Supplementary Information

Efficient Delivery of Chlorin e6 into Ovarian Cancer Cell with Octalysine Conjugated Superparamagnetic Iron Oxide Nanoparticle for Effective Photodynamic Therapy

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Drug Release: The release profile of SPION-PG-Lys_8/Ce6 at different pH values was evaluated according to the method established previously (L. Zhao et al, Biomaterials, 2014, 35, 5393).

Cell Culture: SKOV3 (human ovarian adenocarcinoma) cell line was purchased from ATCC (LOT: 59487901) and NIH3T3 (mouse fibroblast) cell line was provided by Ms. Yajuan Zou of Shanghai Jiao Tong University. SKOV3 cells and NIH3T3 cells were grown in McCoy's 5A medium and Dulbecco's Modified Eagle Medium (DMEM), respectively, supplemented with 10% fetal bovine serum (FBS) and 100 U/mL penicillin and 1.0% streptomycin. For cell culture, the cells were incubated at 37 °C in 5% CO_2.

Cytotoxicity Assay of SPION-PG-Lys_8: SKOV3 cells cultured in 96-well plates (1×10^4 cells per well) were treated with SPION-PG-Lys_8 of different concentrations in culture medium (100 μL per well). After incubation for 24 h, the culture medium was removed and washed twice with 1 × PBS. Cell toxicity was then assayed using CCK-8 kit as described in the manual provided by the kit manufacture (Dojindo Molecular Technologies, Japan).

Prussian Blue Staining of Iron: The SKOV3 cells (5×10^4 cells per well in 4-well LabTek® chamber slides) were incubated with SPION, SPION-PG, SPION-PG-Lys_8 or SPION-PG-Lys_8/Ce6 of different concentrations. After incubation for 24 h, the culture medium was removed and the adherent cells were washed thrice with 1 × PBS, and fixed with 4% phosphate buffered formaldehyde. Prussian blue staining kit (Muto Pure Chemicals, Japan) was used for

staining of internalized iron oxide nanoparticles. Briefly, the fixed cells were washed thrice with 1 × PBS, incubated with 1:1 mixture of 2% potassium ferrocyanide and 2% hydrochloric acid for 30 min, and counterstained with 1% Safranin O for 2 min. The stained cells were imaged under Nikon DIAPHOT 300 microscope (Nikon, Japan).

Confocal Fluorescence Microscopy: The SKOV3 cells (5×10^4 cells per well in 4-well LabTek chamber slides) were incubated with Ce6 or SPION-PG-Lys_8/Ce6 with a normalized Ce6 concentration of 4.0 μg/mL. After incubation for 8 h, the culture medium was removed and the adherent cells were washed thrice with 1 × PBS, and fixed with 4% phosphate buffered formaldehyde. After staining the cell nuclei with Hoechst 33342 dye (Life Technologies, USA), the cells were imaged under Olympus FV1000-D confocal microscope (Olympus, Tokyo, Japan).

Subcellular Localization of SPION-PG-Lys_8/Ce6: 2×10^5 SKOV3 cells were cultured on 35 mm glass bottom dish (Corning, USA) for 24 h and transiently transfected with DsRed2-Mito plasmid (Clontech, USA) using Fugene HD (Roche Diagnostics, Germany) to label mitochondria. After transfection for 24 h, cells were washed thrice with 1 × PBS. Next, SPION-PG-Lys_8/Ce6 was added to culture dishes with the concentration of 4.0 Ce6 μg/mL and cells were further incubated for 8 h. Cells were washed twice with 1 × PBS and medium was replaced with fresh medium containing 75 nM LysoTracker Green DND-26 (Life Technologies, USA). After incubation in 37°C for 30 min, cells were washed and covered with 1 × PBS, and were imaged under the confocal microscope.

Flow Cytometry: SKOV3 cells cultured in 12-well plates (1×10^5 cells per well) were treated with Ce6 or SPION-PG-Lys_8/Ce6 of different durations or concentrations in culture medium (1 mL per well). Then, cells were washed with 1 × PBS twice, lifted by incubation with trypsin and concentrated, and then redispersed in 1 × PBS (0.5 mL) for FACS analysis (FACSCalibur, USA).

Western Blot: To investigate whether SPION-PG-Lys_8/Ce6 induces autophagy, cells were treated with 4.0 μg/mL or 40 μg/mL SPION-PG and SPION-PG-Lys_8 for 14 h. Cells were then washed twice with 1 × PBS and cultured in fresh medium containing 50 nM Bafilomycin A1 (Baf), an autophagy inhibitor that inhibits vacuolar type H(+)-ATPase (V-ATPase), for another 16 h. Cells were lysed with RIPA buffer supplemented with protease inhibitor cocktail (Roche, USA) on ice for 30 min and cell lysates were centrifuged at 10,000 *g* for 25 min. Supernatant was collected and protein concentration was determined with BCA assay (Pierce Biotechnology, USA).

20 μg of each sample was revolved to SDS polyacrylimde gels and transferred to PVDF membrane. The membrane was blocked in 5% non-fat milk in Tris-buffered saline/0.1% Tween-20 (TBST) for 30 min at room temperature. LC3 and β-actin were detected by incubating the blots with mouse anti-human LC3 antibody (MBL, M152, 1:1,000) and mouse anti-human β-actin antibody (Santa Cruz Biotechnology, 47778, 1:10,000) overnight at 4°C. After washing with TBST three times, the blots were incubated with goat anti-mouse secondary antibody (Jackson ImmunoResearch Laboratories, goat anti-mouse conjugated to horseradish peroxidase, 1:10,000) for 1 h at room temperature. Blots were developed using ECL (SuperSignal West Pico, Thermo Scientific) and analyzed using a LAS-4000 IR multi-color image analyzer (Fujifilm, Tokyo, Japan). Protein levels were semi-quantified by calculating band density using Image-J software.

In Vitro PDT: SKOV3 cells cultured in 96-well plates (1×10^4 cells per well) were treated with Ce6 or SPION-PG-Lys_8/Ce6 of different concentrations in culture medium (100 μL per well). After incubation for 24 h in the dark, cells were washed with 1 × PBS twice and then cultured in growth medium. The treated cells in the culture medium were irradiated using a 660-nm LED with an optical dose of 16.8 J/cm^2 (Optocode LED/660, Japan). After incubation in the dark for another 16 h, Cell toxicity was assayed using CCK-8 kit.

ROS Production Assay: SKOV3 cells cultured in 12-well plates (1×10^5 cells per well) were treated with SPION-PG-Lys_8 (2.5 μg/mL), Ce6 or SPION-PG-Lys_8/Ce6 (0.25 μg Ce6/mL) in culture medium (1 mL per well). After incubation for 24 h, cells were washed with 1 × PBS twice and then cultured in culture medium. Next, cells were divided to 2 groups; one group was irradiated with 660-nm LED (16.8 J/cm^2), whereas the other group was cultured in the dark. After washing with 1 × PBS once, cells were incubated in 20 μM H2DCFDA (Invitrogen, USA) in Opti-MEM (Life Technologies) for 45 min. After removal of the loading buffer, the cells were incubated in prewarmed culture medium for 30 min. Cells were lifted by incubation with trypsin and concentrated, and then redispersed in 1 × PBS (0.5 mL) for FACS analysis. The data were analyzed by software (WinMDI 2.9) and geometric means were used for quantification.

Mitochondrial Membrane Potential (MMP) Assay: MMP levels were measured in the same way to that of ROS production assay using tetramethylrhodamine, methyl ester (TMRM, Invitrogen, USA) as a probe. Fluorescence microphotographs were captured under bright field and TexRed channels with same exposure time and setting using Keyence Biozero BZ-8100 microscopy (Keyence, Japan). The fluorescence intensity of TMRM reflects the levels of mitochondrial membrane potential.

Statistical Analysis: Statistical analysis was performed with Prism software (Graphpad, San Diego, CA, USA) using one-way analysis of variance (ANOVA) or two-way ANOVA with Bonferroni's post test. $p < 0.05$ was considered statistically significant.

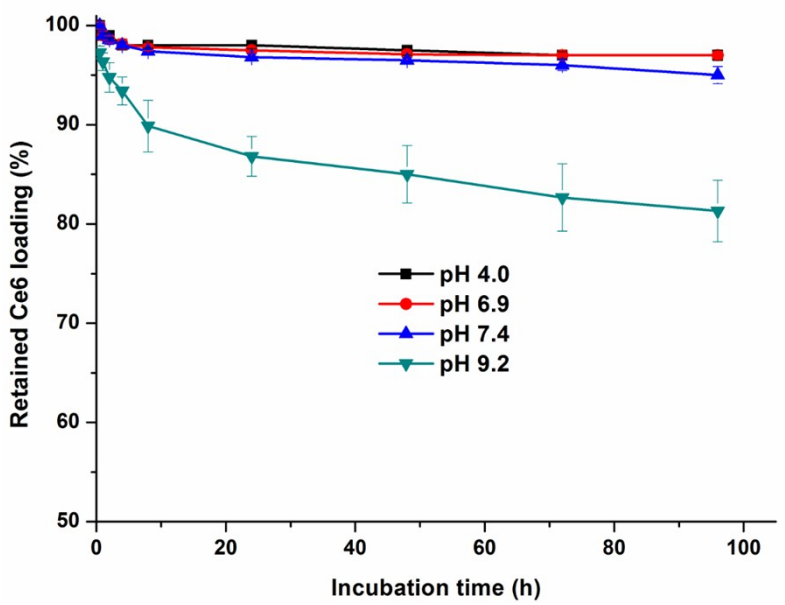

Fig. S1 Release of Ce6 from SPION-PG-Lys$_8$/Ce6 in buffer solutions with different pH values at 37 ^{o}C (n = 3, mean ± SD).

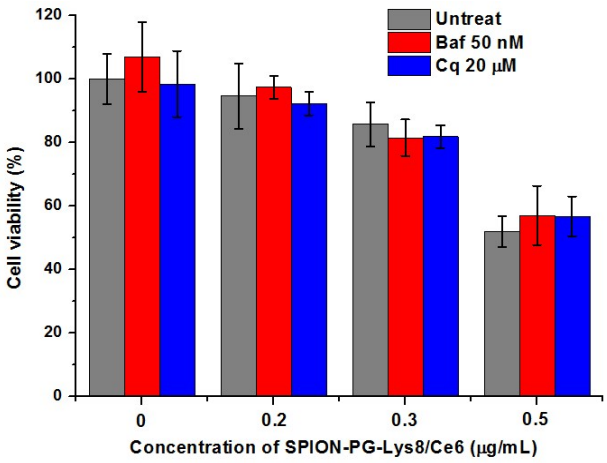

Fig. S2 PDT of SKOV3 cells treated with SPION-PG-Lys$_8$/Ce6 at different concentrations using 50 nM Baf or 20 nM CQ as autophagy inhibitors. The result was expressed as the percentage of untreated cells (n = 3, mean ± SD).

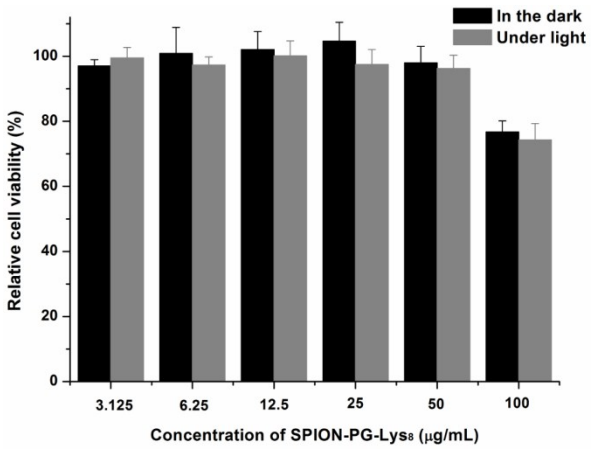

Fig. S3 Cell viability of SKOV3 cells treated with SPION-PG-Lys_8 of different concentrations for 24 h in the dark and under 660-nm light irradiation (16.8 J/cm^2) (n = 3, mean ± SD).

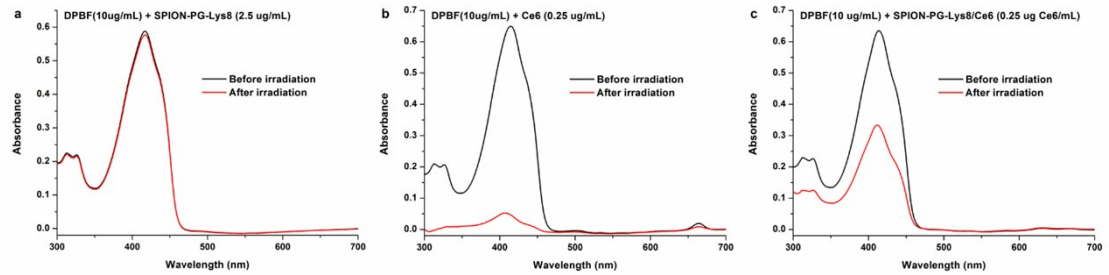

Fig. S4 Absorption spectra of DPBF mixed with (a) SPION-PG-Lys_8 (2.5 μg/mL); (b) Ce6 (0.25 μg/mL) and (c) SPION-PG-Lys_8/Ce6 (0.25 μg Ce6/mL) before and after 660-nm light irradiation (16.8 J/cm^2). The experiments were carried out according to the method reported in the literature (L. M. Rossi et al, Langmuir 2008, 24, 12534).

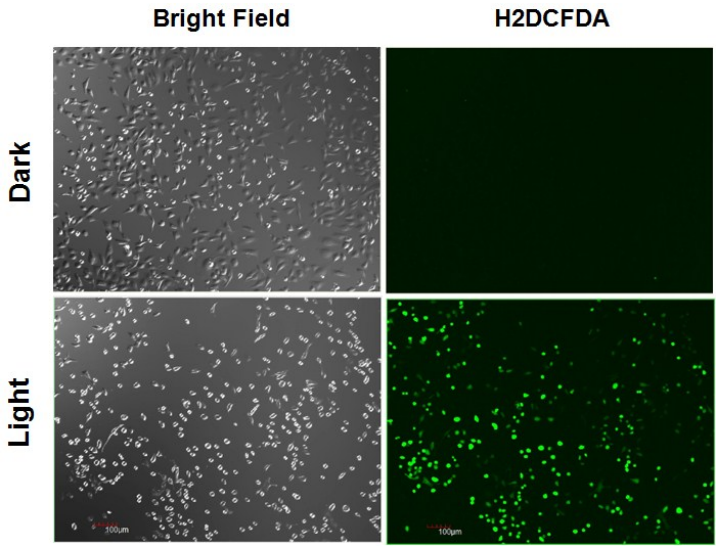

Fig. S5 Fluorescent microphotographs of SKOV3 cells treated with SPION-PG-Lys_8/Ce6 (0.25 μg Ce6/mL) and subsequently incubated with 20 μM H2DCFDA. The bright green fluorescence can be only detected in the cells after photo-irradiation, confirming that the ROS generation was triggered by light.