Published in final edited form as:

Nanotoxicology. 2013 June ; 7: 353–366. doi:10.3109/17435390.2012.662249.

Influence of simulated gastro-intestinal conditions on particle-induced cytotoxicity and interleukin-8 regulation in differentiated and undifferentiated Caco-2 cells

Kirsten Gerloff^{1,4}, Dora I.A. Pereira², Nuno Faria², Agnes W. Boots¹, Julia Kolling¹, Irmgard Förster³, Catrin Albrecht¹, Jonathan J. Powell², and Roel P.F. Schins^{1,*}

¹Particle Research, IUF - Leibniz Research Institute for Environmental Medicine, Düsseldorf, Germany

²Biomineral Research Group, MRC Human Nutrition Research, Elsie Widdowson Laboratory, Cambridge, UK

³Molecular Immunology, IUF - Leibniz Research Institute for Environmental Medicine, Düsseldorf, Germany

⁴Immunity, Infection and Inflammation Program, Mater Medical Research Institute and the University of Queensland, Mater Health Services, South Brisbane, QLD, Australia

Abstract

Novel aspects of engineered nanoparticles offer many advantages for optimizing food products and packaging. However, their potential hazards in the gastrointestinal tract require further investigation. We evaluated the toxic and inflammatory potential of two types of particle that might become increasingly relevant to the food industry, namely SiO₂ and ZnO. The materials were characterized for their morphology, oxidant generation and hydrodynamic behavior. Cytotoxicity and interleukin-8 mRNA and protein expression were evaluated in human intestinal Caco-2 cells. Particle pre-treatment under simulated gastric and intestinal pH-conditions resulted in reduced acellular ROS formation but did not influence cytotoxicity (WST-1 assay) or IL-8 expression. However, the differentiation status of the cells markedly determined the cytotoxic potency of the particles. Further research is needed to determine the *in vivo* relevance of our current observations regarding the role of particle aggregation and the stage of intestinal epithelial cell differentiation in determining the hazards of ingested particles.

Keywords

SiO₂; ZnO; Caco-2; differentiation; digestion

Introduction

The production of functional foods is a fast growing field and the use of inorganic food additives therein, as well as in conventional foods, is widely accepted. Several microparticulate metal oxides have been used as food coloring agents (e.g. E171, titanium

*Corresponding author: Roel P. F. Schins, PhD, IUF - Leibniz Research Institute for Environmental Medicine, Auf'm Hennekamp 50, D-40225 Düsseldorf, Germany, tel: + 49 - 211 - 3389 - 269, fax: + 49 - 211 - 3389 - 331, roel.schins@uni-duesseldorf.de.

Declaration of interest

The authors would like to report no conflict of interests. The authors are entirely responsible for the content and writing of the manuscript.

dioxide; E172, iron oxides and hydroxides), acidity regulators (e.g. E530, magnesium oxide) or anti-caking agents (e.g. E551, silicon dioxide) for many years ([homepage food.gov.uk](http://homepage.food.gov.uk)). Currently, the use of similar mineral oxides, in smaller nanoparticulate sizes, is also receiving much interest as “nano-sizing” alters the properties of the materials and offers new applications for the food industry. Indeed, ZnO is already attracting attention in nanoparticulate form for the development of novel food packaging products due to its antimicrobial and UV-absorbent properties (Chaudhry *et al.*, 2008). Furthermore, amorphous SiO₂ is not only known as a food additive and found, in part, in its nanoform in milk powders, instant soups etc, but also widely used in cosmetics and pharmaceutical products (Chaudhry *et al.*, 2008; Schmid and Riediker, 2008; Dekkers *et al.*, 2010). An average Western diet provides a large intake of various mineral oxide microparticles, typically 10¹²-10¹⁴ particles per person per day (Lomer *et al.*, 2001 and 2004). Recently, Dekkers and colleagues estimated a “worst case scenario” intake of nanosilica as 124 mg/day (Dekkers *et al.*, 2010). Thus, nanoparticle intake is becoming significant as food applications continue to grow.

The altered properties of nano-sized materials is generating some concern as it is well recognized that their highly increased specific surface area enhances their reactivity and may similarly, therefore, influence aspects of their toxicity and inflammatory potential (reviewed in Oberdörster *et al.*, 2005; Borm *et al.*, 2006). Certain nanoparticles (especially those based on reactive metals e.g. Fe, Zn etc.) proffer a unique capability to generate reactive oxygen species (ROS) such as superoxide (O₂^{•-}), hydroxyl radicals (HO[•]) or hydrogen peroxide (H₂O₂) with the associated induction of cellular oxidative stress (Oberdörster *et al.*, 2005 and 2007; Xia *et al.*, 2006; Unfried *et al.*, 2007). This ROS formation can be triggered both directly by the specific surface chemistry of particles (Brown *et al.*, 2001; Stoeger *et al.*, 2009) or indirectly, for example by acting on ROS-producing cellular components such as membrane-bound NADPH oxidase enzymes and mitochondria (Unfried *et al.*, 2007). In turn, ROS formation and oxidative stress are implicated in the inflammatory effects of nanoparticles by activating pro-inflammatory signaling cascades. Particle-induced inflammation is typically characterized by the recruitment and activation of ROS producing phagocytic cells including monocytes and neutrophils, which exacerbates oxidative stress and tissue damage (Schins and Knaapen, 2007). Herein, IL-8 is considered to play a key role, as it is a major neutrophil recruiting chemokine (Kunkel *et al.*, 1991; Mitsuyama *et al.*, 1994).

Toxic effects of particles are widely described in the lung, whereas considerably fewer data are available for the gastrointestinal tract. The human intestine undergoes constant regeneration, whereupon undifferentiated enterocytes, generated from stem cells, migrate from the crypts to the villus (small intestine) or the surface epithelium (colorectum) within 5 days. On their way, they begin to differentiate and become postmitotic before undergoing apoptosis followed by cell shedding (Ramachandran *et al.*, 2000; Sancho *et al.*, 2004). The Caco-2 cell line is derived from colonic epithelial adenocarcinoma cells. When grown under normal cell culture conditions, and after reaching confluence, they differentiate into small intestine enterocytes as the cells become polarized and express many of the characteristics of these enterocytes (e.g. tight junctions, microvilli and membrane transporters) (Chantret *et al.*, 1988; Chopra *et al.*, 2010). Therefore this cell line finds a use as a model for *in vitro* studies looking at uptake of nutrients and pharmaceuticals. Without phenotypic differentiation these cells still resemble human colonic cancer cells in a wide range of their gene expression patterns (Säaf *et al.*, 2007). Both the differentiated and the undifferentiated cells are frequently used for toxicity studies (undifferentiated cells: Velarde *et al.*, 1999; Gerloff *et al.*, 2009, 2012; Rawlison *et al.*, 2010; differentiated cells: Wahl *et al.*, 2008; Pfaller *et al.*, 2010), although the differences in both cell models might lead to differential cellular responses towards toxins.

The uptake of nanoparticles can occur via different mechanisms (reviewed in Unfried *et al.*, 2007) and varies with the nature of the material and also the cell type. The gastrointestinal tract features Peyer's Patch lymphoid tissue, rich in M-cells which are specialized and differentiated epithelial cells highly capable of taking up particles (reviewed in Powell *et al.*, 2010). Other mechanisms must also be relevant as, for example, previous work has shown that 40 % of 60 nm polystyrene particles that were taken up could be recovered in non-lymphoid tissue of Sprague-Dawley rats after 5 days of gavage and, presumably, therefore, mainly entering via regular enterocytic epithelial cells (Hillery *et al.*, 1994).

Recently, we have investigated the cytotoxic and DNA damaging potential of various types of nanoparticles in undifferentiated Caco-2 cells (Gerloff *et al.*, 2009; 2012). Amongst other materials, we included amorphous SiO₂ as well as a ZnO powder with a large specific surface area. Both particle types were toxic to undifferentiated Caco-2 cells (Gerloff *et al.*, 2009). Therefore, in the current study we have compared effects in differentiated and undifferentiated cells and assessed the additional impact of simulated gastrointestinal digestion of particles prior to cellular exposure. Emphasis was also placed on a thorough characterization of the morphology, solubility and ROS-generating capacity of the particles, as well as on the measurement of their hydrodynamic size distribution characteristics under the conditions of cell treatment.

Methods

Materials

Two types of particle were used in the present study: amorphous SiO₂ (fumed; Sigma, Germany) with a mean primary diameter of 14 nm and specific surface area (SSA) of 200 m²/g and ZnO (Nanoscale Materials Inc., US) with a reported crystallite size of 10 nm and SSA 70 m²/g. The ZnO sample was kindly provided by Dr. Duffin (University of Edinburgh) who reported a SSA of 48.3 m²/g by independent analysis (personal communication). Trypsin, Dulbecco's Ca²⁺/Mg²⁺-free phosphate buffered saline (PBS), ethylenediaminetetraacetic acid disodium salt dehydrate (EDTA), para-nitrophenyl phosphate (pNPP), glycine, ZnCl₂, MgCl₂, BCA-assay kit (bicinchoninic acid- and copper(II) sulphate solution), hydrogen peroxide (H₂O₂), nitric acid (HNO₃), deferoxamine (DFO), sucrose, fetal calf serum (FCS) and bovine serum albumin (BSA) were all purchased from Sigma (Germany). Minimum essential Medium (MEM) with Earle's salts was obtained from Invitrogen (Germany). DC Protein Assay kit and iScript cDNA Synthesis Kit were purchased from BioRad Laboratories (USA). RNeasy Mini kit and QuantiFast SYBR Green PCR Kit were obtained from Qiagen (Germany). Calibration standard solutions (zinc or silicon solution 1000 ppm) were obtained from Fisher Scientific (UK), 1-hydroxy-3-carboxy-pyrrolidine (CPH) was purchased from Alexis Biochemicals (Germany). Cell Proliferation Reagent WST-1 and Lactate dehydrogenase (LDH) Cytotoxicity Detection Kit were purchased from Roche (Germany) and human IL-8 ELISA kit was obtained from Sanquin (The Netherlands). Spartan Syringe filters 0.2 µm were purchased from Whatman (Germany), ultrafiltration units (Vivaspin, 3kDa MWCO) were obtained from Sartorius Stedim Biotechnology (Germany). Interleukin-1β (IL-1β) was obtained from R&D Systems (USA). All other chemicals were from Merck (Germany). All assay kits were used according to the manufacturer's protocol.

Morphological characterization

The morphology of the particles was evaluated by transmission electron microscopy (TEM). The powders were suspended in ethanol and a drop of the resultant suspension was placed and allowed to dry on a 300-mesh copper grid for analysis on a FEI Philips CM100 TEM at 80 kV.

Characterization of particle suspensions

The freshly-prepared particle suspensions were analyzed using elemental mass analysis for both their soluble and insoluble (i.e. particulate) fractions. The latter was further subdivided into fractions < 200 nm, containing individual primary particles and/or small aggregates (generated by fusion of primary particles) and/or small agglomerates (undispersed clusters of aggregates) (Donaldson *et al.*, 2005), and fractions above 200 nm, consisting of large aggregates and/or agglomerates. Therefore, samples of (i) the initial particle suspension (1 mg/ml serum free cell culture media), (ii) the suspension after 10 min of sonication as detailed below, and (iii) the final dilution as used in the cell culture experiments (i.e. 20 µg/cm²), were filtered through a 0.2 µm syringe filter followed by ultrafiltration with a 3 kDa unit. This approach was applied to the original particles as well as to those that were subjected to the digestion assay according to the procedure described below. Each fraction was analyzed by inductively coupled plasma optical emission spectrometry (ICP-OES) for quantitative evaluation of elemental Si or Zn following overnight digestion in concentrated HNO₃.

Inductively coupled plasma optical emission spectrometry (ICP-OES)

Samples were diluted in 5% HNO₃ and their elemental content was analyzed using a JY2000-2 ICP Optical Emission Spectrometer (Jobin Yvon Horiba). External calibrations (0.1-20 ppm) were carried out for the determination of Si (251.611 nm) and Zn (202.551 nm).

Determination of hydrodynamic size

The hydrodynamic particle size of the sonicated materials was determined by dynamic light scattering (DLS), using a Zetasizer Nano-ZS (Malvern Instruments, UK). DLS determines the size of small nanoparticles (down to 0.5 nm diameter) by measuring their Brownian motion and relating it to their particle sizes. Due to the nature of the measurement, DLS is only appropriate for samples free of sedimenting particles (e.g. removed by centrifugation) and typically less than a few hundred nanometers. The hydrodynamic particle size of the large fraction was determined by static light diffraction (SLD), using a Mastersizer 2000 (Malvern Instruments, UK). SLD determines the angle at which light from a laser is scattered by particles and relates it to particle size. This technique is not appropriate for small nanoparticles but, instead, can accurately determine the size of larger aggregates/agglomerates. Both size measurements were reported in volume, which can be related to the mass of particles, rather than intensity, which is an instrumental output of limited experimental relevance.

These measurements were performed only for the native ZnO and SiO₂ suspensions immediately following their sonication (SLD) or following sonication and before and after centrifugation at 200 g for 5 min (DLS).

Culture and treatment of the cells

The human colon adenocarcinoma cell line, Caco-2, was obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, ACC 169) GmbH, Germany, and grown in MEM with Earlé's salts and Non Essential Amino Acids, supplemented with 20 % FCS, 1 % L-glutamine and 30 IU/ml penicillin-streptomycin. For the experiments, cells were trypsinized at near confluence and 4×10⁴ cells per cm² were seeded into 6-well tissue culture plates (for ELISA and qRT-PCR) or 96-well tissue culture plates (for the WST-1 assay) and grown overnight (undifferentiated cells) or for 10 days (differentiated cells). Prior to the experiments, the cells were incubated overnight (20 h) in serum free medium. All experiments were performed between cell passages 5 to 30. The SiO₂ and ZnO particles

were suspended in serum-free culture medium. Two treatments were applied to the cells: (a) the suspension was sonicated (Sonorex TK52 water-bath; 60 Watt, 35 kHz) for 10 min and then directly added to the cells at the indicated concentrations or (b) particles were pre-incubated under simulated gastrointestinal pH-conditions (see below) and then applied to the cells.

Alkaline phosphatase activity assay

Alkaline phosphatase activity is a well-known marker of Caco-2 cell differentiation (Chantret *et al.*, 1988). Cells were grown for either 1 or 10 days and harvested upon thorough washing with PBS using a lysis buffer containing 50 mM Tris-HCl (pH 7.0) and 1 % Triton X-100. Alkaline phosphatase activity was then determined as described by Nemoto *et al.* (2009), with a total reaction time of 60 minutes. Total amount of protein was determined using the commercially available BCA (bicinchoninic acid) protein assay according to the manufacturer's protocol (Sigma). Data were performed from 5 independent samples per group and are expressed as alkaline phosphatase activity (mU/mg).

Particle uptake

Cells were incubated in triplicate wells with 10, 20 or 40 $\mu\text{g}/\text{cm}^2$ particles at 37 °C for 1 h. The cell monolayer was washed three (SiO_2) or four (ZnO) times with DPBS containing 2 mM EDTA to remove loosely bound (i.e. removable) particles. The content of Si and Zn in the wash solutions was monitored by ICP-OES. Only background Si levels were detected in the wash solution from the second wash onwards, whereas ~6 % of Zn was still found recovered within the 4th wash solution (data not shown). For the latter, further wash steps were not undertaken as we wished to prevent detachment of the cells. Two milliliters $\text{HNO}_3/\text{H}_2\text{O}_2$ was added to the cell monolayer to lyse the cells overnight. After homogenization by shaking, the Si or Zn content was determined by ICP-OES. To ensure that particles were not taken up by cells due to abnormal permeability of membrane damaged cells the LDH content of the media supernatants, after treatment, was determined using a commercial diagnostic kit according to the manufacturer's instructions (LDH leakage in each treatment < 5 %; data not shown). Additionally, triplicate wells were lysed with 2 ml 0.5 M NaOH for 15 min and homogenized by shaking for determination of total cell protein by the DC (detergent compatible) protein assay. Particle uptake was expressed in μg of Si or Zn per mg of cell protein.

Uptake inhibition

Two independent methods were used to inhibit particle uptake by the differentiated Caco-2 cells. For the inhibition of active energy-dependent particle uptake by destruction of microtubules, cells were incubated at 4 °C with the particles for 1 h on an ice slab in the incubator and washed with ice cold buffer. To block clathrin AP2-mediated endocytosis, cells were treated in hypertonic serum free cell culture medium with 0.45 M sucrose for 1 h. After the incubation period the particle uptake was determined as described above.

Simulation of gastrointestinal digestion

The SiO_2 and ZnO particles were used either directly after their suspension in serum-free medium and subsequent sonication (hereafter referred to as native particles, i.e. SiO_2 and ZnO) or, alternatively, were pre-treated under gastro- and intestinal pH-conditions (referred to hereafter as "digestion simulated" (DS) particles, $\text{SiO}_2\text{-DS}$ and ZnO-DS). The digestion simulated particles were not sonicated throughout the whole procedure to mimic physiological conditions as closely as possible. The SiO_2 and ZnO particles were incubated at a concentration of 1 mg/ml for 30 min in 25 ml gastric solution (34 mM NaCl / HCl solution, pH 2.7). This approach was used to mimic the gastric pH upon ingestion of a small

meal containing particles as food additives. Following this, a 3.75 ml sample was taken for characterization of dispersability of the particles (solubility and aggregation/agglomeration) as described above, the remaining suspension was neutralized to pH 7.5 with 2.5 ml intestinal solution, (a carbonate/bicarbonate buffer [50 mM] at pH 9.5 including 1.68 g of NaCO_3 , 7.16 g NaHCO_3 and 4 g NaCl in 2 l H_2O) and incubated for a further 30 min. After the second incubation period the suspensions were characterized for dispersability as before. The simulated digestion was conducted at 37 °C with constant shaking. Finally, the samples were appropriately diluted in serum-free media to obtain the final particle suspension that Caco-2 cells were incubated with.

ROS measurements by Electron Paramagnetic Resonance (EPR)

For detection of reactive oxygen species (ROS) formation induced by the particles, 20 and 80 $\mu\text{g}/\text{cm}^2$ of SiO_2 , ZnO , $\text{SiO}_2\text{-DS}$ and ZnO-DS particles were preincubated for 30 or 60 minutes with the spin probe 1-hydroxy-3-carboxy-pyrrolidine (CPH) (0.5 mM in 10 μM DFO) for specific superoxide detection and analyzed using a MiniScope MS200 Spectrometer (Magnetech, Berlin, Germany) at room temperature. The following instrumental settings were used: Magnetic field: 3360 G; sweep width: 97 G; scan time: 60 sec; number of scans: 1; modulation amplitude: 2000 mG. Data shown are calculated from the average peak amplitude of CPH characteristic triplet spectrum and expressed in arbitrary units (AU).

Cytotoxicity

Cytotoxicity was determined by the tetrazolium salt WST-1 for measurement of the metabolic activity of the cells. The cleavage of the tetrazolium salt WST-1 to formazan dye via mitochondrial dehydrogenases in the cells was measured using a commercial WST-1 diagnostic kit (Roche, Germany) in 96-well culture plates. Cells were incubated with 0.3125, 1.25, 5, 20 and 80 $\mu\text{g}/\text{cm}^2$ native or digestion simulated particles for 4 and 24 h as described above. After the incubation period with the particles, the cells were incubated for a further 2 h (undifferentiated Caco-2 cells) or 30 min (differentiated cells) with WST-1 and analyzed as per the manufacturer's protocol. The reduced incubation time of the assay reagent for the differentiated cells, being metabolically more active, was required to avoid saturation effects in the spectrophotometric analysis. The particle types used in our current study have been carefully evaluated for potential assay interferences with various toxicity tests, and artefacts could be excluded for the WST-1 assay (Wilhelmi *et al.*, in press).

Enzyme-linked immunosorbent assay (ELISA)

To measure IL-8 release, cells were treated with 20 $\mu\text{g}/\text{cm}^2$ native or digestion simulated particles or the positive control IL-1 β (10 ng/ml) for 4 and 24 h. Cell culture medium was collected, centrifuged (6000 g, 5 min at 4 °C) and supernatants were frozen at -20 °C until analysis. IL-8 activity was determined by a commercial ELISA kit according to the supplier's recommendations (Sanquin, The Netherlands).

RNA isolation and quantitative Real-Time reverse-transcription polymerase chain reaction (qRT-PCR)

Cells were treated with 20 $\mu\text{g}/\text{cm}^2$ native or digestion simulated particles for 4 h as described above. After the treatment period, cell monolayers were washed two times with PBS. Total RNA was isolated using a commercial RNeasy kit according to the manufacturer's instructions and 0.5 μg per sample was transcribed into complementary DNA using iScript cDNA Synthesis Kit according to the protocol. Total amount of mRNA was analyzed in the Rotor-Gene Q real-time cycler (Qiagen) in 47 cycles, using 7.5 μl QuantiFast SYBR Green PCR Kit, 25 ng cDNA and 0.5 μM primers. Human β -Actin was

used as the internal reference gene. Primer sequences for *IL-8* were 5'-ACT CCA AAC CTT TCC ACC C-3' (forward) and 5'-CCC TCT TCA AAA ACT TCT CCA C-3' (reverse), and for *β-Actin* 5'-CCC CAG GCA CCA GGG CGT GAT-3' (forward) and 5'-GGT CAT CTT CTC GCG GTT GGC CTT GGG GT -3' (reverse).

Statistics

All means were calculated from three independent experiments and each condition was investigated in triplicate wells in each experiment. The error bars represent standard deviation (SD). Analysis of statistical significance was done by unpaired Student's t-test with * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ as levels of significance. Cytotoxicity data were analyzed using ANOVA with Dunnett post-hoc comparison with * $p < 0.01$ and ** $p < 0.001$. TC_{50} values were analyzed by log-linear regression.

Results

The morphology of the SiO_2 and ZnO particles was evaluated by TEM (Figure 1). Both materials tended to aggregate/agglomerate. ZnO was composed of near-spherical and needle-like particles (Figure 1B), whereas SiO_2 consisted of spherical particles only (Figure 1A).

Although the above could have been explained by drying artefacts for TEM analysis, when suspended in cell culture medium, both particle types were still found to appear as aggregates/agglomerates; soluble material varied between 1 and 7 % and negligible quantities were found in the < 200 nm fraction (Figure 2A). The presence of large aggregates/agglomerates in the post-sonicated suspension was further confirmed by DLS and SLD measurement. Rapid sedimentation of particles was observed by DLS measurement (Figure 2B). Therefore, the suspension was centrifuged and size distribution of particles in the supernatant was evaluated. For SiO_2 a peak is present at 80 nm, whereas no small particles were recovered in the ZnO supernatant (Figure 2C). As a result, accurate dynamic light scattering measurements were not possible for ZnO and only limited for SiO_2 , since DLS is based on the determination of Brownian motion and requires the absence of sedimenting particles in the sample. Consequently, size analysis was repeated using SLD, which revealed a peak at approximately 50 μm for SiO_2 and at about 9 μm for ZnO (Figure 2D).

The capability of differentiated Caco-2 cells to take up the particles within one hour incubation was assessed by ICP-OES analysis (Figure 3). Both SiO_2 and ZnO appeared to be taken up in a dose dependent manner, but on a mass basis ZnO uptake was much higher than for SiO_2 (Figure 3A, B). Particle uptake was not apparently due to increased cell permeability as, after the 1 h incubation, none of the doses tested induced significant leakage of LDH (< 5 %) or, therefore, disruption of the cell membrane (data not shown). To investigate possible uptake mechanisms, the particle-treated cells were either incubated on ice to reduce microtubule-dependent endocytosis or incubated with 0.45 M sucrose, since hypertonic conditions are known to inhibit clathrin AP2-mediated endocytosis (Figure 3C, D). The incubation on ice caused a slight reduction of the SiO_2 uptake, although this did not reach statistical significance. There was no reduction of the ZnO uptake when the cells were incubated at 4 °C. Similarly, the hypertonic conditions did not significantly influence the uptake of either type of particle. Taken together these data indicate that, besides uptake, considerable outer cell membrane adhesion occurred, especially for ZnO, which could not be fully removed by washing.

To simulate gastrointestinal digestion processes, which could potentially alter the toxicity of particles after their oral intake, we developed an *in vitro* assay. The amount of Si and Zn was

determined in samples collected during all steps of this assay, namely, particle treatment in gastric buffer, the additional treatment in neutralized intestinal buffer and the final diluted particle suspensions used for the cellular experiments (Figure 4). The agglomeration patterns of the final dilutions of the digestion simulated particles (SiO₂-DS and ZnO-DS; Figure 4) were similar to those of the native particles (SiO₂ and ZnO; Figure 2A), implying little alteration of aggregation/agglomeration. Surprisingly, treatment under gastric pH conditions did not strongly increase solubility of the ZnO particles used in this study.

To determine the effect of the simulated digestion on the surface reactivity of both types of particle, their ability to generate superoxide anion radicals (O₂^{•-}) was determined by EPR Spectroscopy using the spin probe CPH. Figure 5A shows the time- and concentration-dependent potential of both the native and the digestion simulated SiO₂ and ZnO particles to generate O₂^{•-}. Interestingly, the formation of superoxide was significantly reduced after simulated digestion of both particles. Representative spectra of CPH control, digestion simulated and native SiO₂ (80 µg/cm², 60 min incubation with CPH) are depicted in Figure 5B.

The differentiation status of the Caco-2 cells was evaluated by alkaline phosphatase activity evaluation, a well-known marker of differentiation of this cell line and regularly used to prove enterocyte differentiation (Chantret *et al.*, 1988; Speckmann *et al.*, 2011). A highly significant difference in enzyme activity was observed between the proliferating cells that were used 1 day post seeding in comparison to the cells that were grown for 10 days (Figure 6), hence confirming the differentiated status of the later cells.

To investigate whether the simulated gastric and intestinal digestion had an influence on the toxic potency of the particles, we performed the WST-1 assay after incubation of Caco-2 cells with the native and digestion simulated particle suspensions. These experiments were performed both in undifferentiated and differentiated Caco-2 cells in order to address the possible impact of cell differentiation status on particle-induced toxicity. The cytotoxic responses induced by the native and digestion simulated particles in the undifferentiated versus the differentiated cells were evaluated after 4 h as well as 24 h treatment. Results for the undifferentiated cells (i.e. at 70-80 % confluence) are shown in Panels A and B, while effects in the differentiated cells are depicted in Panels C and D of Figures 7 (SiO₂ treatment) and 8 (ZnO treatment). SiO₂ and SiO₂-DS were both found to reduce cell viability only in undifferentiated Caco-2 cells. For the 24 h incubation, significant effects in undifferentiated Caco-2 cells were even observed at low concentrations (5 µg/cm²). In contrast, the differentiated cells were unaffected by SiO₂ or by SiO₂-DS (Figure 7). The TC₅₀ values as obtained for the different treatment regimens are shown in Table 1.

Contrary to these observations, ZnO was found to be cytotoxic to both undifferentiated and differentiated cells after 24 h incubation, although considerably more so in the former (Figure 8). The TC₅₀ values (24 h) in the undifferentiated cells were 2 ± 2 µg/cm² for both ZnO and ZnO-DS (Table 1). For the differentiated cells, significantly higher TC₅₀ values were obtained being 9 ± 1 µg/cm² and 10 ± 3 µg/cm² for ZnO and ZnO-DS, respectively. However, the TC₅₀ values did not differ between the native and the digestion simulated ZnO, indicating that toxicity of the particles was mostly dependent on cell differentiation status (Table 1). At 4 h, only the undifferentiated cells showed significantly reduced viability after treatment with ZnO, at doses of 5 µg/cm² or higher. For all conditions tested, the simulated digestion of the particles did not significantly influence their toxicity as evaluated by the WST-1 assay.

To address potential pro-inflammatory effects of the particles, the mRNA expression and protein secretion of IL-8 was determined in both undifferentiated and differentiated Caco-2

cells. Results are shown in Figure 9 for the effects of SiO₂ and SiO₂-DS and in Figure 10 for ZnO and ZnO-DS. It should be emphasized that data are expressed on a logarithmic scale for the y-axis. In the undifferentiated Caco-2 cells, SiO₂ strongly induced mRNA expression of IL-8, with levels that were similar to those provoked by the positive control (IL-1 β). The upregulation was even higher after treatment with SiO₂-DS. In differentiated cells, however, SiO₂ incubation could only induce a slight albeit non-significant upregulation of IL-8 mRNA expression. The effect was significantly lower than that induced in undifferentiated cells (Figure 9A). IL-8 protein release from the undifferentiated cells after 24 h was significantly increased after treatment with both the native and the digestion simulated SiO₂ particles, however it should be noted that at this late time point, the viability of the undifferentiated cells was strongly reduced by the SiO₂ (Figure 9B). In the differentiated cells, an increase of IL-8 protein secretion upon treatment with SiO₂ and SiO₂-DS was also observed, but this did not reach statistical significance.

Incubation with ZnO resulted only in a slight upregulation of IL-8 mRNA levels in both undifferentiated and differentiated cells (Figure 10A). Undifferentiated cells did not respond to stimulation by ZnO in terms of IL-8 secretion, which most likely is explained by the cytotoxic effect of the material at the tested concentration (i.e. 20 $\mu\text{g}/\text{cm}^2$) (Figure 10B). Remarkably however, despite this reduced metabolic activity, a marked secretion of IL-8 could still be observed in differentiated cells after 24 h incubation with ZnO and ZnO-DS. Due to the large inter-experimental variation, probably also caused by interfering cytotoxic effects, this did not reach statistical significance (note the logarithmic scale of the y-axis).

Discussion

For the present study we characterized SiO₂ and ZnO particles by several independent methods, namely TEM, fractionated filtration combined with elemental analysis, laser diffraction and EPR spectroscopy. By TEM we observed that both particle types occurred as aggregates and/or agglomerates of varying sizes. In serum free medium (used for biological testing) aggregation/agglomeration of both materials could be confirmed by fractionated filtration as well as laser diffraction. It is well-known that in (nano)particle dispersions, the actual object size can differ significantly from the size of the primary particles and their aggregates due to agglomeration processes. Notably, size distribution measurements by SLD confirmed the presence of large agglomerates in suspension. Accurate determination was possible since SLD can measure sizes from approximately 70 nm to hundreds of micrometers. In contrast, DLS, which is suitable for size measurements of small nanoparticles (down to 0.5 nm), showed immediate agglomeration. DLS requires the absence of sedimenting particles and therefore limited its suitability for the measurement of the particles used in our study. Another technical aspect that should be considered when using light diffraction techniques is the masking of small particles by larger ones. For example, the 50 μm particles observed for SiO₂ by SLD could be concealing smaller particles of a few hundred nanometers which are detected by DLS.

Aggregated and agglomerated particles retain the majority of their large combined surface area despite losing many of their size specific properties, and many of the adverse biological effects appear to be driven by the surface area of the material (Oberdörster *et al.*, 2005; Borm *et al.*, 2006; Singh *et al.*, 2007). Aggregation of particles is generally associated with a decrease in their uptake (Tiede *et al.*, 2008). Nevertheless, for many types of particles which typically occur in aggregated forms, uptake into epithelial cells has still been shown to be considerable and of biological relevance (Churg, 1996). Thus, aggregates of TiO₂ have been observed within vacuoles, vesicles and multivesiculated bodies upon treatment of human lung epithelial cells (Stearns *et al.*, 2001; Singh *et al.*, 2007). Here, we tried to determine the uptake (into and onto) of both our materials by differentiated Caco-2 cells. The proportion of

uptake appeared to be dependent on the treatment dose. Possible uptake mechanisms include adsorptive and clathrin-mediated endocytosis. Involvement of endocytotic processes was determined by applying a low incubation temperature of 4°C, and thus a lowering of the energy availability (Moriya and Linder, 2006). Clathrin-mediated endocytosis was aimed to be more specifically inhibited by introducing hypertonic conditions via addition of sucrose, preventing the interaction of clathrin with AP2 (San Martin *et al.*, 2008; Kalgaonkar and Lönnerdal, 2009). Here, only a decrease of the incubation temperature indicated a reduction in SiO₂ uptake, although, this did not reach statistical significance. Sucrose treatment did not reduce internalization of either SiO₂ or ZnO, while low temperature had no influence on ZnO uptake. This suggests that either other uptake mechanisms, that are not temperature or toxicity dependent, might play a role in the internalization of both materials, such as phagocytosis, macropinocytosis or diffusion (Unfried *et al.*, 2007), or, especially for ZnO, mainly cell membrane adherence occurs. The negative surface charge of both SiO₂ and ZnO suggests strong extracellular adhesion. In future projects, uptake of both materials into the cell needs to be further evaluated, i.e. by TEM analysis. However, this was beyond the scope of the current study.

To analyze the cytotoxic and pro-inflammatory potential of our materials upon passage through the gastrointestinal tract, we developed an *in vitro* assay based upon physiological pH conditions. The gastric pH ranges from 1.5-2.0 in the fasting state and might rise up to pH 7.0 after ingestion of a meal, while sodium and chloride are the most prominent elements within gastric acid (Powell *et al.*, 1994; Lindahl *et al.*, 1997; Hörter and Dressman, 2001). As particles are mainly used as food additives, we used a NaCl-based HCl buffer with pH 2.7, simulating the pH conditions arising upon ingestion of a light meal. It should be noted that ZnO is discussed to be rather soluble, and therefore Zn²⁺ ions are often argued to be a key factor in the particle's cytotoxic potential (Franklin *et al.* 2007; Wong *et al.* 2010). Therefore it was surprising that treatment of our material under gastric pH conditions did not strongly enhance its solubility compared to the untreated material. However, the short incubation time of 30 minutes at acidic conditions could be a limiting factor here, but is a physiologically relevant time point, as it reflects the duration of the chyme in the stomach.

To mimic the subsequent pancreatic bicarbonate output, the suspension was neutralized with a carbonate/bicarbonate buffer to a pH of 7.5, which is representative of the intestinal conditions (Evans *et al.*, 1988; Hörter and Dressman, 2001). In the final particle suspensions, both particles showed similar dispersion behavior and their dispersion was independent of the digestion simulation treatment. In terms of mass, the majority of the particles were highly aggregated/agglomerated. However, one needs to take into account that the < 200 nm fraction, although representing only a small proportion of the total particle mass, likely represents a dominant fraction in terms of particle number. For example, a hypothetical spherical particle of 10 µm diameters has the same mass of 10⁶ spherical particles of 100 nm diameters (Oberdörster *et al.*, 2005).

In contrast to size distribution and solubility, the simulated digestion of the particles markedly affected their ROS generating capacity. Using EPR, we observed a significant reduction of O₂^{•-} generation by treated particles in comparison to the native materials. This may be explained by surface modifications during acidic treatment, such as surface chemistry alterations and removal of possible contaminants including trace iron that might support O₂^{•-} formation by these materials (Fubini, 1998). Comparable effects have been reported for cobalt chrome particles, for which cyto- and genotoxic properties were found to be largely decreased upon macrophage phagocytosis, presumably due to surface modifications induced by the low pH in the lysosomes of these cells (Papageorgiou *et al.*, 2008).

Although the simulated digestion affected acellular ROS generation, it did not influence the cytotoxic potency of the particles. However, cytotoxicity was found to depend on the cell's differentiation status. To this extent, we used the WST-1 assay, which was already successfully applied in earlier studies in our lab (Gerloff *et al.*, 2009) and found feasible for both types of particles tested (Wilhelmi *et al.*, in press). SiO₂ only reduced cell viability in undifferentiated cells whereas ZnO appeared toxic to both undifferentiated and differentiated Caco-2 cells after 24 h treatment. Interestingly, the lack of early toxicity towards differentiated cells (after 4 hours) appears to be pointing to a delayed toxicity, which might be partly caused by the formation of Zn²⁺ ions over time, as for example following uptake into the lysosomes and therefore into an acidic environment (Studer *et al.*, 2010). Moreover, for differentiated cells, a higher dose of particles seems to be required to achieve comparable toxic effects on the cells. It should be noted however that upon confluence and subsequent differentiation, an approximate 3-fold higher amount of cells was counted than in undifferentiated (semi-confluent) Caco-2 cell cultures. Therefore more cells face the same amount of particles and, as such, this influences the toxic effect measured by the WST-1 assay. However, even if one considers a 3-fold adjustment of the dose (expressed per unit cell number), marked differences in sensitivity remain present for both samples (see Table 1 and Figures 7 and 8). Exemplary, the lowest concentrations of ZnO and SiO₂ which triggered significant toxicity at 4 h and 24 h, respectively differ (on a mass basis) by at least a factor 16. Thus, the observed contrasts in sensitivity are not solely explained by mere differences in cell number in both culture models, but by cell differentiation dependent contrasts in the appearance of the Caco-2 cells. Our observations stand in line with the work of Anelli and colleagues, showing a 2-fold difference in cytotoxicity to undifferentiated versus differentiated Caco-2 cells after treatment with lymphokine-activated natural killer cells (Anelli *et al.*, 1993). This suggests that the state of differentiation of these cells may influence the response to toxic agents. Recently, this hypothesis was supported by work of Speckmann and colleagues, who have shown that the extracellular antioxidant selenoprotein P was markedly upregulated in differentiated compared to semi-confluent Caco-2 cells (Speckmann *et al.*, 2010), which might protect the cells against oxidative stress-induced cell damage.

The differentiation status of intestinal epithelial cells is a constant process in the healthy human gut and known to be imbalanced in inflammatory bowel diseases (Huang *et al.*, 1997; Ramachandran *et al.*, 2000; Sancho *et al.*, 2004). Differentiation of intestinal epithelial cells has been shown to reduce their pro-inflammatory responsiveness to IL-1 β at early time points (2-12 hours) (Huang *et al.*, 1997; Böcker *et al.*, 2000). In our study we found similar effects with IL-1 β after 4 h incubation, since the induction of IL-8 mRNA expression was dramatically lower in differentiated versus undifferentiated Caco-2 cells. These contrasts in mRNA expression were not reflected on the level of secreted IL-8. Interestingly, we observed a high pro-inflammatory potential of SiO₂, predominantly in undifferentiated cells. However, it should be kept in mind that at this late time point, SiO₂ induced cytotoxicity in the undifferentiated cells only, which might interfere with the absolute protein secretion. In contrast, ZnO induced IL-8 secretion in differentiated cells only. The lack of secreted IL-8 from undifferentiated cells after 24 hours is most likely caused by the marked cytotoxicity observed at the concentration used for the IL-8 expression analyses. Here, the increased cell number of the differentiated compared to the undifferentiated cells might play a role in the differential outcomes rather than the mere state of differentiation. For our future investigations we have therefore planned to address the pro-inflammatory effects of ZnO in both cell models at equi-(non)toxic concentrations. Herein, we also aim to address the involvement of signal transduction mechanisms that are considered to regulate IL-8 secretion in Caco-2 cells (Bernotti *et al.*, 2003).

Although the gastrointestinal assay applied in our current work is a rather simplistic approach, it allows for a more realistic judgment on the hazard of ingested particles. The biological effects of the native particles were found to be rather comparable to the more physiologically treated (i.e. digestion simulated) particles. Of course, this will not necessarily be the case for other particle types that differ in terms of size, chemical composition and/or solubility. Therefore one should always consider including a “digestion protocol” when potential hazards of ingested particles are being investigated. Obviously, our current assay is limited to investigating pH effects of the gastrointestinal tract. It does not take into account the presence of proteins or various chemical or biological compounds including other endogenous or exogenous particles in the chyme, and most importantly, the mucus layer which represents the first physical and chemical barrier (McGuckin *et al.*, 2009). Physiological proteins may further alter the reactivity of particles by masking their reactive surface and hence reducing their ROS generating capacity. Finally, the intestinal environment may also support particle de-aggregation, and therefore, it is yet unknown to which extent epithelial cells will be exposed *in vivo* to the large aggregates of the particles as investigated here.

Conclusions

In conclusion, we have pointed out the necessity of using the appropriate methods for particle characterization when aggregation and/or agglomeration occur. The presented results consist of a first *in vitro* effort to address both the influence of digestion and cell differentiation on the toxic potential of nanoparticles. We have shown that the food-relevant particles SiO₂ and ZnO exerted toxic and inflammatory effects in human intestinal Caco-2 cells. These effects mainly depended on the differentiation status of the cells: undifferentiated Caco-2 cells were more sensitive towards particle toxicity and might therefore serve as an initial screening tool. However, as differentiated Caco-2 cells more closely resemble the physiological intestinal epithelium, the combination of both cell models might be the most useful tool for toxicity test batteries. In contrast, the effect of a simulated gastrointestinal digestion was negligible despite its impact on the ROS generating properties of the particles. Further research is needed to determine the relevance of the state of differentiation of intestinal epithelial cells for ingested particles *in vivo*, and future digestion assays should furthermore include the influence of key proteins and bacteria of the intestinal bacterial flora, to mimic particle ingestion as closely as possible.

Acknowledgments

This study was financially supported by a grant from the German Research Council (DFG Deutsche Forschungsgemeinschaft - Graduate College GRK-1427) and a DFG Research Fellowship (GE-2328/1-1), awarded to KG. The authors acknowledge Emma Thomas-McKay for conducting the TEM analysis of the particles. Moreover, the authors thank Dr. Klaus Unfried (IUF, Düsseldorf) for providing primers for human IL-8 and β -Actin.

KG thanks the MRC Human Nutrition Research Elsie Widdowson laboratory in Cambridge (UK) for hosting her during 3 months to conduct part of the research presented here.

References

- Anelli R, Placido R, Sambuy Y, Bach S, Di Massimo A, Colizzi V. Cytotoxic activity of human lymphocytes against differentiated intestinal tumour cell lines. *Immunology*. 1993; 78(1):166–9. [PubMed: 8436403]
- Bernotti S, Seidman E, Sinnett D, Brunet S, Dionne S, Delvin E, Levy E. Inflammatory reaction without endogenous antioxidant response in Caco-2 cells exposed to iron/ascorbate-mediated lipid peroxidation. *Am J Physiol Gastrointest Liver Physiol*. 2003; 285:G898–906. [PubMed: 12842821]

- Borm PJA, Robbins D, Haubold S, Kuhlbusch T, Fissan H, Donaldson K, Schins RPF, Stone V, Kreyling W, Lademann J, et al. The potential risks of nanomaterials: a review carried out for ECETOC. Part Fibre Toxicol. 2006; 3:11. [PubMed: 16907977]
- Böcker U, Schottelius A, Watson JM, Holt L, Licato LL, Brenner DA, Sartor RB, Jobin C. Cellular differentiation causes a selective down-regulation of interleukin (IL)-1beta-mediated NF-kappaB activation and IL-8 gene expression in intestinal epithelial cells. J Biol Chem. 2000; 275(16):12207–13. [PubMed: 10766857]
- Brown DM, Wilson MR, MacNee W, Stone V, Donaldson K. Size-dependent proinflammatory effects of ultrafine polystyrene particles: a role for surface area and oxidative stress in the enhanced activity of ultrafines. Toxicol Appl Pharmacol. 2001; 175:191–9. [PubMed: 11559017]
- Chantret I, Barbat A, Dussaulx E, Brattain MG, Zweibaum A. Epithelial polarity, villin expression, and enterocytic differentiation of cultured human colon carcinoma cells: a survey of twenty cell lines. Cancer Res. 1988; 48(7):1936–42. [PubMed: 3349466]
- Chaudhry Q, Scotter M, Blackburn J, Ross B, Boxall A, Castle L, Aitken R, Watkins R. Applications and implications of nanotechnologies for the food sector. Food Addit Contam Part A Chem Anal Control Expo Risk Assess. 2008; 25(3):241–58. [PubMed: 18311618]
- Chopra DP, Dombkowski AA, Stemmer PM, Parker GC. Intestinal epithelial cells in vitro. Stem Cells Dev. 2010; 19(1):131–42. [PubMed: 19580443]
- Churg A. The uptake of mineral particles by pulmonary epithelial cells. Am J Respir Crit Care Med. 1996; 154:1124–40. [PubMed: 8887617]
- Dekkers S, Krystek P, Peters RJ, Lankveld DX, Bokkers BG, van Hoeven-Arentzen PH, Bouwmeester H, Oomen AG. Presence and risks of nanosilica in food products. Nanotoxicol. Sep 24.2010 Epub ahead of print.
- Donaldson K, Tran L, Jimenez LA, Duffin R, Newby DE, Mills N, MacNee W, Stone V. Combustion-derived nanoparticles: a review of their toxicology following inhalation exposure. Part Fibre Toxicol. 2005; 2:10. [PubMed: 16242040]
- Evans DF, Pye G, Bramley R, Clark AG, Dyson TJ, Hardcastle JD. Measurement of gastrointestinal pH profiles in normal ambulant human subjects. Gut. 1988; 29(8):1035–41. [PubMed: 3410329]
- Franklin NM, Rogers NJ, Apte SC, Batley GE, Gadd GE, Casey PS. Comparative toxicity of nanoparticulate ZnO, bulk ZnO, and ZnCl₂ to a freshwater microalga (*Pseudokirchneriella subcapitata*): The importance of particle solubility. Environ Sci Technol. 2007; 41(24):8484–8490. [PubMed: 18200883]
- Fubini B. Surface chemistry and quartz hazard. Ann Occup Hyg. 1998; 42(8):521–30. [PubMed: 9838865]
- Gerloff K, Albrecht C, Boots AW, Förster I, Schins RPF. Cytotoxicity and oxidative DNA damage by nanoparticles in human intestinal Caco-2 cells. Nanotoxicol. 2009; 3(4):355–364.
- Gerloff K, Fenoglio I, Carella E, Kolling J, Albrecht C, Boots AW, Förster I, Schins RPF. Distinctive toxicity of TiO₂ rutile/anatase mixed phases nanoparticles on Caco-2 cells. Chem Res Toxicol. 2012 accepted for publication.
- Hillery AM, Jani PU, Florence AT. Comparative, quantitative study of lymphoid and non-lymphoid uptake of 60 nm polystyrene particles. J Drug Target. 1994; 2(2):151–6. [PubMed: 8069593]
- Hörter D, Dressman JB. Influence of physicochemical properties on dissolution of drugs in the gastrointestinal tract. Adv Drug Deliv Rev. 2001; 46(1-3):75–87. [PubMed: 11259834]
- Huang N, Katz JP, Martin DR, Wu GD. Inhibition of IL-8 gene expression in Caco-2 cells by compounds which induce histone hyperacetylation. Cytokine. 1997; 9(1):27–36. [PubMed: 9067093]
- [Accessed on 17 June 2011] <http://www.food.gov.uk/safereating/chemsafe/additivesbranch/enumberlist>
- Kalgaonkar S, Lönnerdal B. Receptor-mediated uptake of ferritin-bound iron by human intestinal Caco-2 cells. J Nutr Biochem. 2009; 20(4):304–11. [PubMed: 18602806]
- Kunkel SL, Standiford T, Kasahara K, Strieter RM. Interleukin-8 (IL-8): the major neutrophil chemotactic factor in the lung. Exp Lung Res. 1991; 17(1):17–23. [PubMed: 2013270]
- Lindahl A, Ungell AL, Knutson L, Lennernäs H. Characterization of fluids from the stomach and proximal jejunum in men and women. Pharm Res. 1997; 14(4):497–502. [PubMed: 9144738]

- Lomer MC, Harvey RS, Evans SM, Thompson RP, Powell JJ. Efficacy and tolerability of a low microparticle diet in a double blind, randomized, pilot study in Crohn's disease. *Eur J Gastroenterol Hepatol*. 2001; 13(2):101–6. [PubMed: 11246607]
- Lomer MC, Hutchinson C, Volkert S, Greenfield SM, Catterall A, Thompson RP, Powell JJ. Dietary sources of inorganic microparticles and their intake in healthy subjects and patients with Crohn's disease. *Br J Nutr*. 2004; 92(6):947–55. [PubMed: 15613257]
- McGuckin MA, Eri R, Simms LA, Florin TH, Radford-Smith G. Intestinal barrier dysfunction in inflammatory bowel diseases. *Inflamm Bowel Dis*. 2009; 15(1):100–13. [PubMed: 18623167]
- Mitsuyama K, Toyonaga A, Sasaki E, Watanabe K, Tateishi H, Nishiyama T, Saiki T, Ikeda H, Tsuruta O, Tanikawa K. IL-8 as an important chemoattractant for neutrophils in ulcerative colitis and Crohn's disease. *Clin Exp Immunol*. 1994; 96(3):432–6. [PubMed: 8004812]
- Moriya M, Linder MC. Vesicular transport and apotransferrin in intestinal iron absorption, as shown in the Caco-2 cell model. *Am J Physiol Gastrointest Liver Physiol*. 2006; 290(2):G301–9. [PubMed: 16179601]
- Nemoto E, Koshikawa Y, Kanaya S, Tsuchiya M, Tamura M, Somerman MJ, Shimauchi H. Wnt signaling inhibits cementoblast differentiation and promotes proliferation. *Bone*. 2009; 44(5):805–12. [PubMed: 19442631]
- Oberdörster G, Oberdörster E, Oberdörster J. Nanotoxicology: an emerging discipline evolving from studies of ultrafine particles. *Environ Health Perspect*. 2005; 113(7):823–39. [PubMed: 16002369]
- Oberdörster G, Stone V, Donaldson K. Toxicology of nanoparticles: a historical perspective. *Nanotoxicol*. 2007; 1:2–25.
- Papageorgiou I, Shadrick V, Davis S, Hails L, Schins R, Newson R, Fisher J, Ingham E, Case CP. Macrophages detoxify the genotoxic and cytotoxic effects of surgical cobalt chrome alloy particles but not quartz particles on human cells in vitro. *Mutat Res*. 2008; 643(1-2):11–9. [PubMed: 18614183]
- Pfaller T, Colognato R, Nelissen I, Favilli F, Casals E, Ooms D, Leppens H, Ponti J, Stritzinger R, Puentes V, Boraschi D, Duschl A, Oostingh GJ. The suitability of different cellular in vitro immunotoxicity and genotoxicity methods for the analysis of nanoparticle-induced events. *Nanotoxicol*. 2010; 4(1):52–72.
- Powell JJ, Whitehead MW, Lee S, Thompson RPH. Mechanisms of gastrointestinal absorption: dietary minerals and the influence of beverage ingestion. *Food Chem*. 1994; 51:381–388.
- Powell JJ, Faria N, Thomas-McKay E, Pele LC. Origin and fate of dietary nanoparticles and microparticles in the gastrointestinal tract. *J Autoimmun*. 2010; 34(3):J226–33. [PubMed: 20096538]
- Ramachandran A, Madesh M, Balasubramanian KA. Apoptosis in the intestinal epithelium: its relevance in normal and pathophysiological conditions. *J Gastroenterol Hepatol*. 2000; 15(2):109–20. [PubMed: 10735533]
- Rawlinson LA, O'Brien PJ, Brayden DJ. High content analysis of cytotoxic effects of pDMAEMA on human intestinal epithelial and monocyte cultures. *J Control Release*. 2010; 17(1):84–92. [PubMed: 20457190]
- San Martin CD, Garri C, Pizarro F, Walter T, Theil EC, Núñez MT. Caco-2 intestinal epithelial cells absorb soybean ferritin by mu2 (AP2)-dependent endocytosis. *J Nutr*. 2008; 138(4):659–66. [PubMed: 18356317]
- Sancho E, Batlle E, Clevers H. Signaling pathways in intestinal development and cancer. *Annu Rev Cell Dev Biol*. 2004; 20:695–723. [PubMed: 15473857]
- Sääf AM, Halbleib JM, Chen X, Yuen ST, Leung SY, Nelson WJ, Brown PO. Parallels between global transcriptional programs of polarizing Caco-2 intestinal epithelial cells in vitro and gene expression programs in normal colon and colon cancer. *Mol Biol Cell*. 2007; 18(11):4245–60. [PubMed: 17699589]
- Schins RP, Knaapen AM. Genotoxicity of poorly soluble particles. *Inhal Toxicol*. 2007; 19(Suppl 1): 189–98. [PubMed: 17886067]
- Schmid K, Riediker M. Use of nanoparticles in Swiss Industry: a targeted survey. *Environ Sci Technol*. 2008; 42(7):2253–60. [PubMed: 18504950]

- Singh S, Shi T, Duffin R, Albrecht C, van Berlo D, Höhr D, Fubini B, Martra G, Fenoglio I, Borm PJ, Schins RP. Endocytosis, oxidative stress and IL-8 expression in human lung epithelial cells upon treatment with fine and ultrafine TiO₂: role of the specific surface area and of surface methylation of the particles. *Toxicol Appl Pharmacol.* 2007; 222(2):141–51. [PubMed: 17599375]
- Speckmann B, Pinto A, Winter M, Förster I, Sies H, Steinbrenner H. Proinflammatory cytokines down-regulate intestinal selenoprotein P biosynthesis via NOS2 induction. *Free Radic Biol Med.* 2010; 49(5):777–85. [PubMed: 20542496]
- Speckmann B, Bidmon HJ, Pinto A, Anlauf M, Sies H, Steinbrenner H. Induction of glutathione peroxidase 4 expression during enterocytic cell differentiation. *J Biol Chem.* 2011; 286(12):10764–72. 25. [PubMed: 21252226]
- Stearns RC, Paulauskis JD, Godleski JJ. Endocytosis of ultrafine particles by A549 cells. *Am J Respir Cell Mol Biol.* 2001; 24(2):108–15. [PubMed: 11159043]
- Stoeger T, Takenaka S, Frankenberger B, Ritter B, Karg E, Maier K, Schulz H, Schmid O. Deducing in vivo toxicity of combustion-derived nanoparticles from a cell-free oxidative potency assay and metabolic activation of organic compounds. *Environ Health Perspect.* 2009; 117:54–60. [PubMed: 19165387]
- Studer AM, Limbach LK, Van Duc L, Krumeich F, Athanassiou EK, Gerber LC, Moch H, Stark WJ. Nanoparticle cytotoxicity depends on intracellular solubility: comparison of stabilized copper metal and degradable copper oxide nanoparticles. *Toxicol Lett* 1. 2010; 197(3):169–74.
- Tiede K, Boxall AB, Tear SP, Lewis J, David H, Hasselov M. Detection and characterization of engineered nanoparticles in food and the environment. *Food Addit Contam Part A Chem Anal Control Expo Risk Assess.* 2008; 25(7):795–821. [PubMed: 18569000]
- Unfried K, Albrecht C, Klotz LO, von Mikecz A, Grether-Beck S, Schins RPF. Cellular responses to nanoparticles: target structures and mechanisms. *Nanotoxicol.* 2007; 1:52–71.
- Velarde G, Ait-Aissa S, Gillet C, Rogerieux F, Lambre C, Vindimian E, Porcher JM. Use of the caco-2 model in the screening of polluting substance toxicity. *Toxicol In Vitro.* 1999; 13(4-5):719–22. [PubMed: 20654540]
- Wahl B, Daum N, Ohrem HL, Lehr CM. Novel luminescence assay offers new possibilities for the risk assessment of silica nanoparticles. *Nanotoxicol.* 2008; 2(4):243–251.
- Wilhelmi V, Fischer U, van Berlo D, Schulze-Osthoff K, Schins RP, Albrecht C. Evaluation of apoptosis induced by nanoparticles and fine particles in RAW 264.7 macrophages: Facts and artefacts. *Toxicol In Vitro.* Dec 14.2011 Epub ahead of print.
- Wong SW, Leung PT, Djurisi AB, Leung KM. Toxicities of nano zinc oxide to five marine organisms: Influences of aggregate size and ion solubility. *Anal Bioanal Chem.* 2010; 396(2):609–618. [PubMed: 19902187]
- Xia T, Kovochich M, Brant J, Hotze M, Sempf J, Oberley T, Sioutas C, Yeh JJ, Wiesner MR, Nel AE. Comparison of the abilities of ambient and manufactured nanoparticles to induce cellular toxicity according to an oxidative stress paradigm. *Nano Lett.* 2006; 6:1794–807. [PubMed: 16895376]

Figure 1. Morphological characterisation of silica dioxide and zinc oxide particles
Transmission Electron Microscopy images of (A) SiO₂ and (B) ZnO.

Figure 2. Characterisation of particle suspensions

SiO₂ and ZnO particles were characterised for aggregate/agglomerate formation and solubility in serum free cell culture medium via fractionated filtration and quantitative element analysis (A). Final dilution = particle concentration used for cell incubation (20 μg/cm²). Size distribution of post-sonicated SiO₂ and ZnO measured by DLS before (B) and after centrifugation (C) as well as size distribution (post-sonication) measured by Mastersizer (D). Values in A are expressed as mean and standard deviation, n=2-3.

Figure 3. Particle uptake and uptake inhibition in Caco-2 cells

The uptake and adhesion of SiO_2 (A) or ZnO (B) was determined in differentiated Caco-2 cells after treatment for 1 h at 10, 20 and 40 $\mu\text{g}/\text{cm}^2$. Inhibition of uptake of SiO_2 (C) or ZnO (D) particles was determined after 20 $\mu\text{g}/\text{cm}^2$ treatment by incubation at 4°C (ice) or introducing hypertonic conditions (sucrose, B). Values are expressed as mean and standard deviation, n=3.

Figure 4. Particle characterisation after gastro-intestinal pH-treatment

SiO₂ and ZnO particles were characterised for aggregate/agglomerate formation and solubility via fractionated filtration and quantitative element analysis after incubation in gastric pH-conditions followed by intestinal pH-conditions. Final dilution = particle concentration used for cell incubation (20 µg/cm²) in serum free cell culture medium. Values are expressed as mean and standard deviation, n=2-3.

Figure 5. Acellular ROS formation by SiO₂ and ZnO particles

The formation of superoxide anion radicals was determined by EPR after 30 and 60 min pre-incubation with the spin probe CPH. 20 and 80 µg/cm² native or digestion simulated SiO₂ or ZnO were used (A). Representative spectra of CPH-control, 80 µg/cm² native or digestion simulated SiO₂ after 60 min pre-incubation (B). Values are expressed as mean and standard deviation, n=3.

* p < 0.05, ** p < 0.01 and *** p < 0.001

Figure 6. Alkaline phosphatase activity measured in undifferentiated and differentiated Caco-2 cells as marker for enterocytic differentiation

n=5.

** $p < 0.01$

Figure 7. Effects of SiO_2 on the viability of undifferentiated and differentiated Caco-2 cells
 Undifferentiated (A, B) and differentiated (C, D) Caco-2 cells were incubated with 0.3125, 1.25, 5, 20 and 80 $\mu\text{g}/\text{cm}^2$ native or digestion simulated SiO_2 particles for 4 (A, C) and 24 (B, D) hours. Metabolic competence of the cells was determined via conversion of WST-1 as a marker of cell viability. Values are expressed as mean and standard deviation, n=3.
 * p < 0.01 and ** p < 0.001 versus control for native particles
 # p < 0.01 and ## p < 0.001 versus control for digestion simulated particles

Figure 8. Influences of ZnO on the viability of undifferentiated and differentiated Caco-2 cells Undifferentiated (A, B) and differentiated (C, D) Caco-2 cells were incubated with 0.3125, 1.25, 5, 20 and 80 $\mu\text{g}/\text{cm}^2$ native or digestion simulated ZnO particles for 4 (A, C) and 24 h (B, D). Metabolic competence was measured as conversion of WST-1 as a marker of cell viability. Values are expressed as mean and standard deviation, n=3.

* p < 0.01 and ** p < 0.001 versus control for native particles

p < 0.01 and ## p < 0.001 versus control for digestion simulated particles

Figure 9. Effects of SiO₂ particles on IL-8 mRNA expression and protein secretion after treatment of undifferentiated and differentiated Caco-2 cells

Induction of IL-8 mRNA after 4 h incubation with 20 $\mu\text{g}/\text{cm}^2$ native or digestion simulated SiO₂ was determined in undifferentiated and differentiated Caco-2 cells via qRT-PCR (A). The secretion of IL-8 after 4 and 24 h incubation with 20 $\mu\text{g}/\text{cm}^2$ native or digestion simulated SiO₂ was determined in undifferentiated and differentiated Caco-2 cells by ELISA (B). IL-1 β (10 ng/ml) was used as a positive control. Values are expressed as mean and standard deviation, n=3.

* p < 0.05, ** p < 0.01 and *** p < 0.001 versus control

+ p < 0.05 and ++ p < 0.01 native versus digestion simulated particles

p < 0.05 and ## p < 0.01 differentiated versus undifferentiated cells

Figure 10. Effects of ZnO particles on IL-8 mRNA expression and protein secretion after treatment of undifferentiated and differentiated Caco-2 cells

Induction of IL-8 mRNA after 4 h incubation with 20 $\mu\text{g}/\text{cm}^2$ native or digestion simulated ZnO was determined in undifferentiated and differentiated Caco-2 cells via qRT-PCR (A). The total secretion of IL-8 after 4 and 24 h incubation with 20 $\mu\text{g}/\text{cm}^2$ native or digestion simulated ZnO was determined in undifferentiated and differentiated Caco-2 cells by ELISA (B). IL-1 β (10 ng/ml) was used as a positive control. Values are expressed as mean and standard deviation, n=3.

* $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ versus control

++ $p < 0.01$ native versus digestion simulated particles

$p < 0.05$ differentiated versus undifferentiated cells

Table 1

		SiO ₂		ZnO	
		undifferentiated	differentiated	undifferentiated	differentiated
4 h	native particles	> 80	> 80	19 ± 4	> 80
	DS-particles	> 80	> 80	14 ± 2	> 80
24 h	native particles	9 ± 6	> 80	2 ± 2	9 ± 1
	DS-particles	10 ± 4	> 80	2 ± 2	10 ± 3

TC50 values of SiO₂ and ZnO in undifferentiated and differentiated Caco-2 cells upon treatment for 4 and 24 hours. Values are expressed as mean $\mu\text{g}/\text{cm}^2 \pm$ standard deviation, n=3.