Electronic Supplementary Material (ESI) for Journal of Materials Chemistry B.

SUPPORTING INFORMATION:

3rd Generation Poly(ethylene imine)s for Gene Delivery

Tanja Bus,[a,b,†] Christoph Englert,[a,b,†] Martin Reifarth,[a,b,c,d] Philipp Borchers,[a,b]
Matthias Hartlieb,[a,b,#] Antje Vollrath,[a,b] Stephanie Hoeppener,[a,b]
Anja Traeger,[a,b,] Ulrich S. Schubert[a,b,]**

[a] Laboratory of Organic and Macromolecular Chemistry (IOMC), Friedrich Schiller University Jena, Humboldtstrasse 10, 07743 Jena, Germany

[b] Jena Center for Soft Matter (JCSM), Friedrich Schiller University Jena, Philosophenweg 7, 07743 Jena, Germany

[c] Institute of Physical Chemistry and Abbe Center of Photonics, Friedrich Schiller University Jena, Helmholtzweg 4, 07743 Jena, Germany

[d] Leibniz Institute of Photonic Technology, Albert-Einstein-Strasse 9, 07745 Jena, Germany

[†] The authors contributed equally to this work

[#] Current address: Department of Chemistry, University of Warwick, Gibbet Hill Road, Coventry, CV4 7AL, UK.

Email: ulrich.schubert@uni-jena.de, anja.traeger@uni-jena.de

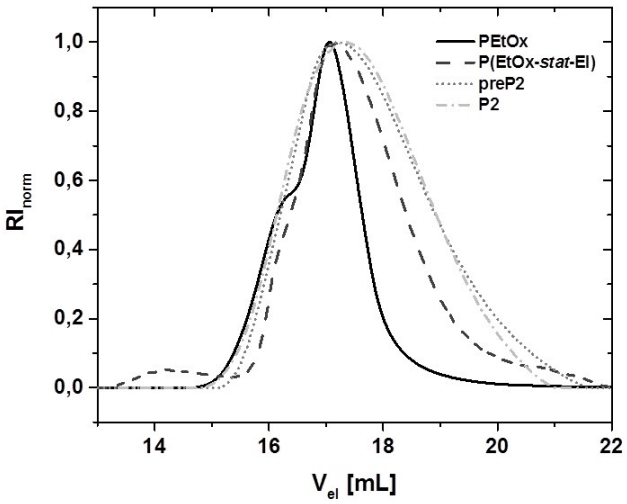

Figure S1. Size exclusion chromatography traces of the starting homopolymer **PEtOx** and the copolymers **P(EtOx-*stat*-EI)**, **preP2** and **P2** (*N*,*N*-dimethylacetamide, 0.21% LiCl, calibration: polystyrene).

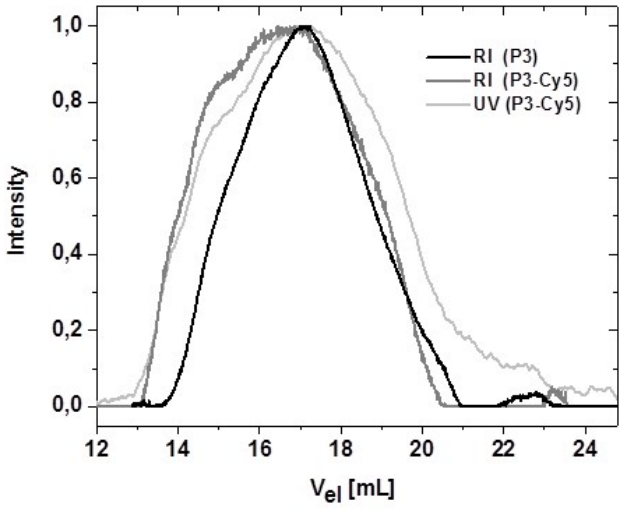

Figure S2. Size exclusion chromatography elugrams of the labeled copolymer **P3** (**P3-Cy5**) in comparison to the unlabeled starting material (**P3**) (*N*,*N*-dimethylacetamide, 0.21% LiCl, calibration: polystyrene).

Hemocompatibility of PEI-based polyplexes

The erythrocyte aggregation of the PEI copolymers was performed in parallel with high molar mass lPEI polymers as positive controls. **lPEI** show membrane-perturbing activity at high concentrations (100 µg mL^{-1}) leading to the aggregation of erythrocytes as indicated in the photospectrometrically measurement and by light microscopy. This effect was not seen with the copolymers **P1** to **P3**.

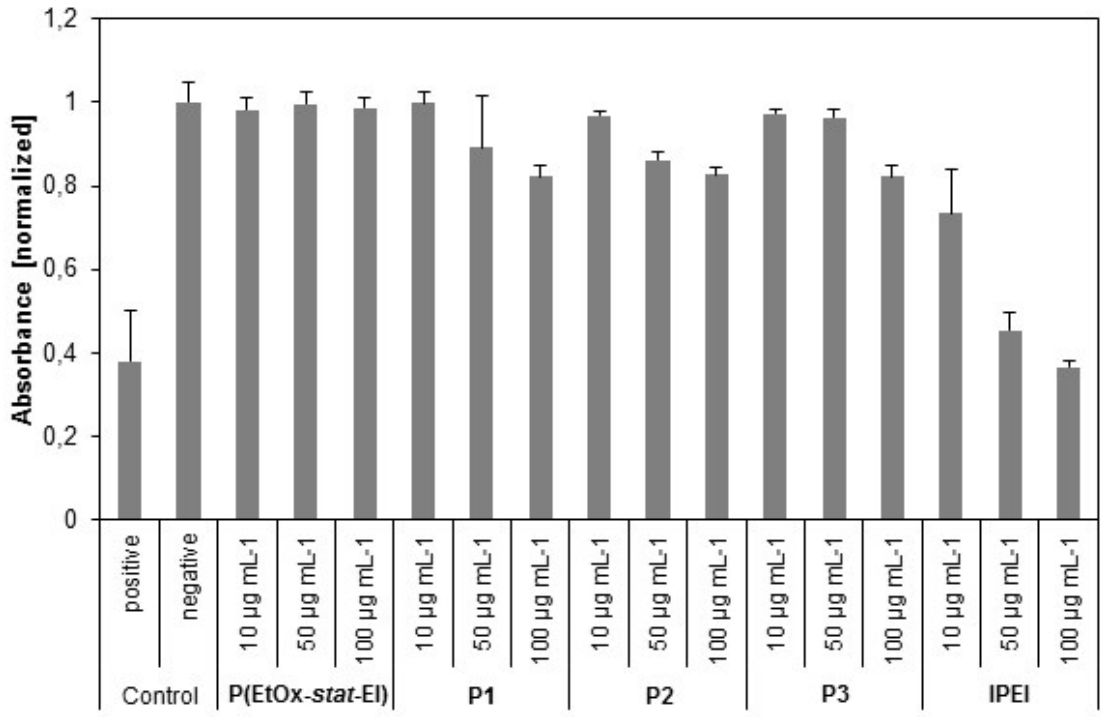

Figure S3. Erythrocyte aggregation of the tested polymers at indicated concentrations. bPEI (25 kDa) served as positive control resulting in high aggregation formation and PBS as negative control. Values represent the mean ± S.D. (n=3).

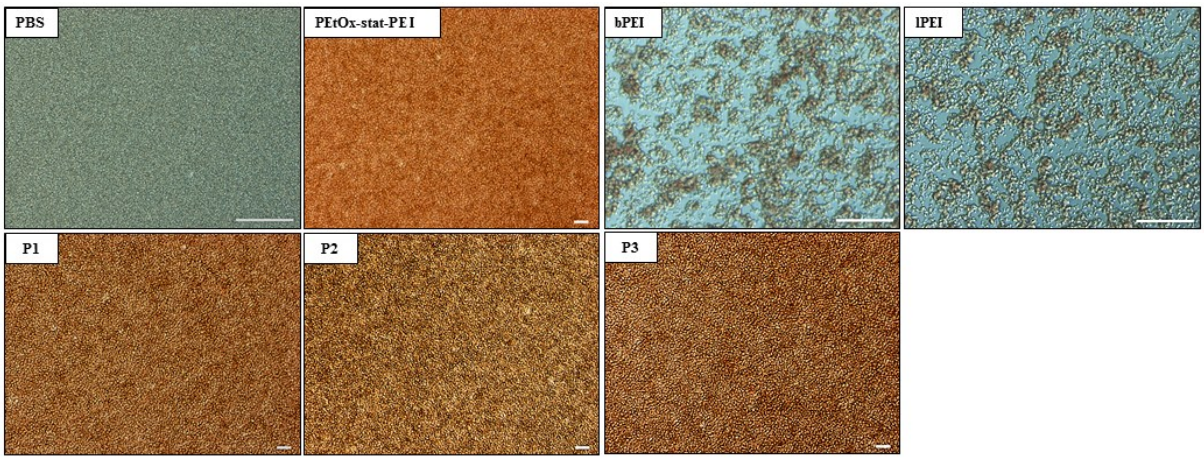

Figure S4. Light microscopy of erythrocyte aggregation of the polymers **P1** to **P3**, **PEtOx** and both **lPEI** polymers. PBS served as negative control, while bPEI (25kDa) was served as positive control. Scale bar = 50 µm.

Interaction of polymers with genetic material

The polyplex dissociation assay was performed aside from heparin with poly(methacrylic acid) (PMAA) (DP = 200) as competing factor. To keep equal conditions, same PMAA concentrations as for heparin were used during the measurement.

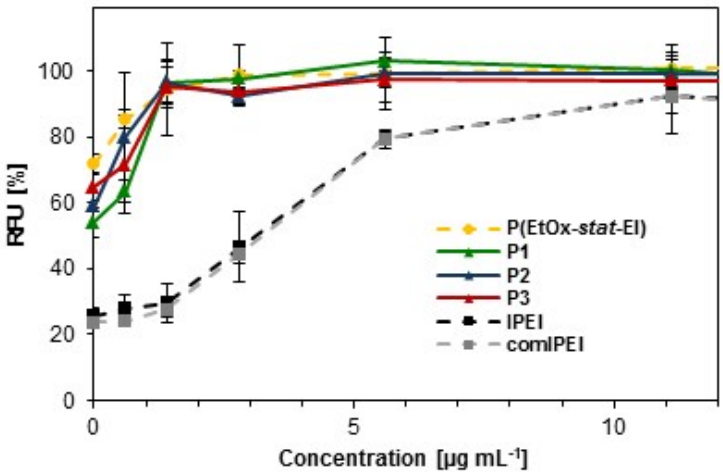

Figure S5. Dissociation assay of polyplexes formed with pDNA at N/P 30 and with increasing PMAA concentrations, which correlates to heparin concentrations.

Analysis of polyplex uptake and transfection of cells

The uptake and transfection studies were performed with HEK cells and pDNA encoding the EGFP (enhanced green fluorescence protein) or with YOYO-labeled pDNA. Transfection efficiency was determined by measuring the amount of viable cells (PI stained) expressing EGFP after 24 h *via* flow cytometry, whereas non-transfected cells served as negative control. To determine the amount of EGFP expressing cells, the histogramm of control cells was used and the percentage of cells within the gated area was defined as transfection efficiency in percentage.

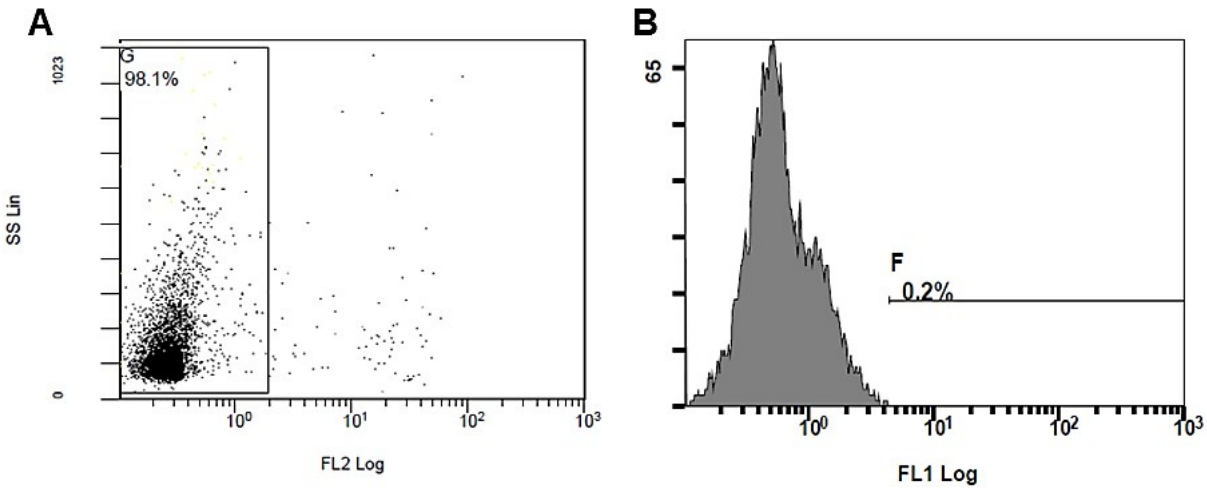

Figure S6. Flow cytometry measurements. A) Dot-plot of PI stained HEK cells for determining cell viability. FL2 Log represents red fluorescence of PI stained cells. All cells within the specified area G represent all measured viable cells. B) Histogramm of non-transfected cells served as control. FL1 Log represents green fluorescence by EGFP expression.

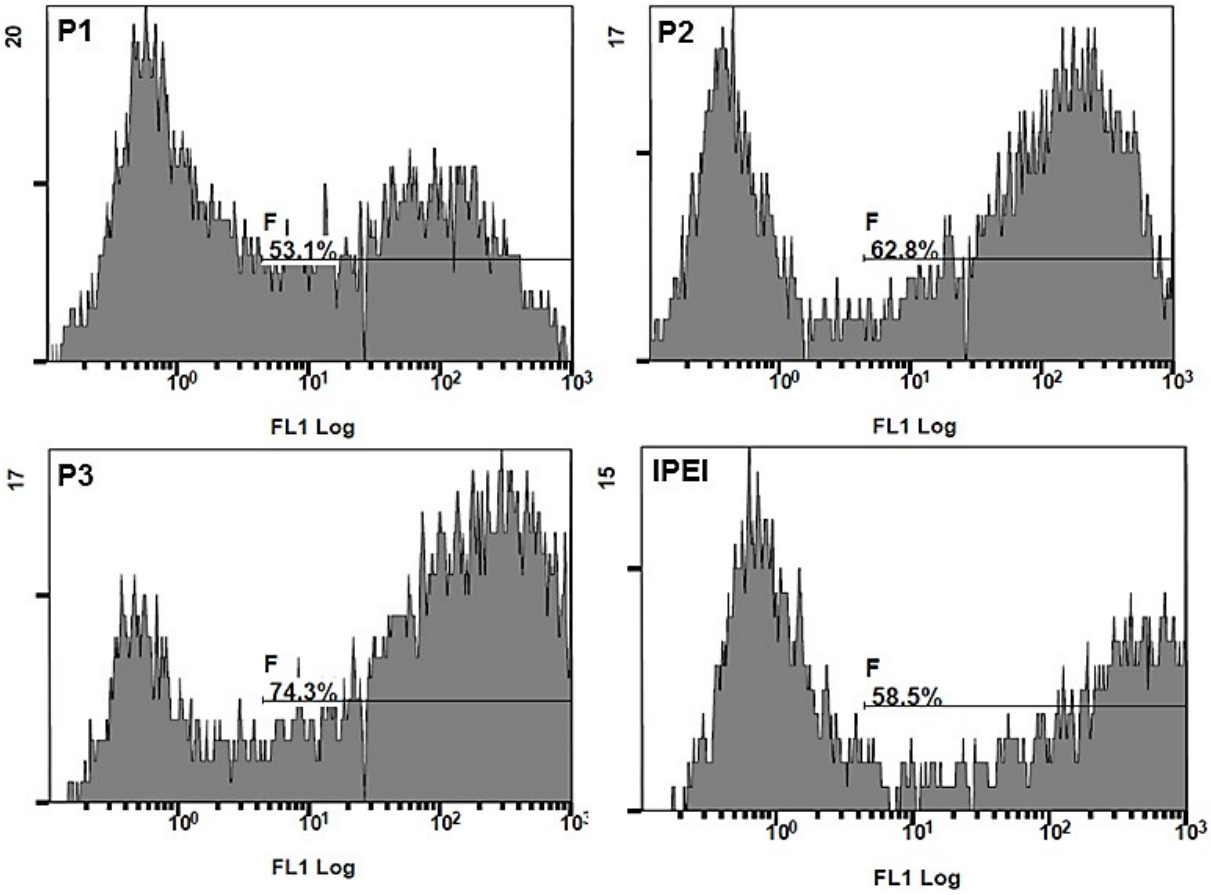

Figure S7. Histograms of flow cytometry measurements determining positive EGFP-expressing HEK cells after 24 h post-transfection with **P1** to **P3** and lPEI (N/P 30). Only viable HEK cells (PI staining) were gated. FL1 Log represents green fluorescence by EGFP expression.

To investigate the uptake mechanism in detail, cells were treated at different conditions with bafilomycin (proton pump inhibitor) or at 4 °C and 37 °C.

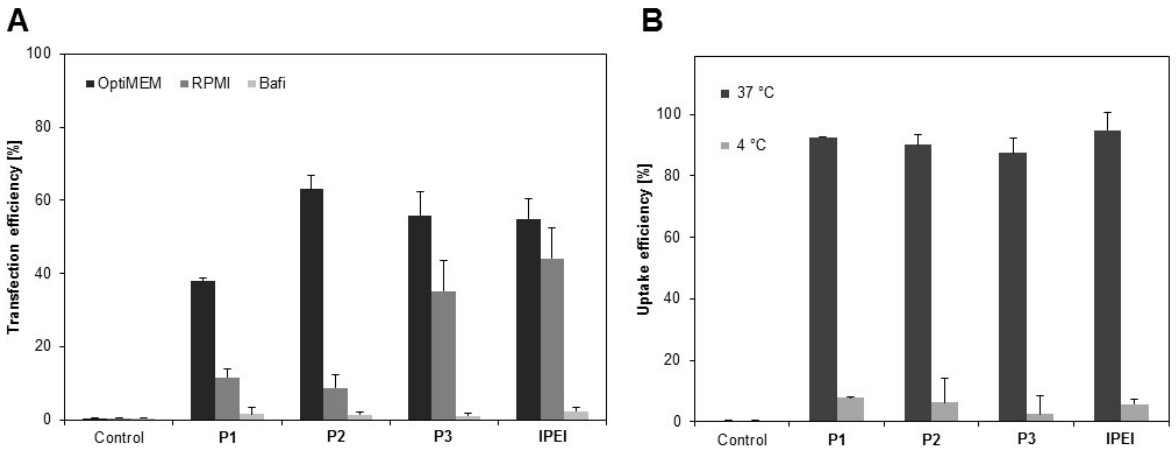

Figure S8. A) Uptake study: amount of cells taken up YOYO-1 labeled pDNA after 4 h at different temperatures (4 °C and 37 °C) using the copolymers **P1** to **P3** and **lPEI** (N/P ratio 30) as controls. Values represent the mean (n = 3). B) Comparison of the transfection efficiency of **P1** to **P3** and **lPEI** for adherent HEK cells in serum reduced (OptiMEM) and serum containing media (RPMI + 10% FCS) as well as after bafilomycin treatment at N/P 30. Values represent the mean (n = 3).

Live cell imaging

Confocal as well as structured illumination microscopy were used to investigate the uptake process of polyplexes in more detail and for visualization purposes. Therefore, non-treated control HEK cells as well as **P3** polyplexes added to HEK cells in serum reduced media were analyzed.

Control: pDNA transfection

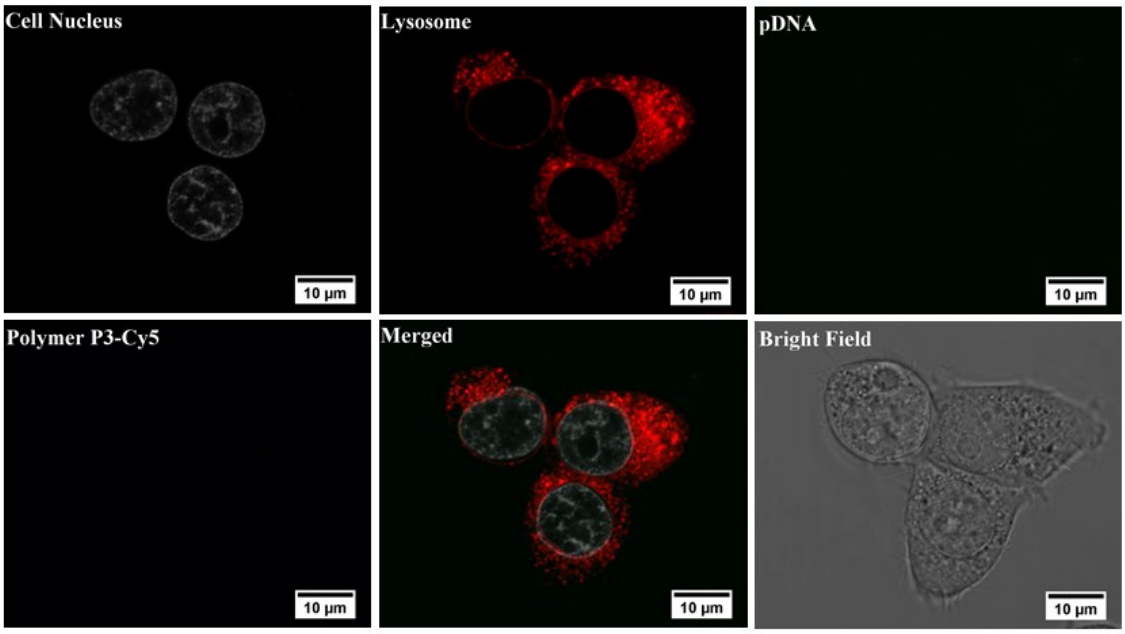

Figure S9. Uptake studies: HEK cells in serum reduced media without polyplexes served as negative controls. The cells were analyzed after 4 h *via* confocal laser scanning microscopy. The cell nucleus was stained with Hoechst 33342, the lysosomes with LysoTracker Red.

P3-Cy5: pDNA transfection

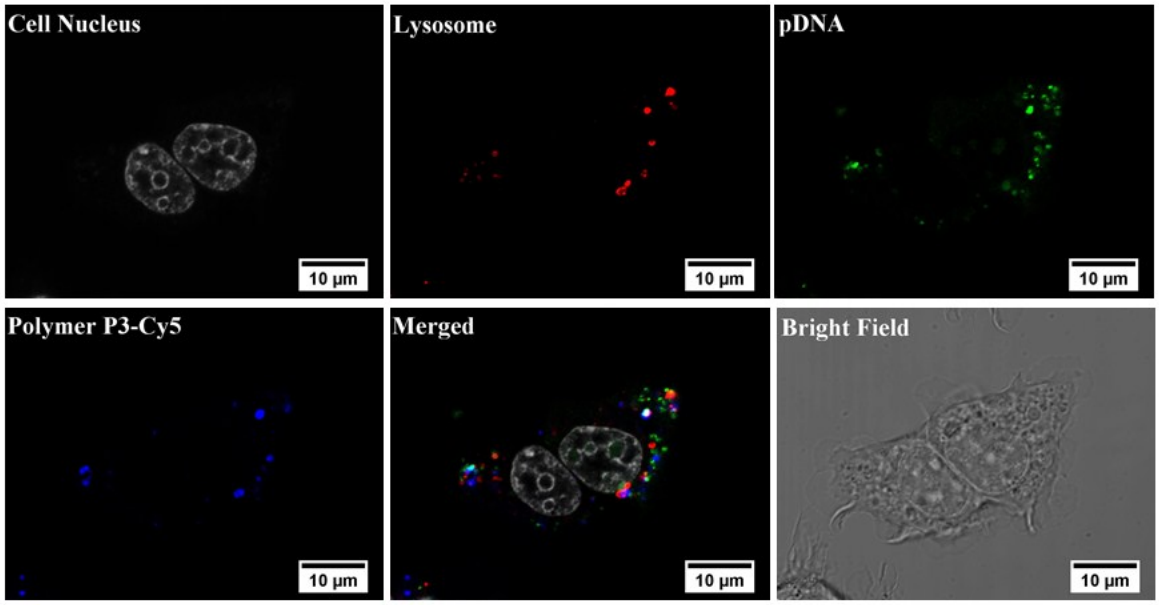

Figure S10. Uptake studies: Pure YOYO-labeled pDNA was added to HEK cells in serum reduced media. The cells were analyzed after 4 h *via* confocal laser scanning microscopy. The cell nucleus was stained with Hoechst 33342, the lysosomes with LysoTracker Red.

Structured illumination microscopy (SIM)

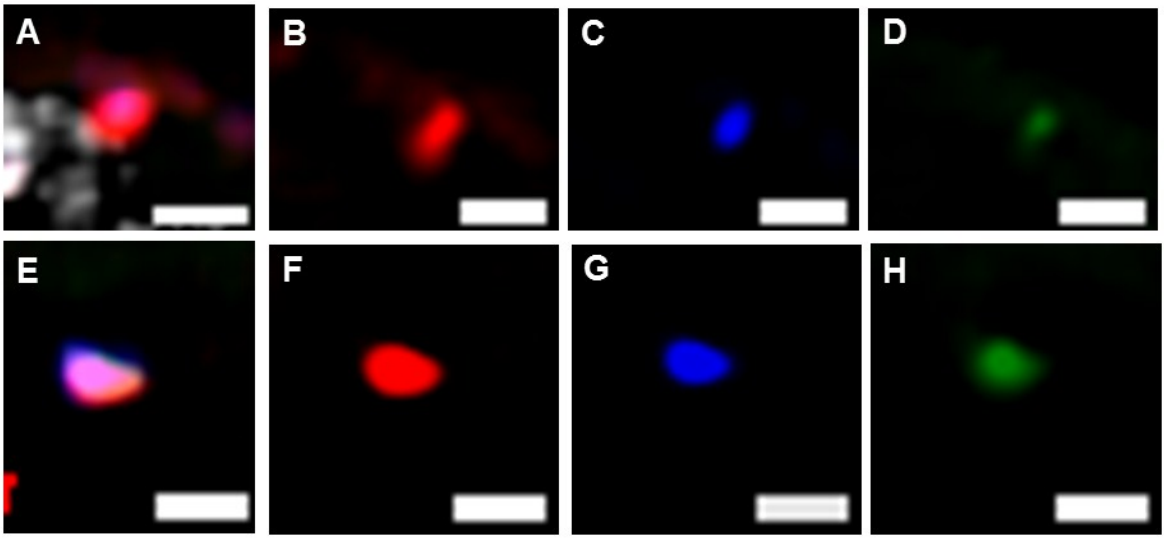

Figure S11. Magnified SIM images of endosome bearing polyplexes formed with **P3** in the presence of DNA (SIM data, deconvolved, acquired with 63x Oil Obj. 1.4 NA). Red; Lysosomal membrane (RFP). Green: pDNA labeling (YOYO-1). Blue: Polymer labeling (Cy5). A and E: Merged channels. B-D, F-H: split channels. Scale bars = 1 µm,

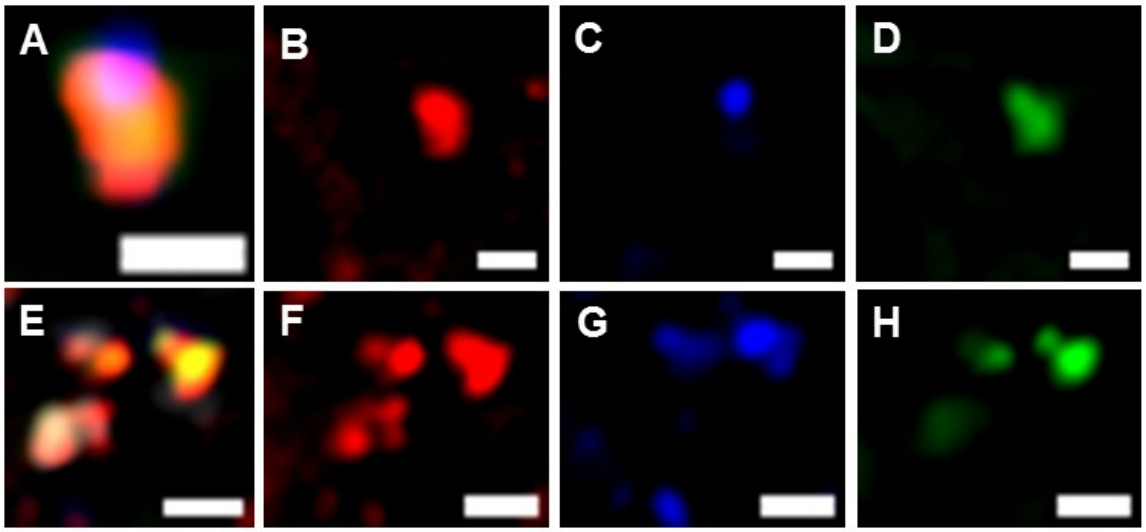

Figure S12. Magnified SIM images of endosome bearing polyplexes formed with **lPEI** in the presence of DNA (SIM data, deconvolved, acquired with 63x Oil Obj. 1.4 NA). Red; Lysosomal membrane (RFP). Green: pDNA labeling (YOYO-1). Blue: Polymer labeling (Cy5). A and E: Merged channels. B-D, F-H: split channels. Scale bars = 1 µm,

Scanning transmission electron microscopy (STEM)

To obtain deeper insights into the uptake mechanism and the fate of polyplexes inside the cell as well as the endosomal environment, scanning transmission electron microscopy (STEM) were performed. The images display a section (thickness of the resin slice: 80 nm) through the cell and sizes are determined by a two-dimensional section through the cell. This can only conditionally make a statement of the actual size of the three-dimensional vesicle. More than 5 sections (and ~ 10 vesicles) of different cells were analyzed to evaluate our findings.

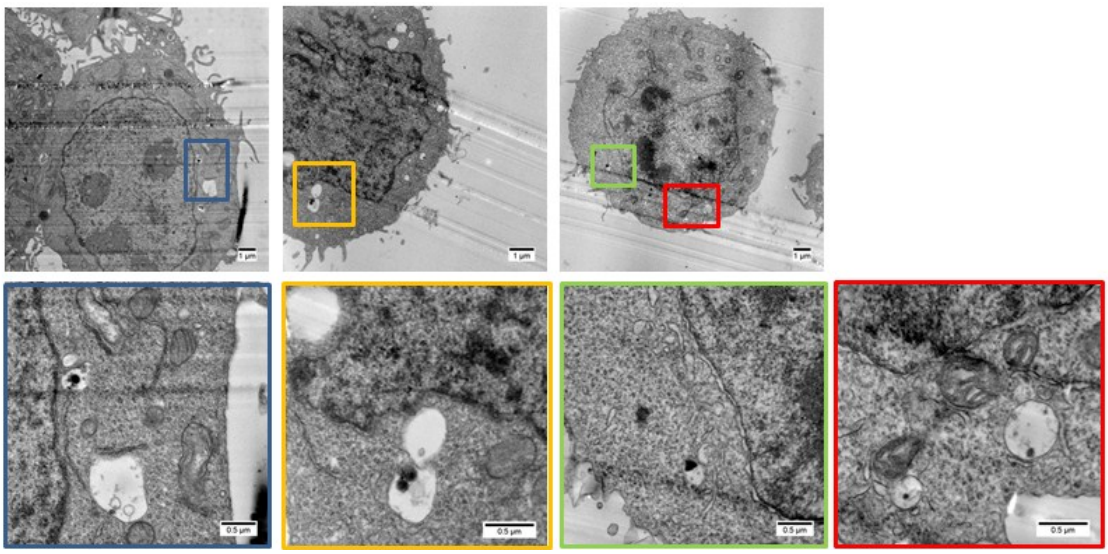

Figure S13. STEM images of polyplex uptake in HEK cells at standard conditions. Polyplexes were formed with **P3** and pDNA. Cells were harvested after 4 h.

Macropinocytosis inhibitor

For inhibition experiments, cells were treated with 100 µM 5-(*N*-ethyl-*N*-isopropyl)amiloride (EIPA) in standard culture media 30 min prior to polyplex addition. Subsequently, **P3** and lPEI polyplexes were added to the cells and incubated for further 4 h. Afterwards, the cells were harvested and analyzed as described above *via* flow cytometry or were further prepared for STEM imaging.

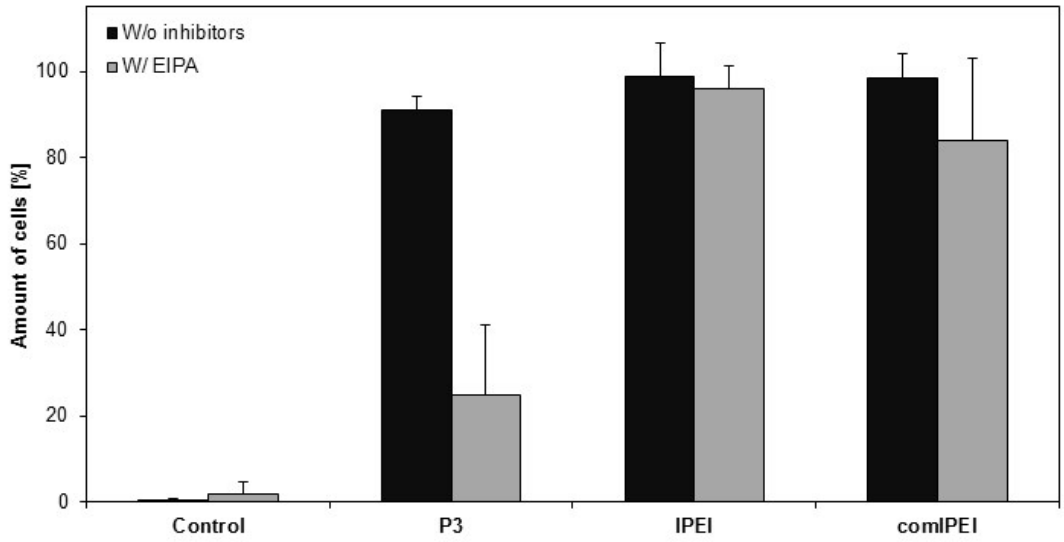

Figure S14. Polyplex uptake (YOYO-labeled pDNA) in HEK cells after treatment with EIPA (macropinocytosis inhibitor).

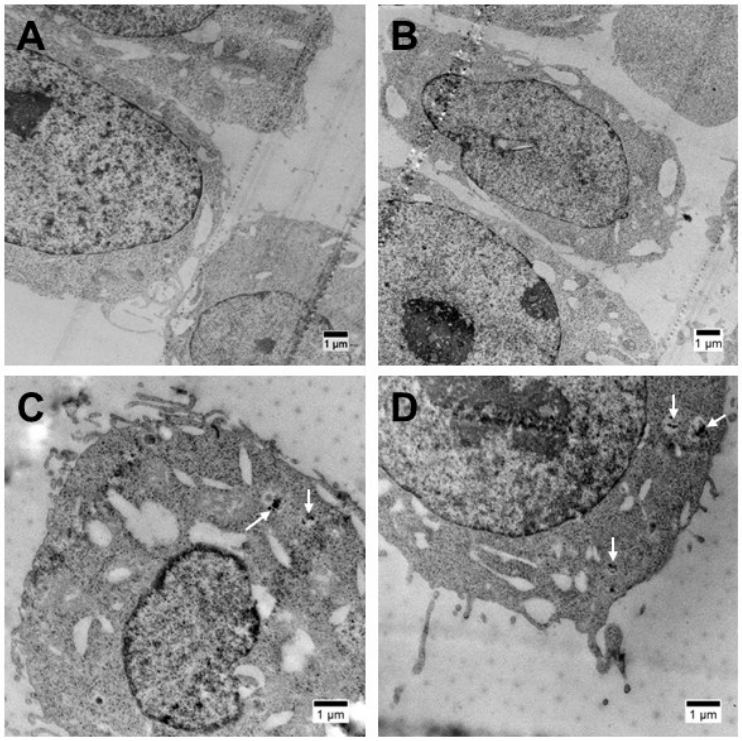

Figure S15. STEM images of polyplex uptake in HEK cells after treatment with EIPA (macropinocytosis inhibitor). A-B) Uptake of **P3** polyplexes. C-D) Uptake of **lPEI** polyplexes. White arrows indicate vesicles with polyplexes.

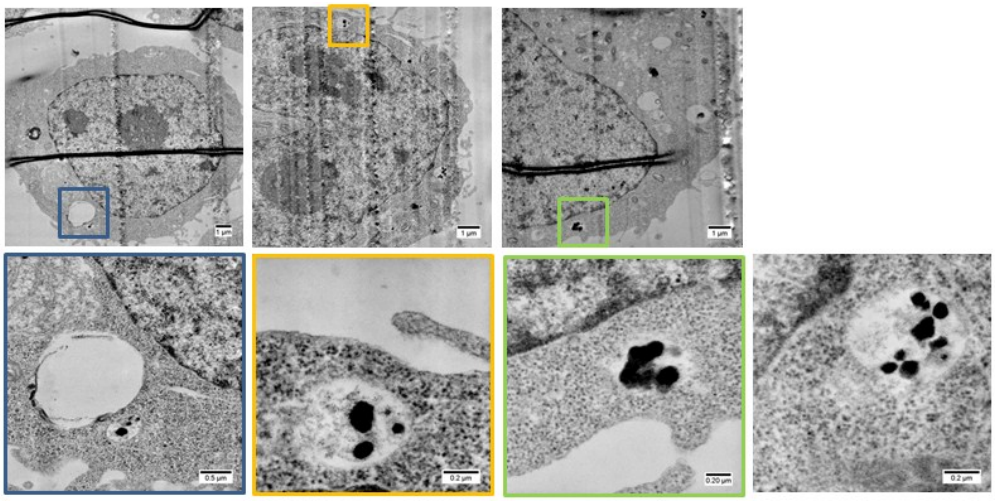

Figure S16. STEM images polyplex uptake in HEK cells at standard conditions. Polyplexes were formed with **lPEI** and pDNA. Cells were harvested after 4 h.

siRNA delivery

A stable GFP-expressing CHO cell line was transfected with the polymers **P1** to **P3** as well as PEI using scrambled siRNA as negative control. The knockdown of EGFP was analyzed *via* flow cytometry by measuring the MFI of all viable cells (PI staining).

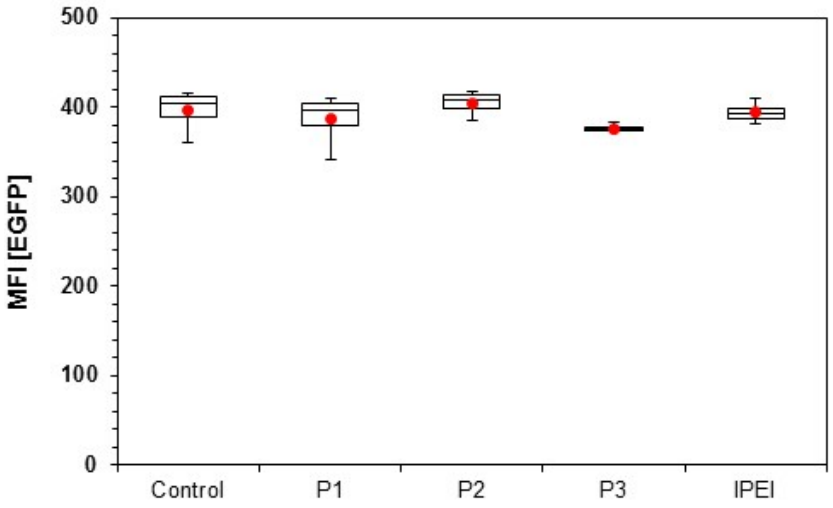

Figure S17. siRNA transfection efficiency mediated by **P1** to **P3** as well as PEI polyplexes at N/P 30 after 72 h. Stable EGFP-expressing CHO cells were transfected with scrambled siRNA served as negative control. The values represent the mean ± S.D., $n \geq 3$.

Comparison of lPEI and commercial lPEI25k (comlPEI, Sigma Aldrich)

The cytotoxicity tests of the PEI copolymers were performed in parallel with high molar mass lPEI polymers as positive controls. Fully hydrolyzed PEtOx, thus lPEI as well as the commercially available lPEI (25 kDa, **comlPEI**) obtained from Polysciences were used. The synthesized lPEI shows a higher cytotoxicity (IC_{50} at ~ 4 µg mL^{-1}), whereas the commercial PEI reaches 50% cell viability at 25 µg mL^{-1}. The reduced cytotoxicity could be attributed to residual, N-acyl groups from polymerization, which is also stated by the supplier.[39] Furthermore, the hemolysis and the erythrocyte aggregation assay were performed with both PEIs. Both polymers show membrane-perturbing activity at high concentrations (100 µg mL^{-1}) leading to hemoglobin release and the aggregation of erythrocytes. This effect was not seen with the copolymers **P1** to **P3**.

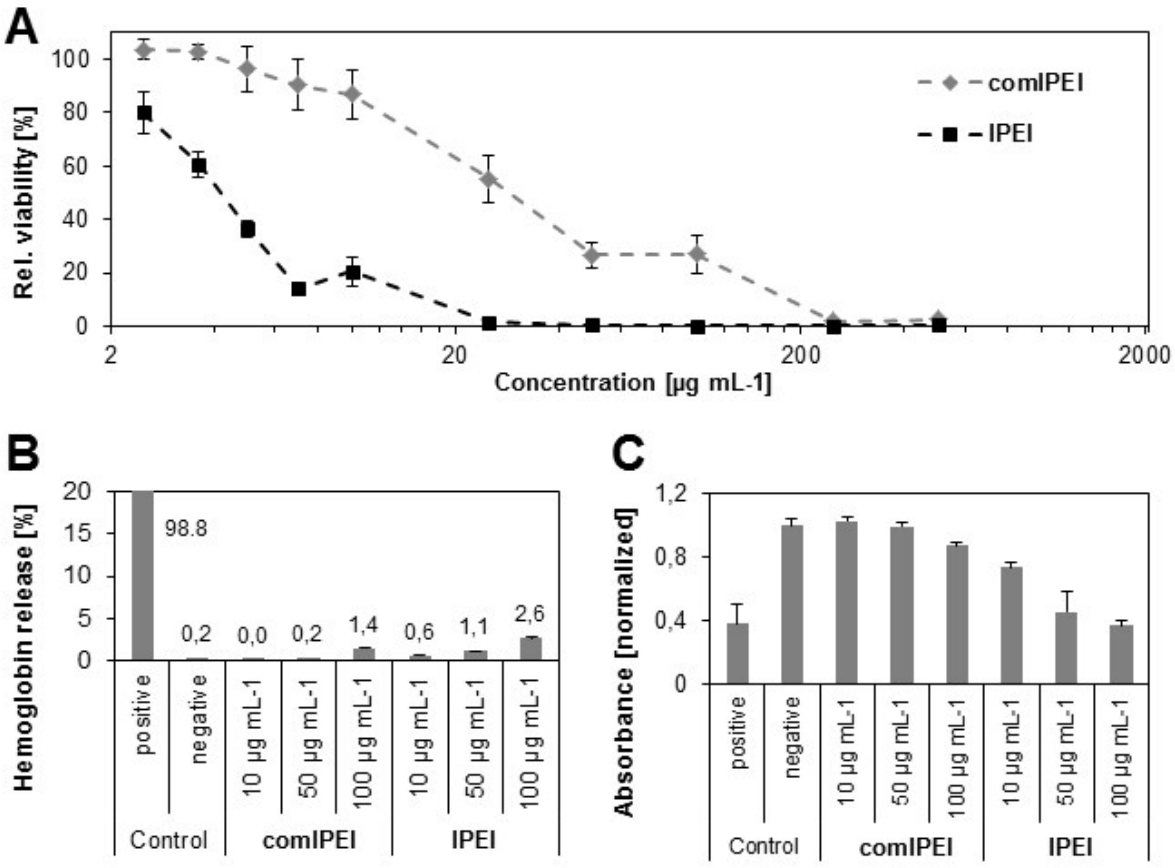

Figure S18. Comparison of **lPEI** and commercially available PEI (**comlPEI**, Polysciences). A) Cytotoxicity assay treating L929 cells with the synthesized **lPEI** as well as **comlPEI** at indicated concentrations. B) Hemolysis assay of erythrocytes after incubation with polymers at indicated concentrations. Triton X-100 served as positive control (100% hemolysis) and PBS as negative control. C) Erythrocyte aggregation of the tested polymers at indicated concentrations. bPEI (25 kDa) served as negative control resulting in high aggregation formation and PBS as negative control. Values represent the mean ± S.D. (n=3).

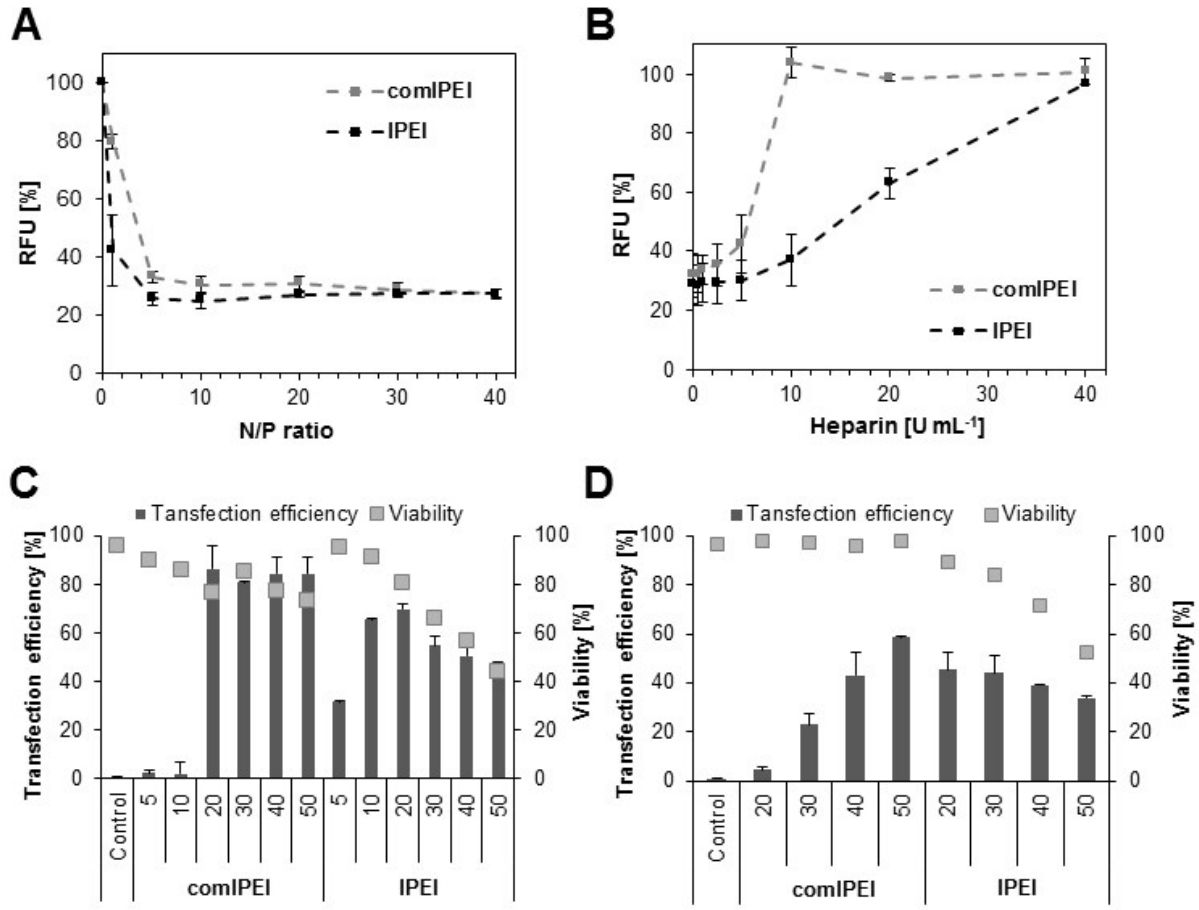

Figure S19. Comparison of **lPEI** and commercially available PEI (**comlPEI**, Polysciences). A) Complexation affinity (EBA) of mentioned polymers using pDNA at the indicated N/P ratios. B) Dissociation assay with heparin of polyplexes formed with pDNA at N/P 30. C-D) Transfection efficiency of both PEI polymers for adherent HEK cells in serum reduced (C) as well as serum containing (D) media at different N/P ratios after 24 h. Values represent the mean ± S.D. (n=3).

Besides the synthesized lPEI, comlPEI was used as control for the ethidium bromide quenching assay (Figure S18A). Both polymers show a high complexation affinity with pDNA, while a faster polyplex formation of lPEI could be detected at N/P 5. Regarding the heparin dissociation assay, comlPEI achieved a full decomplexation of genetic material at a heparin concentration of 10 U mL^{-1} (Figure S19B). For the complete release of pDNA (100% RFU) from lPEI polyplexes, 40 U mL^{-1} heparin was required. The uptake and transfection studies were

performed with HEK cells and pDNA encoding the EGFP (enhanced green fluorescence protein). Transfection efficiency was determined by measuring the amount of cells expressing EGFP after 24 h *via* flow cytometry. ComlPEI shows high TE > 80% at N/P ratios of 20 to 50 in serum reduced media, which is comparable to other studies. It has to be mentioned that with increasing N/P ratio, i.e. the polyplex concentration, the cell viability is reduced. This effect could be prevented using serum containing media for transfection, whereas a significant reduction of up to 70% (at N/P 20) of TE is occured.

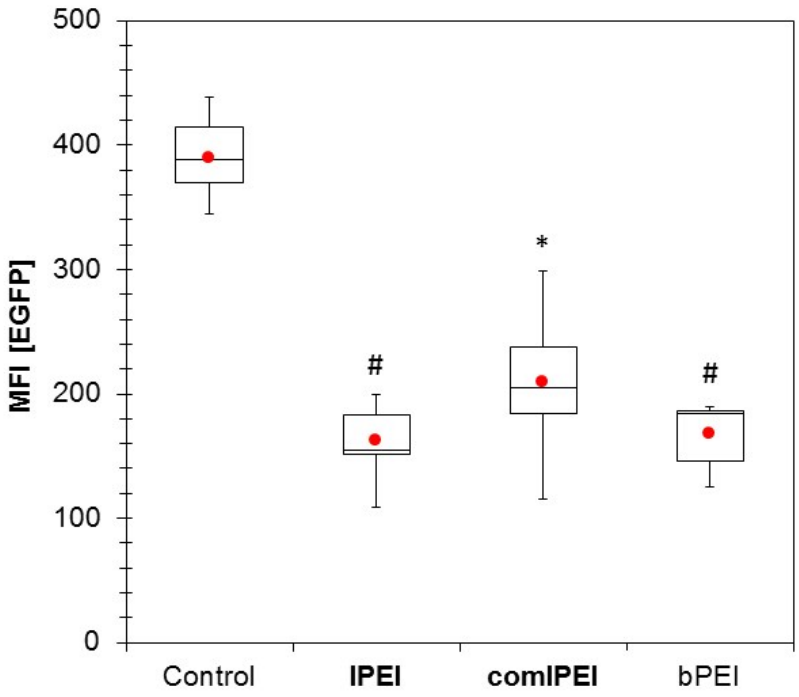

Figure S20. siRNA transfection efficiency mediated by PEI polyplexes at N/P 30 after 72 h. Stable EGFP-expressing CHO cells were transfected with polyplexes formed using siRNA able to knock down *egfp*. Statistical analysis (t-test) was used to compare the MFI of the control with PEI, * represents $p < 0.05$ and # $p < 0.005$. The values represent the mean ± S.D., n ≥