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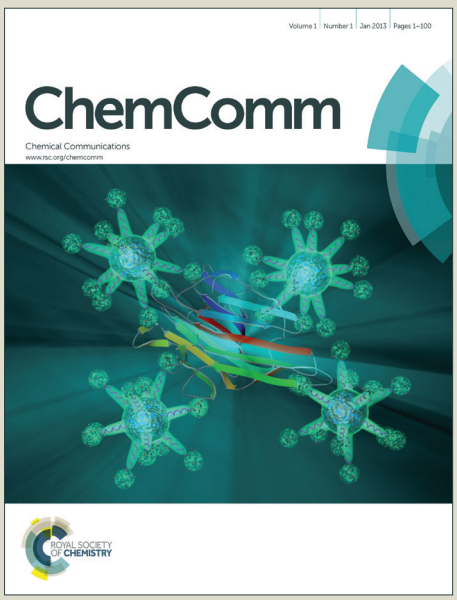

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PCR Quantification of SiO_2 Particle Uptake in Cells in the ppb and ppm range via silica encapsulated DNA barcodes

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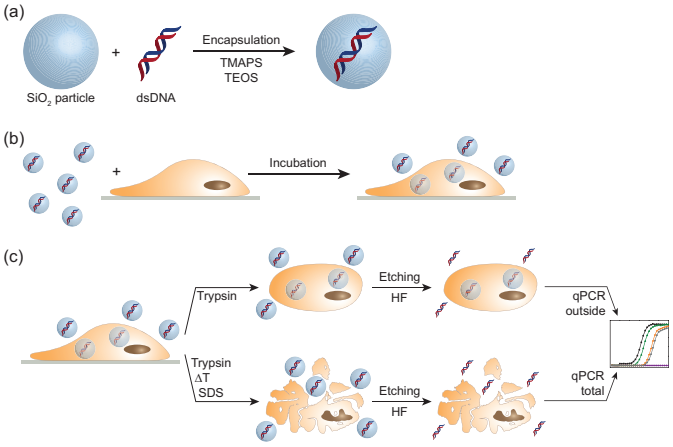
Marcus Hoop,[a] Daniela Paunescu,[a] Philipp R. Stoessel,[a] Fritz Eichenseher,[b] Wendelin J. Stark,[a] and Robert N. Grass*[a]

There is a strong interest in studying the cellular uptake of silica nanoparticles, particularly at medically relevant concentrations (ppb-ppm range) to understand their toxicology. At present, uptake analysis at these exposure levels is impeded by the high silica background concentration. Here we describe the use of DNA encapsulated within silica particles as a tool to quantify silica nanoparticles in *in vitro* cell-uptake experiments at low concentrations (down to 10 fg / cell).

SiO_2 is the second most abundant compound found in nature.[1] Humans are exposed to SiO_2 particles everyday, generated mainly by combustion processes (fly ash).[2] Additionally, amorphous silica (nano)particles, commonly known as aerosil or silica gel, are produced commercially at megaton scales[3] and have been utilized as polymer fillers, cosmetic ingredients, food- and pharmaceutical additive (E551) for more than 60 years.[4] Silica based materials are increasingly studied for use in biomedical applications,[5] such as drug delivery,[6] bio-imaging,[7] and as a cellular marker.[8] Therefore, there is a long-standing interest in understanding the interaction between these materials and human health. During the recent public awareness of nanotoxicology these efforts have been intensified and various studies on the health effects of silica particles have been conducted *in vitro* and *in vivo*.[9] An important factor of particle – toxicology is the understanding of particle uptake into living cells. As the physical behaviour of particles in fluids, most prominently aggregation, depends strongly on the particle concentration (second order rate equation),[10] the uptake of particles into cells should be studied at relevant exposure concentrations,[11] which are usually in the sub ppm range.[12] While the uptake of more exotic metal oxides (e.g. ceria) can be measured at these concentrations by standard techniques (ICP-MS), the ubiquitous presence of silica impedes reliable measurements at low concentrations. To circumvent this silica particle uptake has been measured by electron or confocal microscopy of dye loaded silica particles.[13-16]

As these techniques are time intensive and particle quantification is still challenging at low particle loadings, we investigated the possibility of utilizing DNA loaded silica particles as a quantification tool. The novel technique takes its cue from virus analysis. There, the presence and concentration of viruses in a biological compartment are related to the amount of viral DNA (or RNA) found in the compartment following lysis of the virus. In human diagnostics quantitative polymerase chain reaction (qPCR) detection of viruses in blood is possible down to a single infective unit per µl and has been established as the gold-standard for many viruses (e.g. HIV).[17] Paunescu et. al[18] recently developed a technique for the encapsulation of DNA within silica particles and showed that the particles can be quantified in water utilizing qPCR analysis following a mild fluoride degradation (='lysis') of the particles. Herein, we



Scheme 1 (a) Encapsulation of dsDNA into silica by a core-shell approach (b) Incubation of cellular model system – A549 – with different concentrations of SiO_2 particles. (c) Quantification of SiO_2 particle uptake by cells.

report the utilization of these DNA encapsulates as quantifiable model compounds for measuring the uptake of silica into biological compartments.

The encapsulates (Scheme 1a) carrying artificial double strand (ds)DNA amplicons (113bp, see SI for sequence and experimental procedure) were synthesized as previously described.[18] Following the synthesis procedure, the particles were heavily agglomerated, with an average hydrodynamic particle size of > 2 µm. In this state the particles resemble the structure of the commercially produced silica, Aerosil OX50 as visible from TEM images (Figure S1). The DNA comprising particles were deagglomerated utilizing a ball-milled to achieve particles of 200 nm hydrodynamic size (Table S4; Fig. S1). Both the agglomerated (comprising >1'000 primary particles per agglomerate) and the deagglomerated (1-5 primary particles per agglomerate, see particle size distribution Figure S2) particles were utilized in the uptake-study. The formed particles were further analysed in terms of particle size distribution, zeta-potential and resistance of the encapsulated DNA against aggressive radical oxygen species (ROS) to validate the stability of the encapsulates (see Table S4 and Paunescu et al.[18]). For visual validation of the particle uptake into the cells, we also synthesized encapsulates from fluorescent silica particles utilizing the same methodology.

For visual and qualitative particle uptake, we cultivated adenocarcinomic human alveolar basal epithelial cells (A549, Supplementary Information) in 6 well plates for 24 hours. This cell type was utilized as it has a history in nanoparticle uptake studies, and comparative data from ceria is available.[19] Sets of cells were incubated with 150 ppm of both agglomerated (2 µm) and deagglomerated (~200 nm) fluorescent SiO_2 particles for six hours. Then, cells were imaged with confocal microscopy at different depth levels (z). A 3-dimensional cell reconstruction (Supplement video) as well as the cell images at different height sections illustrate the uptake of agglomerated and deagglomerated particles into A549 cells. As indicated in literature the microscopic images demonstrate a higher uptake rate of smaller particles compared to larger agglomerates. Furthermore, the images confirm the adhesion of particles on the cell membrane (Fig. 1b)[13] as well as the accumulation of particles in cell compartments (Fig. 1b & 1e).[15] Hence, we qualitatively validated the uptake of our synthesized fluorescently labelled SiO_2 particles in A549 cells. These findings justify the use of A549 cells in order to quantify the uptake efficiency of SiO_2 particles

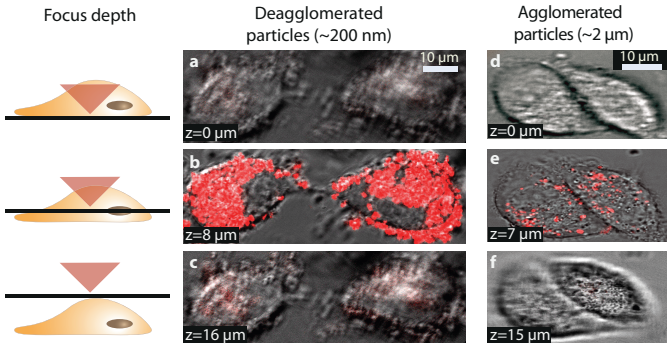


Fig. 1 Confocal Laser Scanning Microscopy pictures. Cells incubated with deagglomerated (a-c) and agglomerated (d-f) fluorescence labelled particles with a concentration of 150 ppm at different imaging height levels.

by our cellular model. The images also show the problem of particle attachment to the outer cell membrane making a discrimination of *internal* and *external* particles difficult.

In a second row of experiments we utilized marker DNA comprising silica particles in exchange for the fluorescently marked silica particles in order to build a quantitative silica-uptake tool. After dissolution of the particles by buffered oxide etch solutions, the amount of marker DNA present in the sample could be used as a direct measure for the concentration of the particles as previously shown for aqueous samples.[18] In order to discriminate between particles inside and outside of the cell, we took advantage of the natural cell membrane, which is generally regarded as impermeable to DNA (see Scheme 1). Following cellular exposure for 6 hours, the DNA present in particles free in solution, as well as DNA present any particles attached to the cell surface (particles *extern* of cell) was released into the extracellular environment by dissolving the particles in a fluoride buffer. qPCR analysis of the marker DNA in the extracellular solution allowed a quantification of the particles originally present therein. Assuming intactness of the cell membrane, any DNA released from particles within the cell, would remain therein throughout this procedure. To measure the total amount of DNA present in the sample (*internal* and *external* of the cell) the cells were lysed by SDS at elevated temperatures and the particles were collected and purified by repeated centrifugation and resuspension in water. The marker DNA present was thereafter quantified following particle dissolution by the fluoride buffer.

As the intactness of the cell membrane is of great importance for the presented method, we investigated the cell viability following various treatments. Both the effect of the particles themselves, as well as any effect of the fluoride buffer was, therefore, investigated with a calcein-AM assay. Results (Figure S3) indeed show that the viability of the cells is neither significantly influenced by the presence of the particles, nor, by the buffered oxide etch solutions. The resistance of the cells to short (minutes) exposure to the fluoride containing buffer may seem surprising at first (high *in vivo* toxicity of

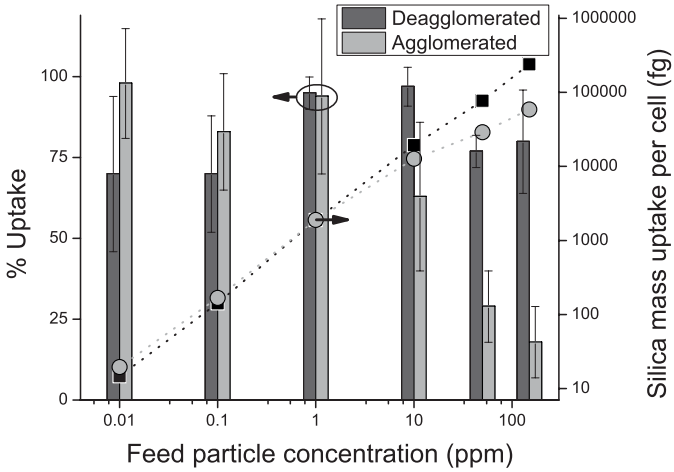


Fig. 2 Uptake of silica particles into A549 cells at feed concentrations ranging over 4 orders of magnitude. The difference between small (deagglomerated, ~200 nm) particles and large (agglomerated ~2 µm) is evident at high (> 10 ppm) feed concentrations in terms of relative uptake (left axis). Details on calculation of data from qPCR results and negative controls are given in the Supporting Information.

fluoride), but goes in line with the application of fluoride comprising solutions and gels (formulations comprising up to 2 wt% fluoride) in dentistry and the slow (days) toxic response of cells to high concentrations of NaF.[20]

In order to show the applicability of the method, silica particle uptake was quantified for agglomerated (ca. 2 µm hydrodynamic size) and deagglomerated (~200 nm) particles at various initial particle concentrations ranging over four orders of magnitude from 10 ppb (10 µg / l) to 150 ppm (150 mg / l = 150'000 ppb). Whereas for the smaller particles there was no effect of the particle concentration on the fraction of particles taken up (> 80%), the initial particle concentration had a pronounced effect for the larger agglomerates (Fig. 2); For small initial particle concentrations (< 1 ppm) the behaviour was very similar to the 200 nm particles with near to quantitative uptake, but for higher initial concentrations only a fraction of the applied particles were taken up (raw data in Table S1-S3 and Figure S4&S5). At 150 ppm only ~ 20 % of the agglomerated particles initially present were internalized by the cells, which is also confirmed by the fluorescence spectroscopy images (Figure 1). A possible explanation for this effect is the significantly smaller diffusion constant of the larger aggregates as previously shown in many studies utilizing fluorescently labelled particles[14,15] and the effect of aggregation[21] on cell uptake as recently shown by Fayol et al.[22]

Our experimental setup outlined in this report describes a fast and convenient methodology for the detection of SiO_2 particle uptake. Based on the highly potent qPCR technology the method allows quantification of labelled nanoparticles in the supernatant and in the cell down to the ppb level, equivalent to 10 fg of silica taken up per cell and, therefore, not only requires less infrastructure, but is also more sensitive than the currently applied fluorescence based methods. The method may in future also allow the tracking of administered silica particles in histologic specimens taken from biopsies and serve as a broadly applicably tool in nanotoxicology studies.

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Notes and references

[a]*Institute for Chemical and Bioengineering, ETH Zurich, 8093 Zurich, Switzerland. Fax: +41 44 633 10 83; Tel: +41 44 633 63 34; E-mail: robert.grass@chem.ethz.ch.*
[b]*Institute of Food, Nutrition and Health, ETH Zurich, 8092 Zurich, Switzerland.*

† Electronic Supplementary Information (ESI) available: Materials and Methods, material characterization, details on calculation of particle uptake from qPCR data, raw qPCR data and supplement video of 3D construction of fluorescent nanoparticle uptake; See DOI: 10.1039/b000000x/

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