Electronic Supplementary Information

NIR-Absorbing Dye Functionalized Hollow Mesoporous Silica Nanoparticles for Combined Photothermal-Chemotherapy

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Experimental section

Materials: Acetic anhydride, anhydrous dichloromethane, anhydrous dimethylformamide (DMF), 5-bromovaleric acid, *N,N'*-dicyclohexylcarbodiimide (DCC), (dimethylamino propyl)carbodiimide hydrochlorid, *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride (15 mg), dimethylaminopyridine, DL-dithiothreitol (DTT), doxorubicin hydrochloride (DOX·HCl), glutathione (GSH), *N*-hydroxysuccinimid, 1,1,2-trimethylbenz[e]indole, and all other conventional solvents were purchased from Sigma-Aldrich and used without purifications. PEG2K-SH was obtained from Yarebio Ltd (Shanghai, China). 2-Chloro-1-formyl-3-hydroxymethylene cyclohexene, 3-(pyridin-2-yldisulfanyl)propanoic acid, and mono-(6-ethylenediamine-6-deoxy)-β-cyclodextrin were prepared according to procedures reported in literature.[1-3]

Instruments: ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectra were recorded on a Bruker BBFO-400 using deuterated solvents. Transmission electron microscopy (TEM) images were collected on JEM-1400 (JEOL) at 100 kV. Fluorescence spectra were measured by fluorescence spectrophotometer (RF5301PC, Shimadzu, Japan). UV−vis−NIR absorption spectra were recorded on UV-3600 spectrophotometer (Shimadzu). X-ray photoelectric spectroscopy (XPS) was obtained on a Phoibos 100 spectrometer with a SPECS monochromatic Mg X-ray radiation source (wide-range and high resolution S 2p scans at 12.53 kV). N_2 adsorption-desorption isotherms were conducted using Quantachrome Instruments Autosorb-iQ (Boynton Beach, Florida USA) with extra-high pure gases. The samples were moved into a sample cell and dried under vacuum at 60 °C and 120 °C by using the "outgasser" function for 6 h before the measurements. The specific surface areas were determined from the adsorption data in low pressure range following the Brunauer−Emmett−Teller (BET) model, and pore size was calculated using the Barret-Joyner-Halenda (BJH) method. Thermogravimetric analysis (TGA) was performed using a TGA-Q500 recorded from 100 to 1000 °C in an air flow at a heating rate of 10 °C min^{-1}. CLSM images were acquired by a Leica TCS confocal microscope with a Nikon Eclipse

TE2000-S objective. Flow cytometry experiments were conducted by BD LSRFortessa X-20 cell analyzer. The temperature of the irradiated suspensions was determined by an infrared thermal imaging device (Testo 868, Testo Inc.). HPLC analysis of DOX content was performed with a Shimadzu analytical HPLC system using the Waters XSelectTM HSS C18 (5 μm, 4.6 × 250 mm) column as the stationary phase with UV detection at 480 nm at 30 °C. Acetonitrile/water binary system containing 0.5% trifluoroacetic acid was used as the mobile phase. HPLC analysis was performed using gradient elution method, starting with 95% water to 5% water and back to 95% water over a period of 40 min. Flow rate was maintained at 1 mL min^{-1} throughout the analysis.

Preparation of Ad-dye-PEG

Synthesis of 1: The compound **1** was prepared according to procedures reported in literature.[4]

Synthesis of 2: 5-Bromovaleric acid (2.72 g, 15 mmol), compound **1** (3.00 g, 15 mmol), 4-dimethylaminopyridine (244 mg, 2.0 mmol), and *N,N'*-dicyclohexylcarbodiimide (3.71mg, 18 mmol, 1.2 eqv) were mixed in anhydrous dichloromethane (150 mL). The solution was stirred at room temperature overnight. Then, the solution was filtered and the solvent was removed by rotary evaporation. The residue was purified by column silica gel chromatography with the eluents of hexane/ethyl acetate (8/1, v/v) to obtain the compound **2** as the white solid. Yield: 56%. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 1.56-1.68 (m, 6H), 1.72-1.83 (m, 8H), 1.87-1.95 (m, 2H), 2.15 (S, 3H), 2.37 (t, 2H), 3.41 (t, 2H), 3.61 (t, 2H), 4.18 (t, 2H). ^{13}C NMR ($CDCl_3$, 400 MHz): 23.51, 30.48, 31.97, 33.06, 33.24, 36.40, 41.44, 58.07, 64.49, 72.51. TOF HRMS: calcd. for $C_{17}H_{28}BrO_3$ $[M + H]^+$, 359.1222; found, 359.1222.

Synthesis of 3: 1,1,2-Trimethylbenz[e]indole (1.05 g, 5.0 mmol) and compound **2** (1.47 g mg, 7.5 mmol) were dissolved in dichlorobenzene (10mL) and stirred under 110 °C for 4 h. Then, the mixture was added into the diethyl ether (50 mL) and the precipitation was collected by vacuum filtration. The product was purified by column silica gel chromatography with the eluent of dichloromethane and obtained as pale powder. Yield: 36%. ^{1}H NMR (DMSO-d_6, 400 MHz): δ 1.42-1.52 (m, 12H), 1.66-1.78 (m, 8 H), 1.94 (m, 2H), 2.00 (s, 3H), 2.95 (s, 3H), 3.49 (t, 2H), 4.04 (t, 2H), 4.61 (t, 2H), 7.70-7.82 (m, 2H), 8.15 (d, 1H), 8.22 (d, 1H), 8.30 (d, 1H), 8.37 (d, 1H). TOF HRMS: calcd. for $C_{32}H_{42}NO_3$ $[M]^+$, 488.3165; found, 488.3160.

Synthesis of 4: Compound **3** (0.567 g, 1 mmol), 2-chloro-1-formyl-3-hydroxymethylene cyclohexene (0.086 g, 0.5 mmol), and sodium acetate (0.164 g, 2 mmol) were heated in acetic anhydride (15 mL) at 70 °C for 2 h. The green solution was cooled down, and then poured into saturated solution of sodium chloride. The green solid was filtered and collected. The crude product was purified by column silica gel chromatography with the eluent of dichloromethane to obtain the compound **4** as a dark green solid. Yield: 40 %. ^{1}H NMR (DMSO-d_6, 400 MHz): δ 1.49-1.64 (m, 12 H), 1.65-1.71 (m, 12H), 1.81-2.13 (m, 28H), 2.45 (t, 2H), 2.79 (s, 4H), 3.57 (t, 4H), 4.14 (t, 4H), 4.39 (t, 4H), 6.33 (d, 2H), 7.44-7.51 (m, 4H), 7.61 (t, 2H), 7.91-7.97 (m, 4H), 8.12 (d, 2H), 8.44 (d, 2H). TOF HRMS: calcd. for $C_{32}H_{42}NO_3$ $[M]^+$, 1111.6331; found, 1111.6377.

Synthesis of Ad-dye-PEG: PEG2k-SH (4.00 g, 2 mmol), compound **4** (1.19 g, 1 mmol) and TEA (200 μL, 1.44 mmol) were mixed in anhydrous DMF (5 mL) under the protection of N_2. The mixture was heated at 40 °C overnight and precipitated with diethyl ether. The residue was collected by filtration and purified by column silica gel chromatography with the eluents

of dichloromethane/methanol (20/1, v/v) to obtain the compound Ad-dye-PEG. Yield: 49%. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 1.48-1.64 (m, 12H), 1.65-1.72 (m, 12H), 1.80-2.15 (m, 28H), 2.46 (t, 2H), 2.73 (s, 4H), 3.01 (t, 2H), 3.32-3.39 (s, 3H), 3.42-3.86 (m, 184 H), 4.16 (t, 4H), 4.37 (t, 4H), 6.34 (d, 2H), 7.40-7.75 (m, 6H), 7.61 (t, 2H), 7.89-8.01 (m, 4H), 8.12 (d, 2H), 8.89 (d, 2H).

Preparation of HMSNs-COOH

Template-free thiol-modified hollow mesoporous nanoparticles (HMSNs-SH) were prepared according previous literature procedures.[5,6] Disulfide bond with terminal carboxylic acid group was immobilized. Basically, HMSNs-SH (0.5 g) was dispersed in the mixture of ethanol (30 mL) and acetic acid (1.2 mL). Then, the suspension was added with 3-(pyridin-2-yldisulfanyl)propanoic acid (0.5 g) and stirred at room temperature for 48 h. The resultant disulfide bond containing nanocarriers (HMSNs-COOH) were centrifuged, washed exclusively with ethanol, and dried under vacuum.

Preparation of HMSNs-CD@DOX

For the DOX loading, the synthesized HMSNs-COOH (20 mg) and DOX (15 mg) were dispersed in distilled water (4 mL) assisted by ultrasonication, and then the suspension was stirred gently away from light for 24 h. The DOX-loaded HMSNs were collected through centrifugation, washed with phosphate buffer (pH = 7.4) to remove any unloaded DOX.

Then, the nanoparticles were suspended in phosphate buffer (4 mL, pH = 7.4), and *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride (15 mg) and *N*-hydroxysuccinimide (12 mg) were added to the above suspension. The obtained mixture was stirred at room temperature for 0.5 h. After this, mono-(6-ethylenediamine-6-deoxy)-β-cyclodextrin (15 mg) was added to the suspension and stirred for 24 h. The product HMSNs-CD@DOX was collected through centrifugation, washed thoroughly with methanol, and then dried under vacuum.

In a similar way, HMSNs-COOH was directly modified with β-CD without DOX loading and the resulted nanoparticles were denoted as HMSNs-CD.

Preparation of HMSNs-Dye@DOX

Ad-dye-PEG (0.15 g) was dissolved in deionized water (2 mL), and this solution was subsequently added into the HMSNs-CD@DOX suspension in deionized water (4 mL). The dye was complexed on the surface of HMSNs through strong β-CD/adamantane host-guest interaction. After stirring at room temperature overnight, the product HMSNs-Dye@DOX was collected through centrifugation, washed thoroughly with methanol, and then dried under vacuum.

For the preparation of HMSNs-Dye without DOX loading, HMSNs-CD was employed to complex with Ad-dye-PEG in a similar way.

***In vitro* drug release**

To evaluate the DOX loading efficiency of the nanocarriers, HMSNs-Dye@DOX (2.0 mg) was treated with DTT (10 μM) in DMSO (2.0 mL) at 25 °C in the dark for 24 h, and the supernatant was collected after the centrifugation. The remaining nanoparticles were

repeatedly washed with DMSO until the supernatant was nearly colorless. The collected supernatants were combined, and the total amount of extracted DOX was determined by the HPLC analysis (Fig. S11).

The reduction-responsive release of DOX from HMSNs-Dye@DOX was determined by fluorescence spectrophotometer when exposed to deionized water with and without the GSH stimulus. Briefly, HMSNs-Dye@DOX suspension (1.5 mL) was firstly placed into dialysis bag (MWCO = 12 000) and dialyzed against deionized water (25 mL) in the presence of GSH (0, 5, and 10 mM, respectively) at 37 ^{o}C in the dark. At different time intervals, a portion of medium (4 mL) was taken out and the intensity of its fluorescence emission at 560 nm under the excitation wavelength of 480 nm was monitored using the fluorescence spectrometer, while the same amount of fresh buffer solution was added back. After conducting the release experiments for three independent times, the mean value was obtained as the final result.

Cellular uptake study

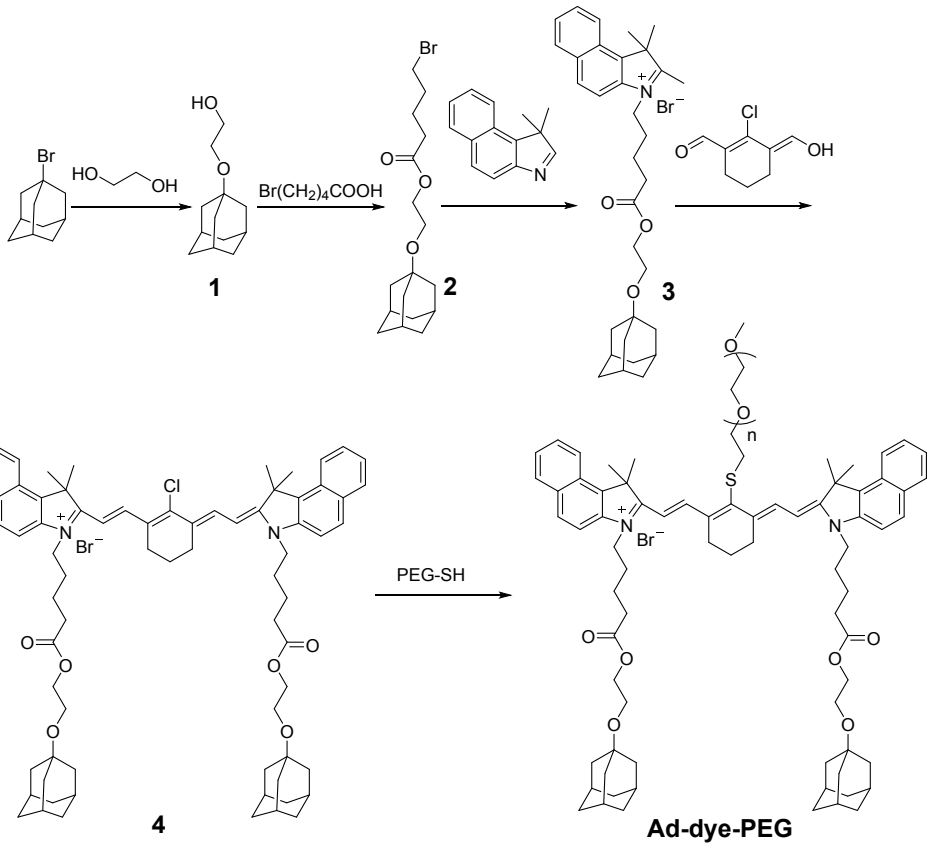
CLSM was employed to observe the intracellular release behavior of HMSNs-Dye@DOX within DOX-sensitive human cervical carcinoma HeLa cells and DOX-resistant ovarian carcinoma A2780/DOX^{R} cells. HeLa cells were incubated in Dulbecco's modified Eagle medium, while A2780/DOX^{R} cells were seeded in RPMI 1640 medium supplemented with 10% fetal bovine serum, penicillin (100 U mL^{-1}), and streptomycin (100 μg mL^{-1}). Cells were seeded in a six-well tissue culture plate (2 mL medium) at a density of 2.0×10^5 cells per well. After culturing at 37 °C and 5% CO_2 for 24 h, an appropriate amount of HMSNs-Dye@DOX suspension with an equivalent DOX concentration (5 μg mL^{-1}) was added into the cell culture medium. After further incubation for 8 h, the culture medium was removed, and the cells on microscope plates were washed three times with phosphate buffered saline (PBS). In addition, cells treated with free DOX were incubated as a control. Finally, the samples were stained with 4',6-diamidino-2-phenylindole (DAPI, blue color) for 5 min and then mounted for observation with CLSM.

In flow cytometry studies, HeLa cells were seeded in a six-well tissue culture plate (2 mL medium) at a density of 2.0×10^5 cells per well in DMEM (2 mL) for 20 h and then treated with and without GSH-OEt (10 mM in cell medium) for 2 h. Cells were washed with PBS one time and incubated in the fresh culture medium. After this, free DOX or HMSNs-Dye@DOX was added, all of which was maintained at equivalent DOX concentration (1 μg mL^{-1}) for every well. After the incubation for 2 h, the cells were washed with PBS for two times and treated with trypsin (0.5 mL) for 2 min. Then, fresh medium (0.5 mL) was added to each culture well, and the cells were collected *via* centrifugation at 2000 rpm for 3 min. After one more round of washing with PBS through centrifugation, the cells were suspended in PBS (1 mL) and subjected to the flow cytometry analysis.

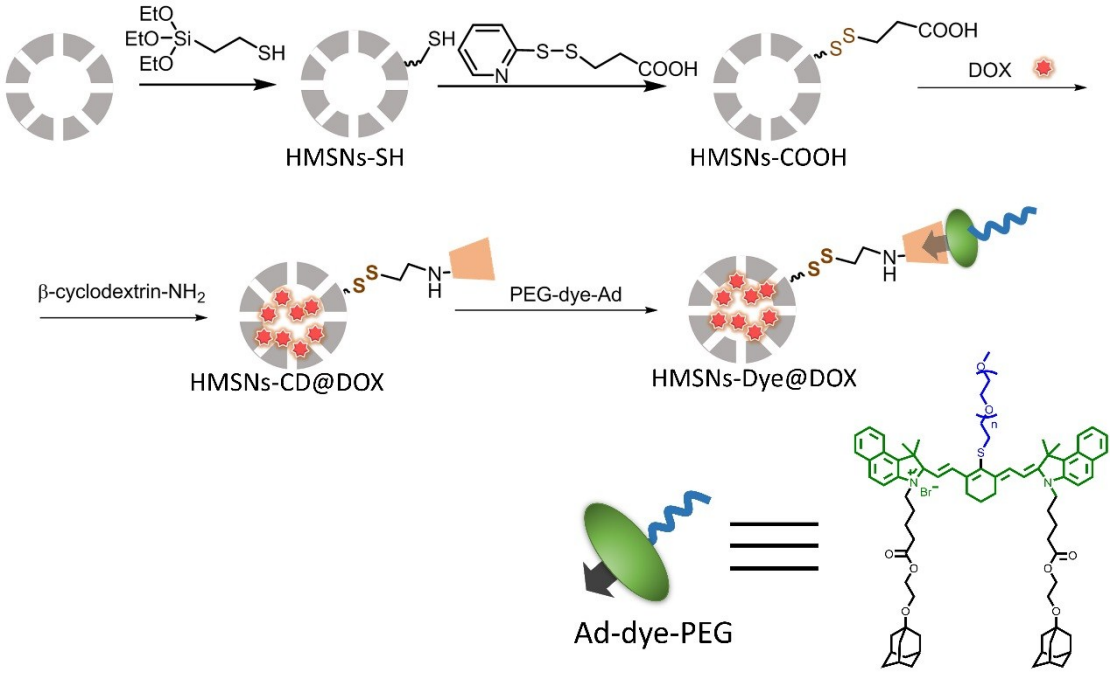
***In vitro* cytotoxicity study**

The anticancer efficacy of HMSNs-Dye@DOX on proliferations of HeLa cells and A2780/DOX^{R} cells with or without NIR irradiation was quantitatively investigated by using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Briefly, the cells were seeded in 96-well plates (100 μL medium) and incubated. When the cell confluence reached around 60−70%, the medium was replaced with fresh one (90 μL), and then HMSNs-

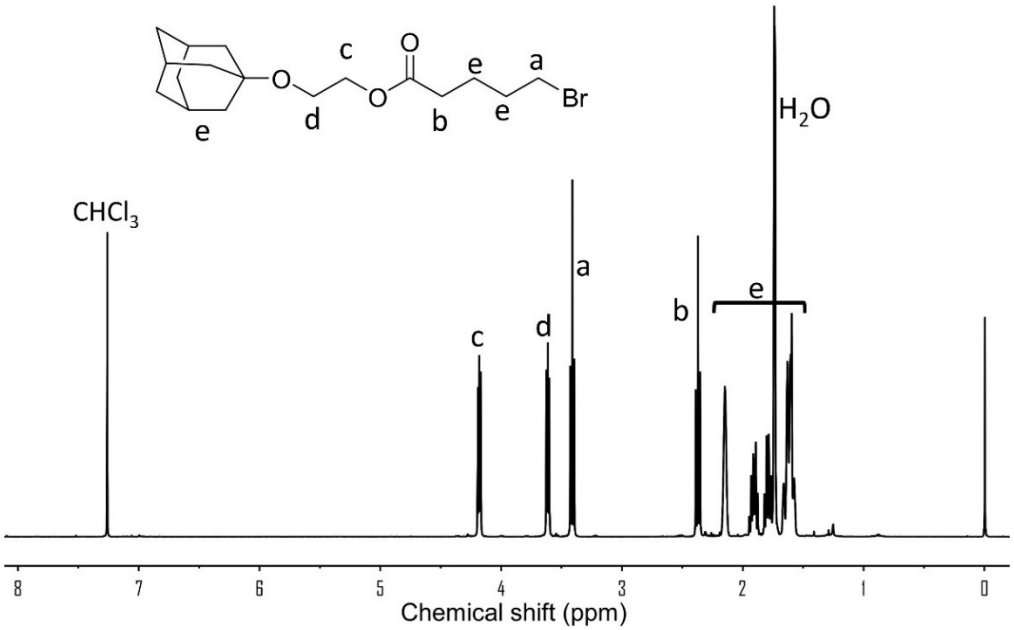
Dye@DOX suspension (10 μL each) with various concentrations were added. After 4 h of incubation, the cultural medium was replaced, and the cells were illuminated by 808 laser at certain power densities for 5 min. After the incubation for 24 h, the medium was again replaced with fresh one, followed by the addition of MTT solution (10 μL, 5 mg L^{-1}). The cells were incubated for another 4 h, and then the medium was replaced by DMSO (150 μL) to dissolve the resulted purple crystals. Finally, optical densities of the samples were measured using a microplate reader (infinite M200, TECAN) at 490 nm. The cell viability was calculated based on the following equation: $(A_{sample}/A_{control}) \times 100\%$, where A_{sample} and $A_{control}$ represent the absorbance of the sample and control groups, respectively.

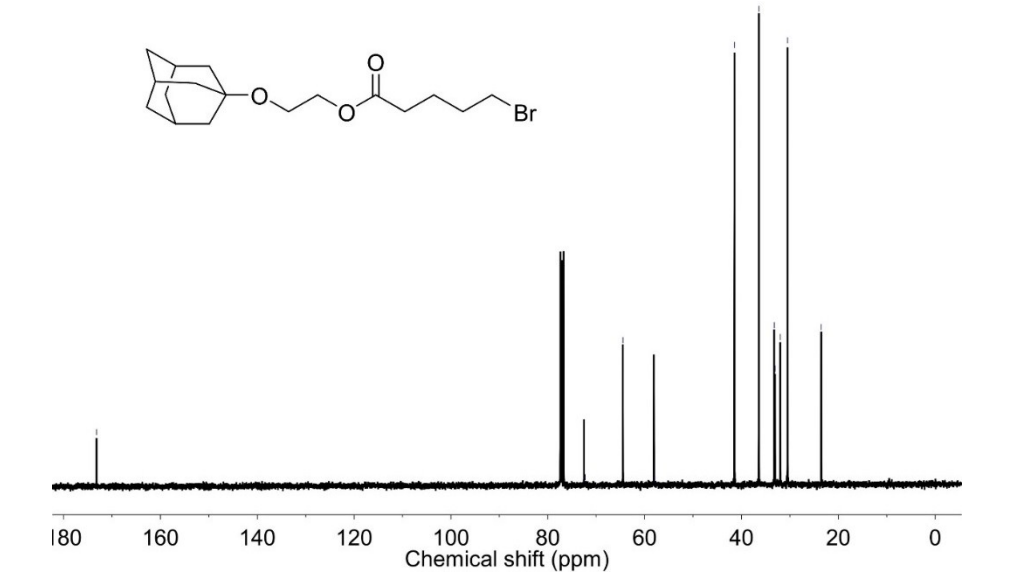


Scheme S1. Synthetic route of adamantane functionalized PEGylated dye Ad-dye-PEG.



Scheme 2. Synthetic route of HMSNs-Dye@DOX for combined photothermal-chemotherapy.





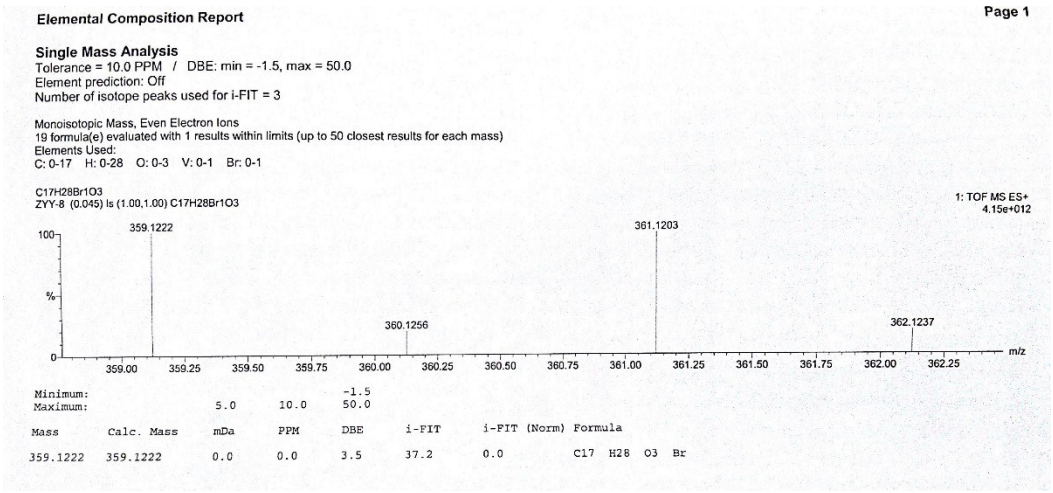
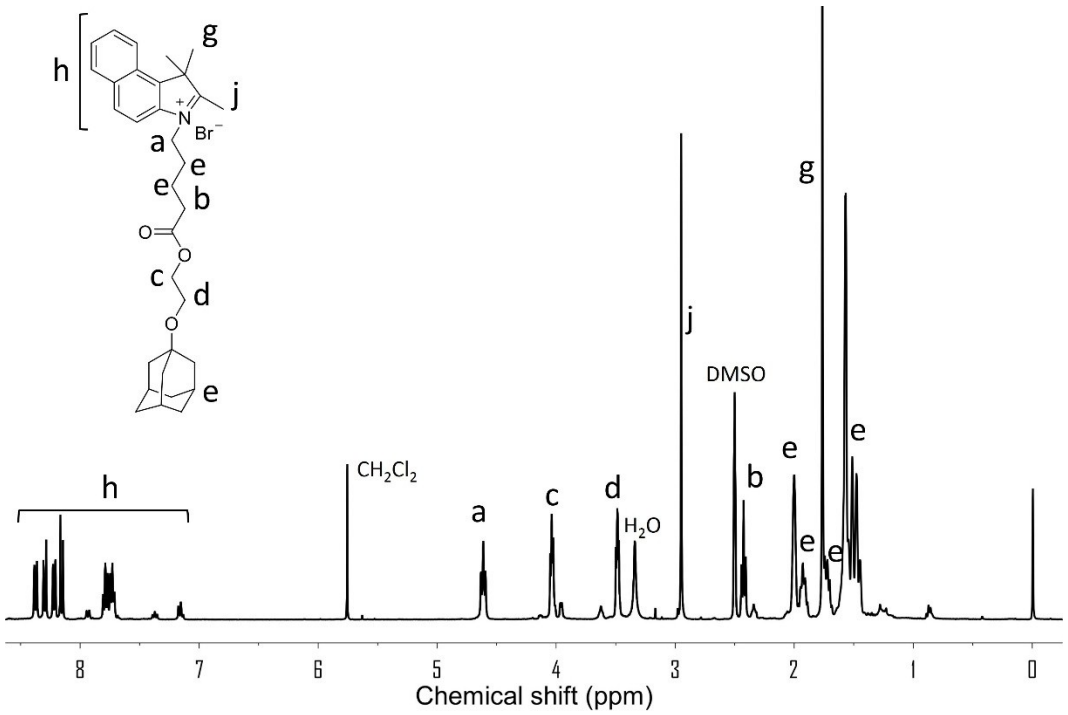


Fig. S1. ^{1}H NMR (top), ^{3}C NMR (middle) and HRMS (down) spectra of compound **2** (solvent in NMR: $CDCl_3$).



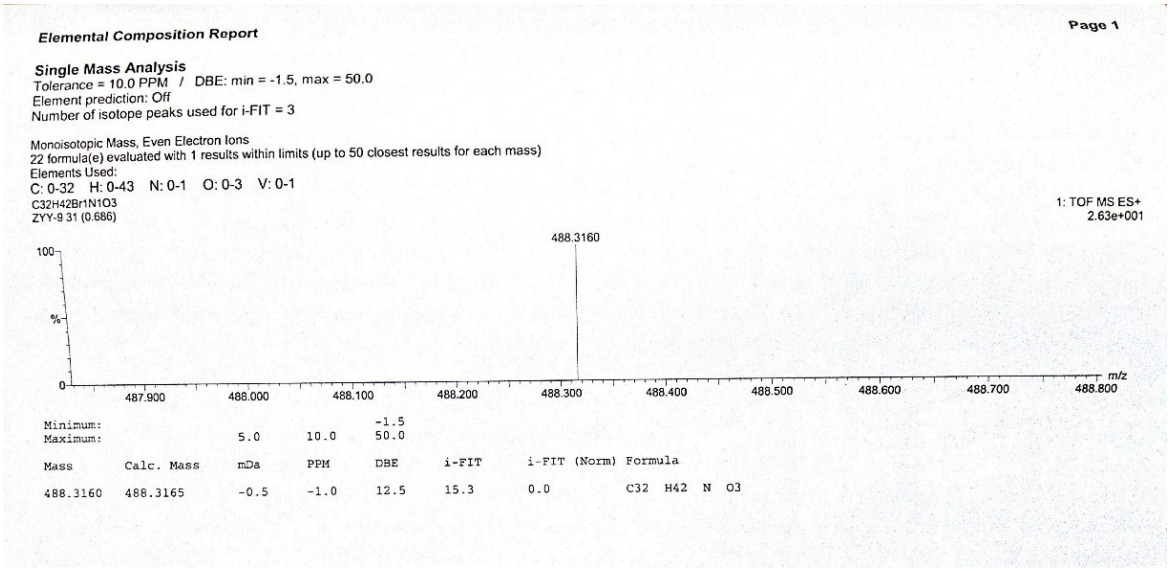
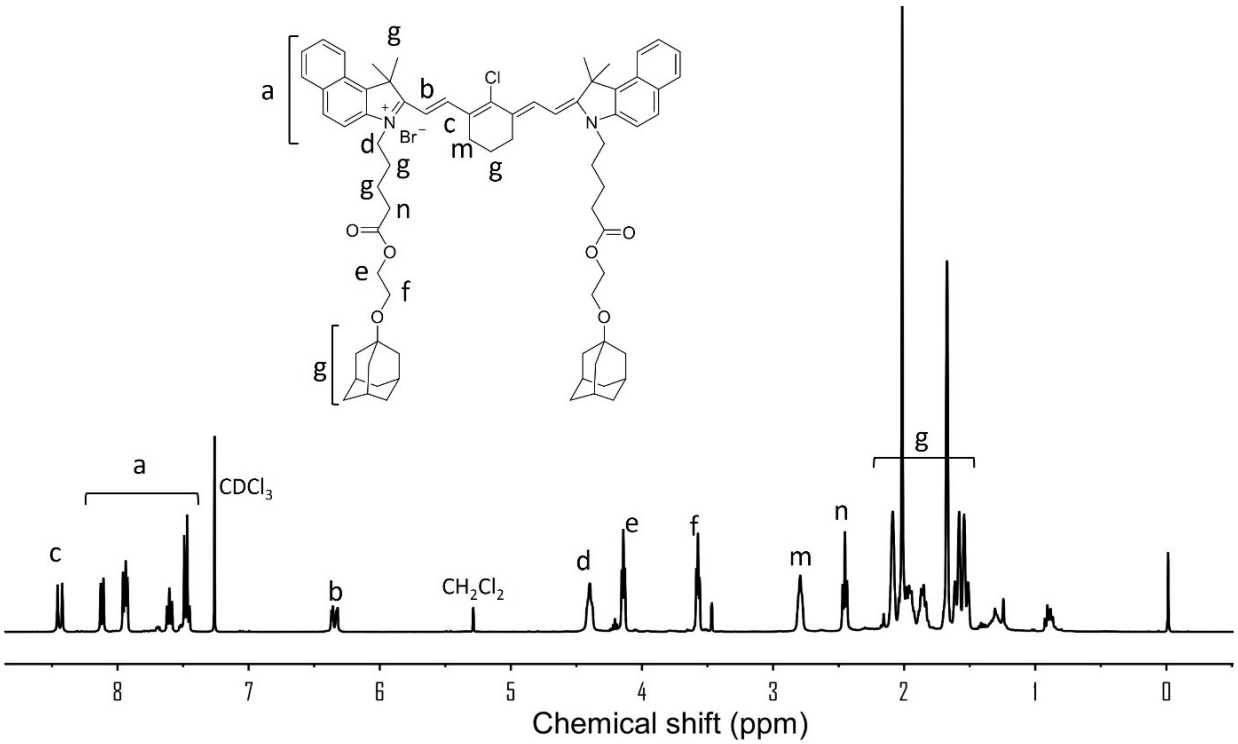


Fig. S2. [1]H NMR (top) and HRMS (down) spectra of compound **3** (solvent in NMR: DMSO-d_6).



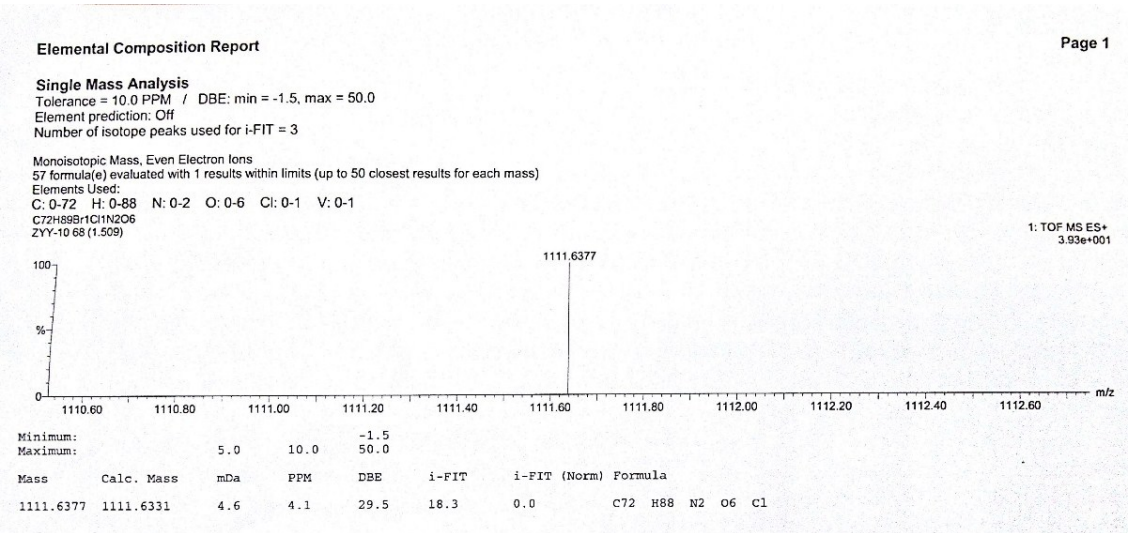


Fig. S3. ^{1}H NMR (top) and HRMS (down) spectra of compound **4** (solvent in NMR: DMSO-d_6).

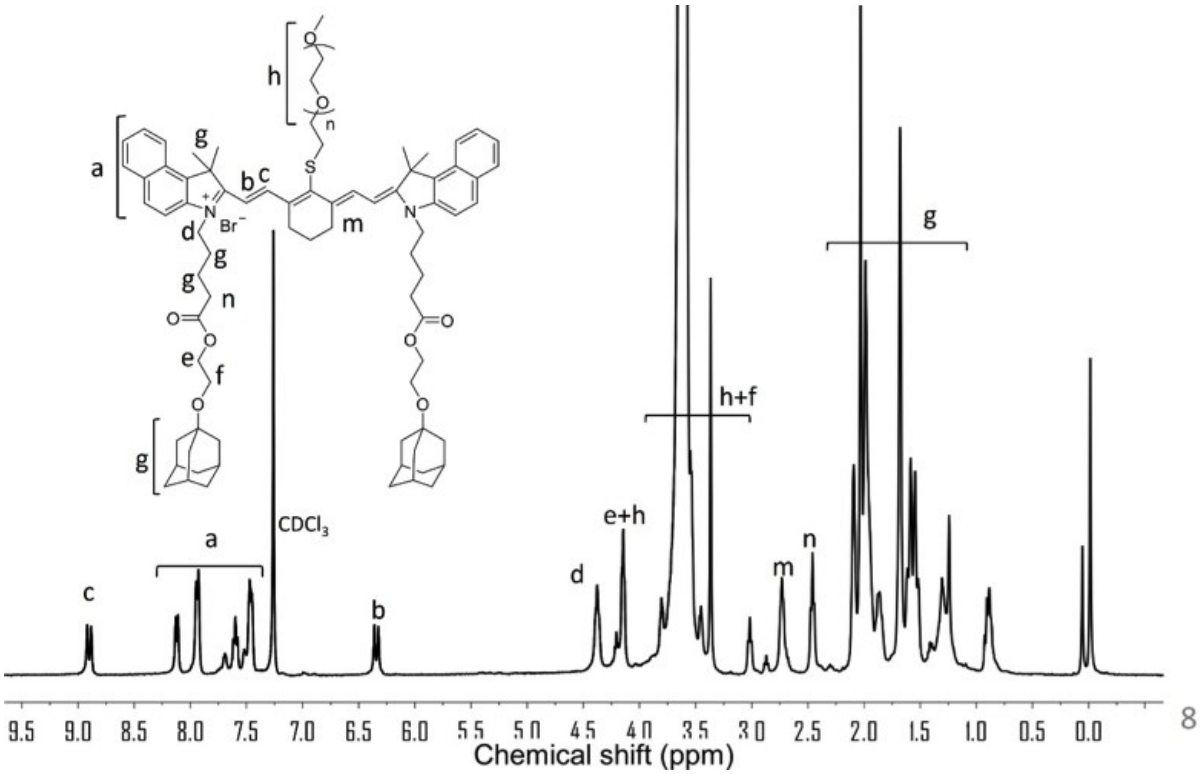


Fig. S4 ^{1}H NMR spectrum of compound Ad-dye-PEG (solvent in NMR: $CDCl_3$).

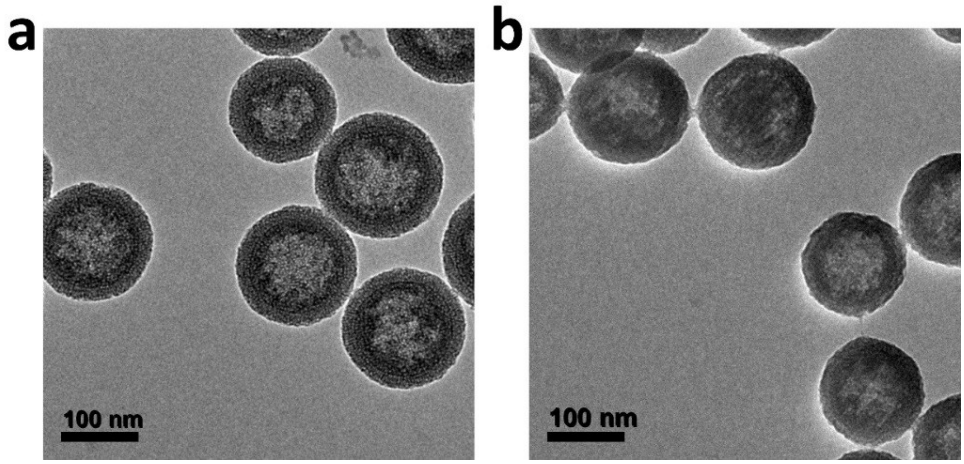


Fig. S5 Enlarged TEM images of (a) HMSNs-COOH and (b) HMSNs-Dye@DOX.

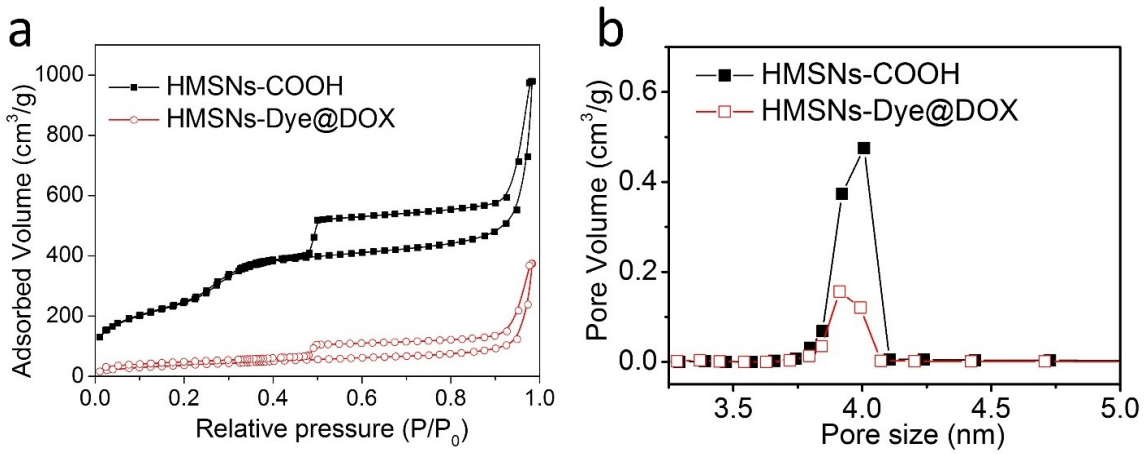


Fig. S6. (a) N_2 adsorption/desorption isotherms of HMSNs-COOH and HMSNs-Dye@DOX. (b) Pore size distributions of HMSNs-COOH and HMSNs-Dye@DOX obtained by the BJH method.

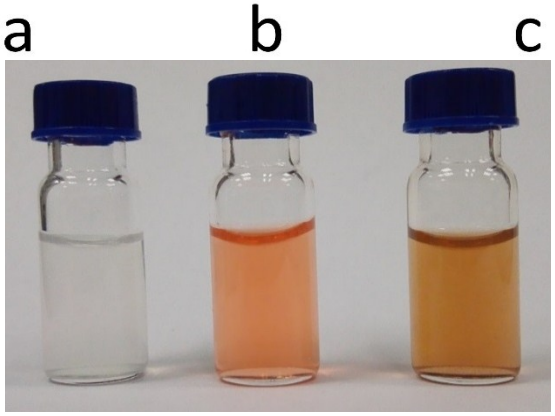


Fig. S7 Photos of (a) HMSNs-COOH, (b) HMSNs-CD@DOX, and (c) HMSNs-Dye@DOX nanoparticles dispersed in deionized water.

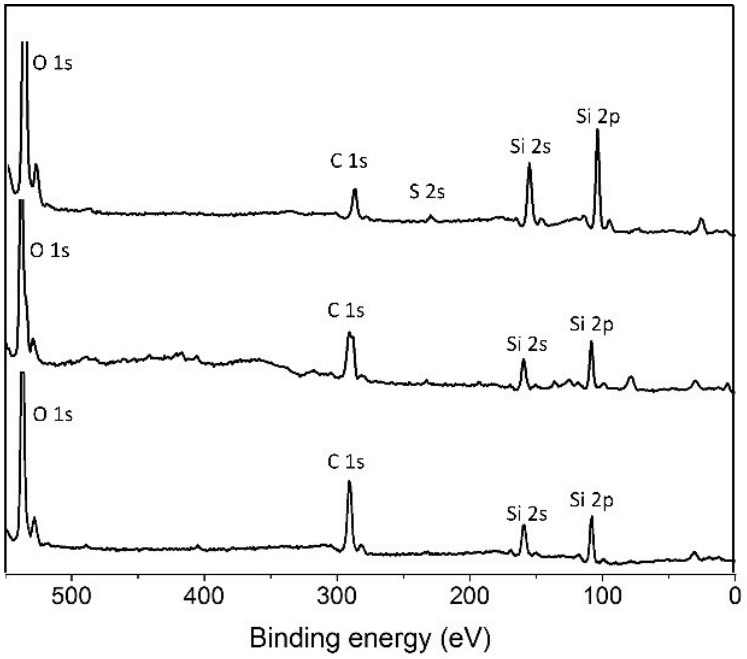


Fig. S8 XPS spectra of (a) HMSNs-COOH, (b) HMSNs-CD@DOX, and (c) HMSNs-Dye@DOX.

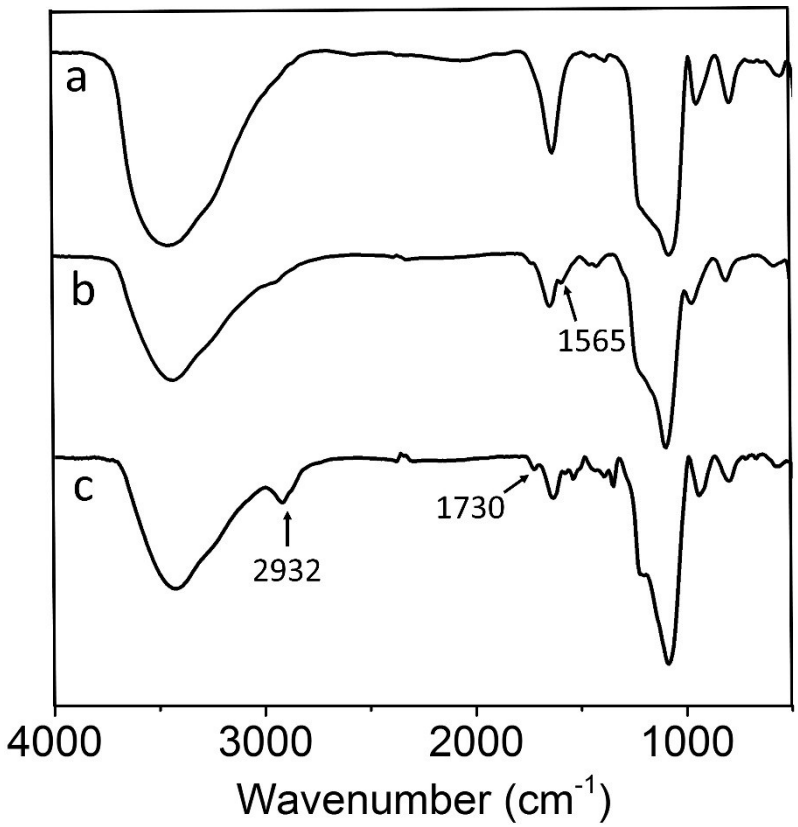


Fig. S9 FT-IR spectra of (a) HMSNs-COOH, (b) HMSNs-CD@DOX, and (C) HMSNs-Dye@DOX.

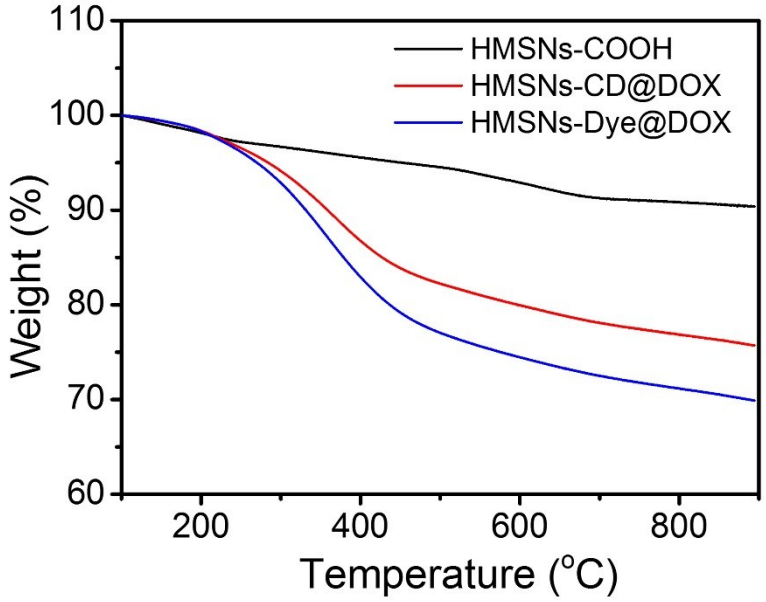


Fig. S10 TGA curves of HMSNs-COOH, HMSNs-CD@DOX, and HMSNs-Dye@DOX.

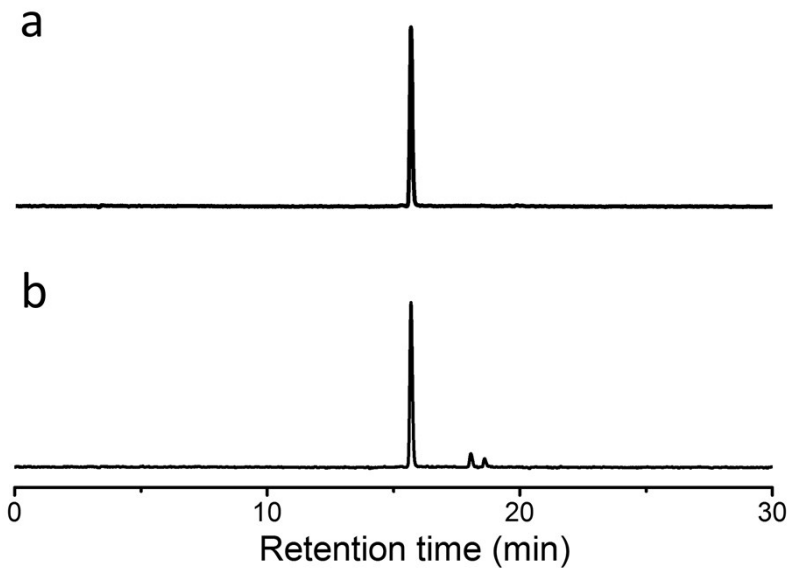


Fig. S11 HPLC elution curves of (a) free DOX, and (b) DOX extracting solution from HMSNs-Dye@DOX.

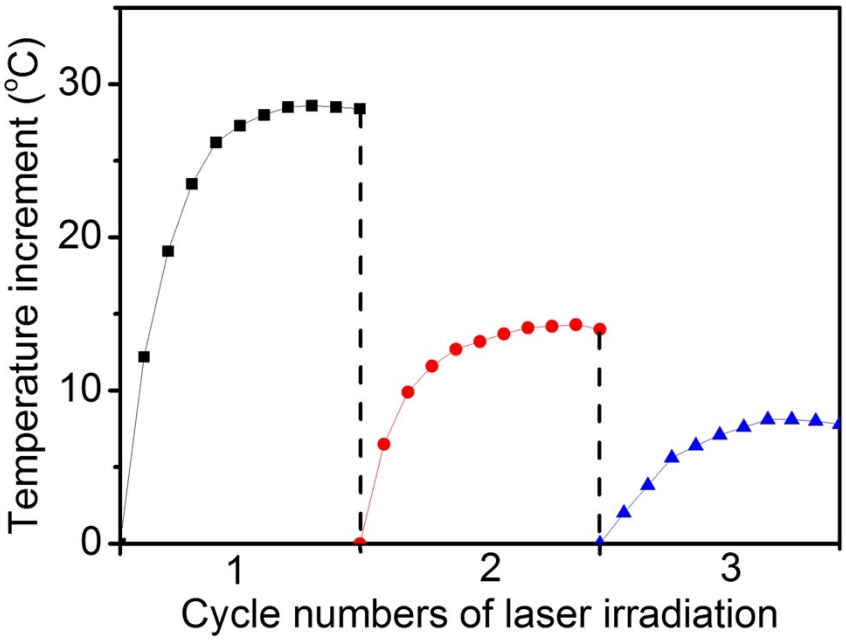


Fig. S12 Temperature increments of HMSNs-Dye@DOX (0.5 mg mL^{-1}) suspension under the 808 nm laser irradiation at the power density of 1.0 W cm^{-2} for 3 cycles (5 min of irradiation for each cycle).

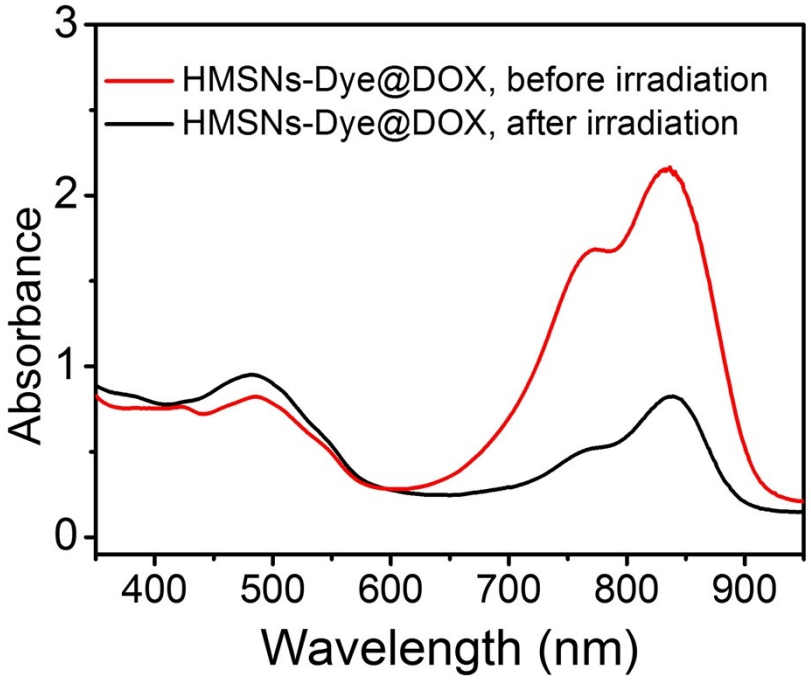


Fig. S13 UV−vis−NIR absorbance spectra of HMSNs-Dye@DOX suspension before and after 3 cycles of laser irradiation (5 min each, 808 nm, 1.0 W cm^{-2})

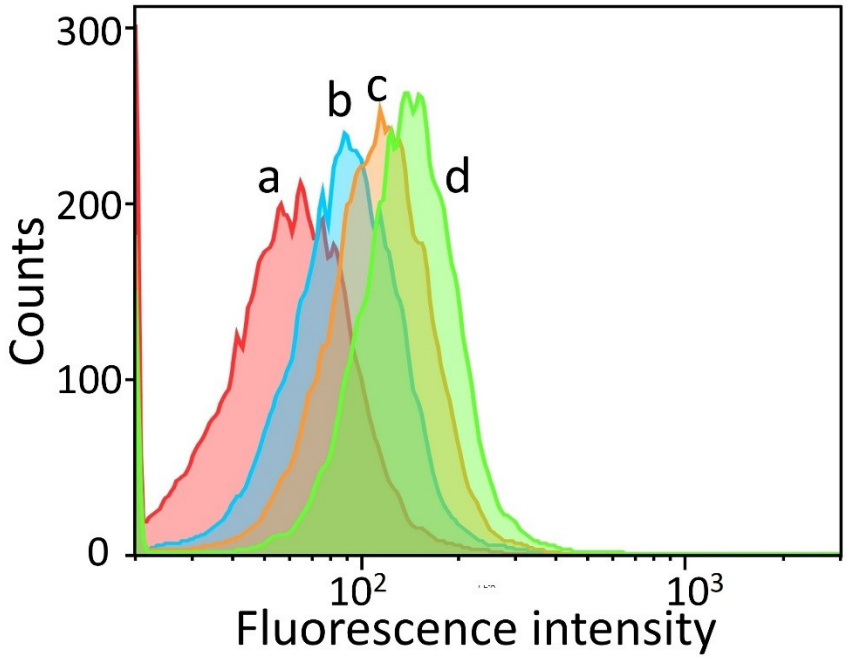


Fig. S14 Flow cytometric profiles of HeLa cells incubated with HMSNs-Dye@IR825 and free DOX for 2 h: (a) control, (b) HMSNs-Dye@IR825, (c) HMSNs-Dye@IR825, cells pretreated with 10 mM GSH-OEt, and (d) free DOX. The cells without any treatment were used as the control, and the DOX intensity was recorded through the propidium iodide (PI) channel.

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