Electronic Supplementary Material (ESI) for ChemComm.

Supplementary Information

PCR Quantification of SiO_2 Particle Uptake in Cells in the ppb and ppm range via silica encapsulated DNA barcodes

Marcus Hoop,[a] Daniela Paunescu,[a] Philipp R. Stoessel,[a] Fritz Eichenseher,[b] Wendelin J. Stark,[a] and Robert N. Grass*[a]

[a] Institute for Chemical and Bioengineering, ETH Zurich, CH-8093 Zurich, Switzerland.

[b] Institute of Food, Science, and Nutrition, ETH Zurich, CH-8092 Zurich, Switzerland.

E-Mail: robert.grass@chem.ethz.ch

Synthesis of fluorescently labelled RITC-SiO_2 particles

The synthesis of fluorescently labelled SiO_2 particles is based on the procedure described by Zukoski et. al[1], and modified following Jiang[2]. Shortly, 28 mg of Rhodamine Isothiocyanate (RITC) were applied to 44 mg 3-aminopropyltriethoxysilane, 5 mL ethanol, and stirred overnight. Then, 176 mL ethanol, 7.7 mL ammonia, and 4.6 mL ddH_2O were added and stirred for additional 6 h. 7.7 mL TEOS were added, followed by incubation for 2 h, and addition of 1.7 mL ddH_2O and 10.2 mL TEOS.

Functionalization of SiO_2 particles

First, SiO_2 (147 nm with 50 mg/mL) (SiO_2-R-B1181, micro particles GmbH) and synthesized RITC-SiO_2 particles were centrifuged and washed twice with isopropanol. The particles were dispersed in 2.5 mL isopropanol. Functionalization was conducted by adding 25 µL N-trimethxylsilylpropyl-N,N,N-trimethyl-ammonium chloride (TMAPS, 50% MeOH, ABCR GmbH; Cat. no. 168658). The suspension was stirred for 24 h at room temperature. Finally, particles were purified by sedimentation and resuspension in 2.5 mL isopropanol.

Preparation of dsDNA

The dsDNA for adsorption on functionalized SiO_2 particles was prepared by adding 50 µL ssDNA (DAP1, 100 µM, Microsynth) and 50 µL ssDNA-RC (DAP-RC-1, 100 µM, Microsynth) to 100 µL of Annealing TE-Buffer (10 mM Tris, 50 mM NaCl, 1mM EDTA, pH 8.0), giving 25 µM dsDNA solution. Annealing reaction was performed at 95°C for 5 min, followed by cooling at room temperature for 45 min.

Sequence:

ssDAP 1 (5'-3'): ATT CAT GCG ACA GGG GTA AGA CCA TCA GTA GTA GGG ATA GTG CCA AAC CTC ACT CAC CAC TGC CAA TAA GGG GTC CTT ACC TGA AGA ATA AGT GTC AGC CAG TGT AAC CCG AT

ssDAPRC1 (5'-3'): ATC GGG TTA CAC TGG CTG ACA CTT ATT CTT CAG GTA AGG ACC CCT TAT TGG CAG TGG TGA GTG AGG TTT GGC ACT ATC CCT ACT ACT GAT GGT CTT ACC CCT GTC GCA TGA AT

Fossilization of dsDNA in SiO_2 particles

The encapsulation of DNA was performed following Paunescu et. al[3,4]. 35 µL of functionalized SiO_2 particles were added to 1 mL ddH_2O, and 20 µL dsDNA. The suspension was mixed and centrifuged for 2 min, the supernatant got discarded, followed by resuspension of the particles in 1 mL ddH_2O. After two additional washing steps, the resulting functionalized particle pellet, with bounded dsDNA, was resuspended in 500 µL ddH_2O, 0.6 µL TMAPS, and 0.6 µL TEOS (>99.9%, Alfa Aesar; Cat. no. 7810-4), followed by sonification, and 4 h shaking at room temperature. Then, 4 µL of TEOS were added to the suspension and reaction mixture was shaken for 96 h. After completion of the reaction, the particles were centrifuged and washed twice with 1 mL ddH_2O. The pellet was resuspended in 100 µL ddH_2O, giving a final concentration of 17.5 mg/mL fossilized dsDNA SiO_2 particles.

Ball milling of SiO_2 particle suspension

Due to agglomeration of synthesized SiO_2 particles with encapsulated dsDNA, deagglomerated suspensions were obtained by ball milling with ZrO_2 milling balls in a Fritsch premium line Pulverisette 2 minutes at 800 rpm (1 cycle, 0 pause). After milling, the obtained suspension was washed twice with ddH_2O.

Particle analysis

Dynamic Light Scattering (DLS)

Particle size of SiO_2 particles was measured by dynamic light scattering (DLS) in situ at a fixed angle of $\theta = 173°$ using Zetasizer Nano (Malvern, Worcestershire, UK), equipped with laser beam of wavelength $\lambda_0 = 633$ nm, and Ratiolab® PMMA cuvettes (Ratiolab GmbH, Dreieich, Germany). The Z-average is calculated by fitting the autocorrelation function with cumulant method.

Zeta Potential

Zeta potential has been determined with Zetasizer Nano (Malvern, Worcestershire, UK), by measuring electrophoretic mobility by Laser Doppler Effect.

Particle size by analytical centrifugation

The nanoparticle size of SiO_2 particles was determined by volume-weighted sedimentation analysis ($\rho = 2.2$ g·cm^{-3}, LUMisizer®, LUM GmbH. Berlin Germany). The analytical photocentrifuge allows measuring the intensity of the transmitted light as function of time and position over the entire sample length simultaneously.

Radical Stability

5 μL of the respective SiO_2 particle suspension, before and after milling, were treated with 2.5 μL of L-ascorbic acid solution (20 mM), 12.5 μL H_2O_2 solution (20 mM), and 17.5 μL $CuCl_2$ (500 μM). The reaction was stopped by adding 17.5 μL EDTA (100 mM). Then, the solution was mixed with 20 μL buffered oxide etch in order to release encapsulated DNA. Consequently, solutions with dissolved particles were dialyzed on dialysis filter (Millipore 0.025 μm, VSWP) for dsDNA purification by qPCR.

Microscopy

Scanning Electron Microscopy (SEM):

Particle morphology and homogeneity of dsDNA fossilized non-fluorescence SiO_2 particles was investigated by Scanning Electron Microscope (SEM). Particles were sputter-coated with platinum layer (Leica EM SCD005). Samples were visualized by FEI NovaNanoSEM 540, at 5 kV.

Scanning Transmission Electron Microscopy (STEM):

Particle morphology and homogeneity of dsDNA fossilized fluorescence and non-fluorescence SiO_2 particles, before and after ball milling, was investigated by Scanning Transmission Electron Microscopy (STEM). Particles were dispersed in isopropanol. Samples were dried on a copper/carbon grid and visualized by FEI NovaNanoSEM 540, at 30 kV.

Confocal Fluorescence Microscopy

A549 cells were seeded at $3x10^5$, 24 h prior to the measurements. Then, cells were incubated for 6 h with RITC-SiO_2 particles, before and after ball milling, at concentrations of 150 ppm. Images at various z-dimension frames were obtained, after washing cells twice with PBS, using Leica TCS SPE confocal microscope (Leica Microsystems GmbH, Wetzlar, Germany), operated by Leica LAS AF interface. Pinholes were set to 1 Airy, to ensure strict confocal images. Emission bandwidth was set to 542 nm and 602 nm.

Cell culture and treatment with SiO_2 particles

The human adenocarcinoma alveolar basal epithelial (A549) cell line was cultivated in cell culture medium composed of DMEM (Invitrogen), 10% FCS (Invitrogen), 100 U/mL Antibiotic-Antimycotic mix (Invitrogen) in TC Flasks 150 cm^2 (TPP) at 37°C in a humidified, 5% CO_2 incubator. For the investigation of SiO_2 particle uptake, A549 cells were plated in 6-well TC Plates (VWR) at a density of $5x10^5$ cells per well in 3 mL cell culture medium and allowed to attach for 24 h. At a confluence of 90 %, freshly dispersed silica particle suspension in culture medium were prepared and diluted to appropriate experimental concentrations (0.01; 0.1; 1; 10; 50; 150 µg/mL [ppm]). 1 mL of the respective particle concentration was immediately sonicated. Culture media was aspirated and particle suspension was applied to the cells. Non-exposed cells were used as controls. An exposure time of 6 h was chosen for all investigations.

The experiment was conducted using two wells with identical amounts of cells and SiO_2 particle concentrations. The first well was used to investigate particles present within the media as well as attached to the cell surface. Therefore, cell media containing non-uptaken particles was removed from the well. Cells within the well were trypsinized with 0.25 % Trypsin / 0.53 mM EDTA (1 mL) (Invitrogen) and centrifuged at 300 g for 10 min. The supernatant of the pelleted cell suspension as well as the previously removed cell media were combined and centrifuged at 21.500 g. The pellet was resuspended in buffered oxide etch (20 µL for 0.01 – 0.1 ppm; 40 µL for 1 ppm – 150 ppm) (HF/NH_4F, 0.34 g NH_4F and 10 g HF (0.8% in ddH_2O)) to release encapsulated dsDNA from the SiO_2 particles. This buffered oxide etch solution was added to the cell pellet to release the remaining encapsulated dsDNA of particles on the cell surface and diluted in ddH_2O (1:10). The supernatant was used for dsDNA quantification by qPCR.

The second well was investigated for particles present within and outside the cell. The cell media was removed and centrifuged at 21,500 g, resulting in a particle pellet. The cells were trypsinized and added to the particle pellet. For cell lysis, 10% SDS solution (1 mL) was added and incubated for 90 min at 95°C. The solution containing released particles was washed with 1 mL ddH_2O, followed by centrifugation at 21,500 g. After three washing steps, the particles were collected in buffered oxide etch solution (20 µL for 0.01 – 0.1 ppm; 40 µL for 1 ppm – 150 ppm), diluted in ddH_2O (1:10), and the released dsDNA in the supernatant was quantified by qPCR

.

Quantification via quantitative Polymerase Chain Reaction (qPCR)

For qPCR the following primers were used:

fPrimer: ATT CAT GCG ACA GGG GTA AG

rPrimer: ATC GGG TTA CAC TGG CTG AC

The qPCR samples were prepared by mixing 10 µl of PCR master mix (Roche, Lightcycler 480 SYBR Green I Master Mix), 7 µl of ddH2O, 2 µl Primer mix (forward and reverse primers, 5 µM each), and 1 µl of sample. qPCR was performed utilizing a Roche LightCycler® 96 system with 45 cycles (15 s at 95°C, 30 s at 56°C, 30 s at 72°C). qPCR was quantified using the corresponding amplicons at known concentrations.

Calculation of uptake data from qPCR data

The procedure outlined above resulted in three qPCR readings (Cycle thresholds, Ct): Ct_c of the control, where only particles were processed (no cells); Ct_t, where the total amount of particles was measured in the presence of cells; and Ct_o, measuring only the amount of particles outside of the cells. The cell-free control was utilized to check that there was no particle loss during cell work-up.

Using a previously recorded standard curve of the particles in water (with known concentrations of particles in water), the individual Ct readings were converted into particle concentrations of the control (c_c) total (c_t) and outside (c_o), respectively. Standard curves were recorded for the agglomerated and deagglomerated particles individually, as the experiments were performed on different days. The agglomerated particle standard curve was y=3.86x+11.98; the deagglomerated particle standard curve y=3.46x+11.98, in both cases x is the logarithm of the particle concentration. Standard curves were recorded from 7 data-points, R^2>0.99.

The percentage of particle uptake was calculated as:

$$\%\ \boldsymbol{Uptake} = \frac{\boldsymbol{c_t - c_o}}{\boldsymbol{c_t}}$$

The mass of particles taken up per cell was calculated by the mass of material applied (1 ml at the given concentration) multiplied by the uptake percentage and corrected for the average cell number per well of 5E5.

As an example, 20% of the initially present particles were taken up when exposing the cells to 150 ppm of agglomerated particles. Therefore, 150 µg of particles were applied to 500'000 cells = 0.0003 µg per cell, of this 20% were taken up, which is 0.00006 µg of particles per cell = 60'000 fg).

Table S1: Raw data for agglomerated particles, averages of three data points each

Agglomerated				
ppm	Ct particles Inside	Ct particles Outside	Ct particles control	Dilution factor
0.01	25.80	32.05	25.51	2
0.1	22.77	25.86	22.22	2
1	19.69	24.49	19.36	2
10	15.79	17.71	15.67	2
50	12.98	13.57	12.78	4
150	10.55	10.91	10.46	4

Table S2: Raw qPCR data for deagglomerated particles, averages of three data points each

De-agglomerated				
ppm	Ct particles Inside	Ct particles Outside	Ct particles control	Dilution factor
0.01	25.61	27.40	25.26	2
0.1	21.92	23.76	22.00	2
1	19.77	24.18	19.74	2
10	16.00	21.11	15.91	2
50	13.75	15.96	13.35	4
150	11.83	14.22	11.74	4

No template and negative controls

Every experiment was accompanied by measurements of no template control (control of qPCR efficiency) and a zero-particle-control, where cells were exposed to no particles (effect of cellular debris on qPCR after centrifugation steps).

The values recorded are given in the table below and show that there was no effect of the cellular debris on the qPCR controls.

Table S3. Raw qPCR data for no template control

Agglomerated			Deagglomerated		
	Sample	C(t)		Sample	C(t)
qPCR w/o nanoparticles Blank qPCR	1	33.27	qPCR w/o nanoparticles Blank qPCR	1	28.76
	2	33.93		2	28.52
	3	33.59		3	29.35
	1	32.43		1	28.03
	2	33.67		2	28.89
	3	33.58		3	28.94

Calcein-AM cell viability assay

A549 cells were plated in 96-well microplates (TPP) at a cell density of $1x10^4$ cells per well in 100 µL DMEM. Cells were allowed to attach for 24h. Afterwards, cells were exposed to various SiO_2 particle concentrations (0.01 – 150 ppm) in 100 µL culture media for 6 h. Culture media was aspirated and cells were washed twice with PBS. Afterwards, each well was treated with 4 µM calcein-AM (Roche) and incubated for 30 min at 37°C and 5% CO_2. Fluorescence measurements were performed with micro-titer plate reader (Infinite f200 Tecan) using excitation and emission wavelength at 480 nm and 535 nm. Fluorescence results are proportional to the integrity of cell membranes. As a negative control, cells without SiO_2 particle exposure were used.

The influence of buffered oxide etch on cell integrity was investigated by exposing a cell pellet ($1x10^6$ cells – corresponds to the cell density in 6 well plates with 90% confluence) to buffered oxide etch (40 µL), followed by dilution with ddH_2O (1:10), and wash with PBS. Calcein-AM test was performed as described above.

Table S4 Properties of the particles utilized in this study.

	Agglomerated particles		De-agglomerated particles	
	Fluorescent	Non-fluorescent	Fluorescent	Non-fluorescent
Average Hydrodynamic diameter (nm)[a]	2'180[a]	2'920[a]	340[a]	240[a] 116[b]
Zeta Potential (mV)	n.a.	n.a.	-23	-27
Radical stability	> 90 %	> 90 %	> 90 %	> 90 %

[a]From Light-scattering
[b]From analytical centrifugation

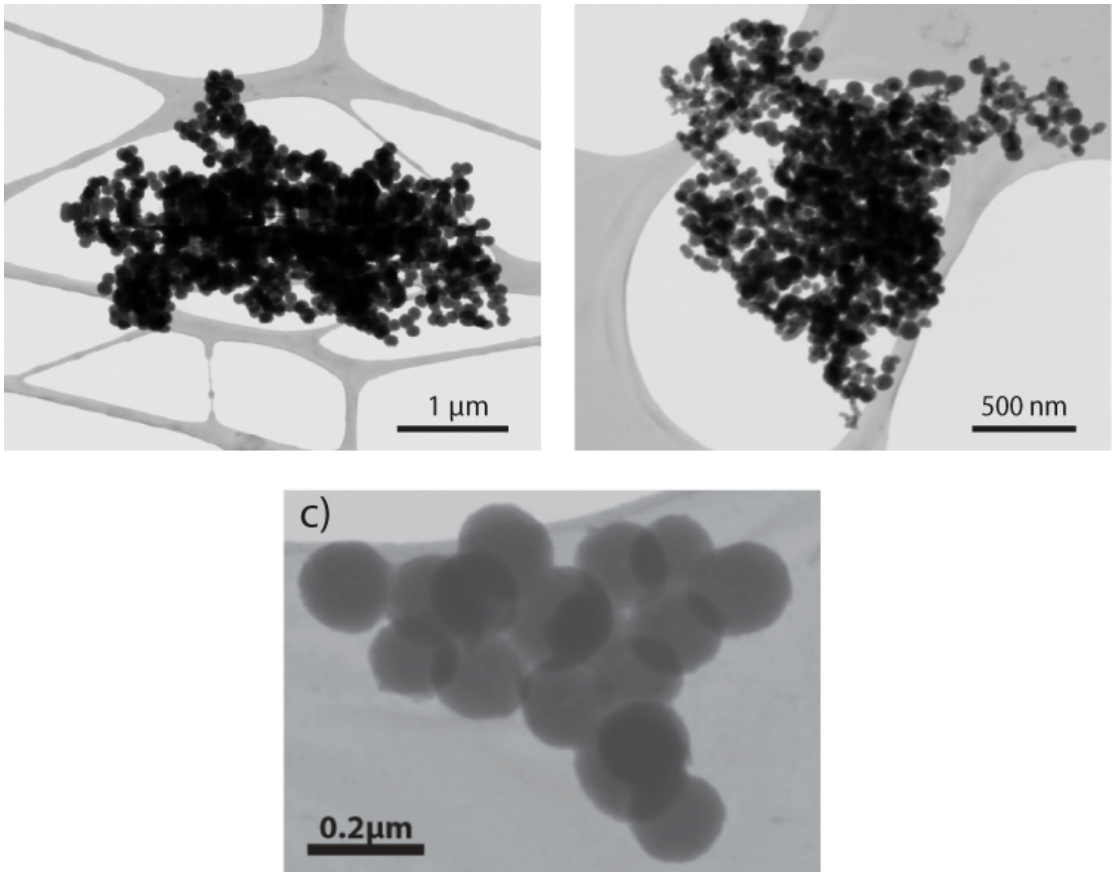

Figure S1 STEM images of agglomerated particles utilized in this study (a) compared to commercial Aerosil Ox50 particles (b). Breakup of the agglomerates (deagglomeration) was simulated in a ball mill resulting in significantly smaller aggregates (c).

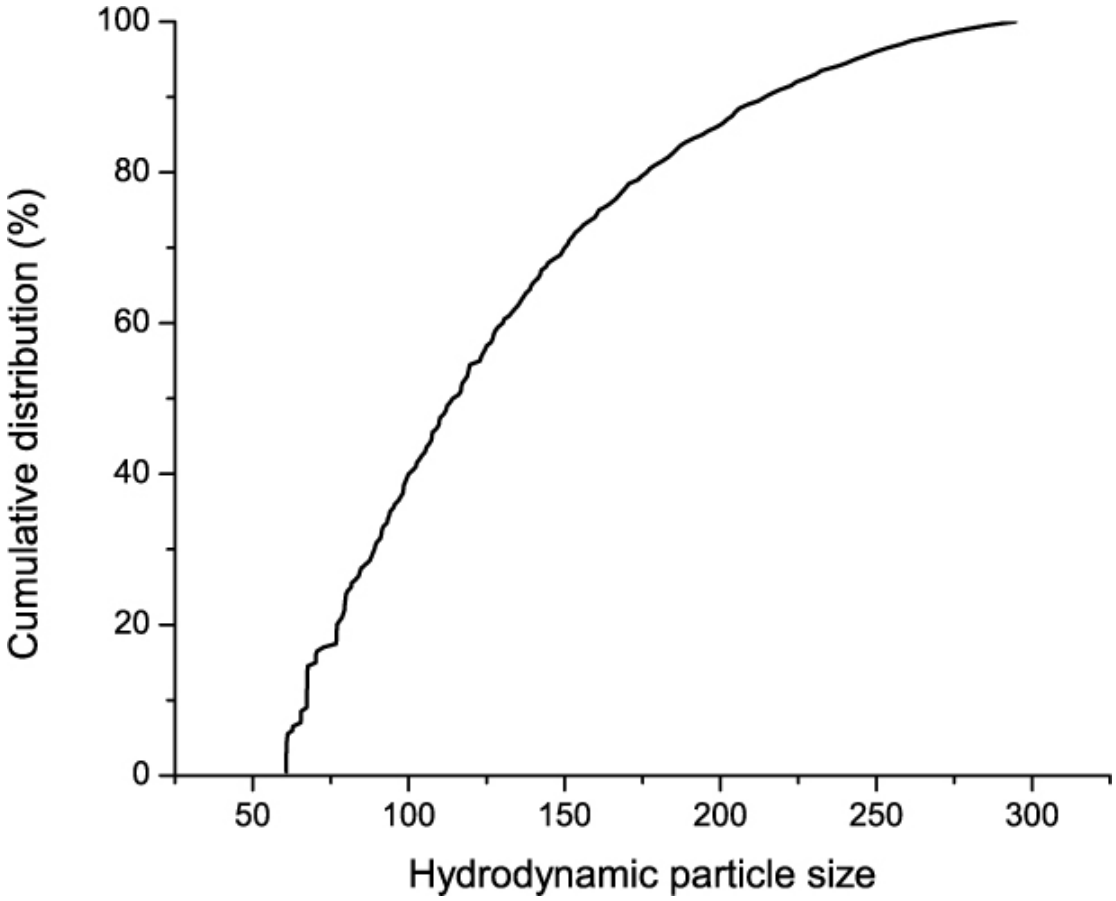

Figure S2: Cumulative particle size distribution of deagglomerated particles as measured by the analytical centrifugation technique.

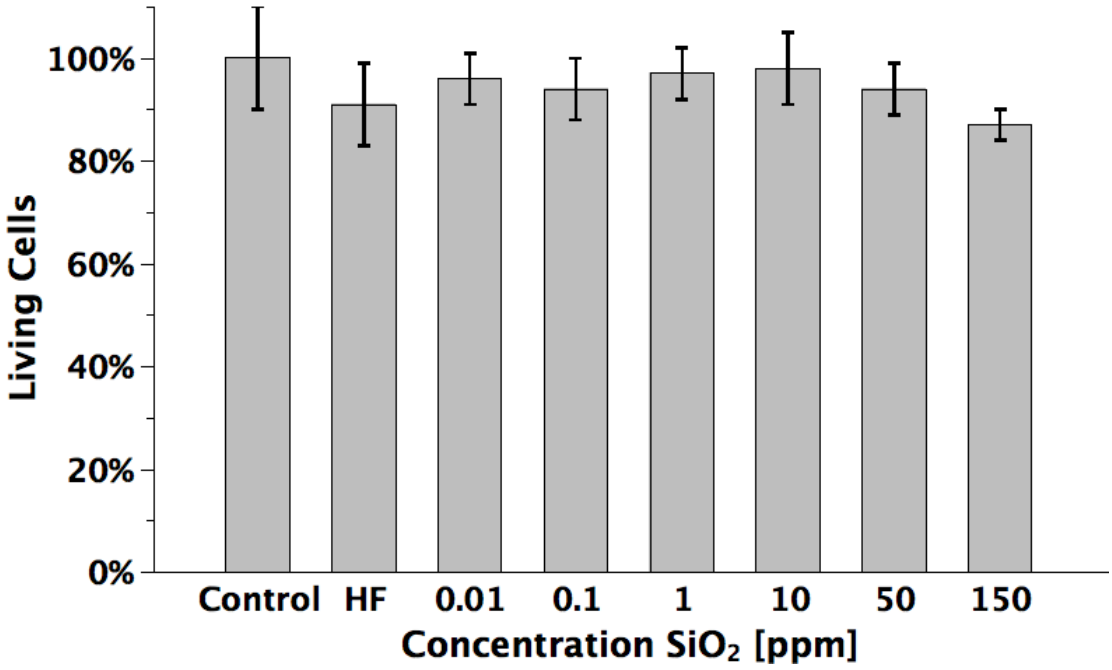

Figure S3 Cell viability Test with calcein-AM. Results indicate no significant influence of neither particle concentration nor buffered oxide etch (BOE) on cell viability.

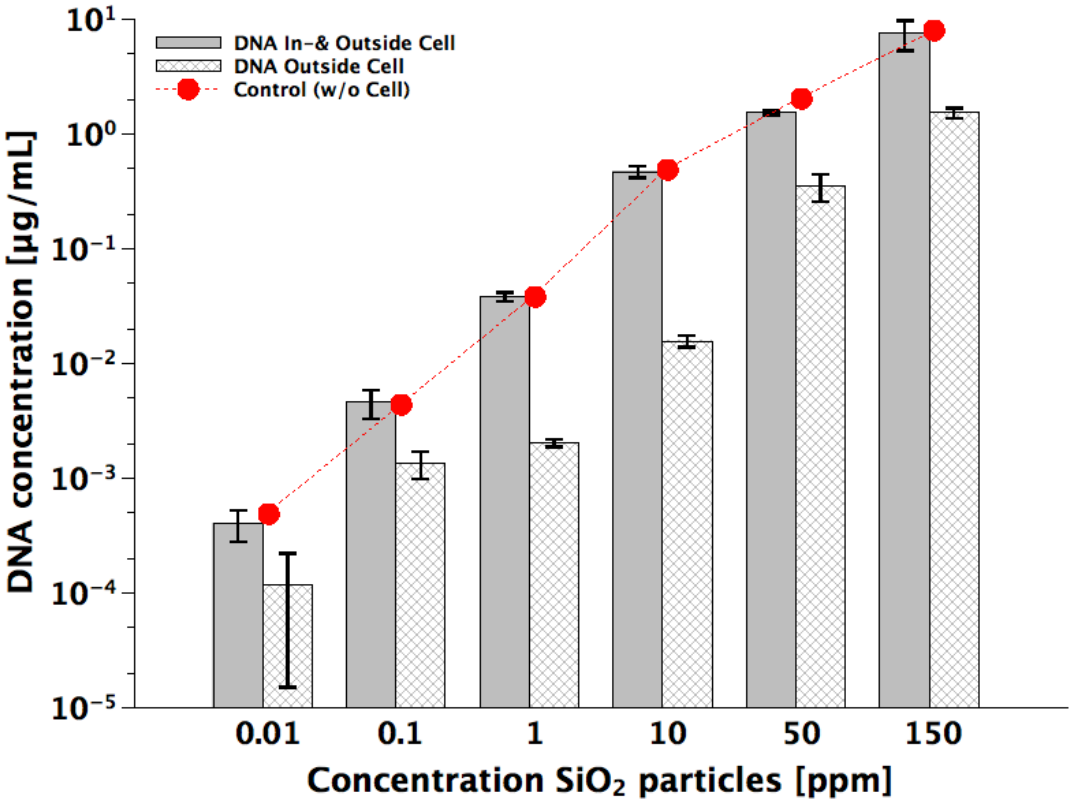

Figure S4 Equivalent DNA concentration (not normalized) determined by qPCR for different concentrations of deagglomerated particles (~200nm). Bars indicate particle concentrations for cell experiments. Detection of particles was possible down to 0.01 ppm. dsDNA concentration of particles within the media and uptaken by the cell indicate the overall dsDNA present within the sample (grey bar). Controls (red points) showed no significant difference to the dsDNA concentration measured in and outside the cell, confirming the detection capability of the described method.

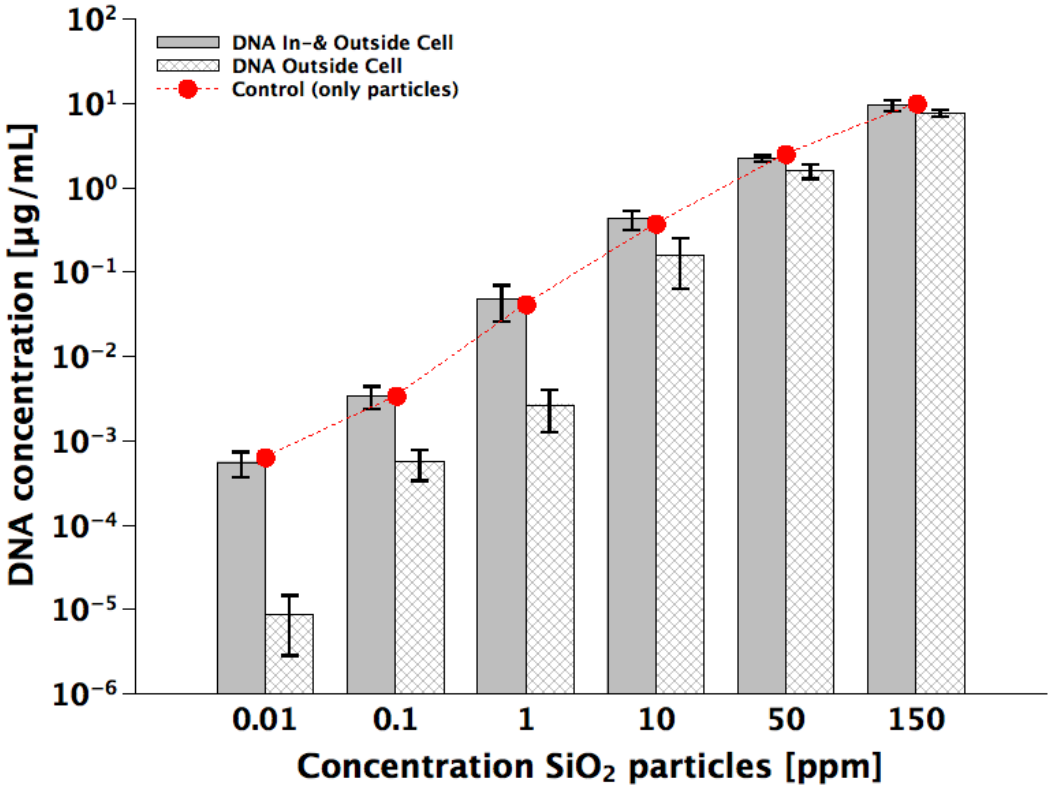

Figure S5 Equivalent DNA concentration (not normalized) determined by qPCR for different concentrations of agglomerated particles (~2 µm). Particle concentrations for cell experiments are indicated by bars. Detection of particles was possible down to 0.01 ppm. dsDNA concentration of particles within the media and uptaken by the cell indicate the overall dsDNA present within the sample (grey bar). Controls (red points) showed no significant difference to the dsDNA concentration measured in and outside the cell, confirming the detection capability of the described method.

References:

1. G. H. T. Bogush, M.A.; Zukoski, C.F., *Journal of Non-Crystalline Solids*, 1988, 104, 95-106.
2. S. Jiang, Recipes for Stöber Silica Particles, http://web.mit.edu/sjiang2/www/Resources/stober_recipes.pdf, Accessed Sep. 24 2013.
3. D. Paunescu, R. Fuhrer and R. N. Grass, *Angewandte Chemie Int. Ed.*, 2013, **52**, 4269-4272.
4. D. Paunescu, M. Puddu, J. O. Soellner, P. R. Stoessel and R. N. Grass, *Nature Protocols*, 2013, **8**, 2440-2448.