm) before (*t*=0) and after (*t*=final) polymerization. The theoretical number-average molar mass ($M_{n,th}$) was then calculated using equation (6):

$$M_{n,th} = \frac{[M]_0 p M_M}{[CTA]_0} + M_{CTA} \qquad (6)$$

Where $[M]_0$ and $[M]_0$ are the initial concentrations of monomer and chain transfer agent (CTA), respectively, M_M and M_{CTA} are the molecular weight of the monomer and CTA, respectively, and *p* is the monomer conversion.

Table S1. Amount of different substances used for polymerization of PGPAms and PAEAms.

	$PGPAm_8$	$PGPAm_{22}$	$PGPAm_{43}$	$PGPAm_{89}$	$PGPAm_{94}$	$PAEAm_9$	$PAEAm_{24}$	$PAEAm_{45}$	$PAEAm_{96}$
Monomer	diBocGPAm	diBocGPAm	diBocGPAm	diBocGPAm	diBocGPAm	BocAEAm	BocAEAm	BocAEAm	BocAEAm
$DP_{n,target}$	10	25	50	100	100	10	25	50	100
m_{CTA} added (mg)	38.2	15.41	4.13*[a]	4.48	3.25[a]	68.4	27.0	6.12[a]	5.44[a]
n_{CTA} added (moles)	1.60×10^{-4}	6.46×10^{-5}	1.24×10^{-5}	1.88×10^{-5}	9.74×10^{-6}	2.87×10^{-4}	1.13×10^{-4}	1.83×10^{-5}	1.63×10^{-5}
$m_{monomer}$ added (mg)	597.7	599.9	230.7	700.6	362.7	600.2	602.4	194.2	348.6
$n_{monomer}$ added (moles)	1.61×10^{-3}	1.61×10^{-3}	6.20×10^{-4}	1.89×10^{-3}	9.74×10^{-4}	2.80×10^{-3}	2.81×10^{-3}	9.07×10^{-4}	1.63×10^{-3}
m_{V65b} added (mg)	3.18	3.23	0.65	3.01	0.515	5.40	2.90	0.63	0.42
n_{V65b} added (moles)	1.23×10^{-5}	1.25×10^{-5}	2.51×10^{-6}	1.17×10^{-5}	1.99×10^{-6}	2.09×10^{-5}	1.12×10^{-5}	2.43×10^{-6}	1.63×10^{-6}
Dioxane added (uL)	835.6	841.9	185.5	1029.4	540.9	949.9	951.1	283.4	563.7
DMAc added (uL)	375.9	365.8	430.6	428.5	222.3	420.8	369.6	297.8	269.8
PABTC/V65b	13.0	5.2	4.9	1.6	4.9	13.7	10.1	7.6	10.0
T (°C)	45	45	50	45	45	50	50	50	60
Time (min)	540	540	360	300	300	300	300	360	240

Table S2. Amount of different substances used for polymerization of PDMAEAms and PDMAPAms.

	$PDMAEAm_8$	$PDMAEAm_{22}$	$PDMAEAm_{45}$	$PDMAEAm_{88}$	$PDMAPAm_{11}$	$PDMAPAm_{24}$	$PDMAPAm_{38}$	$PDMAPAm_{71}$
Monomer	DMAEAm	DMAEAm	DMAEAm[a]	DMAEAm[a]	DMAPAm[a]	DMAPAm[a]	DMAPAm[a]	DMAPAm[a]
$DP_{n,target}$	10	25	49	99	14	30	50	99
m_{CTA} added (mg)	67.1	25.1	8.25[b]	5.97[b]	50.3	19.9	7.49[b]	5.33[b]
n_{CTA} added (moles)	2.81×10^{-4}	1.05×10^{-4}	2.47×10^{-5}	1.79×10^{-5}	2.11×10^{-4}	8.35×10^{-5}	2.24×10^{-5}	1.60×10^{-5}
$m_{monomer}$ added (mg)	400.7	371.8	174.0	252.2	461.0	383.3	175.5	248.9
$n_{monomer}$ added (moles)	2.82×10^{-3}	2.62×10^{-3}	1.22×10^{-3}	1.77×10^{-3}	2.95×10^{-3}	2.46×10^{-3}	1.12×10^{-3}	1.59×10^{-3}
m_{V65b} added (mg)	5.77	3.05	1.21	0.93	5.07	2.11	1.16	0.85
n_{V65b} added (moles)	2.23×10^{-5}	1.18×10^{-5}	4.67×10^{-6}	3.60×10^{-6}	1.96×10^{-5}	8.17×10^{-6}	4.49×10^{-6}	3.27×10^{-6}
Dioxane added (uL)	461.4	229.0	514.4	574.1	242.3	202.5	483.5	577.0
DMAc added (uL)	209.6	233.5	141.9	140.9	249.7	206.0	136.8	34.5
PABTC/V65b	12.6	8.9	5.3	5.0	10.8	10.2	5.0	4.9
T (°C)	60	60	50	60	60	60	50	60
Time (min)	240	240	420	240	240	240	420	240

[a] Monomer was not purified

[a] Polymerization using PBTC-NHS

Typical Attachment of DY-635 to PAm Polymers.

New batches of polymers were used in each instance (Aq.-SEC, P2VP standards): $PAEAm_{96}$, $M_{n,SEC}$ = 12.8 kg mol^{-1}, Đ = 1.16; $PGPAm_{94}$, $M_{n,SEC}$ = 9.2 kg mol^{-1}, Đ = 1.25; $PDMAEAm_{94}$, $M_{n,SEC}$ = 10.3 kg mol^{-1}, Đ = 1.26; $PDMAPAm_{111}$, $M_{n,SEC}$ = 11.9 kg mol^{-1}, Đ = 1.51. The respective polymer, a 2 mg mL^{-1} solution of DY-635 amine in HPLC grade DMF, a 2 mg mL^{-1} solution of HBTU in HPLC grade DMF and a 2 mg mL^{-1} solution of NMM in HPLC grade DMF were added to a screw-cap vial equipped with a magnetic stirring bar (Table S3).The reaction was left to stir in the dark for 24 h. The solution was diluted in ultrapure water and dialyzed against first H_2O/MeOH (4/1) for 2 days and then against H_2O for 3 days. The polymer was obtained as a blue solid following lyophilization. For $PDMAEAm_{94}$, a 5 mg mL^{-1} solution of DY-635 amine and a 1 mg mL^{-1} solution of NMM were used. Regarding dye attachment to $PAEAm_{96}$, the DY-635 NHS-ester was used.

Table S3. Amount of different substances used for attachment to DY-635 amine to PAms

Substance	Mass mg	Amount of substance mol
$PGPAm_{94}$	39.6	1.35×10^{-6}
DY-635-Amine	0.99	1.39×10^{-6}
HBTU	0.84	2.02×10^{-6}
NMM	0.41	4.04×10^{-6}
$PAEAm_{96}$	29.0	1.31×10^{-6}
DY-635-NHS	0.99	1.35×10^{-6}
NMM	0.40	3.93×10^{-6}
$PDMAEAm_{94}$	21.3	1.57×10^{-6}
DY-635-Amine	1.16	1.57×10^{-6}
HBTU	0.97	2.35×10^{-6}
NMM	0.48	4.7×10^{-6}
$PDMAPAm_{111}$	22.7	1.34×10^{-6}
DY-635-Amine	0.99	1.34×10^{-6}
HBTU	0.83	2.02×10^{-6}
NMM	0.41	4.03×10^{-6}

N*/P Ratio Calculations.

The N*/P ratio was defined as the ratio of the total amount of protonatable amines in polymer solution in relation to the total amount of phosphates in the pDNA solution.

The volume of polymer needed to prepare polyplexes with 15 μg mL^{-1} pDNA at different N*/P ratios was calculated as described by the following equations:

$$V_{total} \cdot P = V_{poly} \cdot N_{poly}$$

$$V_{poly} = \frac{V_{total} \cdot P}{N_{poly}}$$

$$V_{poly} = V_{total} \cdot \frac{n_{pDNA} \cdot P}{n_{poly} \cdot N}$$

$$V_{poly} = V_{total} \cdot \frac{m_{pDNA} \cdot P \cdot M_{poly}}{m_{poly} \cdot N \cdot M_{pDNA}}$$

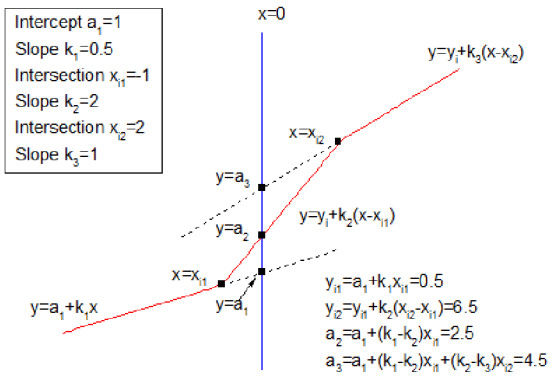
Where V_{total}, P, V_{poly} and N_{poly} are the total required volume, the total number of phosphates of the pDNA, the required volume of polymer and the total number of active amines of the polymer, respectively.

Heparin dissociation assay.

Table S4. Kinetic cycle protocol for automated heparin addition by the microplate reader

Kinetic cycle	**Repetitions**	***Addition of heparin***		***Orbital shake***	***Incubation***	***Measurement***
		V / µL	Stock Solution / U mL^{-1}			
1	2	5	100	10 s	10 min, 37°C	λ_{Ex} = 525 nm / λ_{Em} = 605 nm
2	1	15	100	10 s	10 min, 37°C	λ_{Ex} = 525 nm / λ_{Em} = 605 nm
3	3	5	500	10 s	10 min, 37°C	λ_{Ex} = 525 nm / λ_{Em} = 605 nm
4	1	10	500	10 s	10 min, 37°C	λ_{Ex} = 525 nm / λ_{Em} = 605 nm

The heparin concentration needed to release the maximum of pDNA was calculated with OriginPro, Version 2018b (OriginLab Corporation, US) using a piecewise linear function with three segments fitted to the respective data ($n \geq 3$) of each polymer, keeping the value of the first and third slopes constant at 0 (Figure S1). The values for the heparin concentration (x_{i2}) required to release maximum pDNA (a3) were read off the equation.



$$y = a_1 + k_1 x \qquad \left(x < x_{i_1}\right)$$

$$y = y_{i_1} + k_2\left(x - x_{i_1}\right) \qquad \left(x_{i_1} \le x < x_{i_2}\right)$$

$$y = y_{i_3} + k_3\left(x - x_{i_2}\right) \qquad \left(x \ge x_{i_2}\right)$$

$$y_{i_1} = a_1 + k_1 x_{i_1}$$

$$y_{i_2} = y_{i_1} + k_2\left(x_{i_2} - x_{i_1}\right)$$

Figure S1. Equations used by OriginPro, Version 2018b to provide the piecewise linear fit functions for the polymers. Screenshots taken from the software.

Dynamic Light Scattering.

The size (diameter) of the polyplexes was investigated by dynamic light scattering (DLS). The polyplexes were prepared at N*/P 30 in 100 μL HBG buffer as described above. Measurements were conducted on a Zetasizer Nano ZS (Malvern Instruments, Germany) with a He–Ne laser operating at a wavelength of 633 nm. Each sample was measured in quintuplicates with three runs of 30 s at 25 °C after an equilibration time of 30 s. The counts were detected at an angle of 173°. The mean particle size was approximated as the effective (z-average) diameter and the width of the distribution as the polydispersity index of the particles (PDI) obtained by the cumulants method assuming a spherical shape. Data are expressed as mean ± SD of two independent determinations.

Determination of Cytotoxicity in HEK293T Cells.

The HEK293T cells were seeded at 10^5 cells per well in a 24-well plate and incubated in medium containing 10 mM HEPES for 24 h. 1 h after medium change, cells were treated with polymers at concentrations equal to N*/P 30 and incubated for additional 4 h. Cells on the same plate incubated with 10% (v/v) in growth medium served as non-treated controls. The medium was replaced by a 10% (v/v) alamarBlue™ solution in fresh culture medium, prepared according to the manufacturer's instructions. Following an incubation for 4 h at 37 °C, the fluorescence was

measured at λ_{Ex} = 570 / λ_{Em} = 610 nm. The non-treated control cells were referred to as 100 % viability. Values below 70 % were regarded as cytotoxic. Data are expressed as mean ± SD of at least three independent determinations.

Determination of CC_{50} and Cell Viability at N*/P 30.

With OriginPro, version 2018b a logistic function was fitted to the data of each polymer with the following equation (7):

$$y = \frac{A_1 - A_2}{1+(x/x_0)^p} + A_2 \qquad (7)$$

Where A_1 and A_2 are the initial and the final values, respectively, x_o is the center and p is the power of the curve. For polymers reaching ≥ 50% toxicity, A_1 and A_2 were kept constant at 1 and 0, respectively. For polymers not reaching 50% toxicity, A_2 and p were kept constant at 0 and 1.5, respectively. With the obtained equations, the "polymer concentration needed to kill 50% of the cells" (CC_{50}) or the "cell viability at N*/P 30" were calculated by substituting y with 50 or x with the concentration of the respective polymer at N*/P 30, respectively.

Polyplex Uptake with CLSM and Image Processing.

For uptake studies *via* confocal laser scanning microscopy (CLSM), HEK293T cells were seeded and cultured as described above in glass-bottomed dishes (CellView cell culture dishes with four compartments, Greiner Bio-One) and analyzed following incubation with polyplexes containing YOYO-1 labeled pDNA (0.31 nmol per 1 μg pDNA) and indicated polymers for 4 h. To image intracellular distribution in living cells, Hoechst 33342 was added for 10 min to stain cell nuclei. Prior to imaging, trypan blue was added to a final concentration of 0.04% to quench fluorescence of YOYO-1 outside the cells. Live cell imaging was performed using a LSM880, Elyra PS.1 system (Zeiss, Germany) applying the argon laser for excitation at 488 nm (0.2%) and 405 nm

(0.5%), emission filters for 410-479 nm (Hoechst) and 508-553 nm (YOYO-1) with gains of 750 and 800, respectively. For magnification, a 40 × 1.4 NA plan apochromat oil objective was applied. Images were acquired using the ZEN software, version 2.3 SP1 (Zeiss, Germany). The experiments were performed at least twice. All images were processed in batch mode using ImageJ, version 1.52.[4] They were resized with a scaling factor of 2 in x- and y-dimension and bicubic interpolation. Regarding the Hoechst-channel, the images were processed as follows: The background was corrected using the rolling ball background subtraction tool applying a sliding paraboloid with a radius of 23.5 pixels without previous image smoothing. The contrast was enhanced automatically with a normalization of 0.01% saturation. For YOYO-1 fluorescence, only the background was corrected applying a sliding paraboloid with a radius of 7 pixels following image smoothing. For the overlay image, both channels were merged.

Lactate Dehydrogenase Release (LDH) Assay.

To analyze membrane interactions of the polymers with HEK293T cells, the LDH release assay was performed using the CytoTox-ONE™ assay (Promega, Germany) according to the manufacturer's instructions. Briefly, cells were seeded at a density of 10^5 cells per well in 24-well plates and treated with polyplexes as described for uptake studies (including YOYO-1 for pDNA staining, having no influence on the performed assay, see Figure S16). Following incubation for 4 h, while cells were used to analyze uptake efficiency *via* flow cytometry, the supernatant was transferred to a new 96-well plate as a triplicate and allowed to cool down to room temperature. Subsequently, the substrate mixture including assay buffer was added and incubated at room temperature for 10 min. After the addition of the stop solution fluorescence intensity was measured at λ_{Ex} = 560 nm / λ_{Em} = 590 nm. For the positive control (100 % LDH release), cells were incubated with 0.2% Triton X-100 for 30 min prior to analysis. Cells incubated with only pDNA

and YOYO-1 were used as negative control (0% LDH release). The LDH release of the polymers was calculated as follows (8):

$$\text{LDH release / \%} = \frac{F_{Sample} - F_0}{F_{Positive\ control} - F_0} \cdot 100 \quad (8)$$

Where F_{sample}, F_0, and $F_{Positive\ control}$ represent the fluorescence intensity of a given sample, medium without cells, and of the Triton X-100 treated cells, respectively.

FURTHER RESULTS

Characterization of Polymers.

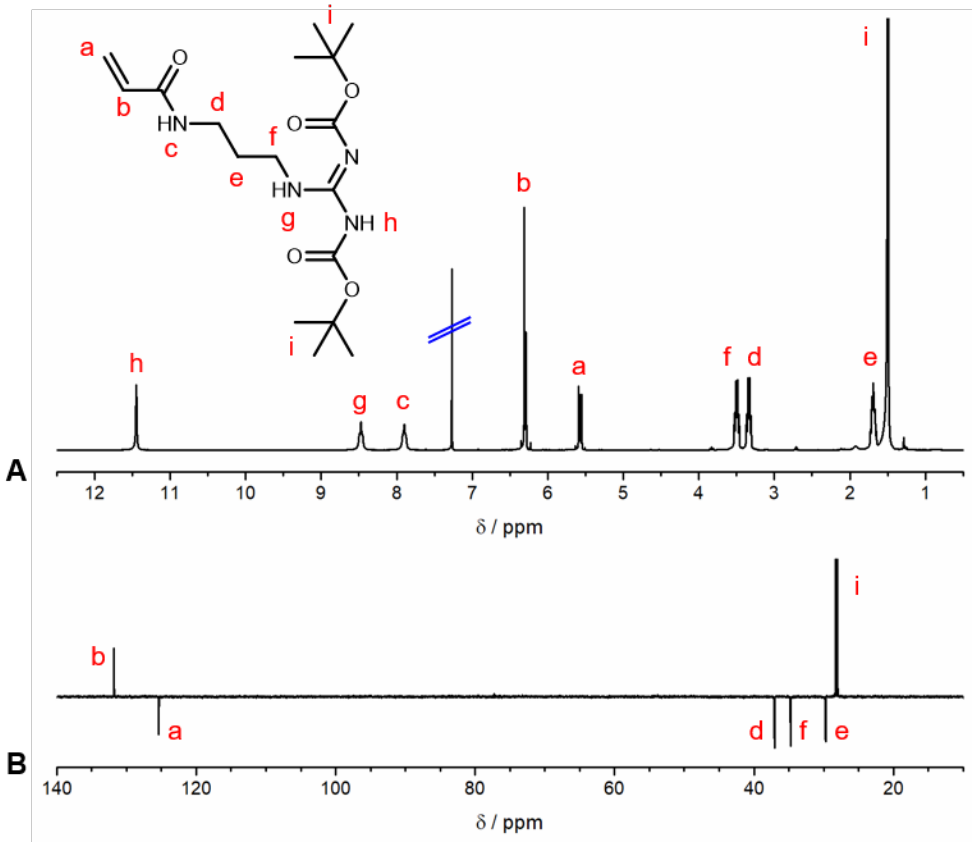


Figure S2. NMR spectra of GPAm^{diBoc}.

^{1}H **(A)** and DEPT ^{13}C **(B)** NMR spectra of GPAm^{diBoc} in $CDCl_3$.

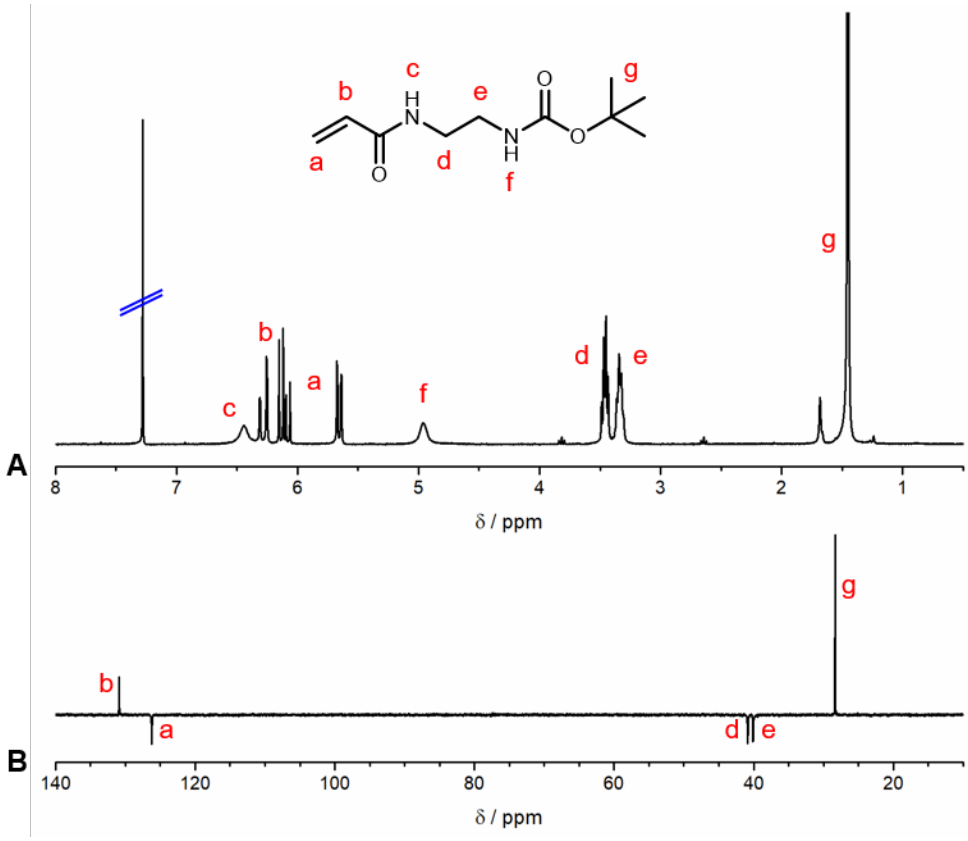


Figure S3. NMR spectra of $AEAm^{Boc}$.

^{1}H **(A)** and DEPT ^{13}C **(B)** NMR spectra of $AEAm^{Boc}$ in $CDCl_3$.

Table S5. Summary of (protected) cationic homopolymers prepared *via* RAFT polymerization.

Polymer		Protected polymers		
	DP$_n$ [a]	$M_{n,th}$ [b]	$M_{n,SEC}$ [c]	$Đ$ [c]
		(kg mol^{-1})		
PGPAm$_8$	8	3.3	5.1	1.11
PGPAm$_{22}$	22	8.4	9.9	1.11
PGPAm$_{43}$	43	16.2	14.7	1.22
PGPAm$_{94}$	94	35.0	27.4	1.31
PAEAm$_9$	9	2.2	4.3	1.11
PAEAm$_{24}$	24	5.4	8.3	1.11
PAEAm$_{45}$	45	9.9	16.1	1.09
PAEAm$_{96}$	96	20.8	26.3	1.15
PDMAEAm$_8$	8	-	-	-
PDMAEAm$_{22}$	22	-	-	-
PDMAEAm$_{45}$	45	-	-	-
PDMAEAm$_{88}$	88	-	-	-
PDMAPAm$_{11}$	11	-	-	-
PDMAPAm$_{24}$	24	-	-	-
PDMAPAm$_{38}$	38	-	-	-
PDMAPAm$_{71}$	71	-	-	-

[a] Determined *via* ^{1}H NMR.
[b] Determined using equation 6.
[c] Determined *via* DMAc-SEC with PMMA standards.

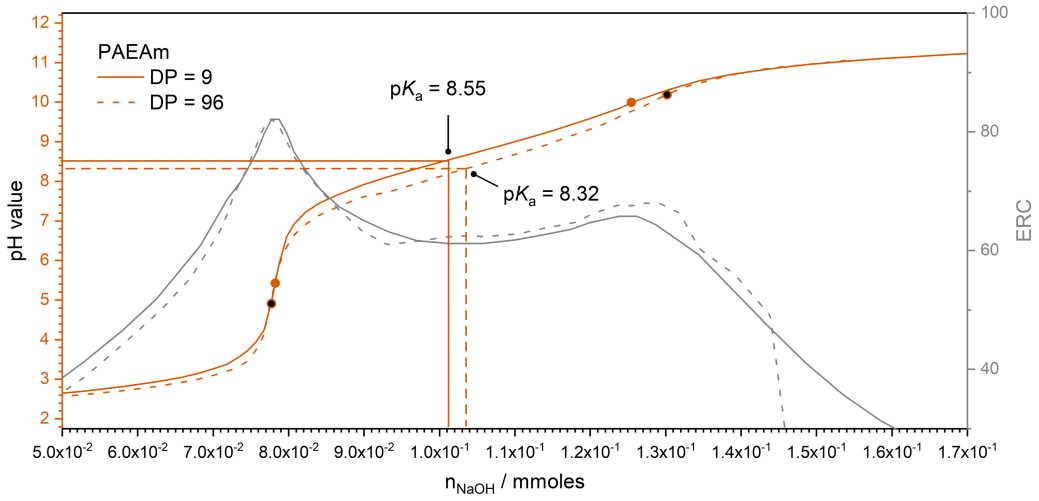


Figure S4. Titration curves and equivalence point recognition criterion for titrations of PAEAm polymers.

Polymers were dissolved at 1 mg mL^{-1} in 125 mM NaCl and titrated against 0.1 M NaOH up to pH 11. A new batch of PAEAm (PAEAm$_{96}$, Aq.-SEC, P2VP standards: $M_{n,SEC}$ = 12.8 kg mol^{-1}, $Đ$ = 1.16) was used in this case.

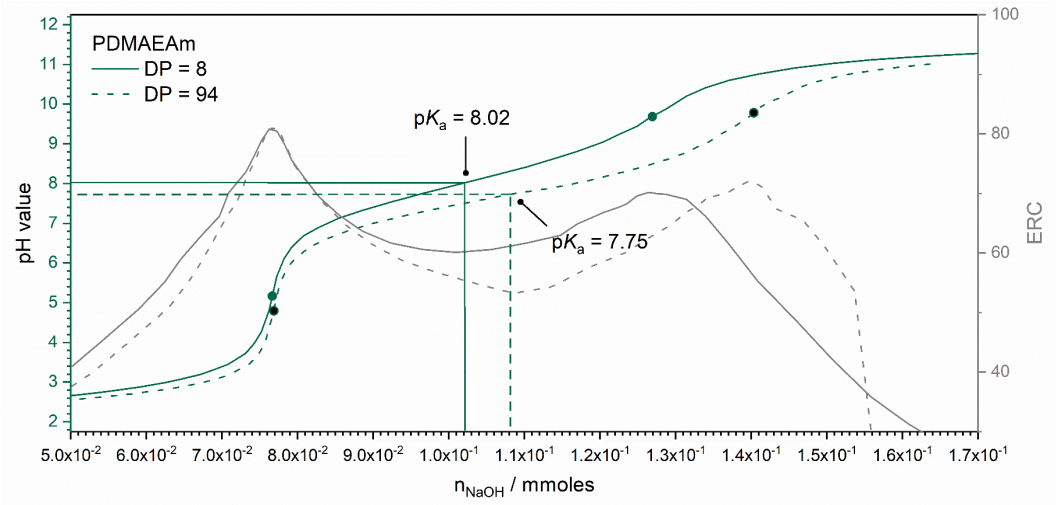


Figure S5. Titration curves and equivalence point recognition criterion for titrations of PDMAEAm polymers.

Polymers were dissolved at 1 mg mL^{-1} in 125 mM NaCl and titrated against 0.1 M NaOH up to pH 11. A new batch of PDMAEm (PDMAEAm$_{94}$, Aq.-SEC, P2VP standards: $M_{n,SEC}$ = 10.3 kg mol^{-1}, $Đ$ = 1.26) was used in this case.

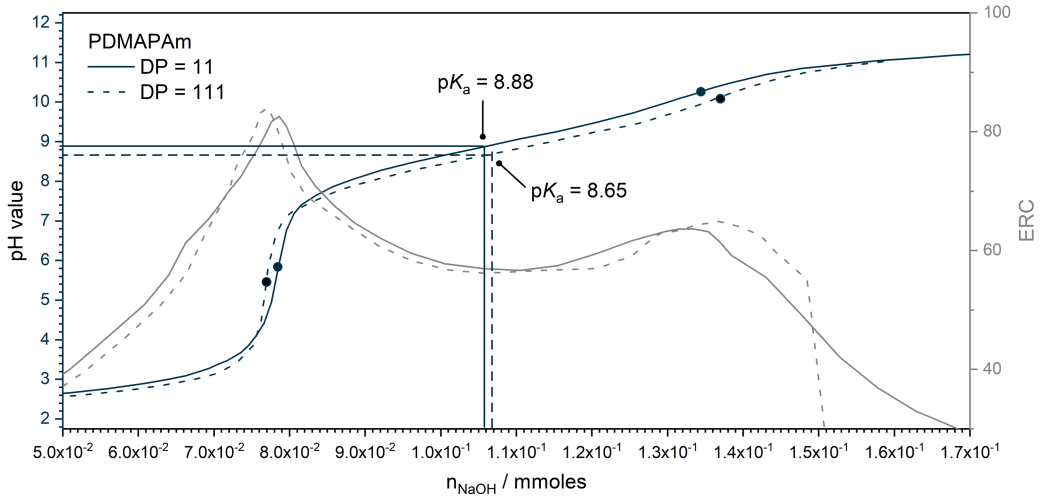


Figure S6. Titration curves and equivalence point recognition criterion for titrations of PDMAPAm polymers.

Polymers were dissolved at 1 mg mL^{-1} in 125 mM NaCl and titrated against 0.1 M NaOH up to pH 11. A new batch of PDMAPAm (PDMAPAm$_{111}$, Aq.-SEC, P2VP standards: $M_{n,SEC}$ = 11.9 kg mol^{-1}, $Đ$ = 1.51) was used in this case.

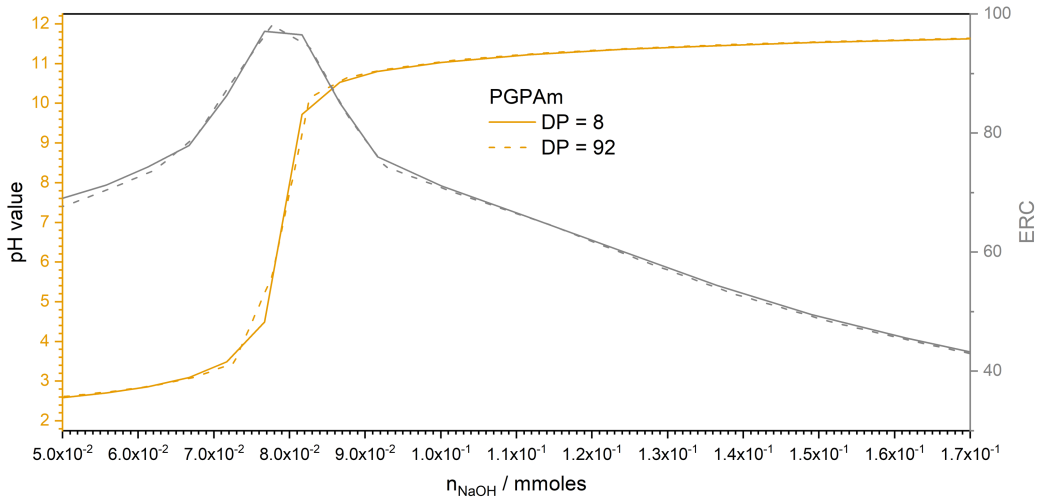


Figure S7. Titration curves and equivalence point recognition criterion for titrations of PGPAm polymers.

Polymers were dissolved at 1 mg mL^{-1} in 125 mM NaCl and titrated against 0.5 M NaOH up to pH 12. A new batch of PGPAm (PGPAm$_{92}$, Aq.-SEC, P2VP standards: $M_{n,SEC}$ = 10.6 kg mol^{-1}, $Đ$ = 1.24) was used in this case.

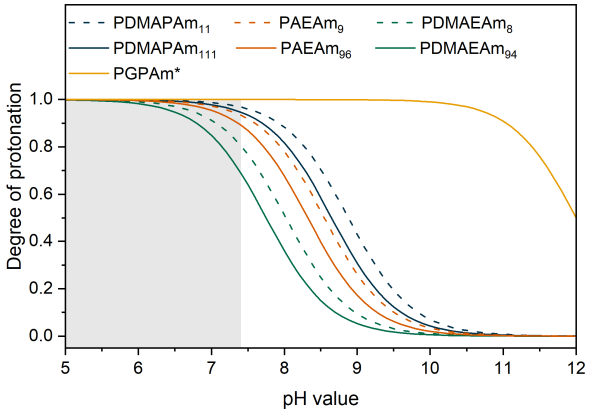


Figure S8. Theoretical determination of the degree of protonation.

The pH for the cationic polymer library based on their pK_a values calculated using the Henderson–Hasselbalch equation (1). Since the pK_a of PGPAm could not be determined the curve is generated assuming a pK_a of 12. The grey region designates the physiologically relevant pH window.

Biological Results.

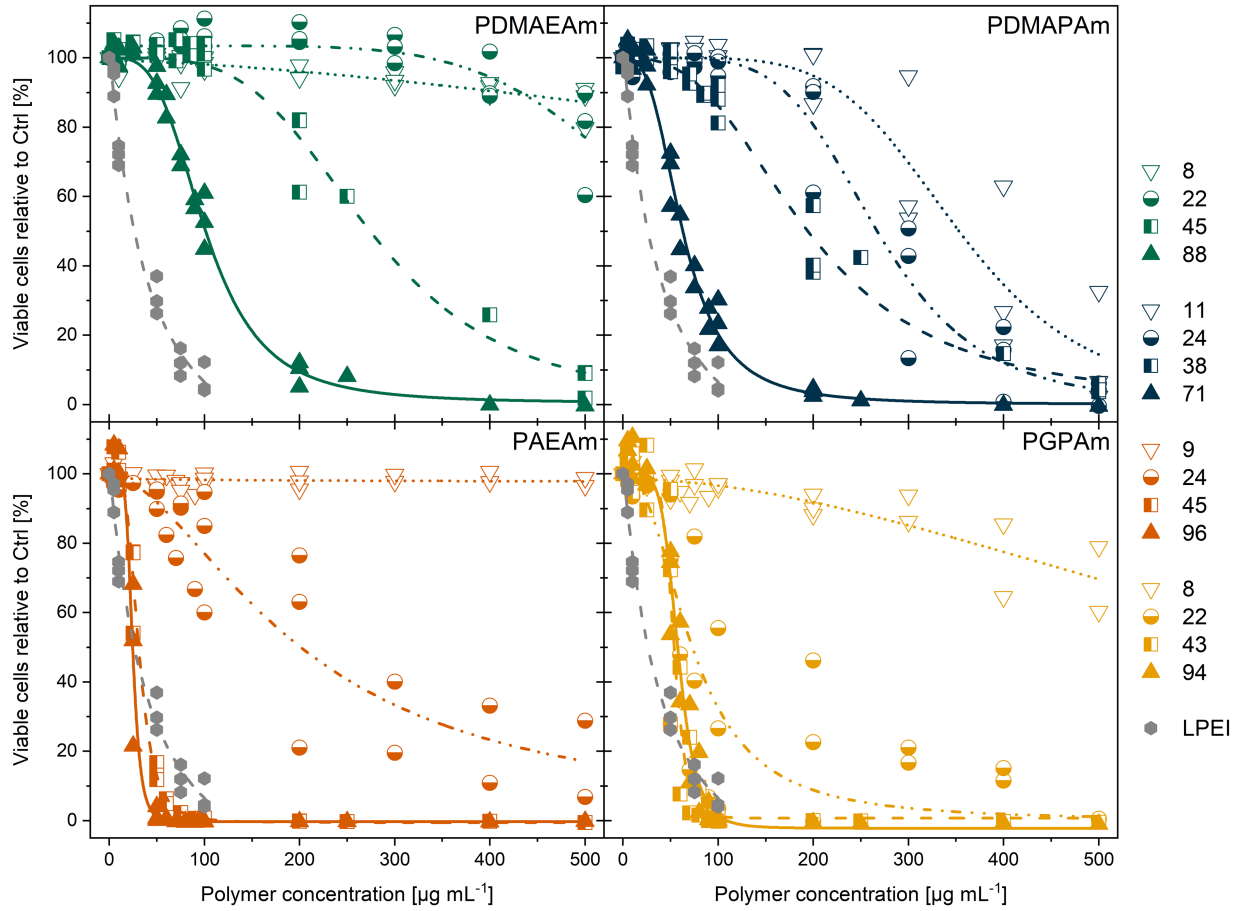


Figure S9. Cytotoxicity of PAm homopolymers in L929 cells.

Metabolic activity was measured in L929 cells using the alamarBlue™ assay following incubation with (A) PDMAEAm, (B) PDMAPAm, (C) PAEAm, (D) PGPAm polymers at indicated concentrations for 24 h. Dots represent values of single repetitions and lines represent logistic fit functions calculated via OriginPro (n = 3).

Table S6. Toxicity and concentration at N*/P 30 and CC_{50} values calculated *via* non-linear fit.

ID	DP	Viability at N*/P 30 / % (c in µg mL^{-1})	CC_{50} / µg mL^{-1}
PAEAm	9	99 (19)	> 500
	24	99 (17)	201.27
	45	93 (16)	30.92
	96	95 (16)	24.59
PDMAEAm	8	99 (23)	> 500
	22	105 (21)	> 500
	45	100 (20)	274.26
	88	100 (20)	101.22
PDMAPAm	11	100 (24)	353.24
	24	100 (22)	267.26
	38	100 (22)	194.31
	71	96 (22)	62.80
PGPAm	8	99 (27)	> 500
	22	92 (25)	72.93
	43	100 (24)	53.72
	94	99 (24)	57.41
PEI	600	88 (6)	22.73

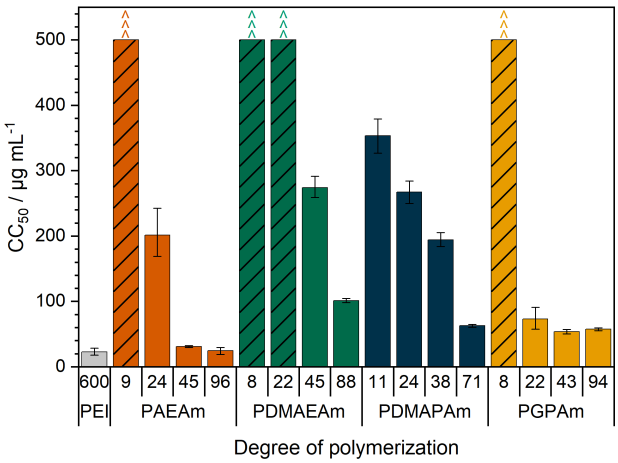


Figure S10. Cytotoxicity of PAm homopolymers of different DP and amino group. Metabolic activity was measured in L929 cells after 24 h incubation with PAm polymers at indicated concentrations (Figure S8) using the alamarBlue™ assay. Values represent CC_{50} values calculated after fitting the resulting toxicity values to a logistic function ± 95% CI (n = 3). Upwards pointing arrows and striped columns indicate CC_{50} values above 500 μg mL^{-1}.

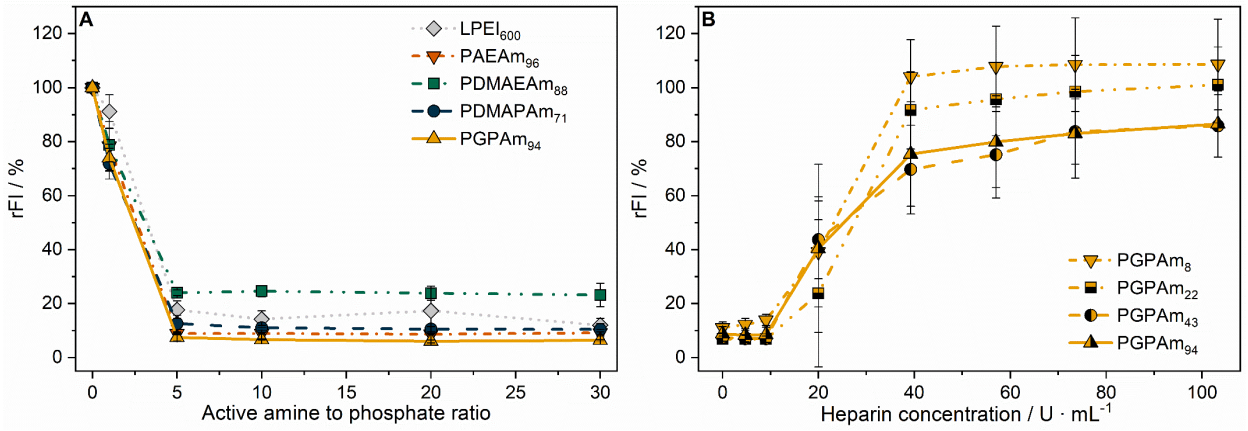


Figure S11. Polyplex formation and stability tests with pCMV-GFP pDNA and PAm homopolymers.

(A) EBA of polymers with highest DP at different N*/P ratios in HBG buffer showing strong pDNA binding of all polymers. Values represent mean ± SD (n ≥ 3). (B) HRA of polyplexes formed with P(GPAm) polymers at N*/P 30 using heparin as a competing polyanion showing the reversible binding of the polyplex. Values represent mean ± SD (n ≥ 3).

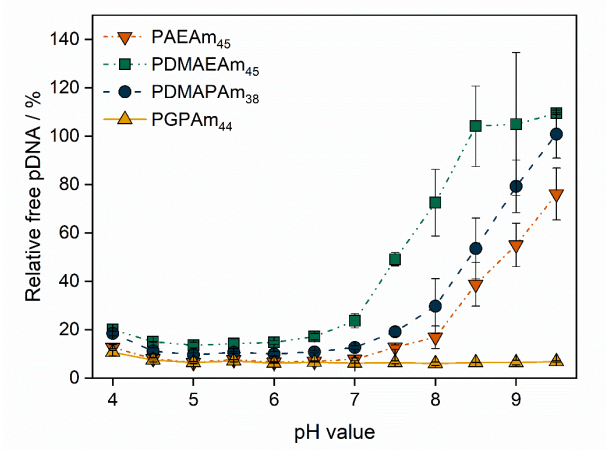


Figure S12. Polyplex formation with pCMV-GFP pDNA and polyacrylamide-homopolymers at different pH values.

EBA of polymers with second highest DP at N*/P 30 in HBG buffer of different pH showing a good pDNA binding of all polymers from pH 4 to pH 7 but differences between the polymers at pH > 7. Values represent mean ± SD (n ≥ 2).

Table S7. Size determination of formed polyplexes via DLS.

Polyplexes were formed using 15 μg mL^{-1} DNA at N*/P ratio of 30 in HBG buffer.

Polymer	DP	Z-Average (d / nm)	PdI	Main peak (d / nm)	Intensity-weighted % of main peak
PAEAm	9	127 ± 11	0.09 ± 0.00	135 ± 13	100 ± 0
	24	47 ± 7	0.20 ± 0.06	54 ± 6	98 ± 2
	45	40 ± 0	0.28 ± 0.02	43 ± 1	92 ± 3
	96	33 ± 1	0.27 ± 0.01	44 ± 1	92 ± 8
PDMAEAm	8	69 ± 1	0.11 ± 0.02	73 ± 2	100 ± 0
	22	115 ± 2	0.08 ± 0.01	123 ± 5	100 ± 0
	45	49 ± 3	0.22 ± 0.03	58 ± 3	100 ± 0
	88	58 ± 20	0.22 ± 0.02	66 ± 23	96 ± 3
PDMAPAm	11	57 ± 1	0.29 ± 0.01	61 ± 2	89 ± 4
	24	61 ± 0	0.34 ± 0.01	71 ± 7	54 ± 3
	38	39 ± 2	0.24 ± 0.01	51 ± 0	87 ± 1
	71	32 ± 1	0.24 ± 0.01	39 ± 1	87 ± 1
PGPAm	8	63 ± 1	0.09 ± 0.00	68 ± 2	100 ± 0
	22	50 ± 18	0.20 ± 0.01	58 ± 19	100 ± 0
	43	37 ± 4	0.23 ± 0.01	46 ± 6	100 ± 0
	89	35 ± 0	0.23 ± 0.01	42 ± 1	99 ± 1
PEI	600	59 ± 22	0.25 ± 0.04	60 ± 11	82 ± 1

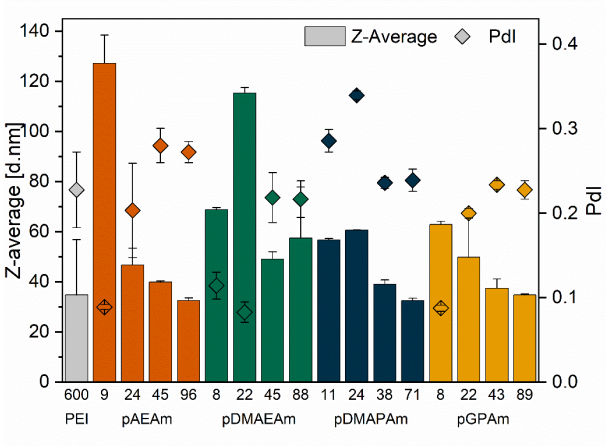


Figure S13. Size determination of formed polyplexes of PAm library of different DP *via* DLS.

Polyplexes were formed using 15 μg mL^{-1} DNA at N*/P ratio of 30 in HBG buffer.

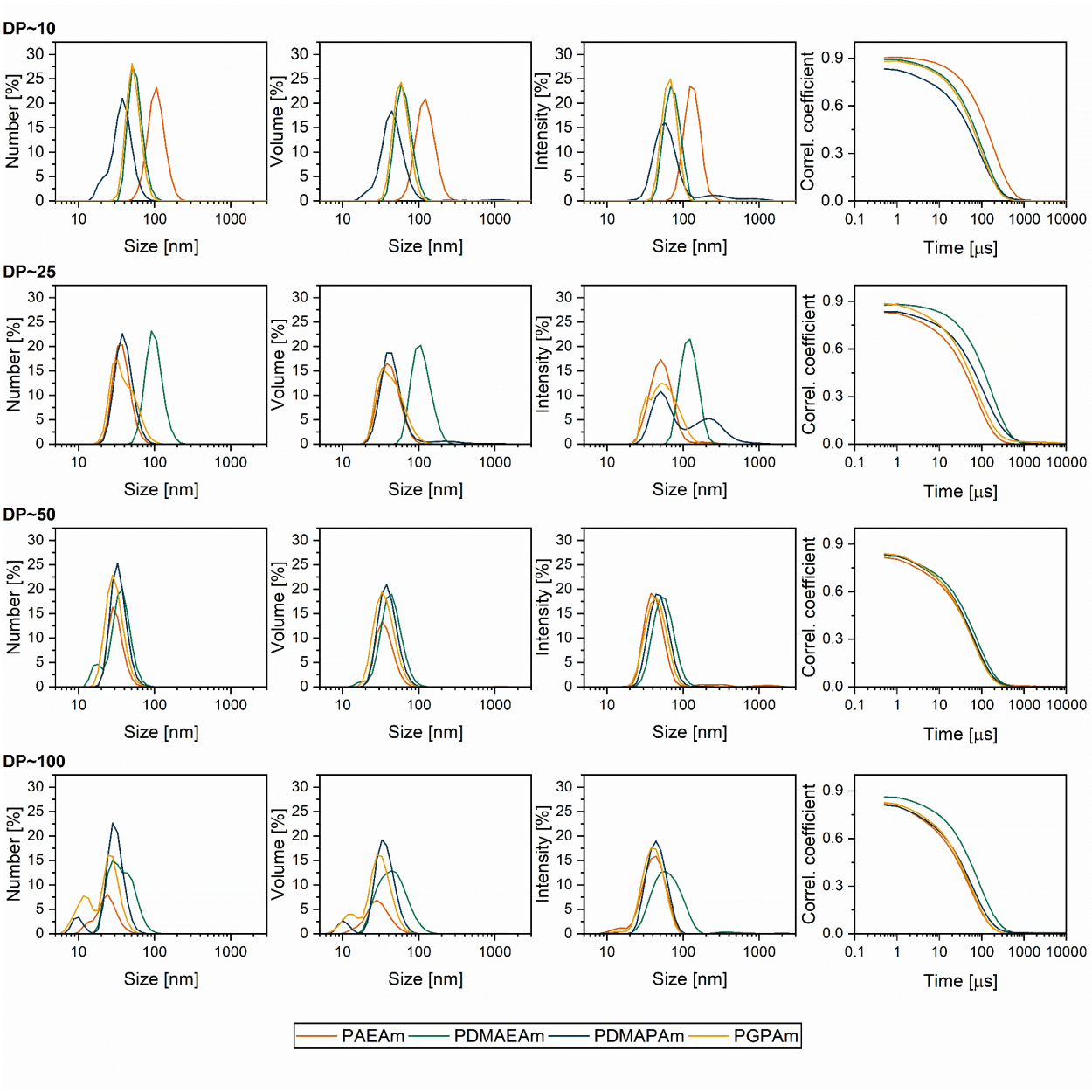


Figure S14. DLS traces of polyplexes formed with PAm polymers and 15 μg mL^{-1} DNA at N*/P 30 in HBG buffer.

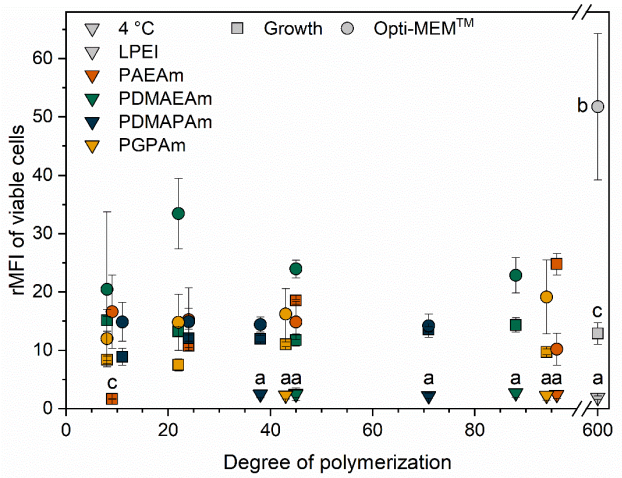


Figure S15. Influence of degree of polymerization on polyplex uptake in HEK293T cells.

Flow cytometry following incubation with polyplexes of YOYO-1-labeled pDNA and polymers at N*/P 30. Incubation was in growth medium at 37°C for 4 h (G), in serum-reduced Opti-MEM™ at 37 °C for 4 h (OM) or in growth medium at 4 °C for 4 h (4 °C). Cells incubated with labeled pDNA served as control (rMFI = 1). Values represent mean ± SD (n ≥ 3). a: significant difference to the same polymer in G, b: significant difference to all polymers in OM, c: significant difference to same polymer in OM ($p < 0.001$)

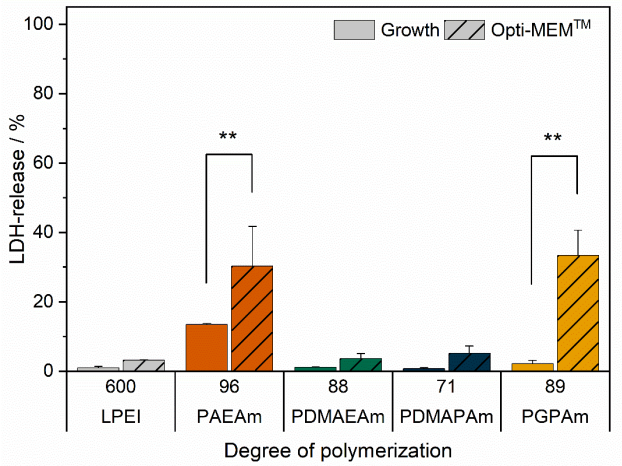


Figure S16. LDH release assay with PAm polyplexes in HEK293T cells.

Cells were incubated with YOYO-1 labeled polyplexes at N*/P 30 in growth medium or Opti-MEM™ for 4 h. Values represent mean ± SD (n ≥ 1). **: significant difference ($p < 0.001$).

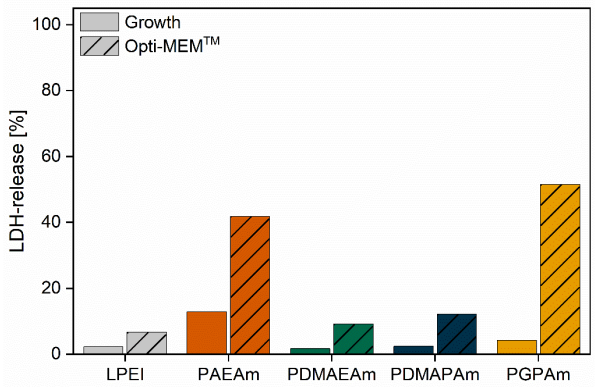


Figure S17. LDH release assay with HEK293T cells.

Cells were incubated with polyplexes (not labeled) at N*/P 30 in growth medium or Opti-MEM™ for 4 h and showed no difference to the results with YOYO-1 labeled polyplexes. Determination was performed once.

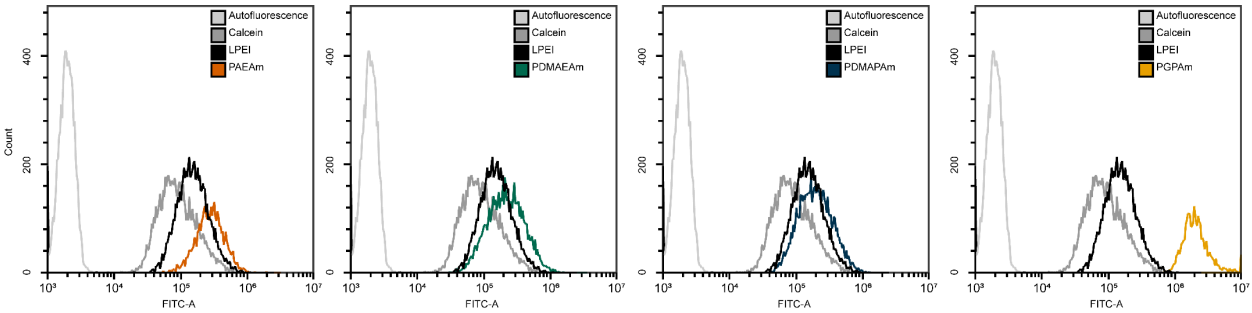


Figure S18. Flow cytometry analysis of calcein release assay.

Representative plots of the calcein channel following flow cytometry of HEK293T cells incubated with the respective polymers at equal amine concentrations (≙ N*/P 30) in Opti-MEM™ for 4 h.

Table S8. Data used for determination of the squared Pearson's correlation coefficient (R^2).

Title of factor in correlation graph	Data
Transfection	Viable EGFP-fluorescent HEK293T cells in Opti-MEM in %
Molar mass[a]	Theoretical molar mass without counter ion in g mol^{-1}
Cationic moiety[b]	C/N ratio of the different amine moieties (3 for tertiary amines, 1 for primary amines, 0.6 for guanidinium)
Toxicity[b]	CC_{50} values in µg mL^{-1} polymer following 24 h incubation in L929 cells
pDNA-binding[b]	Results of EBA as rFI at N*/P 30
pDNA-release[b]	Heparin concentration needed to release maximum amount of DNA at N*/P 30 in U/mL
Uptake[b]	rMFI values of viable HEK293T cells following 4 h incubation in Opti-MEM
Hemolysis[b]	Hemolysis in % of positive control Triton X-100 at pH 6
Aggregation[b]	Negative control PBS relative to respective polymer at pH 6
LDH release[b]	Mean of LDH release in Opti-MEM in % of positive control Triton X-100
Calcein-release[b]	Viable calcein-fluorescent HEK293T cells in Opti-MEM in %-
pDNA-binding[b]	Results of EBA as rFI at N*/P 30
BMP-binding[b]	rFI values of lipid-polymer-binding assay at BMP-concentration of 1 mM

[a] Data of all PAm polymers were considered
[b] Only data of longest PAm polymers were considered.

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