Supplementary information

Assessment of nanoparticles' safety: corrected absorbance-based toxicity test

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Materials and Methods

IONPs: IONPs were synthesized following a protocol described previously,[1] by co-precipitation alone (IONPs named CP) or in combination with a hydrothermal treatment performed at 120 °C for 24 h (IONPs named CP+HT).

In vitro toxicity study: HeLa cells were cultured in DMEM (Life Technologies) and LnCaP cells were cultured in RPMI 1640 medium (ATCC), both supplemented with 10 % fetal bovine serum (Life Technologies) and 1.5 % 10'000 U ml^{-1} Penicillin Streptomycin (Life Technologies). To determine the cell viability, the MTS and PrestoBlue® tests were used. HeLa (20'000 cells per well) and LnCaP (40'000 cells per well) cells were cultured in 96-well plates at 37 °C, and exposed to 100 µl of different concentrations of CP or CP+HT (0, 20, 40, 60, 80, 100 and 200 μg_{Fe} ml^{-1}) for 24 h. Cells treated only with medium served as negative controls. After 24 h incubation, the supernatant of each well were removed. For the MTS test, 100 µl of MTS solution (CellTiter 96® AQueous One Solution Cell Proliferation Assay from Promega, diluted 6 times in medium) was added to the cells. After 2 h incubation, the absorbance of the formazan product was measured with a microplate reader (Tecan Infinite M200) at a wavelength of 490 nm. For the PrestoBlue® test, 100 µl of PrestoBlue® Cell Viability Reagent (ThermoFisher Scientific; diluted 10 times in medium) was added to the cells. After 1 h incubation, the fluorescence of the resofurin product was measured with the microplate reader at an excitation wavelength of 535 nm and an emission wavelength of 615 nm. All experiments were performed in triplicates. Results are given as means (with standard deviations) of the values obtained in these triplicates.

UV-visible spectroscopy: The absorbance of NPs' suspensions (CP and CP+HT at concentrations of 0, 20, 40, 60, 80, 100 and 200 μg_{Fe} ml^{-1} in DMEM or RPMI 1640 medium) were measured by UV-visible spectroscopy in Brand® UV-cuvettes with a Cary 100 Bio spectrometer between 190 and 900 nm. The average time was set to 0.1 s, the data interval to 1 nm and the scan rate to 600 nm min^{-1}. The absorbance was plotted in function of the NPs' concentration at 490 nm and linear regression lines were obtained from these calibration curves.

Agglomerate diameter measurement: The volume hydrodynamic diameters of 1 ml of suspensions of CP or CP+HT in RPMI or DMEM medium containing 10 % fetal bovine serum were measured at room temperature in acrylic cuvettes (Sarstedt) with a Zetasizer Nano ZS (Malvern Instruments). The hydrodynamic diameters were obtained from the average of 3 x 12 measurements. The refractive index of γ-Fe_2O_3 and absorbance were set to 2.95 and 0.1, respectively. The main peaks were approximated to be the main agglomerate diameter.

Agglomerate density measurement: 1 ml of CP or CP+HT at 100 μg_{Fe} ml^{-1} were dispensed into TPP PCV tubes (Techno Plastic Products, Trasadingen, Switzerland) and centrifuged at 2000 rpm for 1 h (Eppendorf centrifuge 5702 R, A-4-38 rotor). Agglomerate pellet volumes were measured using a TPP ''easy read'' measuring device and the agglomerate density of nine samples per condition were calculated as previously described:[2]

$$\rho_{agg} = \rho_{media} + \left[\left(\frac{c_{NP}V_{tot}}{V_{pellet}SF}\right)\left(1 - \frac{\rho_{media}}{\rho_{NP}}\right)\right],$$

where ρ_{agg}, ρ_{media} and ρ_{NP} are the densities of the agglomerate, media and NPs, c_{NP} is the NPs' concentration, V_{tot} is the total volume in the TPP PCV tube (1 ml) and V_{pellet} is the volume of the pellet measured after centrifugation. *SF* is the stacking factor, which is the fraction of the pellet volume occupied by agglomerates. For the family of agglomerating metal oxides, such as IONPs, the *SF* value can be approximated to 0.64, which is the theoretical value for random close stacking, as previously reported.[2] The obtained results for the agglomerate densities of CP and CP+HT are given in Table S1.

Deposited mass calculation: The deposited mass in function of time for different NPs' concentrations and different agglomerate diameters was estimated with the *In vitro* Sedimentation, Diffusion and Dosimetry (ISDD) model.[3,4] The parameters used for the calculations are given in Table S1. The deposited dose (c_{dep}) was calculated as followed for the "24 h" time point:

$$c_{dep} = \frac{m_{dep}}{V},$$

where m_{dep} is the deposited mass of NPs obtained with the ISDD and V is the volume of medium in the well (0.1 ml). 24 h is the duration that cells were incubated with IONPs for the MTS test.

Correction of the cell viability obtained by MTS: The absorbance of the deposited dose was obtained from the calibration curves measured by UV-visible spectroscopy. This was done for both DMEM and the RPMI 1640 medium. The absorbance of deposited IONPs in DMEM was then subtracted from the absorbance of the MTS solution to calculate the viability of HeLa cells. The absorbance of deposited IONPs in the RPMI 1640 medium was subtracted from the absorbance of the MTS solution to calculate the viability of LnCaP cells.

Table S1: Parameters used to calculate the deposited mass by ISDD.

	CP	CP+HT
IONPs' diameter (nm)	8.0 ± 1.9	21.5 ± 6.3
IONPs' mean hydrodynamic diameter in 10 mM HNO_3 (nm)	16.1 ± 4.5	30.2 ± 9.1
IONPs' density (g ml^{-1})	4.92	4.92
IONPs' concentration (μg_{Fe} ml^{-1})	20, 40, 60, 80, 100, 200	20, 40, 60, 80, 100, 200
IONPs' concentration (mg_{Fe2O3} ml^{-1})	0.05, 0.11, 0.17, 0.23, 0.29, 0.57	0.05, 0.11, 0.17, 0.23, 0.29, 0.57
Depth of the well plate (mm)	3.125	3.125
Volume of medium (ml)	0.1	0.1
Temperature (K)	310	310
Viscosity (Pa s)	0.00074	0.00074
Medium density (g ml^{-1})	1	1
Agglomerate diameter (nm)	70	100, 150, 200, 1500
Agglomerate density (g ml^{-1}) for 0.1 mg ml^{-1}	3.48	1.62

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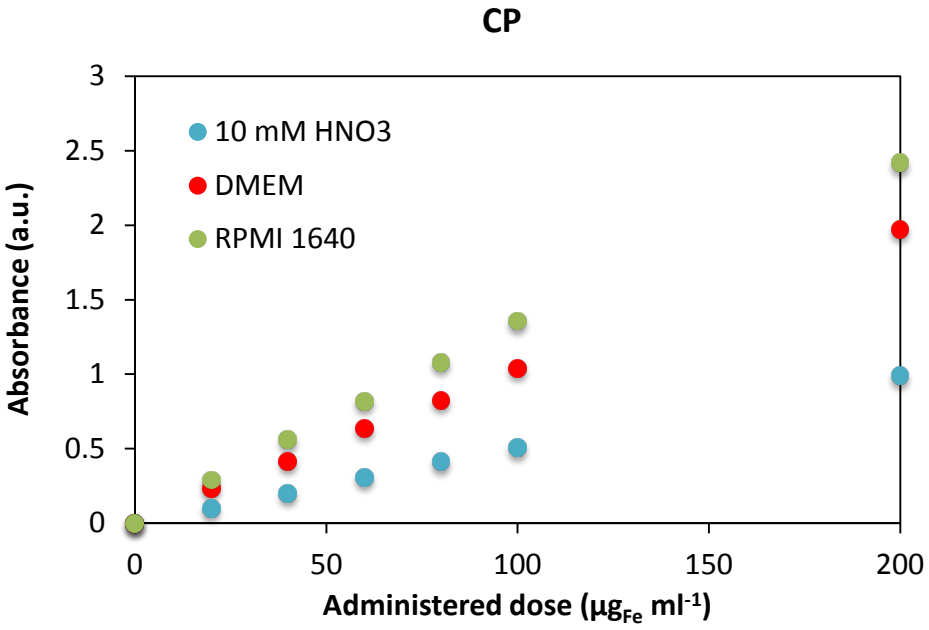

Fig. S1: Absorbance of CP dispersed in 10 mM HNO_3, DMEM and RPMI 1640 media measured by UV-visible spectroscopy at 490 nm at concentrations corresponding to the ones used for the MTS assay (0, 20, 40, 60, 80, 100 and 200 μg_{Fe} ml^{-1}).

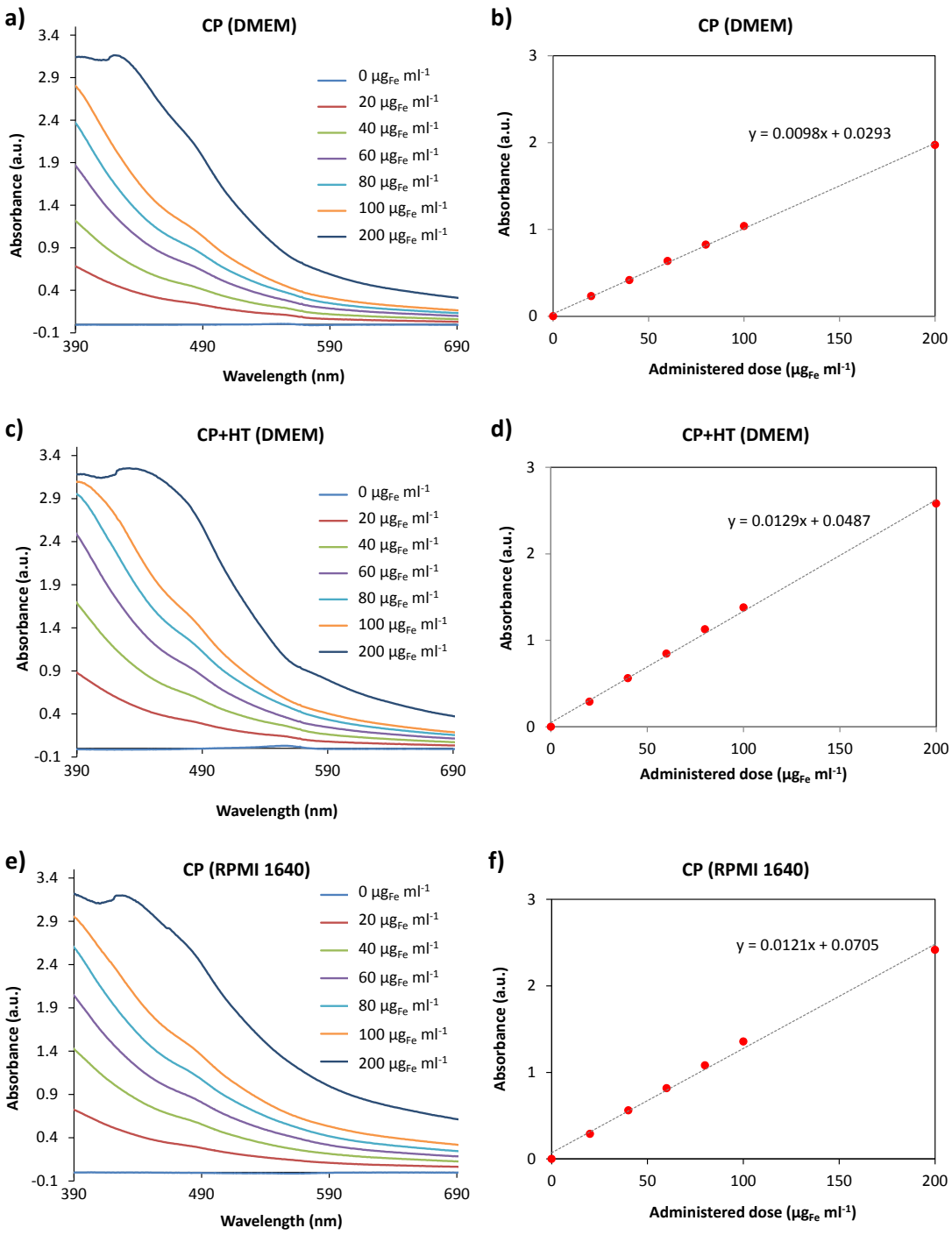

Fig. S2: Absorbance of CP (a and e) and CP+HT (c) at administered doses used for the MTS test (0, 20, 40, 60, 80, 100 and 200 μg_{Fe} ml^{-1}) measured by UV-visible spectroscopy. The spectra were measured in DMEM (a and c) and in RPMI 1640 media (e). (c, d and f) The corresponding calibration curves for CP (b and f) and CP+HT (d) at 490 nm, which is the wavelength at which the colored formazan product of the MTS test was measured. The grey dashed lines represent the linear regression lines. The equations of the linear regressions are also given.

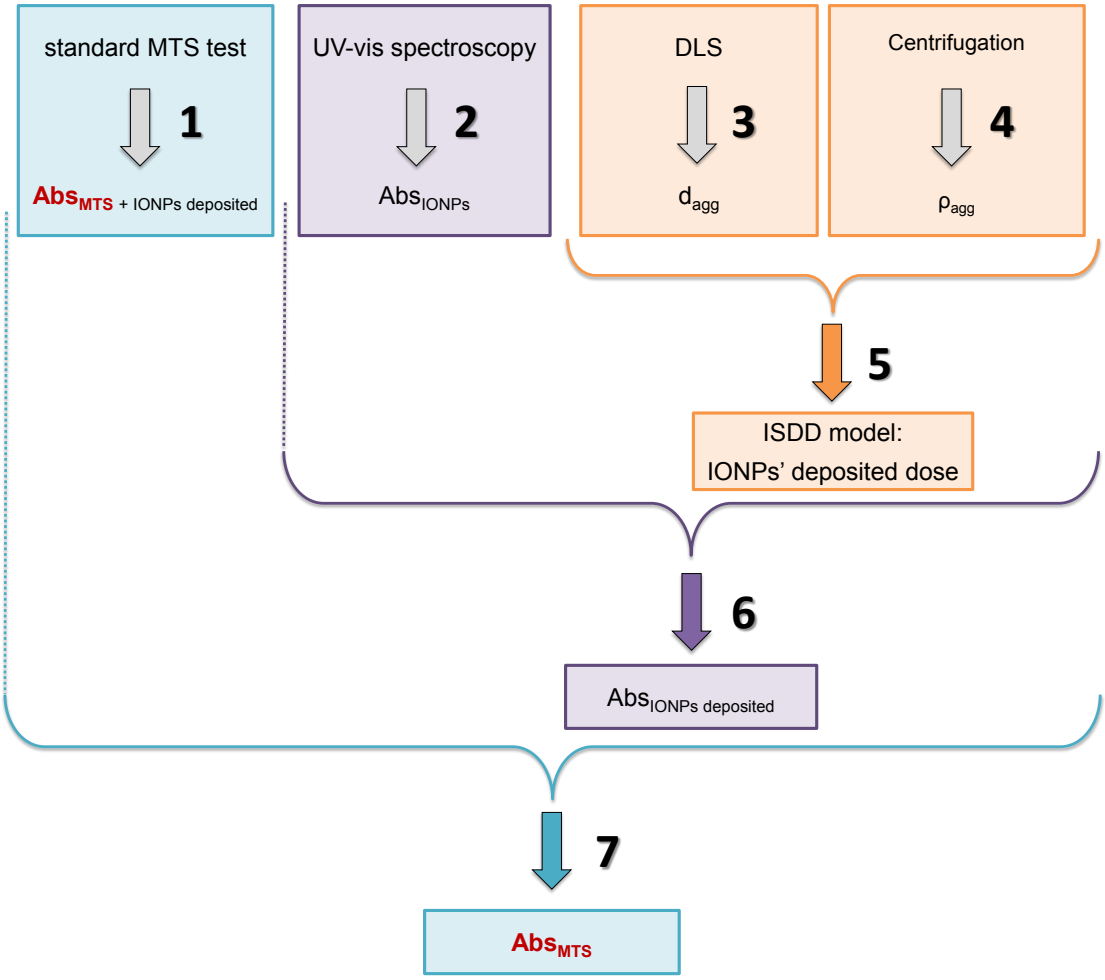

Fig. S3: Scheme of the procedure to correct the absorbance of the MTS test with the deposited dose of IONPs. (1) The MTS absorbance ($Abs_{MTS + IONPs\ deposited}$) is measured, which comprise of the wanted results of the MTS and the unwanted results of IONPs (absorbance of MTS + deposited IONPs). (2) The absorbance of IONPs in the same medium as the MTS test (Abs_{IONPs}) is measured by UV-visible spectroscopy at the administered doses. The calibration curve of the absorbance of IONPs at 490 nm in function of the administered dose can be drawn. (3) The volume hydrodynamic diameters of IONPs in the medium used for the MTS test is measured by dynamic light scattering (DLS). The main peak is approximated to be the agglomerate diameter (d_{agg}). (4) The volume of the pellet obtained after centrifugation of IONPs' suspensions in packed cell volume tubes was measured. The agglomerate density (ρ_{agg}) can be calculated. (5) d_{agg} and ρ_{agg} can be entered in the ISDD model, resulting in the deposited mass and dose of IONPs. (6) The absorbance of the deposited dose of IONPs at 490 nm ($Abs_{IONPs\ deposited}$) is read in the calibration curve obtained from step 2. (7) The corrected absorbance of *only* the MTS (Abs_{MTS}) is calculated by subtracting $Abs_{MTS + IONPs\ deposited}$ by $Abs_{IONPs\ deposited}$.

References

1D. Bonvin, H. Hofmann and M. Mionic Ebersold, *J. Nanoparticle Res.*, 2016, **18**, 376.

2G. DeLoid, J. M. Cohen, T. Darrah, R. Derk, L. Rojanasakul, G. Pyrgiotakis, W. Wohlleben and P. Demokritou, *Nat. Commun.*, 2014, **5**, 3514.

3G. M. DeLoid, J. M. Cohen, G. Pyrgiotakis, S. V. Pirela, A. Pal, J. Liu, J. Srebric and P. Demokritou, *Part. Fibre Toxicol.*, 2015, **12**, 32.
4P. M. Hinderliter, K. R. Minard, G. Orr, W. B. Chrisler, B. D. Thrall, J. G. Pounds and J. G. Teeguarden, *Part. Fibre Toxicol.*, 2010, **7**, 36.