Electronic Supplementary Information (ESI)

Firmly anchored photosensitizer Chlorin e6 to layered double hydroxide nanoflakes for highly efficient photodynamic therapy *in vivo*.

Li Yan,[a,c] Zhigang Wang,[d] Xianfeng Chen,[b]* Xiao-Jun Gou,[a] Zhenyu Zhang,[c] Xiaoyue Zhu,[c] Minhuan Lan,[c] Wei Chen,[c] Guangyu Zhu[d] and Wenjun Zhang [c]*

[a] Antibiotics Research and Re-evaluation Key Laboratory of Sichuan Province, Sichuan Industrial Institute of Antibiotics (SIIA), Chengdu University, Chengdu, Sichuan, PR China.

[b] Institute for Bioengineering, School of Engineering, The University of Edinburgh, King's Buildings, Mayfield Road, Edinburgh EH9 3JL, United Kingdom.

[c] Center of Super-Diamond and Advanced Films (COSDAF) and Department of Physics and Materials Science, City University of Hong Kong, Hong Kong SAR, PR China.

[d] Department of Biology and Chemistry, City University of Hong Kong, Hong Kong SAR, PR China.

Corresponding authors:
xianfeng.chen@oxon.org; apwjzh@cityu.edu.hk

Experimental Section

Chemicals and Characterization. Chlorine e6 (Ce6) was from J&K chemical (China). NaOH and $MgCl_2$ were purchased from International Laboratory (U.S.A.). Sodium dodecyl sulfate, N-(3-Dimethylaminopropyl)-N-ethylcarbodiimide hydrochloride, methylene chloride, N-cetyl-N,N,N-trimethylammonium, and methotrexate were ordered from Acros (U.S.A.). $AlCl_3$, (3-Aminopropyl) triethoxysilane, and dimethyl sulfoxide were from Sigma Aldrich (U.S.A.). Dulbecco's modified eagle medium (DMEM), fetal bovine serum (FBS), phosphate-buffered saline (PBS), MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide), and penicillin/streptomycin were obtained from Life Technologies (U.S.A.). 96-well cell culture plates and cell culture dishes were from Corning (U.S.A.). TEM images were taken on Philips CM200. The UV-Vis absorption spectra were recorded using Ultra-Violet Visible Scanning Spectrophotometer (Shimadzu 1700). Fourier transform infrared spectroscopy (FTIR) spectra were taken with a Perkin-Elmer 2000 FTIR spectrometer. Powder X-ray diffraction (XRD) patterns were recorded using a Smartlab instrument with Cu-Ka radiation.

Preparation of LDH-SDS nanoparticles. LDH-SDS was prepared according to previous reports with modification.[1] Briefly, 80 mL of 0.15 M sodium hydroxide water solution and 4 mmol of SDS were added to 20 mL of water solution containing 2.0 mmol of $MgCl_2$ and 1.0 mmol of $AlCl_3$. The obtained mixture was vigorous stirring for 10 minutes. After that, the solution was centrifuged and the precipitate was washed with water followed by re-dispersing in 80 ml of water for incubation at 80 ℃ for 24 hours under N_2 protection. The LDH-SDS nanoparticles were collected by centrifuge at 4000 rpm for 5 minutes and dried by freeze-drying.

Preparation of LDH-Ce6 hybrid. To conjugate Ce6 to APTES, 3.8 mg of Ce6 was mixed with 50 μL of APTES in 2 mL DMSO for 48 hours in presence of EDC under N_2 protection. 10 mL of methylene chloride and 100 mg of CTAB were mixed at ~ 45 ^oC. Then, the as-prepared Ce6-APTES and CTAB solution were quickly mixed with 50 mg of LDH-SDS nanoparticles under N_2 protection. Next, the mixture was being ultrasonicated for half hour at ~ 45 ^oC. Subsequently, the solution was kept stirring for 48 hours at ~ 45 ^oC. Finally, the product was obtained by centrifugation followed by sequential washing with methylene chloride, ethanol and Millipore water.

Preparation of LDH-Ce6-PEG hybrid. To conjugate PEG-COOH to APTES, 2.5 mg of PEG was mixed with 25 μL of APTES in 1 mL DMSO for 48 hours in presence of EDC under N_2 protection. To conjugate Ce6 to APTES, 3.8 mg of Ce6 was mixed with 50 μL of APTES in 2 mL DMSO for 48 hours in presence of EDC under N_2 protection. Then, 10 mL of methylene chloride and 100 mg of CTAB were mixed at ~ 45 ^oC. Then, the as-prepared Ce6-APTES, PEG-APTES and CTAB solution were quickly mixed with 50 mg of LDH-SDS nanoparticles under N_2 protection. Next, the mixture was being ultrasonicated for half hour at ~ 45 ^oC. Subsequently, the solution was kept stirring for 48 hours at ~ 45 ^oC. Finally, the product was obtained by centrifugation followed by sequential washing with methylene chloride, ethanol and Millipore water.

Singlet oxygen generation ability analysis. The method is according to a previous reported protocol.[2] Briefly, 100 μL of 0.05 mg/mL DPBF solution in DMSO was added into each well in a 96-well plate. Ce6 or LDH-Ce6 containing 60 ng of Ce6 was added to different wells. The plate was irradiated by a 650 nm LED lamp (7 mW/cm^2). After irradiation, the absorbance of each well at 410 nm was measured by a microplate reader (Biotek Powerwave xs).

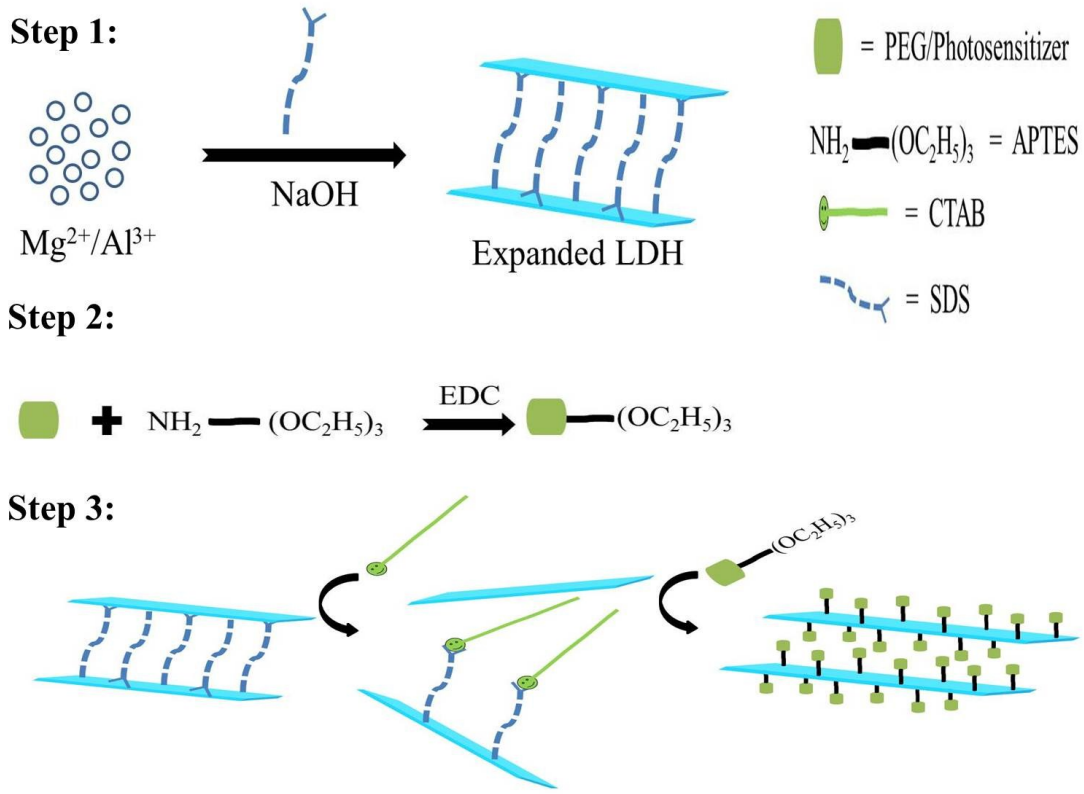
Laser scanning confocal microscopy of intracellular Ce6 delivery and ROS detection. A549 cell suspension was seeded to a sterile glass coverslip in a 35-mm tissue culture dish for 24 hours. Then the cell culture medium was removed and a fresh medium containing Ce6 and LDH-Ce6 with final concentration of Ce6 at 3 μg/mL was added following by post-culture for 4 or 24 hours. Then, the cells were stained with 10 μM of H_2DCFDA (Life Technologies) in PBS for 40 min. Then, the staining solution was removed and the complete medium was added to each dish. The cells treated with Ce6 or LDH-Ce6 hybrid were irradiated at 650 nm for 15 min at 7 mW/cm^2. Finally, after washing with PBS, the coverslip was mounted onto slides for laser scanning confocal microscopy. Imaging was performed on a confocal microscope (Leica TCS SPE).

Cell viability measurement. As a typical protocol, A549 cells were washed twice with PBS. A549 cells suspended in DMEM (with 10% FBS, 1% penicillin/streptomycin) were plated into 96-well plates (100 μL DMEM; 1,500-3,000 cells per well). The cells were incubated at 37 °C for 24 hours before further treatment. Then, another 100 μL of DMEM containing various concentrations of Ce6 or LDH-Ce6 was added into 96-well plates for additional 4 or 24 hours incubation. After certain periods of incubation, the original medium in each well was removed and 200 μL of fresh DMEM (with 10% FBS, 1% penicillin/streptomycin) were added into each well. The cells treated with Ce6 or LDH-Ce6 hybrid were irradiated at 650 nm for 10 min at 7 mW/cm^2 and incubated for further 24 hours. Finally, 180 μL of DMEM (without FBS) and 20 μL of MTT stock solution (5 mg mL^{-1} in PBS) were added and incubated for 4 hours. Then the medium containing MTT was completely removed, followed by adding 200μL DMSO to each well. Cell viabilities were quantified by measuring the absorbance of the product at 540 nm with a BioTek Powerwave XS microplate reader.

Photo-stability analysis of Ce6 and LDH-Ce6. Ce6 and LDH-Ce6 hybrid with concentration of 46 μg/mL were irradiated under 650 nm at 7 mW/cm^2 for a certain period of time. The absorbance was measured by a UV-Visible Spectrophotometer (Shimadzu 1700).

In vivo **photodynamic therapy.** Tumor bearing BALB/C mice were developed by injection of 4T1 cells to BALB/C mice (2.5×10^6 4T1 cells in 150 μL PBS). At 10 days after inoculation of the cancer cells, the mice were randomly divided into 5 groups (n=4) (defined as treatment Day 1). 200 μL 83.3 μg Ce6 containing formations (LDH-Ce6 nanoflakes, Free Ce6 and LDH-Ce6 Mix) and PBS solution were injected into the different groups of mice via the tail vein at treatment Day 1, 4 and 7. The tumors were irradiated by 200 mW 650 nm laser for 5 minutes at treatment Day 1, 3, 5, 7, 9 and 11.

The tumor size and body weight were monitored at 2 days interval for two weeks. Then, the heart, liver, spleen, lung, kidney and tumor were harvested, and all tissues were fixed in a 10% formalin solution, embedded in paraffin, sectioned, and stained with hematoxylin and eosin for analyzing. All the experiments were performed in compliance with the National Act on the Use of Experimental Animals (China) and the guidelines issued by Sichuan Province and Chengdu University. All the experiments were approved by the Animal Ethics Committee of Chengdu University.



Scheme S1. Schematic illustration of the preparation of LDH-Ce6 hybrid nanoflakes. Step 1: LDH nanoparticles with expanded interlayer spacing were fabricated through the addition of SDS in the co-precipitation process. Long chain SDS was used to expand LDH gallery from original 0.77 to 2.60 nm. Step 2: Ce6 molecules were covalently linked to (3-aminopropyl) triethoxysilane (APTES) in the presence of EDC as a catalyst. Step 3: APTES linked Ce6 was reacted with SDS expanded LDH in the presence of CTAB to get LDH-Ce6. The role of CTAB is to extract SDS molecules from LDH interlayers. Ce6 molecules can be covalently congugated through APTES anchoring to LDH surface.

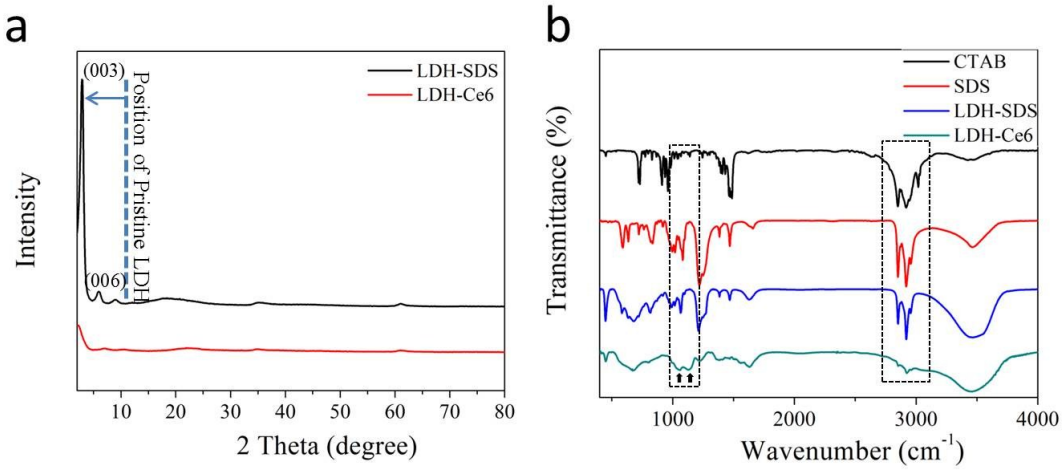


Fig. S1. a) The powder X-ray diffraction patterns (XRD) of SDS expanded LDH (LDH-SDS) and LDH-Ce6 hybrid nanoflakes (LDH-Ce6). b) Fourier transform infrared (FTIR) spectroscopy of N-cetyl-N,N,N-trimethylammonium (CTAB), sodium dodecyl sulfate (SDS), SDS expanded LDH (LDH-SDS), and LDH-Ce6 hybrid nanoflakes (LDH-Ce6).

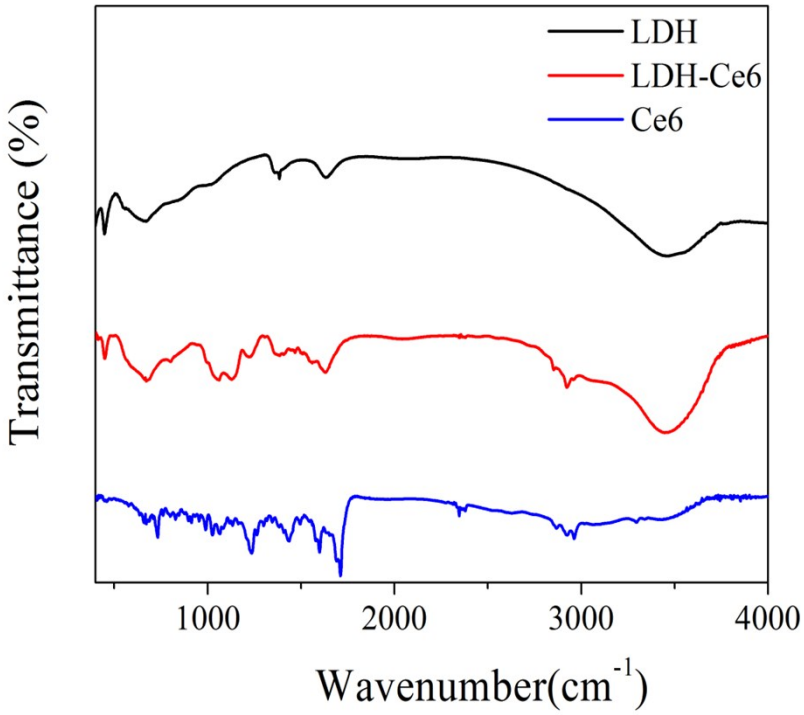


Fig. S2. Fourier transform infrared (FTIR) spectroscopy of pristine LDH (LDH), LDH-Ce6 hybrid nanoflakes (LDH-Ce6) and Ce6 powder (Ce6).

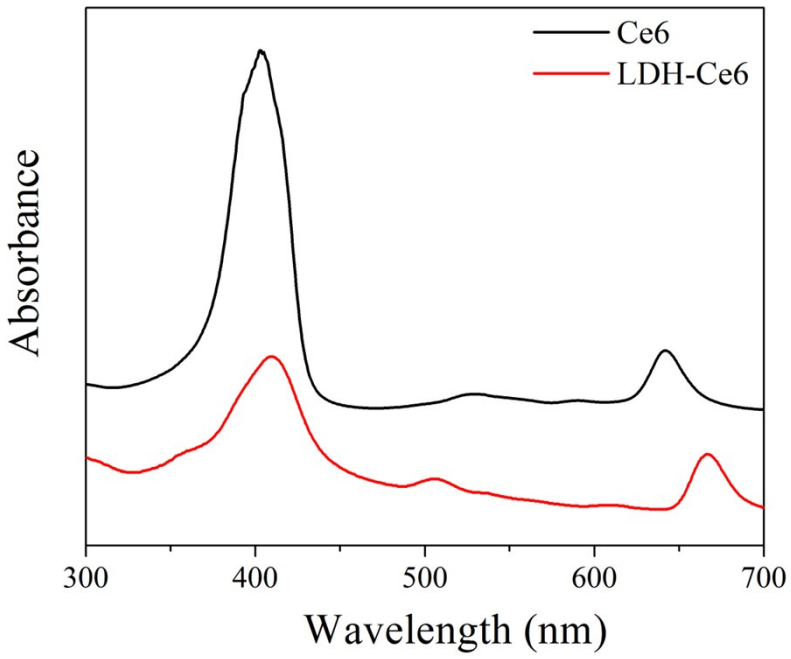


Fig. S3. UV-Vis absorption spectra of free Ce6 (Ce6) and LDH-Ce6 hybrid nanoflakes (LDH-Ce6).

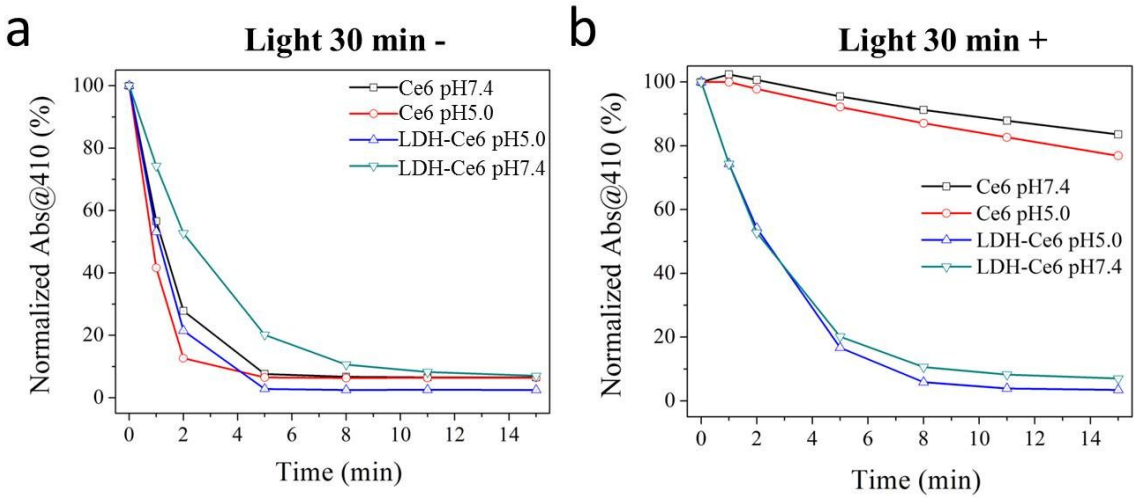


Fig. S4. Singlet oxygen generation analysis. Free Ce6 (Ce6) and LDH-Ce6 hybrid nanoflakes (LDH-Ce6) were under 650 nm irradiation. a) no and b) 30 minutes 650 nm light was irradiated before the test.

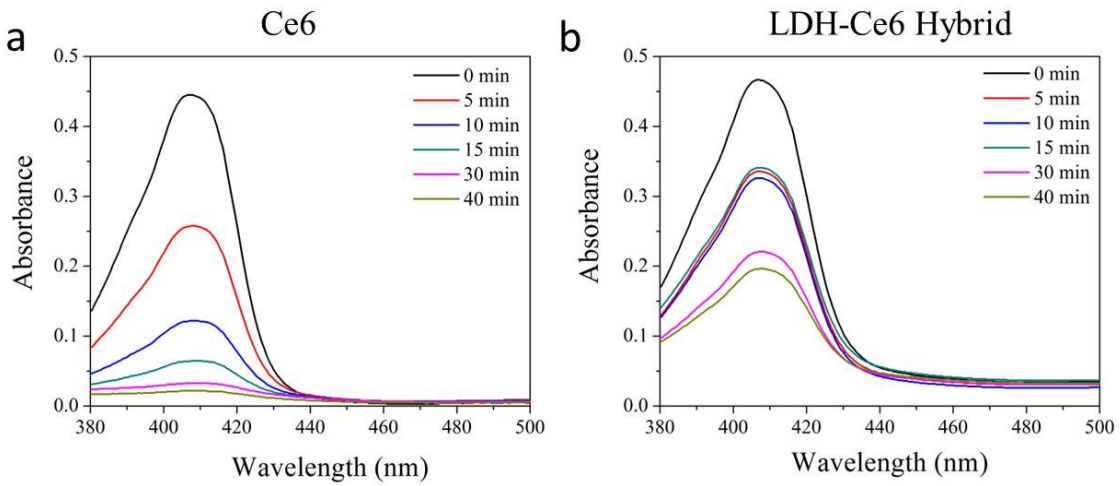


Fig. S5. Time-dependent absorbance change caused by 650 nm light irradiation: a) free Ce6 solution (Ce6), b) LDH-Ce6 hybrid nanoflakes.

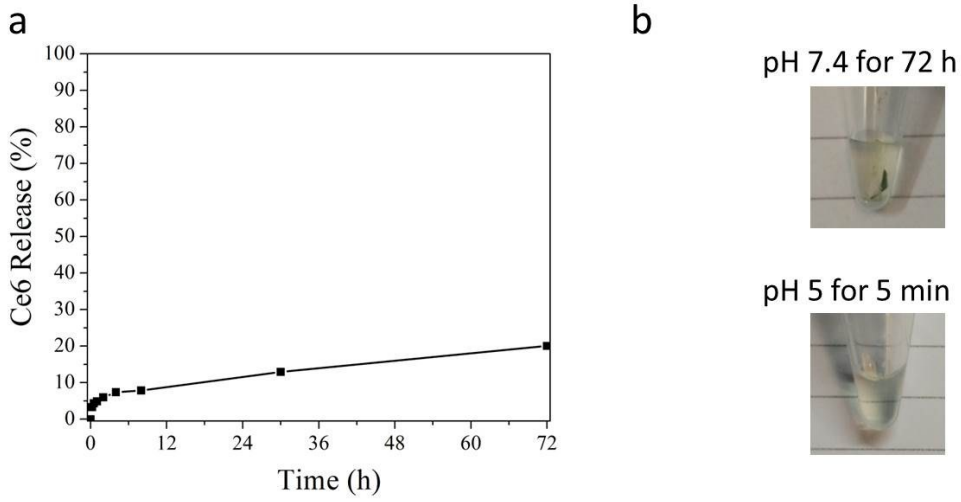


Fig. S6. a) Release profile of Ce6 from LDH-Ce6 hybrid in pH 7.4 PBS. b) The digital images of LDH-Ce6 treated with pH 7.4 or pH 5 buffers and centrifuged @12,000 rpm for 5min. After we keep LDH-Ce6 in aqueous solution at pH 7.4 for 72 hours, we can still centrifuge the sample to get precipitation of LDH-Ce6. In great comparison, if we leave LDH-Ce6 in water at pH 5 for 5 minutes, we cannot get any precipitation even at high speed centrifugation, implying the double hydroxides structure is broken at acidic environment and there is no nanomaterial left. When we use TEM to observe the sample, no nanostructure could be identified.

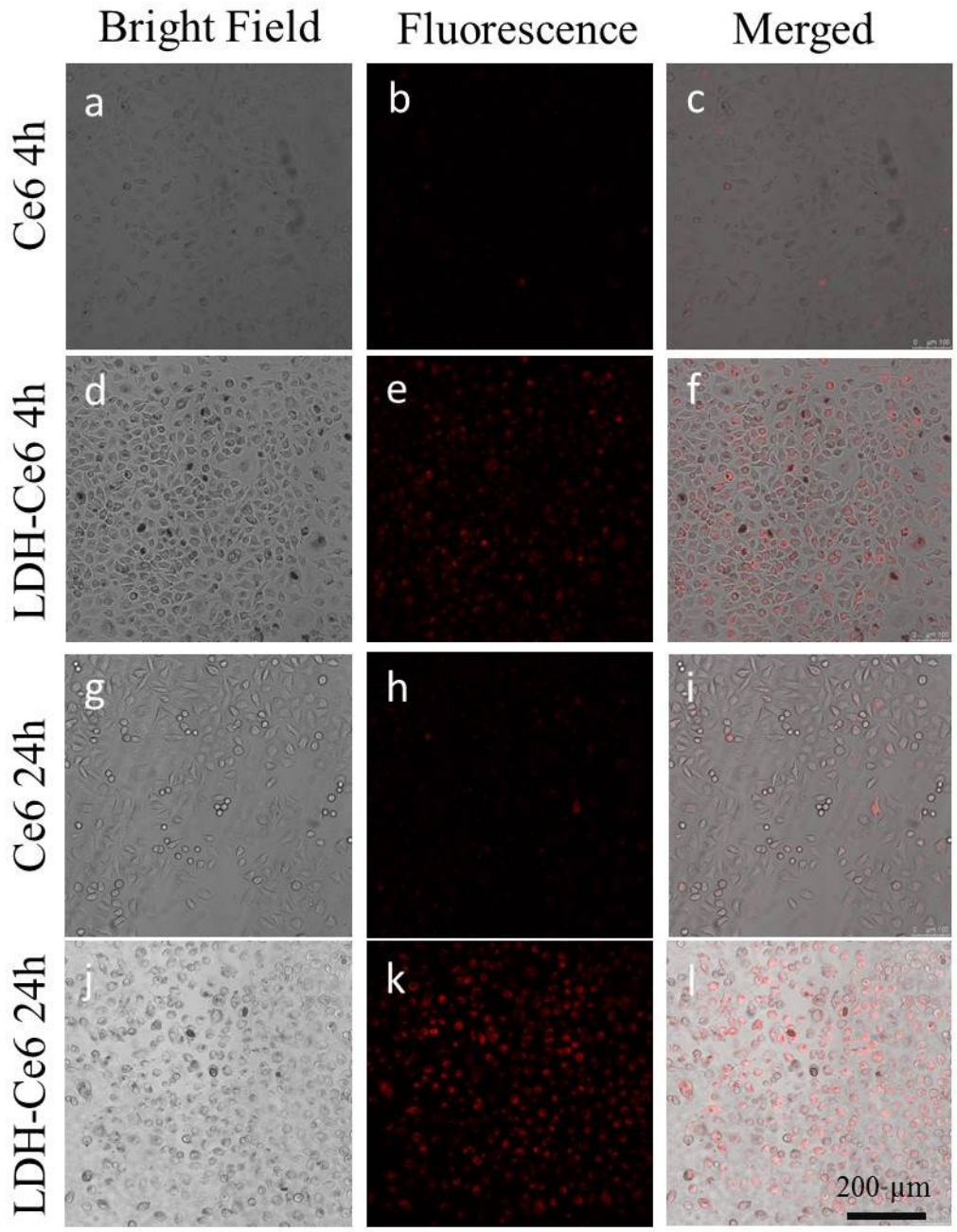


Fig. S7. Laser scanning confocal microscopy images of A549 cells after 4 (a-f) and 24 (g-l) hours Ce6 and LDH-Ce6 hybrid nanoflakes incubation. The cells were irradiated for 15 minutes (wavelength at 650 nm and power of 7 mW/cm^2) before confocal microscopy observation. The zeta potential of LDH-Ce6 is positively charged (about +30 mV).

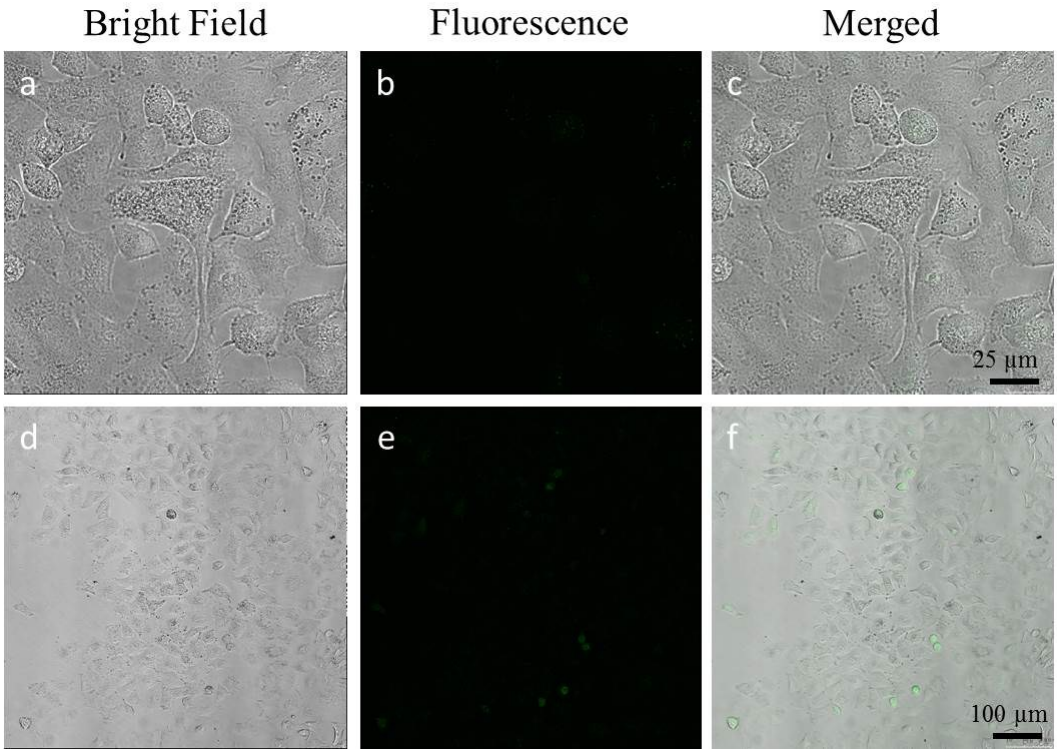


Fig. S8. Laser scanning confocal microscopy images of H_2DCFDA-stained A549 cells irradiated by 650 nm light for 10 minutes. This is a control of the experiment described in Fig. 2. When H_2DCFDA-stained A549 cells were irradiated by 650 nm light for 10 minutes without addition Ce6/LDH-Ce6, the generation of ROS was negligible.

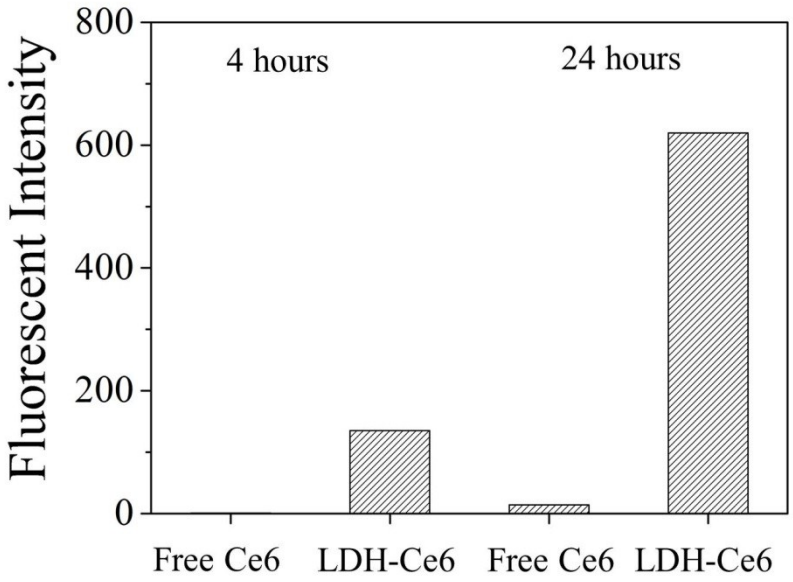


Fig. S9. Quantitative analysis of the ROS fluorescence signals collected in Fig. 2.

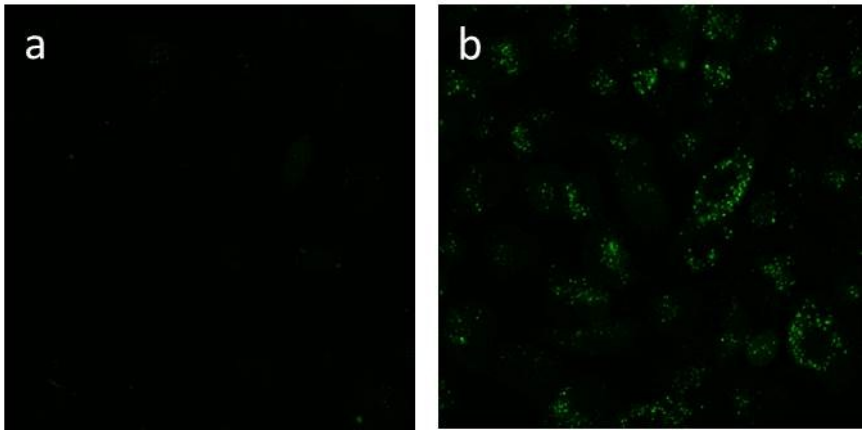


Fig. S10. Laser scanning confocal microscopy images of H2DCFDA-stained A549 cells after 4 (a) and 24 (b) hours incubation with Ce6 followed by 10 minutes 650 nm irradiation. For 24 hours incubation, the cell viability of free-Ce6 and LDH-Ce6 treated group is highly concentration dependent. At a high concentration, a relatively high amount of free Ce6 can enter cancer cells after 24 hours incubation and generate enough ROS under irradiation to kill cancer cells. Fig.2 shows images at a low magnification, so it is not clear enough to observe the uptake of Ce6 in cells. This figure is the high magnification fluorescence image of cellular ROS generated by free Ce6. Although the cellular uptake is significantly lower than the LDH-Ce6 treated group (Fig. 2), the generated ROS is still able to dramatically kill cancer cells when high concentration of Ce6 is used to incubate cells for 24 hours.

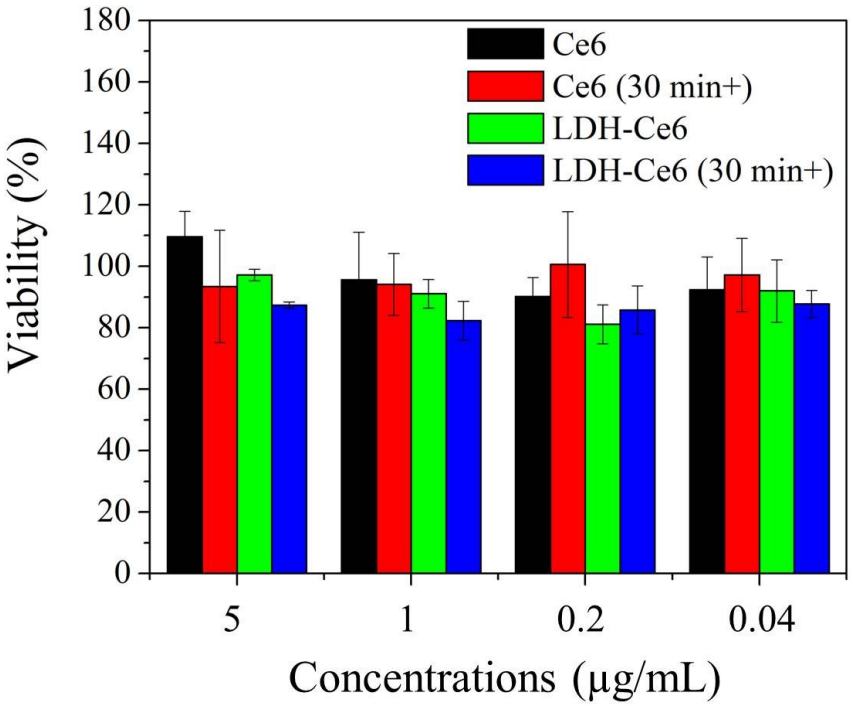


Fig. S11. Dark toxicity of free Ce6 (Ce6), LDH-Ce6 hybrid (LDH-Ce6), 30 minutes 650 nm light pre-irradiated free Ce6 (Ce6 (30 min+)) and 30 minutes 650 nm light pre-irradiated LDH-Ce6 hybrid (LDH-Ce6 (30 min+)). In all groups under different conditions, the cell viability remains high. This indicates that under dark environment, free Ce6 and LDH-Ce6 hybrid have very low toxicity.

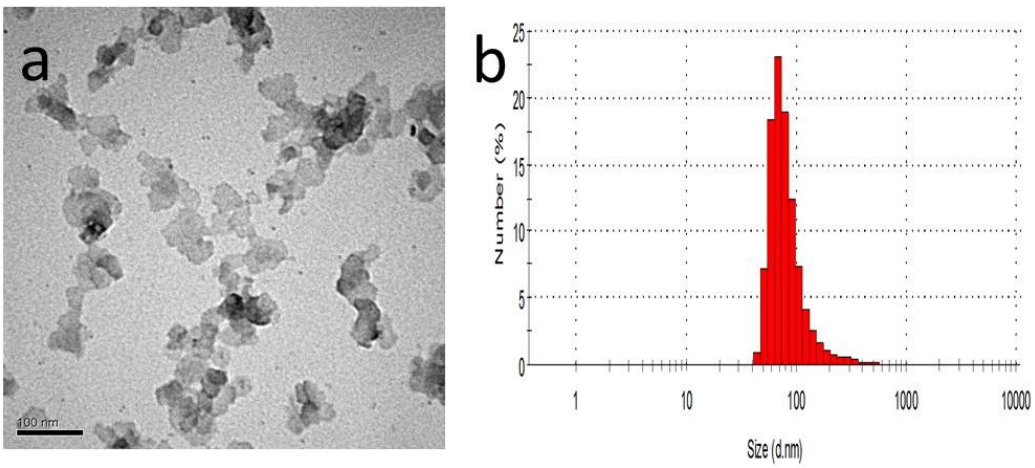


Fig. S12. a) TEM images and b) Dynamic light scattering measured size distribution of LDH-Ce6-PEG hybrid nanoflakes. The particle morphology and size of LDH-Ce6-PEG are very similar with LDH-Ce6.

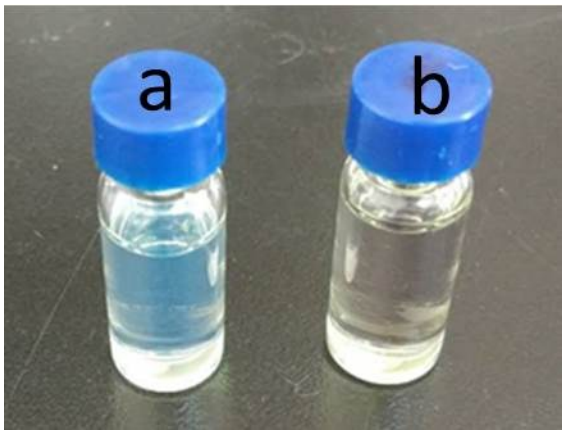


Fig. S13. a) DPBF and b) DPBF and Ce6 after 1 hour room light exposure. Significantly diminished DPBF were found, indicating room light can generate ROS.

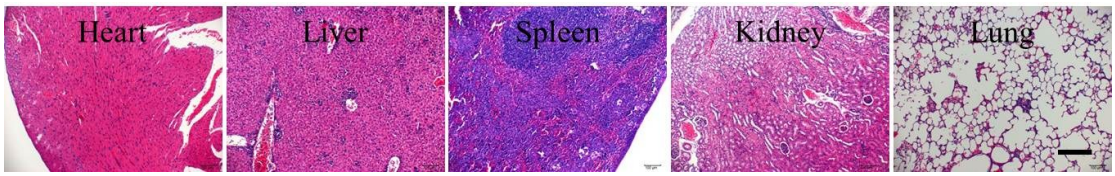


Fig. S14. H&E staining images of major organs (heart, liver, spleen, lung, and kidney).

References

1. Z.P. Xu, G.S. Stevenson, C.Q. Lu, G.Q. Lu, P.F. Bartlett and P.P. Gray, *J. Am. Chem. Soc.* 2006, **128**, 36.
2. Z. Wang, R. Ma, L. Yan, X. Chen and G. Zhu, *Chem. Commun.* 2015, **51**, 11587.