Electronic Supplementary Information

An aqueous method for the controlled manganese (Mn^{2+}) substitution in superparamagnetic iron oxide nanoparticle for contrast enhancement in MRI

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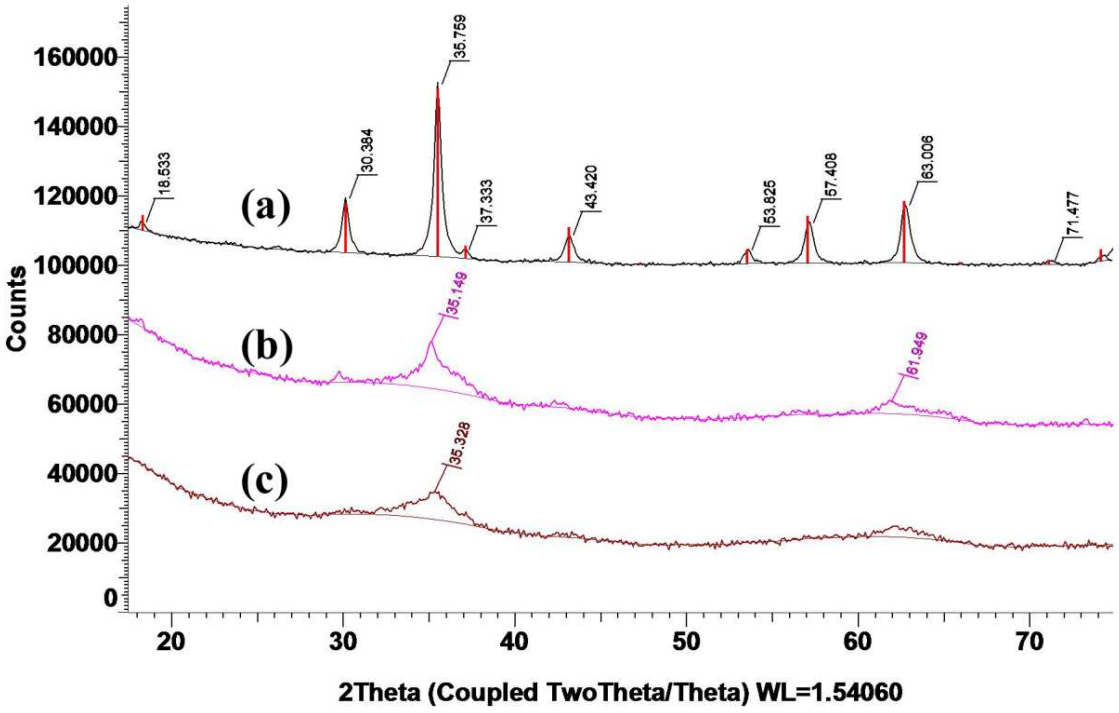

Figure S1. XRD patterns for the samples recorded with Cu $K\alpha_1$ radiation. a) SPION b)MnIO80 c)MnIO90.

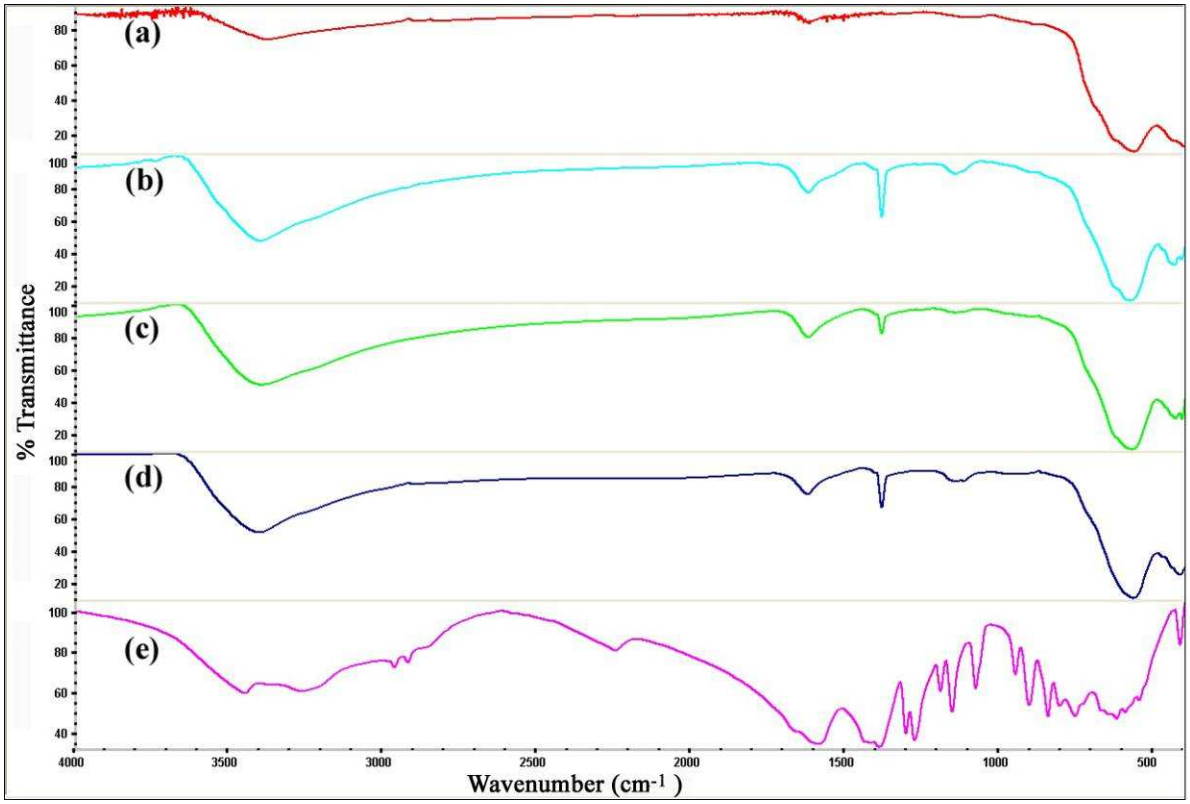

Figure S2. FTIR spectra of the freeze dried powder samples of a) SPION b) MnIO25 c) MnIO50 d) MnIO75 e) TSC.

Cell culture

HeLa (human cervical carcinoma) cells were cultured in Dulbecco's Modified Eagle's Medium – High Glucose (DMEM -HG) with 10% Fetal Bovine Serum (FBS), 50 units ml^{-1} of penicillin and 50 μg ml^{-1} of streptomycin. All reagents were sourced from Invitrogen, India and cell culture lab ware from NUNC, Denmark. Cells were seeded and maintained at 37°C and 5% CO_2 atmosphere and experiments performed at 80% confluence.

Cytotoxicity - Alamar Blue Assay and Light Microscopic Technique

The cytotoxicity of SPION and MnIOs were evaluated *via* alamar blue (AB) assay. The AB assay was used to assess cell viability and proliferation based on the reduction potential of metabolically active cells. The mitochondrial reductase enzymes in living cells are active and it changes the alamar blue to pink color on the basis of live cell activity. In this study, HeLa cells were seeded in a transparent 96 well plates and exposed to the nanoparticle [SPION and MnIO25, MnIO50 and MnIO75] dispersions at concentrations ranging from 0.5mg to 3mg for 24h. After the exposure, 100μl of alamar blue reagent (invitrogen assay protocol) was added in each well and incubated for 4h at 37°C. The fluorescence was measured at 560nm excitation and 590nm emission wavelengths using a plate reader (HIDEX Chameleon) and expressed in percentage activity of live cells versus control. The experiments were carried out in triplicate for each nanoparticle concentrations. The 24h material exposed cell structure was also evaluated *via* bright field microscopy. Microscopic observations and cell imagery acquired with a Leica DMIL microscope (Leica, Germany) support the non – cytotoxic nature of the particles.

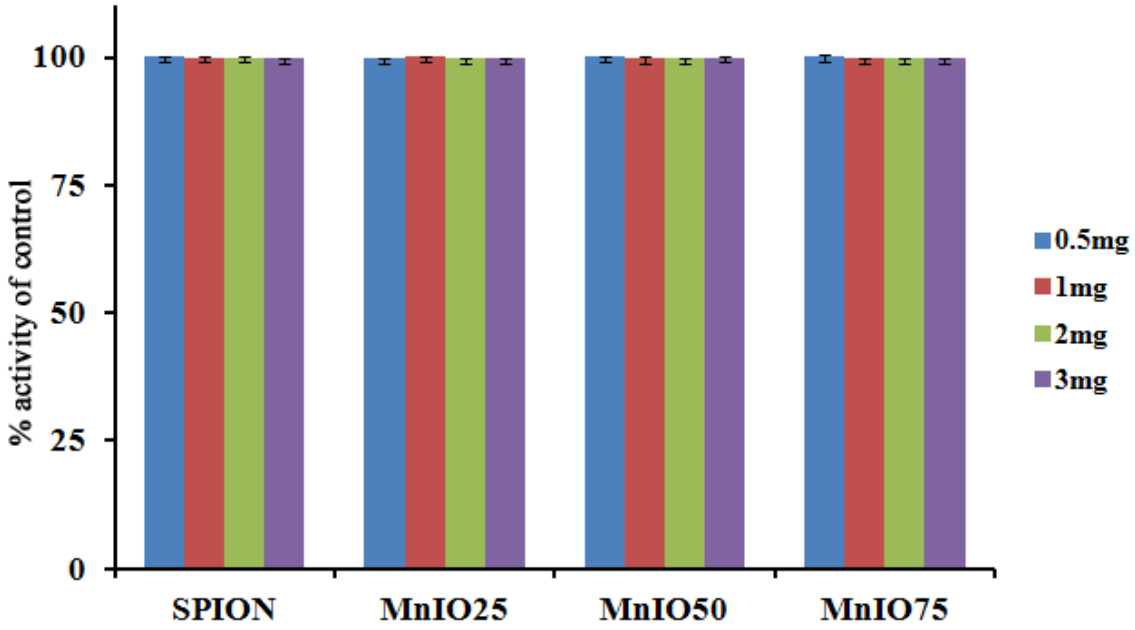

Figure S3. Cell Activity at 24hrs contact with SPION and MnIOs evaluation via Alamar Blue assay.

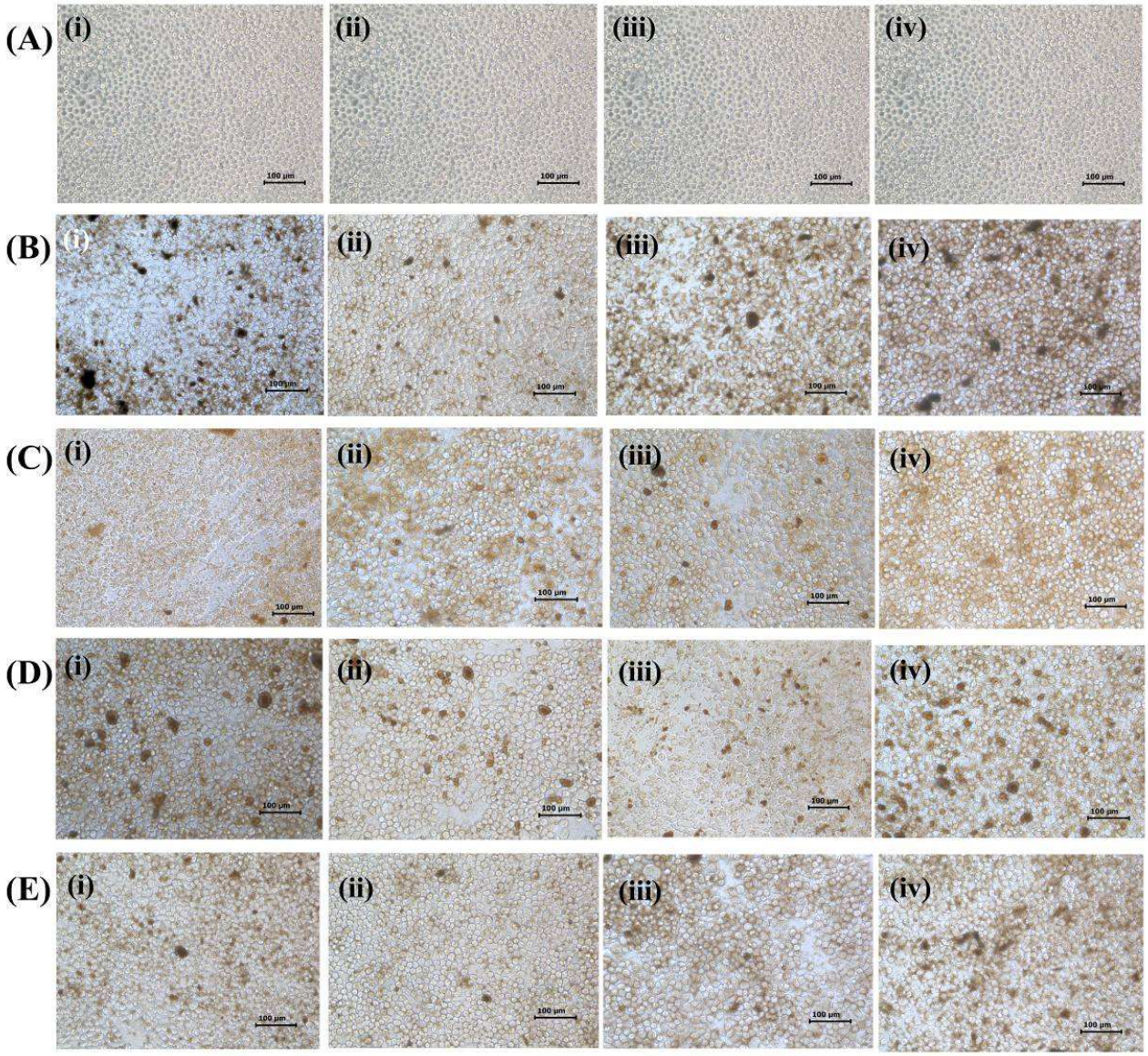

Figure S4. Phase Contrast Micrographs of HeLa cells after 24h incubation with nanoparticles. (A) Control, (B) SPION, (C) MnIO25, (D) MnIO50, (E) MnIO75 and (i), (ii), (iii), (iv) are 0.5mg, 1mg, 2mg, 3mg concentrations of corresponding materials.

Haemolysis assay

Blood from human volunteer was collected into the anticoagulant, ACD (acid citrate dextrose). ACD blood was prepared by adding 1mL of ACD solution to 9mL of fresh human blood. The blood compatibility experiments were based on the standard protocol ISO10993-4:2002(E).

Percentage haemolysis

Samples /controls (1mg) were added into each of the $10mm^2$ polystyrene wells, followed by addition of 1ml blood and incubated for a period of 2h under agitation at 70 ± 5 rpm using an environ shaker thermostat (Kuhner shaker, Switzerland) at 35 ± 2^oC. Three empty polystyrene culture dishes were exposed to blood as reference. The total haemoglobin in the whole blood samples were measured using automatic haematology analyzer (sysmex-K 4500). The free haemoglobin liberated into the plasma after exposure to materials was measured using Diode array spectrophotometer (Hewlett Packard 8453) and the percentage haemolysis was calculated using the formula (Free Hb/ total Hb)× 100. Haemolysis expressed as a percentage of haemoglobin release was performed to assess the effect of nanocrystals on the blood cell membranes.

Clotting time

Whole human blood was collected from an un-medicated healthy donor without anti-coagulant and was transferred immediately to glass tubes for clotting time analysis. Briefly, 1mg of each sample was mixed with 1ml of whole blood and time required for clot formation was observed manually by tilting the tube at fixed time intervals measured using a stopwatch. The time period between the addition of the samples to whole blood and first visible clot formation was taken as the clotting time. Negative and positive controls were also treated identically.

Sample	Haemolysis (%)	Clotting time (s)
MnIO25	0.01±0.002	220±28
MnIO50	0.01±0.001	245±34
MnIO75	0.04±0.005	245±27
SPION	0.03±0.003	235±26
Negative control	0.00±0.000	255±30
Positive control	0.52±0.008	165±28

Table S1 Percentage haemolysis values of blood cells after 2hrs incubation with MnIOs at 37^{o}C and clotting time measurement assessing the MnIOs contacted blood samples.

Clotting time of whole blood was compared to samples incubated with nanoparticles. On comparison of nanoparticle exposed samples to negative control there is no significant deviation from observed values in both cases. The preservation of the normal clotting cascade is perceived.

RBC aggregation

Red blood cell concentration was collected from whole blood in ACD by centrifugation at 3000rpm for 15min. 1mg of each of the samples, positive and negative control were added to 1ml of 1:10 normal saline diluted red cell concentrates and were incubated in Environ shaker thermostat for 30min at 35 ± 2^{o}C. Aggregation was observed using 40x objective of Leica phase contrast DMIL microscope (Leica, Germany) after 1: 100 dilution of the mixture with normal saline.

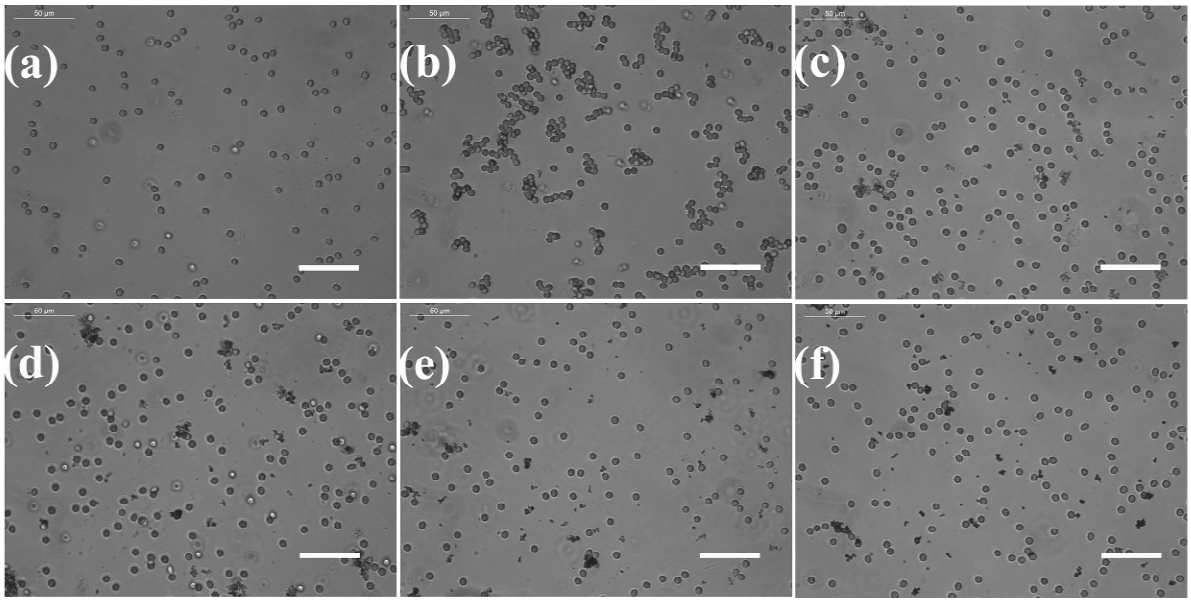

Figure S5. Phase contrast microscopic view 400X (Scale bar 50µm) of erythrocytes after incubation with a) Negative control (Normal saline) b) positive control (polyethylenimine) c) MnIO25 d) MnIO50 e) MnIO75 f)SPION.

WBC aggregation

WBCs were isolated from citrate human whole blood by gradient centrifugation using Histopaque (sigma-1077). Cells were carefully collected and diluted with normal saline to obtain a concentration of 5000-10000 cells per microlitre. 1mg of each samples, positive and negative controls were then added to 1ml of count adjusted WBC and were incubated in Environ shaker thermostat for 30min at 35 ± 2^{o}C. Aggregation was observed using 40x objective of Leica phase contrast microscope after 1: 100dilution of the mixture with normal saline.

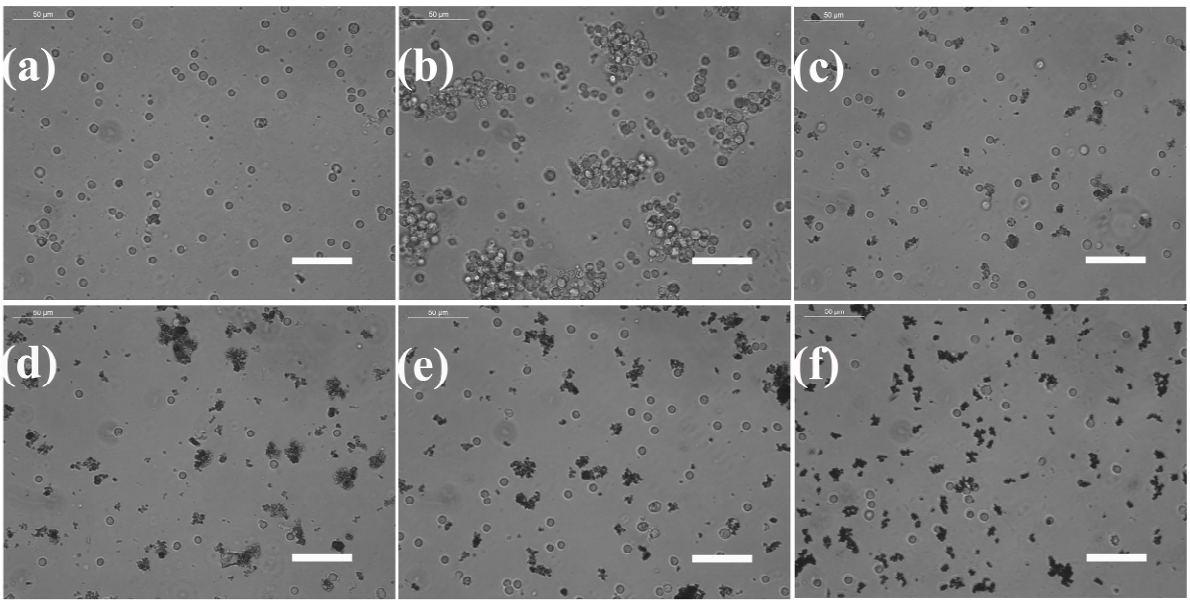

Figure S6. Phase contrast micrographs of leukocytes after incubation with MnIOs a) Negative control normal saline b) positive control (polyethylenimine) c) MnIO25 d) MnIO50 e) MnIO75 f) IO. Scale bar 50μm

Platelet aggregation

Platelets were isolated from citrate human whole blood via gradient centrifugation using Histopaque. Cells were carefully collected and diluted with platelet poor plasma to obtain a concentration of 2.0 - 2.5x 10^8/cells. 1mg each of the samples, positive and negative controls were then added to 1ml of count adjusted platelet solution and were incubated in Environ shaker thermostat for 30min at 35 ± 2^{o}C. Aggregation was observed using 40x objective of Leica phase contrast microscope after 1: 100 dilution of the mixer with normal saline.

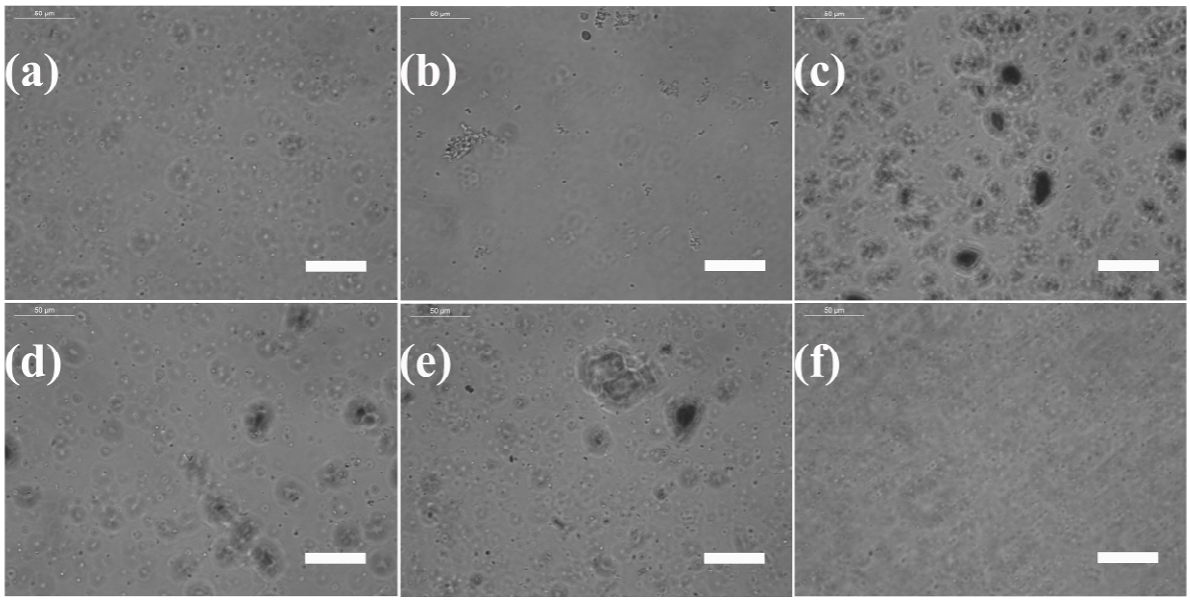

Figure S7. Phase contrast micrographs of platelets after incubation with MnIOs a) Negative control (normal saline) b) positive control (polyethylenimine) c) MnIO25 d)MnIO50 e) MnIO75 f)IO. Scale bar 50µm

Cell uptake

To evaluate the cell uptake of SPION and MnIOs, the cells were incubated with 50µg of SPION and MnIOs at 37^{o}C for 24h. After incubation, the cells were washed with sterile PBS buffer and fixed with 3.7% paraformaldehyde for 4h. The prussian blue staining was carried out with 7:3 volume ratios of 10% potassium ferrocyanide and 10% hydrochloric acid. After 20 min the cells were washed thrice with distilled water. Further, cells were counterstained by nuclear fast red (NFR) to visualize cell nuclei. Coverlsip was mounted in DPX and images were captured on a Leica DMIL microscope.

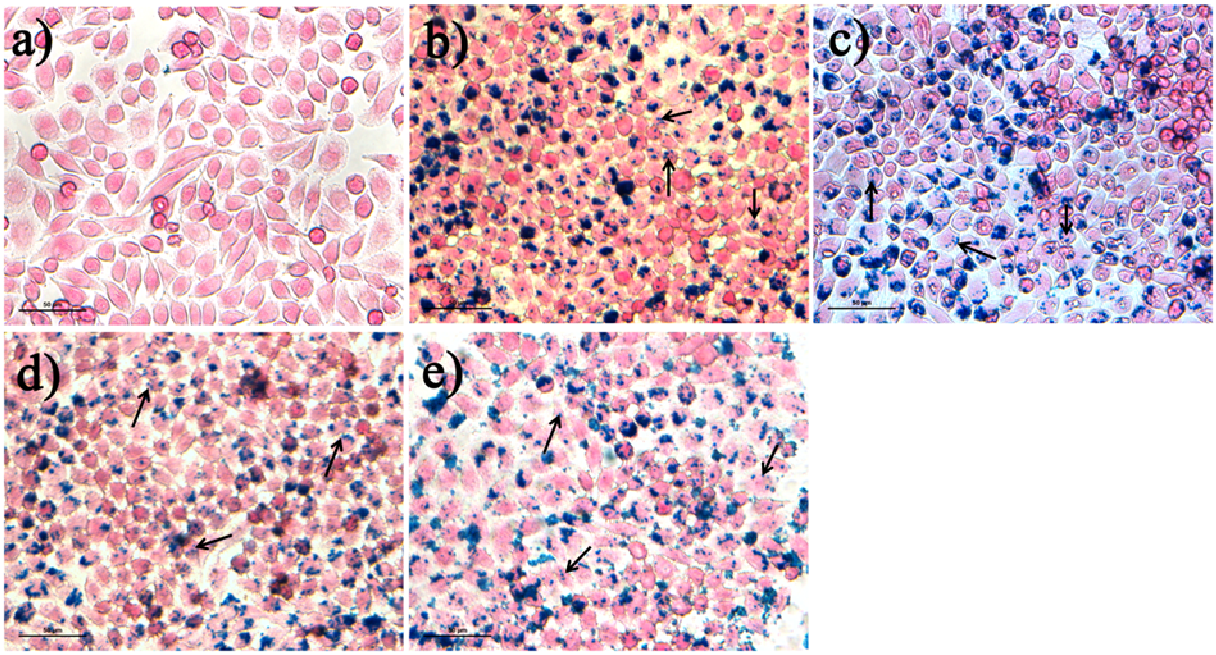

Figure S8. HeLa cells incubated for 24 h with SPION and MnIOs nanoparticles and stained with Prussian Blue. Blue indicates iron based nanoparticle uptake. Samples are (a) Control (b) SPION (c) MnIO25 (d) MnIO50 (e) MnIO75 respectively. Uptake of the SPION and MnIOs are clearly seen and it marked by arrows

The ferric iron (Fe^{3+}) in the presence of ferrocyanide ion is precipitated as the intense colored and highly water insoluble complex, potassium ferric ferrocyanide prussian blue which is contrasted against the background of Nuclear Fast Red. Cells post – exposure with vivid blue stained vesicles in the cytoplasm. Particle uptake was not discriminated on the basis of material composition but indicated uptake of SPION as well as MnIOs by cells.