

Electronic Supplementary Information

Synthesis of Functionalized Dipeptide for Targeted Delivery and pH Sensitive Release of Chemotherapeutics

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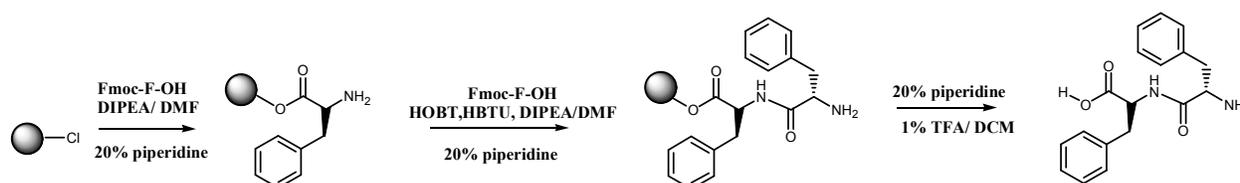
These authors contributes equally to this work

1. General method and results

Doxorubicin was purchased from Sangong Biotech Inc. (Shanghai, China). All the chemicals used in this study were analytical reagents obtained from commercial sources and were used without further purification. An Electron Spray Ionization (ESI) mass spectrum was recorded by Finnigan LCQ Advantage ion trap mass spectrometer (Thermo Fisher Corporation) that was equipped with a standard ESI source. ¹H NMR spectra were recorded by a Bruker Model AV 300 system. In order to record fluorescence spectra, an F-4600 fluorescence spectrophotometer (Hitachi High-Technologies Corporation, Japan) was used. An IX71 fluorescence microscope (Olympus, Japan) was used for cell imaging. Transmission electron microscopic (TEM) images were acquired by a Hitachi H7650 transmission electron microscope with a CCD imaging system at an acceleration voltage of 120 kV.

Syntheses and Characterizations of FF

Scheme S1. Scheme S1. The synthetic route for FF



Synthesis of L: Compound Phe-Phe (FF) was synthesized by solid phase peptide synthesis (SPPS) method using 2-chlorotrityl chloride resin and the corresponding Fmoc-protected amino acids with side chains properly protected. The first amino acid was loaded onto the resin at about 0.5 mmol/g of resin. Subsequently, the capping reagent (DCM: MeOH: DIPEA = 17: 2: 1) was used to ensure all the active sites of the resin were protected. The solution of 20% piperidine in DMF was used to remove the Fmoc group, the next Fmoc-protected amino acid was coupled to the free amino group using HBTU as the coupling reagent. The growth of the peptide chain followed the established Fmoc SPPS protocol. Compound FF was purified by HPLC. ¹H NMR (400 MHz, DMSO) δ 8.89, 8.09, 7.95, 7.42 – 7.16, 4.52, 4.04, 3.16 – 3.07, 2.98, 2.93, 2.55, 2.50 (Figure S1). MS: calculated for 312.15, obsvd. ESI-MS: m/z 313.16. (Figure S3).

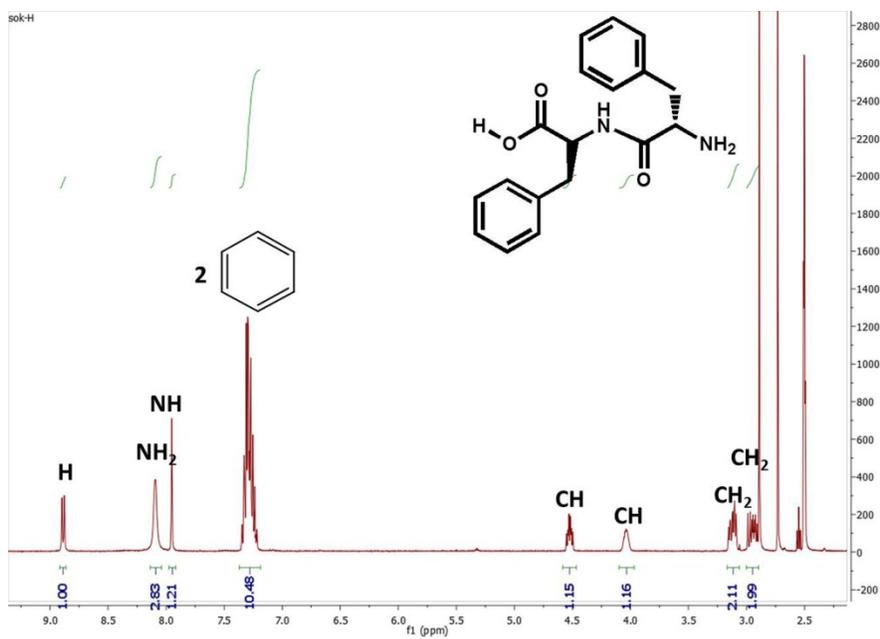


Figure S1. ¹H NMR spectrum of FF in *d*₆-DMSO.

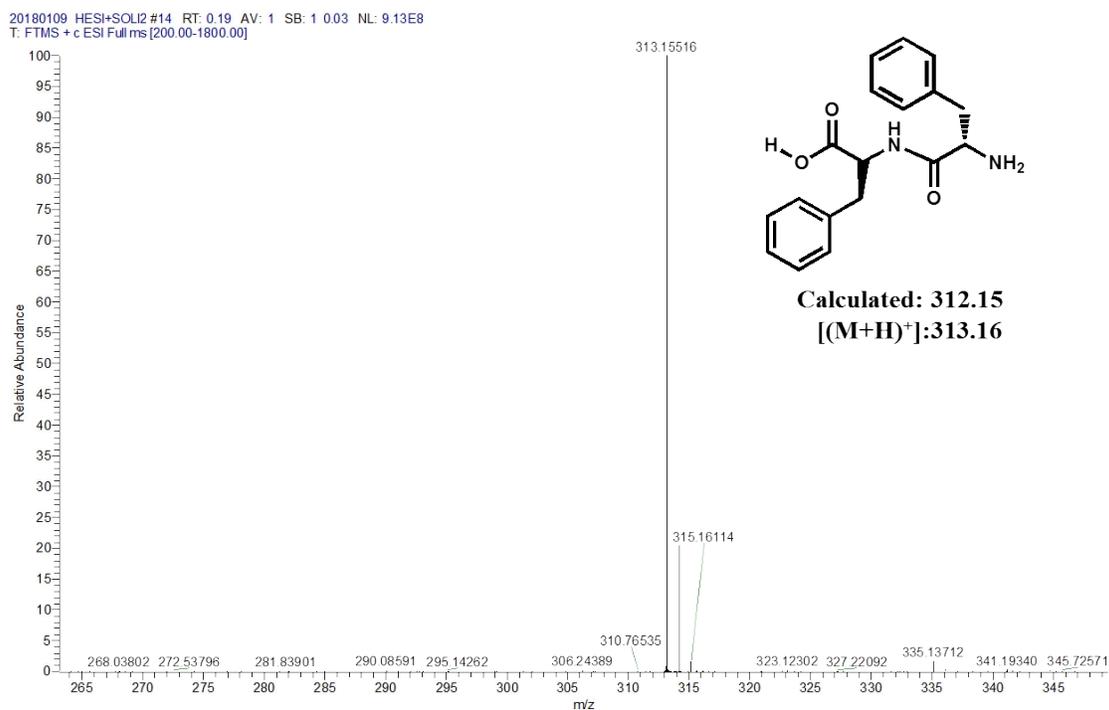
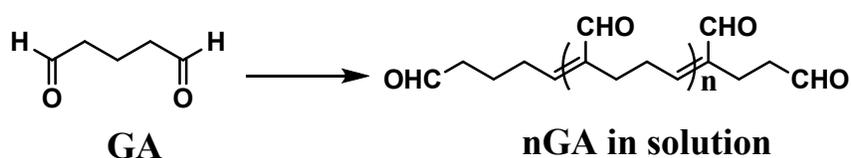


Figure S2. ESI/MS spectrum of FF.

Synthesis and characterization of diphenylalanine peptide and its NPs

Diphenylalanine peptide (FF) was synthesized by using a solid phase peptide synthesis method as described before with some modifications¹. The detailed method of preparation has been illustrated in scheme 1 of supplementary file. After successful synthesis, the FF was purified by HPLC method. The purified FF was characterized using ¹H NMR and ESI-MS. Further, we achieved the controlled assembly of FF into unilocular nanoparticles (FF-NPs) by dissolving 1 mg of FF in minimum amount of 1,1,1,3,3,3-hexa fluoro2-propanol (HFP) and further adding 1 mL of diluted 0.06% of glutaraldehyde (GA) solution². The reaction was carried out for 24 h to yield a yellow precipitate. The scheme of the self-assembly of FF-NPs assisted with GA (oligomer in solution) has been illustrated in Scheme 2.

Scheme S2. A) glutaraldehyde in aqueous solution



Synthesis and characterization of FA-FF-NPs

FA conjugated FF-NPs (FA-FF-NPs) were synthesized by carbodiimide reaction with assistance of EDC^{3,4}. The FA was first conjugated with N-Boc-ethylenediamine for which carboxyl group of FA was activated initially using EDC and NHS as described before. Briefly, 135 mg of FA was dissolved in DMSO followed by addition of NHS and EDC. The reaction mixture was stirred overnight and the side product of urea was removed through filtration. Following which 0.250 μ L of TEA was added subsequent to which Boc-ethylenediamine in 2.5 mL of DMSO was added to the mixture in a molar ratio of 1:1 with FA and stirred again for overnight. Thus, N-Boc-ethylenediamine folate was collected after washing the product with acetone and diethyl ether. Next, the ethylenediamine-folate was obtained from N-Boc-ethylenediamine folate by following the method reported in literature⁴. Briefly, N-Boc-ethylenediamine folate was dissolved in TFA with stirring which was subsequently removed using rotavapor and DCM. Successively the precipitate was dissolved in DMF and was further precipitated using TEA. The precipitate was washed with acetone and diethyl ether several times to finally obtain yellow colored ethylenediamine-folate.

Finally, the above prepared FF-NPs were further decorated with FA, for which ethylene diamine folate was used. To conjugate FF-NPs with FA, the 20 mg of FF-NPs were dispersed in 10 mL of ultrapure water and the carboxyl group of FF-NPS was activated using EDC and NHS. The reaction was carried out at ambient temperature for 3 h. Subsequently the pH was increased to 8 by the assistance of TEA. Further ethylene diamine folate was added to FF-NPs in a molar ratio 1:2 and the reaction was carried out at 35°C for 4 h. After the reaction, the unreacted ethylene diamine folate was removed using DMSO and resulting FA-FF-NPs were washed with ultrapure water several times and finally collected after lyophilization. The schematic illustration of FA-FF-NPs synthesis has been represented in scheme 3. The FA-FF-NPs were further characterized using FTIR as shown in figure S3.

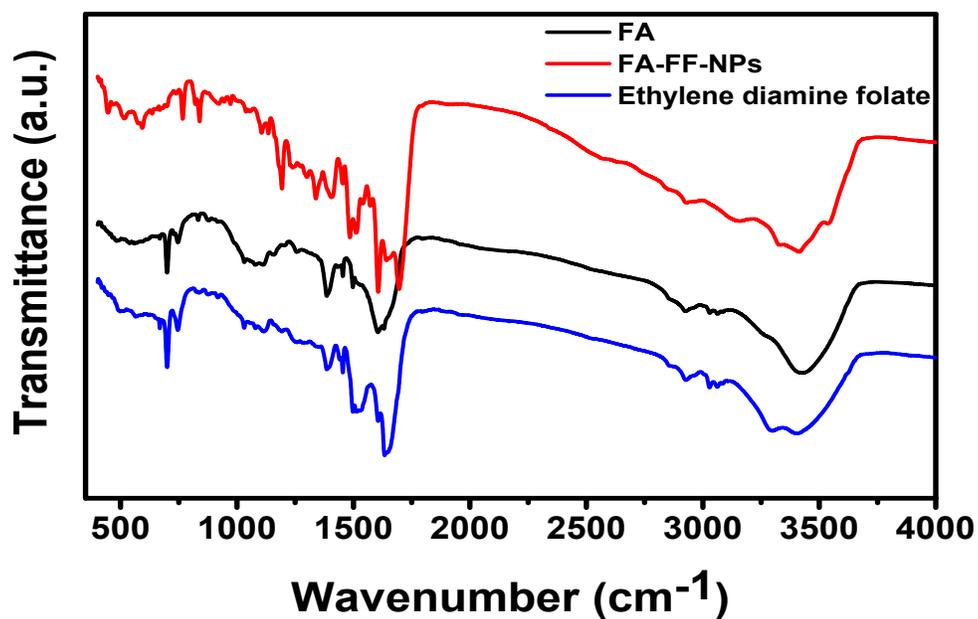
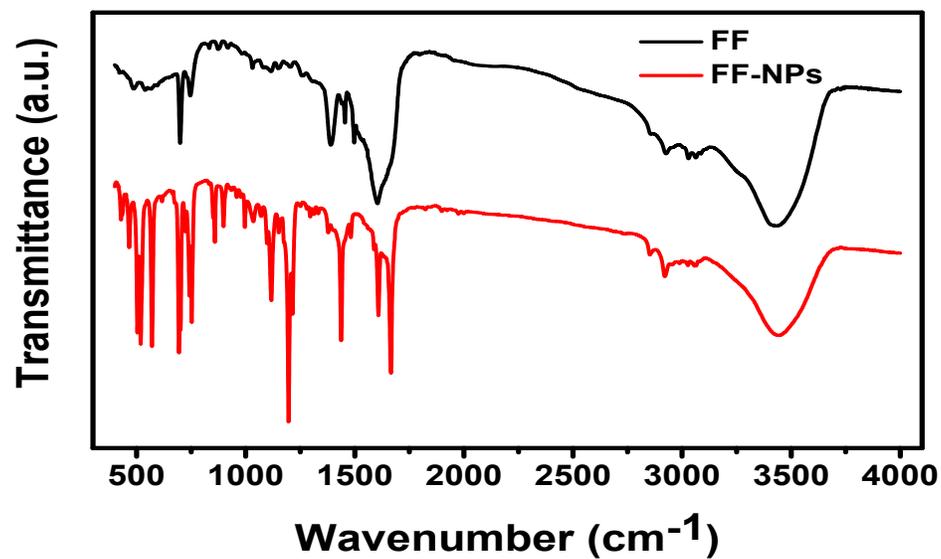


Figure S3. FTIR spectra at the top showing characteristic peaks of FF and FF-NPs whereas the FTIR spectra below shows the characteristic peaks of folic acid (FA) and ethylene diamine folate and folic acid conjugation FF-NPs (FA-FF-NPs)

We further evaluated the conjugation of FA to FF-NPs using FTIR by determining the functional groups and the changes which might have appeared during the interaction of FF and FA. The significant broad bands of FA at 3424 cm^{-1} was due to the O–H stretching vibrations which might even have overlapped –NH₂ stretching vibrations and was not clearly observed. The prominent band at 1631 cm^{-1} corresponds to C=O stretching vibrations. The ethylene diamine folate exhibited significant two peaks in broad bands at 3413 cm^{-1} and 3293 cm^{-1} that represent broad O–H and N–H stretching vibrations respectively. The peak of N–H stretching vibration was prominent ethylene diamine folate. Also the shift of C=O stretching vibration which was now observed at 1653 cm^{-1} due to the formation of new amide bond. The results confirm the formation of ethylene diamine folate. Further, FTIR spectra of FA-FF-NPs revealed the peak at 3409 cm^{-1} , corresponding to O–H stretching vibrations of FA-FF-NPs and significant C=O stretching vibrations at 1671 cm^{-1} . The new peak was generated at 1606 cm^{-1} corresponding to the introduction of Schiff base C=N stretching present in FA-FF-NPs. The results further confirm the conjugation of FA to FF-NPs to generate FA functionalized FF-NPs (FA-FF-NPs).

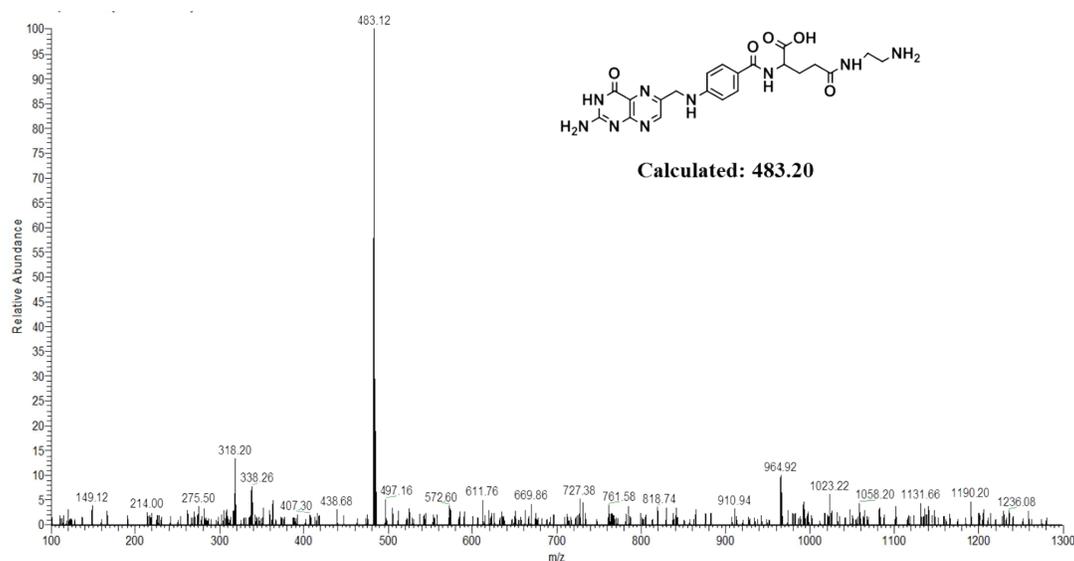


Figure S4. ESI/MS spectrum of ethylene diamine folate.

Synthesis and characterization of FA-FF-Dox and FF-Dox NPs

Dox was loaded during the self-assembly of FF-NPs by using co-precipitation method with some modifications⁵. The loading of Dox was optimized by using various concentration of DOX (5-10% of FF). The Dox was added before the self-assembly of FF and its crosslinking with GA. The loading was carried out at $40\text{ }^{\circ}\text{C}$ in ultrasonic water bath. The FF-Dox NPs was resulted after overnight incubation at $37\text{ }^{\circ}\text{C}$. Free Dox, and HFP were separated from FF-Dox NPs by washing and using ultrafiltration tube (MW cut-off, 100,000 Da) at $2000\times\text{g}$ for 15 min. Finally obtained FF-Dox was kept at $4\text{ }^{\circ}\text{C}$.

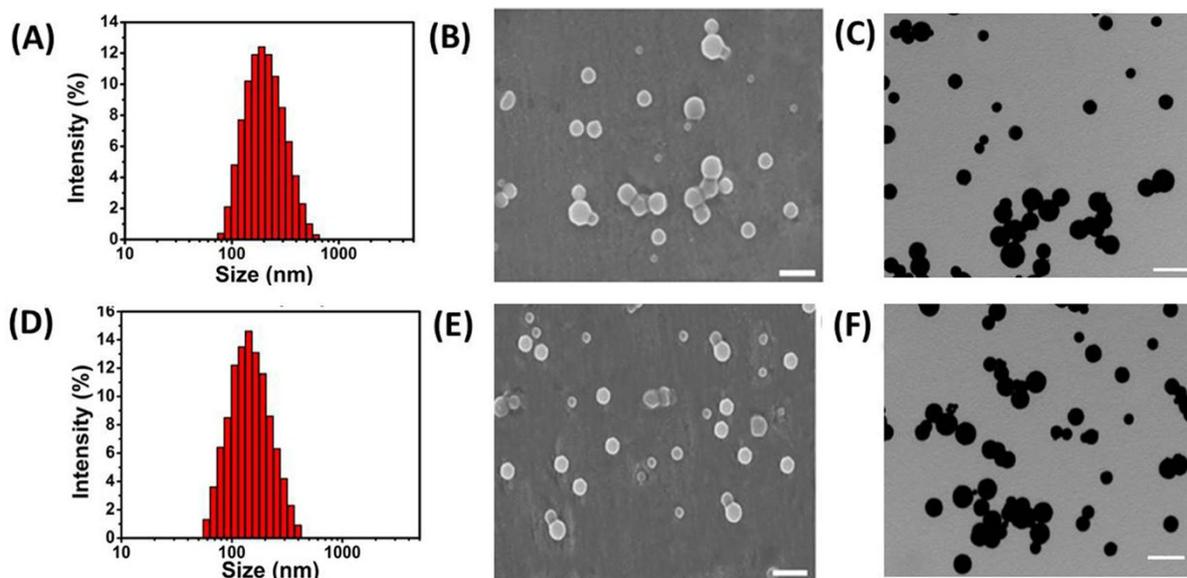


Figure S5. (A) Size distribution graph of FA-FF-Dox (B) scanning electron microscopic image of FA-FF-Dox (C) transmission electron microscopic image of FA-FF-Dox. (D) Size distribution graph of FF-Dox (E) scanning electron microscopic image of FF-Dox (F) transmission electron microscopic image of FF-Dox. The scale bar of SEM represents 500 nm and scale bar of TEM represents 500 nm).

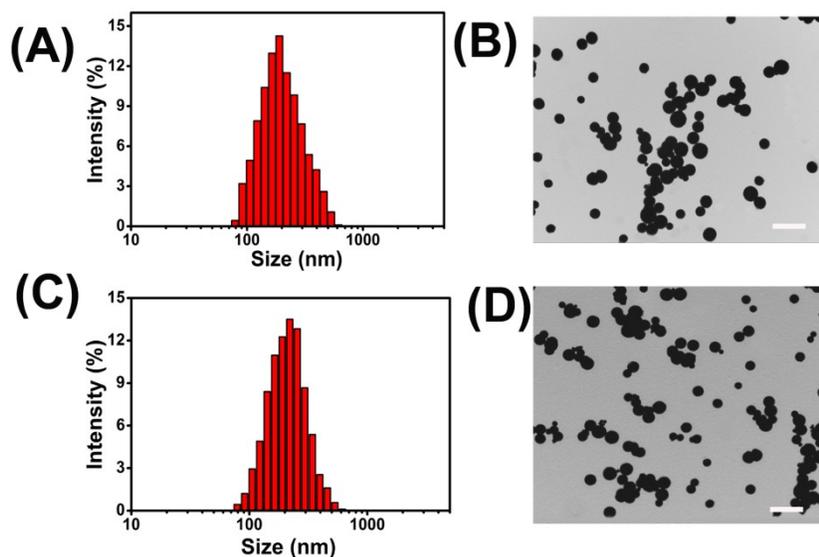


Figure S6. (A) Size distribution graph of FA-FF-NPs (B) transmission electron microscopic image of FA-FF (C) Size distribution graph of FF-NPs (D) transmission electron microscopic image of FF-Dox. The scale bar of TEM represents 1 μ m.

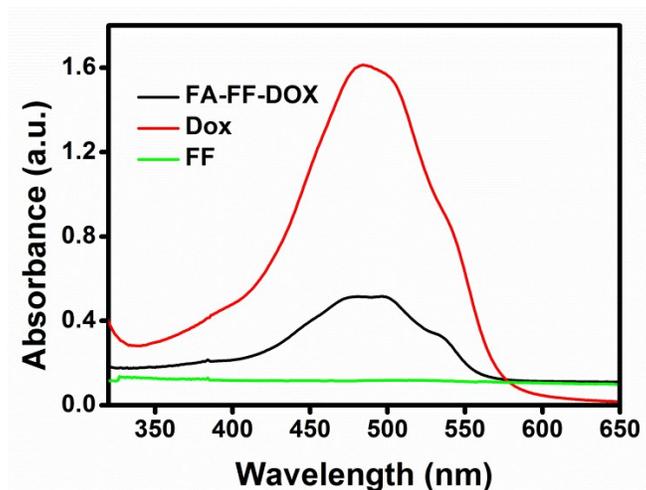


Figure S7 UV-vis spectra of FF, Dox and FA-FF-DOX (1:6) in PBS buffer. (pH 7.4, 10mM).

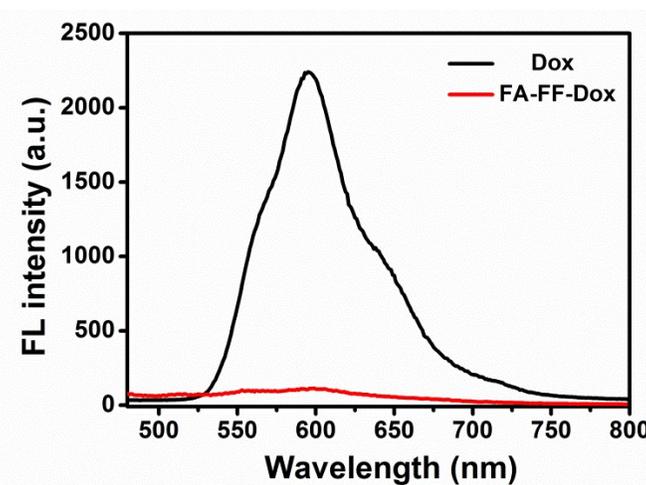


Figure S8 Fluorescence Emission ($\lambda_{ex}=480\text{nm}$) of Dox and FA-FF-DOX in PBS buffer. (pH 7.4, 10mM).

Drug loading efficiency and drug loading content

To evaluate the percent drug loading efficiency (DLE%) and drug loading content (DLC%) in FF-DOX and FA-FF-DOX a standard calibration curve of Dox solution was prepared using a fluorescence spectrophotometry and the readings were obtained at Ex/Em: 480/590 nm. The amount of Dox present in FF-DOX and FA-FF-DOX were calculated based on the fluorescent intensity of their respective solution after stirring 5 mg of NPs in water for 15 min. DLE% and DLC% were calculated as follows:

$$DLE (\%) = \frac{\text{Weight of Dox in FF - Dox or FA - FF - Dox} \times 100}{\text{Weight of Dox used initially}}$$

$$DLC (\%) = \frac{\text{weight of Dox in FF - Dox or FA - FF - DOX} \times 100}{\text{weight of FF - Dox or FA - FF - DOX}}$$

Dox release profile in vitro

The release of FF-Dox and FA-FF-Dox was studied in an in vitro environment mimicking the physiological pH 7.4 of blood and slightly acidic pH 5.5 representing the endosome pH (5-5.5). The release of these particles was also compared with the release of free Dox in the same conditions. To investigate the release pattern the NPs and the free drug at an equivalent concentration of Dox were dispersed in 5 mL of release medium and were placed in a dialysis bag. The dialysis bag was further placed in 200 mL of different release medium with stirring maintained at the temperature of 37°C. The samples were collected at pre-determined time interval and the quantity of medium was maintained by adding fresh medium. The collected samples were analyzed using fluorescence spectrophotometry with excitation and emission at 480/590 nm.

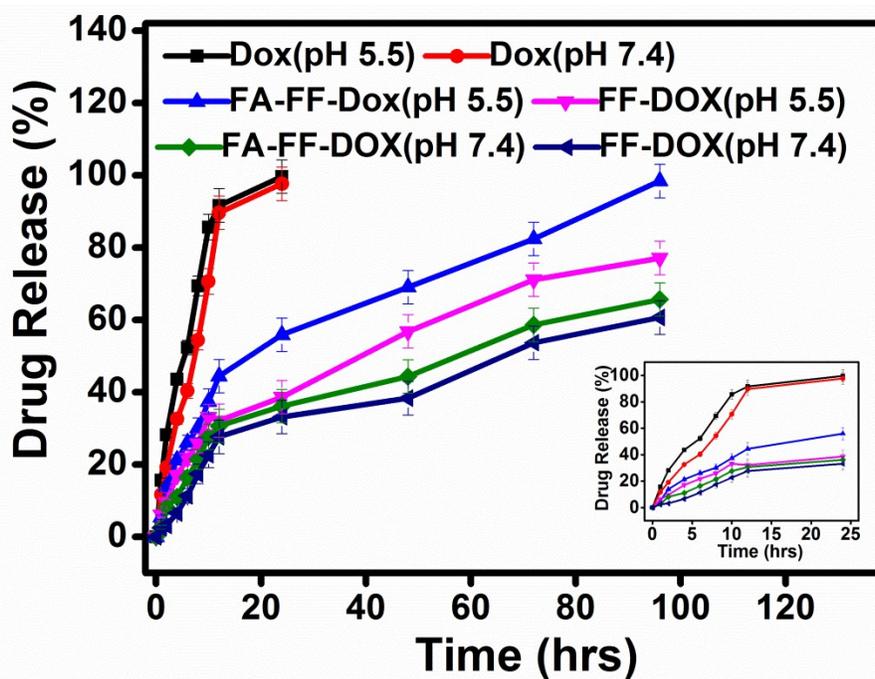


Figure S9 In vitro release studies revealing the release of different formulations at physiological pH 7.4 and acidic pH 5.5 at 37 °C.

Cell culture

In order to conduct cell studies we cultured cervical cancer cell lines HeLa. The HeLa cells were grown using dulbecco's modified eagle medium (DMEM) flourished using 10% fetal bovine serum and augmented with combination of antibiotic penicillin-streptomycin 1%. The cells were incubated at 5% CO₂ with temperature maintained at 37°C.

Cytotoxicity assay

To evaluate the cytotoxicity caused by free Dox, FF-Dox and FA-FF-Dox, we employed 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) test. 96-well cell culture plates were used to grow cervical cancer HeLa cells at a density of 3×10^3 cells/well in humid atmosphere. The solution of free Dox, FF-Dox and FA-FF-Dox having equivalent concentrations (6.25, 12.5, 25, and 50 μ M) were mixed in to each well cautiously. These cultured plates were

incubated in humid atmosphere for different time (24 and 48 h). Afterwards, 10 μL of MTT solution in a concentration of 5 mg/mL was added to each test groups. Subsequent to the incubation for another 4 h, DMSO (100 μL) was added to every well of 96 well plate. The data was recorded by using enzyme-linked immunosorbent assay at 490 nm. In order to calculate the cell viability percentage we used viability formula.

$$\text{Viability (\%)} = \frac{\text{mean of absorbance value of treatment group} \times 100}{\text{mean of absorbance value of control}}$$

$$\text{Viability (\%)} = (\text{mean of absorbance value of treatment group} \times 100) / (\text{mean of absorbance value of control})$$

We also evaluated the targeting ability of FA modified peptide delivery system by calculating the half maximal inhibitory concentration (IC_{50}) after 24 h and 48 h of treatment. FA-FF-Dox unveiled the highest anti-proliferation corresponding to its active targeting ability and enhanced cellular uptake, recording an IC_{50} value of 24.63 μM and 17.72 μM for the 24 h and 48 h respectively. However, the non-specific delivery of Dox and FF-Dox resulted in significantly less IC_{50} in the whole study. The results displayed IC_{50} values 41.71 μM and 33.63 μM for free Dox, whereas FF-Dox indicated 33.12 μM and 29.16 μM for 24 h and 48 h respectively. The comparatively low IC_{50} of Dox than FF-Dox might be attributed to reduced uptake of free drug in the cells. Moreover, the nanoparticles could sustain the P-glycoprotein efflux pump whereas the free drug could be efflux out of the cells⁶. The results clearly demonstrated the superior efficacy of FA-FF-Dox. The results established that FF modification with FA in FA-FF-Dox had significantly enhanced Dox concentration in cells due to active targeting and FRs mediated endocytosis⁷. Figure S10 A&B illustrates the cell viability of HeLa cells after the treatment for 24h and 48 h respectively with equivalent concentration of Dox. These results also support the *in vitro* release studies of FA-FF-Dox where the Dox release at pH mimicking the intracellular low pH resulting in high accumulation of Dox within the cells.

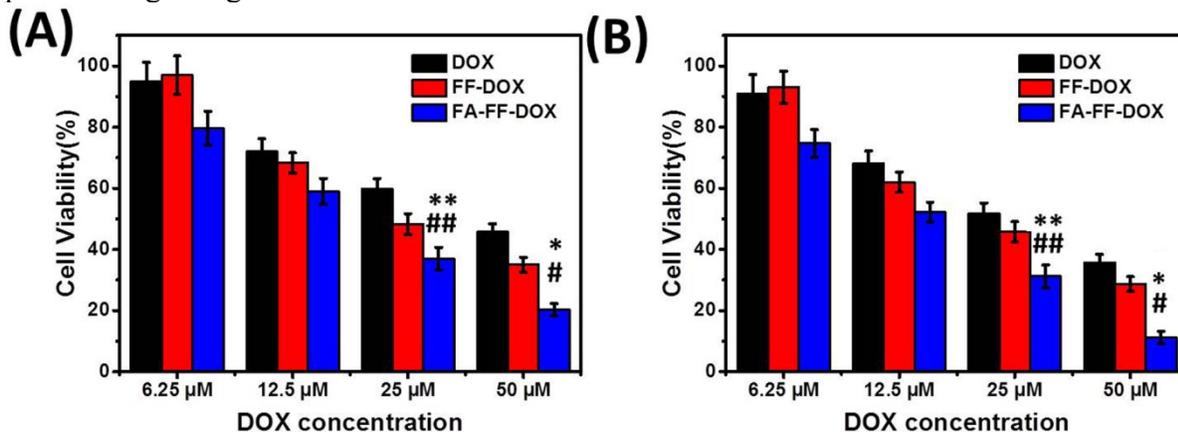


Figure S10. MTT assay of Dox, FF-Dox and FA-FF-Dox on HeLa cells incubated with different concentration for (A) After 24 h # p < 0.05 FA-FF-Dox vs FF-Dox, * p < 0.05 FA-FF-Dox vs Dox at 50 μM concentration and ### p < 0.05 FA-FF-Dox vs FF-Dox, ** p < 0.05 FA-FF-Dox vs Dox at 25 μM concentration. (B) after 48 h # p < 0.05 FA-FF-Dox vs FF-Dox, * p < 0.01 FA-FF-Dox vs Dox at 50 μM concentration and ### p < 0.05 FA-FF-Dox vs FF-Dox, ** p < 0.05 FA-FF-Dox vs Dox at 25 μM concentration.. These experiments were performed in triplicate. Error bars represent standard deviations.

Imaging and FACS studies for cell uptake

For cell imaging, cultured HeLa cell dishes were washed with excess of PBS followed by addition of free Dox, FF-Dox and FA-FF-Dox in serum-free DMEM in each cell-cultured dish. The cells were imaged by a fluorescence microscope after 4 h of incubation.

Briefly, 1×10^6 HeLa cells/well were plated in glass bottom cell culture dishes of 15 mm. The cells were treated with free Dox, FF-Dox and FA-FF-Dox for 4 h, at an equal dose of Dox. Subsequently, the free Dox and NPs were removed from the cells by several washings with PBS and the cells were fixed using ice cold methanol. To observe the nucleus and better localization of the Dox, the cells were stained with Hoechst before imaging. The intracellular localization was observed through imaging the cells by IX71 fluorescence microscope (Olympus, Japan) to evaluate the intracellular uptake.

Fluorescence activated cell sorting (FACS) was used to estimate the intracellular uptake of particles. The HeLa cells seeded in a 6 well tissue culture plate at a density of 1×10^6 cells/well were treated with free Dox, FF-Dox and FA-FF-Dox for 4 h, at an equal dose of Dox. Subsequently, the cells were rinsed with PBS to remove free drug and particles and the cells were collected by utilizing a minimum amount of trypsin. Afterwards the uptake of drug and particles were analyzed based on the fluorescent intensity of Dox using BD Biosciences FACSCalibur flow cytometer.

We further extended our studies to quantitatively estimate the intracellular uptake of Dox and to evaluate any advantage of functionalized FA-FF-Dox over other formulations. After treating the HeLa cells (1×10^5 cells/well in a 6 well plate) with free Dox, FF-Dox and FA-FF-Dox having equivalent amount of Dox (6.25, 12.5 and 25 $\mu\text{g/mL}$), the cells were incubated for 4 h at 37 °C. Afterwards, the cells were rinsed with PBS to wash off free drug and particles, lysed and centrifuged at 1000 g for 5 min. The supernatant was collected and analyzed using UV-Vis spectrophotometer to estimate the amount of Dox present in the solution.

To further verify the advantage of FA-FF-Dox over non-specific delivery of FF-Dox and free Dox, fluorescent microscope and FACS uptake studies were performed at equivalent concentration of Dox. The cells transfected with FA-FF-Dox revealed superior targeting potential which exhibited high fluorescent intensity within the cells as revealed in figure 11A.

The results were corresponding to its FRs binding affinity achieved between FA-FF-Dox and FRs compared to without modified peptides (FF-Dox) and free Dox into the cells. Unmodified FF-Dox and Dox showed no specific binding. The FACS study results further verified that the endocytosis of FA-FF-Dox was significantly higher with 2.44 fold and 8.57-fold increase in comparison with FF-Dox and free Dox respectively (Figure 11 B & C).

Further, the quantitative estimation of intracellular Dox agreed with the above mentioned results and significantly higher intracellular Dox was estimated in the cells treated with FA-FF-Dox at all the concentrations. The results of intracellular Dox uptake has been illustrated in Figure 11D. All the above results clearly suggest that the FA-FF-Dox has advantage over non-functionalized peptide particles and were much superior to free Dox. The results could be attributed to FRs mediated endocytosis and intracellular release and also corresponding to the positive charge on the particles which could have generated electrostatic interaction between particles and negatively charged cells.

To study the targeting ability of FA-FF-Dox and its endocytosis mediated by FRs 1×10^6 HeLa cells/well were plated in glass bottom cell culture dishes of 15 mm. Further, the cells were treated with different concentrations of free folate along with FF-Dox and FA-FF-Dox having equivalent amount of Dox 25 $\mu\text{g/mL}$, the cells were incubated for 4 h at 37 °C. Uptake of FF-Dox and FA-FF-Dox was estimated quantitatively based on the amount of Dox present in the cells. The cells were rinsed with PBS to wash off free drug and particles, lysed and centrifuged at 1000 g for 5 min. The supernatant was collected and analyzed using UV-Vis spectrophotometer to estimate the amount of Dox present in the solution.

As shown in Fig. S12, the uptake of FA-FF-Dox was inhibited by excess free FA present in the solution. However the uptake of FF-Dox was not influenced by the addition of FA. The results

clearly suggest that presence of free FA in higher concentration clearly inhibited FA-FF-Dox suggesting the role of FRs in their endocytosis. The results obtained were in accordance with the other qualitative and quantitative cell uptake studies, where in absence of FA free media significantly higher uptake has been observed for FA-FF-Dox in comparison with FF-Dox. Remarkably, no alteration in binding ability of FA to FRs has been reported even after covalent conjugation to small molecules, proteins.

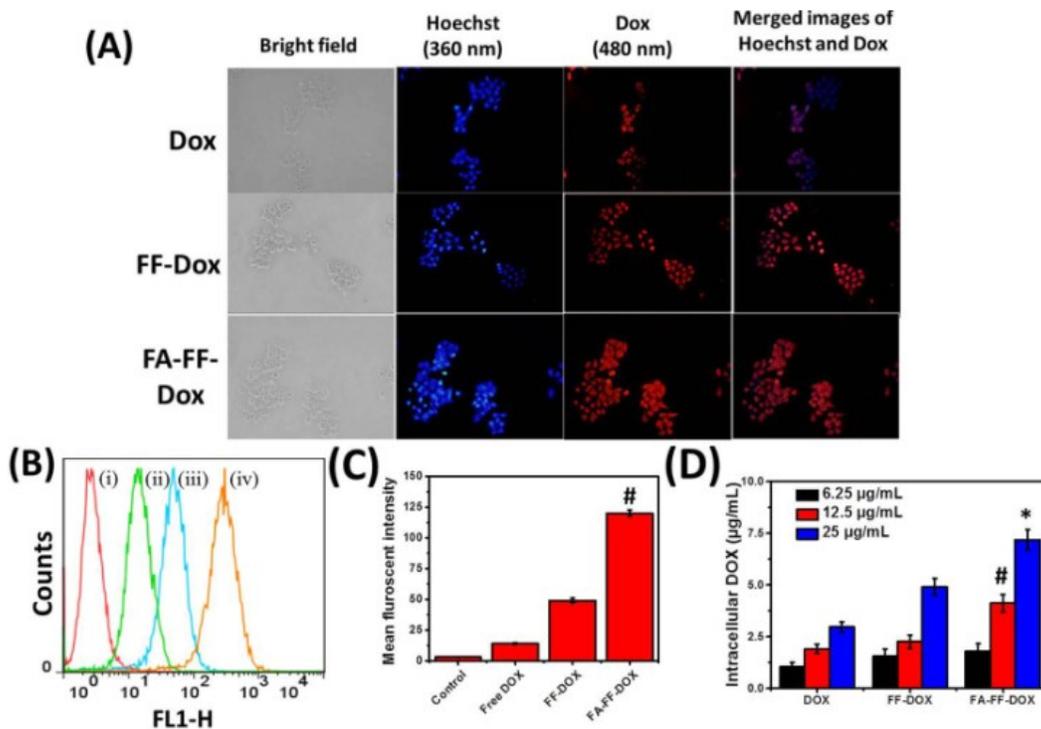


Figure S11. (A) Fluorescence uptake of free Dox, FF-Dox and FA-FF-Dox by treating HeLa cells with free Dox, FF-Dox and FA-FF-Dox after 4 hr. (B & C) represents cell uptake studies using Fluorescence-activated cell sorting (FACS) (i) Control (ii) Dox (iii) FF-Dox (iv) FA-FF-Dox and their mean fluorescent intensity. $n=3$, # $p < 0.01$ FA-FF-Dox vs FF-Dox. (D) Quantitative analysis of intracellular Dox after 4 h of treatment with Dox, FF-Dox and FA-FF-Dox. # $p < 0.05$ FA-FF-Dox vs FF-Dox at 12.5 µg/mL and * $p < 0.05$ FA-FF-Dox vs FF-Dox at 25 µg/mL.

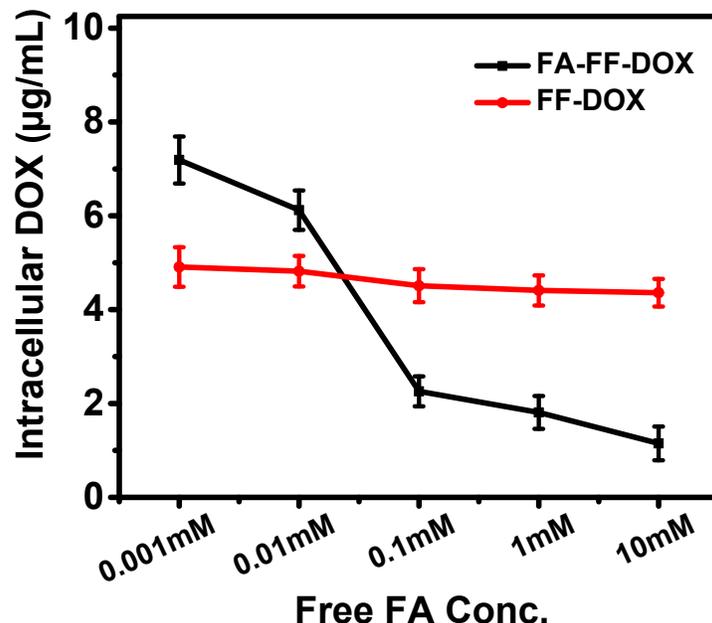


Figure S12. Quantitative analysis of intracellular Dox after 4 h of treatment with FF-Dox and FA-FF-Dox in presence of free FA at different concentrations

Tumor model establishment

In order to study the in-vivo efficacy of the developed FA-FF-DOX, animal experiments were conducted using 5 week-old BALB/c nude mice (20 g), whereas pharmacokinetics and biodistribution studies were conducted in 4 week-old male Wistar rats (70 g). The animals were kept according to the ethical guidelines provided by the local ethical committee. The experimental protocols were preapproved by the University of Science and Technology of China Animal Care and Use Committee (USTCACUC1801025). Cervical cancer HeLa cells were used to generate the tumor models in both mice and rats which were injected subcutaneously (1×10^6 / animal) to the right hind leg of each mouse. The animals were kept under observation after cells inoculation. The tumor volume when reached $\sim 150 \text{ mm}^3$ in mice and $\sim 500 \text{ mm}^3$ in rats, the animals were further divided in different groups according to the protocol.

Pharmacokinetics and Biodistribution studies

The pharmacokinetics and biodistribution of developed formulations were studied in tumor bearing male Wistar rats. Once the tumor was developed, the rats were divided into 3 groups, Group I was with administered with free DOX, Group II administered with FF-DOX and Group III was administered FA-FF-DOX intravenously at equivalent 10 mg/kg dose. Via retro orbital plexus, 500 μL of blood was drawn at predetermined time intervals in a heparinized tubes and the plasma was separated simultaneously. Further, the animals were sacrificed and vital organs including heart, liver, kidney, spleen and tumor were collected at 1 h, 4 h, 8 h, 12 h, and 24 h (n=5 for each time point). The total Dox concentration was determined via HPLC method described earlier. Briefly the 100 μL volume of plasma was carefully precipitated by 400 μL of acetonitrile/methanol solvent, 5 min vortexed and then centrifuged for 15min at 8000 rpm rate. The supernatant were collected and dried. After which it was reconstituted with 50 μL of mobile phase and subjected for further HPLC analysis. For organ distribution analysis, the collected organs were washed, dried and homogenized using PBS. The drug was extracted and analyzed as stated above.

The fate of free Dox, FF-Dox and FA-FF-Dox in plasma was determined after intravenous administration at the dose of 10 mg/kg, the pharmacokinetic study was conducted. The 24 h profile of plasma has been presented in Figure S13, with the data illustrated in Table S3. It was observed that the free Dox was rapidly absorbed from the blood and distributed to the organs; therefore, its concentration in plasma could not be detected after 12 h. However the FF-Dox and FF-FF-Dox circulation time was more and was detected till 24 h of study. FA-FF-Dox group had 6.7-fold and 1.43-fold enhanced AUC_{0-t} as compared to Dox and FF-Dox group respectively. On other hand the average half-life ($t_{1/2}$) calculated for FA-FF-Dox was 3.1- and 1.11-times prolonged and mean residence time (MRT) was 3.58- and 1.13-times enhanced in comparison to free Dox and FF-Dox group respectively. Hence, these findings depicted that in FA-FF-Dox group the drug retained in the body for long time and could be accumulated at tumor targeted site via EPR effect. The lower free Dox half-life and MRT as compared to NPs may be due to rapid distribution of drug in the body and fast elimination via kidney which cause lower concentration of drug in blood. In FA-FF-Dox and FF-Dox group, the Dox was detected till the end of the study. The previous studies stated that lower surface charge of NPs may prolonged blood circulation time by decreasing the process of opsonisation⁸.

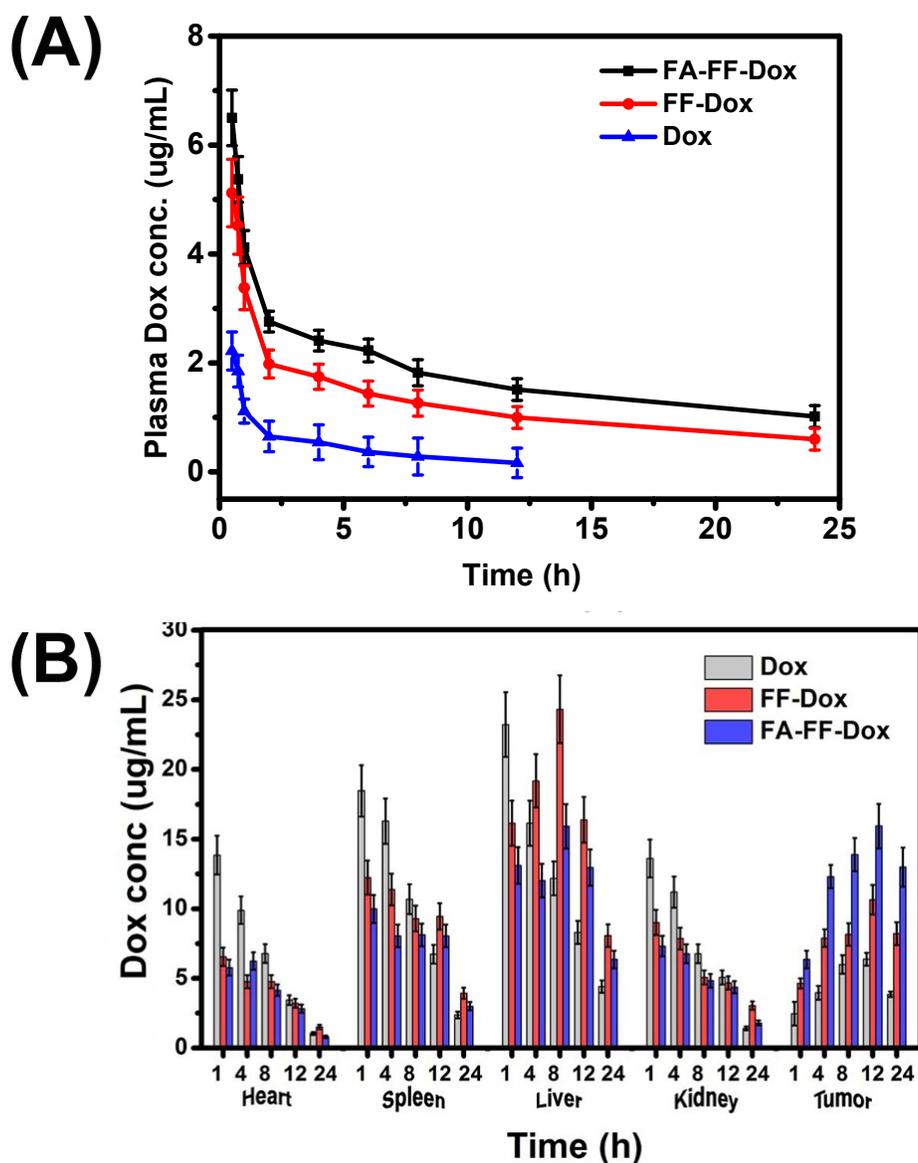


Figure S13: (A) Pharmacokinetic profile of free Dox, FF-Dox and FA-FF-Dox in plasma on intravenous administration at equivalent dose of Dox (10 mg/kg). (B) Organ distribution of Dox, FF-Dox and FA-FF-Dox on intravenous administration at equivalent dose of Dox (10 mg/kg, n=3)

Dox concentration in different organs was further analyzed and the results have been presented in figure S13B. The distribution profile of Dox was found to be different from the FF-Dox and FA-FF-Dox. The Dox was widely distributed in all the organs at high concentration in initial time points with clearance with the time, whereas the distribution of the FF-Dox and FA-FF-Dox was low initially. The clearance of Dox from organs was decreased over time in contrast the concentration of FF-Dox and FA-FF-Dox, which increased over time. This was also observed in the plasma profile where the fast clearance of Dox from blood was seen which might have distributed in the organs in contrast to FF-Dox and FA-FF-Dox.

The concentration of Dox, FF-Dox and FA-FF-Dox increased in tumors with time and was significantly high for FA-FF-Dox. The results might be attributed to the target-specific and receptor-mediated delivery of FA-FF-Dox in comparison with FF-Dox and Dox. The results were further confirmed by bio-distribution imaging which also reveals the similar pattern of Dox distribution.

In-vivo biodistribution imaging and antitumor efficacy

Subsequent to the tumor development, mice were randomly divided in two subsets, one set of mice were used for in vivo biodistribution imaging while another set of mice were used to evaluate the antitumor efficacy of developed particles. Both the sets of mice were further divided in to four groups (n=6). Group I was assigned as control and was administered with PBS. Group II, Group III and Group IV were treatment group and were administered with free Dox solution, FF-Dox and FA-FF-Dox with an equivalent concentration of 10 mg/kg Dox respectively.

For in vivo imaging the mice were intravenously administered with a single dose of PBS, free Dox, FF-Dox and FA-FF-Dox respectively. The animals were sedated using isoflurane gas in oxygen flow. Fluorescence images were captured by Xenogen IVIS® spectrum system at predetermined time intervals (1, 4, 12, 24 h) and analyzed by a Living Image 4.5.2 software package. After the completion of the study, the mice were sacrificed using CO₂ to collect the vital organs and tumor for ex vivo imaging.

For antitumor efficacy studies all the groups of mice received corresponding dosage through intravenous injection at day 1, 4 and 8. The mice were administered with PBS, free Dox, FF-Dox and FA-FF-Dox at an equivalent Dox dose of 10mg/kg. The mice were in continuous observation and were monitored for any change in their weight and tumor volume every other day. At the end of study the animals were sacrificed and the organs included tumors were isolated for H & E staining.

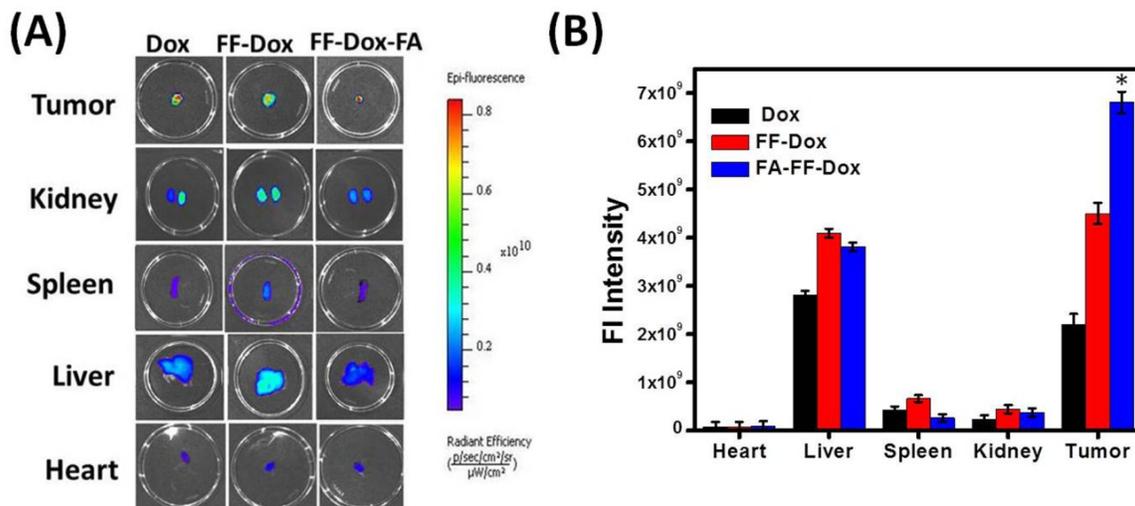


Figure S14. Ex vivo fluorescence images and quantification of fluorescence intensity Dox, FF-Dox and FA-FF-Dox in various organs at 24 h determined by the fluorescence intensity of Dox (Ex/Em: 500/620 nm). # $p < 0.05$ FA-FF-Dox vs FF-Dox after 24 h of treatment.

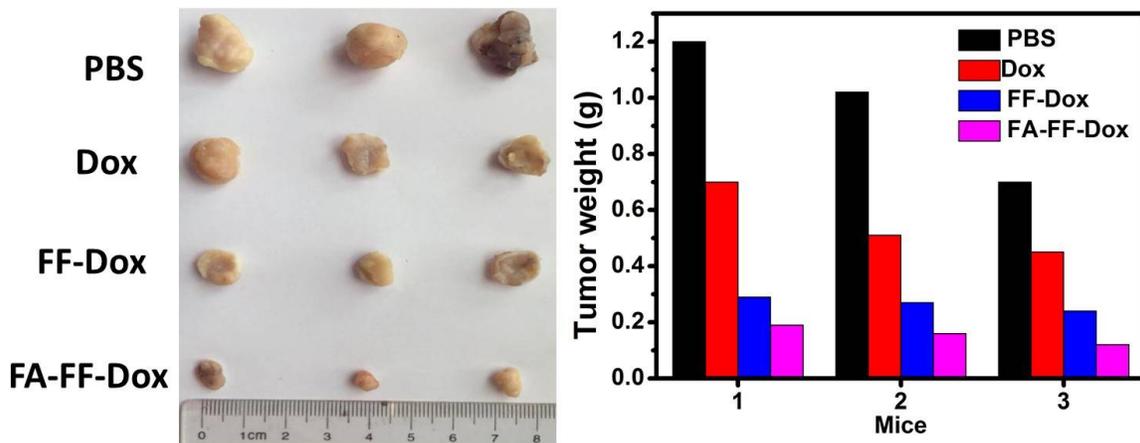


Figure S15. Left, ex vivo images of the tumors dissected from the tumor-bearing nude mice at day 15 after being intratumorally injected with PBS, Dox, FF-Dox and FA-FF-Dox. Right figure shows tumor morphology, tumor weight of mice has been presented in the left.

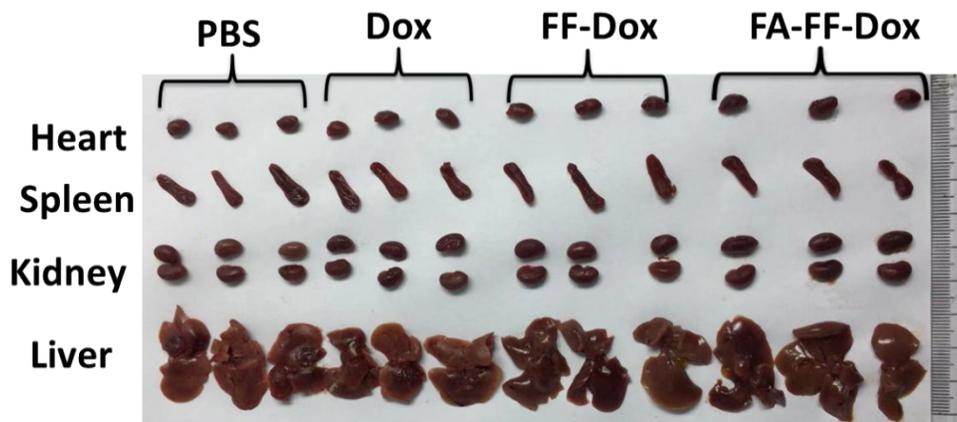


Figure S16. Ex vivo images of the fresh organs extracted from 4 groups of nude mice after being dissected at day 15.

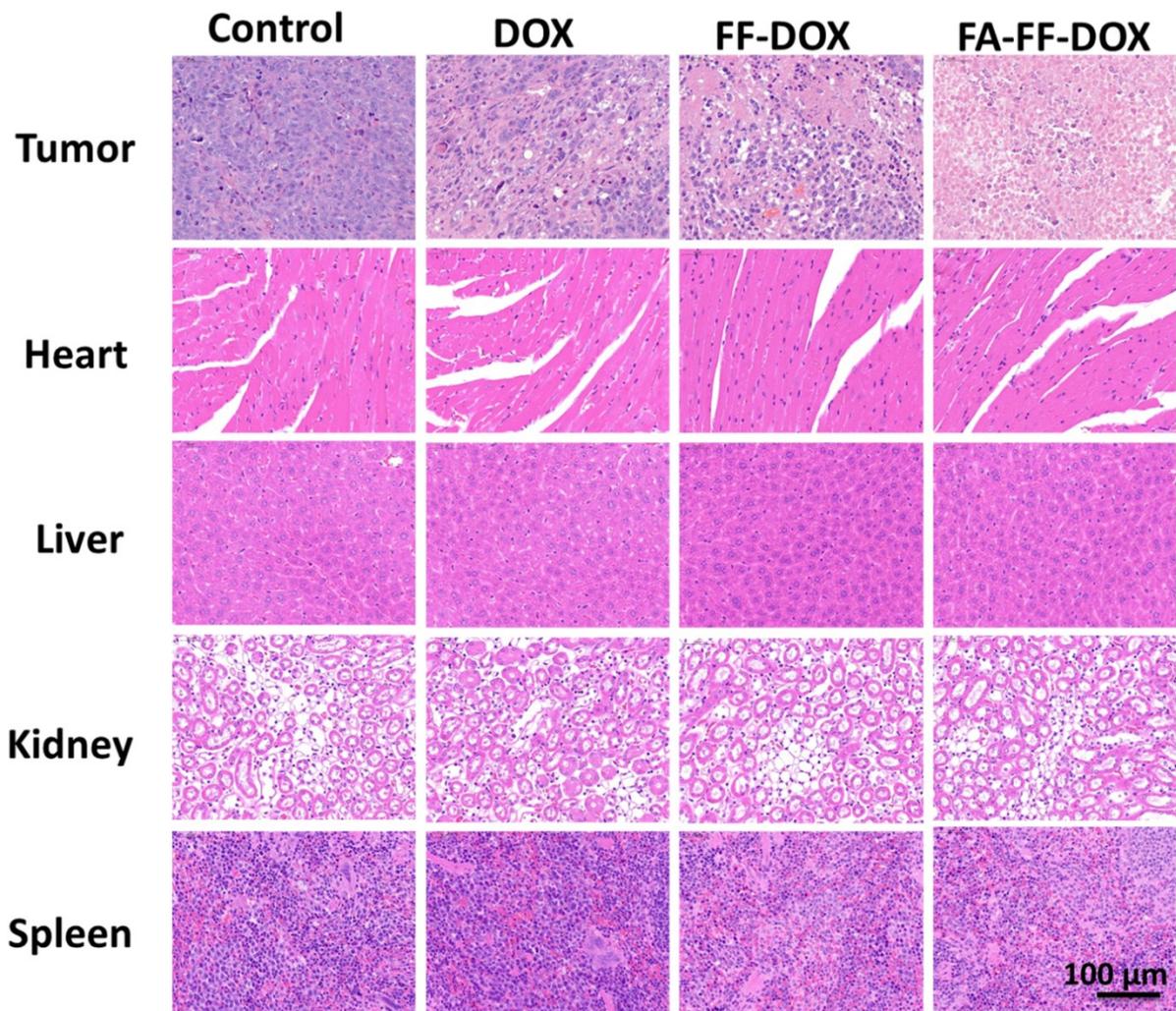


Figure S17. Histological (H&E) images (40× lens of Nikon microscope) of tumor and other major organs excised from the treated group of mice after they were sacrificed. Scale bars: 100 μm

Table S1: HPLC condition for analysis of FF

Time (min)	Flow (mL/min)	H ₂ O% (0.1% TFA)	CH ₃ CN% (0.1% TFA)
0	3.0	70	30
3	3.0	70	30
35	3.0	10	90
37	3.0	10	90
38	3.0	70	30
40	3.0	70	30

Table S2: HPLC condition for purification of FF

Time (min)	Flow (mL/min)	H ₂ O% (0.1% TFA)	CH ₃ CN% (0.1% TFA)
0	12.0	50	50
3	12.0	50	50
35	12.0	5	95
37	12.0	5	95
38	12.0	50	50
40	12.0	50	50

Table S3: Pharmacokinetic parameters observed after intravenous administration of Dox, FF-Dox and FA-FF-Dox at the dose of 10 mg per kg body weight in mice.

Parameter	Dox	FF-Dox	FA-FF-Dox
AUC _{0-t} (h.µg/mL)	6.76 ± 2.73	31.50 ± 5.27	45.80 ± 8.92 ^{*#}
C _{max} (µg/mL)	2.22 ± 0.28	5.12 ± 0.75	6.51 ± 0.63
T _{max} (h)	0.5	0.5	0.5
MRT (h)	5.860	19.17	25.29 [‡]

Data represented as mean ± S.D. (n=3) * P < 0.05 and [#]P < 0.01 compared to group treated with FF-Dox and free Dox respectively. Whereas [‡]P < 0.0101 compared to group treated with free Dox

References

1. R. Merrifield, *Journal of the American Chemical Society*, 1964, **86**, 304-305.
2. H. Zhang, J. Fei, X. Yan, A. Wang and J. Li, *Advanced Functional Materials*, 2015, **25**, 1193-1204.
3. A. F. Trindade, R. F. Frade, E. M. Macoas, C. Graca, C. A. Rodrigues, J. M. Martinho and C. A. Afonso, *Org Biomol Chem* 2014, **12**, 3181-3190. doi: 3110.1039/c3184ob00150h. Epub 02014 Apr 00110.
4. C. Figliola, E. Marchal, B. R. Groves and A. Thompson, *RSC Advances*, 2019, **9**, 14078-14092.
5. H. Zhang, J. Fei, X. Yan, A. Wang and J. Li, *Advanced Functional Materials*, 2015, **25**, 1193-1204.
6. A. Lamprecht and J.-P. Benoit, *Journal of Controlled Release*, 2006, **112**, 208-213.
7. M. Sharma, R. Malik, A. Verma, P. Dwivedi, G. S. Banoth, N. Pandey, J. Sarkar, P. R. Mishra and A. K. Dwivedi, *Journal of biomedical nanotechnology*, 2013, **9**, 96-106.
8. H. Kang, S. Mintri, A. V. Menon, H. Y. Lee, H. S. Choi and J. Kim, *Nanoscale*, 2015, **7**, 18848-18862.