Supplementary Information:

Dual-self-recognizing, stimulus-responsive and carrier-free methotrexate-mannose conjugate nanoparticles with highly synergistic chemotherapeutic effects

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■ Materials and methods

Materials

Methotrexate (MTX), folic acid (FA), Mannose (MAN), and Lectin (LT) were purchase from Shanghai Macklin Biochemical Co., Ltd (China). 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and 4-dimethylaminopyridine (DMAP) were brought from Sigma-Aldrich (St. Louis, MO,

USA). Cell Counting Kit-8 (CCK-8), calcein-acetoxymethyl ester (calcein AM), propidium iodide (PI), and 4',6-diamidino-2-phenylindole (DAPI) were acquired from Sigma-Aldrich (St. Louis, MO, USA). 4-chloro-7-nitro-1, 2, 3-benzoxadiazole (NBD-Cl) and indocyanine green N-Hydroxysuccinimide (ICG-NHS) ester were obtained from Xi'an Ruixi Biotechnology co., Ltd (Xi'an, China). Apoptosis kit with annexin V-FITC and PI were provided from Thermo Fisher Scientific (Waltham, MA, USA). Ethanol (EtOH) and dimethyl sulfoxide (DMSO) was purchased from Xiamen kezhan Reagent Glass Instrument Co., Ltd. (China). Deionized (DI) water was used in the whole experiments. Unless otherwise stated, the whole chemical reagents were of analytical grade, commercially available, and used as received without further purification. Phosphate-buffered saline (PBS), fetal bovine serum (FBS), FA-scarce Roswell ParkMemorial Institute (RPMI) 1640 (RPMI 1640 medium without FA), penicillin/streptomycin, trypsin/ethylenediamine tetra-acetic acid (EDTA), and penicillin/streptomycin were obtained from Hyclone (USA). All experiments were performed according to the relevant laws and institutional guidelines. The experiments were conducted in accordance with the guidelines issued *via* the Ethical Committee of Xiamen University.

Synthesis of MTX-MAN conjugate.

MTX (454.5 mg, 1.0 mmol), EDC·HCl (249.2 mg, 1.3 mmol), and DMAP (183.2 mg, 1.5 mmol) were dissolved in 10 mL of DMSO and then the mixture was stirred at 0 ℃ under nitrogen. After stirring for 0.5 h, MAN (198.2 mg, 1.1 mmol) dissolved in DMSO solution was added dropwise and then the mixture was stirred for 24 h at room temperature in the dark. Afterwards, the reaction mixture was precipitated in excess cold ethyl acetate, and EtOH was exploited to wash the precipitate three times to remove the unreacted MAN. The crude product was further purified by column chromatograph using ethyl acetate and methanol (10:1) as the eluent. The product was collected and dried under vacuum to obtain a yellow solid (435.0 mg, 65%).

Characterization of MTX-MAN conjugate.

The Fourier Transform infrared (FT-IR) spectrum was carried out on a Bruker

IFS-55 infrared spectrometer (Bruker, Switzerland). The proton nuclear magnetic resonance (^{1}H-NMR) spectra were implemented on a Bruker AVANCE III 500MHz NMR spectrometer (Bruker, Germany) with DMSO-d_6 as solvents. The ultraviolet-visible (UV-vis) absorption spectra were gained using a Perkin Elmer Lambda 750 UV-vis-near-infrared spectrophotometer (Perkin-Elmer, Norwalk CT). The X-ray diffraction (XRD) pattern was tested with a Philips X'Pert Pro Super X-ray diffractometer (Philips, Netherlands). The differential scanning calorimetry (DSC) curves and thermal gravity analysis (TGA) curves were DSC analysis (DSC 204F1, Netzsch, Selb, Germany). The electron spray ionization mass spectrum (ESI-MS) was administrated using a ESI-MS spectrometer (Esquire3000 plus spectrometer, Bruker Co., Zurich, Switzerland). The matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) was performed on a Microflex LRF MALDI-TOF MS (Bruker Daltonics, USA). The high-resolution MS were obtained using Bruker Daltonics Apex-ultra 7.0 T Fourier transform ion cyclotron resonance mass spectrometer with an Apollo ESI source (Bruker Daltonics, USA) spectrometer.

Preparation of MTX-MAN NPs

MTX-MAN NPs were prepared *via* an anti-solvent method. In brief, 10 mg of MTX-MAN conjugate was dissolved in 2 mL of DMSO at room temperature. Then, 10 mL of DI water was slowly injected dropwise into the solution of MTX-MAN conjugate and stirred slightly for 0.5 h. Afterwards, the solution was dialyzed *via* DI water for 24 h (molecular weight cutoff = 1,000 DA, Slide-A-Lyzer, Thermo Scientific, USA), during which the water was replaced every 2 h. The volume of the solution was increased to 10 mL with the addition of DI water to obtain a solution with a concentration of 0.5 mg/mL for further experiments.

Characterization of MTX-MAN NPs

The morphology was observed using transmission electron microscopy (TEM, JEM2100, JEOL, Tokyo, Japan) working at an accelerating voltage of 200 kV, field-emission scanning electron microscope (FE-SEM, Hitachi SU-70, Tokyo, Japan) working at an accelerating voltage of 5 KV, and atomic force microscopy (AFM,

Multimode 8, Bruker, USA) working in tapping mode. Energy dispersive spectrometer (EDS) and element mapping were carried out on a high-resolution transmission electron microscopy (HRTEM, FEI, Talos 200s) working at 200 kV and FE-SEM (Hitachi SU-70, Tokyo, Japan) working at an accelerating voltage of 20 KV. The Hydrodynamic diameter (D_h), polydispersity index (PDI), and zeta potential were determined *via* dynamic light scattering (DLS) and electrophoretic light scattering (ELS) by a Malvern Zetasizer 2000 (Malvern, UK), and data were shown by mean ± standard deviation (SD). High-performance liquid chromatography (HPLC) was performed on an Agilent 1100 liquid chromatograph (Agilent Technologies, CA, USA).

Measurement of Critical Aggregation Concentration

To measure the critical aggregation concentration (CAC) of MTX-MAN NPs, pyrene was used as a fluorescence probe. In brief, 6.0×10^{-4} mg/mL of pyrene acetone solution was formulated and subsequnetly MTX-MAN NPs was doubly diluted to different concentration from 0.8 μM to 0.00156 μM. After that, 20 μL of the pyrene solution was injected dropwise into MTX-MAN NPs of a series of concentration. Then, the resulting solution was sonicated for 45 min and placed overnight at room temperature to volatilize the acetone. The emission spectra of all samples were determined using a FluoroMax-4 Spectrofluorometer (HORIBA Jobin Yvon, USA) at 335 nm excitation wavelength and 5 nm slit width. Finally, the fluorescent intensity of all samples at 360 nm was recorded.

***In vitro* colloidal stability**

To evaluate the *in vitro* colloidal stability of MTX-MAN NPs, water, PBS, and serum were used as the physiological media. In brief, the in *vitro* colloidal stability of MTX-MAN NPs was determined in DI water, PBS (PH 7.4), and RPMI1640 medium containing FBS. The colloidal stability of MTX-MAN NPs in different physiological media for predesigned incubation time points was evaluated *via* determining the hydrodynamic diameter, zeta potential, and morphology using DLS, ELS, and SEM.

***In vitro* drug release**

In vitro release behaviors of MTX from MTX-MAN NPs were assessed *via* a

series of simulated physiological conditions. Typically, 3 mL of MTX-MAN NPs was transferred into a dialysis bag (molecular weight cutoff = 1,000 DA, Slide-A-Lyzer, Thermo Scientific, USA) with/without esterase (5 mg, 30 U/mL), which was immersed into 57 mL of PBS at different pH values (7.4 and 5.0). Afterwards, 2 mL of the external PBS was withdrawn and renewed with 2 mL of fresh PBS immediately according to the predesigned time periods. The release amount of MTX was determined using HPLC method as depicted in our previous reports.[1] To precisely determine the amount of MTX, the conditions were as follows: stationary phase, Luna C18 column (250 mm×4.6 mm, 5 μm); temperature, 25℃; mobile phase, HPLC grade acetonitrile: 0.04 M potassium dihydrogen phosphate (pH 4.5) (12/88, v/v), freshly prepared, filtered through a 0.22 mm Millipore membrane filter before using, and degassed using a sonication method; elution flow rate, 1.0 mL/min; detection wavelength, 303 nm.

Cell culture

Human breast carcinoma cell line MCF-7 was chosen for the following experiments because of its folate (FA)/Lectin (LT)-receptor overexpression. Human lung carcinoma cell line A549 ascribing to its FA receptor underexpression and LT receptor overexpression. Normal human hepatocytes cell line L02 was chosen due to its FA/LT-receptors-underexpression. Three kinds of cell lines were cultured in FA-free RPMI 1640 medium containing 10% FBS, 100 μg/mL of streptomycin, 4 mM of stable glutamine, and 100 U/mL of penicillin. Cells were cultured in an incubator (Thermo Fisher Scientific Inc., USA) with 5 % CO_2 under 37 ℃ in humidified atmosphere.

***In vitro* dual-recognizing cellular uptake**

To determine the *in vitro* dual-recognizing cellular uptake of MTX-MAN NPs, MTX, MAN, and MTX-MAN conjugate was labeled with NBD-Cl with a very small molecular structure *via* a nucleophilic substitution reaction (named as MTX^{NBD}, MAN^{NBD}, and MTX-MAN^{NBD}). MCF-7 cells (FA/LT receptors overexpression), A549 cells (FA receptor underexpression and LT receptor overexpression), and L02 cells (FA/LT receptors-nonexpression) were seeded into 6-well plates at a density of

5.0 × 10^5 cells/well, incubated at 37 ℃ for 24 h, and then treated with MTX^{NBD}, MAN^{NBD}, and MTX-MAN^{NBD} NPs at equivalent NBD concentration according to the predesigned incubation time periods at 37℃. Afterwards, culture medium was removed and cells were washed thrice time using PBS and subsequently fixed by 4% paraformaldehyde for 30 min. Next, nuclei were stained using DAPI for 20 min. Finally, cells were imaged by a Leica SP5-STED CLSM with excitation at 488 nm for NBD and 360 nm for DAPI.

Flow cytometry analysis

Flow cytometry analysis was performed to quantitatively measure the *in vitro* dual-recognizing cellular uptake of MTX-MAN NPs. MCF-7 cells, A549 cells, and L02 cells were seeded into 6-well plates at a density of 2.0 × 10^5 cells/well. After incubation for 24 h, cells were treated with MTX^{NBD}, MAN^{NBD}, and MTX-MAN^{NBD} NPs at equivalent NBD concentration for predesigned incubation time intervals at 37℃. After that, cells were detached with trypsin/EDTA, suspended in PBS with 10% FBS, centrifuged at 2, 000 rpm for 5 min at 4 ℃, and re-suspended in fluorescence-activated cell sorting (FACS) buffer. Finally, the cellular fluorescence intensity of NBD was recorded by a FACSCalibur flow cytometer (Becton Dickinson, USA) and subsequently the results were analyzed by Cell Quest software.

Subcellular Localization

MCF-7 cells were seeded into 24-well plate at a density of 1.0 × 10^4 cells/well. After culture for 24 h, cells were incubated with fresh FBS-free cell culture medium containing MTX-MAN^{NBD} NPs (20 μg /mL of MTX-MAN^{NBD}) at 37 ℃. According to predetermined time intervals, cells were incubated with 0.3 μM Lysotracker red DND-99 for 30 min and fixed with 4% paraformaldehyde for 30 min. Then, cells were washed with cold PBS, stained with DAPI for 15 min, and observed using a Leica SP5-STED CLSM.

***In vitro* cytotoxicity**

The CCK-8 assay was performed to quantitatively evaluate the therapy efficiency. Briefly, MCF-7, A549, and L02 cells were seeded in 96-well plates at a density of 5.0 × 10^3 cells/well and then cultured for 24 h. The cell culture medium was removed and

then replaced by 200 μL of fresh medium containing MTX, MAN, MTX/MAN mixture, and MTX-MAN NPs at the same drug concentration for 24 h incubation. The cell viability was evaluated *via* CCK-8 in accordance with the manufacturer's instructions. The absorbance was measured using a microplate reader (Bio Tek ELX800, USA) at 490 nm and a reference wavelength of 630 nm. Besides, the half-maximal inhibitory concentrations (IC_{50}) were calculated *via* SPSS Statistics 20.0 software.

***In vitro* apoptosis**

Tumor cell-inhibiting effect of MTX-MAN NPs was also evaluated using the Annexin V-FITC Apoptosis Detection Kit (BD Biosciences). In brief, MCF-7 cells (1.0×10^5 cells/well) were seeded in 6-well plates. After culture for 24 h, cells were respectively treated with PBS (control), MTX, MAN, MTX/MAN mixture, and MTX-MAN NPs for 12 h. The subsequent procedures were conducted in accordance with the manufacturer's suggested procedures. Cells were analyzed by flow cytometry (FACSCalibur, BD Biosciences, USA) and CellQuest/FlowJo (Tree Star, USA) software.

Living cells and dead cells staining

MTX-MAN NPs-induced tumor cell death was further verified *via* calcein AM/PI costaining assay. In brief, living and dead cells were stained with calcein-AM and PI, respectively. Cell culture medium containing PBS (control), MTX, MAN, MTX/MAN mixture, and MTX-MAN NPs were used for culturing MCF-7 cells (1.0×10^5 cells/well) for 4 h. Subsequently, Living cells and dead cells were staining *via* a live/dead staining kit according to the manufacturer's instructions and subsequently observed with a fluorescence microscope (Leica Microsystems, Germany).

Animals

Study protocols involving animals were approved by the Institutional Animal Care and Use Committee of Xiamen University. BALB/c nude mice (5 weeks, male, 18-20 g) and Sprague-Dawley (SD) rats (adult male, 180-200 g) were brought from Experimental Animal Laboratory of Cancer Research Center of Xiamen University.

***In vivo* imaging (fluorescence/photoacoustic (PA) imaging)**

To evaluate the *in vivo* dual-self-recognizing ability toward FA/LT-receptor-overexpression solid tumors, MCF-7 human breast carcinoma tumor-bearing nude mice (BALB/c) model was built and subsequently assessed *via* the *in vivo* fluorescence/PA research. In brief, 1.0×10^6 MCF-7 cells were suspended in 100 μL of PBS and subcutaneously inoculated into the right lower limb of the tumor-bearing nude mice. When the tumors grew until about 100 mm^3, 200 μL of ICG, MTX^{ICG}, and MTX-MAN^{ICG} NPs at the same concentration of ICG (200 μg/mL) were *i. v.* injected into the MCF-7 tumor-bearing nude mice *via* the tail vein. After *i. v.* injection for 0, 1, 3, 6, 12, 16, 24, 36, and 48 h, *in vivo* fluorescence images were collected by the IVIS Lumina II System (Caliper Life Sciences, USA) and then semiquantitatively analyzed *via* the Living Image Software. After *i. v.* injection for 48 h, the MCF-7 tumor-bearing nude mice were immediately executed by euthanasia and subsequently the primary organs (heart, liver, spleen, lung, and kidney) and tumor tissues were harvested. Afterwards, *ex vivo* fluorescence images were also gained using the IVIS Lumina II System (Caliper Life Sciences, USA) and fluorescence intensity was semiquantitatively analyzed using the Living Image Software.

In addition, to further verify the *in vivo* dual-self-recognizing ability of MTX-MAN NPs, *in vivo* PA imaging was implemented on a Vevo LAZR PA system (Vevo®LAZR-X, VisualSonics, Canada). Briefly, When the tumors grew until about 100 mm^3, the MCF-7 tumor-bearing nude mice were *i. v.* injected by 100 μL of ICG, MTX^{ICG}, and MTX-MAN^{ICG} NPs at an equivalent concentration of ICG (200 μg/mL) *via* the tail vein and then real-time PA signals in the tumor tissues were collected at 0, 6, 12, 24, and 48 h post-*i. v.* injection. After *i. v.* injection for 48 h, the MCF-7 tumor-bearing nude mice were immediately sacrificed *via* euthanasia and subsequently the primary organs and tumor tissues were separated. Next, *ex vivo* PA images were also collected by the Vevo LAZR PA system (Vevo®LAZR-X, VisualSonics, Canada). After *ex vivo* PA imaging, the tumors were fixed in 10% neutral buffered formalin and subsequently embedded in paraffin. Tumor tissues were stained using DAPI to label all nuclei of tumor cells. The fluorescence images of the tumor sections were collected using a fluorescence microscope (Nikon Eclipse TE

300, Tokyo, Japan).

***In Vivo* pharmacokinetic**

To verify the long circulation life of MTX-MAN NPs, the in *vivo* pharmacokinetic analysis was administrated. Briefly, SD rats were stochasticly divided into two groups (n = 4): (A) MTX group and (B) MTX-MAN NPs group. The SD rats were *i. v.* injected with 200 μL of MTX and MTX-MAN NPs (8 mg/kg), respectively. 200 μL of blood samples were drawn from retro orbital choroid plexus of SD rats according to the predesigned time points under mild anesthesia and collected into a heparinized tube. The plasma was acquired by centrifugation at 3,000 rpm for 15 min and strored at -20℃ till further analysis. The drug concentration in each plasma sample was determined by HPLC method as described above. Finally, pharmacokinetic parameters were calculated via fitting the blood drug pharmaceutical concentrations to a two-compartment model using WinNonlin®Professional Network Edition, version 5.2 (Pharsight Corp, Sunnyvale, California).

***In vitro* MAN-enhanced antitumor activity**

To evaluate the *in vitro* MAN-enhanced antitumor activity of MTX-MAN NPs, MCF-7 tumor-bearing nude mice model (BALB/c) was established by subcutaneously inoculating 1.0×10^6 MCF-7 cells into the right lower limb. When the tumors grew until about 100 mm^3, the tumor-bearing nude mice were randomly divided into five groups (n = 5 per group): (A) 0.9% NaCl group as negative control, (B) MTX group, (C) MAN group, (D) MTX/MAN mixture group, and (E) MTX-MAN NPs group. The MCF-7 tumor-bearing mice were *i. v.* injected with 200 μL of 0.9% NaCl, MTX (9.08 mg/kg), free MAN (3.60 mg/kg), MTX/MAN mixture (12.68 mg/kg), or MTX-MAN NPs (12.32 mg/kg) at the equivalent dosage of MTX or MAN, respectively on days 0, 2, 4, 6, 8, 10, 12, 14, and 16 post-injection. Afterwards, the tumor size of every mouse were tested by a caliper every 2 days for 3 weeks and the tumor volume was calculated as $V = (\text{tumor length} \times \text{tumor width}^2)/2$. The relative tumor volume was calculated as V/V_0 (V_0: the initial tumor volume) at the beginning of the treatment. Survival rate of mice was calculated every-day for 3 weeks. The weight of every mouse was recorded every 2 days for 3 weeks using electronic balance. After the 21st

day of therapy, the blood samples were collected for serum biochemical analyses. After that, all mice were sacrificed via euthanasia and subsequently the subcutaneous tumors and primary organs (heart, liver, spleen, lung, and kidney) were separated, weighed, and washed with 0.9% NaCl thrice.

Next, to assess the tissue destruction as well as cell apoptosis after therapy, the above-collected tumors were cut into small pieces, fixed in the 10% formalin, and embedded in paraffin blocks. Subsequently, the tissues embedded in paraffin were sectioned at 8 mm, stained with H&E, and observed under a light microscopy (Nikon Eclipse Ci, Tokyo, Japan).

***In Vivo* Biosafety**

Hemolysis assay were exploited to determine biomaterials to red blood cells and biocompatibility. In brief, 5 mL of blood samples were collected from retro orbital choroid plexus of SD rats under mild anesthesia. Red blood cells were obtained by centrifugation (3500 rpm, 10 min, and 4 ℃) and then washed three times with PBS (pH 7.4) until the supernatant became colorless. Afterwards, 200 μL of cell suspension was diluted to 10% (v/v) of cell suspension.

1 mL of MTX, MTX, MAN, MTX/MAN mixture, and MTX-MAN NPs were injected dropwise into 200 μL of cell suspension, respectively, acting as the experimental groups. According to the same method as described above, 1 mL of DI water and PBS were set as negative control and positive control, respectively. After that, all samples were incubated at 37 ℃ for 12 h and subsequently centrifuged at 3500 rpm for 10 min at 4 ℃ to obtain the supernatant. Finally, the UV absorption of the obtained supernatant at 541 nm was determined using a microplate reader. HP (hemolysis percentage) was calculated according to the following formula:

$$HP\ (\%) = (As\text{-}As_{(0)}\text{-}\ Ac_{(-)})/(Ac_{(+)}\text{-}A_{C(-)}) \times 100\%$$

In the above formula, As, $As_{(0)}$, $Ac_{(+)}$, $Ac_{(-)}$ depicted the UV absorption of the experimental groups containing red blood cells, the experimental groups without red blood cells, the positive control group, and the negative control group, respectively.

In addition, to evaluate the *in vivo* biosafety of MTX-MAN NPs, the pathobiology of the above-collected organs was also conducted on the H&E staining.

Besides, the above-collected blood was further used to analyze the biochemical indicators of liver functions such as total serum protein (TP), serum albumin (ALB), aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP) and kidney functions such as uric acid (UA) and creatinine (Cr).

Statistical analysis

Quantitative results are expressed as mean ± SD value. Statistical differences among groups are analyzed using one-way ANOVA analysis followed by Tukey's post-test. $P < 0.05$ is presented by a single asterisk (*) and is considered significant. $P < 0.01$ is presented by a double asterisk (**) and is considered highly significant.

Supplementary Data:

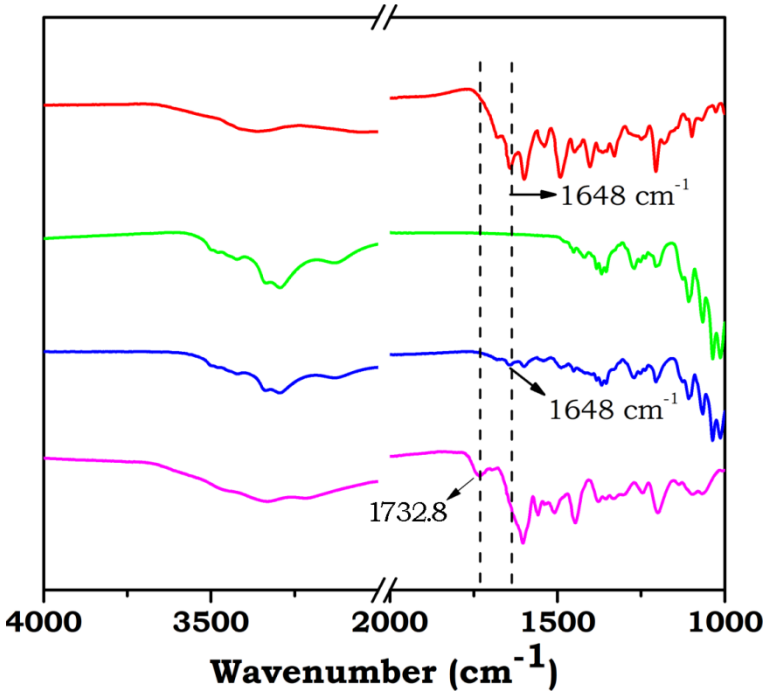

Figure S1. Detailed FTIR spectra of MTX, MAN, MTX/MAN mixture, and MTX-MAN conjugate.

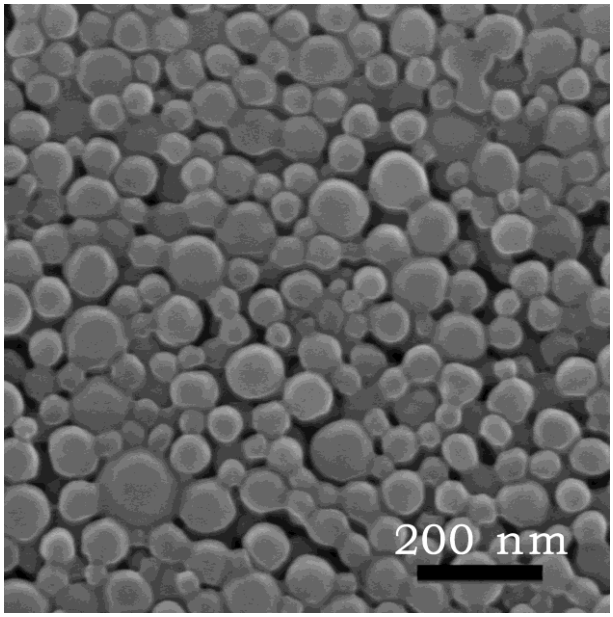

Figure S2. SEM image of MTX-MAN NPs at 1 mg/mL of MTX-MAN congjugate.

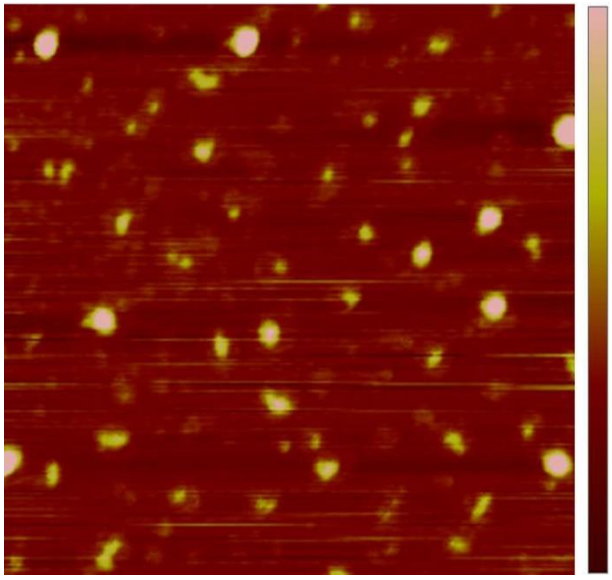

Figure S3. AFM image of MTX-MAN NPs dispersed in DI water.

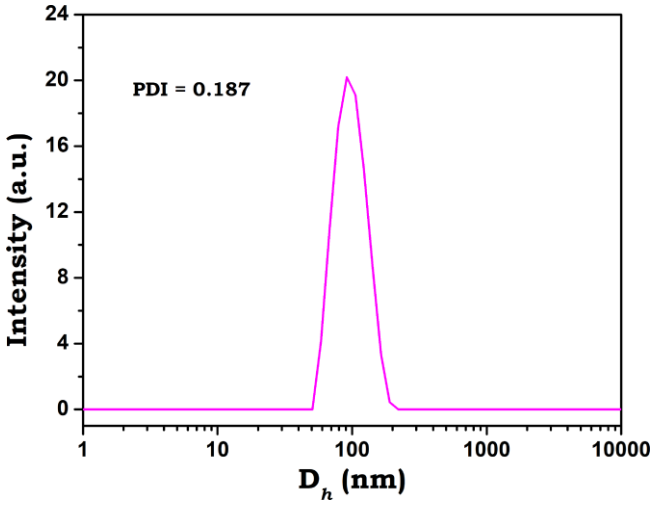

Figure S4. D_h of MTX-MAN dispersed in DI water measured *via* DLS.

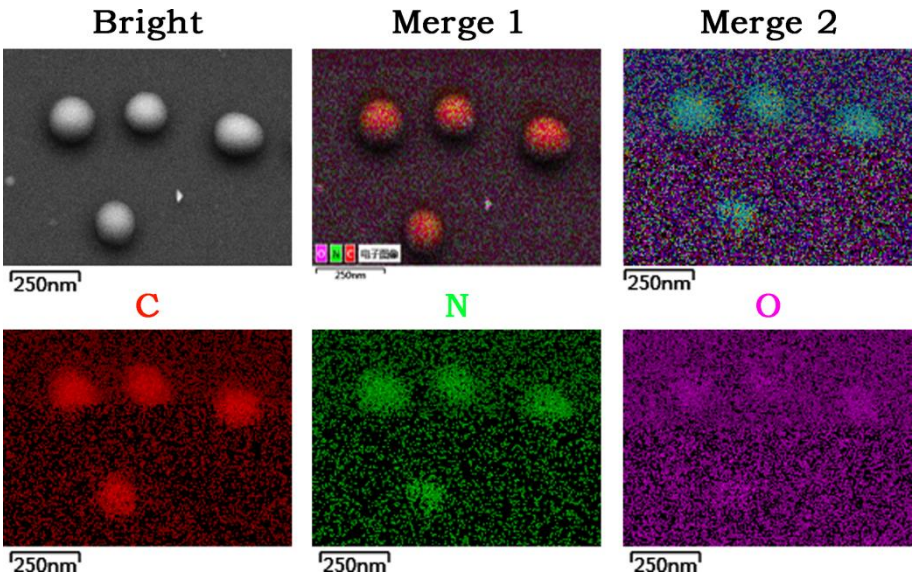

Figure S5. SEM-EDS mapping of MTX-MAN NPs.

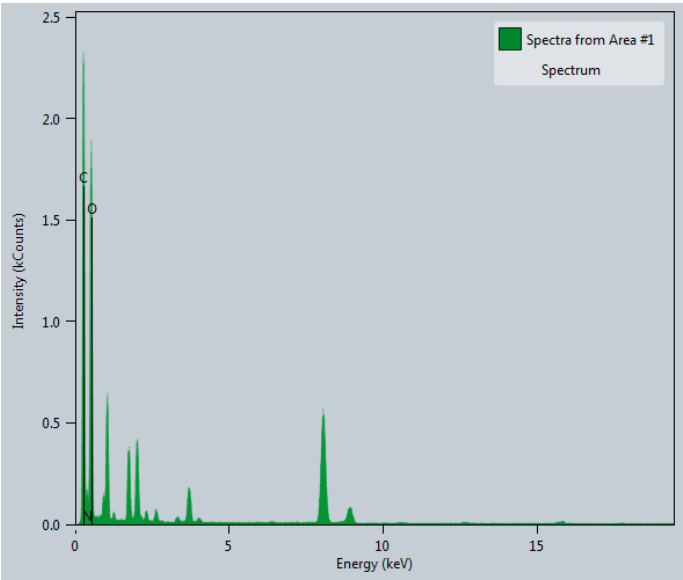

Figure S6. TEM-EDS spectrum of MTX-MAN NPs.

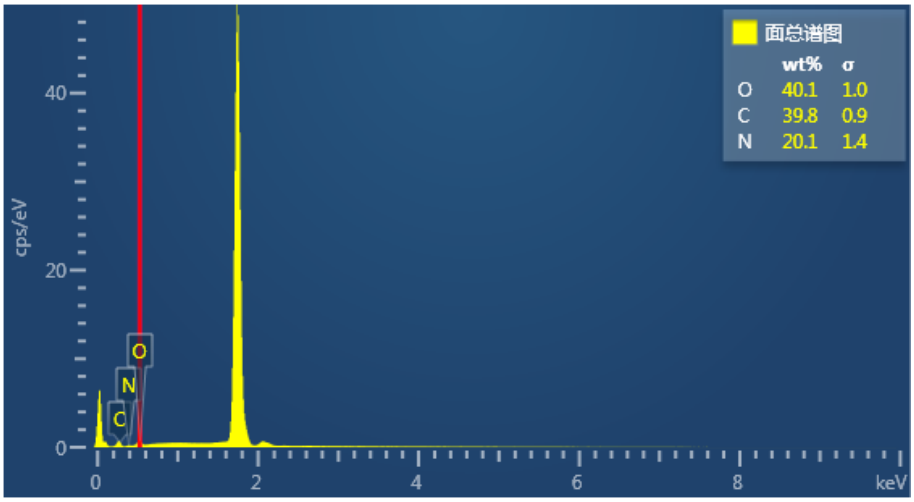

Figure S7. SEM-EDS spectrum of MTX-MAN NPs.

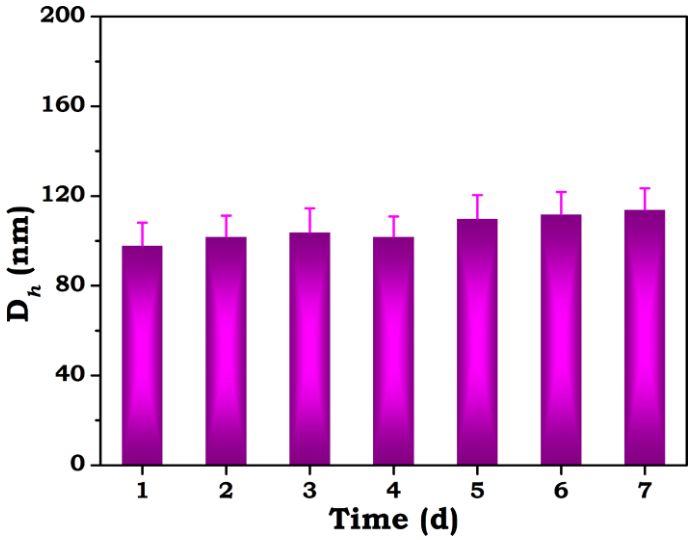

Figure S8. D_h of MTX-MAN NPs dispersed in DI water for 7 d. Error bars present SD (n = 3).

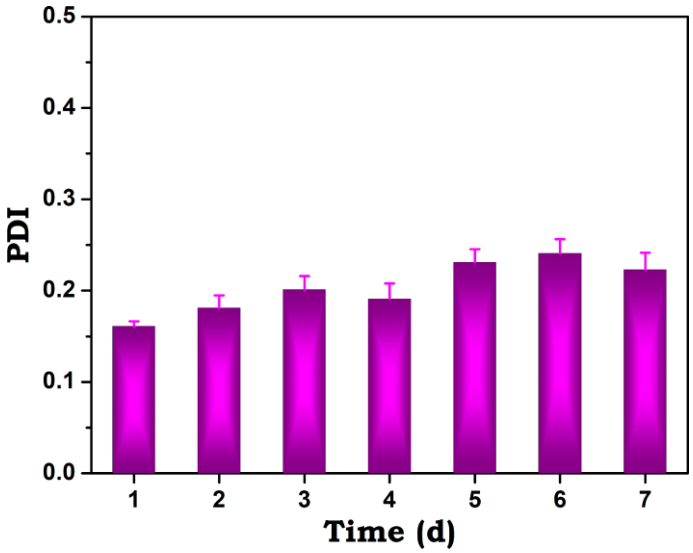

Figure S9. Change of polydispersity index (PDI) of MTX-MAN NPs dispersed in DI water for 7 d. Error bars indicate SD (*n* = 3).

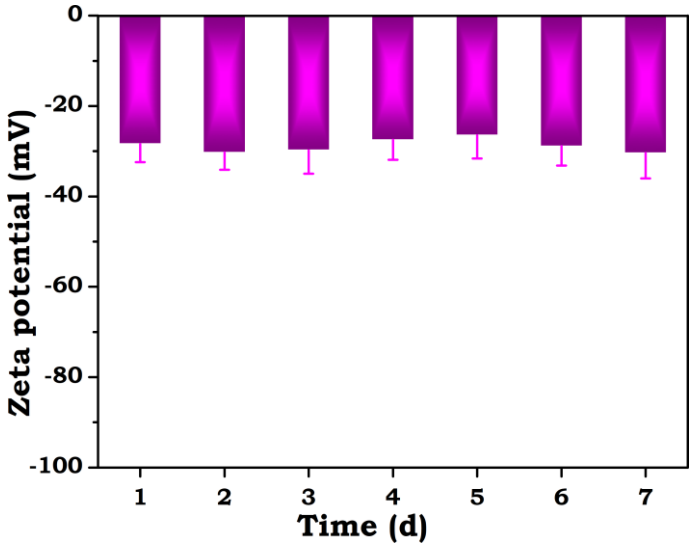

Figure S10. Change of zeta potential of MTX-MAN NPs dispersed in DI water for 7 d. Error bars present SD (n = 3).

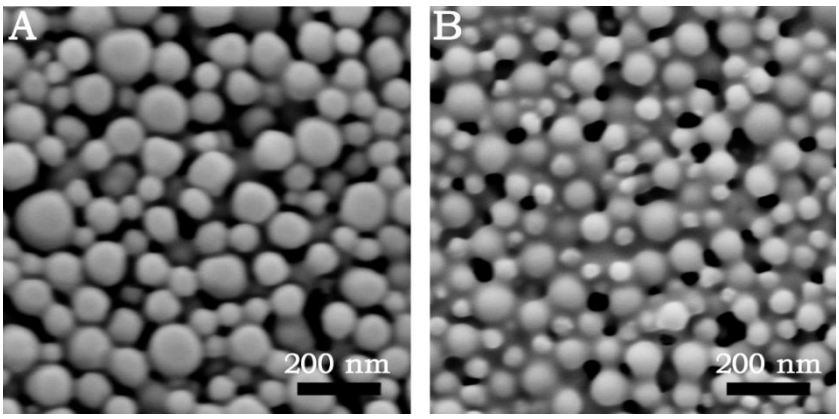

Figure S11. SEM images of MTX-MAN NPs dispersed in DI water for (A) 1 d and (B) 7 d.

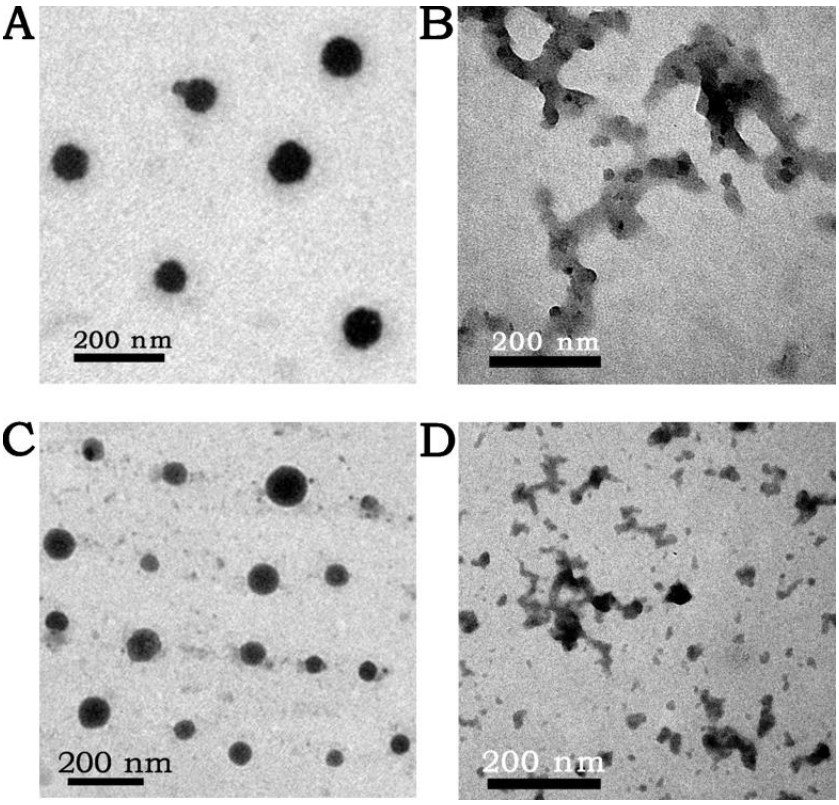

Figure S12. lysosomal acidity-/esterase-induced disassembly of MTX-MAN NPs. TEM images of MTX-MAN NPs at (A) pH 7.4 without esterase, (B) pH 5.0 without esterase, (C) pH 7.4 with esterase, and (D) pH 5.0 with esterase, respectively.

Formulation	IC_{50} (μM)
MTX	369.21 ± 7.2
MAN	809.67 ± 4.5
MTX/MAN	190.93 ± 8.8
MTX-MAN NPs	100.21 ± 3.5

Table S1. IC_{50} values of MCF-7 cells incubated *via* various concentrations of MTX, MAN, MTX/MAN mixture, and MTX-MAN NPs after 24 h. IC_{50} was calculated *via* using the probit regression method using SPSS Statistics 20.0 software. Error bars present SD (n = 3).

Formulation	IC50 (μM)
MTX	1758 ± 9.6
MAN	559.4 ± 5.2
MTX/MAN	338.7 ± 4.8
MTX-MAN NPs	171.5 ± 3.4

Table S2. IC50 values of A549 cells incubated *via* various concentrations of MTX, MAN, MTX/MAN mixture, and HA-SS-MTX NPs after 24 h. IC_{50} was calculated *via* using the probit regression method using SPSS Statistics 20.0 software. Error bars present SD (n = 3)

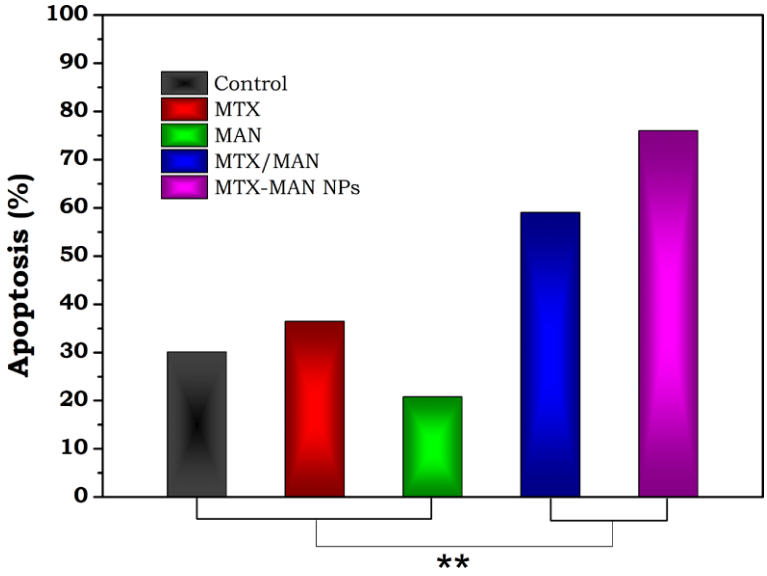

Figure S13. Apoptotic rate including early and late apoptotic cells of PBS, MTX, MAN, MTX/MAN mixture, and MTX-MAN NPs. $^{**}P < 0.01$.

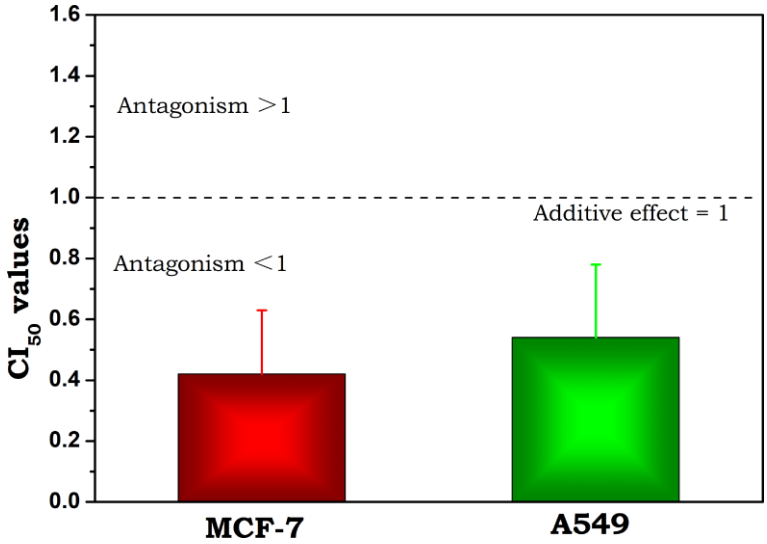

Figure S14. CI_{50} values of drug combinations in MTX-MAN NPs towards MCF-7 and A549 cells for 24 h treatment. CI values <1 indicate synergism, CI values equal to 1 indicate an additive effect, and CI values >1 indicate antagonism. Error bars indicate SD (n = 3).

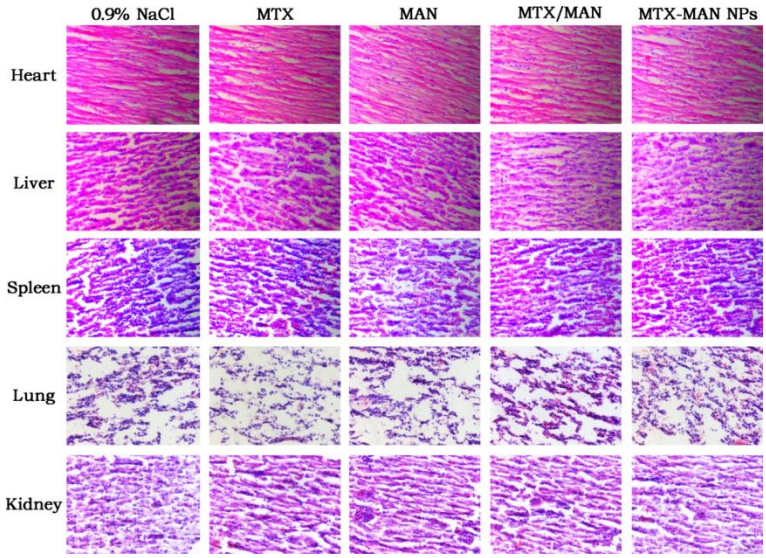

Figure S15. Representative H&E stained images of primary organs (heart, liver, spleen, lung, and kidney) of MCF-7 tumor-bearing nude mice after different therapy.

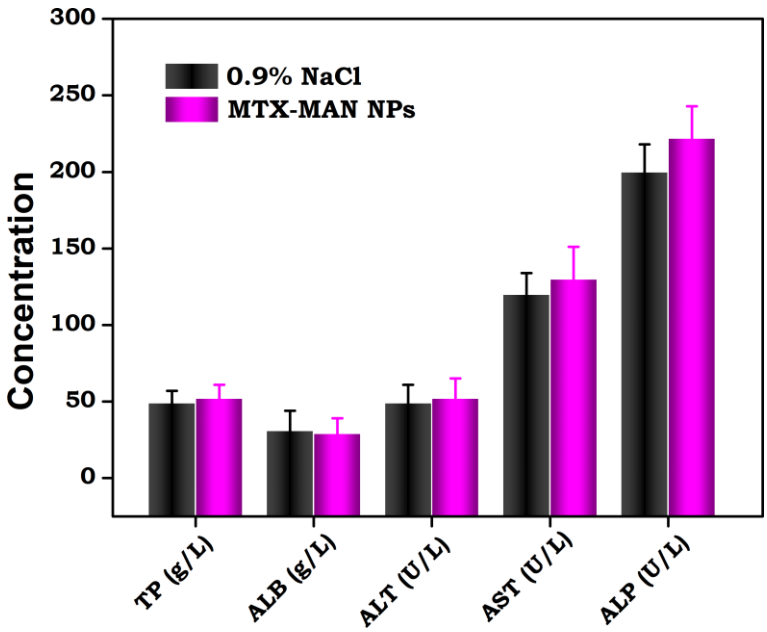

Figure S16. The serum biochemical indicators of liver functions gained from HeLa tumor-bearing nude mice at 21 days after *i. v.* injection with 0.9 NaCl% and MTX-MAN NPs. Error bars present SD (n = 5 per group).

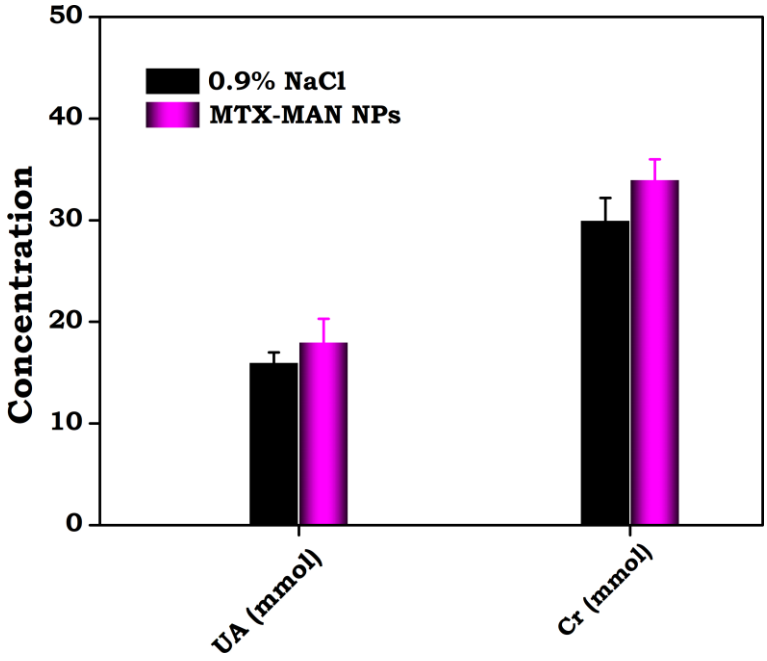

Figure S17. The serum biochemical indicators of kidney functions gained from HeLa tumor-bearing nude mice at 21 days after *i. v.* injection with 0.9 NaCl% and MTX-MAN NPs. Error bars present SD (n = 5 per group).

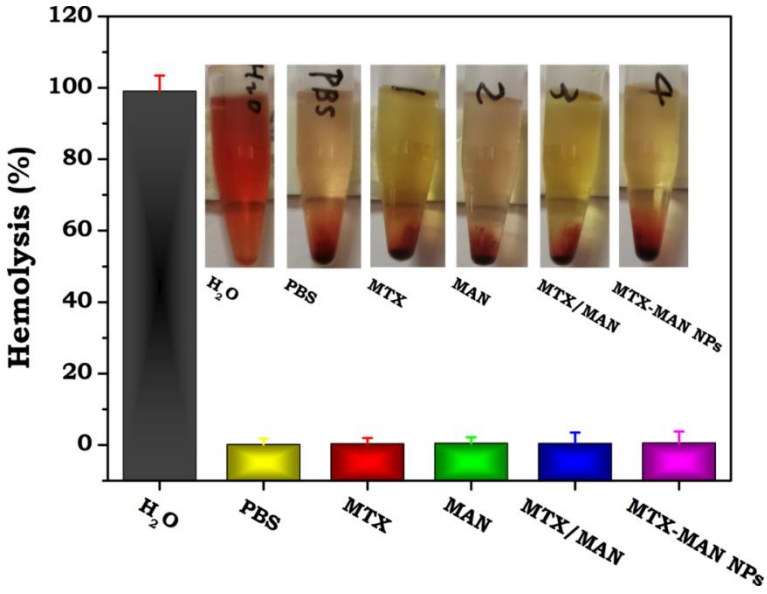

Figure S18. Hemolysis percentage of DI water, PBS (pH 7.4), MTX, MAN, MTX/MAN mixture, and MTX-MAN NPs. inset: photographs of DI water, PBS (pH 7.4), MTX, MAN, MTX/MTX-MAN mixture, and MTX-MAN NPs dispersed in red blood cells. Error bars present SD (n = 3).

References

[1] Li Y, Lin J, Ma J, et al. Methotrexate-Camptothecin Prodrug Nanoassemblies as a Versatile Nanoplatform for Biomodal Imaging-Guided Self-Active Targeted and Synergistic Chemotherapy[J]. ACS Appl Mater Interfaces, 2017, 9(40): 34650-34665.