Electronic Supplementary Information (ESI†)

Surface engineered Fe_3O_4 nanomagnets for pH-responsive delivery of gemcitabine hydrochloride and *in-vivo* tracking by radiolabeling

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1. Materials

Ferrous chloride tetrahydrate, ferric chloride hexahydrate and gemcitabine hydrochloride (GEM) were purchased from Sigma Aldrich, USA. Sodium tripolyphosphate (STTP), ammonia solution (25%) and hydrogen chloride (HCl) were bought from SRL Pvt. Ltd., India. Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum (FBS), MTT reagent and dialysis membrane-60 were procured from Himedia Laboratories Pvt. Ltd., India. A549 and MCF-7 cells were purchased from National Centre for Cell Science (*NCCS*), Pune, India. The acetate buffer (AB, pH 4 and 5) and phosphate buffered saline (PBS, pH 7.4) were prepared using standard protocols. All chemicals used were of AR grade unless otherwise specified. All the aqueous solutions were prepared using deionised water from a Millipore-Milli Q system (resistivity ~18 MΩ cm).

1.1. Synthesis and structural characterization of PPNMs

PPNMs were prepared through co-precipitation of ferrous and ferric ions (1:2 molar ratio) in presence of 25 % ammonia followed by in-situ coating of sodium tripolyphosphate as reported elsewhere [1]. X-ray diffraction (XRD) analysis was performed on PPNMs in the range 2θ = 20-70^o for identification of phase formation (Phillips PW1729 diffractometer using Cu K_α). The infrared spectrum was recorded on a Fourier-transform infrared spectrometer (FTIR, Bomen MB series) for

monitoring the organic modification. The transmission electron micrographs were taken by Philips CM 200 TEM. The field dependent magnetization measurement was carried out by Physical Property Measurement System (PPMS, Quantum Design). Dynamic light scattering measurements were performed by a Malvern 4800 Autosizer employing a 7132 digital correlator to obtained the hydrodynamic diameter of particles. The surface charge was measured by Zetasizer nanoseries, Malvern Instruments.

1.2. Drug loading and release studies

The interaction of GEM with PPNMs were investigated by zeta-potential and UV-visible absorption spectroscopic studies. The methanolic solution of GEM (10 μg/ml) was added to aqueous dispersion of different amounts of PPNM (50, 100, 200, 400 and 800 μg, total volume fixed to 1 ml, pH of the solution = 6) and mixed thoroughly by shaking under vortex at room temperature for 24 h. Then, the GEM loaded PPNMs (GEM-PPNMs) were separated by using external magnet and supernatant was collected for absorbance studies. The obtained GEM-PPNMs were washed carefully to remove physically adsorbed drug. The absorbances of supernatant and washed solution were measured using JASCO V-650, UV-visible spectrophotometer against the calibration curve of GEM prepared under similar conditions. The loading efficiency (w/w %) was determined as reported elsewhere [2]. The drug-particle interactions were also performed at pH 4 and 8 with drug to particle ratio of 1:40.

For release study, the loading was carried out, at an increased scale (with drug to particle ratio of 1:40), by vortexing 0.1 ml of methanolic solution of GEM (1 mg/ml) with 1 ml of the aqueous suspension (pH 6) of PPNMs (4 mg/ml) for 24 h in dark. The pH dependent release of drug from GEM-PPNMs was performed under reservoir (r)-sink (s) conditions at 37^{o}C. The GEM-PPNMs (4 mg) were dispersed into 5 ml of release medium (pH 4, 5 or 7.4) and then put into a dialysis bag. The dialysis was carried out against 100 ml of respective release medium under stirring at 37^{o}C. 1 ml of the external medium was taken out at fixed interval of times for absorbance studies. The equivalent amount of fresh medium was added to the external medium to maintain the sink conditions. The percentage of drug

released was obtained by monitoring the absorbance at 269 nm using a plate reader (Synergy™ H1 Multimode microplate reader, BioTek, Germany) against the standard plot prepared under similar conditions. The standard deviation shown in the plot was determined by performing each experiment in triplicate.

1.3. Cytotoxicity, cellular uptake and cell death studies

The A549 (human lung cancer) and MCF-7 (human breast cancer) cells were maintained in DMEM supplemented with 10% FBS, 100 units/mL penicillin and 100 mg/mL streptomycin (complete media) under 5% CO_2 at 37°C. The cytotoxicity of GEM and GEM-PPNMs was investigated at different concentration of drug ranging from 0 to 10 μM. Cells (10000) were seeded overnight in 96 well plates containing 200 μl of compete media in a CO_2 incubator. Then, different concentrations of drugs were added to the cells and were incubated for another 24 or 48 h in culture conditions. Cell viability was obtained by MTT assay as reported earlier [1].

The cellular uptake of PPNMs was studied in MCF-7 cells microscopically by prussian blue staining method. Cells were seeded in 6 well plate to obtain a confluent culture of cells. The cells were then treated with 200 μg of PPNMs for 3 h in culture conditions. Thereafter, the cells were washed with PBS (twice), followed by fixing with 4% paraformaldehyde at 4°C for 30 min. A freshly prepared 1:1 mixture of 5% potassium ferrocyanide and 5% HCl (in PBS) was added to cells and incubated at room temperature for 20 min. Then, the cells were visualized by bright field microscopy.

The cell death study was performed by acridine orange-ethidium bromide (AO-EB) assay. A549 and MCF-7cells were seeded in 6 well plate followed by treatment with GEM and GEM-PPNMs having drug concentration of 2.5 μM for 48 h. After treatment, the cells were washed with PBS, and then treated with 1 μL of acridine orange (1 μg/mL) and 1 μL of ethidium bromide (1 μg/mL). The photographs were obtained using fluorescence microscope (radical fluorescence microscope) at 20 X.

2. Results

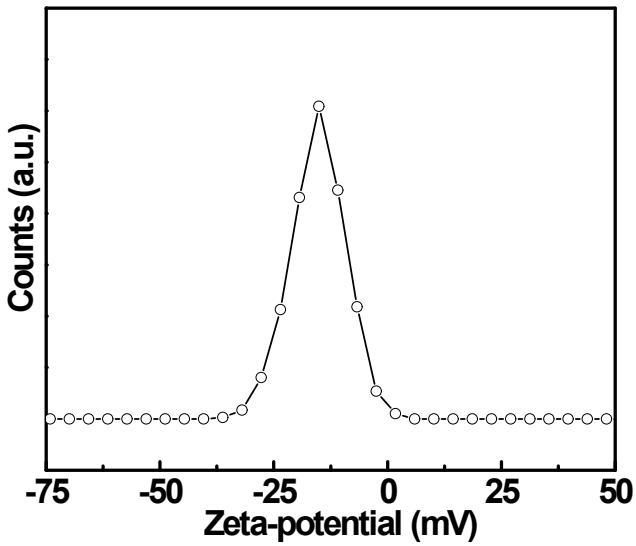

Fig. S1. Zeta-potential plot of bare MNPs in physiological medium.

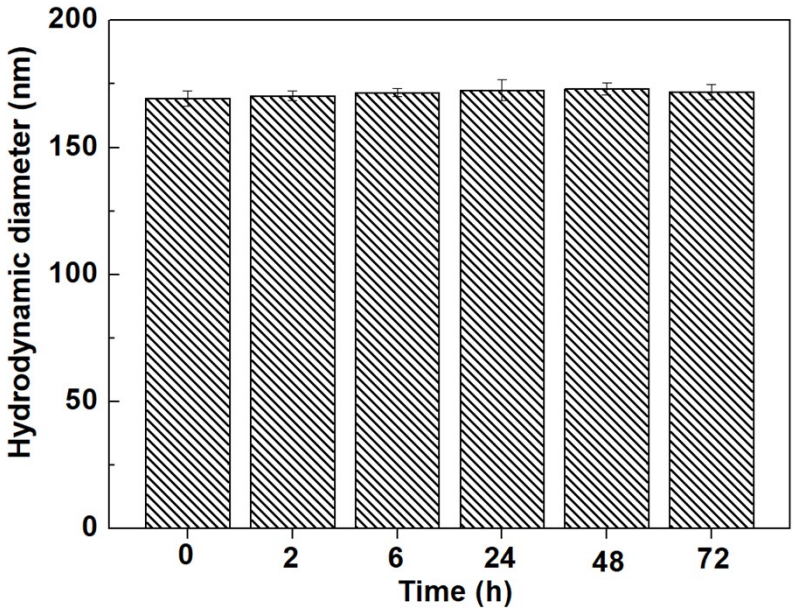

Fig. S2. Time dependent variations of hydrodynamic diameter of PPNMs in physiological medium obtained from DLS studies.

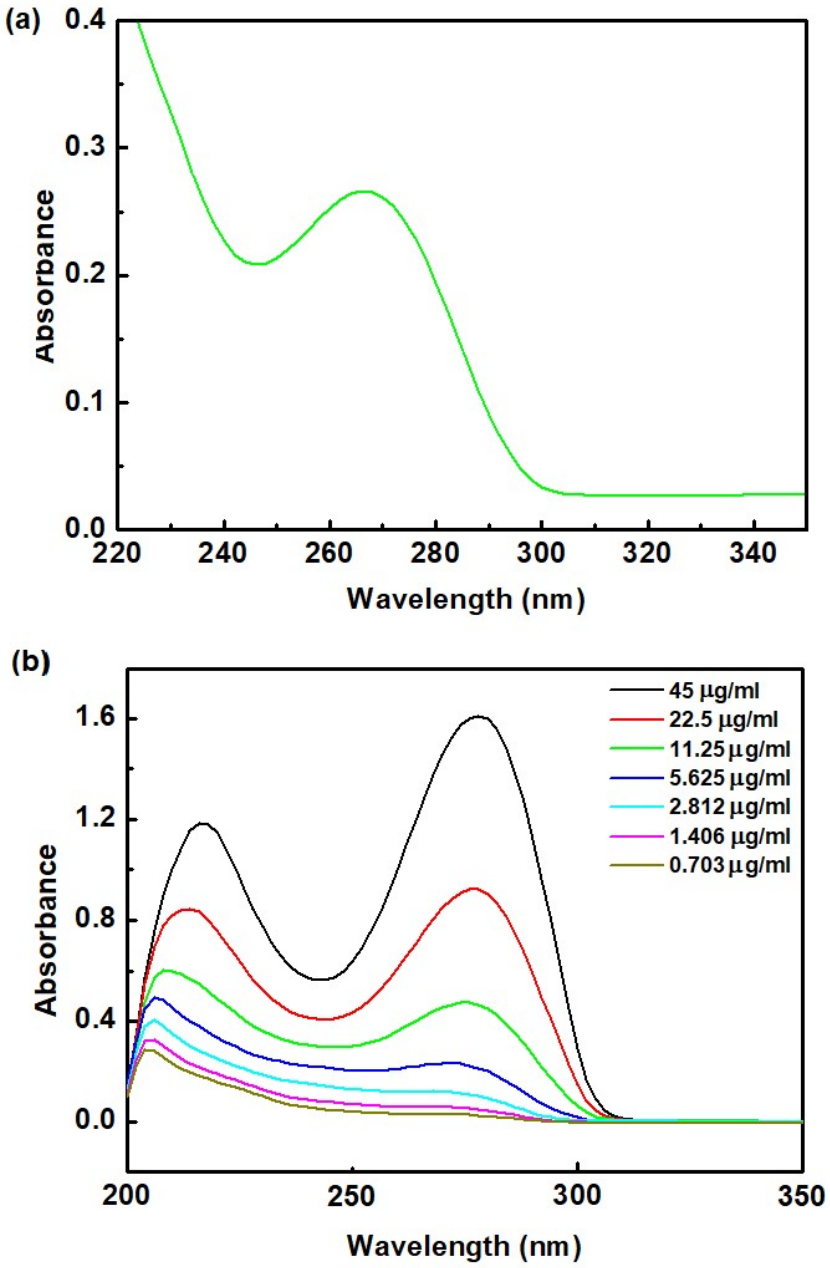

Fig. S3. UV-visible absorption spectra of (a) supernatant solution obtained after magnetic separation of GEM-PPNMs (drug loading experiment was performed with 10 µg/ml of gemcitabine at drug to particle ratio of 1:40) and (b) pure gemcitabine at different concentration (standard curve used for determination of loading efficiency). The washed drug was also considered for calculation of loading efficiency.

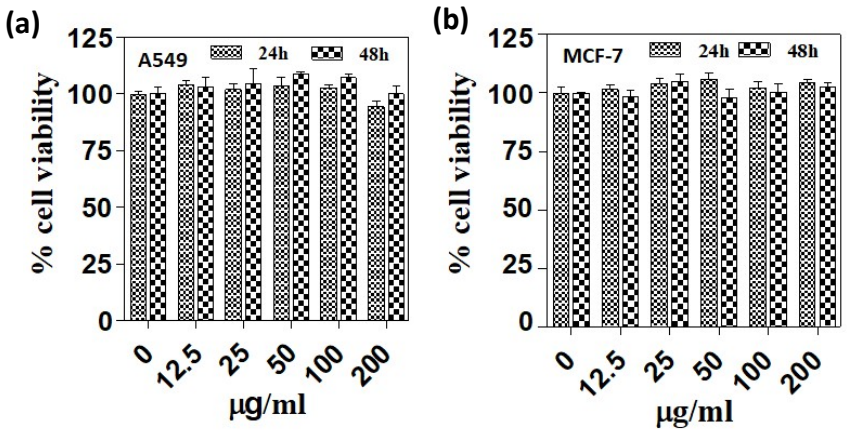

Fig. S4. Viabilities of A549 and MCF-7 cells incubated in medium containing different concentrations of PPNMs for (a) 24 h and (b) 48 h. Mean values were expressed as mean ± SEM for n = 3 in each experimental group.

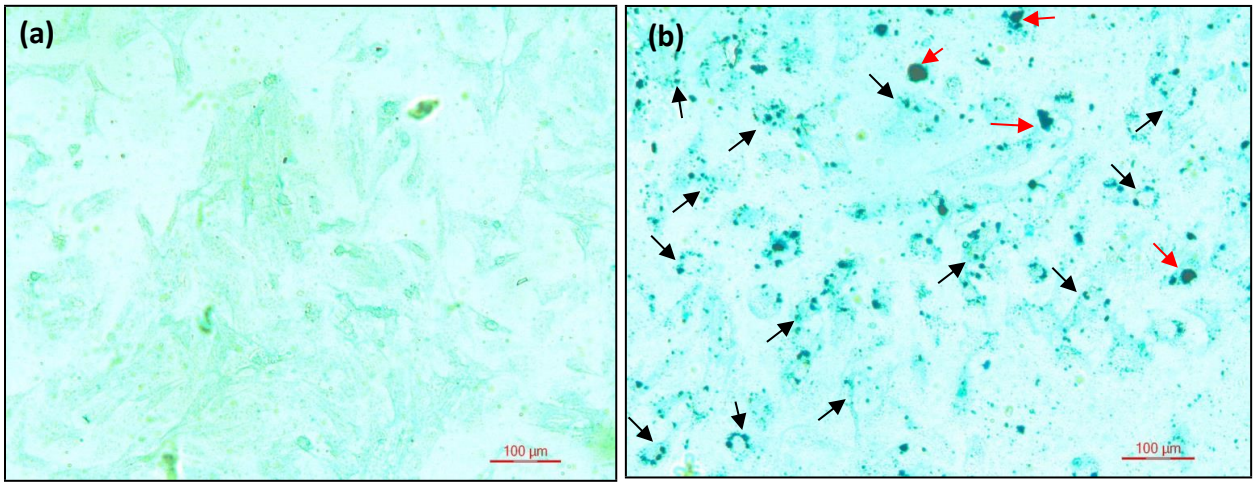

Fig. S5. Microscopy images of MCF-7 cells studied by prussian blue staining: (a) control cells without PPNMs and (b) cells treated with 200 µg PPNMs. Black arrows indicate PPNMs up taken by cells, whereas red arrow show a few positions of cell surface attachment of PPNMs.

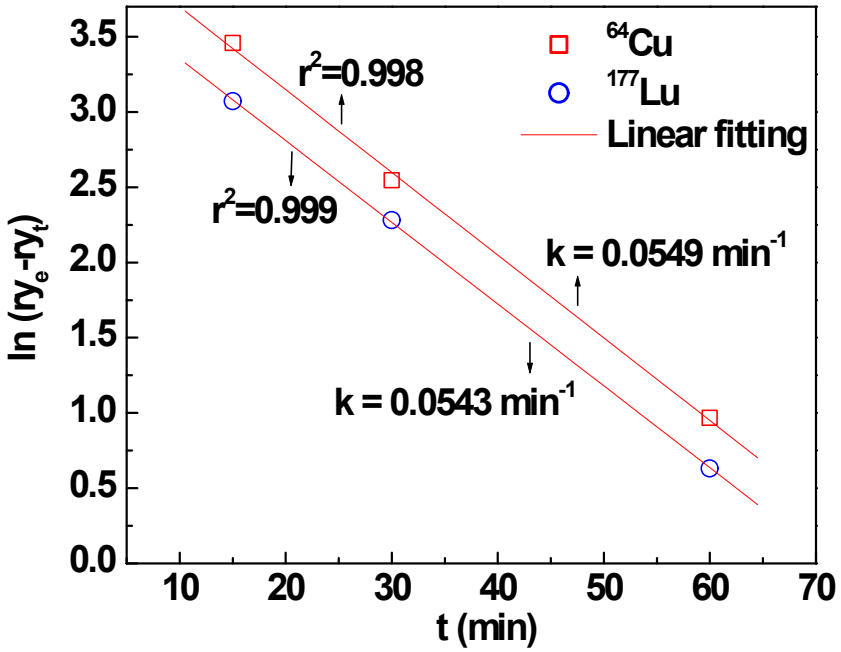

Fig. S6. Pseudo first order kinetic model [ln (ry_e-ry_t) vs. t) on radiolabeling of PPNMs with ^{64}Cu and ^{177}Lu (PPNMs concentration of 1 mg/mL). The pseudo first order kinetic model is represented as "ln (ry_e-ry_t) = ln ry_e-kt", where k is the pseudo-first order rate constant, and ry_e and ry_t are the values of the radiolabeling yield at equilibrium and at time 't' respectively.

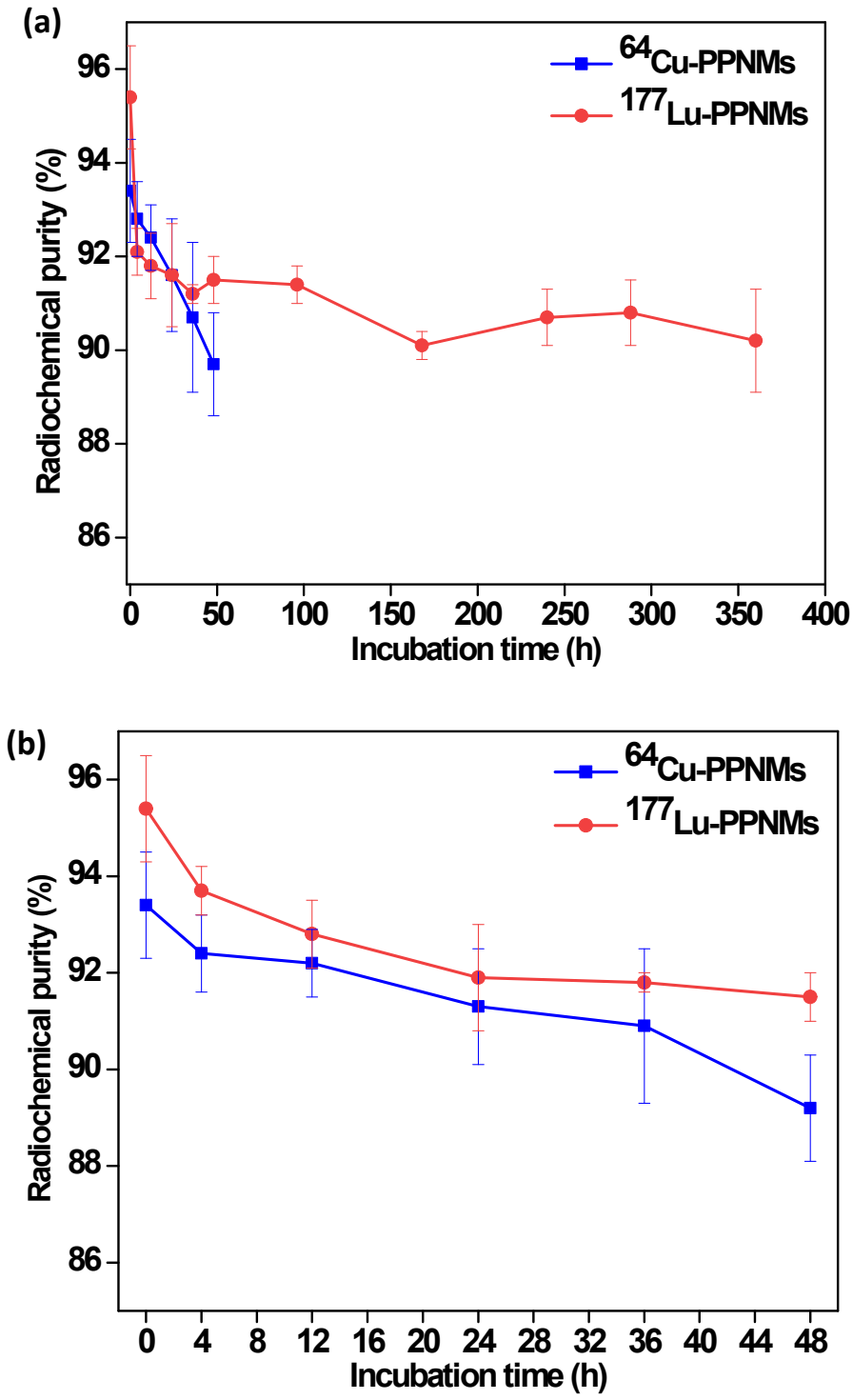

Fig. S7. The percentage of radioactivity leached out from the radiolabeled formulations in (a) saline and (b) rat serum.

Table S1. Surface charge of GEM-PPNMs incubated with BSA protein for different time showing protein-particle interaction.

Sample	Surface charge of sample in 0.01M PBS (pH 7.4)	Surface charge of sample incubated with BSA (0.025 mg/ml) in 1 ml of 0.01 PBS (pH 7.4)			
		0 h	24 h	48 h	96 h
GEM-PPNMs	-3.3 mV	-2.9 mV	-2.3 mV	-2.2 mV	-3.8 mV

References

1. J. Majeed, K. C. Barick, N. G. Shetake, B. N. Pandey, P. A. Hassan, A. K. Tyagi, Water-dispersible polyphosphate grafted Fe_3O_4 nanomagnets for cancer therapy, *RSC Adv.* 2015, **5** 86754-86762.
2. B. Dutta, A. Nema, N. G. Shetake, J. Gupta, K. C. Barick, M. A. Lawande, B. N. Pandey, I. K. Priyadarsini, P. A. Hassan, Glutamic acid-coated Fe_3O_4 nanoparticles for tumor-targeted imaging and therapeutics, *Mater. Sci. Eng. C* 2020, **112,** 110915.