Supporting Information

T_1- and T_2-weighted magnetic resonance dual contrast by single core truncated cubic iron oxide nanoparticles with abrupt cellular internalization and immune evasion

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Materials

Sodium Oleate (NaOL, >97.0%(T)) and Tri-n-octylamine (TOA, >98.0%(GC)(T)) were purchased from TCI America. Methoxy-PEG acetic acid (MeO-PEG-COOH, MW 5K, PEG >95.0%) was purchased from JenKem Technology. Iron(III) chloride hexahydrate ($FeCl_3.6H_2O$, ACS reagent, 97.0–102.0%), Dopamine hydrochloride (≥98.5%), Hexane (anhydrous, 95%), Ethyl alcohol, Pure (EtOH, 200 proof, for molecular biology), N,N-Dimethylformamide (DMF, anhydrous, 99.8%), Sodium nitrite (ACS reagent, ≥97.0%), Sulfuric acid (99.999%), Methanol (MeOH, ACS reagent, ≥99.8%), (1-Cyano-2-ethoxy-2-oxoethylidenaminooxy)dimethylamino-morpholino-carbenium hexafluorophosphate (COMU, 97%), 4-Methylmorpholine (NMM, purified by redistillation, ≥99.5%), Diethyl ether (Et_2O) anhydrous, ≥99.7%), Dimethyl sulfoxide (ACS reagent, ≥99.9%), and Petroleum ether (ACS reagent) were purchased from Sigma-Aldrich. Oleic acid (99%) was purchased from Alfa Aesar. EDC (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride) was purchased from Thermo Scientific. Alexa Fluor 555 Cadaverine and DAPI (4',6-diamidino-2-phenylindole) and Prolong Diamond Antifade Mountant with DAPI were purchased from Invitrogen. NHS (N-hydroxysuccinimide) was purchased from ProteoChem. Deuterium oxide (D2O, for NMR, 99.8% D), Chloroform-d (C D Cl3, for NMR, 99.8 atom % D) were purchased from ACROS Organics. Dulbecco's Modified Eagle Medium (DMEM), Eagle's Minimum Essential Medium (EMEM), RPMI-1640, Iscove's Modified Dulbecco's Medium (IMDM) were purchased from ATCC. Human platelet lysates (hPL) was purchased from EMD Millipore. Iron AA Standard (1000 ppm Fe in 3% HCl) was purchased from RICCA chemical company.

Instrumentation

TEM images of tNCIOs were recorded using a JEOL JEM-2100F transmission electron microscope (JEOL Ltd.) operating at 200 kV. For the preparation of the TEM grid sample, 25 μL of diluted oleic acid-coated nanoparticles was dropped on 'carbon film only on 200 mesh, Copper' grid (Ted Pella, Inc.), and allowed to dry overnight. Also, the powder X-ray diffraction (XRD) measurements of tNCIOs and ND-PEG-tNCIOs were performed using a Rigaku SmartLab X-ray Diffractometer (Rigaku Corporation) equipped with a Cu Kα radiation source (λ=1.5406 Å) operating at an accelerating potential of 40 kV and a tube current of 44 mA.

The ATR-FTIR spectra of tNCIOs and ND-PEG-tNCIOs in pellet form were measured using a Bruker Tensor 27 (Bruker Corporation) in the range of 100–4000 cm^{-1}. Each pellet was prepared by mixing 199 mg of KBr and 2 mg of the sample. The TG and DSC data of the powder samples were obtained by PerkinElmer STA 6000 Simultaneous Thermal Analyzer (PerkinElmer, Inc.) in the temperature range of 30–600 °C with a ramping rate of 10 °C/min, and in the presence of constant synthetic air stream flow of 80 ml/min. The hydrodynamic diameter was determined by DLS technique, and Zeta potential was performed in a Malvern Zetasizer Nanoseries Nano-ZS (Malvern Instruments, Malvern, UK) operated at a helium-neon laser wavelength of 633 nm and power of 4 mW. For measurements, 1 ml of each diluted sample dispersed in the solvent was added to a DLS cuvette and Zeta potential capillary cell.

The magnetic field and temperature dependent magnetic measurements, M(H) and M(T), were measured in the physical property measurement system (PPMS) DynaCool (Quantum Design, Inc.) using the vibrating sample magnetometry (VSM) technique. The powder sample (1–10 mg) was used in the polypropylene VSM powder sample holder (part no. 4096-388, Quantum Design, Inc.) for these measurements. ^{1}H and ^{13}C solution NMR spectra were obtained

by using Bruker Ascend Aeon 700 (Bruker Corporation) at 700 MHz in $CDCl_3$ and d_6-DMSO solvents using tetramethylsilane (TMS) as an internal standard. The UV-vis optical fluorescence spectra of the ND-PEG-tNCIOs-(AF555Cdv) solution were recorded using a SHIMADZU RF-5301PC spectrofluorophotometer (Shimadzu Corp.). The solution was excited with 555 nm wavelength light using a 150W Xenon lamp, and the emission spectra were recorded in the range of 560–700 nm with 5 nm of excitation and emission slit widths.

The MR longitudinal and transverse relaxivities (r_1 and r_2) measurements were conducted using a NMReady-60PRO benchtop relaxometer (Nanalysis Corp. Canada). The ND-PEG-tNCIOs were dispersed in three different media—deionized water (DI H_2O), 10% human platelet lysates (hPL) in PBS 7.4 (PBS-hPL), and 1×10^6 CCRF-CEM cells (human acute lymphoblastic Leukemia cells) per ml PBS 7.4 (PBS-CEM). The concentration of Fe ([Fe]) of 0, 0.2, 0.4, 0.6, 0.8 and 1.0 mM was maintained in each medium and scanned applying 1.41 T at 30 °C to obtain the longitudinal relaxation time (T_1) and transverse relaxation time (T_2) values. The reciprocal of T_1 and T_2 values were plotted against the [Fe], yielding a linear plot. By determining the slopes, the corresponding relaxivity was obtained.

The T_1-and T_2-weighted *in vitro* MR phantom images were obtained using Agilent 4.7 T MRI scanner in the Advanced Magnetic Resonance Imaging and Spectroscopy Facility (AMRIS), McKnight Brain Institute, National High Magnetic Field Laboratory (NHMFL), University of Florida, Gainesville, Florida. The 1×10^6 cultured CCRF-CEM cells per ml of PBS 7.4 were incubated with [Fe]=0, 0.2, 0.4, 0.6, 0.8, 1.0 mM for 4 hours at 37 °C (in the presence of 5% CO_2). And 250 µl of each sample was transferred to the aliquot for image acquisition. The PBS 7.4 with 10% of hPL and in DI H_2O were used with similar [Fe] for the image acquisition. The T_1- and T_2-weighted phantom images were obtained using multislice spin-echo imaging

(MSSEI) sequence, and the operating parameters were TR/TE=12000/34 ms (for T_1-weighted) and TR/TE=12000/24 ms (for T_2-weighted), field of view (FOV)=60, phase=40, slice=3, thickness=1.50 mm).

The cell lines used in this study were purchased from American Type Culture Collection (ATCC). A549 (human lung adenocarcinoma epithelial cells), MDA-MB-231 (human breast adenocarcinoma epithelial cells), and Hela (human cervix adenocarcinoma epithelial cells) were cultured in Dulbecco's Modified Eagle Medium (DMEM). CCRF-CEM (human acute lymphoblastic Leukemia cells) and CRL-9855 (human peripheral blood monocytes/macrophages) were cultured in RPMI-1640 and Iscove's modified Dulbecco's medium (IMDM) respectively. All media were supplemented with 5% human platelet lysate (hPL) and 1% Streptomycin/Penicillin, and the cells were incubated at 37 °C in presence of 5% CO_2.

The fluorescence confocal images were obtained using a Nikon Eclipse Ti Series Inverted Microscope (Nikon Instruments Inc.). The DAPI (4ˈ,6-diamidino-2-phenylindole) was excited with 405 nm laser diode illumination and the Alexa Fluor 555 Cadaverine was excited with a 561 nm Diode Pump Solid State laser. For the time-dependent cellular uptake study, approximately 1×10^6 of CCRF-CEM cells with 1mM of [Fe] were incubated in RPMI-1640 for 0, 2, 4, 6, 8, 12, and 24 hours. The similar condition was followed for 5×10^5 of CRL-9855 incubated in IMDM. Also, for the concentration-dependent cellular uptake study, the corresponding cells were incubated for 4 hours with 0, 0.2, 0.6, 1.0 mM of [Fe] in the FALCON 6 well tissue culture plate (Fisher Scientific). Since both cells exhibit suspension culture properties, they were fixed by adding 1 ml of 4% paraformaldehyde (PFA) and were gently resuspended for 15 min. The samples were washed three times using PBS 7.4 via centrifugation at 3000 g for 3 min. The 10 µl

of the washed sample was dropped on the glass micro slides (VWR International), and 5 µl of Prolong Diamond Antifade Mountant with DAPI was mixed followed by immediate placement of coverslips. Each sample was triplicated. The edges of the coverslips were covered using nail polish to avoid the formation of air bubbles. All the experiments were conducted at room temperature.

Inductively coupled plasma-optical emission spectrometry (ICP-OES) was used for the quantification of uptaken [Fe] into the targeted cells (CCRF-CEM and CRL-9855). The cells were washed three times with PBS 7.4 and were digested in 3% HCl overnight at room temperature. The Calibrations were performed using ionic Fe standards in HCl from Iron AA Standard (1000 ppm of Fe in 3% HCl) ranging from 0 to 100 parts per million (ppm). The Fe content of the resulting solutions was measured by PerkinElmer Optima 8000 Optical Emission Spectrometer using ICP continuous technique. The number of Fe was determined by dividing the number of Fe calculated by the number of the cells and was averaged. All experiments were conducted in three independent replicates for each treatment.

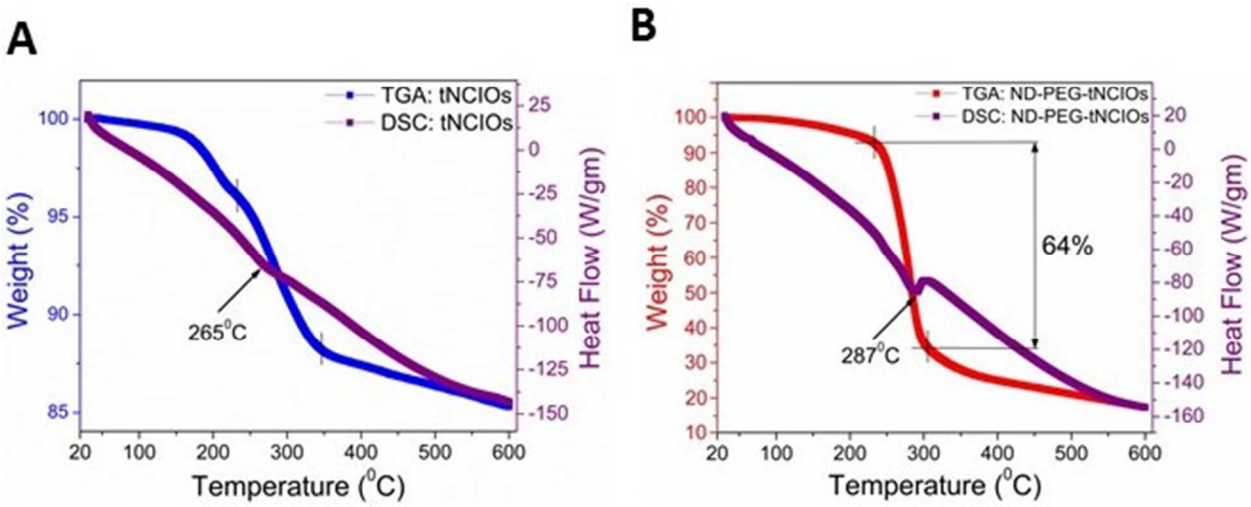

Figure S1. TGA and DSC profile of (**A**) tNCIOs and (**B**) ND-PEG-tNCIOs.

1. Grafting density calculation:

The grafting density was estimated using the following equation:

$$\sigma_{TGA} = \frac{\left(\frac{Weight\,\%\,of\,shell}{Weight\,\%\,of\,core}\right)(Volume\,of\,single\,nanoparticle)\rho_{core}N_A}{(Molecular\,Weight)_{shell}\,(Surface\,area\,of\,single\,nanoparticle)} \quad \text{.....(2)}$$

According to TGA profile (Figure S1B),

Weight % of shell = 64%, Weight % of core = 18%, Volume of single nanoparticle = $(12\ nm)^3$ = 1728 nm^3, Density of nanoparticle (ρ_{core}) = 5.26 gm/cm^3 = 5.26×10^{-21} gm/nm^3, Avogadro's constant (N_A) = 6.022×10^{23} mol^{-1}

Molecular weight of shell = molecular weight of PEG(5K)+molecular weight of ND

= 5K+198.178 gm/mol ~ 5200 gm/mol

Surface area of single nanoparticle = $6\times (12\ nm)^2$ = 864 nm^2

Substituting all these values in equation (1),

σ_{TGA}= 4.329 chains/nm^2 ~4 chains/nm^2

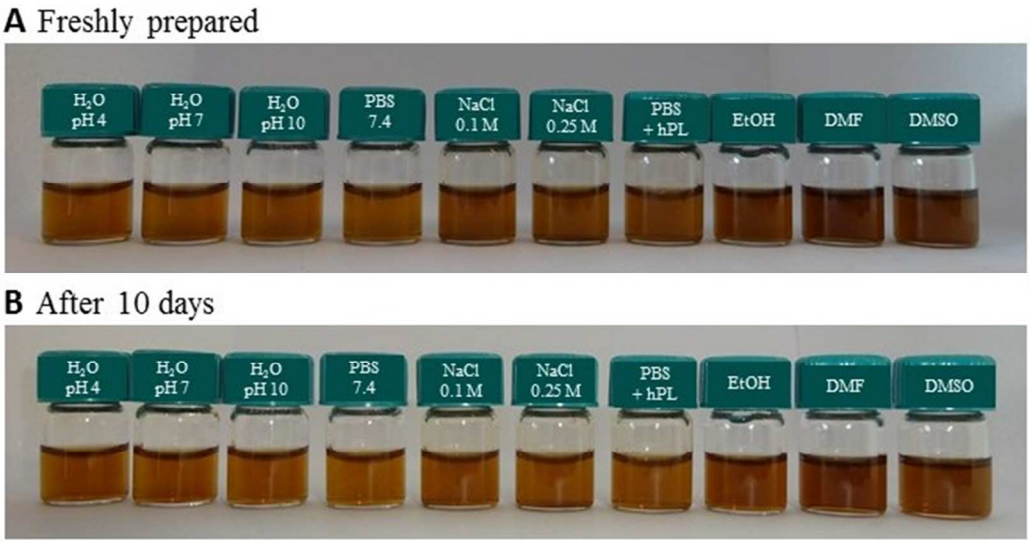

Figure S2. Optical images of ND-PEG-tNCIOs dispersed in (from left to right respectively) H_2O (pH 4, 7, 10), PBS pH 7.4, NaCl (0.1, 0.15 M), PBS + hPL, EtOH, DMF and DMSO. (**A**) Freshly prepared solutions and (**B**) Solutions after 10 days.

2. Calculation of the size of a nanoparticle:

Langevin's equation:

$$M(H)= M_S \times L(\beta)=M_S\left[Coth(\beta)-\frac{1}{\beta}\right]\ldots\ldots(3)$$

Here, $L(\beta)=\left[Coth(\beta)-\frac{1}{\beta}\right]$ is Langevin's function

$$M(H) = M_S\left[Coth\left(\frac{\mu H}{k_B T}\right)-\left(\frac{k_B T}{\mu H}\right)\right] = M_S\left[Coth\left(\frac{M_{bulk}VH}{k_B T}\right)-\left(\frac{k_B T}{\mu H}\right)\right],$$

Here, M_S =Saturation magnetization of sample, $M_{bulk=}$ Saturation magnetization of bulk material

If "D" be the diameter of the particle,

$$M_S=\left[\frac{M_{bulk}V}{\left\{\rho\left(\frac{\pi D^3}{6}\right)\right\}}\right],\ D=\left[\frac{M_{bulk}V}{\left\{\rho\left(\frac{\pi M_S}{6}\right)\right\}}\right]^{\frac{1}{3}},$$

Volume of single nanoparticle= $(12\ nm)^3$=1728 nm^3

$$=\left[\frac{(100\ emugm^{-1})(1728\ nm^3)}{\left\{5.26gmcm^{-3}\left(\frac{\pi\times 82\ emugm^{-1}}{6}\right)\right\}}\right]^{\frac{1}{3}}=14.71\ nm \sim 15\ nm$$

This theoretical value contradicts to experimental (12 nm) owing to the particles' size distribution, anisotropy properties, interparticle interaction (dipolar and exchange).

3. Calculation of the anisotropy constant (K_{eff}) and anisotropy energy (E_a):

(1) For ND-PEG-tNCIOs;

$$K_{eff}=\left[\frac{25k_B T_B}{V_p}\right]\ldots\ldots(4)$$

k_B=1.38×10^{-23} m^2kgSec^{-2}K^{-1} and T_B=255 K

$$=\left[\frac{25\times1.38\times10^{-23}\mathrm{m^2kgSec^{-2}K^{-1}}\times255\mathrm{K}}{1728\ \mathrm{nm^3}}\right]=\left[\frac{25\times1.38\times255\times10^{-23}m^2kgSec^{-2}}{1728\times10^{-27}m^3}\right]$$

$$=\left[\frac{50911.46\ \mathrm{kgSec^{-2}}}{m}\right]=\left[\frac{1}{\mathrm{m^3}}\frac{50911.46\mathrm{kgm^2}}{\mathrm{Sec^2}}\right]=\left[\frac{1}{10^6\ \mathrm{cm^3}}\frac{50911.46\ \mathrm{kgm^2}}{\mathrm{Sec^2}}\right]$$

$$=\left[\left(\frac{1\ \mathrm{kgm^2}}{10^7\mathrm{Sec^2}}\right)\frac{10\times50911.46}{\mathrm{cm^3}}\right]=\left[1\ \mathrm{erg}\times\frac{10\times50911.46}{\mathrm{cm^3}}\right]=\left[\frac{509114.6\ \mathrm{erg}}{\mathrm{cm^3}}\right]$$

$$=5.09\times10^5\ \frac{\mathrm{erg}}{\mathrm{cm^3}}$$

Anisotropy energy of ND-PEG-tNCIOs (E_a)= $K_{eff}V$= $\left(5.09\times10^5\ \frac{\mathrm{erg}}{\mathrm{cm^3}}\right)\times(1728\ \mathrm{nm^3})$ = 8.795×10^{-20} joule

(2) For tNCIOs; No T_B observed within 10-325 K. High anisotropy is expected.

4. Calculation of the Néél relaxation time:

(1) For ND-PEG-tNCIOs;

$$\tau_N=\ \tau_0\exp\left[\frac{K_{eff}V}{K_BT}\right]\text{.....(5)}$$

Here, pre-exponential factor of the Néel relaxation time expression,

$\tau_0 \approx 10^{-9}-10^{-12}\ Sec$, k_B=1.38×10^{-23} m^{2}kgSec^{-2}K^{-1} and T=300 K

$$=\left(10^{-12}\ \mathrm{Sec}\right)\exp\left[\frac{\left(5.6\times10^5\frac{\mathrm{erg}}{\mathrm{cm^3}}\right)\times1728\ \mathrm{nm^3}}{1.38\times10^{-23}\mathrm{m^2kgSec^{-2}K^{-1}}\times300\mathrm{K}}\right]$$

$$=(10^{-12}\ \mathrm{Sec})\exp\left[\frac{\left\{5.6\times10^5\times\frac{\left(\frac{1\ \mathrm{kgm^2}}{10^7\mathrm{Sec^2}}\right)}{10^{21}\mathrm{nm^3}}\right\}\times1728\ \mathrm{nm^3}}{1.38\times10^{-23}\mathrm{m^2kgSec^{-2}K^{-1}}\times300\mathrm{K}}\right]$$

$$=(10^{-12}\ \mathrm{Sec})\exp\left[23.374\right]$$

$$=1.41\times10^{-2}\ \mathrm{Sec}$$

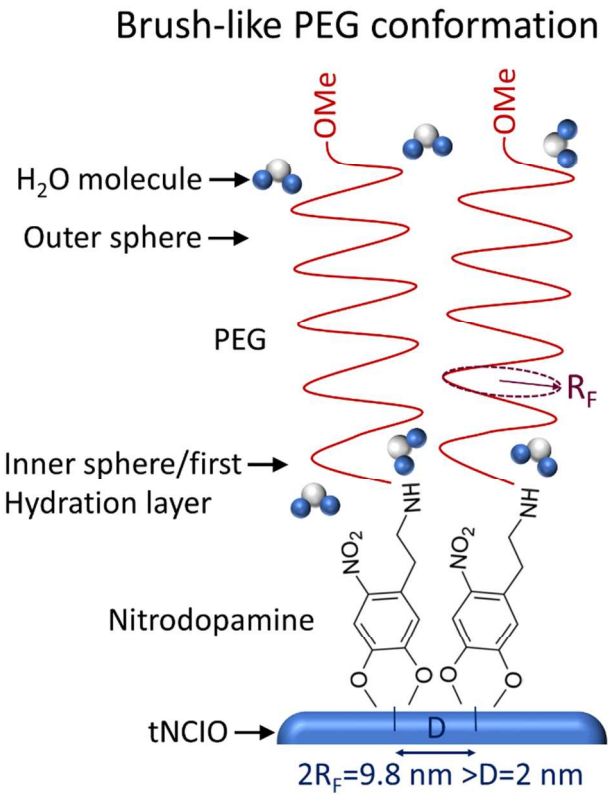

Figure S3. Schematic representation of brush-like conformation of PEG with Flory radius (R_F) higher than the inter ligand distance (D).

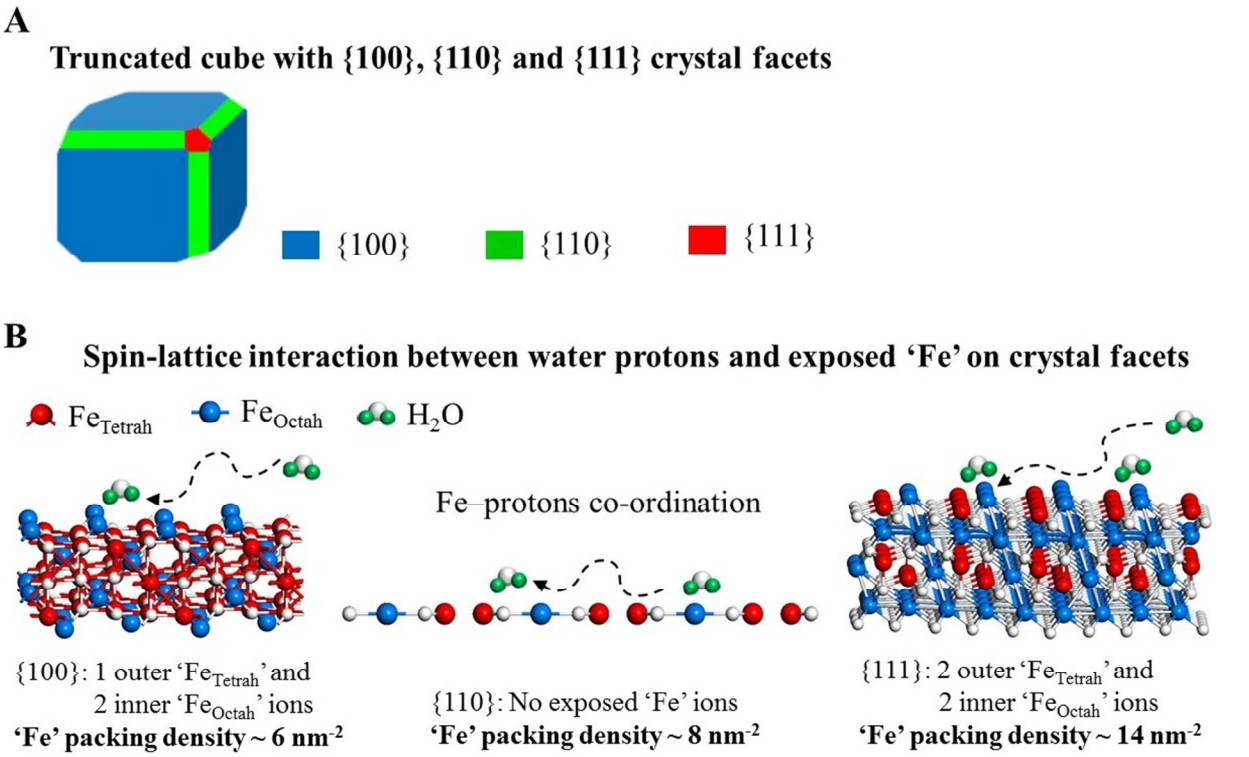

Figure S4. (**A**) The schematic diagram of the truncated cubic structure with three exposed crystal facets: {100}, {110}, and {111} and (**B**) The schematic representation of the co-ordination of the 'Fe' atoms of those crystal facets and the water protons that facilitate the spin-lattice relaxation.

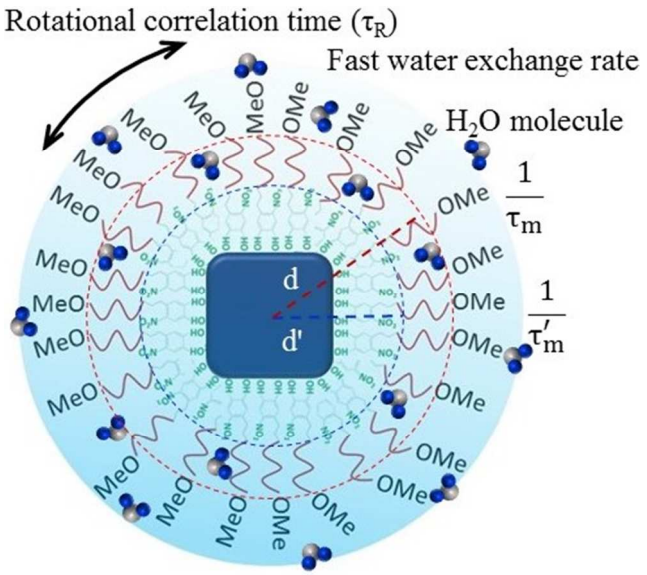

Figure S5. First and second hydration layers (inner and outer spheres) formed by brush-like conformation of PEG (5K) in ND-PEG-tNCIO shown in blue and red dotted circles respectively. The respective radii are 'd' and 'd′'. τ_R: rotational correlation time of ND-PEG-tNCIOs (τ_R) and τ_m: exchange correlation time.

5. Calculation of the interspacing distance (D) between ND-PEG and Flory radius (R_F):

The grafting density of the ND-PEG on tNCIOs= 4 chains/nm^2

Surface are of one face= 12×12 nm^2 = 144 nm^2

Total no. of ND-PEG chain on one face=144×4 chains=576 chains

Assuming the chains are arranged uniformly over the face,

the footprint of the chains on one surface= $\sqrt{576\ nm^2}$ = 24 nm^2

The interspacing distance (D) between the attachment points (D)= $\frac{24\ \mathrm{nm}^2}{12\ \mathrm{nm}}$= 2 nm

Flory radius (R_F)= (length of PEG monomer) $\left(\text{number of monomers}\right)^{\frac{3}{5}}$ = $\alpha n^{\frac{3}{5}}$

= (3.5 Å) $(81)^{\frac{3}{5}}$ = 48.88 Å≈ 4.9 nm

So, $2R_F > D$, gives brush-like conformation of ND-PEG on tNCIOs.

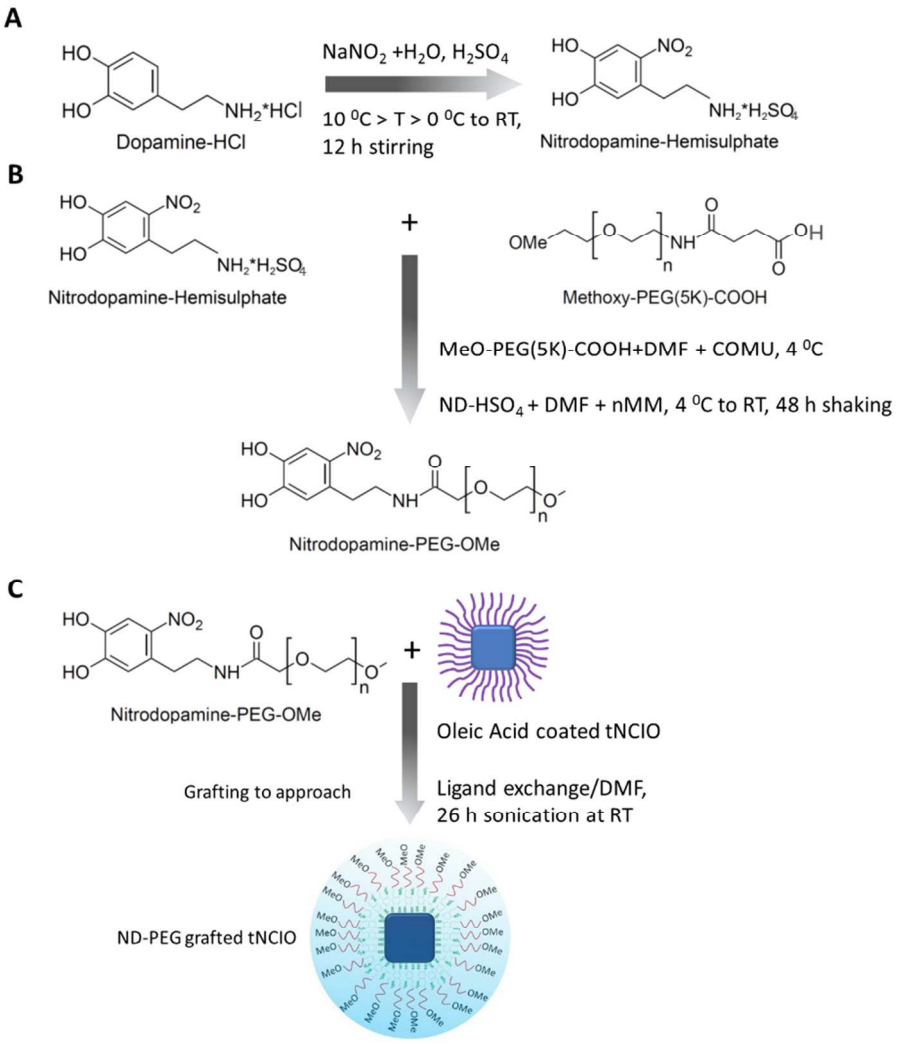

Figure S6. Schematic representation of the surface functionalization of tNCIOs. (**A**) Synthesis of Nitrodopamine-Hemisulphate. (**B**) Synthesis of Nitrodopamine-PEG(5K)-MeO. (**C**) ND-PEG functionalized tNCIO via ligand exchange followed by grafting to approach.

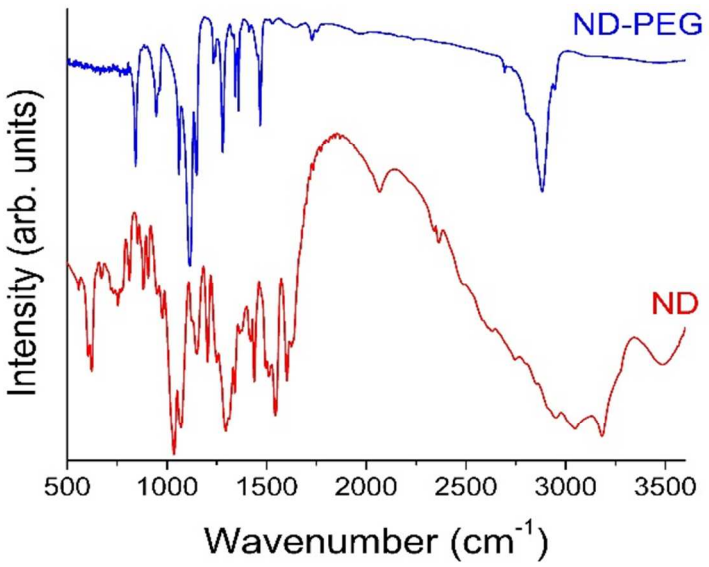

Figure S7. ATR-FTIR spectra of ND and ND-PEG. The peaks position for ND (in cm^{-1}): 557, 604, 623, 670, 754, 811, 852, 882, 906, 951, 976, 1034, 1070, 1148, 1205, 1250, 1295, 1312, 1340, 1364, 1419, 1438, 1496, 1510, 1544, 1602, 1625, 2066, 2341, 2363, 3046, 3182, 3183, 3484. The peaks position for ND-PEG (in cm^{-1}): 843, 947, 1060, 1115, 1147, 1234, 1279, 1343, 1359, 1412, 1454, 1467, 1529 (CONH), 1641 (CONH), 1727 (COOH), 1753, 1967, 2694, 2738, 2804, 2862, 2882, 2946.

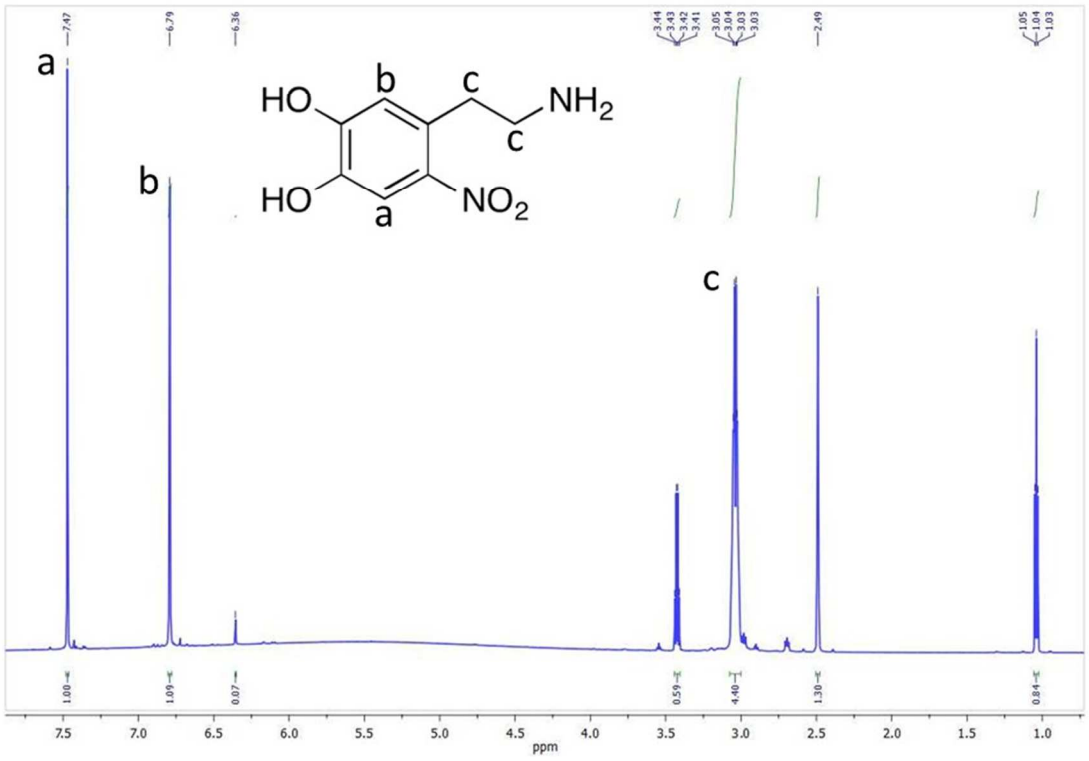

Figure S8. ^{1}H-NMR (DMSO-d_6, 700 MHz, ppm) spectrum of ND. 7.47 (s b, 1H, OH), 6.79 (s, 1H, Ph), 3.04 (s, 4H, CH_2). The broad peak between 6 to 5 ppm are associated to the primary amine and the hydroxyl groups of ND. The presence of this broad peak could be due to the higher concentration of ND and the bridging between the hydroxyl groups in ND and the primary amine of adjacent ND. The peaks at 3.42 ppm and 1.04 ppm indicate the presence of traces of ethanol that was used for cleaning. The peak at 2.49 ppm is associated to the solvent (DMSO-d_6).

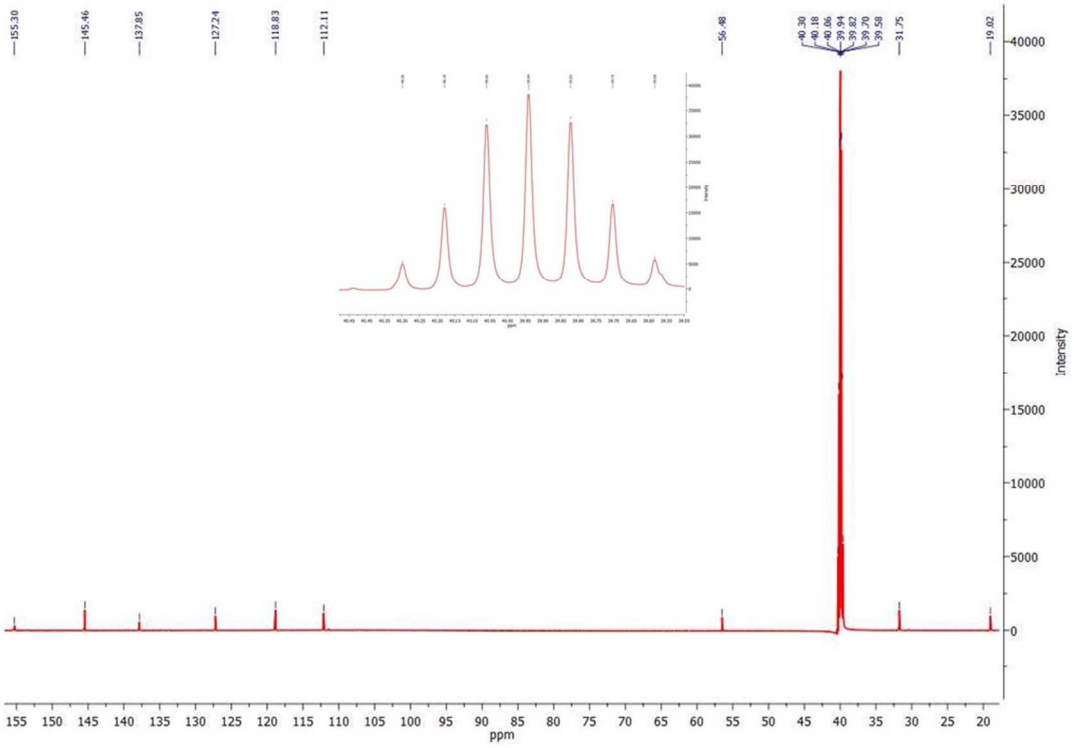

Figure S9. ^{13}C-NMR (DMSO-d6, 700 MHz, ppm) spectrum of ND. 155.30 (C), 145.46 (C), 137.85 (C), 127.24 (C), 118.83 (CH), 112.11 (CH), 39.94 (CH_2), 31.75 (CH_2). The peaks at 56.48 ppm and 19.02 ppm are associated to the ethanol that was used for cleaning. The peak associated to the hexane (which was used for cleaning) was observed at 31.75 ppm.

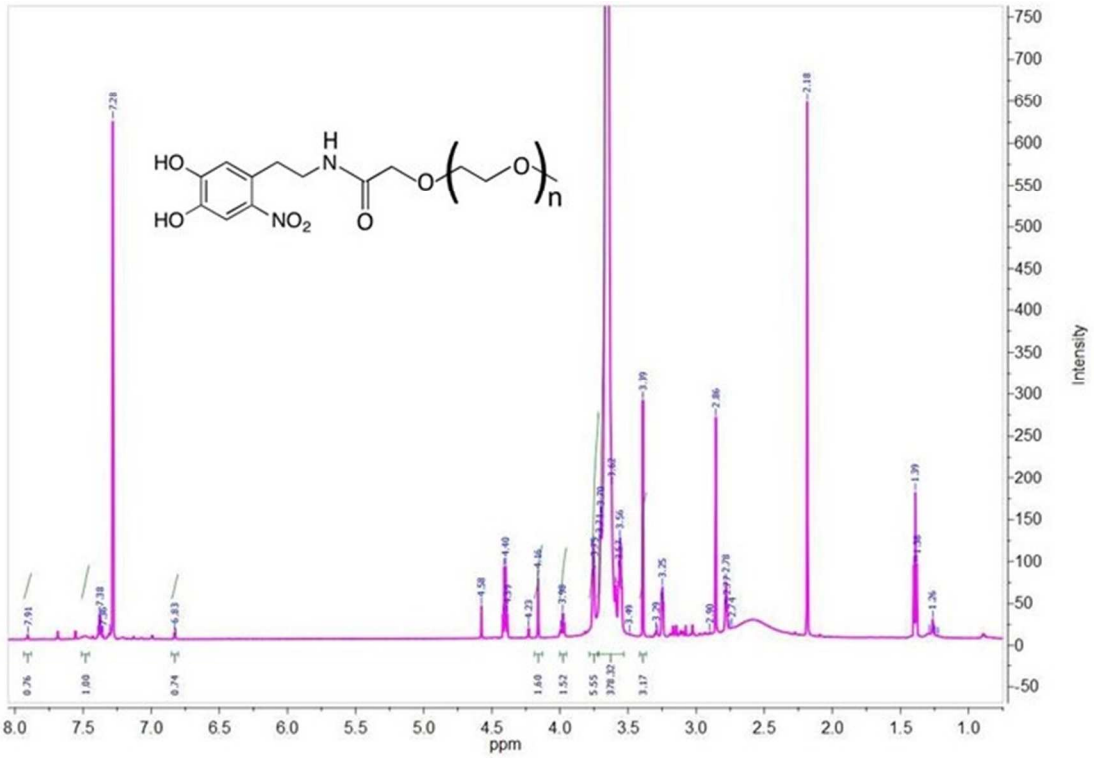

Figure S10. ^{1}H-NMR ($CDCL_3$, 700 MHz, ppm) spectrum of ND-PEG. 7.62 (s, 1H, Ph), 7.28 (s, 1H, CONH), 6.83 (s, 1H, Ph), 4.23 (s, 2H, CH_2CONH), 3.62 (t, 452H, EG-CH_2), 3.39 (s, 6H, OCH_3). Traces of ethanol was detected (1.39 ppm and 1.26 ppm).

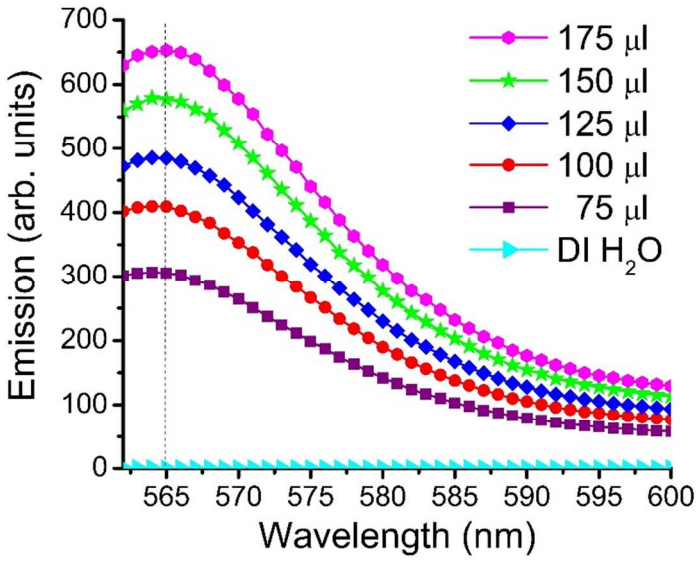

Figure S11. Emission spectra of ND-PEG-tNCIOs-AF555Cdv dispersed in DI H_2O at different concentrations. 1 mg of sample was dispersed in 1 mL of DI H_2O as the source solution. Then 1 ml of measurement samples were prepared by adding 175, 150, 125, 100 and 75 µL of source sample in DI H_2O. The broad emission band centered near to 565 nm is associated with the emission band of AF555Cdv.

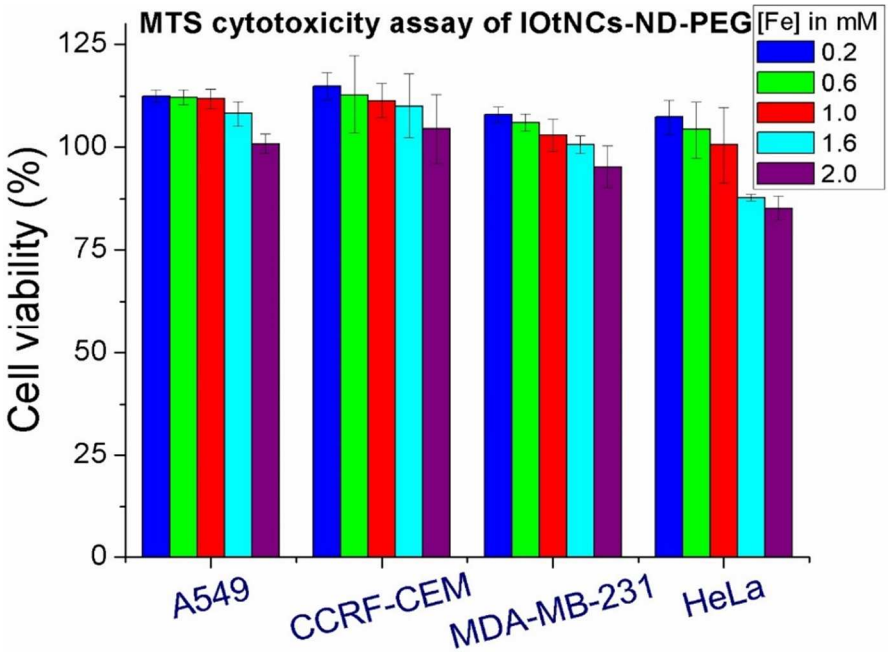

Figure S12. The MTS toxicological cellular assays. ND-PEG-tNCIOs were incubated with A549, CCRF-CEM, MDA-MB-231, HeLa carcinoma cells for 24 hours at 37°C in presence of 5% CO_2. The [Fe] was maintained 0.2, 0.6, 1.0, 1.6, 2.0 mM in each cell line. The nanoparticles are not toxic upto [Fe]=1.0 mM in all cell lines showing prolific cell viability.