Electronic Supplementary Information to the paper

Biodegradable polymer network encapsulated polyplex for DNA delivery

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1. Synthesis of copolymer.

   1.1 Poly(propylene oxide) (PPO) macroinitiator for ATRP.

   Commercially available PPO (MW 4000, degree of polymerization 69) was reacted with 3 mol equiv. of 2-bromoisobutyryl bromide in dry dichloromethane(DCM) in the presence of triethylamine (3 mol equiv.) for 24 h at 20 ◦C. The insoluble hydrobromide salt was eliminated by filtration, and DCM was then evaporated. The reaction product was dissolved in THF. The solution was stirred over activated carbon, which was eliminated by filtration, the organic solvent being ultimately evaporated. The macroinitiator was added to water at pH = 9 (turbid solution), and the macroinitiator was extracted several times with DCM. The organic solution was dried with magnesium sulfate. It was recovered by filtration, and the solvent was finally distilled off. Degree of esterification *ca.* 98% was calculated from the proton NMR.

   1.2 Poly(dimethylaminoethyl methacrylate)-block-poly(propylene oxide)-blockpoly(dimethyl-aminoethyl methacrylate) (PDMAEMA-PPO-PDMAEMA) triblock copolymer.

   ATRP of DMAEMA was initiated by $BrPPO_{69}Br$ in methanol, with the BiPy/CuCl/$CuCl_2$ catalytic system. As a typical example, 0.99 g $BrPPO_{69}Br$ (0.23mmol), 0.1432 g BiPy (0.92 mmol), 0.0454 g CuCl (0.46 mmol) and 0.0062 g $CuCl_2$ (0.046 mmol) were degassed by three times repeated vacuum/argon cycles, dissolved in methanol (1.93mL) and purged with dry argon under stirring for 60 min. Then, 1.93mL freshly distilled and degassed DMAEMA (0.0115 mol) was added, followed by polymerization at 60 ◦C for 6 h. The reaction mixture was poured in hot water (60 ◦C). After separation, the copolymer was re-dissolved in methanol and the solution was eluted through a silica column in order to remove the Cu(II) catalyst. Finally, methanol was distilled off, and the copolymer was dried in vacuum at 50 ◦C. The copolymer composition and molar mass characteristics were determined using proton NMR and SEC (degree of polymerization of PDMAEMA block = 13, Mw/Mn = 1.26).[1]

2. Coating of polyplex *via* seeded radical polymerization.

The polyplex solution was heated to 60 ^{o}C and purged with nitrogen under vigorous stirring for 45 min. NIPAM (5 mmol) and N,N'bis(acryloyl)cystamine (BAC) (0.5 mmol) were dissolved in water/methanol mixture, purged with nitrogen and added into the polyplex solution. After that, 2,2'-azobis(2-methylpropyonamidine) dihydrochloride (1 mmol) was added and the reaction was carried out at 60 ^{o}C for 5 h. Finally, the reaction solution was dialyzed against pure water (MWCO 12 000 Da).

3. Dynamic light scattering measurements.

The measurements were carried out at 37 ^{o}C on a Zetasizer Nano-ZS instrument (Malvern Instruments), equipped with a He–Ne laser (λ = 633 nm) at a scattering angle of 173^{o}. The zeta potentials were calculated from the obtained electrophoretic mobility at 25 ^{o}C by the Smoluchowski equation:

$$\xi=4\pi\eta\nu/\varepsilon$$

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

4. Transmission electron microscopy.

A drop of samples solution was deposited on a TEM copper grid coated with a Carbon film, and the solvent was allowed to evaporate. A JEOL JEM-2100 electron microscope was used at an accelerating voltage of 200 kV.

5. Plasmid DNA preparation and gel electrophoresis

Plasmid pEGFP-C2 (4700bp, Clontech Laboratories, Mountain View, CA) encoding for the eGFP (green flourescent protein) was used in the transfection experiments. The plasmid was amplified in *E. coli* DH5α strain in LB medium. The isolation and purification of the plasmid was carried out by Sigma's GenElute^{TM} Plasmid Midiprep Kit. The concentration and quality of the isolated pEGFP-C2 plasmid were determined by $A_{260/\,280}$ ratio and by agarose gel electrophoresis.

The polyplex formation and stability was checked by (0.8 %w/v) agarose electrophoresis stained with ethidium bromide (0.5 μg/ml).

6. Transfection of HEK 293 cells

Human Embrionic Kidney cells (HEK 293, ATCC CRL-1573, kindly provided by Assoc. Prof. Anastas Gospodinov, IMB-BAS) were seeded in 3 ml growth medium (DMEM-F12 supplemented with 10% FBS; 100 μg/ml streptomycin, 100 IU/ml penicillin and 40 μg/ml gentamicin) at density 1x10^{6} cells/well in 6-well plates 24 h prior to transfection. One hour prior transfection the medium was replaced with 3ml OptiMEM (Gibco, USA) conditioned media containing 12.5 mM $CaCl_2$ and 50 μM

$ZnCl_2$. The different amounts of the polyplex were mixed with two-times concentrated HeBS (Hepes-Buffered Saline, 50mM HEPES, 280mM NaCl, 1.5mM Na_2HPO_4, pH 7.05) buffer, vortexed and incubated at room temperature for 20 min. After incubation the transfection solution was added to the cells in different volumes (from 100 µl to 500 µl). Cells were further incubated for 20 hours in a CO_2-incubator at 37^{o}C. Following that, the medium was removed and 3 ml of fresh DMEM-F12 was added. The transfection efficiency was measured after 24h or 48h. Cells were harvested by trypsinization and re-suspended in PBS. The expression of eGFP was measured by flow cytometry using FACS Calibur flow cytometer (BD Biosciences, USA). The parameters of the FACS device were set according to fluorescence intensity of negative control cells (non-transfected). All cells that showed green fluorescence intensity above the intensity of the negative control were regarded as transfected. For a positive control we used cells transfected with pEGFP-C2 by Lipofectamin^{TM} 2000, according to the manufacturer's instructions (Invitrogen, USA).

The transfection efficiency was also verified by fluorescent microscopy. Carl Zeiss AM240 microscope equipped with fluorescin filter, and ApoTome camera was used. Results shown are representative of at least independent three experiments.

7. Cytotoxicity studies

The cytotoxicity of the block copolymer and polyplex against human HEK 293 cells was investigated using 3-(4,5- dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay[2] . The MTT assay is a common method for evaluating biomaterial toxicity based on the mitochondrial activity, which influences metabolic activity and cell viability. HEK 293 cells growing in logarithmic phase were harvested and subsequently plated into 96-well microtitre plates (Nunc, Wiesbaden, Germany) at a density of $1x10^5$ cells/well in DMEM-F12 media. Following 24 h, the copolymer or polyplex solutions at different concentrations were added to the cells and further incubated for up to 48h. 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 five times

8. Statistical analysis

The data were evaluated by analysis of variance (ANOVA) followed by Tukey's post-hock test. Differences in the results at the level of $p<0.05$ were considered statistically significant. The statistical analysis was carried out using the PASW 18.0 statistical software package (IBM) for Windows.

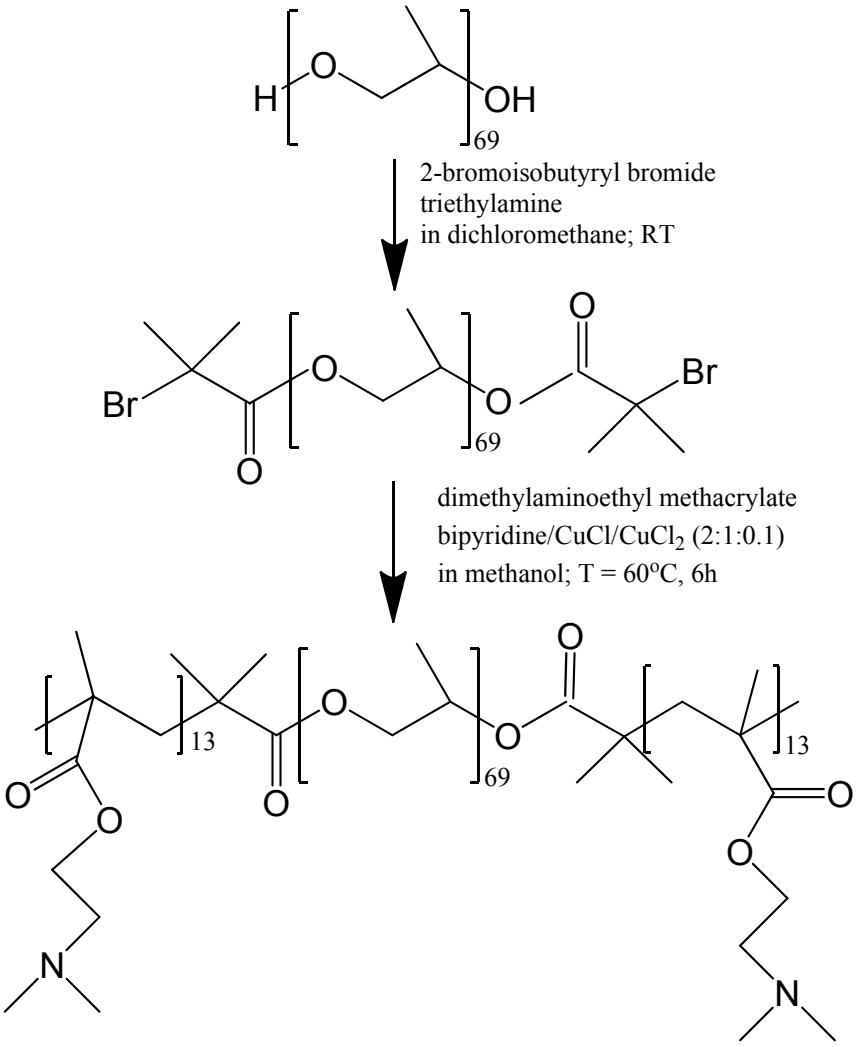

Figure S1. Schematic representation of the synthesis or PDMAEMA-PPO-PDMAEMA triblock copolymer.

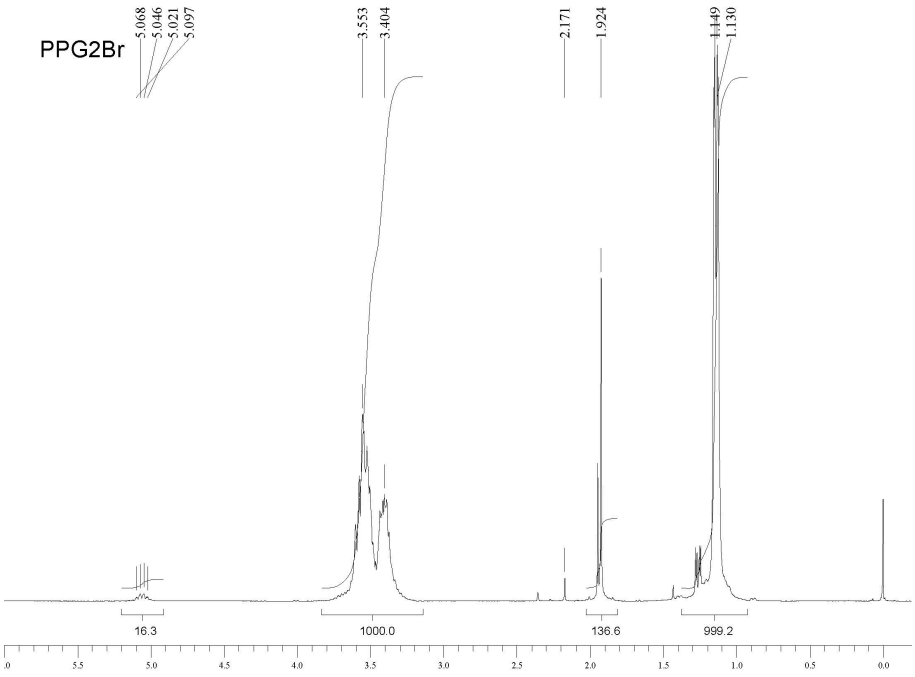

Figure S2. Proton NMR spectrum of Br-PPO_{69}-Br macroinitiator in d-chloroform.

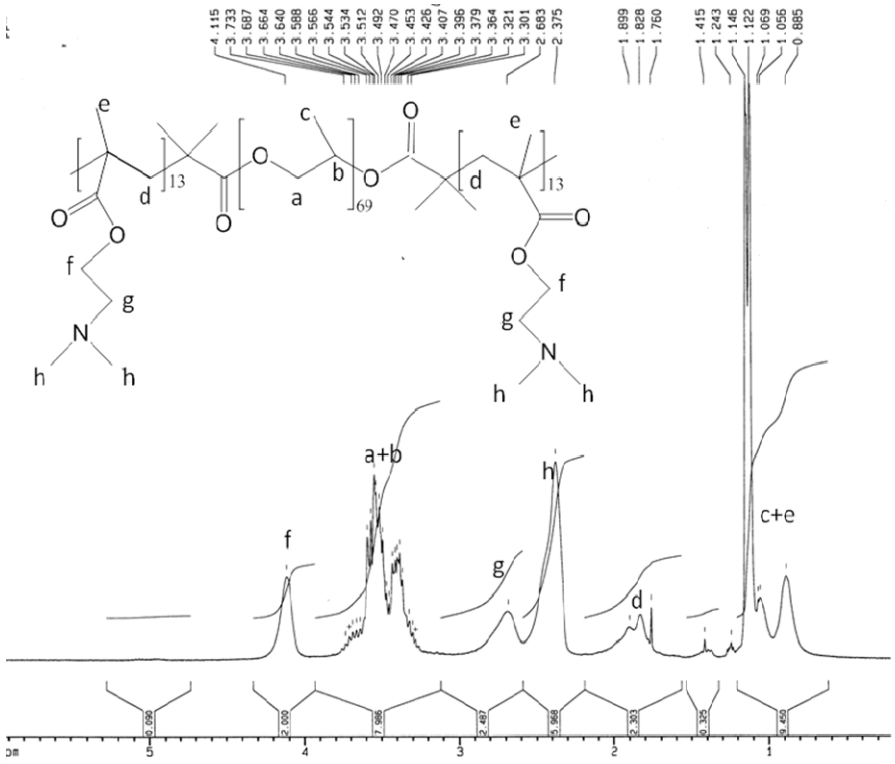

Figure S3. Proton NMR spectrum of $PDMAEMA_{13}$-PPO_{69}-$PDMAEMA_{13}$ triblock copolymer in d-chloroform. The copolymer composition was calculated from PPO protons (2H, -O–C<u>H</u>$_2$-CH-) at 3.566 ppm and (1H, -O–CH$_2$-C<u>H</u>-) at 3.407 ppm (a+b) and PDMAEMA protons (2H, -CH$_2$-C<u>H</u>$_2$-N-) at 4.115 ppm (f).

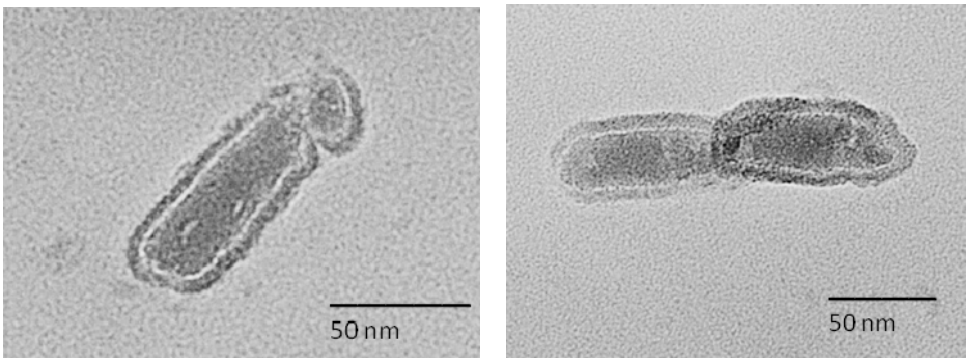

Figure S4. Representative TEM micrographs of pDNA/cationic micelles complex.

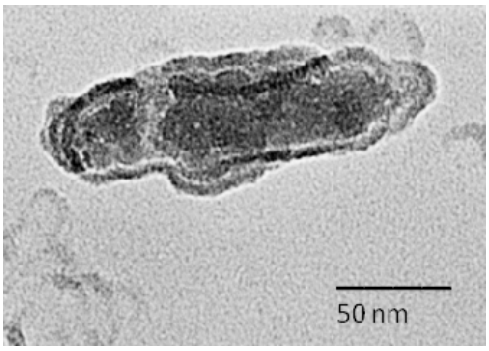

Figure S5. Representative micrograph of encapsulated pDNA/cationic micelles complex.

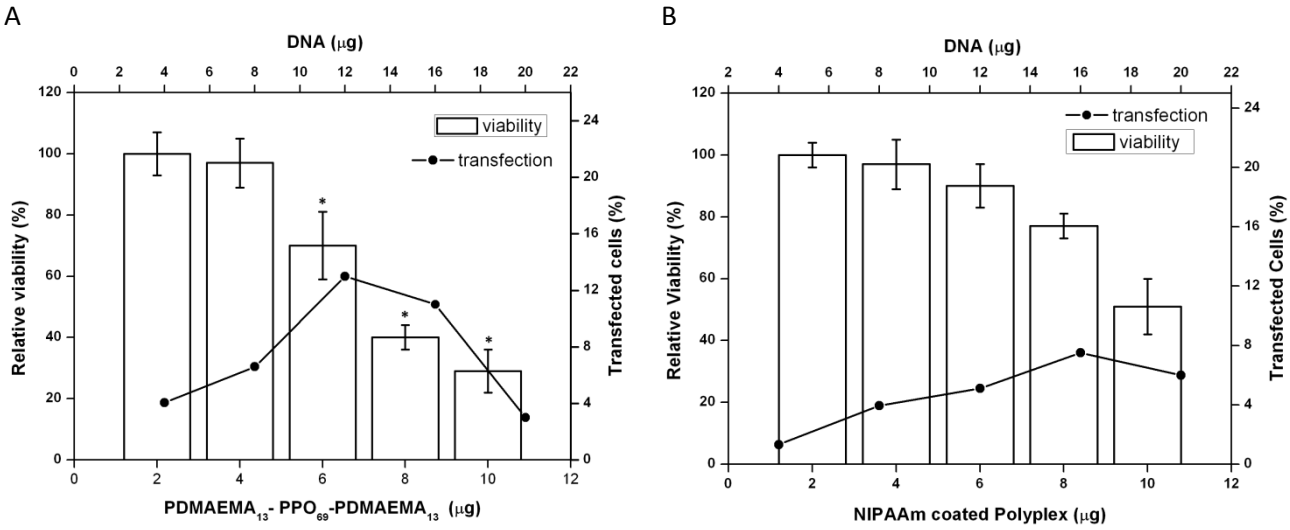

Figure S6. Transfection efficiency of naked polyplex (A) and PNIAAm coated polyplex (B). HEK 293 cells were transfected with different amount pEGFP-C2 containing polyplexes and 24h following transfection the percentage of GFP-positive cells were assessed by flow cytometry and the viability of the cells was measured by MTT assay at same time. Data are means of 3

independent experiments, and error bars represent the SD. *, $p<0.05$ against control, non-transfected cells.

References:

1. P. Petrov, C.B. Tsvetanov and R. Jerome, *Polym. Int.*, 2008, **57**, 1258.

2. T. Mosmann, *J. Immunol. Methods*, 1983, **65**, 55.