

Electronic Supplementary Information

A two-pronged photodynamic nanodrug to prevent metastasis of basal-like breast cancer

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Experimental Section

Materials

Epalrestat (Epa) was purchased from TCI (Shanghai) Development Co., Ltd., and chlorin e6 (Ce6) was obtained from Beijing J&K Technology Co., Ltd. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and 1,3-diphenylisobenzofuran (DPBF) were bought from Aladdin Industrial Corporation. 2',7'-Dichlorofluorescein diacetate (DCFH-DA) was obtained from Sigma-Aldrich. Hoechst 33342, N-Cadherin Rabbit Polyclonal Antibody, Alexa Fluor 488-Labeled Goat Anti-Rabbit IgG (H+L), E-Cadherin Mouse Monoclonal Antibody and Alexa Fluor 488-Labeled Goat Anti-Mouse IgG (H+L) were all purchased from Beyotime Biotechnology Co., Ltd.

Cell culture

Human basal-like breast cancer MDA-MB-231 cells were incubated in RPMI 1640 medium containing 10% fetal bovine serum (FBS), 1% streptomycin and 1% penicillin at 37 °C in a humidified atmosphere containing 5% CO₂.

Animals

SCID mice (female, 5-6 weeks old) were purchased from Beijing HFK Bioscience Co., Ltd. All animal experiments were conducted under the approval of Shandong University Animal Experiment Ethics Review and the Health Guide for the Care and Use of Laboratory Animals of National Institutes. All mice received care in accordance with international ethics guidelines.

Preparation and characterizations of Epa/Ce6 NPs

Nanoprecipitation method was applied to prepare co-assembled Ce6/Epa NPs. In brief, 100 μ L Ce6 in DMF (50 mg mL⁻¹) was mixed with 100 μ L Epa in DMF (50 mg mL⁻¹), and then the mixture was slowly added into 5 mL deionized water under vigorous stirring at 1000 rpm. After 20 min, it was dialyzed against 2 L of deionized water to remove DMF and further filtered with a 0.45 μ m

membrane. For comparison, Ce6 and Epa sample were also prepared in a similar way in which 200 μL Ce6 in DMF (25 mg mL^{-1}) or 200 μL Epa in DMF (25 mg mL^{-1}) was slowly added into 5 mL deionized water under stirring at 1000 rpm for 20 min. UV-Vis spectra of Ce6/Epa NPs in water and mixed solvent (The volume ratio of DMF and water was 9:1) were recorded by a UV-Vis spectrophotometer (UV-8000S, Metash). The morphological observation of Ce6/Epa NPs was conducted with a transmission electron microscopy (TEM, JEM-200CX). The particle size distribution, physiological stability in 10% FBS and zeta potential of Epa/Ce6 NPs were measured by a Malvern Zetasizer Nano ZS. The release behavior of Epa from Ce6/Epa NPs was studied using dialysis method.

Singlet oxygen ($^1\text{O}_2$) detection

DPBF was occupied to detect the $^1\text{O}_2$ generated in Ce6-mediated PDT. If DPBF reacted with $^1\text{O}_2$, its characteristic absorbance at around 420 nm would decrease. Briefly, 2 mL of Ce6 (2 $\mu\text{g mL}^{-1}$), Epa (1.6 $\mu\text{g mL}^{-1}$) or Ce6/Epa NPs (2 $\mu\text{g mL}^{-1}$ for Ce6 and 1.6 $\mu\text{g mL}^{-1}$ for Epa) sample containing DPBF (15 $\mu\text{g mL}^{-1}$) were irradiated with 660 nm laser (100 mW cm^{-2}) for different periods. For comparison, blank sample containing DPBF was set as negative control. Because Ce6 and Epa themselves had weak absorbance at around 420 nm at this concentration, Ce6, Epa and Ce6/Epa NPs without DPBF were also irradiated with the same procedure. $A_t/A_0 \times 100\%$ was calculated to indicate the remaining DPBF. A_t referred to the difference in absorbance of every sample with or without DPBF after being irradiated for a specified period while A_0 represented the absorbance difference of every sample at the beginning. The smaller A_t/A_0 was, the more $^1\text{O}_2$ produced.

Cellular uptake assay

The uptake behaviors of Ce6 and Ce6/Epa NPs were analyzed via flow cytometry. MDA-MB-231 cells were seeded in 6-well plates at a density of 2×10^5 cells per well and incubated for 24 h. Afterwards, the cells were cultured in fresh medium containing equivalent dose of Ce6 or Ce6/Epa NPs at a final Ce6 concentration of 10 $\mu\text{g mL}^{-1}$. After incubation for 1, 2, 4 or 6 h, the cells were washed, harvested and resuspended in PBS for flow cytometer (BD FACSCelesta).

Cellular ROS measurement

A ROS probe, DCFH-DA, was applied to evaluate the ROS production in this Ce6-mediated PDT. Fluorescent DCF oxidized by ROS could be measured by a flow cytometer. Briefly, MDA-

MB-231 cells were seeded in 6-well plates at a density of 2×10^5 cells per well and incubated for 24 h. Afterwards, the cells were cultured in fresh medium containing equivalent dose of Ce6 ($2 \mu\text{g mL}^{-1}$) or Ce6/Epa NPs. After incubation for 4 h, the cells were washed, incubated with $20 \mu\text{M}$ DCFH-DA in RPMI 1640 medium (without FBS) for 20 min and then irradiated with 660 nm laser at a power intensity of 100 mW cm^{-2} for 2 min or not. For fluorescence imaging, the cells were also stained with Hoechst 33342 ($5 \mu\text{g mL}^{-1}$) for 15 min. For flow cytometry analysis, the cells were washed and collected.

MTT assay

The dark cytotoxicity and photodynamic cytotoxicity of Epa, Ce6 and Epa/Ce6 NPs were determined by MTT assay. Briefly, MDA-MB-231 cells were seeded in 96-well plates at a density of 7×10^3 cells per well for 12 h incubation. Afterwards, the cells were cultured in fresh medium containing serial concentrations of Epa, Ce6 or Ce6/Epa NPs. The concentrations ranged from 0.8 to $8.1 \mu\text{g mL}^{-1}$ for Epa and from 1.0 to $10.0 \mu\text{g mL}^{-1}$ for Ce6, respectively. After incubation for 4 h, the cells were washed, cultured with fresh medium and irradiated with 660 nm laser at a power intensity of 100 mW cm^{-2} for 2 min or not. After 20 h, $10 \mu\text{L}$ MTT in PBS solution (5 mg mL^{-1}) was added to each well and incubated for another 4 h. Then, the supernatant was cleaned up, and $200 \mu\text{L}$ of DMSO was added. Finally, a microplate reader was utilized to record the absorbance at 490 nm of each well, and then the cell inhibition ratio was calculated based on the following equation.

$$\text{Cell inhibition ratio (\%)} = \frac{A_{\text{negative control}} - A_{\text{sample}}}{A_{\text{negative control}} - A_{\text{blank}}} \times 100\%$$

Where $A_{\text{negative control}}$ referred to the absorbance of untreated cells. A_{blank} was the absorbance of well without cells.

EMT inhibition evaluation

E-cad and N-cad expression in different-treated MDA-MB-231 cells were evaluated with immunofluorescence analysis. Briefly, MDA-MB-231 cells were seeded in 6-well plates at a density of 2×10^5 cells per well and incubated for 24 h. Afterwards, the cells were cultured in fresh medium containing equivalent dose of Epa ($8.1 \mu\text{g mL}^{-1}$) or Ce6/Epa NPs. After incubation for 24 h, the cells were washed, stained with Hoechst 33342 for 15 min and then fixed. For E-cad expression analysis, the cells were incubated with E-Cadherin Mouse Monoclonal Antibody for 1 h and Alexa

Fluor 488-Labeled Goat Anti-Mouse IgG (H+L) for another 1 h. For N-cad expression analysis, the cells were incubated with N-Cadherin Rabbit Polyclonal Antibody for 1 h and Alexa Fluor 488-Labeled Goat Anti-Rabbit IgG (H+L) for another 1 h. All samples were observed with fluorescence microscope.

Would healing assay

MDA-MB-231 cells were seeded in 6-well plates at a density of 2×10^5 cells per well and then incubated until the cells reached more than 90% confluency. Then, the medium was discarded, and wounds were drawn. The wells were washed to remove the falling cells, and then Epa ($8.1 \mu\text{g mL}^{-1}$), Ce6 ($10 \mu\text{g mL}^{-1}$) or Ce6/Epa NPs in medium (containing 2% FBS) was added. The wound of every sample was captured with a microscope. After 24 h, the wounds were captured again.

Bio-distribution of Ce6/Epa NPs *in vivo*

Live animal *in vivo* imaging system (IVIS Kinetic) was applied to investigate the bio-distribution of Ce6/Epa NPs *in vivo*. 5×10^6 MDA-MB-231 cells (0.1 mL, containing 50% matrigel) were subcutaneously inoculated in the second pair of breast pads on the left of female SCID mice. When the tumors reached around 200 mm^3 , the mice were injected with Ce6 (3 mg kg^{-1}) or Ce6/Epa NPs via tail vein, respectively. At the predetermined time, the fluorescence signal was collected with a live animal *in vivo* imaging system. The isolated major organs were excised 24 h post administration for signal collection.

***In vivo* anti-tumor effects and anti-metastasis evaluation of Ce6/Epa NPs**

For *in vivo* therapeutic efficacy evaluation of the well-designed Ce6/Epa NPs, 5×10^6 MDA-MB-231 cells (0.1 mL, containing 50% matrigel) were subcutaneously inoculated in the second pair of breast pads on the left of female SCID mice. When the tumors reached around 50 mm^3 , the mice were randomly divided into three groups (NS, Ce6/Epa NPs and Ce6/Epa NPs + NIR), and every group was intravenously injected with normal saline (NS), Ce6/Epa NPs (3 mg kg^{-1} for Ce6 and 2.4 mg kg^{-1} for Epa) or equivalent Ce6/Epa NPs with NIR laser irradiation (660 nm , 100 mW cm^{-2} , 5 min) after 8 h post administration, respectively. During the treatment, the body weight was measured every two days, and the tumor volume was also monitored with the following equation: $\text{length} \times \text{width}^2/2$. After the last treatment, some tumors were dissected out and digested with collagenase IV, hyaluronidase and DNase at 37°C . Then, the tumor cells were stained with FITC Anti-Human CD24 Antibody and PerCP/Cy5.5 Anti-Mouse/Human CD44 Antibody to analyze

cancer stem cell (CSC), stained with E-Cadherin Mouse Monoclonal Antibody and Alexa Fluor 488-Labeled Goat Anti-Mouse IgG (H+L) to determine E-cad expression or stained with N-Cadherin Rabbit Polyclonal Antibody and Alexa Fluor 488-Labeled Goat Anti-Rabbit IgG (H+L) to determine N-cad expression. After a month of the last treatment, the lungs were excised and fixed with 4% polyoxymethylene. Metastatic tumor nodules in lung were observed and sectioned for H&E staining.

Statistical analysis

Ordinary one-way ANOVA analysis was performed. Statistical significance values were referred as follows: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$.

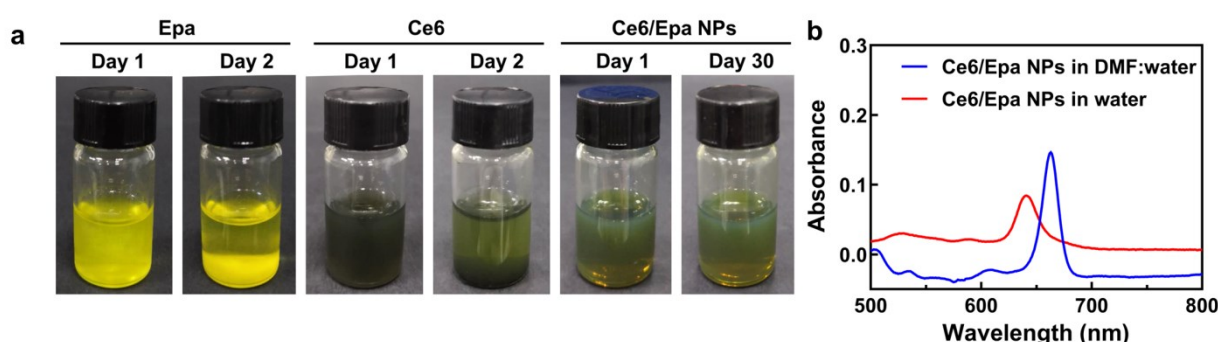


Fig. S1 (a) Photographs of Epa, Ce6 and Ce6/Epa NPs. (b) UV-Vis spectra of Ce6/Epa NPs in mixed solvent (The volume ratio of DMF and water was 9:1) and water.

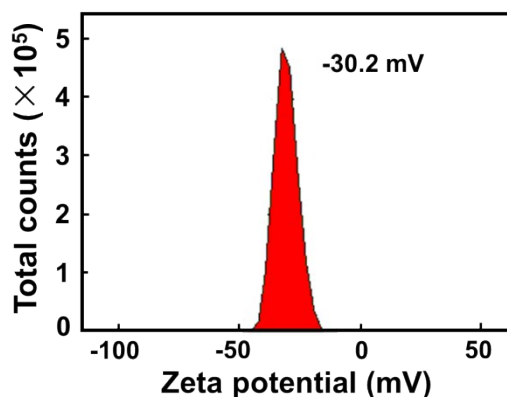


Fig. S2 The zeta potential of Ce6/Epa NPs.

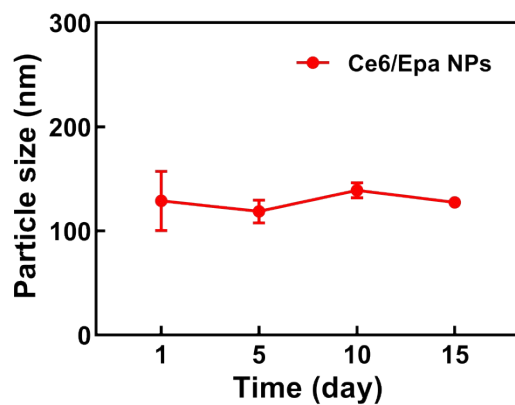


Fig. S3 The particle size change of Ce6/Epa NPs in 10% FBS (n=3).

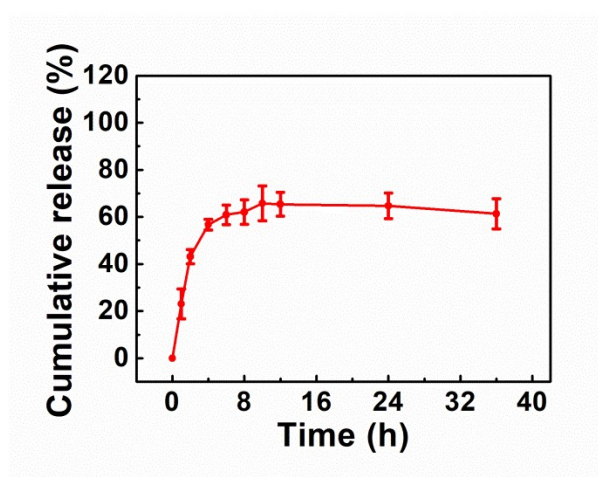


Fig. S4 The cumulative release behavior of Epa (n=3).

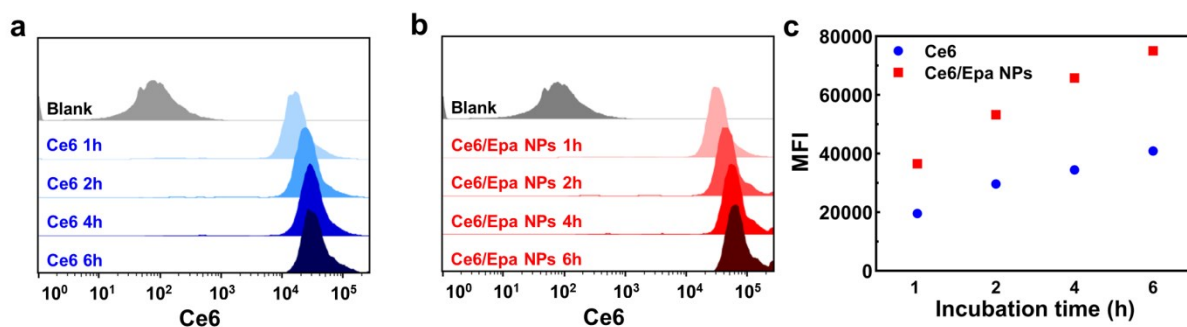


Fig. S5 The uptake behaviors of (a) Ce6 and (b) Ce6/Epa NPs and (c) the mean fluorescence intensity (MFI) after incubation for 1, 2, 4 or 6 h.

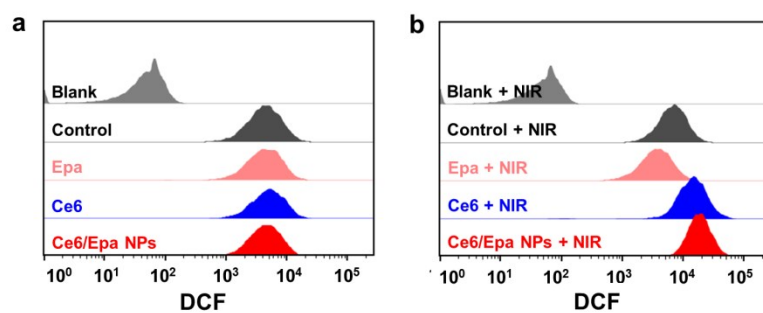


Fig. S6 Flow cytometry to evaluate intracellular ROS generating in different-treated cells (a) without or (b) with NIR laser irradiation.