Supporting Information

Fluorinated Chitosan-Mediated Intracellular Catalase Delivery for Enhanced Photodynamic Therapy of Oral Cancer

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Supplementary Experimental Section:

General methods, materials and instruments

All reactions were carried out under Argon atmosphere if no additional indication. All solvents were processed according to standard methods prior to use and reagents were used as purchased without purification. Chitosan and chlorin e6 were purchased from Sigma-Aldrich. Tracker Green (lysosome probe), Hoechst and apoptosis detection kit were purchased from Thermo Fisher, and 3-(4, 5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) was purchased from Sigma. Dulbecco's Modified Eagle Medium (DMEM) was purchased from Gibco. Fetal bovine serum (FBS), penicillin and streptomycin were obtained from Gibco.

^{1}H-NMR spectra were recorded on 400 MHZ Bruker® and ^{13}C-NMR spectra were recorded on 100 MHz Bruker®. Data for ^{1}H-NMR are recorded as follows: chemical shift (δ, ppm), multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet or unresolved, coupling constant(s) in Hz, integration). Data for ^{13}C-NMR are reported in terms of chemical shift (δ, ppm). The measurement of UV-vis absorption spectra was carried out using a UV-vis absorption spectrometer (Shimadzu, UV-1700, Japan). PL spectra were collected using a Perkin-Elmer LS-55 equipped with a xenon lamp excitation source and a Hamamatsu (Japan) 928 PMT, using 90^{o} angle detection for solution samples. Hydrodynamic diameter and size distribution were measured by dynamic light scattering (DLS) with Zetasizer Nano S (Malvern Instruments Ltd, Worcestershire, UK) at room temperature. Transmission electron microscopy (B-TEM, Tecnai G2 Spirit Biotwin) studies were performed to investigate the morphology and

size of nanoparticles. The in vitro cell imaging and immunofluorescence were measured by laser confocal fluorescence microscopy (TCS SP8 STED 3X, Leica, Instrumental Analysis Center of Shanghai Jiao Tong University). The cell apoptosis analysis was performed by flow cytometry (BD Biosciences). The in vivo animal imaging was measured by chemiluminescence fluorescence image analysis system (PerkinElmer/*IVIS spectrum, Instrumental Analysis Center of Shanghai Jiao Tong University).

Synthesis of Synthesis of Fluorinated Chitosan

Tridecafluoroheptanoic acid (1 mmol, 364 mg) was dissolved in dimethyl sulfoxide (DMSO, Adamas) and then mixed with 1-(3-(dimethylamino)propyl)-3-ethylcarbodiimide hydrochloride (229 mg, 1.2 equiv) and NHS (138 mg, 1.2 equiv) under stirring in the dark at room temperature for 4 h to obtain activated tridecafluoroheptanoic acid. Chitosan (500 mg) was then dissolved in 1% acetic acid solution and mixed dropwise with activated tridecafluoroheptanoic acid under stirring in the dark for 24 h. To obtain fluorinated chitosan, a dialysis bag (MWCO: 1.0 KDa) was applied for purification in double distilled water for 72 h. The obtained polymer fluorinated chitosan (FC) was characterized by ^{19}F NMR.

Synthesis of FC-Ce6 Conjugate

Ce6 (59.6 mg, 0.1 mmol) was dissolved in dimethyl sulfoxide (DMSO, Adamas) and then mixed with 1-(3-(dimethylamino)propyl)-3-ethylcarbodiimide hydrochloride (22.9 mg, 1.2 equiv) and NHS (13.8 mg, 1.2 equiv) under stirring in the dark at room temperature for 4 h to obtain activated Ce6. FC (50 mg) was then dissolved in 1% acetic

acid solution and mixed dropwise with activated Ce6 under stirring in the dark for 24 h. To obtain purified FC-Ce6 conjugate, a dialysis bag (MWCO: 1.0 KDa) was applied for purification in double distilled water for 72 h. The obtained FC-Ce6 conjugate was characterized by ^{1}H NMR.

Preparation of Nanoparticles

FC-Ce6 was dissolved in PBS, then 10 mg mL^{-1} Catalase in PBS was added. The solution was kept in an ice bath in darkness for another 2 h. Subsequently, the mixture was dialyzed overnight at 4 °C (MWCO = 100 kD). The reactant was then subjected to ultrafiltration centrifugation at 1000 × g at 4 °C for 10 min. After filtration and purification, the product was stored at -20 °C for later use.

Cell culture

The oral cancer cell line Cal-27 was cultured in Dulbecco's Modified Eagle Medium (DMEM). The culture medium contains 10% fetal bovine serum (FBS) and antibiotics (50 units mL^{-1} and 50 units mL^{-1} streptomycin) at 37°C under a humidified atmosphere containing 5% CO_2 for 24 h and then replaced the fresh medium.

***In vitro* cell uptake and cytotoxicity study**

Cal-27 cells were seeded in confocal dishes and cultured for 24 h in DMEM culture medium. CAT-FC-Ce6 nanoparticles (Ce6: 10 μM) and nucleus probe (Hoechst) were added to each dish and cultured for 1, 2, 4 and 6 h, then the culture medium was removed and Hoechst (5 μg/mL, 500 μL) in fresh culture medium was added for incubation another 15 min. Cells were washed with PBS three times, and 1 mL of PBS was finally added before imaging. Cells were imaged with a 20 × objective lens. The

fluorescence of Hoechst was excited with a 405 nm laser with emission collected at 420-450 nm. The fluorescence of Ce6 was excited with a 633 nm laser with emission collected at 650-700 nm.

For cytotoxicity study, Cal-27 cells were seeded in 96-well plates. Twelve hours later, cells were treated with different groups at various concentrations with or without light irradiation (60 mW/cm^2, 10 min) and incubated for 72 h. Then, 20 μL of MTT solutions (5 mg/mL) were added to the 96-well plates. After 4 h incubation at 37°C, the medium was replaced with 200 μL of DMSO. The obtained solution was measured in a Bio Tech Synergy H4 at a wavelength of 570 nm.

Colocalization study of CAT-FC-Ce6 and lysosomes

Cal-27 cells were cultured in confocal dishes (2 × 10^5 cells per well) and cultured for 24 h. The culture medium was then removed before the cells were washed with PBS. The cells were then incubated with CAT-FC-Ce6 nanoparticles (equivalent Ce6 concentration: 10 μM) at 37 ^{o}C for 12 h. Cells being incubated with CAT-CS-Ce6 nanoparticles were used as controls. Thereafter, the medium was replaced with fresh medium containing 60 nM LysoTracker DND-26 (400 μL) and incubated for another 30 min. Then the medium was replaced with fresh PBS and observed by confocal microscopy (Leica TCS SP8 STED 3×). The fluorescence of Ce6 was excited with a 633 nm laser with emission collected at 650-700 nm. The fluorescence of Lyso Tracker was excited using a 488 nm laser with emission collected at 500-540 nm.

Cell apoptosis assay

Apoptosis of Cal-27 cells was measured by FITC-Annexin V/propidium iodide (PI)

(Thermo-Fisher) method. Cal-27 cells (2×10^5 cells/well) were incubated with different groups with or without light irradiation (60 mW/cm^2, 10 min). After 48 h incubation, FITC-Annexin V (5 μL) /PI (1 μL) was used to stain with the cells. The cells without any treatment were used as control. The samples were analyzed with a BD LSR Fortessa flow cytometer.

Intracellular ROS determination

Cal-27 cells (4×10^5) with DMEM culture medium were seed in a culture dish. After 12 h, cells were treated with different groups with or without white light illumination (60 mW/cm^2, 10 min) and incubated for another 12 h. Then, cells were washed by cold PBS (500 μL) and incubated with 100 μL DCFH-DA probe at 37^{o}C for 30 min. After that, cells were washed with cold PBS again. Fluorescence intensity was measured by laser confocal fluorescence microscopy (Leica) at excitation wavelength 488 nm and emission wavelength was collected at 510-550 nm.

Animals and tumor models

Experiment protocols involving animals were authorized by the Animal Ethics Committee of Shanghai Jiao Tong University School of Medicine (Approved number: A2018057). Balb/c male nude mice (4 weeks of age) was purchased from Chinese Academy of Science (Shanghai). The male nude mice were injected subcutaneously with 200 μL of cell suspension containing 5×10^6 Cal-27 cells. The tumor volume was allowed to grow to about 100 mm^3 before *in vivo* experiment.

***In vivo* imaging and biodistribution study**

Cal-27 bearing nude female mice (n = 3) were intravenously injected via tail vein with

200 μL of CAT-FC-Ce6 nanoparticles (5 mg/kg); mice without any treatment were used as negative control. Fluorescence intensity was monitored using an *in vivo* imaging system (PerkinElmer/*IVIS spectrum, Instrumental Analysis Center of Shanghai Jiao Tong University) with appropriate wavelength. (λ_{ex} = 640 nm, λ_{em} = 660 nm).

***In vivo* pharmacodynamic study**

The tumor-bearing nude mice were randomly divided into five groups (n = 5). Mice in various groups were intravenously injected via the tail vein with saline (200 μL), FC-Ce6, CAT-CS-Ce6 and CAT-FC-Ce6 with or without 650 nm laser irradiation (60 mW/cm^2) for 10 min once every three days for 30 days. The length and width of tumor and the body weight of mice were measured before every injection during the therapy. The tumor volume was calculated using the formula

$$V\ (mm^3) = 1/2 \times \text{length (mm)} \times \text{width (mm)}^2$$

After 30 days' treatment, some of mice were sacrificed and tumors were separated, weighed, and photographed. Additionally, the tumors were cut into small pieces, fixed in 40% formalin, and embedded in the paraffin. Then, the tissues were sectioned for histopathological analysis with H&E staining, immunohistochemical analysis and TUNEL immunofluorescence assay. For histology analysis, tissues were fixed in 4% paraformaldehyde and embedded in paraffin. Paraffin-embedded serial sections (5 μm) were obtained, stained with hematoxylin solution and eosin Y solution (H&E) to assess histological alterations by microscope (Olympus). In immunohistochemical studies, the paraffin embedded tumor sections were mounted on glass slides, heated for 30 min at 60 °C. Followed by incubations in a bath at room temperature, the deparaffinized slides

were rehydrated by graded alcohols (100%, 95%, 85%, 75%) and washed in distilled water. Pretreated with blocking medium for 5 min, the slides were incubated with primary anti-Bcl-2 antibody (1:500, ABcam) in antibody diluent solution for 30 min at room temperature and then 4 °C for 12 h. After washing with distilled water (1 mL) three time, the slides were counterstained using Aquatex (Merck, Germany) and observed by microscope (Olympus). Tissues apoptotic cells were assessed by a terminal transferase dUTP nick-end labeling (TUNEL) assay kit (In Situ Cell Death Detection Kit, Roche, USA) according to manufacturer's protocol. The paraffin-embedded 5-μm-thick tumor sections were mounted on glass slides, heated for 30 min at 60 °C. Followed by incubations in a bath at room temperature, the deparaffinized slides were rehydrated by graded alcohols (100%, 95%, 85%, 75%) and washed in distilled water. The slides were treated with freshly prepared 2% H_2O_2 for 10 min at room temperature, and washed three times with distilled H_2O for 2 min. Then slides were covered by 20 μg/mL of proteinase K solution (1 mL) for 15 min at room temperature and washed with distilled H_2O three times subsequently. The slides were blotted away excess water carefully, and then incubated with TdT equilibration buffer (2.5 mM Tris-HCl (pH 6.6), 0.2 M potassium cacodylate, 2.5 mM $CoCl_2$, 0.25 mg/mL bovine serum albumin (BSA)) for 10 min at room temperature. After removing the TdT equilibration buffer, the slides were incubated with TdT reaction buffer for 30 min at 37°C. TdT buffer was carefully covered with a glass coverslip and incubated for 30 min at room temperature. The slides were incubated in 2×SSC (300 mM NaCl, 30 mM sodium citrate) for 10 min to stop the reaction. After washing with 1x PBS (1 mL), the slides were incubated with

2% BSA solution for 30-60 min at room temperature. After washing with 1× PBS (1 mL) again, the slides were then covered with TdT staining buffer for 30 min in the dark. After washed with PBS and stained with Hoechst 33342, the slides were washed with PBS again, dried in air, and attached coverslips by Vectashield antifade mounting medium. The tissue sections were examined by laser confocal fluorescence microscopy (Leica) at an excitation wavelength of 488 nm with emission wavelength at 510-550 nm. Hoechst 33342 was excited with a 405 nm laser with emission collected at 420-460 nm.

Statistics study

Quantitative data were presented as mean ± standard error of the mean (SEM). ANOVA analysis was used for multiple comparisons, and Student's t-test was used for two-group comparisons. The differences in survival in each group were analyzed by the Kaplan–Meier method, and the P-value was determined by the log-rank test. All statistical analyses were carried out using GraphPad Prism 5.0. (P-value: *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

Supplementary Figures:

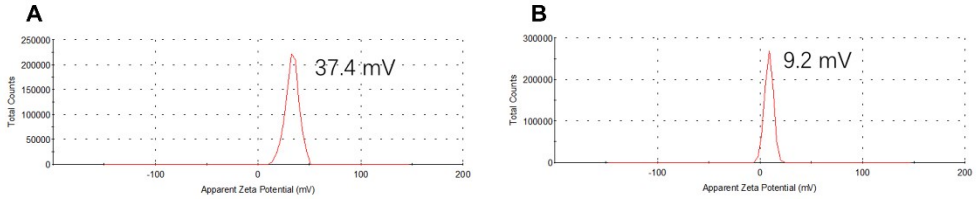

Figure S1. Zeta potential of chitosan (A) and CAT-FC-Ce6 nanoparticles (B) in deionized water. After modification, the zeta potential was decreased from 37.4 mV to 9.2 mV.

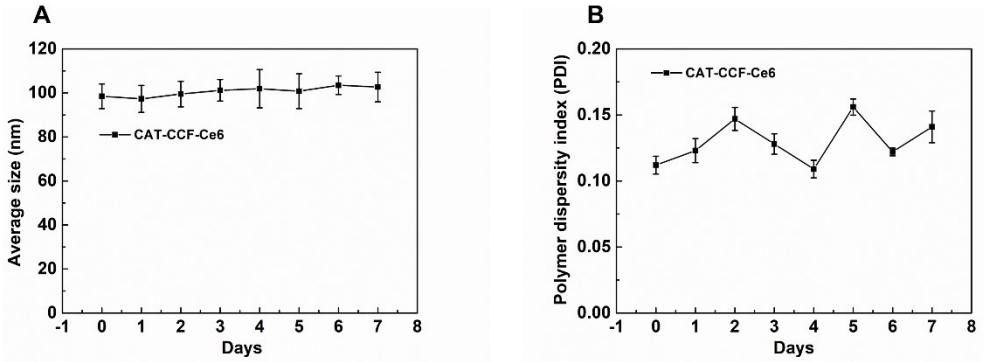

Figure S2. Size change and PDI change of CAT-FC-Ce6 nanoparticles when incubating them in PBS buffers for different days. Data represent mean values±standard deviation, n = 3.

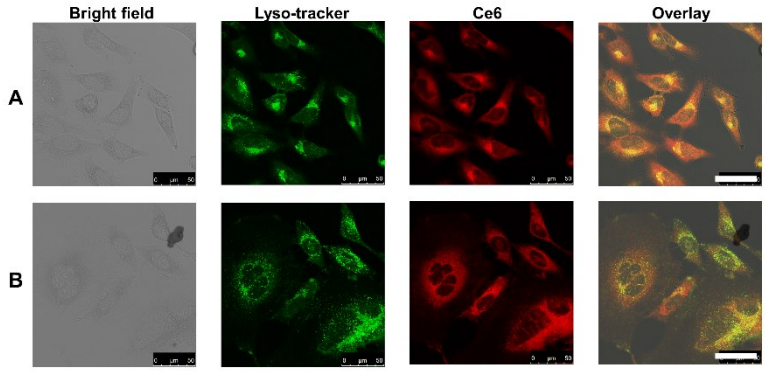

Figure S3. Confocal imaging of Cal-27 cells costained with lysosome tracker (green: 15 μL added to 200 μL culture medium), then added CAT-CS-Ce6 and CAT-FC-Ce6 nanoparticles (200 μL) (red), respectively. (A) CAT-CS-Ce6 nanoparticles; (B) CAT-FC-Ce6 nanoparticles. For lysosome tracker, the excitation wavelength was 488 nm, and the emission was collected between 500 to 540 nm. For Ce6, the excitation wavelength was 633 nm, and the emission was collected between 650 to 700 nm.

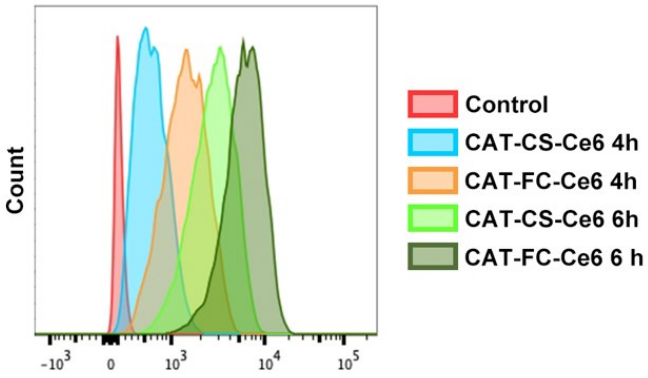

Figure S4. Flow cytometry analysis of Cal-27 cells incubated with 10μM CAT-CS-Ce6 or CAT-FC-Ce6 (based on the concentration of Ce6) for different time intervals.

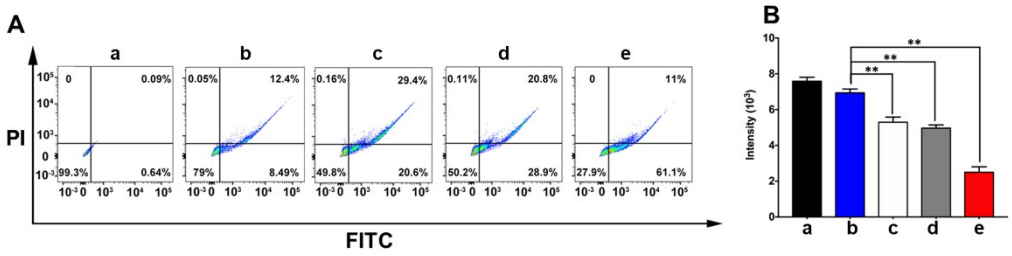

Figure S5. (A) Cell apoptosis study of Cal-27 cells after incubation with different formulation with or without 650 nm laser irradiation. (B) The phosphorescence signal of the oxygen probe reflecting dissolved oxygen in the culture medium of CAl-27 cells treated with different formulations. The statistical significance level is *p<0.05, **p<0.01. a, Ce6; b, CS-Ce6; c, CCF-Ce6; d,CAT-Ce6; e: CAT-CCF-Ce6.

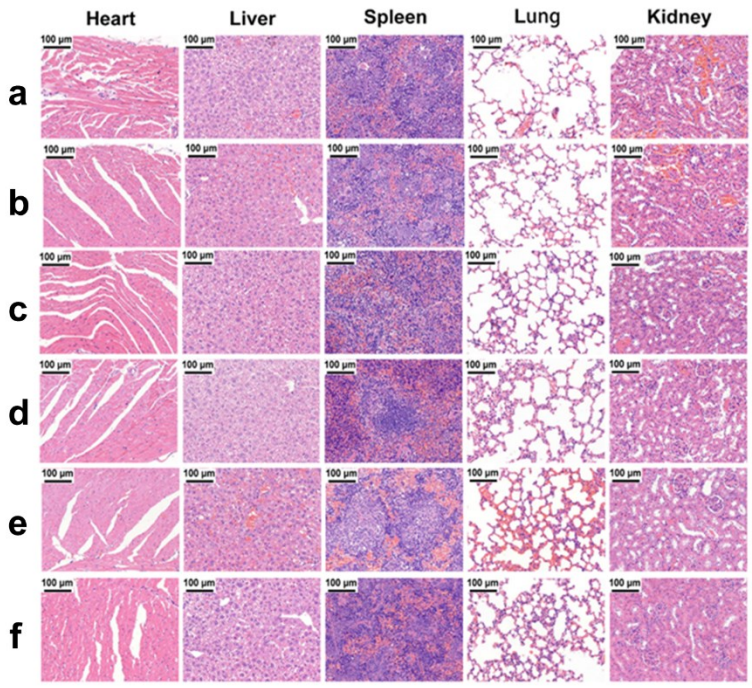

Figure S6. Microscopic images of H&E-stained sections of the major organs (heart, liver, spleen, lung, kidney) after tumor-bearing nude mice. HE staining results showed no organ toxicity after treatment by different agents both under dark or laser irradiation. The scale bar is 50 μm. a, saline group; b, Ce6 (laser); c, FC-Ce6 (laser); d, CAT-CS-Ce6 (laser); e, CAT-CCF-Ce6 (dark); f, CAT-CCF-Ce6 (laser).

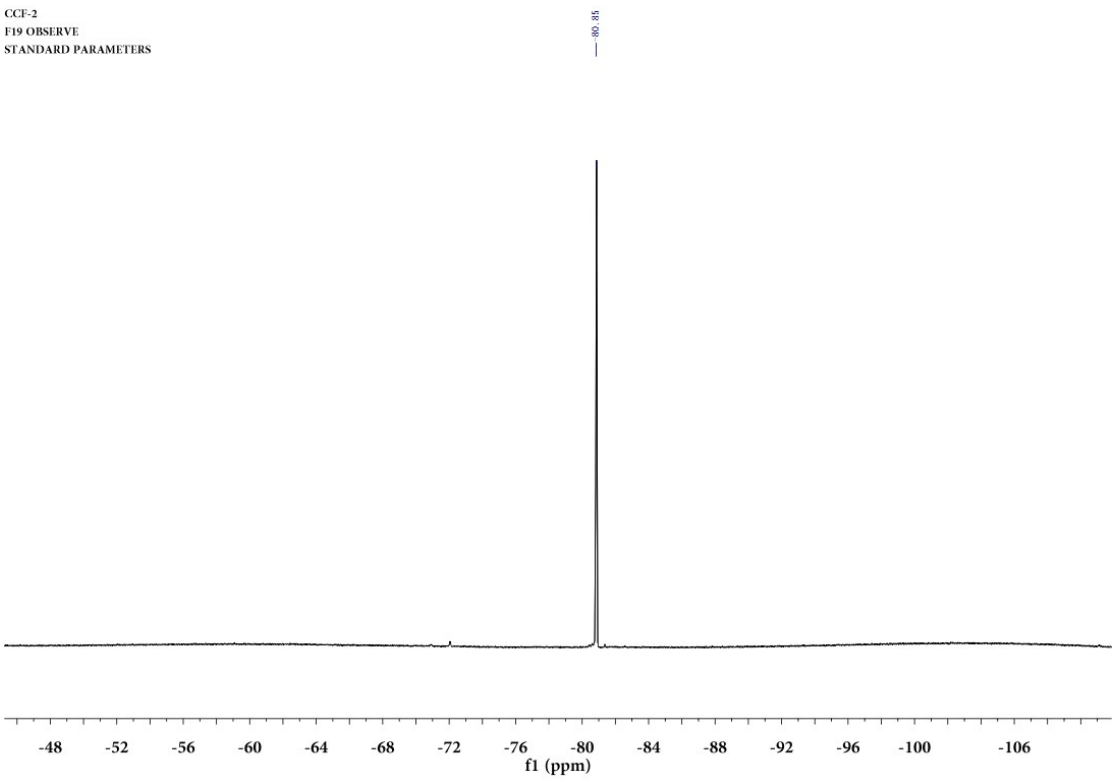

Figure S7. ^{19}F NMR spectrum of FC (D_2O, CF_3COOH).