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Supporting Information

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Efficacy Dependence of Photodynamic Therapy Mediated by Upconversion Nanoparticles: Subcellular Positioning and Irradiation Productivity

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1. Materials and methods

1.1 Materials

(3-aminopropyl)triethoxysilane (APTS), tetraethoxysilane (TEOS)， Igepal CO-520 (average molecular weight 441), protoporphyrin IX (PpIX, 95%), 1, 3-Diphenyl-isobenzofuran (DPBF, 97%), fluorescein diacetate, propidium iodide (FDA/PI), 9,10-anthracenediyl-bis(methylene)dimalonic acid (ABMDMA), 4, 6-diamidino-2 phenylindole (DAPI) and Rhodamine 123 (Rh123) were purchased from Sigma-Aldrich Co.. Folic acid (FA, 96%), (3-Carboxypropyl)triphenylphosphonium Bromide (TPP, 97%) were purchased from J&K Scientific Ltd. Lipofectamine® 2000 was purchased from Life Technologies Co. 1-Ethyl-3-(3-dimethyllaminopropyl)carbodiimide hydrochloride (EDC, >99%), N-Hydroxysuccinimide (NHS, >99%) were purchased from Shanghai Medpep Co., Ltd. Anhydrous ethanol (>99.7%), chloroform (>99%), dimethyl sulfoxide (DMSO, >99%), cyclohexane (>99.5%), methanol (>99%) , oxalyl chloride(≥98%) were

purchased from Sinopharm Chemical Reagent Co., Ltd. β-$NaYF_4$: Yb, Er (UCNs) with ~40nm size was prepared following the method previously developed by us.[1]

1.2 Synthesis of UCNs@SiO_2-PpIX

In the first step for all synthesis, 10mg UCNs were dispersed in 15 ml cyclohexane and 0.5 ml Igepal CO-520 were added and stirred for 30min. Then 20μl (30 mM) PpIX solution (H_2O: DMSO=1:1) were added in and sonicated for about 20min until uniform emulsion was formed.

For TEOS or non-covalent bound APTS as precursor, TEOS (20 μl) or APTS (20 μl) were then added dropwise into the emulsion under stirring, respectively, followed by the adding of 80 μl ammonia.

In parallel, synthesis was also performed using covalent bound APTS-PpIX as precursor. APTS-PpIX complex was prepared following the route reported by Rossi[2]. Typically, PpIX (100 mg) was first activated by oxalyl chloride (3 ml) under a dry nitrogen atmosphere. Afterwards, the excess of oxalyl chloride was evaporated before APTS (1 ml) was added for reaction. The as-prepared solution was directly used for next steps without removing extra APTS. Different volume of APTS-PpIX complex (6 μl, 3 μl, 2 μl, 1 μl, respectively) were added in the emulsion and sonicated for about 30min, followed by adding of 80 μl ammonia and 15 μl TEOS successively.

The emulsion was then protected from light and stirred at room temperature for 48h. The resultant nanoparticles were collected by centrifugation (8500 rpm, 6 min) and washed with ethanol three times before finally dissolving in the ethanol at a concentration of 1 mg/ml for storage. The size and morphology of the nanocrystals were observed with a JEOL JEM-2100

transmission electron microscope (TEM) operated at 200 kV. The fluorescence spectra were measured by Shimadzu RF5301PC luminescence spectrometer with an external 980nm continuous wave laser source. The luminescence decays at 540nm and 660nm were measured respectively with 980 nm pulse wave laser excitation by FLSP920 spectrometers (Edinburgh instruments). All the luminescence decay curves were fitted using a single exponential function.

1.3 Surface modification with FA and TPP

10mg FA dissolving in 2 ml DMSO was added in an EDC/NHS aqueous solution (46 mg EDC and 28mg NHS dissolving in 12 ml water). The mixture was stirred for 1 hour at room temperature before adding UCNs@SiO_2-PpIX water solution (4 ml, 1 mg/ml). The system went on reacting under stirring in dark overnight. The modification of TPP (20 mg) followed same procedure, only except that TPP was dissolved in water and doses of EDC/NHS doubled. After the finish of reaction, the nanoparticles were washed with water 5 times and redispersed in 4 ml phosphate buffer solution (PBS).

1.4 Determination of the PpIX loading contents

PpIX loading content, defined as the weight ratio between PpIX and UCNs, was quantified by UV-Vis analysis (UV-2550, Shimadzu). The absorbance of PpIX at 410 nm was measured to determine the drug content in the solution using an established calibration curve. A UV-Vis analysis of blank silica coated UCNs was also performed as a control that showed no absorption at 410 nm, indicating lack of interference by UCNs and silica at the concentrations used for characterization.

1.5 Measurement of reactive oxygen species (ROS) and $^1O_2$ production

Acetonitrile solution of UCNs@SiO_2-PpIX (1 ml, 1 mg/ml) was placed in a 1 ml quartz cuvette and absorption spectrum was measured firstly. Then DPBF acetonitrile solution (20 μl, 8 mM) was added and mixed by ultrasonic dispersion. The mixture was measured the absorption spectra after irradiation by a 980 nm laser (1W, continuous wave, Xi'An Sapling Institute of Laser Technology) for a certain time. The ROS generation efficiency was evaluated by the UV-Vis absorption intensity decrease rate of DPBF at around 400nm.

For the measurement of $^1O_2$, an aqueous solution of different samples (2.5 ml, 1 mg/ml) was added in a 4ml quartz cuvette and mixed with 200 μl DMSO solution of 9,10-anthracenediyl-bis(methylene)dimalonic acid (ABMDMA) (0.25 mg/ml). The as-prepared mixture was irradiated with a 980 nm laser at 1 W/cm^{-2} for a certain period with recording the UV-Vis absorption spectra. The singlet oxygen generation was evaluated by the decay of UV-Vis absorption intensity decrease of ABMDMA at about 400 nm.

1.6 Cellular uptake and fluorescence confocal microscope imaging

HeLa cells (1×10^4 cells/ml) were seeded on glass coverslips (10mm×10mm), placed in 60-culture plate, and grown for 24 h. Then UCNs dispersing in culture media (200μg/ml) was added. After incubating for 4 h, the cells were washed twice with PBS. After that, the cells were stained with DAPI (2μg/ml) and observed under a two photon confocal microscope (Leica TSC SP8, Germany) equipped with a 980 nm femtosecond pulse laser. The cells were also trypsinized and were counted by an auto cell counter (Counstar, Inno-Alliacne Biotech, U.S.). The amount of Y^{3+} ions taken up by cells was measured by inductively coupled plasma

atom emission spectrometer (ICP-AES, iCAP 6000 Radial, THERMO), based on which UCNs amount per cell was calculated.

1.7 Transmission electron microscopy of cells

HeLa cells (1×10^4 cells/ml) were seeded into BD Falcon 35mm dish and cocultured with nanoparticles for 4h. The cells were then dislodged with curette and collected by centrifugation at 1500rpm for 5 min. The media was discarded and cells were fixed with 2.5% glutaraldehyde in PBS, washed with 0.1M PBS three times and postfixed in 1% osmium tetraoxide. Next the cells were dehydrated in ascending concentrations of ethanol before infiltration and embedment in epoxy resins (Epon 812) at 60°C for 48 h, Ultrathin sections prepared with a LKB-1 Ultracut were lightly stained with 1% uranyl acetate and lead citrate. Finally, the cells were observed with 120KV Transmission Electron Microscope (Tecnai G2 Spirit Biotwin, FEI).

1.8 *Mitochondrial Imaging*

HeLa cells (1×10^4 cells /ml) were grown on cover slips placed in 6-well tissue culture plates and cocultured with nanoparticles for 4h before washing twice with PBS. After that, the cells were stained with mitochondrial specific fluorescent dye Rh123(10 μg/mL) and DAPI (2μg/ml) successively. The cells were washed with PBS thoroughly before fixed with 4% p-formaldehyde for 15 min. The cover slips were mounted cell-side-down on glass slides with fluoromount G media and visualized under a two photon confocal microscope (Leica TSC SP8, Germany) with DAPI (λ_{ex}=385 nm, λ_{em}=470 nm), Rh123 (λ_{ex}=488 nm, λ_{em}=530 nm) and PpIX (λ_{ex}=552 nm, λ_{em}=660 nm). The fluorescent micrographs were analyzed with Plugin coloc 2, ImageJ software.

1.9 Photodynamic effect on cancer cells

HeLa cells were seeded in 96-well culture plate (BD Falcon, U.S.) at a density of 5000 cells per well and cultured in the condition with 5% CO_2 at 37°C for 24h. Then 150 μl culture media containing UCNs-PpIX (200 μg/ml) replaces the media. After 4 h incubation, the cells were exposed to 1 W/cm^2 NIR laser. An interval of 5 minutes was set for every 5 minutes' irradiation, until the total irradiation time is accomplished. The temperature of cell culturing system treated with irradiation was measured during the irradiation process by an InfraPro1 noncontact thermometer (Oakton Instruments, Vernon Hills, IL, USA), showing that the increase of temperature is lower than 0.5°C. After another 24 h culture, the MTT assay was used to assess the cell viability and fluorescence-activated cell sorting (FACS) technique on cytometry was performed with FITC Annexin V Apoptosis Detection Kit I (BD, U.S.) to analyze the damage mechanism.

For MTT assay, the cells were washed twice with (PBS) and then cocultured with media solution of trypan blue (0.5mg/ml) for another 4 h. After discarding the medium, 150 mL of DMSO was added to dissolve the precipitates and the resulting solution was measured absorbance at 570 nm with a reference wavelength of 630 nm using a microplate reader (Varioskan Flash, Thermo Scientific).

For FACS test, HeLa cells in aforementioned were detached and collected by 0.25% Trypsin solution without EDTA (BD, U.S.), and washed twice with PBS solution. Then cells were resuspended in PBS with a concentration of 1 x 10^6 cells/ml; 100 μl of the suspension was drawn out and cocultured with 5μl FITC Annexin V as well as PI in dark at room temperature for 15 min; After adding of 400μl more PBS, cells were analyzed by flow

cytometry immediately. Data was analyzed through software of FlowJo_V10 (Treestar, Inc., CA, USA).

1.10 In vivo PDT efficacy of UCN and Tumor histology examination

BALB/C nude mice were purchased from Shanghai Laboratory Animal Center, Chinese Academy of Sciences (SLAC, Shanghai, China). All animal experiments were carried out in compliance with the Animal Management Rules of the Ministry of Health of the People's Republic of China and the guidelines for the Care and Use of Laboratory Animals of Shanghai Jiao Tong University. HeLa cells (5×10^6) were subcutaneously injected into the nude mice (n = 4). As the tumors grew up to a diameter of about 1 cm, the PBS solution of UCN was injected into the tumor of the mouse (100 μL, 2 mg/mL).

After 1 h of the injection, the tumor sites of nude mice were locally irradiated 30 min. After 24 hours, the sites were irradiated for another 30 min with the same laser. In each time of irradiation, the tumor were exposed to 1 W/cm^2 of 980 nm NIR laser, and 5 minutes interval were set for every 5-minute irradiation, The temperature at the irradiation site was measured before, during and after irradiation with an infrared thermometer (InfraPro1, Oakton Instruments, USA). The result shows after 30 min irradiation, the increase temperature at tumor site is lower than 1°C, ruling out the photothermal effect.

The change in tumor volume was monitored after treatment. Tumor volume was calculated as length$\times$(width)^2/2.

To further investigate the PDT effects in the tumor tissues, all mice were sacrificed and the tumors were collected for histology analysis 12 days after treatment. Tumor tissues of the mice in the control group and treatment group were isolated from the mice, fixed with 10%

neutral buffered formalin and embedded in paraffin. The sliced organs and tumor tissues were stained with Hematoxylin and Eosin (H&E) and observed by a microscope.

2. Figures

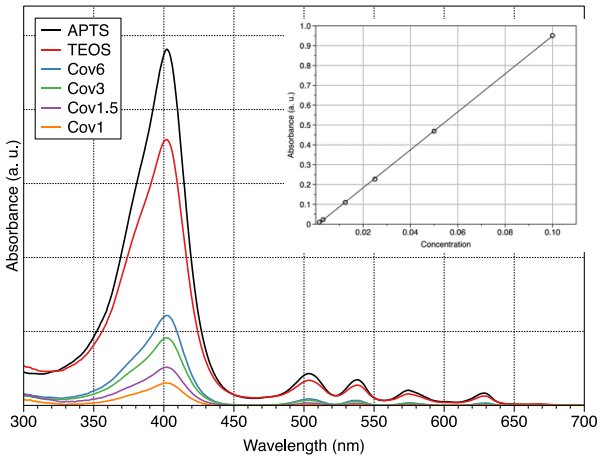


Figure S1. UV-Vis spectra of supernatant after the synthesis of UCNs@SiO_2-PpIX, showing the uncoated amount of PpIX calculated from a standard concentration-absorption curve (inset). The difference between feeding and uncoated amount of PpIX indicates the loaded amount of PpIX in samples.

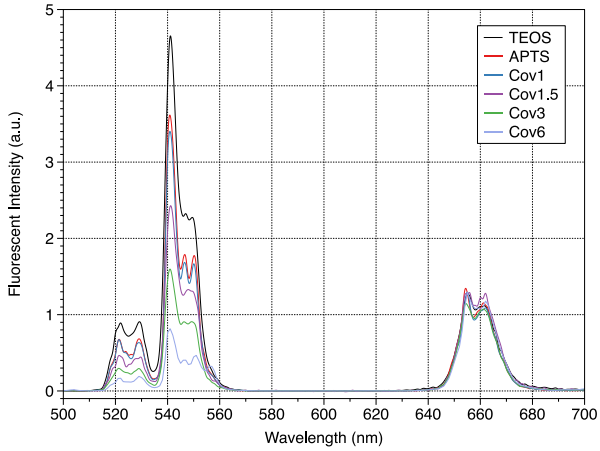
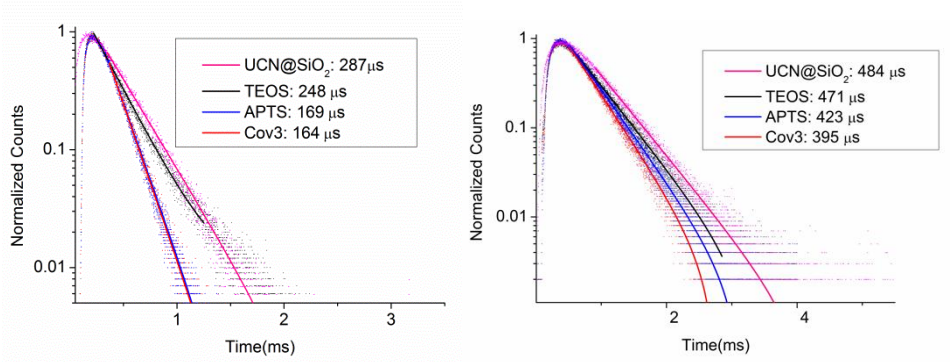


Figure S2. Fluorescent spectra of different samples. Owing to that the absorption of coated PpIX is in the range from UV to ~620nm, the fluorescent peak at ~655nm almost kept the same, while energy of the emission from ~520nm to ~550nm was transferred to PpIX with a

certain efficiency, and the efficiency was calculated according to the decrease of fluorescent intensity at ~542nm.



(a) (b)

Figure S3. Luminescence decay of the UCNPs and different samples at (a) 540 nm, (b) 660nm.

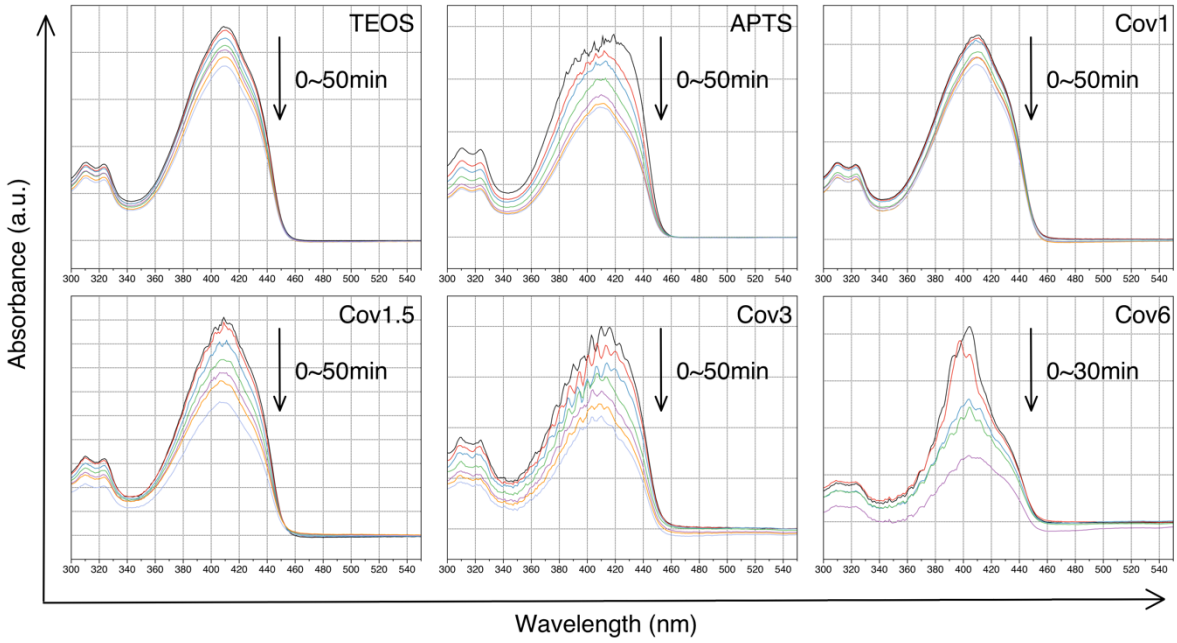


Figure S4. UV-Vis spectra of DPBF mixing with different samples, under irradiation for different time with a 980 nm continuous laser. Each absorption curves have subtracted the curve of corresponding sample, which leads to a demonstration of the absorption decay of pure DPBF. The fluctuation of some samples curves is due to relatively high PpIX loading, which results in the higher UV-Vis absorption of corresponding sample as a base line.

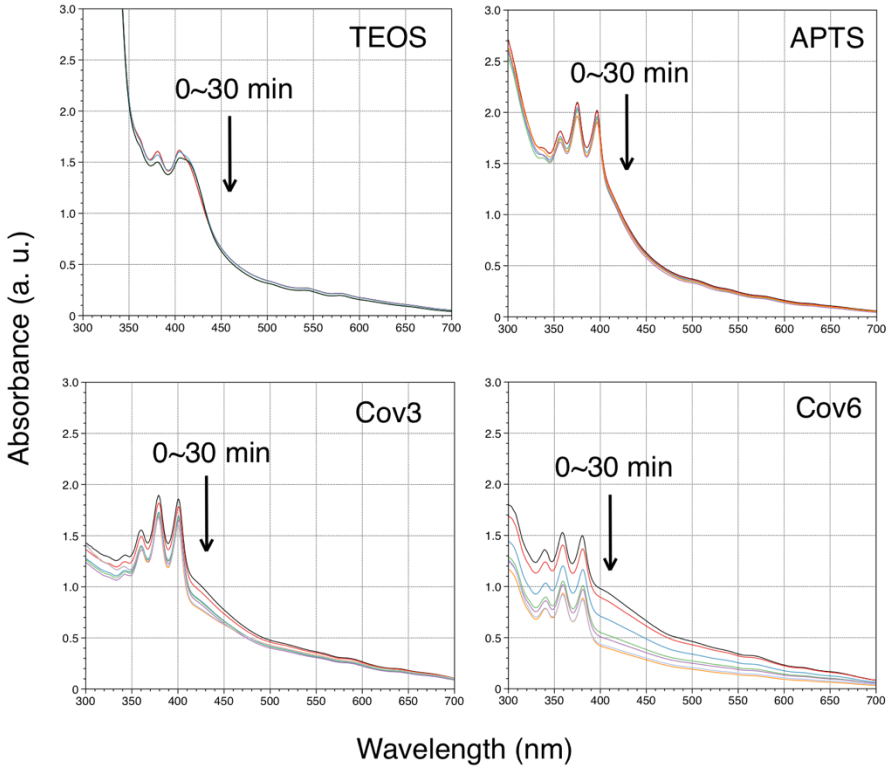


Figure S5. The UV-Vis spectra of ABMDMA mixing with different samples under irradiation for different time with a 980 nm laser

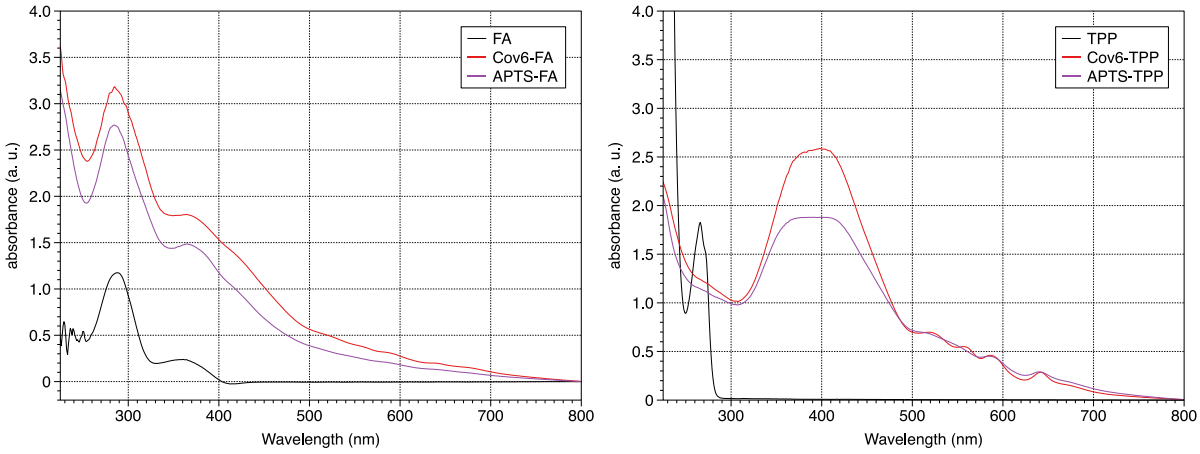


Figure S6. Left panel: UV-Vis spectra of FA, and FA modified samples APTS and Cov6, respectively. The existence of specific absorption peak at ~283nm clearly shows the successfully grafting of FA on the samples. Right Panel: UV-Vis spectra of TPP, and TPP modified samples APTS and Cov6, respectively. As the absorption of TPP is in the UV range,

the absorption of samples show an obvious increase when the wavelength is shortened to ~260nm, indicating the conjugation of TPP.

References:

[1] Y. Sui, K. Tao, Q. Tian, K. Sun, Interaction between Y^{3+} and oleate ions for the cubic-to-hexagonal phase transformation of $NaYF_4$ nanocrystals, *J. Phys. Chem. C*, **2012**, *116*, 1732-1739.

[2] L. M. Rossi, P. R. Silva, L. L. R. Vono, A. U. Fernandes, D. B. Tada, M. S. Baptista, Protoporphyrin IX nanoparticle carrier: preparation, optical properties, and singlet oxygen generation, *Langmuir* **2008,** *24*, 12534-12538