Supporting Information

A novel magnetic drug delivery nanocomplex of cisplatin-conjugated Fe_3O_4 core and a PEG-functionalized mesoporous silica shell nanocomposites for enhancing drug delivery efficiency of cancers

Chandrababu Rejeeth, [a*, †] Raju Vivek, [a†] and Soundarapandian Kannan [a, b]

[a] *Proteomics & Molecular Cell Physiology Laboratory, Department of Zoology, School of Life Sciences, Bharathiar University, Coimbatore-641 046, TN, India.*

[b] *Department of Zoology, Periyar University, Salem-636 001, TN, India.*

[†]*Current address: Department of Biomedical Engineering, Shanghai Jiao Tong University, Shanghai, China.*

Materials

Iron (III) acetylacetonate (Fe (acac) $_3$, 99.9%), oleylamine (70%), diphenyl ether (99%), methy 3-mercaptopropionate ($HSCH_2CH_2COOCH_3$, 98%), hydrazine monohydrate ($N_2H_4 \bullet H_2O$, 98%), tetraethylorthosilicate (TEOS, 98%), 3-aminopropyltriethoxysilane (APTES, 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. Cis-diamminedichloroplatinum (II) (CDDP 98%) was obtained from BBI. Sodium hydroxide (NaOH), ammonium hydroxide ($NH_3.H_2O$, 25-28 % 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 Himedia Lab Chemicals Mumbai, India. The aforementioned chemicals without denotation are of analytical grade and used as received without further purification. Dulbecco's Modified Eagle Medium (DMEM) and fetal bovine serum (FBS) were purchased from Invitrogen Bangalore, India. Human breast carcinoma MCF-7 and Human cervical carcinoma HeLa cell line were purchased from National Centre for Cell Science (NCCS), Pune, India.

Methods

1. Synthesis of Fe_3O_4 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 hour under N_2 atmosphere, then

quickly heated to 300°C at a heating rate of 20°C/ min, and aged at this temperature for 1 hour. 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°C under vacuum evaporator.

2. Functionalization of Fe_3O_4 nanoparticles

The prepared Fe_3O_4 nanoparticles (150 mg) were dispersed in 25 ml of diphenyl ether to form a colloid solution by sonication. Methyl 3-mercaptopropionate (35 µl) was then added to the colloid solution and refluxed at ~ 260°C for 1 hour. Subsequently, the solution was cooled to 100°C and 150 µL of hydrazine monohydrate ($N_2H_4 \bullet H_2O$) was added drop wise to the solution, while continuously stirring the solution for 2 hours. 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 hours. 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-CDDP nanoparticles

The Fe_3O_4-$NHNH_2$ nanoparticles (120 mg) were dispersed by sonication in 25 ml of methanol containing 0.5 ml of acetic acid to form a colloid solution. CDDP (10 mg) was then added to the colloid solution while stirring. The reaction was carried out at room temperature for 48 hours. The resulted CDDP-loaded magnetite nanoparticles (Fe_3O_4-CDDP) were isolated with an 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. CDDP-loading capacity

The CDDP loading capacity reported our previous [1] work and some modification as follows: 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 CDDP loading efficiency is calculated as follows:

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

The free CDDP weight ($W_{\text{free CDDP}}$) in the supernatant is determined using the Lambert-Beer law. The CDDP loading efficiency estimated using the above equation is 56.9% ($W_{\text{feed CDDP}}$ =10 mg) and the amount of CDDP grafted per 1 mg Fe_3O_4 nanoparticles is 64.5 µg/mg. However, due to the presence of mosoporous silica shell functioned with PEG, the CDDP loading capacity of the prepared porous drug carrier system is only 16.0

μg/mg, calculated directly from its absorption value of UV-*vis* spectra due to the good hydrophilicity.

5. Synthesis of core/shell Fe_3O_4-CDDP /mpSiO_2 nanocomposite

The obtained Fe_3O_4-CDDP dispersion (5 ml) was mixed with 20 ml of isopropanol. The solution was then treated by ultra-sonication for 30 min. 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 hours. The obtained Fe_3O_4-CDDP/mpSiO_2 nanocomposite was isolated by centrifugation and washed four times with water and ethanol to eliminate the homogeneous silica nucleus. Finally, the Fe_3O_4-CDDP/mpSiO_2 core/shell nanocomposite was redispersed in 20 ml of deionized water.

6. Etching the mesoporous silica shell

PEG (Mw~ 8,000, 4 g) was added to the solution of the prepared Fe_3O_4-CDDP/ mpSiO_2 under stirring. The mixture was heated up to 100^{o}C for 3 hours 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-CDDP/ mpSiO_2-PEG core/shell nanocomposite were finally dispersed in 10 ml of deionized water or dried at room temperature.

7. Nanocomposite Characterization

Morphology and structure of the nanocomposite were characterized by using a JEOL JEM-2100 transmission electron microscope (TEM). Samples for TEM were prepared by placing a drop of ethanol dispersion onto a carbon-coated copper grid and dried at room temperature. Fourier transform infrared (FT-IR) spectra were collected with a Thermo Nicolet 6700 spectrophotometer with a resolution of 4 cm^{-1}. The samples for FT-IR 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 ^{o} C for 12 hours. 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 magnetic property was measured on a Lakeshore 7300 vibrating sample magnetometer at room temperature. Absorbance in the MTT assay was read by a Biotek Elx 800 micro plate reader.

8. Folic acid-modified Fe_3O_4-CDDP/ mpSiO_2-PEG core/shell nanocomposite

The solution of the Fe_3O_4-CDDP/mpSiO_2-PEG core/shell nanocomposite was mixed with 20μl 3-aminopropyltriethoxysilane (APTES) 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 48 hours at room temperature. The obtained folic acid-modified Fe_3O_4-CDDP/mpSiO_2-PEG core/shell nanocomposite were isolated with an Nd-Fe-B magnet and washed with deionized water several times.

9. Intracellular uptake of the Fe_3O_4-CDDP/ mpSiO_2-PEG core/shell nanocomposite

To demonstrate the localization of the drug-loaded nanoparticles in the cells, N-1-(3-triethoxysilypropyl) -N'-fluoresceylthiourea (FITC-APTES), prepared by stirring fluorescein isothiocyanate (FITC) in ethanol solution of APTES in the dark for 24 hours, was mixed with the Fe_3O_4-CDDP/ mpSiO_2-PEG core/shell nanocomposite to label FITC. Then the FITC-labeled nanoparticles loaded with CDDP (5 μg/ ml) were incubated with HeLa and MCF-7 cell for 3 hours. The cells in the wells were washed three times with PBS and then visualized by fluorescence microscope after being stained with DAPI.

10. In vitro drug release experiment

Fe_3O_4-CDDP nanoparticles and Fe_3O_4-CDDP/MPS-PEG nanocomposite were suspended, respectively, in 4 ml PBS buffer (pH=7. 4) with a concentration of 0.1 mg/ml at 37^oC 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 72 hours, the nanoparticle suspensions were centrifuged at 3000 rpm to isolate the nanoparticles from the released CDDP and PBS solutions. The released CDDP was analyzed by monitoring the absorbance at 482 nm, and the amount was determined by the calibration curve of CDDP in PBS ($C_{\mu g/ml}$ = 42.7758Abs + 0.2244), range from 0 to 25 μg/ml with R2 = 0.9999.

11. Intracellular release of CDDP from the nanocarriers

The MCF-7 and HeLa cells were cultured in 12-well plates (1×10^5 cells per well) overnight, then the culture medium were replaced with fresh culture medium containing drug carrier particles and incubated in a fully humidified atmosphere at 37 °C containing 5% CO_2, respectively. The final concentration of CDDP cultured with cells was all at 5 μg mL^{-1}. After 1-12 hours treatment, the medium were replaced with PBS and the fluorescence of CDDP in the cells was observed under a fluorescence microscope.

12. Cytotoxicity of the prepared drug carrier nanocomposite

To determine cytotoxicity/viability, the MCF-7 cells were plated at a density of 1×10^4 cells/well in a 96-well plate at 37^oC in 5% CO_2 atmosphere. After 24 hours of culture, the medium in the wells was replaced with the fresh medium containing the nanoparticles of

varying concentrations. After 24 hours, 20µl of MTT dye solution (5 mg/ml in phosphate buffer pH 7.4) was added to each well. After 4 hours of incubation at 37°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 cell culture medium without nanoparticles was calculated by $[A_{test}] / [A_{control}] \times 100\%$.

Supporting Figures

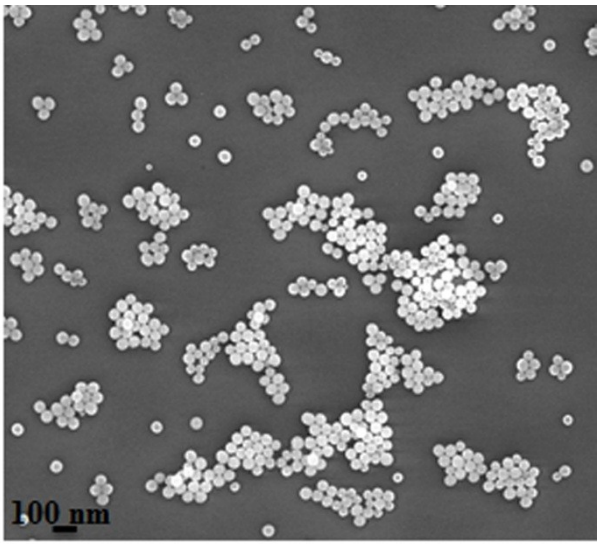

Fig. S1 SEM images of the Fe_3O_4 nanoparticles

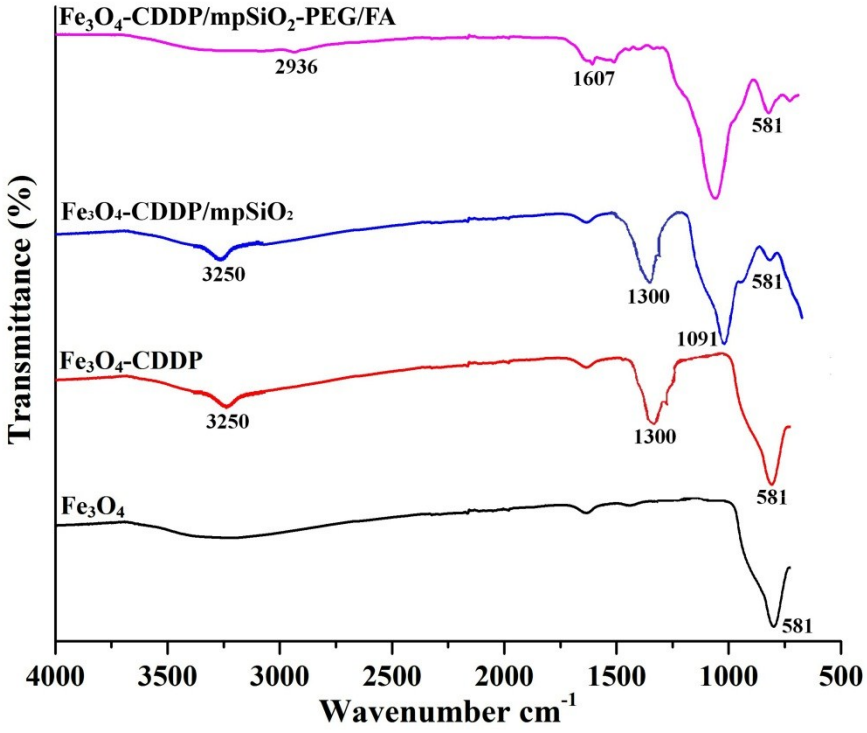

Fig. S2 FT-IR spectra of the as-synthesized Fe_3O_4-CDDP/mpSiO_2-PEG nanocomposite .

Fourier transform infrared spectroscopy (FT-IR) was also used to confirm the Fe_3O_4-CDDP/mpSiO_2-PEG composite formation. As revealed in Fig. S1, Fe_3O_4 spheres have one strong characteristic peak at 581cm^{-1} corresponding to the vibration of Fe–O bond. After coated with CDDP, two additional strong absorption peaks appeared. While the peak at 1300 cm^{-1} is attributed to the stretching of $NHNH_2$ bond CDDP linker [2], another peak at 3250 cm^{-1} arise from the amine bond vibration. Taken together, the Fe_3O_4 nanoparticles are successfully functionalized with a hydrazide end-group, convenient for grafting of CDDP in the next step. Similarly, FT-IR results also confirm the conjugation of mesoporous silica Si-O-Si bond peak at 1091cm^{-1}. The well-resolved vibrational peak at 1607 cm-1 assigned to the N-H bending vibration of the CONH group was present in the spectrum. Therefore, the FTIR technique further confirmed that FA ligands have been successfully grafted onto Fe_3O_4-CDDP/mpSiO_2 nanocomposite.

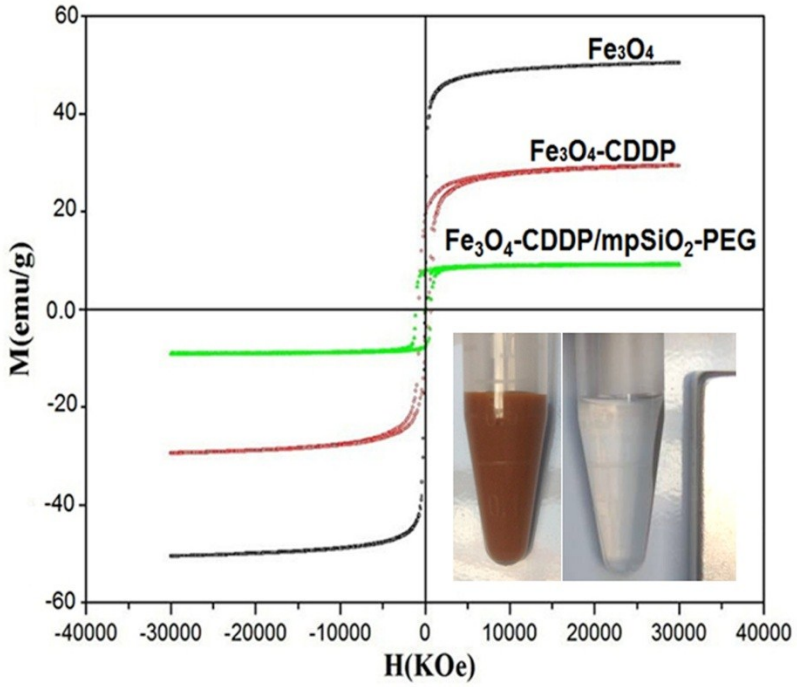

Fig.S3 Photographs of an aqueous solution of the Fe_3O_4-CDDP/mpSiO_2-PEG core/shell nanocomposite attracted by an external magnet and their dispersion on removal the external magnetic field

Fig.S3 shows an application of an external magnetic field to the container with the Fe_3O_4-CDDP/mpSiO_2-PEG core/shell nanocomposite, the nanocomposite are attracted towards the magnet and attached to the wall of the container in close proximity of the magnet and the dispersion becomes clear. Removal of the external magnetic field and shaking leads to the complete recovery of the dispersion (inset) Fig S3, confirming that the prepared CDDP-loaded magnetic delivery system is sensitive to an external magnetic field and shows superparamagnetic property [3]. 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-CDDP/mpSiO_2-PEG (green line), Fe_3O_4- CDDP (redline) and Fe_3O_4 (black line) nanoparticles. Room temperature, specific magnetization (M) versus applied magnetic field (H) curve measurement of the Fe_3O_4-CDDP/mpSiO_2-PEG nanocomposite gives a saturation magnetization value (Ms) of 9.2 $emug^{-1}$ higher than that of the dense silica shell coated Fe_3O_4-CDDP nanocomposite (25 $emug^{-1}$). Fe_3O_4 alone was (55 $emug^{-1}$). These results are in agreement with our expectation that the presence of the porous silica shell is beneficial to magnetic manipulation.

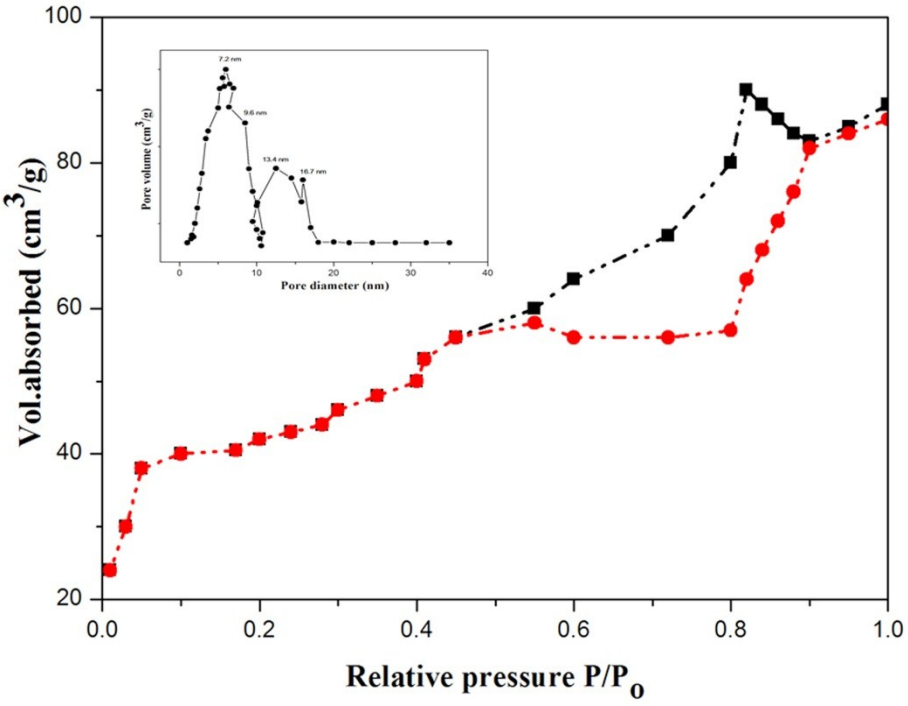

Fig.S4 N_2 adsorption-desorption isotherms and pore size distribution (inset) of the Fe_3O_4-CDDP/mpSiO_2-PEG core/shell nanocombosite.

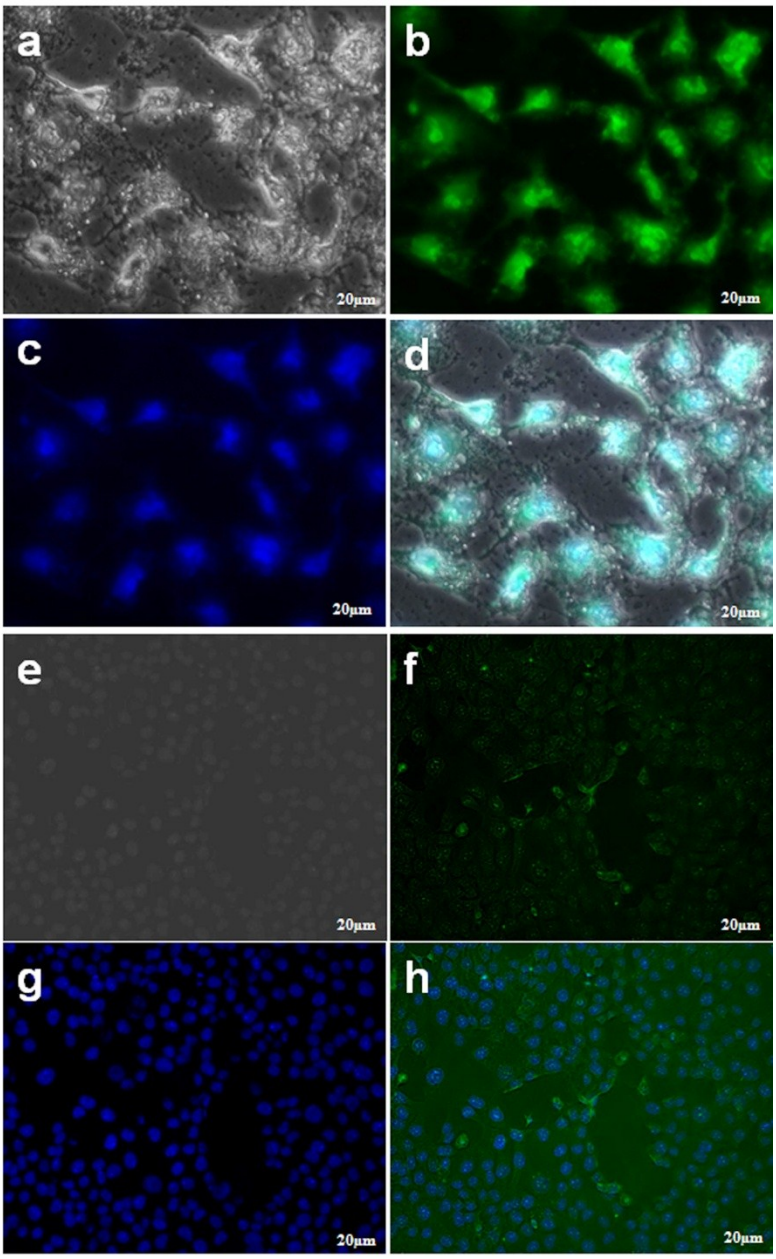

Fig.S5 Fluorescence microscope images of HeLa and MCF-7 cells incubated with the Fe_3O_4-CDDP/mpSiO_2-PEG core/shell nanoparticles labeled with FITC for 3h: a, b, c and d Hela cells e, f, g and h MCF-7 cells. (a, e) bright field; (b, f) green fluorescence from FITC; (c, g) blue fluorescence from DAPI in the nuclei (cell nuclei were stained with DAPI); (d, h) the merge.

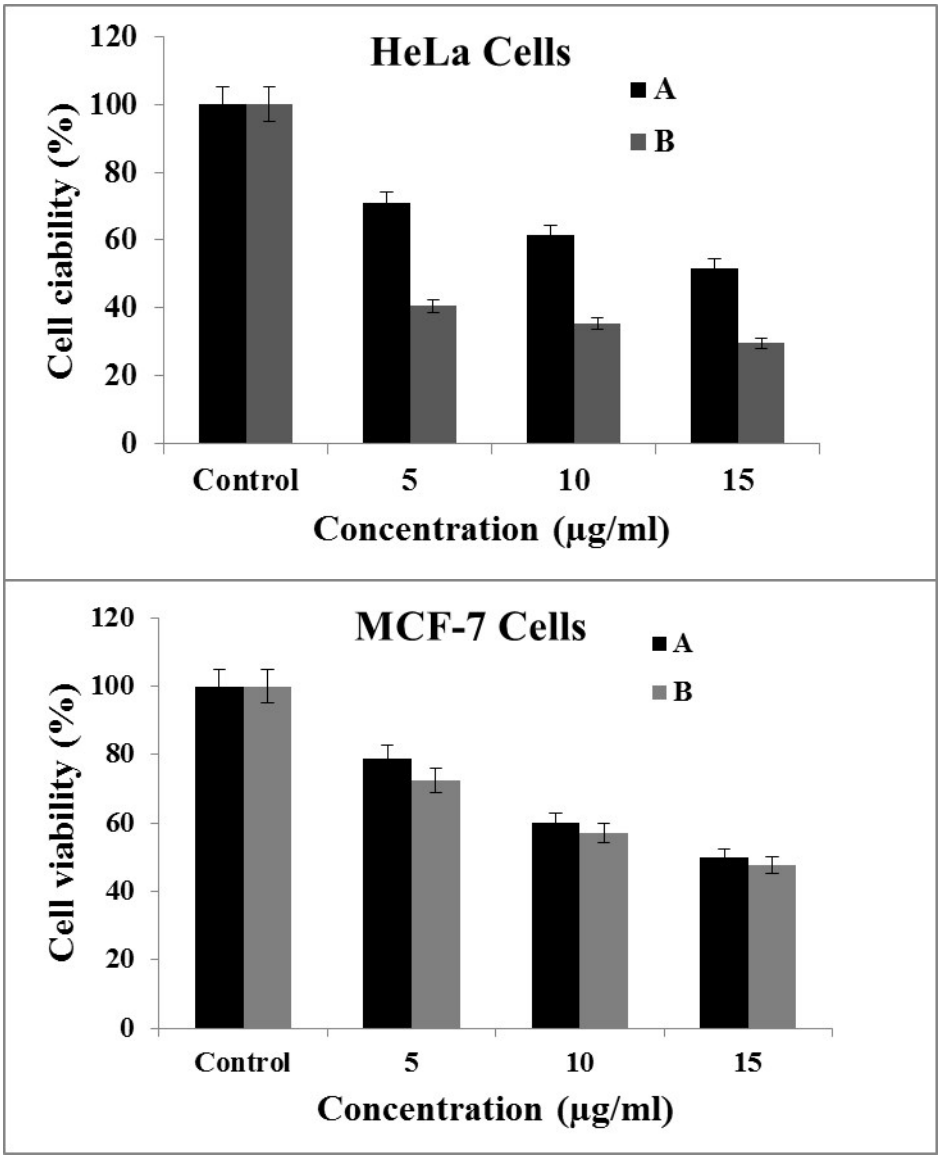

Fig.S6 Relative cell viability of HeLa and MCF-7 cells incubated with the Fe_3O_4-CDDP/mpSiO_2-PEG core/shell nanocomposite without FA (a) and with FA (b) for 24hours.

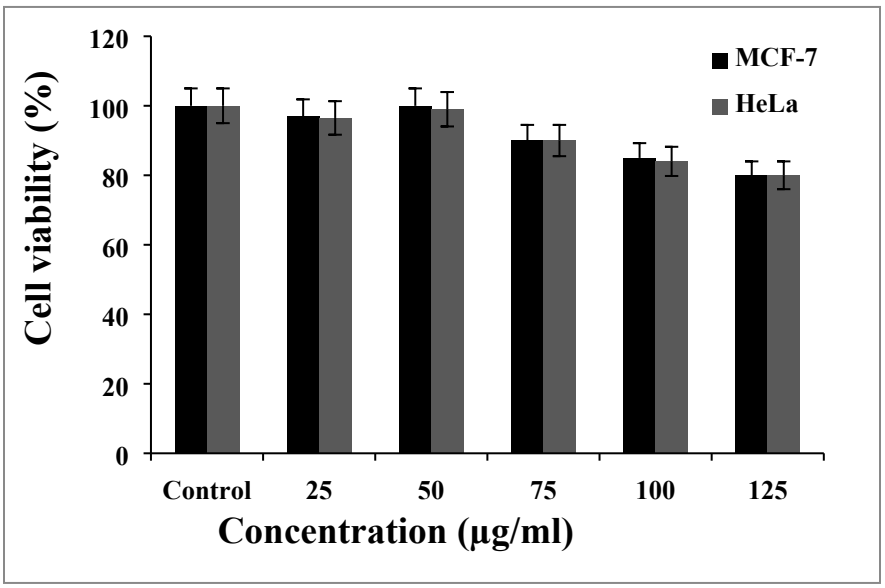

Fig.S7 Relative cell viability of Hela and MCF-7 cells incubated with the Fe_3O_4/mpSiO_2-PEG core/shell nanocomposite of different concentrations for 24 hours.

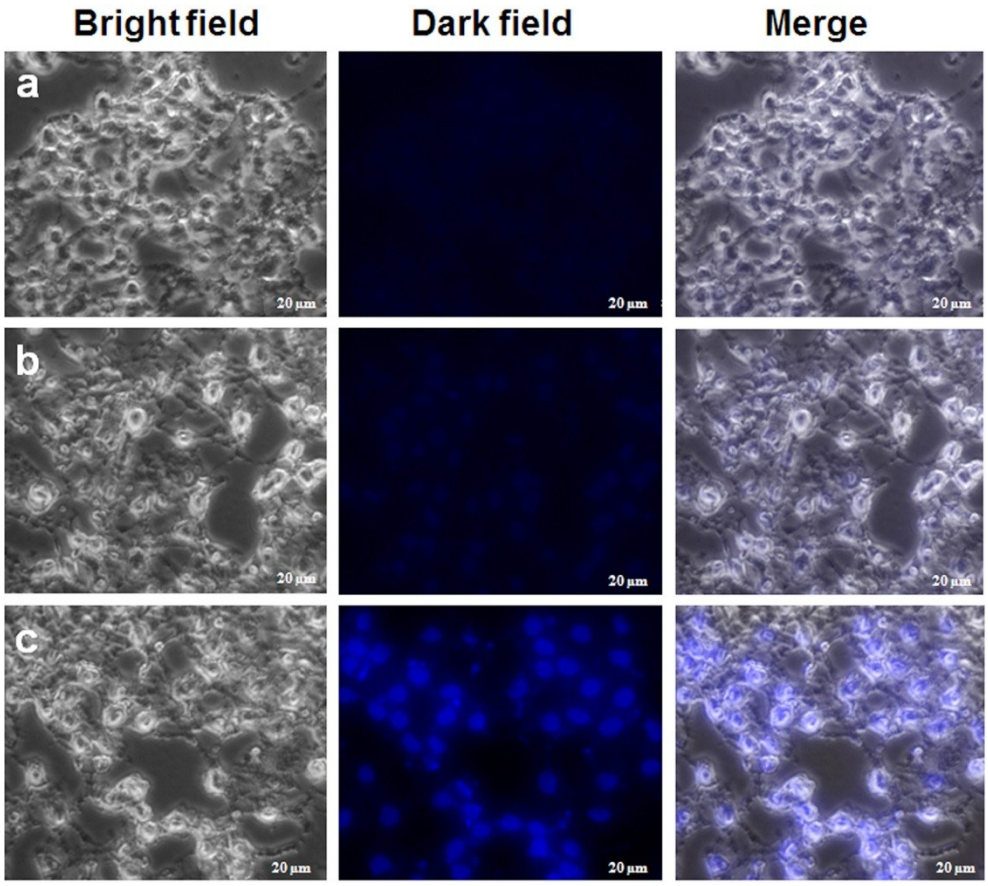

Fig. S8 Micrograph of HeLa cells treated with Fe_3O_4-CDDP/mpSiO_2-PEG core/shell nanocomposite. HeLa cells were incubated with 10 µM and labeled with DAPI to show nuclear morphology. (a) Control HeLa cells; (b) 3 hours incubated HeLa cells and (c) 12 hours incubated HeLa cells showing nuclear morphological changes.

Reference

1. C. Rejeeth, T.C. Nag, S. Kannan, *Cancer Nano.* 2013, **4**, 127-136.

2. P. D. Marcato, W. J. Favaro, N. Duran, *Curr Cancer Drug Tar.,* 2014, **14**, 476.

3. S. Mohapatra, S. R. Rout, R. Narayan, T. K. Maiti, *Dalton. Trans.,* 2014, **43**, 15850.