Supporting Information

Synthesis of a novel magnetic drug delivery system composed of doxorubicin-conjugated Fe_3O_4 nanoparticle cores and a PEG-functionalized porous silica shell

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Materials

Iron(III) acetylacetonate ($Fe(acac)_3$, 99.9%), oleylamine (>70%), diphenyl ether (99%), methyl 3-mercaptopropionate ($HSCH_2CH_2COOCH_3$, ≥98%), hydrazine monohydrate ($N_2H_4•H_2O$, ≥98%), tetraethylorthosilicate (TEOS, 98%), 3-aminopropyltriethoxysilane (APTS, 99%) poly(ethylene glycol) (PEG, Mw ~ 8,000), 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC, >98.5%), *N*-hydroxysuccinimide (NHS, 98%), fluorescein isothiocyanate isomer I (FITC, ≥90%), and folic acid (≥97%) were purchased from Sigma-Aldrich. Doxorubicin hydrochloride (DOX·HCl, ≥98%) was obtained from BBI. Sodium hydroxide (NaOH), ammonium hydroxide ($NH_3·H_2O$, 28 % by weight in water), disodium hydrogen phosphate (Na_2HPO_4), potassium dihydrogen phosphate (KH_2PO_4), ethanol, 2-propanol, acetic acid, and hydrochloride (HCl, 37.5%) were purchased from Beijing Chemical Co. The aforementioned chemicals without denotation are of analytical grade and used as received without further purification. RPMI 1640 culture medium and fetal bovine serum (FBS) were purchased from Invitrogen. Human cervical carcinoma HeLa cells and Human lung cancer A549 cell line were kindly gifted by Prof. Haiyan Liu of Soochow University.

Experimental

1. Preparation of Magnetite Nanoparticles.

Fe (acac) $_3$ (3 mmol) was dissolved in 10 mL of diphenyl ether and 20 mL of oleylamine. The solution was dehydrated at 110^{o}C for 1 h under N_2 atmosphere, then quickly heated to 300 ^{o}C at a heating rate of 20 ^{o}C/ min, and aged at this temperature for 1 h. After the reaction, the solution was allowed to cool down to room temperature. The Fe_3O_4 nanoparticles were extracted upon the addition of 50 mL of ethanol, followed by centrifuging and drying at 60 ^{o}C under vacuum.

2. Functionalization of Magnetite Nanoparticles.

The as-prepared Fe_3O_4 nanoparticles (140 mg) were dispersed in 20 mL of diphenyl ether to form a colloid solution by sonication. Methyl 3-mercaptopropionate (33 μL) was then added to the colloid solution and refluxed at ~ 259^{o}C for 1 h. Subsequently, the solution was cooled to 100^{o}C and 145 μL of hydrazine monohydrate (N_2H_4•H_2O) was added dropwise to the solution, while continuously stirring the solution for 2 h. The resulting nanoparticles were separated by centrifuging at 15,000 rpm for 10 min, washed with methanol, and dried at 60°C under vacuum for 24 h. As a consequence of the above procedure, the Fe_3O_4 nanoparticles functionalized with -$NHNH_2$ group on the surface (Fe_3O_4-$NHNH_2$) were obtained.

3. Synthesis of Fe_3O_4-DOX nanoparticles

The Fe_3O_4-$NHNH_2$ nanoparticles (100 mg) were dispersed by sonication in 20 mL of methanol containing 0.5 mL of acetic acid to form a colloid solution. Doxorubicin (10.3 mg) was then added to the colloid solution while stirring. The reaction was carried out at room temperature for 48 h. The resulted DOX-loaded magnetite nanoparticles (Fe_3O_4-DOX) were isolated with a Nd-Fe-B magnet and washed with methanol until the supernatant became colorless, and finally

dried at room temperature under vacuum. All of the supernatants were collected and diluted to 250 mL with methanol in a capacitance flask for evaluation of the drug loading efficiency by means of UV-vis spectroscopy.

4. Synthesis of core/shell Fe_3O_4-DOX /SiO_2 nanoparticles

The obtained Fe_3O_4-DOX dispersion (4 mL) was mixed with 20 mL of isopropanol. The solution was then treated by ultrasonication for 30min. Under continuous stirring, 0.5 mL of ammonia solution (28%) and 200 μl of TEOS were consecutively added to the reaction mixture. The reaction was allowed to proceed at room temperature for 24 h. The obtained Fe_3O_4-DOX /SiO_2 nanoparticles were isolated by centrifugation, and washed four times with water and ethanol to eliminate the homogeneous silica nucleus. Finally, the Fe_3O_4-DOX/SiO_2 core/shell nanoparticles were redispersed in 20 mL of deionized water.

5. Etching the silica shell

PEG (Mw~ 8,000, 4 g) was added to the solution of the as-prepared Fe_3O_4-DOX/SiO_2 under stirring. The mixture was heated up to 100 ^oC for 3 h to load PEG, and then cooled to room temperature. Under magnetic stirring, 4 mL of sodium hydroxide aqueous solution (0.1 g/mL) was added to the above solution to etch the silica shell for 60 min. The solution was then cleaned with repeated cycles of water dilution and centrifugation. The obtained Fe_3O_4-DOX/pSiO_2-PEG core/shell nanoparticles were finally dispersed in 10 mL of deionized water or dried at room temperature.

6. ***DOX-loading capacity***

The amount of the drug grafted to the Fe_3O_4 nanoparticles was estimated by UV-vis spectral measurement. Considering spectral measurement may be interfered by the presence of the

nanoparticles, the DOX loading efficiency is calculated as follows:

$$\text{DOX-loading efficiency (\%)} = 100\ (W_{\text{feed DOX}} - W_{\text{free DOX}}) / W_{\text{feed DOX}}$$

The free DOX weight ($W_{\text{free DOX}}$) in the supernatant is determined using the Lamber-Beer law.[1] The DOX loading efficiency estimated using the above equation is 56.9% ($W_{\text{feed DOX}}$ =10.3 mg) and the amount of DOX grafted per 1 mg Fe_3O_4 nanoparticles is 64.5 μg/mg. However, due to the presence of porous silica shell functionalized with PEG, the DOX loading capacity of the prepared porous drug carrier system is only 16.3 μg/mg, calculated directly from its absorption value of UV-vis spectra due to the good hydrophilicity

7. *Folic acid-modified Fe_3O_4-DOX/pSiO_2-PEG core/shell nanoparticles*

The solution of the Fe_3O_4-DOX/pSiO_2-PEG core/shell nanoparticle was mixed with 20μL 3-aminopropyltriethoxysilane (APTS) to result the $–NH_2$ functionalized nanoparticles, which then reacted with the –COOH groups of folic acid in the presence of EDC and NHS for 48h at room temperature. The obtained folic acid-modified Fe_3O_4-DOX/pSiO_2-PEG core/shell nanoparticles were isolated with a Nd-Fe-B magnet and washed with deionized water several times.

8. Intracellular uptake of the Fe_3O_4-DOX/pSiO_2-PEG core/shell nanoparticles

To demonstrate the localization of the drug-loaded nanoparticles in the cells, N-1-(3-triethoxysilypropyl)-N'-fluoresceylthiourea (FITC-APTS), prepared by stirring fluorescein isothiocyanate (FITC) in ethanol solution of APTS in the dark for 24 h, was mixed with the Fe_3O_4-DOX/pSiO_2-PEG core/shell nanoparticles to label FITC. Then the FITC-labeled nanoparticles loaded with DOX (5 μg/ mL) was incubated with HeLa cell for 3 h. The cells in the wells were washed three times with PBS and then visualized by fluorescence microscope after being stained by DAPI.

9. In vitro drug release experiment

Fe_3O_4-DOX nanoparticles and Fe_3O_4-DOX/pSiO_2-PEG were suspended, respectively, in 4 mL PBS buffer (pH=7.4) with a concentration of 0.1 mg/mL at 37^{o}C under constant stirring. The solution pH was adjusted by titration of 1.0 M HCl to achieve pH values of 5. After incubation for 1, 2, 3, 5, 8, 12, 24, 36, 48 and 72h, the nanoparticle suspensions were centrifuged at 3000 rpm to isolate the nanoparticles from the released DOX and PBS solutions. The released DOX was analyzed by monitoring the absorbance at 482 nm, and the amount was determined by the calibration curve of DOX in PBS ($C_{\mu g/mL}$=42.7758Abs+0.2244), range from 0 to 25 μg/mL with R^2=0.9999.

10. Intracellular release of DOX from the nanocarriers

The HeLa and A549 cells were cultured in 12-well plates (1×10^5 cells per well) overnight, then the culture medium was replaced with fresh culture medium containing drug carrier particles and incubated in a fully humidified atmosphere at 37 ^{0}C containing 5% CO_2 , respectively. The final concentration of DOX cultured with cells was all at 5 μg mL^{-1}. After 1-12 hours treatment, the medium was replaced with PBS and the fluorescence of DOX in the cells were observed under a fluorescence microscope.

11.Cytotoxicity of the prepared drug carrier nanoparticles

To determine cytotoxicity/viability, the HeLa cells were plated at a density of 1×10^4 cells/well in a 96-well plate at 37^{o}C in 5% CO_2 atmosphere. After 24 h of culture, the medium in the wells was replaced with the fresh medium containing the nanoparticles of varying concentrations. After 24h, 20μL of MTT dye solution (5 mg/mL in phosphate buffer pH 7.4) was added to each well. After 4 h of incubation at 37^{o}C in 5% CO_2 for exponentially growing

cells and 15 min for steady-state confluent cells, the medium was removed. Formazan were solubilized with 150μL of DMSO and the solution was mixed under vigorous stirring to dissolve the reacted dye. The absorbance of each well was read on a microplate reader at 490 nm. The spectrophotometer was calibrated to zero absorbance, using culture medium without cells. The relative cell viability (%) related to control wells containing cells culture medium without nanoparticles was calculated by $[A_{test}]/[A_{control}] \times 100\%$.

Characterization

Morphology and structure of the nanoparticles were characterized by using a Tecnai-G20 transmission electron microscope (TEM). Samples for TEM were prepared by placing a drop of water dispersion onto a carbon-coated copper grid and dried at room temperature. Fourier transform infrared (FTIR) spectra were collected with a Thermo Nicolet 6700 spectrophotometer with a resolution of 4 cm^{-1}. The samples for FTIR measurement were prepared by dispersing of the nanoparticles in a KBr pellet. N_2 adsorption-desorption isotherms, and pore-size distributions were obtained using a Micromeritics ASAP 2020 analyzer at 77K. Prior to measurements, samples were degassed at 180 ^oC for 12 h. Uv-vis spectra were recorded on a Lambda 25 UV-vis spectrophotometer. Cell lines were cultured with a water-jacketed CO_2 incubator (Thermo 3111). Cells were visualized by a Nikon TI-E fluorescent microscope. The crystallographic data were obtained on an X'Pert-Pro MRD X- ray diffractometer with Cu Kα radiation (I =1.5418 Å). The magnetic property was measured on a Lakeshore 7300 vibrating sample magnetometer at room temperature. Contact angle of the nanoparticles was measured using a Dataphysics OCA20 contact-angle system at ambient temperature. Absorbance in the MTT assay was read by a Biotek Elx 800 microplate reader.

Supporting Figures

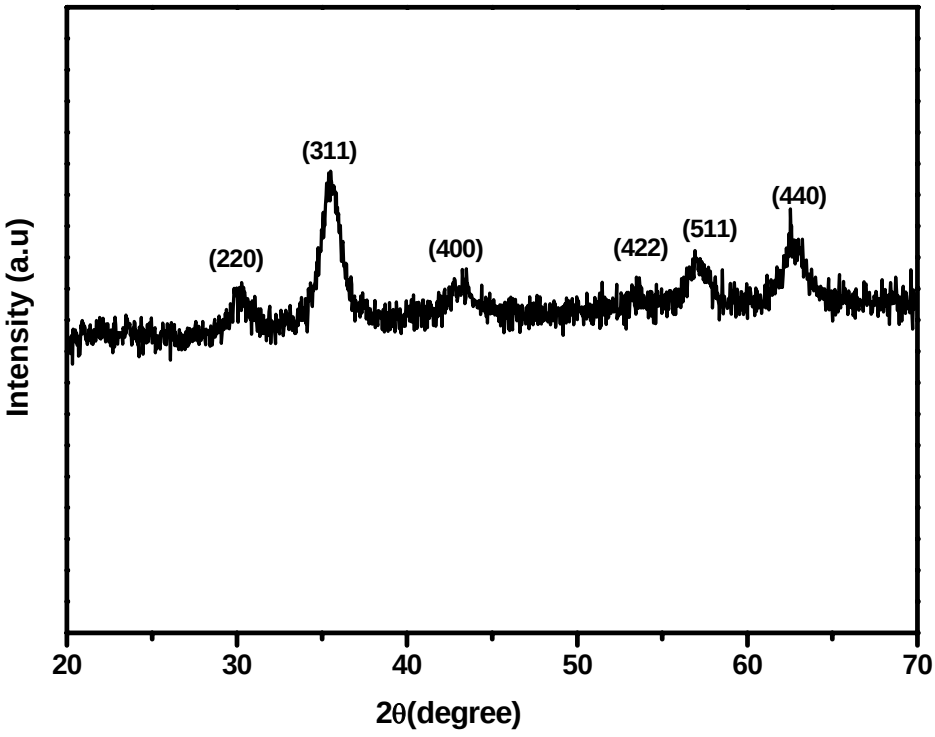

Fig.S1 XRD pattern of the as-prepared Fe_3O_4 nanoparticles

Fig.S1 shows the XRD pattern of the as-prepared Fe_3O_4 nanoparticles. All diffraction peaks in the XRD pattern of the as-prepared magnetite nanoparticles can be easily indexed to be a pure cubic inverse spinel structure, which matches well with the reported data (JCPDS: 65-3107), indicating the formation of Fe_3O_4 phase.

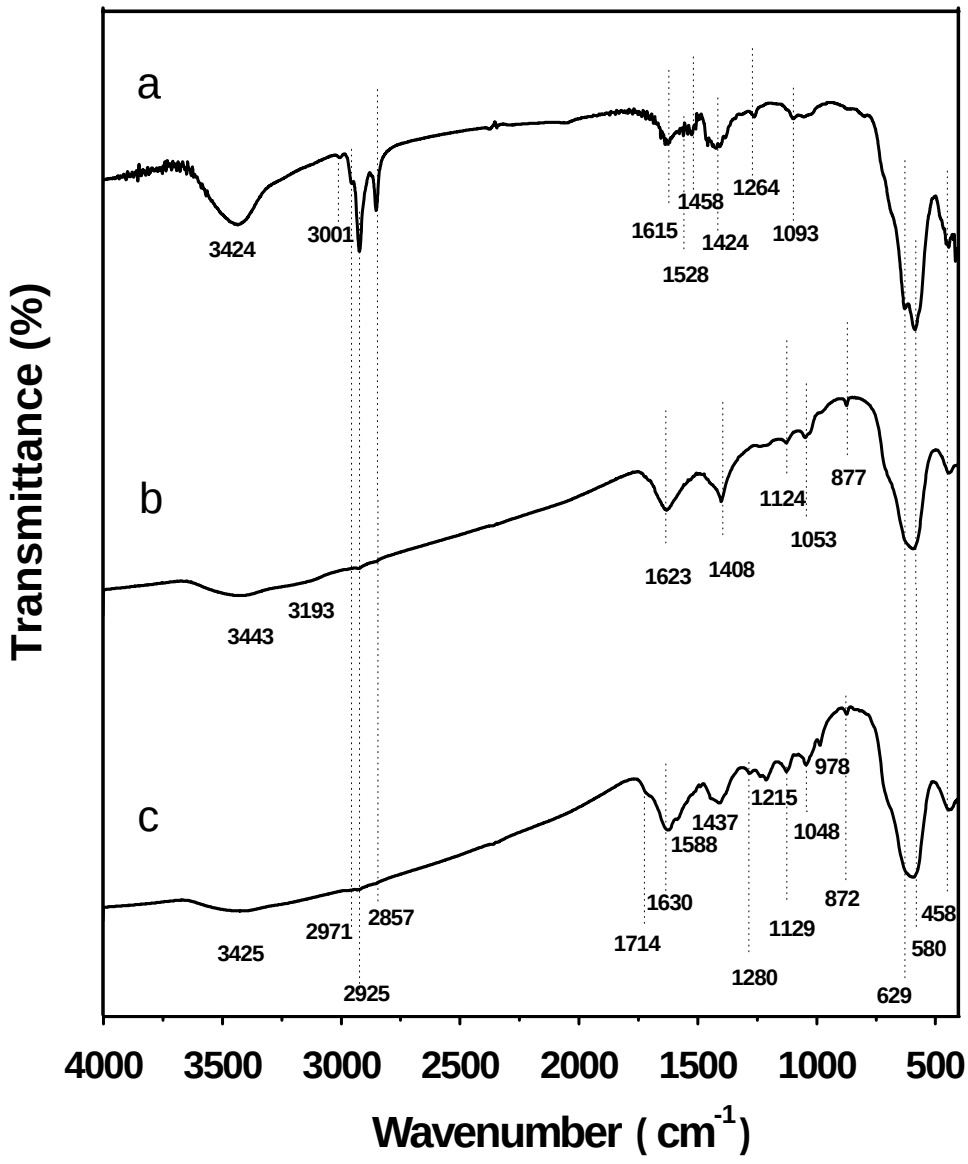

Fig.S2 FTIR spectra of the as-synthesized hydrophobic Fe_3O_4 nanoparticles (a), the Fe_3O_4-$NHNH_2$ nanoparticles (b), and the Fe_3O_4 -DOX nanoparticles(c).

Fig.S2 shows FTIR spectra of the as-synthesized hydrophobic Fe_3O_4 nanoparticles (a), the Fe_3O_4-$NHNH_2$ nanoparticles (b) and the Fe_3O_4-DOX (c). The absorption bands at 633, 580, and 458 cm^{-1} in all spectra, characteristic of the Fe-O bond of Fe_3O_4, imply the presence of the Fe_3O_4

nanoparticles in each step. Curve a of Fig.S2 gives the FTIR spectrum of the as-synthesized hydrophobic Fe_3O_4 nanoparticles. The absorption bands at 3424 cm^{-1} (ν(N-H)), 3001 cm^{-1} (ν(=C-H)), 2970, 2925 and 2857 cm^{-1} (ν_{as}(C-H), ν_s(C-H)), 1615 cm^{-1} (δ (N-H)), 1458 and 1424 cm^{-1} ($\delta_{as}(CH_3)$, $\delta_s(CH_3)$), and 1093 cm^{-1} (ν(C-N)) are originated from oleylamine, the surface ligand. The absorption band at 1528 cm^{-1} is assigned to Fe-N bonds, suggesting that $-NH_2$ coordinates with Fe (III) on the surface of the nanoparticles.[2] The characteristic bands of FTIR spectra of Fe_3O_4-$NHNH_2$ (Fig. S2b) include ν(N-H···H) (3443, 3193 cm^{-1}), ν(C-H) (2925, 2857 cm^{-1}), ν(C=O) (1623 cm^{-1}), $\nu_{as}(-NH_3^+)$ (1408 cm^{-1}) , ν(C-N) (1053 cm^{-1}) and δ (N-H) (877 cm^{-1}). In addition, the bands due to C-H stretching mode in the range from 2800 to 3000 cm^{-1} become significantly weak, indicating a change of the hydrocarbon chain length from C_{18} (oleylamine) to C_3 ($HSCH_2CH_2CONHNH_2$). Taken together, the Fe_3O_4 nanoparticles are successfully functionalized with a hydrazide end-group, convenient for grafting of DOX in the next step. Similarly, FTIR results also confirm the conjugation of DOX to magnetic nanoparticles via reaction of hydrazide groups of the Fe_3O_4-$SCH_2CH_2CONHNH_2$ with carbonyl groups of DOX (Fig.S2c). A feature peak at 1630 cm^{-1} is assigned to -C=N- bonds resulting from the reaction of hydrazide group of the Fe_3O_4-$SCH_2CH_2CONHNH_2$ with carbonyl group at the 13-keto position of DOX. The characteristic bands at 1714 cm^{-1}, 1588 cm^{-1} and 1280 cm^{-1} correspond to DOX. They are ascribed to the stretching vibration of unreacted carbonyl group at the 13-keto position, the stretching vibration of carbonyl groups located at the anthracene ring and the skeleton vibration of DOX molecule, respectively. While the other characteristic bands outlined in Fig.S2b continue to be present (Fig. S2c), demonstrating that DOX is chemically conjugated on the surface of the magnetite nanoparticles.

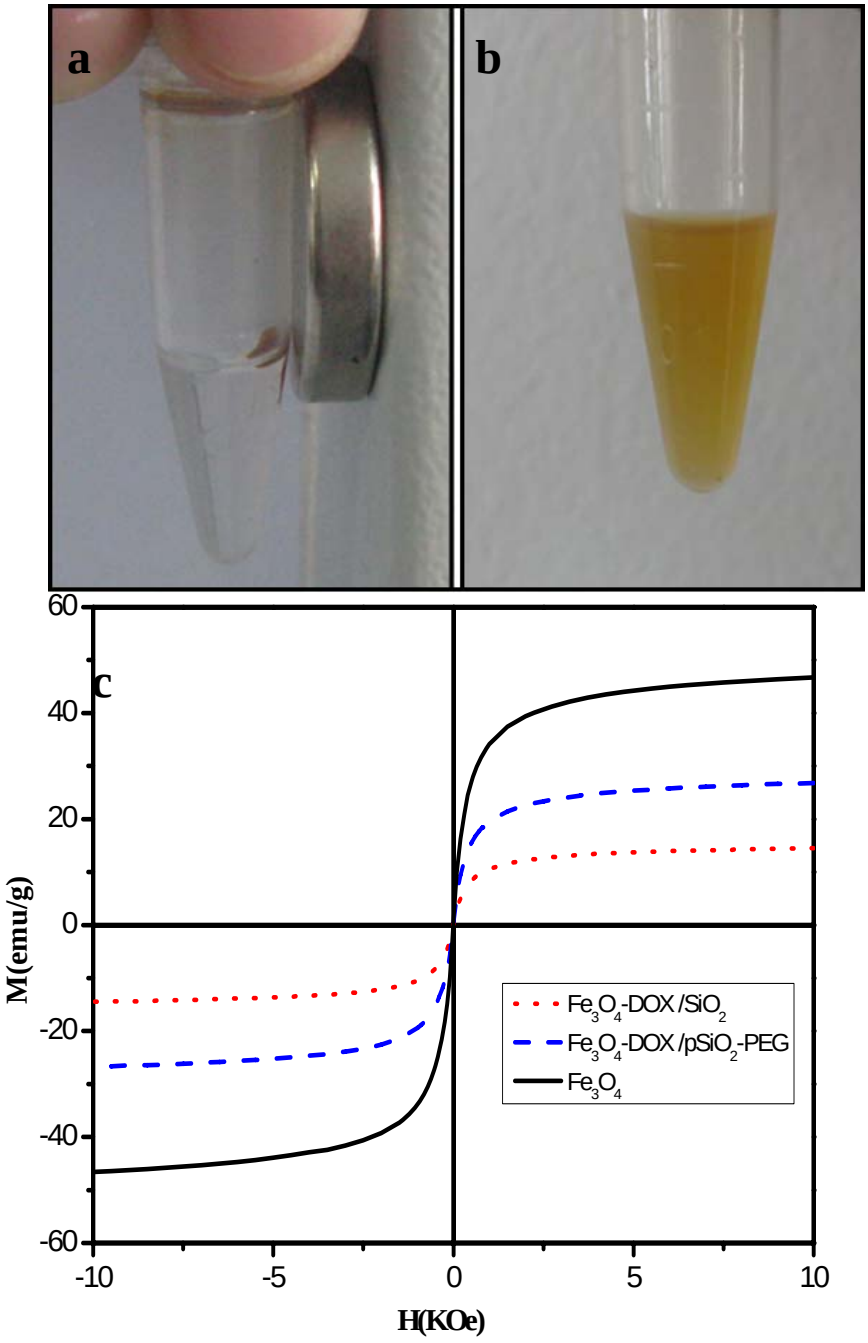

Fig.S3 Photographs of aqueous solution of the Fe_3O_4-DOX/pSiO_2-PEG core/shell nanoparticles attracted by an external magnet (a) and their dispersion on removal the external magnetic field (b), Magnetization curve for the Fe_3O_4-DOX/pSiO_2-PEG (dash line), the Fe_3O_4-DOX/SiO_2 (dot line) and the Fe_3O_4 (solid line) nanoparticles (c).

Fig.S3 shows photographs of the prepared Fe_3O_4-DOX/pSiO_2-PEG core/shell nanoparticles attracted by an external magnet (a) and their dispersion on removal the external magnetic field (b). On application of an external magnetic field to the container with the Fe_3O_4-DOX/pSiO_2-PEG core/shell nanoparticles, the nanoparticles are attracted towards the magnet and attached on the wall of the container in close proximity of the magnet and the dispersion becomes clear (Fig.S3a). Removal of the external magnetic field and shaking leads to the complete recovery of the dispersion (Fig.S3b), confirming that the prepared DOX-loaded magnetic delivery system is sensitive to an external magnetic field and shows superparamagnetic property.

The other merit of converting dense silica shell to porous one lies in increased magnetization value, as evidenced by the magnetization curve for the Fe_3O_4-DOX/pSiO_2-PEG (dot line), Fe_3O_4-DOX/SiO_2 (dash line) and Fe_3O_4 (solid line) nanoparticles illustrated in Fig.S3c. Room temperature specific magnetization (M) versus applied magnetic field (H) curve measurement of the Fe_3O_4-DOX/pSiO_2-PEG nanoparticles gives a saturation magnetization value (Ms)of 26.8 $emug^{-1}$, higher than that of the dense silica shell coated Fe_3O_4-DOX nanocomposite (14.5 $emug^{-1}$). These results are in agreement with our expectation that the presence of the porous silica shell is beneficial to magnetic manipulation.

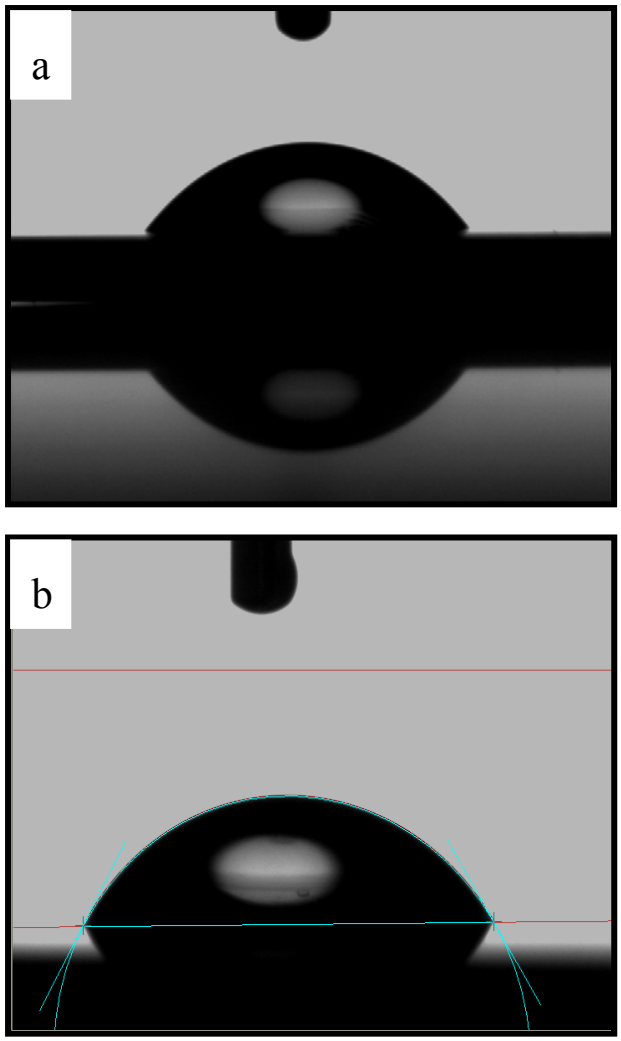

Fig.S4 Wettability of a 2 μL water droplet (a) and the water dispersion of the Fe_3O_4-DOX/pSiO_2-PEG core/shell nanoparticles (b) on a silicon substrate surface.

Fig.S4 shows the wettability of a 2 μL water droplet (a) and the water dispersion of the prepared Fe_3O_4-DOX/pSiO_2-PEG core/shell nanoparticles (b) on the silicon substrate surface. Their apparent contact angles are measured to be 51.3 ^{o} and 58.6 ^{o}, respectively. The contiguous contact angles of the prepared nanoparticle water dispersion and water, both smaller than 90^{o}, indicate good hydrophilicity.

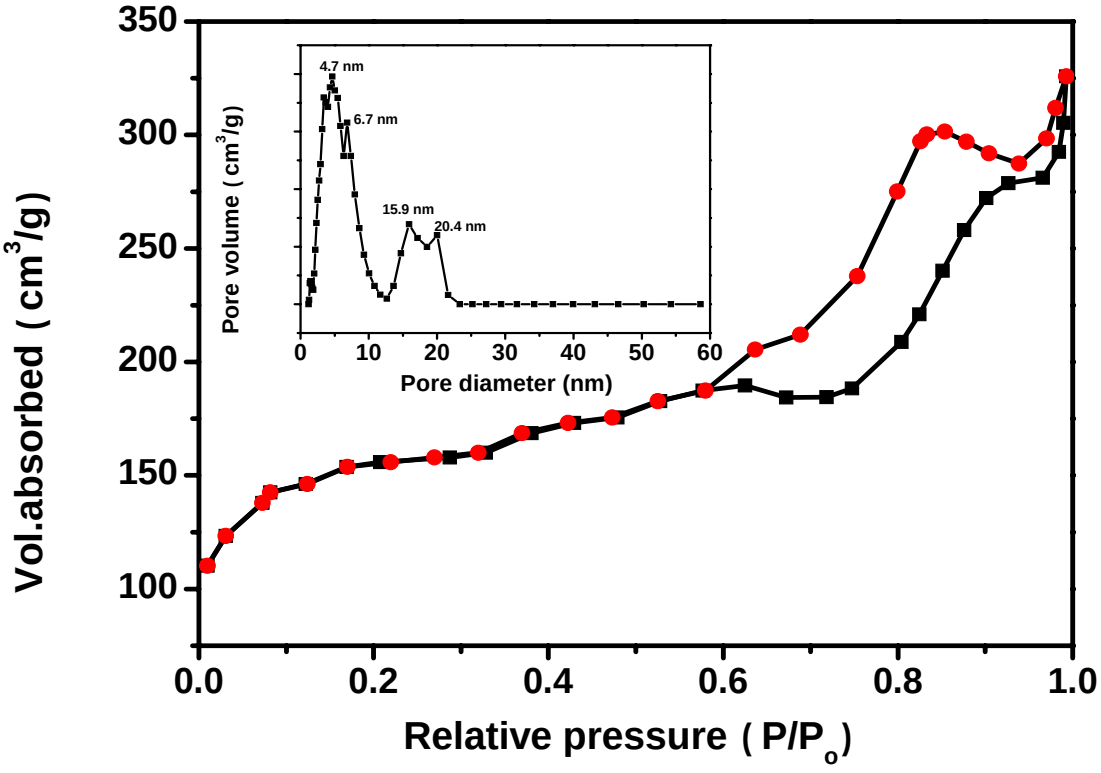

Fig.S5 N_2 adsorption-desorption isotherms and pore size distribution (inset) of the Fe_3O_4-DOX /pSiO_2-PEG core/shell nanoparticles.

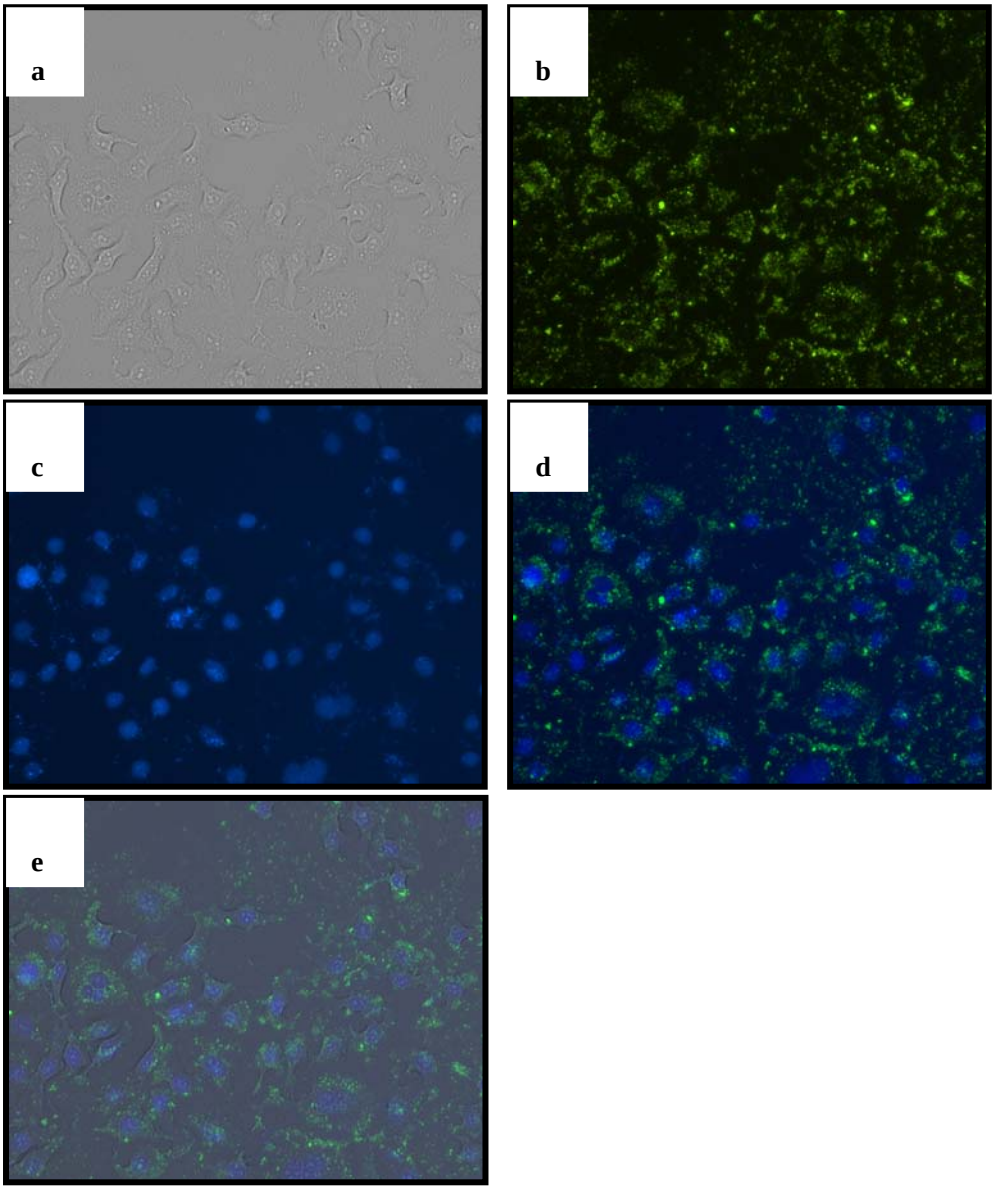

Fig.S6 Fluorescence microscope images of HeLa cells incubated with the Fe_3O_4-DOX/pSiO_2-PEG core/shell nanoparticles labeled by FITC for 3h: (a) bright field; (b) green fluorescence from FITC; (c) blue fluorescence from DAPI in the nuclei (cell nuclei were stained with DAPI); (d) the merge images of (b) and (c); (e) the merge images of (a) and (d).

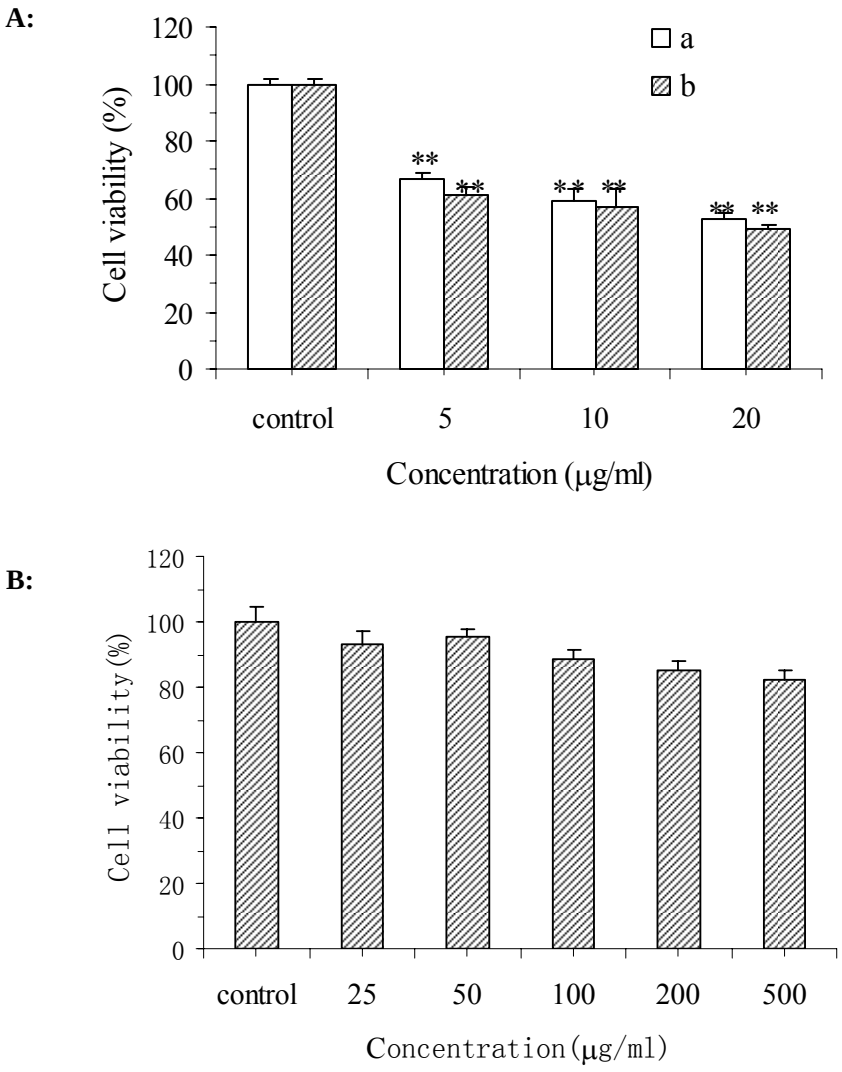

Fig.S7 (A) Relative cell viability of HeLa cells incubated with the Fe_3O_4-DOX/pSiO_2-PEG core/shell nanoparticles without FA (a) and with FA (b) for 24h; (B) Relative cell viability of HeLa cells incubated with the Fe_3O_4 /pSiO_2-PEG core/shell nanoparticles of different concentrations for 24 h.

Cytotoxicity of the FA modified Fe_3O_4-DOX/pSiO_2-PEG core/shell nanoparticles is higher than that of the corresponding nanoparticles without FA modification, indirectly confirming the specific uptake of the FA-targeted drug delivery system by HeLa cells via receptor-mediated

endocytosis. It is noted that the Fe_3O_4 /pSiO_2-PEG core/shell nanoparticles without loading of DOX have very low cytotoxicity in a wide range of concentrations (25μg/ml to 500μg/ml), as shown in Fig. S7B. All these facts strongly suggest the effective antitumor activity of DOX released from the carriers in the cells.

References:

1. D. H. Williams and L. Fleming, *Spectroscopic methods in organic chemistry, 4th edn*. New York, McGraw-Hill, 1989.

2. Z. C. Xu, C. M. Shen, Y. L. Hou, H. J. Gao and S. H. Sun, *Chem. Mater.*, 2009**, 21**, 1778.