Supporting Information:

Determination of size- and number-based concentration of silica nanoparticles in a complex biological matrix by on-line techniques

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S-1. Experimental details.

Experimental set-up. Asymmetric flow Field-Flow Fractionation (AF4) system (AF2000, Postnova), equipped with an analytical ceramic-frit channel with 350µm spacer and loaded with 10kDa regenerated cellulose membrane (all from Postnova) was connected on-line to a range of detectors. In detail, the outlet from the AF4 was connected to the inlet port of photodiode array (PDA) UV-vis detector (Accela, Thermo Scientific) *via* PEEK tubing (blue, 0.010" internal diameter). The outlet from PDA was connected to the inlet port of MALS (PN3621, Postnova) with PEEK tubing (blue, 0.010"). The outlet from MALS was connected to a 'T connector' with PEEK tubing (red, 0.005") of length appropriate to increase the overall pressure of the system (with AF4 outer flow rate of 0.5ml/min for aqueous carrier) measured with the AF4 software (AF2000 1.1.0.23, Postnova) to 10 bars. One of the two outlets of the 'T connector' was connected to ICP-MS (7700, Agilent) *via* PEEK tubing (blue, 0.010"), not leading directly to the nebuliser but connected to another 'T connector' allowing introduction of an internal standards solution (*via* peri pump and orange/red/orange tubing, 0.19mm). The outlet from the second 'T' was connected to a nebuliser with PFA tubing (0.5mm). Second outlet from the first 'T connector' was connected with PEEK tubing (blue, 0.010") to a switching valve (High Pressure Solenoid valve, NResearch Inc). The two outlets from the valve were leading to NTA (NS500, NanoSight) or waste tank *via* PEEK tubing (blue, 0.010"). The valve was connected to the NTA's PC and software controlled.

Instrument operating conditions. AF4 outer flow rate was kept at 0.5ml/min at all times, whilst the system pressure at 10 bars. Filtered through 0.1µm PVDF membrane, 5mM Tris-HCl pH 7.4 solution was used as a carrier. The system was flushed with 10% methanol in water (disconnected from the ICP-MS and with the switching valve opened to the waste tank) after every 3 injections and at the end of the day. The system was controlled with AF2000 1.1.0.23 software (Postnova) and the following method was used to separate the sample. Focusing step: time 4min, x-flow 3ml/min; elution step: x-flow 3ml/min for 1.5min, then linear decay to x-flow 0ml/min over 15min and additional 15min with x-flow 0ml/min. The injection loop was 21.8µl, whilst the temperature in the AF4 channel was kept at 25°C. PDA was set to monitor the UV range at 220nm and 280nm wavelengths with the Xcalibur 2.1.x Data System software (Thermo Scientific), with the data recorded by the AF2000 1.1.0.23 software. MALS was operating at 80% laser power and also controlled by the AF2000 1.1.0.23. Both, UV and MALS detectors were calibrated and normalized following Postnova's instructions against BSA and PS standards, respectively, supplied by Postnova. Collision reaction cell ICP-MS was operating in the hydrogen mode. Data were recorded in time resolved analysis mode with an integration time of 0.5s with MassHunter B.01.01.software (Agilent). Two isotopes of silicon *m/z* 28 and 29 and one for germanium *m/z* 72 (internal standard) were monitored. Data was acquired automatically using an electronic trigger. NTA and the switching valve were controlled by the NTA2.3 software (NanoSight). NTA's EMCCD camera levels were set to 14, no filters were used. Software was set to record movies following manual trigger in a sequence: valve to NTA, wait 15s, valve to waste, record 30s, valve to NTA, etc. over the duration of the AF4 method. NTA instrument was calibrated for size and concentration measurements by the manufacturer's representative against a polystyrene standard. Since the calibrant and analyzed particles show very similar scattering properties, the depth of field of view is comparable. In the NS500 model, the depth of field of the microscope and the intensity of light are user independent and kept constant.

Sample preparation. Plain silica nanoparticles (30R50, Klebosol), aminated at the Hungarian Academy of Sciences and ampouled at JRC-IRMM under the EMRP NanoChOp project were used as base material in this study. The ampoules contained around 9ml of 2.5g/kg material. More details on the material are available on the project's website or from the project's coordinator (e-mail: Heidi.Goenaga-Infante@lgcgroup.com). Under sterile conditions, 50mg of the material was added to 10g of fetal bovine serum (FBS Gold, PAA Laboratories), vortexed for 15s and measured immediately.

Nanoparticles size determination. Two detectors were used to measure the size of the particles present in the sample. NTA movies were analyzed with NTA2.3 software (NanoSight), with the detection threshold set to 8, minimum expected particle size of 100nm and the background correction kept on. The remaining parameters were set to automatic. MALS data were evaluated with the AF2000 1.1.0.23 software (Postnova) using sphere fit model and 17 out of 21 available angles. Results shown in Table 1 are the average ± stdev of three measurements performed under repeatability conditions of the main fraction of particles present in the sample.

Nanoparticles concentration determination. The concentration of particles in terms of silicon content in the main size fraction was determined with ICP-MS using post-column quantification approach explained in Supplementary Information S-1. From the quantified silicon content, sample recovery rate (Supplementary Information S-4) and estimated primary particle size, the number of particles present in the main size fraction was calculated (Supplementary Information S-2). The number of particles present in the sample was also measured directly with NTA. The particle number obtained from all individual movies (analyzed with NTA2.3 as explained in the previous section) was plotted against the sample elution time giving a concentration over time fractograms, as shown in Figure 2. Using a sum of trapezoid approximation and with the known sample flow rate into the detector, the total number of particles present in the eluted fraction was calculated. For both, ICP-MS and NTA the split flow rates were measured and accounted for in the calculations of particle number. Results shown in Table 1 are the average ± stdev of three measurements performed under repeatability conditions of the main fraction of particles present in the sample.

Nanoparticles molecular weight determination. The molecular weight was measured directly with MALS using the AF2000 1.1.0.23 (Postnova) software for data processing, as explained in the previous section on nanoparticles size determination or calculated from the primary particle size, post-column quantification and mass balance data (Supplementary Information S-4) as explained in Supplementary Information S-2. Results shown in Table 1 are the average ± stdev of three measurements performed under repeatability conditions of the main fraction of particles present in the sample.

S-2. Post-column quantification of silicon in NP suspensions.

The content of silicon in the samples was monitored with ICP-MS (7700, Agilent) operating in a hydrogen mode and connected on-line to AF4 (AF2000 MT, Postnova) and NTA (NS500, NanoSight) systems. Samples eluting from the AF4 channel were quantified for the content of silicon present by a post-column calibration approach, as described previously[1,2]. 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 normalized against internal standard regression parameters are shown below (for *m/z* 28).

Table S-1. Regression parameters.

Regression Parameters	
Slope	58.7
Intercept	0.15
Linearity (R^2)	0.99994

AF4 fractograms normalized 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.

References:

(1) Nischwitz V.; Goenaga-Infante, H. *J. Anal. At. Spectrom.* **2012**, *27*, 1084–1092.

(2) Nischwitz, V.; Berthele, A.; Michalke, B. *J. Anal. At. Spectrom.* **2010**, *25*, 1130–1137.

S-3. Analysis of the NP core size and theoretical calculations of NP number.

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 80kV 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 **Supplementary Figure S-1** below). A value of 82nm, which is the main peak from the size distribution histogram, was taken forward for the NP molecular weight calculations.

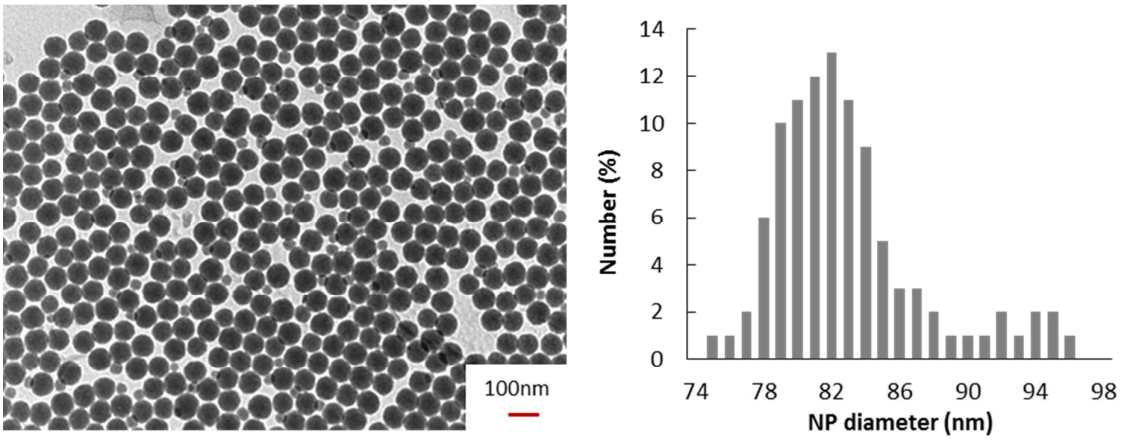

Figure S-1. Representative TEM micrograph and a corresponding size distribution histogram of the main particle population.

NP number in the sample was calculated from the weight of NP, assuming density of around 2.0g/cm^3 (2.0±0.1g/cm^3, estimated empirically, data not shown) and diameter of around 82nm (TEM), and the weight of SiO_2 in the sample, calculated from the ICP-MS (total amount of silicon in the separated fraction), taking the absolute recovery rate of the material (around 65.5%, **Supplementary Information S-5**) under consideration. The summary of calculations is shown in **Supplementary Table S-2** below. The weight of SiO_2 and number of particles in the sample are shown as average ± stdev (n=3) of the total amount of silicon in separated fractions.

Table S-2. Theoretical calculations of NP number.

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
2.0	60	4	$1.99 \cdot 10^{-1}$	$2.89 \cdot 10^{5}$	$5.77 \cdot 10^{-16}$	$2.54 \pm 0.12 \cdot 10^{-3}$	$4.4 \pm 0.21 \cdot 10^{12}$

From the hypothetical weight of single NP of around 82nm (**Supplementary Table S-3**), the MW of 'bare' NP was calculated ($3.47 \cdot 10^8$g/mol). However, the NP in fetal bovine serum would have albumin (main component of serum) adsorbed on their surface. According to the literature data, roughly 780 protein molecules could adsorb to a single NP[1], increasing the **MW of NP** to **$4.01 \pm 0.03 \cdot 10^8$g/mol**, when MW of protein [$(6.8 \pm 0.4) \cdot 10^4$g/mol; average ± stdev, n=3)] determined with MALS is taken under consideration.

References:

(1) Brewer, S. H.; Glomm, W. R.; Johnson, M. C.; Knag, M. K.; Franzen, S. *Langmuir*, **2005**, *21*, 9303–9307.

S-4. ICP-MS analysis of the total silicon content in stock NP suspension and comparison study of sample mineralization versus nebulization of the nanoparticulate form for ICP-MS analysis of nanomaterials.

NP suspensions were digested using Ethos microwave (Milestone, Sorisole, Italy). In detail, 0.2g of NP suspension was weighed in Teflon digestion vessels. Next, 4ml of nitric acid (UpA, ROMIL), 4ml of hydrogen peroxide (UpA, ROMIL), 1ml of Milli-Q water and 35µl of hydrofluoric acid (UpA, ROMIL) were subsequently added. Digestion was performed by increasing the temperature in the vessels to 180°C, over 15min and keeping the samples at this temperature for additional 20min before allowing the system to cool down to room temperature. The digests were transferred to 50ml plastic tubes and filled with Milli-Q water to a total weight of 40g. 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 used as an internal standard. Quantification was performed by external calibration. The internal and calibration standard elemental solutions were purchased from NIST. The silicon content was measured from two individual ampoules on two different days in duplicates. The results obtained for *m/z* 28 are shown in a table below.

Note: The obtained value of 1186µg/g of silicon, equals to 2.54±0.21g/kg of silica, as expected for this material.

Table S-3. Total silicon content in NP stock suspension upon digestion.

Ampoule	Replicate	Si [mg kg^{-1}]	Average [mg kg^{-1}]*	Stdev [mg kg^{-1}]*
#0205	1	1113	1186	95
	2	1124		
#0009	1	1252		
	2	1257		

* n=4

NP stock suspensions were diluted approximately 4650 times gravimetrically with 0.43% nitric acid (UpA, ROMIL), to mimic the content of suspension introduced into ICP-MS during on-line AF4/ICP-MS/NTA experiments, where the acid containing internal standards is mixed with a sample eluting from the AF4 channel before going into the nebuliser.

NP digests were prepared as described above. Digested samples were diluted ~23.26 times gravimetrically to a final concentration of nitric acid around 0.43%, to match the content of acid in NP suspension samples.

The content of silicon in NP digests and NP suspensions 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 used as an internal standard. Quantification was performed by external calibration. The internal and calibration standard elemental solutions were purchased from NIST. The silicon content was

measured from two individual ampoules in duplicates on the same day; n=4 (NP digests and NP suspensions were prepared from the same ampoules). The results obtained for *m/z* 28 are shown in table below.

Table S-4. NP suspensions versus digests determined for micro mist nebuliser (n=4).

Sample	^{28}Si [mg kg^{-1}]		Efficiency [%]	
	Average	Stdev	Average	Stdev
NP suspension	876	46.5	80.6	4.3
NP digests	1087	36.2		

S-5. Mass balance calculations.

Based on the total content of silicon (**Supplementary Table S-3**), recoveries from the AF4/ICP-MS system for the main silicon fraction were 80.8 ± 3.9% (average ± stdev, n=3) when no separation force (or cross flow) was applied and 67.6 ± 3.5% (average ± stdev, n=3) when a cross flow of 3ml/min (linear decay to 0ml/min) was applied, in both cases for the same sample of silica particles suspended in serum. In theory, when no separation force is applied, the total injected silicon should quantitatively be recovered from the AF4 channel. However, based on peak area comparison, only about 81% of the total silicon injected onto the AF4 was detected by ICP-MS without cross flow. This could be explained by the observed differences in transportation and/or nebulization efficiency between the silica particles and the silicon ions used as calibrants for post column quantification using ICP-MS detection (**Supplementary Table S-4**). The remaining 13.2% bias from a 100% recovery (for separated sample) could be attributed to silicon losses due to the interactions with the AF4 membrane and/or silica particles dissolution (being the dissolved silicon transported by the cross flow to waste), since no flush out of silicon during the wash step (which could suggest adsorption to the PEEK tubing) was observed.