Electronic Supplementary Material (ESI) for Toxicology Research.

Supporting Information:

Changes of silica nanoparticles upon internalisation by cells: size, aggregation/agglomeration state, mass- and number-based concentrations

D. Bartczak[†*], J. Davies[†], C. Gollwitzer[‡], M. Krumrey[‡], H. Goenaga-Infante[†]

[†]LGC Limited, Queens Road, Teddington, Middlesex, TW11 0LY, United Kingdom.

[‡]Physikalisch-Technische Bundesanstalt (PTB), Abbestrasse 2-12, D-10587 Berlin, Germany.

*e-mail for correspondence: dorota.bartczak@lgcgroup.com

Table of contents:

S1. EDAX analysis of internalised NP.

TEM grids prepared as described in the Experimental Section (main text) were imaged with FEI Technai-12 Transmission Electron Microscope operating at 80 kV voltage and equipped with TEAM™ EDS Apollo XLT X-ray microanalysis platform (Biomedical Imaging Unit, Southampton, UK). Representative EDAX spectra are shown in **Figure S1** below. Note: copper and carbon signals come from the TEM grid.

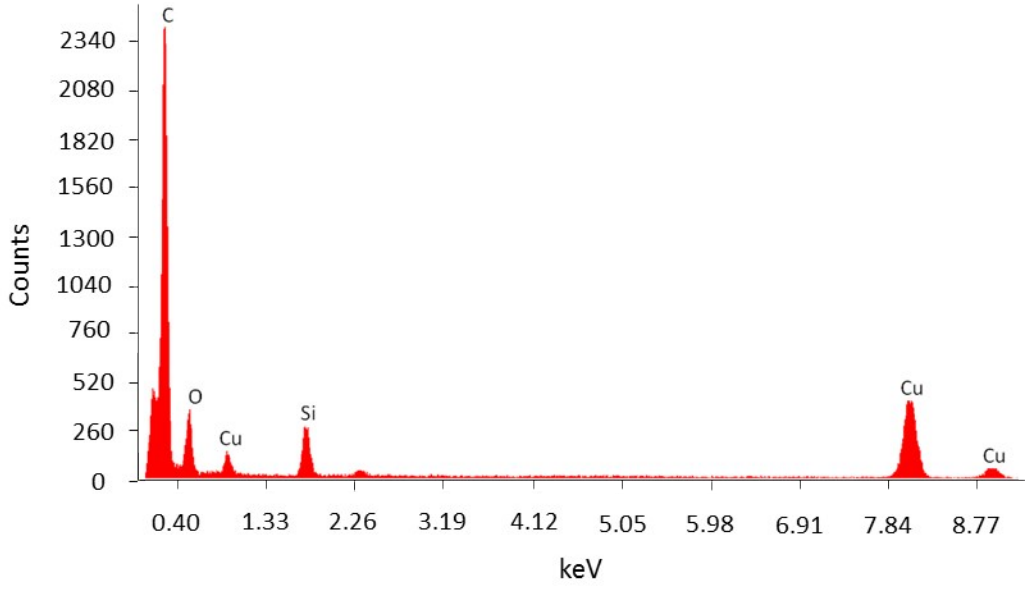

Figure S1. Representative EDAX spectra of internalised silica particles.

S2. Quantification of internalised NP as mass-fraction of silicon.

Cell suspensions prepared according to a procedure described in the Methods Section were digested using Ethos microwave (Milestone, Sorisole, Italy) following removal of non-internalised particles and cell culture media. In detail, 0.2 g of cell suspension in accutase was weighed in Teflon digestion vessels. Next, 4 ml of nitric acid (UpA, ROMIL), 4 ml of hydrogen peroxide (UpA, ROMIL), 1 ml of Milli-Q water and 35 µl of hydrofluoric acid (UpA, ROMIL) were subsequently added. Digestion was performed by increasing the temperature in the vessels to 180 °C, over 15 min and keeping the samples at this temperature for additional 20 min before allowing the system to cool down to room temperature. The digests were transferred to 50 ml plastic tubes and filled with Milli-Q water to a total weight of 40 g. Samples were diluted 4 times gravimetrically just before analysis.

Total silicon content in digested samples was determined by collision reaction cell ICP-MS (Agilent 7700) operating in a hydrogen mode. Isotopes ^{28}Si, ^{29}Si were monitored and ^{72}Ge (ROMIL) was used as an internal standard mixed with the sample *via* T-piece. Quantification was performed by external calibration with NIST SRM 3150. NIST SRM 3150 was also used as QC, which was digested along with the samples, with (105 ± 6)% recovery rate obtained (mean ± stdev, n=2). Plain silica particles were used as an additional QC of particulate nature with (104 ± 3)% recovery rate obtained (mean ± stdev, n=2). Cell suspension containing the same number of cells but no particles was used for blank correction. The results obtained for *m/z* 28 were recalculated for 8'000'000 cells/ml of cell suspension or percent of introduced into cells silicon and are shown in **Table 1**, main text.

S3. Zeta-potential analysis of plain and aminated silica suspensions in simple water matrix.

Prior to analysis, plain and aminated material was diluted around 1500 times with ultrapure water (18.2 MΩ.cm at 25 °C). Zeta-potential was measured with Nanoparticle Tracking Analysis (NTA) Platform (NS500, Malvern Instruments Ltd.). The instrument was switched on at least 30 min before the measurements. The temperature was set and maintained at 25 °C (± 0.1 °C) throughout the measurements. The applied voltage was 24 V. Movies were recorded over 30 - 60 s depending on the depth of view, with 30 s equilibration time prior to each measurement. Camera levels were set to 9. No filters were used. Performance of the instrument was checked daily with NIST RM 8013 (Au nanoparticles, nominal average diameter 60 nm) diluted ~50 times with ultrapure filtered water (18.2 MΩ.cm at 25 °C). The following parameters were fixed: viscosity was set to 0.8905 mPa.s, detection threshold was set to 25 and minimum particle size was set to 30 nm, blur and minimum track length were set to automatic. A minimum of 700 completed tracks were recorded per measurement. The recorded movies were analyzed with NTA 3.0 software and the data was further processed with Excel 2010. The results shown in **Table S3** below are the average ± stdev of n = 9 measurements. Representative, zeta-potential distribution graphs are shown in **Figure S3** below.

Table S3. Zeta-potential of plain and aminated silica (average ± stdev, n = 9).

Sample	Zeta-potential (mV)	pH
aminated silica	-3.6 ± 0.7	5.2 ± 0.1
plain silica	-39.1 ± 4.1	5.6 ± 0.2

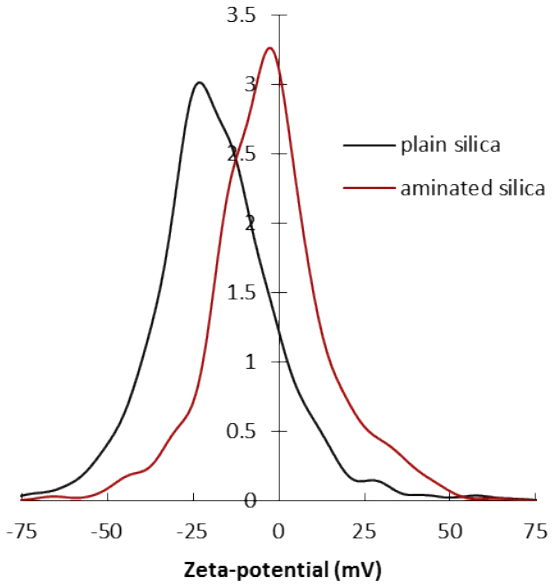

Figure S3. Representative zeta-potential distribution graphs for plain and aminated silica.

S4. Size analysis of plain and aminated silica suspensions in simple water matrix.

Prior to analysis, plain and aminated material was diluted gravimetrically between 1500 - 2500 times with ultrapure water (18.2 MΩ.cm at 25 °C). Hydrodynamic diameter was measured with Nanoparticle Tracking Analysis (PTA) Platform (NS500, Malvern Instruments Ltd.). The instrument was switched on at least 30 min before the measurements. The temperature was set and maintained at 25 °C (± 0.1 °C) throughout the measurements. Movies were recorded over 60 s. Camera levels were set to 9. No filters were used. Performance of the instrument was checked daily with gold particles (NIST RM 8013, nominal diameter 60 nm) diluted ~50 times with ultrapure filtered water (18.2 MΩ.cm at 25 °C). The following parameters were fixed: viscosity was set to 0.8887 mPa.s, detection threshold was set to 4, whilst other parameters were set to automatic. A minimum of 1000 completed tracks were recorded per measurement. Recorded movies were analysed with NTA 3.0 software and the data was further processed with Excel 2010. The results shown in **Table S4** below are the average ± stdev of n = 9 measurements, whilst representative size distribution graphs are shown in **Figure S4**.

Table S4. Average diameter of silica NPs measured with PTA.

Sample	Diameter (nm)	
	water	**cell culture media**
aminated silica	80 ± 2	102 ± 6
plain silica	81 ± 2	91 ± 7

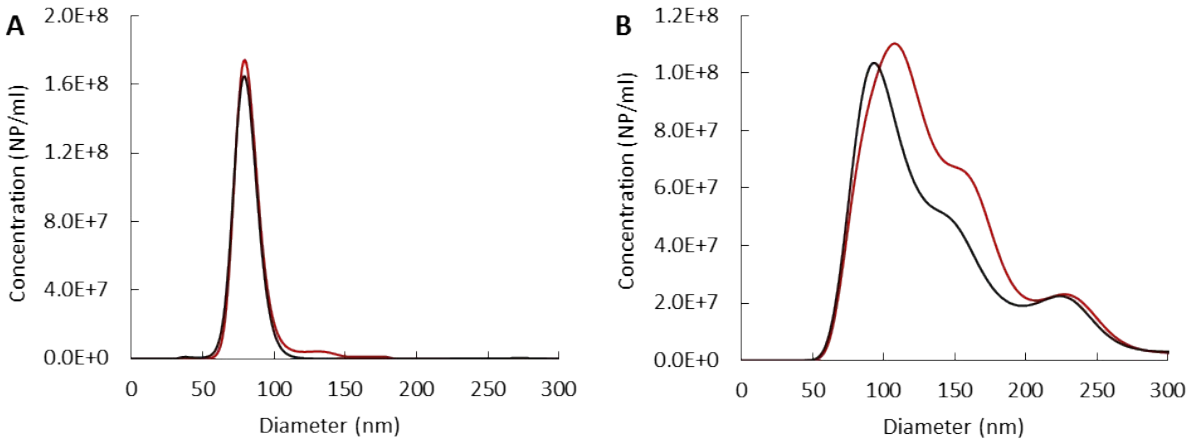

Figure S4. Representative size distribution graphs for plain (black line) and aminated (red line) silica suspended in water (**A**) and cell culture media (**B**).

S5. SAXS analysis of internalised particles.

The SAXS curves were fitted with a model for solid spherical particles with a Gaussian size distribution, an additional population of smaller particles with a lognormal distribution, and a constant background in intensity. From the model fit, the mean size of the dominant population and the corresponding standard uncertainty can be extracted, which is displayed in **Figure S5**. It is clear that the matrix of lysed cells did not hinder the size determination by SAXS, neither in the spiked samples, nor in the particles that have been internalised by the cells. The data also agrees with dilutions of both nanomaterials in pure water and with the result of the TEM imaging (section **S6**).

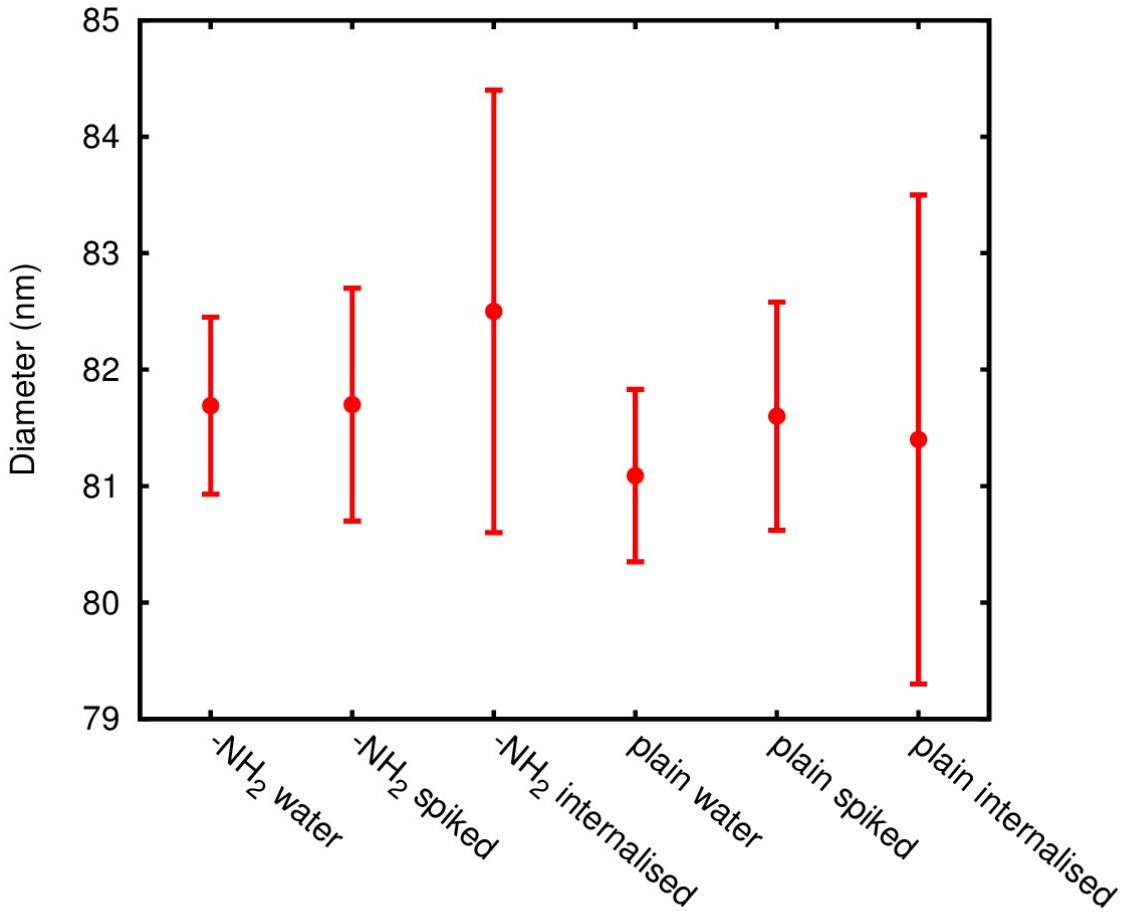

Figure S5. Comparison of the core size determined by SAXS for plain and aminated silica particles between spiked and internalised samples and simple dilutions in pure water (mean ± standard measurement uncertainty, k=1).

S6. Analysis of the NP core size.

The size of NP core was determined by TEM analysis. A droplet of NP stock solution was deposited on a TEM grid (carbon film on 400-mesh copper, Agar Scientific) and air dried. TEM grids were imaged with FEI Technai-12 Transmission Electron Microscope operating at 80 kV voltage (Biomedical Imaging Unit, Southampton, UK). The obtained images were processed with ImageJ software. A total of 10 images were processed and over 500 individual NP were counted (summary is shown in **Figure S6** below). A value of 82 nm, which is the mode of the main peak from the size distribution histogram, was taken for the average NP size and used for the NP number calculations.

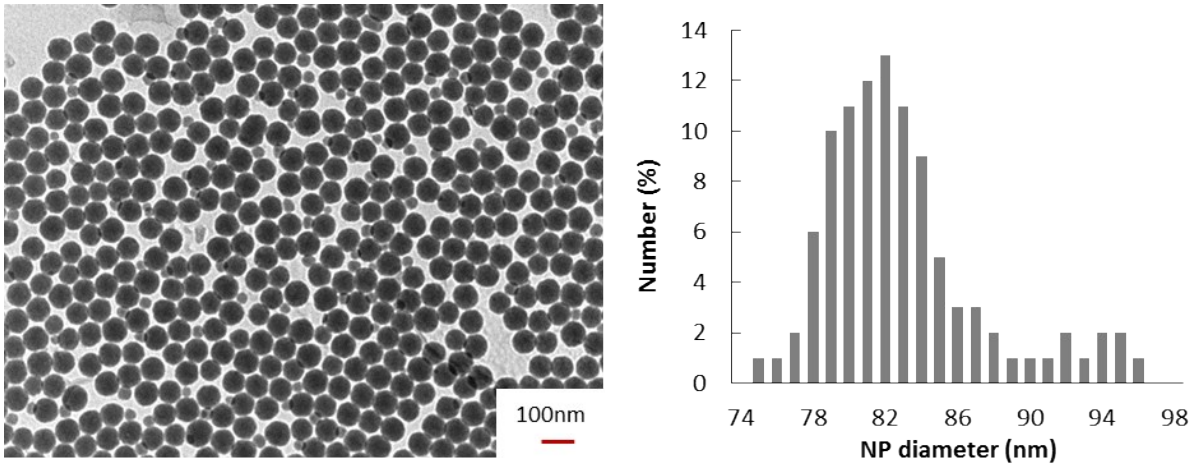

Figure S6. Representative TEM micrograph and a corresponding size distribution histogram of the main particle population.

S7. Post-channel Si quantification in lysates and the equivalent particle number calculation.

The content of silicon in the samples was monitored with ICP-MS (7700, Agilent Technologies) operating in a hydrogen mode and connected on-line to AF4 (AF2000 MT, Postnova Analytics). Samples eluting from the AF4 channel were quantified for the content of silicon by a post-column calibration approach, as previously described[1]. Briefly, calibration was performed by replacing the post-column diluting nitric acid/internal standard mix with calibration standards, containing the same amount of nitric acid (UpA, ROMIL) and internal standard (Ge, *m/z* 72 was monitored) but increasing concentrations of elemental silicon (*m/z* 28 and *m/z* 29 were monitored). The internal and calibration standard elemental solutions were purchased from NIST. Elemental rather than particulate form of silicon was used as calibrant, since this is the only type of reference material certified for silicon content available on the market at the time the experiments were performed. Same flow rates going into the nebuliser were used during the calibration step and sample analysis. Obtained from the calibration curve and normalised against internal standard regression parameters are shown below (for *m/z* 28).

Table S7A. Regression parameters.

Regression Parameters	
Slope	78.88
Intercept	-0.05
Linearity (R^2)	0.9999

AF4 fractograms normalised against internal standard were converted into mass flow fractograms using the calculated regression parameters and measured sample flow rates. Total peak area present in the background corrected fractograms was calculated using sum of trapezoid approximation and the concentration of silicon in the sample was calculated from the total injected volume and dilution factor of the injected sample. The summary of the obtained results is shown in **Table S7B**.

Table S7B. Content of silicon in spiked internalised samples determined with AF4/ICP-MS (average ± stdev; n = 4).

Material	**Si content (μg/g)**			
	Spiked	**Internalised**		
		fraction 1	**fraction 2**	**fraction 3**
aminated silica	219 ± 9	n/a	21 ± 1	54 ± 3
plain silica	253 ± 8	2.8 ± 0.2	14 ± 1	29 ± 1

NP number in the sample was calculated from the weight of NP, assuming density of around 2.0 g/cm^3 (2.0 ± 0.1 g/cm^3, estimated empirically, data not shown) and diameter of around 82 nm (analysis of the core, **section S-6**) for spiked plain and aminated silica and the weight of SiO_2 in the

sample, calculated from the AF4/ICP-MS results shown in **Table S7B** above (by multiplying the quantifies amount of Si by a factor of ~2.146 to account for the weight of oxygen). The example of calculations for spiked samples is shown in **Table S7C** below, whilst the summary of the obtained results in **Table S7D**. **Table S7E** shows sample recovery rates. The weight of SiO_2 and number of particles in the sample are shown as average ± stdev from four replicate measurements of the total amount of silicon in separated fractions.

Table S7C. Theoretical calculations of NP number in AF4/ICP-MS fractions.

Material	Density (g/cm^3)	MW of single component (g/mol)	Atoms per unit cell	Volume of unit cell (nm^3)	Volume of NP (nm^3)	Weight of NP (g)	Weight of SiO_2 in the sample (g)	NP number in the sample (NP/g)
aminated	2.0	60	4	$1.99 \cdot 10^{-1}$	$2.89 \cdot 10^{5}$	$5.77 \cdot 10^{-16}$	$(5.4\pm0.2) \cdot 10^{-4}$	$(1.4\pm0.1) \cdot 10^{11}$
plain							$(4.7\pm0.2) \cdot 10^{-4}$	$(8.2\pm0.3) \cdot 10^{11}$

Table S7D. Measured with AF4/ICP-MS particulate silicon mass-fraction and equivalent number of particles (average ± stdev, n = 4).

Material	Expected Si content (µg/g)	Measured particulate silicon content (µg/g)				Equivalent particle number (NP/g)*			
		Spiked into lysates	Internalised by cells			Spiked into lysates	Internalised by cells		
			fraction1	fraction2	fraction3		fraction1	fraction2	fraction3
plain silica	257.6±72.0	219.4±8.9	n/a	21.2±1.2	53.9±2.9	$(8.2\pm0.3)\cdot10^{11}$	n/a	$(2.3\pm0.1)\cdot10^{10}$	$(6.6\pm0.4)\cdot10^{9}$
aminated silica	391.8±61.9	253.2±7.6	2.8±0.2	13.6±1.1	28.9±0.7	$(6.3\pm0.2)\cdot10^{11}$	$(3.4\pm0.2)\cdot10^{11}$	$(1.6\pm0.1)\cdot10^{10}$	$(6.4\pm0.2)\cdot10^{9}$

* assuming diameter of internalised fractions based on MALS measurements.

Table S7E. Sample recovery rate calculations from the expected Si content, based on the silicon content measured with AF4/ICP-MS (average ± stdev, n = 4).

Material	Recovery rate as mass of particulate silicon (%)*			
	Spiked into lysates	Internalised and processed by cells		
		fraction1	fraction2	fraction3
plain silica	85.2±3.5	n/a	8.2±0.5	20.9±1.1
aminated silica	64.4±0.8	0.7±0.1	3.5±0.3	7.4±0.2

* from the expected Si content, as shown in Table 4.

References:

1 V. Nischwitz and H. Goenaga-Infante, *J Anal At Spectrom*, 2012, **27**, 1084-1092; V. Nischwitz, A. Berthele and B. Michalke, *J Anal At Spectrom*, 2010, **25,** 1130-1137.

S8. Particle number determined with PTA.

NP number in the sample was determined with PTA and the summary of obtained results is shown in **Table S8** below.

Table S8. Particle number and recovery rates from expected particle number determined with PTA (average ± stdev, n = 4).

Sample		Particle number (NP/g)		Recovery rate (%)*	
		plain silica	**aminated silica**	**plain silica**	**aminated silica**
Spiked		$(1.02\pm0.04)\cdot10^{12}$	$(1.07\pm0.03)\cdot10^{12}$	98.9±4.0	68.1±2.0
Internalised	**fraction1**	n/a	$(4.28\pm0.16)\cdot10^{9}$	n/a	0.3±0.1
	fraction2	$(2.43\pm0.07)\cdot10^{11}$	$(2.37\pm0.13)\cdot10^{11}$	23.6±0.7	15.2±0.8
	fraction3	$(8.68\pm0.38)\cdot10^{10}$	$(1.08\pm0.03)\cdot10^{11}$	8.4±0.4	6.9±0.2
	fraction4	$(7.38\pm0.34)\cdot10^{10}$	$(6.16\pm0.10)\cdot10^{10}$	7.2±0.3	3.9±0.1
	fraction5	$(3.15\pm0.35)\cdot10^{10}$	$(7.39\pm0.67)\cdot10^{9}$	3.1±0.3	0.5±0.1

* from the expected particle concentration (plain $1.03\cdot10^{12}$ NP/g; aminated $1.57\cdot10^{12}$ NP/g)