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Integration of phospholipid-hyaluronic acid-methotrexate nanocarrier assembly and amphiphilic drug-drug conjugate for synergistic targeted delivery and combinational tumor therapy

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Materials and methods

1.1. Materials. All chemical reagents were of analytical grade and used without further purification unless otherwise stated. Deionized (DI) water was used throughout. Sodium hyaluronic acid (molecular weights = 8000 Da) was purchased from Shandong Freda Biopharm Co. Ltd. (China). 1, 2-distearoyl-sn-glycero-3-phosphoethanolamine (DSPE) was provided by Avanti Polar Lipids. Methotrexate (MTX, purify ≥ 98.0%) and folic acid (FA, purify ≥ 96.0%) were purchased from Bio Basic Inc. (Markham, Ontario, Canada). 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), N, N'-dicyclohexylcarbodiimide (DCC), N-hydroxysuccinimide (NHS), and 4-dimethylaminopyridine (DMAP) were purchased from Sigma-Aldrich Co., Ltd. (St. Louis, MO, USA). 1'-dioctadecyl-3, 3, 3', 3'-tetramethylindotricarbocyanine iodide (DiR), 1, 1'-dioctadecyl-3, 3, 3', 3'-tetramethylindodicarbocyanine perchlorate (DiD), YOYO-3, and LysoTracker Green were obtained from Molecular Probes Inc. (Eugene, OR, USA). 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) was purchased from Amresco (Solon, OH, USA). Dulbecco's modified Eagle's medium (DMEM) and 0.25% trypsin were purchased from M&C Gene Technology (Beijing, China). Penicillin-streptomycin and trypsin-ethylenediamine tetra-acetic acid (EDTA) were purchased from Hyclone (USA). Fetal bovine serum (FBS) was purchased from Gibco Life Technologies (AG, Switzerland).

1.2. Synthesis of HCPT-MTX drug-drug conjugate. To synthesize HCPT-MTX conjugate, MTX (454.5 mg, 1.0 mmol), DCC (247.4 mg, 1.2 mmol), and DMAP (146.6 mg, 1.2 mmol) were added to 15 mL of anhydrous dimethylformamide (DMF). After stirring for 30 min at 0°C under argon, HCPT (400.8 mg, 1.1 mmol) dissolved in DMF solution was added dropwise and the mixture was stirred for 48 h at 25°C in darkness. After that, the reaction mixture was filtered to remove the white solid (dicyclohexylurea, DCU) and the filtrate was dried under vacuum. Afterwards, the residue was precipitated in excess cold ethyl acetate, and DCM was used to wash the precipitate three times to remove free HCPT. The crude product was further purified by column chromatograph using the mixture of ethyl acetate and methanol (10:1) as

the eluent. The product was collected and then dried under vacuum to obtain a yellow solid. Yield: ~62%.

1.3. Synthesis of DSPE-HA-MTX amphiphilic polymer prodrug. The DSPE-HA amphiphilic polymer was synthesized via amidation reaction between amino groups of DSPE and carboxyl groups of HA. Briefly, HA (2.40 g, 0.3 mmol), EDC·HCl (0.29 g, 1.5 mmol), and NHS (0.17 g, 1.5 mmol) were dissolved in anhydrous formamide and gently stirred for 30 min at 0°C under argon to activate the carboxylic groups of HA. DSPE (0.45 g, 0.6 mmol) was added in 40 mL of *tert*-butanol/deionized water (10%, v/v) containing triethylamine (0.06 mg, 0.6 mmol) and stirred at 60 °C until the solution became transparent. The obtained DSPE solution was slowly dropped into the above HA solution. The reaction was stirred at 60°C under argon for another 24 h. Subsequently, the reaction solution was dialyzed (MWCO = 1000 Da) against ethanol for 24 h followed by dialysis against distilled water for 48 h. Finally, the solution was filtered to remove other impurities through 0.45 μm millipore filter (Millipore, Bedford, MA), and then lyophilized at -80℃ to obtain DSPE-HA. Yield: ~90%.

The DSPE-HA-MTX amphiphilic polymer prodrug was synthesized via esterification between hydroxyl groups of DSPE-HA and carboxyl groups of MTX. Specially, MTX (90.8 mg, 0.2 mmol), EDC·HCl (57.5 mg, 0.3 mmol), and DMAP (36.7 mg, 0.3 mmol) were dissolved in 10 mL of anhydrous dimethylsulfoxide (DMSO) and stirred for 30 min at 0°C under argon to activate the carboxylic groups of MTX. Then the DSPE-HA conjugate in DMSO/water was slowly injected into the above MTX solution and stirred for 48 h at ambient temperature away from light. The resultant mixture was dialyzed against excess amount of $NaHCO_3$-Na_2CO_3 buffer solution (pH = 10) followed by dialysis against deionized (DI) water. Finally, the dialyzed solution was lyophilized at -80°C for 24 h. Yield: ~75%.

1.4. Characterization of HCPT-MTX drug-drug conjugate and DSPE-HA-MTX amphiphilic polymer prodrug. The ^1H NMR nuclear magnetic resonance (NMR) spectrum was determined on a Bruker AV400 MHz NMR spectrometer (Bruker, Billerica, MA, USA). The Fourier transform infrared spectroscopy (FTIR) spectrum

was performed on a Bruker IFS-55 infrared spectrometer (Bruker, Switzerland). The X-ray diffraction (XRD) spectrum was recorded by an X-ray diffractometer (Phillips X'pert Pro Super, Panalytical, Almelo, Netherlands). The ultraviolet-visible (UV-vis) absorption spectrum was recorded with a Perkin Elmer Lambda 750 UV-vis-near-infrared spectrophotometer (Perkin-Elmer, Norwalk CT). The matrix-assisted laser desorption/ionization time-of-flight mass (MALDI-TOF-MS) spectrum was acquired by a Bruker Reflex III MALDI-TOF mass spectrometer (Bruker Daltonics, Bremen, Germany). The fluorescence spectrum was recorded with a FluoroMax-4 Spectrofluorometer (HORIBA Jobin Yvon Inc., NY, USA).

1.5. Preparation of HCPT-MTX-loaded DSPE-HA-MTX (HCPT-MTX@DHM) nanoparticles. The HCPT-MTX-loaded DHM nanoparticles (HCPT-MTX@DHM) were prepared via an anti-solvent precipitation augmented by ultrasonication. Briefly, 100 mg of DSPE-HA-MTX polymer prodrug was dispersed in 40 mL of distilled water. Then, 20.8 mg of HCPT-MTX in 8 mL of anhydrous dimethyl sulphoxide (DMSO) was added dropwise into 40 mL of DSPE-HA-MTX dispersion under ultrasonication at 200 W for 10 min with 10 s intervals in an ice bath using a probetype ultrasonicator. After that, the obtained nanoparticles were dialyzed against DI water with a dialysis bag (MWCO = 3500 Da) followed by centrifugation at 4000 rpm for 10 min to remove the unincorporated HCPT-MTX. The supernatant was filtrated through polycarbonate membranes with 0.45 μm pore size, and the filtrate was stored at 4°C for use. The HCPT-MTX-loaded DSPE-HA (HCPT-MTX@DH) nanoparticles were prepared with the same procedure except that DSPE-HA-MTX was replaced by DSPE-HA. In addition, the concentration of HA-MTX in HCPT-MTX@DHM nanoparticles was the same as the concentration of HA in HCPT-MTX@DH nanoparticles.

The DiD dye-loaded DSPE-HA or DSPE-HA-MTX nanoparticles were prepared with the same procedure except that drug was replaced by DiD dye (excitation wavelength: ~630 nm), and were used for *in vitro* cell imaging. The DiR dye-loaded DSPE-HA or DSPE-HA-MTX nanoparticles were prepared with the same procedure except that drug was replaced by DiR dye (excitation wavelength: ~745 nm), and

were used for *in vivo* animal imaging.

1.6. Characterization of HCPT-MTX@DHM nanoparticles. The hydrodynamic particle size, polydispersity index (PDI), and surface charge were determined by dynamic light scattering (DLS) using a Malvern Zetasizer Nano-ZS (Malvern Instruments, Worcestershire, U. K.). The zeta potential was determined by electrophoretic light scattering (ELS) using a same equipment. The morphology was visualized by scanning electron microscopy (SEM, LEO1530VP, Carl Zeiss AG, Germany) operated at an acceleration voltage of 20 kV and transmission electron microscopy (TEM, JEM2100, JEOL, Japan) operated at an acceleration voltage of 200 kV.

1.7. Drug encapsulation efficiency and drug-loading capacity. To quantify the amount of HCPT (encapsulated) or MTX (encapsulated and conjugated) loaded within HCPT-MTX@DHM nanoparticles, the lyophilized HCPT-MTX@DHM nanoparticles were dissolved in anhydrous DMSO by ultrasonication. After the filtration by 0.45 and 0.22 μm filter membrane, a part of the filtrate was analyzed for the determination of drug-loading content through a high performance liquid chromatography (HPLC, Agilent Technologies, Waldbronn, Germany) with a UV-vis detector. [1, 2] The encapsulation efficiency of HCPT-MTX@DHM nanoparticles was calculated as (weight of loaded HCPT-MTX drug)/ (weight of total HCPT-MTX drug) × 100%. The drug-loading capacity of HCPT-MTX@DHM nanoparticles was calculated as (weight of loaded drug)/ (weight of drug-loaded nanoparticles) × 100%. The HCPT-MTX@DH nanoparticles were used as a control.

1.8. *In vitro* stability. For the storage stability study, HCPT-MTX@DHM nanoparticles were stored at 4°C over 14 days, and the particle size and zeta potential were measured at 0, 4, 7, 10, and 14 days. For the stability study under physiological conditions, HCPT-MTX@DHM nanoparticles were suspended in phosphate buffer saline (PBS) and 10% FBS at 37 °C over 48 h, and the particle size was measured at 0, 12, 24, and 48 h. The HCPT-MTX@DH nanoparticles were used as a control.

1.9. *In vitro* drug release. The dual-drug release characteristics of HCPT-MTX@DH or HCPT-MTX@DHM nanoparticles were determined by a dialysis method. Briefly,

1 mL of HCPT-MTX@DH or HCPT-MTX@DHM (0.5 mg/ml) was placed in a dialysis bag (molecular weight cut-off of 1000 Da) with/without the addition of esterase (30 U/mL, Aldrich chemicals) and then immersed into 49 mL of buffer saline (pH 7.4 and pH 5.5) in an incubator shaker (100 r/min) at 37°C. At predetermined time intervals, 1 mL of the release medium were withdrawn for analysis and an equivalent volume of the release medium was replenished. The amount of HCPT and MTX released was determined by fluorescence method and high-performance liquid chromatography (HPLC) method, respectively. The conditions of HPLC method were as follows: stationary phase, Hypersil ODS column (250 mm × 4.6 mm, 5 μm); temperature, 25°C; elution flow rate, 1.0 mL/min; detection wavelength, 303 nm; mobile phase, HPLC grade acetonitrile/0.04 M potassium dihydrogen phosphate (pH 4.5) (12/88, v/v).[1]

The cumulative release amount of HCPT and MTX from HCPT-MTX@DH or HCPT-MTX@DHM was calculated using eqn (1).

$$\text{Cumulative release}(\%) = \frac{1\times \sum_{i=1}^{n-1} \text{Ci} + 50\times \text{Cn}}{\text{weight of drug in nanosystems}} \times 100\% \quad (1)$$

where Ci means the concentration of HCPT or MTX drug in dialysate at i time.

1.10. Cell culture. MCF-7 human breast cancer cell lines and 4T1 murine breast cancer cell lines with high expression of both CD44 and folate receptors were originally obtained from American Type Culture Collection (ATCC). MCF-7 and 4T1 cells were cultured in the Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS), 100 IU/mL of penicillin, and 100 μg/mL of streptomycin in an incubator under an atmosphere of 5% CO_2 at 37°C. Murine fibroblast NIH-3T3 cells (normal cell lines) with low expression of both CD44 and folate receptors were used as a control.

1.11. *In vitro* cellular uptake. MCF-7 cells (1×10^5 cells) were seeded in a 6-well plate and cultured at 37°C for 24 h in a humidified atmosphere with 5% CO_2. The cells were incubated with DiD-labeled DH or DHM nanoparticles for the same time periods at 37°C. After that, the culture medium was removed, and the cells were

washed by PBS thrice and fixed with 4% of paraformaldehyde for 15 min. Finally, the cells were stained with YOYO-3 for 10 min. All of cell images were immediately observed using Leica TCS SP5 confocal laser scanning microscopy (CLSM, Leica Microsystems, Germany). In addition, to investigate the folate/HA receptor-mediated internalization of DH or DHM nanoparticles, MCF-7 cells was incubated with the excess of free FA, HA, or FA/HA for 2 h prior to the addition of DiD-labeled DH or DHM nanoparticles.

1.12. Flow cytometry analysis. Quantitative cellular uptake of DiD-labeled DH or DHM nanoparticles was measured by flow cytometry analysis. MCF-7 and 4T1 cells were seeded at a density of 2×10^5 cells per well into 6-well plates and further cultured for 24 h. The cells were incubated with DiD-labeled DH or DHM nanoparticles at an equivalent DiD concentration for the same time periods. Then, the culture medium was removed and the cells were washed with PBS for three times. After that, the cells were detached with trypsin/EDTA, suspended in PBS with 10% FBS, harvested by centrifugation at 2000 rpm for 5 min at 4°C, and resuspended in fluorescence-activated cell sorting (FACS) buffer. The data were detected on a FACSCalibur flow cytometer (Becton Dickinson, USA) and the data were analyzed using Cell Quest software.

1.13. *In vitro* cytotoxicity assay. *In vitro* cytotoxicity against MCF-7, 4T1, and NIH-3T3 cells of nanosystems was evaluated by an MTT assay (Sigma-Aldirich, USA). Briefly, three types of cells were seeded in 96-well plates at a density of 8×10^3 cells per well. After incubation for 48 h, the culture medium was replaced with fresh DMEM. Afterwards, DH nanocarriers, DHM nanocarriers, free HCPT, free MTX, free HCPT/MTX mixture, free HCPT-MTX conjugate, HCPT-MTX@DH nanoparticles, and HCPT-MTX@DHM nanoparticles at an equivalent HCPT concentration were added into the wells for 36 h incubation. Afterwards, 10 μL of MTT solution (5 mg/mL) was added to each well and incubated for another 4 h. The medium was removed and 150 μL of DMSO was added into each well to dissolve the formazan. The absorbance of each plate was determined using a microplate reader at a test wavelength of 570 nm to obtain the optical density (OD) value. The relative cell

viability was calculated as follows. Cell viability (%) = $OD_{treated}/OD_{control} \times 100\%$, where $OD_{treated}$ represents the optical density of the cells treated by different drug formulations, and $OD_{control}$ represents the optical density of the cells by culture mediums.

1.14. *In vitro* hemolytic effect. Approximately 2 mL of blood was taken from the orbital venous plexus of a male Sprague-Dawley rat and centrifuged at 3000 rpm for 5 min. The plasma supernatant was removed, and the erythrocytes were resuspended in 0.9% NaCl solution. The DH or DHM nanocarriers were incubated with the 2% (w/v) red blood cells suspension at 37°C for 4 h with different concentrations. Then the red blood cells were removed by centrifugation, 150 μL of the supernatant was pipetted into a 96-well plate, and the absorbance was measured at 540 nm using a microplate reader. The results were expressed as percentage of hemolysis with the assumption that Triton X-100 caused 100% hemolysis and 0.9% NaCl solution caused 0% hemolysis.

1.15. Animal and tumor xenograft models. Female BALB/c nude mice (20± 2 g, 4-6 weeks old) and male Sprague-Dawley rats (200 ± 20 g) were purchased from Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China). All the animals were acclimatized in a laminar flow room at controlled temperature of 25 ± 2°C, relative humidity of 50-60%, and 12 h light-dark cycles with standard diet ad libitum for 1 week prior to experiment. All experimental procedures comply with the Guidelines and Policies for Ethical and Regulatory for Animal Experiments as approved by the Institutional Animal Care and Use Committee of Xiamen University.

1.16. *In vivo* fluorescence imaging and photoacoustic (PA) imaging. To determine the *in vivo* biodistribution, the BALB/c nude mice bearing MCF-7 tumor model was established. Briefly, 2×10^6 MCF-7 cells were inoculated subcutaneously into the right armpit of the nude mice. When the tumor exceeded 200 mm^3, the MCF-7 tumor-bearing mice were randomly divided into 2 groups, and intravenously injected with DiR-doped HCPT-MTX@DH and HCPT-MTX@DHM nanoparticles at an equivalent DiR concentration, respectively. The *in vivo* distribution and tumor accumulation of DiR-doped HCPT-MTX@DH and HCPT-MTX@DHM

nanoparticles were recorded at the pre-scheduled post-injection time intervals using an *in vivo* imaging system (IVIS Spectrum 200, Perkin-Elmer Co., MA, USA). Mice were sacrificed at 36 h after post-injection, and the tissues were excised and observed by the imaging system. The fluorescence intensity of different tissues was quantified as the sum of all the detected photon counts within the region of interest (ROI) in the unit of [photo/cm^2/s].

In vivo PA imaging was obtained with an Endra Life Sciences Unveils Nexus 128 Photoacoustic Scanner (Ann Arbor, USA), which produced a 3D photoacoustic image by a hemispherical ultrasonic detector with 128 identical ultrasonic transducers spirally installed on the surface. PA signals at the tumor sites were recorded using a PA system after the intravenous injection of 200 μL of DiR-doped HCPT-MTX@DH and HCPT-MTX@DHM nanoparticles at equivalent DiR concentration into MCF-7 tumor-bearing mice via the tail vein.

1.17. *In vivo* antitumor efficiency. *In vivo* antitumor activity was evaluated using MCF-7 tumor-bearing BALB/c nude mice models. Briefly, 0.2 mL of cell suspension containing 2× 10^6 MCF-7 cells was inoculated subcutaneously into the right armpit of the BALB/c nude mice (4-6 weeks, 18-22 g). When the tumor reached to around 200 mm^3, the tumor-bearing mice were randomly divided into five groups. Mice were intravenously administrated with 0.2 mL of 0.9% NaCl (control group), free HCPT/MTX (8.18 mg/kg), free HCPT-MTX (8.00 mg/kg), HCPT-MTX@DH (8.00 mg/kg of HCPT-MTX), or HCPT-MTX@DHM (8.00 mg/kg of HCPT-MTX) via the tail vein every 3 days for 4 times. The tumor size and body weight of the mice were monitored during treatment. Tumor size was measured every 2 or 3 days by a caliper, and tumor volume (V) was calculated by the following formula: V (mm^3) = 0.5 × L × W^2, where L and W represent the largest diameter and the smallest diameter, respectively.

On the 22th day, all animals were sacrificed, and the tumors and major organs (i. e., liver, heart, spleen, kidney, and lung) were excised, weighted, fixed with 4% paraformaldehyde overnight, and embedded in paraffin. After that, the 5.0 μm thick slices of tumor and major tissues were stained by hematoxylin and eosin (H&E)

according to the manufacturer's protocol, and the stained tumor sections were observed by an optical microscopy (DM5500B, Leica).

1.18. Statistical analysis. All quantitative data were described as the mean values plus/minus standard deviation (mean ± SD) unless otherwise noted. Statistical significance was tested using Student's *t*-test or SPSS 19.0 software. A value of $P < 0.05$ was considered as statistically significant. A value of $P < 0.01$ was considered as highly statistically significant. A value of $P < 0.001$ was considered as very highly statistically significant.

References

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2. Y. Li, J. Lin, Y. Huang, Y. Li, X. Yang, H. Wu, S. Wu, L. Xie, L. Dai and Z. Hou, *ACS Appl. Mater. Interfaces*, 2015, **7**, 25553-25559.

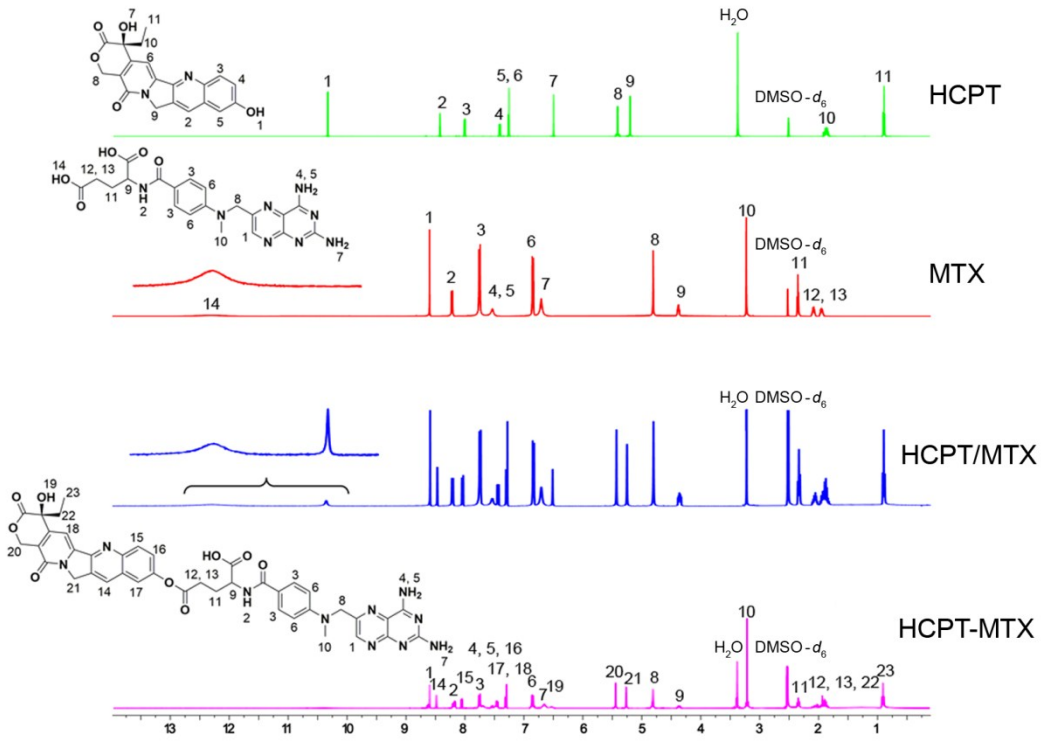

Figure S1. ^{1}H NMR spectra of HCPT, MTX, HCPT/MTX mixture, and HCPT-MTX drug-drug conjugate (DMSO-d_6 as solvent).

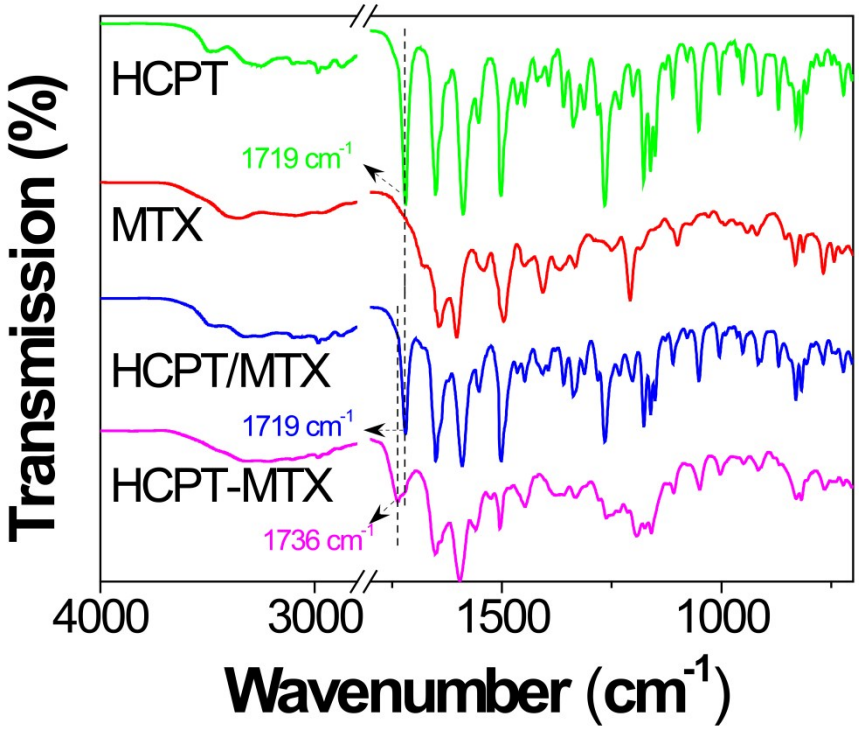

Figure S2. FTIR spectra of HCPT, MTX, HCPT/MTX mixture, and HCPT-MTX drug-drug conjugate.

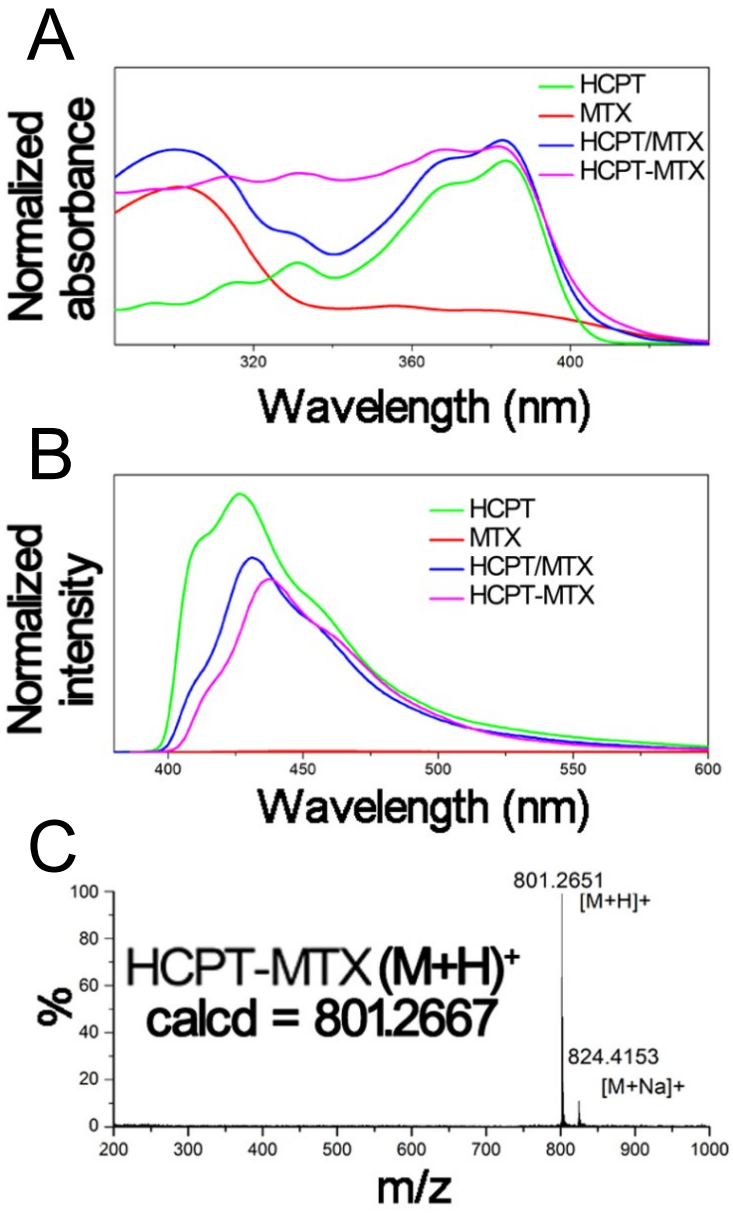

Figure S3. (A) UV-vis absorption and (B) fluorescence emission spectra of HCPT, MTX, HCPT/MTX mixture, and HCPT-MTX drug-drug conjugate (DMSO as solvent). (C) MS spectrum of HCPT-MTX drug-drug conjugate. The molecular weight of HCPT-MTX (*m/z*, $[M-H]^+$) is 801.2651, which is well in line with the calculated value (*m/z*, $[M-H]^+$, 801.2667).

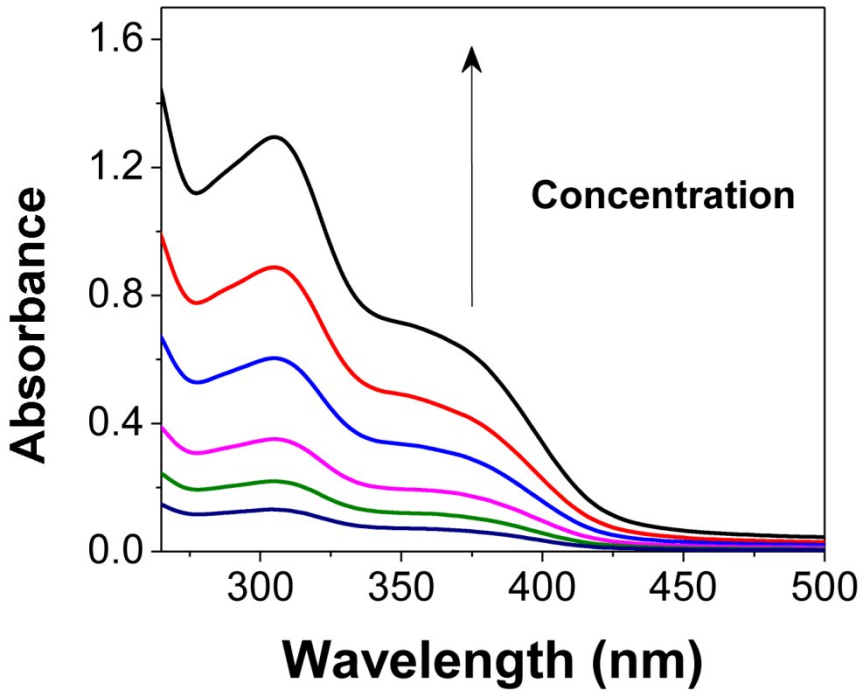

Figure S4. UV-vis absorbance spectra of DHM nanoparticles (water as solvent).

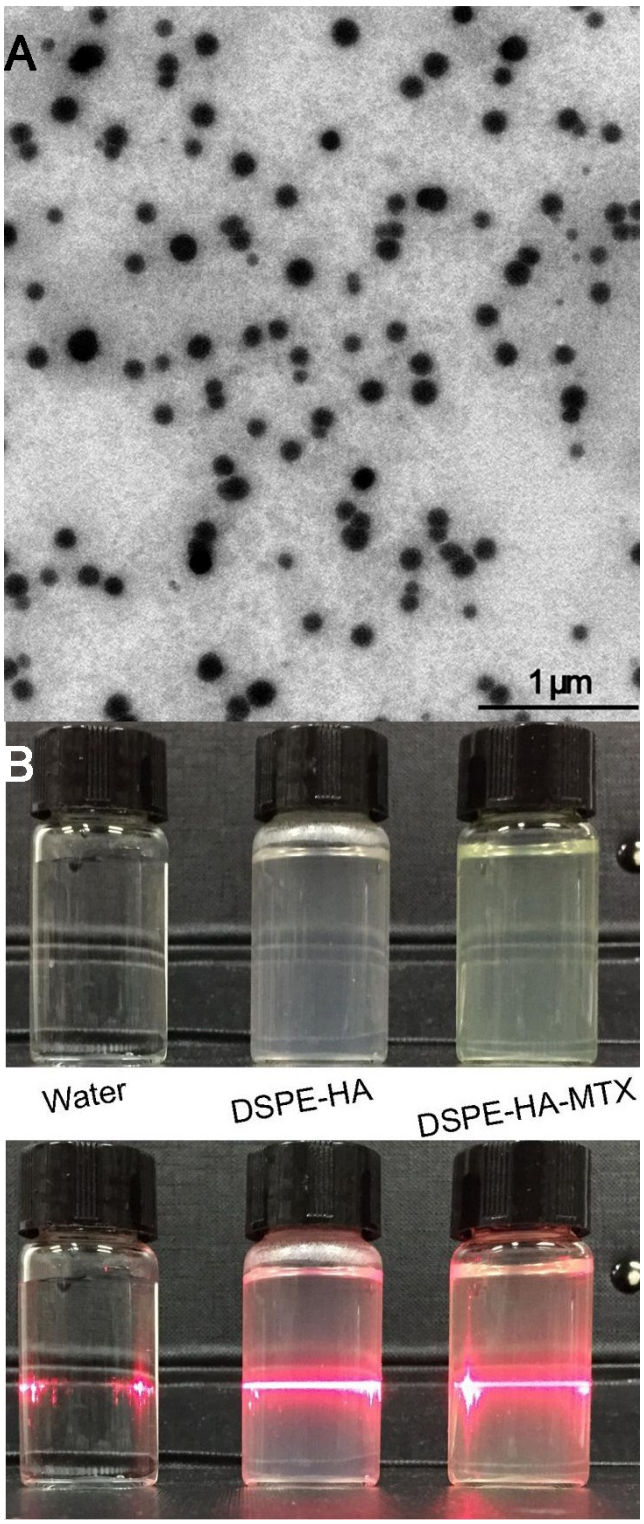

Figure S5. (A) Enlarged TEM image of DHM nanoparticles. (B) Photographs and tyndall effect of DH and DHM nanoparticles dispersion.

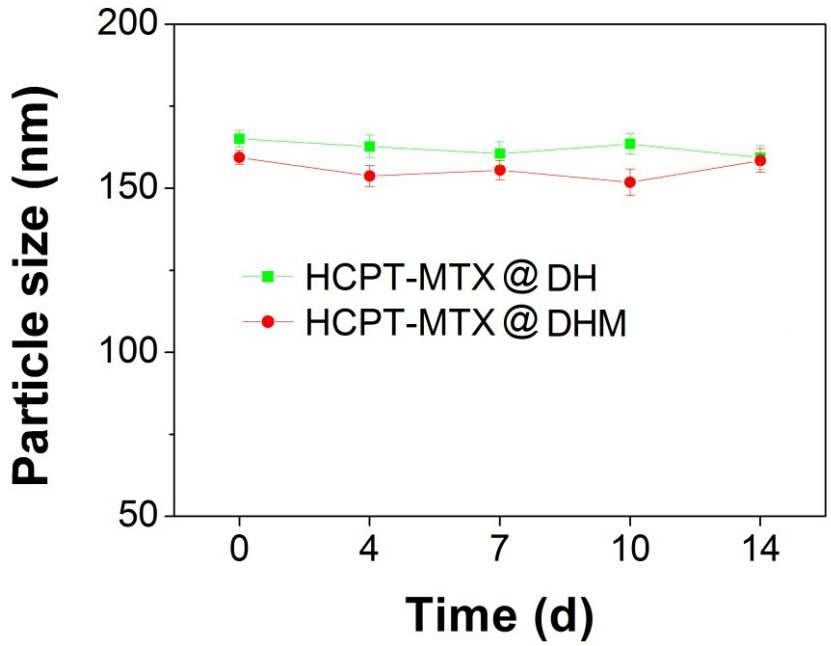

Figure S6. The hydrodynamic particle size of HCPT-MTX@DH and HCPT-MTX@DHM nanoparticles during the storage at 4 °C for 14 days. At different time intervals (0, 4, 7, 10, and 14 d), the hydrodynamic particle size was determined by DLS. Error bars indicate SD (n = 3).

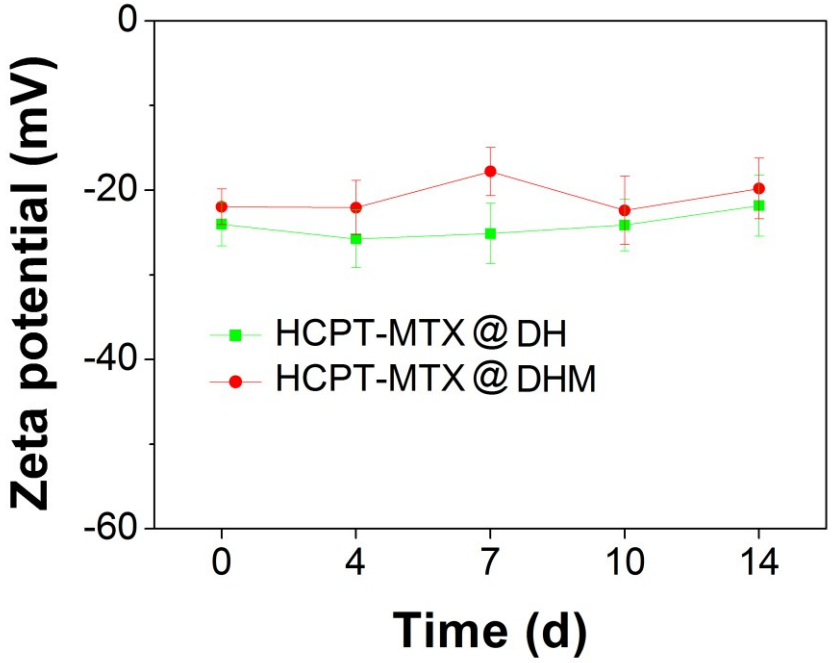

Figure S7. The zeta potential of HCPT-MTX@DH and HCPT-MTX@DHM nanoparticles during the storage at 4 °C for 14 days. At different time intervals (0, 4, 7,

10, and 14 d), the zeta potential was determined by DLS. Error bars indicate SD (n = 3).

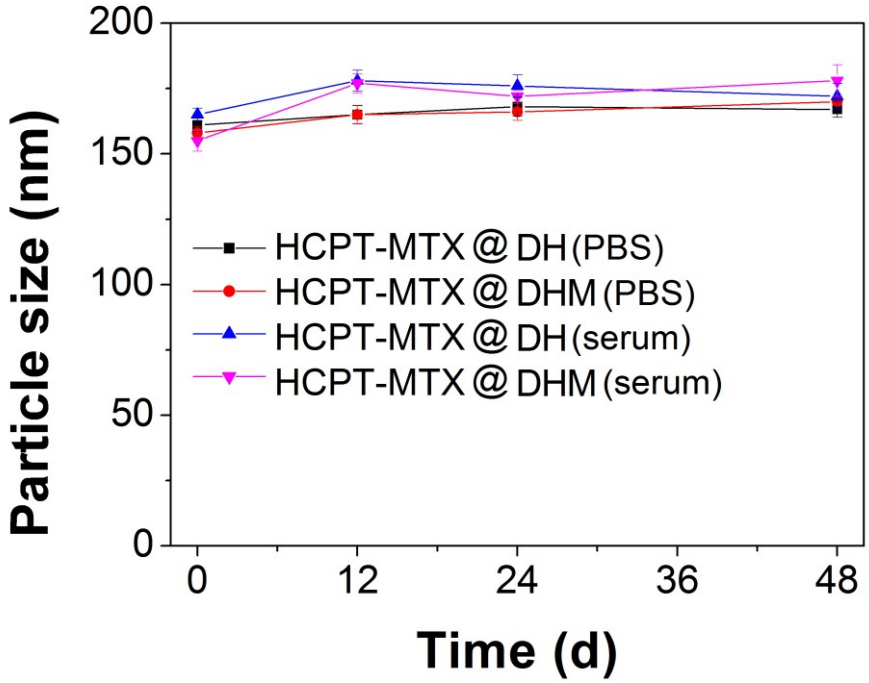

Figure S8. Change of the hydrodynamic particle size of HCPT-MTX@DH and HCPT-MTX@DHM nanoparticles dispersed in PBS buffer and 10% fetal bovine serum (FBS) for 48 h. At different time intervals (0, 12, 24, and 48 h), the hydrodynamic particle size was determined by DLS. Error bars indicate SD (n = 3).

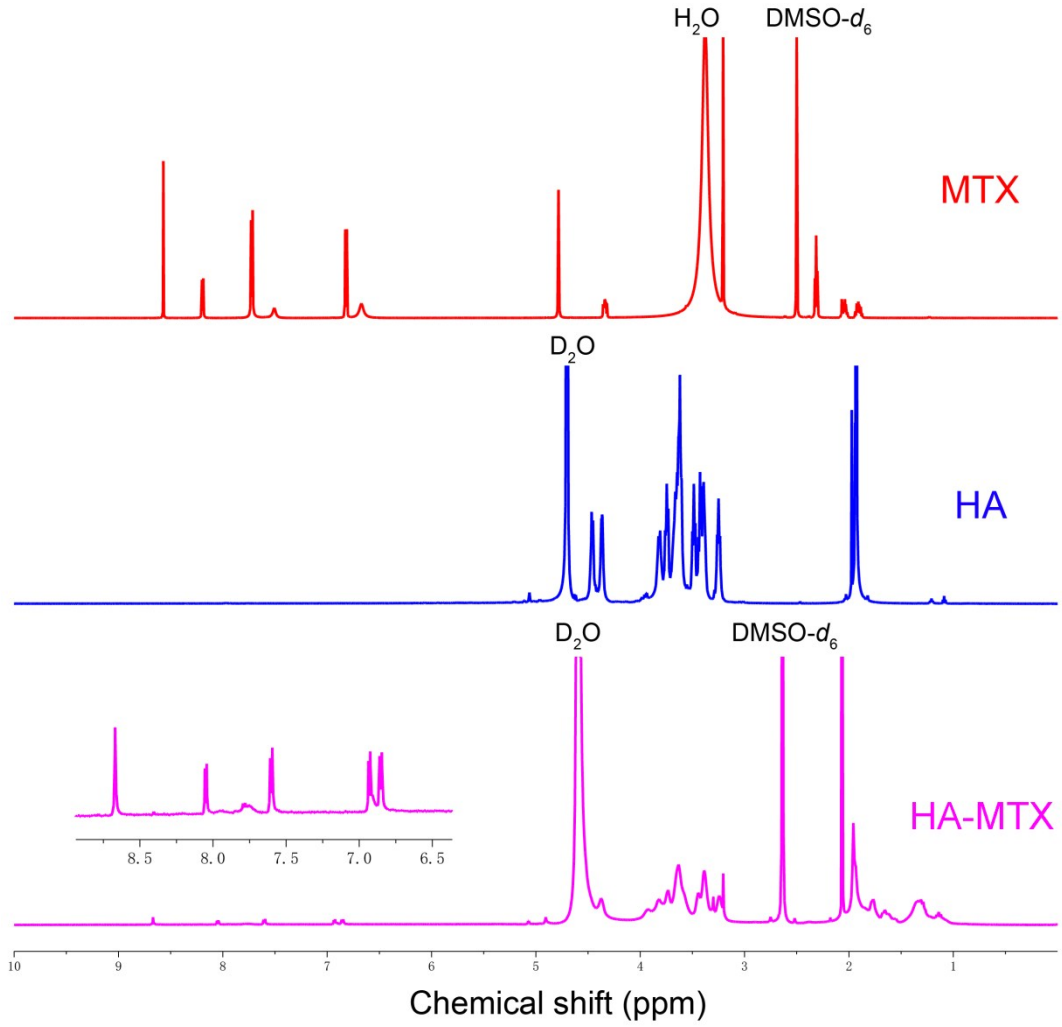

Figure S9. ^{1}H NMR spectra of MTX (DMSO-d_6 as solvent), HA (D_2O as solvent), and MTX-HA conjugate (DMSO-d_6/D_2O as solvent).

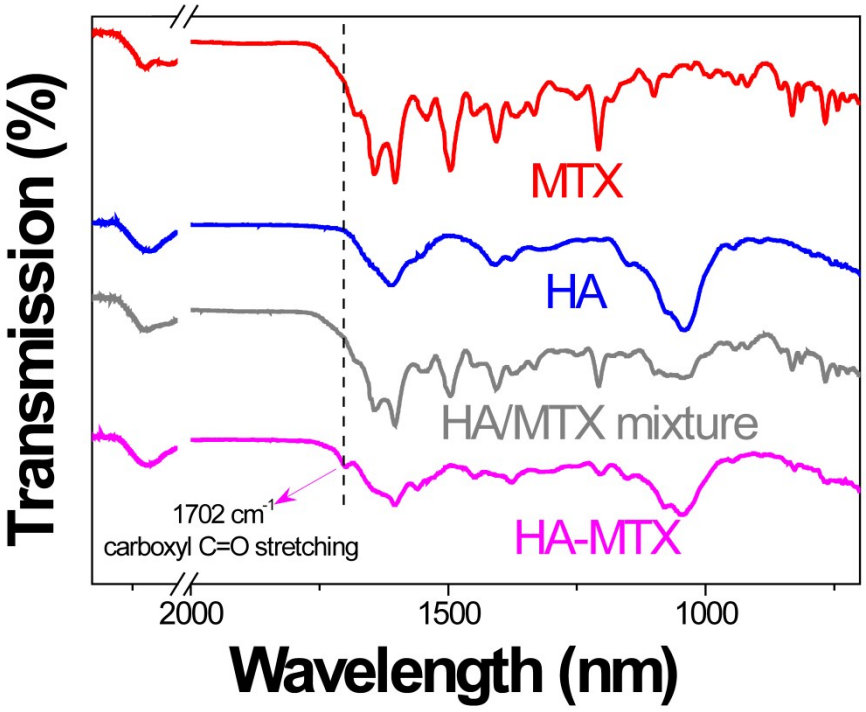

Figure S10. FTIR spectra of MTX, HA, HA/MTX mixture, and HA-MTX conjugate.

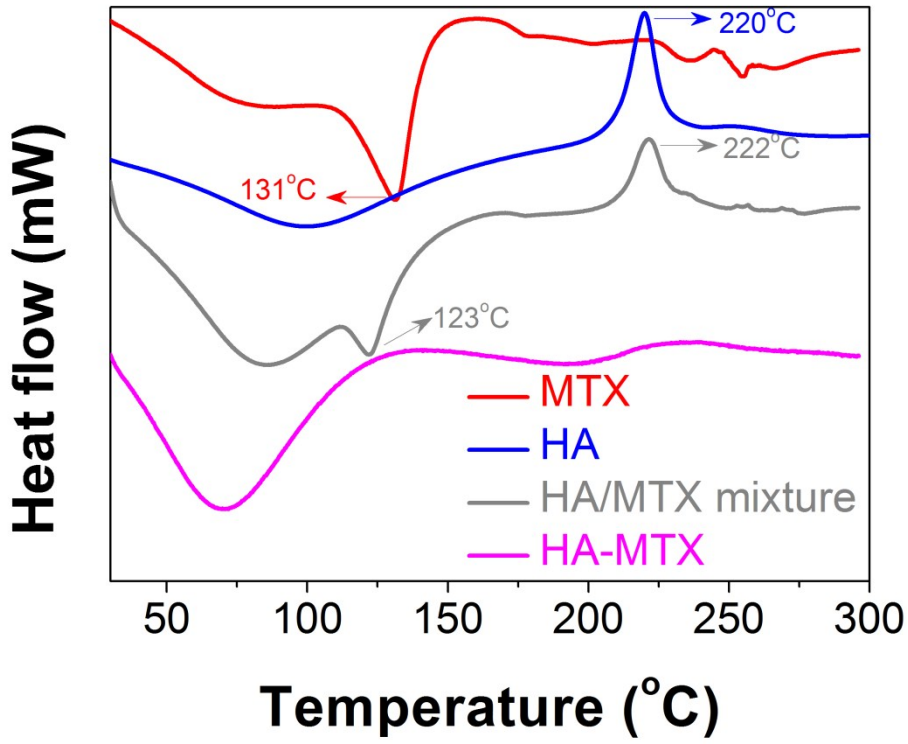

Figure S11. DSC spectra of MTX, HA, HA/MTX mixture, and HA-MTX conjugate.

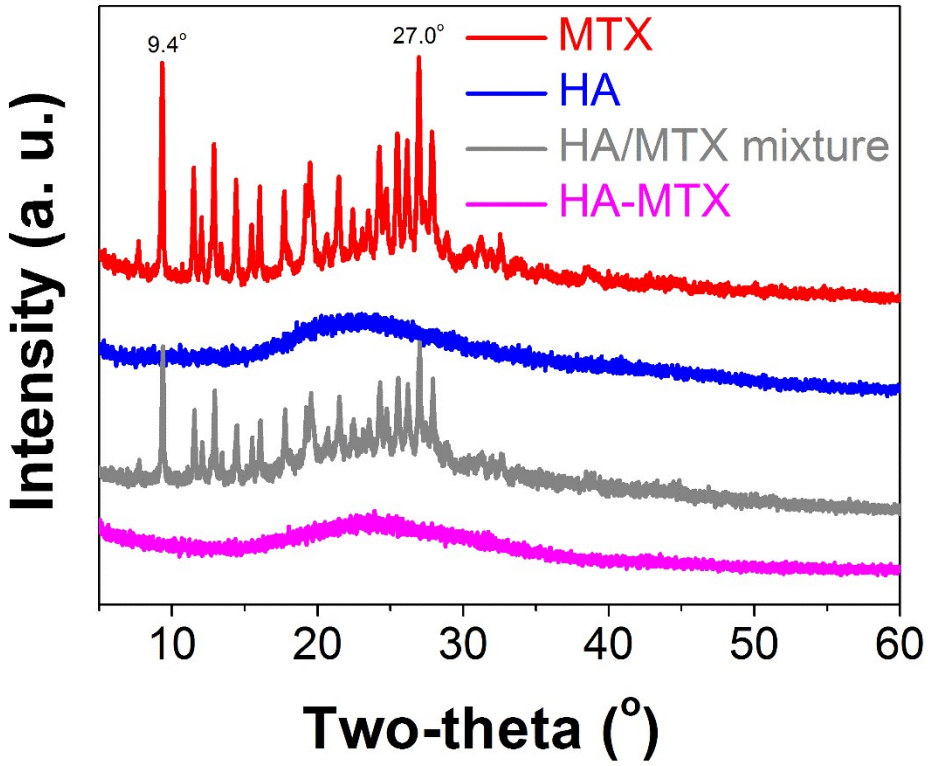

Figure S12. XRD spectra of MTX, HA, HA/MTX mixture, and HA-MTX conjugate.

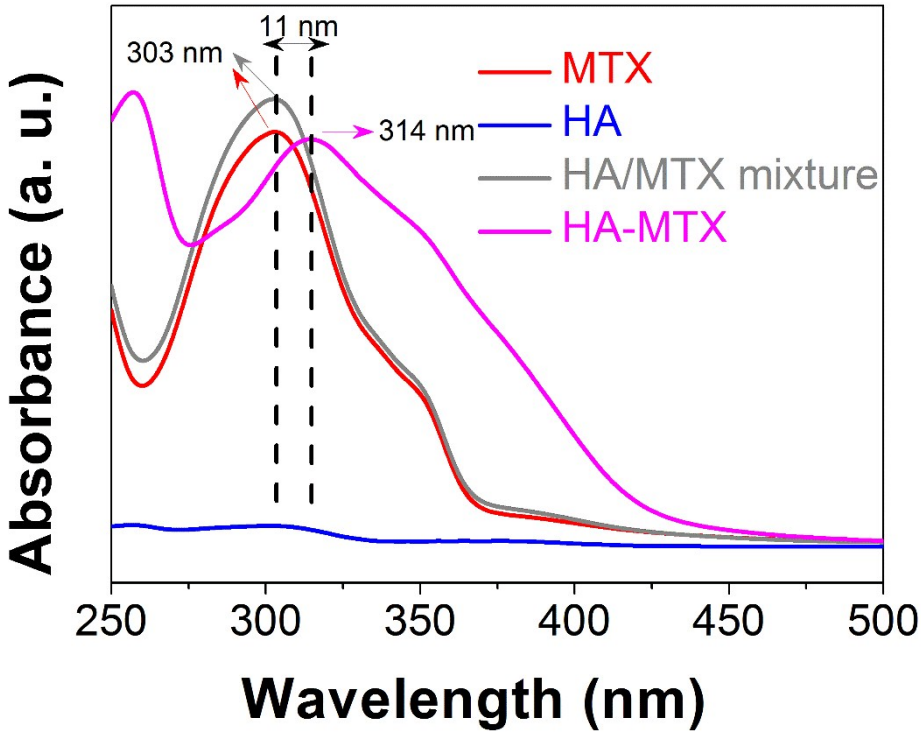

Figure S13. UV-vis absorption spectra of MTX, HA, HA/MTX mixture, and HA-MTX conjugate (water as solvent).

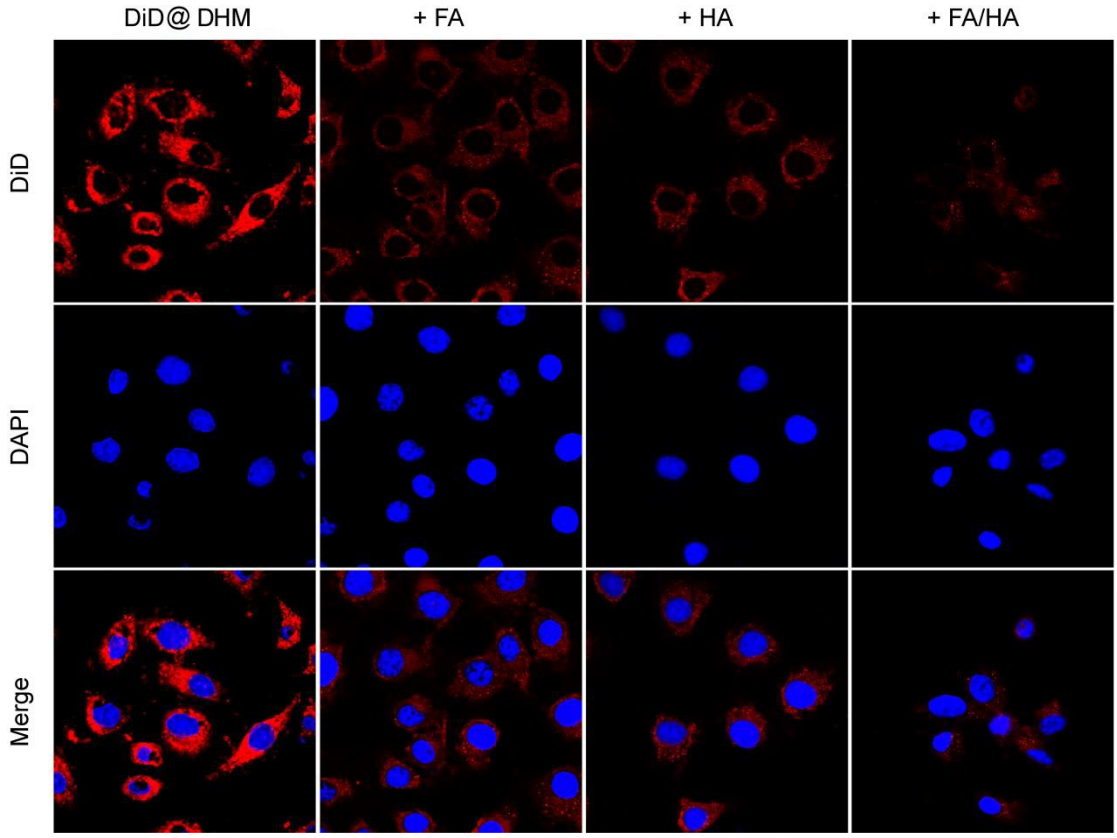

Figure S14. *In vitro* cell uptake of DHM nanoparticles towards 4T1 cells. CLSM images of 4T1 cells treated with DiD@DHM with/without the pretreatment of free FA, HA, or FA/HA mixture for 6 h.

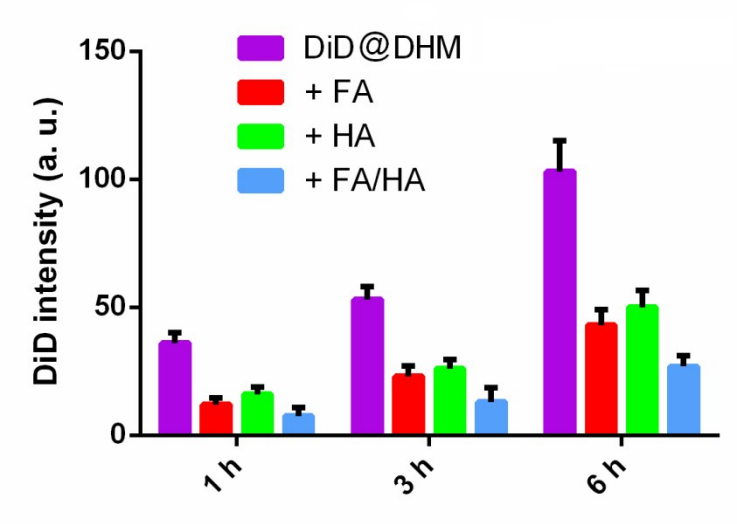

Figure S15. Mean fluorescence intensity of 4T1 cells treated with DiD@DHM with/without the pretreatment of free FA, HA, or FA/HA mixture for 1, 3, and 6 h determined by flow cytometry. Error bars indicate SD (n = 4).

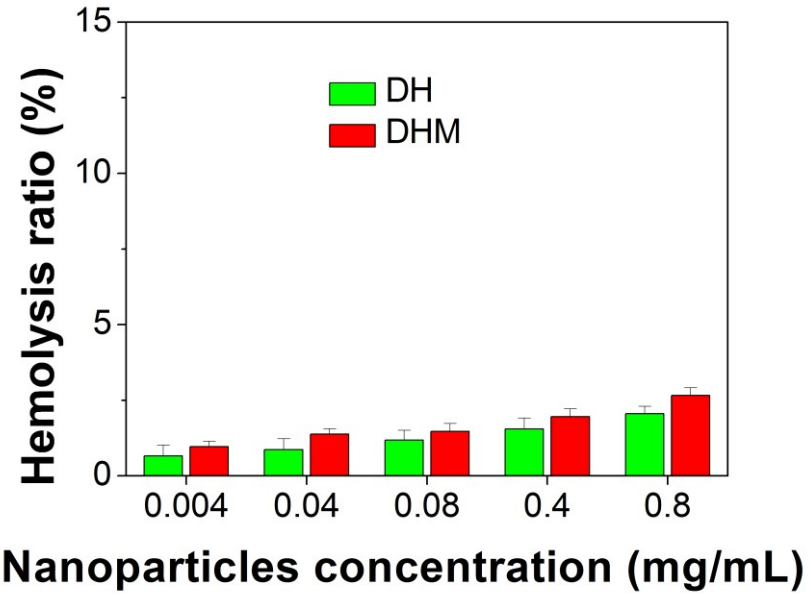

Figure S16. Hemolysis ratios of DH and DHM nanoparticles at various concentrations. Error bars indicate SD (n = 6).