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Supporting Information

Photosensitizer-conjugated polymeric nanoparticles for redox-responsive fluorescence imaging and photodynamic therapy

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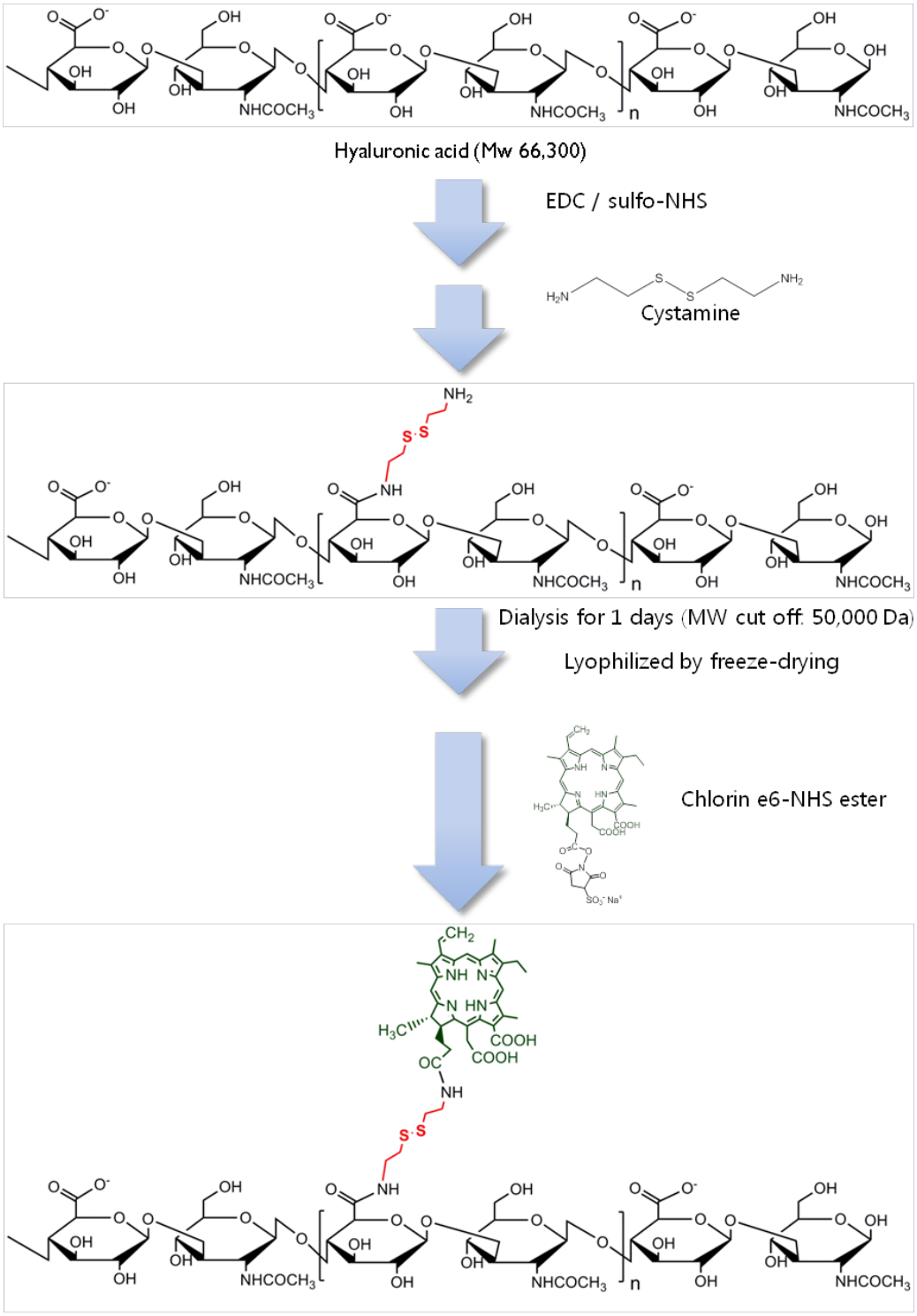

Fig. S1. Synthesis of HA-Ce6 conjugates with disulfide linker

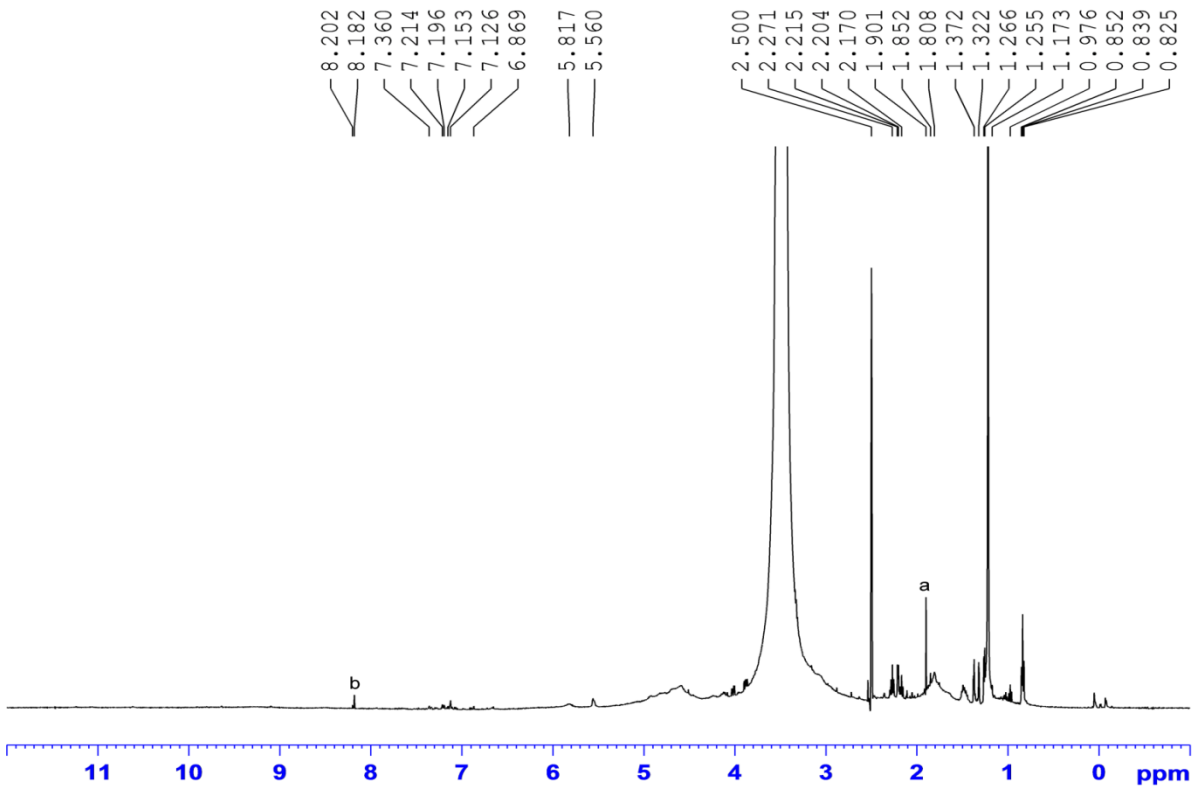

Figure S2. ^{1}H-NMR spectrum of HA-Ce6 conjugate in DMSO-d6. The proton of the acetamido methyl group of the HA backbone and 3-CH=CH_2 of Ce6 were observed at (a) d = 1.9 ppm and (b) 8.2 ppm, respectively.

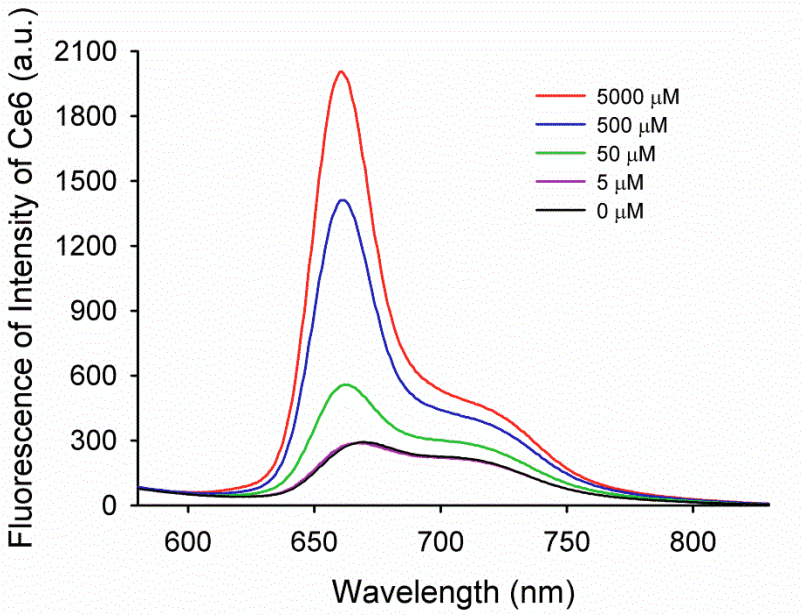

Fig. S3. Fluorescence intensity spectra of HA-Ce6 NPs at 2 μM of Ce6 equivalent after incubation in 0–5 mM DTT solution for 5 h (Ex. 400 nm).

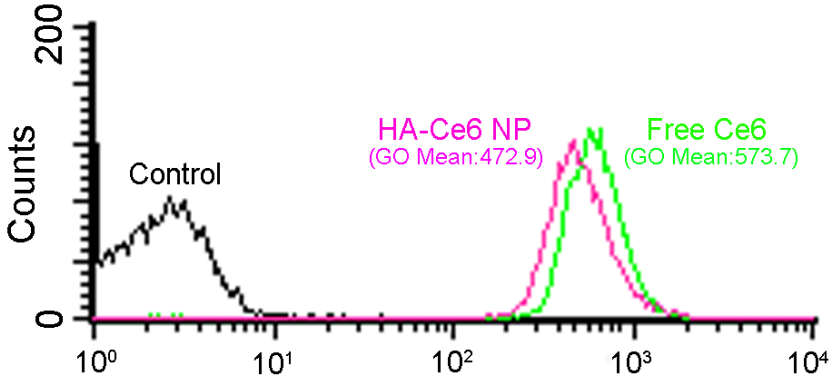

Fig S4. Fluorescence-activated cell sorting (FACS) of U-87MG cells treated with free Ce6 or HA-Ce6 NPs for quantitative analysis of redox-responsive fluorescence imaging and photodynamic therapy. Cells were incubated with 2 μM of Ce6 equiv. for 18 h.

Experimental Section

Preparation of HA-Ce6 nanoparticles: Hyarulonic acid (HA, MW 66,300 Da) was purchased from Lifecore Biomedical (MN, USA). 1-Ethyl-3(3-dimethylaminopropyl) carbodiimide (EDC), sulfo-N-hydroxysulfosuccinimide (sulfo-NHS), and cystamine dihydrochloride were purchased from Sigma-Aldrich (MO, USA). Chlorin e6 (Ce6) and dialysis membranes (MW cut-off: 50,000 Da) were purchased from Frontier Scientific (UT, USA) and Spectrum Laboratories (CA, USA), respectively.

Amine-functionalized HA was obtained by conjugating cystamine dihydrochloride with the carboxylic acid components of HA using standard EDC/NHS chemistry. Briefly, 200 mg HA was dissolved in sodium phosphate buffer (pH 7.4, 10 mM, 18 mL); EDC (240 mM, 0.5 mL) and sulfo-NHS (250 mM, 0.5 mL) were sequentially added to the HA solution. After 30 min, cystamine dihydrochloride (27 mg), dissolved in sodium phosphate buffer (pH 7.4, 10 mM, 1 mL), was added to the activated HA solution, and the reaction was allowed to proceed overnight at room temperature. The reactant was then dialyzed against deionized (DI) water and lyophilized. To conjugate the amine-functionalized HA and Ce6, the carboxylic acid of Ce6 (20 mg, 10 mL) was activated with EDC (34 mM) and sulfo-NHS (35 mM) in DMSO. Aminated HA (50 mg) was dissolved in DMF:H_2O cosolvent (1:1 v/v, 5 mL), mixed with the activated Ce6 solution, and then reacted for 18 h at room temperature. The resulting HA-Ce6

solution was dialyzed against phosphate buffer (pH 7.4, 10 mM) and DI water for 1 day, after which the solution was lyophilized. The final products were analyzed by UV/Vis spectrometry (DU730, Beckman Coulter, Brea, CA), fluorescence spectrometer, and ^{1}H-NMR.

Characterization of HA-Ce6 nanoparticles: The morphology of HA-Ce6 nanoparticles was observed by Field Emission-Scanning Electron Microscopy (FE-SEM; JEOL 7001F, JEOL Ltd. Japan). The surface charge and hydrodynamic size of HA-Ce6 NPs were characterized using a zeta potential/particle sizer (Malvern Instrument, Malvern, UK). For the analyses of optical properties, HA-Ce6 NPs were dispersed in PBS (6.7 mM, pH 7.4, NaCl 154 mM) and free Ce6 was dissolved in 1% (v/v) Tween/PBS. Fluorescence spectra were recorded on a multifunctional microplate (Tecan, Safire2) with excitation at 400 nm.

To calculate the concentration of Ce6 in the nanoparticles, the absorbance of HA-Ce6 NP (dissolved in 0.1 M NaOH / 0.1% SDS) was measured at 400 nm. Ce6 has a molar extinction coefficient of 1.5×10^{5} M^{-1} cm^{-1} at 400 nm.[1]

Analysis of fluorescence intensity and singlet oxygen generation: To observe fluorescence quenching and recovery, free Ce6 was dissolved in 1% (v/v) Tween/PBS to prevent self-quenching due to aggregation.HA-Ce6 NPs were dissolved in PBS. In this study, a concentration equivalent to 2 μM Ce6 was used. A cleavable disulfide linkage of HA-Ce6 NPs permits release of Ce6 with an increasing dithiothreitol (DTT) concentration that apparently results in the recovery of the fluorescence signal. Fluorescence measurements were performed by measuring the intensity of free Ce6 released from the HA-Ce6 NPs after the addition of 0–5 mM DTT. The excitation wavelength was 400 nm and the emission wavelength was 430 to 700 nm. The time-dependent fluorescence response was measured at 5, 60, 120, 180, 240, and 300 min after adding 0–5 mM DTT. To evaluate the inhibitory and recovery characteristics with respect to singlet oxygen generation, singlet-oxygen-detecting reagent (Singlet Oxygen Sensor Green, Molecular Probes) was dissolved in PBS (saturated with oxygen gas) containing HA-Ce6 NPs treated with various concentrations of DTT for 5 h. The final concentration of SOSG reagent in the test solution was maintained at 1 μM. Each solution was irradiated with a CW laser beam at 670 nm (irradiation dose rate 68 mW/cm^{2}). Releative SOG of HA-Ce6 NPs after treatment with 0–5 mM DTT was anlayzed by

measuring the increase in SOSG fluorescence during 30 sec light illumination with a 670 nm laser. All experiments were performed in triplicate.

Cytotoxicity test: U-87MG human malignant glioma cell lines were obtained from the American Type Culture Collection (Rockville, MD, USA), and maintained in Dulbecco's Modified Eagle Medium (GIBCO®, Invitrogen) supplemented with 10% (v/v) fetal bovine serum (FBS) and 1% antibiotic-antimycotic in a humidified 5% CO2 incubator at 37°C. The cells were seeded in a 96-well plate at 1×10^4 cells/well and incubated for 24 h for cell attachment. Then, free Ce6 and HA-Ce6 NPs were diluted in cell culture medium containing 10% FBS to obtain equivalent concentrations of 1 μM, 5 μM, 10 μM, and 20 μM Ce6. The existing culture medium was replaced with 200 μL fresh medium containing the photosensitizers, and the cells were incubated for 24 h. After the cells were washed twice, a fresh cell culture medium was added. Then viability of the U-87MG cells was analyzed using a CCK-8 solution. The absorbance was measured at 450 nm (reference = 650 nm) using a microplate reader (Tecan Safire 2, Switzerland). Untreated control cells served as 100% viable cells, and the medium served as the background. Data are expressed as the mean (SD) of 4 data samples.

In vitro Phtotoxicity test: The cells were seeded in a 96-well plate at 1×10^4 cells/well and incubated for 24 h for cell attachment. Then, free Ce6 and HA-Ce6 NPs were diluted in cell culture medium containing 10% FBS to obtain equivalent concentrations of 0.1 μM, 0.5 μM, 1 μM, and 2 μM Ce6. The existing culture medium was replaced with 200 μL fresh medium containing the photosensitizers, and the cells were incubated for 24 h. After the cells were washed twice, fresh cell culture medium was added. The cells were irradiated with a 670 nm CW laser at 5 J/cm^2 and 50 mW/cm^2. Viability of the U-87MG cells was analyzed using a CCK-8 solution. Absorbance was measured at 450 nm (reference = 650 nm) using a microplate reader (Tecan Safire 2, Switzerland). Untreated control cells served as 100% viable cells, and the medium served as the background. Data are expressed as the mean (SD) of 4 data samples.

In vitro cellular uptake and fluorescence activation test: U-87MG cells were plated at a density of 1×10^5 cells/well onto a LabTek II Chambered Coverglass (Nalge Nunc International Corp.) and incubated for 24 h to induce cell attachment. Free Ce6 or HA-Ce6

NP was added to fresh DMEM medium supplemented with 10% fetal bovine serum (FBS) with an equivalent concentration of 2 μM Ce6. After incubation for 18 h, the cells were washed 3 times and fresh culture medium containing 100 nM Lyso-Tracker Blue-DND 22 (Molecular Probe, Inc., Netherland) was added for 30 min. Fluorescence images of Ce6 (excitation: 405 nm, emission: 650 nm long-pass filter) and Lyso-Tracker (excitation: 405 nm, emission: 422-570 nm) were acquired by confocal scanning-laser microscopy (CSLM, ZEISS LSM 510 META).

To quantify the intracellular uptake of free Ce6 and HA-Ce6 NPs, flow cytometric analysis (FACS) with an excitation laser at 633 nm was used. U-87MG cells were plated at 1 × 10^5 cells/well onto 6-well plates and incubated for 24 h. Then, the existing cell culture medium was replaced with fresh medium containing free Ce6 or HA-Ce6 NPs at the equivalent concentration of 2 μM Ce6. After incubation for 18 h, the cells were washed 3 times with cell culture medium, harvested, and transferred to FACS tubes.

References

[1] M. R. Hamblin, J. L. Miller, I. Rizvi, *Cancer Res.* **2001**, *61*, 7155-7162.