Electronic Supplementary Material (ESI) for Chemical Communications

Supporting Information

A Graphene Oxide/Photosensitizer Complex as an Enzyme-Activatable Theranostic

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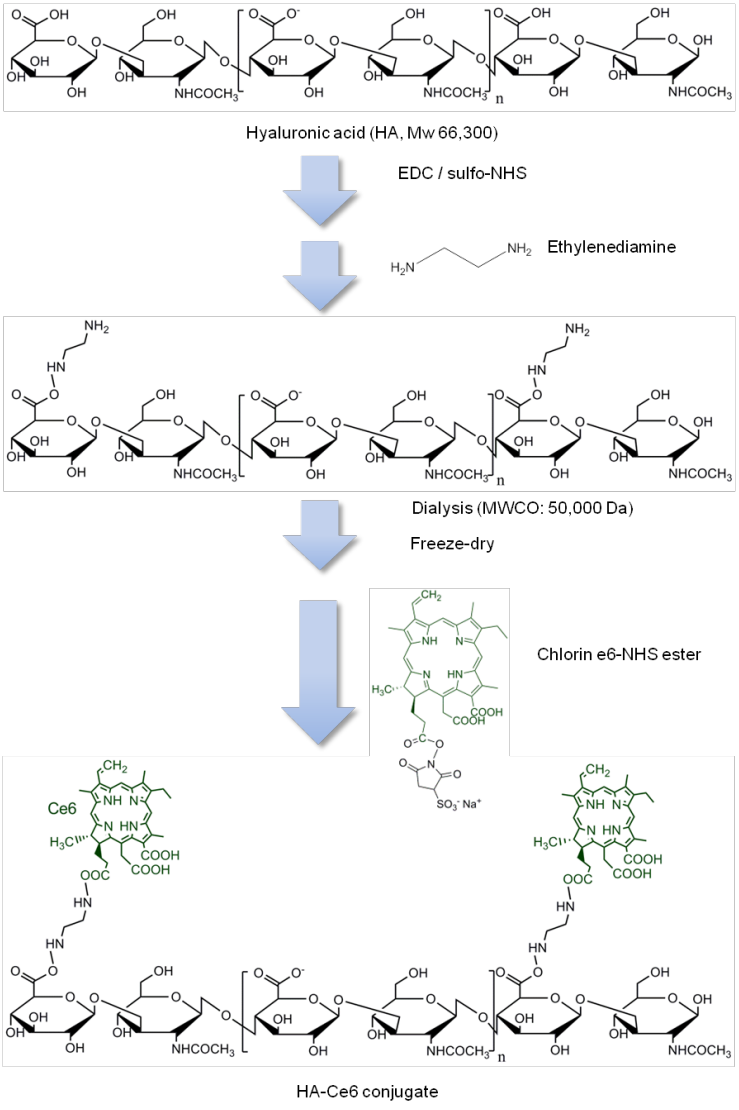

Fig. S1. Synthesis of Ce6-conjugated HA (HA-Ce6). The molar ratio of conjugated Ce6 versus HA in the HA-Ce6 conjugate was calculated to be 1.5:1.

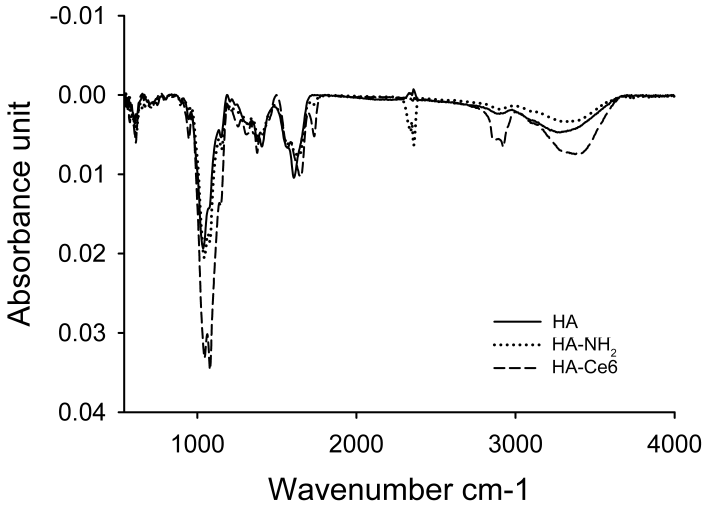

Fig. S2. FT-IR spectra of HA, HA-NH_2, and HA-Ce6. After conjugation of HA with Ce6, strong characteristic peaks were observed between 950 and 1200 cm^{-1} (C-O stretching vibration), between 1500 and 1700 cm^{-1} (carbonyl and carboxyl bands), at 2900 cm^{-1} (C-H stretching vibration), and at 3400 cm^{-1} (O-H stretching vibration), indicating successful formation of the HA-Ce6 conjugate.

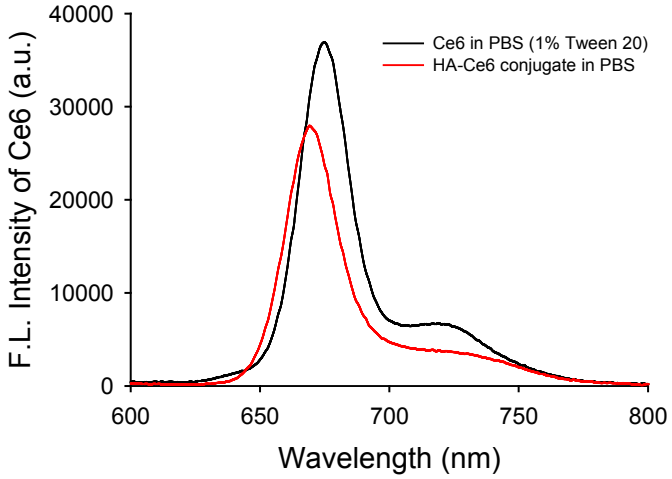

Fig. S3. Fluorescence spectra of free Ce6 and HA-Ce6 (final concentration: 1.8 μM Ce6 equiv.; excitation: 400 nm). HA-Ce6 conjugate was dissolved in PBS (6.7 mM, pH 7.4, 154 mM NaCl). Free Ce6 was dissolved in PBS containing 1% (v/v) Tween 20 to obtain monomeric Ce6 without any aggregation. The fluorescence of HA-Ce6 was slightly lower than that of monomeric Ce6 (i.e., 76% of free Ce6 in Tween 20-containing PBS).

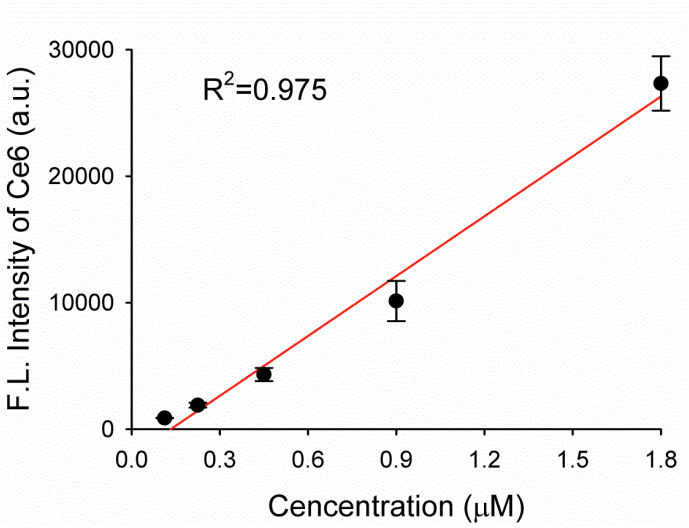

Fig. S4. Standard curve of concentration (Ce6 equivalent) vs. fluorescence intensity.

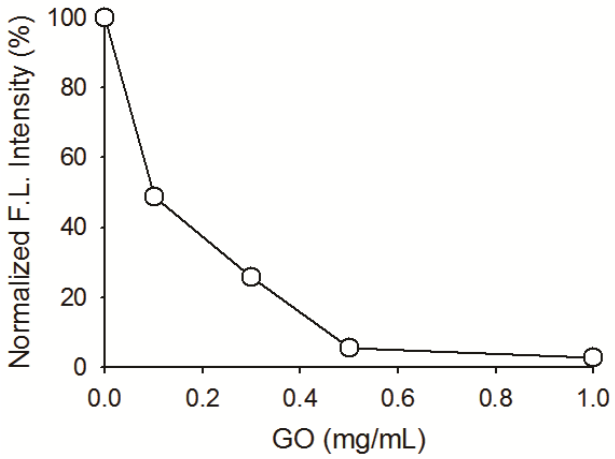

Fig. S5. Normalized fluorescence intensity of HA-Ce6 (final concentration: 1.8 μM Ce6 equiv.) in the presence of different concentrations of GO, ranging from 0 to 1.0 mg/mL (excitation: 400 nm, emission: 670 nm).

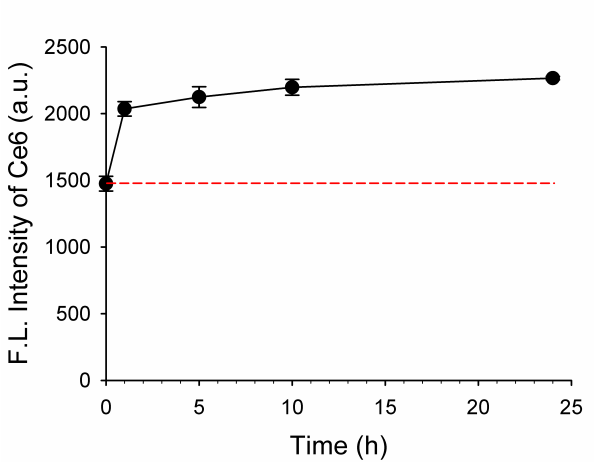

Fig. S6. Stability test of GO-HA-Ce6 in serum conditions. GO-HA-Ce6 (1.8 μM Ce6 equiv.) was dissolved in PBS and PBS containing 10% (v/v) fetal bovine serum (FBS), and the changes in fluorescence intenstity were then monitored for 24 h. Closed circle: fluorescence intensity of GO-HA-Ce6 in PBS containing 10% FBS. Dotted red line: fluorescence intensity of GO-HA-Ce6 in PBS.

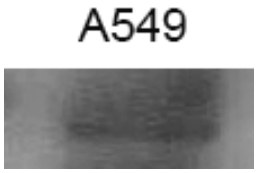

Fig. S7. Western blotting in cell lysates. Whole cell lysate (5 μg) of A549 cells was used for SDS-PAGE and transferred into nitrocellulose membrane. The membrane was blocked with 5% skim milk in PBST (PBS + 0.1% Tween 20) for 1 h. The primary and secondary antibodies used in this test were anti-HYAL1 antibody (rabbit polyclonal, 1 μg/mL) and donkey polyclonal antibody to rabbit IgG-HRP conjugates (1/000 dilution), respectively. Antibodies were purchased from Abcam Inc. (Cambridge, UK).

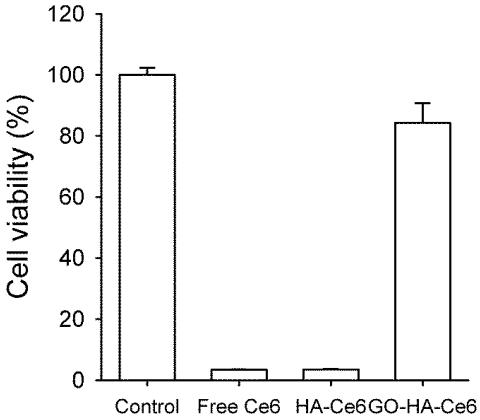

Fig. S8. *In vitro* phototoxicity test performed in the presence of photosensitizers in the extracellular space. A549 human lung cancer cells were seeded in a 96-well plate at a density of 1×10^4 cells/well and incubated for 24 h to allow for cell attachment. The cell culture media was then exchanged with fresh RPMI 1640 media containing Ce6, HA-Ce6, and GO-HA-Ce6 at 1 μM Ce6 equivalent. After 30 min, the cells were irradiated with a 670-nm CW laser (50 mW/cm^2, 10 J/cm^2), washed 3 times, and further incubated overnight. Cell viability was then measured using a cell counting kit. Previously, it was reported that the cellular uptake of porphyrin derivatives, including Ce6, is significantly lower in the presence of serum than it is in the absence of serum, because nonspecific binding to serum prevents adsorption of the photosensitizers on cell membrane and thereby inhibits intracellular uptake of the photosensitizers [1]. Therefore, we dissolved Ce6, HA-Ce6, and GO-HA-Ce6 in fresh RPMI 1640 media without serum to facilitate adsorption of the photosensitizers on the cell membrane, and then treated A549 cells with the media containing photosensitizers. As a result, most of the cells incubated with free Ce6 and HA-Ce6 died, due to SOG by photosensitizers located near the cell membrane. In contrast, cells incubated with GO-HA-Ce6 showed only 16% cell death, indicating the inhibition of nonspecific phototoxicity.

References

1. R. M. Bohmer, G. Morstyn, Cancer Res., 1985, 45, 5328-5334; (b) B. Cunderlikova, L. Gangerskar, J. Moan, J. Photochem. Photobiol. B, 1999, 53, 81-90; (c) Y. Choi, R. Weissleder, C.H. Tung, ChemMedChem, 2006, 1, 698-701.

Experimental Section

Preparation of HA-Ce6 conjugate

Hyarulonic acid (HA, MW 66,300 Da) was purchased from Lifecore Biomedical (MN, USA). 1-Ethyl-3(3-dimethylaminopropyl) carbodiimide (EDC), N-hydroxysulfosuccinimide (sulfo-NHS), and ethylene diamine hydrochloride 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 ethylenediamine 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, 10 mL), and then EDC (1 M, 0.125 mL) and sulfo-NHS (100 mM, 0.5 mL) were sequentially added to the HA solution. After 15 min, ethylenediamine dihydrochloride (9.4 mg), dissolved in sodium phosphate buffer (pH 7.4, 10 mM, 0.5 mL), was added to the activated HA solution, and the reaction was allowed to proceed for 2 h at room temperature. The reactant was then dialyzed against deionized (DI) water and lyophilized. Next, for conjugation of amine-functionalized HA and Ce6, the carboxylic acid of Ce6 (5.3 mg) was activated with EDC (1 M, 0.125 mL) and sulfo-NHS (100 mM, 0.5 mL) in DMSO. Aminated HA (25 mg) was dissolved in DMF:H_2O cosolvent (1:1 v/v, 2.5 mL), mixed with the activated Ce6 solution, and then reacted for 24 h at room temperature. The resulting HA-Ce6 solution was dialyzed against phosphate buffer and DI water for 1 day, after which the solution was lyophilized. The final products were analyzed using a UV/Vis spectrometer (DU730, Beckman Coulter, Brea, CA), fluorescence spectrometer, and Fourier transform infrared (FT-IR) spectrometer. To calculate the conjugation ratio of Ce6 in HA, the absorbance of the HA-Ce6 conjugate (dissolved in 0.1 M NaOH/0.1% SDS aqueous solution) was measured at 400 nm. Ce6 is known to have a molar

extinction coefficient of 1.5×10^5 $M^{-1}cm^{-1}$ at 400 nm.[31] The mole ratio of conjugated Ce6 versus HA was calculated to be 1.5:1.

Preparation and characterization of HA-Ce6 loaded graphene oxide (GO-HA-Ce6)

GO was purchased from Graphene Laboratories Inc. (Calverton, NY, USA). As-received GO suspensions (1 mg/mL) were sonicated in DI water for 1 h and then mixed with HA-Ce6 dissolved in DI water at room temperature in the dark overnight. Then, unbound excess HA-Ce6 was purified by repeated centrifugation at 16000 rpm for 30 min followed by dialysis in DI water to obtain the final suspension of HA-Ce6-loaded GO (GO-HA-Ce6). The final product was redispersed in DI water and stored at 4°C. The loading concentration of HA-Ce6 on GO was calculated by measuring the absorbance peak at 400 nm and subsequently subtracting the absorbance of GO at the same wavelength. For the surface characterization of GO-HA-Ce6, we used a high-resolution field-emission scanning electron microscope (FE-SEM). GO-HA-Ce6 samples for FE-SEM observation were prepared by applying a drop of the suspension to a glass slide, drying the sample, and subsequently spin-coating the sample with a thin gold film under vacuum. Measurements of the hydrodynamic size of GO-HA-Ce6 in PBS (0.1 mg/mL) were performed using a zeta potential/particle sizer (Malvern Instruments, Worcestershire, UK). The optical properties of GO, GO-HA-Ce6, and free HA-Ce6 were evaluated with a UV/Vis spectrophotometer. Fluorescence analyses was carried out on a multifunctional microplate reader (Safire2; Tecan, Männedorf, Switzerland) with an excitation of 400 nm, with all solutions dispersed in PBS (6.7 mM, pH 7.4, NaCl 154 mM). To evaluate the inhibitory and recovery characteristics with respect to SOG, a singlet-oxygen-detecting reagent (Singlet Oxygen Sensor Green, Molecular Probes, NY, USA) was dissolved in PBS (saturated with oxygen gas) containing free HA-Ce6 or GO-HA-Ce6. Final concentrations of free HA-Ce6 and the GO-HA-Ce6 complex were maintained at 1.8 μM Ce6

equiv. Each solution was irradiated with a 670-nm CW laser (irradiation dose rate, 100 mW cm^{-2} and irradiation dose, 20 J cm^{-2}). All experiments were performed in triplicate.

Enzyme-responsive recovery of fluorescence and SOG in the GO-HA-Ce6 complex

HAdase (800 unit/mL) was added to the GO-HA-Ce6 complex (1.8 μM Ce6 equiv.) dissolved in PBS, and the solution was gently stirred for 3 h at 37°C. To observe the effects of inhibition of HAdase, the HAdase inhibitor 6-O-palmitoyl-L-ascorbic acid (1–100 μM) was employed. HAdase, pre-incubated with HAdase inhibitor at 37°C for 30 min, was added to the GO-HA-Ce6 complex (1.8 μM Ce6 equiv.) dissolved in PBS and incubated for 3 h. Finally, fluorescence intensity and SOG were analyzed. All experiments were performed in triplicate.

Temperature increases in the GO-HA-Ce6 suspension during irradiation with an 810-nm laser

Prior to laser irradiation, samples were kept at room temperature for at least 30 min to equilibrate with the ambient temperature. Then, the samples were irradiated with an 810-nm laser at 1 W/cm^2 or 4 W/cm^2 for 8 min. Temperature changes were recorded in real-time using an infrared thermographic camera (Thermovision A40, FLIR, Wilsonville, OR). Finally, temperature variation was analyzed using commercial software (ThermaCam Research, Boston, MA).

In vitro cell studies

A549 human lung cancer cells were seeded in a 96-well plate at a density of 1×10^4 cells/well and incubated for 24 h to allow for cell attachment. Then, free Ce6, free HA-Ce6,

and GO-HA-Ce6 were diluted with fresh RPMI 1640 medium supplemented with 10% FBS to obtain a concentration of 1.8 μM Ce6 equiv. The existing culture medium was replaced with fresh medium containing the photosensitizers. After incubation for 24 h, the cells were washed 3 times, and fresh culture medium was added. For PDT, laser irradiation of the cells was conducted with a 670-nm laser at a dose rate of 50 mW/cm^2 and a dose of 4 J/cm^2. The cells were incubated for an additional 24 h, and cell viability was measured using a cell counting kit (Dojindo Laboratories). For untreated control groups, the same volume of fresh culture medium was added to the plate. Cell viability was calculated as a percentage relative to untreated control cells.

To conduct PTT, A549 cells were plated at a density of 1×10^4 cells/well. Cells were treated with fresh culture medium containing free Ce6, HA-Ce6, or GO-HA-Ce6 at a concentration of 1.80 μM Ce6 equiv. After incubation for 24 h, the cells were washed 3 times, and fresh culture medium was added. Laser irradiation of the cells was conducted with an 810-nm laser at a dose rate of 4 W/cm^2 and a dose of 250 J/cm^2 to induce photothermally enhanced cell death. After incubating the cells for an additional 24 h, cell viability was measured using a cell counting kit.

For combined PTT and PDT, cells were incubated with fresh culture medium containing GO-HA-Ce6 for 24 h. The cells were then washed 3 times, and fresh culture medium was added. Laser irradiation of the cells was conducted with an 810-nm CW laser at a dose rate of 4 W/cm^2 and a dose of 250 J/cm^2, followed by irradiation with a 670-nm CW laser at a dose rate of 50 mW/cm^2 and a dose of 4 J/cm^2. After incubation for an additional 24 h, cell viability was measured in order to analyze combined the PTT and PDT effect.

Statistical analysis

Unless otherwise specified, statistical analysis was done using student's t test. Differences were considered statistically significant when *P* value was less than 0.05. Results are expressed as the mean ± SD.