Pheomelanin-coated iron oxide magnetic nanoparticles: a promising candidate for negative T_2 contrast enhancement in magnetic resonance imaging

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Electronic Supplementary Information

Synthesis was carried out using commercially available reagents.

Iron (III) chloride ($FeCl_3$ .6(H_2O), 97%), iron (II) chloride ($FeCl_2$, 98%), ammonium hydroxide (NH_4^+OH), potassium hydroxide (KOH), 3,4-dihydroxy-L-phenylalanine (L-Dopa), ethanol, tyrosinase from mushroom, and L-cysteine were all purchased from Sigma-Aldrich.

Synthesis of pheomelanin

The preparation of pheomelanin was based on previous work performed by Chedekel and co-workers[1], with modifications, and involved the reaction of L-Dopa with the amino acid L-cysteine in the presence of tyrosinase acting as a catalyst. The preparation involved the solubilisation of L-Dopa (0.36 mmol) in an aqueous phosphate buffer solution (PBS) (0.1 M), pH = 7.4 at 37ºC. After complete dissolution of the precursor reagent, the minimum

amount of tyrosinase was added and one minute later an orange-coloured was noted due to the subsequent formation of dopaquinones[2]. In the next step, 0.71 mmol of amino acid previously dissolved in the minimum amount of PBS possible was added. The medium was kept under magnetic stirring for 48 h and then 5 mL of HCl (1 M) was added to precipitate the final brown product. The product was then centrifuged to obtain the solid, which was purified for recrystallization in a hot medium using acetone/distilled water to eliminate the L-Dopa and non-polymerized cysteine. Finally, the solvent that remained in the product and the solution was washed twice in deionized water, freezing overnight for lyophilisation to obtain a fine brownish powder which was used for the characterization studies and synthesis of the IOMNPs coating.

Synthesis of Fe_3O_4@Pheo magnetic nanoparticles

The pheomelanin-coated iron oxide (Fe_3O_4@PHEO) magnetic nanoparticles were prepared by the co-precipitation method with modifications[3]. Firstly, the pheomelanin solution was prepared in a beaker (80 mL), containing 100 mg of pheomelanin solubilized in 30 mL of deionized water with magnetic stirring and a certain volume of alkaline solution (0.1 M KOH) was added slowly until pH 9.0 was reached. The reaction system was then kept under stirring overnight. The pheomelanin-coated IOMNPs were obtained as follows. Briefly, stock solutions of 4.0 mmol (1.233 g) of $FeCl_3 6.H_2O$ and 2.0 mmol (0.253 g) of $FeCl_2$ diluted in HCl (1 M) were placed in a 250-mL three-necked round-bottle flask with 100 mL of deionized water and heated to 90º C. In the next step, 10 mL of ammonium hydroxide solution (25 vol.%) were injected rapidly into the reaction vessel. The pheomelanin solution was added to the medium in 40 s and the reaction system was kept under vigorous stirring for 30 min, in inert atmosphere. An immediate change in the colour of the solution (to

black) was noted, indicating the formation of Fe_3O_4@Pheo magnetic nanoparticles. The reaction medium was then cooled to room temperature and the nanoparticles were rapidly isolated by magnetic decantation and washed several times with deionized water until neutralization of the solution (pH = 7.0). Finally, the magnetic nanoparticles were maintained under freezing conditions overnight and then subjected to lyophilisation and further characterization.

Physical measurements:

For the infrared analysis a JASCO FT/IR 4100 spectrometer was used and the spectra were obtained with 64 scans for each sample at a resolution of 4 cm^{-1} within the baseline correction, and for the background reading KBr tablets (100 mg) were used. Transmission electron microscopy (TEM) and selected area electron diffraction (SAED) images were taken on a JEM-1011 TEM microscope at an acceleration voltage of 100 kV. X-ray powder diffraction (XRD) measurements were obtained on a Siemens D500 diffractometer, using Cu Kα ($\lambda_{CuK\alpha}$ = 1.5418 Aº) radiation at a scanning rate of °/min in the 2θ range of 20 to 80° with 0.05º steps (40 kV, 40 mA). Magnetization measurements obtained from a dried pheomelanin-coated IOMNP sample, pressed and held in a cylindrical holder, on a vibrating sample magnetometer (VSM; Model Microsense EV9). The variation of the magnetic moment was carried out by altering the applied field from 20,000 Oe (2T) to -20,000 Oe (-2 T) at 300 K. The iron concentration was determined using a flame atomization atomic absorption (FAAS) spectrophotometer (model PinAAcle 900T, PTAS11120701 Perkin Elmer, USA). The thermogravimetric analysis (TGA) was performed under nitrogen at a heating rate of 10ºC/min from room temperature up to 900ºC

using a Shimadzu TGA-50 analyser. The XPS spectra were collected on a Thermo Scientific™ ESCALAB™ 250Xi X-ray photoelectron spectrometer and the instrument was equipped with a monochromatized AlKα source (1486.6 eV). The sample was analysed as a powder. The radiation was focused on a 900 μm spot size and the sample was charge compensated by an electron gun. The spectra were acquired with an energy step of 1 eV while the Fe2p, O1s and C1s peaks were acquired with an energy step of 0.05 eV. Dynamic light scattering (DLS) measurements were performed on a Malvern 3000HSA Zetasizer analyser, equipped with a He/Ne laser of 633 nm wavelength.

Supplementary Figures and Tables

Table S1: Fe_3O_4 d-spacing diffraction planes obtained from SAED analysis.

(hkl)	d-spacing (nm)
(220)	0.2965
(311)	0.2508
(400)	0.2079
(511)	0.1606
(440)	0.1464

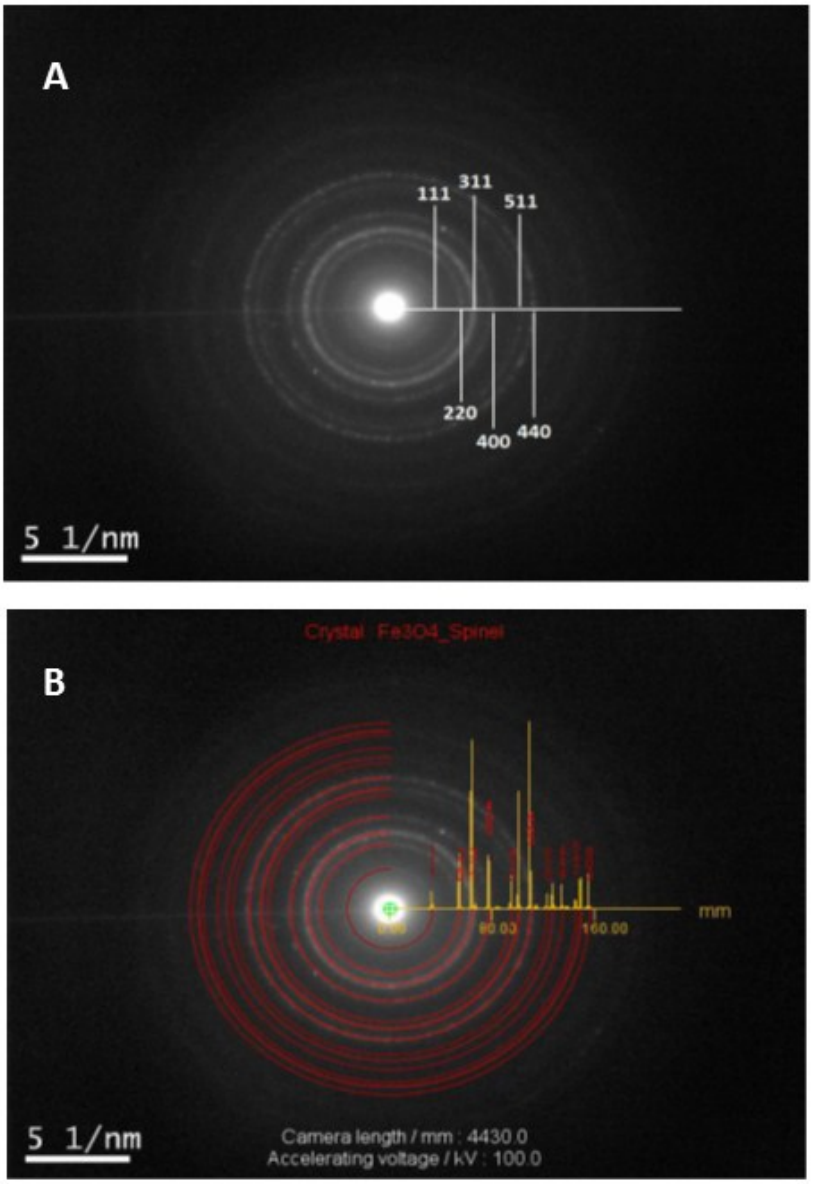

Figure S1: SAED measurements with the diffraction planes labelled (A) and indexed pattern of magnetite matching that of the pheomelanin-coated IOMNPs sample (B).

The X-ray powder diffraction (XRD) analysis of the sample was performed to characterize the crystallite size, phase and crystallization of the IOMNPs and the pheomelanin coating. Figure 1C shows that all peaks of the IOMNPs coated with pheomelanin are in good agreement with the cubic structure [space group: Fd-3mS] known for the Fe_3O_4 crystal pattern (JCPDS: 88-0315), indicating that the samples were highly crystalline with 100% of Fe_3O_4 phase present in the core. According to the full width at half

maximum (FWHM) of (311) reflections, the average size of the Fe_3O_4 nanocrystalline particles was calculated to be 12.7 nm. Furthermore, the crystal cell dimension from (311) reflections was calculated to be a = 0.83632 nm using for the quantitative analysis the Rietveld approach and TOPAS software.[4]

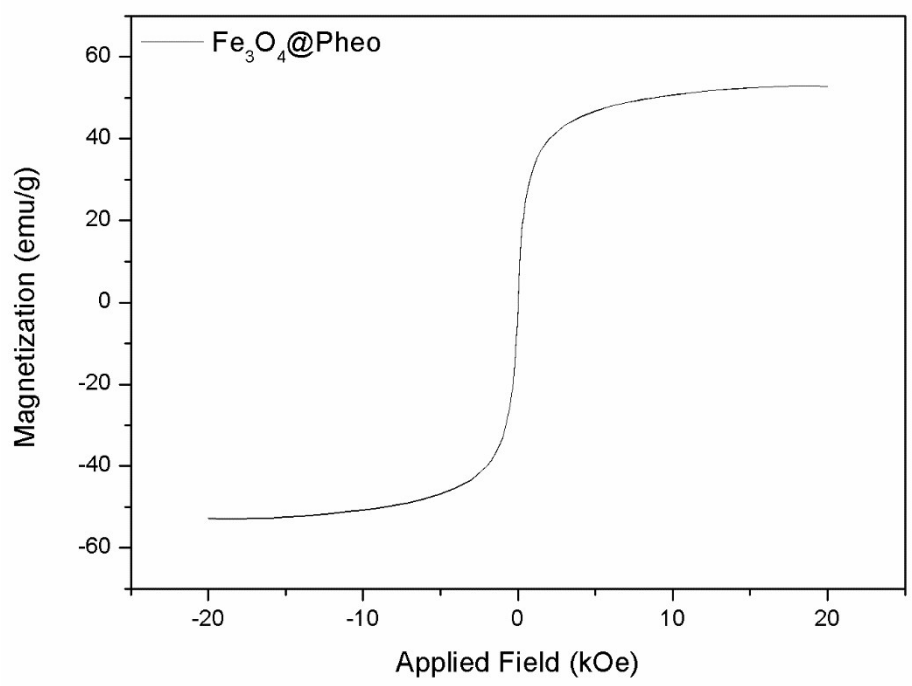

Figure S2: VSM analysis of IOMNPs coated with pheomelanin.

Table S2. Saturation magnetization (*Ms*) of IOMNPs coated with different stabilizers.

	Core diameter (nm)	***Ms* (emu/g)**	**Reference**
Fe_3O_4@Pheo	10-12	52.5	This study
Fe_3O_4@Oleate	8.9–12.2	65.39	[5]
Fe_3O_4@Humic acid	10	70	[6]
Fe_3O_4@Dopamine	9-10	56	[7]
Fe_3O_4@Alginate	5-10	52	[8]

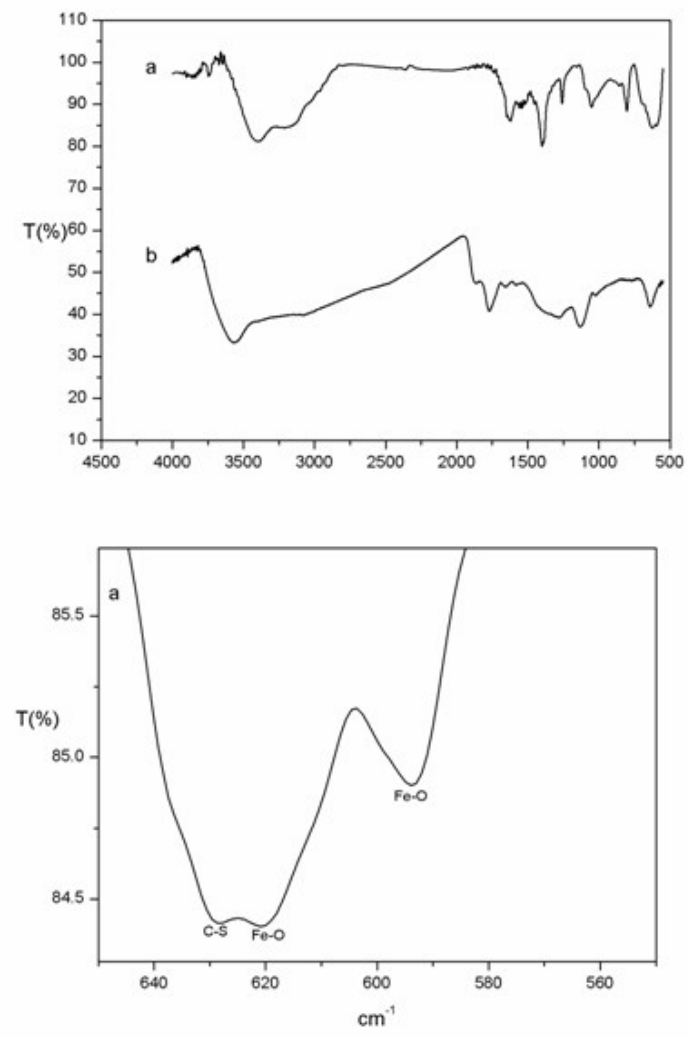

Figure S3 - FT-IR spectra for pheomelanin (b) and coated iron oxide magnetic nanoparticles (a). Inset indicates Fe-O normally present in magnetite.

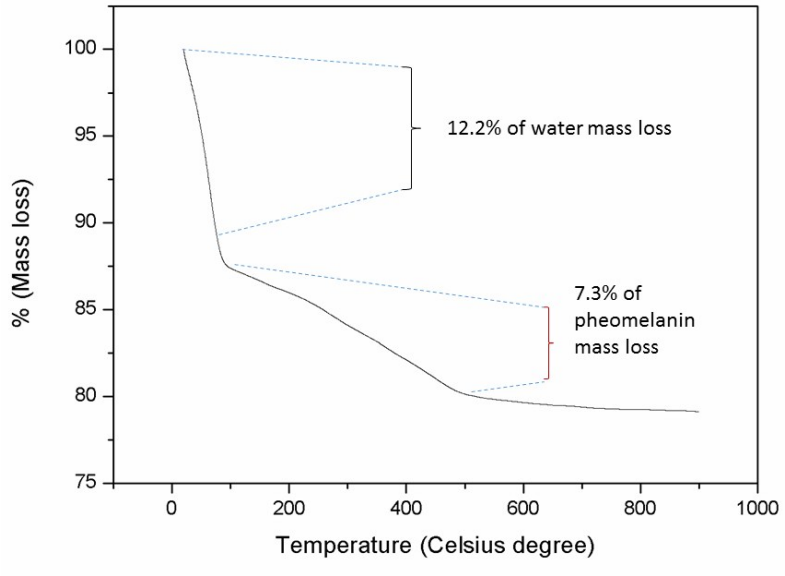

Figure S4: Thermogravimetric analysis showing the degree of coating associated with the mass loss of pheomelanin-coated IOMNPs.

Table S3. Energy-dispersive X-ray (EDX) spectroscopy of pheomelanin-coated IOMNPs.

	C-K	**O-K**	**S-K**	**Fe-K**
Net Counts	299	12590	532	15324
Intensity	-	-	-	-
Weight (%)	5.11	31.39	0.62	62.89
Atom (%)	12.04	55.53	0.54	31.88
Error (%)	+/- 1.69	+/- 0.79	+/- 0.11	+/- 0.47

Table S4. Carbon, hydrogen, nitrogen and sulphur contents of the pheomelanin sample determined by elemental analysis.

	C%	**H%**	**N%**	**S%**	**O%**
Cys-Dopa (pheomelanin)	45.8	4.3	7.7	16.0	26.2
Fe3O4@Pheo*	3.9	0.87	1.25	0.6	-

- Corresponding to a mass of 2.1 mg (0.01 mM) of L-Dopa melanin.

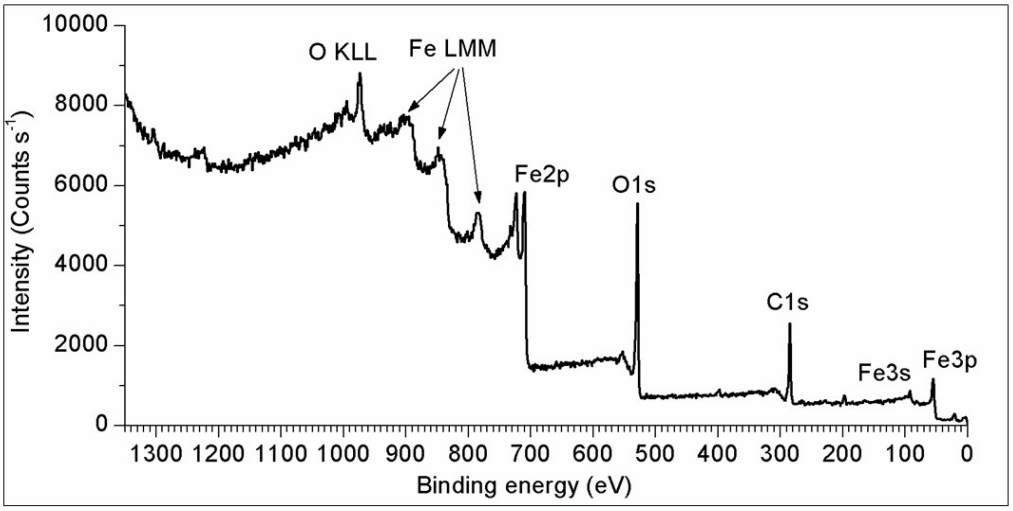

Figure S5: XPS spectrum for pheomelanin-coated IOMNPs.

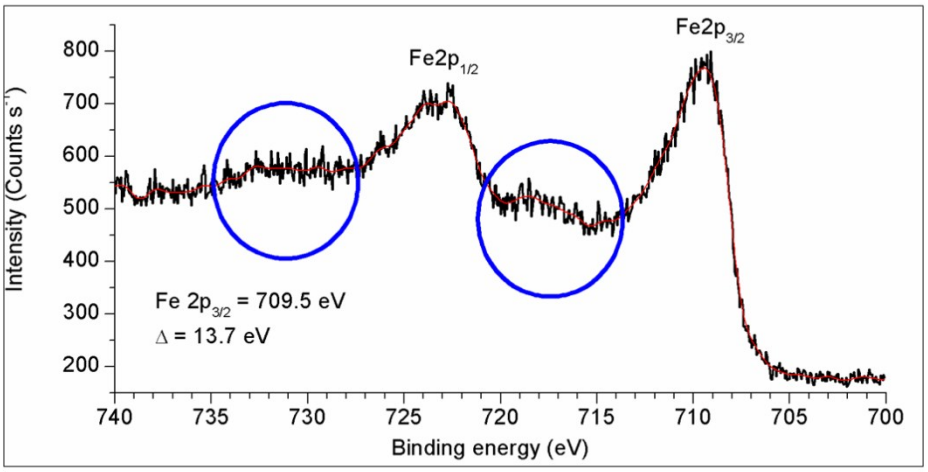

Figure S6: XPS spectrum showing $Fe2p_{1/2}$ and $Fe2p_{3/2}$.

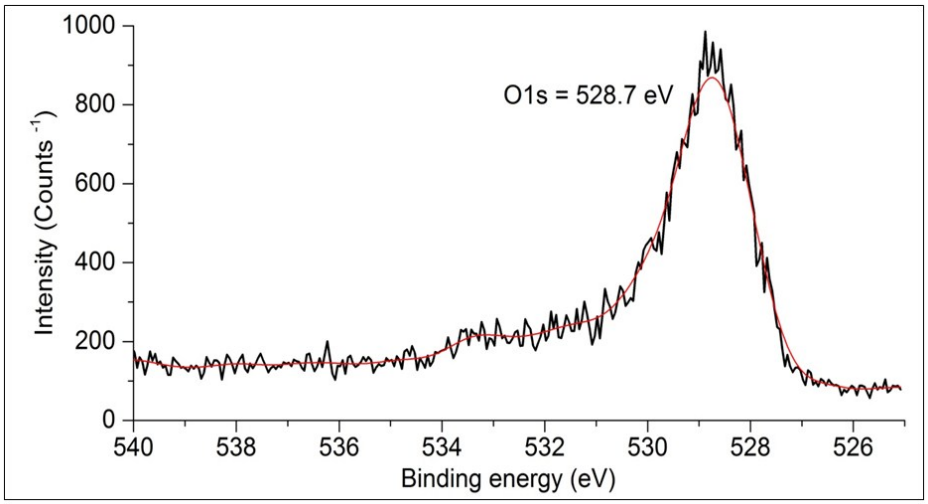

Figure S7: XPS spectrum for Fe-O.

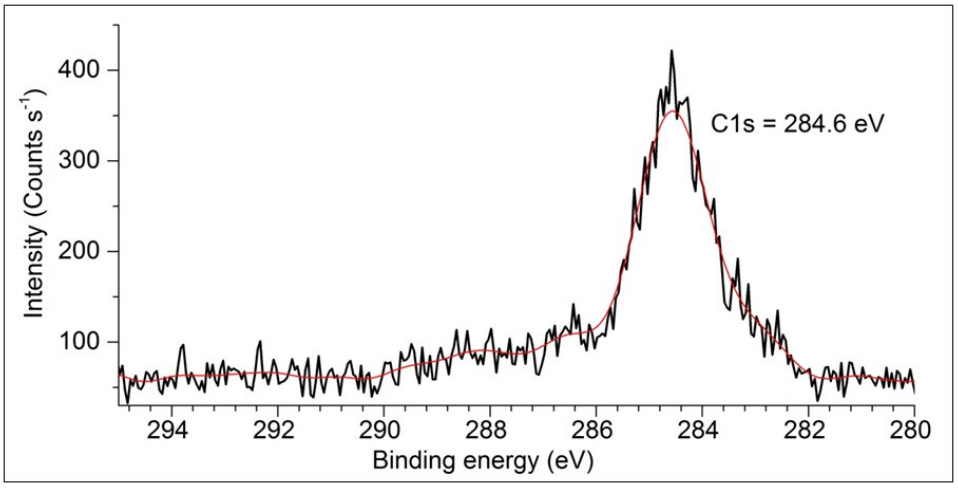

Figure S8: XPS spectrum for Fe-C

Preparation of agar gel phantom for MRI analysis

The preparation of IOMNP suspensions on agar gel phantom for acquisition of the MRI images was carried out according to the described method.[9] Suspensions with IOMNP concentrations of 0, 5, 10, 25 and 50 $\mu g.mL^{-1}$) were analysed by FAAS analysis and maintained in PBS (phosphate buffer pH = 7.2). An agar solution of 2.5% w/v was then prepared by heating 250 mg of agar in 10 mL of PBS at 80ºC for 20 min. To prepare the gels, 160 µl of the solution was mixed with 840 µL of the suspension of IOMNPs at each concentration, and this solution was preheated at 60ºC to prevent gel formation while mixing occurs. The agar gel and the IOMNPs suspensions were mixed under heating in microcentrifuge tubes (plastic tubes of 1.5 mL) while turning the tubes upside down several times. An aliquot of 250 µl of the mixture was then quickly transferred to another microcentrifuge tube and allowed to cool to room temperature.

Measurements of imaging characteristics of magnetic nanoparticles in gel phantom

After the preparation, the tubes containing IOMNPs in gel-agar suspension (250 µL) were positioned near the isocentre in a Sigma Excite 1.5 T - General Electric 1.5 T MRI scanner within a quadrature transmit–receive head coil with a homogeneous B1 field to obtain the MRI images. The host software (Weasis DICOM viewer, Eclipse Public License - v 1.0) was used for visualization/analysis of the images. To estimate the transverse relaxation time (T_2) for each sample, coronal images (TH = 2 mm) were

acquired at various echo times (*TE*) from 10 to 240 ms with a repetition time (*TR*) of 10000 ms. Similarly, the *T1* relaxation time for each sample was measured by varying *TR* between 100, 200, 400, 800, 1600, 3200, 6400, and 10000 ms while keeping *TE* constant at 10 ms. After acquiring the images, the magnitudes of the image intensities were measured within manually-drawn regions of interest (ROIs) for each of the samples. Longitudinal and transverse relaxation rates, respectively, R_1 ($1/T_1$) and R_2 ($1/T_2$) were calculated by mono-exponential curve fitting of the signal intensity vs. time (*TE* or *TR*) data (using Origin 8.0 software). The fitting of the curve for the relaxation rate R_2 ($1/T_2$) against the intensity (I(t)) with that for the iron concentration [Fe], is given by the following equation:

$$I(t) = I(t=0)*\exp(-T_E/T_2) \qquad (1S)$$

where I(t=0) is measured by transversal magnetization ($M_{x,y}$)

The fitting of the curve for the linear dependency, plotting the relaxation rates $1/T_1$ or $1/T_2$ as a function of the iron concentration for the magnetite magnetic nanoparticles, was carried out using the following equations for the longitudinal and transversal relaxivity (r_1 and r_2, respectively) as a function of the iron concentration, respectively.

$$1/T_1 = 1/T_{1,0} + r_1[\mathrm{Fe}] \qquad (2S)$$

where:

$1/T_1$ is the relaxation rate of the sample

$1/T_{1,0}$ is the relaxation rate of the control

$$I(t) = I(t=0)*(1- \exp(-TR/T_1)) \qquad (3S)$$

where: I(t=0) is the longitudinal magnetization (M_o) of the IOMNPs.

For the transverse relaxation rate R_2:

$$1/T_2 = 1/T_{2,0} + r_2[\mathrm{Fe}] \qquad (4S)$$

where $1/T_2$ is the relaxation rate of the sample and $1/T_{2,0}$ is the relaxation rate of the control.

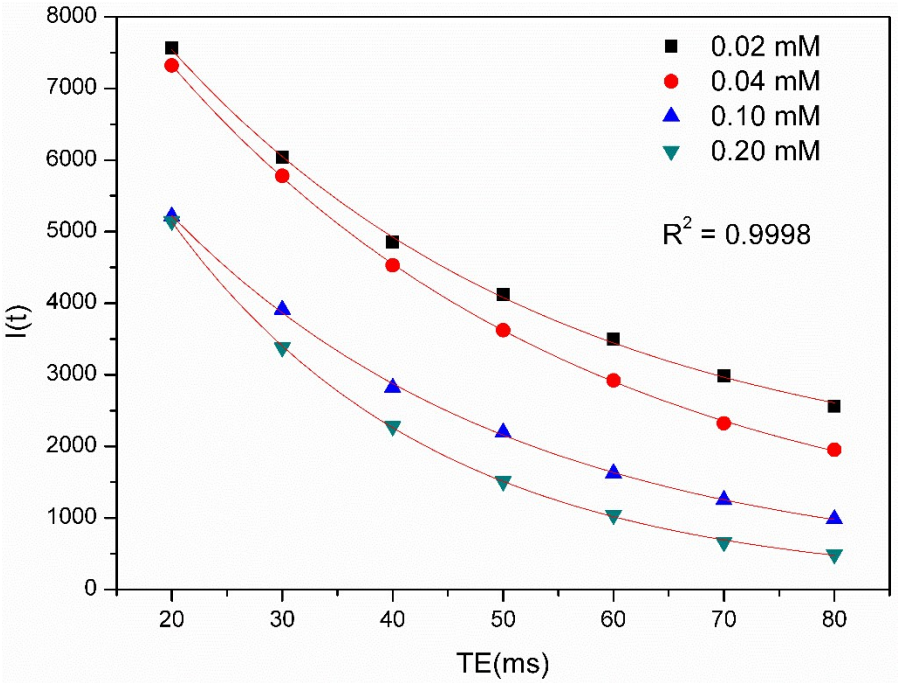

Figure S9: Relaxation curve $1/T_2$ with signal intensity (*I(t)* versus T_E with different iron concentrations.

Stability of pheomelanin-coated IOMNPs

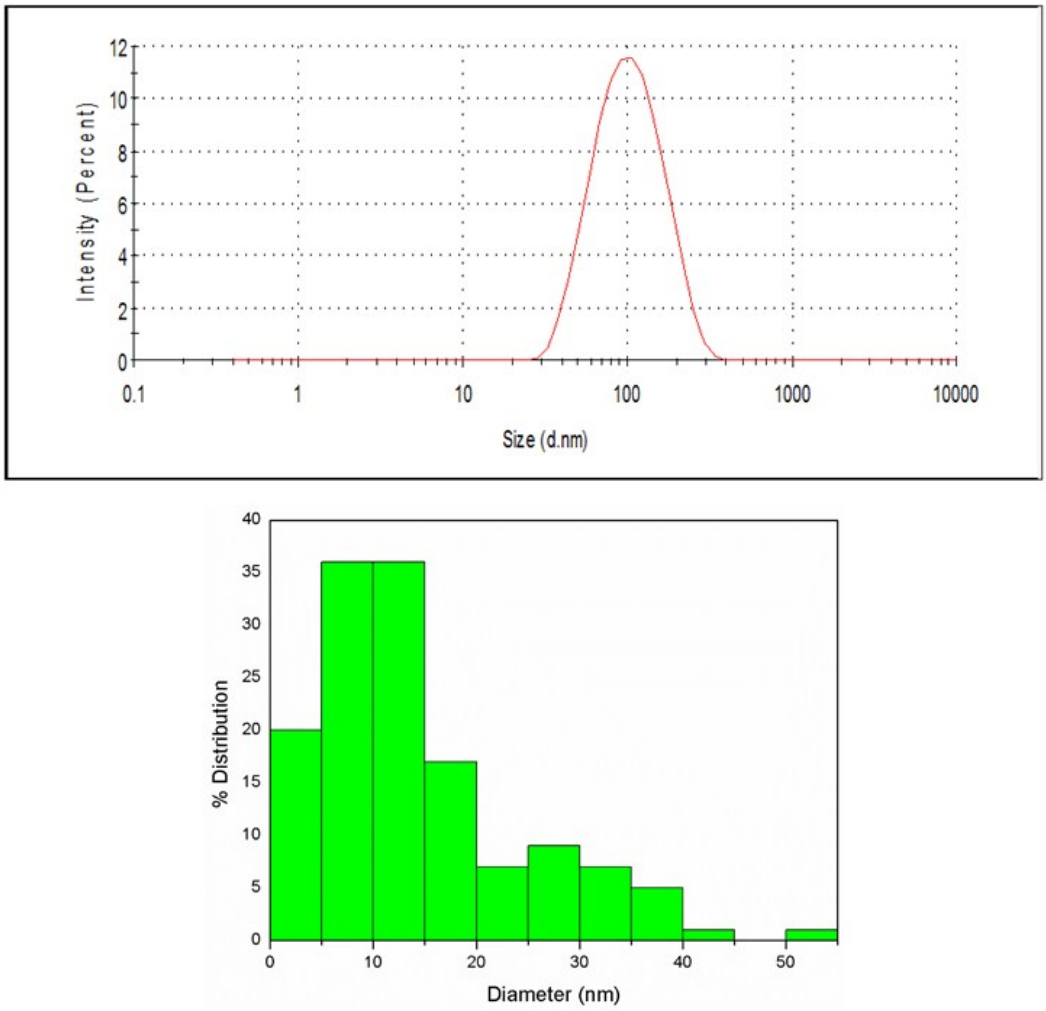

Figure S10: (Top) DLS analysis of pheomelanin-coated IOMNPs with mean hydrodynamic diameter (90.45 nm) and (PDI) polydispersity index (0.179) in buffer solution (*pH*=8.0) and (Bottom) plot of TEM results for the size distribution of IOMNP core diameter.

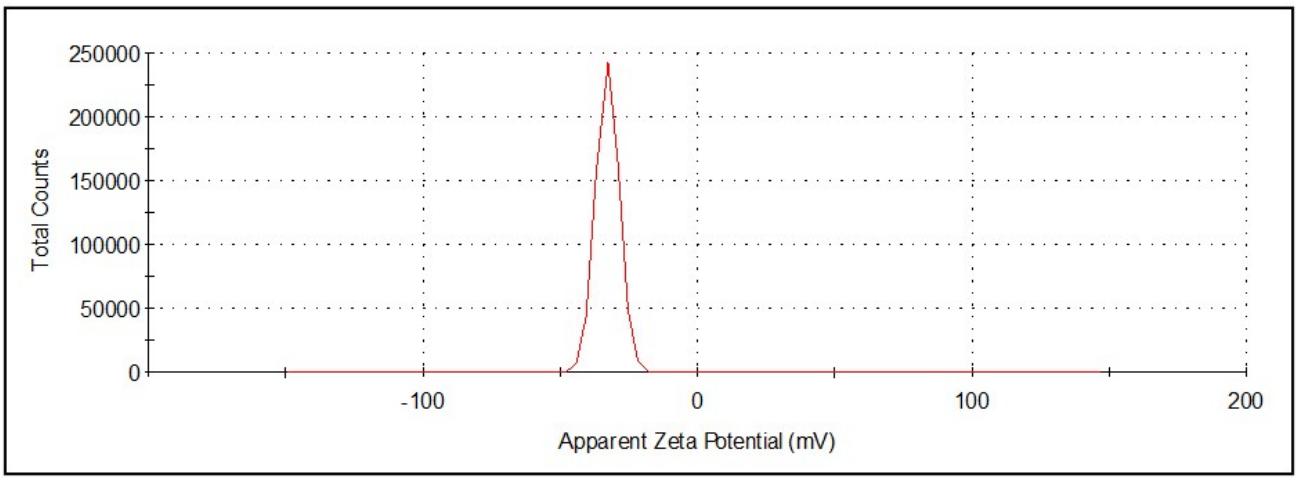

Figure S11: Zeta potential surface analysis of pheomelanin-coated IOMNPs (zeta potential ζ of -38.1mV) stabilized in buffer solution (*pH*=8.0).

Cytotoxicity against MCF-7 cells

Human breast carcinoma-derived MCF-7 cells were obtained from the Rio de Janeiro cell bank, Brazil. They were cultured at 37 ºC under a 5% CO_2 atmosphere with 95% air humidity. Cells were grown in DMEM supplemented with 10% FBS, penicillin (100 U/mL) and streptomycin (100 μg/mL). Cytotoxicity was measured using the MTT assay[10]. Briefly, 104 cells/well were plated onto 96-well plates. At confluence, the cells were exposed to nanoparticles (5, 10, 25, 50, 75 and 100 ppm) for up to 24 h. The cells were then washed twice with PBS and incubated for 2 h with MTT (0.5 mg/mL). The formazan crystals were solubilised by adding DMSO (100 μL/well), and the coloured solutions were read at 550 nm. Three independent experiments were conducted, and the results are presented as cell viability.

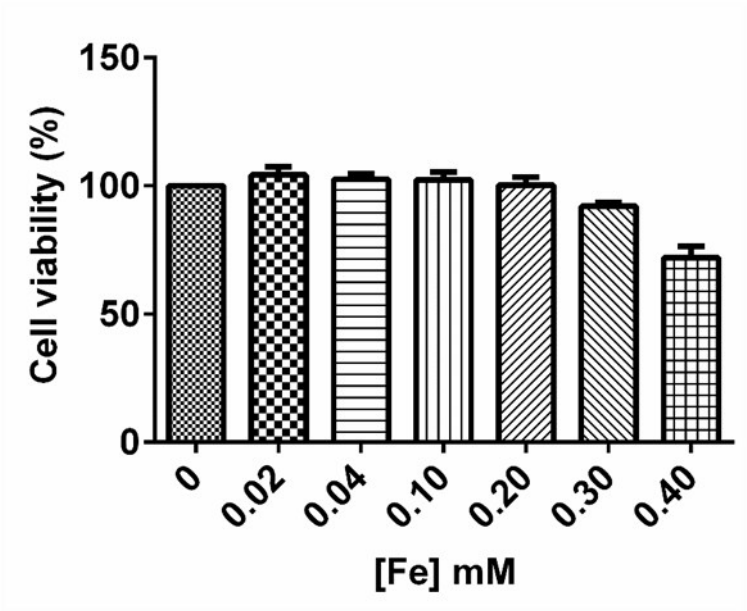

Figure S12: MTT assay of pheomelanin-coated IOMNPs showing cell viability in function of the iron concentration.

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