Supplementary information

Neglected nano-effects of nanoparticles in the interpretation of their toxicity

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Experimental section

In vitro toxicity study

HeLa cells were cultured in DMEM (Life Technologies) 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 PrestoBlue® test was used. HeLa cells (20'000 cells per well) were cultured in 96-well plates at 37 °C, and exposed to 100 µl of different administered concentrations of S1 or S2 (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 was removed and 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. Results are reported as a function of the following parameters:

(i) the administered mass m_A (μg_{Fe}), which is the administered concentration times the administered volume (100 µl)

(ii) the deposited mass m_D (μg_{Fe}), which was estimated with the *In vitro* Sedimentation, Diffusion and Dosimetry (ISDD) model, as previously reported[1–3] (parameters used for the calculations are given in Table S1)

(iii) the number of administered IONPs (N_A), which was estimated as follows:

$$N_A = \frac{m_A}{mass\ of\ one\ IONP} = \frac{m_A}{\rho_{Fe2O3} * \frac{4}{3}\pi r^3}$$

where r is the radius of the IONPs and ρ_{Fe2O3} is the density of Fe_2O_3 (4.92 g cm^{-3}).

(iv) the number of deposited IONPs (N_D), which was estimated as follows:

$$N_D = \frac{m_D}{mass\ of\ one\ IONP} = \frac{m_D}{\rho_{Fe2O3} * \frac{4}{3}\pi r^3}$$

where r is the radius of the IONPs and ρ_{Fe2O3} is the density of Fe_2O_3 (4.92 g cm^{-3}).

(v) the surface area of the administered IONPs (S_A), which was estimated as follows:

$$S_A = m_A * SSA_{BET}$$

where SSA_{BET} is the specific surface area obtained by Brunauer-Emmett-Teller (BET) analysis.

(vi) the surface area of the deposited IONPs (S_D), which was estimated as follows:

$$S_D = m_D * SSA_{BET}$$

where SSA_{BET} is the specific surface area obtained by Brunauer-Emmett-Teller (BET) analysis.

(vii) the cross section area of the administered IONPs (CSA_A), which was estimated as follows:

$$CSA_A = N_A * (surface\ of\ cross\ section\ of\ one\ IONP) = N_A * \pi r^2$$

where *r* is the radius of the IONPs.

(viii) the cross section area of the deposited IONPs (CSA_D), which was estimated as follows:

$$CSA_D = N_D * (surface\ of\ cross\ section\ of\ one\ IONP) = N_D * \pi r^2$$

where *r* is the radius of the IONPs.

(ix) the number of layers of the administered IONPs (NL_A), which was estimated as follows:

$$NL_A = \frac{CSA_A}{surface\ of\ one\ well}$$

where the surface of one well is 32 mm^2 for a 96 well plate.

(x) the number of layers of the deposited IONPs (NL_D), which was estimated as follows:

$$NL_D = \frac{CSA_D}{surface\ of\ one\ well}$$

where the surface of one well is 32 mm^2 for a 96 well plate.

Agglomerate diameter measurement

The volume hydrodynamic diameters of 1 ml of suspensions of S1 and of S2 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 diameters.

Agglomerate density measurement

1 ml of CP or CP+HT at 100 $\mu g_{Fe}\ ml^{-1}$ 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:[4]

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

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.[4] The obtained results for the agglomerate densities of S1 and S2 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.[1,2] 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.

Supplementary note

For example, if one studies a property *Y* for two NPs' samples with the same *m* and the same chemical composition (thus density, i.e. the same volume, *V*) but with different *d*, this would be considered as a study of *Y* as a function of one experimental variable, *d*. It may look like the other experimental parameters (besides *d*) are constant, such as *m* and *V*. However, NPs with the same *m* and *V* but different *d* have different *S*, *N* etc. Therefore, if *Y* depends on *S* and/or *N*, then the difference in *Y* of two NPs' samples with the same *m* (i.e. *V*) and different *d* would not be caused only by the studied *d* but also by the difference in *S* and/or *N*. In other words, we would in fact study *Y* as a function of minimum two simultaneously changing and mutually correlated variables (*d* and also *S* and/or *N*), which would not be simultaneously controlled. However, the essential point of a basic experimental design is to study *Y* as a function of *one* variable *X* while keeping all other experimental parameters constant. Therefore, an interpretation of the results of such a study of *Y* could be easily erroneous.

Supplementary tables

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

	S1	**S2**
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

Table S2. Values of parameters of IONPs in sample S1 considered in this study.

Administered concentration (μg_{Fe} ml^{-1})	Administered mass m_A (μg_{Fe})	Deposited mass m_D (μg_{Fe})	Surface area of m_D, S_D (mm^2)	Number of IONPs in m_D (-)	Cross section area of m_D (mm^2)	Number of layers of m_D per well (-)
0	0	0.00	0.00	0.00E+00	0.00	0.00
20	2	0.64	109.02	4.85E+17	24.39	0.76
40	4	1.28	218.04	9.70E+17	48.78	1.52
60	6	1.92	327.05	1.46E+18	73.17	2.29
80	8	2.56	436.07	1.94E+18	97.56	3.05
100	10	3.20	545.09	2.43E+18	121.95	3.81
200	20	6.40	1090.18	4.85E+18	243.90	7.62

Table S3. Values of parameters of IONPs in sample S2 considered in this study.

Administered concentration (μg_{Fe} ml^{-1})	Administered mass m_A (μg_{Fe})	Deposited mass m_D (μg_{Fe})	Surface area of m_D, S_D (mm^2)	Number of IONPs in m_D (-)	Cross section area of m_D (mm^2)	Number of layers of m_D per well (-)
0	0	0.00	0.00	0.00E+00	0.00	0.00
20	2	0.52	43.23	2.18E+16	7.55	0.24
40	4	1.04	86.46	4.36E+16	15.10	0.47
60	6	1.56	129.68	6.54E+16	22.65	0.71
80	8	2.08	172.91	8.72E+16	30.20	0.94
100	10	2.60	216.14	1.09E+17	37.75	1.18
200	20	5.20	432.28	2.18E+17	75.49	2.36

Table S4. Number of spherical NPs in 1 µg of indicated nanomaterials with different density.

Material	Density	Diameter 2 nm	Diameter 20 nm	Diameter 200 nm
Gold	19.30 g/cm^3	1.24E+06	1238	1
Silver	10.49 g/cm^3	2.28E+06	2277	2
γ-Fe_2O_3	5.24 g/cm^3	4.56E+06	4558	5
Fe_3O_4	5.17 g/cm^3	4.62E+06	4620	5
TiO_2	4.23 g/cm^3	5.65E+06	5647	6
$CaCO_3$	2.71 g/cm^3	8.81E+06	8814	9
Silica	2.65 g/cm^3	9.01E+06	9013	9
Polystyrene	1.04 g/cm^3	2.30E+07	22967	23

Table S5. Number of spherical NPs in 1 mg of indicated nanomaterials with different density.

Material	Density	Diameter 2 nm	Diameter 20 nm	Diameter 200 nm
Gold	19.30 g/cm^3	1.24E+09	1.24E+06	1238
Silver	10.49 g/cm^3	2.28E+09	2.28E+06	2277
γ-Fe_2O_3	5.24 g/cm^3	4.56E+09	4.56E+06	4558
Fe_3O_4	5.17 g/cm^3	4.62E+09	4.62E+06	4620
TiO_2	4.23 g/cm^3	5.65E+09	5.65E+06	5647
$CaCO_3$	2.71 g/cm^3	8.81E+09	8.81E+06	8814
Silica	2.65 g/cm^3	9.01E+09	9.01E+06	9013
Polystyrene	1.04 g/cm^3	2.30E+10	2.30E+07	22967

Supplementary figures

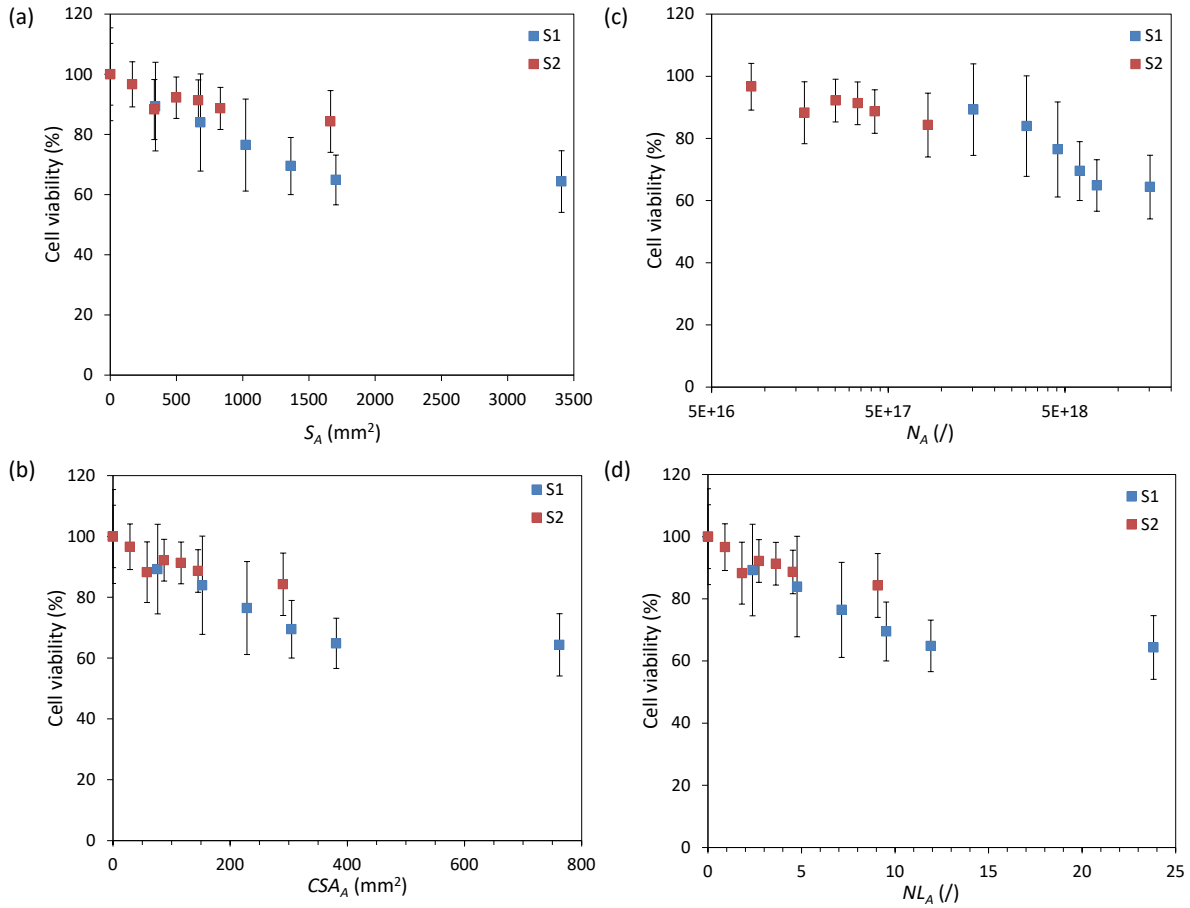

Fig. S1 Viability of HeLa cells incubated with different administrated mass, m_A, of IONPs in S1 and S2 was measured with the PrestoBlue® test. Surface area of m_A (S_A), cross-section area of m_A (CSA_A), NPs' number of m_A (N_A) and number of layers of m_A per well (NL_A) were calculated for each value of m_A for S1 and S2. The same cell viabilities as given in Fig. 1 are shown as a function of S_A (a), CSA_A (b), N_A (c) and NL_A (d).

Acknowledgements

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