

Supporting information:

Hyperbranched poly(ethylenimine-*co*-oxazoline) by thiol-yne chemistry for non-viral gene delivery: investigating the role of polymer architecture

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Static Light Scattering

The incremental refractive index, dn/dC , was determined by measuring the refractive index of the polymer over a range of concentrations. The RI was determined using a Shodex RI detector, operating at a wavelength of 632 nm. Multiplying the gradient of the plot of RI vs concentration by the refractive index of the solvent (water = 1.3325) and dividing by the RI constant of the instrument (-1,398,000) gives the dn/dC of the polymer.

Light scattering measurements were obtained using an ALV-CGS3 system operating with a vertically polarised laser with wavelength $\lambda = 632$ nm. The measurements were taken at 20 °C over a range of scattering wave vectors ($q = 4\pi n \sin(\theta/2)/\lambda$, with θ the angle of observation and n the refractive index of the solvent). The Rayleigh ratio, R_θ , was determined using eq. 1,

$$R_\theta = \frac{I_{\text{solution}}(\theta) - I_{\text{solvent}}(\theta)}{I_{\text{toluene}}(\theta)} \cdot \left(\frac{n_{\text{solvent}}}{n_{\text{toluene}}} \right)^2 \cdot R_{\text{toluene}} \quad (1)$$

where I_{solution} , I_{solvent} and I_{toluene} are the scattering intensities of the solution, solvent and reference (toluene) respectively, n is the refractive index ($n_{\text{water}} = 1.333$, $n_{\text{dmf}} = 1.431$, $n_{\text{toluene}} = 1.496$) and R_{toluene} the Rayleigh ratio of toluene ($R_{\text{toluene}} = 1.35 \times 10^{-5} \text{ cm}^{-1}$ for $\lambda = 632.8$ nm).

The optical constant, K , is defined by eq. 2, where N_a is Avogadro number and dn/dC is the incremental refractive index.

$$K = \frac{4\pi^2 n_{\text{solvent}}^2}{\lambda^4 N_a} \left(\frac{\partial n}{\partial C} \right)^2 \quad (2)$$

At a given concentration the Rayleigh ratio, R_θ , is related to the apparent molecular weight of the sample, given by eq. 3. It is only at infinite dilutions, where the interactions between scattering particles are negligible, that the apparent molecular weight is equal to the true molecular weight. Multiple concentrations were measured and a plot of linear regression used to determine the apparent molecular weight at $\text{conc.} = 0$ mg/mL. Data and dn/dC values for all polymers are shown in the supplementary information as Figures S10-S15.

$$\frac{KC}{R_\theta} = \frac{1}{M_a} \cdot \left(1 + \frac{q^2 \cdot R_g^2}{3} \right) \quad (3)$$

DLS/Zetapotential

Dynamic light scattering measurements of resulting polymers and polyplexes at various N/P ratios were carried out using a Malvern nanoZS zetasizer instrument (scattering angle of 173°, 10 mW He-Ne laser). For polyplex formation, appropriate amount of polymer stock solution and DNA stock solution were mixed and made up to a total volume of 1 mL in PBS (final concentration of polymer was 1 mg/mL, in all solutions). The resulting solutions were vortexed, incubated for 30 minutes at room temperature, and analysed at 25 °C. Each sample was run in triplicate and data was acquired using the software (Malvern Zetasizer) provided. Zeta potential measurements were carried out of the same DLS samples at various N/P ratios using the same instrument, and Malvern disposable folded capillary cell (DTS1070) cuvettes.

Atomic Force Microscopy

AFM images were taken using an Asylum Research MFP-3D Stand Alone atomic force microscope with an extended z-range of 40 µm, with closed loop scanning in *x* and *y* over a range of 90 µm. 20 µL of polymer/pDNA complexes in Hepes buffer solution (4 mM Hepes, 10 mM NaCl, and 2 mM MgCl₂ (pH 7.4)) containing 0.08 µg of pDNA at various N/P ratios were dropped onto freshly cleaved mica sheets for 5 min, then rinsed with distilled water several times and dried naturally in air overnight. The tapping mode was used for all measurements.

pH titration

Potentiometric titration was performed manually at room temperature with a micropipette to control the added volume and a pH meter (HI2211 Hanna Instruments) to determine the pH. The pH of the polymer solutions (with 0.15 M NaCl) was set at 2.0 with concentrated HCl, and the solutions titrated with NaOH at 0.1 M or 0.2 M in various added volumes (from 0.01 mL to 0.2 mL) in order to obtain a constant increase of pH between each addition. 40 mL of polymer solution (1 mg/mL polymer) was used for each potentiometric titration experiment. For comparison, branched PEI (25 kDa) dissolved in 0.15 M NaCl aqueous solution adjusted to pH 2.0, was also titrated using the same method.

Ethidium Bromide displacement assay

Polyplex formation of DNA with cationic polymers was followed using quenching of the ethidium bromide fluorescence, as described in the literature. DNA (7.5 µg/mL) and EB (0.4 µg/mL) were dissolved in HEPES buffer, pH 7, and incubated for 10 min at room

temperature. 100 μL of the DNA+EB solution was transferred to the wells of a 96-well plate containing different polymer concentrations. Fluorescence was measured after 20 min of incubation with the polymer solution using a Biotek Synergy HTX fluorescence microplate reader (Ex. 525 nm, Em. 605 nm). Control samples containing only DNA and EB were used to calibrate the measurements. Relative fluorescence = $(F_{\text{SAMPLE}} - F_{\text{DNA}}) / (F_{\text{DNA+EB}} - F_{\text{DNA}})$.

Agarose gel electrophoresis

Agarose gels (1% w/v) were prepared with agarose and $0.5 \times$ TAE buffer. The solution was cooled on the bench for 5 minutes and 100 μL of 0.5 $\mu\text{g}/\text{mL}$ ethidium bromide solution was added. The mixture was poured into the casted agarose tray and a comb inserted. The gel was left to set for a minimum of 30 minutes at room temperature. The agarose gels were run in $0.5 \times$ TAE buffer. The final gel was visualized under UV illumination at 365 nm using a UVP benchtop UV transilluminator system. Polyplexes of DNA were prepared at various N/P ratios. DNA stock solution of 60 $\mu\text{g}/\text{mL}$ was prepared in PBS, and polymer stock solution of 300 $\mu\text{g}/\text{mL}$. For polyplex formation, the appropriate amount of polymer stock solution and DNA stock solution were mixed and made up to a total volume of 100 μL in PBS (final concentration of DNA was 0.030 $\mu\text{g}/\mu\text{L}$ in all solutions). Polyplexes were vortexed and incubated at room temperature for 30 min. Prior to loading, 30 μL of loading buffer was added to each sample and 20 μL of polyplexes were loaded into the agarose gel wells. Gel electrophoresis was performed at 100 V for 30 minutes.

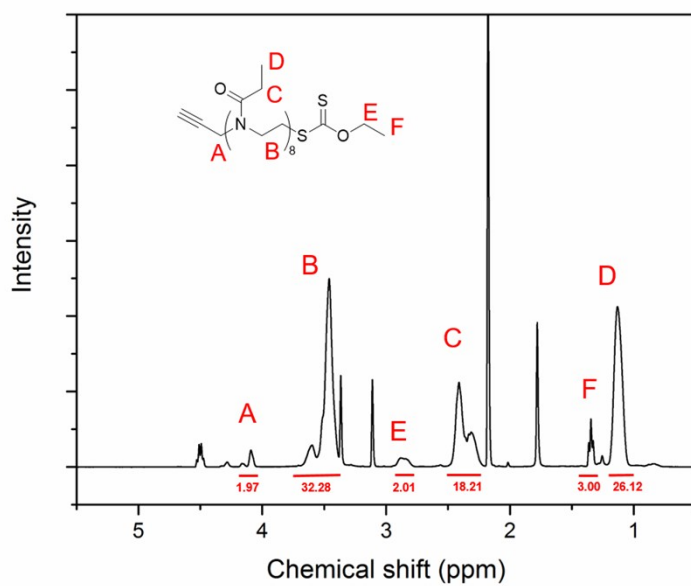


Figure S1. ¹H NMR spectrum (400 MHz, CDCl₃) of xanthate-protected thiol-yne macromonomer synthesized by cationic ring opening polymerization of 2-ethyl-2-oxazoline monomer.

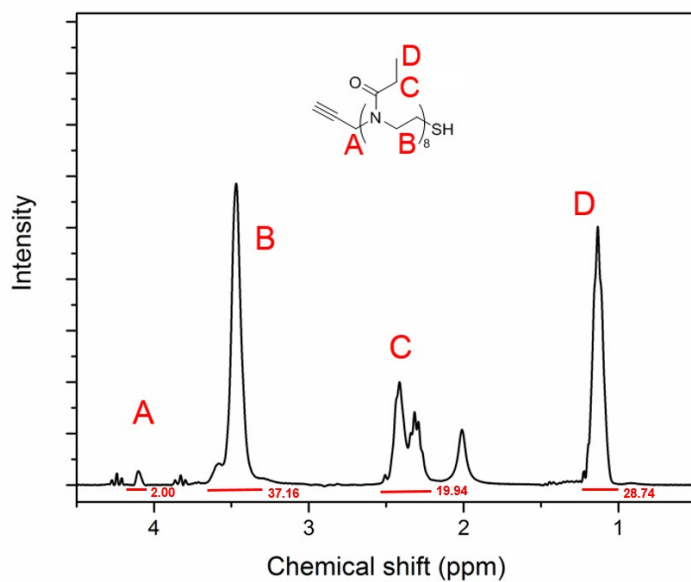


Figure S2. ¹H NMR spectrum (400 MHz, CDCl₃) of thiol-yne macromonomer synthesized by cationic ring opening polymerization of 2-ethyl-2-oxazoline monomer, and xanthate removal.

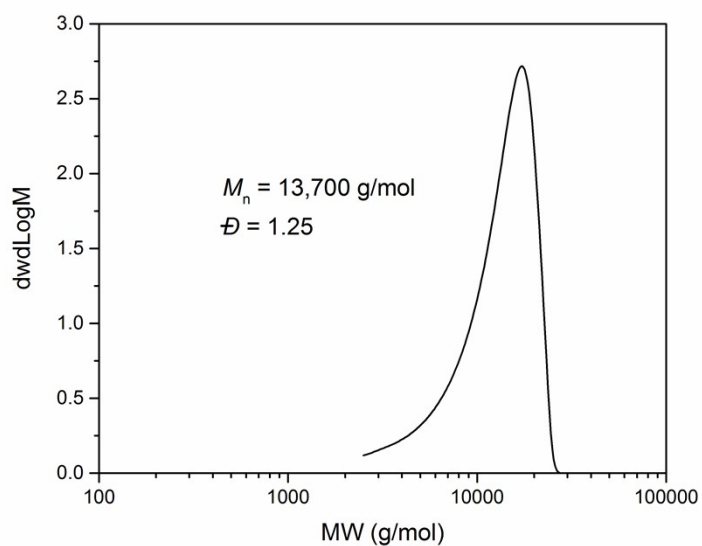


Figure S3. Size exclusion chromatogram (DMF +NH₄BF₄ additive eluent, PMMA calibration) of linear POx control polymer synthesized by cationic ring opening polymerization of 2-ethyl-2-oxazoline monomer.

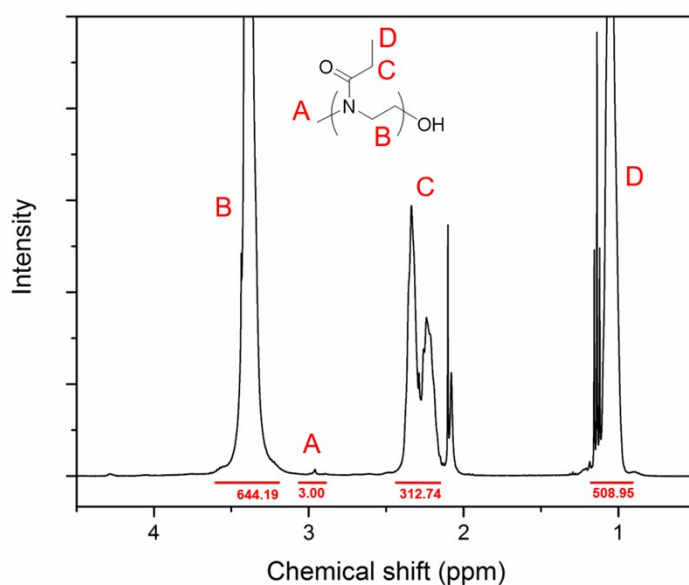


Figure S4. ¹H NMR spectrum (400 MHz, CDCl₃) of linear POx control polymer synthesized by cationic ring opening polymerization of 2-ethyl-2-oxazoline monomer.

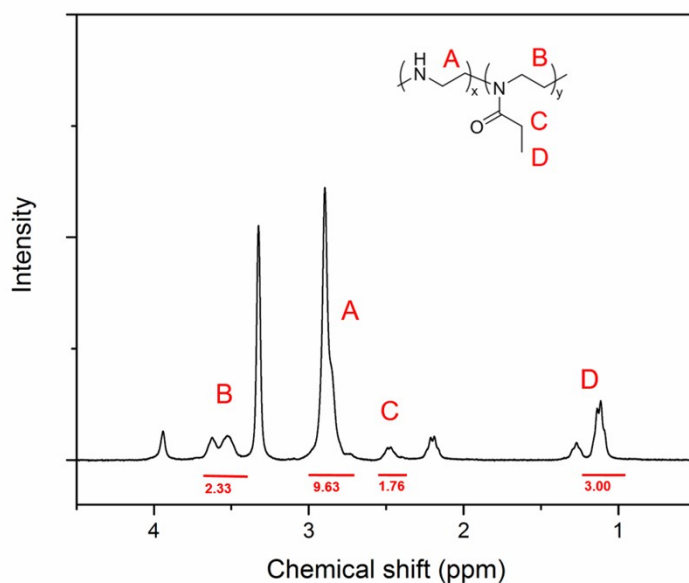


Figure S5. ^1H NMR spectrum (400 MHz, CDCl_3) of linear POx PEI 81% control polymer synthesized by cationic ring opening polymerization of 2-ethyl-2-oxazoline monomer, and subsequent hydrolysis in 1M HCl.

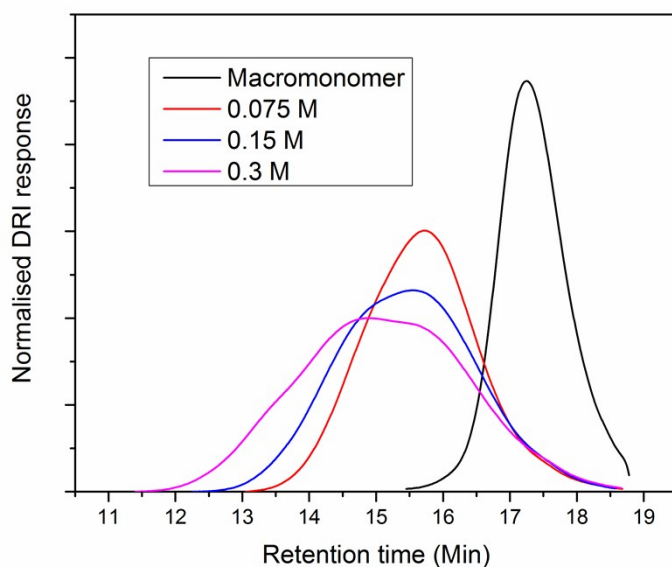


Figure S6. Size exclusion chromatogram (DMF + NH_4BF_4 additive eluent, PMMA calibration) of xanthate-protected thiol-yne macromonomer synthesized by cationic ring opening polymerization of 2-ethyl-2-oxazoline monomer, and subsequent hyperbranched polymers formed at various monomer concentrations.

	$M_{n,SEC}$ (g/mol)	$M_{w,SEC}$ (g/mol)	\bar{D}	DB
Monomer	1200	1500	1.19	
0.075 M	5400	8200	1.51	0.83
0.15 M	5900	10500	1.78	0.83
0.3 M	6800	16400	2.41	0.87

Table S1. Size exclusion chromatography data (DMF +NH₄BF₄ additive eluent, PMMA calibration) of xanthate-protected thiol-yne macromonomer synthesized by cationic ring opening polymerization of 2-ethyl-2-oxazoline monomer, and subsequent hyperbranched polymers formed at various monomer concentrations. Degrees of branching (DB) were calculated from ¹H NMR spectroscopy (400 MHz, CDCl₃) as exemplified in Figure 1d and Figure S7.

Table S2. Size-exclusion chromatography data (DMF +NH₄BF₄ additive eluent, PMMA calibration) for photopolymerisation of poly(2-ethyloxazoline) thiol-yne macromonomer with varying irradiation times.

	$M_{n,SEC}$ (g/mol)	$M_{w,SEC}$ (g/mol)	\bar{D}
Monomer	1,200	1,500	1.19
0.5 hr	2,900	4,800	1.67
1 hr	3,900	8,300	2.11
2 hr	4,200	17,000	4.07
4 hr	4,700	23,800	5.10
8 hr	4,700	23,600	5.05
8 hr purified	11,600	28,400	2.45

$$\begin{aligned}
 DB &= \frac{\text{#####no. of dendritic units} + \text{no. of #terminal units}}{\text{Total no. of units}} \\
 &= \frac{1 - L}{\frac{16.00}{16}} \\
 &= \frac{1 - 0.13}{1} \\
 &= 0.87
 \end{aligned}$$

Figure S7. Example of calculation for degree of branching using ^1H NMR spectroscopy (400 MHz, CDCl_3). Example calculation using NMR spectrum from Figure 1d and reference of 16.00 from $M_{n,\text{NMR}}$ of 800 for the macromonomer.

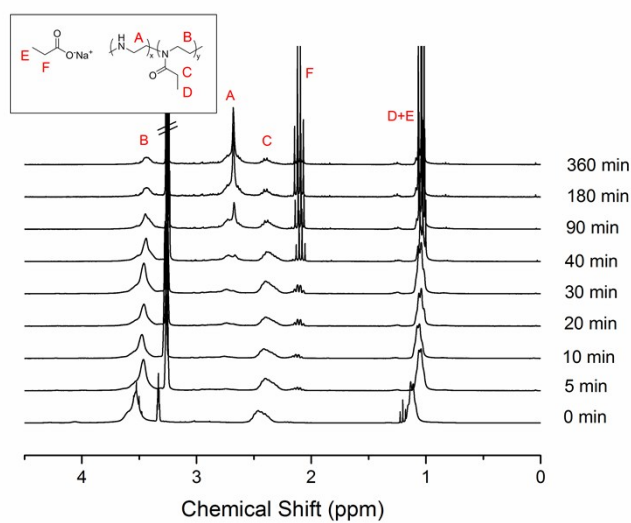


Figure S8. Hydrolysis kinetic study of HB POx (^1H NMR spectra (400 MHz, CDCl_3)) in 1M HCl,

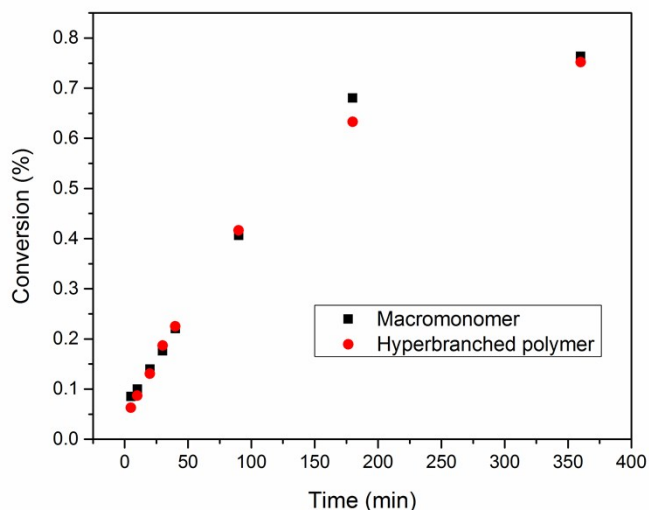


Figure S9. Hydrolysis kinetic study of both POx macromonomer and HB PEtOx (^1H NMR spectrum (400 MHz, CDCl_3)) in 1M HCl, percentage hydrolysis calculated from integral of propionic acid salt in crude hydrolysis product.

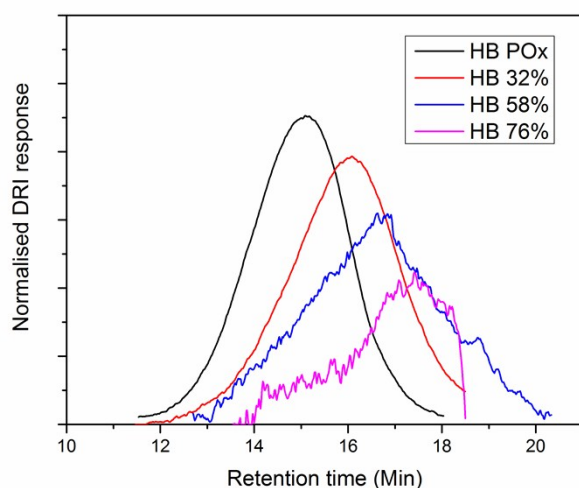


Figure S10. Size exclusion chromatograms (DMF + NH_4BF_4 additive eluent, PMMA calibration) of purified hydrolysed hyperbranched polymers and HB POx, showing previously reported low signals and increased retention times due to increased polymer cationic charges.

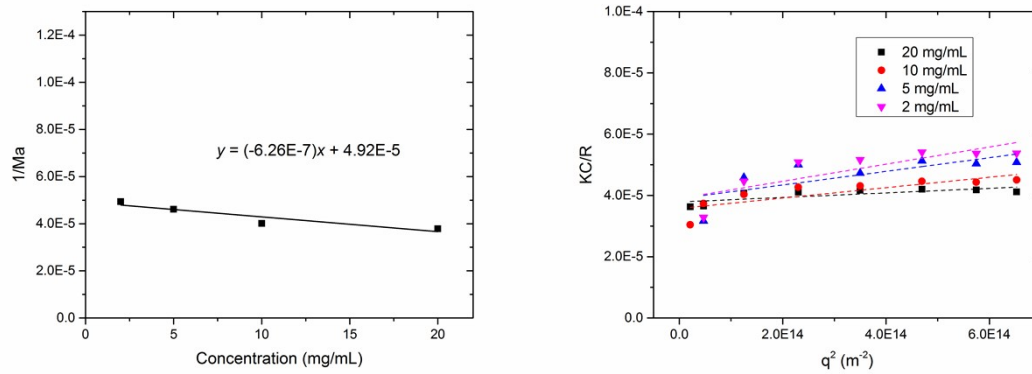


Figure S11. Evolution of KC/R of HB POx in water as a function of q^2 and concentration obtained by Static Light Scattering. $MW = 20,400$ g/mol, $dn/dC = 0.143$.

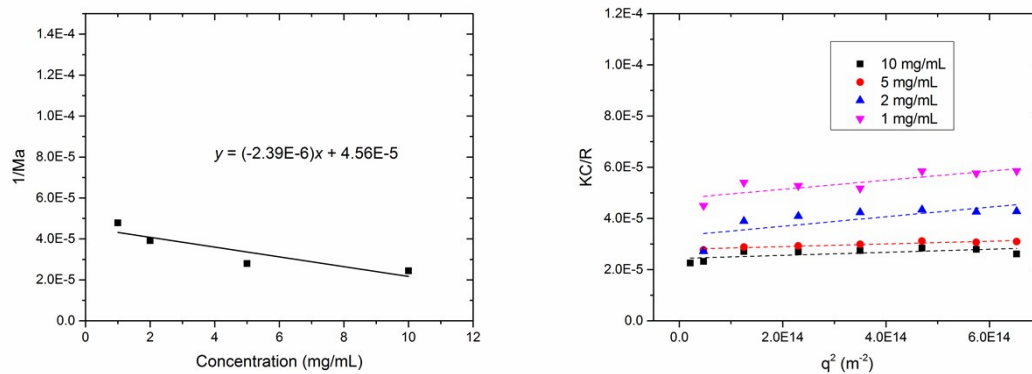


Figure S12. Evolution of KC/R of HB 32% in water as a function of q^2 and concentration obtained by Static Light Scattering. $MW = 21,900$ g/mol, $dn/dC = 0.175$.

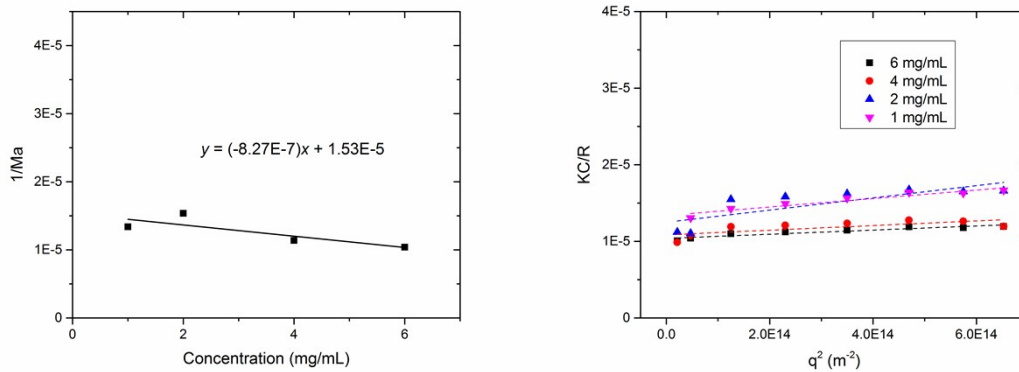


Figure S13. Evolution of KC/R of HB 58% in water as a function of q^2 and concentration obtained by Static Light Scattering. $MW = 65,300$ g/mol, $dn/dC = 0.185$.

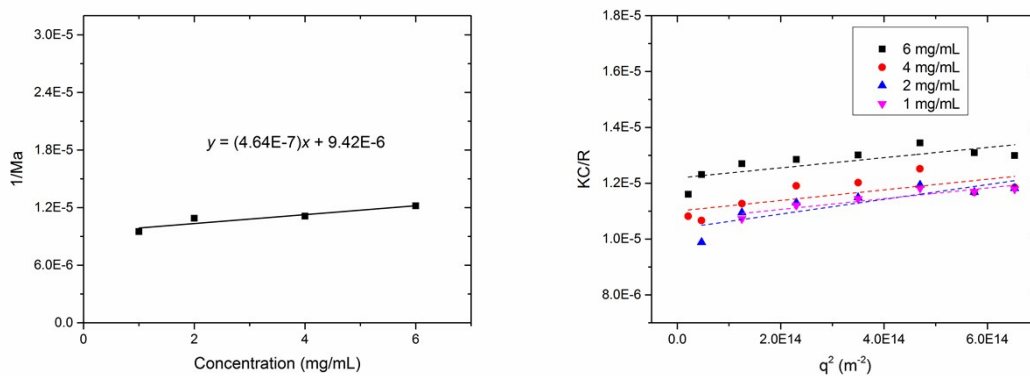


Figure S14. Evolution of KC/R of HB 76% in water as a function of q^2 and concentration obtained by Static Light Scattering. $MW = 106,200$ g/mol, $dn/dC = 0.198$.

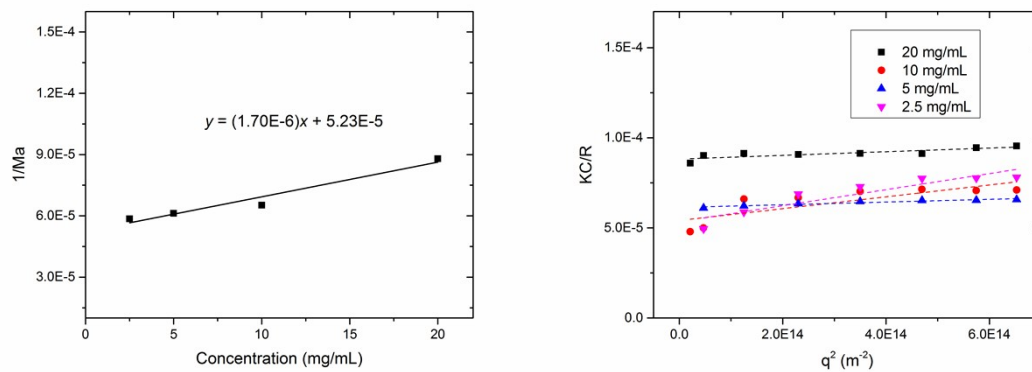


Figure S15. Evolution of KC/R of linear POx control in water as a function of q^2 and concentration obtained by Static Light Scattering. $MW = 19,100$ g/mol, $dn/dC = 0.143$.

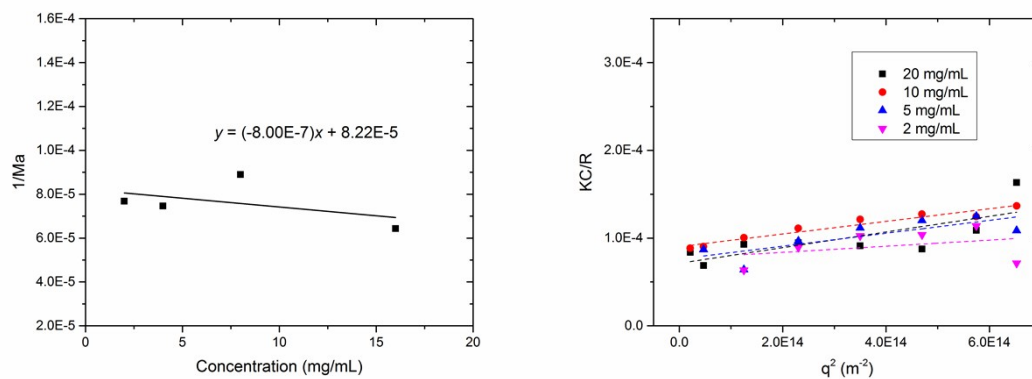


Figure S16. Evolution of KC/R of linear POx PEI 81% control in water as a function of q^2 and concentration obtained by Static Light Scattering. $MW = 12,400$ g/mol, $dn/dC = 0.128$.

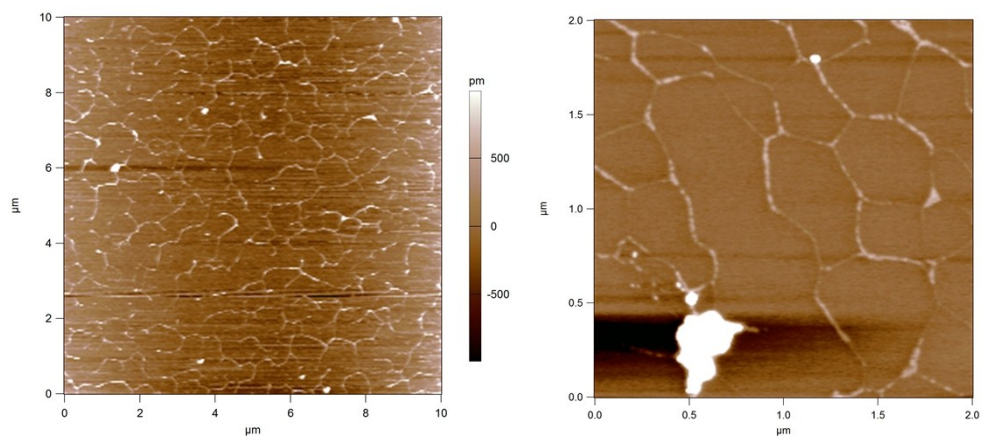


Figure S17. AFM images of pDNA on mica surface, at two different resolutions.

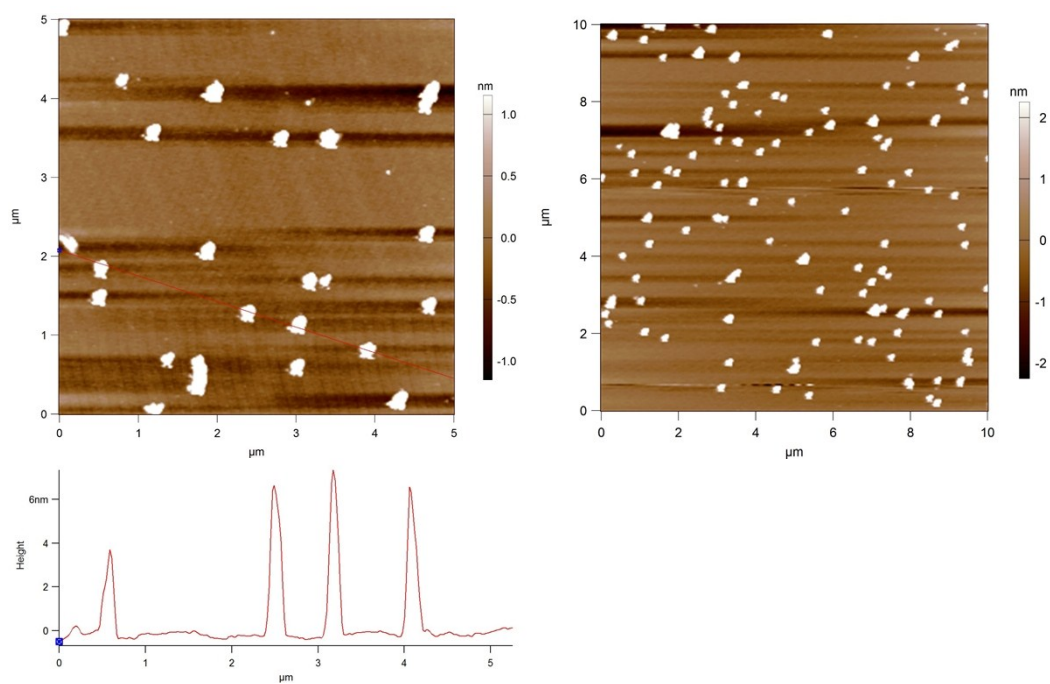


Figure S18. AFM images of linear 81% control polymer pDNA polyplex (N/P 20) on mica surface, at two different resolutions. Height profile included.

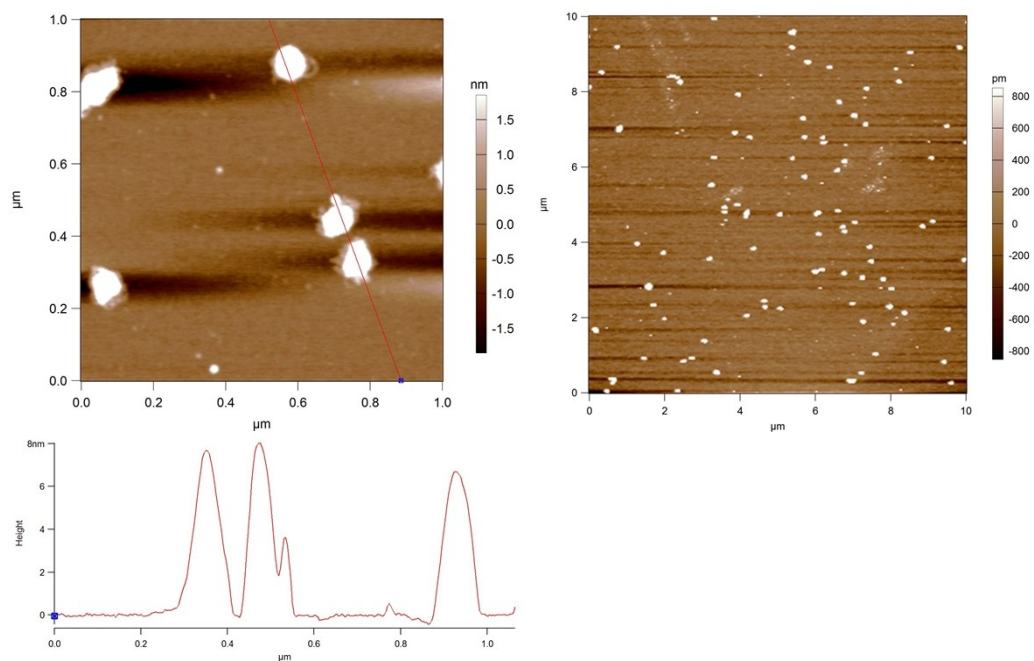


Figure S19. AFM images of hyperbranched POx PEI 76% polymer pDNA polyplex (N/P 20) on mica surface, at two different resolutions. Height profile included.

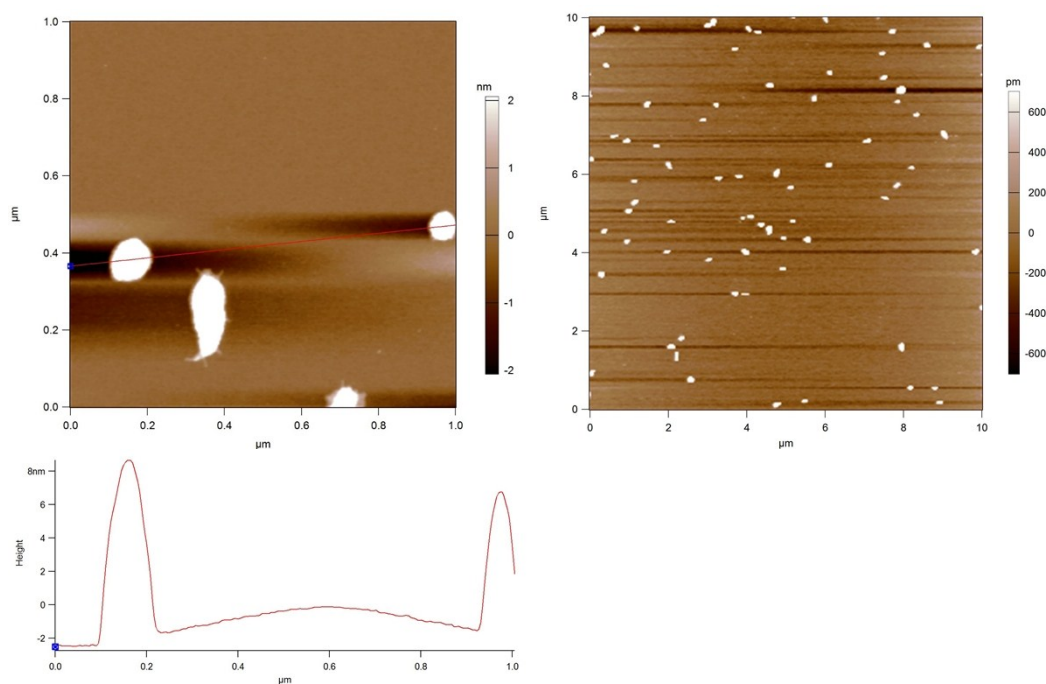


Figure S20. AFM images of bPEI polymer pDNA polyplex (N/P 20) on mica surface, at two different resolutions. Height profile included.

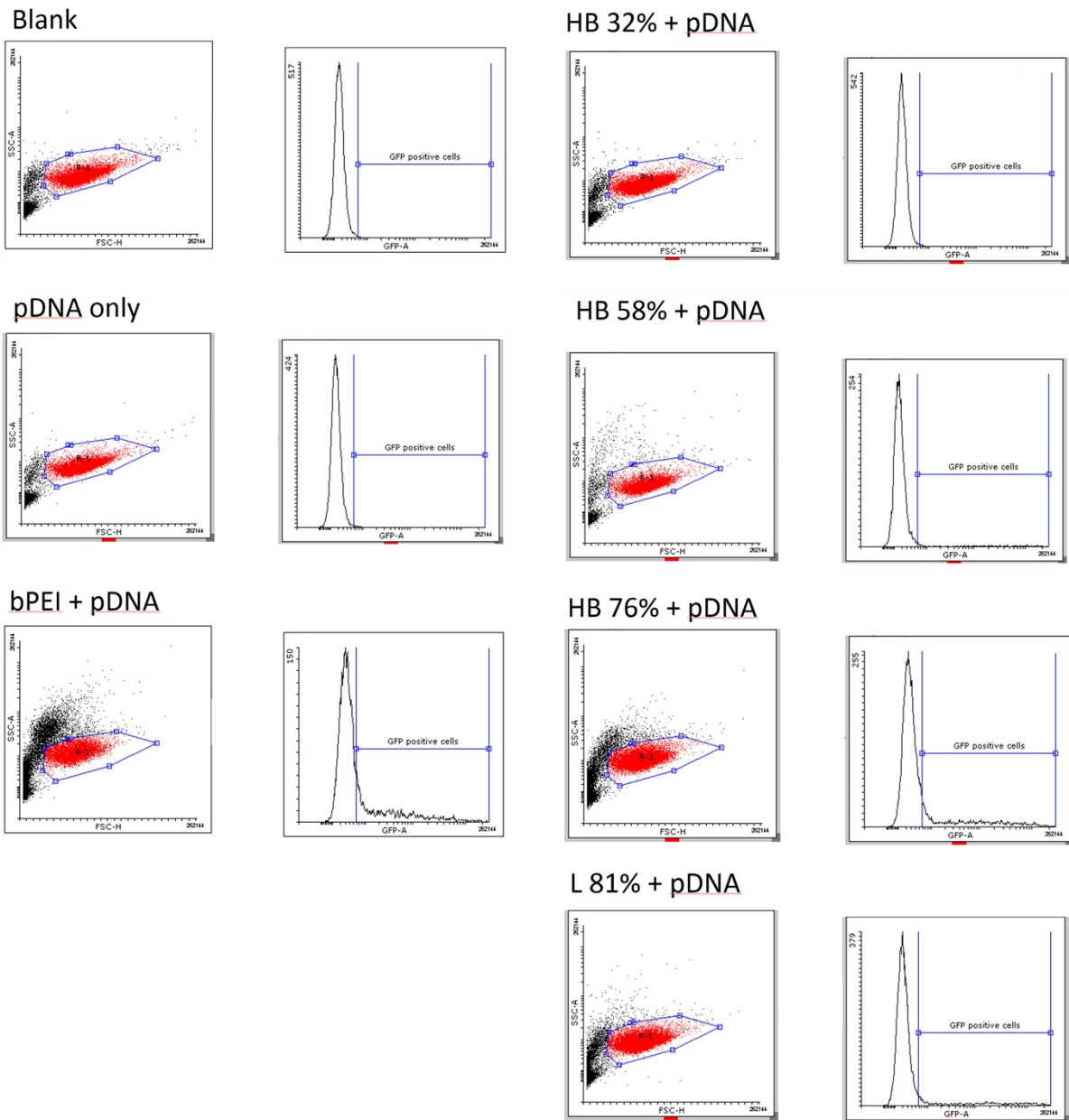


Figure S21. Representative (two separate experiments where each sample was measured in duplicate ($n = 4$)) dot plots and histograms of flow cytometry measurements determining positive GFP-expressing HEK cells after 24 h post-transfection with bPEI and synthesised polymers (all N/P 20).