Supporting Information for

Multipronged Design of Theranostic Nanovehicles with Endogenous and Exogenous Stimuli-responsiveness for Precise Cancer Therapy

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1. Materials

N-[5-(phenylamino)-2,4-pentadienylidene]aniline monohydrochloride, ethylene glycol, 1,2-dichlorobenzene, 2-bromoethanol, 1,1,2-trimethyl-1[H]-benz[e]indole, N,N'-Methylenebis(2-propenamide) (MBA) and N,N-diisopropylethylamine (DIEA) were obtained from J&K China Chemical Ltd. (Beijing, China). Triethylamine (TEA), hydrazine hydrate and sodium hydride were purchased from Kemiou Chemical Co., Ltd. (Tianjin, China). Pyrrole, carbon disulfide and acryloyl chloride were available from Aladdin (Shanghai, China). N-Isopropylacrylamide, sodium chloroacetate and N,N-methylenebisacrylamide were obtained from Sigma-Aldrich (China). Chloroacetic Acid and benzyl chloride were purchased from TCI (Shanghai, China). Fetal bovine serum (FBS) and DMEM culture medium were commercially available from Sigma-Aldrich (China). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT, Dojindo, Japan) was used in the cell cytotoxicity assay. Hela cells were obtained from Life Technologies (Carlsbad, CA). All chemicals and organic solvents were of analytical grade with desiccation before use.

2. Synthesis and characterization

2.1. Synthesis of MACyanine

The Synthetic route of MACyanine was shown in Figure S1. Firstly, 1,1,2-trimethyl[1H]-benz[e]indole-2-ethanol was synthesized. 2-bromoethanol (330 mg, 2.615 mmol) and 1,1,2-trimethyl-1[H]-benz[e]indole (400 mg, 1.911 mmol) were dissolved in 1,2-dichlorobenzene (5 mL). The solution was subjected for refluxing at 110 °C for 18 h. The obtained mixture was cooled to room temperature, which was precipitated in a large excess of cold ether for precipitation and was collected by filtration. The final product was dried in vacuum to yield the purple black

powder. (Yield: 570 mg, 78%).

Secondly, N-[5-(phenylamino)-2,4-pentadienylid-ene]aniline monohydrochloride (142 mg, 0.5 mmol) and DIEA (130 mg, 1.0 mmol) were dissolved in DCM (2 mL). A solution of Ac_2O (60 mg, 1.0 mmol) in DCM (250 μL) was added dropwise into the reaction mixture at 0 °C for 5 min. The resulting clear solution was stirred for another 1 h. Then the resulting solution was added dropwise to a refluxing solution of 1,1,2-trimethyl[1H]-benz[e]indole-2-ethanol (380 mg, 1.132 mmol) and sodium acetate (160 mg, 1.95 mmol) in acetonitrile and water mixture (10 mL) with volume ratio of 19:1. The mixture was refluxed for 16 h at 85 °C. The reaction mixture was cooled to room temperature, followed by addition of water and dichloromethane, the combined organic layer was dried over anhydrous Na_2SO_4, concentrated and dropped into cold ether. A green powder was obtained. (Yield: 76 mg, 21%).

Finally, the aforementioned obtained product (65 mg, 0.1 mmol) was dissolved in 1,2-dichlorobenzene solution (10 mL) with TEA (404.8 mg, 4 mmol) under nitrogen. Acryloylchloride (181 mg, 2 mmol) in 1,2-dichlorobenzene (5 mL) was added dropwise to the solution, and the mixture was stirred for another 30 min at 0 °C. The solution was further stirred at 25° C for overnight. The crude product was precipitated in an excess of hexane and filtrated, extracted with dichloromethane and water. The organic layer was dried over anhydrous Na_2SO_4. After evaporation of dichloromethane, the residue was precipitated in an excess of hexane twice to generate the resultant product, which was dried in vacuum to a light green powder. (Yield: 26 mg, 25%). ^{1}H NMR (400 mHz, TMS, DMSO-d_6, ppm, Figure S3), δ: 8.17-8.32 (m, 2H, Naphthalene), 8.05 (m, 2H, Naphthalene), 7.71 (d, 2H, Naphthalene), 7.51 (m, 2H, Naphthalene), 6.55 (m, 4H, -CH=CH-CH), 6.14 (d, 2H, CH_2=CH-), 5.91-6.03 (m, 2H, CH_2=CH-), 5.85 (d, 1H, CH_2=CH-), 4.58 (s, 8H, -CH_2-CH_2-O), 1.89 (s, 12H, -CH_3).

2.2. Synthesis of 2-[(methoxy-carbonyl)methoxy]ethyl methyl acrylate (MCME-MA)

The Synthetic route of MCMEMA was shown in Figure S2. Ethylene glycol (300 mL), chloroacetic acid (94.5 g, 1 mol) and NaOH (80 g) were combined to obtain sodium hydroxyethoxyacetate. Then H_2SO_4 and methanol were added to obtain methylester of hydroxyethoxyacetic acid, which was transformed to 2-[(methoxy-carbonyl)methoxy]ethyl methyl acrylate by using acrylic chloride. (Yield: 100.6g, 40%) ^{1}H NMR (400 mHz, TMS, $CDCl_3$, ppm, Figure S4), δ: 6.44 (d, 1H,

CH_2=CH-), 6.16 (q, 1H, CH_2=CH-), 5.85 (d, 1H, CH_2=CH-), 4.29-4.40 (m, 2H, -(=O)-O-CH_2-), 4.16 (s, 2H, -CH_2-O-), 3.79-3.87 (m, 2H, -O-CH_2-(=O)), 3.76 (s, 3H, -OCH_3)

2.3. Synthesis of RAFT chain transfer agent, benzyl 1-pyrrolecarbodithioate

Sodium hydride (0.48 g, 0.02 mol) was dissolved in dimethyl sulfoxide (20 mL) and pyrrole (1.34 g, 0.02 mol) was added dropwise to the solution, which was stirred for 30 min at 25 °C. Then carbon disulfide (1.52 g, 0.02 mol) was added. The mixture was subjected to stir at room temperature for a further 0.5 h and benzyl chloride (2.53 g, 0.02 mol) was added, followed by addition of water (20 mL) and diethyl ether (50 mL) after 1 h. The aqueous layer was extracted with diethyl ether (20 mL) twice. The combined extracts was dried over anhydrous $MgSO_4$ and evaporated to dryness under reduced pressure. The crude product was chromatographed on silica eluting with 5% ethyl acetate in petroleum spirits to obtain the yellow oil. (Yield: 2.34 g, 50%). ^{1}H NMR (400 mHz, TMS, $CDCl_3$, ppm), δ: 4.60 (s, 2H, CH_2Ph), 6.30 (m, 2H, pyrrole-H3), 7.40 (m, 5H, CH_2Ph), 7.70 (m, 2H, pyrrole-H2).

2.4. Synthesis of p(NIPAM-*co*-MACyanine-*co*-MCMEMA) copolymer

Copolymers were obtained by RAFT polymerization. NIPAM (340 mg, 3.00 mmol), MBA(38.54 mg, 0.25 mmol), MCMEMA (94 mg, 0.4995 mmol), MACyanine (18 mg, 0.025 mmol), and AIBN (1.6 mg, 0.001 mmol) was dissolved in anhydrous DMF(1.8 mL). RAFT chain transfer agent (11.65 mg, 0.05 mmol) in anhydrous methanol (0.6 mL) was added to the mixture, and the resultant mixture was then degassed, purged with N_2 and heated at 70 °C for 24 h. The reaction quenched with an ice bath. The resultant solution was dialyzed against ethanol and water (cut-off 3.5 kDa MW) for 3 days, followed by lyophilization to obtain p(NIPAM-*co*-MACyanine-*co*-MCMEMA) copolymer.

2.5. Synthesis of hydrazine functionalized p(NIPAM-*co*-MACyanine-*co*-MCME-MA) copolymer

p(NIPAM-*co*-MACyanine-*co*-MCMEMA) copolymer (81.2 mg) was dissolved in DMSO (5 mL). Hydrazine hydrate (250 μL) was added dropwise to the solution, and the mixture was stirred for 24 h at 25 °C. The resultant was dialyzed against water (cut-off 3.5 kDa MW) for 2 days, followed by lyophilization to obtain hydrazine functionalized p(NIPAM-*co*-MACyanine-*co*-MCMEMA) copolymer.

2.6. Synthesis of p(NIPAM-*co*-MACyanine-*co*-MCMEMA)-*g*-DOX copolymer

Hydrazine functionalized p(NIPAM-*co*-MACyanine-*co*-MCMEMA) copolymer

(30 mg) and DOX·HCl (4 mg) were dissolved in 5 mL anhydrous dimethylformamide, and added 5 μL phosphoric acid to the mixture, which was stirred in the dark at 25°C for 48 h. Then, 50 μL trimethylamine (TEA) was added and stirring continued for another 0.5 h. The mixture was dialyzed against weak alkaline PBS (pH 8.0) (cut-off 3.5 kDa MW) for 48 h, followed by lyophilization. The final product was obtained and confirmed by ^{1}H NMR.

2.7. Characterization of monomers and copolymers

^{1}H NMR spectra of the synthetic monomers and copolymers were measured at room temperature on a 400 MHz Bruker Avance-400 spectrometer (400 MHz, Bruker, Freemont, CA). $CDCl_3$, DMSO-d_6 and D_2O were used as solvents. Hydrodynamic size were measured at 37 °C. The zeta potential of samples was measured with Brookhaven Zeta-PALS instrument. A UV spectrophotometer (UV-2550) and fluorescence spectrophotometer (RF-5301PC) from Shimadzu were used to determine the UV absorption and fluorescence emission spectra.

2.8. Morphology of NMM-DOX nanoparticle

By utilizing scanning electron microscope (SEM) to investigate the morphology of NMM-DOX nanoparticle, which was performed on a JSM-6 700F (JEOL Ltd., Japan) instrument at an acceleration voltage of 10 kV. A drop of NMM-DOX dispersion solution was deposited onto a double-polished SiO_2 slice, followed by drying at 37 °C for 3 days. The specimens were manufactured by coating the above samples with a thin gold layer.

2.9. Photothemal effect

Photothermal effect was determined through the measurement of temperature under irradiation. NMM-DOX at the MACyanine concentrations of 0, 1, 2, 4, 9 and 17 μg/mL were placed under irradiation (785 nm, 1.5 W cm^{-2}). The temperature of solution was monitored within 5 min irradiation using a thermocouple.

2.10. Temperature-dependent size distribution

The nanoparticle size was evaluated by dynamic light scattering (Malvern Instruments, Southborough, MA). Monitor the change of the nanoparticle size at specific temperature intervals (20-50 °C).

2.11. In vitro DOX release

Release behaviors of DOX from the nanoparticle were carried out using dialysis method. 3 mL of NMM-DOX dispersion solution (polymer concentration: 2.0 mg/mL) was transformed to a dialysis bag (cut-off 3.5 kDa MW) and dialyzed against 20 mL

of release buffer PBS pH 5.4 (100 mM) or PBS pH 7.4 (100 mM) in an air contrast temperature oscillator shaker at 37 °C or 45 °C. Each 4.0 mL sample was taken at specific time intervals followed by the addition of fresh medium. The concentration of DOX were measured using UV-vis (485 nm) analysis. All measurements were performed in triplicate in the dark. For NIR-light-triggered drug release, the amounts of DOX from NMM-DOX at 4 μg mL^{-1} MACyanine were also evaluated under NIR light irradiation (1.5 W cm^{-2}, 10 min) at different release time points (0, 1, 3, 5 and 7 h) at 37 °C.

2.12. Cell culture and cellular uptake

Hela cells were cultured in culture medium (DMEM) containing 10% FBS, 1% penicillin streptomycin, and incubated at 37 °C in a 5% CO_2 atmosphere. Cells were seeded onto 35 mm glass-bottom dishes and allowed to grow until a confluence of 80%. Prior to experiments, the medium was removed and the adherent cells were washed twice with PBS to remove the remnant growth medium. The Free-DOX, NMM and NMM-DOX were then added to the plates. After 6 h of incubation, NMM-DOX further suffered from 10 min irradiation at 785 nm or not. Then, the cells were incubated for another 30 min. Afterwards, the cells were washed three times with PBS and then fixed by 75% ethanol for 20 min, which were further washed twice with PBS and the cell nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI) and incubated at 37 °C for 0.5 h. The stained cells were then washed three times with PBS buffer and used for bioimaging subsequently. Under a Nikon A1 confocal laser scanning microscopy (CLSM), DOX were excited at 488 nm and the emission was collected at 552-617 nm. The excitation of MACyanine was 638 nm and the emission was collected at 662-737 nm. No background fluorescence of cells was detected under the setting condition.

2.13. Time-lapse cell imaging

Hela cells were plated in 35 mm glass-bottom culture dishes, grown to 80% confluence for 24 h at 37 °C, and then washed twice with PBS. After NMM-DOX (125 μg/ml) were added into the dishes, living cells were cultured at 37 °C for 0.5, 1, 2, 3, 4, 5, 6 and 8 h. At specific time points, living cells were observed and analyzed using the Nikon A1 confocal laser scanning microscopy (CLSM).

2.14. Cell viability

The cytotoxicity of nanoparticle was evaluated via the MTT assay in 96-well plates. Hela cells were seeded at a density of 5×10^3 cells per well and the cells were

cultured for 24 h in culture medium (100 μL of medium per well). Then, the NMM-DOX were added into the cell culture media. After incubation for 6 h, followed by 10 min irradiation at 785 nm or not. After another 18 h of incubation, the cell viability was evaluated using MTT assay. L929 cells were also seeded at a density of 5×10^3 cells per well. NMM were added into the cell culture media. Optical density was read on a microplate reader at 570 nm in triplicate. Relative cell viability was calculated as a percentage, compared with that of untreated control.

3. Supplementary Figures

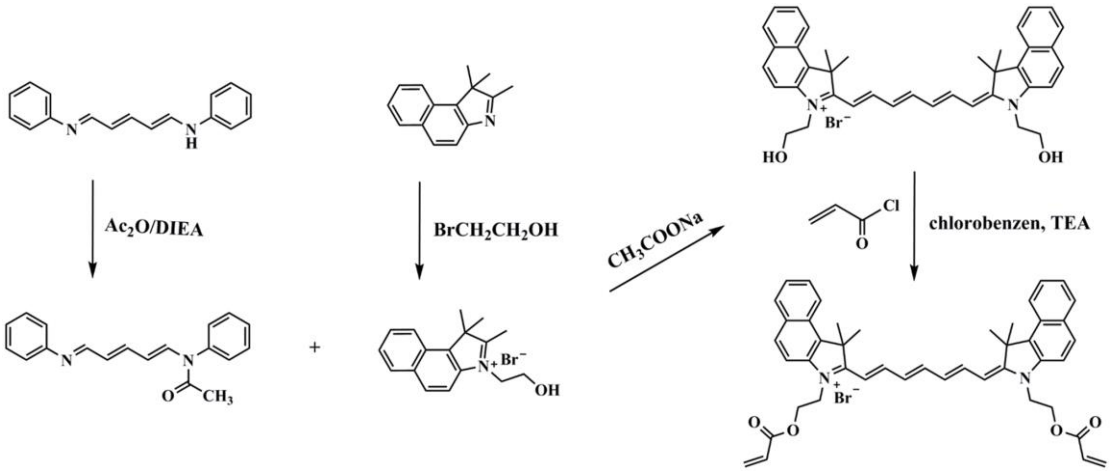

Figure S1. Synthetic route of MACyanine.

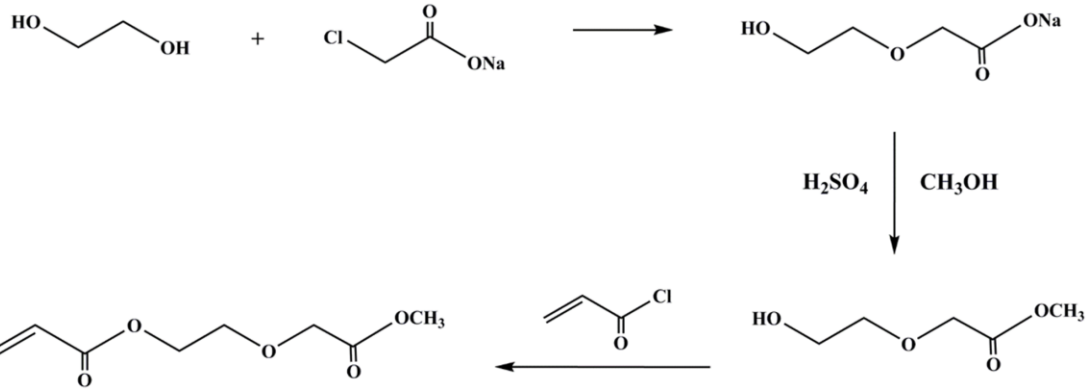

Figure S2. Synthetic route of MCMEMA.

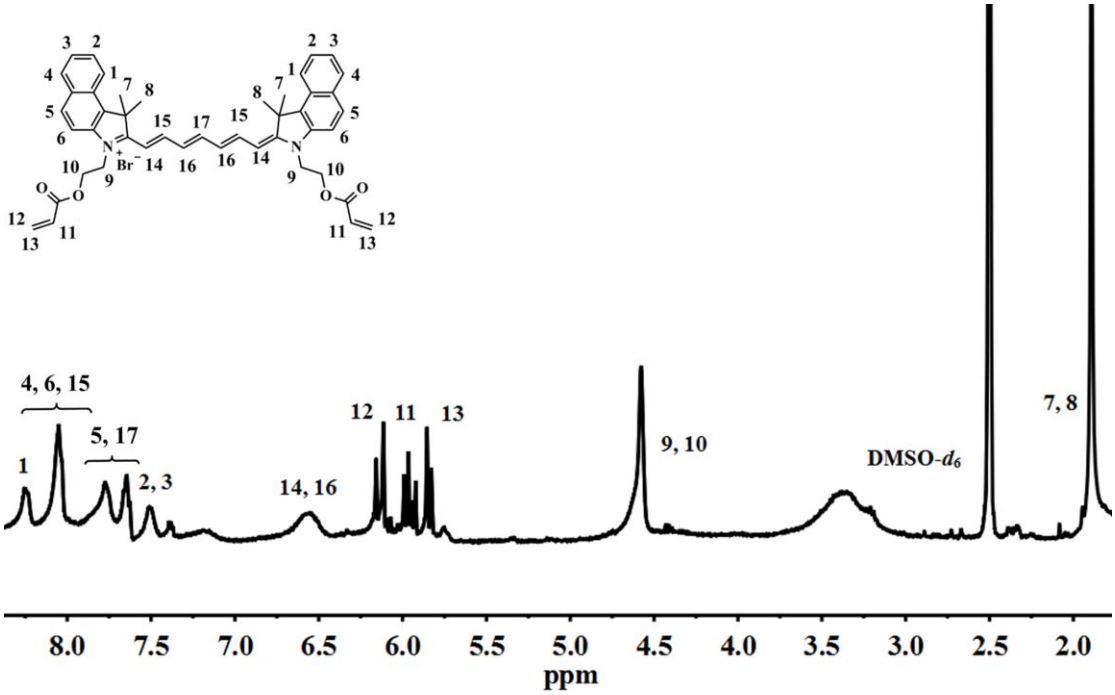

Figure S3. ^{1}H NMR spectrum of MACyanine.

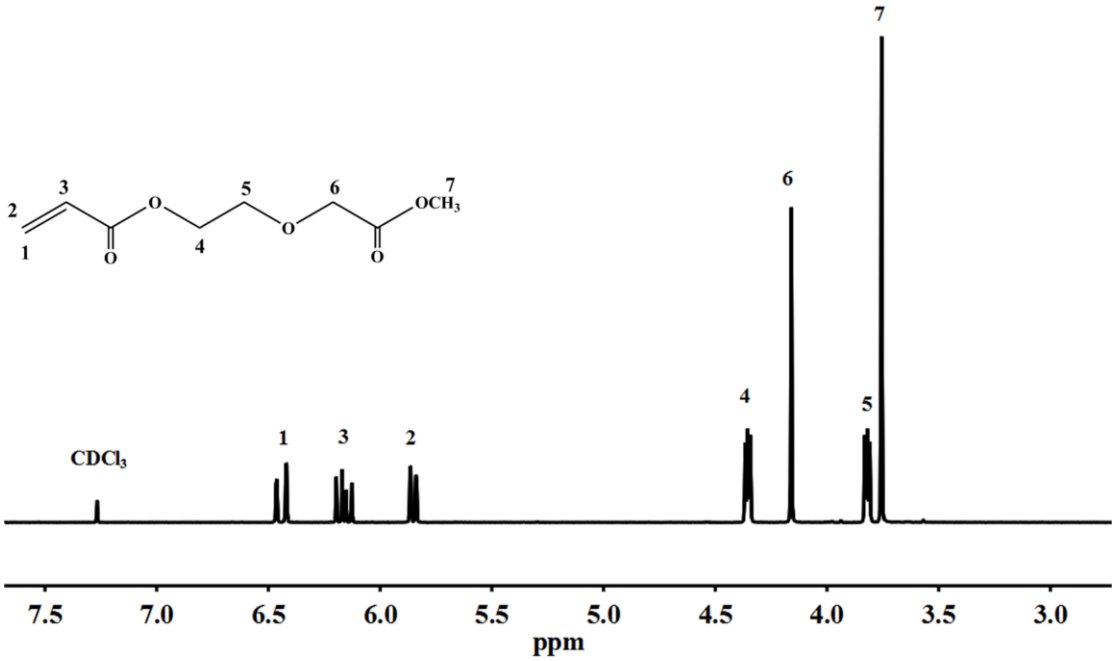

Figure S4. ^{1}H NMR spectrum of MCMEMA.

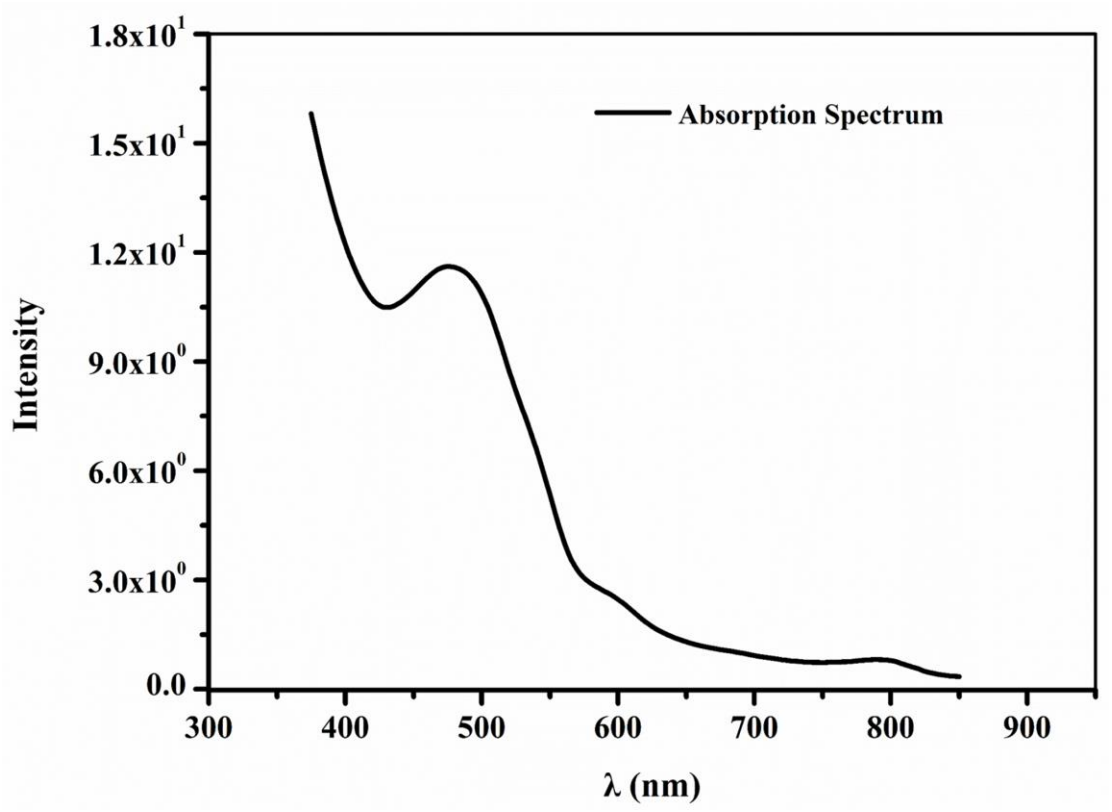

Figure S5. UV-vis spectrum of NMM-DOX.

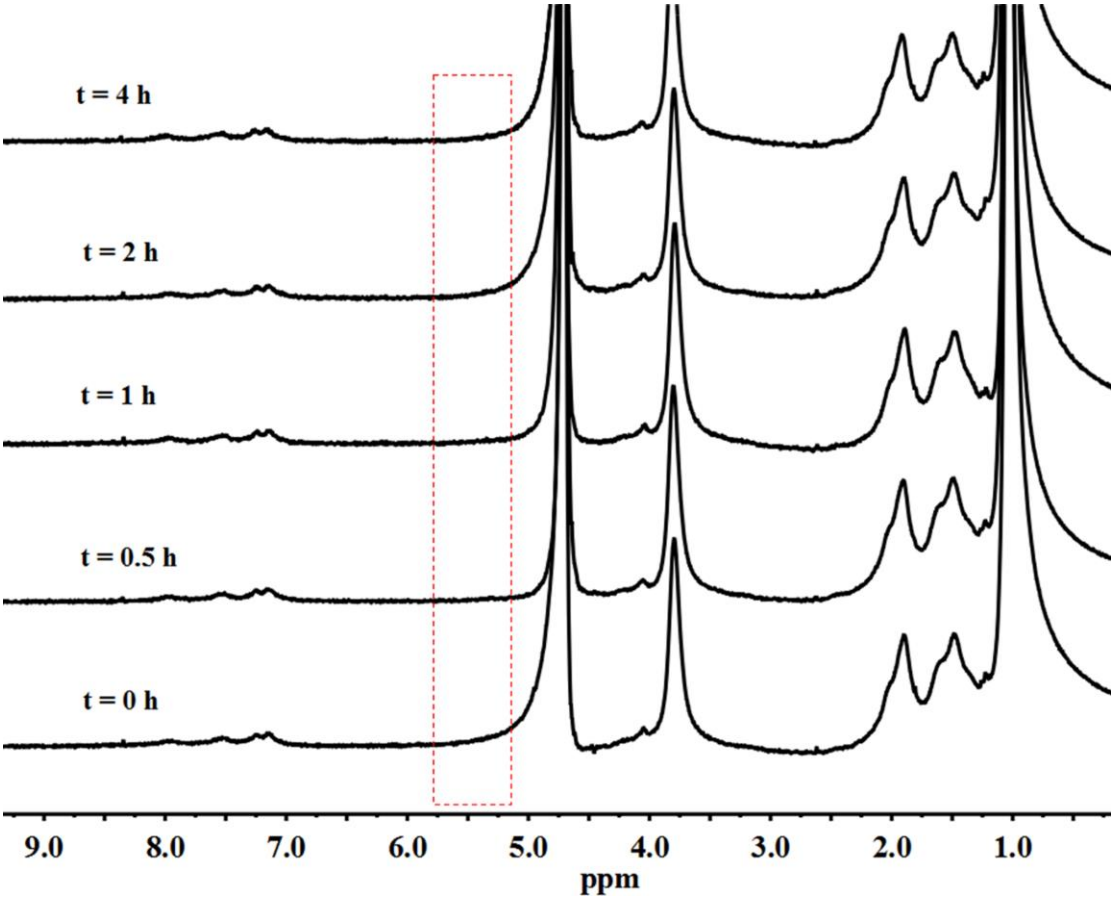

Figure S6. ^{1}H NMR spectra of NMM-DOX in D_2O at pH 7.4.

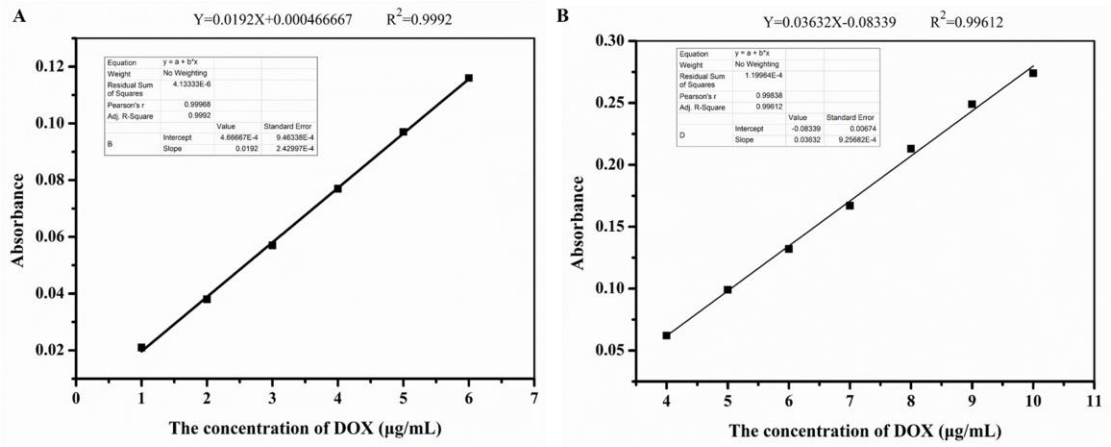

Figure S7. The calibration curve of DOX in (A) H_2O and (B) in DMSO.

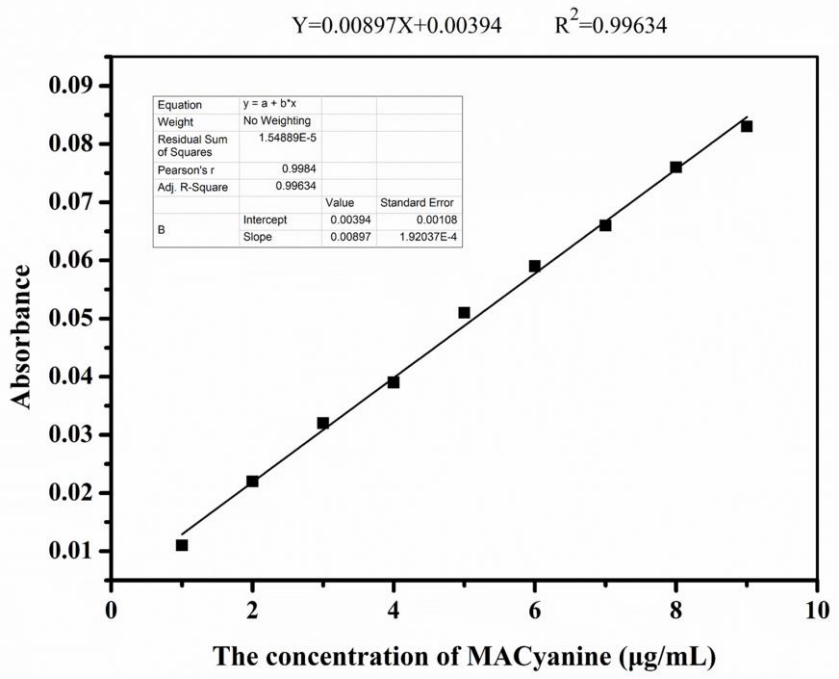

Figure S8. The calibration curve of MACyanine in DMSO.