Supporting information

Construction of a targeted photodynamic nanotheranostic agent using upconversion nanoparticles coated with the ultrathin silica layer

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Contents

1. Chemicals and Materials

Rare earth oxides, including Y_2O_3, Yb_2O_3 and Er_2O_3 with purities large than 99.99% were obtained from Sigma-Aldrich Corp. 1-Octadecene, oleic acid and MB were provided by the Aladdin Reagent, Ltd. (Shanghai, China) while the rest chemical reagents of analytical grade were provided by Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). An OKP purification system (Shanghai Laikie Instrument Corp., China) was used to prepare ultrapure water for all sample solutions. Mice were bought from Hubei Biossci Biotechnology Co. Ltd. (Wuhan, China). In addition, the animal care and handing procedures were reviewed and approved by the Animal Care and Use Committee of Linyi University.

2. Characterizations

The size and morphology of the prepared UCNPs and UCNPs@SiO_2 were characterized by the transmission electron microscope (TEM, model: JEM-2010, JEOL). The X-ray diffraction (XRD) experiments were obtained on a D8 ADVANCE X-ray powder diffractometer with CuKα radiation (λ=1.5405Å). A fluorescence spectrophotometer (mode: F-4600, Hitachi) equipped with an external 980 continuous wave laser was used to record the upconversion luminescence spectra. An Uv-Vis

spectrophotometer (model: Cary 60, Agilent) was used to obtain the Uv-Vis absorption spectra. The Zeta-size nano instrument (Zen 3600, Malvern Instruments Ltd.) was used to provide the ζ potential of the nanoprobes. The fluorescent images were achieved on a Leica TCS SP5 two-photon laser scan confocal microscope with an objective lens (×40). CCK-8 test was performed on a Thermo Scientific Multiskan Mk3 microplate reader. Flow cytometry (cytoflex, beckmancoulter, America) was used to record the cellular fluorescence information.

3. Preparation of the nanoprobe

3.1 Preparation of UCNPs@SiO_2/MB

The preparation of the oleic acid protected $NaYF_4$:Yb^{3+}, Ho^{3+}/Tm^{3+} UCNPs were based a previously reported procedure.[1,2] Then, UCNPs@SiO_2/MB were prepared according to a modified procedure reported by Fan et al. to obtain the thin silica layer.[3] The detailed preparation process was as follows: 10 mL cyclohexane was mixed with 0.660 mL of Igepal CO-520 (NP-5) and stirred for 1 h to form the reverse micelles. Afterward, 0.450 mmol oleic acid protected UCNPs were added and the system was vigorously stirring for 1 h to promote a ligand exchange between oleate and NP-5, leading to the UCNPs entrapped in the water pool.[4] Then, 225 uL MB aqueous solution (4 mg/mL) and 60 uL ammonia (30%) was added dropwise, respectively, and stirred for 2 h. Finally, 90 uL tetraethyl orthosilicate (TEOS) and 20 uL (3-aminopropyl)triethoxysilane (APTES) was slowly added into the system and the silica layer grew on the surface of UCNPs for 24 h. Partially hydrolyzed and negatively charged TEOS transferred into the hydrophilic interior of reverse micelle to interact with the positively charged MB. Experienced classic hydrolysis and condensation, UCNPs@SiO_2/MB was obtained.

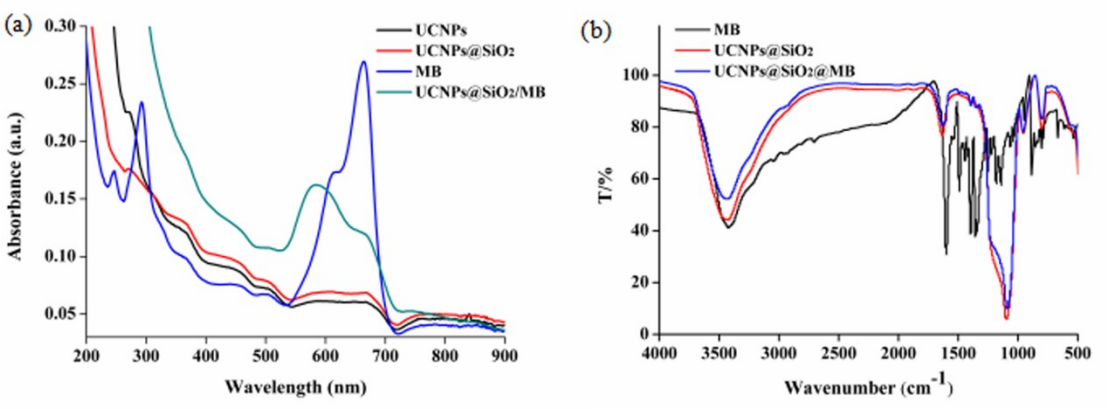

Figure S1: (a) Uv-Vis spectra of UCNPs, UCNPs@SiO_2, MB, UCNPs@SiO_2/MB; (b) FT-IR spectra of MB, UCNPs@SiO_2, UCNPs@SiO_2/MB

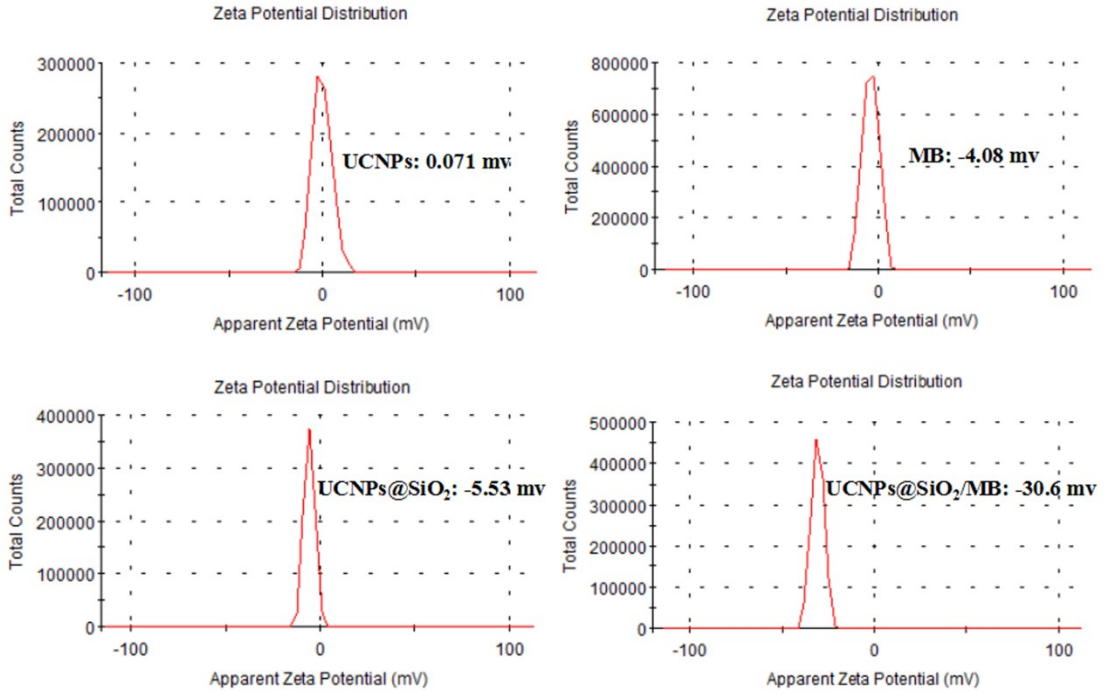

Figure S2: Zeta-potential analysis of UCNPs; MB; UCNPs@SiO_2; UCNPs@SiO_2/MB.

3.2 Preparation of UCNPs@SiO_2/MB@PEG-FA

Briefly, COOH-PEG-FA, NHS and EDC were mixed in dimethyl sulfoxide (DMSO) for activation in a molar ratio of 1:2.5:1 (7.2, 18 and 7.2 μmol) for 1 h.[3] And then, 250 uL of the activated mixture was reacted with 2 mL UCNPs@SiO_2/MB for 12 h under slow vibration. The obtained UCNPs@SiO_2/MB@PEG-FA was purified with

ultrapure water by centrifugation, and finally dispersed in PBS buffer solution (pH-7.5).

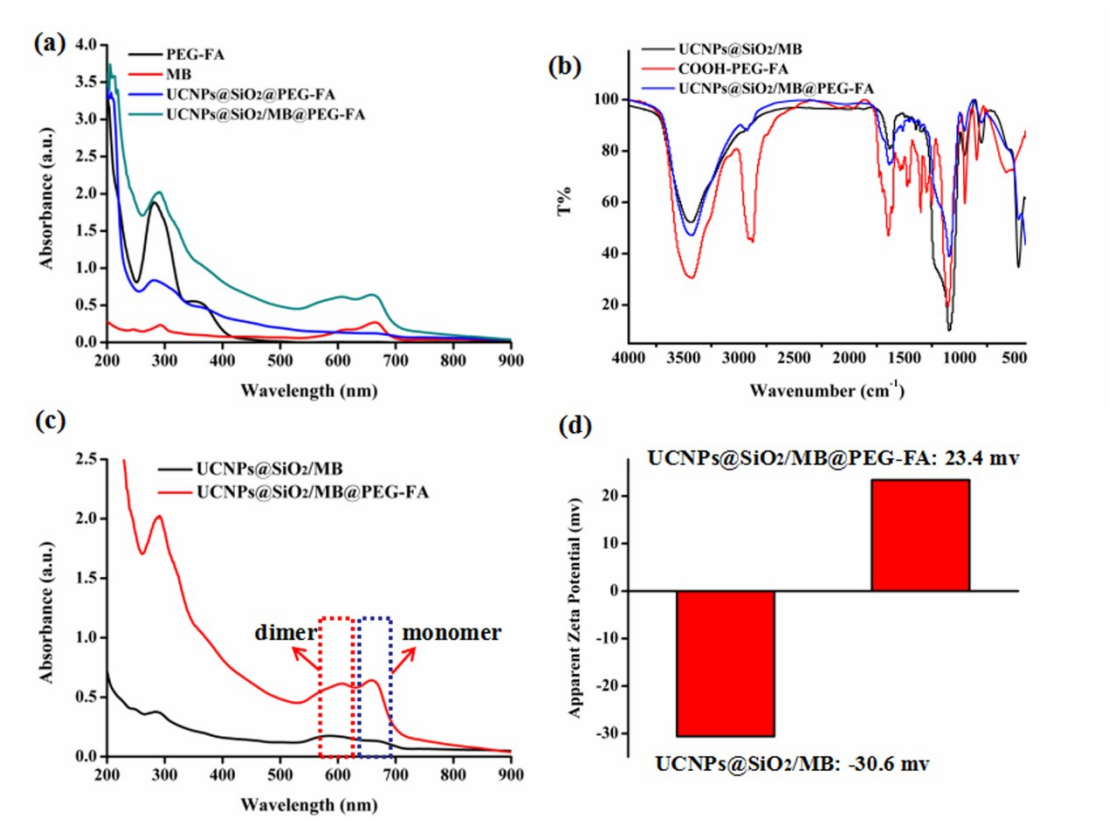

Figure S3: (a) Uv-Vis Spectra of PEG-FA, MB, UCNPs@SiO_2@PEG-FA; UCNPs@SiO_2/MB@PEG-FA; (b) FT-IR analysis of UCNPs@SiO_2/MB, COOH-PEG-FA and UCNPs@SiO_2/MB@PEG-FA; (c) Uv-Vis Spectra of UCNPs@SiO_2/MB; UCNPs@SiO_2/MB@PEG-FA; (d) Zeta-potential analysis of UCNPs@SiO_2/MB and UCNPs@SiO_2/MB@PEG-FA

4. Evaluation of the prepared nanoprobe

4.1 Cytotoxicity evaluation

The CCK-8 assay was used to evaluate the *in vitro* cytotoxicity of the prepared UCNPs@SiO_2/MB@PEG-FA nanoprobe. In brief, MCF-7 cancer cells at a density of 15000 cells per well were cultured in 96-well flat-bottom microtiter plates. Then, different concentrations of the UCNPs@SiO_2/MB@PEG-FA nanoprobe (0-0.12 mg/mL) were added into the corresponding wells, respectively, and each

concentration was examined in four parallel wells. After 24 h, MCF-7 cancer cells were washed with PBS for several times to remove excess nanoprobe and then incubated with 10 μL of CCK-8 at 37°C for another 2 h. Finally, the microplate reader was used to detect the absorbance of MCF-7 cancer cells at 450 nm. Cell viability (%) was calculated according to the equation: Cell Viability (%) = Mean Absorbance $_{\text{treated wells}}$/Mean Absorbance $_{\text{control wells}}$×100%.

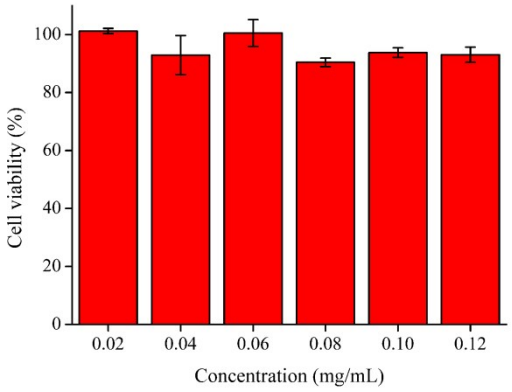

Figure S4: Cell viability (%) under different concentrations of the UCNPs@SiO_2/MB@PEG-FA nanoprobe.

4.2 Cellular uptake and localization

Dulbecco's modified Eagle's medium (DMEM) supplied with heat-inactivated fetal bovine serum (FBS) (10%), penicillin (100 U/mL), and streptomycin (100 U/mL) was used to culture MCF-7 cells under 37°C in humidified air containing certain CO_2 (5%). The MCF-7 cancer cell was transferred on glass coverslips and cultured for 12 h. Then, 80 μg/mL of the UCNPs@SiO_2/MB@PEG-FA was added and incubated the MCF-7 cancer cell for 6 h. After washed with PBS buffer (pH=7.4) for several times, MCF-7 cancer cell were imaged immediately with a two-photon confocal lasers scanning microscope with an objective lens (×40). The fluorescence information of the UCNPs@SiO_2/MB@PEG-FA nanoprobe was recorded at the wavelength range of 450-510 nm and 515-575 nm, respectively, excited by a 980 nm laser.

In addition, the positive Zeta-potential of the prepared

UCNPs@SiO_2/MB@PEG-FA nanoprobe (+23.4 mv) promoted most of the prepared nanoprobe to distribute in the mitochondria (Figure S5), which was beneficial for the ROSs produced by MB to damage mitochondria and induce the mitochondria-mediated cell apoptosis.

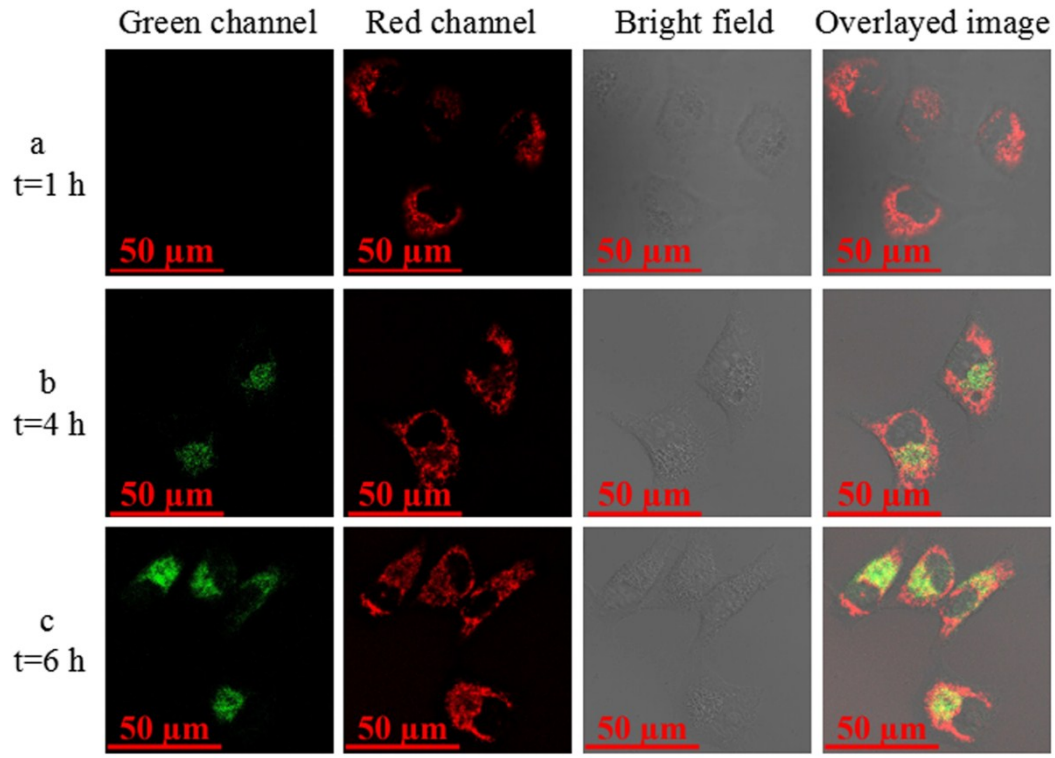

Figure S5: CLSM images of MCF-7 cells treated with 80 μg/mL of the prepared nanoprobe for (a) 1 h; (b) 4 h; (c) 6 h, respectively. Green channel was collected at 450-510 nm under the excitation of 980 nm to obtain the UCNPs information; Red channel was collected at 650-720 nm under the excitation of 633 nm to obtain the mitochondria information (fluorescent dye, the MitoTracker@ Deep Red); Bright field to obtain the cell information.

4.3 Detection of ROSs

4.3.1 Detection of ROSs produced by the UCNPs@SiO_2/MB@PEG-FA nanoprobe in aqueous solution

The dye, 9,10-anthracenediyl-bis(methylene)dimalonic acid (ABDA), is a commonly-used indicator to investigate the generation of ROSs, which could irreversibly react with ROSs to cause a decay of the fluorescence intensity at 407 nm.[5] ABDA in

DMSO (1 mL, 10 μL) was added into UCNPs@SiO_2/MB@PEG-FA solution (1 mg/mL, 1 mL) and then the mixture was irradiated with 980 nm continuous wave laser for 65 min. The first measurement was performed after 5 min of irradiation. ROSs production was confirmed via detecting the fluorescence emission of ABDA at 407 nm under the excitation at 380 nm.

4.3.2 *In vitro* ROSs detection

For *in situ* observation, MCF-7 cancer cells were cultured in the glass coverslips for 12 h. Then, the prepared UCNPs@SiO_2/MB@PEG-FA nanoprobe (80 μg/mL) incubated MCF-7 cancer cells for 6 h. Afterwards, MCF-7 cancer cells were stained with 1.0 mL DMEM containing 2'7'-dichlorofluorescin diacetate (DCFH-DA, *V:V*=5000:1) for 20 min. After washed with PBS buffer (pH=7.4) for several times, MCF-7 cancer cells were immersed in 1 mL PBS and observed under the two-photon laser scan confocal microscope. The fluorescence information of the oxidation product of DCFH-DA, DCF, was recorded at green channel (500 nm to 570 nm) under the excitation at 488 nm.

4.4 Observation of the changes of the mitochondrial membrane potential ($\Delta\psi_m$)

$\Delta\psi_m$ was detected as the manufacturer's instructions. Briefly, MCF-7 cancer cells were incubated with the prepared UCNPs@SiO_2/MB@PEG-FA nanoprobe (80 μg/mL) for 6 h, then collected and divided into two groups. One was irradiated with 980 nm continuous wave laser (1.5 W/cm^2, 4 min) as the experimental group, and the other was used for the control group without further treatment. MCF-7 cancer cells in two groups were washed with serum-free culture medium and then incubated with DMEM containing JC-1 working solution (*V:V*=40:1) for 20 min. After washing with ice-cold staining buffer twice, MCF-7 cancer cells were resuspended in 500 μL PBS and analyzed by the flow cytometry.

To *in situ* observe the $\Delta\psi_m$ change, MCF-7 cancer cells were incubated with 80

μg/mL of the UCNPs@SiO_2/MB@PEG-FA nanoprobe for 6 h and then washed with PBS for several times to remove the excess nanoprobe. MCF-7 cancer cells were incubated with 1 mL JC-1 staining solution for 20 min, washed with the ice-cold staining buffer and observed under the two-photon laser scan confocal microscope. The fluorescence information of JC-1 was recorded at green channel (λ_{ex}=488 nm, λ_{em}=500-550 nm) and red channel (λ_{ex}=561 nm, λ_{em}=580-640 nm), respectively.

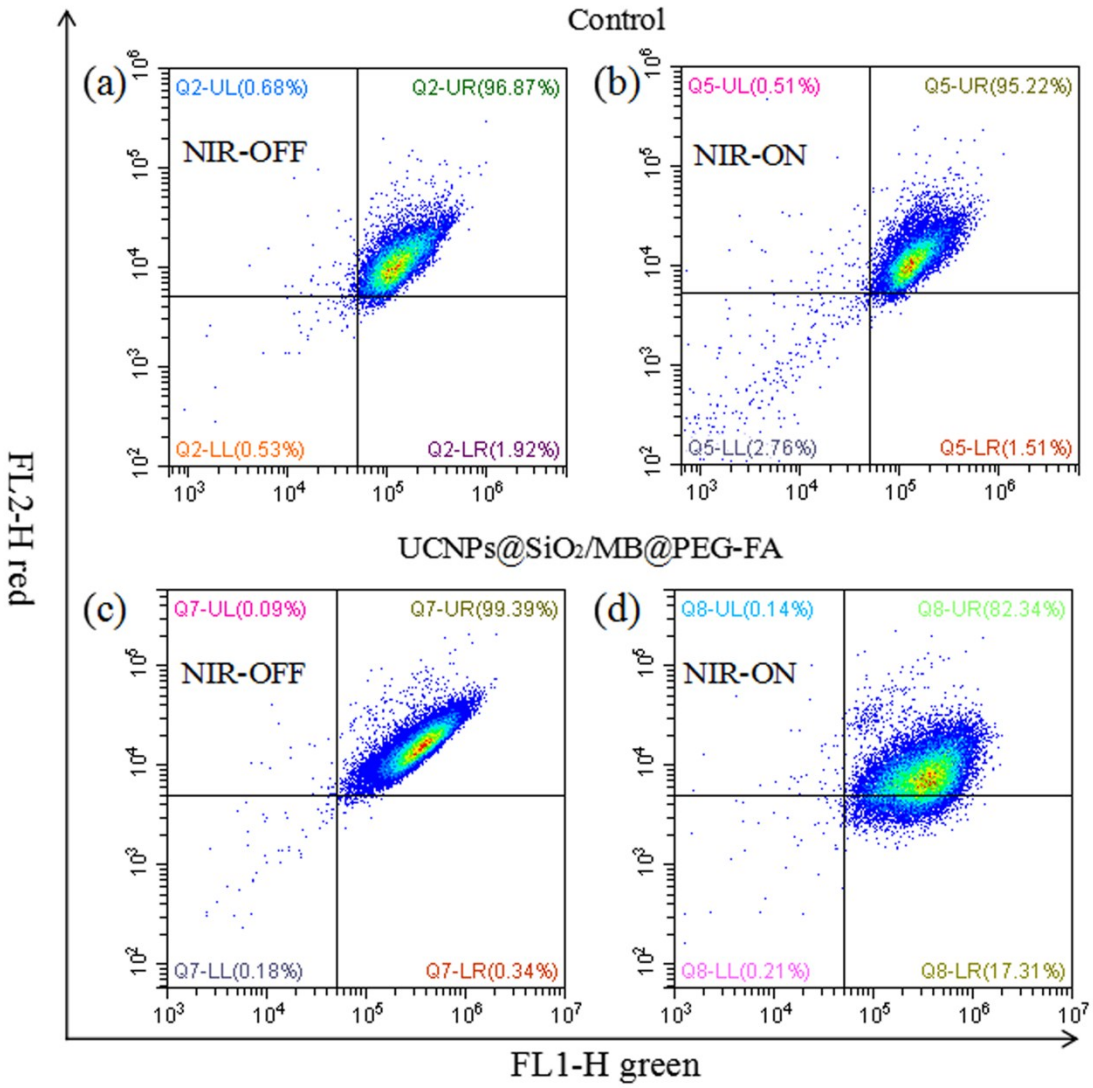

Figure S6: Flow cytometry to evaluate the $\Delta\Psi_m$ change of MCF-7 cancer cells treated with the prepared UCNPs@SiO_2/MB@PEG-FA nanoprobe using JC-1 staining. MCF-7 cancer cells treated with PBS, and then irradiated with the 980 nm continuous wave laser (1.5 W/cm^2) for 0 min (a) and 4 min (b); MCF-7 cancer cells treated with the prepared UCNPs@SiO_2/MB@PEG-FA nanoprobe and then irradiated with the 980 nm continuous wave laser (1.5 W/cm^2) for 0 min

(c) and 4 min (d).

4.5 PDT efficacy assay in living cells

MCF cancer cells were cultured in 96-well microtiter plates at 37°C in 5% CO_2. After 24 h, the above MCF-7 cancer cells were incubated with 80 μg/mL of the prepared UCNPs@SiO_2/MB@PEG-FA nanoprobe for 12 h, washed with PBS to remove the excess nanoprobe and then irradiated with the 980 nm continuous laser for 4 min (1.5 W/cm^2). Next, 10 μL of CCK-8 was added and incubated with the cells at 37°C for another 2 h. Finally, the microplate reader was used to detect the absorbance of MCF-7 cancer cells at 450 nm.

Continuous 980 nm laser would produce the biological heating problem due to water absorption and possibly induce cell death, which was provided by the MTT assay. As shown in Figure S7a, the irradiation intensity (1.5 W/cm^2) used in this experiment would induce negligible cell death even at the irradiation time of 50 min while the cell death became worse with stronger laser intensity and longer irradiation time. To avoid the heating problem on the cell viability, weak laser intensity (1.5 W/cm^2) and short irradiation time (4 min with an interval of 1 min) was used to guarantee the negligible cytotoxicity of the laser.

To investigate the PDT efficacy of the prepared UCNPs@SiO_2/MB@PEG-FA nanoprobe in living cells, three control groups were designed: (a) control (only treated with PBS); (b) the nanoprobe without laser irradiation; (c) PBS and laser irradiation (1.5 W/cm^2, 4 min).

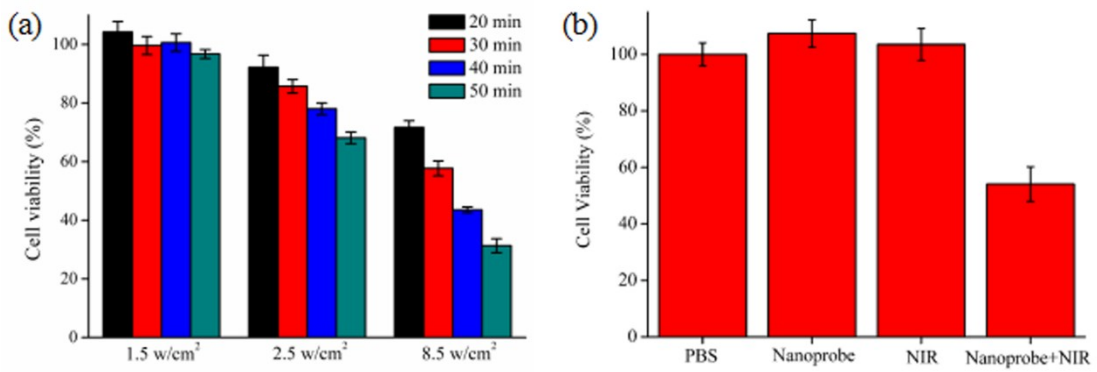

Figure S7: (a) The cell viability of MCF-7 cancer cells with different irradiation intensity and time; (b) the cell viability of MCF-7 cancer cells with the prepared nanoprobe, UCNPs@SiO_2/MB@PEG-FA.

4.6 Effects of the amount of MB

To investigate the effects of the amount of MB on the ability of ROSs generation and the therapeutic efficacy of UCNPs@SiO_2/MB@PEG-FA, different volumes of MB aqueous solution (4 mg/mL) were added to prepare UCNPs@SiO_2/MB and then obtain the corresponding UCNPs@SiO_2/MB@PEG-FA nanoprobes. As shown in Figure S8a, with more MB molecules added, the prepared UCNPs@SiO_2/MB@PEG-FA nanoprobe produced increasing ROSs while the increase was not obvious with added volume of MB aqueous solution more than 225 uL, proving enough photosensitizers were introduced. The amount of MB molecules also made an important effect on the therapeutic efficacy of UCNPs@SiO_2/MB@PEG-FA (Figure S8b).

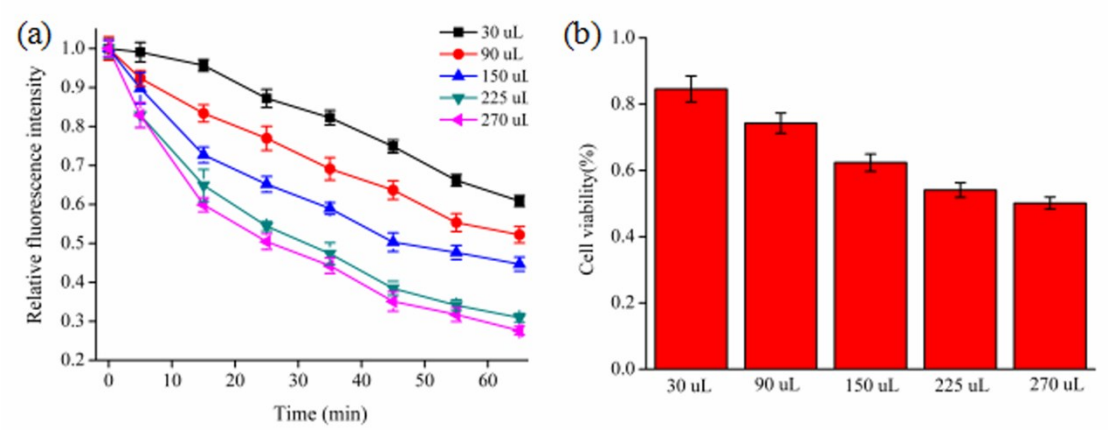

Figure S8: (a) ROSs production (b) cell viability of the UCNPs@SiO_2/MB@PEG-FA nanoprobes with different added volume of MB aqueous solution.

4.7 *In vivo* PDT efficacy assay

The right leg of the Balb/c nude mouse (6 weeks, around 20 g) was injected with 50 μL PBS containing 1×10^6 cancer cells. Experiments were performed when tumors reached at the tumor volume of 80-100 mm^3. The tumor-bearing mouse was injected

with the UCNPs@SiO_2/MB@PEG-FA nanoprobe (50 uL, 8 mg/mL) in the tumor section. After 12 h, the tumor section was irradiated under 980 nm laser (1.5 W/cm^2) for 4 min at an interval of 1 min. The tumor volume (V=length×$width^2$/2) was recorded every two days over a period for 14 days. At the seventh day, the mouse could be injected with the nanoprobe and irradiated under the laser again. Finally, the mouse was sacrificed, and the tumor section was sliced for hematoxylin and eosin (H&E) staining and tunel staining. Other three control groups were designed to investigate the PDT efficacy of the prepared UCNPs@SiO_2/MB@PEG-FA nanoprobe: (a) control (only injected with PBS); (b) laser irradiation alone; (c) only injected with the nanoprobe.

Reference:

[1] X. Y. Song, Z. H. Yue, J. Y. Zhang, Y. X. L. Jiang, Z. H. Wang and S. S. Zhang, *Chem. Eur. J.,* 2018, **24**, 6458.

[2] X. Y. Song, J. Y. Zhang, Z. H. Yue, Z. H. Wang, Z. H. Liu and S. S. Zhang, *Anal. Chem.*, 2017, **89**, 11021.

[3] W. P. Fan, B. Shen, W. B. Bu, F. Chen, Q. J. He, K. L. Zhao, S. J. Zhang, L. P. Zhou, W. J. Peng, Q. F. Xiao, D. L. Ni, J. N. Liu, and J. L. Shi, *Biomaterials*, 2014, **35**, 8992.

[4] F. Chen, S. J. Zhang, W. B. Bu, Y. Chen, Q. F. Xiao, J. N. Liu, H. Y. Xing, L. P. Zhou, W. J. Peng and J. L. Shi, *Chem. Eur. J.,* 2012, **18**, 7082.

[5] N. N. Idris, M. K. Gnanasammandhan, J. Zhang, P. C. Ho, R. Mahendran and Y. Zhang, *Nat. Med.,* 2012, **18**, 1580.