The effect of settling on cytotoxicity evaluation of SiO₂ nanoparticles

Anastasia Spyrogianni^a, Georgios A. Sotiriou^{b,c}, Davide Brambilla^b, Jean-Christophe Leroux^b,
Sotiris E. Pratsinis^{a*}

^a Particle Technology Laboratory, Institute of Process Engineering, Department of Mechanical and Process Engineering, ETH Zurich, Zurich, Switzerland

^b Drug Formulation and Delivery Laboratory, Institute of Pharmaceutical Sciences, Department of Chemistry and Applied Biosciences, ETH Zurich, Zurich, Switzerland

^c Department of Microbiology, Tumor and Cell Biology, Karolinska Institutet, Stockholm, Sweden

*Corresponding author: sotiris.pratsinis@ptl.mavt.ethz.ch

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Abstract

The *in vitro* cytotoxicity of nanomaterials is commonly assessed by monitoring cell viability under exposure to their suspensions. The actual nanoparticle dose delivered to cells for the same nominal nanoparticle concentration may depend on their settling rate. The goal here is to evaluate quantitatively the importance of settling for the *in vitro* cytotoxicity evaluation of aerosol-made, fractal-like SiO₂ nanoparticles that have similar agglomerate size and structure but varying primary particle diameter. Their deposition rate is measured by UV-visible spectroscopy and their *in vitro* cytotoxicity is evaluated in both standard upright and inverted murine macrophage culture configurations. Silicas of larger primary particles settle faster and appear more cytotoxic than those of smaller ones in standard upright cell cultures. In contrast, with inverted cell cultures all silicas show similar and much lower cytotoxicity revealing the impact of nanoparticle settling in such assessments that are benchmarked with H₂O₂ solutions and nanosilver suspensions. When the measured settled fractions are accounted for, the reduction in cell viability depends on the surface of SiO₂ nanoparticles deposited onto cells regardless of primary particle size. This study quantitatively demonstrates the importance of nanoparticle settling for *in vitro* studies of the widely used fumed SiO₂ and highlights that settling measurements combined with inverted-upright cell cultures can be useful tools in nanotoxicology studies.

Keywords: Aerosol-made nanoparticles; dosimetry; sedimentation; UV-vis; inverted - upright cell cultures

1. Introduction

The *in vitro* cytotoxicity of nanomaterials on cell cultures is commonly assessed by monitoring the viability (or functions) of cells exposed to nanomaterial suspensions. Even though for relatively heavy nanoparticles settling may determine the nanoparticle dose reaching such cell cultures (Cohen et al., 2015; Teeguarden et al., 2007), its implications on the cellular uptake (Cho et al., 2011; Limbach et al., 2005) and cytotoxicity ranking (Pal et al., 2015) of nanomaterials have not been widely addressed. For example, Limbach et al. (2005) showed that the cellular uptake was higher for larger (in terms of both primary particle and agglomerate sizes) than smaller CeO₂ nanoparticles, consistent with the higher settling rate of larger ones. This was shown also for Au nanoparticles of various sizes, shapes and surface-coatings by Cho et al. (2011) who used a standard “upright” and an “inverted” cell culture configuration, *i.e.* with the cells placed either at the bottom or top, respectively, of particle suspensions to decouple nanoparticle gravitational sedimentation from diffusion. Higher cellular uptake (and transfection efficacy) in upright than in inverted cell culture configurations was observed also by Giger et al. (2013) for PEGylated calcium phosphate nanoparticles of ~200 nm hydrodynamic diameter, indicating again the importance of nanoparticle settling.

As aerosol-made nanoparticles are typically in an agglomerated or aggregated shape (Eggersdorfer & Pratsinis, 2014), quantifying their settling or deposition rate is quite challenging, primarily due to the largely unknown relationship between agglomerate particle transport and structure (Johnson et al., 1996; Limbach et al., 2005). Hinderliter et al. (2010) developed first the *In vitro* Sedimentation, Diffusion and Dosimetry (ISDD) model to calculate the deposited (or delivered) doses on cell cultures for spherical (non-agglomerated) and agglomerated nanoparticles. This model was used also in other studies, *e.g.* for (mostly) spherical fluorescently-labelled polystyrene beads (Khanbeigi et al., 2012) or for flame-made fractal-like nanoparticles (SiO₂, Ag/SiO₂, CeO₂, Fe₂O₃, TiO₂) (Cohen et al., 2013).

To overcome the need for an assumption for the structure of fractal-like agglomerates in the ISDD model, DeLoid et al. (2014) measured the effective density of various flame-made fractal-like (*e.g.* SiO₂, Fe₂O₃, CeO₂, TiO₂) and also non-agglomerated (*e.g.* Au) nanoparticles. This density was used in a revised ISDD model to show that the difference between nominal (or administered) and deposited

nanomaterial dose depends on the primary particle and/or agglomerate sizes and densities (Cohen et al., 2014; DeLoid et al., 2014; Pal et al., 2015). Furthermore, using the deposited instead of the nominal nanoparticle mass changed the *in vitro* cytotoxicity ranking of H₂O₂-oxidized carbon nanohorns, carbon black, TiO₂, CeO₂, Ni, MnO_x and Ag nanoparticles in human monocyte/macrophage leukemia cells (Pal et al., 2015). This led to a better correlation of the *in vitro* cell death with the *in vivo* lung inflammation data when the deposited dose was used (Pal et al., 2015).

The aim of this study is to evaluate quantitatively the importance of particle settling for the *in vitro* cytotoxicity assessment of aerosol-made, fractal-like SiO₂ nanoparticles that have similar agglomerate size and structure but different primary particle diameter. The nanoparticle deposition rates and fractions are measured by UV-visible (UV-vis) spectroscopy (Allouni et al., 2009; Hsiao & Huang, 2013) and benchmarked with theoretical settling rate calculations for fractal-like agglomerates (Johnson et al., 1996; Limbach et al., 2005). The importance of SiO₂ nanoparticle settling or sedimentation on cytotoxicity assessment is demonstrated by using both standard upright (Pratsinis et al., 2013) and inverted (Lee et al., 2014) cultures of murine macrophages, which represent the first line of defense upon systemic exposure (Oberdörster et al., 2005). It is noted that the *in vitro* cytotoxicity of fumed SiO₂ nanoparticles has been studied previously both in serum-free (Gerloff et al., 2009) and serum-containing (Gehrke et al., 2013; Panas et al., 2013) media, typically reporting lower cytotoxicity in the presence of serum for given nominal nanoparticle concentrations. This may be attributed partly to slower deposition rate (Halamoda-Kenzaoui et al., 2015; Spyrogianni et al., 2016), different uptake levels (Lesniak et al., 2012) or inhibition of reactive oxygen species generation and inflammatory responses (Escamilla-Rivera et al., 2016) compared to serum-free conditions. To minimize the contribution of additional variables that can be introduced by the presence of serum proteins, here serum-free conditions are used to understand the effect of nanoparticle settling on the observed cytotoxicity for varying primary particle diameter.

2. Materials and Methods

2.1. Materials

Hydrophilic fumed SiO₂ powders from Evonik Industries (Aerosil®, herein named as “P1”) and from Cabot (Cab-O-Sil®, herein named as “P2”) were used. More specifically, the samples Aerosil® 90, 150, 200 and Cab-O-Sil® EL-90 and M-5 were used. Particle suspensions were prepared in fresh ultrapure water (Milli-Q®, 18.2 MΩ·cm, 25 °C, TOC: 3 ppb), herein named as “milli-Q water”. For cytotoxicity studies, cell culture media and supplements were obtained from Invitrogen (Pratsinis et al., 2013), as described in Supplementary Information (SI). CellTiter® 96 Aqueous One Cell Proliferation Assay (MTS) was obtained from Promega. 96-well plates (Nunc™ 167008, flat bottom, clear), 24-well plates (Nunc™ 142475, flat bottom, clear) and cell culture coverslips (Nunc™ Thermanox™ 174950, 13 mm diameter) were obtained from Thermo Scientific. Limulus Amebocyte Lysate (LAL) assay (QCL-1000) was obtained from Lonza. Hydrogen peroxide (H₂O₂ 30% from Merck Millipore) as well as previously prepared nanosilver (nano-Ag) containing 50wt% SiO₂ (Sotiriou et al., 2014) were used as additional controls. Polystyrene semi-micro and macro cuvettes for DLS and UV-vis spectroscopy were obtained from VWR. Packed cell volume tubes (Catalog No. 87005) and an “easy read” measuring device (Catalog No. 87010) for effective agglomerate density measurements were obtained from TPP (Switzerland).

2.2. SiO₂ characterization

The particle specific surface area (SSA) was measured thrice by N₂ adsorption at 77 K using a five-point Brunauer-Emmet-Teller method (Tristar, Micromeritics) after degassing the samples for at least 1 h at 150 °C in nitrogen (Mueller et al., 2003). The equivalent average primary particle diameter (d_p) was calculated as $d_p = 6/(SSA \cdot \rho_p)$ where ρ_p is the particle density. The SiO₂ powders were dispersed in ethanol and deposited onto a perforated carbon foil supported by a copper grid for transmission electron microscopy (TEM) with a Tecnai F30 ST microscope (FEI, FEG, 300 kV).

2.3. Particle suspensions preparation and characterization

Particle suspensions in serum-free cell culture medium were prepared, following a previously

established protocol (Sotiriou et al., 2014) (detailed description in SI). DLS and zeta potential measurements were performed with a Zetasizer (Nano ZS, Malvern Instruments). Before each size measurement, the suspensions were vortex-mixed and a sample of 1 mL was removed via pipetting from the middle of the suspension and placed in a polystyrene semi-micro cuvette, where it was equilibrated for 30 s prior to measurement. Similar procedure but with 1 mL of sample placed in a disposable folded capillary cell (DTS1061 or 1070, Malvern) was followed for zeta potential measurements. Small peaks in the micron-size range that occasionally appeared in the intensity- but never in the number-based distributions by DLS could be attributed to contamination during handling and were considered negligible. The zeta potential of the as-prepared SiO₂ suspensions in milli-Q water was in the range -38 to -45 mV, indicating relatively stable suspensions (Wengeler et al., 2006), while dilution with serum-free medium reduced their zeta potential (in absolute value) in the range -16 to -22 mV (with measured pH in the range 8.0 - 8.3), consistent with literature (Gehrke et al., 2013).

2.4. UV-visible (UV-vis) spectroscopy

The settling of the SiO₂ agglomerates in serum-free cell culture medium was evaluated by monitoring their optical absorption over time (Allouni et al., 2009; Hsiao & Huang, 2013) at 23 °C, using a Cary Varian 500 UV-vis spectrophotometer. Before each measurement, the suspensions were vortex-mixed and a sample of 2.1 mL was removed via pipetting from the middle of the suspension and placed into a polystyrene macro cuvette. For all measurements, 2.1 mL of a mixture of milli-Q water with serum-free medium (volume ratio 1:3) was used as blank. The absorbance of all SiO₂ suspensions was first scanned from 350 to 700 nm (scan rate: 400.2 nm/min), indicating maximum absorbance at 350 nm. This wavelength (350 nm) was therefore selected to perform kinetic measurements of absorbance up to 24 h. For such measurements, both the sample and the blank cuvette were sealed with plastic paraffin (Parafilm M). The cuvettes were partly-covered (by placing appropriate black covers) in the front, leaving free only an upper suspension layer of ~3 mm height, to ensure that only the absorbance of this layer was monitored. Freshly-prepared suspensions were used for each calibration curve and the subsequent kinetic measurement and up to 3 independent suspensions were prepared for each sample. The settled fraction, f_s , out of the monitored suspension

layer was calculated from Eq. 1:

$$f_s = \frac{C_m(t=0) - C_m(t)}{C_m(t=0)} \quad (1)$$

where C_m is the SiO_2 mass concentration at time t , as determined from the kinetic absorbance measurements and calibration.

2.5. Endotoxin contamination assay

The potential endotoxin contamination of the SiO_2 suspensions was tested, using an endpoint, chromogenic LAL assay that allows the detection of endotoxin spectrophotometrically at 405 - 410 nm (Smulders et al., 2012). All steam-sterilized SiO_2 suspensions in milli-Q water (400 $\mu\text{g/mL}$, prepared as described in SI) and the steam-sterilized milli-Q water employed for cytotoxicity studies were tested for potential endotoxin contamination, using the microplate method of the LAL assay, according to the manufacturer's instructions. LAL reagent water was used as blank and four *E. coli* endotoxin concentrations (0.1, 0.25, 0.5 and 1 EU/mL) were used as controls. The absorbance of the final suspensions (prepared in duplicate) at 405 - 410 nm was measured with a plate reader (Tecan Infinite M200).

2.6. Cell growth

RAW 264.7 murine macrophages were cultured at 37 °C in a humidified atmosphere containing 5% CO_2 and subcultivation was performed every 2 - 3 days (Pratsinis et al., 2013). The cells are routinely tested to verify the absence of mycoplasma contamination.

2.7. Cytotoxicity evaluation in standard upright cell culture configuration

For the SiO_2 cytotoxicity evaluation in RAW 264.7 murine macrophages in standard upright cell culture configuration (in 96-well plates), the cell viability was determined by the MTS assay (Pratsinis et al., 2013), following a previously developed protocol (Sotiriou et al., 2014) (detailed description in SI). Nominal SiO_2 mass concentrations, IC_{50} , (Pratsinis et al., 2013) causing 50% reduction in cell viability (or 50% (cyto)toxicity concentrations, CC_{50} (Ye et al., 2010) or TC_{50} (Napieriska et al., 2009)), were calculated from four-parameter logistic curve fitting to the final cell viability data (Beer

et al., 2006) using GraphPad Prism (GraphPad Software).

2.8. Cytotoxicity evaluation in inverted vs. modified upright cell culture configuration

For the SiO₂ cytotoxicity evaluation in inverted vs. modified upright cell culture configuration, a protocol developed by Lee et al. (2014) was adapted, based on the herein employed protocol of the standard cytotoxicity evaluation (detailed description in SI).

2.9. Effective density by volumetric centrifugation method (VCM)

Effective agglomerate density measurements with the VCM were performed adapting the methodology introduced by DeLoid et al. (2014). SiO₂ suspensions of 100 µg/mL final concentrations in serum-free medium were prepared (from 5000 µg/mL stock suspensions in milli-Q water, which were sonicated at 100 kJ/L and steam-sterilized prior to final dilution with serum-free medium, as described in SI) and 1 mL aliquots were dispensed in packed cell volume tubes to be centrifuged for 1 h at 2300 x g. The pellet volume was measured using an “easy read” measuring device and used to calculate agglomerate effective density, $\rho_{\text{agg,VCM}}$, with a stacking factor of 0.634, as described in (DeLoid et al., 2014). All measurements were performed in triplicate.

2.10. Statistical analysis

The statistical significance of the data was analyzed by One-Way ANOVA (Sidak test) using OriginPro (OriginLab) and GraphPad Prism (GraphPad Software) for IC₅₀ comparison. Differences were considered significant at $p < 0.05$.

3. Agglomerate settling rate: Theory

The settling rate V_s of a fractal-like agglomerate (in the Stokes law regime) can be calculated from Eq. 2 (Johnson et al., 1996; Limbach et al., 2005):

$$V_s = \frac{(\rho_p - \rho_f) \cdot n_p \cdot d_p^3 \cdot g}{18 \cdot \mu \cdot d_m} \quad (2)$$

where ρ_p and ρ_f (kg/m³) are the densities of particles and fluid, respectively, n_p (-) is the number of primary particles per agglomerate, d_p is the average primary particle diameter (monodisperse primary

particles are assumed), g (m/s^2) is the gravitational acceleration, μ ($\text{Pa}\cdot\text{s}$) is the dynamic viscosity and d_m is the agglomerate mobility or hydrodynamic diameter, as determined by DLS (Sorensen, 2011; Wiltzius, 1987). For SiO_2 suspensions in serum-free cell culture media, $\rho_p = 2200 \text{ kg/m}^3$, $\rho_f = 1000 \text{ kg/m}^3$ and μ for water (Hinderliter et al., 2010) are used (with $\mu(23^\circ\text{C}) = 0.00093 \text{ Pa}\cdot\text{s}$ and $\mu(37^\circ\text{C}) = 0.00069 \text{ Pa}\cdot\text{s}$ (Kestin et al., 1978)). The n_p is given by a power law (Eggersdorfer et al., 2012; Sorensen, 2011):

$$n_p = k_m \cdot \left(\frac{d_m}{d_p} \right)^{D_{fm}} \quad (3)$$

where k_m (-) is the scaling prefactor and D_{fm} (-) is the mass-mobility exponent. So, substituting n_p from Eq. 3 in Eq. 2 results in Eq. 4:

$$V_s(d_p, d_m) = \frac{(\rho_p - \rho_f) \cdot g}{18 \cdot \mu} \cdot k_m \cdot d_p^{3-D_{fm}} \cdot d_m^{D_{fm}-1} \quad (4)$$

Fumed SiO_2 agglomerates have a structure similar to those made by diffusion-limited cluster-cluster agglomeration (DLCA) as their measured mass fractal dimension, D_f , of 1.84 ± 0.08 (Martin et al., 1986) or $1.78 - 1.84$ (Camenzind et al., 2010) is close to the theoretical D_f of 1.78 ± 0.1 for particles made by DLCA (Sorensen, 2011). Therefore, for the V_s calculation by Eq. 4 here $k_m = 1.11$ and $D_{fm} = 2.15$ from the literature (Eggersdorfer et al., 2012) for DLCA agglomerates are used.

The average V_s for a SiO_2 agglomerate size distribution of a given d_p can be calculated as:

$$V_{sa} = \frac{\sum_i V_s(d_{m,i}) \cdot N(d_{m,i})}{\sum_i N(d_{m,i})} \Bigg|_{d_p} \quad (5)$$

where $d_{m,i}$ is each agglomerate diameter class, i , and $N(d_{m,i})$ is the corresponding agglomerate fraction of size class i from the distributions by DLS. The intensity-weighted size distributions are selected as they are more reliable than the post-derived number or volume distributions by DLS (Finsy, 1994).

4. Results and Discussion

4.1. Primary particle and agglomerate size and structure

Figure 1a shows the measured and nominal specific surface area, SSA, of fumed silica powders from two commercial producers, P1 (open symbols) and P2 (filled symbols), along with their average or Sauter mean primary particle diameter, d_p , that varies between 14 and 30 nm (upper abscissa). Such silicas consist of nano-sized and narrowly distributed primary particles that are held together in the form of sub-micron aggregates (or hard-agglomerates) (Ulrich, 1971) and micron-sized aggregate-clusters (or soft-agglomerates) (Tsantilis & Pratsinis, 2004), having filamentary or fractal-like morphology (Fig. 1a, insets).

Prior to cytotoxicity evaluation, the SiO_2 powders were dispersed in water by progressively increasing the ultrasonication intensity to break up soft-agglomerates (Kusters et al., 1993) and reduce asymptotically their diameter (SI, Fig. S1), similar to high-pressure dispersion (Wengeler et al., 2006). Figure 1b shows the final agglomerate hydrodynamic or mobility diameter, d_m , (Sorensen, 2011; Wiltzius, 1987) distributions of P1 (open symbols) and P2 (filled symbols) SiO_2 suspensions of various d_p in serum-free cell culture medium, as determined by dynamic light scattering (DLS). These distributions have similar shape and mode independent of d_p and commercial producer. For SiO_2 with $d_p = 14$ nm, the mode d_m (160 - 190 nm) is in good agreement with the average d_m (~200 nm) obtained asymptotically by high-pressure dispersion for such d_p (Wengeler et al., 2006). Furthermore, all SiO_2 suspensions were quite stable, at least, for 24 h, as verified by DLS (SI, Fig. S2).

4.2. Settled SiO_2 mass fraction and agglomerate settling rate

Figure 2a shows the evolution of light absorbance during settling (Allouni et al., 2009; Hsiao & Huang, 2013) of three P1 SiO_2 suspensions of average $d_p = 14, 18$ and 30 nm, having 1000 $\mu\text{g/mL}$ initial concentration in serum-free cell culture medium, at 3 mm below their free surface (inset). This depth is identical to that used for the upright cytotoxicity evaluation hereafter. Agglomerates of the largest d_p have the highest initial absorbance, as expected from the particle extinction coefficient definition (Hinds, 1999). As the agglomerates settle, their number concentration decreases along with

their absorbance (Allouni et al., 2009; Hsiao & Huang, 2013; Plaza et al., 2002).

The kinetic absorbance measurements of Fig. 2a correspond to kinetic SiO₂ concentration measurements through calibration (SI, Fig. S3a) and subsequently, the SiO₂ mass fraction that settles out of the monitored suspension layer, f_s , at a given time is calculated (Eq. 1). Figure 2b shows the settled SiO₂ fraction f_s after 24 h for P1 (open circles) and P2 (filled circles) SiO₂ suspensions having 1000 µg/mL initial concentration in serum-free cell culture medium, as a function of SiO₂ d_p . The settled fraction f_s increases for larger d_p . Since all samples have similar mobility diameter d_m distributions (Fig. 1b), their diffusion coefficients approximated from the Stokes-Einstein equation (Hinds, 1999) are also similar. Therefore, the increasing measured f_s with increasing d_p indicates that the agglomerate settling rate increases for larger d_p . The average settling rate of SiO₂ agglomerates, V_{sa} , calculated from Eqs. 4, 5 is shown in Fig. 2b, confirming the indicated trend of faster settling with increasing d_p for both P1 (open triangles) and P2 (filled triangles) SiO₂ agglomerates. Therefore, a simple model accounting only for gravitational sedimentation (see SI) predicts qualitatively the trend of increasing f_s with increasing d_p from Fig. 2b (circles), as shown in SI, Fig. S4. The calculated f_s by that simple model, however, is lower than the measured f_s . This might be attributed to the assumptions of monodisperse primary particles, negligible van der Waals forces among agglomerates or between agglomerates and medium molecules and fixed agglomerate structural parameters for the entire agglomerate population, *i.e.* assumptions that are commonly made in dosimetry models (DeLoid et al., 2014; Hinderliter et al., 2010; Liu et al., 2015). If diffusion was accounted for in the conditions of Fig. 2a, even lower f_s would be obtained than those of Fig. S4.

It should be noted that for quantitative kinetic absorbance measurements in Fig. 2, absorbance values higher than, at least, 0.1 throughout the measurement are sought because the effect of various uncertainty sources (*e.g.* spectrophotometer drift) at very low absorbance values may become important for long measurement times (Soovali et al., 2006). The minimum possible initial concentrations were 500 µg/mL for $d_p = 30$ nm and 1000 µg/mL for $d_p = 14$ and 18 nm. Settling measurements at various initial concentrations showed that the settled SiO₂ fraction f_s remained rather constant for concentrations up to, at least, 1500 µg/mL (SI, Fig. S3b), consistent with the similar

agglomerate size distributions independent of concentration at a wide range (25 to 2000 $\mu\text{g/mL}$, SI, Fig. S3c). So, the f_s measured at the lowest possible, common (for all d_p) concentration in this range (*i.e.* 1000 $\mu\text{g/mL}$, Fig. 2) will be employed below to calculate the deposited SiO_2 doses at the bottom of 96-well plates filled with 3 mm deep SiO_2 suspensions, assuming constant f_s down to $\leq 100 \mu\text{g/mL}$. It should be noted that the settling measurements are performed at 23 °C, while the cytotoxicity evaluation at 37 °C. The medium viscosity, which is similar to that of water (Hinderliter et al., 2010), is 30% lower at 37 °C than that at 23 °C (Kestin et al., 1978). Since the settling rate V_s is inversely proportional to viscosity (Eq. 4) and the f_s is proportional to V_s (for quiescent settling) (Hinds, 1999), a correction factor of 1.3 is employed to the measured f_s .

The VCM-ISDD model (DeLoid et al., 2014) was also employed to calculate the deposited SiO_2 fractions after 24 h at the conditions of Fig. 2 (SI, Table S1 and Fig. S5), resulting in values that do not predict the trend of increasing f_s with increasing d_p and are lower than those measured here (Fig. 2b). The ISDD model has been validated for Fe_2O_3 agglomerates measuring cell-associated Fe_2O_3 with a magnetic particle detector (Hinderliter et al., 2010) and for neutron-activated flame-made CeO_2 and SiO_2 -coated CeO_2 agglomerates by gamma spectroscopy (DeLoid et al., 2014). The different validation approaches might contribute to differences between the ISDD model and measured f_s here. Furthermore, the ISDD model uses only the average agglomerate diameter (and not the complete distribution) that can also lead to underestimation of the deposited doses, as has been addressed by DeLoid et al. (2015) and Liu et al. (2015).

4.3. Cytotoxicity evaluation in upright cell cultures

Figure 3 shows the relative viability (or mitochondrial activity) of RAW 264.7 murine macrophages as determined by MTS assay (Pratsinis et al., 2013) after 24 h incubation with P1 (open symbols) and P2 (filled symbols) SiO_2 suspensions in standard upright cell culture configurations (in 96-well plates). All employed SiO_2 suspensions were endotoxin free (Smulders et al., 2012) (SI, Fig. S6). Figure 3a shows the cell viability (%) as a function of nominal SiO_2 mass concentration. All silicas reduce the cell viability in a dose-dependent manner, in excellent agreement (for $d_p = 14 \text{ nm}$) with Panas et al. (2013) (diamonds) under similar conditions. For this SiO_2 primary particle size, dose-

dependent cytotoxicity was reported also under different experimental conditions (Gehrke et al., 2013; Gerloff et al., 2009; Panas et al., 2013; Park & Park, 2009; Zhang et al., 2012) while negligible cytotoxicity was observed in some cases (Choi et al., 2009; Gehrke et al., 2013; Panas et al., 2013) (SI, Table S2). The broken line at 50% cell viability in Fig. 3a indicates the so-called 50% (nominal) inhibitory concentrations, IC_{50} , (Pratsinis et al., 2013) which are in the range of 40 to 60 $\mu\text{g/mL}$ for d_p from 30 to 14 nm (SI, Table S3).

So for the same nominal mass concentration, all silicas induce rather similar cytotoxicity regardless of SiO_2 producer though increasing d_p exhibits slightly higher cytotoxicity. The data of Fig. 3a are presented also as a function of nominal SiO_2 surface area concentration in Fig. 3b because this dose metric is often applied for unraveling the cytotoxicity mechanism (Napierska et al., 2009). For constant nominal SiO_2 surface area concentration, the above trend is exacerbated; silicas with larger d_p appear more cytotoxic than those with smaller d_p , also independent of SiO_2 producer.

For constant nominal SiO_2 mass concentration, Gehrke et al. (2013) reported that the reduction of mitochondrial activity and membrane integrity in human colon carcinoma cells HT29 after exposure to the fumed silicas Aerosil 200 (P1, $d_p = 14$ nm here) and OX50 (with $d_p = 55$ nm from nominal SSA) was rather independent of d_p (SI, Table S2). Furthermore, Gazzano et al. (2012) reported that Aerosil 300 (with $d_p = 9$ nm) and OX50 ($d_p = 55$ nm) induced similar membrane damage in MH-S macrophages for constant nominal SiO_2 mass concentration. When, however, the SiO_2 dose was expressed as nominal surface area concentration, OX50 appeared more cytotoxic than Aerosil 300 (Gazzano et al., 2012), consistent with the trend of Fig. 3b here. This had been mainly attributed to the lower total silanol coverage (2.0 vs. 2.5 Si-OH/nm^2 (Mathias & Wannemacher, 1988)) and affinity to water of OX50 than Aerosil 300 (Gazzano et al., 2012).

The fumed silicas selected here, however, have similar total silanol coverage ($\sim 2.5 \text{ Si-OH/nm}^2$) (Mathias & Wannemacher, 1988; Mueller et al., 2003). So the present results may not be explained by silanol coverage. Most interestingly, these results seem to contradict the widespread notion of increasing nanoparticle toxicity with decreasing d_p at constant nominal mass concentration and cytotoxicity rather independent of d_p at constant nominal surface area concentration, as has been

shown with wet-made SiO₂ (Napierska et al., 2009) and other nanomaterials such as TiO₂ (Nel et al., 2006) and Ag (Pratsinis et al., 2013).

Comparing the results of Fig. 3b with those of Fig. 2b, it appears that the increasing cytotoxicity with increasing d_p may be attributed to the higher settled fraction, f_s , of larger than smaller silicas at the bottom of the cell culture wells. So, the measured f_s after 24 h corrected for 37 °C was used to determine the deposited SiO₂ doses (mass and surface area) at the bottom of each cell culture well. Figures 3c,d show the viability data of Figs. 3a,b as a function of the calculated deposited SiO₂ mass (c) and surface area (d) per well. There, the cytotoxicity of SiO₂ follows the general trend expected for nanomaterials. Namely, the cytotoxicity increases with decreasing d_p for constant deposited SiO₂ mass (Fig. 3c) but is rather independent of d_p for constant deposited SiO₂ surface area (Fig. 3d).

Furthermore, this trend is independent of SiO₂ manufacturer.

4.4. Cytotoxicity evaluation in inverted cell cultures

To further evaluate the importance of settling for the upright cytotoxicity assessment of Fig. 3, an inverted cell culture configuration (Cho et al., 2011; Lee et al., 2014) (with coverslips in 24-well plates) was employed in Fig. 4 (open bars) at otherwise identical conditions to Fig. 3a. In fact, the corresponding data from Fig. 3a are also included in Fig. 4 (shaded bars) for a direct comparison. In the inverted configuration, a relatively small reduction in cell viability (down to $70 \pm 20\%$) is observed only at the highest SiO₂ concentrations of 75 - 100 µg/mL for $d_p = 14$ (Fig. 4a) and 18 nm (Fig. 4b), while no reduction of cell viability is observed over the whole concentration range for the SiO₂ of the largest d_p (Fig. 4c). This is in contrast to the strong cell viability reduction for all silicas in the upright configuration (Fig. 4a-c, shaded bars).

In the inverted configuration, the SiO₂ agglomerates can reach the macrophages that are located on top of the suspensions only by Brownian motion and diffusion. So, for a given nominal SiO₂ dose, the actual dose in contact with the cells is lower in the inverted than in the upright configuration where gravitational settling largely determines the agglomerate transport to the bottom of the well plate. This explains why in Fig. 4 all silicas exhibit much lower cytotoxicity in inverted (open bars) than in upright (shaded bars) configuration and highlights the importance of nanoparticle-cell contact for the

SiO₂ cytotoxicity in macrophages.

It should be noted that the inverted cytotoxicity evaluation was always performed in parallel with a modified upright cytotoxicity evaluation (detailed description in SI), verifying that the use of coverslips does not alter the cell viability compared to the standard upright configuration (SI, Fig. S7 vs. Fig. 3a). Figure 5 shows the relative difference in cell viability between inverted (Fig. 4) and modified upright (SI, Fig. S7) configurations for the various SiO₂ sizes and nominal mass concentrations. For most concentrations, this difference is higher for silicas of larger than smaller d_p , since larger silicas settle faster than smaller ones (Fig. 2b). This is consistent with the higher disparity in cellular uptake between inverted and upright configuration for larger than smaller Au nanoparticles reported by Cho et al. (2011). Furthermore, these results indicate that gravitational sedimentation is the dominant transport mechanism (in the direction of gravity) for the investigated agglomerates, consistent with CeO₂ nanoparticles of comparable average agglomerate diameters (> 200 nm) (Limbach et al., 2005). It should be clarified, however, that the relative contribution of diffusion increases for silicas of smaller d_p , as their average agglomerate diameter from Fig. 1b is also slightly smaller.

To validate the employed protocol of the inverted configuration, two positive controls were employed: hydrogen peroxide (H₂O₂) (Cantoni et al., 1989) and nanosilver (nano-Ag) of relatively small crystal size (~8 nm) (Sotiriou et al., 2014). Figure 6 shows the viability (%) of murine macrophages as a function of the positive control concentrations in both inverted (open bars) and modified upright (shaded bars) cell culture configurations. With increasing H₂O₂ concentration the cell viability is reduced and, since H₂O₂ diffuses freely in all directions, the orientation or configuration of the cell culture does not influence its cytotoxicity, as expected. Similarly, the cell viability decreases with increasing concentration of nano-Ag and is independent of cell culture orientation. This is also expected, as the cytotoxicity from nano-Ag of this size is dominated by Ag⁺ ions that are released from its surface (Pratsinis et al., 2013; Sotiriou et al., 2014; Sotiriou et al., 2012; Sotiriou et al., 2011) and diffuse throughout the solution.

5. Conclusions

The effect of nanoparticle settling during standard *in vitro* cytotoxicity evaluation was studied with commercially-available aerosol-made SiO₂ nanoparticles of 14 - 30 nm average primary particle diameter and agglomerate diameter distributions of similar shape and mode (in the range 160 to 260 nm). The agglomerate settling in suspension was quantified by means of UV-vis spectroscopy. Large particles settled faster and appeared more cytotoxic than small ones for given nominal SiO₂ surface area concentrations in standard upright cell culture configuration. When the deposited SiO₂ mass or area was calculated it was found that the cytotoxicity of SiO₂ strongly depends on the surface area of particles that have settled onto cells regardless of primary particle size. This was confirmed by the use of an inverted cell culture configuration where all silicas showed much lower cytotoxicity due to the lower actual dose in contact with the cells. These data quantitatively demonstrate the importance of settling for *in vitro* studies of aerosol-made, fractal-like SiO₂ nanoparticles. Furthermore, this study highlights a simple methodology for *in vitro* dosimetry considerations in nanotoxicology studies, by combining settling measurements with cytotoxicity evaluation in inverted-upright cell cultures.

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Fig. 1. a) Nominal and measured specific surface area (SSA) of fumed silicas from two commercial producers, P1 (open symbols) and P2 (filled symbols), along with the corresponding average primary particle diameter, d_p (upper abscissa). The error bars correspond to the standard deviation (SD) of, at least, three measurements. Insets: representative TEM images of P1 silicas with their characteristic filamentary or fractal-like morphology. b) Agglomerate size distributions of P1 and P2 silicas in serum-free cell culture medium by dynamic light scattering (DLS). The error bars correspond to the SD of one or three sets of 3 - 5 replicate measurements.

Fig. 2. a) Absorbance at 350 nm of P1 SiO_2 suspensions (initially 1000 $\mu\text{g/mL}$) in serum-free cell culture medium at 23 $^\circ\text{C}$ as a function of time (representative measurements are shown for each d_p). The light beam was aligned to monitor the absorbance 3 mm below the free surface of the suspensions (inset). b) Settled SiO_2 fraction, f_s (circles), after 24 h out of the monitored suspension layer in (a) and calculated average settling rate of agglomerates, V_{sa} (triangles), as a function of d_p for P1 (open symbols) and P2 (filled symbols) SiO_2 . The error bars correspond to the SD of, at least, two independent measurements.

Fig. 3. Viability (%) of RAW 264.7 murine macrophages determined by MTS assay after 24 h incubation with P1 (open symbols) and P2 (filled symbols) SiO₂ suspensions in standard cell culture configuration (upright, 96-well plates), as a function of: (a-b) nominal SiO₂ mass (a) and surface area (b) concentrations in serum-free cell culture medium and (c-d) calculated deposited SiO₂ mass (c) and surface area (d) at the bottom of each well within 24 h. Present data (circles) in (a) are in excellent agreement with Panas et al. (2013) (diamonds) for d_p = 14 nm. The error bars correspond to the SD of, at least, three sets of 4 - 6 replicate measurements. Statistically significant (p < 0.05) reduction in cell viability compared to the control (0 µg/mL SiO₂) was observed at all concentrations for d_p = 14 (P1) and 30 nm (P1) and at concentrations ≥ 25 µg/mL for 18 nm and 30 nm (P2) and ≥ 50 µg/mL for 14 nm (P2). Inset in (c): simplified schematic of standard upright cell culture configuration.

Fig. 4. Viability (%) of RAW 264.7 murine macrophages determined by MTS assay, after 24 h incubation with P1 SiO₂ suspensions in inverted (open bars) and upright (shaded bars, same data as in Fig. 3a) cell culture configuration for d_p of 14 (a), 18 (b) and 30 nm (c). For inverted configuration: error bars correspond to the SD of, at least, three sets of 2 - 3 replicate measurements, while statistically significant ($p < 0.05$) reduction in cell viability compared to the control (0 μg/mL SiO₂) was observed at concentrations ≥ 75 μg/mL for 14 nm (a) and at 100 μg/mL for 18 nm (b). Inset in (a): simplified schematic of inverted cell culture configuration.

Fig. 5. Relative difference in cell viability (Viab.) between inverted and modified upright cell culture configurations as a function of nominal SiO₂ mass concentration for P1 SiO₂ of various d_p.

Fig. 6. Viability (%) of RAW 264.7 murine macrophages determined by MTS assay, after 24 h incubation with the positive controls, H₂O₂ solutions and nanosilver suspensions, in both inverted (open bars) and modified upright (shaded bars) cell culture configurations, at various concentrations in serum-free cell culture medium. The error bars correspond to the SD of, at least, three sets of 2 - 3 replicate measurements.