Submitted to ADVANCED MATERIALS

Supporting Information

DOI: 10.1002/adma.

Dual-sensitive Micellar Nanoparticles Regulate DNA Unpacking and Enhance Gene Delivery Efficiency

By *Xuan Jiang, Yiran Zheng, Hunter H. Chen, Kam W. Leong, Tza-Huei Wang, and Hai-Quan Mao**

[*] Corresponding author contact: Prof. Hai-Quan Mao, Department of Materials Science and Engineering and Whitaker Biomedical Engineering Institute, Johns Hopkins University, 206 Maryland Hall, Baltimore, MD 21218, U.S.A. Tel: 410-516-8792; Fax: 410-516-5293; Email: hmao@jhu.edu.

Additional Experimental Methods

Synthesis of PEG_{12K}-b-PPA_{128K} and thiolated PEG_{12K}-b-PPA_{128K} copolymers

PEG_{12K}-*b*-PPA_{128K} was prepared as described in the previous report [1]. For thiolation of this copolymer, PEG_{12K}-*b*-PPA_{128K} (10 mg) polymer was dissolved in 75 mM pH8.0 PBS-2 mM EDTA buffer (purged with argon to remove dissolved oxygen) to achieve a concentration of 20 mg mL^{-1}. Calculated volume of Traut's reagent dissolved in the same buffer at a concentration of mg mL^{-1} was mixed with PEG_{12K}-*b*-PPA_{128K} polymer solution and reacted overnight under stirring. The reaction mixture was then dialyzed against 2 mM EDTA solution (purged with argon) for 24 h through a dialysis membrane with MWCO of 3,500 Da (Spectrapor, Rancho Dominguez, CA). The final product was obtained after lyophilization. The grafting degree of free thiol group was determined by using 4-(amino-sulfonyl)-7-fluoro-2,1,3-benzoxadiazole (ABD-F) reagent according to a reported method [12].

Preparation and characterization of uncrosslinked and dual-sensitive micelles

For a typical preparation of PEG_{12K}-*b*-PPA_{128K}/DNA micelles, VR1255 plasmid DNA solution (250 µL) in DI water at a concentration of 100 µg mL^{-1} was added to an equal volume of PEG_{12K}-*b*-PPA_{128K} solution in DI water at various concentrations, which corresponded to different N/P ratios. The mixture was vortexed for 20 sec, incubated for 30 min at room temperature. For crosslinked micelles, plasmid DNA was incubated with thiolated copolymer similarly [9]; and then the micelle suspension was transferred to a Biotech-grade RC float-dialyzer with MWCO of 3,500 Da (Spectrapor, Rancho Dominguez, CA) and dialyzed against 0.5% (v/v) DMSO for 24 h to oxidize thiol groups. Complete oxidization and removal of residual DMSO was then achieved by dialyzing the micelle solution against DI water for additional 48 h.

The oxidization degrees of thiol groups in the micelles were analyzed with Ellman's reagent [13] at 24 h and 48 h after oxidization in 0.5% (v/v) DMSO/water mixture. The results indicated that the oxidization degree after 48 h (78%) increased only slightly compared with that (73%) after 24 h. Neverless, after the micelles were further crosslinked by aerial oxidization in water for another 48 h, the oxidization of free thiol groups reached completion. Therefore, the aerial oxidization step is necessary for not only the removal of DMSO but also the completion of crosslinking.

The particle size and surface charge of micelles were measured by photon correlation spectroscopy and laser Doppler anemometry, respectively, using a ZS90 Zetasizer (Malvern Instruments, Southborough, MA). For transmission electron microscopy (TEM) imaging of micelles was conducted according to the method described previously [1].

In vitro gene transfection

HEK293 cells (American Type Culture Collection, Manassas, VA) were maintained in Dulbecco's Modified Eagle's Medium supplemented with 10% fetal calf serum at 37°C and 5% CO_2. Cells were seeded in 24-well plates at a density of 8×10^4 cells per well and incubated for one day. The transfections with all nanoparticles were carried out in the presence of 10% fetal calf serum. PEG_{12K}-*b*-PPA_{128K}/DNA micelles or crosslinked micelles were added to each well at a dose of 1 μg of plasmid DNA in 10% serum-containing medium. After 4 h of incubation, the culture media were refreshed with complete serum-containing medium. One day and three days later, cells were washed with 0.5 mL of phosphate buffered saline (pH 7.4) and lysed with a reporter lysis buffer (0.2 mL per well, Promega, Madison, WI). The suspensions were centrifuged at 14,000 rpm for 5 min. Twenty μL of cell lysate supernatant was mixed with 100 μL of luciferase substrate (Promega), and the light units were measured on a luminometer (2020n, Sunnyvale, USA). The concentration of luciferase (ng mL^{-1}) in the suspension was then calculated using a standard curve generated from recombinant luciferase. To evaluate the transfection efficiency on days 5, 7 and 10, another set of cells transfected with nanoparticles were split at 1:5 ratio on day 3 to prevent over-confluence of cells. On day 5, the cells were split again 1:5 ratio and 80% of the cells were harvested for the measurement of luciferase activity. On day 10, all the cells were harvested for the measurement of luciferase activity. To compare the total transgene expression level on days 5, 7 and 10 with that at earlier time points, the luciferase activity obtained on day 5, day 7 and day 10 were multiplied by factors of 5, 25 and 125, respectively. Total transgene expression level was used to compare the relative transfection efficiency, since we observed much slower proliferation of PEI/DNA nanoparticle-transfected cells over the longer culture period in comparison with those transfected with crosslinked micelles and uncrosslinked micelles. By day 7, very few cells remained in the PEI/DNA nanoparticle-transfected wells. This was likely due to the intrinsic toxicity of PEI.

Labeling of plasmid DNA and copolymers for intracellular trafficking study

Plasmid DNA (pEGFP-C1, 4.9 kilobase, Clontech, Mountain View, CA) was labeled with streptavidin-functionalized QD605 (Qdot 605 ITK; Invitrogen, Carlsbad, CA) via a biotin–streptavidin linkage as described previously [14]. The primary amines of PEG_{12K}-b-PPA_{128K} or thiolated- PEG_{12K}-*b*-PPA_{128K} polymer was labeled with N-hydroxysuccinimide–functionalized Cy5 (Amersham Biosciences, Piscataway, NJ) using carbodiimide chemistry. A solution of Cy5 dye was gradually added to aqueous solutions of copolymer and stirred continuously for at least 12 h. Unreacted Cy5 was removed by dialyzing against DI water through a membrane with MWCO of 3,500 Da. The level of Cy5 labeling on copolymer was controlled to 0.5% of total primary amine groups of PEG_{12K}-*b*-PPA_{128K} or thiolated-PEG_{12K}-*b*-PPA_{128K} copolymer analyzed by measuring the absorbance of Cy5 at 650 nm.

Live cell imaging for intracellular trafficking study

HEK293 cells were seeded (1×10^5 cells per well) and grown on chambered coverglasses (4-well Labtek II; Nalge Nunc, Rochester, NY) in complete media (minimal essential medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 50 U mL^{-1} penicillin, and 50 U mL^{-1} streptomycin; Gibco Invitrogen, Carlsbad, CA). Duplicate wells of 60-70% confluent cells

were transfected with QD-FRET polyplexes containing 0.5 µg DNA in 0.5 mL of reduced-serum media (Opti-MEM; Gibco Invitrogen, Carlsbad, CA) at 37°C. At specific time points after transfection (2 and 4 h), the transfection medium was replaced and live cells were imaged on a confocal microscope (LSM 510 Meta; Carl Zeiss, Thornwood, NY) equipped with a × 63 objective (numerical aperture 1.40) and heated stage. Immediately prior to imaging, cells were stained with nuclear dye (Hoechst 33342, Molecular Probes, Carlsbad, CA) and a dye specific for acidic vesicles (LysoTracker Green DND-26; Molecular Probes, Carlsbad, CA). Imaging was performed in the multi-track mode using 405-nm excitation for Hoechst (emission band pass 420–480 nm) and 488-nm excitation for LysoTracker (bandpass 505–530 nm), QD605 (Meta 593–615 nm), and Cy5 (Meta 636–754 nm). The main dichroic was Haupt FarbvTeiler 405/488/543 and the secondary dichroic was Neben Farb Teiler 545. For all cells, image stacks were composed of at least 20 Z-sections, which was optimal for image-based analysis [15].

Quantification of distribution of complexed or unpacked DNA

An image-based quantification method was adapted from a previous report using a pixel-based algorithm [15]. The approach used in this study takes full advantage of the 3D image data stacks obtained by confocal microscopy from which particles are isolated as individual 3D objects. For each time point, at least 30 cells containing at least one particle were randomly selected. This number of cells was determined to be statistically sufficient and representative for image-based analysis. Each individual cell was outlined with a corresponding phase image and the volumes of all internalized objects were determined in voxels where v, the volume per ith particle, was summed over of N total particles to obtain V, the total volume per cellular compartment (Equation 2). The type of each object [t: complexed (C) or unpacked (U) DNA] was identified digitally by QD-FRET, without needing to calculate or normalize FRET efficiencies [14]. Specifically, objects exhibiting FRET-mediated Cy5 signals were considered as complexed DNA within micelles, and objects exhibiting only QD signals were considered as unpacked DNA. Threshold levels were based on background intensities of the cell. The subcellular location [k: cytosol (cyto), endo/lysosome (endo/lyso), or nucleus (nuc)] of each object was determined by colocalization with nuclear and endo/lysosomal-specific dyes and mass balance for the entire cell (Equation 3). Image processing and volume determinations were performed with a custom script using Image J (v1.37; http://rsb.info.nih.gov/ij). Volume fractions of unpacked DNA (f_U) for the entire cell were calculated using Equation 4, which was normalized to all internalized micelles. Volume fractions across all compartments $f_U(k)$ were calculated using Equation 5, which was normalized to all internalized particles of the same type in the cell. Volume fractions in specific compartments $f_U^{'}(k)$ were calculated using Equation 6, which was normalized to all particles in the same compartment. At each time point, the fraction of cells within an analyzed cohort having a particular binned volume fraction of free plasmid DNA (f_U) was calculated to generate histograms as shown in **Figure 4**.

$$V_t(k) = \sum_i^N v_{i,t}\,(k), \qquad \text{(Equation 2)}$$

where $t = U\ (for\ unpacked\ DNA)\ or\ C\ (for\ complexed\ DNA)$

$$V_{t,Total} = V_t(cyto) + V_t(endo/lyso) + V_t(nuc) \qquad \text{(Equation 3)}$$

$$f_U = V_U/[V_{U,total} + V_{C,total}] \qquad \text{(Equation 4)}$$

$$f_U(k) = V_U(k)/V_{U,total} \qquad \text{(Equation 5)}$$

$$f_U^{'}(k) = V_U(k)/[V_U(k) + V_C(k)] \qquad \text{(Equation 6)}$$

where $k = endo/lyso\ (for\ the\ endolysosomal\ compartment)$

$k = cyto\ (for\ the\ cytosols)$

$k = nuc\ (for\ the\ nucleus)$

Additional References:

[12] C. C. Q. Chin, F. Wold, Anal. Biochem. **1993**, *214*, 128-134.
[13] K. Kuwata, M. Uebori, K. Yamada, Y. Yamazaki, Anal. Chem. **1982**, *54*, 1082-1087.
[14] Y. P. Ho, H. H. Chen, K. W. Leong, T. H. Wang, J. Controlled Release **2006**, *116*, 83-89.
[15] H. Akita, R. Ito, I. A. Khalil, S. Futaki, H. Harashima, Mol. Ther. **2004**, *9*, 443-451.

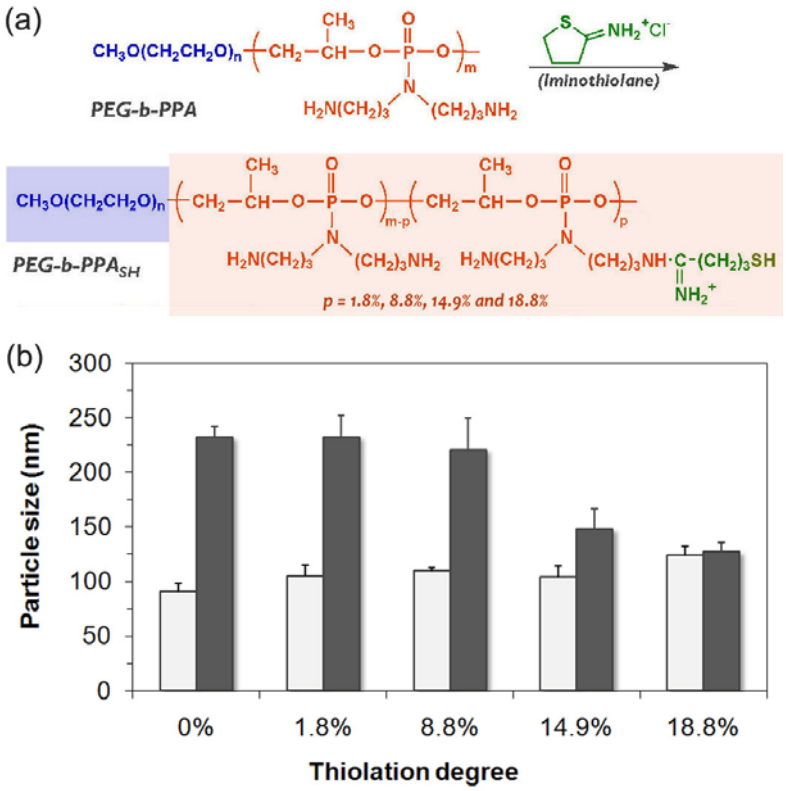

Figure S1. (a). Synthesis of thiolated PEG_{12K}-b-PPA_{128K} copolymers with different thiolation degrees. (b). Complex stability of the crosslinked micelles prepared from copolymers with different thiolation degrees. Average particle size of different sets of crosslinked micelles in deionized water (open bar) and in the presence of 0.15 M NaCl (grey bar).

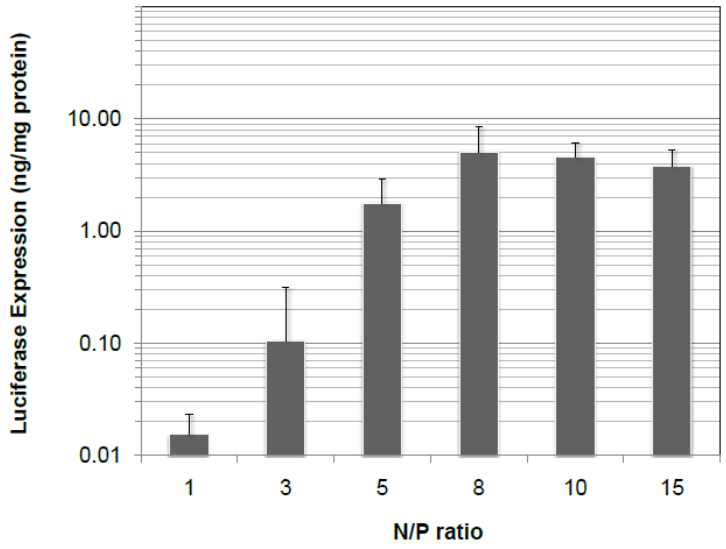

Figure S2. Transfection efficiency of PEG_{12K}-*b*-PPA_{128K}/DNA micelles at different N/P ratios. Each data point represents average ± standard division (n = 4).

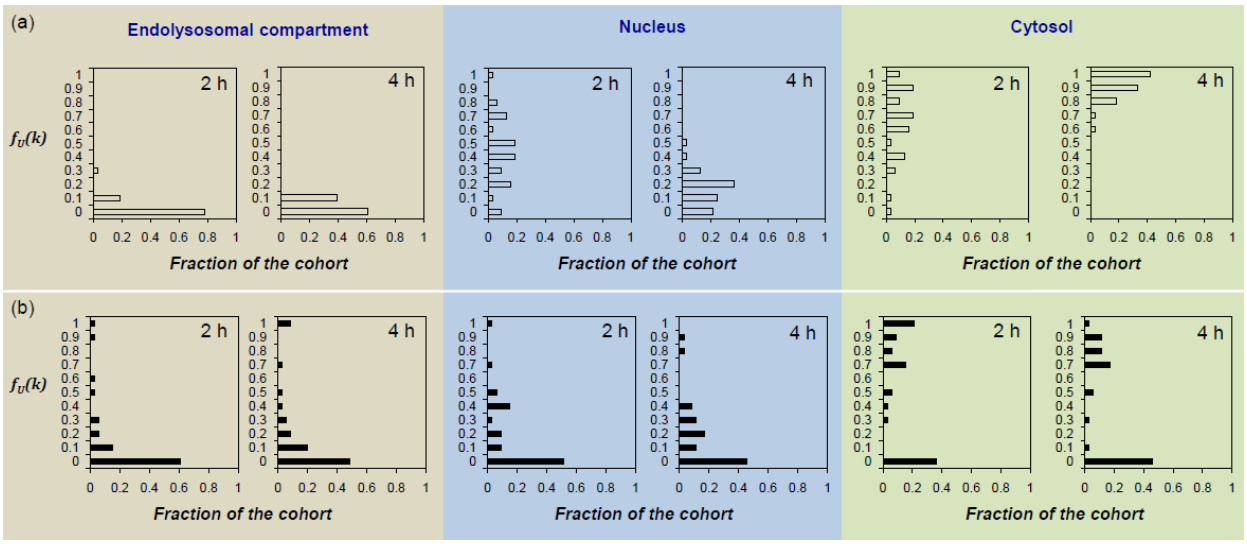

Figure S3. Distribution of unpacked DNA among different compartments from uncrosslinked micelles (a), and dual-sensitive micelles (b) at 2 h and 4 h post-transfection. At each time point, histograms show the fraction of an analyzed cohort of cells (30-34 cells) having a particular binned volume fraction of unpacked DNA $[f_U(k)]$ in the endolysosomal compartments $[f_U(endolyso)]$, cytosolic $[f_U(cyto)]$, and nuclear compartments $[f_U(nuc)]$ as determined by quantitative image-based analysis and normalized to the total unpacked DNA inside the cell (Eq. 5).

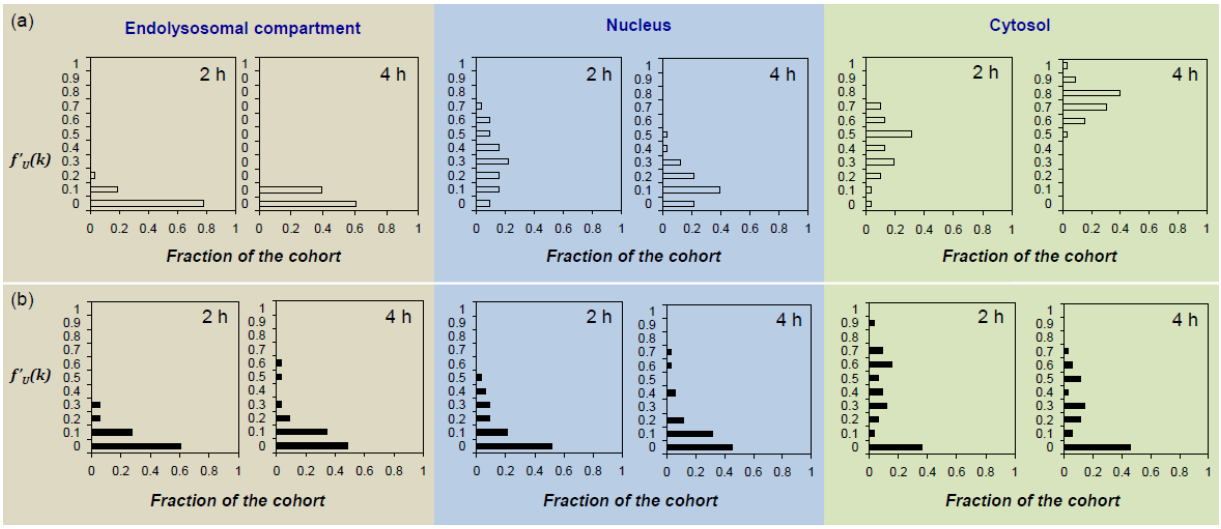

Figure S4. The fraction of unpacked DNA among all DNA in different compartments from uncrosslinked micelles (a) and dual-sensitive micelles (b) at 2 and 4 h post-transfection. At each time point, histograms show the fraction of an analyzed cohort of cells (30–34 cells) having a particular binned volume fraction of unpacked DNA $[f_U^{'}(k)]$ in the endolysosomal $[f_U^{'}(endolyso)]]$, cytosolic $[f_U^{'}(cyto)]$, and nuclear compartments $[f_U^{'}(nuc)]$ as determined by quantitative image-based analysis and normalized to the total amount of DNA taken-up within the compartment (Eq. 6).