Electronic Supplementary Material (ESI) for Polymer Chemistry

Supporting information

Synthesis and characterization of variable conformation pH responsive block co-polymers for nucleic acid delivery and targeted cell entry

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Representative Titration assays

Polymer **(9)** was dissolved [1 mg/mL] in deionized water and NaCl [150 mM]. The titration was performed by addition of aliquots of 0.1 M NaOH with stirring, starting from ~pH 3 to ~pH 11. The back titration was started from the pH value reached at the end of the titration by addition of 10 µL aliquots of 0.1 M HCl until a value of pH 3 was reached. Variations of pH were recorded after each addition.

Apparent pK_a values were determined from mid-points between titration start and equivalence points, utilising first derivatives of the titration curves to aid in measurement of equivalence points. The pK_a values found with this method must be considered as "formal" pK_a as the polymer after pH 5.7 (see Turbidimetry assay below) began to form aggregates.

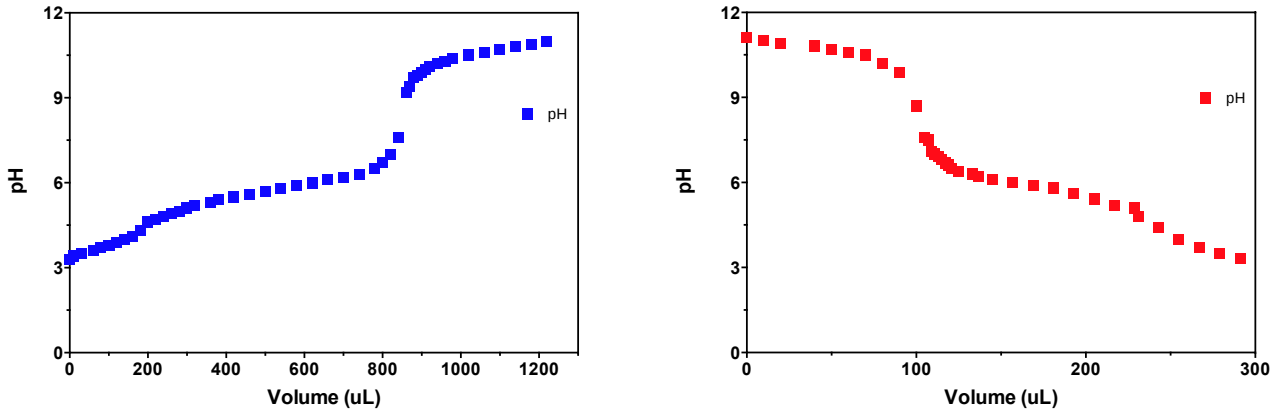

Figure S1. Titration (■) and back titration (■) of polymer (**9**).

Representative Turbidimetry assays

Polymer **9** (1 mg/mL) was dissolved in deionized water and NaCl (150 mM). The value of turbidity (T, %) was registered (λ= 500 nm) to be 100% at pH 3, then, small volumes (10 μL) of 0.1 M NaOH were added until ~pH 10, registering the % T at each addition.

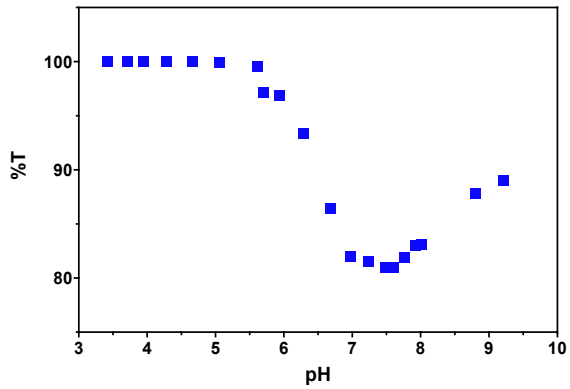

Figure S2. Turbidimetry assay for polymer **9** showing cloud point at ~ pH 5.7

Dynamic Light Scattering studies

Polymer solutions (final concentration [1 mg/mL]) were prepared using 10 mM phosphate buffer, 150 mM NaCl (PBS), or 150 mM NaCl in deionized water. The pH was varied by using aliquots of 0.1 M NaOH or 0.1 M HCl. Analysis was carried out at 25 ºC.

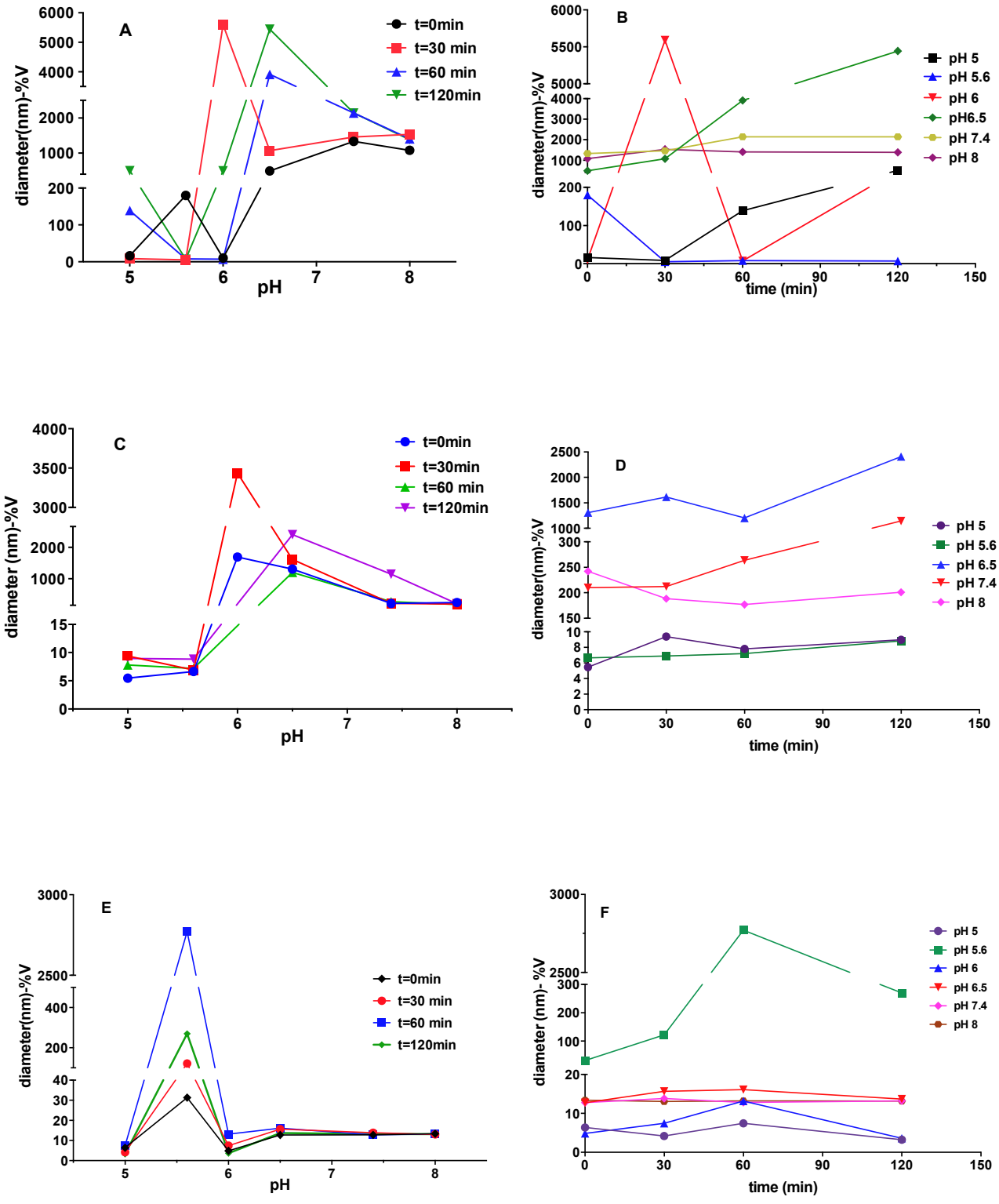

Figure S3. Kinetic profiles of polymer **(6, 9, 10)** by Dynamic Light Scattering analysis at different pH, at 25ºC. Particle diameters were recorded across pH ranges at scheduled times (Panel A, C, E) and over increasing time periods at specified pH (Panel B, D, F).

Polymer response with pH - NMR studies

Polymer (**9**) solutions [1 mg/mL] were prepared in D_2O, 150 mM NaCl. The different values of pH were obtained by increasing the pH using aliquots (5 µL) of NaOD (100 mM in D_2O). Analysis was carried out by ^{1}H-NMR (500 MHz), 256 scans for each sample submitted.

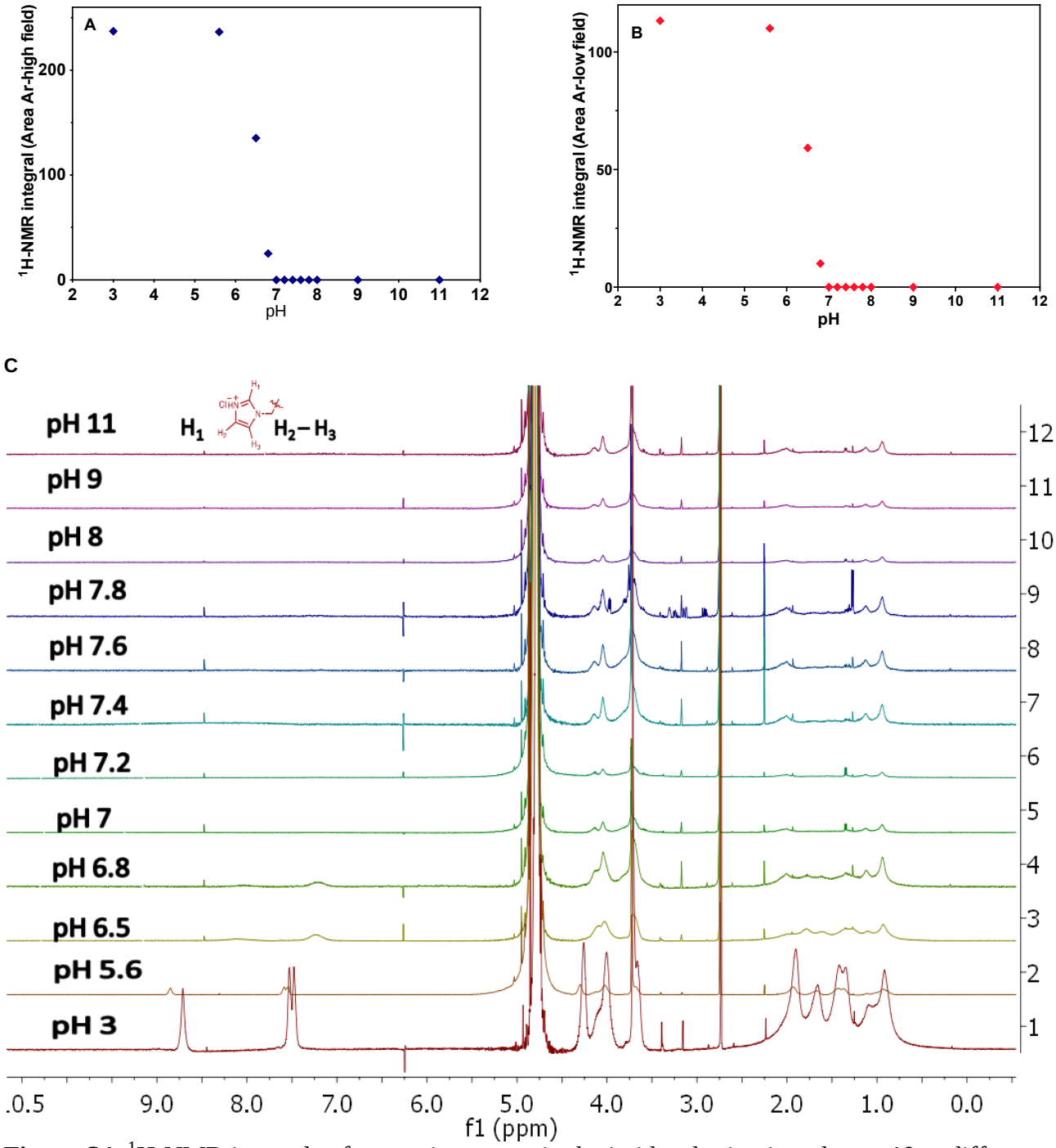

Figure S4. ^{1}H-NMR integrals of aromatic protons in the imidazole ring in polymer **10** at different pH: protons in the "high field" range of the spectra (Panel A); protons in the "low field" of the spectra (Panel B). ^{1}H-NMR spectra of polymer **10** at different pH (Panel C)

Determination of the critical aggregation concentration (CAC)

The CAC of block copolymer **9** was determined using pyrene as a fluorescent probe.[1] The polymer vesicle dispersion was prepared by the pH-switching method described by Lomas *et al*[2]. The dispersion was diluted with 20 mM phosphate, 150 mM NaCl, pH 7.4 yielding different polymer concentrations ranging from 0.2 to 100 μg/mL. Pyrene (5 μL) dissolved in acetone (0.18 mM) was added to 0.75 mL of the polymer dispersions. The samples were incubated overnight at room

temperature in the dark to allow equilibration. Prior to the measurements, the dispersions were incubated at 37 °C for 15 minutes. Fluorescence excitation spectra of pyrene were obtained using a Jasco spectrofluorometer FP-6500. The excitation spectra were recorded at 37 °C from 300 to 360 nm with the emission wavelength set at 390 nm. The excitation and emission band slits were 4 and 2 nm, respectively. The intensity ratio of I_{338}/I_{333} was plotted against the logarithmic concentration of the polymer to determine the CAC.

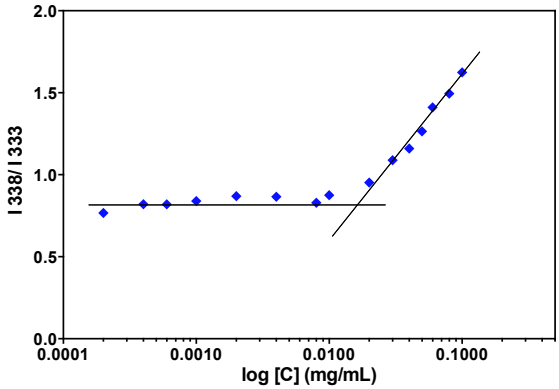

Figure S5. Critical aggregation concentration profile of polymer (**9**).

Polymersome preparations

Polymersomes were prepared using: 99:1, 95:5, 90:10 w/w polymer **9**/**10**, 90:5:5 w/w polymer **9**/**10**/**11** as described in the manuscript. The resulting polymersome dispersions were analyzed by DLS for evaluation of the mean size±standard deviation. The ζ–potentials of polymersomes were obtained following dilution of polymer formulations by 10-fold in high purity water at 25 °C prior to the analysis. ζ–potentials were neutral for the three formulations with different polymer **9**/**10** weight ratio (Figure S8).

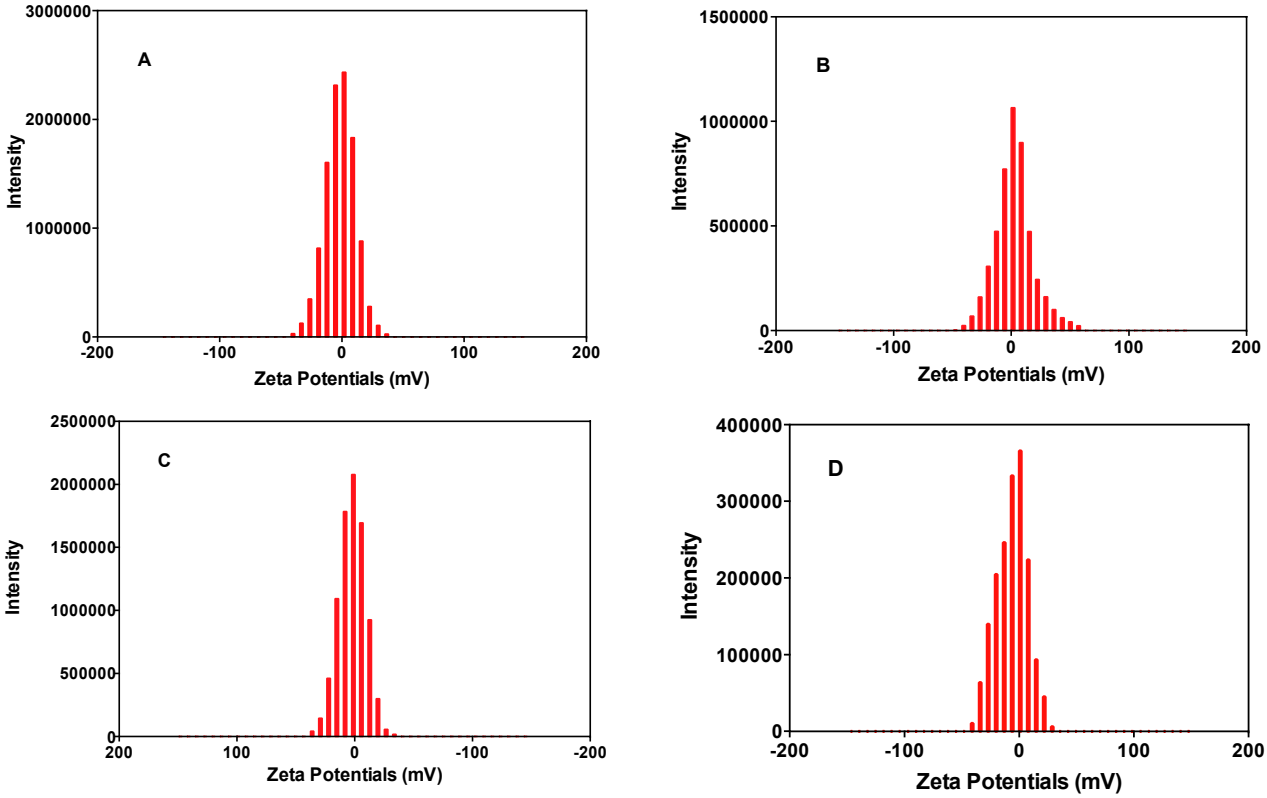

Figure S6. ζ–potential for formulations with different polymer ratios **9**/**10**/**11** w/w: 99:1:0 (panel A); 95:5:0 (panel B); 90:10:0 (panel C); 90:5:5(panel D).

Polymersome stability studies

Polymersome formulations (99:1, 95:5, 90:10 w/w polymers **9**/**10**, 90:5:5 w/w polymers **9**/**10**/**11)** (1 mg/mL) in 20 mM phosphate, 150 mM NaCl, pH 7.4, were incubated at 37 °C and analyzed by dynamic light-scattering (DLS) at scheduled times.

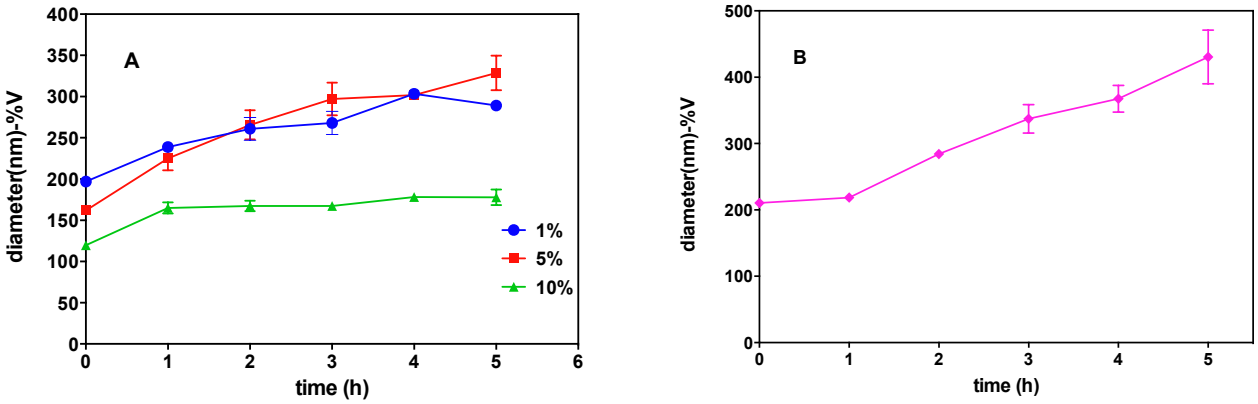

Figure S7. Kinetic stability profile of A) polymer **9**/**10** polymersomes at increasing weight percentage of polymer **10**: 1% (•), 5% (■), 10% (▲) and B) polymersomes assembled with 90:5:5 w/w polymer **9**/**10**/**11**.

Stability of folate decorated polymersomes

Polymersome formulations (90:10 w/w polymers **9**/**11**, 90:5:5 w/w polymers **9**/**10**/**11** and 90:10 polymers **9**/**10**) (1 mg/mL) in 20 mM phosphate, 150 mM NaCl, pH 7.4, were incubated at 37 °C and analyzed by dynamic light-scattering (DLS) at scheduled times.

90:5:5 w/w ratio for polymers **9**/**10**/**11** was found to give stable and well-defined dsDNA-loaded polymersomes. These experiments showed that the formulation obtained with 90:5:5 w/w ratio of polymers **9**/**10**/**11** yielded polymersomes with the size and stability analogous to the empty polymersomes obtained with the 90:10 w/w ratio of polymer **9**/**11**.

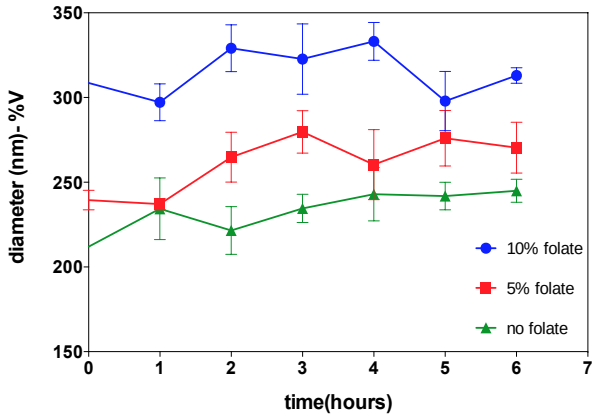

Figure S8. Kinetic stability profile of DNA-loaded polymersomes obtained with 90:10 w/w **9**/**11** (10% folate), 90:5:5 w/w **9**/**10**/**11** (5% folate) and 90:10 w/w **9**/**10** (no folate) at 37°C.

Stability of polymersomes in PBS and 10% fetal bovine serum.

The 90:10 w/w polymer **9**/**10** and 90:5:5 w/w polymer **9**/**10**/**11** formulations (1 mg/mL) were incubated in 20 mM phosphate, 150 mM NaCl, pH 7.4, in the presence of 10% of fetal bovine serum for 8 hours at 37 ºC. At scheduled times, the samples were analyzed by DLS.

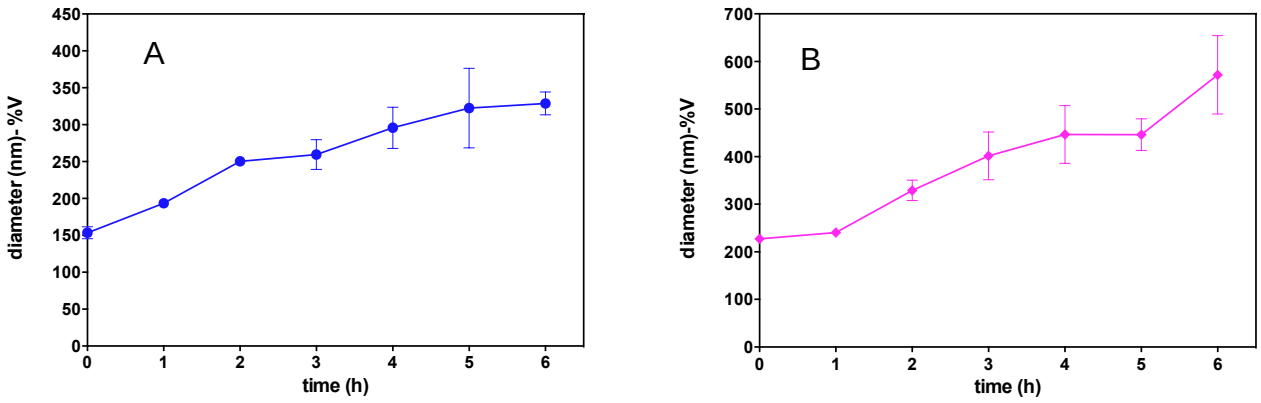

Figure S9. Kinetic stability profile at 37 °C in 10% serum of polymersomes obtained with A) 90:10 w/w polymer **9**/**10** and B) with 90:5:5 w/w polymer **9**/**10**/**11**.

DNA loading studies

A dsDNA, 19-bp oligonucleotide, sequence was used as a model for siRNA. The sequences chosen for the experiment were GAGATGTAAGGCCAGGCCG and its complementary strand. When hybridized, the dsDNA had a total molecular weight of 11.5 kDa.

dsDNA was loaded into polymer dispersions at different N/P feed ratios, where N is the number of imidazole groups of the triblock copolymer and P is the number of phosphate groups of DNA. N/P feed ratios of 10:1 and 1:1 were investigated.

To a solution of 90:10 w/w polymer **9**/**10** mixture (1 mg/mL in 20 mM phosphate, 150 mM NaCl pH 5) was added 27 or 265 μL of DNA solution (100 μM in 10 mM TRIS HCl, 50 mM NaCl, 1 mM EDTA, pH 7.8) to achieve 10:1 N/P and 1:1 N/P feed ratios, respectively. The polymer was induced to self-assemble by increasing the pH to 7.4. The assembly into colloidal nanostructures was confirmed by DLS.

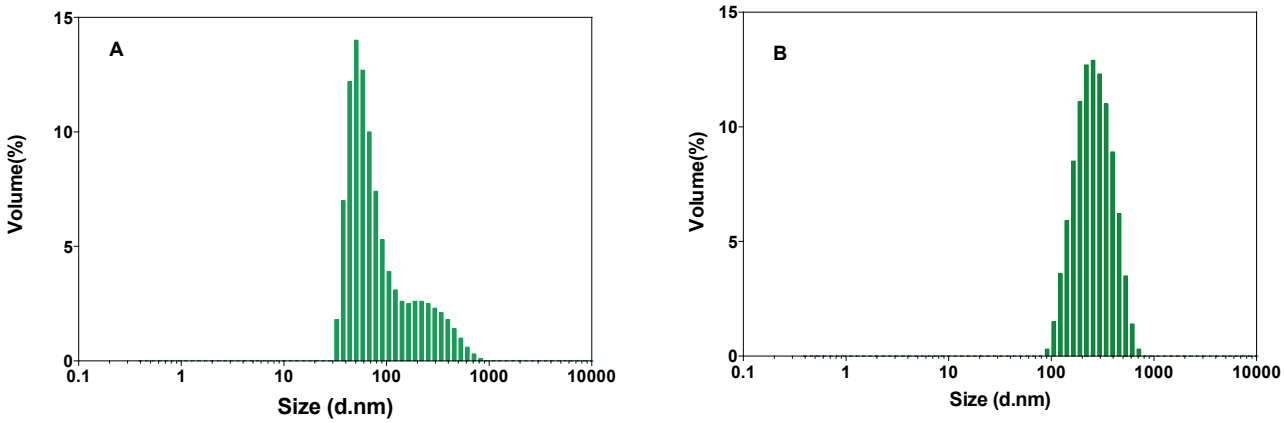

Figure S10. Dynamic light-scattering profile for the DNA-loaded polymersomes before dialysis (Panel A) and after dialysis (Panel B).

Unentrapped dsDNA was removed from polymer assemblies using a Float-A-lyzer® system equipped with a 100 kDa MWCO membrane.. The dialysis of dsDNA-loaded polymersomes was performed for 24 hours against 20 mM phosphate, 150 mM NaCl, pH 7.4. The purification method was validated by introducing in the device free dsDNA or empty polymersomes at the same concentration used for the loading test. dsDNA was completely removed by dialysis in 24 hours while the polymer was retained. dsDNA loaded polymer assemblies with a mean size of 252 nm and 0.151 PDI were obtained.

When the pH of dsDNA-loaded polymer carrier dispersions was lowered to 5, the size analysis showed a complete dissociation of the assemblies.

ζ-potential of polymer formulations loaded with dsDNA diluted 10-fold in high purity water was determined.

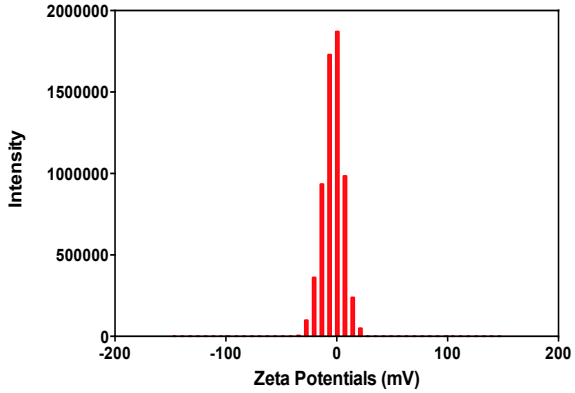

Figure S11. ζ–potential profile of the DNA-polymer formulation with 1:1 N/P feed ratio (-0.172mV) The ζ–potential analysis of an equimolar solution of dsDNA (control) was found to be -34.27 mV.

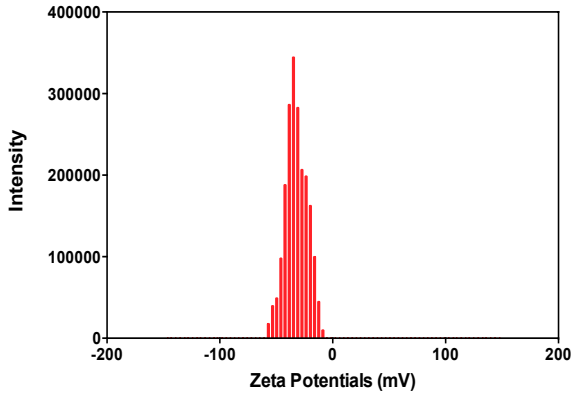

Figure S12. ζ–potential profile of the equimolar dsDNA solution used for the loading.

dsDNA loading quantification within the polymer assemblies was performed by UV-vis spectroscopy at 263 nm after decreasing the pH to 5 by 1 M HCl addition in order to disassemble the polymers and eliminate the contribution of larger particle scattering.

The polymersomes loaded with 10:1 N/P feed ratio showed a negligible loading, while the sample loaded with 1:1 N/P feed ratio was found to have a loading capacity (LC%) and encapsulation efficiency (EC%) of 14% mol/mol.

Transmission Electron Microscopy (TEM)

dsDNA-free and dsDNA-loaded 90:10 w/w polymer **9**/**10** polymersomes were prepared according to a previously described protocol[2] with final polymer concentrations of 2 mg/mL in 20 mM phosphate, 150 mM NaCl, pH 7.4 and analyzed by TEM. Samples were observed in negative staining mode, using small copper grid (400 mesh), covered by a "holey film" carbon layer. Samples were deposited on the grids and the contrast staining was performed with a uranyl acetate solution 1% w/v.

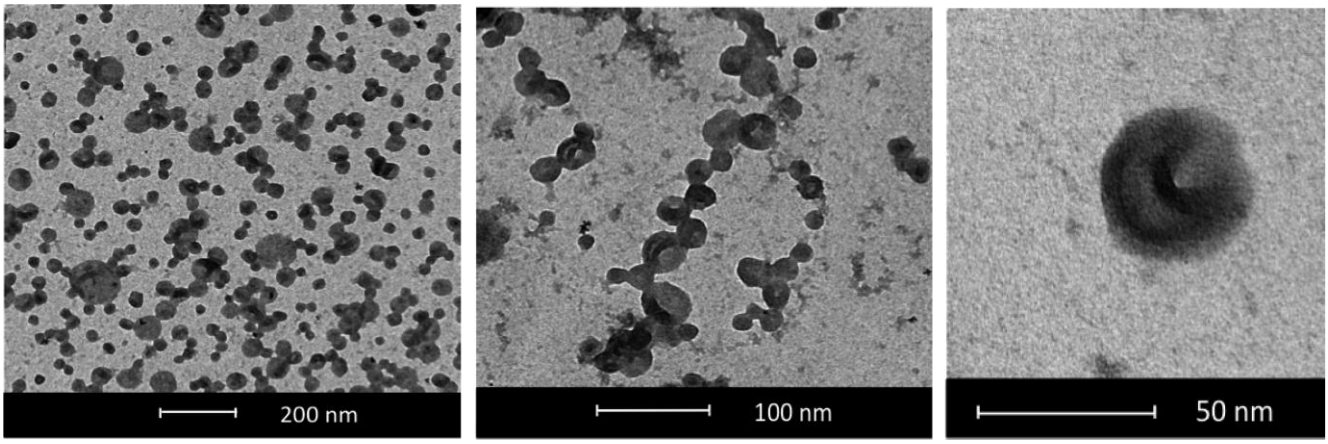

Figure S13. TEM images of dsDNA-free 90:10 w/w polymer **9**/**10** polymersomes at pH 7.4.

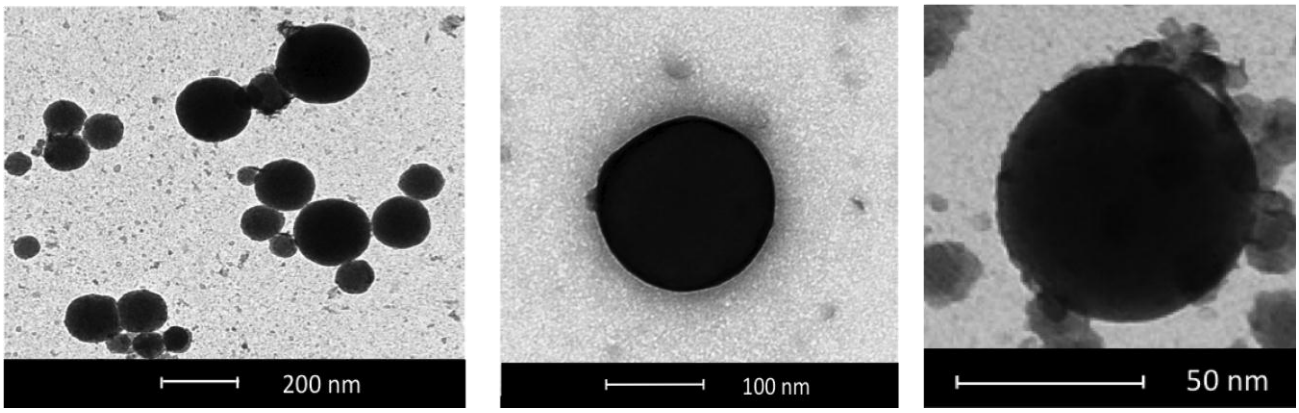

Figure S14. TEM images of dsDNA-loaded 90:10 w/w polymer **9**/**10** polymersomes at pH 7.4.

dsDNA release studies

Release studies were performed in 20 mM phosphate, 150 mM NaCl at pH 7.4 and 5.0.

Two (1 mg/mL) dsDNA-loaded 90:10 w/w polymer **9**/**10** formulations in PBS pH 7.4 were prepared with a 1:1 N/P feed ratio. 1 mL of this solution was transferred in a Float-A-lyzer® MWCO 100 kDa and dialyzed against 20 mM phosphate, 150 mM NaCl at pH 7.4. The second sample was acidified to pH 5 with 0.1 N HCl and dialyzed against 20 mM phosphate, 150 mM NaCl at pH 5.

The release study was performed at 37 °C. At scheduled times the samples were diluted with 20 mM phosphate, 150 mM NaCl pH 5 and spectrophotometrically monitored at 263 nm for DNA concentration.

References

1. G. Kwon, M. Naito, M. Yokoyama, T. Okano, Y. Sakurai and K. Kataoka, *Langmuir*, 1993, **9**, 945-949.
2. H. Lomas, I. Canton, S. MacNeil, J. Du, S. P. Armes, A. J. Ryan, A. L. Lewis and G. Battaglia, *Adv. Mater.*, 2007, **19**, 4238-4243.