Electronic Supplementary Information

Shape Transformation Following Reduction-Sensitive PEG Cleavage of Polymer/DNA Nanoparticles

DOI: 10.1039/

John-Michael Williford,[a,d,e] Yong Ren,[b,d,e] Kevin Huang, [c] Deng Pan,[a] and Hai-Quan Mao[b,d,e] *

[a]Department of Biomedical Engineering, Johns Hopkins University School of Medicine, Baltimore, MD 21205;

[b]Department of Materials Science and Engineering, Johns Hopkins University, Baltimore, MD 21218;

[c]Department of Chemical and Biomolecular Engineering, Johns Hopkins University, Baltimore, MD 21218;

[d]Institute for NanoBioTechnology, Johns Hopkins University, Baltimore, MD 21218;

[e]Translational Tissue Engineering Center, Johns Hopkins University, Baltimore, MD 21287.

* Corresponding author. Email: hmao@jhu.edu

Experimental Methods

Materials

Linear polyethylenimine HCl salt (*l*PEI·HCl, molecular weight of *l*PEI 9 kDa) was a gift from Polymer Chemistry Innovations Inc. (Tucson, AZ). Aminopropyl polyethylene glycol (PEG-NH_2, MW 10 kDa) was purchased from NOF corporation (Irvine, CA). N-Succinimidyl 3-[2-pyridyldithio]-propionate (SPDP) and Sulfosuccinimidyl 6-(3'-[2-pyridyldithio]-propionamido)hexanoate (Sulfo-LC-SPDP) were purchased from ProteoChem, Inc. (Loves Park, IL). Dithiothreitol (DTT) was purchased from Thermo Fisher Scientific Inc. (Waltham, MA). Dichloromethane (DCM), triethylamine (TEA), and all other reagents were purchased from Sigma-Aldrich (St. Louis, MO), unless otherwise specified.

Synthesis of Reduction-Cleavable lPEI-g-PEG copolymer

*l*PEI·HCl (30 mg, 0.377 mmol amine) was dissolved in 3.76 mL DI water. The pH of *l*PEI solution was adjusted to 6 with 1 M NaOH. Sulfo-LC-SPDP (7.94 mg, 0.0151 mmol) was dissolved in 100 µL water rapidly added to the *l*PEI solution. The reaction mixture was vortexed overnight at room temperature. The product was dialyzed in dialysis tubing (MWCO 3500, Spectrapor, Spectrum Labs, CA) against DI water overnight with frequent water changes. The obtained solution was lyophilized to yield *l*PEI-SPDP, and the SPDP content in the polymer was determined by measuring the UV absorption change at 343 nm after addition of DTT solution to a final concentration of 20 mM.

PEG-NH_2 (MW 10,000 Da, 200 mg, 20 µmol) was dissolved in 0.5 mL of anhydrous DCM. 7 µL TEA was added to PEG solution. SPDP (9.37 mg, 30 µmol) was dissolved in 0.5 mL of

DCM and then mixed with PEG-NH_2 solution. The reaction mixture was vortexed overnight. DCM was evaporated by vacuum and remaining reaction mixture was precipitated in 1.5 mL of ether to yield PEG-SPDP. The product was purified by dissolution/precipitation cycle in DCM/ether for 3 times and then vacuumed to remove all solvents. The final PEG-SPDP was characterized by ^{1}H-NMR.

PEG-SPDP (8 mg, 0.8 μmol) was dissolved in 0.1 mL 0.04 M EDTA solution and mixed with 25 μL of 150 mM DTT solution to yield PEG-SH. After vortexing for 2 hours, the reaction mixture was dialyzed against 1 L of 0.04 M EDTA solution overnight to remove excess DTT. The purified PEG-SH solution was then mixed with *l*PEI-SPDP solution at different ratios according to the designed grafting degree. The obtained graft polymer, *l*PEI-*g*-PEG, was characterized by an Agilent 1200 Series Isocratic gel permeation chromatography (GPC) System coupled with a multi-angle light scattering detector (MiniDawn, Wyatt Technology, Santa Barbara, CA). The PDI of the *l*PEI-*g*-PEG copolymers was characterized to be 1.197, 1.413, and 1.342 for 0.5% PEG grafting degree, 1% PEG grafting degree, and 2% PEG grafting degree, respectively.

Synthesis of Non-Cleavable lPEI-g-PEG copolymer

N-hydroxysuccinimidyl ester of methoxy polyethylene glycol hexanoic acid (PEG-NHS, M_n = 10 kDa) was purchased from NOF America Corporation (White Plains, NY).

The *l*PEI·HCl (7.95 mg, 0.1 mmol of amine) was dissolved in 1 mL of DI water, and the pH of the solution was adjusted to 6 through drop-wise addition of 1 M NaOH solution. The solution was then mixed with 40 mg of PEG-NHS and incubated overnight for a designed 2% PEG grafting degree. The reaction mixture was dialyzed against DI water and lyophilized to yield a white foam-like solid with a 95% yield. The molecular weight of the graft copolymer was

characterized by gel permeation chromatography. The PDI of the non-cleavable *l*PEI-*g*-PEG was found to be 1.368.

Degradation of lPEI-g-PEG copolymer under reducing conditions

To investigate the concentration-dependent degradation of the copolymer, *l*PEI-*g*-PEG (1%) samples (0.5 mg for each sample) were dissolved in 1 mL of DTT solutions at final concentrations of 0.1, 0.3, 1, 3, 10, 30 and 100 mM, respectively. The samples were incubated for 1 h, and the degradation of polymer was characterized by GPC. To investigate the time-dependent degradation of the copolymer, *l*PEI-*g*-PEG (1%) samples (0.5 mg for each sample) were dissolved in 1 mL of DTT solution at the concentration of 1 mM and incubated for 0.5, 0.75, 1, 2, 3, 4, 16 and 24 h, respectively. The degradation of the polymers was characterized by GPC where fraction of degradation is equal to the cleaved disulfide bond number divided by the total disulfide bond number.

Preparation of plasmid DNA

Plasmid DNA, VR1255C (6400 kb), encoding the gene for firefly luciferase driven by the cytomegalovirus promoter, was kindly provided by Vical (San Diego, CA). Plasmid DNA was amplified in DH5α E. coli and was purified using an EndoFree Giga Kit (Qiagen, Valencia, CA) and dissolved at 1 mg/mL in endotoxin-free TE buffer.

Preparation of lPEI-g-PEG/DNA Nanoparticles

For a typical nanoparticle preparation, 10 μg DNA was diluted in 100 μL DI water for a final concentration of 100 μg/mL DNA. A solution of *l*PEI-*g*-PEG was diluted to 100 μL in DI water

to give a final N/P ratio (ratio of amine in *l*PEI to phosphate in DNA) of 8. The polymer solution was added to the DNA solution and mixed by rapid pipetting, after which the polymer/DNA mixture was incubated for 10 min prior to further use to allow for nanoparticle formation.

Transmission Electron Microscopy (TEM) Imaging of Nanoparticles

Samples for TEM imaging were prepared by adding 10 μL of *l*PEI-g-PEG/DNA nanoparticle solution onto an ionized nickel grid covered with a carbon film. After 10 min, the solution was pipetted away, and a 6-μL drop of 2% uranyl acetate was added to the grid. After 30 s, the solution was removed, and the grid was left to dry at room temperature. The samples were then imaged using a Technai FEI-12 electron microscope.

Nanoparticle Zeta Potential Measurements

Nanoparticle zeta potential was characterized using a Zetasizer Nano ZS90 (Malvern Instruments, Southborough, MA). An aliquot of 10 μg DNA nanoparticle solution was diluted to 800 μL with DI water, added to a DTS1060-folded capillary cell, and measured in the automatic mode

Nanoparticle Shape Transformation and Kinetics

Nanoparticles were prepared according to the typical preparation described above. To evaluate the nanoparticle shape transformation, dithiothreitol (DTT) was dissolved in DI water to give a concentration of 150 mM. DTT solution was then added to the nanoparticle solution to give a final DTT concentration of 5 mM. Characterization of nanoparticle shape transformation and PEG cleavage was then characterized using TEM imaging and zeta potential measurements after 2 h incubation with DTT. Nanoparticle sizes were characterized from TEM images using

Image J 1.44. Aspect ratios were calculated by dividing the length of the nanoparticle by the diameter. At least 100 nanoparticles were measured from TEM images at each condition. For nanoparticle shape transformation kinetics, particles were prepared as previously described, and 5 mM DTT was added to the solution. At pre-defined time points, maleimide solution was added to the nanoparticle solution at a final concentration of 10 mM to quench the DTT reaction. TEM imaging was then performed to analyze nanoparticle shape.

Nanoparticle stability in salt and serum-containing media

Nanoparticles were prepared as previously described. To test the stability in salt, a determined amount of NaCl solution (5 M) was added to the nanoparticle solution to give a final salt concentration of 150 mM. Particles were incubated for 2 h, after which TEM imaging was performed to evaluate nanoparticle shape. To test the stability in serum, nanoparticle solution was incubated in 10% (v/v) fetal bovine serum for 2 h, after which TEM imaging was performed.

Gel Electrophoresis of lPEI-g-PEG/DNA Nanoparticles

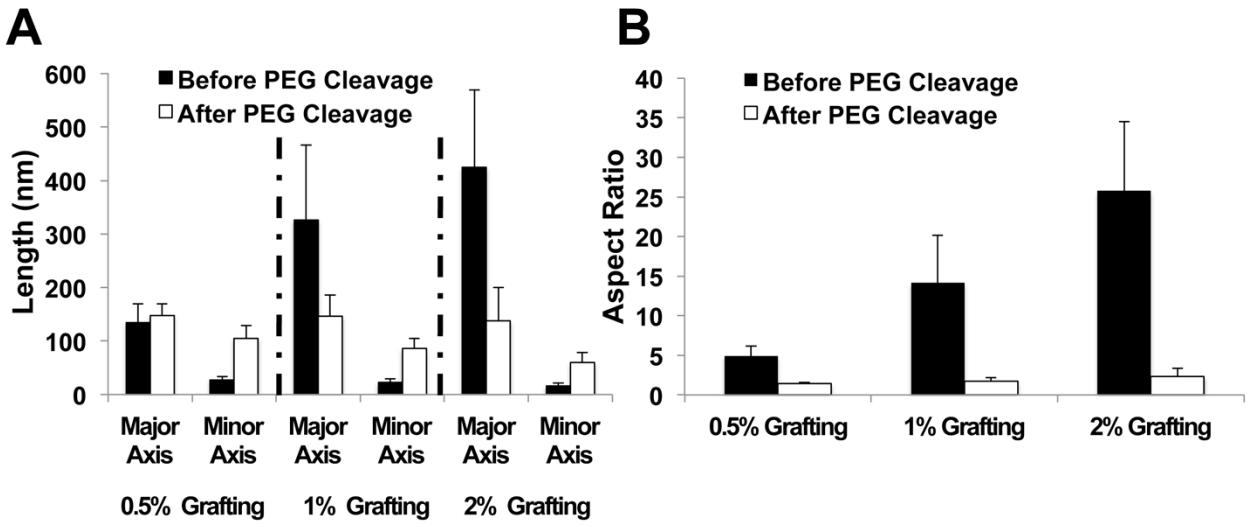
To evaluate the DNA compaction ability of the nanoparticles, gel electrophoresis was performed. Nanoparticle samples were either incubated in the presence or absence of 5 mM DTT for 2 h, after which 0.5 μg DNA dose was added to each well and analyzed by electrophoresis at 80 V for 45 min on a 0.8% (w/v) agarose gel. DNA bands were visualized under a UV transilluminator.

In Vitro Transfection of lPEI-g-PEG/DNA Nanoparticles

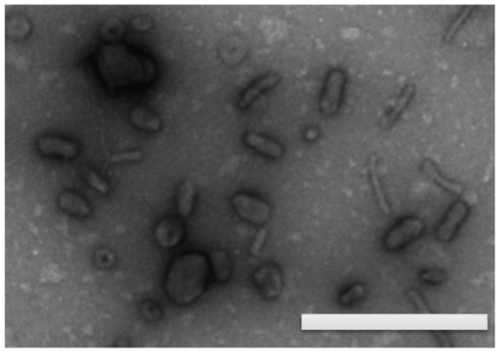
HeLa cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 100 U/mL Penicillin / 100 μg/mL

Streptomycin at 37°C and 5% CO_2. At 24 h prior to the experiment, cells were seeded in 24-well plates at a density of 5×10^4 cells/well. Nanoparticle solutions were pre-incubated in the presence or absence of 5 mM DTT, after which 2 μg DNA dose was added to each well. The culture media was replaced 4 h after nanoparticle addition. 48 h later, media was removed, cells were washed with 1× PBS (pH 7.4), and 200 μL reporter lysis buffer (Promega, Madison, WI) was added to each well. Cells were then subjected to two freeze-thaw cycles. 20 μL cell lysate from each well was assayed using a luciferase assay kit (Promega, Madison, WI) on a luminometer (20/20n, Turner BioSystems, Sunnyvale, CA). The luciferase activity was converted to the amount of luciferase expressed using a recombinant luciferase protein (Promega) as the standard and normalized against the total protein content in the lysate using a BCA assay (Pierce, Rockford, IL)

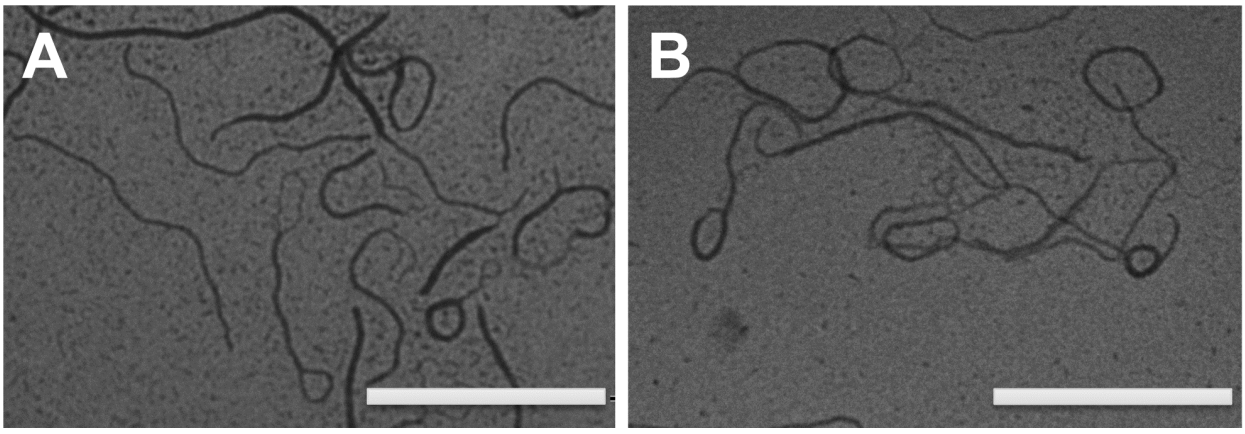
Supplementary Figures



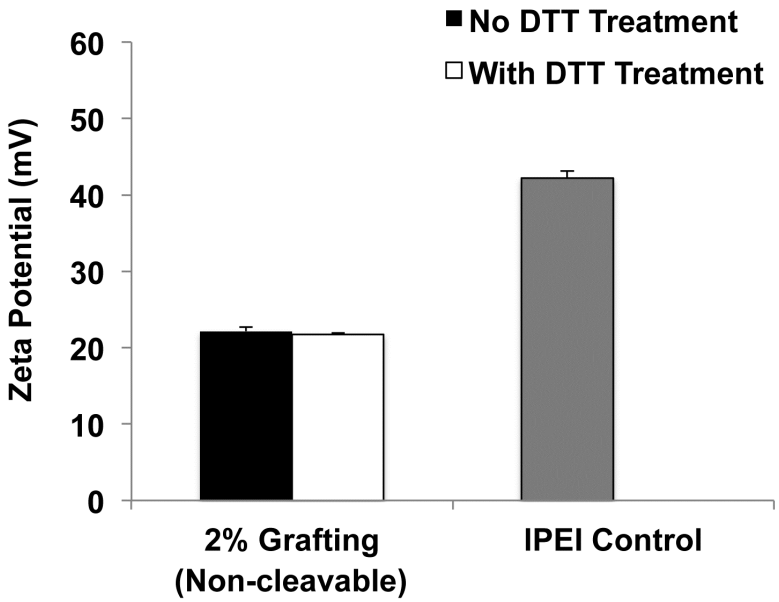
Supplementary Figure S1. (A) Average major and minor axis lengths and (B) aspect ratios of *l*PEI-*g*-PEG/DNA nanoparticles before and after PEG cleavage. Each bar represents mean ± standard division (n >100 particles, from TEM images).



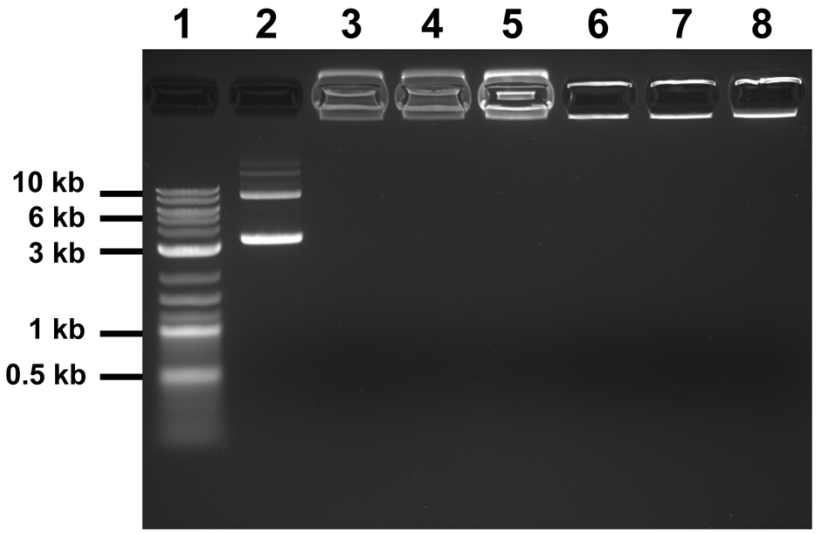
Supplementary Figure S2. TEM images of *l*PEI/DNA nanoparticles. All scale bars represent 500 nm.



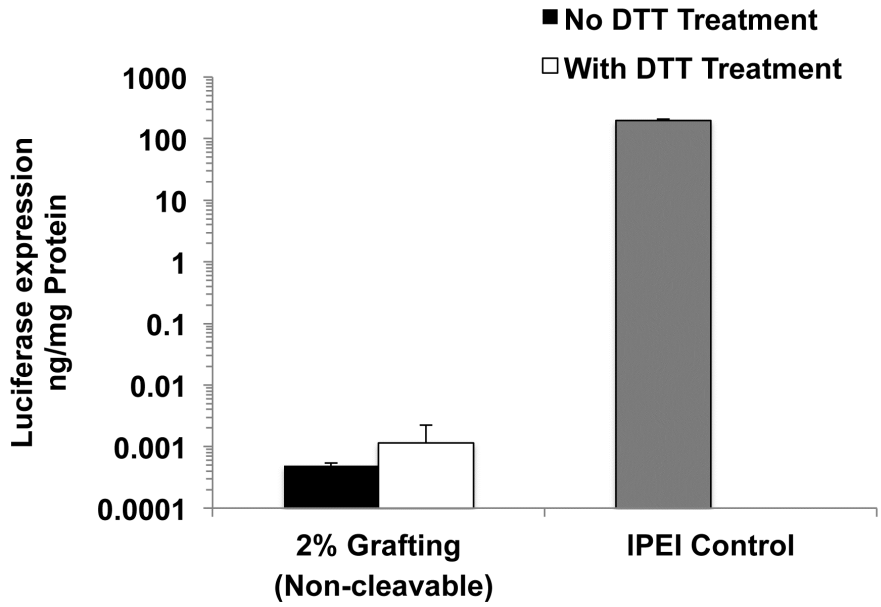
Supplementary Figure S3. TEM images of non-cleavable *l*PEI-*g*-PEG/DNA nanoparticles (A) before incubation and (B) after incubation with 5 mM DTT for 2 h. All scale bars represent 500 nm.



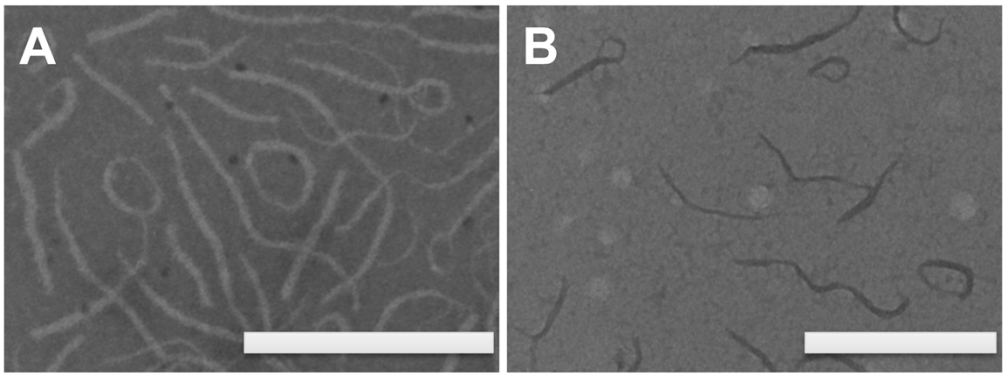
Supplementary Figure S4. Average zeta potential of non-cleavable *l*PEI-*g*-PEG/DNA nanoparticles in the presence and absence of 5 mM DTT. Each bar represents mean ± standard deviation (n = 3).



Supplementary Figure S5. Stability of *l*PEI-*g*-PEG/DNA nanoparticles before and after PEG cleavage as characterized by gel electrophoresis. Lane 1: DNA ladder; Lane 2: VR1255 *p*DNA control; Lanes 3–5: *l*PEI-*g*-PEG/DNA nanoparticles with 0.5%, 1%, and 2% PEG grafting before PEG cleavage by 5 mM DTT; Lanes 6–8: *l*PEI-*g*-PEG/DNA nanoparticles with 0.5%, 1%, and 2% PEG grafting degree after PEG cleavage by 5 mM DTT.



Supplementary Figure S6. Transfection efficiency of non-cleavable *l*PEI-*g*-PEG/DNA nanoparticles in the presence and absence of 5 mM DTT as compared to to *l*PEI/DNA control nanoparticles in HeLa cells. Each bar represents mean ± standard deviation (n=4).



Supplementary Figure S7. TEM images of *l*PEI-*g*-PEG/DNA nanoparticles following incubation with (A) 150 mM NaCl and (B) 10% FBS for 2 h. All scale bars represent 500 nm.