Electronic Supplementary Information for:

A mild and versatile approach for DNA encapsulation

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1. General Experimental Details.

1.1. Materials.

All starting materials were purchased from Aldrich unless otherwise indicated. The solvents were purified applying standard procedures. Milipore® Type I water form Direct-Q® 3 – UV System, equipped with BioPak ultrafiltration cartridge was used in the polyplex formation and stabilization experiments.

N^{ϵ}-(benzyloxycarbonyl)-L-lysine *N*-carboxyanhydride (ZLLys-NCA*)* was prepared from Z-L-lysine and triphosgene in ethyl acetate applying the advanced purification procedure described by Poché at al.[1] Yield: 86%.

Salmon sperm DNA (D-1626; M_w ~ 2000 bp) was received form Sigma-Aldrich. A stock solution of 100 μg/ml DNA was prepared in ultra pure water (>18 MΩ) and used for polyplex formation.

1.2. Methods.

^{1}H NMR spectra were obtained using Bruker Avance II+ 600 MHz instrument.

Aqueous gel permeation chromatography (GPC) was performed on a set of CATSEC (Eprogen Inc., USA, and Eichrom Techn. Inc., USA) columns 100, 300, 1 000, and 4 000 Å, calibrated versus polyoxyethylene (PEO) narrow molar mass standards; the mobile phase was 0.2 M CH_3COOLi, 0.2 M CH_3COOH with pH≈4.6 and a flow rate of 0.25 mL/min; the column and DRI detector temperature was 40°C. GPC measurements of poly(*N*-isopropylacrylamide) (PNIPAM)-macroinitiator and the hybrid block copolymer were performed in *N,N*-dimethylformamide (DMF) at a flow rate 1mL/min on a guard (Polymer Laboratories) + GRAM 100 Å (Polymer Standards Service) + 2 x Mixed-C (Polymer Laboratories) column system, calibrated versus polystyrene (PS) narrow molar mass standards; the refractive index detector was Δ*n*-2010 RI from WGE Dr. Bures.

UV-vis absorption spectra were taken on a Beckman Coulter DU® 800 spectrophotometer. Quartz cells with a path length of 1 cm were used.

Dynamic light scattering (DLS) measurements were carried out at 25 and 40°C on a Zetasizer Nano-ZS instrument (Malvern Instruments), equipped with a He-Ne laser (λ = 633 nm) with a scattering angle of 173°. The ζ-potentials were calculated from the obtained electrophoretic mobility at 25°C by the Smoluchowski equation:

$\zeta = 4\pi\eta\upsilon/\varepsilon$,

where η is the solvent viscosity, υ is the electrophoretic mobility, and ε is the dielectric constant of the solvent.

Transmission electron microscopy (TEM) images were obtained using HRTEM JEOL JEM-2100 (200 kV) instrument.

Atomic force microscopy (AFM) images were obtained from Multimode V Scanning Microscope (Veeco, CA).

The polyplex formation was checked by (0.6 $\%_{w/v}$) agarose electrophoresis stained with ethidium bromide (0.5 µg/ml).

Ethidium bromide displacement assay for the block copolymer was performed following a literature procedure.[2]

The cytotoxicity of the block copolymer, polyplex and stabilized polyplex against human cervical cancer cells (HeLa S3, ATCC: CCL 2.2) was investigated using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The MTT assay is a common method for evaluating biomaterial toxicity based on the mitochondrial activity, which influences metabolic activity and cell viability.[3] HeLa cells were cultured in DMEM (Dilbecco's Modified Eagle Medium, Applichem, Germany) supplemented with 10% (v/v) FBS (Fetal Bovine Serum, BioWhittaker, USA), penicillin (100 µg/ml), streptomycin (100 µg/ml) and 4 mM l-glutamine at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. Cells were routinely checked for mycoplasma contamination, by DAPI staining (Roche Diagnostics, Mannheim, Germany) and found to be free of it.

In the logarithmic growth phase the cells were harvested and subsequently plated into 96-well microtitre plates (Nunc, Wiesbaden, Germany) at a density of $1x10^4$ cells/well in DMEM media. Following 24 h, copolymer and polyplex solutions at different concentrations were incubated separately with the cells for further 24 h. Following the incubation periods MTT solution (10 µl; 5 mg/ml) was added to each well and the plates were re-incubated for 3 h. The MTT- formazan product was dissolved in isopropanol and the absorption at 550/630 nm was measured on an ELISA plate reader (Bio-Tek Instruments Inc., USA). MTT assay was performed eight times.

Data were reported as means ± standard deviation (SD). Differences among the mean were compared by one-way analysis of variance (ANOVA) followed by the Tucky posthoc test for multiple comparisons using PASW Statistics 18.0 software package (IBM) for Windows. The level of significance was set at $\mathbf{p} = 0.05$.

2. Synthetic Details.

2.1. Poly(N-isopropylacrylamide)-b-poly(L-lysine) (PNIPAM-b-PLLys).

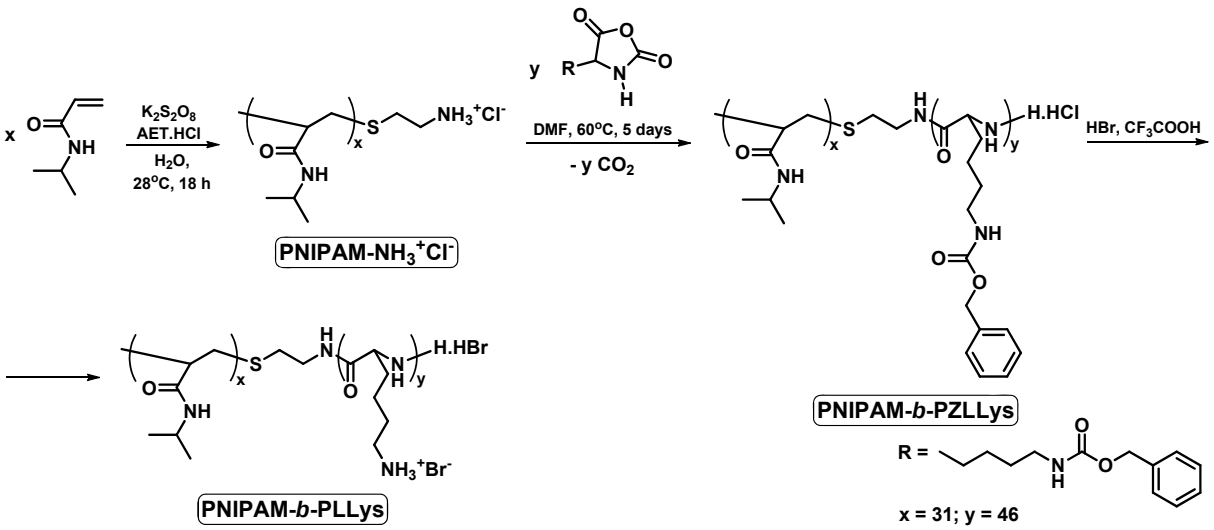


Figure S1. Synthesis of hybrid block copolymer PNIPAM-*b*-PLLys

PNIPAM-$NH_3^+Cl^-$. The macroinitiator was synthesized using a literature procedure.[4] NIPAM (5 g, 44.18 mmol) was dissolved in 45 mL of water and was degassed for 60 min. Separately, $K_2S_2O_8$ (0.24 g, 0.89 mmol) and 2-aminoethanethiol hydrochloride (AET.HCl, 0.25 g, 2.2 mmol) were dissolved in 5 mL and 2 mL of water respectively and degassed for 60 min. The latter two solutions were added rapidly to the monomer solution. The reaction was allowed to proceed at 28°C for 18 h. The product was dialyzed against distilled water and recovered through lyophilization. Yield: 4.6 g (92%). GPC in DMF (vs. PS standards): M_w/M_n = 1.90. ^{1}H NMR (D_2O): δ 1.11 (CH-(CH_3)$_2$); δ 1.30-1.75 (CH_2-CH); δ 1.85-2.15 (CH_2-CH); δ 2.78 (-S-CH_2-CH_2-NH_3^+); δ 3.15 (-S-CH_2-CH_2-NH_3^+); δ 3.86 (CH-(CH_3)$_2$). M_n=3 500 (31 r.u.) –titration of the terminal primary amine groups.

PNIPAM-*b*-PLLys. The hybrid block copolymer was synthesized through ammonium-mediated ring-opening polymerization of ZLLys-NCA[5] initiated by PNIPAM-$NH_3^+Cl^-$. The macroinitiator (0.6 g, 0.17 mmol) was dissolved in 10 mL of DMF and degassed. Separately, 1.57 g (5.14 mmol) of ZLLys-NCA were dissolved in 16 mL of DMF and degassed. The two solutions were combined *via* transfer needle and stirred under a dry argon atmosphere. The polymerization was performed at 60°C for 5 days. The solvent was evaporated and the residue was extracted with methanol to give PNIPAM-*b*-PZLLys. Yield: 1.62 g, 76%. GPC in DMF

(vs. polystyrene standards): M_w/M_n = 1.8. ^{1}H NMR (DMSO-d_6): δ 1.05 (CH-(CH_3)$_2$); δ 1.20-1.70 (CH_2-CH + αCH-(CH_2)$_3$); δ 1.98 (CH$_2$-CH); δ 2.95 (αCH-(CH$_2$)$_3$CH_2 + S-CH$_2$-CH_2-NH + S-CH_2-CH$_2$-NH); δ 3.85 (CH-(CH$_3$)$_2$); δ 4.21 (αCH-NH); δ 4.99 (Z-CH_2); δ 6.85-7.38 (αCH-(CH$_2$)$_4$-NH + S-(CH$_2$)$_2$-NH + NH-CH-(CH$_3$)$_2$ + C$_6$H$_5$); δ 7.90-8.15 (αCH-NH).

As the molar mass of the macroinitiator PNIPAM-$NH_3^+Cl^-$ is known, the experimental degree of peptide polymerization was calculated from the ratio of the intensities of methyne protons from isopropyl groups of the PNIPAM chain at 3.85 ppm and methylene protons at 4.99 ppm (Z-protecting group). The calculated value for DP_{Lys} was 46.

The Z-protecting groups were removed from the peptide block of PNIPAM-*b*-PZLLys following the general procedure: 0.67 g of the polymer (1.9 mmol of protecting groups) was dissolved in 5 mL of trifluoroacetic acid. Then 2 mL of 4N HBr in glacial acetic acid were added. The reaction mixture was stirred for an hour at room temperature followed by the addition of 10 mL of chilled water. The mixture was extracted several times with chilled diethyl ether. The aqueous layer was neutralized with 10N NaOH and dialyzed against distilled water. The product was recovered through lyophilization. Yield: 0.42 g, 75%. ^{1}H NMR (D$_2$O): δ 1.11 (CH-(CH_3)$_2$); δ 1.33-1.83 (CH_2-CH + αCH-(CH_2)$_3$); δ 1.97 (CH$_2$-CH);δ 2.97 (αCH-(CH$_2$)$_3$CH_2); δ 3.86 (CH-(CH$_3$)$_2$); δ 4.27 (αCH-NH).

2.2. α-Acryloyl-ω-folate-polyoxyethylene (Ac-PEO-FA).

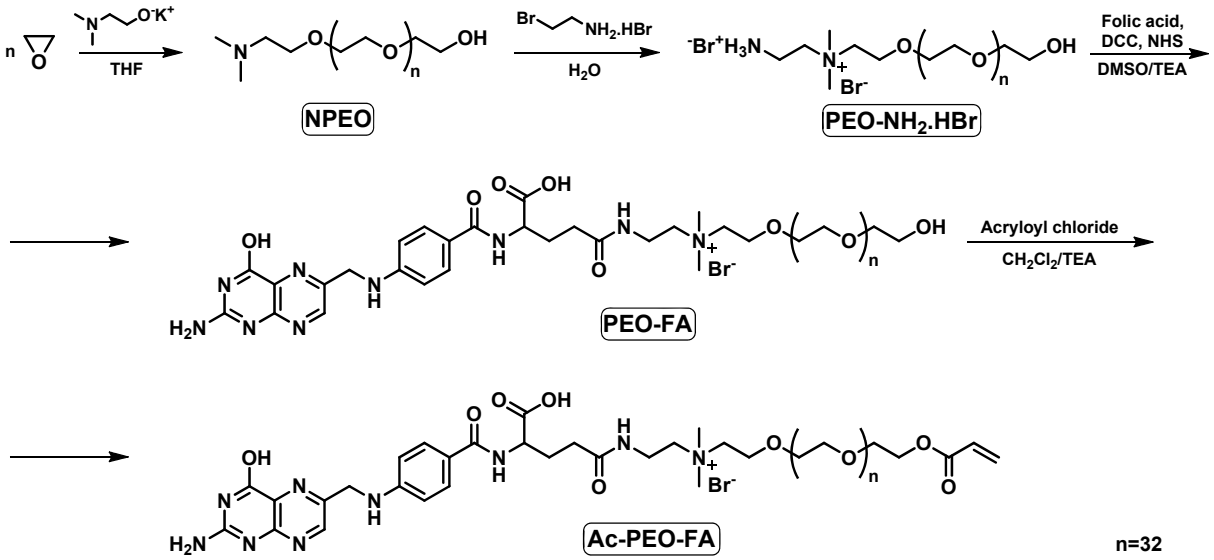


Figure S2. Synthesis of folate-terminated PEO-macromonomer (Ac-PEO-FA).

α-Dimethylaminoethylpolyoxyethylene (NPEO) was prepared by anionic polymerization of ethylene oxide in THF initiated by potassium alkoxide of *N*,*N*-dimethylethanolamine as previously described.[6] Aqueous GPC (vs. PEO standards): M_n = 1 350, M_w/M_n = 1.12. ^{1}H NMR ($CDCl_3$): δ 2.24 ($(CH_3)_2$-N); δ 2.49 ($(CH_3)_2$-N-CH_2); δ 3.5-3.75 (O-CH_2CH_2-O).

The number average molar mass determined by ^{1}H NMR from the relative intensities of oxyethylene protons at 3.5-3.75 ppm and dimethylamine protons at 2.24 ppm was M_n=1 400.

PEO-NH$_2$.HBr. Heterobifunctional polyoxyethylene bearing terminal primary amine and hydroxyl groups was synthesized through quaternization of the tertiary amine group of NPEO (5 g, 3.57 mmol) with 2-bromoethylamine hydrobromide (0.73 g, 3.93 mmol) in 20 mL of water. The reaction mixture was kept for 48 h at 60°C. The product was recovered by lyophilization. ^{1}H NMR (D_2O): δ 2.89 ($(CH_3)_3$-N); δ 3.34 (N-CH_2-CH_2-NH_3^+); δ 3.58-3.85 (O-CH_2CH_2-O + N-CH_2-CH_2-NH_3^+).

PEO-FA. PEO-folate conjugate was obtained through the following reaction procedure: Folic acid (0.28 g, 0.625 mmol) was dissolved in 70 mL of dimethyl sulfoxide (DMSO) followed by the addition of *N*-hydroxysuccinimide (NHS, 0.18 g, 1.56 mmol) and *N.N'*-dicyclohexylcarbodiimide (DCC, 0.13 g, 6.25 mmol). The reaction mixture was stirred at room temperature in dark for 30 min. Finally, a solution of PEO-NH_2.HBr in 3 mL of DMSO and 1.5 mL of triethylamine were added. The reaction was left to proceed overnight at

room temperature. The cloudy solution was filtered, concentrated and the product was precipitated in diethyl ether. Then it was redissolved in aqueous alkaline solution (pH~8.5, adjusted with 2 M Na_2CO_3) and dialyzed. The product was recovered by lyophilization. Yield: 0.68 g (55%). ^{1}H NMR (D_2O): δ 1.97-2.26 (NH-C(O)-CH_2-CH_2-CH(COOH)); δ 2.88 ((CH_3)$_3$-N); 3.33-3.85 (N-CH$_2$-CH_2-NH + CH_2CH_2-O + N-CH_2-CH$_2$-NH); δ 4.26 (NH-C(O)-CH$_2$-CH$_2$-CH(COOH)); δ 4.48 (C_6H_5-NH-CH_2); δ 6.46 (C(O)-ArH-3 + C(O)-ArH-5); δ 7.59 (C(O)-ArH-2 + C(O)-ArH-6); δ 8.66 (1H, substituted pteridine ring).

Ac-PEO-FA. Folate conjugated PEO macromonomer was synthesized applying the following procedure: PEO-FA (0.13 g, 0.065 mmol) was dispersed in 0.5 mL of dichloromethane. Solutions of acryloyl chloride (0.1 mL, 1.23 mmol) in 0.2 mL of dichloromethane and triethylamine (0.17 mL, 1.25 mmol) in 0.2 mL of dichloromethane were added dropwise simultaneously. The reaction mixture was stirred overnight at room temperature in dark. The solvent was evaporated. The residue was dissolved in water, filtered and dialyzed. The product was recovered by lyophilization. Yield: 0.1 g (75%). NMR (D_2O): δ 1.97-2.26 (NH-C(O)-CH_2-CH_2-CH(COOH)); δ 2.86 ((CH_3)$_3$-N); 3.30-3.95 (N-CH$_2$-CH_2-NH + CH_2CH_2-O + N-CH_2-CH$_2$-NH); δ 4.24 (NH-C(O)-CH$_2$-CH$_2$-CH(COOH)); δ 4.30 (CH$_2$-CH_2-O-C(O)-CH=CH$_2$); δ 4.48 (C_6H_5-NH-CH_2); δ 5.95 (CH=C(H)H); δ 6.19 (CH=C(H)H); δ 6.40 (C=(H)H); δ 6.46 (C(O)-ArH-3 + C(O)-ArH-5); δ 7.62 (C(O)-ArH-2 + C(O)-ArH-6); δ 8.63 (1H, substituted pteridine ring).

The amount of conjugated folic acid was quantified by UV-vis spectroscopy by comparing the absorbance of Ac-PEO-FA in phosphate buffer (pH 7.24) at 344 nm with a previously constructed folic acid calibration curve. The molar extinction coefficient value of 7150 mol^{-1} dm^3 cm^{-1} at λ=344 nm was determined from the calibration curve. The results indicated almost complete folate functionalization of the PEO-macromonomer.

3. Polyplex Formation.

Polyplexes were prepared by dropwise addition of 3 mL from the DNA solution (2000 bp, 100μg/mL) to 3 mL aqueous polymer solutions of different concentrations. The ratio

N/P was varied from 0.6:1 to 5:1. The polyplexes were gently stirred at room temperature for 30 min and then samples for characterization were withdrawn.

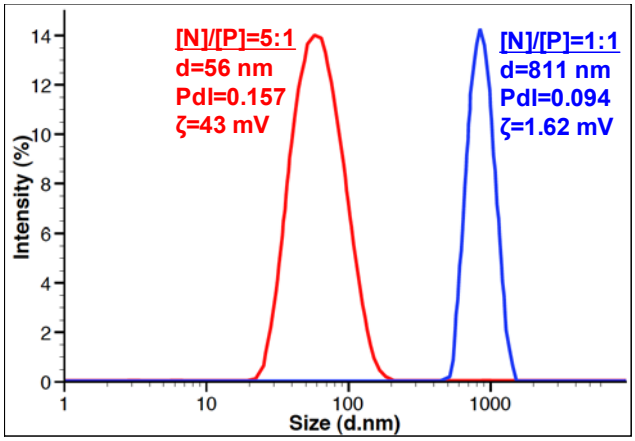


Figure S3. Size distribution of polyplexes formed at N/P=1:1 and 5:1.

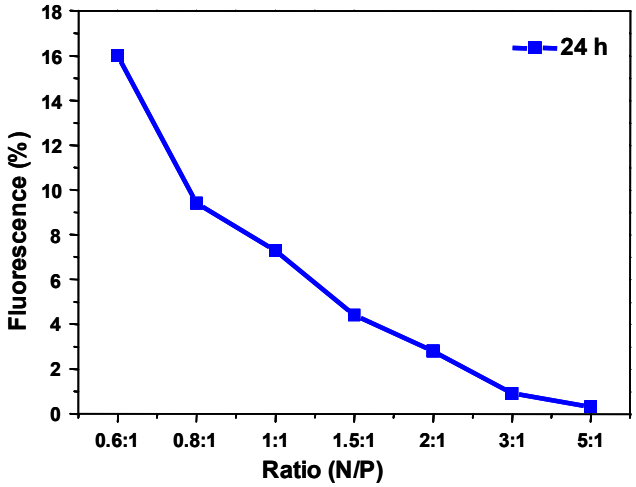


Figure S4. Ethidium bromide displacement assay for PNIPAM-*b*-PLLys. The ability of the hybrid block copolymer to condense DNA was evaluated at different N/P ratios.

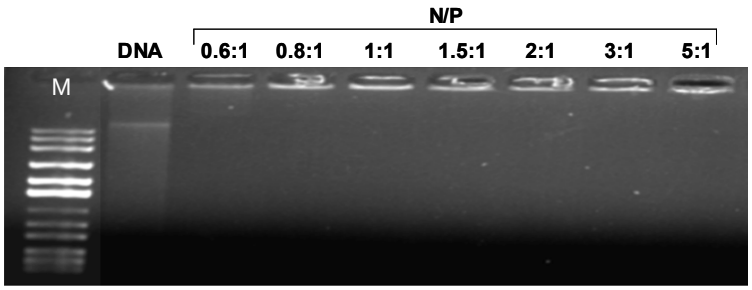


Figure S5. Gel retardation assay of the polyplexes at different N/P ratios.

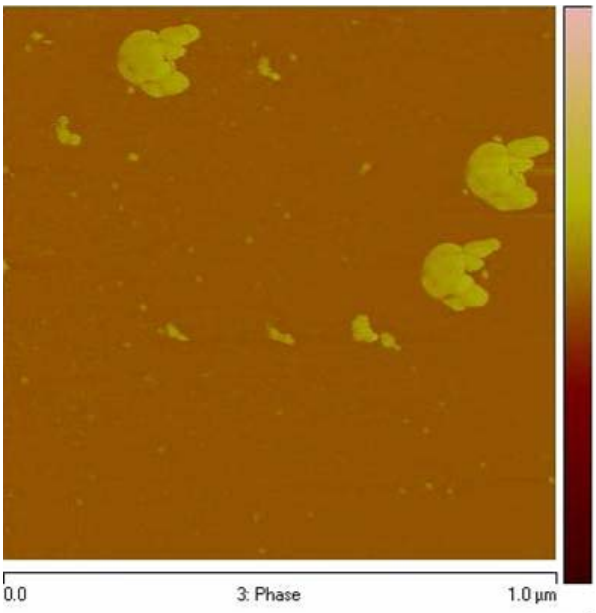


Figure S6. AFM image of polyplex (N/P=5:1).

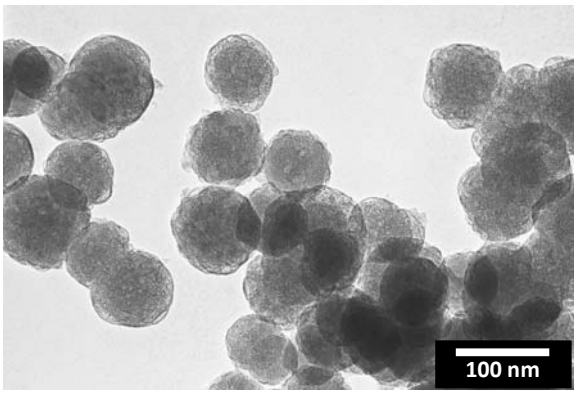
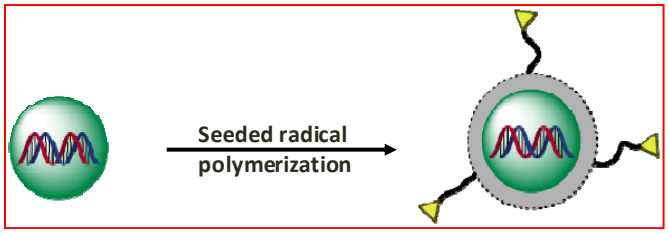


Figure S7. TEM image of polyplex (N/P=5:1).

4. Polyplex Stabilization.



Typically, the polyplex solution (5 mL, 0.265 mg/mL) was immersed in preheated to 70°C oil bath. Under vigorous stirring an aqueous solution (0.125 mL) of monomer, cross-linker and/or folate conjugated macromonomer was added. The ingredient concentrations in the total volume were as follows:

- 1-4 mM monomer (NIPAM);

- 1.46-1.9 mM cross-linkers (*N*,*N*-methylenebis(acrylamide) (BIS), polyethylene glycol diacrylate (PEG-DA), *N*,*N*′-bis(acryloyl)cystamine (BAC) or combinations between them);
- 0.13 mM acryloyl-PEO-folate conjugate (Ac-PEO-FA).

The solution was degassed for 30 min, followed by the injection of 75 µl from the 0.1 M initiator solution in H_2O/MeOH=75:25% (v/v). The water soluble 2,2′-Azobis(2-methylpropionamidine) dihydrochloride (AAPH) was used to initiate the seeded radical copolymerization. The cross-linked polymer shell was formed for 5 h at 70°C. Finally, the reaction solution was dyalized against ultra pure water.

The folate content in the polymer shell was determined by quantitative UV-vis spectroscopy. Molar extinction coefficient ε=7150 mol^{-1} dm^3 cm^{-1} at λ=344 nm was used for the calculations. Up to 2 wt% of folate residues were introduced on the surfaces of the stabilized polyplexes.

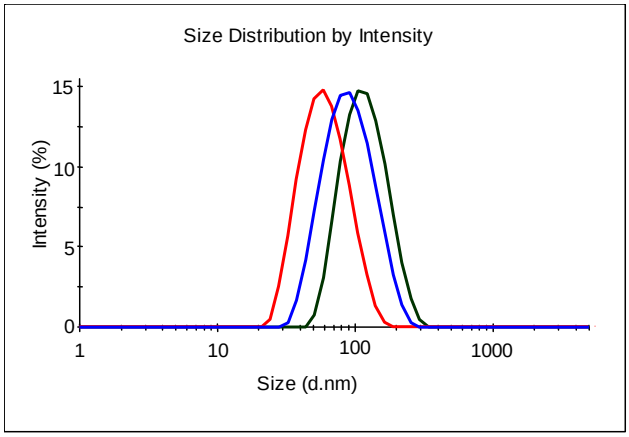


Figure S8. Size distribution of: (—) polyplex N/P=5:1 (d=64.0 nm; PdI: 0.16; ζ=38.3 mV); (—) stabilized polyplex using 2 mM NIPAM for the shell formation (d=82.5 nm; PdI: 0.14; ζ=16.5 mV), and (—) stabilized polyplex using 4 mM NIPAM for the shell formation (d=124 nm; PdI: 0.15; ζ= 9.6 mV).

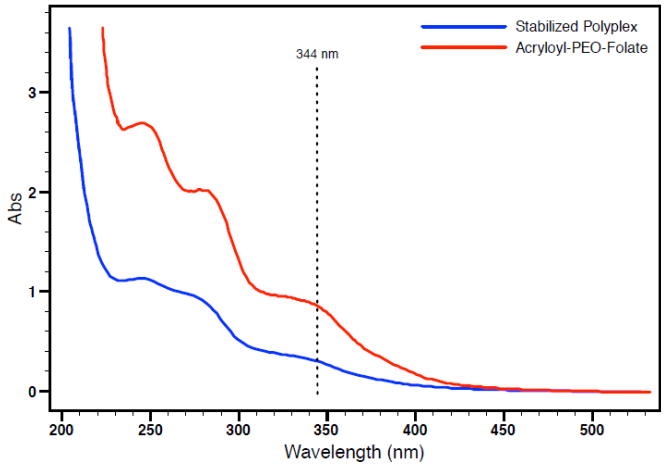


Figure S9. UV spectra of acryloyl-PEO-folate (Ac-PEO-FA) and stabilized polyplex with attached folate targeting ligand.

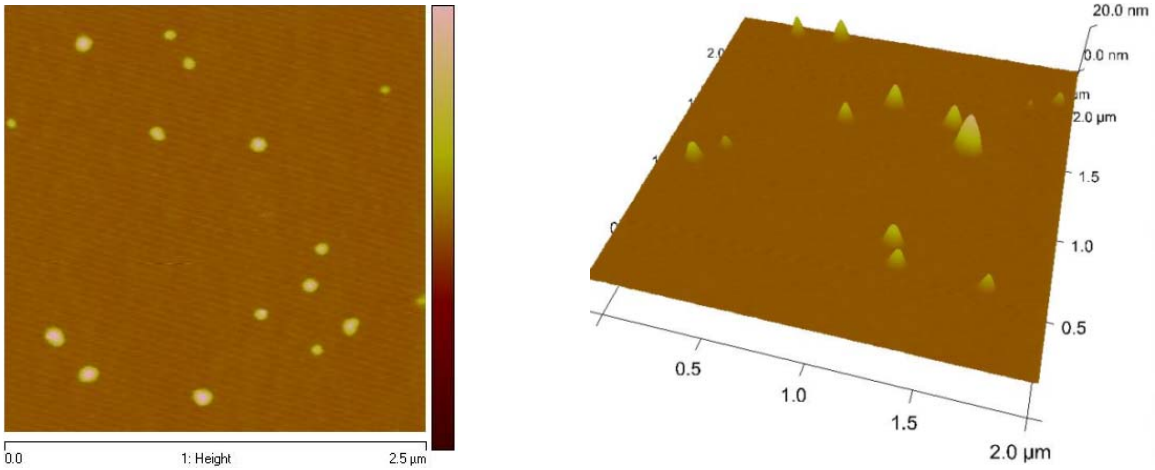


Figure S10. AFM image of stabilized polyplex.

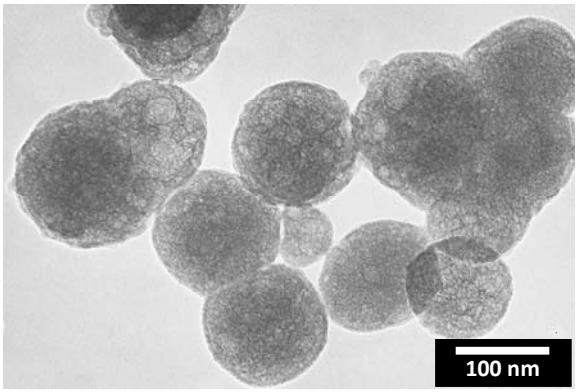


Figure S11. TEM image of stabilized polyplex.

5. Cytotoxicity Evaluation (MTT Assay).

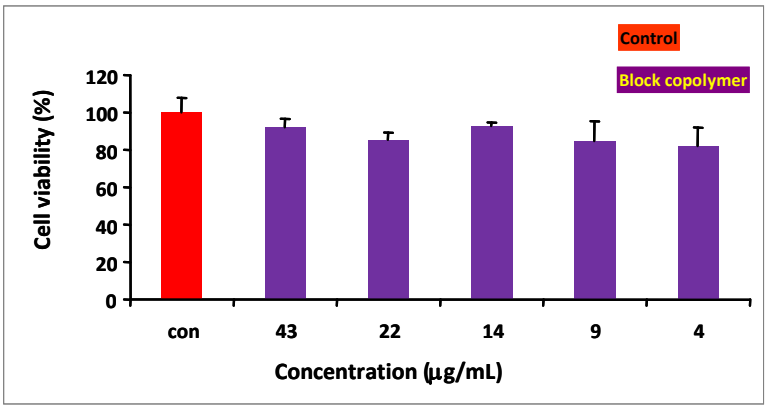


Figure S12. Viability of HeLa cells as a function of block copolymer (PNIPAM-*b*-PLLys) concentration determined by MTT assay after 24 h of incubation at 37°C (n=8, $p<0.05$).

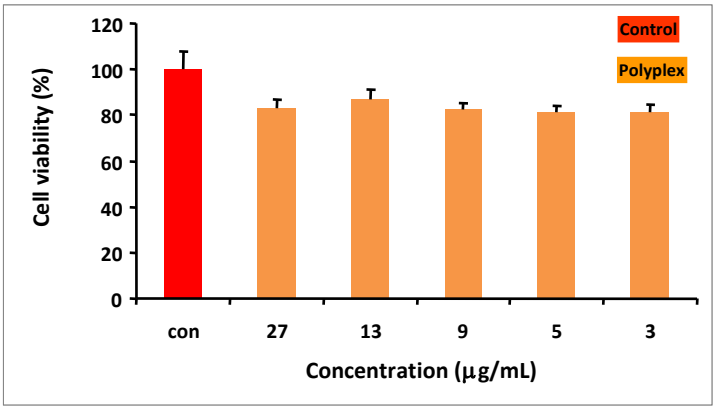


Figure S13. Viability of HeLa cells as a function of polyplex (N/P=5:1) concentration determined by MTT assay after 24 h of incubation at 37°C (n=8, $p<0.05$).

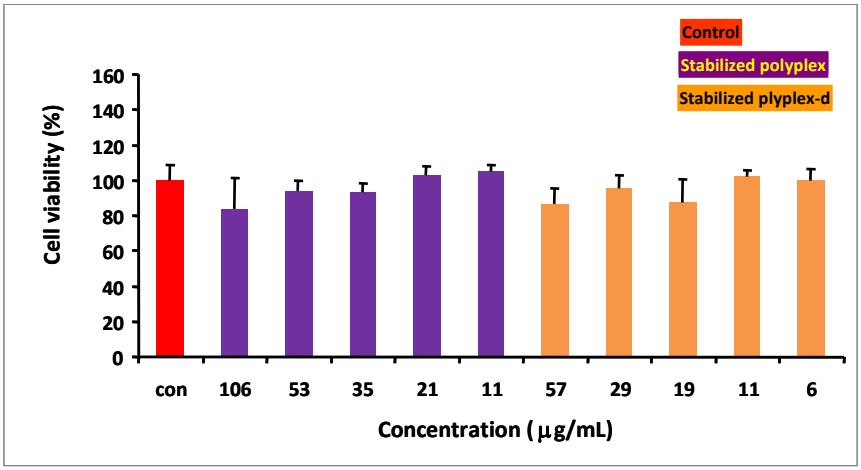


Figure S14. Viability of HeLa cells as a function of stabilized polyplex concentration before and after dialysis determined by MTT assay (24 h of incubation at 37°C, n=8, $p<0.05$).

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