Supporting Information

Self-assembly of folic acid dextran conjugates for cancer chemotherapy

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Materials and Methods

Materials: Dextran (DEX) with average molecular weight (Mw) 40 kDa was purchased from Sinopharm China (Shanghai, China). Chitosan oligosaccharide (CSO) with Mw 5 kDa was purchased from JinHu Crust Product Co., Ltd. Pullulan with Mw 100 kDa was purchased from Widely Co., Ltd. (Hubei, China). Hyaluronic acid (HA) with Mw 50 kDa, hydroxyethyl cellulose (HEC) with Mw 50 kDa, folic acid (FA, 99%), hydroxybenzotriazole (HOBT, 99%) and N-ethyl-N'-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDCI, 98%) were purchased from J&K Chemical Reagent Inc. (Shanghai, China). Doxorubicin (99%) was purchased from Beijing Huafenglianbo Technology Co., Ltd. (Beijing, China). All other chemicals were of analytical grade and used as received, except for dimethyl sulfoxide (DMSO) which was dried with 4 Å molecular sieves before use.

Synthesis and characterization of DEX-FA

DEX-FA was synthesized via esterification reaction between γ-carboxylic acid group of FA and hydroxyl group of DEX. Briefly, FA (1 g, 2.3 mmol) was dissolved in 20 mL of DMSO. To the solution, EDCI (1.32 g, 6.9 mmol), HOBT (0.93 g, 6.9 mmol), and DEX (1 g, 0.025 mmol) were added. The resulting mixture was then vigorously stirred at 45 ℃ for 48 hours. After that, the reaction mixture was cooled to room temperature, dialyzed against PBS buffer (6.7 mmol/L, pH 7.4) for three days (MWCO: 4500 Da), and lyophilized for further use. The structure of DEX-FA was confirmed by ^{1}H-NMR and FT-IR. ^{1}HNMR spectra were recorded on a nuclear magnetic resonance spectrometer (Ascend TM 600 MHZ, Bruker) by using tetramethylsilane (TMS) as an internal reference. FT-IR spectra were recorded on a flourier transform infrared spectrometer (Vertex70, Bruker) with an attenuate total reflection (ATR) accessory. The degree of substitution (DS) of FA in the synthesized DEX-FA was determined by ^{1}H-NMR and calculated as follow:

$$DS_{FA} = \frac{\frac{I_c \times 441}{\frac{I_{1-5}}{5} \times 162}}{} \times 100\% \qquad (1)$$

Where I_c is the integral for the protons of poison ring (as shown in Figure S1C) in FA, I_1-I_5 is the integral for the protons of I_1-I_5 in Dextran between 3.2 and 4.0 ppm. The DS of FA in the synthesized DEX-FA calculated by Eq. (1) is 46.4 wt%, corresponding to 79 FA

molecules per DEX.

Synthesis of CSO-FA

CSO-FA was synthesized via acylation reaction between γ-carboxylic acid group of FA and amino group of CSO. Briefly, FA (1 g, 2.3 mmol) was dissolved in 20 mL of DMSO. To the solution, EDCI (1.32 g, 6.9 mmol), HOBT (0.93 g, 6.9 mmol), and CSO (1 g, 0.2 mmol) were added. The resulting mixture was then vigorously stirred at 45 ℃ for 48 hours. After that, the reaction mixture was cooled to room temperature, dialyzed against PBS buffer for three days (MWCO: 4500 Da), and lyophilized for further use. The structure of CSO-FA was confirmed by ^{1}H-NMR and FT-IR. The DS of FA in the synthesized CSO-FA was determined by ^{1}H-NMR and calculated as follow:

$$DS_{FA} = \frac{\frac{I_c \times 441}{\frac{I_{1-6}}{6} \times 160}}{} \times 100\% \quad (2)$$

Where I_c is the integral for the protons of poison ring (as shown in Figure S2A) in FA, I_1-I_6 is the integral for the protons of I_1-I_6 in Dextran between 3.2 and 4.0 ppm. The DS of FA in the synthesized CSO-FA calculated by Eq. (2) is 36.2 wt%, corresponding to 7 FA molecules per CSO.

Synthesis of HA-FA

HA-FA was synthesized via esterification reaction between γ-carboxylic acid group of FA and hydroxyl group of HA. Briefly, FA (1 g, 2.3 mmol) was dissolved in 20 mL of DMSO. To the solution, EDCI (1.32 g, 6.9 mmol), HOBT (0.93 g, 6.9 mmol), and HA (1 g, 0.02 mmol) were added. The resulting mixture was then vigorously stirred at 45 ℃ for 48 hours. After that, the reaction mixture was cooled to room temperature, dialyzed against PBS buffer for three days (MWCO: 4500 Da), and lyophilized for further use. The structure of HA-FA was confirmed by ^{1}H-NMR and FT-IR. The DS of FA in the synthesized HA-FA was determined by ^{1}H-NMR and calculated as follow:

$$DS_{FA} = \frac{\frac{I_c \times 441}{\frac{I_{1-11}}{11} \times 379}}{} \times 100\% \quad (3)$$

integral for the protons of I_1-I_{11} in HA between 3.0 and 4.0 ppm. The DS of FA in the

synthesized HA-FA calculated by Eq. (3) is 30.7 wt%, corresponding to 50 FA molecules per HA.

Synthesis of Pullulan-FA

Pullulan-FA was synthesized via esterification reaction between γ-carboxylic acid group of FA and hydroxyl group of pullulan. Briefly, FA (1 g, 2.3 mmol) was dissolved in 20 mL of DMSO. To the solution, EDCI (1.32 g, 6.9 mmol), HOBT (0.93 g, 6.9 mmol), and pullulan (1 g, 0.01 mmol) were added. The resulting mixture was then vigorously stirred at 45 ℃ for 48 hours. After that, the reaction mixture was cooled to room temperature, dialyzed against PBS buffer for three days (MWCO: 4500 Da), and lyophilized for further use. The structure of pullulan-FA was confirmed by ^{1}H-NMR and FT-IR. The DS of FA in the synthesized pullulan-FA was determined by ^{1}H-NMR and calculated as follow:

$$DS_{FA} = \frac{\frac{I_c \times 441}{\frac{I_{1-18}}{18} \times 162}}{} \times 100\% \qquad (4)$$

Where I_c is the integral for the protons of poison ring (as shown in Figure S2C) in FA, $I_{1\text{-}18}$ is the integral for the protons of I_1-I_{18} in Pullulan between 3.2 and 4.0 ppm. The DS of FA in the synthesized Pullulan-FA calculated by Eq. (4) is 48.5 wt%, corresponding to 214 FA molecules per Pullulan.

Synthesis of HEC-FA

HEC-FA was synthesized via esterification reaction between γ-carboxylic acid group of FA and hydroxyl group of HEC. Briefly, FA (1 g, 2.3 mmol) was dissolved in 20 mL of DMSO. To the solution, EDCI (1.32 g, 6.9 mmol), HOBT (0.93 g, 6.9 mmol), and HEC (1 g, 0.02 mmol) were added. The resulting mixture was then vigorously stirred at 45 ℃ for 48 hours. After that, the reaction mixture was cooled to room temperature, dialyzed against PBS buffer for three days (MWCO: 4500 Da), and lyophilized for further use. The structure of HEC-FA was confirmed by ^{1}H-NMR and FT-IR. The DS of FA in the synthesized HEC-FA was determined by ^{1}H-NMR and calculated as follow:

$$DS_{FA} = \frac{\frac{I_c \times 441}{\frac{I_{1-6}}{6} \times 162}}{} \times 100\% \qquad (5)$$

Where I_c is the integral for the protons of poison ring (as shown in Figure S2D) in FA, I_{1-6} is the integral for the protons of C_1-C_6 in HEC between 3.2 and 4.0 ppm. The DS of FA in the synthesized HEC-FA calculated by Eq. (5) is 30.9 wt%, corresponding to 51 FA molecules per HEC.

Titration of DEX-FA conjugates

Acid-base titration was carried out to determine the pKa value of DEX-FA and the amount of titratable carboxylic acid groups. 2 mg DEX-FA conjugates were dissolved in 4 ml deionized water at pH 11 adjusted by 0.1 M NaOH, followed by sequentially adding 0.001 M HCl into the conjugates solution. After each addition of HCl, the pH value of the solution was recorded using a pH meter. By plotting pH value as a function of the volume of added HCl and differentiating the obtained plot, the pKa value of DEX-FA and the amount of titratable carboxylic acid groups in FA can be obtained.

Preparation and characterization of DEX-FA NPs

DEX-FA NPs were prepared by adjusting pH. Briefly, 1 mg DEX-FA was suspended in 1 mL of deionized water. To the suspension, NaOH solution was added to adjust the pH to 12.0. Then, the solution was stabilized for 5 minutes. Next, HCl solution was added into the mixture solution to adjust the pH to 7.4 in 1 second. DEX-FA NPs were thus obtained. The hydrodynamic diameter and count rate were measured by dynamic light scattering (DLS, Nano-ZS90, Malvern). The morphology of the prepared DEX-FA NPs at different pH buffer solutions were characterized by transmission electron microscopy (TEM, Tecnai G2, FEI, Holland) operated at an accelerating voltage of 100 KV. The TEM samples were prepared by placing a small drop of the prepared DEX-FA suspension onto a carbon-coated copper grid and dried at room temperature followed by negatively stained with phosphotungstic acid at the concentration of 2% (w/w) for 3 min.

The effect of FA grafting rate on DEX-FA self-assemble

DEX-FA, with 8.4 wt%, 12.7 wt%, 28.6 wt, 46.4 wt%, 53.7 wt% and 62.1 wt% grafting rate of FA, was suspended in PBS buffer to achieve 1 mg/mL DEX-FA solution, respectively. Then, NaOH was added to adjust the pH to 12.0. The solution was stabilized for 5 minutes. Next, HCl solution was added into the mixture solutions to adjust the pH to 7.4 in 1 second. The count rate and hydrodynamic diameter were measured by DLS.

The effect of pH on DEX-FA self-assembly

12.0 mg DEX-FA (FA grafting rate of 46.4 wt%) was suspended in 12.0 mL PBS buffer. The suspension was equally distributed into 6 groups. HCl or NaOH solution were added to adjust the pH to 5.7, 8.0, 9.0, 10.0, 11.0, respectively. The count rate was measured by DLS.

The effect of pH adjusting time on DEX-FA NPs

6.0 mg DEX-FA (FA grafting rate of 46.4 wt%) was suspended in 6.0 mL PBS buffer. The suspension was equally distributed into 3 groups. Then, NaOH was added to each group to adjust the pH to 12.0. The solutions were stabilized for 5 minutes. Next, HCl solution was added into the mixture solutions to adjust the pH back to 7.4 in 1, 3, and 10 seconds, respectively. The hydrodynamic diameter was measured by DLS.

The effect of DEX-FA concentration on DEX-FA NPs

DEX-FA (FA grafting rate of 46.4 wt%) was suspended in PBS buffer to achieve 0.5 mg/mL, 1.0 mg/mL, 2.0 mg/mL, 4.0 mg/mL, 8.0 mg/mL DEX-FA PBS buffer solution, respectively. Then, NaOH was added to adjust the pH to 12.0. The solutions were stabilized for 5 minutes. Next, HCl solution was added into the mixture solutions to adjust the pH to 7.4. The hydrodynamic diameter was measured by DLS.

The effect FA grafting rate on CSO-FA self-assemble

CSO-FA, with 8.2 wt%, 15.4 wt%, 21.8 wt, 32.6 wt%, 53.5 wt% and 68.0 wt% grafting rate of FA, was suspended in PBS buffer to achieve 1 mg/mL CSO-FA solution, respectively. Then, NaOH was added to adjust the pH to 12. The solutions were stabilized for 5 minutes. Next, HCl solution was added into the mixture solutions to adjust the pH to 7.4 in 1 second. The count rate and hydrodynamic diameter of each group were measured by DLS.

The effect of pH on CSO-FA self-assemble

12 mg CSO-FA (32.6 wt%) was suspended in 12 mL PBS buffer. The suspension was equally distributed into 6 groups. HCl or NaOH solution were added to adjust the pH to 5.7, 8.0, 9.0, 10.0, and 11.0, respectively. The count rate was measured by DLS.

Interaction between DOX and FA

1.4 mg DOX was dissolved in 14 mL PBS buffer to achieve 14 mL DOX solution

(DOX:100 μg/mL). Then, certain amounts of FA were dissolved in PBS buffer to achieve 200 μg/mL,150 μg/mL, 100 μg/mL, 70 μg/mL, 40 μg/mL, and 10 μg/mL FA solution, respectively. Next, FA solution with different concentration was mixed with the 100 μg/mL DOX solution at volume ratio of 1:1 (mL:mL), respectively. The absorbance of the each mixed sample was measured by UV/Vis spectrophotometer. To avoid the fluorescence quenching of DOX at high concentration, above-mentioned DOX and FA solutions were ten times diluted. FA solution with different concentration (20 μg/mL, 15 μg/mL, 10 μg/mL, 7 μg/mL, 4 μg/mL, 1 μg/mL) was mixed with the 10 μg/mL DOX solution at volume ratio of 1:1 (mL:mL). Emission spectra of each mixed sample was monitored by fluorescence spectrophotometer at the excitation wavelength of 480 nm.

Interaction between DOX and DEX-FA

A certain amount of DOX, DEX-FA, and DOX@DEX-FA were firstly selected and dissolved in 7.4 PBS buffer to achieve DOX, DEX-FA and DOX@DEX-FA PBS solution, respectively (DOX:16 μg/mL, DEX-FA:270 μg/mL, DOX@DEX-FA:286μg/mL). DOX concentration were kept the same in DOX group and DOX@DEX-FA group (DOX:16 μg/mL). The absorbance of each sample at pH 7.4 was measured by UV/Vis spectrophotometer. Emission spectra of each sample was monitored by fluorescence spectrophotometer at the excitation wavelength of 480 nm. Next, part of the solvents were taken out and HCl was added to adjust the pH to 5.5. The absorbance of each sample at pH 5.5 was measured by UV/Vis spectrophotometer. Emission spectra of each sample was monitored by fluorescence spectrophotometer at the excitation wavelength of 480 nm.

Preparation and characterization of DOX@DEX-FA NPs

DOX-loaded DEX-FA (DOX@DEX-FA) NPs were also prepared by adjusting pH. Firstly, 50.0 mg DEX-FA was suspended in 10.0 mL of deionized water. To the suspension, NaOH solution was added to adjust the pH to 9.0. DEX-FA solution was thus obtained. Then, 2.0 mL of DOX containing DMSO solution (DOX: 7.0 mg/mL) was added and the resulting mixture was sonicated for 5 min by using a probe-type ultrasonic device (Scientz-Ⅱ D, Ningbo Scientz Biotechnology Co., Ltd). Next, HCl solution was added into the mixture solution to adjust the pH to 7.4 in 1 second. The obtained solution was then dialyzed against PBS buffer (6.7 mmol/L, pH 7.4) for two days (MWCO: 14000 Da) to

remove the free DOX. The DOX@DEX-FA NPs were thus prepared. The hydrodynamic diameter and count rate were measured by DLS. The morphology of the prepared DEX-FA at different pH were characterized by TEM operated at an accelerating voltage of 100 KV. The TEM samples were prepared by placing a small drop of the prepared DOX@DEX-FA NPs suspension onto a carbon-coated copper grid and dried at room temperature followed by negatively stained with phosphotungstic acid at the concentration of 2% (w/w) for 3 min. The drug loading of DOX was quantified by UV/Vis spectrophotometer. Briefly, certain amounts of DOX@DEX-FA was dissolved in DMSO. The absorbance of DOX at 480 nm was used to determine DOX drug loading.

The effect of DOX to DEX-FA mass ratio on DOX@DEX-FA NPs

DEX-FA (46.4 wt%) was suspended in PBS buffer to achieve 1 mg/mL DEX-FA PBS suspension. The suspension was distributed into 5 groups. Then, NaOH was added to adjust the pH to 9.0. Subsequently, 0 mg, 0.05mg, 0.1 mg, and 0.2 mg DOX were added to each suspension, respectively. Next, the mixed solutions were sonicated for 5 min using a probe-type ultrasonic device. HCl solution was added into the mixture solution to adjust the pH to 7.4 in 1 second. The obtained solution was then dialyzed against PBS buffer (6.7 mmol/L, pH 7.4) for two days (MWCO: 14000 Da) to remove the free DOX. The DOX@DEX-FA was thus achieved. The Zeta potential and hydrodynamic diameter were measured by DLS. The drug loading of DOX was quantified by UV/Vis spectrophotometer. Briefly, certain amounts of DOX@DEX-FA was dissolved in DMSO. The absorbance of DOX at 480 nm was used to determine the drug loading of DOX.

In vitro drug release

The release behaviors of DOX from DOX@DEX-FA NPs were studied by using a dialysis method at 37 ℃, in pH 7.4 PBS buffer (10.0 mmol/L), pH 6.8 PBS buffer (10.0 mmol/L) and pH 5.5 PBS buffer (10.0 mmol/L). Briefly, 0.5 mL of DOX@DEX-FA NPs (DOX: 2.0 mg/mL) solution was placed in a dialysis tube (MWCO: 3500 Da), and the tube was immersed in 50.0 mL of release media and shaken at a speed of 150.0 rpm. At desired intervals, 1.0 mL of release media were taken out and replenished with equal volume of fresh media. The amount of DOX releasing was determined by UV/Vis spectrophotometer at 480 nm. The releasing experiments were conducted in triplicate. The whole releasing

process was shielded from light.

Stability of DOX@DEX-FA NPs

DOX@DEX-FA NPs in PBS buffer (pH 7.4, 6.7 mmol/L) (1mg/mL) and in FBS (10%) (1mg/mL) were incubated at 37 ℃ for 7 days. The diameter of each sample was measured by DLS every day.

Cell culture

The murine breast cancer cells 4T1 cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 100.0 U penicillin and100.0 μg/mL streptomycin at 37 ℃ in 5% CO_2. Human lung carcinoma cells A549 were cultured in RPMI 1640 medium supplemented with 10% FBS, 100.0 U penicillin and 100.0 μg/mL streptomycin at 37 ℃ in 5% CO_2.

Cellular uptake

4T1 and A549 cells were seeded on 12-well plates at a cell density of 10^5 cells per well. Each drug was tested in three wells. After incubating the cells with free DOX and DOX@DEX-FA NPs for 1 hour, the medium was removed. The cells were washed with PBS buffer (pH 7.4, 6.7 mM) for three times and trypsinized for cell counting followed by ultrasonication. The intracellular DOX were extracted with DMSO and measured by fluorescence spectrometer.

Quantification of intracellular DOX amount

To investigate the active targeting ability of DOX@DEX-FA NPs, 4T1 cells with overexpressed FRs were selected. A549 cells with no expression of FRs were used as a negative control. The amount of intracellular DOX was first measured. Briefly, 4T1 and A549 were seeded on 12-well plates at a cell density of 10^5 cells per well. Each drug was tested in three wells. After incubated the cells with free DOX, DOX@DEX-FA NPs for 1 hour, the medium was removed and the cells were washed with PBS buffer (pH 7.4, 6.7 mM) for three times and tripsinized for cell counting followed by ultrasonication. The intracellular DOX were extracted with DMSO and measured by fluorescence spectrometer. Competitive inhibition effect of FA on the uptake of DOX and DOX@DEX-FA NPs was studied as follows: 4T1 cells and A549 cells were pre-incubated with 200 μg/mL FA for 1 hour. Then the medium was removed, and the cells were incubated with DOX and

DOX@DEX-FA NPs (5.0 μg/mL as DOX) for 1 hour. The cells were collected and measured by fluorescence as follow.

Intracellular Trafficking

Intracellular trafficking was performed on 4T1 cancer cells. 4T1 cells were seeded on cell culture dishes with a glass bottom at a cell density of 2×10^5 cells per well and incubated at 37 ℃ overnight. After incubating the cells with 1 mL solution containing DOX or DOX@DEX-FA NPs (equivalent to 5 μg DOX/mL) for 0.5 hour, 1 hour and 2 hour, respectively, the medium was removed and the cells were washed with PBS buffer (pH 7.4, 6.7 mmol L^{-1}) three times. Then, the cells were fixed with 4% paraformaldehyde and stained with DAPI. The prepared samples were observed with confocal laser scanning microscopy (CLSM).

***In vitro* antitumor activity**

In vitro antitumor activity of different formulations against 4T1 and A549 was evaluated by using MTT assays. The cells harvested at the logarithmic growth phase were seeded on 96-well plates at a cell density of 5×10^3 cells per well. After incubating the cells with different formulations at various DOX concentrations for 48 hours, the medium was removed. Cells were washed with PBS buffer (pH 7.4, 6.7 mM) for three times and replenished with fresh medium. After that, 20 μL of MTT (5 mg/mL) was added to each well and incubated with the cells for 4 h. Then the medium was removed and 150 μL of DMSO was added to dissolve the formed crystals. The absorbance was determined at the wavelength of 492 nm by using a microplate reader (381C Microplate Reader).

Animal subjects

Female BALB/c mice were purchased from Hubei Research Center of Experimental Animals. All experimental procedures, the animal use and care protocols were carried out under a protocol approved by the Animal care and Use Committee of Huazhong University of Science and Technology.

Pharmacokinetic Study

Male Kunming mice (8-9 weeks of age) were randomly divided into two groups with three mice in each group. DOX and DOX@DEX-FA were administrated to each group via the tail vain at dose of 4 mg DOX/kg body weight. At desired intervals (5, 10, 30 min, 1, 2,

4, 8, 12, and 24 h), 150 μL blood samples were collected from the retro-orbital sinus and transferred to EDTA.2K-treated tubes. The blood samples were centrifuged at 3500 rpm for 10 min to obtain the plasma samples. 300 μL methanol was added to 30 μL plasma sample, which was sonicated for 5 min, and then the samples were further centrifuged at 4000 rpm for 5 min. 250 μL supernatant was taken and sheltered from light at room temperature overnight to remove methanol. Then, 100 μL DMSO was added to the dried sample, which was further sonicated for 5 min to dissolve all extracted DOX. The fluorescence intensity of DOX was measured at 485/590 nm using a microplate reader (FlexStation 3, Molecular Devices, USA). With the measured fluorescence intensity, the concentration of DOX in each plasma sample was calculated according to the standard curve.

***Ex vivo* NIR fluorescence imaging and biodistribution**

4T1 tumor-bearing mice were randomly divided into 3 groups, with 3 mice in each group. Free DOX, DOX@DEX-FA NPs and FA+DOX@DEX-FA NPs were injected to each group by intravenous administration (equivalent to 4 mg/kg DOX body weight). In the FA+DOX@DEX-FA NPs group, FA (equivalent to 2 mg/kg FA body weight) was firstly injected by intravenous administration 2 hours before injection of DOX@DEX-FA NPs. The biodistribution of DOX and DOX@DEX-FA was investigated by using DOX Channel *ex vivo* imaging. Mice were sacrificed 48 hours post administration. Then tumors and the major organs including heart, liver, spleen, lung and kidney were harvested for *ex vivo* fluorescence imaging. As for mice injected with free DOX, DOX@DEX-FA NPs and FA + DOX@DEX-FA NPs, the fluorescence images were collected on DOX channel (excitation at 500 nm, emission ranging from 550-650 nm).

DOX biodistribution quantification

4T1 tumor-bearing mice were randomly assigned to 3 groups, with 3 mice in each group. Free DOX, DOX@DEX-FA NPs, and FA+DOX@DEX-FA NPs were injected to each group by intravenous administration (equivalent to 4 mg/kg DOX body weight). In the FA+DOX@DEX-FA NPs group, FA (equivalent to 2 mg/kg FA body weight) was firstly injected by intravenous administration 2 hours before injection of DOX@DEX-FA NPs. Mice were sacrificed in 48 hours post administrated, and major organs including heart, liver

spleen, lung, kidney, and tumors were harvested and homogenized. Then, the DOX of each group was extracted with DMSO. The extracted DOX was quantified by fluorescence spectrometer.

***In vivo* antitumor effect**

Female BALB/c mice (20-22 g) were inoculated subcutaneously with 4T1 tumor cells (each with 1×10^6 4T1 tumor cells) on the right lower back. When the 4T1 tumor volumes reached about 0.1 cm^3, mice were randomly assigned to 5 groups (n=6) and intravenously injected with PBS, free DEX-FA, free DOX, FA+DOX@DEX-FA NPs and DOX@DEX-FA NPs (4.0 mg/kg DOX, 67.4 mg/kg DEX-FA). The FA+DOX@DEX-FA group was 2 hours pre-injected with FA (2.0 mg/kg). The second and third administrations with the same procedure were conducted 3 days and 6 days after the first injection, respectively. Tumor volume of mice from each group were measured every 2 days. At the end of the test, mice from each group were sacrificed. The blood samples were harvested for serological analysis. The tumors were weighted, imaged and analyzed by H&E staining and Ki67 staining. The organs, including heart, liver, spleen, lung and kidneys, were also harvested and analyzed by H&E staining.

Chronic toxicity evaluation *in vivo*

At the end of the experiments, the blood samples were harvested for serological analysis. The organs including heart, liver, spleen, lung, and kidney were also harvested and analyzed by H&E staining.

Mice *in vivo* lifetime

Female BALB/c mice (20-22 g) were inoculated subcutaneously with 4T1 tumor cells (each with 1×10^6 4T1 tumor cells) on the right lower back. When the 4T1 tumor volumes reached about 0.3 cm^3, mice were randomly assigned to 5 groups (n=6) and intravenously injected with PBS, free DEX-FA, FA+DOX@DEX-FA NPs, free DOX and DOX@DEX-FA NPs (4.0 mg/kg DOX, 67.4 mg/kg DEX-FA). The FA+DOX@DEX-FA group was 2 hours pre-injected with FA (0.5 mg/kg). The second, third and fourth administrations with the same procedure were conducted 4, 7 and 11 days after the first injection, respectively. Tumor volume of mice from each group were measured every 2 days. Mice lifetime were not recorded until the last mice was dead.

Statistical analysis

All data was presented as the mean value ± SEM. Statistical analysis was performed by Statistical Product and Service Solutions (SPSS) with independent samples T-test. Statistical significance was established at $P < 0.05$. ↓ $P < 0.05$, ↓↓ $P < 0.01$, ↓↓↓ $P < 0.001$.

Supplementary Figures

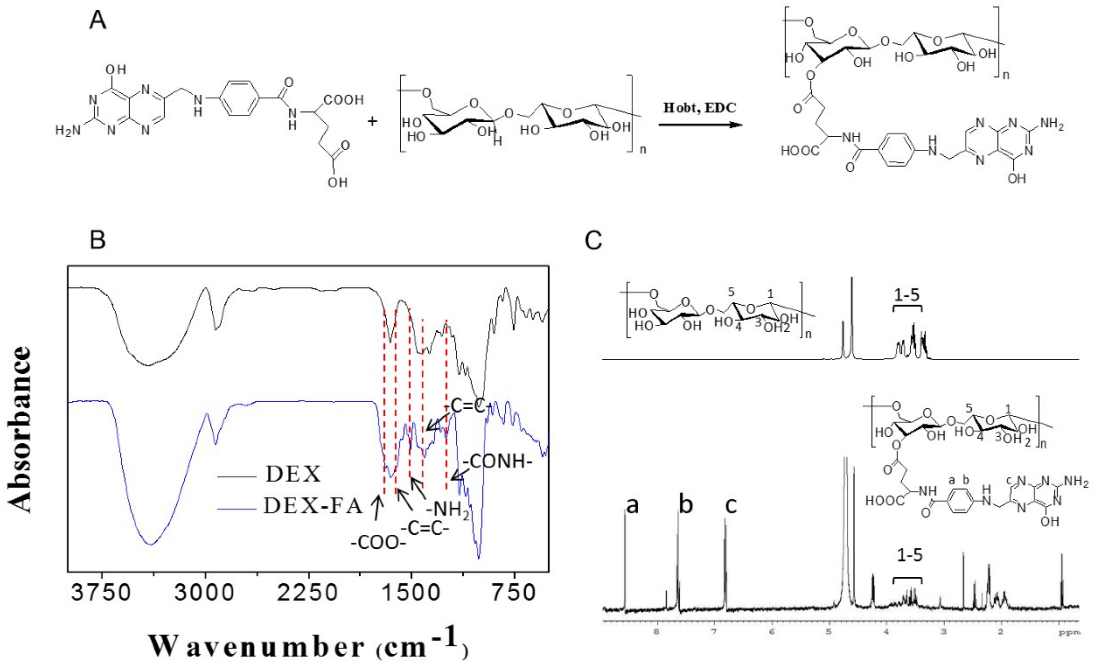

Figure S1. (A) Synthetic route of DEX-FA; (B) FTIR of DEX and DEX-FA; (C) ^{1}H-NMR of DEX and DEX-FA.

DEX-FA was synthesized by grafting FA onto DEX through ester bond, as shown in Figure S1. The successful synthesis of DEX-FA was validated by FT-IR spectra and ^{1}H-NMR. The FT-IR spectra of DEX-FA show the characteristic band of C=O stretching vibration of ester bond of DEX-FA at 1731 cm^{-1}, Figure S1B. The characteristic band of -C=C- stretching vibration of FA at 1610 cm^{-1} and 1410 cm^{-1}, the characteristic band of -NH_2 of FA at 1510 cm^{-1} and -CONH- stretching vibration of FA at 1255 cm^{-1}, indicating the successful grafting of FA onto DEX. The characteristic resonance signals of FA at 8.5 (a), 7.6 (b), and 6.8 ppm (c) appear in ^{1}H-NMR spectra of DEX-FA, Figure S1C, indicating the successful grafting of FA onto DEX. The grafting rate of FA of DEX-FA is determined by

^{1}H-NMR, is 46.4 wt%. The ^{1}H-NMR and FT-IR spectra consistently reveal that DEX-FA are successfully synthesized with well-defined chemical structure.

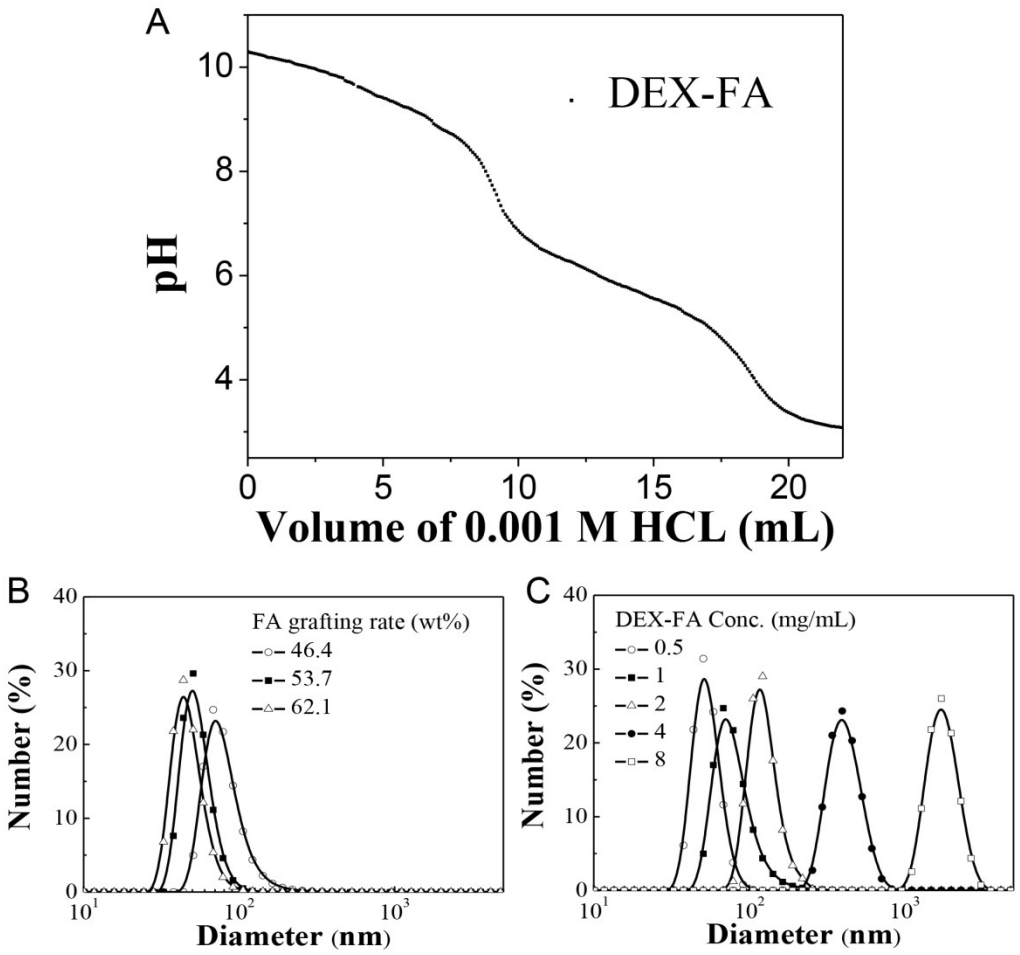

Figure S2. (A) Titration curve of DEX-FA; (B) Size distribution of DEX-FA NPs in condition of different FA grafting rates as measured by DLS; (C) Size distribution of DEX-FA NPs in condition of different DE-FA NPs Concentration.

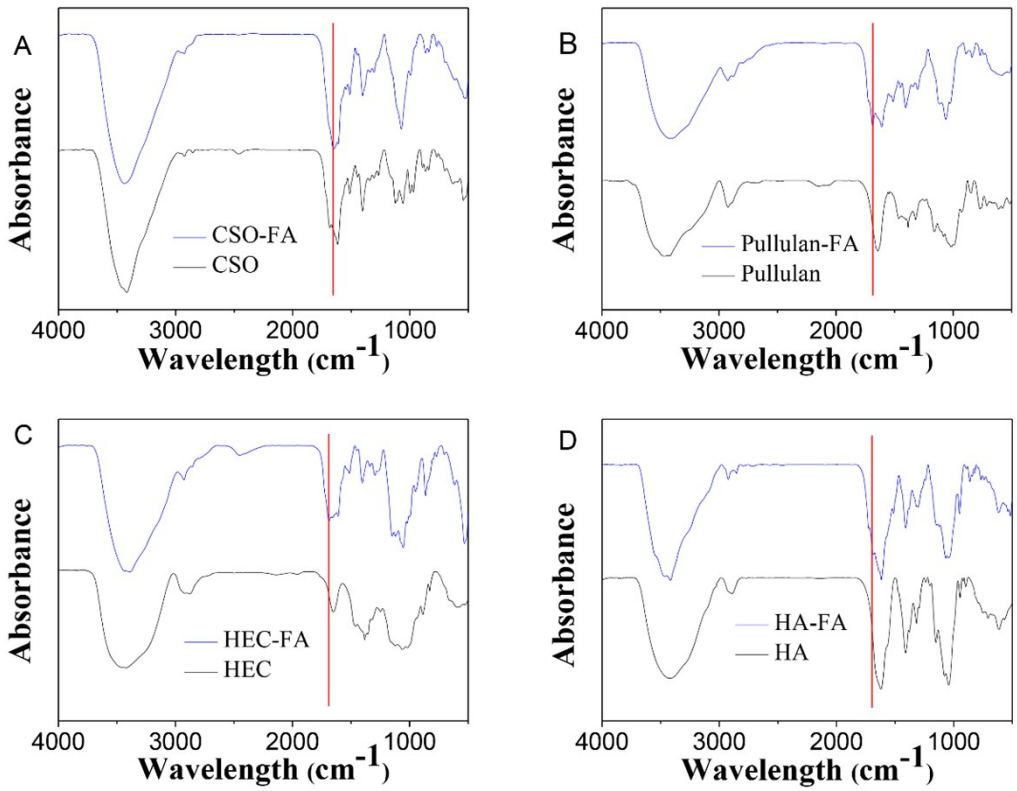

Figure S3. (A) FTIR of CSO and CSO-FA; (B) FTIR of HA and HA-FA; (C) FTIR of pullulan and pullulan-FA; (D) FTIR of HEC and HEC-FA.

The successful synthesis of CSO-FA, HA-FA, Pullulan-FA and HES-FA were validated by FTIR and ^{1}H-NMR. As shown in Figure S3A, a new peak appeared in 1650 cm^{-1} was ascribed to -CONH- flexural vibration of CSO-FA, indicating the FA was successfully grafted onto CSO. In Figure S3B, Figure S3C and Figure S3D, new peaks appeared in 1720 cm^{-1} were ascribed to -COO- flexural vibration of Pullulan-FA, HEC-FA, and HA-FA, indicating FA were successfully grafted onto Pullulan, HEC and HA. The successful synthesis were further verified by ^{1}H-NMR (Figure S4A, S4B, S4C, S4D), the characteristic resonance signals of FA at 8.5 (a), 7.6 (b), and 6.8 ppm (c) appear in ^{1}H-NMR spectra of CSO-FA, pullulan-FA, HA-FA and HEC-FA. The grafting rate of CSO-FA, HA-FA, pullulan-FA and HEC-FA were calculated by ^{1}H-NMR, was 36.2 wt%, 30.7 wt%, 48.5 wt%, 30.9 wt%, respectively.

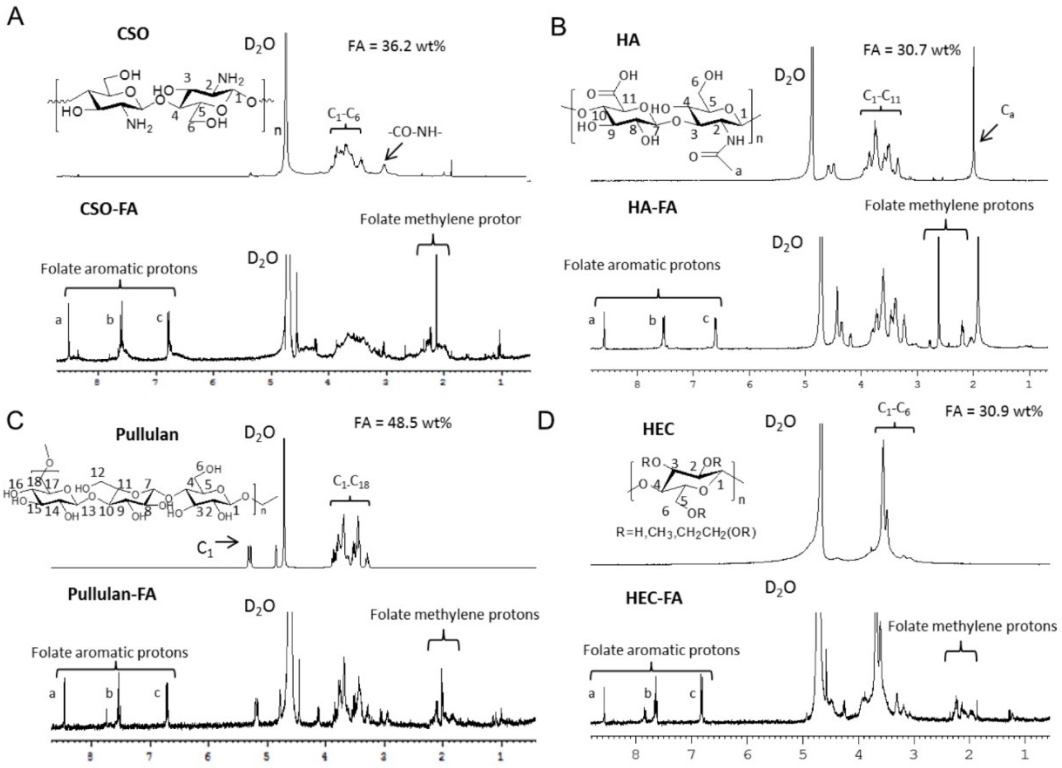

Figure S4. (A) ^{1}H-NMR of CSO and CSO-FA; (B) ^{1}H-NMR of HA and HA-FA; (C) ^{1}H-NMR of pullulan and pullulan-FA; (D) ^{1}H-NMR of HEC and HEC-FA.

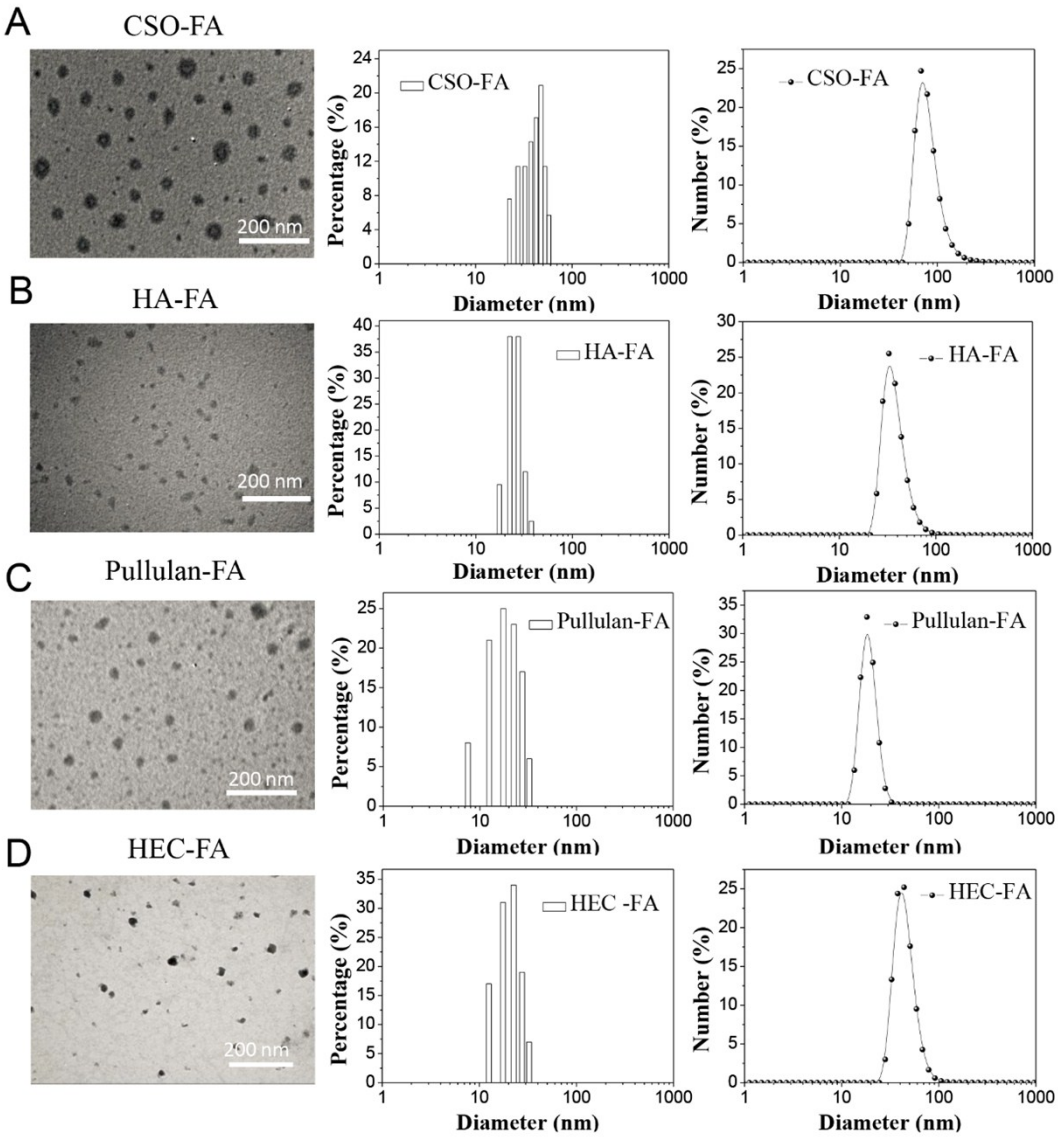

Figure S5. FA-guided self-assembly of CSO-FA, HA-FA, Pullulan-FA and HEC-FA NPs. (A) TEM image, TEM diameter statistics and diameter distribution of CSO-FA NPs fabricated by adjusting pH. (B) TEM image, TEM diameter statistics and diameter distribution of HA-FA NPs fabricated by adjusting pH. (C) TEM image, TEM diameter statistics and diameter distribution of Pullulan-FA NPs fabricated by adjusting pH. (D) TEM image, TEM diameter statistics and diameter distribution of HEC-FA NPs fabricated by adjusting pH. The scale bar is 200 nm.

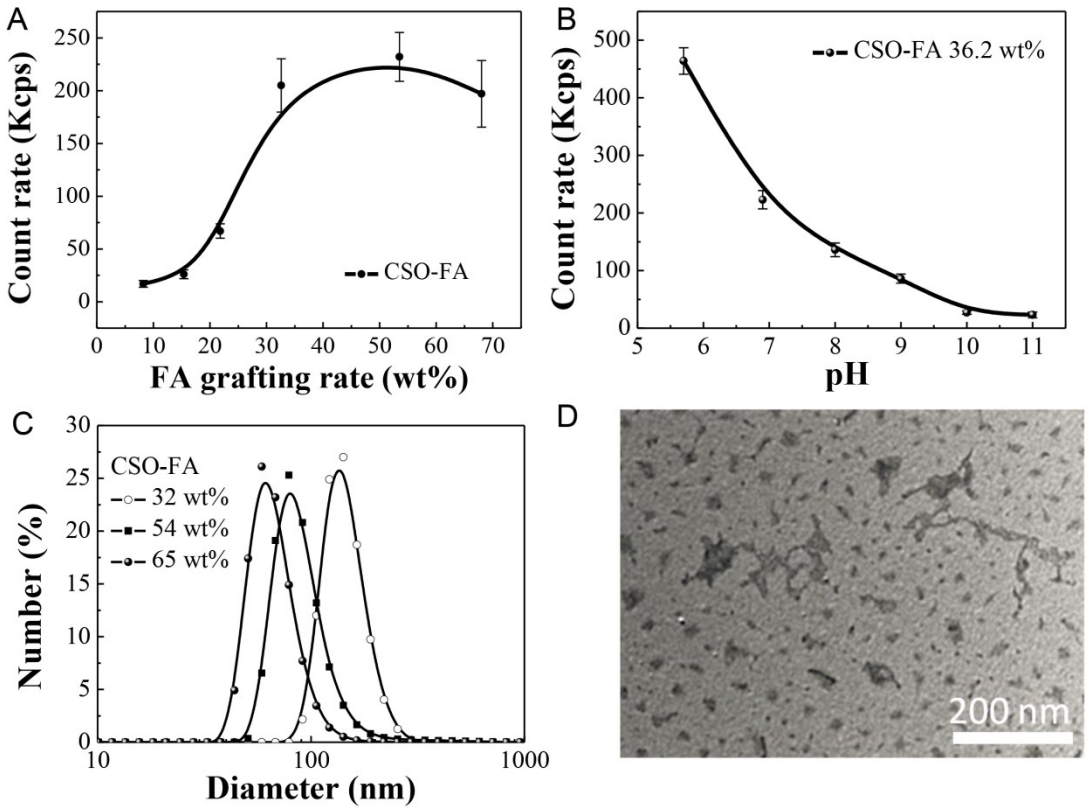

Figure S6. (A) Count rate of DEX-FA NPs fabricated by adjusting pH in condition of different FA grafting rate; (B) Count rate of CSO-FA at different pH; (C) Diameter distribution of CSO-FA NPs in condition of different FA grafting rates; (D) TEM image of CSO-FA in pH 9.0. Data is presented as mean ± SEM, n=3. The scale bar is 200 nm.

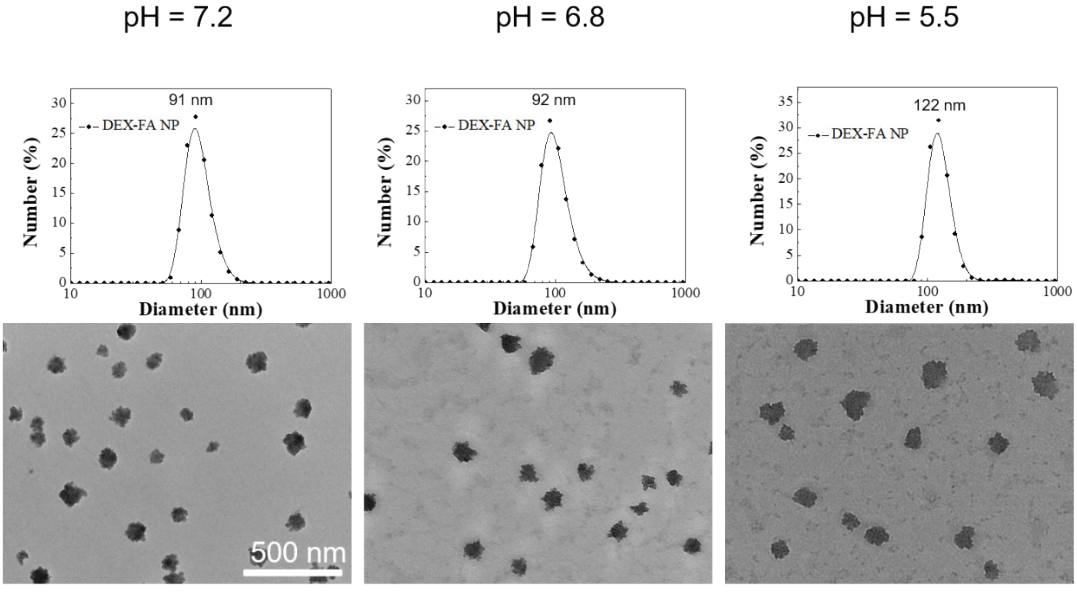

Figure S7. Size distribution, as measured with DLS, and TEM images of DEX-FA NPs

fabricated by adjusting pH from 9.0 to 7.2, pH from 7.2 to 6.8, and pH from 7.2 to 5.5. The scale bar is 500 nm and applied for all images. Monodispersed nanoparticles are obtained at three different pH, and the diameter does not change significantly from pH 7.2 to 6.8 but increase around 30 nm when pH is decreased from 7.2 to 5.5. As pH 5.5 is lower than the pKa value of DEX-FA, more DEX-FA conjugates are needed to form stable nanoparticles.

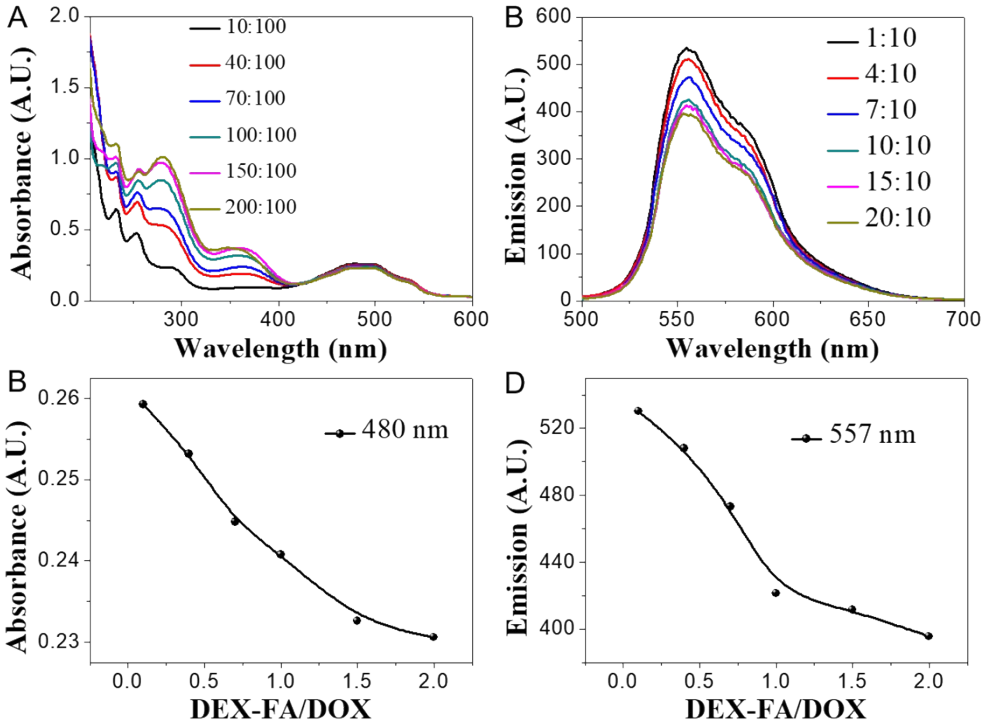

Figure S8. Interactions between DEX-FA and DOX. (A) UV-Vis spectra of DEX-FA and DOX with varied DEX-FA/DOX ratio (w/w). (B) Fluorescence of DEX-FA and DOX with varied DEX-FA/DOX ratio (w/w). (C) Absorbance of DEX-FA and DOX at 480 nm with varied DEX-FA/DOX ratio (w/w). (D) Emission FA and DOX at 577 nm with DEX-FA/DOX ratio (w/w).

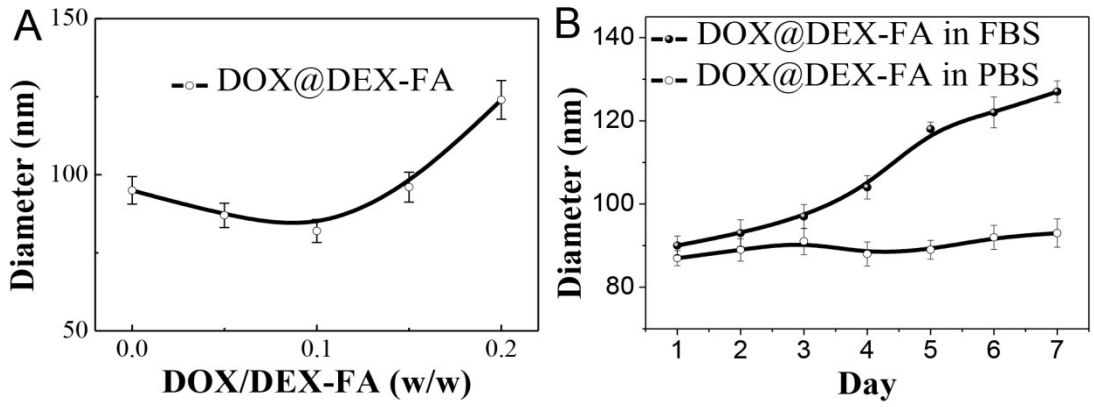

Figure S9. (A) Diameter of DOX@DEX-FA NPs in the condition of different DOX/DEX-FA (w/w) as measured by DLS; (B) Diameter variation of DOX@DEX-FA NPs in PBS buffer and 10% FBS in 7 days as measured by DLS. Data is represented as mean ± SEM, n=3.

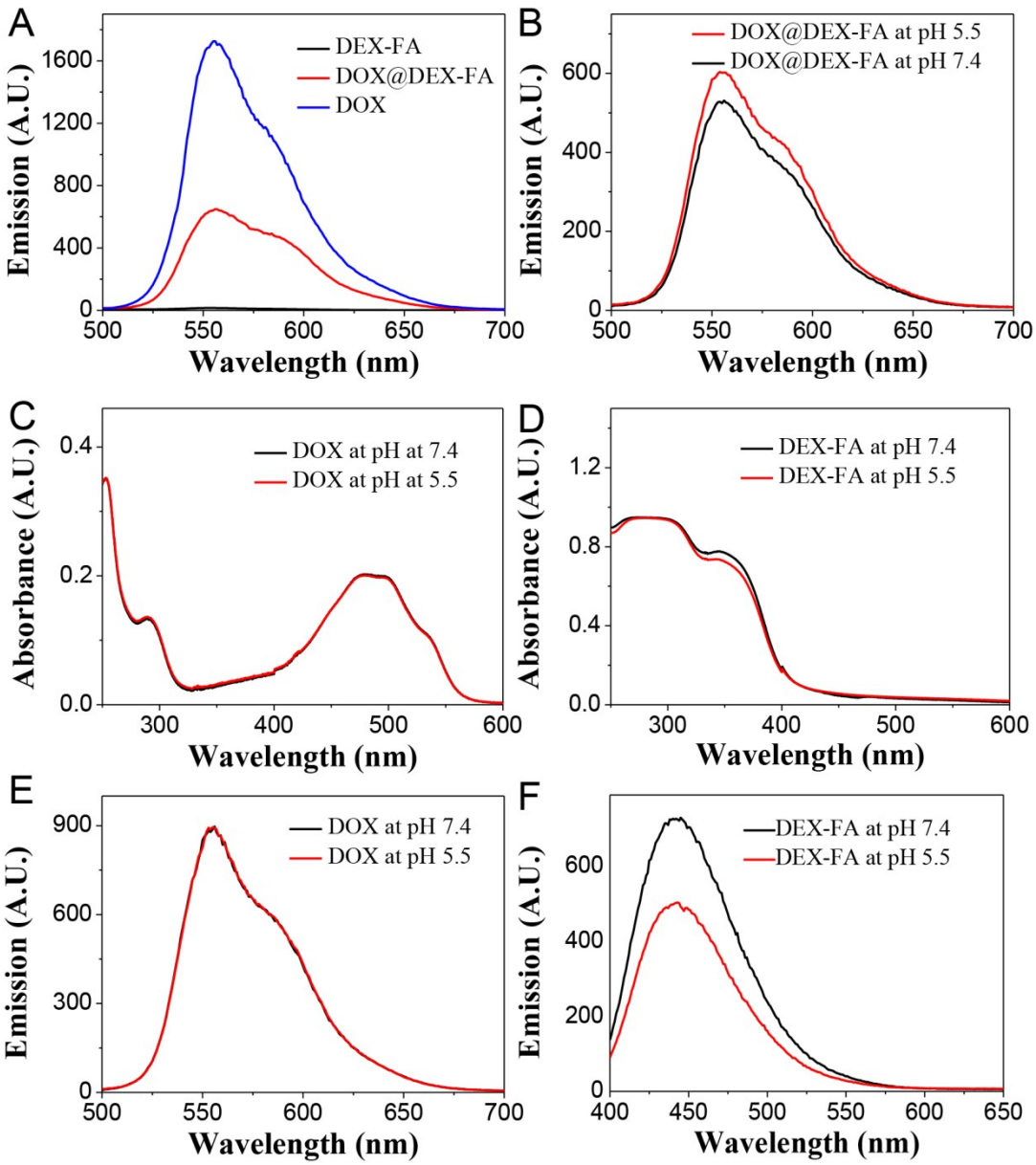

Figure S10. Interactions between DEX-FA and DOX. (A) Fluorescence spectra of DEX-FA NPs, DOX@DEX-FA NPs and DOX in deionized water at pH 7.4; (B) Fluorescence spectra of DOX@DEX-FA NPs in deionized water at pH 7.4 and 5.5; (C) UV-Vis spectra of DOX in deionized water at pH 7.4 and 5.5; (D) UV-Vis spectra of DEX-FA in deionized water at pH 7.4 and 5.5; (E) Fluorescence spectra of DOX in deionized water at pH 7.4 and 5.5; (F) Fluorescence spectra of DEX-FA in deionized water at pH 7.4 and 5.5.

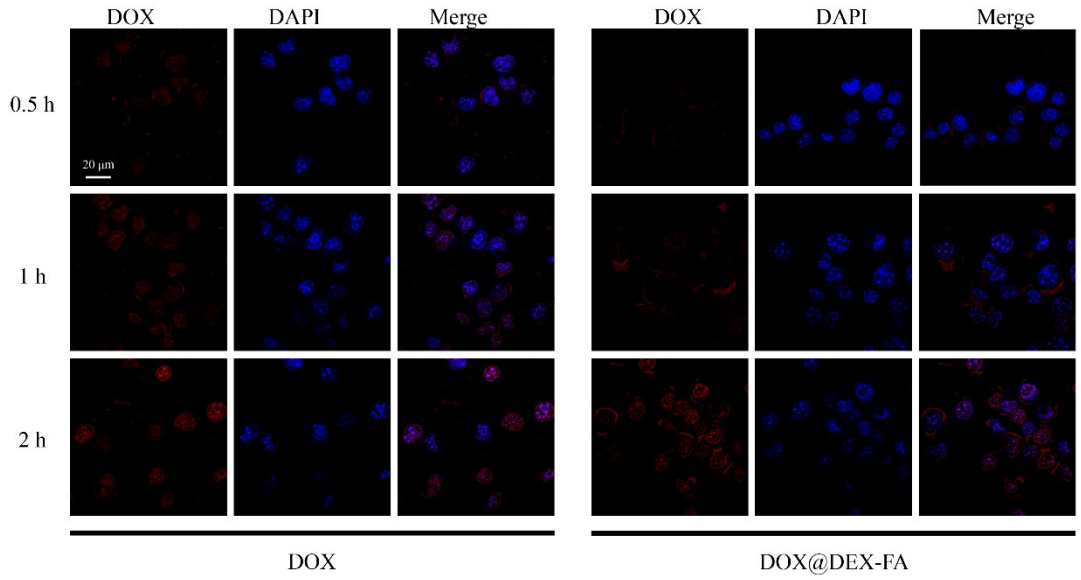

Figure S11. Intracellular trafficking of DOX and DOX@DEX-FA as a function of time. The scale bar is 20 μm and applied for all images.

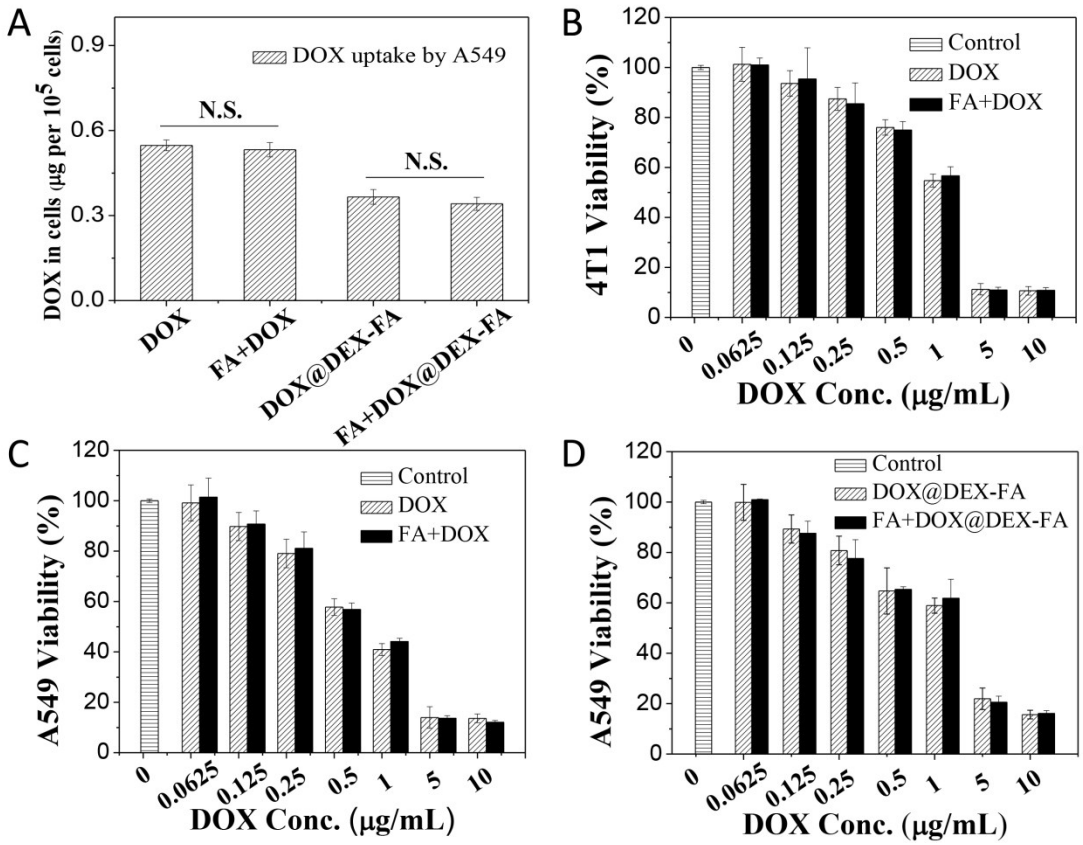

Figure S12. FA-mediated cellular uptake and in vitro cytotoxicity of DOX@DEX-FA NPs. (A) Cellular uptake of DOX by A549 cell (DOX: 5 μg/mL, FA: 200 μg/mL) for 1 hour; (B) *In vitro* cytotoxicity of different groups against 4T1 cells as a function of DOX concentration

after incubating for 48 hours (DOX: 5 μg/mL, FA 200 μg/mL); (C) In vitro cytotoxicity of different groups against A549 cells as a function of DOX concentration after incubating for 48 hours (DOX: 5 μg/mL, FA 200 μg/mL); (D) In vitro cytotoxicity of different groups against A549 cells as a function of DOX concentration after incubating for 48 hours (DOX: 5 μg/mL, FA 200 μg/mL).

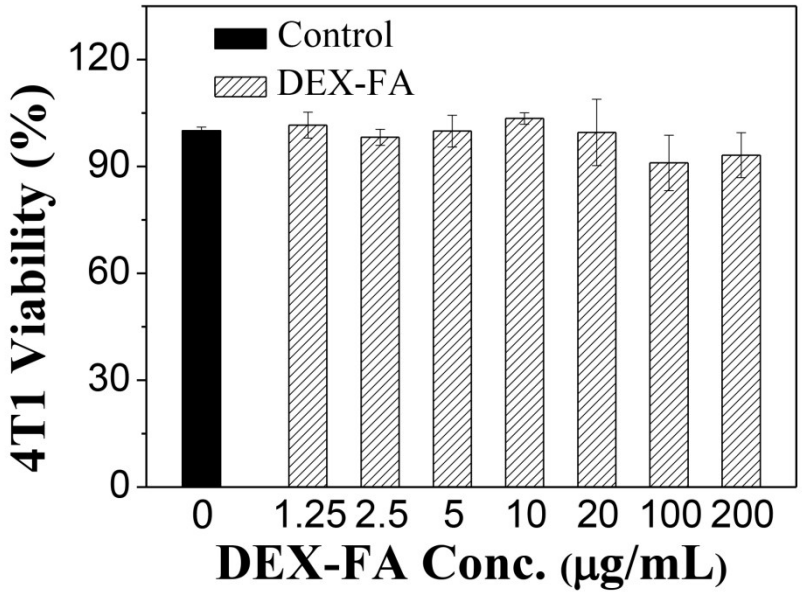

Figure S13. MTT assay of DEX-FA as a function of DEX-FA concentrations. Data is presented as mean ± SEM, n=3.

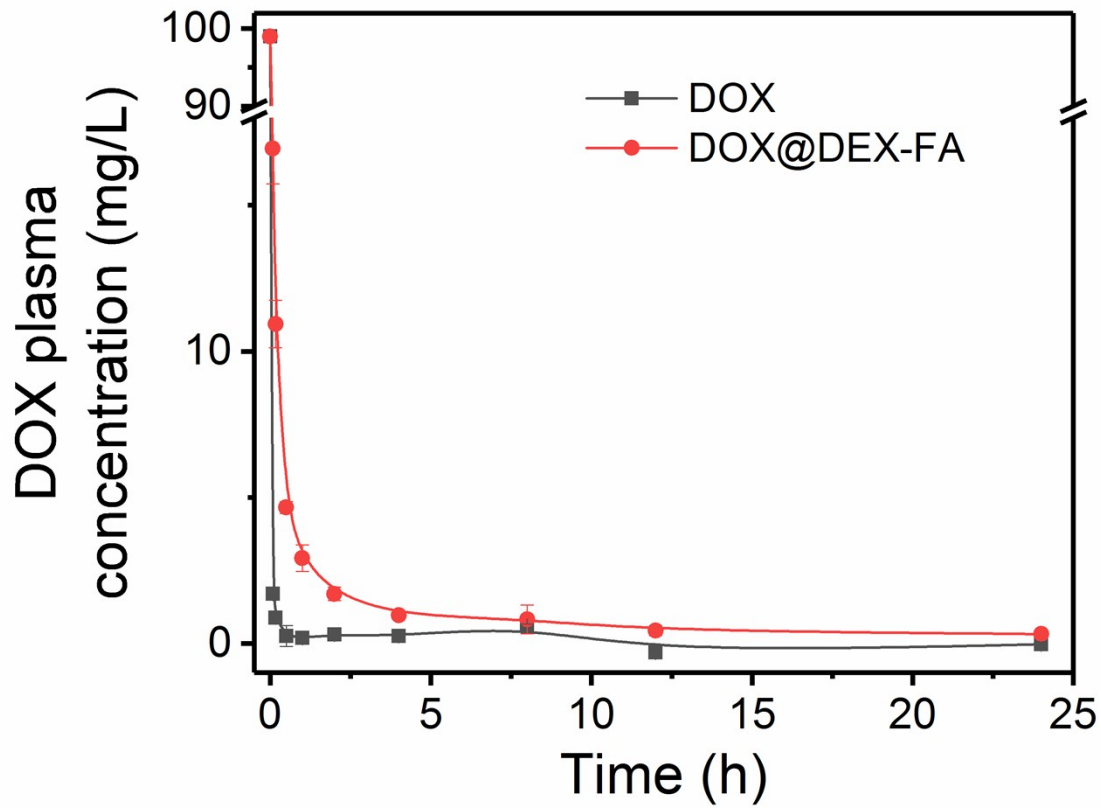

Figure S14. Mean plasma concentration of DOX after administration of DOX and DOX@DEX-FA NPs. Data represent the mean ± SEM, (n=3).

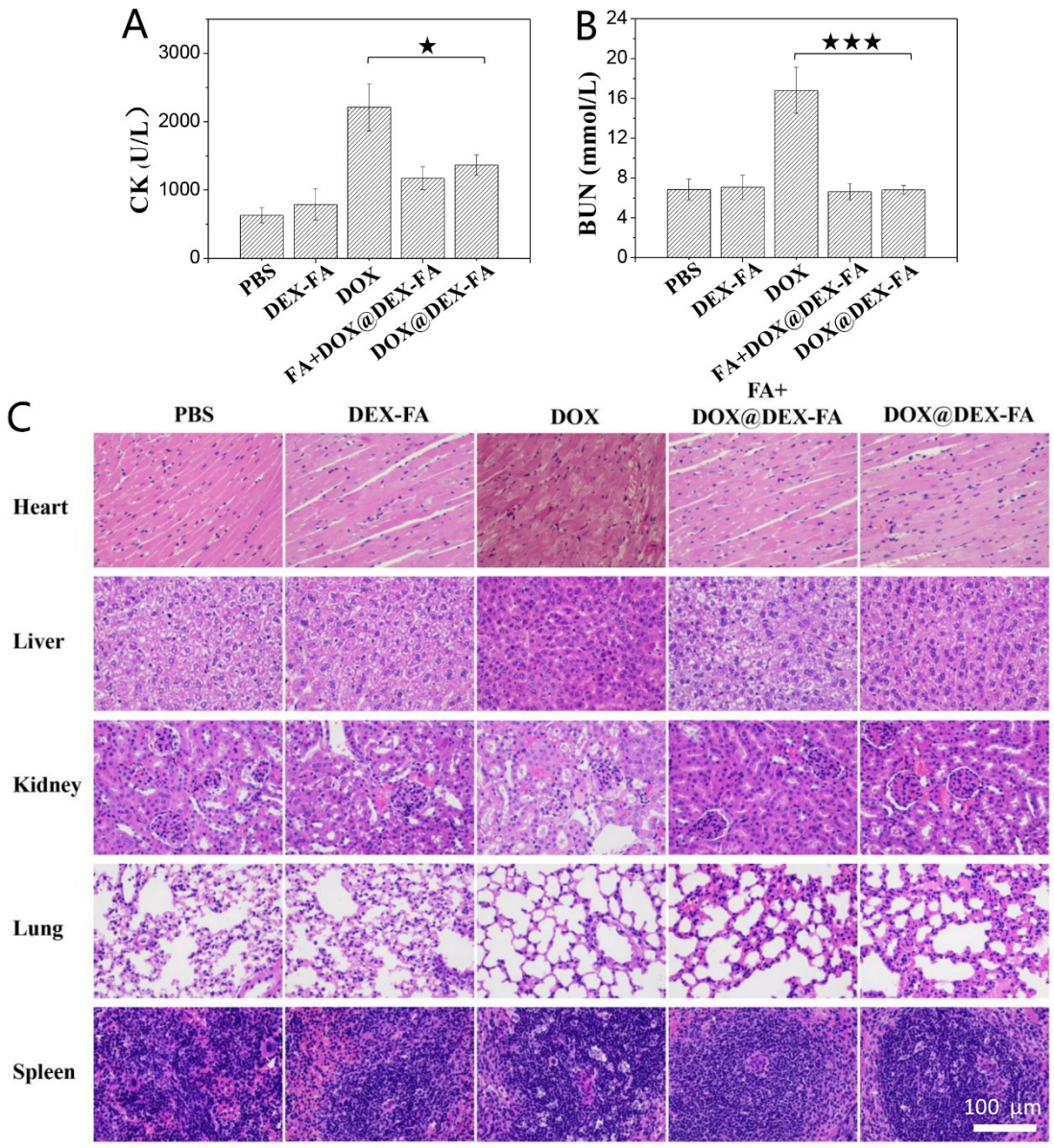

Figure S15. Acute toxicity analysis. (A) CK and (B) BUN level of different groups. Data is presented as mean ± SEM, n=6. 🡻 $P < 0.05$, 🡻🡻🡻 $P < 0.001$. (C) H&E staining of heart, liver, spleen, lung, and kidneys of mice from different groups (PBS, DEX-FA, DOX, FA+DOX@DEX-FA, DOX@DEX-FA NPs) (DOX: 4 mg/kg body weight, FA: 2 mg/kg body weight). The scale bar is 100 μm and applied for all images in (C).

Table S1. Diameter and Zeta potential of DEX-FA, CSO-FA, Pullulan-FA, HA-FA and HEC-FA NPs.

	DEX-FA	CSO-FA	Pullulan-FA	HA-FA	HEC-FA
Diameter (nm)	67±2.1	86±4.5	36±3.7	45±1.9	53±2.7
Zeta potential (mv)	-26±1.4	-21±0.7	-16±1.2	-19±2.3	-21±2.4

Table S2. Characterization of DOX@DEX-FA NPs with the variation weight ratio of DOX/DEX-FA.

DOX/DEX-FA (w/w)	**0**	**0.05**	**0.1**	**0.15**	**0.2**
Diameter (nm)	95±4.4	87±3.9	82±3.7	96±4.8	124±6.2
Drug loading (wt%)	0	3.6±0.3	5.6±0.2	6.8±0.4	7.2±1.1
Zeta potential (mv)	-24±1.9	-21±1.6	-17±1.8	-15±1.2	-12±0.8

Table S3. IC_{50} (μg/mL) of DOX, FA+DOX, DOX@DEX-FA, and FA+DOX@DEX-FA.

IC_{50} (μg/mL)	**4T1**	**A549**
DOX	1.180	0.887
FA+DOX	1.196	0.875
DOX@DEX-FA	1.030	1.328
FA+DOX@DEX-FA	1.733	1.259

Table S4. Pharmacokinetic parameters of DOX after administration of DOX and DOX@DEX-FA.

Parameters	DOX	DOX@DEX-FA
Dosage (mg DOX/kg)	4	4
$t_{1/2}$ (h)	4.45±0.57	4.40±0.32
CL (L/h/kg)	0.87±0.10	0.16±0.02 [a]
$AUC_{(0\rightarrow\infty)}$ (mg·h/L)	4.68±0.53	23.27±2.16 [a]

Note: $t_{1/2}$: half-life time; CL: clearance rate; AUC: area under the curve. a $p<0.005$ compared to DOX group.

Table S5. Median survival day of 4T1-bearing mice after receiving different formulations.

	PBS	**DEX-FA**	**DOX**	**FA+DOX@DEX-FA**	**DOX@DEX-FA**
Median survival (day)	30	32	33	34	39