Electronic Supplementary Material (ESI) for Biomaterials Science.

Supporting Information for

A Potent, Minimally Invasive and Simple Strategy of Enhancing Intracellular Targeted Delivery of Tat Peptide-conjugated Quantum Dots: Organic Solvent-based Permeation Enhancer

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This Supporting Information document contains the following:

EXPERIMENTAL METHODS

Materials. QDs-PEG (catalogue number QMG-600) and QDs-COOH (catalogue number QSH-540) were purchased from Ocean Nanotech. Tat peptide (sequence YGRKKRRQRRR) was purchased from ChinaPeptides. Tat peptide-conjugated QDs (QDs-Tat) were formed by conjugating QDs-COOH with Tat peptide (molar ratio of QDs-COOH to Tat peptide used in the bioconjugation reaction 1:20) via 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) chemistry. EDC, N-hydroxysuccinimide (NHS), and chloroquine were purchased from Sigma-Aldrich. Dimethyl sulfoxide (DMSO), dimethylformamide (DMF), ethanol, acetone, and tetrahydrofuran (THF) were purchased from Sinopharm Chemical Reagent. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), DiO, and calcein were purchased from KeyGEN BioTECH. Hoechst 33342 was purchased from ThermoFisher Scientific. Cytochalasin D was purchased from Shanghai BaiLi Biotechnology. Doxorubicin (DOX) was purchased from Sigma-Aldrich. Cell lines (HeLa, MCF-7, A549, and Hep G2 cells) and their culture media were purchased from KeyGEN BioTECH.

Live cell spinning-disk confocal microscopy. HeLa cells were used for most of the cellular experiments and were maintained in Dulbecco's modified eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) at 37 ^{o}C with 5% CO_2, as recommended by the manufacturer. Other cell lines used (MCF-7, Hep G2, and A549) were also cultured in conditions recommended by the manufacturer. Live cell imaging studies were performed using a live cell spinning-disk confocal imaging system

consisting of a cell incubation chamber (IX3W, Tokai Hit), an epi-fluorescent microscope (IX-83, Olympus), a spinning-disk confocal system (Andor), and an electron multiplying charge-coupled device (EMCCD) camera (Evolve 512, Photometrics). The cells were first seeded at ~35% confluency (in the log phase of cell growth) on a glass bottom cell culture dish (0.17 mm thickness for the glass bottom) (Nest, China). Following an 24 h incubation period at the conditions of 37 ^{o}C and 5% CO_2, the cell culture medium was replaced by a dispersion of water-soluble QDs (2 nM, QDs-Tat, unless specified otherwise) with (or without) organic cosolvent (1% DMSO unless specified otherwise). After incubation with QDs for a specific time duration (24 h unless specified otherwise), cell culture medium was withdrawn and cells were washed by phosphate buffered saline (PBS) three times to remove the QDs outside the cells or adsorbed to the cell surface before imaging. To counter-stain the cell nucleus, right before imaging (at a particular time point of cellular transport), the fluorescent dye Hoechst 33342 (blue fluorescent color, 5 μM in cell culture medium) was incubated with live cells for 20 min. Image processing and analysis was conducted using the MetaMorph and Image J software.

Measurement of intracellular targeting. Intracellular targeting of QDs to the cell nucleus was measured as the ratio of the amount of QDs delivered to the cell nucleus to the total amount of QDs in the cell. The measurement was performed with the live cell spinning-disk confocal imaging system. The amount of QDs in the cell nucleus or cell was quantified by measuring the fluorescence intensity generated by the QDs in the respective area.

Endocytosis inhibition studies. To inhibit endocytosis by low temperature, cells were incubated at 4 ºC in the cell culture medium for 1 h. Subsequently the medium was replaced with a nanoparticle formulation (in cell culture medium). To inhibit endocytosis by chemical means, cells were treated with cytochalasin D-containing cell culture medium (2 μM) for 30 min. Subsequently the medium was replaced with a nanoparticle formulation (in cell culture medium). After 1 h of nanoparticle delivery, the cells were washed with phosphate buffer solution five times before imaging using the live cell spinning-disk confocal microscopy system.

Cell membrane leakage studies (LDH release assay). HeLa cells were seeded on 96-well plates (Corning Costar, China) with the density of 6000 cells/well. Following 24 h of cell culture, the culture medium was replaced by the formulation for testing (e.g. 2 nM QDs-Tat with 1% DMSO; 200 μL/well, in complete DMEM). Following 24 h of incubation, the supernatant (120 μl/well) was collected and the released lactate dehydrogenase (LDH) was analyzed by a commercial kit (C0017, Beyotime, China). The LDH release value by the cells treated with the LDH releasing agent was set as 100% LDH release (i.e., total LDH amount in intact cells); the LDH release value by untreated cells was set as negative control. Optical density was measured by an ELISA reader (RT-6000, Rayto, China) at a wavelength of 490 nm (test) and 630nm (reference). The relative LDH release is defined by the ratio of LDH released over total LDH in intact cells. Cell samples with less than 10% LDH release are regarded as cells with intact cell membranes, following well-established criterion.[1-3] All samples were run in quintuplicate.

Cell viability analysis (MTT assay). HeLa cells were seeded on 96-well plates (Corning Costar, China) with the density of 6000 cells/well. After 24 h of incubation, the cell culture medium was replaced by the formulation for testing (e.g. 2 nM QDs-Tat with 1% DMSO; 200 μL/well, in complete DMEM). Following cell culture for 24 h, 20 μl 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution (5 mg/ml) and 180 μl DMEM were added to each well and incubated for 4 h at 37^{o}C. After the medium was removed, the insoluble formazan crystals were dissolved in 150 μl/well DMSO and measured spectrophotometrically in an ELISA reader (RT-6000, Rayto, China) at a wavelength of 570 nm. The relative cell viability (%) compared to that of the control well containing cell culture medium only (in addition to the cells) was calculated by the optical density of the test well divided by that of the control well. All samples were run in quintuplicate.

Vesicle colocalization study. The lipophilic fluorescent dye DiO (5 μM) was incubated with live cells for 30 min to label intracellular vesicles. At given time points, the cells were washed with PBS for three times before imaging with the live cell spinning-disk confocal imaging system. Colocalization with fluorescent nanoparticles was quantified using the Mander's correlation coefficient.

Vesicle membrane integrity study. The water-soluble fluorescent dye calcein was used to examine the integrity of intracellular vesicles. After 30 min incubation with cells, calcein molecules (50 μM) were internalized into intracellular vesicles by endocytosis. At this concentration (with intact vesicles), the fluorescence of calcein self-quenches, and punctate and weak fluorescence is shown. If vesicles are broken,

calcein molecules are released to the cytoplasm, and show diffuse and strong fluorescence.

Pair-correlation function (pCF) microscopy. Live cell spinning-disk confocal microscopy was performed to collect images (videos, image capture frame rate 1000 frames/sec) for pCF data analysis. Data analysis was performed using a MATLAB computer program written in-house (available upon request). The program is similar to that described in the references.[4,5] The main difference of our program from that in the references[4,5] is that our program incorporates data from a spinning-disk scan. Briefly, fluorescence intensities at different locations and different time points are presented using a carpet representation in which the x-coordinate corresponds to location point along a line (pixels) drawn on the image view and the y coordinate corresponds to time. The pCF for two points at a distance δr as a function of the transit time τ was calculated by the following equation:

$$G(\tau,\delta r) = \frac{\langle F(t,0) \times F(t+\tau,\delta r)\rangle}{\langle F(t,0)\rangle\langle F(t,\delta r)\rangle} - 1$$

The maximum of the derived pCF profile was determined as the average time that a particle took to travel the given distance. To study the vesicle escape transit time in a given direction, a line was drawn in the direction and the pCF profile was calculated for two location points on the line, one of which was just inside the vesicle and the other of which was just outside the vesicle.

Intracellular delivery of the anticancer drug doxorubicin (DOX). Loading of DOX to QDs-Tat was conducted by adapting the method described in reference[6]. Briefly, a nanoparticle dispersion (QDs-Tat with or without 1% DMSO) was mixed

with DOX at 4 ºC for 12 h to load DOX onto nanoparticle surface by electrostatic adsorption.[6] Drug-loaded nanoparticles were purified by dialysis (molecular weight cutoff 100k Dalton). The removed free drug was measured by UV-Vis absorption spectroscopy against a calibration curve (absorption at 480 nm). Drug loading was then quantified based on the amount of the free drug removed. In addition, an alternative method was used to quantify the drug loading by measuring the fluorescent intensity of DOX (at 598 nm fluorescent emission peak) on the nanoparticle surface. It was found that these two methods yielded nearly identical results in drug loading measurement. The drug loading efficiency (the percentage of the amount of the drug successfully loaded relative to the total amount of the drug added) for all nanoparticle formulations was found to be 40%~50%. The amount of drug molecules loaded per particle was estimated to be ~45. Triplicate samples were studied for each different formulation. Drug-loaded nanoparticle dispersions (in cell culture medium) were incubated with HeLa cells. At different time points after the incubation started, cell viability was measured by MTT assay, and distributions of nanoparticles and DOX were imaged by spinning-disk confocal microscopy.

References

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Description of Supplementary Videos

Supplementary Video 1 Targeted delivery of QDs-Tat to the cell nucleus with the assistance of 1% DMSO. The video is a collection of 3D reconstruction confocal images showing colocalization of cell nucleus (blue, stained by the nucleus dye Hoechst 33342) with QDs-Tat (green). Colocalization of the cell nucleus and QDs-Tat leads to the composite color watchet blue.

Supplementary Video 2 Targeted delivery of the anticancer drug doxorubicin (DOX) to the cell nucleus with the assistance of QDs-Tat and 1% DMSO. The video is a collection of 3D reconstruction confocal images showing colocalization of cell nucleus (blue, stained by the nucleus dye Hoechst 33342) with QDs-Tat (green) and DOX (red).