Supplementary Material (ESI) for Molecular BioSystems
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Supplementary Information

Combination Dual Responsive Polypeptide Vectors for Enhanced Gene Delivery*

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Peptide purification and characterization

Crude peptides 1-6 synthesized by Alta Bioscience (Birmingham, UK) using standard Fmoc procedures were then purified by Preparative RP-HPLC. Briefly, all crude peptides were analyzed by analytical RP-HPLC eluted with the gradient 0-100% of MeCN/water (60:40) plus 0.05% TFA for 60 minutes. The methods used in analytical RP-HPLC were then written separately for separating all peaks for each peptide sample. Analytical RP-HPLC runs were performed on a Luna (Phenomenex), C_{18}, 250 mm × 4.6 mm ID, with 10 µm pore size column.

Small amounts (~1mg) of crude peptides were dissolved in water and 10 µl were injected at 1.0 ml/min flow rate and monitored the UV absorbance at 210 nm. After finding the optimum method, all fractions were collected and their mass was determined by ElectroSpray Mass Spectrometry (ESI-MS). Preparative RP-HPLC (Phenomenex), C_{18} with 250 mm × 21.2 mm ID and 10 µm pore size, was utilized for peptide purification. Crude peptides 20 mg were dissolved with 700 µl water and injected onto a column using MeCN plus 0.05% TFA as an elution solvent at 10 ml/min flow rate. The methods applied for purifying peptides were those used in analytical HPLC. Fractions of the peptides were collected, concentrated by rotary evaporation, lyophilized, purged with nitrogen or helium and stored dry at -20°C. The lyophilized samples were then analyzed by ESI-MS. The structures of samples were determined using ^{1}H NMR 300 MHz (Bruker AC300). Purity of purified peptides was further analyzed by analytical RP-HPLC. All purified peptides used in further experiment were between 97.35-99.59% pure.

Peptide 1 (CK_8C): ^{1}H NMR δ (D_2O, 300 MHz, ppm): 4.61 (t, *J* = 5.23Hz, 1H, -CO-C$\underline{H}$-N-), 4.36-4.24 (m, 9H, -CO-C$\underline{H}$-N-), 2.98 (t, *J* = 6.99 Hz, 20H, CH-C_3H_6-C$\underline{H}_2$-NH_2 -C$\underline{H}_2$-SH), 1.79-1.66 (m, 32H, CH-C$\underline{H}_2$-CH_2-CH_2-CH_2-NH_2, CH-CH_2-CH_2-C$\underline{H}_2$-CH_2-NH_2), 1.45-1.42 (m, 16H, CH-CH_2-C$\underline{H}_2$-CH_2-CH_2-NH_2)

Peptide 2 (CK_4H_4C): ^{1}H NMR δ (D_2O, 300 MHz, ppm): 8.63-8.61 (m, 4H, Im, -N=C$\underline{H}$-NH-), 7.32-7.27 (m, 4H, Im, -C-CH-N-), 4.69-4.63 (m, 4H, -CO-C$\underline{H}$-N-), 4.54 (t, 1H, *J* = 5.73 Hz,-CO-C$\underline{H}$-N-), 4.35 (t, *J* = 5.73 Hz, 1H, -CO-C$\underline{H}$-N-), 4.33-4.21 (m, 4H, -CO-C$\underline{H}$-N-), 3.21-3.10 (m, 10H, -NH-CH-C$\underline{H}_2$-,-C$\underline{H}_2$-SH), 3.07-2.92 (m, 10H, CH-C_3H_6-C$\underline{H}_2$-NH_2, -C$\underline{H}_2$-SH), 1.74-1.66 (m, 16H, CH-C$\underline{H}_2$-CH_2-CH_2-CH_2-NH_2, CH-CH_2-CH_2-C$\underline{H}_2$-CH_2-NH_2), 1.45-1.38 (m, 8H, CH-CH_2-C$\underline{H}_2$-CH_2-CH_2-NH_2)

Peptide 3 ($CK_2H_2K_2H_2C$): ^{1}H NMR δ (D_2O, 300 MHz, ppm): 8.63-8.62 (m, 4H, Im, -N=C$\underline{H}$-NH-), 7.31-7.26 (m, 4H, Im, -C-CH-N-), 4.67-4.64 (m, 4H, -CO-C$\underline{H}$-N-), 4.49 (t, 1H, *J* = 5.59 Hz,-CO-C$\underline{H}$-N-), 4.34-4.21 (m, 5H, -CO-C$\underline{H}$-N-), 3.25-3.10 (m, 10H, -NH-CH-C$\underline{H}_2$-, -C$\underline{H}_2$-SH), 3.08-2.89 (m, 10H, CH-C_3H_6-C$\underline{H}_2$-NH_2, -C$\underline{H}_2$-SH), 1.77-1.65 (m, 16H, CH-C$\underline{H}_2$-CH_2-CH_2-CH_2-NH_2, CH-CH_2-CH_2-C$\underline{H}_2$-CH_2-NH_2), 1.45-1.35 (m, 8H, CH-CH_2-C$\underline{H}_2$-CH_2-CH_2-NH_2)

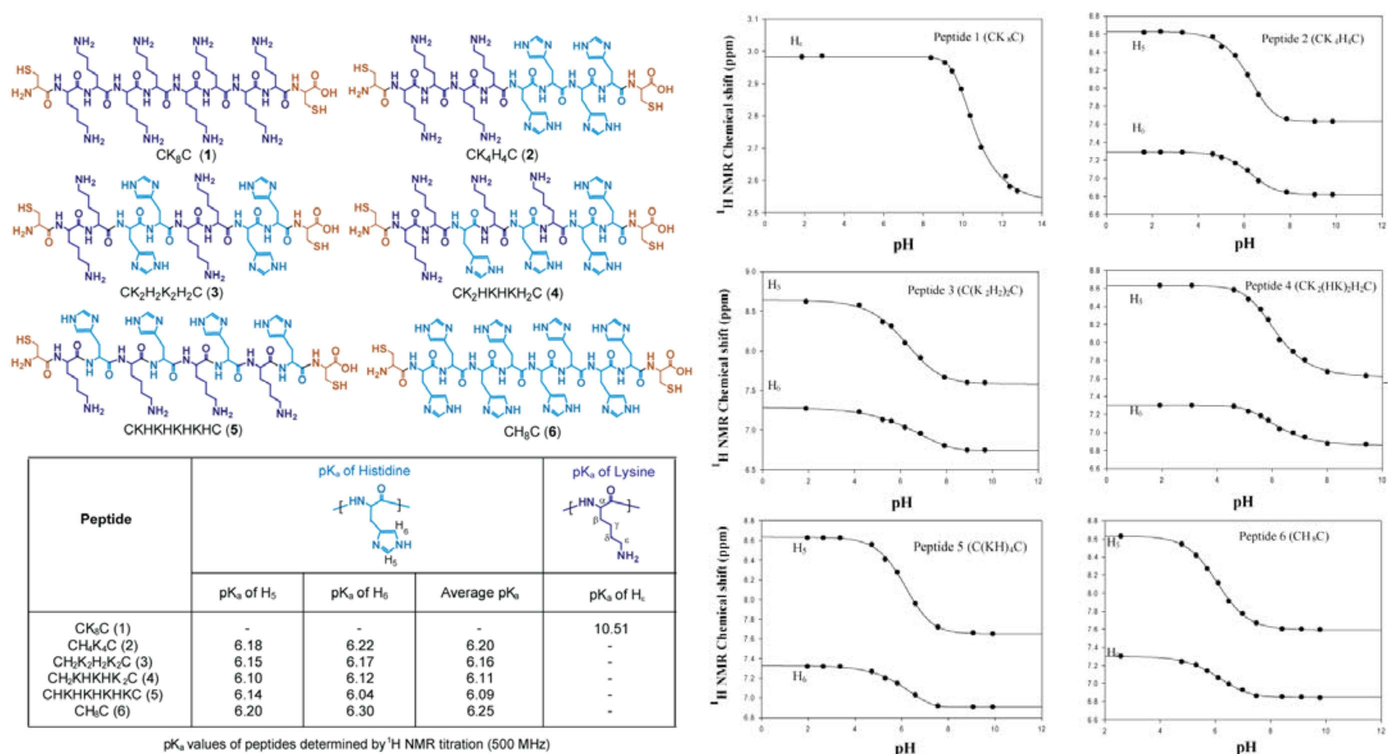
Peptide 4 (CK_2HKHKH_2C): ^{1}H NMR δ (D_2O, 300 MHz, ppm): 8.64-8.62 (m, 4H, im, -N=C$\underline{H}$-NH-), 7.32-7.28 (m, 4H, im, -C-CH-N-), 4.69-4.63 (m, 4H, -CO-C$\underline{H}$-N-), 4.53 (t, 1H, *J* = 5.66 Hz,-CO-C$\underline{H}$-N-), 4.35 (m, 1H, -CO-C$\underline{H}$-N-), 4.33-4.27 (m, 4H, -CO-C$\underline{H}$-N-), 3.26-3.03 (m, 10H, -NH-CH-C$\underline{H}_2$-,-C$\underline{H}_2$-SH), 2.98-2.90 (m, 10 H, CH-C_3H_6-C$\underline{H}_2$-NH_2, -C$\underline{H}_2$-SH), 1.76-1.65 (m, 32H, CH-C$\underline{H}_2$-CH_2-CH_2-CH_2-NH_2, CH-CH_2-CH_2-C$\underline{H}_2$-CH_2-NH_2), 1.43-1.35 (m, 8H, CH-CH_2-C$\underline{H}_2$-CH_2-CH_2-NH_2)

Peptide 5 (CKHKHKHKHC): ^{1}H NMR δ (D_2O, 300 MHz, ppm): 8.64-8.63 (m, 4H, Im, -N=C$\underline{H}$-NH-), 7.34-7.30 (m, 4H, Im, -C-CH-N-), 4.69-4.63 (m, 4H, -CO-C$\underline{H}$-N-), 4.55 (t, 1H, *J* = 5.62 Hz,-CO-C$\underline{H}$-N-), 4.33-4.24 (m, 5H, -CO-C$\underline{H}$-N-), 3.22-3.07 (m, 10H, -NH-CH-C$\underline{H}_2$-, -C$\underline{H}_2$-SH), 3.01-2.94 (m, 10 H, CH-C_3H_6-C$\underline{H}_2$-NH_2, -C$\underline{H}_2$-SH), 1.74-1.64 (m, 16H, -CH-C$\underline{H}_2$-CH_2-CH_2-CH_2-NH_2, -CH-CH_2-CH_2-C$\underline{H}_2$-CH_2-NH_2), 1.45-1.38 (m, 8H, -CH-CH_2-C$\underline{H}_2$-CH_2-CH_2-NH_2).

Peptide 6 (CH_8C): ^{1}H NMR δ (D_2O, 300 MHz, ppm): 8.64-8.63 (m, 8H, Im, -N=C$\underline{H}$-NH-), 7.28-726 (m, 8H, Im, -C-CH-N-), 4.73-4.63 (m, 8H, -CO-C$\underline{H}$-N-), 4.45 (t, 1H, *J* = 5.38 Hz,-CO-C$\underline{H}$-N-), 4.17 (t, *J* = 5.37 Hz, 1H, -CO-C$\underline{H}$-N-), 3.00-2.84 (m, 20H, -NH-CH-C$\underline{H}_2$-, -C$\underline{H}_2$-SH)

Determination of pK_a of peptides

NMR titration was used for the determination of the pK_a values of the histidine moieties. 10 mg of each peptide was dissolved in 0.75 ml of deuterated water (D_2O). Aliquots of 100 mM NaOD solution (20 µl) was added to the peptide solution and then mixed by vortex for 2 minutes. The pH values were measured using a thin stem stainless pH reference probe (ISFET electrode). The NMR spectra were recorded using ^{1}H NMR (500 MHz). The NMR sample was left in the NMR instrument for 10 minutes at 27°C. The chemical shifts of H_5 and H_6 of the imidazole rings of histidine residues and $H_ε$ of lysine residues were measured as a function of pH. The pK_a values were determined by plotting the change of chemical shift against the pH values.



Peptide	pKa of Histidine			pKa of Lysine
	pKa of H_5	pKa of H_6	Average pKa	pKa of H_ϵ
CK_8C (1)	-	-	-	10.51
CH_4K_4C (2)	6.18	6.22	6.20	-
$CH_2K_2H_2K_2C$ (3)	6.15	6.17	6.16	-
CH_2KHKHK_2C (4)	6.10	6.12	6.11	-
CHKHKHKHKC (5)	6.14	6.04	6.09	-
CH_8C (6)	6.20	6.30	6.25	-

Figure S1 – Structures and NMR titration plots for vectors 1-6

Oxidative polymerization

The reaction mixtures were performed in various concentrations of the peptides (18, 30 and 60 mM). Peptides were dissolved in PBS (75mM, 0.5X PBS) and 30% DMSO (~70 fold molar excess in respect to thiol groups) to form 18, 30 and 60 mM of the peptide concentrations in 100 µl total volumes. The reactions were incubated at ambient. At 0, 2, 4, 6, 8, 10, 12, 24, 30, 36 and 48 hr 5 µl aliquot was removed the reaction quenched by adding 40 µl of 17 µM AET (2-aminoethainthiol). The progress of the polymerization was measured by monitoring the increase in molecular weight of the resulting polymer by Gel Permeation Chromatography (GPC).

Gel Permeation Chromatography (GPC)

The quenched reactions were analyzed by Size Exclusion Chromatography (SEC). The SEC analysis was performed using CATSEC-300 (250 mm x 4.5 mm ID) column eluted with 200 mM NaCl with 0.1% TFA. Ten microliters of the samples were injected with 0.5 ml/min flow rate. Commercially available poly-L-lysine (PLL) (Sigma) samples (5-128 kDa) were used to estimate the molecular weight.

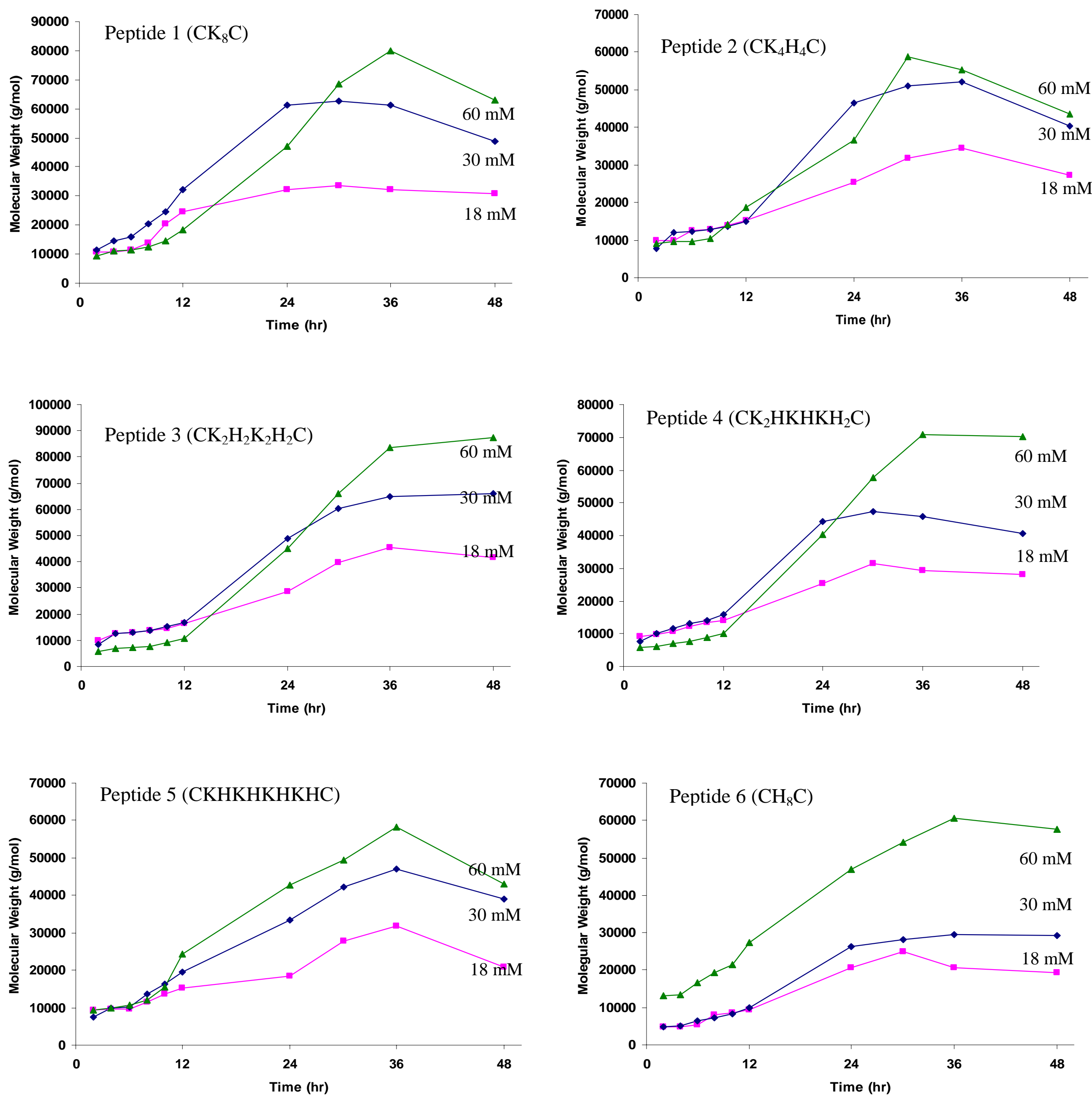


Figure S2 – Oxidative polymerization of peptides 1-6 at various peptide concentrations at room temperature. The molar masses were obtained from GPC using PLL at pH 5.6 (128 kDa) as standard curve (all data are an average of triplicate experiments).

Polymer purification

The reaction mixture was diluted in HEPES (15 ml, 15 mM, pH 7.4). The polymers were purified on centrifugal filter concentrators with molecular weight cut-off of 10,000 (Centricon Plus 2, Amicon Bioseparation, Millipore, cellulose membrane, UK). The mixture was spun down to 150 µl at 4000 rpm repeated three times for this procedure. For the final time of the centrifugation, the sample was spun down until a final volume of 400-500 µl obtained. After purification of the polymers, the molecular weight of the polymers and the removal of DMSO and other impurities were verified by GPC.

Formation of polypeptide-DNA complexes

Reducible Polypeptides (RPC1-5) were dissolved in 10 mM HEPES buffer, pH 7.4 and stock solutions diluted to give the desired N:P ratio based on the number of protonatable amino-groups. Plasmid DNA (pCMV-Luc, 500 µl, 40 µg/ml) was added to an eppendorf tube, followed by the polypeptide solution (500 µl) and the resulting suspension of complexes was mixed by inverting the tube 5 times. The complexes were allowed to equilibrate for 1 hr at ambient temperature.

Gel shift assays

Polyplexes were run on 1 % agarose gels in 0.5x TBE buffer containing 5 µl of 10mg/ml EtBr. For some assays, the polyplex solutions were prepared by mixing with 5mM or 20mM GSH or 25mM DTT. The mixtures were incubated at 37°C for 1 hr before adding 0.5M or 1M NaCl. Run conditions were 110 volt for 30 minutes in 0.5x TBE. The pCMV-Luc was loaded into gel in the equal amount with the pDNA in the polyplex samples.

Dynamic Light Scattering

Hydrodynamic radii of the polymer-DNA complexes were measured via scattered light recorded at 90° angle to incident radiation in a Viscotek 802 dynamic light scattering (DLS) instrument equipped with a 50 mW internal laser operating at a wavelength of 830 nm. From standard auto correlation functions, measured diffusion coefficients were related to particle hydrodynamic radius via the Stokes-Einstein Equation. In addition, it was assumed that particles were spherical and non-interacting. Measurements quoted are the averages of triplicate samples of six replicates. Radii quoted are averages for samples where > 90 % of the scattered light in terms of numbers of particles was from complexes within the size range quoted unless otherwise stated. In some experiments, particle size data was recorded on Malvern Zetasizer 3000Hs in PCS mode.The polyplex solutions were prepared in cuvettes by adding 50 µl of the polyplex solution into 550 µl of 18 milliQ water. Zetapotential measuremrnts were also recorded on the Malvern Zetasizer 3000Hs using the Zeta mode to monitor the polyplex charge. For these experiments, the polyplex solutions were prepared in a 5 ml vial by adding 100 µl of the polyplex solution into 3 ml of 10mM hepes buffer, pH 7.4. The buffer solutions were filtered through a 0.2 µm membrane before use.

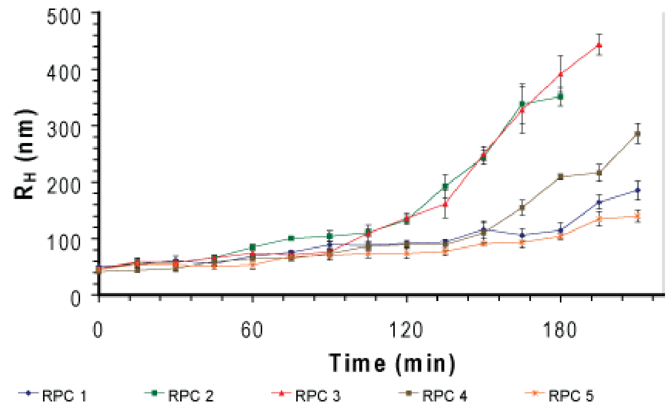


Figure S3 Hydrodynamic radii of RPC1-5 polyplexes following addition of GSH (20 mM)

Atomic Force Microscopy

An AFM liquid cell and oxidation-sharpened NP-S tips on a V-shaped, silicon nitride cantilever, with a spring constant of around 0.1 N/m (Nanoprobe, Veeco Instruments) and resonant frequency between 9 and 10 kHz were used. All AFM imaging was carried out using a Tapping Mode on a Veeco Nanoscope (IIIa) MultiMode system (Veeco Instruments, Santa Barbara, CA). Topographical images were taken at 512X512 pixel resolution, plane-flattened, and analyzed by the computer program Nanoscope Software v 5.12.

Polypeptide(A-F)-pEGFP-C1 complexes at a 5:1 monomer/nucleotide molar ratio in HEPES buffer (10 mM, 30 μL, pH 7.4) were incubated at room temperature prior to immobilization onto freshly cleaved muscovite mica (Agar Scientific,Essex, UK) and being imaged directly in liquid.

For time-course experiments, polypeptide A-DNA complex (120 µL, (97.8 µg/mL, N/P=5) was deposited on freshly cleaved $NiCl_2$ modified mica and incubated for 10 minutes before being washed two times with deionized water (2 x 200 µL). An aliquot of the fluid (100 µL) in the cell was then exchanged with GSH (20mM, 100 µL). The time for this exchange was defined as time zero and images were then captured according to stated times.

AFM Studies of Polypeptide-DNA Complexation

Incubation of all the polypeptides with plasmid DNA resulted in the formation of polyplexes with predominantly spherical architectures, although other conformations were apparent in AFM due most likely to the nature of imaging soft polyelectrolyte complexes in aqueous phase. The polylysine-analogue pCK_8C was observed to form complexes with a number of alternate architectures, including rods and coils, consistent with the more strongly protonated backbone of this vector, and hence an increased rigidity of the polymer chains resulting from charge-charge repulsion.

Peptide	Width of complex with pEGFP-C1			Height		
Peptide (**1**)	158.14	±	17.23	7.93	±	1.97
Peptide (**5**)	122.57	±	14.12	14.94	±	4.20
RPC1-	109.14	±	8.80	9.19	±	2.66
RPC2	91.43	±	13.75	17.47	±	2.35
RPC3-	85.29	±	8.67	11.86	±	2.04
RPC4	83.57	±	2.88	10.14	±	1.71
RPC5	79.71	±	13.19	10.09	±	2.51

Table S1 – AFM analysis of polypeptide vector complexes

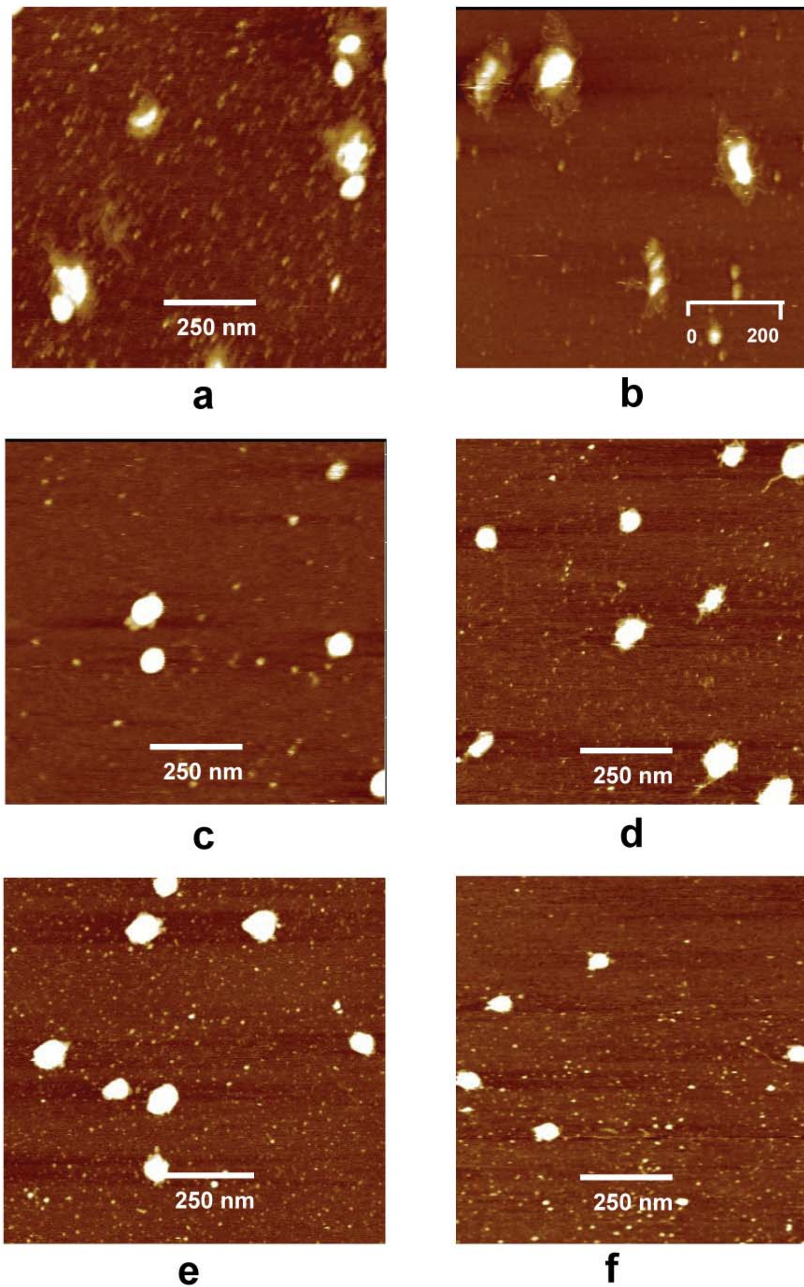
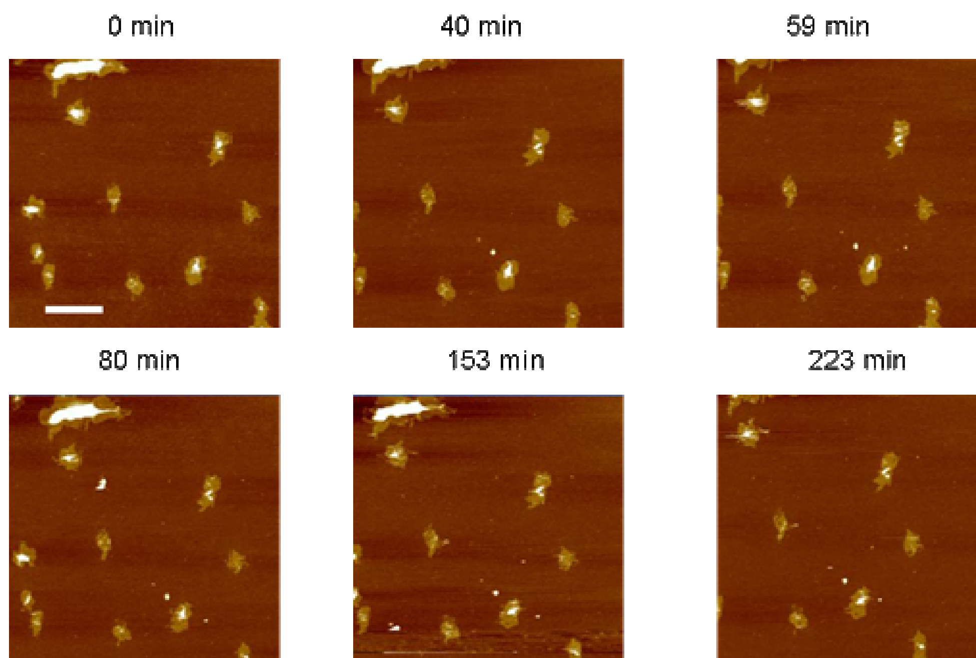


Figure S4 Aqueous phase atomic force microscopy of polypeptide-DNA complexes at N:P 5:1. Images show complexes of pEGFP-C1 with (a,b) RPC1 p(CK_8C); (c) RPC2; (d) RPC3, (e) RPC4 and (f) RPC5. Note that RPC1 (pCK_8C) displays several morphologies consistent with the more rigid structure of the fully protonated polylysine backbone at pH 7.4, as seen clearly in plate **b** at higher magnification.



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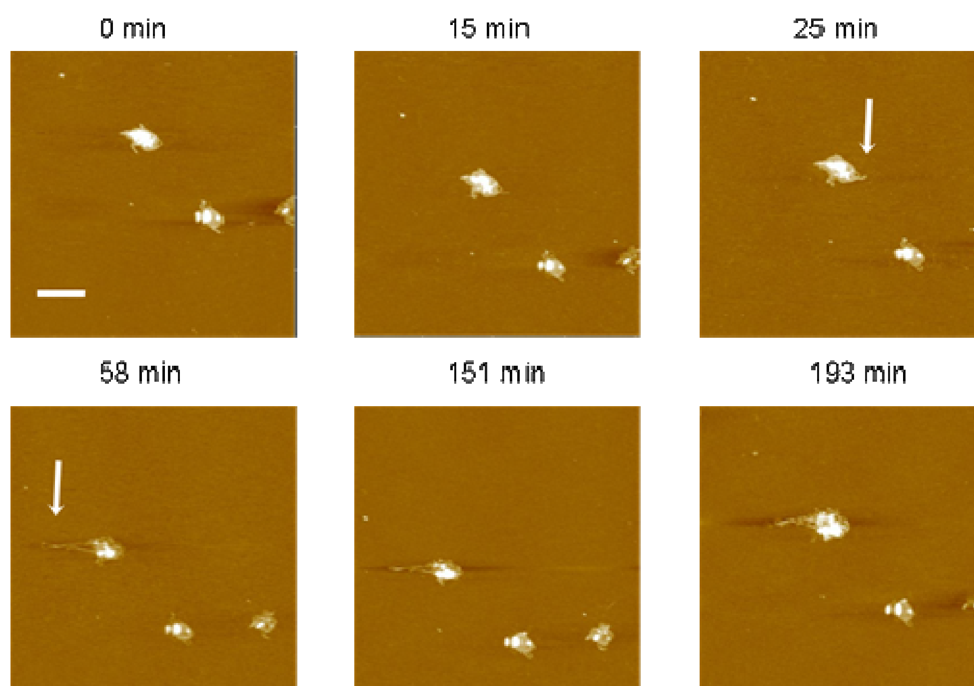

a

Figure S5 – Effect of glutathione on PLL-DNA complexes (plates **a**) and on RPC-1-DNA (poly(CK_8C)-DNA) complexes. Polyplex unpackaging to reveal strands of free DNA takes place only for RPC-1-DNA complexes following treatment with GSH

Transfection assays

Polyplex solutions of RPCs 1-5 and PLL(70 kDa) were prepared with plasmid DNA encoding for luciferase (pCMV-Luc) at nitrogen:phosphate ratios (N:P) of 5, polyplex solutions of PEI was prepared at N:P 10. Cells for transfection experiments (A549 and bEND3) were plated into 96 well plates in DMEM or RPMI to obtain 10 x 10^3 cells/well and incubated at 37°C, 5% CO_2 for 24 hr prior to the transfection. Polyplexes were added directly to the well plates After 4 h, the mixture containing polyplexes was discarded and replaced with 200 ml per well of fresh media. Cells were cultured for 24 h prior to analysis of reporter gene expression. Fluorescence was measured on a $Victor^2$ plate reader (Wallac) at 360 nm excitation and 460 nm emission wavelengths and background values subtracted.

In some experiments, cells were incubated in the presence of 1) 100 µM BSO, 1) 5mM GSH-MEE, 3) 100 µM CQ and 4) the combination between 5mM GSH and 100 µM CQ as described below.

1) Buthionine sulfoximine (BSO) solution (10 µL of 1.1 mM) was added to cells after 2 hr of cell plating, then incubated with cells for 24 hr before transfection.
2) 100 µL of 5mM Glutathione-monoethyl ester (GSH-MEE) solution was added into cells after cells washing with PBS, and then incubated for 3 hr before transfection.
3) 100 µL of 100 µM Chloroquine (CQ) solutions was added into cells after cells washing with PBS, and then incubated for 1 hr before transfection.
4) 100 µL of 5mM Glutathione-monoethyl ester (GSH-MEE) solution was added into cells after cells washing with PBS, then incubated for 2 hr. 10 µL of 1.1 mM chloroquine solution was then added and incubated for further 1 hr before transfection.

Effect of extracellular Glutathione

A459 cell lines were incubated with 100 µM Chloroquine or 5mM Glutathione for 1 and 3 hr, respectively before performing the transfections. Polyplexes were added directly into cells without cell washing. The transfection results in all cases were less effective, when glutathione was added to the extracellular milieu **(Figure S6).**

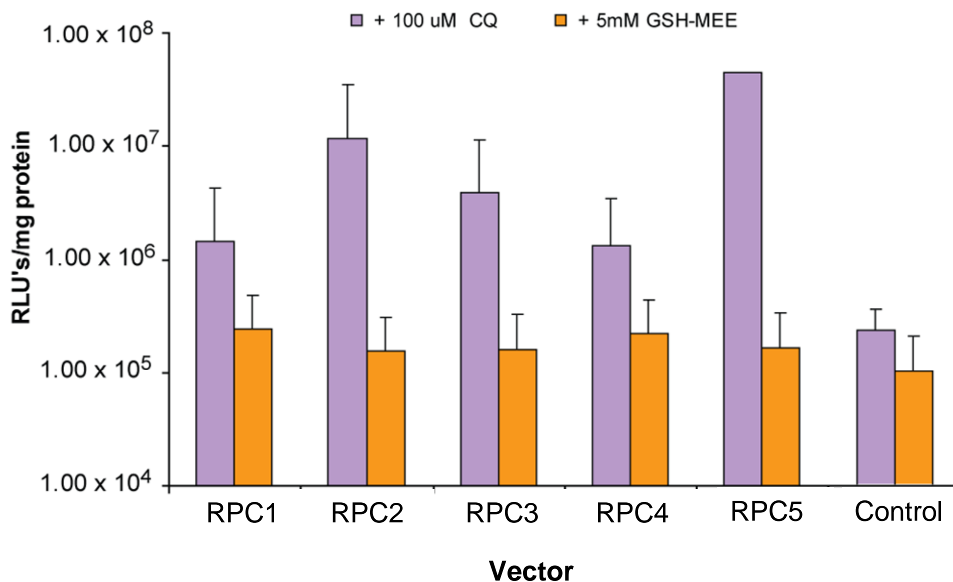

Figure S6 – Effect of extracellular GSH

Cell viability assays

The MTS assay for cell viability was conducted using the CellTiter 96® Assay kit from Promega. Full instructions and protocols for this assay are available from the manufacturer (http://www.promega.com/paguide/chap4.htm#title3)