Supporting Information

A pH-responsive Folate Conjugated Magnetic Nanoparticle for Targeted Chemo-thermal Therapy and MRI Diagnosis

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a. Characterization techniques

X-ray diffraction (XRD) patterns were recorded on a PANalytical's X'Pert PRO diffractometer with Cu Kα radiation. The crystallite sizes were estimated using Scherrer calculator of X'Pert High Score Plus software. The electron microscopy images were taken by JEOL (HR-TEM). UV-visible spectra were recorded with a UV-visible spectrophotometer (Cecil, Model No. CE3021). The fluorescence spectra of samples were recorded using Hitachi F 2500 fluorescence spectrophotometer. The field dependent magnetization and zero field cooled-field cooled (ZFC-FC) measurements at an applied field of 100 Oe were carried out by physical property measurement system (PPMS, Quantum Design). In order to evaluate the specific absorption rate, amount of Fe in suspension of FA-MNPs was obtained by ICP-AES analysis (Spectro Arcos, German). The R_2 relaxivity of the FA-MNPs were scanned using a multi-echo

T_2-weighted fast spin echo pulse (FSE) imaging sequence (TR/TE=3500/15, 30, 45, 60, 75 and 90 ms, slice thickness = 0.1 mm) by a 3 Tesla clinical MRI scanner.

b. Drug Loading and Release

Doxorubicin hydrochloride (DOX) was used as an anticancer drug to evolute the drug loading and release study of the FA-MNPs. The fluorescence spectroscopy was used to examine the interaction between pure DOX and FA-MNPs. For the drug loading experiment, the aqueous suspension of different concentration of FA-MNPs (0, 20, 40, 60, 80 and 100 μg from a stock suspension of 1 mg/ml of FA-MNPs) was added to 1 ml of DOX solution (10μg/ml). This solution was mixed systematically at room temperature for 15 min. After magnetic separation of drug loaded FA-MNPs, the fluorescence spectra of the supernatant were recorded using Hitachi F-2500 fluorescence spectrophotometer. The fluorescence intensities of supernatants against that of pure DOX solution were used to establish the loading efficiency. The loading efficiency (w/w %) was calculated using the following relation:

$$\% \text{ Loading efficiency} = I_{DOX} - I_S - I_w \times 100/I_{DOX}$$

Where, I_{DOX} is the fluorescence intensity of pure DOX solution, I_S the fluorescence intensity of supernatant and I_W the fluorescence intensity of washed DOX (physically adsorbed DOX molecules)

For release study, the loading was accomplished by incubating 0.5 ml of aqueous solution of DOX (1 mg/ml) with 1 ml of the aqueous suspension of FA-MNPs (10 mg/ml) for 24 h in dark. Drug loaded FA-MNPs were separated from free drug molecules through magnetic separation and washing with milli Q water. The pH-responsive drug release studies were executed at different pH conditions. The drug-loaded DOX-MNPs (5 mg) were immersed into 2 ml of acetate buffer medium at different pH 4.3, 5.6 and 7.3 and then placed it into a dialysis

bag. The release of DOX from DOX-MNPs in the dialysis was done against 100 ml of phosphate buffered saline (PBS) under continuous stirring at 37℃. After a fixed interval of time, 1 ml of the external PBS is replaced with the fresh PBS. The released amount of doxorubicin was determined by evaluating the fluorescence intensity at $\lambda_{excitation}$= 490 nm and $\lambda_{emission}$= 535±35 nm using Perkin Elmer 1420 multi label counter. The released amount of DOX was calculated against the standard plot prepared under similar condition.

c. Cytotoxicity Study

SulphorhodamineB B (SRB) assay was used to evaluate cytotoxicity of the FA-MNPs and DOX-MNPs on human cervical cancer cells (HeLa). The cells were seeded into 96 well plates at densities of 1×10^4 cells per well for 24. Subsequently different concentrations of FA-MNPs, DOX and DOX-MNPs were added to cells and incubated for next 24 h. In addition to this, different concentration of DOX (1 to 10 µg/ml) was also incubated for 24 h. Consequently, the cells were washed thrice with PBS and processed for SRB assay to determine the cell viability. Further, cells were fixed with a solution of 10% trichloroacetic acid and stained with 0.057% SRB and washed with 1% acetic acid. Cell-bound dye was extracted with 10mM buffered Tris buffer solution (pH 10.5) and then the absorbance was measured at 560 nm using a plate reader. The cell viability was calculated using the following formula:

$$\% \text{ cell Viability} = (\text{Absorbance of sample}/\text{Absorbance of control}) \times 100$$

d. Cellular uptake

Cellular uptake analysis was performed in the 24 well plate in HeLa and L929 cells, with nearly 1×10^5 cells per well. After 24 h, the HeLa cells were washed with PBS and incubated with FA-MNPs for 3 and 24 h. After incubation, cells were washed with PBS several times to remove uninternalized particles. Subsequently Cells were trypsinized and centrifuged at 3000 rpm for 5

min. The obtained cell plate was dissolved in dilute HCl. Cellular uptake study of FA-MNPs was also carried out in L929 cells with 24 h incubation as a negative control experiment.

e. Cell morphological evaluation of apoptosis

To identify morphological indication of apoptosis, the cell nuclei were stained with fluorescent nuclear dye, ethidium bromide (EtBr). In brief, 1×10^5 cells were seeded in the 12 well plates on the cover slip. After 24h, cells were treated with 100 μg/ml of DOX-MNPs and equivalent amount of DOX. The cells were incubated for 3, 24 and 48 h with the DOX and DOX-MNPs. After the incubation, the cells were washed thoroughly with PBS, fixed with 70% ethanol and further incubated with EtBr (10 mM) for 5 min. Again the cells were washed with PBS, mounted on a glass slide and then examined with an IX81 FV500 laser scanning confocal microscope.

f. In vitro magnetic hyperthermia experiments

The heating ability of FA-MNPs was evaluated from the time dependent calorimetric measurements using hyperthermia instrument. 2 mg/ml suspension of FA-MNPs was subjected for the heating ability under appropriate conditions to reduce the heat loss. The AC magnetic field (AMF) of 338 Oe at a fixed frequency of 265 kHz was used to evaluate the specific absorption rate (SAR). The SAR was calculated using the following equation:

$$SAR = C * (\Delta T/\Delta t) * (1/m_{Fe})$$

Where, C is the specific heat of solvent (C =4.18 J/g℃), ΔT / Δt is the initial slope of the time-dependent temperature curve and $m_{\text{FA-MNPs}}$ is FA-MNPs concentration in 1 ml of water.

Furthermore, for the investigation of *in vitro* magnetic hyperthermia, HeLa cells (1×10^6) were seeded in 6 well plates followed by treatment with FA-MNPs, DOX and DOX-MNPs for 24 h. Subsequently, cells were washed three times with PBS to remove non-internalized

particles. Then the cells were trypsinized and centrifuged. Cell pellets were further dispersed in 1ml of fresh culture media and were subjected to ACMF (338 Oe, 10 min.) using induction heating system. After ACMF exposure, cells were further cultured for 24 h. Cell viability was performed using SRB assay. Cells were treated under different conditions and referred in the text as follows: (a) control HeLa cells without any treatment, (b) cells with FA-MNPs under ACMF (FA-MNPs +ACMF), (c) cells with DOX (d) cells with (DOX-MNPs), and (e) cells with DOX loaded FA-MNPs under ACMF (DOX-MNPs + ACMF).

g. Evaluation of HeLa cell death mechanisms by Annexin V-FITC and PI assay

An Annexin V-FITC and PI assay was used for the detection of apoptotic cells. The assay procedure was followed according to the instructions given in the Annexin V-FITC apoptosis detection kit (Invitrogen). 1×10^6 cells per experiment have been taken. After 24 h, cells were treated with 2mg/ml of FA-MNPs, DOX-MNPs in the presence ACMF for 10 min and kept in the incubator for 24 h. Then, cells are washed with cold PBS. Cells were centrifuged and the supernatants were removed. Cell plates were again suspended in 100 µl of binding buffer. Cells were stained with 5 µl of Annexin V-FITC and 1 µl of propidium iodide (PI) at room temperature in the dark and kept for 30 min. The stained cells were again diluted by the same binding buffer and directly analyzed by the fluorescence-activated cell sorting method (FACS, BD FACS Aria, and USA).

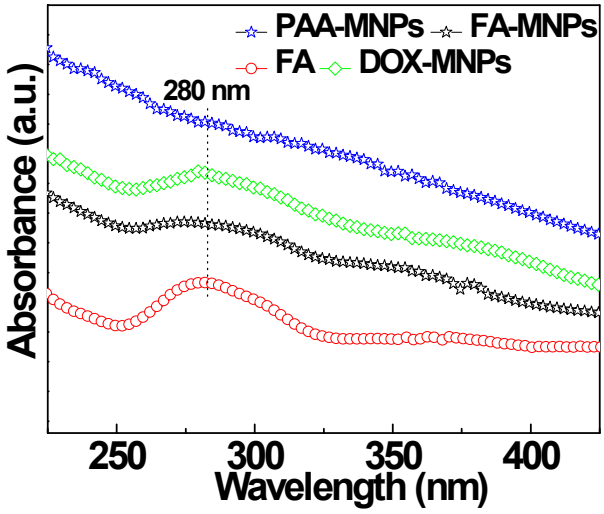

Fig.S1.UV-visible spectra of FA, FA-MNPs and DOX-MNPs.

UV-visible spectra of FA, FA-MNPs clearly shows the characteristic peaks of FA at 280 and 370 nm on the spectrum of FA-MNPs, which suggests that FA ligands have been grafted by amide reaction. However, a bathochromic shift could be noticed compared to those of FA, which reveals the modification in the environment of the FA between the free and grafted states.

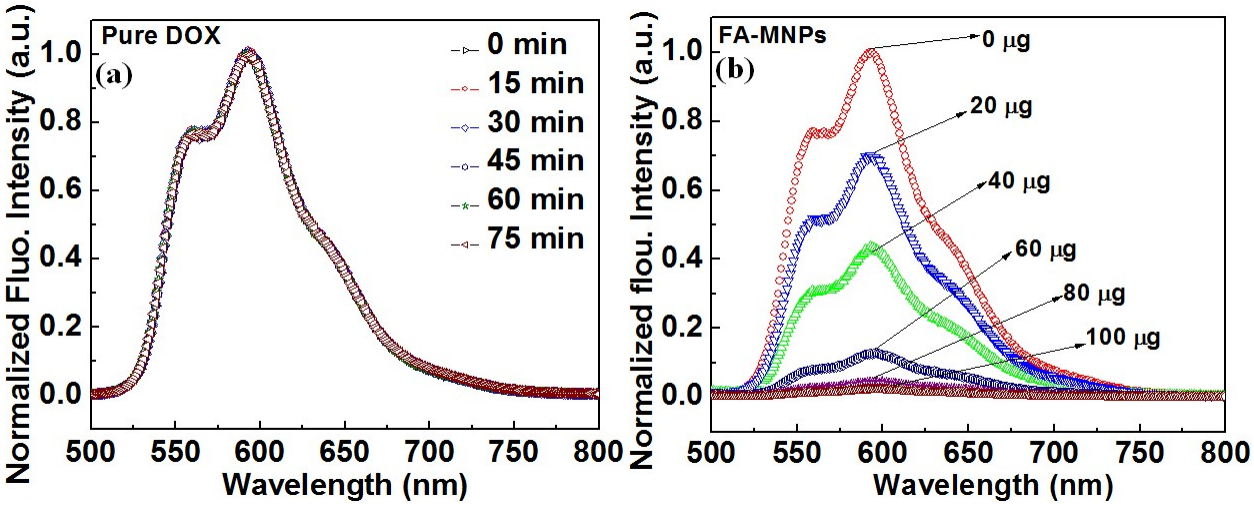

Fig. S2. Fluorescence spectra of (a) pure DOX and (b) Fluorescence spectra of DOX with the addition of FA-MNPs.

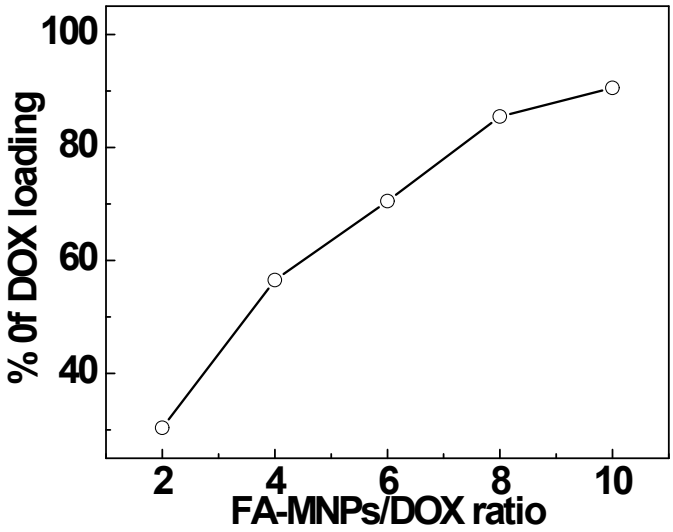

Fig.S3. Loading efficiency of FA-MNPs

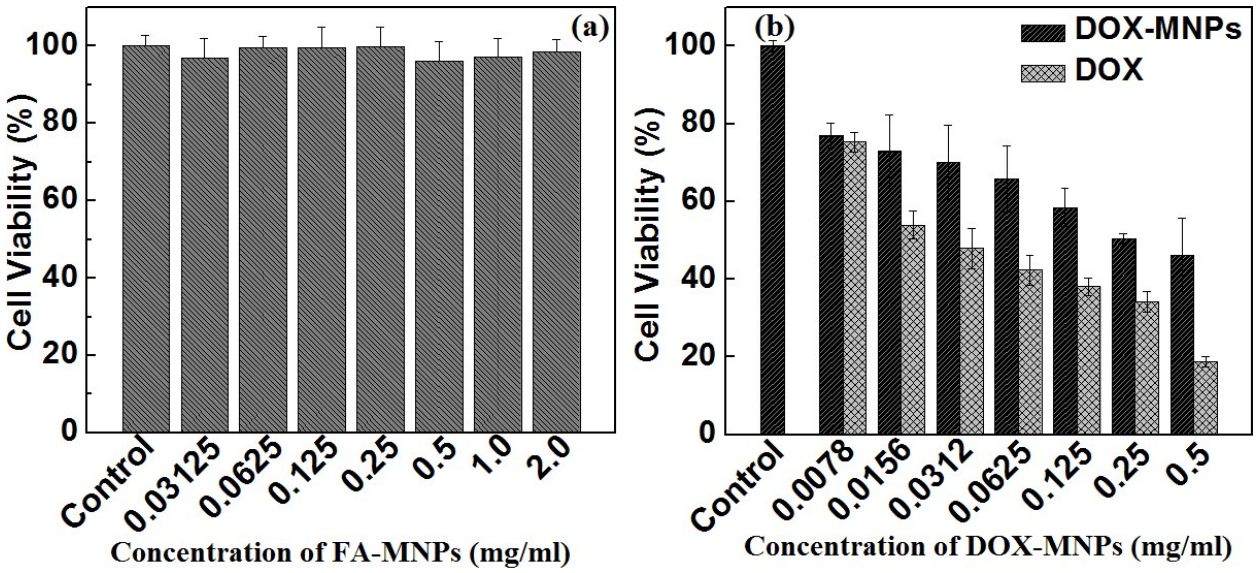

Fig.S4 (a) Cell Viability of FA-MNPs and (b) Cytotoxicity study of DOX and DOX-MNPs of HeLa cells after 24 h incubation, respectively.

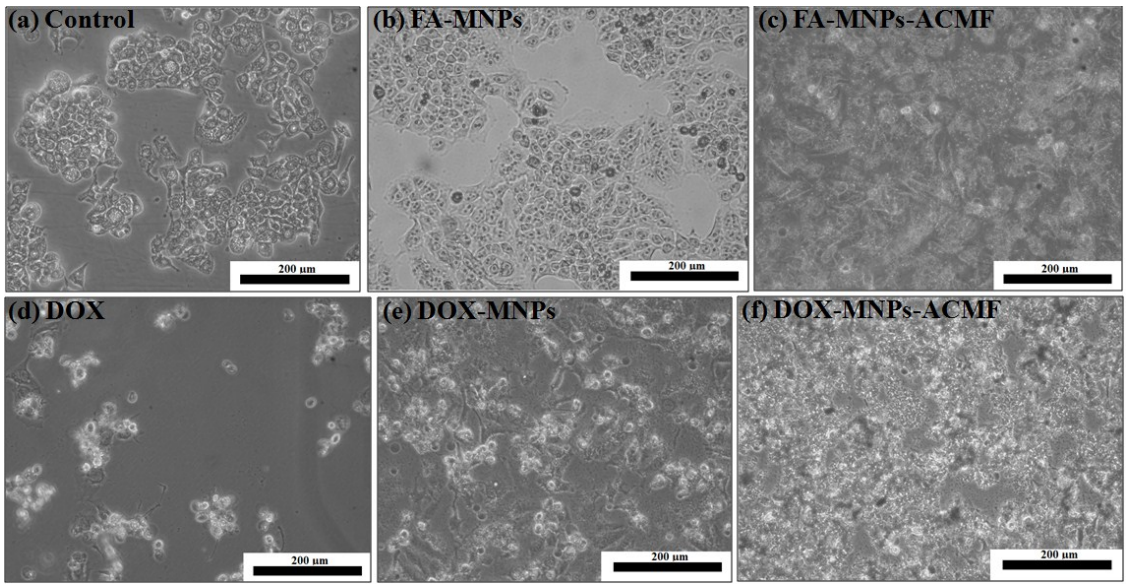

Fig.S5. Representative Micrographs of HeLa cells for (a) control, (b) FA-MNPs, (c) FA-MNPs with ACMF, (d) DOX, (e) DOX-MNPs, (f) DOX-MNPs with ACMF.

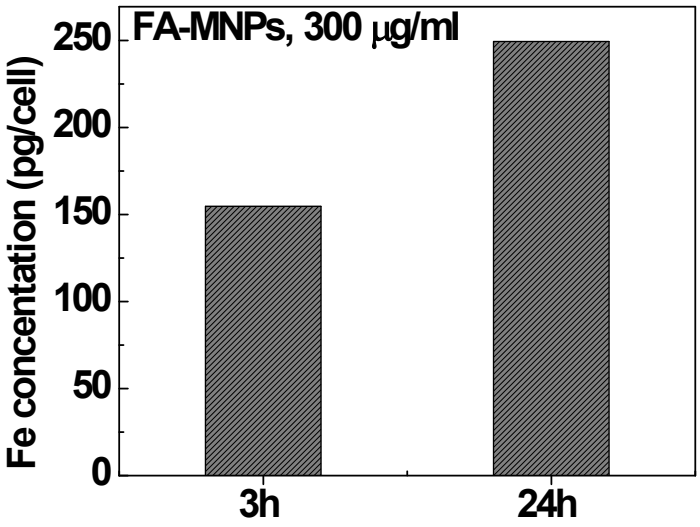

Fig. S6. Quantification of cellular uptake of FA-MNPs by measuring the iron content per cell using ICP-AES analysis for different time periods.

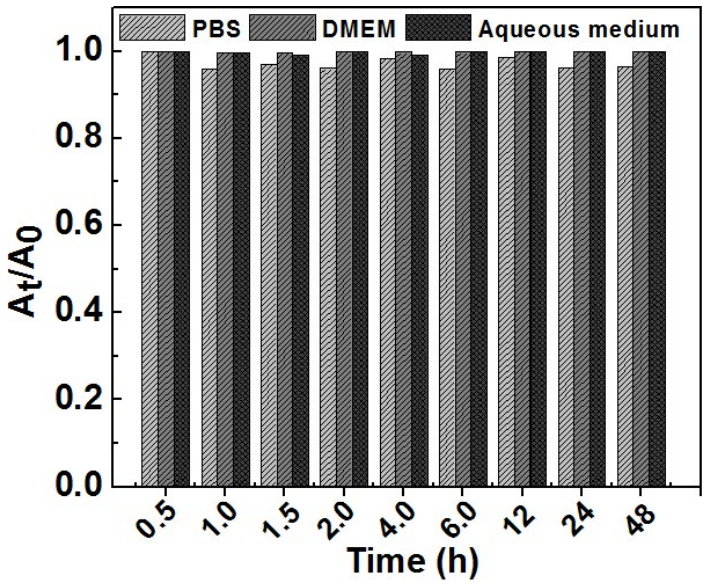

Fig. S7. (a) Normalized UV absorbance (At/A0) vs. time plot of FA-MNPs (0.1 mg/ml) at wavelength of 350 nm in PBS, aqueous and cell culture medium (At = absorabnce at time 't' and A0 =absorbance at t = 0)

Protein resistance properties of FA-MNPs:

Table S1. Zeta-potential of FA-MNPs incubated with BSA for different time.

FA-MNPs (0.1 mg/ml) in 5 ml of 0.1 M PBS (pH 7.3)	FA-MNPs (0.1 mg/ml) incubated with BSA (0.1 mg/ml) in 5 ml of 0.1 M PBS (pH 7.3)			
	30 min	2 h	6h	24 h
-24.29	-24.03	-24.22	-25.54	-25.58