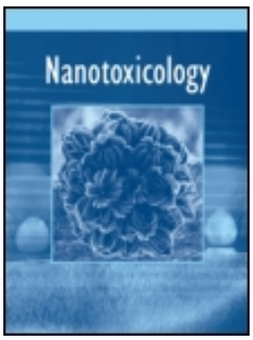

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Genotoxicity evaluation of amorphous silica nanoparticles of different sizes using the micronucleus and the plasmid *lacZ* gene mutation assay

MARGRIET V. D. Z. PARK[1,2], HENNY W. VERHAREN[1], EDWIN ZWART[1], LYA G. HERNANDEZ[1], JAN VAN BENTHEM[1], ANDREAS ELSAESSER[3], CLIFFORD BARNES[3], GEORGE MCKERR[3], C. VYVYAN HOWARD[3], ANNA SALVATI[4], ISEULT LYNCH[4], KENNETH A. DAWSON[4], & WIM H. DE JONG[1]

[1]*Laboratory for Health Protection Research, National Institute for Public Health and the Environment (RIVM), Bilthoven, The Netherlands,* [2]*Department of Health Risk Analysis and Toxicology, Maastricht University, Maastricht, The Netherlands,* [3]*Nano Systems Biology, Centre for Molecular Bioscience, University of Ulster, Coleraine, UK, and* [4]*Centre for BioNano Interactions, School of Chemistry and Chemical Biology, University College Dublin, Belfield, Dublin, Ireland*

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Abstract

We investigated the potential of four well-characterized amorphous silica nanoparticles to induce chromosomal aberrations and gene mutations using two *in vitro* genotoxicity assays. Transmission electron microscopy (TEM) was used to verify the manufacturer's nominal size of 10, 30, 80 and 400 nm which showed actual sizes of 11, 34, 34 and 248 nm, respectively. The 80 (34) nm silica nanoparticles induced chromosomal aberrations in the micronucleus assay using 3T3-L1 mouse fibroblasts and the 30 (34) and 80 (34) nm silica nanoparticles induced gene mutations in mouse embryonic fibroblasts carrying the *lacZ* reporter gene. TEM imaging demonstrated that the majority of nanoparticles were localized in vacuoles and not in the nucleus of 3T3-L1 cells, indicating that the observed DNA damage was most likely a result of indirect mechanisms. Further studies are needed to reveal these mechanisms and to determine the biological relevance of the effects of these particular silica nanoparticles *in vivo*.

Keywords: *Genotoxicity, nanoparticles, silica, micronucleus, mutagenic effects*

Introduction

The element silicon (Si) mainly exists as silicon dioxide (silica, SiO_2) with either amorphous or crystalline molecular structures (Figure 1 in Supplementary information available online). As the main component of sand and rocks, silica is one of the most abundant materials on earth (Merget et al. 2002). Amorphous silica can be synthetically produced and has a wide commercial use in various applications. Amorphous silica products such as gels and sols (colloidal suspensions) are manufactured for applications such as food additives and microelectronics (Barik et al. 2008). Due to its relative biocompatibility, silica has also been used in the formulation of artificial implants (Areva et al. 2007).

Since the development of nanotechnology, the nanoform of silica materials has gained interest for a wide variety of applications. Surface charge modified hydrophobic amorphous silica nanoparticles have qualified for approval as a novel form of nanobiopesticides for horticultural and crop plants (Barik et al. 2008). Silica nanomaterial is in the top five of nanomaterials explicitly referenced in nanotechnology consumer products, mostly in sporting goods and cosmetics (Hansen et al. 2008; Woodrow Wilson International Center for Scholars 2009). Their inert nature and small size make silica nanomaterials particularly useful to the pharmaceutical industry as drug carriers, bio-imaging agents and gene transfection vectors (Santra et al. 2004; Bharali et al. 2005; Gemeinhart et al. 2005; Trewyn et al. 2007;

Correspondence: Margriet Park, Laboratory of Health Protection Research, National Institute of Public Health and the Environment, PO Box 1, 3720 BA, Bilthoven, The Netherlands. Fax: +31 30 274 4446. E-mail: margriet.park@rivm.nl

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Barik et al. 2008; Slowing et al. 2008). The diversity of these applications can be anticipated to result in an increased level of exposure to a heterogeneous mixture consisting of silica nanoparticles differing in size, structure, specific surface area, shape, charge and other characteristics. Several of these characteristics have been claimed to play a role in determining the toxic effects of nanoparticles, particularly size and surface area, but there appears to be no consensus (Hamoir et al. 2003; Limbach et al. 2005; Duffin et al. 2007; Monteiller et al. 2007; Yang et al. 2009). As a consequence of not knowing which nanoparticle characteristic(s) may play a role in eliciting toxic effects, regulatory frameworks are contemplating whether the safety of all these different types of nanoparticles should be evaluated separately, or whether extrapolation of safety data from one type to another is possible. In view of the existing uncertainty a case by case approach is advocated (Scientific Committee on Emerging and Newly Identified Health Risks [SCENIHR] 2009).

Our current research is focused on investigating whether nanoparticle size might be one of those characteristics determining the toxicity of amorphous silica nanoparticles. We have previously evaluated four well-characterized amorphous silica nanoparticles of the same source in the embryonic stem cell differentiation assay (Park et al. 2009a). This assay is validated as an *in vitro* developmental toxicity test discriminating between chemicals in three classes of embryotoxicity (Genschow et al. 2004). While silica nanoparticles of 11 nm inhibited the differentiation of embryonic stem cells into contracting cardiomyocytes, those of 248 nm elicited no effect. In addition, this study included two silica nanoparticles of 34 nm with different zetapotentials, and only one of these inhibited the differentiation of embryonic stem cells into contracting cardiomyocytes, indicating that factors other than nanoparticle size contributed to the biological effects in this assay.

We have now evaluated the potential size-related effects of these same silica nanoparticles in a series of genotoxicity studies. We have previously reported that none of these silica nanoparticles elicited a response in the single cell gel electrophoresis (comet) assay using 3T3-L1 mouse fibroblasts, performed by two independent laboratories (Barnes et al. 2008). This indicator test is commonly used to assess whether chemicals induce single- or double-strand DNA breaks, which may or may not be reversible.

In the current study, we further investigated the genotoxic potential of these silica nanoparticles by performing two genotoxicity assays which are commonly used in regulatory frameworks to measure chromosomal aberrations and gene mutations; both irreversible genotoxic endpoints. The micronucleus assay detects chromosomal aberrations and was performed in 3T3-L1 mouse fibroblasts, while gene mutations were measured in mouse embryonic fibroblasts isolated from pU288 plasmid *LacZ* mice, containing *lacZ* as a reporter gene (MEF-*lacZ*). To our knowledge, these two assays have not previously been applied to test whether amorphous silica nanoparticles induce genotoxic effects.

Materials and methods

Silica nanoparticles

The spherical amorphous silica nanoparticles used in this study were obtained from Glantreo, Ltd, Cork, Ireland. The particles were synthesized via the Stöber method without any stabilizer (Stöber et al. 1968). To avoid potential interference from synthesis residues and solvents, nanoparticles were dialyzed extensively against a very large excess of pure MilliQ water. The obtained nanoparticle stock solutions tested negative for the presence of endotoxin and bacterial or fungal infection, as determined by the *Limulus Amoebocyte* Lysate (Gel-clot) assay and inoculation on Columbia sheep blood agar plates (Oxoid Ltd, Hants, UK), respectively (data not shown).

The primary size of the four tested silica nanoparticles was determined by means of Transmission Electron Microscopy. Hydrodynamic size by means of Dynamic Light Scattering (DLS) and the zetapotential in deionized water were determined with the Malvern 3000HS Zetasizer photon correlation spectrophotometer. More details on the characterization methods have been described earlier (Barnes et al. 2008).

Shortly before the start of each experiment, silica nanoparticles were thoroughly vortexed (20 s at 1500/min), diluted in distilled water and dispersed in the same cell culture medium used to routinely culture the cells, i.e., containing 10% Fetal Calf Serum.

Cell culture

The mouse embryonic fibroblast cell line 3T3-L1 was obtained from the Nofer Institute of Occupational Medicine, Lodz, Poland, and was the same cell line used for the comet assay published previously (Barnes et al. 2008). The 3T3-L1 cell line is derived from a Swiss albino mouse and to the best of our knowledge this cell line has not been genetically modified. Therefore, we expect the 3T3-L1 cell to

have a normal genetic background and to be genetically stable.

Cells were routinely cultured at 37°C and 5% CO_2 in Dulbecco's Modified Eagle Medium (DMEM), high glucose with glutamax (Gibco 61965-059) supplemented with 10% Foetal Calf Serum (Gibco 16010-159), 1% Penicillin-Streptomycin solution (10,000 U/ml/10,000 μg/ml, Gibco 15140), and 1% non-essential amino acids (Gibco 11140).

Mouse embryonic fibroblasts (MEF-*LacZ*) were isolated from 13.5 day old embryos from transgenic C57BL/6 mice containing multiple copies of the pUR288 plasmid with the bacterial *lacZ* gene as a reporter gene in their genome, as described previously (Mahabir et al. 2009). Aliquots of 3×10^6 cells in 1 ml cell culture medium were kept at −80°C for at least 24 h and were then stored in liquid nitrogen. Before the start of the experiment, cells were thawed and cultured for one week in 175 cm^2 flasks at 37°C under 10% CO_2 and 3% O_2, undergoing 1 passage. The cell culture medium used for both cell culture and experiments with MEF-*LacZ* consisted of DMEM (Gibco 31885) supplemented with 1% Modified Eagles Medium Non-Essential Amino Acids (Gibco 11140), 2% Penicillin-Streptomycin (Gibco 15070) and 10% Fetal Bovine Serum (Biocell).

Cytotoxicity in MEF-LacZ cells

The effect of the silica nanoparticles on the metabolic activity of the MEF-*LacZ* cells after 24 h exposure to silica nanoparticles was evaluated using the WST-1 cytotoxicity assay. Cells were seeded in 96-well tissue grade microtiter plates at a density of $5–8 \times 10^3$ cells per well and cultured for 24 h in a humidified atmosphere at 37°C, 10% CO_2 and 3% O_2. The following day, cells were exposed to silica nanoparticles in cell culture medium containing 10% FCS at concentrations ranging from 0.3–100 μg/ml in triplicate wells. In each cytotoxicity experiment, cells exposed to 10% distilled water or 5% DMSO served as solvent and positive controls, respectively. After 24 h exposure, cells were incubated with 10 μl Cell Proliferation Reagent WST-1 (Roche, Almere, The Netherlands) at 37°C for 3 h. The absorbance of the wells was measured using a SpectraMax® 190 scanning multiwell spectrophotometer (Molecular Devices, Sunnyvale, USA) at a wavelength of 440 nm. Data were acquired using SoftMax Pro 5 software (Molecular Devices, Sunnyvale, USA). Interference of the silica nanoparticles with the WST-1 assay was experimentally excluded, as reported previously (Park et al. 2009a). At least three independent experiments were performed.

Nanoparticle uptake in 3T3-L1 cells

To study the uptake of silica nanoparticles in 3T3-L1 fibroblasts, cells were seeded into 25 cm^2 cell culture flasks at a density of $1–2 \times 10^5$ cells/cm^2 and allowed to grow for 24 h (37°C, 5% CO_2). Subsequently, cells were exposed for 16 h to silica particles in cell culture medium containing 10% FCS at a concentration of 50 μg/ml. Control samples were not exposed to nanoparticles. After incubation with nanoparticles the cells were dislodged and centrifuged into a pellet. The pellet was chemically fixed with a pre-warmed combination of 2.5% glutaraldehyde and 2% formaldehyde prepared in 0.1 M sodium cacodylate buffer before post-fixation in 1% aq osmium tetroxide (Agar Scientific). The cells were rinsed with PBS and subsequently dehydrated in a graded ethanol series. The samples were then infiltrated in increasing proportions of Low Viscosity Spurr resin (SPI Supplies) and were allowed to polymerise at 70°C for 24 h. After polymerization of the resin block, 100–300 nm sections (depending on the nanoparticles size) were cut with a diamond knife (Diatome) using an ultramicrotome (PowerTome XL, RMC). The sections were transferred onto standard TEM grids and imaged in a Transmission Electron Microscope (Tecnai G^2 Spirit, FEI).

In vitro micronucleus test in 3T3-L1 cells

The *in vitro* micronucleus test is commonly used to evaluate whether chemicals have the potential to induce chromosomal aberrations consisting of clastogenic (chromosome breaks) or aneugenic (chromosome loss) effects. During mitosis, these chromosomes or chromosome fragments become separated from the nucleus in nuclear envelopes, which are visible as micronuclei (MN) in the cell. Exposure of the cells to the cytokinesis blocking agent cytochalasin B enables the identification of MN in binucleated cells, representing chromosomal damage. In addition, the % of cytostasis based on the cytokinesis block proliferation index (CBPI) represents a measure of cytotoxicity resulting from the treatment.

3T3-L1 cell treatment. 1×10^6 3T3- L1 cells were seeded in a volume of 5 ml cell culture medium in a 25 cm^2 tissue culture flask. The cells were incubated for 24 h at 37°C under 5% CO_2 to form a semi- confluent monolayer. Before treatment with nanoparticles, cells were washed once with Dulbecco'sphosphate-buffered saline, containing KCl, KH_2PO_4, NaCl, Na_2HPO_4 without calcium or magnesium (DPBS). Cytochalasin B (6 μg/ml) and

silica nanoparticles in cell culture medium containing 10% FCS were added simultaneously for 24 h at final concentrations of 4, 40 or 400 μg/ml in single cultures. The cell and nanoparticle test concentrations 4 and 40 μg/ml are the same as the concentrations used in the comet assay published earlier. We also included an extra concentration of 400 μg/ml to enable testing up to cytotoxic concentrations. Cells treated with distilled water and cytochalasin B served as a negative control. Bleomycin (BLM) was included as a positive control clastogen at a final concentration of 2 μg/ml. Cells were incubated for 1.5 cell cycles, i.e., 24 h.

Slide preparation and staining. After treatment, cells were harvested with trypsin (0.5%) and transferred to 15 ml vials. Cytospin preparations (5 min at 500 rpm) were made using a Cytospin 3 (Thermo Shandon Ltd, Runcorn, Cheshire, UK). The optimal cell volume for the cytospin was determined based on the cell density of a number of test slides, and was generally 100 μl. After drying, the slides were fixed with methanol (70%) for 5 min and stained for approximately 20 min with a 6% Giemsa solution.

Scoring of micronuclei. For a reliable measurement, MN were scored in at least 1000 binucleated cells for every treatment. Simultaneously, the effect of a treatment on cell division was measured by assessing the % cytostasis scored in at least 2000 cells per treatment. The % cytostasis was calculated as:

$$100 - 100 * ((CBPI_T - 1)/(CBPI_C - 1))$$

where: CBPI = ((# mononucleate cells) + (2 * # binucleate cells) + (3 * # multinucleate cells))/(Total # cells); T = nanoparticle treatment; C = solvent control. Results were obtained from three independent experiments.

LacZ gene mutation test in MEF-LacZ cells

In vitro gene mutation tests in mammalian cells are routinely used to detect gene mutations induced by chemicals. For the current experiments, embryonic fibroblasts isolated from pU288 plasmid *LacZ* mice were used because they contain multiple copies of the pUR288 plasmid with the bacterial *lacZ* gene as a reporter gene in their genome. The advantage of using these particular cells compared to other commonly used cells is that they are capable of detecting various mutagenic events, including point mutations and deletions induced by chemicals (Mahabir et al. 2009). After exposure of the cells to the test substance (silica nanoparticles in this case), plasmids are isolated from the genome and transfected into *Escherichia coli C* (Δ*lacZ*/*galE*$^-$) cells (Figure 2 in Supplementary information, available online). The *E. coli* cells are plated onto both a non-selective and a mutant selective plate. The non-selective plate contains 5-bromo-4-chloro-3-indolyl-ß-D-galactopyranoside (X-gal) and is used to determine overall plasmid rescue efficiency. Blue colonies are formed when X-gal is metabolized by ß-galactosidase present in cells containing an intact *lacZ* product from the transfected plasmid. The selective plate contains phenyl-ß-D-galactopyranoside (P-gal), which is toxic to the cells when metabolized by ß-galactosidase and subsequent enzymes. The colonies growing on the selective plate result from cells carrying a plasmid with a mutation in the *lacZ* gene, which hampers ß-galactosidase activity (Dollé et al. 1996).

Cell treatment. MEF-*lacZ* cells were seeded in 10 cm Petri dishes containing 1×10^6 cells and a final volume of 5 ml cell culture medium. The cells were incubated for 24 h at 37°C under 10% CO_2 and 3% O_2. Subsequently, the cells were washed once with DPBS and fresh cell culture medium containing 10% FCS and silica nanoparticles at concentrations ranging from 4–400 μg/ml was added in duplicate cultures per experiment. Distilled water was used as a vehicle control and N-nitroso-N-methylurea (MNU) as a positive control. After 16 h treatment, cells were collected in 1 ml phosphate buffered saline (PBS) using a cell scraper. The cell suspension was centrifuged at 1200 rpm and 4°C for 5 min and the pellet was stored at −20°C until the *lacZ* plasmid rescue assay was performed.

LacZ plasmid rescue and determination of mutant frequency. The *lacZ* plasmid rescue and determination of the mutant frequency was performed as described previously (Dollé et al. 1996). Briefly, total genomic DNA containing (parts of) the *lacZ* gene was isolated from the cells using phenol extraction, followed by digestion with the restriction enzyme HindIII. The *lacZ* containing plasmids were rescued from the digest using magnetic beads coated with the *lacZ*/*lacI* fusion protein. After elution from the beads, the plasmids were transfected into an electrocompetent *E. coli* strain C (Δ*lacZ* /*galE*−). Small fractions of the bacterial samples (dilution factor 200, 400 or 1000) were plated on non-selective X-galactosidase plates to determine the plasmid rescue efficiency. The remainder was plated on selective P-glycosidase plates to select for mutants. The *lacZ* mutant frequency was calculated by dividing the number of mutants by the total number of rescued colonies × dilution factor.

Results were obtained from at least three independent experiments.

Acellular redox activity of silica nanoparticles

The acellular redox activity of the nanoparticles was evaluated using the dithiothreitol (DTT) assay. The DTT assay determines the general redox activity of a sample by measuring the stimulus (silica nanoparticles in this case)-dependent oxidation of DTT, a dithiol. The assay measures the formation of reactive oxygen species (ROS) by a known amount of DTT. During this reaction, DTT is consumed, followed by its reaction with 5,5′-dithiobis-(2-) nitrobenzoic acid (DTNB) to form 2-nitro-5-mercaptobenzoic acid which can be measured by a spectrophotometer.

Silica nanoparticles (10 μg) were incubated with 100 nM DTT in a 0.5 M potassium phosphate buffer at pH 7.4 for 0, 15, 30 or 45 min at 37°C with a total volume of 1 ml per time-aliquot and transferred to an equal volume of cold 10% trichloroacetic acid solution after the indicated incubation time. Aliquots were transferred to 1 ml 0.4 M Tris-HCl buffer (pH 8.9) containing 25 μl 10 mM 5,5′-dithiobis-(2-) nitrobenzoic acid (DNTB) and the absorbance was read at 412 nm on a spectrophotometer within 30 min. Results were obtained from two independent experiments.

Generation of ROS in MEF-LacZ cells

ROS are generated by cells as by-products of normal cellular activity, but an increase in ROS in response to stress factors may lead to an overload of the antioxidant capacity, and may result in cell damage. Non-fluorescent 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCF-DA) can be used to measure the generation of ROS. Upon entering the cell, the acetate groups are cleaved off, and the remaining compound H_2DCF remains inside the cell where it is oxidized by reactive species to the fluorescent compound DCF.

MEF-*LacZ* cells were seeded in 96-well tissue grade microtiter plates at a density of 5×10^3 cells per well and cultured in a humidified atmosphere at 37°C, 3% O_2 and 10% CO_2. When the wells had reached approximately 80% confluence, cell culture medium was removed and cells were exposed to 100 μl of nanoparticle suspension in cell culture medium containing 10% FCS (0.3–100 μg/ml) for 4 h, in triplicate wells. The 4 h time point was selected after pilot studies using exposure periods ranging from 15 min to 24 h, and is in agreement with many other published studies using this assay (reviewed by Park et al. 2009b). In each experiment, cells exposed to 10% distilled water served as solvent control. Cells exposed to cell culture medium containing 10 μg/ml lipopolysaccharide (LPS) and 10 μg/ml phorbol myristate acetate (PMA) served as a positive control.

The stock solution of H_2DCF-DA (40 μM in DMSO) was diluted 4,000 times with PBS to yield a 10 μM working solution. After 4 h exposure to silica nanoparticles, the cell culture medium was removed, cells were washed with warm PBS and incubated with freshly prepared 10 μM H_2DCF-DA probe in PBS for 45 minutes at 37°C, 3% O_2 and 10% CO_2, while protected from light. Subsequently, probe that was not taken up by the cells was removed by washing the cells with warm PBS. A final aliquot of 100 μl of PBS was added to each well and fluorescence was measured at an excitation wavelength of 485 nm and an emission wavelength of 520 nm using a FLUOstar OPTIMA microplate reader (BMG LABTECH, Offenburg, Germany). At these wavelengths, silica nanoparticles alone in PBS did not affect fluorescence. Results were obtained from at least two independent experiments.

Data analysis

Data from the *LacZ* gene mutation test in MEF-*LacZ* cells were analyzed by means of the non-parametric Kruskall-Wallis test and Mann-Whitney test where appropriate. Data from the micronucleus test were analyzed by means of the Pearson's Chi square test. Data from the WST-1 test were analyzed using PROAST software version 23.2 (RIVM, Bilthoven, The Netherlands) (Slob 2002). The following exponential dose-response model was fitted to the data: $y = a^{\star}\exp(bx)$, where y denotes the metabolic activity of the cells, and x the concentration of the test compound. Experiment ($n = 3$) was defined as a covariate for a in the model. The IC_{20} was defined as the silica nanoparticle concentration resulting in a 20% decrease of metabolic activity as compared to the solvent control group.

Results

Particle characterization

The silica nanoparticles were specified by the manufacturer as having diameters of 10, 30, 80 and 400 nm. However, TEM measurements revealed that the diameters of the 80 and 400 nm deviated considerably from these specifications, i.e., 34 and 248 nm, respectively (Table I). Therefore, the TEM measured sizes will be included in the designation of the particles,

Table I. Characterization of silica nanoparticles shortly after dispersion in deionized water.

			In deionized water	
Nominal size [nm]	TEM[a] size [nm]	Zeta potential ζ [mV]	DLS[b] size [nm]	PDI[c]
10	11.0 (2.2)	−43.3 (11.8)	103.1 (1.9)	0.792 (0.011)
30	33.7 (4.0)	−33.7 (3.0)	77.9 (1.1)	0.259 (0.094)
80	33.7 (6.1)	−10.6 (0.9)	65.9 (1.7)	0.374 (0.068)
400	247.9 (24.1)	−49.1 (0.5)	269.0 (7.1)	0.049 (0.020)

Values given are mean (standard deviation). [a]Transmission Electron Microscopy measurements are the average of at least 100 nanoparticles; [b]Dynamic Light Scattering (DLS) measurements are the average of at least 5 runs each containing 10 sub-measurements. [c]PDI = polydispersity index.

i.e., 10 (11) nm, 30 (34) nm, 80 (34) nm and 400 (248) nm hereafter. The 30 (34) nm and the 80 (34) nm particles had significantly different zeta-potentials, so both materials were included in the assays. Furthermore, the polydispersity index and dynamic light scattering measurements revealed that the 10 (11) nm silica nanoparticles were highly aggregated in distilled water.

Silica nanoparticle uptake in 3T3-L1 cells

TEM images of 3T3-L1 cells show that silica nanoparticles of all sizes tested were taken up into the cells after 16 h of exposure to 50 μg/ml. Nanoparticles appeared to be mainly located in vesicles and although these vesicles were sometimes detected near the cell nucleus, no nanoparticles were actually observed in the cell nucleus (Figure 1).

In vitro micronucleus test in 3T3-L1 cells

The purpose of the micronucleus test is to identify chromosome aberrations by measuring the formation of micronuclei (MN) containing lagging chromosome fragments (clastogenic effects) or whole chromosomes (aneugenic effects). These chromosomal fragments are not able to migrate to the spindle poles during mitosis (nuclear division). A nuclear envelope forms around (each of) the chromosome fragment and is noticeable as a small micronucleus.

As indicated by the increased cytostasis, cell proliferation was significantly inhibited in 3T3- L1

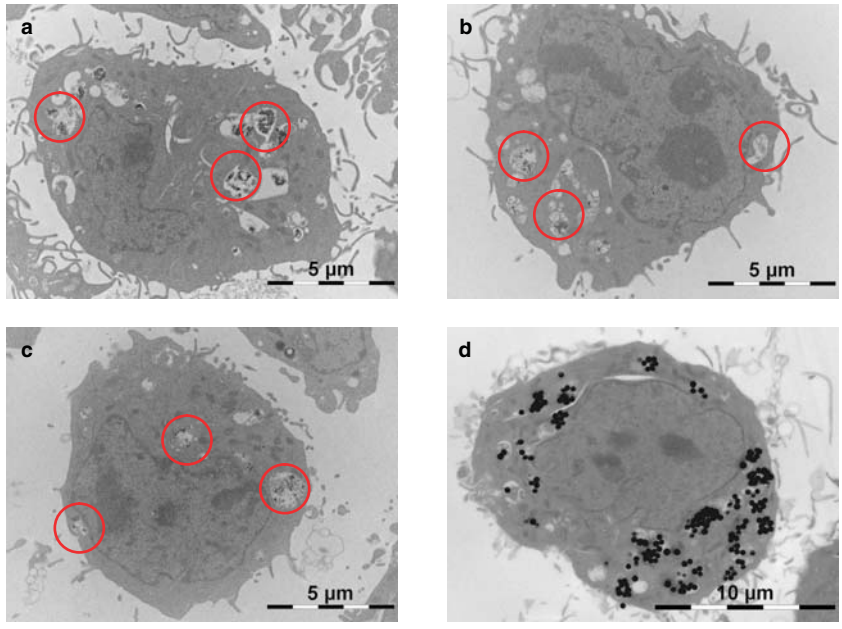

Figure 1. TEM images demonstrating uptake of silica nanoparticles of (a) 10 (11) nm, (b) 30 (34) nm, (c) 80 (34) nm and (d) 400 (248) nm by 3T3-L1 cells.

cells at the highest test concentration of 400 μg/ml for all silica nanoparticle sizes, but not at lower concentrations (Figure 2). At non-cytotoxic concentrations, a minor but statistically significant and dose-related increase in binucleated cells with MN was observed in cells exposed to the 80 (34) nm silica nanoparticle only. Exposure to the positive control bleomycin resulted in an approximately four-fold increase in binucleated cells with MN compared to the solvent control in every experiment (data not shown).

LacZ gene mutation test in MEF-LacZ cells

Gene mutations such as point mutations or base shifts are not detected by the micronucleus assay. Therefore, a gene mutation test was performed in mouse embryonic fibroblasts (MEF-*lacZ*) containing *lacZ* as a reporter gene. This test is capable of detecting various mutagenic events, including point mutations and chromosomal rearrangements (Mahabir et al. 2009).

Silica particles of 10 (11) and 400 (248) nm did not induce an increase in mutant frequencies (Figure 3). A dose related increase in mutant frequency was observed in MEF-*LacZ* cells exposed to silica nanoparticles of 30 (34) nm and 80 (34) nm, which was statistically significant for the 30 (34) nm only. Compared to the solvent control, the increase was at most three-fold for the 30 (34) nm particle and two-fold for the 80 (34) nm particle. The positive control MNU induced a seven-fold increase in mutant frequency compared to the solvent control (data not shown).

Cytotoxicity in MEF-LacZ cells

To determine whether the observed genotoxic effects of 30 (34) and 80 (34) nm silica nanoparticles occurred at concentrations below cytotoxic concentrations, a WST-1 assay was performed. Metabolic activity of MEF-*LacZ* cells was slightly decreased after exposure to 30 (34) nm silica nanoparticles (Figure 4). Modelling of the WST-1 dose response data in MEF-*LacZ* cells showed that metabolic activity of exposed cells was decreased to 80% of that of control cells at a concentration of 85 μg/ml (Figure 5).

Redox potential and generation of reactive oxygen species

The potential contribution of reactive oxygen species to the observed increase in the occurrence of mutant frequencies in MEF-*LacZ* cells after exposure to 30 (34) nm and 80 (34) nm silica nanoparticles was investigated by determining the induction of ROS both in an acellular and in a cellular system. The metabolism of H_2DCF by reactive oxygen species in MEF-*LacZ* cells exposed to 30 (34) nm and 80 (34) nm silica nanoparticles was not increased at concentrations up to 100 μg/ml (Figure 6). However, the positive control (10 μg/ml LPS and 10 μg/ml PMA) only induced a two-fold increase in ROS, indicating the assay was not very sensitive in this cell type. The metabolism of DTT in the acellular system was not increased by any of the silica nanoparticles (data not shown).

Discussion and conclusion

To investigate potentially size-dependent effects of silica nanoparticles in genotoxicity assays, we aimed to include four different sizes of nanoparticles. However, characterization of the particles by electron microscopy revealed that the measured diameter of the nanoparticle specified as 80 nm by the manufacturer was in fact 34 nm, similar to the nanoparticle specified as 30 nm which also measured 34 nm. The manufacturer's nominal sizes are based on the expected size from the synthesis recipe, and are as such no more than guidance sizes. The nanoparticles had significantly different surface charges, so both materials were included in the genotoxicity assays and will be referred to as 30 (34) nm and 80 (34) nm nanoparticles hereafter.

An important issue recently re-emphasized is that genotoxic effects are not relevant at high cytotoxic concentrations, as high levels of cytotoxicity may induce chromosomal damage as a secondary effect (Rossman 2009). For the micronucleus assay, cytotoxicity in 3T3-L1 cells was determined by assessing cytostasis for each treatment, which provides an accurate method of quantifying the effect on cell proliferation and the cytotoxic activity of a treatment. At the highest concentration tested (400 μg/ml) of each silica nanoparticle, cytostasis was significantly increased compared to solvent control, indicating high cytotoxicity at this concentration. Cytostasis at the other test concentrations was not affected, confirming earlier results using a variety of cell viability tests of the same 30 (34), 80 (34) and 400 (248) nm silica nanoparticles in the same 3T3-L1 cells, as reported together with the comet assay (Barnes et al. 2008). In the absence of cytotoxicity, the 4 and 40 μg/ml test concentrations were relevant for genotoxicity evaluation.

Cytotoxicity of silica nanoparticles in MEF-*lacZ* cells was also low or absent at concentrations up to 100 μg/ml when determined with the WST-1 assay. However, the WST-1 and analogous assays used to assess cytotoxicity have been claimed to

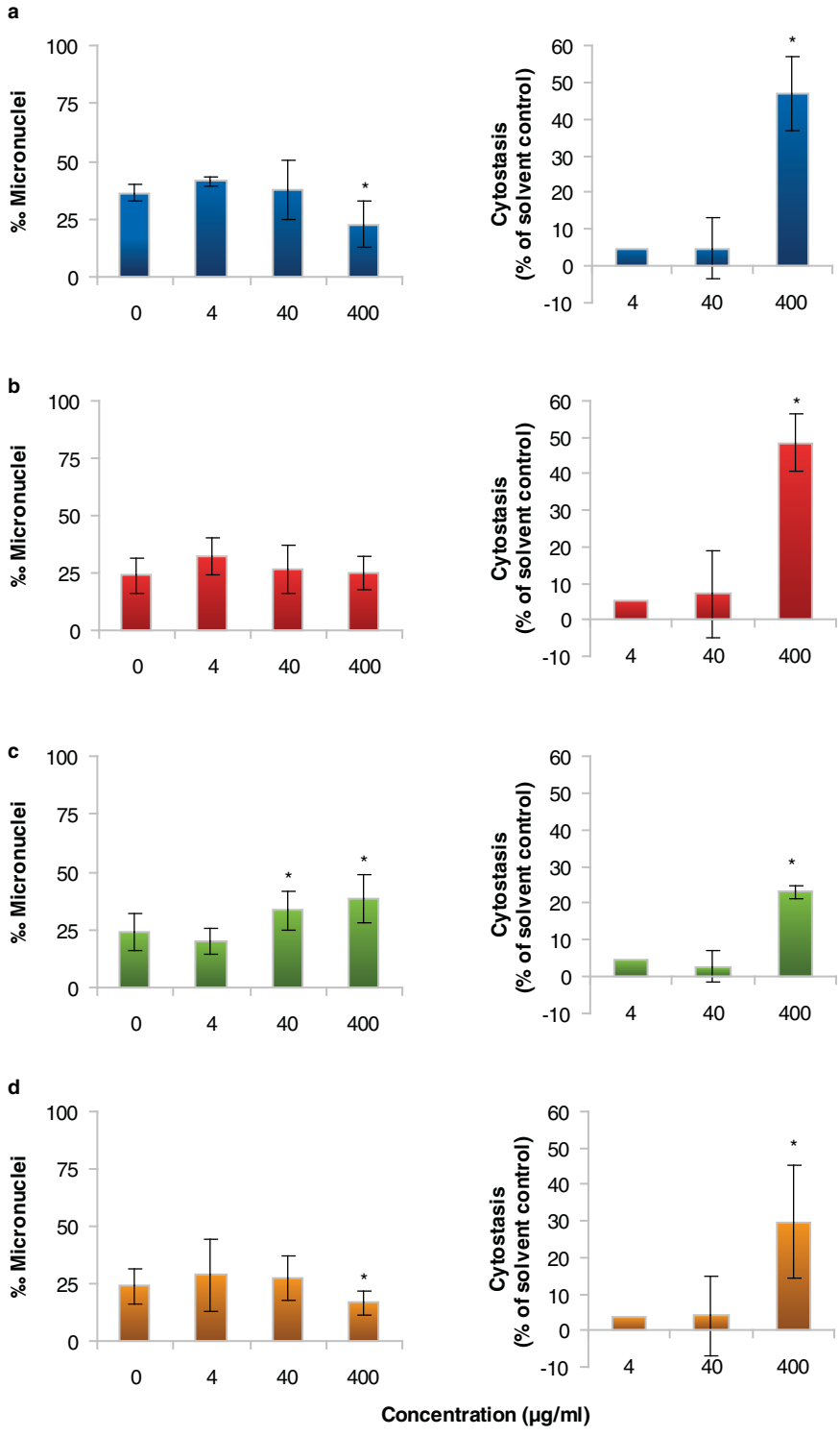

Figure 2. Frequency of binucleated cells with micronuclei and ratio binucleated to mononucleated 3T3-L1 cells exposed to (a) 10 (11) nm, (b) 30 (34) nm, (c) 80 (34) nm, and (d) 400 (248) nm silica nanoparticles. *Indicates a statistically significant difference compared to the solvent control ($p < 0.05$).

underestimate cytotoxicity because the metabolic activity of cells undergoing apoptosis does not necessarily decrease until the last stage of apoptosis (Rossman 2009). The use of the clonal survival assay may be a more appropriate assay for cytotoxicity, but is not compatible with the MEF-*lacZ* cells used in our studies, which are difficult to clone in the absence of intercellular contact. Nevertheless, our cytotoxicity

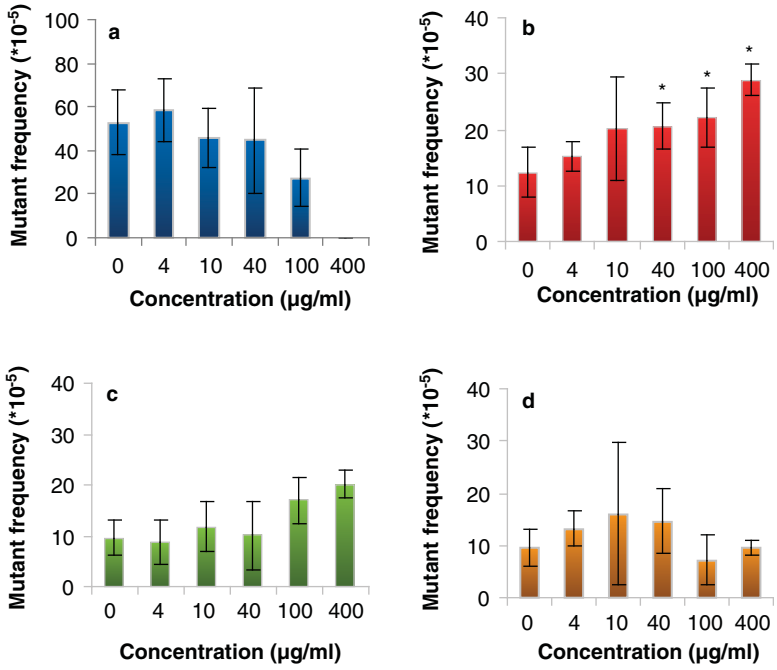

Figure 3. Mutant frequencies in MEF-*LacZ* cells exposed to (a) 10 (11) nm, (b) 30 (34) nm, (c) 80 (34) nm, and (d) 400 (248) nm silica nanoparticles. *Indicates a statistically significant difference compared to the solvent control ($p < 0.05$).

results are in agreement with results of various other *in vitro* studies with silica nanoparticles (Brunner et al. 2006; Chang et al. 2007; Chung et al. 2007; Adili et al. 2008; Fahmy and Cormier 2009; Lu et al. 2009). Using a number of different cytotoxicity assays, these studies reported minimal cytotoxicity of amorphous silica nanoparticles in epithelial cells and fibroblasts at concentrations of up to 100 µg/ml. Evaluation of genotoxicity at concentrations up to 100 µg/ml of silica nanoparticles in MEF-*LacZ* cells is therefore unlikely influenced by high cytotoxicity.

We previously reported that these silica nanoparticles elicited no response in the comet assay at concentrations up to 40 µg/ml (Barnes et al. 2008). The comet assay gives an indication of the potential of chemicals to induce single- or double-strand DNA breaks. Our results were consistent with the negative outcome in the comet assay after exposure of A549 human lung epithelial cells to luminescent silica nanoparticles (50 nm) for up to 72 h (Jin et al. 2007). In contrast, silica nanoparticles were found to be genotoxic in cultured breast cancer cells (MCF-7) (Pacheco et al. 2007). The comet assay is a highly sensitive test, and positive responses in the comet assay do not necessarily point toward genotoxicity since the DNA strand breaks detected may be reversible. On the other hand, a negative outcome of the comet assay does not necessarily prove the absence of genotoxicity, since DNA strand breaks are only one form of DNA damage that a chemical may induce.

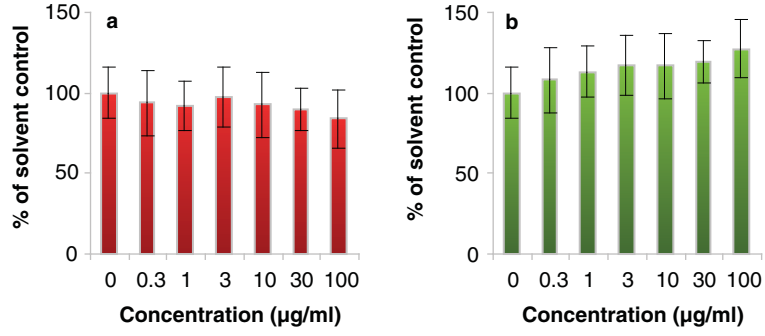

Figure 4. Metabolic activity of MEF-*LacZ* cells exposed to (a) 30 (34) nm, and (b) 80 (34) nm silica nanoparticles, as measured by the WST-1 assay.

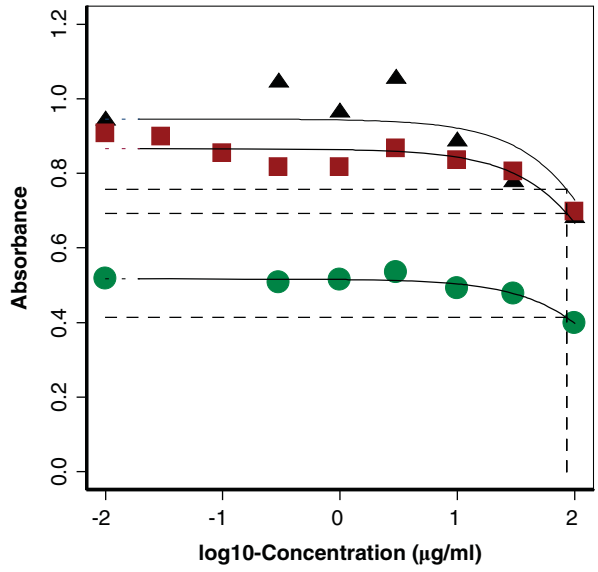

Figure 5. Dose-response curves of the metabolic activity of MEF-*LacZ* cells as a function of the 30 (34) nm silica nanoparticle concentration. Data was fitted with the exponential dose-response model: y = a*exp(bx). Dashed lines represent IC_{20} value, i.e., the concentration at which the metabolic activity of exposed cells decreased 20% compared to control cells. Different lines and symbols represent different experiments. For each experiment MEF-*LacZ* cells were obtained from different embryos, which may contribute to the variable background in metabolic activity between the experiments.

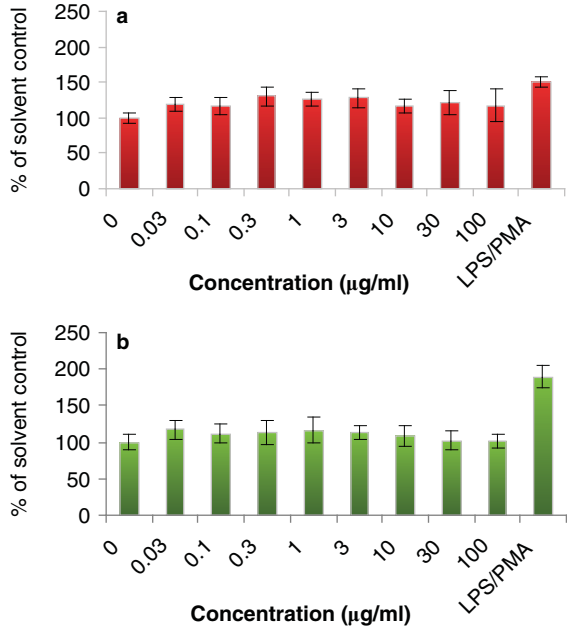

Figure 6. Generation of reactive oxygen species in MEF-*LacZ* cells exposed to (a) 30 (34) nm, and (b) 80 (34) nm silica nanoparticles, as measured by the H_2DCF-DA assay.

Therefore, evaluation of the genotoxic potential of chemicals or, in this case, nanoparticles requires the use of assays detecting additional and irreversible genotoxicity endpoints.

The micronucleus assay is a commonly used test for evaluating the potential of chemicals to induce chromosomal aberrations, which may consist of chromosome breaks or whole chromosome loss. To the best of our knowledge, this is the first study to evaluate amorphous silica nanoparticles in this assay. Using the same cell type, storage and exposure protocol as the previously reported comet assay, amorphous silica nanoparticles of 80 (34) nm induced a minor, but dose-related and statistically significant increase in chromosomal aberrations in 3T3-L1 mouse fibroblasts, while the other silica nanoparticles elicited no effect. Similar to our studies with amorphous silica, Wang et al. (2007) reported genotoxicity of crystalline silica particles in the *in vitro* micronucleus assay, while the comet assay was negative. It needs to be emphasized that, although both these assays detect DNA strand breaks, the endpoints do not completely overlap. In a different type of chromosomal aberration assay, silica-coated magnetic nanoparticles of 50 nm did not induce chromosomal aberrations in Chinese hamster lung fibroblasts (Kim et al. 2006). The different outcome for the 80 (34) nm nanoparticles in our study is surprising, since the TEM measured diameter of this nanoparticle was similar to that of the 30 (34) nm nanoparticle, which did not induce chromosomal aberrations. We have previously reported that these two nanoparticles also elicited different responses in the embryonic stem cell test (Park et al. 2009a), suggesting that characteristics other than primary size contribute to the potential of these nanoparticles to elicit effects in these assays.

Our micronucleus assay protocol involved the use of cytochalasine B as a cytokinesis blocker, which may inhibit the endocytosis of certain nanoparticles (Papageorgiou et al. 2007). However, using a protocol similar to the one used in our study, increased frequencies of binucleated cells with MN were also found after exposure to aluminum oxide and anatase TiO2 nanoparticles (Falck et al. 2009; Di Virgilio et al. 2010). This indicates that endocytosis of these nanoparticles was not completely inhibited, although the effects of these nanoparticles may have been underestimated.

The gene mutation assay is regularly used to evaluate a different genotoxicity endpoint than the micronucleus assay, specifically the potential of a chemical to induce gene mutations. This genotoxicity endpoint is commonly evaluated *in vitro* in a mammalian cell gene mutation tests with a reporter gene or in the bacterial reverse mutation assay (Ames test). We have evaluated for the first time the potential of silica

nanoparticles to induce gene mutations in mouse embryonic fibroblasts containing *LacZ* as a reporter gene, which is capable of detecting various mutagenic events such as point mutations and deletions induced by chemicals (Mahabir et al. 2009). Silica nanoparticles of 30 (34) and 80 (34) nm induced a dose related increase in gene mutations in MEF-*LacZ* cells, while those of 10 (11) nm and 400 (248) nm did not. Similar methodology was used in studies investigating the mutagenicity of single walled carbon nanotubes, C_{60} fullerenes, carbon black and diesel exhaust particles, but only the latter two induced an increase in mutant frequencies (Jacobsen et al. 2007, 2008). In addition, silica-coated magnetic nanoparticles of 50 nm were not genotoxic in the Ames test (Jin et al. 2007). However, the Ames test has been claimed to be less suitable for the evaluation of nanoparticles, since not all particles may be able to cross the bacterial membrane (Landsiedel et al. 2009).

The lack of genotoxicity observed in nanoparticles of 10 (11) nm and 400 (248) nm may be attributed to their larger hydrodynamic size in cell culture medium. We have previously assessed and reported the agglomeration status of these silica nanoparticles by means of DLS in cell culture medium containing FCS at different time points (shortly after dispersion, 24 h and five days after dispersion). The DLS data demonstrate that all particles are to some extent agglomerated/aggregated in cell culture medium, and the state of agglomeration/aggregation varies with different time points after dispersion. Therefore, rather than a permanent agglomeration 'status', the agglomeration in cell culture medium actually appears to be a very dynamic process, and the relevant time point for determining the agglomeration 'status' is not straightforward. Nevertheless, the 10 (11) nm silica nanoparticles were highly aggregated in distilled water and this aggregation persisted in cell culture medium for embryonic stem cells (Park et al. 2009a). In the current study, the larger particle size of the 400 (248) nm particles and the high level of aggregation of the 10 (11) nm particles did not appear to prevent their uptake in 3T3-L1 cells. However, at equal-mass concentrations for all particles, the number of particles (aggregates) available in the cell culture medium is lower for larger or highly aggregated particles. This in turn may lead to a lower number of cells in the cell population being affected by 10 (11) and 400 (248) nm particles compared to the better dispersed 30 (34) and 80 (34) nm particles.

Our TEM studies further demonstrated that silica nanoparticles taken up by 3T3-L1 cells were mainly located in vacuoles, but the presence of silica nanoparticles in the cell nucleus could not be confirmed. In the micronucleus assay, the presence of micronuclei was scored in at least 1000 binucleated cells for every treatment in each of the three experiments. On average, micronuclei were observed in 3.3% of the 3T3-L1 cells treated with 40 μg/ml 80 (34) nm silica nanoparticles, i.e., in approximately 100 cells. Several thousands of cell transects have been examined by means of TEM throughout our studies, but silica nanoparticles have never been observed within a nuclear profile. It should be noted that individual 10 (11) nm silica nanoparticles are too small to be visualized in a nuclear profile by means of TEM. Nevertheless, our findings are consistent with others using fluorescently labeled or luminescent amorphous silica nanoparticles and crystalline silica particles, which were found to be almost exclusively located in lysosomes in regions near to but not in the cell nucleus (Gemeinhart et al. 2005; Jin et al. 2007; Li et al. 2007; Kayle et al. 2010). In contrast, one study reported the nuclear translocation of fluorescently labeled silica nanoparticles sized between 40 and 70 nm (Chen and von Mikecz 2005).

For the time being, we are uncertain of the mechanism(s) that would explain the genotoxic responses observed with the 30 (34) nm and 80 (34) nm silica nanoparticles when their localization was primarily observed in vacuoles outside the cell nucleus. We can only postulate from the literature that nanoparticles may alter two pathways that may result in indirect DNA damage (Gonzalez et al. 2008; Singh et al. 2009).

Firstly, the release of transition metal ions and/or the high surface area associated with nanoparticles can promote the generation of ROS, resulting in oxidative DNA damage. This pathway can be attained without the presence of the nanoparticles themselves in the cell nucleus, as has been demonstrated for CoCr particles (Papageorgiou et al. 2007). Exposure of human lung epithelial cells (A549) to amorphous silica did not induce an increase 7,8-dihydro-xodeoxyguanine, a marker of oxidative damage (Jin et al. 2007). In addition, results from both acellular and cellular assays indicated that the reactivity of silica nanoparticles used in our study was low and did not result in generation of high levels of ROS either in solution or inside cells. However, given that the methodology used to measure ROS is rather insensitive, we cannot rule out the possibility that ROS may be involved in the induction of genotoxic effects.

Secondly, indirect DNA damage may result from mechanical hindrance of the mitotic process of the cells rather than the direct induction of DNA strand breaks, due to the presence of vacuoles containing nanoparticles in the cytoplasm of the cells during mitosis. Interferences with mitotic processes have been reported for various

nanoparticles, including silica nanoparticles (Pernodet et al. 2006; AshaRani et al. 2009a, 2009b; Villanueva et al. 2009; Wang et al. 2009). For instance, exposure of human lung fibroblasts and glioblastoma cells to starch-coated silver nanoparticles induced the formation of chromosomal aberrations and micronuclei, which was associated with significant cytoskeleton deformations, down-regulation of actin binding proteins and cell cycle arrest in the gap2/mitosis phase (AshaRani et al. 2009a, 2009b). Modification of the actin structure of human dermal fibroblasts exposed to citrate/gold nanoparticles was suggested to be the result of mechanical hindrance by the unusually large number of vacuoles (Pernodet et al. 2006). Mechanical hindrance may also induce indirect DNA damage by affecting microtubules, which are important in cellular transport of proteins required for DNA repair processes (Gonzalez et al. 2008). In our study, differences in number of vacuoles between control cells and those exposed to 50 μg/ml nanoparticles of different sizes were not qualitatively obvious. However, detailed quantitative studies of vacuole numbers are needed to exclude the contribution of mechanical hindrance by nanoparticles to the observed effects in the genotoxicity assays.

So far, our studies indicate that the 30 (34) and 80 (34) nm amorphous silica nanoparticles may not be as inert as previously thought. In light of the current and anticipated applications of silica nanoparticles in the pharmaceutical industry and in the food sector, *in vivo* studies are needed to further evaluate the relevance of the effects found *in vitro*. The 10 (11) and 400 (248) nm silica nanomaterials did not show any genotoxic effect in either assay. Therefore, it needs to be emphasized that not all silica nanoparticles have the potential to be genotoxic, and this may rather depend on as yet unidentified specific nanoparticle characteristics. Nanoparticles which are similar in both chemical composition and primary size exerted different effects in embryonic stem cell differentiation and micronucleus assays, suggesting that safety evaluation of nanoparticles needs to consider additional nanoparticle characteristics. The identification of additional characteristic(s) of nanoparticles that may determine their response in toxicity assays would help to assess the relevance of our findings to human exposure to these and other types of silica nanoparticles. A better understanding of the mechanism by which these effects occurred may help to identify these nanoparticle characteristics. Identification of toxicity-determining nanoparticle characteristics can also aid producers in the development of safe silica nanoparticles. In the meantime, caution is needed when comparing results across studies using different types of silica nanoparticles.

In summary, genotoxic effects of 80 (34) nm silica nanoparticles were observed in 3T3-L1 cells and of the 30 (34) and 80 (34) silica nanoparticles in MEF-*lacZ* cells as determined by the micronucleus assay and the *LacZ* gene mutation assay, respectively, at non-cytotoxic concentrations. The effects were not observed for all four silica nanoparticles, despite their similar chemical composition. In addition, even silica nanoparticles of similar TEM measured primary size (34 nm) elicited different effects in the micronucleus assay, suggesting that nanoparticle characteristics other than chemical composition and primary size play an important role. All nanoparticles were taken up by 3T3-L1 cells, but the presence of silica nanoparticles in the cell nucleus could not be demonstrated by TEM imaging, indicating that the effects were not caused by a direct interaction of the silica nanoparticles with the DNA, but rather by an indirect mechanism. The involvement of ROS cannot be excluded, although the silica nanoparticles had low redox potential and exposure of the MEF-*LacZ* cells did not result in the generation of high levels of ROS. The possibility of mechanical hindrance of mitotic processes as an indirect mechanism underlying these effects needs to be investigated. The question remains whether the observed effects are biologically relevant for human exposure to these 30 (34) nm and 80 (34) nm silica nanoparticles. *In vivo* studies may reveal whether the effects found *in vitro* can be reproduced *in vivo* at relevant exposure concentrations. In addition, the nanoparticle characteristics determining these effects need to be elucidated in order to assess the relevance of these findings for human exposure to a wide variety of silica nanoparticles.

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Supplementary information available online

Supplementary Figures 1 and 2.