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Role of size and surface area for pro-inflammatory responses to silica nanoparticles in epithelial lung cells: Importance of exposure conditions

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ABSTRACT

The present study compared non-crystalline silica particles of nano (50 nm)- and submicro (500 nm)-size (Si50 and Si500) for the potential to induce cytokine responses in bronchial epithelial lung cells (BEAS-2B). The cell cultures were exposed to equal mass and surface area concentrations of the two particles in different exposure media; LHC-9 and DMEM:F12. The state of agglomeration was different in the two media; with marked agglomeration in LHC-9 and nearly no agglomeration in DMEM:F12. On a mass basis, Si50 was more potent than Si500 in inducing cytokine responses in both exposure media. In contrast, upon exposure to similar surface area concentrations, Si500 was more potent than Si50 in DMEM:F12. This might be due to different agglomeration/sedimentation properties of Si50 versus Si500 in the two media. However, influence of differences in particle reactivity or particle uptake cannot be excluded. The data indicated no qualitative changes in the cytokine gene-expression patterns induced by the two particles, suggesting effects through similar mechanisms. These aspects might be of importance for interpretation of *in vitro* studies of nanomaterials.

1. Introduction

The production of different engineered nanoparticles (NPs), defined as particles with a size less than 100 nm in one dimension, has increased dramatically (Nohynek et al., 2010). The large varieties of NPs are used in a wide range of applications, from consumer products to medical devices (Moghimi et al., 2005). However, with the increasing use of NPs concerns have been raised with regard to the potential adverse impacts of NPs exposure on human health. A major task is to acquire knowledge about characteristics important for inducing adverse health effects of NPs, to minimize the need for testing every single type of NP. Toxicological effects of NPs are now intensively investigated in both *in vivo* and *in vitro* systems (Eom and Choi, 2009; Lin et al., 2006; Napierska et al., 2009; Warheit et al., 2007).

The importance of the relative large surface area of nano-sized particles compared to larger-sized particles has received a lot of attention, showing an apparent linear relationship between cellular responses and increasing surface area. Thus, the greater potency of different nano-sized particles (including polystyrene, SiO_2, TiO_2, MnO_2, $BaSO_4$, carbon black) compared to similar, but larger particles tends to disappear in comparisons of equal surface areas, as shown both by *in vitro* and *in vivo* studies (Brown et al., 2001; Duffin et al., 2007; Lison et al., 1997; Monteiller et al., 2007; Napierska et al., 2009; Oberdorster et al., 1994; Sager et al., 2008; Singh et al., 2007; Stoeger et al., 2006; Tran et al., 2000; Waters et al., 2009). Overall, this indicates particle surface area to be an important metric. In most studies, however, the importance of particle surface area has been assessed by re-calculating results obtained from exposure with equal mass concentrations of particles with different sizes, and not by actually exposing to equal particle surface area concentrations. This implies that comparisons often involve extrapolation of effects obtained by low surface area concentrations of large particles to effects obtained by high surface area concentrations of small particles, based on the assumption of linear concentration-effect relationships. Such an approach involves considerable uncertainties, in particular since concentrations-effect curves seldom are truly linear.

Studies with different quartz particles (nanoscale- and fine quartz) by Warheit et al. (2009, 2007), indicate that the toxicity of different particles is more dependent upon particle surface activity effects than particle size and surface area. Notably, both *in vitro* and *in vivo*, cells encounter NPs embedded in a protein corona, that might profoundly alter their surface reactivity, their agglomeration state, uptake in cells and their cellular responses, including inflammatory responses, cytotoxicity and DNA-damage (Ehrenberg et al., 2009; Lundqvist et al., 2008). Thus, the responses to nanoparticles seem highly dependent on exposure conditions, such as cell culture media (Veranth et al., 2008). Moreover, it is a crucial question whether particles in the nano-size area might possess inherently different material/molecular properties than larger

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particles of similar chemical composition, and might interact differently with cells due to size-dependent variations in particle uptake or receptor interactions.

The rapid increase in development and use of different nanomaterials represents a considerable challenge from a toxicological perspective. Development of screening strategies to identify potential hazards has been suggested, in which *in vitro* cell-based assays are likely to play a central part (Oberdorster et al., 2005). *In vitro* tests are both much more cost- and time-efficient than animal studies. In addition, efforts to reduce and replace animal studies favor use of cell culture models. The most commonly used *in vitro* cell models are the submerged cell cultures. A considerable proportion of what we know about particle toxicity derives from studies on submerged cells. However, reports suggest that the choice of cell culture media may influence particle-induced effects (Veranth et al., 2008).

In the present study we have compared the potential of a nano-sized non-crystalline silica particle (nominal size 50 nm, Si50) and a larger submicro silica particle (nominal size 500 nm, Si500) of similar composition, to induce cytokine responses in a bronchial epithelial cell line (BEAS-2B). The importance of particle size and surface area were examined with emphasis on whether choice of cell culture media may affect the outcome, by exposure to both equal mass and equal surface area concentrations in two different cell culture media. The possibility that the nano-sized silica particles (Si50) might induce a qualitatively different cytokine gene expression pattern compared to the larger silica particles (Si500) was also investigated.

2. Methods

2.1. Preparation of particle samples

Two different kinds of silica particles were used. One 50 nm silica nanoparticle, Si50 (GK 1890843-02) and one silica submicro particle, Si500 (GK 0850343T). The silica particles were provided by Kisker-Biotech (Kisker-Biotech GmbH & Co, Steinfurt, Germany). Stock solutions were made by diluting the particles in distilled water to a concentration of 2–5 mg/ml. The particles were sonicated and PBS (final dilution 1×) and BSA (final concentration 0.15%) were added, according to Bihari et al. (2008).

2.2. Transmission Electron Microscopy (TEM)

The morphology and size of the silica particles were assessed by TEM using a Jeol JEM 1220 (Tokyo, Japan). The sizes of the particles in aqueous media were determined by diluting the stock solution in water or in LHC-9 medium (final concentration 100 μg/ml), and pipetting 50 μl of these suspensions on formvar-coated 200 mesh copper TEM-grids. The droplets were left to dry at 30 °C for 10 min, and then the grids analyzed. Pictures of the silica particles were acquired by a digital camera connected to the instrument.

2.3. Dynamic light scattering (DLS)

The hydrodynamic sizes of silica particles were analyzed by a Zetasizer-nano (Malvern Instruments Ltd., UK) according to the manufacturer's instruction. DLS-measurements were performed by diluting the particles (final concentration 100 μg/ml) in sterile water, or in different culture media instantly after dispersion and after 1, 2 and 20 h. The samples containing the particles were kept at 37 °C between the measurements.

2.4. The particle surface area

The surface areas of the silica particles were determined by the single-point Brunauer, Emmett and Teller (BET) procedure on a customized apparatus developed according to literature specification (Thomas and Thomas, 1996).

2.5. Zeta-potentials

The charge of the silica particles was measured in water, using the Zetasizer-nano. The zeta-potentials measurements were performed instantly after diluting the particles to a concentration of 100 μg/ml in sterile water.

2.6. Cell culture and exposure conditions

BEAS-2B cells, a SV-40-transformed human bronchial epithelial cell line (European Collection of Cell Cultures (ECACC), Salisbury, UK) were maintained in LHC-9 medium (Gibco, Life Technologies, NY14072, USA) in collagen (pureCol™)-coated flasks (Advanced BioMatrix, Inc, California 92150-2403, USA) in a humified atmosphere at 37 °C with 5% CO_2, with refreshment of medium every second day. Two days prior to exposure, BEAS-2B cells (passages 8–50) cells were plated into collagen (pureCol™)-coated 35 mm 6-well culture dishes (300.000 cells per well) in LHC-9. For the cytokine experiments the cells were exposed to similar mass (0–200 μg/ml) and to similar surface area concentration (130 cm^2/ml) of the silica particles for 24 h. The mRNA experiments were performed after 6 h exposure to 200 μg/ml of Si50 and to 200 and 1440 μg/ml of Si500. The high concentration of Si500 (1440 μg/ml) corresponds to a surface area of 130 cm^2/ml, which is similar as 200 μg/ml for Si50. For some of the experiments the culture media was changed to DMEM:F12 (Gibco, Life Technologies, NY14072, USA) without any serum supplement one day prior to exposure.

2.7. Cytokine analysis

Cell culture media were collected and centrifuged at 300×*g* to remove cell debris and at 8000×g to remove floating silica particles. IL-6 and IL-8 protein levels were determined by sandwich ELISA according to the manufacturer's guidelines (Biosource International, Camarillo, CA, USA). Absorbance was measured and quantified by a plate reader (TECAN Sunrise) equipped with a dedicated software (Magellan V 1.10).

2.8. Cell viability

The cytotoxicity was evaluated by lactate dehydrogenase (LDH) release. The LDH concentration was measured in media after 20 h of exposure, and compared to maximal release (high control, cells exposed to 2% Triton X-100 in media for 1 h). The calculations of percentage cytotoxicity were performed by using the absorbance values (Abs) from the sample, background control (media) and high control in following equation:

$$\text{Cytotoxicity}\ (\%) = (\text{Abs sample} - \text{Abs background})/(\text{Abs high control} - \text{Abs background}) * 100$$

The measurements were done according to the manufactures (Roche, Germany).