

Superparamagnetic iron oxide nanocargoes for combined cancer thermotherapy and MRI applications

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Experimental

Synthesis of Iron oxide Fe₂O₃ nanoparticles (IONPs)

Iron oxide Fe₂O₃ nanoparticles are prepared by a sol–gel method modified under supercritical conditions of ethyl alcohol (EtOH) and reported in our recent publication.^{1,2} In brief 6 g of iron (III) acetylacetonate [C₁₅H₁₂FeO₆] is dissolved in a 36 ml of methanol. Magnetic stirring is applied to dissolve iron precursor in methanol, the solution is placed in an autoclave and dried under supercritical conditions of EtOH. The supercritical conditions of ethanol were T_c = 243 °C and P_c = 63.3 bar. The temperature programmer is used to control heating of the autoclave. The particles size is tuned by time agitation of magnetic stirring. Three agitation times of 15 min can produce nanoparticles with average diameters of 14 nm.

Synthesis of polymer micelle loaded magnetic nano cargoes (PMNCs)

To form the polymeric micelle on IONPs, initially IONPs are functionalized with bis(*p*-sulfonatophenyl)phenylphosphine) (BPS). In brief, 20 mg IONPs are dispersed in 20 mL double distilled water, in this 10 mg BPS solution prepared in 1 mL DD water added drop wise and resulting solution kept on magnetic stirrer for 24 h. BPS coated IONPs centrifuged and washed three times with mili-pore water and dried in vacuum. In the second step BPS coated IONPs were incubated in mPEG-SH(MW-356.5) with ratio of 1:50 (IONPs: mPEG-SH) on magnetic stirrer at 40 °C for 24 h to allow formation of polymeric micelle structure on IONPs. The solution was centrifuged, and then the thick-colored NPs bottom layer was collected and resuspended in 0.5×TBE. This step was repeated at least three times to wash off free mPEG molecules.

Biocompatibility study

Cell toxicity study

The *in vitro* cytotoxicity study of IONPs and mPEG@IONPs was performed on MCF7 cells in accordance with our previous study.^{3,4} The MCF7 cells were grown in DMEM (Dulbecco's Modified Eagle Medium) supplemented with 10% v/v fetal bovine serum (FBS), kanamycin (0.1 mg/mL), penicillin G (100 U/mL), and sodium bicarbonate (1.5 mg mL⁻¹) at 37°C in a 5% CO₂ atmosphere. After 24, the 10 μL MTT dye (obtained from EZ-CYTOX- Enhanced Cell Viability Assay Kit) was added into each well, including the control wells without NPs. The plates were incubated for 3 h at 37 °C in a 5% CO₂ atmosphere with or without both NPs and growth media as blank. Finally, absorbance at 450 nm was monitored without any further cell washing process according to manufacturer's protocol and the cell viability was calculated. The experiments were replicated three times and the data were graphically presented as mean ± standard deviation (SD). The relative cell viability (%) compared with the control well containing cells without nanoparticles were calculated by the equation: $\frac{[A_{\text{absorbance}}]_{\text{tested}}}{[A_{\text{absorbance}}]_{\text{control}}} \times 100$.

Hemolysis assay

Fresh human blood stabilized with heparin was used for the hemolytic activity of bare and mPEG capped IONPs. A pre-treatment was conducted to obtain HRBCs for hemolysis assay

according to literature.⁵ Briefly, HRBCs were isolated from the blood by centrifugation at 1000 rpm for 20 min at 4°C and repeatedly rinsing with PBS five times. The isolated HRBCs were diluted ten times with PBS buffer. The diluted HRBC suspension (0.1 ml) was added to 1 ml water (positive control, complete hemolysis), PBS only (negative control, no hemolysis), or PBS buffer containing various concentration of bare and capped IONPs from 0.1 to 1.0 mg mL⁻¹. After a gentle shaking, the mixtures were incubated for 3 and 20 h at room temperature in triplicates. The tubes were centrifuged at 5000 rpm for 1 min. The supernatants were used to record the spectra using Perkin Elmer Lambda 25 UV-vis spectrometer. The hemolysis percentage of samples was calculated by dividing the difference in absorbance (at 541 nm) between the sample and the negative control by the difference in absorbance between the positive and negative controls.

FITC labelling and Cellular uptake of mPEG@IONPs

To prepare fluorescent labeled mPEG@IONPs are prepared according protocol.⁶ In brief, 10 mg mPEG capped IONPs are dispersed in 5 mL DD water by ultrasonication. 10 mg FITC dissolved in 1 mL ethanol-acetone (1:1) solution. The prepared FITC solution is added drop wise in IONPs solution and overall mixture was kept for 24 h on magnetic stirrer. In dark After 24 h incubation FITC labelled IONPs are separated by magnetic decantation and washed three times by DD water to remove unbound FITC. The uptake of fluorescent-labeled (FITC) IONPs was investigated in MCF7 cells using confocal microscopy (LEICA TCS SP5, Germany). Cells (1 × 10⁵ mL⁻¹) were incubated with 100 µg mL⁻¹ of FITC labelled IONPs for 10 h in µ-Dish (35 mm, high) Glass bottom obtained from ibidi Germany. After that, cells were fixed with 4% paraformaldehyde for 15 min, washed three times with PBS and finally confocal acquisitions were performed at a magnification of 40×.

Loading and release of anticancer drug (Doxorubicin)

To encapsulate DOX into nanomicell EDC/NHS coupling chemistry is used. Initially 10 mg DOX is dissolved in 1 mL DD water, to this solution EDC (5 mg,) in 1 mL of H₂O, NHS (10 mg,) was added drop wise and solution kept at 4 °C overnight on magnetic stirrer. Further the prepared solution is added into 10 mg mPEG capped IONPs solution made in 10 mL water and final solution is kept for more 24 h on stirring at 4 °C. All DOX conjugation experiments are carried in dark conditions. The DOX encapsulated particles were separated magnetically, washed thoroughly with milli-pore H₂O. In order to characterize the doxorubicin loading directly on IONPs, the particles were dispersed in 5 ml of DI water (1 mg mL⁻¹) and UV absorbance measurements were performed on the hybrid suspension. All the absorbance measurements were performed using a Cary 60 UV-vis spectrophotometer (Agilent Technologies, Inc., USA). The doxorubicin was quantified by measuring the absorbance at 480 nm. Fluorescence measurements on free DOX and conjugated DOX (Ex. 480 nm, Em. 590 nm) were performed using a Fluorolog-3 spectrofluorometer to calculate the loading efficiency. The loading efficiency (w/w%) was calculated using the following equation⁷

$$\% \text{loading efficiency} = \frac{I_{DOX} - I_S - I_W}{I_{DOX}} \times 100 \quad (1)$$

where, I_{DOX} is the fluorescence intensity of only DOX solution, I_S the fluorescence intensity of DOX conjugated IONPs and I_W the fluorescence intensity of washed DOX (physically adsorbed DOX molecules). The release behavior of DOX from the PMNCs was investigated in water with different pH values. Briefly, PMNCs samples with different pH (5 to 9) media

were diluted in water and mounted into a shaking bed at 37 °C with a rotation speed of 150 rpm. To monitor the release behavior, samples were periodically taken out at desired time intervals and subjected to magnetic decantation. The DOX concentration in the supernatant was analysed using a UV/vis spectrophotometer and spectrofluorometer, parallelly. The concentration of DOX is estimated by a standard DOX concentration curve generated from a series of DOX solutions with various concentrations. The amount of DOX released from the PMNCs was the amount of DOX in the supernatant detected by UV-vis spectrometer.

***In vitro* drug release in cancer cells**

A total of 1×10^5 MCF7 cells were reseeded on μ -Dish (35 mm, high) Glass bottom obtained from ibidi Germany and incubated for 24 h. The growth medium was then replaced by PMNCs ($100 \mu\text{g mL}^{-1}$) and control sample i.e. only DOX ($1 \mu\text{g mL}^{-1}$). After incubation for another 10 h, cells were rinsed three times with PBS. Next, cells were fixed with 4% formaldehyde in PBS for 20 min and then washed with PBS three times. For DOX delivery studies, MCF7 cells were stained with 10 μL (10 mM) DAPI for 10 min to mark the cell nuclear. The cells were washed with PBS three times and these micro dishes were observed with a LEICATCS SP5 confocal microscope (Leica Microsystems, Germany). DAPI was visualized with excitation wavelength of 405 nm, while DOX was visualized upon exciting at 511 nm. High magnification images of MCF7 cells were obtained with the 40x oil-immersion lens.

Cancer cell killing by PMNCs

MCF7 cells were seeded into a 96-well plate with a density of 1×10^5 cells per well. After incubation for 24 h, the cell culture medium was changed with a fresh medium containing various doses of PMNCs and free drug DOX. Cells were then allowed to incubate for different time intervals upto 24 h and MTT assay was performed. Briefly, cell culture media were aspirated and MTT in FBS free DMEM (10 mM) were added. After incubating for additional 3 h, MTT solution was removed and 100 μL DMSO was added to dissolve the newly formed formazan crystals. Finally, absorbance at 570 nm was monitored and the cell viability was calculated. The experiments were replicated three times and the data were graphically presented as mean \pm standard deviation (SD). The relative cell viability (%) compared with the control well containing cells without nanoparticles were calculated by the equation: $[\text{A}_{\text{absorbance}}]_{\text{tested}} / [\text{A}_{\text{absorbance}}]_{\text{control}} \times 100$.

***In vitro* hyperthermia on cancer cells**

MCF7 cells were mixed with the PMNCs with a 1 mg mL^{-1} concentration to evaluate their *in-vitro* hyperthermia performance. During the procedure, AC magnetic fields remained switched on until the upper hyperthermia temperature limit of 43-44 °C was attained. All *in-vitro* hyperthermia studies were performed at applied frequency of 265 kHz and the AC magnetic field (AMF) was kept at 300 Oe. The media containing IONPs and PMNCs suspended with 1 mL cancer cells (2×10^5 cells/mL) was placed at the center of the coil. The samples containing NPs and cells were heated for 60 min and the temperature of the system was maintained at 43-44 °C during whole experiment. Immediately after exposure, cells were placed in 96 well plates in triplicates and incubated for 24 h. The cell viability was measured using MTT assay while, simultaneously, the cell samples were incubated in slide petri dishes containing 2 mL DMEM for further confocal microscopy imaging after FITC, PI and DAPI staining.

Reactive oxygen species (ROS) assay

The level of ROS production induced by PMNCs coupled MFH treatment on MCF7 cells was measured using the 5-(and-6)-chloromethyl-2,7-dichloro-dihydrofluorescein diacetateacetyl ester (H₂DCFDA) assay. Immediately after MFH treatment, the cells were placed in 96 well plates (black bottom) divided into two groups to study the time dependent ROS generation (3 h and 24 h). 10 μ L H₂DCFDA (10 mM prepared in cell culture grade DMSO) was added and the plates were placed in dark condition for an additional 1h. Then, the cells were washed with PBS three times and rinsed with 100 μ L PBS. The fluorescence intensity of each sample was assessed using a spectrofluorometer with an excitation wavelength $\lambda_{\text{excitation}} = 488$ nm and emission $\lambda_{\text{emission}} = 525$ nm. The samples were measured in triplicates and the data obtained from this analysis were reported in terms of percentage of the fluorescence intensity of the control.

MRI study

T₂ relaxation times of IONPs were measured for different concentrations of NPs using a 3T clinical MRI scanner (General Electric Healthcare, USA). Samples with different concentrations of NPs were prepared by diluting them with Milli-Q water. T₂-weighted images were obtained according to SE protocol with the following parameters: TR (repetition time) = 5000 ms; (echo time) = 30, 40, 60, 80, 100, 150 and 200 ms. Relaxation rates (1/T₂) were measured and plotted against the concentrations of NPs. The relaxivities (r₂) were then obtained from the slope of these curves. T₂ relaxation time for each concentration was estimated via fitting the decay curve by using the exponential relation $I(\text{TE}) = I_0 e^{-(\text{TE}/T_2)}$, where TE is the echo time and I(TE) is the MRI signal intensity at each TE. The r₂ relaxation values (mM⁻¹ s⁻¹) were calculated from the slope of the linear plots of 1/T₂ versus the NPs concentrations.

Characterization

X-ray powder diffraction (XRD) measurements are performed by PANalytical Xpert Pro diffractometer with Cu-k radiation ($\lambda = 1.54178$ Å) in the range $2\theta = 20 - 70^\circ$ at 0.02° step. The average crystallites size is calculated by the Williamson-Hall method. Mössbauer spectra have been recorded at room temperature (RT) in standard transmission geometry, using a constant acceleration signal spectrometer equipped with ⁵⁷Co source in a rhodium matrix. Magnetic characterization of powder samples and colloidal suspensions are performed in a Quantum Design MPMS-5S SQUID magnetometer. The Fourier transform infrared spectroscopy (FTIR) measurements were carried out on a Perkin-Elmer spectrometer (Model No.783, USA) in the range 400 to 4000 cm⁻¹. The particle size and shape was determined by transmission electron microscopy (TEM, Philips CM200 model, operating voltage 20-200kV, resolution 2.4 Å). Zeta potential measurements were performed in water by using a PSS-NICOMP-380 ZLS (USA) particle sizing system. HCl and NaOH were used to adjust the pH of water from 2 to 12. The reported zeta potential values are an average of three measurements. Induction heating of IONPs for hyperthermia application was performed in plastic microcentrifuge tube (1.5 mL) by using an induction heating unit (Easy Heat 8310, Ambrell; UK) with a 6 cm diameter (4 turns) heating coil. A provision of water circulation in the coil was made to maintain an ambient temperature. The water-suspended IONPs (1 mL) were placed at the center of the coil, and 265 kHz AC current was applied. The particles with different concentrations were ultrasonicated for 5 min to disperse in the carrier fluid. Samples were heated for 10 min with the desired

current (200 to 600 A that can generate magnetic field of 83.8 to 502.8 Oe, respectively). Temperature was measured using optical fiber probe with an accuracy of ± 0.1 °C.

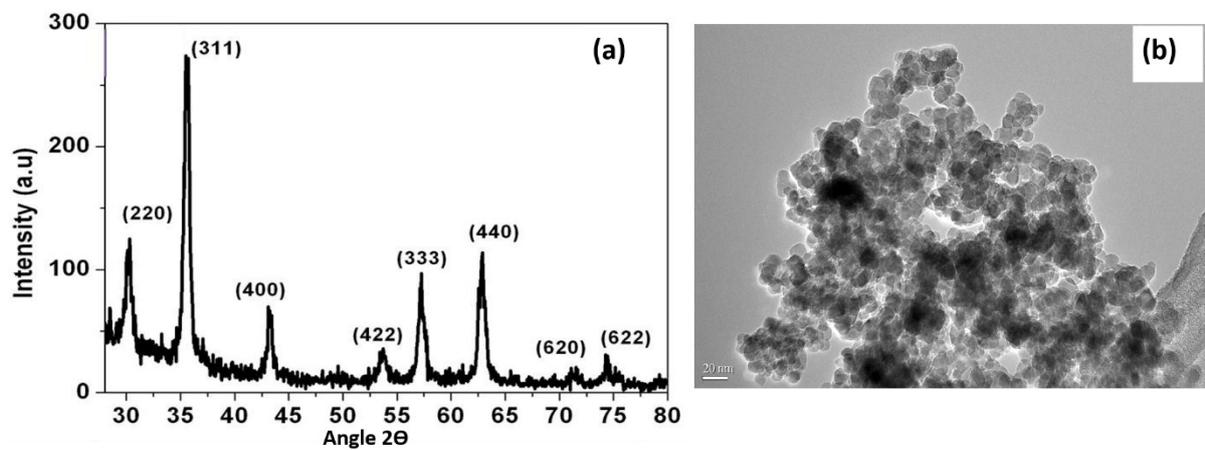


Fig. S1. XRD and TEM results of IONPs

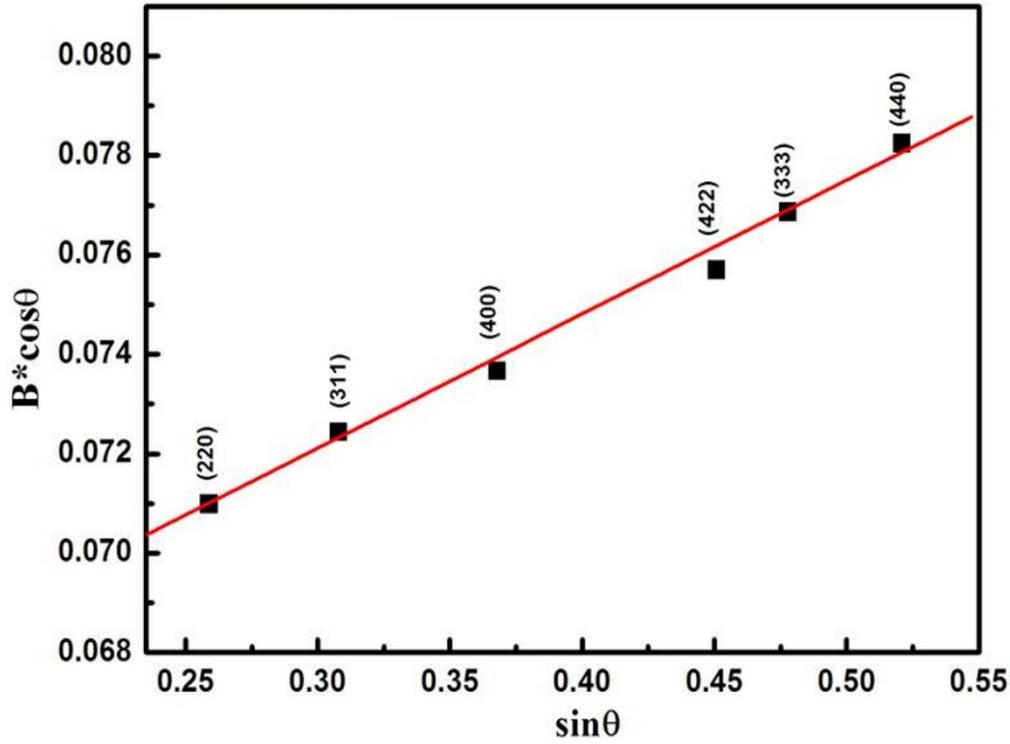


Fig. S2. Crystal size determination of γ -Fe₂O₃ by the Williamson-Hall method.

The analysis includes two steps:

First step: the width () of every peak was measured as the integral breadth. The instrumental **broadening** (β_{inst}) was determined from polycrystalline silicon standard. The peak breadth due to sample (strain + size), B was calculated according to Gaussian profile:

$$B^2 = \beta_{exp}^2 - \beta_{inst}^2 \quad (1)$$

Second step: the crystalline size and internal strain were obtained by fitting the Williamson-Hall equation :

$$B \cos \theta = \frac{K \lambda}{D} + 4\epsilon \sin \theta \quad (2)$$

Where D is the coherent scattering length (crystallite size); K is a constant whose value is approximately 0.9; B the integral width of the sample (in rad) calculated in the first step and ϵ the inhomogeneous internal strain (in %).

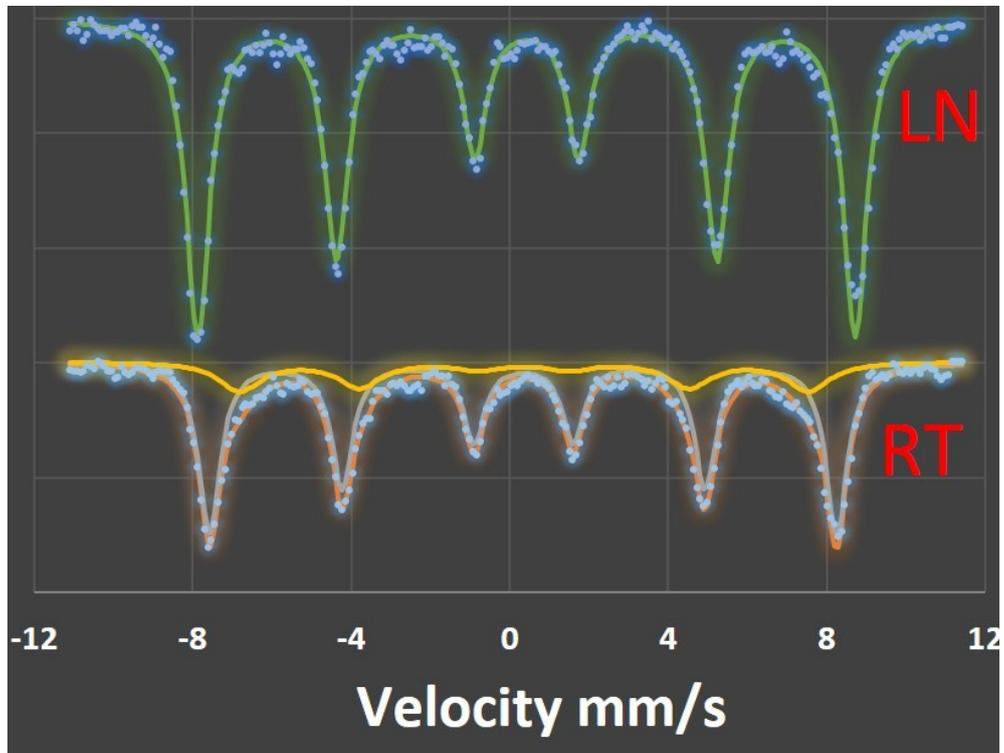


Fig. S3 Mössbauer spectra of the synthesized sample of IONPs (γ - Fe_2O_3) at liquid nitrogen and room temperature.

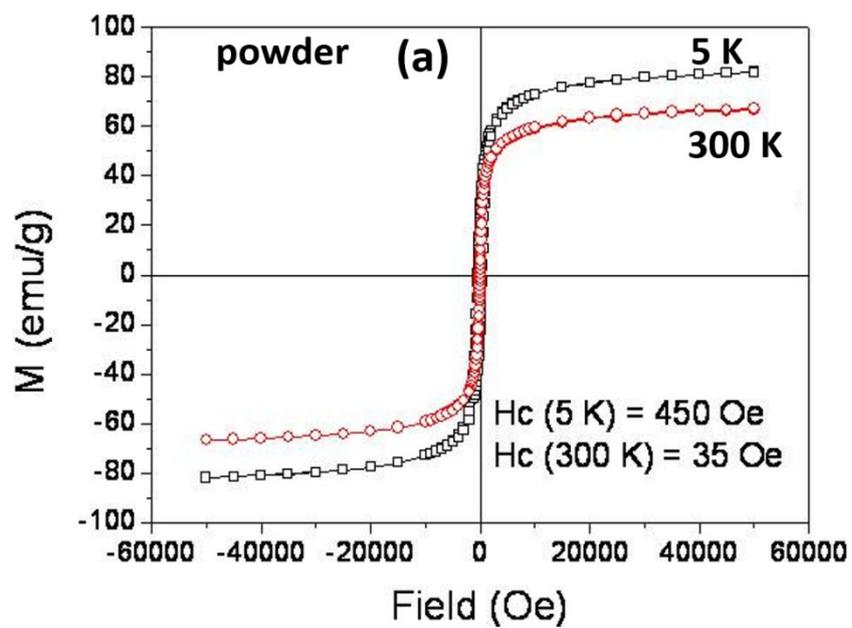


Fig. S4. M vs H measurements of IONPs powder sample at 5 and 300K.

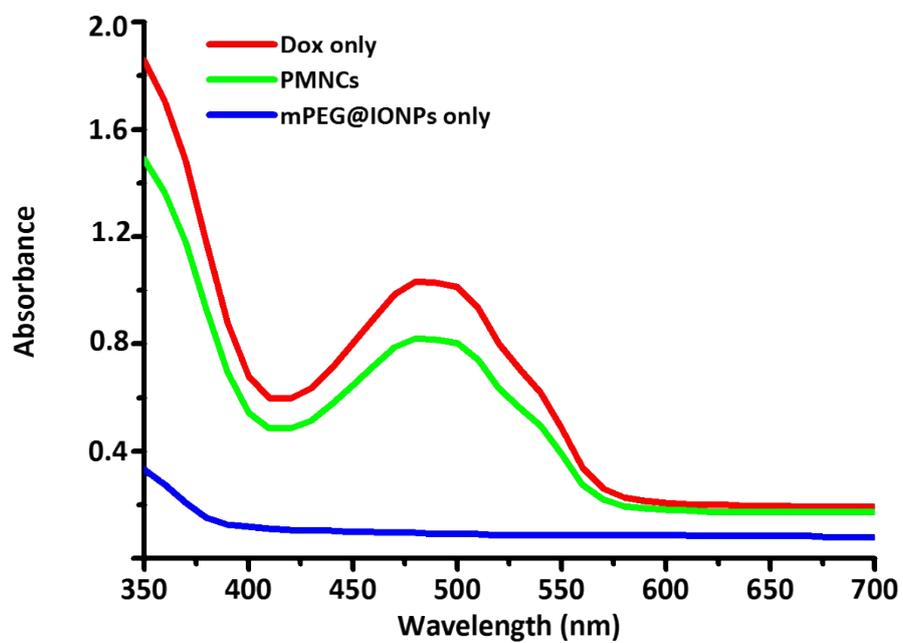


Fig. S5. UV absorbance spectra of DOX, PMNCs and mPEG@IONPs in water.

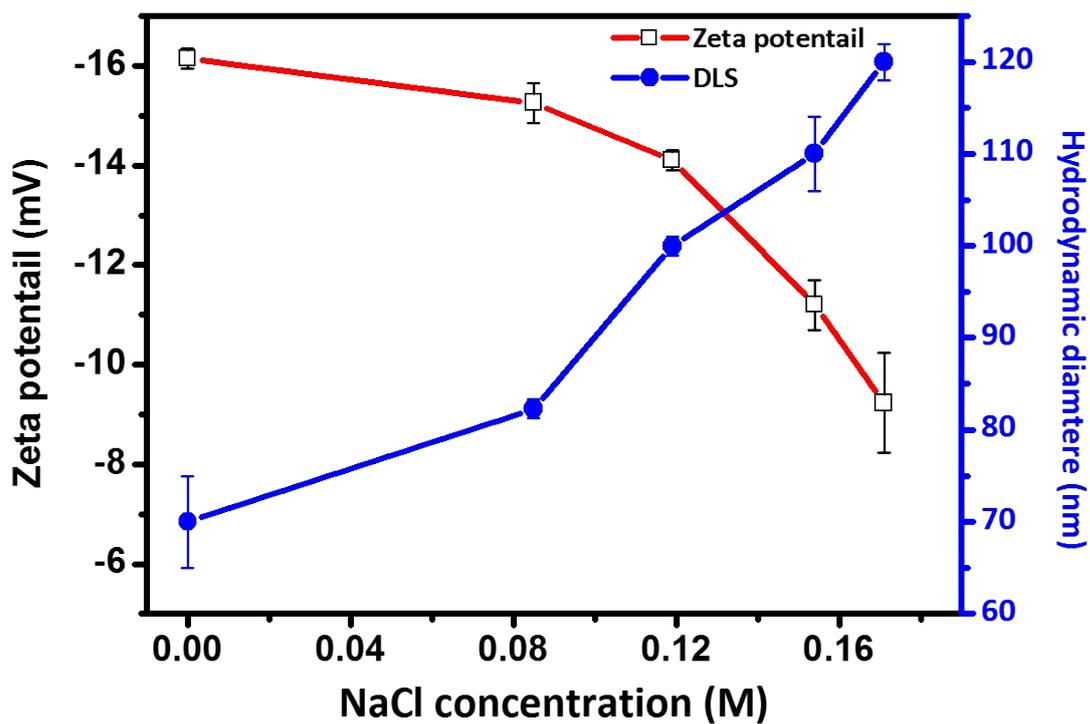


Fig. S6. NaCl concentration dependent zeta potential and DLS results of PMNCs in water, pH is maintained at 7.4.

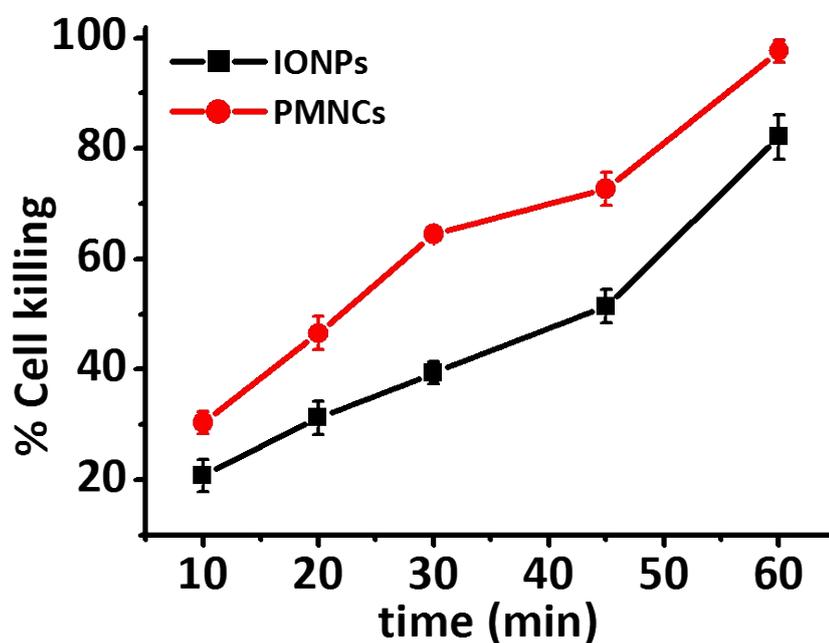


Fig. S7 Cancer cell killing by magnetic hyperthermia generated by only IONPs and PMNCs. The cells were treated with only IONPs and PMNCs with concentration 1 mg mL^{-1} for 10 to 60 min followed by the application of an alternating magnetic field (300 Oe, 267 KHz). After treatment, the cells were incubated in culture conditions for 24 h. The proportion of cell viability is calculated by using MTT assay.

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