Electronic Supplementary Material (ESI) for Biomaterials Science.

Detailed Investigation on How the Protein Corona Modulates the Physicochemical Properties and Gene Delivery of Polyethylenimine (PEI) Polyplexes

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I) Reagents

4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES; #H3375), bovine serum albumin (BSA, # V900933), albumin–fluorescein isothiocyanate conjugate (FITC-BSA, #A9771), branched polyethylenimine with a molecular weight of 25 kDa (PEI, 25 kDa, #408727), tetramethylrhodamine isothiocyanate (#T3163), fluorescein isothiocyanate (#F7250), monensin sodium hydrate (#46468), nigericin sodium salt (#N7143), dextran-tetramethylrhodamine (dextran-TRITC, M_w = 155 kDa, #T1287) were purchased from Sigma-Aldrich. Chlorpromazine hydrochloride (#sc-202537), genistein (#sc-3515), wortmannin (#sc-3505), and cytochalasin D (#sc-201442) were purchased from Santa Cruz Biotechnology®. SNARF®-1 carboxylic acid, acetate, succinimidyl ester (#S-22801), CellLight® Early Endosomes-GFP, BacMam 2.0 (#C10586), CellLight® Tubulin-GFP, and BacMam 2.0 (#C10613) were purchased from Thermo Fisher Scientific. Dextran-TRITC (M_w=10 kDa, #D1816) was obtained from Invitrogen. pGL4.13 luciferase plasmid and the luciferase assay system were purchased from Promega (Madison, WI). Green fluorescent protein (eGFP)-encoding plasmid (pEGFP-N1) was kindly provided by Zhejiang University School of Medicine. The plasmids were propagated in Escherichia coli DH5α and extracted using the Endo-Free Plasmid Kit (Qiagen, Hilden, Germany). LabelIT® Tracer™ Intracellular Nucleic Acid Localization Kit, Cy5™5 (#MIR 7021) was purchased from Mirus Bio. All chemicals were used as received. Milli-Q water with a resistance greater than 18.2 MΩ cm $^{-1}$ was used for all experiments.

II) Synthesis of Materials

II.1) Fluorescence-Labeling of DNA and PEI

eGFP-encoding plasmids were labelled with Cy5 according to the manufacturer's manual (Mirus Bio) with slight modification. Briefly, 10 µL of Label IT® Tracker™ was reacted with 50 µg of DNA for 3 h at 37°C.

For PEI labelling with SNARF-1, PEI (1.4 mg, 31.82 µmol) was dissolved in 250 µL of 0.1 M $NaHCO_3$ buffer. Subsequently SNARF-1 succinimidyl ester (200 µg, 0.34 µmol) dissolved in 50 µL of dimethyl sulfoxide (DMSO) was added and the mixture was incubated overnight in the dark. SNARF-1 was colorless in DMSO but showed deep purple in $NaHCO_3$ buffer. Unreacted dye was removed by using PD-10 desalting columns (Cat. No.: 17-0851-01, GE Healthcare) eluted with milli-Q water. The amount of PEI was estimated via the copper(II) polyplexation method.[1] The amount of attached dye was calculated according to Beer–Lambert's law, using the molar absorbance coefficient ε of SNARF-1 at pH> 10 of 45,000 $M^{-1}cm^{-1}$. The calculated labeling density of SNARF-labelled PEI was 0.118 µmol SNARF/mg PEI. Note that actual PEI concentration is underestimated by this method because it is based on the determination of free amino groups, and thus the amino groups used to conjugate SNARF were not counted.

II.2) Polyplex Formulation

Stock solution of PEI was prepared in 10 mM HEPES-NaOH buffer (pH 7.4) at a mass concentration of C_{PEI} = 38.4 µg/mL. Stock solution of plasmid DNA (including luciferase and eGFP-encoding plasmids) was prepared at a mass concentration of C_{DNA} = 40 µg/mL in 10 mM

HEPES-NaOH buffer (pH 7.4). PEI/DNA polyplexes were formulated by adding PEI solutions into an equal volume of DNA solution at desired amino to phosphate (N/P) ratio and the mixture was immediately vortexed for 5 seconds. Polyplexes were then incubated for 20 min at room temperature before use. The N/P ratios were calculated according to the following formula:

$$N/P = c_N/c_P = \frac{C_{PEI}/44}{\frac{9.9\%}{31} \times C_{DNA}}$$

c_N refers to molar concentration of nitrogen (originating from the amino-groups of PEI). c_P refers to phosphate molar concentration of DNA. C_{PEI} refers to the mass concentration of PEI. C_{DNA} refers to the mass concentration of DNA. The molecular weight of the PEI repeat unit is 44 Da (= g/mol), and thus c_N is calculated as $C_{PEI}/44$ Da. The average mass concentration of phosphate in DNA is about 9.9% and its molecular weight is 31 Da. Therefore, c_p is calculated as $\frac{9.9\%}{31} \times C_{DNA}$. According to the formula a PEI/DNA mass ration of 1/1, as used in most of this work, corresponds to a N/P ratio of ≈ 7.

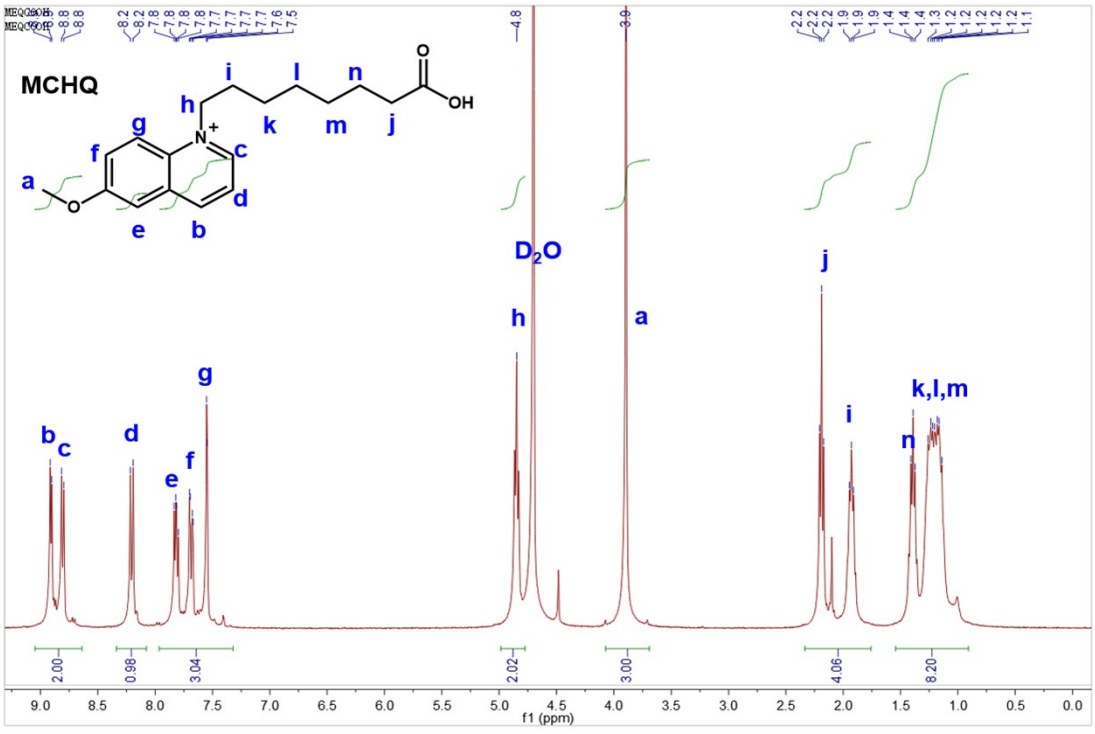
For PEI/DNA coating with BSA or fetal bovine serum (FBS), a protein stock solution was prepared in 10 mM HEPES-NaOH buffer (pH 7.4). One volume of protein solution of appropriate concentration was added into two volumes of PEI/DNA polyplex solution. The solution was immediately vortexed for 5 s. The PEI/DNA/protein polyplexes were further incubated for 20 min before use. We refer to the polyplexes as $P_{X/Y/Z}$ according to their PEI/DNA/protein mass ratios X/Y/Z.

II.3) Synthesis of 6-Methoxy-(7-(N-succinimidyl)-oxycarbonyl-heptyl)-quinolinium bromide

6-Methoxy-(7-(N-succinimidyl)-oxycarbonyl-heptyl)-quinolinium bromide (MCHQ-OSu) was synthesized by a two-step method according to the literature.[2] 6-Methoxyquinoline (4.78 g, 30 mmol) was mixed with 8-bromooctanoic acid (6.69 g, 30 mmol) in a 100 mL round bottomed flask. The mixtures were heated at 110 ℃ for 14 h. After cooling to room temperature (RT),

acetone (150 mL) was added. The black solid was sonicated for 2-3 h and stirred for another 16 h to get a white precipitate and a black supernatant. The solid was collected by filtration, and washed twice with acetone, twice with diethyl ether. The product 6-methoxy-N-(7-carboxyhexyl)-quinolinium bromide (MCHQ) was obtained after drying in vacuo to obtain a light brown solid (9.02 g, 78.6% yield). The compound was characterized with nuclear magnetic resonance: ^{1}H NMR (400 MHz, D_2O, δ): 8.9 (dd, 2H), 8.2 (d, H), 8.0–7.3 (m, 3H), 4.9 (s, 2H), 3. (s, 3H), 2.1 (dt, 4H), 1.5–0.9 (m, 8H). The ^{1}H NMR spectrum and peak assignments are shown in **Figure S1**. ^{13}C NMR (101 MHz, D_2O, δ):179, 159, 146, 145, 132, 128, 122, 120, 108, 58, 56, 34, 29, 28, 25, 24. The ^{13}C NMR spectrum and peak assignments are shown in **Figure S2**.

Figure S1. The ^{1}H NMR spectrum and peak assignments of MCHQ in D_2O.



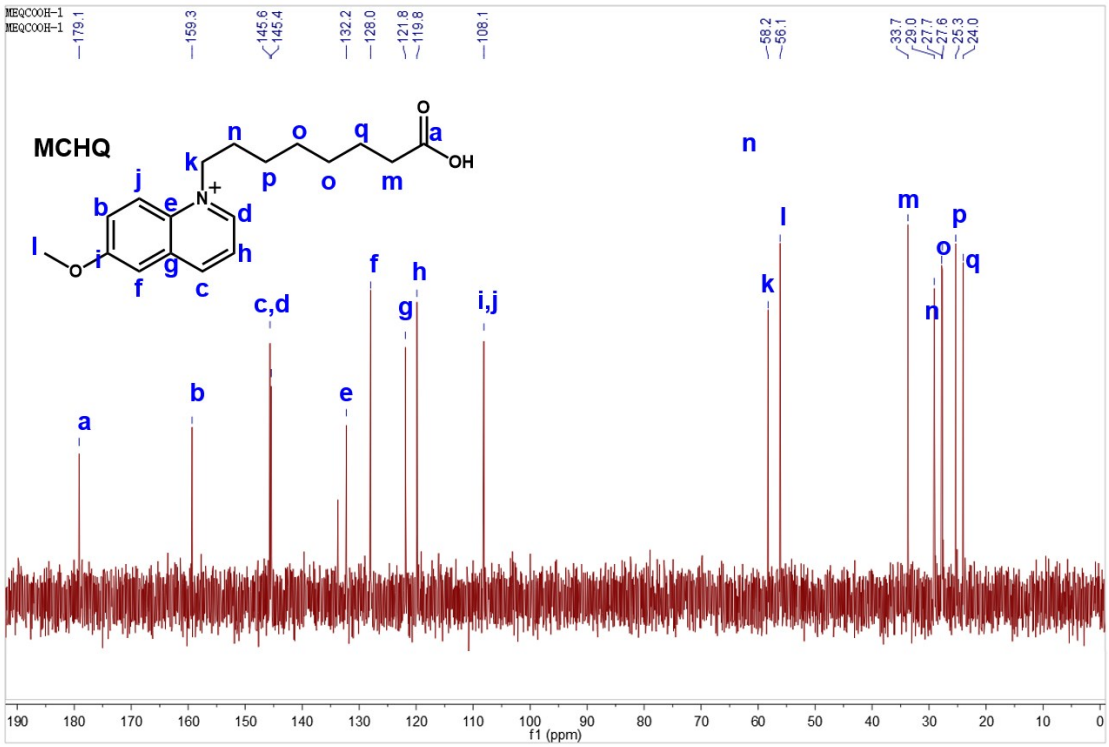


Figure S2. The ^{13}C NMR spectrum and peak assignments of MCHQ in D_2O.

MCHQ (2.29 g, 6 mmol), N,N-dicyclohexylcarbodiimide (3.09 g, 15 mmol) and N-hydroxysuccinimide (1.73 g, 15 mmol) were dissolved in dimethylformamide (DMF, 80 mL) and stirred for 19 at RT in the dark. Afterwards, the precipitate containing the by-product dicyclohexylurea was removed by filtration and the solvent was evaporated in vacuo. Acetonitrile (25 mL) was added and the mixture was further stirred for 1.5 h. The precipitate containing unreacted N,N-dicyclohexylcarbodiimid and N-hydroxysuccinimide was again removed by filtration and the solvent was concentrated in vacuo to 15 mL. The mixture was added dropwise to a strongly stirred solution of ethyl acetate/hexane (500 mL, 3:1 v/v volume ratio). The viscous precipitate was re-dissolved in acetonitrile (15 mL) and precipitated again in ethyl acetate/hexane (500 mL, 3:1 v/v). The viscous precipitate was dried in vacuo and acetone (30 mL) was added. The solid was obtained by filtration and washed twice with acetone and twice with diethyl ether. The product MCHQ-OSu was obtained after drying in vacuo to obtain a

yellowish solid (2.40 g, 83%).[1]H NMR (400 MHz, DMSO, δ): 9.4 (d, 1H), 9.1 (d, 1H), 8.6 (d, 1H), 8.1 (dd, 1H), 8.0–7.7 (m, 2H), 5.0 (t, 2H), 4.0 (s, 3H), 2.9– 2.6 (m, 6H), 2.0 (s, 2H), 1.5 (d, 9H). The [1]H NMR spectrum and peak assignments are shown in **Figure S3**. [13]C NMR (101 MHz, DMSO, δ): 170, 169, 159, 147, 145, 132, 128, 123, 121, 108, 57, 56, 30, 29, 28, 25, 24. The [13]C NMR spectrum and peak assignments are shown in **Figure S4**.

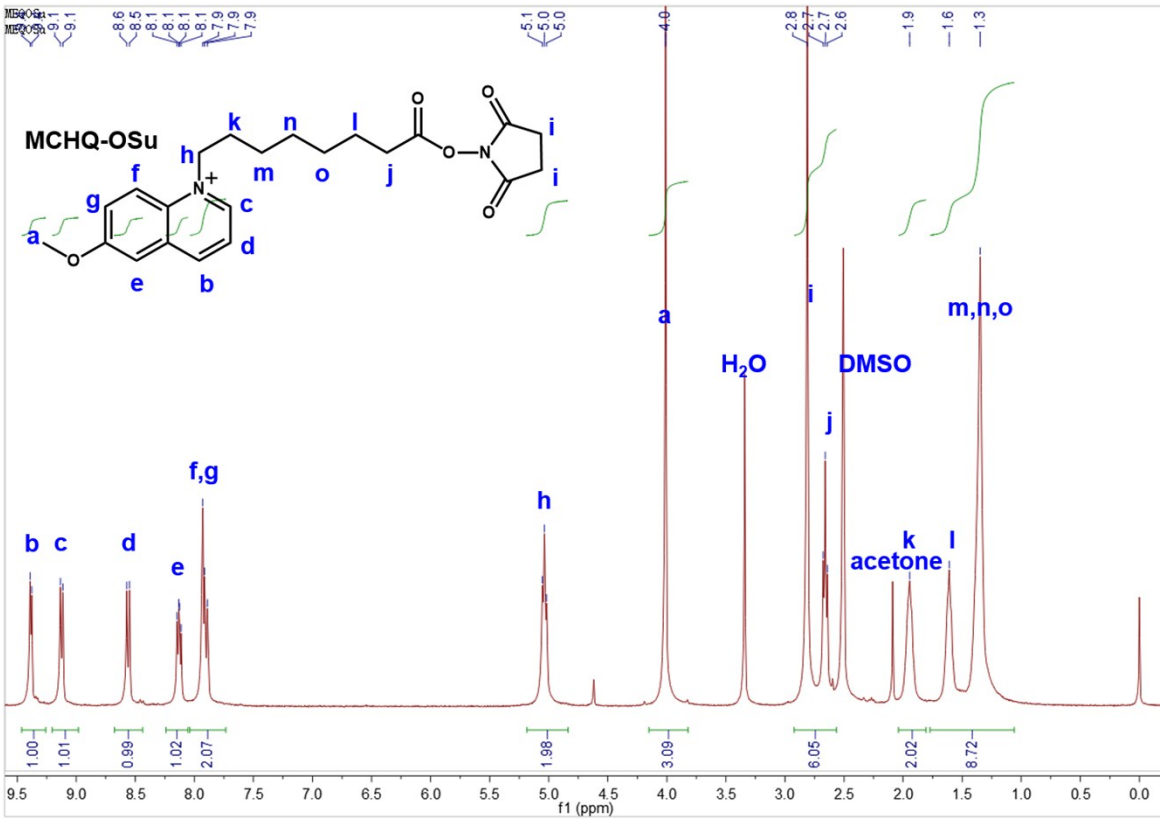


Figure S3. The [1]H NMR spectrum and peak assignments of MCHQ-OSu in δ-dimethyl sulfoxide DMSO.

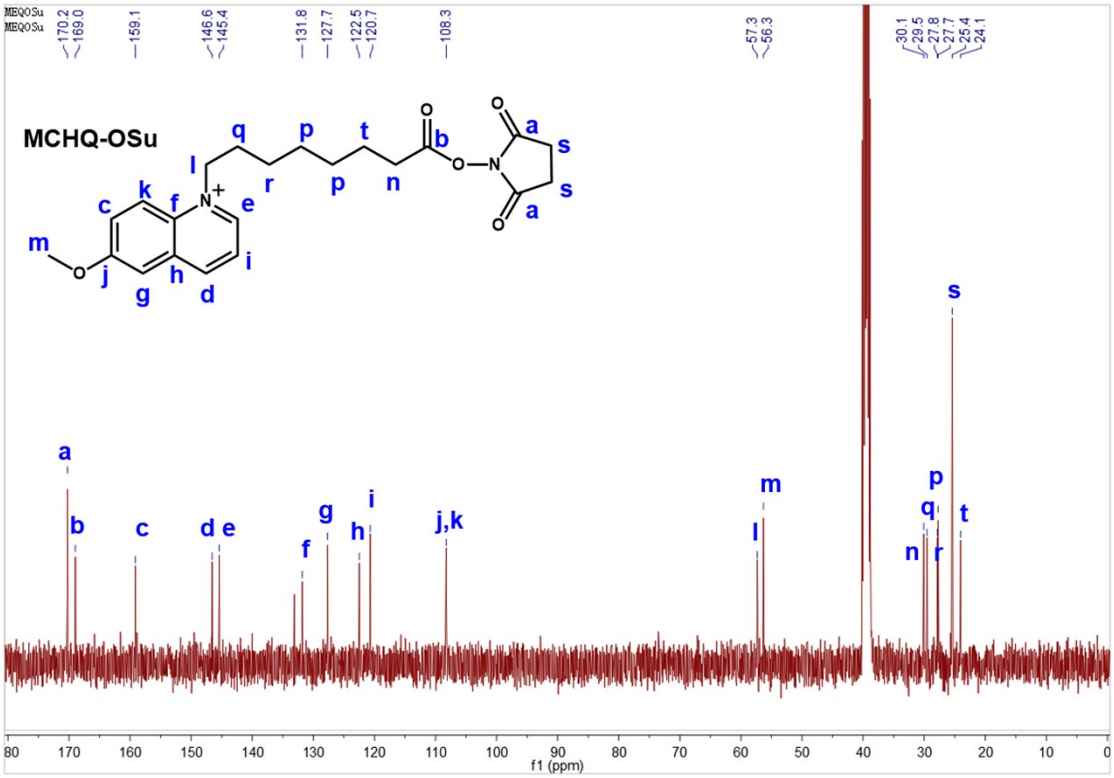


Figure S4. The ^{13}C NMR spectrum and peak assignments of MCHQ-OSu in δ-DMSO.

Absorption $A_{MCHQ}(\lambda)$ and fluorescence excitation and emission $I_{MCHQ}(\lambda)$ spectra of MCHQ are shown in **Figure S5**.

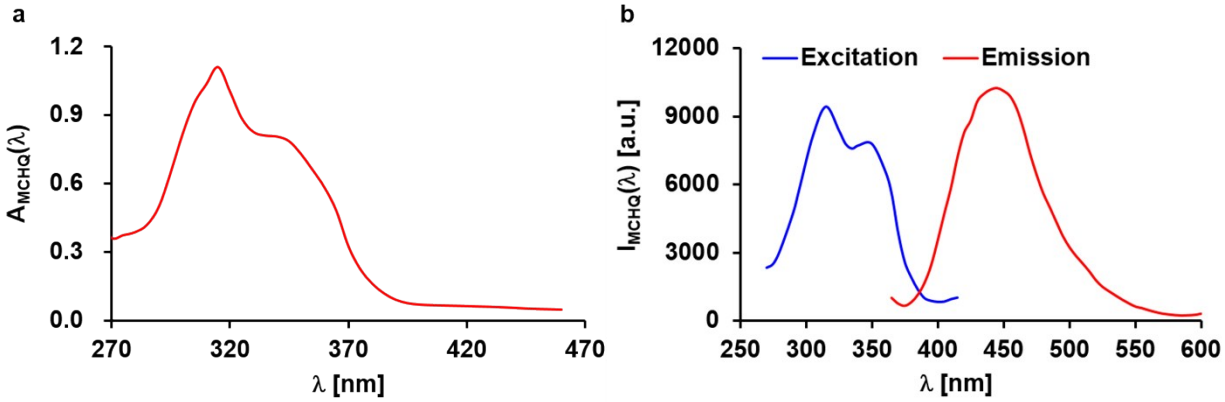


Figure S5. a) Absorption $A_{MCHQ}(\lambda)$ and b) fluorescence excitation and emission spectra $I_{MCHQ}(\lambda)$ of the chloride ion indicator MCHQ (10 μg/mL) in HEPES aqueous solution (10 mM, pH 7.4).

II.4) Synthesis of Gold Nanoparticles

Cationic gold nanoparticles (NPs) were prepared according to the literature[3] and their concentrations were determined by measuring the absorbance according to the literature[4]. Basic characterization data is shown in **Figure S6**.

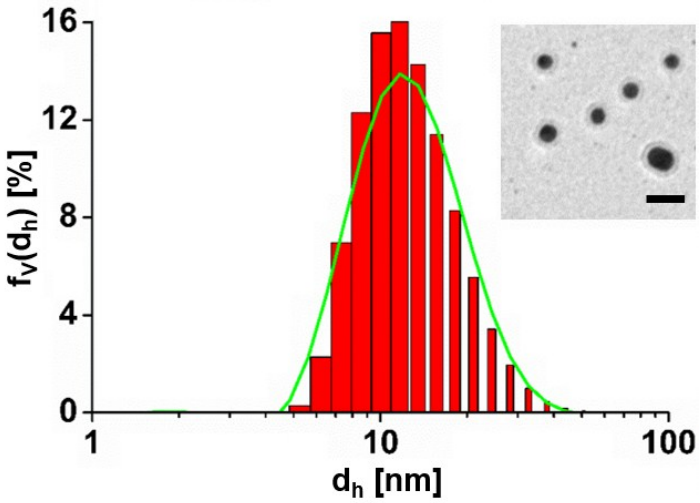


Figure S6. Characterizations of cationic Au NPs. Size distribution $f_V(d_h)$ of the hydrodynamic diameter of the Au NPs as determined from the volume distribution of dynamic light scattering (DLS) experiments. The insert is one selected TEM image. The scar bar corresponds to 20 nm.

II.5) Fabrication of Fluorescein Isothiocyanate-Labeled BSA Nanoparticles

BSA nanoparticles were prepared by the desolvation method according to literature with slight modification.[5] Briefly, BSA was dissolved in Milli-Q water at concentration of 10 mg/mL, and the pH was adjusted by NaOH to 8.8-9.0. 1 mg of fluorescein isothiocyanate (M_W = 389.38 kDa) in 100 μL of ethanol was added to 2 mL of BSA solution (as prepared above) and the mixture was stirred at 500 rpm overnight. On the following day, 3.5 mL of acetone was added dropwise into the mixture at a rate of 1 mL/min under stirring at 500 rpm. The solution became slightly

turbid. 8 μL of 25% (v/v) glutaraldehyde aqueous solution was added immediately to facilitate intra-particle crosslinking and the solution was stirred for 3 h.

Removal of unreacted dye, free BSA, and excess glutaraldehyde was achieved by repeated centrifugation and washing. After centrifugation at 14,000 rpm for 40 min, the supernatant was discard and the precipitate was redispersed in 1.75 mL of 75% (v/v) ethanol via sonication. After two cycles of centrifugation, the pellets were resuspended in 10 mM HEPES-NaOH (pH 7.4) and stored at 4 ℃.

III) Characterization of Polyplexes

III.1) Transmission Electron Microscopy

For transmission electron microscopy (TEM) sample preparation, 50-mesh copper grids were immersed in undiluted polyplex solution for 10 s and excess solution was removed by filter paper. Grids were observed immediately without complete dryness. Images were taken with a JEM-1200EX TEM at a magnification of 20000×.

III.2) Hydrodynamic Diameter and Zeta Potential Measurements

Hydrodynamic diameters d_h and zeta potentials ξ of the polyplexes were measured using dynamic light scattering (DLS) and laser doppler anemometry (LDH). 60 μL of polyplexes were used for size measurements directly at 25 ℃. 120 μL polyplexes were diluted with 780 μL of 10mM HEPES-NaOH buffer (pH 7.4) prior to measuring zeta potentials at 25 ℃. Measurements were performed on a Zetasizer Nano (Malvern Instrument Ltd.). For time-dependent size changes of polyplexes in FBS-free or 10% FBS-supplemented conditions, 2 volumes of PEI/DNA solution were mixed with 1 volume of HEPES or corresponding BSA solution. 20 min after incubation, 6 volumes of culture medium were added and the hydrodynamic diameter or

zeta potential was measured after a certain incubation time t. Data are presented as mean value ± standard deviation (SD) for n = 3 measurements.

Data for polyplexes with PEI/DNA weight ratio of 0.55/1 (N/P = 4) are shown in **Figure S7**. Data for polyplexes with incorporated Au NPs are shown in **Figure S8**.

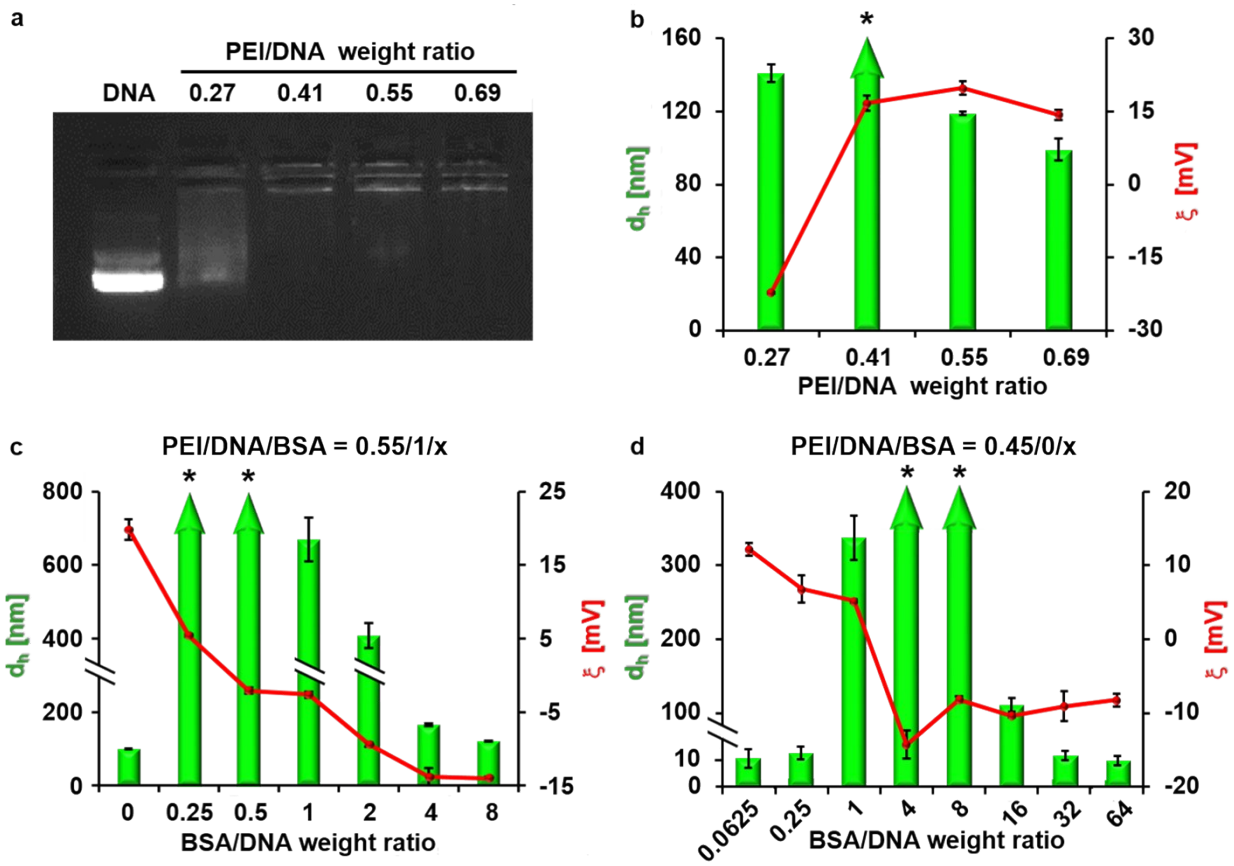


Figure S7. Effects of BSA corona formation on the hydrodynamic diameter d_h, and the zeta potential ξ of PEI/DNA polyplexes. a) Gel electrophoresis, b) hydrodynamic diameter and zeta potential of PEI/DNA polyplexes at different PEI/DNA weight ratios from 0.27 to 0.69 (corresponding to N/P ratios ranging from 2 to 5). *: sizes too large (> 1 µm) to be accurately measured by DLS. c) Effects of protein corona formation on the hydrodynamic diameter and zeta potential of PEI/DNA polyplexes with PEI/DNA weight ratio of 0.55/1 (N/P = 4). A BSA/DNA

weight ratio of X corresponds to polyplexes with a PEI/DNA/BSA weight ratio of 0.55/1/X. d) Effects of protein corona formation on the hydrodynamic diameter and zeta potential of PEI polymer with PEI/DNA weight ratio of 0.45/1 (N/P = 3). A BSA/DNA weight ratio of X is the same with the BSA concentration used in c. No DNA was used in d.

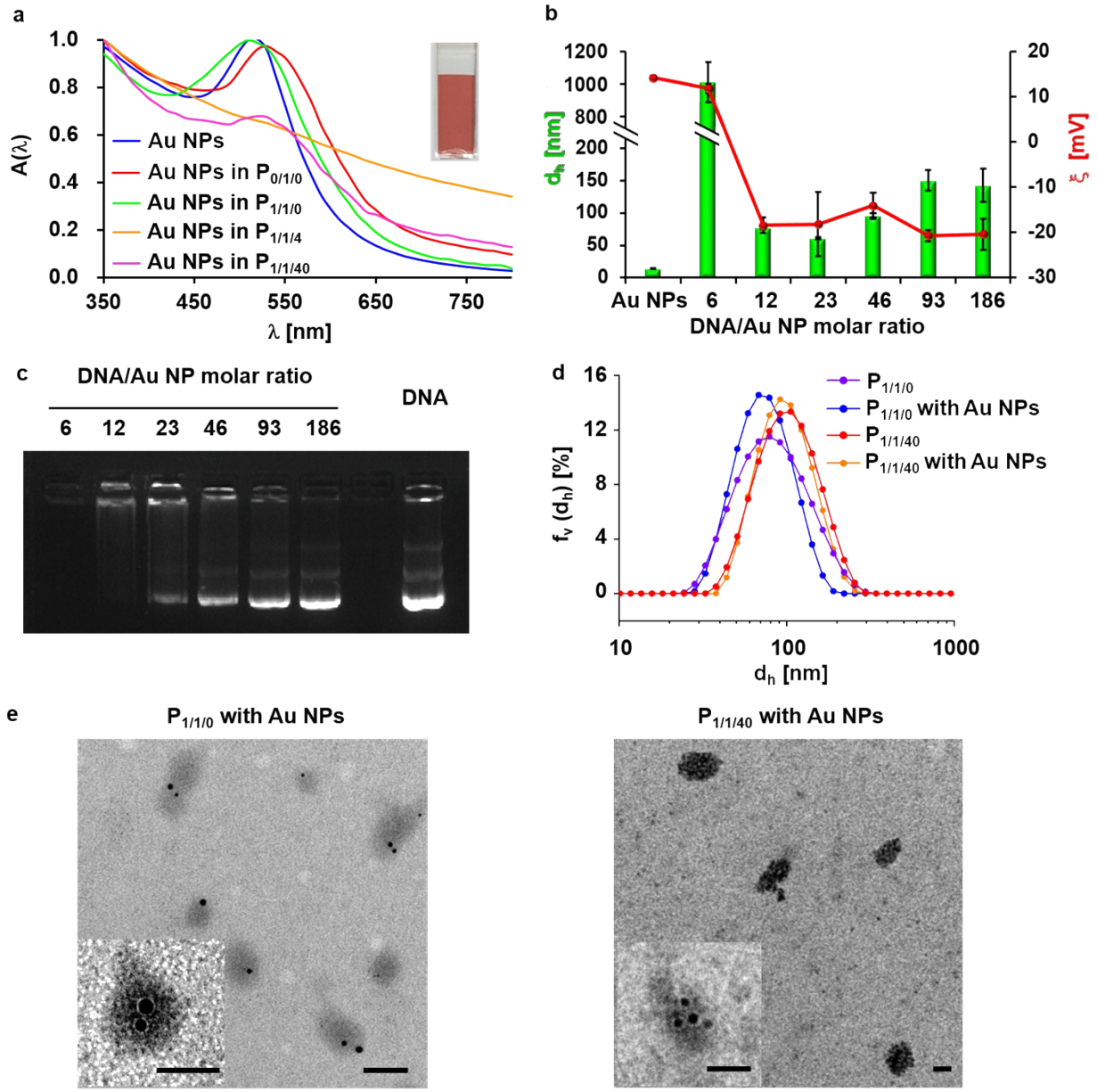


Figure S8. Characterizations of Au NP labeled DNA and its polyplexes with PEI. a) Normalized UV/Vis absorption spectra A(λ) of Au NPs, DNA labeled with Au NPs, and polyplexes made

with Au NP-labelled DNA. The insert is an image of Au NP solution at 4.55 nM. b) Hydrodynamic diameter and zeta potential of Au NPs and DNA labelled with Au NPs at different DNA/Au NP molar ratios. c) Gel electrophoresis of Au NPs and DNA labelled with Au NPs at different DNA/Au NP molar ratios. d) Intensity distributions $f_v(d_h)$ of the hydrodynamic diameters d_h as derived from dynamic light scattering **(**DLS) measurements for $P_{1/1/0}$, $P_{1/1/40}$ polyplexes without and with Au labelling of DNA at DNA/Au NP molar ratio of 23. e) TEM images of $P_{1/1/0}$ (left panel) and $P_{1/1/40}$ with integrated Au NPs (right panel). All scale bars correspond to 50 nm.

III.3) Agarose Gel Electrophoresis

The integrity of PEI/DNA polyplexes at different PEI/DNA weight ratios and DNA leakage from polyplexes after treatment in FBS-free or 10% FBS-supplemented conditions were measured via an agarose gel retardation assay. Only in case DNA is released from the polyplexes, a running band of DNA will be observed, as the polyplexes themselves are too big to run on the gel. For polyplexes treated with culture medium, 2 volumes of PEI/DNA were initially mixed with 1 volume of HEPES-NaOH or BSA solutions. 20 min after incubation, another 6 volumes of culture medium were added and the mixture was incubated for 1 h at room temperature. Twenty microliters of polyplexes were added per well in the gel, which all contained the same amount of DNA (67 ng). Electrophoresis was performed at 110 V for 30 min using 0.7% (w/v) agarose gels with tris acetate-ethylenediaminetetraacetic acid (EDTA) running buffer. 2 μL of ethidium bromide (EB) was added during gel preparation to visualize DNA bands, and images were required via a Fluorescence/Chemiluminescence imaging system (Clinx Science Instruments Co.,Ltd, China). An example is shown in **Figure S9.**

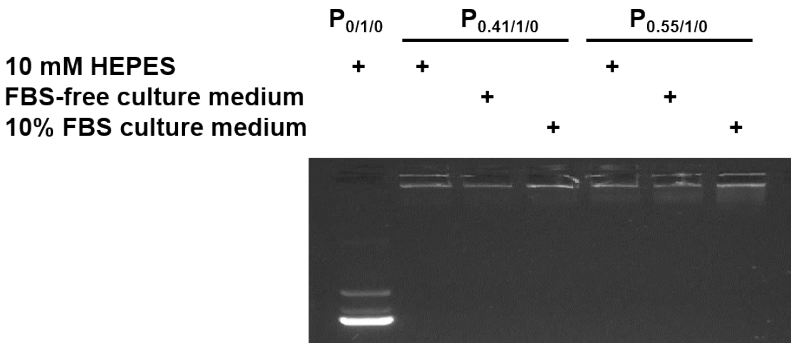


Figure S9. Gel electrophoresis of $P_{0/1/0}$ (i.e. free DNA), $P_{0.41/1/0}$ (N/P = 3), and $P_{0.55/1/0}$ (N/P = 4) after 1 h incubation with 10 mM HEPES, FBS-free or 10% FBS-supplemented medium in RPMI 1640 cell culture medium for 1 h.

III.4) Ethidium Bromide Exclusion Assay

The "compactness", i.e. the packing density of 3 polyplexes before and after treatment with culture medium was measured by the ethidium bromide (EB) exclusion assay.[6] For polyplexes treated with culture medium, 2 volumes of PEI/DNA solution were initially mixed with 1 volume of HEPES-NaOH or BSA solution. 20 min after incubation, another 6 volumes of culture medium were added and the mixture was further incubated for 20 min at room temperature. For polyplexes without treatment with culture medium, 6 volumes of HEPES-NaOH were used instead of culture medium. For each group, 150 μL of sample were mixed with 30 μL of 40 μg/mL EB aqueous solution, and the mixtures were incubated for 40 min at room temperature before measurement. 200 μL of solution was added per well into 96-well black plates and the fluorescent signals were measured by a microplate reader. EB was excited as at 510 nm and emission was recorded at 608 nm. The relative fluorescence change $I_{EB}/ I_{EB,0}$ of EB in the

presence of polyplexes as compared to free DNA was calculated according to the following equation:

$$I_{EB}/I_{EB,0} = \frac{I_{EB+polyplex} - I_{EB\ only}}{I_{EB+free\ DNA} - F_{EB\ only}} \times 100\%$$

where $I_{EB+polyplex}$, $I_{EB\ only}$, $I_{EB+free\ DNA}$ denote the fluorescence intensities of the polyplex sample mixed with EB, pure EB solution, and DNA/EB solution without polymer, respectively.

IV) Polyplex Delivery and Gene Expression

IV.1) Cell Culture Techniques

Human cervical carcinoma HeLa cells were purchased from ATCC (American Type Culture Collection). HeLa cells were maintained in RPMI 1640 medium (Cat. No.: 11875093, Thermo Fisher Scientific) supplemented with 10% heat-inactivated fetal bovine serum (GIBCO), penicillin (100 units/mL), and streptomycin (100 μg/mL) in a humidified atmosphere of 5% CO_2 at 37 ℃.

IV.2) Immunostaining, Cell Imaging, and Uptake Studies by CLSM, Flow Cytometry, and TEM

Cells were seeded on μ-slide 8 well plates (Cat. No.: 80824, Ibidi) at 2.2×10^4 cells per well in 300 μL of culture medium one day prior to use. Cellular structures were optionally immunostained, e.g. with fluorescent markers of known intracellular locations or via transfection with virus expressing eGFP-tagged proteins. The cellular structures, together with the correspondent markers are presented in **Table S1**.

Fluorescence imaging was carried out with confocal laser scanning microscopy (CLSM). Cells were imaged using appropriate laser excitation and filter sets (Hoechst: wavelength of excitation

λ_{ex} = 405 nm and emission recorded with bandpass (BP) from 420-480 nm; eGFP, FITC, LysoTracker Green and Oregon green: excitation λ_{ex} = 488 nm and emission BP 505-550 nm; TRITC: excitation λ_{ex} = 543 nm, emission BP 560-615 nm; Cy5: excitation λ_{ex} = 633 nm, emission recorded with longpass (LP) with 650 nm). The cells depicted in all figures were representative of entire fields observed. The brightness, contrast, excitation laser power, and pinholes were all set the same for all images in the same group. An example is shown in **Figure S10**. From the images, quantitative uptake of polyplexes was studied in terms of fluorescence per cell originating from the Cy5 label of DNA, either by CLSM, or by flow cytometry cf. **Figure S11**. Also, uptake of fluorescence-labelled BSA particles was investigated with CLSM, see **Figure S12**. Alternatively, cells were imaged with transmission electron microscopy (TEM), see **Figure S13**.

Labelled Structure	Dye/Marker/Virus	Final concentration	$T_{labeling}$	λ_{ex}; λ_{em}
Late endosome/ lysosome	LysoTracker® Green	200 nM	1 - 1.5 h	504 nm; 511 nm
Nucleus	Hoechst33342	5 µg/mL	10 min	350 nm; 461 nm
Cell membrane	Wheat germ agglutinin-Alex Fluor 488	20 µg/mL	10 min	495 nm; 519 nm
Actin cytoskeleton	Phalloidin-Oregon Green	5 units/mL	20 min	511 nm; 528 nm
Intracellular vesicles	$Dextran^{TRITC}$ (M_w = 10 kDa)	750 µg/mL	3 h	547 nm; 572 nm
Endosome	CellLight® early	16 µL / 10^4	>16 h	488 nm; 507 nm

	endosomes-GFP	cells		
Tubulin	CellLight® early tubulin-GFP	16 µL / 10^4 cells	>16 h	488 nm; 507 nm

Table S1. Conditions for fluorescence staining of cellular structures. Final concentrations, together with the times of labelling $T_{labelling}$, as well as wavelengths of excitation λ_{ex} and emission λ_{em} are given.

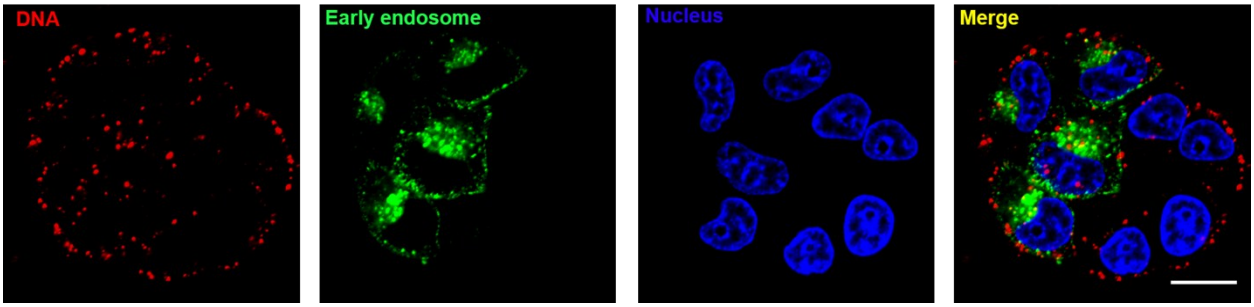


Figure S10. Localization of $P_{1/1/4}$ and early endosomes, as imaged by confocal laser scanning microscopy. HeLa cells were incubated with $P_{1/1/4}$ at 1.4 µg/mL Cy5-labelled DNA in FBS-free medium for 2 h. Cy5-labelled DNA is shown in the red fluorescence channel. Early endosomes tagged with GFP by CellLight® Early Endosomes-GFP are shown in the green fluorescence channel. Nuclei stained with Hoechst 33342 are shown in the blue fluorescence channel. The scale bar corresponds to 20 µm.

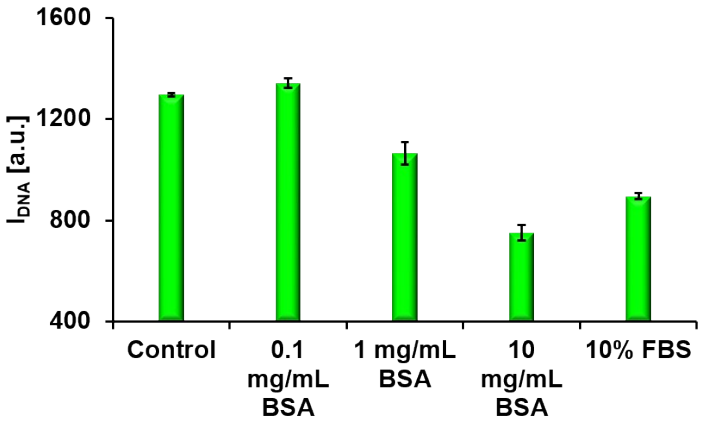


Figure S11. **Competitive uptake of $P_{1/1/40}$ in the presence of BSA or 10% FBS in 1640 RPMI cell culture medium.** HeLa cells were preincubated in 0.1, 1, and 10 mg/mL BSA, or 10% FBS in 1640 RPMI cell culture medium for 1 h, followed by incubation with $P_{1/1/40}$ at 1.4 µg/mL DNA concentration (0.3 µg/mL Cy5-labelled DNA and 1.1 µg/mL DNA) in the presence of BSA or FBS for 2 h. Cellular uptake in terms of Cy5 intensity was measured by flow cytometry. Cells incubated with $P_{1/1/40}$ without BSA or FBS were used as control.

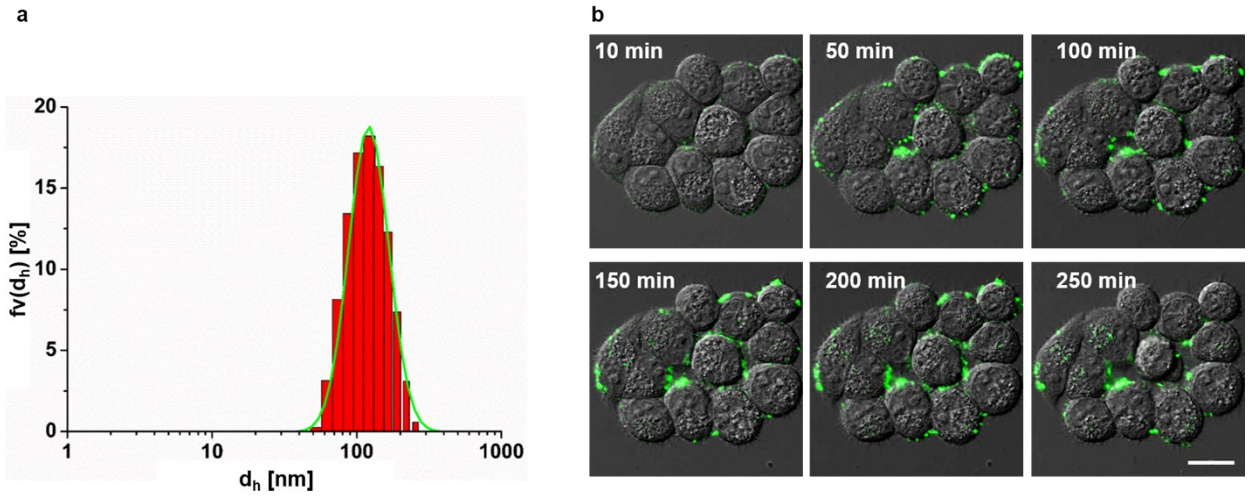


Figure S12. Cellular uptake of fluorescein isothiocyanate-labelled BSA nanoparticles. a) Size distribution $f_V(d_h)$ of the hydrodynamic diameter of the BSA nanoparticles as determined from the volume distribution of DLS experiments. b) Representative images of internalization of

fluorescein isothiocyanate-labelled BSA nanoparticles at different times of incubation. BSA nanoparticles are shown in the green fluorescence channel. The scale bar corresponds to 20 μm.

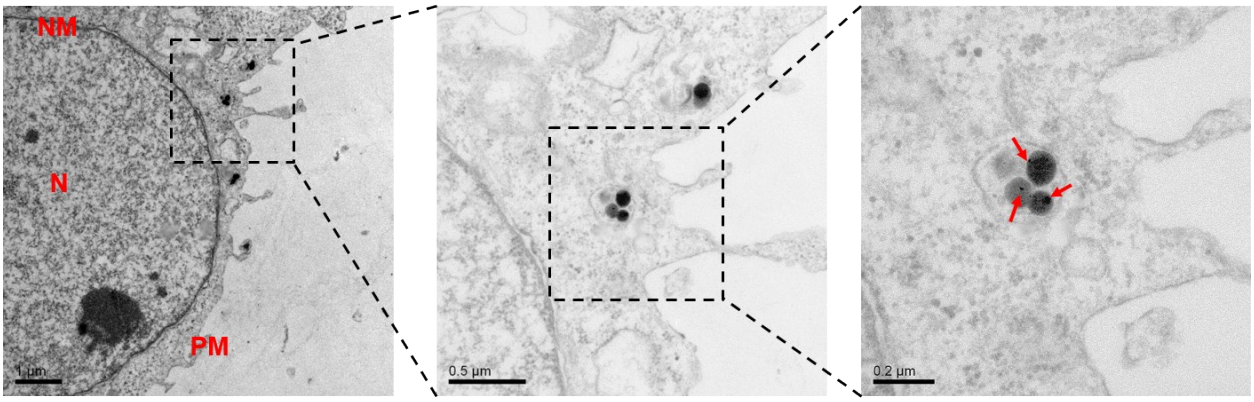


Figure S13. **TEM images of $P_{1/1/40}$ with integrated Au NPs recorded after 2 h incubation in HeLa cells.** PM: plasma membrane. NM: nuclear membrane. N: nucleus. Arrows indicate the Au NPs. The scale bars correspond to 1 μm, 0.5 μm, and 0.2 μm.

IV.3) Gene Transfection

For luciferase gene transfection, luciferase-encoding plasmid, pGL4.13[luc2/SV40] was used. Cells were seeded in 48-well plates at a density of 3.0×10^4 cells per well in 400 μL of 10% FBS-supplemented cell culture medium. Cells were incubated overnight to reach 70~80% cell confluence. Then, the old culture medium was removed and replaced with 400 μL of fresh FBS-free culture medium, and cells were incubated with polyplexes at a concentration of 1.4 μg/mL DNA for 3 h. Afterwards, the medium was replaced with 400 μL of fresh 10% FBS-supplemented culture medium, and cells were further incubated for an additional 24 h. The expression of luciferase was measured in terms of luminescence intensity I_{luci} by an illuminator according to the manufacture manual (Promega). The protein content of the cells was determined by first lysing the cells, and the amount of protein in the lysis solution was measured by the

Bradford protein assay kit (Sangon Biotech, Shanghai). Luminescence due to luciferase was normalized by the amount of protein as determined by the Bradford assay. Experiments were repeated three times (n = 3). Examples are shown in **Figure S14** and **Figure S15**.

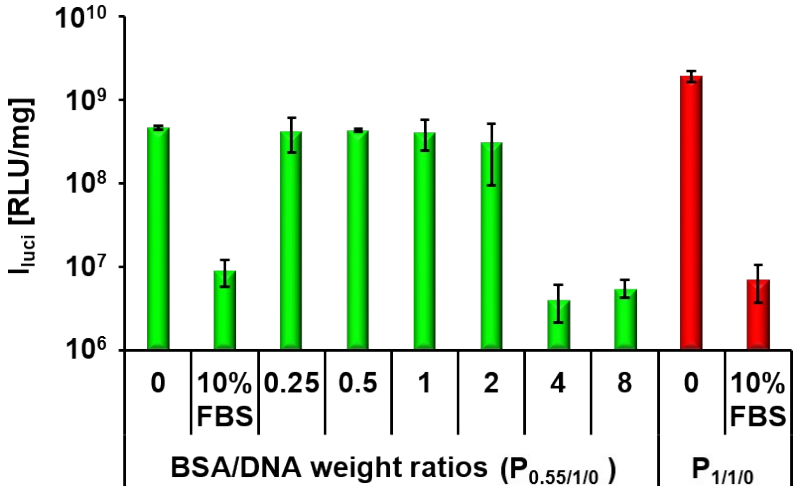


Figure S14. Effects of BSA corona formation on PEI/DNA with 0.55/1 weight ratio (N/P = 4) mediated luciferase expression in HeLa cells. The luciferase expression of PEI/DNA polyplexes (N/P = 4) after incubation with BSA at different BSA/DNA weight ratios. Cells were incubated with the polyplexes at 1.4 μg/mL DNA encoding for luciferase for 3 h in serum free medium, followed by culturing in fresh 10% FBS-supplemented medium for 24 h. The resulting luminescence I_{luci} is quantified in relative luminescence units (RLU) per amount of total cellular protein as RLU/mg.

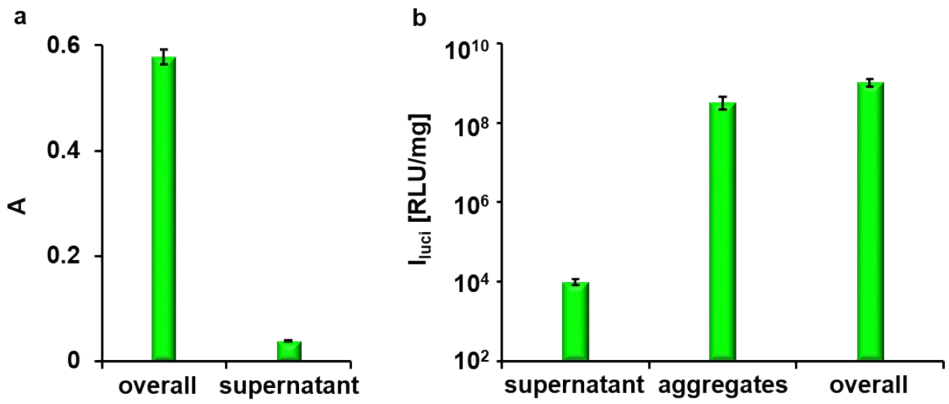


Figure S15. Large aggregates of $P_{1/1/4}$ were responsible for $P_{1/1/4}$ mediated transfection. a) Large aggregates of $P_{1/1/4}$ (1.4 μg/mL luciferase encoding plasmid) were separated via brief centrifugation and the turbidity of supernatant and overall polyplex solution was measured as absorption A at 560 nm. b) Luciferase expression after 3 h incubation of cells with supernatant, aggregates, and original polyplexes in FBS-free medium, followed by 24 h culture in fresh 10% FBS-supplemented medium. The resulting luminescence I_{luci} is quantified in relative luminescence units (RLU) per amount of total cellular protein as RLU/mg.

For eGFP gene transfection measured by flow cytometry, HeLa cells were seeded at a density of 4.4×10^4 cells per well in 24-well plates one day prior to transfection. The medium was then replaced with 500 μL or 1 mL of fresh FBS free culture medium. Cells were incubated with polyplexes at a concentration of 1.4 μg/mL eGFP-encoding DNA for 3 h. Afterwards, the medium was replaced with 500 μL of fresh 10% FBS-supplemented culture medium and cells were further incubated for another 24 h. Cells were washed with cold phosphate buffered saline (PBS) once and were detached from the 24-well plates by 0.05% trypsin. Aspirated culture medium, the PBS used for rinsing and the detached cells were pooled to collect all cells. This cell suspension was then centrifuged at 900×g for 5 min at 4 ℃. The supernatant was discard and cells were resuspended in 500 μL of PBS. The fluorescence of cells due to expressed eGFP was

measured using flow cytometry (BD FACS LSR II) with excitation at 488 nm and recording the emission at 530 nm with a 30 nm bandwidth bandpass. The transfection efficiency was expressed either in terms of the percentage of eGFP-positive cells, or the mean fluorescence intensity per cell or per eGFP-positive cell. Analysis was performed with the FlowJo software.

To visualize the eGPF expression by CLSM, cells were seeded on µ-slide 8 well plates (Ibidi) at 2.2×10^4 cells per well in 300 µL of culture medium one day prior to use. The medium was replaced with 500 µL of fresh FBS-free culture medium. Cells were incubated with polyplexes at a concentration of 1.4 µg/mL DNA for 3 h. Afterwards, cells were further cultured in 300 µL of fresh 10% FBS-supplemented culture medium for 24 h and imaged by a LSM510 confocal system (Zeiss; excitation 488 nm; emission BP 505-570 nm).

IV.4) Effect of Endocytic Inhibitors on Cellular Uptake of Polyplexes and Transfection

For probing suppression of polyplex uptake by endocytic inhibitors HeLa cells were seeded at a density of 4.4×10^4 cells per well in 24-well plates one day prior to transfection. The medium was replaced with 1 mL of fresh FBS-free culture medium. Cells were pretreated with 4 inhibitors in serum-free medium, namely 5 µg/mL of chlorpromazine (an inhibitor of clathrin-mediated endocytosis), 60 µg/mL of genistein (an inhibitor of caveolae-mediated endocytosis), 0.171 µg/mL of wortmannin (an inhibitor of phosphatidylinositol 3-kinases-mediated macropinocytosis), or 5 µg/mL cytochalasin D (an inhibitor of actin-polymerization). For experiments performed at 4℃, microplates were incubated directly on ice (pre-cooled polyplex exposure was also performed on ice). At 1 h post-incubation, polyplexes were added containing 30 ng/mL Cy5-labelled DNA and 1.4 µg/mL unlabeled DNA per well. After 2 h incubation, medium was removed and cells were rinsed with PBS, detached by trypsin, isolated, resuspended in PBS, and analyzed immediately using flow cytometry with excitation at 640 nm and recording

the emission at 670 nm with a 30 nm bandpass. 10,000 cells were counted per exposure condition. Cellular uptake in terms of the percentage of Cy5-positive cells or mean fluorescence intensity per cell were analyzed by FlowJo software.

The influence of inhibitors on luciferase expression was performed similarly. Cells were seeded in 48-well plates at a density of 30,000 cells per well in 0.4 mL medium and were incubated overnight for obtaining 70 - 80% cell confluence. Cells were pretreated with 4 inhibitors in serum-free medium, including 5 μg/mL of chlorpromazine (an inhibitor of clathrin-mediated endocytosis), 60 μg/mL of genistein (an inhibitor of caveolae-mediated endocytosis), 0.171 μg/mL of wortmannin (an inhibitor of phosphatidylinositol 3-kinases-mediated macropinocytosis), or 5 μg/mL cytochalasin D (an inhibitor of actin-polymerization). For incubation at 4℃, cells in the microplates were incubated directly on ice (pre-cooled polyplex exposure was also performed on ice). Afterward, cells were incubated with polyplexes at 1.4 μg/mL for 2 h, followed by further incubation in 400 μL fresh 10% FBS-supplemented culture medium for additional 24 h. Luciferase activity was measured following the steps describe above.

IV.5) Effect of Chlorquine on the Luciferase Expression Capability of $P_{1/1/40}$

Cells were seeded in 48-well plates at a density of 3×10^4 cells per well in 400 μL of 10% FBS-supplemented cell culture medium and incubated overnight to reach 70 - 80% cell confluence. The medium was then replaced with 400 μL of FBS-free culture medium. To evaluate the effect of chlorquine, cells were co-exposed with $P_{1/1/40}$ containing 1.4 μg/mL DNA and various concentrations of chlorquine (10, 20, 40, 60, 80 ,100 μM) for 2 h. Afterwards, the medium was replaced with 400 μL of fresh 10% FBS-supplemented culture medium and cells were further incubated for an additional 24 h. Luciferase activity was measured following the steps describe above. Results are presented in **Figure S16**.

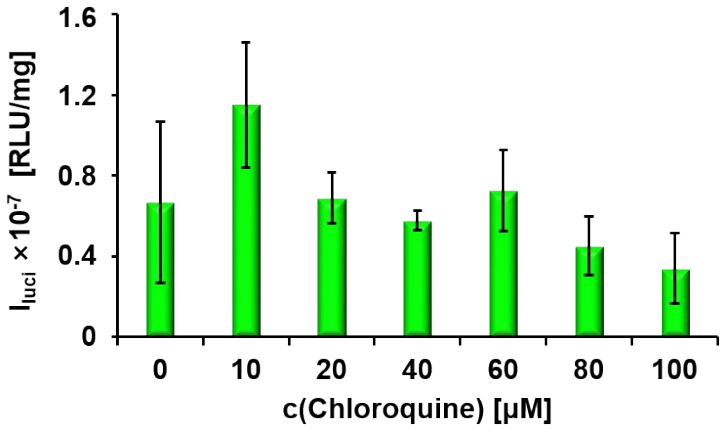


Figure S16. Effects of chloroquine on $P_{1/1/40}$ mediated luciferase expression. HeLa cells were incubated with $P_{1/1/40}$ at 1.4 μg/mL of luciferase encoding plasmid DNA and chlorquine at 10-100 μM in FBS-free medium for 2 h, followed by culturing in fresh 10% FBS-supplemented medium for 24 h. The resulting luminescence I_{luci} is quantified in relative luminescence units (RLU) per amount of total cellular protein as RLU/mg.

IV.6) Cytotoxicity due to Exposure to Polyplexes and Inhibitors

Cytotoxicity caused by administering polyplexes and inhibitors to HeLa cells was investigated by a resazurin-based *in vitro* colorimetric assay[7]. Non-fluorescent resazurin was administered to cells and was metabolized into highly fluorescent resorufin. Increased fluorescence corresponded to high metabolic activity of viable cells, whereas a reduction of fluorescence corresponded to loss of mitochondrial metabolization of resazurin due to cell death. Cells were seeded in 96-well plates at 7,500 cells per well in 200 μL of culture medium. On the following day, polyplexes or inhibitors were administrated with serial dilutions in FBS-free culture medium in 200 μL of culture medium (2 h for nocodazole and 3 h for others). Inhibitors had to be dissolved first in DMSO. The volume fraction of DMSO did not exceed 2% (more than 92% viability in DMSO control). The fraction of HEPES in the polyplex solution did not exceed 30%, and thus solvent triggered toxicity can be excluded. After exposure of cells to polyplexes or inhibitors, the

medium was replaced with 100 µL of fresh 10% FBS-supplemented culture medium and cells were further incubated for additional 24 h. Finally, 10 µL of resazurin was added per well and the cells were incubated for 4 h at 37 ℃. Microplates were then read with a Fluorolog®-3 fluometer equipped with a MicroMax 384 plate reader (both from HORIBA Jobin Yvon). Emission spectra were recorded for all wells using the following parameters: excitation at 525 nm, emission at 575 - 650 nm (step width 1 nm), averaging time 100 ms. The recorded emission I was normalized to the emission I_0 of cells which had not been exposed to polyplexes or inbibitors. The cell viability V was defined as I/I_0. Data are shown in **Figure S17** and **Figure S18**.

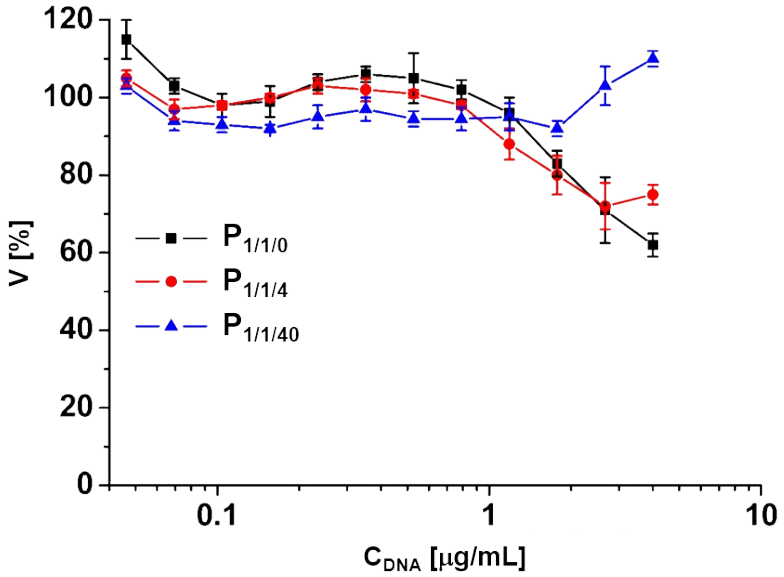


Figure S17. Cell cytoxicity of $P_{1/1/0}$, $P_{1/1/4}$, and $P_{1/1/40}$ to HeLa cells as measured by the resazurin assay. Cells were incubated with polyplexes at a series of DNA concentrations C_{DNA} in FBS-free culture medium for 3 h, followed by culturing in fresh 10% FBS-supplemented medium for 24 h. AlamarBlue® reagent (resazurin) was added at a final concentration of 10% and the cells were further incubated for an additional 4 h to metabolize non-fluorescent resazurin

to fluorescent resorufin. The viability V in terms of normalized fluorescence intensity was measured with a fluorimeter.

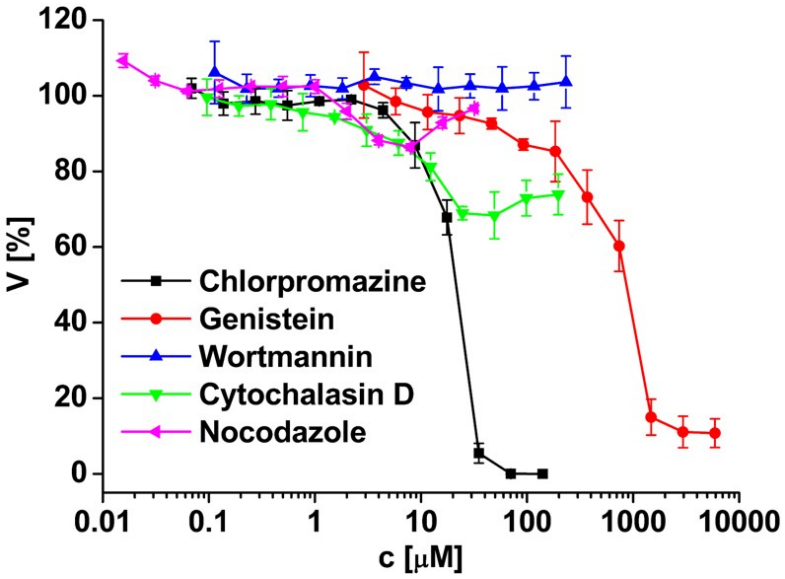


Figure S18. Cytoxicity of chlorpromazine, genistein, wortmannin, cytochalasin D, and nocodazole to HeLa cells in FBS-free medium as measured by the resazurin assay. Cells were incubated with the first 4 different inhibitors for 3 h and with nocodazole for 2 h, followed by culturing in fresh 10% FBS-supplemented medium for 24 h. AlamarBlue® reagent (resazurin) was added at a final concentration of 10% and the cells were further incubated for an additional 4 h to metabolize non-fluorescent resazurin to fluorescent resorufin. The viability V in terms of normalized fluorescence intensity was measured with a fluorimeter. The concentrations of chlorpromazine, genistein, wortmannin, cytochalasin D, and nocodazole used for further experiments were 5 μM, 220 μM, 0.5 μM, 9.8 μM, and 8 μM, respectively.

IV.7) Hemolysis Assay

A hemolysis assay was performed according to a previous report.[8] Fresh human whole blood containing ethylenediaminetetraacetic acid (EDTA) as anticoagulant was centrifuged at 12,000

rpm for 5 min to separate red blood cells (RBCs). RBCs were redispersed in 150 mM NaCl to the original volume of solution and were centrifuged at 12,000 rpm for 5 min. After 2 cycles of centrifugation, trace amounts of leaked hemoglobin were removed and RBCs were diluted in 150 mM NaCl to 10 times of the original volume of the stock solution. Polyplexes or PEI were diluted in 800 μL of 0.1 M sodium phosphate buffer with various pH values (PB, pH 7.4, 6.2, 5.0, prepared from 0.1 M of Na_2HPO_4 and NaH_2PO_4) at a PEI equivalent of 20 μg/mL. 200 μL of RBC stock solution was added to the solution and the mixture was incubated in a 37℃ water bath for 1 h. During the incubation, the mixture was gently mixed by inverting the tube several times. Subsequently cells or debris were removed by centrifugation at 12,000 g for 5 min the absorption A at 541 nm of 200 μL of the supernatant (containing the released contents of lysed cells, hemoglobin is mainly responsible for the absorption at 541 nm) was measured with a microplate reader. The absorption of the supernatants above RBCs in PB (A_0) and in deionized water (A_{100}) was set as 0% and 100% hemolysis, respectively. In deionized water the rupturing (lysis) of red blood cells and the release of their contents into solution is considered to be 100%. The cell hemolysis percentage (i.e. the amount of lysed cells) $P_{hemolysis}$ was calculated as:

$$P_{hemolysis} = \frac{A - A_0}{A_{100} - A_0} \times 100\%$$

All hemolytic experiments were carried out in triplicates. The results are shown in **Figure S19**.

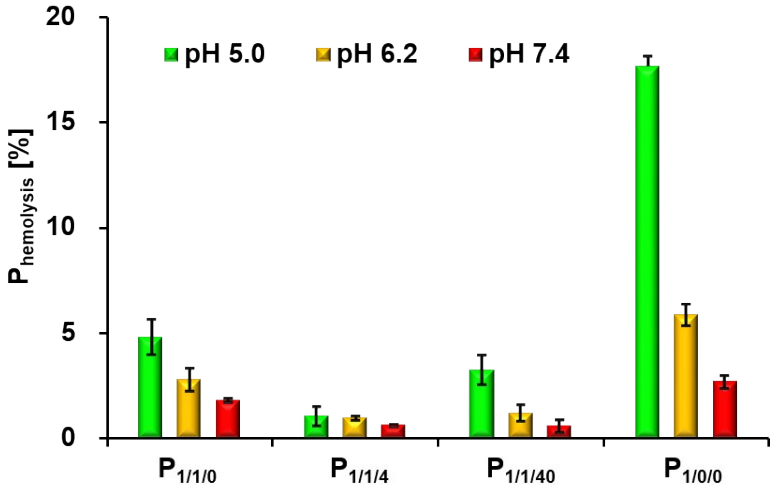


Figure S19. The percentage of hemolysis $P_{hemolysis}$ of $P_{1/1/0}$, $P_{1/1/4}$, $P_{1/1/40}$, and $P_{1/0/0}$ (i.e. free PEI) at pH 5.0, 6.2, and 7.4. The diluted red blood cells were incubated with polyplexes at an equivalent of 30 μg/mL PEI at 37 ℃ for 1 h. The diluted red blood cells at each pH without adding polyplexes or PEI were used as negative control. The diluted red blood cells after adding distilled water were used as positive control. The absorbance of hemoglobin in each sample was normalized to that of negative control, resulting in the percentage of hemolysis $P_{hemolysis}$.

IV.8) Colocalization as Determined by Mander's Coefficient Calculation

All fluorescence images of cells were obtained by CLSM (cf. **Figure S10**). Prior to further processing, shot noise was removed by median filtering (kernel-size: 3x3 pixels) and the background (minimal pixel intensity) was subtracted for each fluorescence channel. In the red channel corresponding to the fluorescence of Cy5-labelled DNA, DNA signals outside cells were erased manually prior to analysis. The integrated pixel intensities in the DNA, lysosome, and BSA channels were measured by the free, open-source image analysis software Cellprofiler v2.2.0.[9] Briefly, the foreground of each channel was identified by the "IdentifyPrimaryObjectes" module using the "MCT-method as "Global" thresholding strategy, and saved as binary objects.

The pixel intensities of each channel were measured based on the obtained objects. Colocalization was processed by calculating Mander's coefficients.[10-12] m_1 (red channel of fluorescence for DNA) and m_2 (green channel of fluorescence for lysosomes or BSA) according to the following equation:

$$m_1 = \frac{\sum_i R_{i,\ coloc}}{\sum_i R_i}, m_2 = \frac{\sum_i G_{i,\ coloc}}{\sum_i G_i}$$

Mander's coe□cients range from 0 to 1, where 1 denotes complete co-localization and 0 represents none. For m_1, $R_{i,\ coloc}$ denotes the intensity of red pixels overlapping with green pixels, and R_i denotes the total intensity of red pixels. Mander's coefficients were calculated by MATLAB. Examples of various correlation coefficients are shown in **Table S**2.

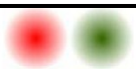
Example	m_1	m_2
	0	0
	0.36	0.36
	1.00	1.00
	0.46	1.00

Table S2. Examples of correlation coefficients. Image adopted from Schweiger et al.[10]

IV.9) Evaluation of the Local pH around polyplexes

The local pH around internalized polyplexes can be measured by labeling PEI[13-15] or DNA[16] with pH-sensitive fluorophores. Ratiometric measurements can be performed by dual labelling with pH-sensitive FITC and pH-insensitive Cy5 or RITC, [13-16] or by single labelling with

SNARF-1.[17] SNARF-1 is a low-molecular-weight pH-sensitive dye, whose emission spectrum undergoes a pH-dependent wavelength shift, thus allowing for using the ratio of the fluorescence intensities at 580 nm and 640 nm as indicator of pH.[18]

Cells were seeded at a density of 4.4×10^4 cells per well in 24-well plates. On the following day, cells were incubated with polyplexes prepared from SNARF-labelled PEI at 1.4 μg/mL DNA. Afterwards, the medium was replaced with 500 μL of fresh 10% FBS-supplemented culture medium and cells were incubated for a certain of time. At predetermined time points (i.e., 1, 2, 3, 4, 10, 24 h post-exposure), cells were rinsed with 150 mM NaCl aqueous solution, trypsinized, pelleted, and resuspended in 150 mM NaCl aqueous solution. To record pH calibration curves, cells were redispersed in 0.5 mL of pH clamp buffers (pH 7.0, 6.5, 6.0, 5.5, 5.0). Buffers were prepared by mixing Dulbecco's phosphate buffered saline (DPBS, pH 7.4) and 2-(N-morpholino)ethanesulfonic acid (MES) buffer (pH 4.0; 50 mM MES, 150 mM NaCl, 4 mM KCl, and 1 mM $MgSO_4$). Ethanol solutions of monensin and nigericin were added into the pH clamp buffers at final concentrations of 20 μM and 10 μM in order to equilibrate the intracellular compartments to the extracellular pH buffer[14, 15]. Cells in the different buffers were measured by flow cytometry with excitation at 561 nm and the emissions at 586 nm (15 nm bandpass) and 670 nm (30 nm bandpass) were recorded. 10,000 cells were counted per sample. The results were analyzed by FlowJo software v10. From these data calibration curves were obtained, relating the fluorescence signal from the SNARF-labelled internalized polyplexes to pH, see **Figure S20**. The response curves were found to be linear. As there was slight difference in the calibration curves with increasing time, we made 2 calibration curves for each polyplex recorded at 3 h for samples with 1, 2, 3, 4, 10 h incubation time, and recorded at 24 h for the samples with 24 h incubation time, respectively.

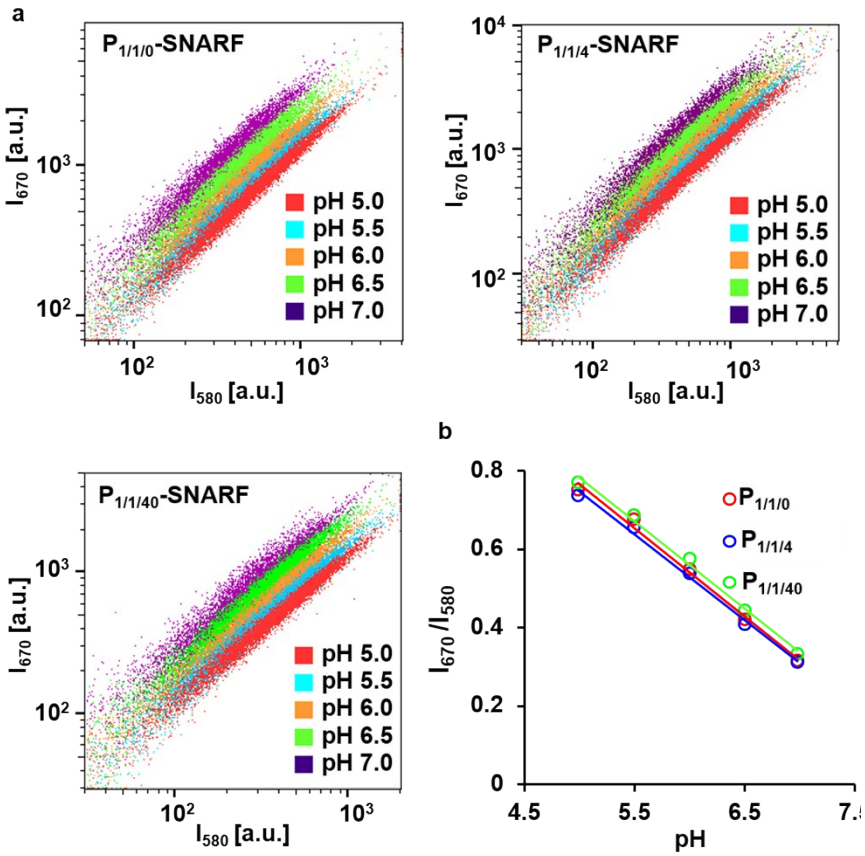


Figure S20. pH calibration curves of SNARF-labelled polyplexes. Cells were incubated with $P_{1/1/0}$, $P_{1/1/4}$ and $P_{1/1/40}$ containing SNARF-labelled PEI in FBS-free medium for 3 and 24 h (3 h incubation followed by 1 h and 21 h in fresh 10% FBS-supplemented medium) at 1.4 µg/mL DNA, respectively. Afterwards, cells were collected and incubated in 0.5 mL of pH clamp buffers (pH 7.0, 6.5, 6.0, 5.5, 5.0) containing monensin (20 µM) and nigericin (10 µM) for 10 min, before analysis by flow cytometry, in which fluorescence emission at 670 nm (I_{670}) and 580 nm (I_{580}) was recorded. a) Dot plots of cell fluorescence at 670 nm versus 580 nm in pH calibration buffers after 24 h incubation. b) pH calibration curves of I_{670}/I_{580} for samples at five pH values.

IV.10) Microinjection Study

Cells were seeded on grid culture dishes (Cat. No.: 80156, µ-Dish, 35mm, low Grid-500, Ibidi) at a density of 5.5×10^4 cells per well one day prior to injection. $P_{1/1/40}$ or $P_{0/1/0}$ (i.e. free DNA) based on eGFP-encoding plasmid DNA was mixed with TRITC-labelled dextran (M_W = 155 kDa) at 10 µg/mL DNA and dextran. The mixture was centrifuged at 12,500 rpm for 10 min to remove large aggregates. A semiautomatic injection system (Eppendorf InjectMan NI 2, Germany) was attached to a micromanipulator system (Eppendorf FemtoJet, Germany). Cytoplasmic and nuclear microinjections were performed using the following parameters of this system: P_i = 90-110 hPa, P_c = 30 hPa, and T_i = 0.3 s. Almost 5.8-15.3 $\times10^4$ copies of pDNA were injected according to the estimations reported by others.[19, 20] At 24 h post-injection, both eGFP and dextran positive cells were observed by CLSM with a 60× oil-immersed objective. Dextran positive cells were divided into cytoplasmic and intranuclear injection groups based on the dextran distribution. The eGFP positive cells in each group were calculated.

IV.11) Data Analysis of Multiple Particle Tracking Experiments

Cells were seeded on µ-slide 8-well plates at 2.2×10^4 cells per well in 300 µL of culture medium one day prior to use. On the following days, the medium was replaced with 500 µL of fresh FBS-free culture medium. Cells were incubated with polyplexes containing 1.4 µg/mL Cy5-labelled DNA for 3 h. Afterwards the culture medium was replaced with fresh 10% FBS-supplemented culture medium. At 4 h post-exposure, multiple particle tracking was performed by recording fluorescence images with CLSM at intervals of 0.98 s for 4 min. Polyplex positions were traced over time in the pixel-based coordinate system by Fiji software using the TrackMate plugin, and the actual displacement x(t), y(t) was calculated by conversion to real scale by multiplication with the conversion factor of pixel size to microns. For analyzing the motion x(t),

y(t) of the polyplexes along their 2-dimensional trajectories, the mean square displacement (MSD) $\Delta r^2(\Delta t) = \Delta x^2(\Delta t) + \Delta y^2(\Delta t)$ was calculated.

$$\Delta r^2(n \times \delta t) = \Delta x^2(n \times \delta t) + \Delta y^2(n \times \delta t)$$

$$= \frac{1}{N-n} \sum_{j=0}^{N-n-1} [x(j \times \delta t + n \times \delta t) - x(j \times \delta t)]^2 + [y(j \times \delta t + n \times \delta t) - y(j \times \delta t)]^2$$

δt is the frame interval, i.e. the time interval between subsequently recorded images (here δt = 0.98 s). N is the total number of frames (i.e. recorded images). n = 0, 1, ..., N-1 and j = 0, 1, ..., N-n-1 are a positive integer numbers. [21] n+1 is the index for the (n+1)-th time frame, which has been recorded at time $\Delta t = n \times \delta t$. $x(j \times \delta t + n \times \delta t) - x(j \times \delta t)$ is the distance the polyplex has travelled in x-direction in the time interval $n \times \delta t$, starting from the time $j \times \delta t$. In this way, $\Delta r^2(\Delta t)$ describes the mean of the square distance a polyplex moves within the time interval Δt along its trajectory. If there is no motion, then $\Delta r^2(\Delta t) = 0$. For a polyplex undergoing active transport, diffusion or subdiffusion, $\Delta r^2(\Delta t)$ can be estimated by the following formulas[21-24]:

diffusive transport: $\Delta r^2(\Delta t) = 4D\Delta t + C_1$

(with diffusion constant D and offset C_1)

active transport: $\Delta r^2(\Delta t) = v^2\Delta t^2 + 4D\Delta t + C_2$

(in addition to diffusion movement with velocity v; offset C_2)

subdiffusive transport: $\Delta r^2(\Delta t) = K\Delta t^\gamma + C_3 \quad (\gamma < 1)$

v is a constant, referring to the average velocity during active transport. D is a constant, referring to a diffusion coefficient. The constants C_1, C_2, C_3 consider the finite spatial and temporal resolution of the setup. All values (i.e. v, D, etc.) were obtained from the $\Delta r^2(\Delta t)$ versus Δt curves. Active transport of polyplexes happened in stop-and-go style, i.e. transport was

interrupted by periods of immobility. We expressed this behavior in terms of mean duration times T. Examples are shown in **Figure S21**.

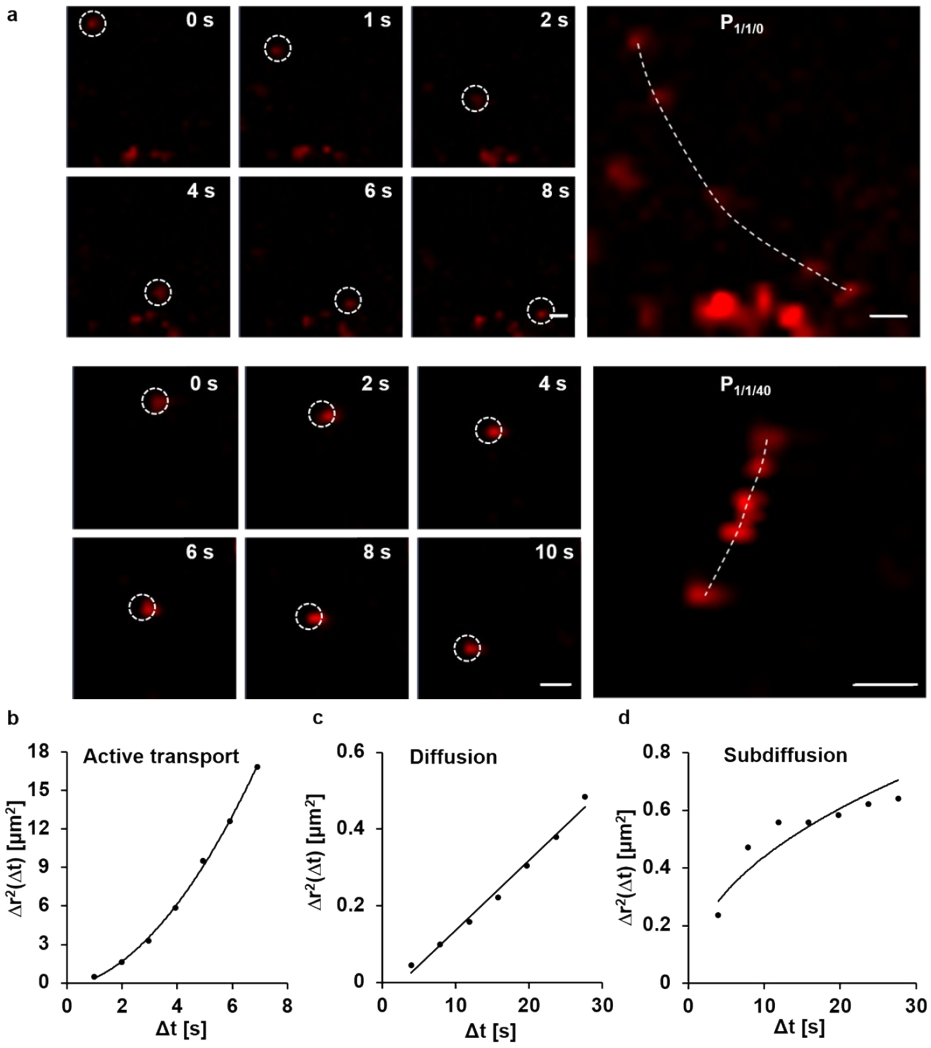


Figure S21. Representative trajectories and MSD fitting curves of $P_{1/1/0}$ and $P_{1/1/40}$ undergoing active transport and diffusion. a) Representative trajectories of $P_{1/1/0}$ (upper lane) and $P_{1/1/40}$ (lower lane) undergoing active transport. All scale bars correspond to 1 µm. b-d) Representative $\Delta r^2(\Delta t)$ versus Δt fits for $P_{1/1/0}$ undergoing b) active transport, c) diffusion, and d) subdiffusion.

IV,12) Sketch of Cellular Uptake Pathways

A plausible sketch of the uptake pathways of the different polyplexes is shown in **Figure S22**.

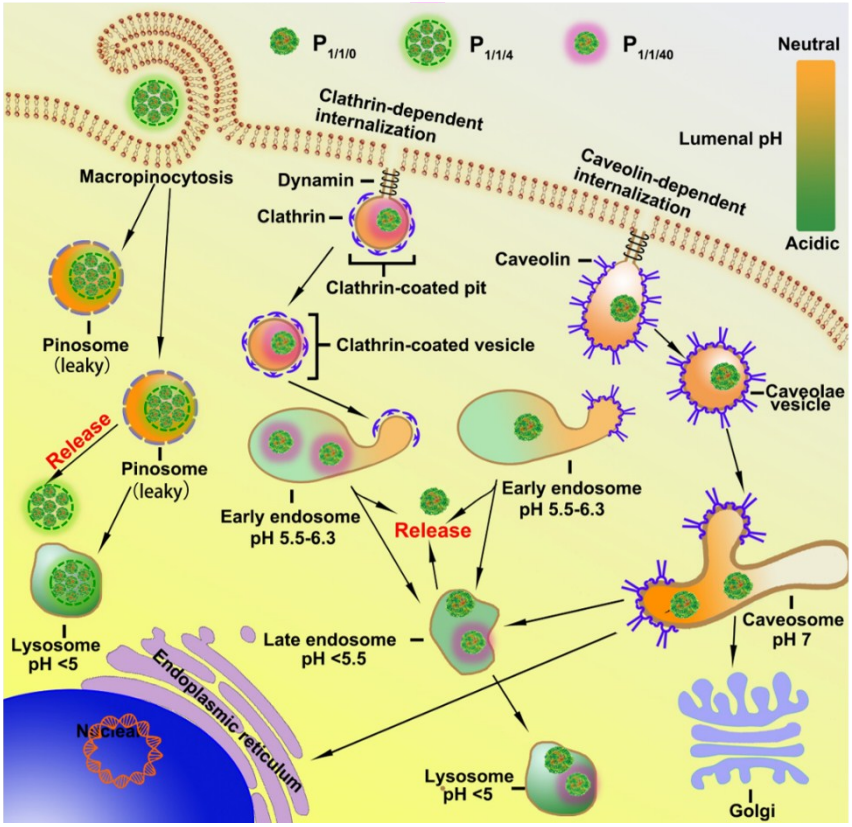


Figure S22. Cellular uptake and intracellular trafficking of $P_{1/1/0}$, $P_{1/1/4}$, and $P_{1/1/40}$ in HeLa cells. $P_{1/1/0}$ and $P_{1/1/4}$ were endocytosed via caveolae-mediated endocytosis and macropinocytosis, respectively, followed by slow lysosome trafficking and efficient vesicle escape, thereby achieving remarkable transfection efficiency. $P_{1/1/40}$ was taken up by digestive clathrin-mediated endocytosis, followed by rapid trafficking to lysosomes and inefficient vesicle escape, thereby having low gene expression.

V) References

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