Supporting Information for

Constructing a Local Hydrophobic Cage in Dye-doped Fluorescent Silica Nanoparticles to Enhance the Photophysical Properties

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1. Experimental section

1.1 Materials

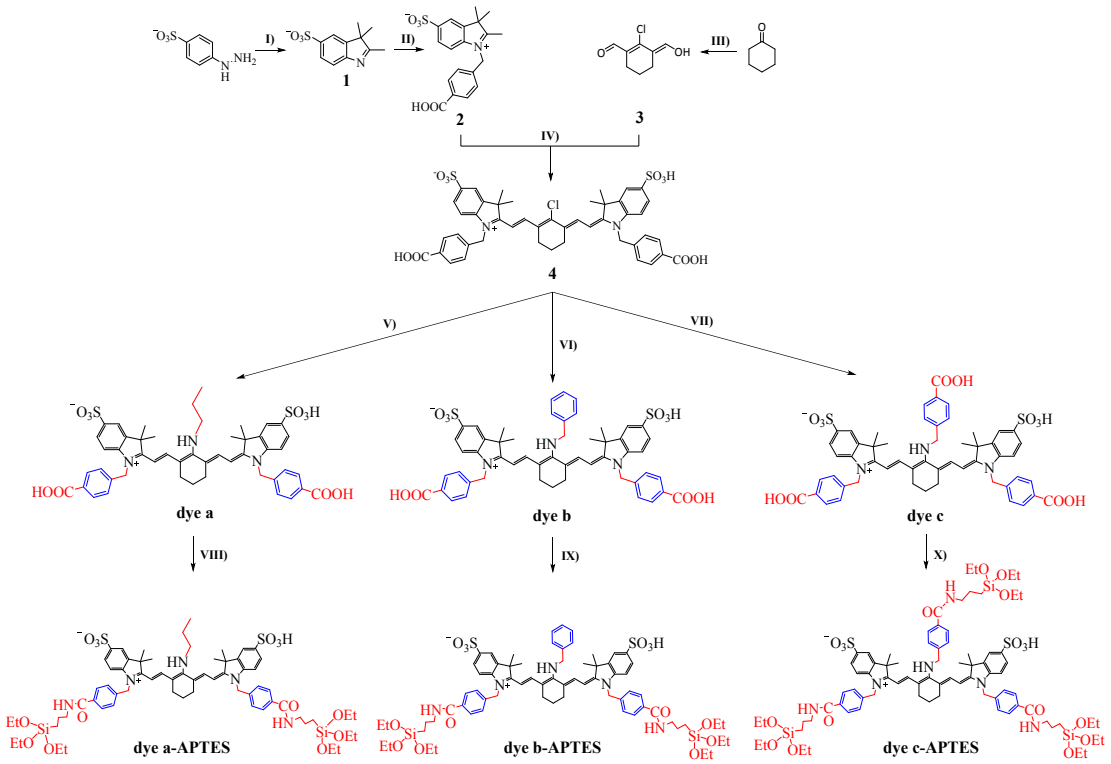
4-Hydrazinobenzenesulfonic acid (98%), 3-methyl-2-butanone (98%), 4-bromomethylbenzoic acid (97%), n-propylamine (98%), benzylamine (99%), 4-(aminomethyl)benzoic acid (97%), cyclohexanone (99%), phosphorus oxychloride (99.5%), 3-aminopropyl)triethoxysilane (APTES, 98%), n-propyltriethoxysilane (PTOS, 98%), n-octyl triethoxysilane (OTES, 97%), n-dodecyltriethoxysilane (DTOS, 95%), tetraethylorthosilicate (TEOS, 97%), EDC·HCl (98%), DMAP (99%) and Triton X-100 were purchased from Sigma-Aldrich without further purifications. All reactions have been carried out under a nitrogen atmosphere unless otherwise noted. N,N-Dimethylformamide (DMF) was dried prior to use with calcium hydride. Ultrapure deionized water from a Milli-Q ultrapure system was used for all synthesis, analysis, separation and purification steps.

1.2 Instruments

^{1}H-NMR spectra were recorded at room temperature using a VARIAN INOVA-400 spectrometer with chemical shifts reported as ppm (in dimethyl sulfoxide (DMSO), tetramethylsilane (TMS) as internal standard. Mass spectrometric data were obtained on a LTQ Orbitrap XL mass spectrometer (Thermo Scientific). Nanosecond fluorescence lifetime was performed on DeltaFlex (HORIBA, UK) with laser excitation at 456 nm. The photostability evaluation in vitro was performed by using W-Halogen lamp irradiation (500 W). The crude product of Cy7 was purified by FLEXA Purification System from Agela Technologies using methanol and deionized

water as mobile phase. The size and shape of the silica nanoparticles were characterized using a transmission electron microscope, TEM (Tecnai F30). Zeta potential measurements of nanoparticles were carried out at 25 °C in ethanol using a Nanozs 90 (Malvern) instrument (λ_{ex} = 640 nm). The adsorption isotherms and pore size distributions for N_2 (99.999%) were conducted using an Autosorb Automated Gas Sorption System supplied by QUANTOCHROME (U.S.). FTIR measurements were performed on a 6700 FTIR spectrometer (Thermo Fisher). Confocal Fluorescence images were performed on a confocal laser scanning microscope (Fluoview FV1000, Olympus). MTT assays were measured on Microplate reader named Multiskan FC from Thermo scientific (Filter: 490 nm).

1.3 Reaction schemes of dye a~c and dye a~c-APTES derivative



Scheme S1 Synthesis of chromophore dye a~c and dye a~c-APTES derivative. I) 3-methyl-2-butanone, AcOH, reflux, 10 h; II) 4-bromomethylbenzoic acid, 1,2-dichlorobenzene,

reflux, 12 h; III) $POCl_3$, DMF, 0 °C, 1 h→70 °C, 1 h, →H_2O, 0 °C, 12 h; IV) NaOAc, Ac_2O, 40 °C, 1 h; V) n-propylamine, DMF, 60 °C, 4 h; VI) benzylamine, DMF, 60 °C, 4 h; VII) 4-(aminomethyl)benzoic acid, DMF, 60 °C, 4 h; VIII, IX, X) 3-aminopropyl)triethoxysilane, EDC·HCl, DMAP, anhydrous DMF, N_2, r.t., 12 h.

Preparation of dye a~c: The reaction process and conditions were shown in Scheme S1. Dye a~c were obtained as purple amorphous powder. The MS (ESI) and ^1H spectras of dye a~c were given in Figure S12~S17.

dye a: ^1H (400 MHz, DMSO-d6): δ 7.92 (4 H, d, *J* = 8.0), 7.67 (2 H, d, *J* = 1.6), 7.57-7.43 (4 H, m), 7.35 (4 H, d, *J* = 8.1), 7.05 (2 H, d, *J* = 8.3), 5.67 (2 H, d, *J* = 12.7), 5.29 (4 H, s), 3.64 (2 H, d, *J* = 8.0), 2.26 (4 H, d, *J* = 6.8), 1.81-1.45 (15 H, m), 1.06 (1 H, t, *J* = 7.0), 0.96-0.87 (3 H, m). MS (ESI) for dye a: m/z calcd. for $C_{49}H_{49}N_3O_{10}S_2^{2-}$ $[M-2H]^{2-}$: 452.04, found: 452.00; $C_{49}H_{48}N_3O_{10}S_2^{3-}$ $[M-3H]^{3-}$: 301.03, found: 301.16.

dye b: ^1H (400 MHz, DMSO-d6): δ 7.92 (4 H, d, *J* = 8.0), 7.59 (2 H, s), 7.54-7.43 (7 H, m), 7.41-7.31 (6 H, m), 7.05 (2 H, d, *J* = 8.3), 5.69 (2 H, d, *J* = 12.6), 5.29 (4 H, s), 4.90 (2 H, s), 2.30 (4 H, t, *J* = 6.4), 1.61 (2 H, h, *J* = 6.8, 5.4), 1.40 (12 H, s). MS (ESI) for dye b: m/z calcd. for $C_{53}H_{50}N_3O_{10}S_2^{-}$ $[M-H]^{-}$: 953.12, found: 952.60; $C_{53}H_{49}N_3O_{10}S_2^{2-}$ $[M-2H]^{2-}$: 476.06, found: 476.06; $C_{53}H_{48}N_3O_{10}S_2^{3-}$ $[M-3H]^{3-}$: 317.04, found: 317.17.

dye c: ^1H (400 MHz, DMSO-d6): δ 8.05 (2 H, d, *J* = 7.8), 7.98 (2 H, d, *J* = 8.2), 7.92 (4 H, d, *J* = 8.1), 7.61-7.56 (4 H, m), 7.52 (2 H, dd, *J* = 8.1, 1.7), 7.33 (4 H, d, *J* = 8.0), 7.06 (2 H, d, *J* = 8.3), 5.71 (2 H, d, *J* = 12.7), 5.30 (4 H, s), 5.03-4.87 (2 H, m), 2.29 (4 H, d, J 7.2), 1.60 (2 H, t, J 6.2), 1.39 (12 H, s). MS (ESI) for dye c: m/z calcd.

for $C_{54}H_{49}N_3O_{12}S_2^{2-}$ $[M-2H]^{2-}$: 498.07, found: 498.00; $C_{54}H_{48}N_3O_{12}S_2^{3-}$ $[M-3H]^{3-}$: 331.71, found: 331.86; $C_{54}H_{47}N_3O_{12}S_2^{3-}$ $[M-4H]^{4-}$: 248.53, found: 248.69.

Preparation of dye a~c-APTES derivative: The reaction process and conditions were shown in Scheme S1. Because the alkoxysilane moiety was severely sensitive to moisture and easy to hydrolyze polymerization, all the products (dye a~c-APTES derivative) were used for further functional silica nanocarrier (FSNPs) formation directly from the reaction pot.

Preparation of FSNP-a~c: The preparation of FSNP-a~c was carried out by reverse microemulsion method. At room temperature, the reverse microemulsion system was prepared by mixing 5.41 mmol Triton X-100, 28.04 mmol n-octanol and 193.60 mmol cyclohexan to stir for 30 min. The prepared dye (a, b or c)-APTES was dissolved in 1.00 ml of ultrapure water under ultrasound. Then the mixture solutions were added dropwise into the reverse microemulsion system to continue stirring for 30 min. Subsequently, 0.90 mmol of TEOS was added at a rate of 0.02 mL/min. Finally, 0.10 mL of NH_4OH (28-30%) was added at a rate of 0.02 mL/min to initiate hydrolysis polymerization. After stirring the mixture for 24 h, FSNP-a, b or c formation was terminated by acetone, and collected by centrifugation at 10,000 rpm. FSNP-a, b or c was washed with absolute ethanol for three times to remove unreacted free components, and then redispersed in ultrapure water.

Preparation of pure SiO_2: The pure SiO_2 without doping any cyanine dyes as control nanoparticles was prepared using the same method of synthesizing *FSNP*-a~c.

Preparation of FSNP-A~C: Similarly, FSNP-A~C was carried out by modified

reverse microemulsion method. At room temperature, the reverse microemulsion system was prepared by mixing 5.41 mmol Triton X-100, 28.04 mmol n-octanol and 193.60 mmol cyclohexan to stir for 30 min. The prepared dye (a, b or c)-APTES was dissolved in 1.00 ml of ultrapure water under ultrasound. Then the mixture solutions and 200 μL hydrophobic organosilicone agents ((different lengths of hydrophobic alkyl chains (C_3-PTOS, C_8-OTES and C_{12}-DTOS)) were added dropwise into the reverse microemulsion system to continue stirring for 30 min. Subsequently, 0.10 mL of NH_4OH (28-30%) was added at a rate of 0.02 mL/min to initiate prehydrolysis. After 12 h of prehydrolysis, 0.90 mmol of TEOS was added at a rate of 0.02 mL/min to form covalently crosslinked silica network through hydrolysis polymerization. After stirring the mixture for 24 h, FSNP-A, B or C formation was terminated by acetone, and collected by centrifugation at 10,000 rpm. FSNP-A, B or C was washed with absolute ethanol for three times to remove unreacted free components, and then redispersed in ultrapure water.

Note: no unexpected or unusually high safety hazards were encountered during the experiment.

2. Optical spectrum studies

2.1 Steady-state absorption spectroscopy

The steady-state absorption spectroscopy was recorded in a quartz cuvette (10 × 10 mm) via a UV-Visible spectrometer named Cary 60 from Agilent Tech at room temperature. Working solutions of FSNPs were prepared by diluting the stock solution in ultrapure deionized water.

2.2 Steady-state fluorescence spectroscopy

The steady-state fluorescence spectroscopy was performed via a fluorometer named Cary Eclipse from Agilent Tech at room temperature. Working solutions of FSNPs were prepared under the same procedures as steady-state absorption test. The data were obtained under the control of a Windows-based PC running the manufacturers' supplied software.

2.3 The evaluation of chemical durability and physiological stability

At room temperature, the chemical durability and physiological stability of FSNPs (0.5 mg mL^{-1}) in phosphate buffer (pH = 7.4, 10 mM), different pH values of phosphate buffer and cell culture medium containing fetal bovine serum (FBS) over 14 d were tested through detecting fluorescence emission.

2.4 The evaluation of photostability

FSNPs were dissolved in 6 mL ethanol, accompanied by magnetic stirring. Sample tubes were exposed to a W-Halogen lamp (500 W) and kept the sample tubes from the lamp at a distance of 30 cm. A cold trap equipped with aqueous solution of sodium nitrite at 50 g L^{-1} was placed in the middle of lamp and sample tubes. Fluorescence

spectra of samples were taken after every 10 min.

2.5 Detection of ROS level in aqueous solution

In singlet oxygen generation measurements, a commercially available ROS probe 1,3-diphenylisobenzofuran (DPBF) was used. Before the measurement, free dyes and FSNPs were respectively dissolved in water to a final concentration with almost the same optical density at 660 nm, and then mixed DPBF at 50 μM. A red LED array (optical density of 1.0 mW/cm^2, λ_{ex} = 660 nm) was used as excitated light source. The absorbance spectra of DPBF at 420 nm were recorded at different intervals.

The relative singlet oxygen quantum yield of free dyes and FSNPs in water were determined compared to methylene blue (MB). Free dyes and FSNPs (Optical densities were adjusted to around 0.2-0.3 at 660 nm) under irradiation (power density of 0.8 mW/cm^2) in the presence of DPBF (50 μM) reduces their optical absorption, indicating formation of the singlet oxygen. Φ_{Δ} values were calculated by the following equation:

$$\Phi\,\Delta = \Phi_{MB} \times \left(\frac{k_{dyes\ or\ FSNPs}}{k_{MB}}\right)\left(\frac{F_{MB}}{F_{dyes\ or\ FSNPs}}\right)$$

Where *k* is the bleach rate of DPBF absorbance (420 nm) with irradiation time, and *F* is the absorption correction factor (Optical densities were adjusted to the same at 660 nm), given that Φ_{MB} = 0.52 in water as reference.[1,2]

3. HeLa cells culture, 3D tumor model of HeLa cells and cells experiment of FSNPs

3.1 HeLa cells culture

HeLa cells were cultured in Dulbecco's Modified Eagle Medium (DMEM; GIBCO) containing 10% fetal bovine serum (FBS; GIBCO) and 1% antibiotics (80 U mL^{-1} penicillin and 0.08 mg mL^{-1} streptomycin; GIBCO). Cultured cells were incubated at 37 °C in an atmosphere of 5% CO_2 in air. HeLa cells and all the biochemical reagents described in this work were purchased from Nanjing Key GEN Bio TECH of China.

3.2 3D tumor models of HeLa cells

HeLa cells were made to aggregate into tumor spheroids using the hanging droplet technique. Briefly, HeLa cells obtained after trypsinization were resuspended in cell culture media at a density of 1.0×10^4 cells/mL. 35 mm dish dishes were filled with 2 mL ultrapure water to provide humidification to the hanging droplets. To form the tumor spheroid, the dispersed cell suspension of 30 μL was seeded on the lid, which were then inverted over the dishes and allowed to incubate for 3~6 days. In the following few days of culture, 10 μL of fresh medium was replaced every day.

3.3 Fluorescence intensity and photostability evaluation of FSNPs in HeLa cells

Measurement of fluorescence intensity in HeLa cells: HeLa cells were seeded in a glass bottom dish (MatTek, 35 mm dish with a 20 mm bottom well) with an amount a density of 1×10^5 cells per dish. After the HeLa cells were cultured for 24 h, cells were co-cultivated with FSNPs (2 μg mL^{-1}) at 37 °C for 4 h. Afterwards, the cells were washed three times with PBS (pH = 7.4). For Cy7 component of FSNPs (red channel),

a standard 635-nm laser was used and image detection range was from 655 nm to 755 nm.

Photostability evaluation in HeLa cells: HeLa cells were seeded in a glass bottom dish (MatTek, 35 mm dish with a 20 mm bottom well) with an amount a density of 1×10^5 cells per dish. After the HeLa cells were cultured for 24 h, cells were co-cultivated with FSNPs (2 μg mL^{-1}) at 37 °C for 4 h. Afterwards, the cells were washed three times with PBS (pH = 7.4). HeLa cells continued to be irradiated for 300 s via time-depend scan mode on CLSM, which was performed on a standard 635-nm laser of OLYMPUS (laser transmissivity: 10%, λ_{em} = 655-755 nm).

3.4 MTT assay of FSNPs

The dark cytotoxicity and phototoxicity of FSNPs were evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays (KeyGEN BioTECH, Nanjing, China). HeLa cells harvested in a logarithmic growth phase were seeded in 96-well plates at 100 μL/well (density of 1×10^4 cells/well) and incubated for 24 h. Then different concentrations of FSNPs were added into each well and incubated for 12 h. After the removal of samples, fresh medium was added and then irradiated by a red LED array at a power density of 50 mW/cm^2 (λ_{ex} = 660 nm) for 15 min. An ice box was disposed below the 96-well plates to eliminate the influence of temperature. MTT assays were measured after being further incubated for 6 h. Taking the same cell culture and staining methods under the premise, the dark cytotoxicity was measured without irradiation at the same time as control. Then the cells were subjected to MTT assays with Microplate reader named Multiskan FC from

Thermo scientific (Filter: 490 nm).

3.5 Annexin V-FITC/PI staining assay.

HeLa cells were plated in 6-well culture plates and cultured for 24 h at 37 °C in an atmosphere of 5% CO_2 in air. Then FSNPs was added at final concentrations of 5 μg mL^{-1}. HeLa Cells without FSNPs were used as control. The plates were further incubated for 4 h, and then the samples were washed three times with PBS. Fresh medium was added in the plates and then irradiated by LED array at a power density of 50 mW/cm^2 (λ_{ex} = 660 nm) for 15 min. An ice box was disposed below the 6-well culture plates to eliminate the influence of temperature. The cells were digested with trypsin and suspended with binding buffer (500 μL, KeyGEN BioTECH, Nanjing, China). Then annexin V-FITC (5μL, KeyGEN BioTECH, Nanjing, China) and propidium iodide (5μL, KeyGEN BioTECH, Nanjing, China) were added and further incubated for 15 min in the dark at room temperature. HeLa cells were immediately analyzed on BD FACSCanto flow cytometer using 488-nm excitation, a 515-545 nm band pass filter for FITC detection and a filter 665-685 nm for PI detection. All measurements were performed in triplicate, and at least three independent experiments were carried out.

3.6 Detection of intracellular ROS levels

Intracellular ROS generation of **FSNPs** was examined by using commercially available ROS probe 2',7'-dichlorofluorescin diacetate (DCFH-DA).[3] Non-fluorescence DCFH-DA could be converted into strong green fluorescent 2,7-dichlorofluorescein by ROS. Briefly, HeLa cells were seeded under the same

procedures as part 3.3. After the HeLa cells were cultured for 24 h, the medium was replaced with fresh medium, followed by addition of FSNP (5 μg mL^{-1}) to continue incubating for 12 h. Then, the cells were washed three times with PBS. Then the HeLa cells were stained with ROS probe (DCFH-DA, KeyGEN BioTECH, Nanjing, China) at the final concentration of 10 μM and consecutively irradiated with a red LED array (λ_{ex} = 660 nm, 50 mW/cm^2) for 5 min. HeLa cells were washed three times with PBS before fluorescence imaging. Then, a standard 488-nm laser was used and image detection range was from 510 nm to 540 nm.

3.7 PDT efficacy of FSNPs in 3D HeLa tumor model

3D tumor spheroids were stained with FSNPs (5 μg mL^{-1}, 4 h of incubation) after 3 days incubation. PDT experiment of 3D tumor spheroids lasts for three days and is irradiated for 15 min day^{-1} with a LED array (λ_{ex} = 660 nm, 50 mW/cm^2).

3.8 Evaluation of intracellular anti-ROS ability of FSNP-C during PDT

HeLa cells were seeded under the same procedures as part 3.3. After the HeLa cells were cultured for 24 h, the medium was replaced with fresh medium, followed by addition of FSNP (5 μg mL^{-1}) and PpIX (10 μM) to continue incubating for 12 h. Then, the cells were washed three times with PBS and fresh medium of 1 mL was added. HeLa cells were irradiated for 5 min with a LED array (λ_{ex} = 660 nm, 50 mW/cm^2). FSNP-c and Cy5 were selected as control groups. A standard 635-nm laser (transmissivity: 10%) was used and image detection range was from 655 nm to 755 nm. The initial fluorescence intensity was adjusted to almost the same level.

4. Supplemental figures

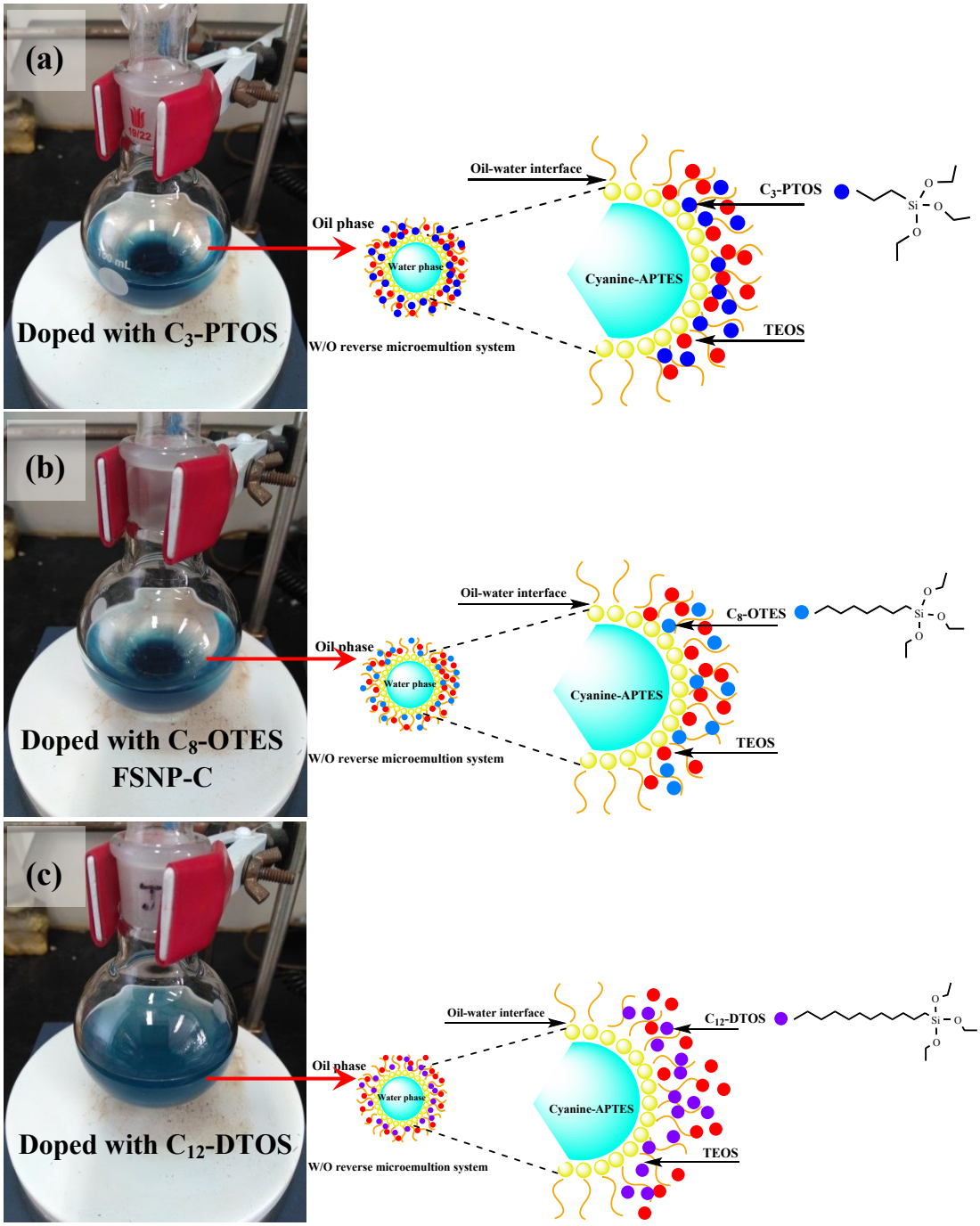


Figure S1. W/O reverse microemulsion with different lengths of hydrophobic alkyl chains (a, C_3-PTOS; b, C_8-OTES; and c, C_{12}-DTOS). The W/O reverse microemulsion system had been demulsified with acetone and allowed to stand for 1 h.

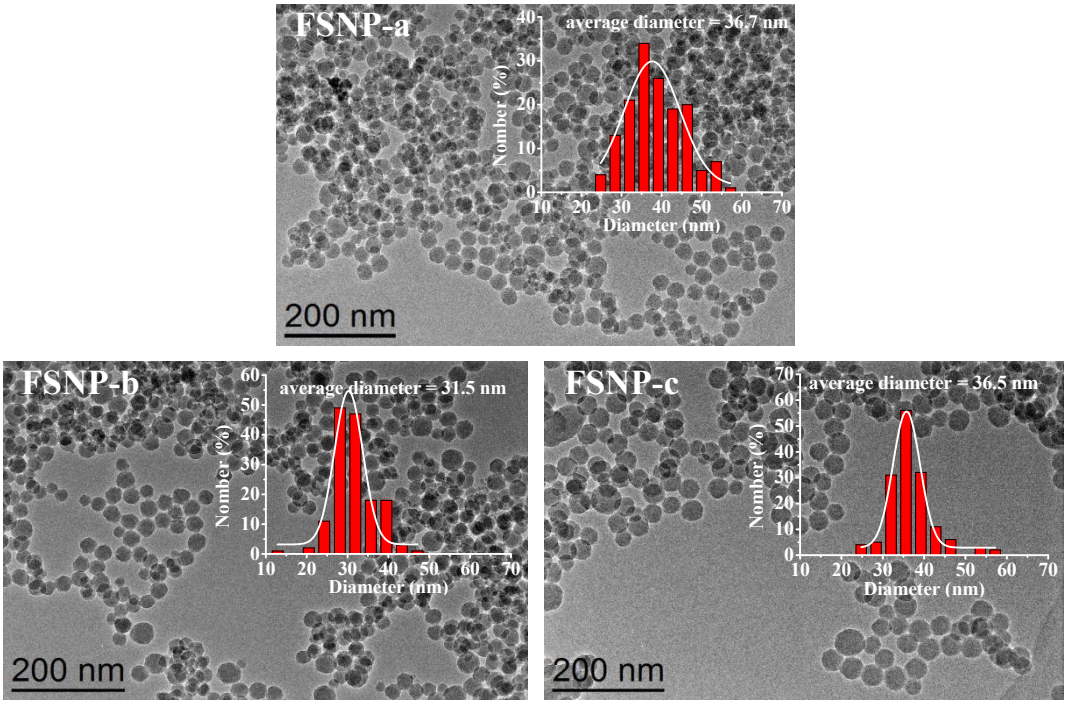


Figure S2. TEM images of FSNP-a~c

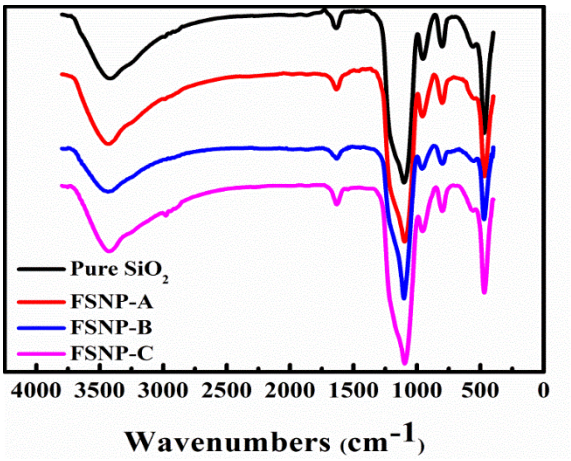


Figure S3. FTIR of FSNP-A~C.

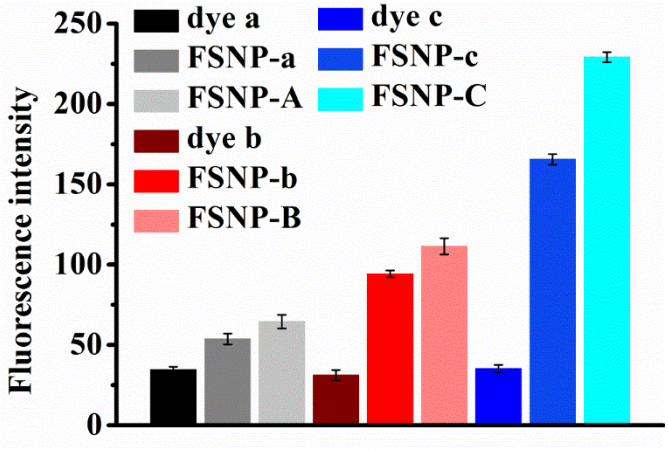


Figure S4. The fluorescent intensity of free dyes and FSNPs in absolute ethanol. All samples were dispersed or dissolved in absolute ethanol to a final concentration with almost the same absorbance (0.01). Error bars represent ±σ for triplicate measurements.

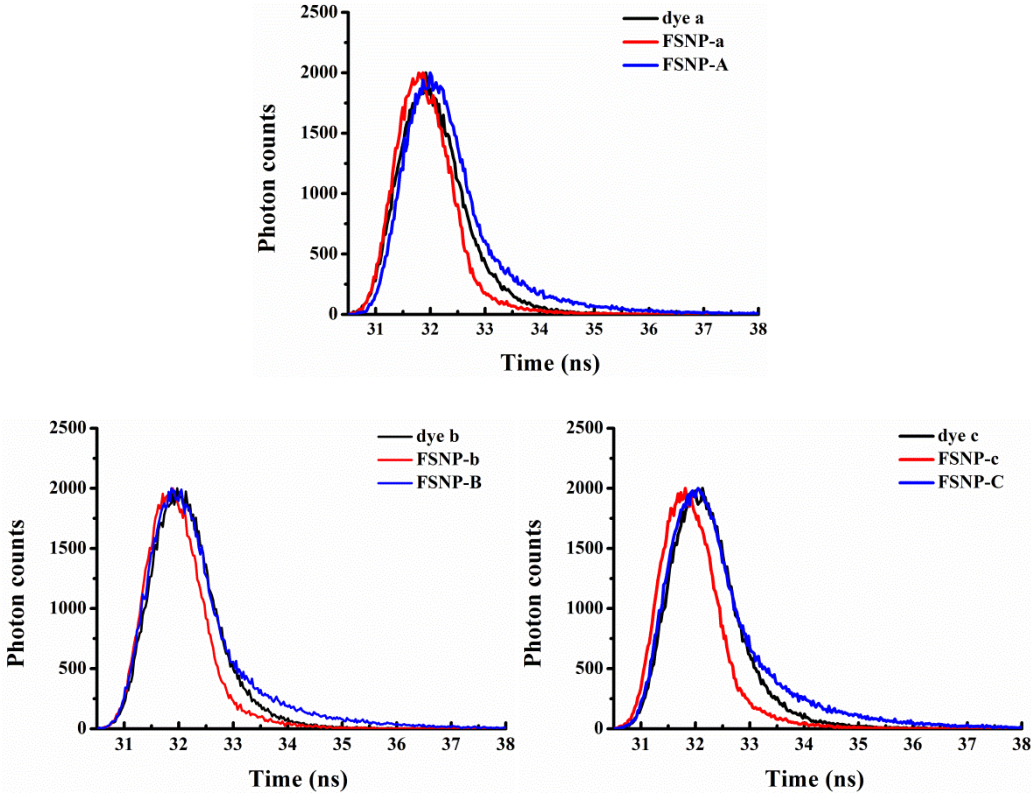


Figure S5. Fluorescence lifetime decays for samples of Free dyes and FSNPs in water.

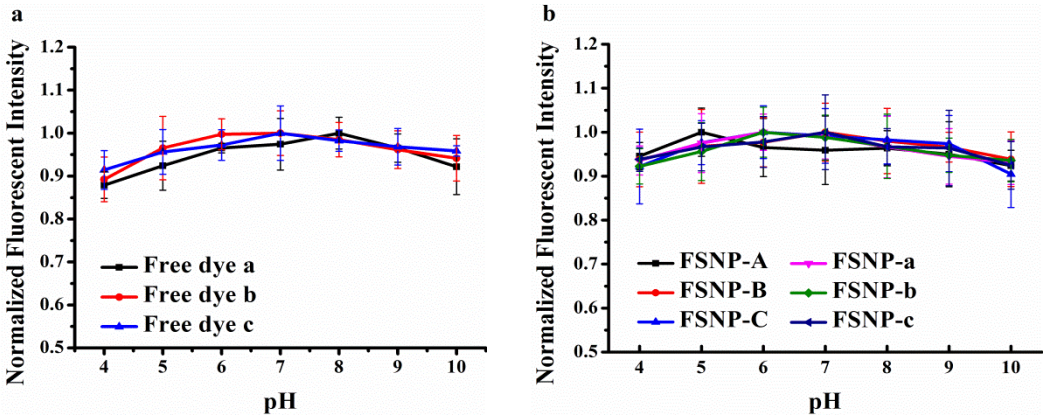


Figure S6. The pH-dependent curve of (a) Free dyes and (b) FSNPs' normalised fluorescent intensity. $C_{\text{Free dye}}$ = 5 μM, C_{FSNPs} = 0.5 mg mL^{-1}.

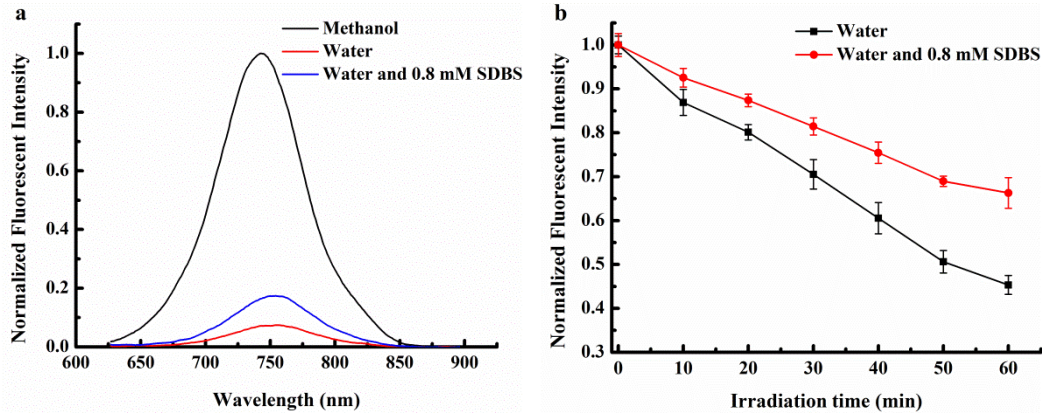


Figure S7. (a) Fluorescence emission spectra of dye c in different solvents; (b) Photostability evaluation of free dye c in different aqueous solution. The concentration of dye c was 20 μM. Sample tubes were exposed to a W-Halogen lamp (500 W).

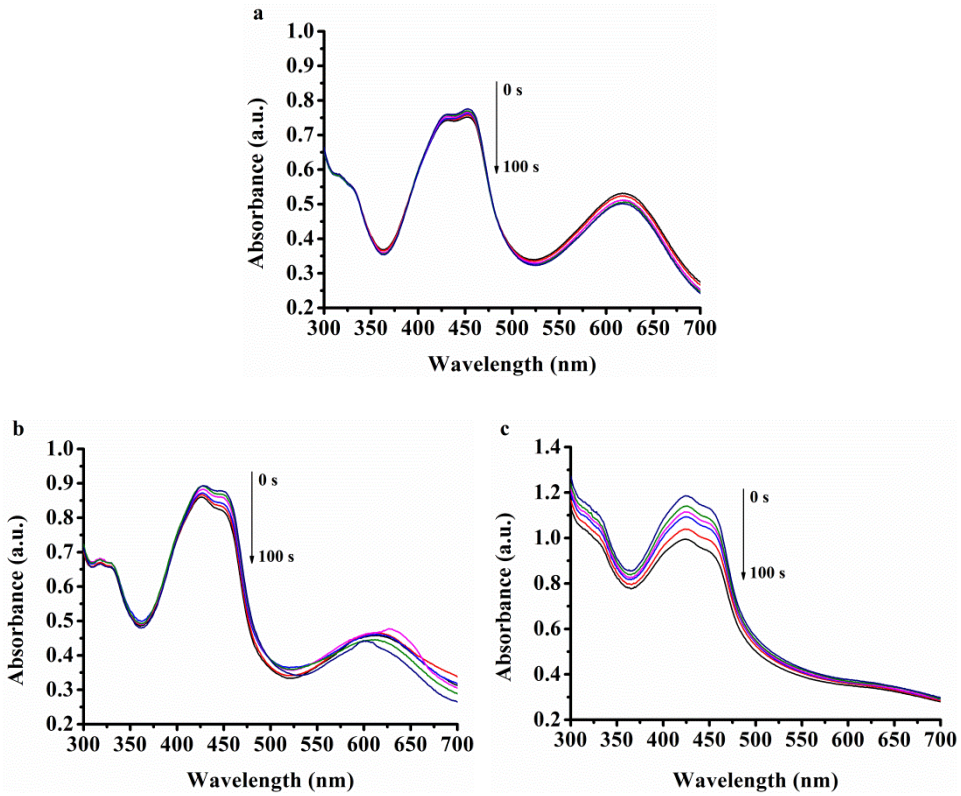


Figure S8. Singlet oxygen generation by dye c (a), FSNP-c (b) and FSNP-C (c) in aqueous solution via monitoring the absorbance decay of DPBF at 420 nm. A LED array: λ_{ex} = 660 nm, 1.0 mW/cm^2. Dye c, FSNP-c and FSNP-C were respectively dissolved in water to a final concentration with almost the same optical density at 660 nm and mixed with DPBF (50 μM).

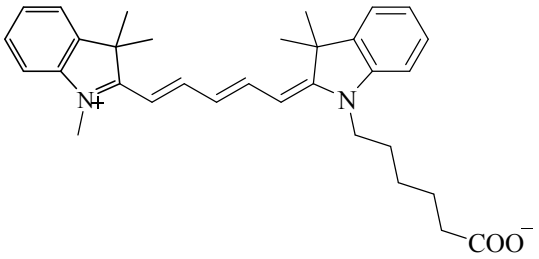


Figure S9. The molecular structure of commercially available Cy5 dye as control for intracellular photostability testing. λ_{ex} = 644 nm, λ_{em} = 665 nm.

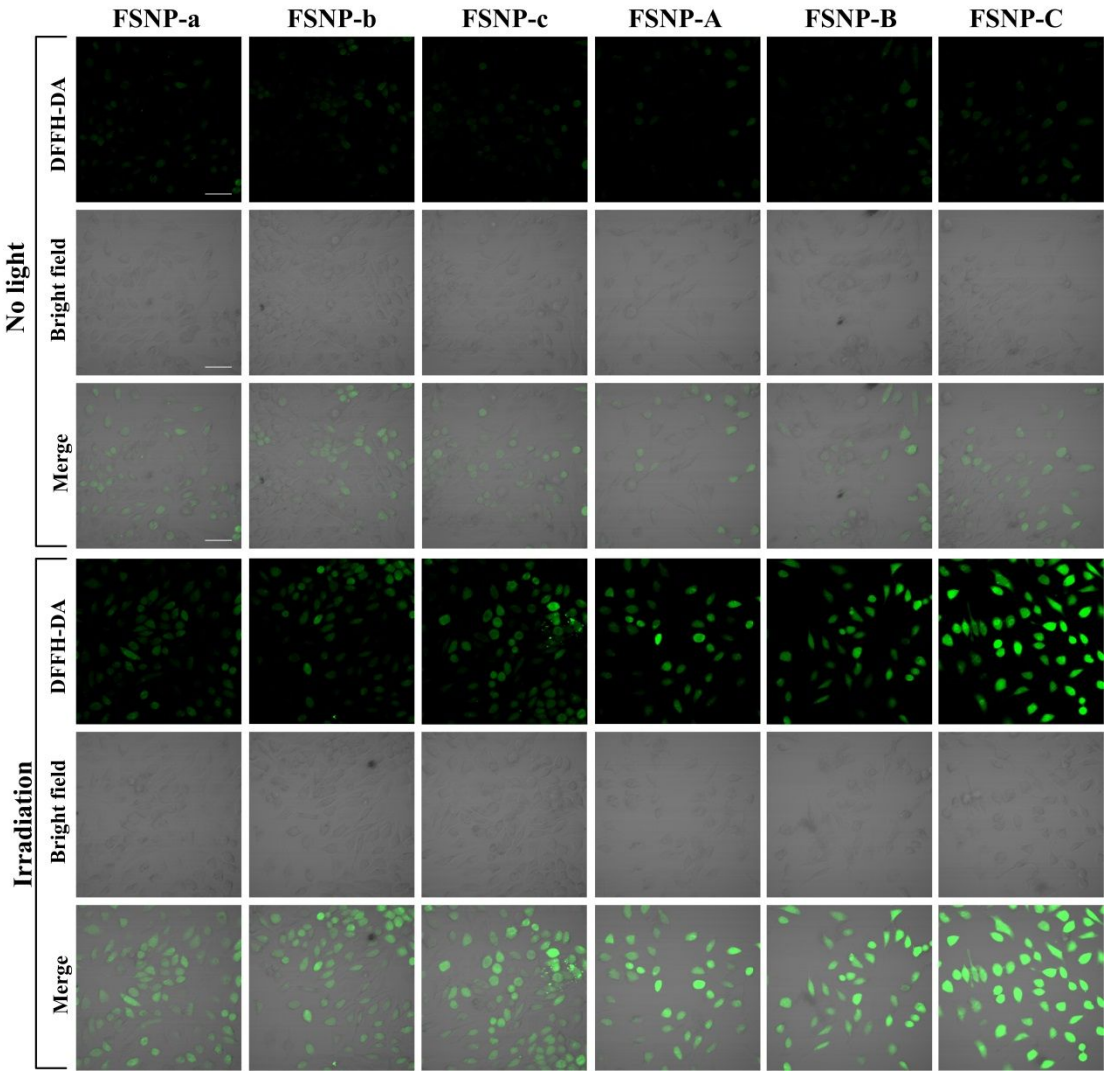


Figure S10. HeLa intracellular ROS detection by DCFH-DA through incubating with different FSNPs. Light source: a LED array (λ_{ex} = 660 nm, optical density = 50 mW/cm^2).

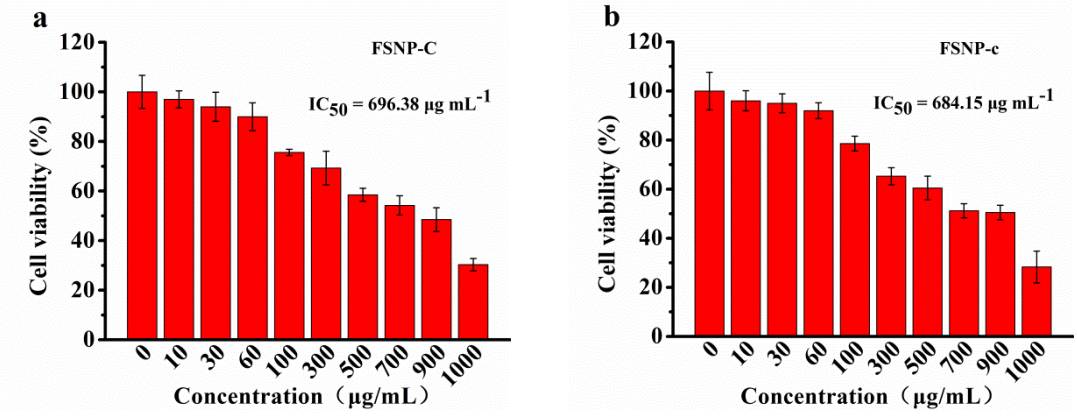


Figure S11. Evaluation of dark cytotoxicity of FSNP-C and FSNP-c on HeLa cells.

5. Supplemental tables

Table S1 Characterization of the prepared FSNP-A~C

FSNPs	Diameter/nm	Zeta potential[a]/mV	Porediameter/nm
FSNP-A	40.8	−24.1 ± 0.5	2.42
FSNP-B	31.5	−23.3 ± 0.7	2.58
FSNP-C	42.6	−23.6 ± 0.9	2.62
Pure SiO_2	36.8	−22.6 ± 0.6	2.61

[a] FSNPs were dispersed in ethanol. Error range is ±σ based on triplicate measurements.

Table S2 Characterization of the prepared FSNP-a~c

FSNPs	Diameter/nm	Zeta potential[a]/mV	Porediameter/nm
FSNP-a	36.7	−23.5 ± 0.6	2.52
FSNP-b	31.5	−24.8 ± 0.8	2.55
FSNP-c	36.5	−23.9 ± 0.8	2.67

[a] FSNPs were dispersed in ethanol. Error range is ±σ based on triplicate measurements.

Table S3 Singlet oxygen quantum yield (Φ_Δ) of the prepared FSNPs [a]

Fluorophores	Φ_Δ	FSNPs	Φ_Δ	FSNPs	Φ_Δ
dye a	0.006 ± 0.002	FSNP-a	0.007 ± 0.001	FSNP-A	0.011± 0.002
dye b	0.007 ± 0.001	FSNP-b	0.007 ± 0.001	FSNP-B	0.013± 0.001
dye c	0.007 ± 0.002	FSNP-c	0.010 ± 0.001	FSNP-C	0.085± 0.002
ICG; IR-787	0.008; 0.007[4]				

[a] Φ_{MB} = 0.52 in water as a reference [1, 2]; Error bars represent ± σ for triplicate measurements.

6. Supplemental spectra

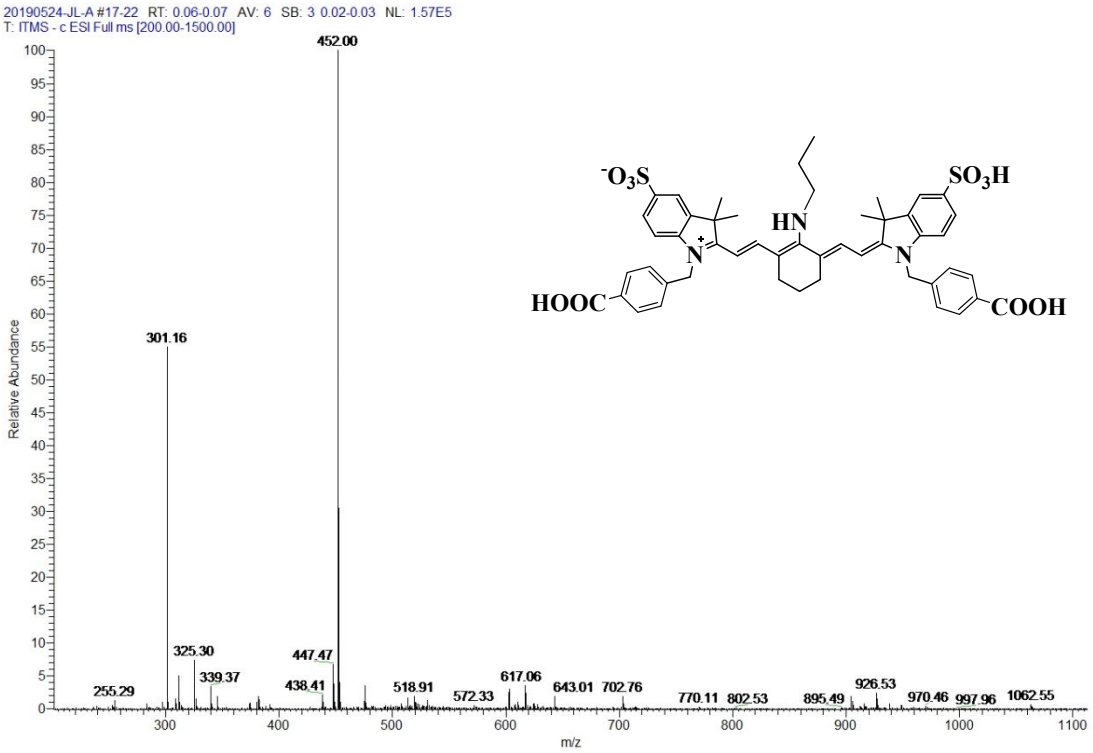


Figure S12. FTMS (ESI^-) of free dye-a.

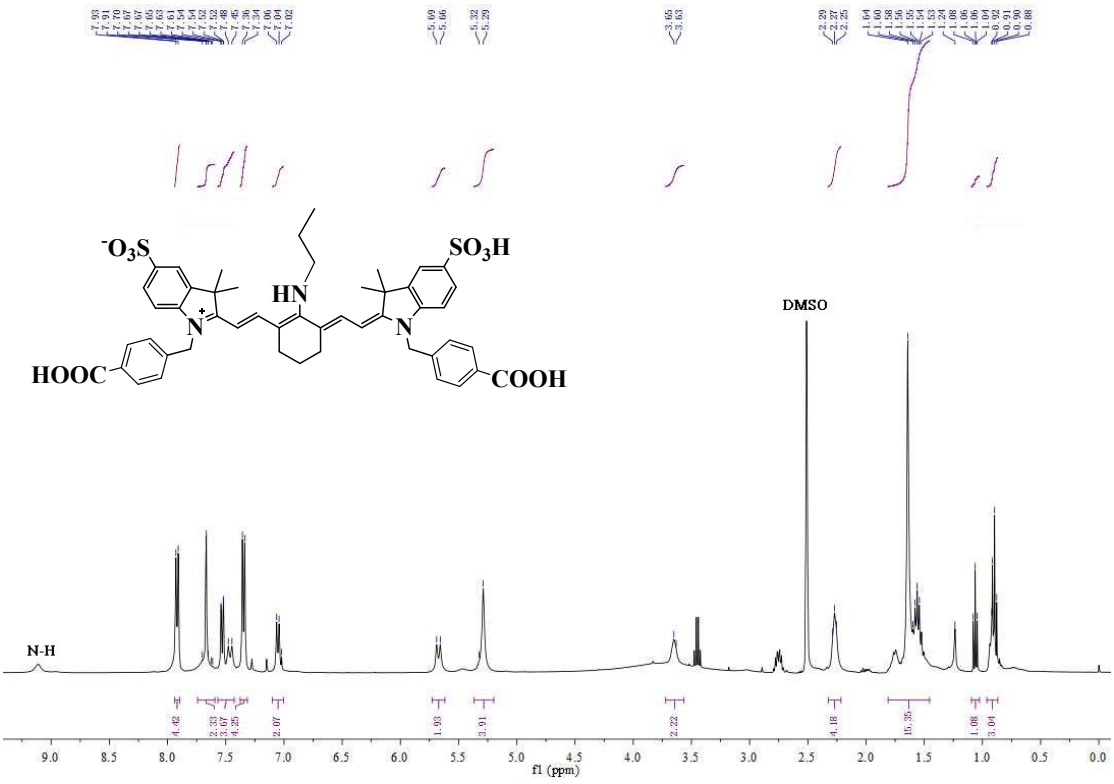


Figure S13. ^1H NMR spectra of dye-a (in DMSO-d6).

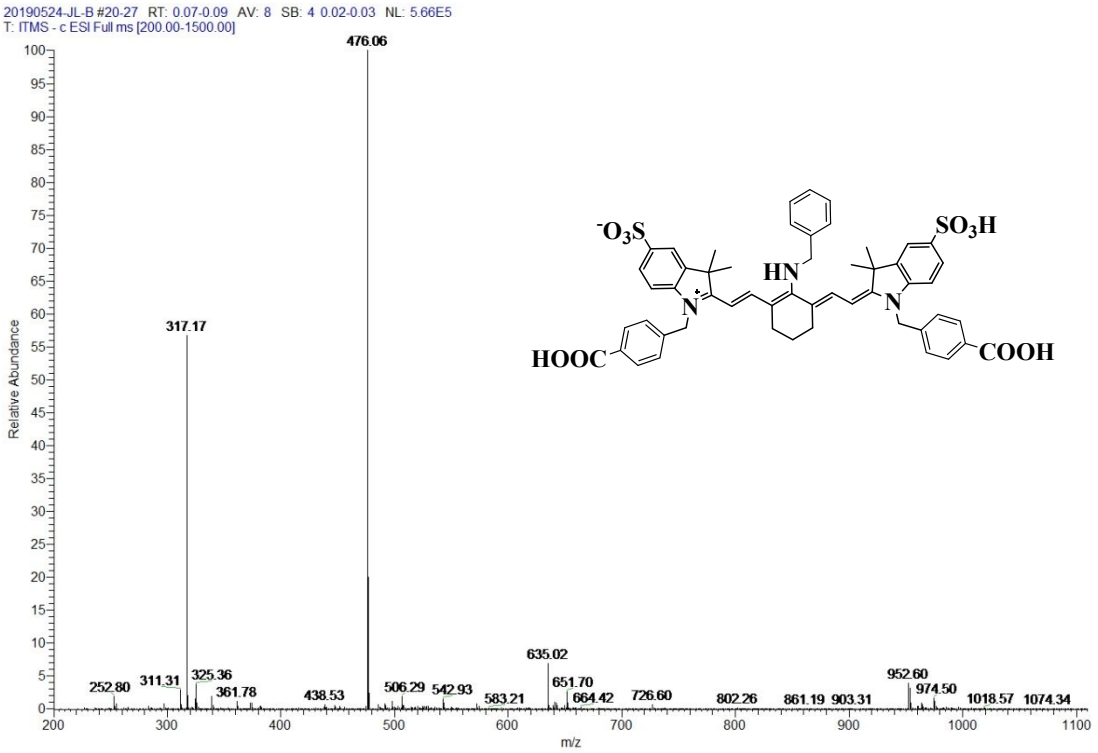


Figure S14. FTMS (ESI$^-$) of free dye-b.

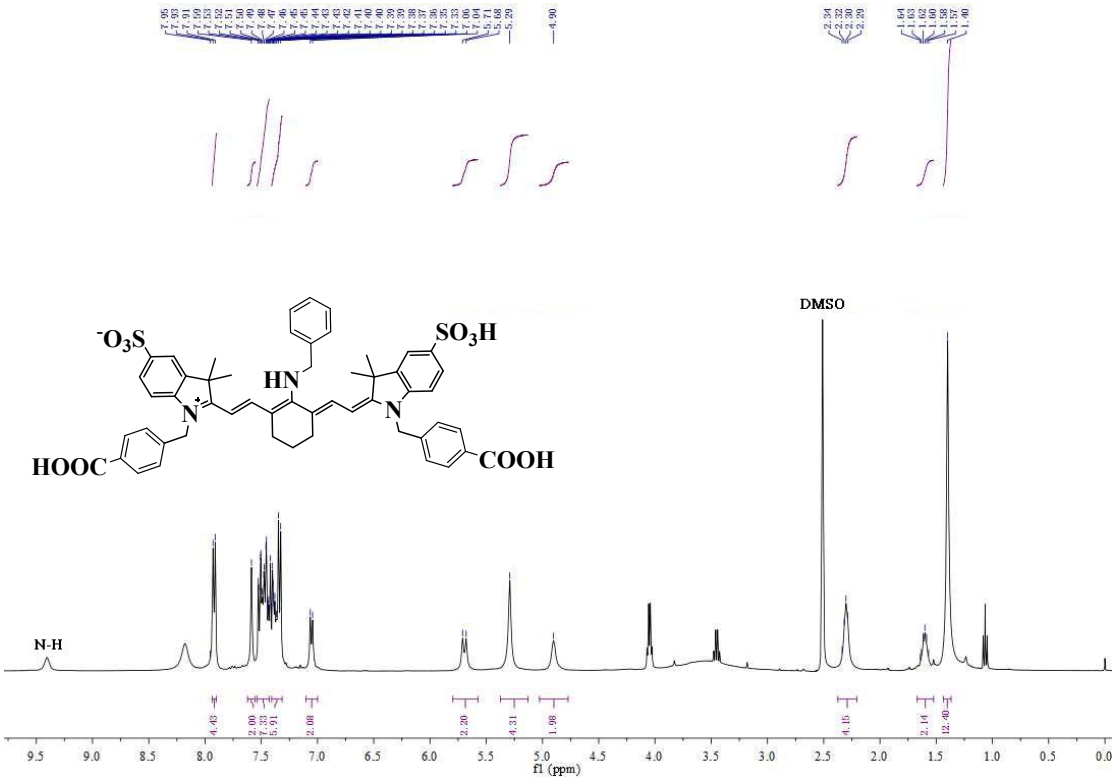


Figure S15. ^{1}H NMR spectra of dye-b (in DMSO-d6).

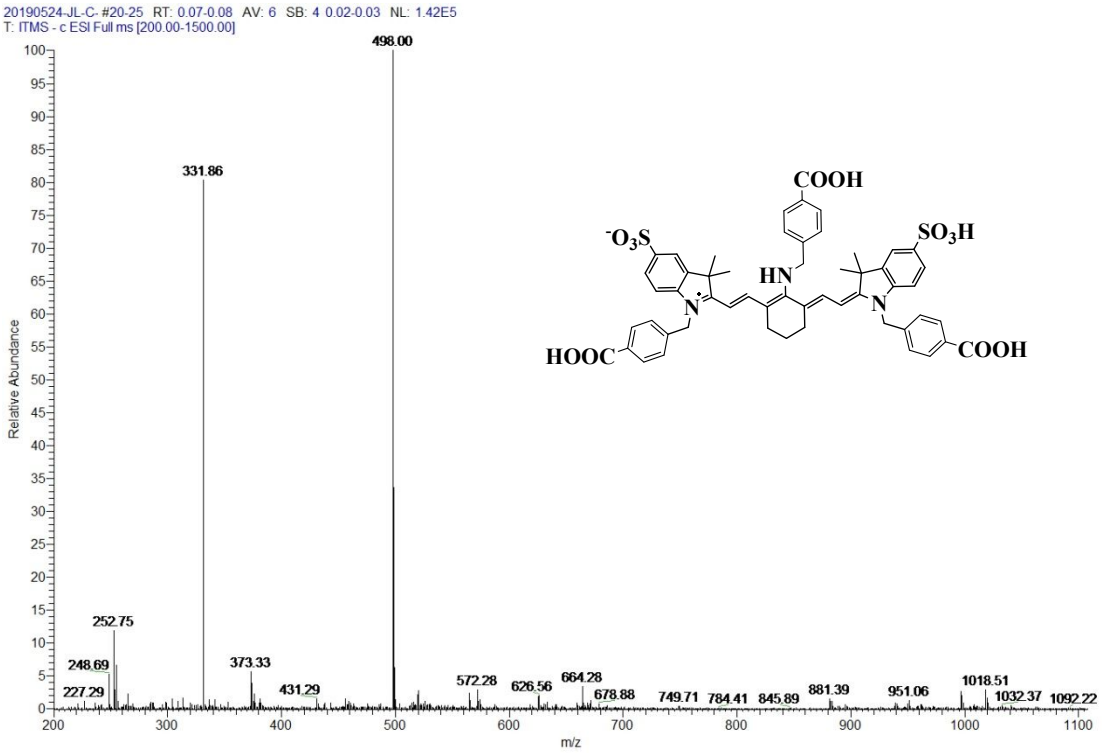


Figure S16. FTMS (ESI^-) of free dye-c.

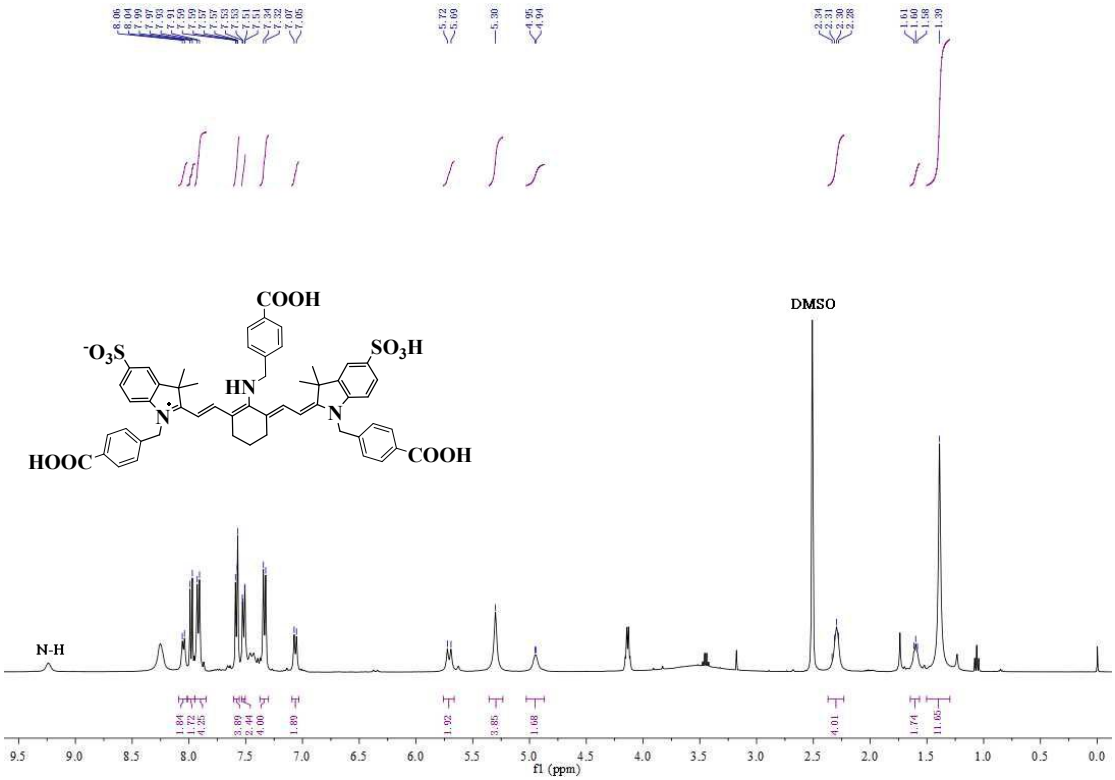


Figure S17. ^1H NMR spectra of dye-c (in DMSO-d6).

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