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Review

The toxicological mode of action and the safety of synthetic amorphous silica—A nanostructured material

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ABSTRACT

Synthetic amorphous silica (SAS), in the form of pyrogenic (fumed), precipitated, gel or colloidal SAS, has been used in a wide variety of industrial and consumer applications including food, cosmetics and pharmaceutical products for many decades. Based on extensive physico-chemical, ecotoxicology, toxicology, safety and epidemiology data, no environmental or health risks have been associated with these materials if produced and used under current hygiene standards and use recommendations. With internal structures in the nanoscale size range, pyrogenic, precipitated and gel SAS are typical examples of nanostructured materials as recently defined by the International Organisation for Standardisation (ISO). The manufacturing process of these SAS materials leads to aggregates of strongly (covalently) bonded or fused primary particles. Weak interaction forces (van der Waals interactions, hydrogen bonding, physical adhesion) between aggregates lead to the formation of micrometre (μm)-sized agglomerates. Typically, isolated nanoparticles do not occur. In contrast, colloidal SAS dispersions may contain isolated primary particles in the nano-size range which can be considered nano-objects. The size of the primary particle resulted in the materials often being considered as "nanosilica" and in the inclusion of SAS in research programmes on nanomaterials. The biological activity of SAS can be related to the particle shape and surface characteristics interfacing with the biological milieu rather than to particle size. SAS adsorbs to cellular surfaces and can affect membrane structures and integrity. Toxicity is linked to mechanisms of interactions with outer and inner cell membranes, signalling responses, and vesicle trafficking pathways. Interaction with membranes may induce the release of endosomal substances, reactive oxygen species, cytokines and chemokines and thus induce inflammatory responses. None of the SAS forms, including colloidal nano-sized particles, were shown to bioaccumulate and all disappear within a short time from living organisms by physiological excretion mechanisms with some indications that the smaller the particle size, the faster the clearance is. Therefore, despite the new nomenclature designating SAS a nanomaterial, none of the recent available data gives any evidence for a novel, hitherto unknown mechanism of toxicity that may raise concerns with regard to human health or environmental risks.

Taken together, commercial SAS forms (including colloidal silicon dioxide and surface-treated SAS) are not new nanomaterials with unknown properties, but are well-studied materials that have been in use for decades.

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1. Introduction and background

Synthetic amorphous silica (SAS) consists of nano-sized primary particles, of nano- or micrometre-sized aggregates and of agglomerates in the micrometre-size range. Hence these materials fall under the definition of nanostructured materials. Spherical nanoparticles may be found in stabilised colloidal SAS suspensions. SAS, including colloidal and surface-treated forms, have widely been used in topical and oral medicines, food and cosmetics for decades without evidence of adverse human health effects. Standard ecotoxicity and toxicity tests generally demonstrated the biological inertness of SAS, and SAS were considered safe if occupational standards and use recommendations are followed (Becker et al., 2009; ECETOC, 2006; IARC, 1997; Lewinson et al., 1994; OECD, 2004). Nevertheless a discussion about hazards and risks of "nanosilica" has recently started calling into question the safety of SAS materials which are made up of primary particles in the nano-size range (Napierska et al., 2010; Dekkers et al., 2010). This discussion has prompted this work to investigate whether the mode of action (MOA) or mechanisms of toxicity of so-called "nano-SAS" or "nanosilica" are different from those of the commercial SAS forms. To this end a systematic literature search was undertaken to identify relevant publications. The studies considered in this review were selected according to commonly accepted criteria of relevance, adequacy, reliability and validity (Klimisch et al., 1997; OECD, 2005). In addition, studies with critical results and those not yet covered in available authoritative reviews (IARC, 1997; OECD, 2004) were included.

2. The different SAS forms under review

There are three main types of silica (silicon dioxide), which are all found under CAS No. 7631-86-9, *i.e.*, (1) crystalline silica, (2) amorphous silica (naturally occurring or as a by-product in the form of fused silica or silica fume), and (3) synthetic amorphous silica (SAS), including silica gel, precipitated silica, pyrogenic (fumed) silica and colloidal silica (silica sol). Only the manufactured forms of amorphous silica (SAS) will be dealt with in the following article, *i.e.*, SAS produced by a wet process and described by CAS number 112926-00-8 (includes silica gel, precipitated silica and colloidal silica) and SAS produced by a thermal process described by CAS number 112945-52-5 (pyrogenic silica). It also includes the surface-treated, hydrophobic SAS types, *i.e.*, silica dimethicone silylate, silica dimethyl silylate and silica silylate (CAS 67762-90-7, 68611-44-9 and 68909-20-6). In general, SAS contains no detectable amounts of crystalline silica (detection limits vary between 0.01 and 0.3% by weight, depending on the method used; ECETOC, 2006, pp. 12–14). SAS also contains fewer impurities than biogenic amorphous silica which is obtained from various sources such as the shell wall of phytoplankton or the epidermis of vegetables, or non-biogenic vitreous amorphous silica. SAS can be distinguished from other forms of amorphous silica by its high chemical purity, the finely particulate nature and by characteristics of the particles observable by electron microscopy, *e.g.*, shape, structure, and degree of fusion (Fig. 1).

SAS may be either hydrated or non-hydrated and contains silicon and oxygen connected in a three-dimensional macromolecular network which imparts a general chemical inertness. All forms of SAS may be surface-modified to produce silica that is more hydrophobic.

The difference between the amorphous and crystalline silica forms arises from the connectivity of the tetrahedral units. Amorphous silica consists of a non-repeating network of tetrahedra, where all the oxygen corners connect two neighbouring tetrahedra. Although there is no long range periodicity in the network there remains significant ordering at length scales well beyond the SiO bond length. The amorphous structure is very "open", *i.e.*, channels exist through which small positive ions such as Na^+ and K^+ can readily migrate.

2.1. Methods of manufacture, composition

Pyrogenic amorphous silica is produced in closed reactors by the hydrolysis of (alkyl)chlorosilanes (*e.g.* $SiCl_4$, $HSiCl_3$. CH_3SiCl_3) in an oxygen/hydrogen flame at temperatures between 1200 and 1600 °C. Nucleation, condensation and coagulation of SiO_2 molecules generate proto-particles of SiO_2 which combine to primary particles. Under the conditions of the reaction zone, primary

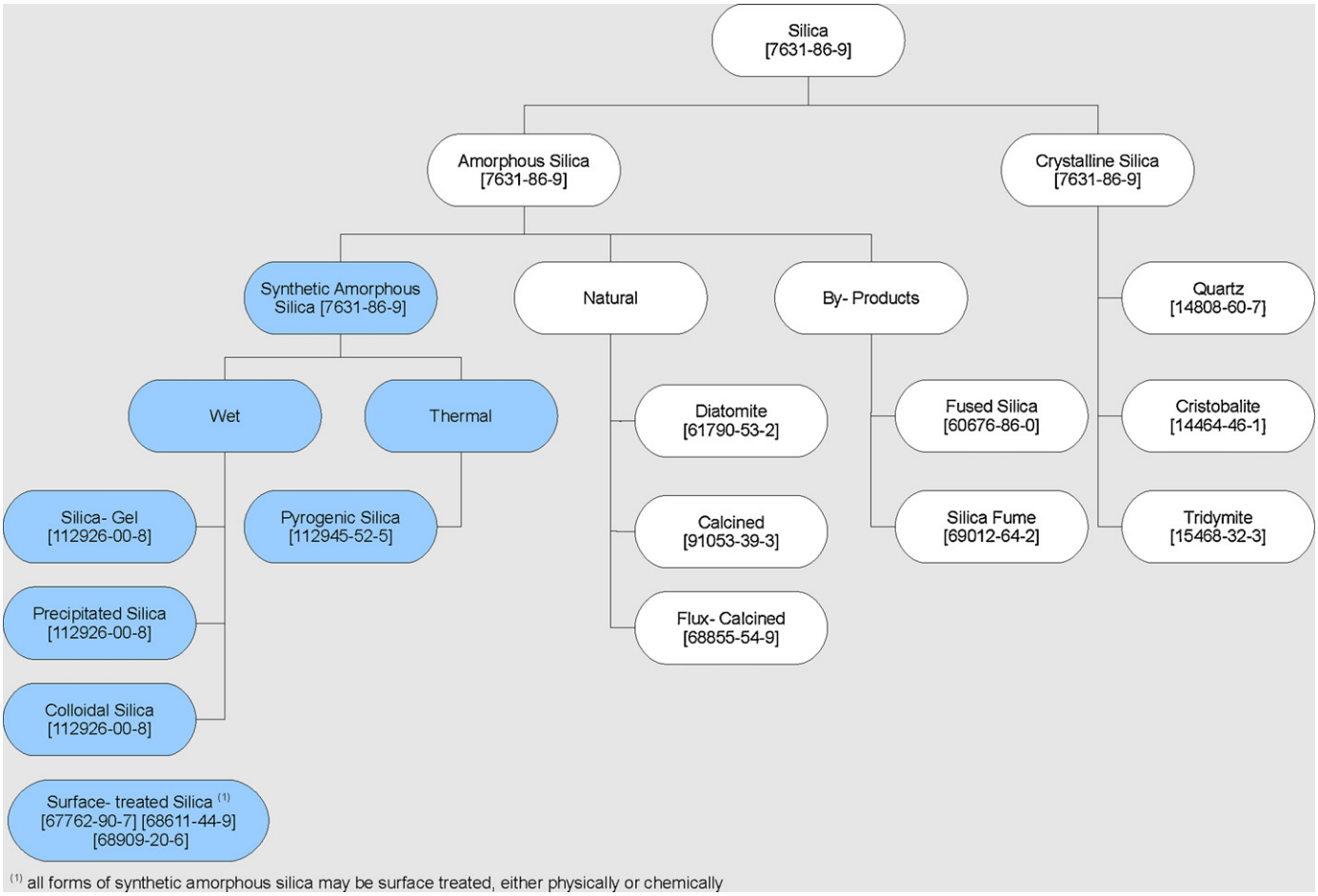

Fig. 1. The various silica forms.
Reproduced with permission by the Members of the Association of Synthetic Amorphous Silica Producers (ASASP).

particles form SiO_2 aggregates; aggregates then form agglomerates of SiO_2. It is important to note that primary particles do not exist outside the reaction zone. The relatively high temperature yields a product that has low water content (Fig. 2).

Precipitated silica and silica gel consist of randomly linked spherical polymerized primary particles. The properties are a result of the size and state of aggregation of the primary particles and their surface chemistry. Precipitated silica and silica gels can be produced from various raw materials. The most relevant process in industry is from sodium silicate solutions by acidification with sulphuric acid to produce a gelatinous precipitate. The precipitate is filtered, washed, dehydrated and milled to produce precipitated silica with typically broad meso/macroporous pore structures reflected in the pore size distribution, or silica gels with generally more narrow microporous or mesoporous structure with average pore diameters between 2 and 50 nm. By controlling the washing, ageing, and drying conditions, the important physical parameters such as porosity, pore size, and surface area can be adjusted to produce a range of different silica gel types with well-defined particle size distributions. Amorphous mesoporous silica with uniformed pores in the size range between 1.5 and 50 nm can be synthesised by reacting tetraethylorthosilicate (TEOS) with a template of surfactant molecules, typically amphiphilic polymers, under either alkaline or acidic conditions. The surfactants are later evacuated from the mesopores by a calcination step or by washing with a solvent. Form and diameter of the mesopores are determined by the type of surfactants used in the synthesis (Mou and Lin, 2000; Napierska et al., 2010).

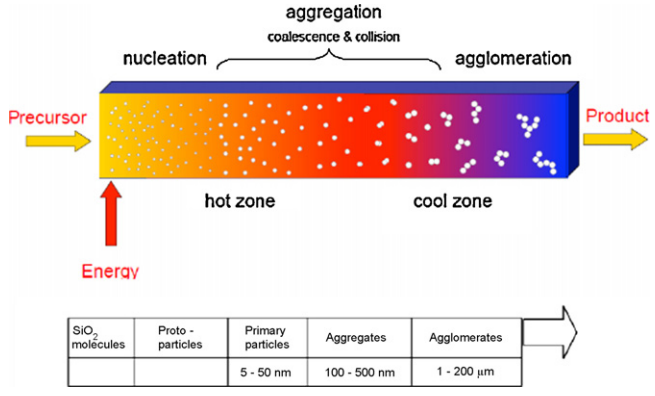

Fig. 2. Particle sizes during the production of pyrogenic SAS from precursors (*e.g.*, $SiCl_4$).

Colloidal silica is usually produced in a multi-step process in which the first step involves the partial neutralisation of an alkali–silicate solution by acidification, electrodialysis, or ion exchange, leading to the formation of silica nuclei, typically in the size range of 1–5 nm. If the pH is reduced below 7 or if salt is added, then the units fuse together in chains to result in silica gel. If, however, the pH is kept slightly on the alkaline side of neutral, then the subunits stay separated, and gradually grow to colloidal silica (silica sols). The maximum concentration at which this step can be carried out is in the range of 10–15%. Higher concentrations will also

result in gelation. The resulting colloidal suspension is stabilised by the addition of KOH, NaOH, NH_3 or HCl in amounts of up to 10% by weight. An alternative method for stabilisation is based on electrostatic repulsion of the particles. Substitution of some of the Si atoms by Al is known to increase the negative colloidal charge, especially at pH ranges below the neutral point leading to higher repulsive forces between the sol particles. The resulting suspension can then be concentrated, usually by evaporation of the liquid phase. Maximum silica concentrations in the end product depend on particle size and range between approximately 30 wt% for 10 nm particles and about 50% for 50 nm particles. Higher concentrated suspensions are not stable. Hydrogen ions from the surface of colloidal silica tend to dissociate in aqueous solution, resulting in a negative charge. Spherical colloidal silica particles in suspension can also be obtained by the Stöber method (Stöber et al., 1968), by which controlled growth of particles of near uniform size and porosity is achieved by hydrolysis of alkylsilicates and subsequent condensation of silicic acid in an ethanolic solution with catalytic amounts of ammonia.

For further details on the manufacture of pyrogenic silica, precipitated silica and silica gel; the reader is referred to the *Best Available Techniques (BAT) Reference Documents* (BREF, 2007).

2.2. Physical and chemical properties

SAS are a distinct, manufactured form of silicon dioxide; they typically contain less than 1% of impurities. Silicon dioxide is described as a white fluffy powder or granules; and is hygroscopic (EFSA, 2009). The tendency to be solvated by water depends on the SAS type, with saturation concentrations usually increasing with increasing surface area. Generally, SAS have a tendency to supersaturate and surface-treated hydrophobic SAS have lower solubility as compared to the hydrophilic forms. For the analysed SAS, the saturation concentration was reached within a few hours (Alexander et al., 1954; Borm et al., 2006a; ECETOC, 2006; Vogelsberger, 1999). Particle size distribution curves and the accuracy of measurements depend on the particular method used, on sample preparation and whether the measurement was performed in solid or liquid phase (for details see ECETOC, 2006; ISO, 2008). Typical physico-chemical properties of the different forms under review here are shown in Table 1 below.

Silanol (Si—OH) groups on the SAS surface render untreated SAS hydrophilic with silanol numbers per square nanometre of SAS surface varying for the different SAS forms between 2 (pyrogenic), up to 6 (precipitated) and up to 8 (gel). A typical treating agent for surface modification is dichlorodimethylsilane, which hydrolyses to form polydimethylsiloxane. Polydimethylsiloxy units bind to surface silanols *via* condensation reactions. On the treated SAS the original treating agent, dichlorodimethylsilane, is no longer detectable. Treated SAS bears on its surface both the hydrophobic entities (polydimethlysiloxy units) and the remaining hydrophilic entities, *i.e.*, surface silanols. The core material is still amorphous silica.

2.3. Nanostructural properties

According to the ISO Core Terms (ISO, 2010) nanomaterials are industrial materials intentionally produced, manufactured or engineered to have unique properties or specific composition at the nanoscale, which is defined as the size range "from approximately 1 nm to 100 nm". Nanomaterials are either nano-objects (nanofibres, nanoplates or nanoparticles with a size of 1–100 nm in at least one dimension) or nanostructured (*i.e.* having an internal or surface structure at the nanoscale) (Fig. 3).

Pyrogenic, precipitated, and gel SAS forms are composed of aggregates and agglomerates of primary particles. Few, if any,

Table 1
Physical and chemical properties of different SAS forms.

Property [units]	Pyrogenic	Precipitated	Colloidal	Surface treated[a]
Space group	Not measurable (amorphous materials)			
SiO_2 content [wt%]	≥99.8	>95%; gel: >95% (dry)	≥99.5	≥99.8
Carbon content [%]	–	–	–	0.5–2
Loss on drying [%]	<2.5	5–7; gel: 2–6	≫2.5	<2.5–7
Melting point [°C]	1600–1725	1600–1725	1600–1725	1600–1725
Density (g/cm^3 @ 20 °C)	2.2	2.2	2.2	2.2–2.7
Tapped (bulk) density [g/L]	30–250	30–500; gel: 1000	29–42	50
Water solubility (saturation) [mg/L] at 37 °C and pH 7.1–7.4)	144–151; (15–68 at 20 °C and pH 5.5–6.6)	141; gel: 127–141	Colloidal dispersions with water	115
pH (1:1 water:ethanol)	3.6–4.5	5–9 gel: 3–8	3.5–4.4 (4% w/v aqueous dispersion)	3.5–4.5
Specific surface area (SSA) BET [m^2/g]	50–400	30–500; gel: 250–1000	50–380 (spherical), ~640 (porous)	150–250
Zeta-potential	Negative	Negative	Usually negative; can be positive with certain stabilisers	Negative
Behaviour towards water	Hydrophilic	Hydrophilic	Hydrophilic	Hydrophobic
Particle size (measured by laser diffraction)				
Primary particle [nm]	5–50	5–100; gel:1–10	7–50	5–20
Aggregate [μm]	0.1–1	0.1–1	0.1–1	0.1–1
Agglomerate [μm]	1–250 (typically 100)	1–250 (typically 100)	1–250 (typically 100)	Mostly > 125
Porosity				
Mean pore size	Microporous	Meso- or macroporous	–	Micro-, meso- or macroporous

Source of data: From ECETOC (2006); Ma-Hock et al. (2007); OECD (2004); Vogelsberger (1999).
[a] All forms of synthetic amorphous silica may be surface-treated, either physically or chemically; treating agents include, among others, dichlorodimethylsilane, polydimethylsiloxanes or hexamethyldisilazane.

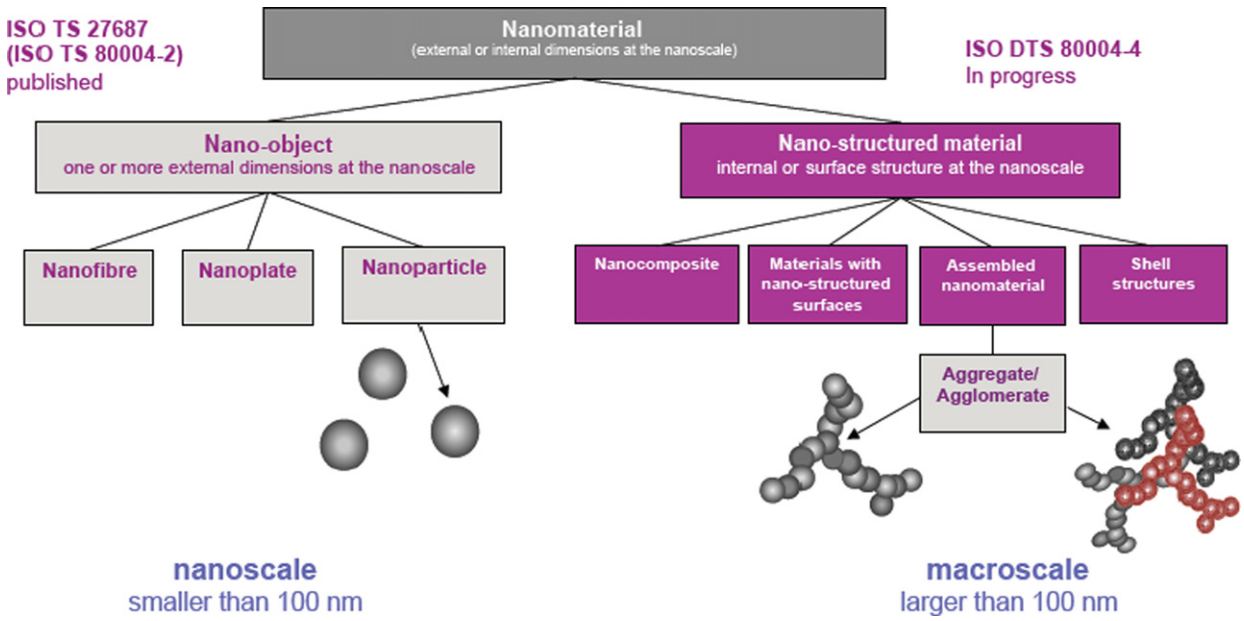

Fig. 3. Definitions.

primary particles would be expected to exist outside of the synthesis reactor. Aggregates consist of strongly bonded or fused particles. The resulting external surface area may be significantly smaller than the sum of calculated surface areas of the individual components (ISO, 2008). SAS aggregates assemble in chains (pyrogenic SAS) or – in liquid phase – in clusters (precipitated and gel forms). Precipitated silica and silica gel contain a larger amount of bound water and tend to agglomerate, causing them to have an even larger particle size. Agglomerates are assemblies of loosely bound particles or aggregates, where the resulting external surface area is similar to the sum of the surface areas of the individual components. Agglomerates are held together by weak forces, such as van der Waals forces and simple physical adhesion forces (ECETOC, 2006; Gray and Muranko, 2006; ISO, 2008). Hence, complex aciniform (grape-like) particle aggregates constitute the smallest inseparable entities in commercial pyrogenic, precipitated and gel SAS. In the vast majority of commercially available grades, these aggregates have no dimensions less than 100 nm. Data from Gray and Muranko (2006) and Ma-Hock et al. (2007) indicate that even for conditions of high-energy dispersion and/or extreme mechanical processing (*e.g.*, uniaxial compression, elastomer mixing, ultrasonication), there is little to no liberation of primary particles.

Colloidal SAS consists of spherical and non-porous silica particles dispersed in a liquid phase, *e.g.*, water. Often, such suspensions are stabilised electrostatically. The particles may remain dispersed, or alternatively, aggregation processes may remove the material from the liquid phase. In practise, even apparently stable dispersions will gradually aggregate out of the aqueous phase over time. Most colloidal silicas are prepared as monodisperse suspensions with particle sizes ranging from approximately 5–100 nm in diameter. Smaller particles are more difficult to stabilise; particles of sizes greater than 150 nm are subject to sedimentation. The sizes of colloidal particles may hence fall within the size definition of nanoparticles.

Consistent with the ISO definition of nanostructured materials as having either an internal or surface structure on the nanoscale (ISO, 2008), manufactured SAS with a surface structure based on nano-sized primary particles can be described as *nanostructured materials*. Because they consist of complex structures of aggregates and agglomerates and usually have no external dimensions of less than 100 nm (when measured by laser diffraction), commercial SAS products – with the exception of colloidal SAS and some nanoscaled aggregates – are neither nanoparticles nor nano-objects.

3. Exposure

High production volumes of SAS and their wide use in a broad variety of applications might lead to significant environmental, occupational and consumer exposure. Solid SAS are used as adsorbents, fillers, thickening agents, anti-caking agents, emulsion stabilisers, free-flow agents and carriers in a variety of industrial and consumer products, including pest control, pharmaceuticals, cosmetics, and food and feed products. Colloidal silica is widely used in coatings, ink receptive papers, metal casting, refractory products, catalysts and as a filter aid in food production.

Emission to the environment may occur during production and use of SAS although the potential amount of anthropogenic SAS released into the aquatic environment is estimated to represent only a small fraction of the dissolved silica naturally present in rivers (OECD, 2004). Analytical data with regard to possible release of SiO_2 particles from nanocomposites, *e.g.* by wear and tear, were not available. Based on a brief, very selective literature review of a few publications, Reijnders (2009) suggested that silica nanoparticles released from nanocomposites might pose an environmental and health risk and therefore proposed some general measures to reduce particle release from composite materials.

Occupational exposure in SAS production is highest during packaging and loading operations, with highest mean values of up to 3 mg/m^3 inhalable dust and up to 1 mg/m^3 respirable dust (OECD, 2004). Under practical occupational conditions, SAS tend to form aggregates and agglomerates of such sizes that will not reach the peripheral areas of the lung. In commercial pyrogenic SAS products, the fraction of particles that may reach the thoracic and alveolar sites was reported to be below 1 vol% (=wt%) (Stintz, 2001). It is important to note that current international guidelines for animal inhalation studies require the test material to be prepared in a way that it is respirable. Under such experimental conditions, the test material is aerosolised applying high shear stresses and mass median aerodynamic diameters [MMAD] range significantly below 10 μm (ideally 1–3 μm). The respirable fraction then accounts for more than 80 vol%. In conclusion, the toxicologically relevant, respirable fraction is much lower in the

products under normal handling and use conditions than under experimental conditions. Surface-treated SAS may be used in perfumes, and hence may be aerosolised during use by consumers (Becker et al., 2009). With typical aerosol particle diameters in the 10–100 μm range, most aerosol particles will not be respirable, but deposited in the nasopharyngeal region. Oral and dermal SAS exposure may arise from the use of personal care products and medicines. Recently, Dekkers et al. (2010) analysed food products with added silica (E551), and estimated the likely oral intake of "nanosilica" *via* food. The authors estimated a daily intake of 124 mg "nanosilica", corresponding to 1.8 mg/kg bw/day for an adult of 70 kg based on products containing E551, although it is stated in the publication itself that ". . . it is not clear whether the food additive E551 contains nano-sized silica." The terminology "nanosilica" as used by Dekkers et al. (2010) was later criticized by Bosch et al. (2011). Silica is usually tightly bound into the matrix of end-use articles, and hence significant exposure of the general population through these products is unlikely.

4. Results of experimental studies

The different forms of SAS have been used as test materials in a number of environmental, ecotoxicological and toxicological studies. Some of these studies were conducted to investigate the toxic potential of SAS while others used SAS as a comparison material in studies on various nanoparticles. Several studies described in the following sections refer to the testing of "nanosilica" *versus* "bulk silica", with some studies highlighting the enhanced biological responses for nano-forms *versus* the findings for larger silica particles. These studies, however, generally refer to the primary particle diameter when classifying some silica products as "nano" rather than a whole-particle dimension that reflects the complex aggregate structures of most silica particles, such as the aggregate diameter. This can lead to the misinterpretation of these study findings as reflecting an effect of particle size while it is well known that silica particles can differ in other toxicologically relevant properties, such as surface area and particle number.

4.1. Solubility/dissolution and aggregation/agglomeration of SAS in biological media

Pyrogenic, precipitated and gel forms of SAS, including surface-treated forms, have been the subject of dissolution testing using a simulated biological medium at 37 °C and pH values near 7 (Roelofs and Vogelsberger, 2004). Depending on the material, the solubility was between 2.3 and 2.7 mmol/L (138–162 mg/L) and was similar to the solubility in water (see above). Roelofs and Vogelsberger (2004) also confirmed that silica has a tendency to supersaturate, *i.e.*, the dissolution rate is more rapid than the precipitation rate. Hence, the different forms of SAS dissolve both in water and in simulated biological systems beyond the equilibrium concentration. Total dissolution can be expected in biological systems where dissolved SAS is quickly removed, such as in the lungs.

Changes in pH, salinity/ionic strength, water hardness, and/or the presence of natural organic matter, may influence SAS particle aggregation and agglomeration. In water, a mean aggregate size of 205 nm was, for example, measured by dynamic light scattering (DLS) for SAS with a reported primary particle size of 14 nm (Adams et al., 2006). Similarly, aggregation was shown for non-stabilised colloidal 10 nm silica particles in distilled water, resulting in an average aggregate size of 103 nm as measured by DLS shortly after dispersion (Park et al., 2010a,b). Lu et al. (2009) note that calcination of mesoporous silica products leads to a non-suspendible aggregate due to interparticle dehydration of surface silanol groups. Therefore, earlier mesoporous silica products synthesized by calcination methods are unsuitable for tests with biological systems.

4.2. Behaviour in the environment and ecotoxicity

4.2.1. Environmental fate

Under normal environmental conditions, silicon dioxide is an inert substance with no known degradation products. At ambient temperature and pH, SAS are slightly soluble in water (Table 1). Due to the known tendency to supersaturate not only solubility but also, in particular, dissolution rates are an important parameter to consider. Amorphous silica hydrosols are very stable at environmental pH values in the presence of alkali metal cations. Between pH values of 7 and 11, alkali cations are able to coagulate silica (Holleman-Wiberg, 2008; Depasse and Watillon, 1970). SAS are not volatile and have no lipophilic character. SAS will therefore settle mainly into soils/sediments and weakly into water. SiO_2 is expected to combine indistinguishably with the soil layer or sediment due to the chemical similarity with inorganic soil matter (OECD, 2004). No adsorption of humic acids was observed on nano-sized SiO_2, neither in the spherical- nor in the porous-form (Yang et al., 2009a,b). Amorphous silica particles are frequently formed during chemical weathering processes of minerals (Farré et al., 2009; Nowack and Bucheli, 2007). Bioavailable forms of silica are dissolved silica [$Si(OH)_4$], silicic acid and silicates. Silicates are found throughout the Earth's lithosphere. The ocean contains a huge reservoir of silica and silicates which are used by a variety of marine organisms (diatoms, radiolarians, sponges) to build up their skeletons. Based on the chemical nature of silica and silicates (inorganic structure and chemical stability of the compound: Si—O bond is highly stable), no photo- or chemical degradation is expected (OECD, 2004). Biodegradation and speciation of SiO_2 (*e.g.*, dissociation or complexation) will not occur in aquatic media under normal conditions, though particle size may change due to aggregation and agglomeration. Due to its inherent physico-chemical properties, such as the absence of lipophilicity as well as the capability of organisms to eliminate absorbed SiO_2 components, bioaccumulation is not to be expected.

4.2.2. Ecotoxicity

In the reviews by the OECD (2004) and the ECETOC (2006), no acute toxicity was reported for fish and daphnia, even after exposures to extremely high concentrations of SAS. Physical effects on daphnia were observed in tests using unfiltered test medium. No effects were found in acute ecotoxicity studies with surface-treated SAS (EPA, 2011). With regard to chronic aquatic toxicity data, the OECD (2004) concluded that although there were no chronic aquatic toxicity data for SAS, there is no evidence of harmful long-term effects due to the known inherent physico-chemical properties, absence of acute toxic effects as well as the ubiquitous presence of silica and silicates in the environment. Tests conducted in terrestrial organisms (German cockroach, Grain weevil) demonstrated a lethal effect after contact at low humidity and when water was not available due to the adsorption of lipids from the insect cuticle followed by dehydration. After ingestion, SAS had no toxic effects (ECETOC, 2006; OECD, 2004). Only results from relevant recent investigations not included in the OECD, ECETOC or EPA evaluations are presented in the following paragraphs. These new studies in bacteria, yeast, algae and mussels confirm the low hazard profile of silica particles and point to the importance of physical and electrostatic interactions between cell walls and particles.

4.2.2.1. Effects on bacteria and yeast. Jiang et al. (2009) compared the toxicity to bacteria of different nano- and micron-sized particles. At the single concentration tested (20 mg/L), SiO_2 particles (LUDOX®[1] CL Al_2O_3 stabilised colloidal silica from Sigma–Aldrich,

[1] LUDOX® is a registered trademark of W.R. GRACE.

primary particle size 20 nm) significantly reduced the survival of Gram-positive *Bacillus subtilis* (−40%), Gram-negative *Escherichia coli* (−58%), and Gram-negative *Pseudomonas fluorescens* (−70%). It was found that the negatively charged bacterial surfaces attracted the positively charged LUDOX® CL particles (+35 mV at pH 6.5) and that the tendency of the particles to attach on the cell wall was greater than the tendency to aggregate together. Similar results were found in the same study with the positively charged Al_2O_3 particles and both LUDOX® CL particles and Al_2O_3 particles were capable of flocculating bacterial cell suspensions soon after mixing.

A suspension in water of SiO_2 particles with a primary particle size of 14 nm (pyrogenic SAS obtained from Sigma–Aldrich, USA; aggregated size in water 205 nm; particles not specified further) inhibited the growth of Gram-positive *B. subtilis* at concentrations ≥1000 ppm (7 ± 4.7% at 1000 ppm, 84 ± 9.9% at 2000 ppm and 99 ± 1.8% at 5000 ppm). Gram-negative *E. coli* bacteria were less sensitive with a growth inhibition of 48 ± 8.5% at 5000 ppm. The presence of light did not significantly increase the toxicity. Increase of the particle size to 930 nm or 60,000 nm did not influence toxicity (Adams et al., 2006).

Silica particles (10–20 nm, purity 99.5%, obtained as dry powder from American Elements, USA), stabilised with a non-toxic dispersant (100 mg Dispex A40/L) did not inhibit oxygen uptake by yeast cells up to the highest tested concentration of 1000 mg/L; however, some damage of the cell membrane was found (Garcia-Saucedo et al., 2011).

4.2.2.2. Effects on daphnids and aquatic midges. Fumed and porous type SiO_2 particles (purchased from Sigma Corp., USA) with specific surface areas of 349.71 and 644.44 m^2/g, and primary particle sizes of 7 nm (fumed) and 10 nm (porous type), respectively (aggregate sizes not reported), did not affect DNA integrity (as measured in the Comet assay), nor growth or reproduction parameters in *Daphnia magna* at the only tested concentration of 1 mg/L. An increase in the mortality rate of *D. magna* was observed after a 96 h-treatment with fumed material (mortality rate 10 ± 8.16%) and porous type material (15 ± 4.08%; controls 5 ± 4.08%). In larvae of the aquatic midge *Chironomus riparius*, an increase in mortality was observed after exposure to the porous-type SiO_2 particles, but growth indicators were not significantly changed (Lee et al., 2009). Because of the high variability in the results reported by Lee et al. (2009), and because only one dose level (1 mg/L) was tested and therefore no dose–response relationship can be established, the relevance of these findings is doubtful.

4.2.2.3. Effects on algae. Fujiwara et al. (2008) report a non-linear, but size-dependent growth inhibition of algae (*Chlorella kessleri)* after a 96 h exposure to suspensions of Na_2O stabilised SiO_2 nanoparticles (Cataloid; 5, 26 and 78 nm). The pH of the culture medium was adjusted to 7.7. The 96 h-EC_{50} values were 0.8 ± 0.6%, 7.1 ± 2.8%, and 9.1 ± 4.7% for materials with primary particle sizes of 5, 26 and 78 nm, indicating an overall very low level of toxicity, even after exposure concentrations that by far exceed current standard testing guideline recommendations. Toxicity was independent of illumination with light. The size of cells increased in the presence of 5 nm particles, and, to a lesser extent in the presence of materials composed of 26 and 78 nm-sized primary particles (as shown by flow cytometry). Coagulation of cells was observed after exposure to the material containing 5 nm particles (1.02%; test conditions not specified further). In a study reported by Ji et al. (2011), SiO_2-nanoparticles showed no significant toxicity in *Chlorella* up to the highest tested concentration of 1000 mg/L.

A low level of toxicity was found in the alga *Scenedesmus obliquus* by Wei et al. (2010), using silica "nano"-particles (primary particle sizes of 10–20 nm, purity 99.5%, purchased from Sigma–Aldrich) and analytical grade silica "bulk particles" (obtained from Shanghai Chemical Reagent Company of China, particle size range 5–10 μm). The 50% effective concentration (EC_{50}) values for growth inhibition at 48, 72 and 96 h were all higher than 200 mg/L, the highest dose tested. Only after exposure to the "nano"-material, the contents of chlorophyll decreased significantly under moderate and high concentrations (50, 100, and 200 mg/L) after 96-h exposure, probably as a result of the adsorption of particle aggregates to the cell walls, which may have inhibited photosynthetic activity and altered the acquisition of light and essential nutrients. As the content of carotenoids (*i.e.*, effective antioxidants) was stable in the alga, a major oxidative stress reaction was excluded by the authors of the study. The alga cells did not change morphologically.

Algal toxicity was found by van Hoecke et al. (2008), who studied interactions between algae cells (*Pseudokirchneriella subcapitata*) and commercial colloidal silica dispersions (LUDOX® LS, primary particle size 12.4 nm, 236 m^2/g and LUDOX® TM40, primary particle size 27 nm, 135 m^2/g). Toxicity was assessed after 72 h of exposure using growth-inhibition experiments; 10 and 20% effect concentrations for growth rate (E_rC10 and E_rC20) were determined, as well as NOEC and LOECs. In addition, "silica bulk material" (silica powder, analytical grade, <62 μm, purchased from Sigma–Aldrich) was tested under identical conditions. Expressed on a mass basis NOEC and LOEC values were 4.6 and 10 mg/L for both LUDOX® materials. Expressed as a surface area, the NOEC and LOEC values for LUDOX® LS were 1.09 and 2.36 m^2/L and for LUDOX® TM40 0.62 and 1.35 m^2/L. The E_rC10 and E_rC20 values were used to compare the toxicities of both particles. Expressed on a mass basis, mean (n = 5) 72-h E_rC10 values (±SD) for LUOOX® LS and TM40 were 10.9 (±4.4) and 15.0 (±4.3) mg/L, respectively. Mean (n = 5) 72-h E_rC20 values (±SD) were 20.0 (±5.0) and 28.8 (±3.2) mg/L, respectively. Expressed as a surface area, mean 72-h E_rC 10 values were 2.6 (±1.0) and 2.0 (±0.6) m^2/L, and 72-h E_rC20 values were 4.7 (±1.2) and 3.9 (±0.4) m^2/L for LS and TM40, respectively. The SiO_2 bulk material was not toxic at the highest tested concentration of 1000 mg/L. According to the study authors, the results demonstrated that ecotoxic effects were correlated with surface area and not with mass. There was no evidence for particle uptake into the cells, rather the particles adsorbed to the cell wall. It is noted that both LUDOX® test materials contained biocides in concentrations of 200 and 500 ppm (=mg/L), respectively. These biocides may have considerably contributed to the algal toxicity seen in this study and the values reported by van Hoecke et al. (2008) should therefore not be associated with pure SiO_2 particles.

Later, van Hoecke et al. (2011) tested LUDOX® aqueous colloidal silica suspensions (obtained from Sigma–Aldrich, *i.e.*, LUDOX® CL-X and the positively charged alumina stabilised LUDOX CL with specific surface areas of 102 m^2/g and 203 m^2/g, respectively, in the alga *P. subcapitata*. In this study, no correlation with the surface area was found. Alumina coated particles showed lower toxicity than bare particles at concentrations ≥46 mg/L, except at pH 6.0. Addition of organic matter decreased toxicity of both particles. Due to the low surface charge, alumina coated particles aggregated in test medium and dissolution and nutrient adsorption characteristics were different and phosphate deficiency could have contributed to the higher toxicity of those particles at pH 6.0–6.8 compared to higher pH values. Again, the biocides and dispersant contained in LUDOX® CL-X may have contributed significantly to the toxicity observed and the values reported by van Hoecke et al. (2011) should therefore not be associated with pure SiO_2 particles.

4.2.2.4. Effects on fish. After injection into the yolk of zebrafish embryos, silica nanowires (55 nm × 2.1 μm) with aspect ratios (*i.e.*, ratio between length and diameter) greater than 1 were found to be highly toxic (LD_{50} = 110 pg/g embryo) and to cause embryo deformities. Spherical SiO_2 particles (particle sizes of 200 and 50 nm,

synthesised by the Stöber method) did however not exhibit any toxic or teratogenic activities at the same concentrations (Nelson et al., 2010).

4.2.2.5. Ex vivo studies. Treatment of mussel haemocytes with 1, 5 or 10 mg/L SiO_2 particles (primary particle size 14 nm, aggregated size in artificial sea water after 1 h 150–1600 nm) did not induce significant cytotoxicity in the neutral red retention (NRR) assay, but stimulated lysozyme release, oxyradical- and NO-production (Canesi et al., 2010).

4.3. Absorption, distribution, elimination and mammalian toxicity

Studies have been summarised by the OECD (2004), the ECETOC (2006), the EPA (2011) and Becker et al. (2009). Epidemiology was reviewed, amongst others, by the ECETOC (2006), IARC (1997), Merget et al. (2002) and McLaughlin et al. (1997). Therefore, only the most relevant and more recent studies are described in detail in the following section.

4.3.1. Absorption, distribution, elimination

A large number of *in vitro* studies have examined the uptake of SAS particles at a cellular level. Shapero and co-workers (Shapero et al., 2011) report time and space resolved uptake studies of 50-, 100 and 300-nm silica particles by A549 human lung epithelial cells. Particles of all sizes were taken up by these cells and found in endosomes of the cells. Also, Yu et al. (2009) found by TEM that SAS particles with average sizes between 30 and 535 nm were all taken up into the cytoplasm of mouse keratinocytes. Similarly, silica particles between 30 and 400 nm were taken up by 3T3-L1 fibroblasts during 24 h of exposure at 50 mg/L and located mostly in vesicles, not in the cell nucleus (Park et al., 2010a,b). Silica particles of different sizes (70, 200, 500 nm) were detected in the cytosol and endosomal compartments of human cervical carcinoma (HeLa) cells; the smaller particles were preferentially localised in lysosomes. No particles were found in mitochondria or nuclei (Al-Rawi et al., 2011). Uptake of 50 nm mesoporous silica particles by HeLa cells was more efficient than uptake of smaller particles (Lu et al., 2009). In none of the studies an uptake of silica nanoparticles in the cell nucleus is reported, except by Chen and von Mikecz (2005) and by Nabeshi et al. (2010), who used fluorescent labelled silica and whose results are therefore not representative for unmodified silicon dioxide particles. Removal of particles from living cells may largely occur by exocytosis (Borm et al., 2006a, 2006b), and has been demonstrated in mammalian cells for mesoporous silica nanoparticles (Slowing et al., 2011).

In vivo, Cho et al. (2009) studied the impact of SAS particle size on tissue distribution and elimination. Fluorescence dye-labelled 50-, 100- and 200 nm silica particles were intravenously injected in mice at a dose of 50 mg/kg bw. The tissue distribution and excretion of the injected particles differed depending on particle size. With increasing particle size, more particles were trapped by macrophages in the liver and spleen. All particles were cleared *via* urine and bile; however, the 50-nm particles were excreted faster than were the 100- and 200-nm particles. Clearance of SAS from the lungs after inhalation exposure is rapid, with silicon levels below the detection limit shortly after exposure (Arts et al., 2007; Lee and Kelly, 1992; Reuzel et al., 1991; Johnston et al., 2000). Most of the SAS is dissolved in the lung fluid, an observation that is consistent with the prediction models of Stöber et al. (2000) and only a minor part of the SAS is removed from the lungs by alveolar macrophages and carried to the oropharyngeal area by the mucociliary escalator or is transported to tracheobronchial lymph nodes.

In conclusion, SAS may enter the body in particulate or dissolved form. Depending on aggregate size and pH, SAS dissolve relatively fast in the body to form silicic acid. The tendency to supersaturate increases dissolution and hence distribution and elimination from the body. There is evidence of ready renal elimination of bioavailable fractions and also of whole particles. After inhalation, oral, intraperitoneal and intravenous exposures, SAS is eliminated from the lung tissues and other organs of experimental animals with no indication of accumulation, even after prolonged exposure to high doses or concentrations.

4.3.2. Acute toxicity

After oral and dermal administration, different SAS forms, including surface-treated SAS did not induce acute toxicity in rats up to the highest dose levels tested. Inhalation exposure to three forms of SAS (precipitated silica, silica gel, pyrogenic silica) on five consecutive days at 1 mg/m^3 for 6 h/day did not cause adverse effects in rats. At 5 mg/m^3, slight histopathological changes and changes in bronchoalveolar fluid (BALF) were found. Measurements at one- and three-months post-exposure to SAS did not reveal changes in BALF parameters. Differences between the three SAS forms were small and confined to the first day post-exposure (Arts et al., 2007). One- or three-day aerosol exposures produced no significant pulmonary inflammatory, genotoxic, or adverse lung histopathological effects in rats exposed to very high particle numbers of SAS (3.7×10^7 or 1.8×10^8 particles/cm^3, corresponding to mass concentrations of 1.8 or 86 mg/m^3 (Sayes et al., 2010). In this study, Sayes and co-workers used a "nanoparticle reactor" capable of producing *de novo* synthesised, aerosolised amorphous silica nanoparticles *via* thermal decomposition of tetraethylorthosilicate (TEOS). The median particle diameters were approximately 30 and 80 nm. Pulmonary toxicity (differential blood cell count, enzymatic activity of lactate dehydrogenase (LDH) and alkaline phosphatase in bronchoalveolar lavage fluid (BALF)) and genotoxicity endpoints (micronuclei induction) were assessed from 24 h up to 2 months after exposure. Kaewamatawong et al. (2005, 2006) compared the pulmonary toxicity of ultrafine and fine colloidal silica particles (average primary particle sizes of 14 and 213 nm) after intratracheal instillation in mice. The smaller particles had a greater ability to induce lung inflammation and tissue damage. Electron microscopy showed both particles on the bronchiolar and alveolar wall surface and in the cytoplasm of alveolar epithelial cells, alveolar macrophages and neutrophils. Mice injected intravenously with laboratory synthesised mesoporous silica with particle sizes of 150, 800 and 4000 nm and pore sizes of 3, 7and 16 nm, respectively, died, probably due to thrombosis (Hudson et al., 2008). In mice, silica particles (70 nm) induced liver injury after intravenous injection at 30 mg/kg bw, while 300- or 800 nm-sized particles had no effect, even at 100 mg/kg bw. Administration of 70 nm particles dose-dependently increased serum markers of liver injury, serum aminotransferase and inflammatory cytokines (Nishimori et al., 2009).

4.3.3. Irritation and sensitisation

Due to its desiccant (hygroscopic) nature, repeated skin contact with SAS can result in dry skin. In humans, symptoms of mechanical irritation of the skin, eye, nose and throat by SAS powder were reported (ECETOC, 2006). Exposure of rats to a high concentration of pyrogenic SAS (27 mg/m^3, 6 h/day for 6 days) resulted in transient changes in breathing parameters during exposure and in nasal and alveolar inflammation (Arts et al., 2008). Surface-treated SAS was not irritating to the rabbit eye or skin (EPA, 2011). "Nanosilica" (primary particle sizes of 7 and 10–20 nm) was not irritating to rabbit skin in a Draize test performed by Park et al. (2010a,b) according to *Korean Food and Drug Administration Guidelines*. Intraperitoneal and subcutaneous injections may produce local tissue reactions and/or granulomas and these routes have therefore not been further explored for medicinal applications of SAS in humans. No

cases of sensitisation in humans have been reported in decades of manufacture and use (information from producers). Furthermore, the chemical composition of SAS does not indicate a sensitising potential.

4.3.4. Repeated dose toxicity

The inhalation of respirable particles of SAS produces a time- and dose-related inflammation response of the lung tissue in animal studies. Exposure of rats for 13 weeks to an average concentration of 1.3 mg/m^3 of pyrogenic SAS resulted in mild reversible pro-inflammatory cell proliferation rather than a pathologically relevant tissue change. Given the low-grade severity of this common lung-tissue response, 1 mg/m^3 can be established as NOAEL and LOEL (sub-chronic, 13 weeks). At the LOAEL (5.9 mg/m^3) signs of adverse effects were found by the microscopic evaluation of tissues (stimulation of collagen production, increase in lung weight, incipient interstitial fibrosis, and slight focal atrophy in the olfactory epithelium). All these effects were reversible following discontinuation of exposure. In the same study also precipitated and surface-treated hydrophobic SAS forms were investigated. All tested forms showed qualitatively the same effects, however, the pyrogenic form induced somewhat more severe inflammatory effects (for details see Reuzel et al., 1991; ECETOC, 2006 and OECD, 2004). A dose-dependent inflammatory response after exposure to colloidal silica was found by Lee and Kelly (1992) and Warheit et al. (1991, 1995) at concentrations ≥50 mg/m^3 (6 h/day, 5 days/week for 2 or 4 weeks). The test material was "Ludox grade CL-X", obtained from Du Pont Chemicals and consisting of approximately 46% silica in water along with about 0.2% sodium oxide and 5% ethylene glycol. About 200 ppm of formaldehyde was present as a biocide. The pH of the liquid was 9 and the average primary particle size was about 22 nm. MMADs of the particles in the test atmosphere were reported as 2.9, 3.3 and 3.7 μm for the 10, 50 or 150 mg/m^3 groups, respectively. Three months after exposure, all biochemical parameters returned to control values. Lung-deposited silica particles were cleared rapidly from the lungs, with half-times of approximately 40 and 50 days for the 50 and 150 mg/m^3 treatment groups, respectively. The lungs did not show formation of fibrotic scar tissue or alveolar bronchiolarisation. The NOEL for Ludox in this study was at 10 mg/m^3. Chen et al. (2008) found that pulmonary inflammation was more severe in old (20 months) rats than in young or adult rats after exposure to amorphous silica particles (purity >99.9%, particle size 37.9 ± 3.3 nm; specific surface area 6.83 × 10^5 cm^2/g, particle number 1.52 × 10^{10} per μg; purchased from Jiangsu Haitai Nano Material Company Limited, Jiangsu/China). The rats were exposed for a period of 4 weeks at a concentration of 24.1 mg/m^3 for 40 min/day. Cardiovascular function changes were observed only in old animals.

Takizawa et al. (1988) tested food-grade micronised SAS by oral administration at dose levels of 0, 1.25, 2.5, and 5% for *ca.* 93 consecutive weeks in mice or for 103 consecutive weeks in rats. No biological or any other meaningful alterations in body weight, food consumption, or physical features were noted. There were no significant dose-related effects in clinical laboratory examinations, and the treatment did not cause gross or microscopic changes in the tissues examined. The occasional presence of neoplasms did not reveal any consistent, dose-related trends in any group. The OECD (2004) derived from this study a NOAEL for chronic oral administration at approximately 2500 mg/kg bw/day. The NOAEL for surface-treated silica in a 6-month dietary study was at 500 mg/kg bw/day, the only dose tested (EPA, 2011).

The toxic effects of nano- and micron-sized silica particles made from rice husk (and hence biogenic amorphous silica, not SAS) were studied by So et al. (2008). As this study is often discussed in the context of "nanosilica in food" it is nevertheless included in this review. The silica particles were about 30–90 nm and 0.5–30 μm in size; their purity given as 99.8%. Groups of male and female Balb/c and female C57BL/6 J mice were fed the particles at 1% in the diet or given the diet alone (controls). After feeding for 10 weeks, the blood of three male and three female Balb/c or three female C57BL/6 J mice was tested biochemically and haematologically. There was no difference between the groups in the tested parameters except for a higher serum alanine aminotransferase (ALT) value in the Balb/c mice treated with the smaller sized particles as compared to the controls (102.5 *vs.* 52.50 U/L). It has to be noted, however, that the high value is well within the normal range of ALT values reported for Balb/C mice in the literature (40.8 ± 6.7–226 ± 105, Hainfeld et al., 2006). Signs indicative of fatty livers were found histologically in selected animals that received the nano-sized particles, while Si contents of livers in both silica-treated groups were "almost the same". From the results, it was suggested by the study authors that "the nano-sized silica particle might have a toxic effect on the liver" even though there was no difference on health parameters after feeding a total amount of 140 g silica/kg mouse. Further to the questionable finding of an increase in ALT values in a very small group of animals, amorphous silica from natural origin was used in this study that may have been contaminated with organic impurities or crystalline silica. The findings reported by So *et al.* (2008), therefore, cannot be used in the assessment of SAS health effects.

In a study on mice by Isoda et al. (2011), (30) or 40 mg/kg bw of 70 nm spherical, non-porous silica particles (not specified further), injected intravenously twice per week for 4 weeks induced liver collagenosis and a 3.5-fold increase in hepatic hydroxyproline content, while 60 mg/kg bw of amino- or carboxyl-modified forms of the same particles did not cause liver fibrosis. Earlier, it was reported that the repeated administration of the unmodified 70 nm particles every three days for 4 weeks caused hepatic fibrosis in mice at 10 mg/kg bw (Nishimori et al., 2009).

From occupational exposure studies, there is no evidence of adverse pulmonary effects from SAS exposure (ECETOC, 2006). Workers in SAS manufacturing industries did not exhibit fibrosis of the lungs (silicosis) or any other permanent respiratory ailments.

4.3.5. Genotoxicity

SAS, including surface-treated SAS, were not mutagenic in standard bacterial test systems with and without metabolic activation (Ames-test) and did not induce chromosomal aberrations in mammalian cells (ECETOC, 2006; EPA, 2011; OECD, 2004).

At highly cytotoxic doses of silica gel (Spherisorb® suspensions at concentrations of 80 and 160 μg/cm^2), a weak induction of micronuclei was found in V79 cells *in vitro*. At doses lower than 40 μg/cm^2, the test material failed to significantly increase the frequency of micronuclei (Liu et al., 1996), suggesting that micronucleus induction was a secondary or indirect result of other cytotoxic processes. Incubation of A549 lung carcinoma cells for 40 h with non-cytotoxic doses of amorphous silica particles synthesised according to the Stöber method (16, 60 and 104 nm) resulted in an increased number of micronuclei which was statistically not significant. In addition, other weak chromosomal effects were observed, but again without reaching statistical significance (Gonzalez et al., 2010). The potential of four differently sized SAS particles (nominal sizes: 10, 30, 80 and 400 nm; actual sizes: 11, 34, 34 and 248 nm) to induce chromosomal aberrations and gene mutations was studied using two *in vitro* genotoxicity assays (Park et al., 2010a,b). The particles had been synthesised with the Stöber-method without stabiliser and were endotoxin-, bacteria- and fungi-free. Only the 80 (34) nm silica nanoparticles induced a weak, but statistically significant increase in the number of chromosomal aberrations in a micronucleus assay using 3T3-L1 mouse fibroblasts (quantitative data not shown in the original publication; test concentrations were 4, 40 or 400 mg/L). The 30

(34) and 80 (34) nm silica nanoparticles induced gene mutations in mouse embryonic fibroblasts carrying the lacZ reporter gene (quantitative data not shown in the original publication, but it is mentioned that the increases were at most three-fold and only for the 80 nm particles statistically significant). 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. DNA damage (most probably as a result of cytotoxicity or indirect mechanisms) was found in Comet assays performed on hamster and human embryonic lung fibroblasts, in a neuronal cell line (without dose-response) and with alumina coated SAS particles in a human breast cell line (Kim et al., 2010; Pacheco et al., 2007; Zhong et al., 1997). Yang et al. (2009a,b) report on a very slight positive effect of SiO_2 particles in a Comet assay, performed on primary mouse embryo fibroblast cells with a material that was described as having "a crystal structure with an average size of 20.2 nm". No genotoxicity was detected in a well-conducted and reproducible Comet assay performed to current standards in mouse fibroblasts with stabilised and non-stabilised LUDOX® materials with positive and negative surface charges (Barnes et al., 2008).

In vivo, SAS were not mutagenic. In rats, no induction of micronuclei was found from 24 h up to 2 months after one- or three-day exposures to *de novo* synthesised, aerosolised amorphous silica nanoparticles at 3.7×10^7 and 1.8×10^8 particles/cm^3, corresponding to mass concentrations of 1.8 or 86 mg/m^3 (Sayes et al., 2010). In rats, no increase in HPRT mutation frequency was detected after 13 weeks of exposure to SAS at 50 mg/m^3 (Aerosil® 200, MMAD 0.81 μm) (Johnston et al., 2000).

Taken together, the results obtained in mutagenicity and genotoxicity tests give no evidence that SAS induce mutations either *in vitro* or *in vivo*. Genotoxicity was observed *in vitro*, usually at dose levels and concentrations that also induced cytotoxicity. No genotoxicity has been found after *in vivo* exposure of experimental animals.

4.3.6. Carcinogenicity

In an oral carcinogenicity study with rats and mice at dietary levels of 1.25, 2.5, and 5% for 102 and 93 weeks, respectively, no evidence of tumour induction by SAS (test material Syloid 244, silica gel) was found (Takizawa et al., 1988). Surface-treated SAS showed no evidence of carcinogenicity in a 24 month dietary study in rats (EPA, 2011).

In one study in rats using intrapleural implantation of two different preparations of synthetic amorphous silica, no increased incidence of tumours was observed (IARC, 1997). Amorphous silica of high surface area (Sigma–Aldrich pyrogenic silica, SSA 210 m^2/g) was used in a study investigating various dusts following repeated weekly intratracheal instillations. After 5 or 10 instillations of silica (each time 3 mg, in total 15 or 30 mg) tumours were found in 0 and 7.9% of rats, respectively. Mortality was 13% and the prevalence of fibrosis was determined as 35% and 76%, but was very high in all study groups, including the two groups of unexposed controls (n = 91 rats), in which 11 cases were found at necropsy (Borm et al., 2004; Morfeld et al., 2006; Valberg et al., 2009).

Recently, Kolling et al. (2011) reported a statistically significant tumour response of 5 out of a group of 53 female Wistar rats (*i.e.*, 9.4%) at 29 months of experimental time after repeated intratracheal instillations of 0.3 mL of a dispersion of amorphous silica in physiological saline (30 mg × 0.5 mg, *i.e.*, in total 15 mg of Aerosil® 150 hydrophilic pyrogenic silica, every 14 days; primary particles size 14 nm; BET surface area 150 ± 15 m^2/g; density *ca.* 2.2 g/m^2; 99.8% SiO_2). The tumours were described as two bronchiolo-alveolar adenomas, two bronichiolo-alveolar carcinomas and one squamous cell carcinoma. The small size of the lung tumours indicated - according to the study authors – that these tumours may have started to develop rather late in life time. The study authors further caution that "...the causation of the tumours observed in rats treated with amorphous silica should be handled with care as it can not be excluded that the high frequency of intratracheal instillations may have added to the development of neoplasias...". There was a significant increase in interstitial fibrosis, inflammatory cell infiltration and bronchiolo-alveolar hyperplasias of the amorphous SiO_2 treated rats. The high toxicity of intratracheally instilled amorphous SiO_2 was shown by the results from bronchioalveolar lavage fluid examinations 9 months after first instillation with leukocyte counts 192-fold higher than the controls. No tumours were observed in the control group treated with physiological saline and there was no difference in mortality between the groups. The positive control, crystalline silica, elicited the greatest magnitude and progression of pulmonary inflammatory reactions, fibrosis and the highest incidence of primary lung tumours (39.6%).

In humans, there is no evidence that SAS is associated with fibrosis of the lungs (silicosis) or cancer of the lung or any other form of cancer. The International Agency on the Research of Cancer (IARC, 1997) has assessed amorphous silica (silicon dioxide without crystalline structure) as not classifiable with regard to its carcinogenicity for humans (Group 3).

Overall, there is no evidence of SAS inducing cancer in animals or humans. The tumour incidence in animals after intratracheal instillation was much lower than that of biopersistent dusts, and was probably caused, as well as the fibrotic reactions, by overload phenomena due to the unphysiological administration of high boluses of the test material. As SAS have not been shown to be mutagenic, no carcinogenic risk is anticipated for the oral, dermal and inhalation routes under exposure conditions that do not induce chronic tissue inflammation.

4.3.7. Reproductive and developmental toxicity

No reproductive or developmental (including teratogenic) effects were observed following the oral administration of food-grade amorphous silica (silica aerogel) in rabbits at 1600 mg/kg bw/day, hamsters at 1600 mg/kg bw/day, mice at 1340 mg/kg bw/day, and rats at 1350 mg/kg bw/day (FDA, 1973). Based on this study and the fact that there were no pathological effects seen in the reproductive organs of male and female rats in repeated dose oral and inhalation studies with surface-treated SAS, the EPA (2011) concluded that there is no need for reproductive and developmental studies with surface-treated silica.

Xue et al. (2006) studied long-term toxicity and reproductive function in groups of 15 male and 20 female Kungming mice treated with silica nanoparticles (prepared in the laboratory from TEOS, primary particle size about 40 nm). Particle suspensions in physiological saline solution were injected in the tail veins of the animals. The authors found that the particles were excreted with the urine. No effect on reproductive function was found.

In conclusion, there is no evidence from limited animal studies that SAS induce reproductive or developmental toxicity.

5. Mode of action

The mode of action (MOA) approach in chemical risk assessment is based on the concept that for an observed effect produced by a given compound it may be possible to hypothesize – based on available data – a sequence of key events that are along the causal path to the effect, *i.e.*, the MOA (Meek, 2009). Once a MOA is established, qualitative and quantitative comparison of each key event between the experimental test systems and humans enables a conclusion as to likely relevance of the MOA for human and environmental risk assessment.

5.1. Critical effects and target organs

5.1.1. Sensitivity of different cell types (in vitro studies)

Certain cell types, such as red blood cells (RBCs) and primary alveolar macrophages seem to be particularly sensitive to SAS toxicity (Costantini et al., 2011; Sayes et al., 2007), while others, particularly those with short doubling times (such as tumour cells) are relatively resistant (Chang et al., 2007; Kim et al., 2010, *cf.* also Table 2) . As described in the following section, this particular toxicity is linked to particular mechanisms of membrane interactions, uptake mechanisms, signalling responses, and vesicle trafficking pathways.

5.1.2. Toxicity targets in vivo

Severe systemic reactions causing deaths in the experimental animals were observed after intraperitoneal or intravenous injections of calcined and non-calcined mesoporous silica. Lung histopathology indicated that thrombosis may have caused the death of the animals (Hudson et al., 2008). Coagulation, thrombosis and vascular dysfunction should therefore be considered as relevant endpoints if particles are to be delivered by these routes.

The only adverse effects found after oral, dermal or inhalation exposures were dryness of skin and mucous membranes, due to the hygroscopic property of SAS, as well as lung toxicity. The latter is considered a critical effect.

The cascade of key events causing thrombosis and lung toxicity *in vivo* after SAS exposure, *i.e.*, the hypothesized modes of action (MOA) of SAS and its relevance to humans are discussed in the following chapter. First, a general overview of SAS interactions with biological media is provided to put these key events into a more general context.

5.2. Key events

5.2.1. Interactions with biological fluids and cells

Silica aggregates or particles can be adsorbed on bacterial cells, aquatic, benthic or terrestrial organisms and damage the outer cell membrane and cuticulae of insects, an effect that has efficiently been exploited in the use of SAS as pest controlling agent. Already in 1966, Nash and co-workers hypothesized that silica toxicity is influenced by particle surface chemistry in that proton-donating groups would denature surrounding proteins (Nash et al., 1966). Due to their surface characteristics, silica particles will adsorb macromolecules (proteins *etc.*) from the surrounding environment (*e.g.*, body fluids) onto their surface. The adsorption process is influenced by surface energy, surface charge and the affinity to specific biomolecules. Hydrophilic silica can effectively adsorb high-molecular proteins of synthetic and natural origin. Dutta and co-workers showed that the protein adsorption profiles for 50–1000-nm amorphous silica particles were comparable (Dutta et al., 2007). Silica particles may also adsorb bronchoalveolar lining fluid components, including lung surfactant and proteins, such as the surfactant protein D (SP-D) (Hamilton et al., 2008). Hence, before inhaled silica particles come into contact with alveolar macrophages, lung surfactant composed of phospholipids and surfactant proteins (SP) could potentially coat the outer surface of the silica particles modifying the surface chemistry and ultimately influence the toxicity (Hamilton et al., 2008). A high specific surface area may promote the adsorption of peptides and proteins contained in the alveolar lining fluid.

Though agglomerated and aggregated particles in the μm range might theoretically be broken down to the size of the primary nanoparticle within the body, research results show the robustness of aggregates and agglomerates to disaggregation, even in the context of high-energy processing (Maier et al., 2006).

5.2.2. Interactions with cell membrane components

The denaturation of cell membrane proteins by proton-donating silanol groups is the major underlying mechanism for membrane damage. Pandurangi et al. (1990) found a strong correlation between surface silanol groups (Si—O—H) and the haemolytic activity of amorphous silica and suggested that the surface hydrogen of silica bonds to protein components of the membrane and subsequently abstracts these proteins from the membrane. The haemolytic activity is highly specific for silanol and seems to depend only on the concentration of negatively charged silanol groups that are accessible by the cell membranes of erythrocytes (Slowing et al., 2009). A strong distortion of the membrane after interaction with silica particles can lead to loss of membrane flexibility and resiliency as well as the release of haemoglobin (haemolysis). The agglutination of erythrocytes can be enhanced due to interaction with aggregates of SAS particles which prevent the electrostatic repulsive interaction of negatively charged cells due to the strong interaction of SAS particles with proteins integrated into the cell membranes (Chuiko, 2003). In contrast, the haemolytic potential of hydrophobic silica particles with a siloxane surface structure is low.

Translocation of particles into cells is dependent on interactions with the cell membrane, *i.e.*, processes of endocytosis (mainly pinocytosis and phagocytosis or receptor-mediated endocytosis). The fusion of endomembranes with phagosomes has long been recognised to occur as part of the normal particle uptake process; particles can be internalised in enterocytes by micropinocytosis (cytopempsis) and be transported in small vesicles through the cells (Volkheimer, 1974). Uptake of particles by the gastrointestinal tract occurs *via* membranous epithelial cells (M-cells) on the intestinal mucosa or by persorption in epithelial cells (Borm et al., 2006b). Silica containing phagosomes may fuse with endosomes during, or shortly after, internalisation. By this mechanism silica particles may cause damage to internal membranes allowing the leakage of endo-lysosomal material into the cytoplasm leading to cytokine release. Particles may also overload the endo-lysosomal system, which could lead to an impairment of lysosomal capacity and interfere with programmed autophagic cell death and breakdown of ingested pathogens. Evidence for an active uptake mechanism of silica particles by actin- and clathrin-mediated endocytosis was found by Chung et al. (2007) and Costantini et al. (2011). Costantini et al. (2011) showed that scavenger receptors on cell surfaces are involved in silica binding and internalisation and that cell contact of silica particles with macrophages was necessary for toxicity. If uptake of silica was driven through the FccRIIA receptor-mediated endocytosis pathway the toxicity of silica in macrophages was drastically reduced. In alveolar type II epithelial cells, heparan sulphate proteoglycans, especially syndeca-1, seem to play a critical role in the attachment and internalisation of positively charged SAS particles (Orr et al., 2009). Syndecan-1 was found to mediate the initial interactions of particles at the cell surface, their coupling with actin filaments across the cell membrane, and their subsequent internalisation. Particle size might be a limiting factor, raising the possibility that positively charged particles smaller than 100 nm might enter the cell *via* another mechanism.

5.2.3. Oxidative stress and inflammation, gene expression profiles

In response to a physical or chemical stressor, cells may produce reactive oxygen species (ROS). Cell injury only results if the amount of ROS produced overloads the normal anti-oxidant capacity of the cell. An increase in cellular ROS production first triggers anti-oxidant defence by the induction of phase II antioxidant enzymes *via* the activation of the antioxidant response element by NF-E2-related factor (Nrf)-2, a key antioxidant transcription factor found, for example, in human lung epithelial cells. At a higher stress level, activation of MAP kinases and NF-κB cascades induces

Table 2
In vitro studies assessing cytotoxicity, ROS production and inflammation.

Cell system	Particle type	Mean primary particle size	Source	Treatment/parameters studied	Results	Reference
Cardiovascular system						
Red blood cells	Cab-O-Sil, 200 m^2/g	nr	Commercial	30 min at RT with 40–2000 mg/L in Dulbecco's phosphate buffered saline	Silanol groups might be involved in the lysis process	Pandurangi et al. (1990)
Human red blood cells	Precipitated SAS (Zeofree 80, purity 98%)	1–3 μm, SSA 77.7 m^2/g	Commercial	Haemolysis of human RBCs (tested at 0.1–26.5 $\mu g/cm^2$), neg control PBS, pos control 1% Triton-X 100	Haemolysis at ≥0.1 $\mu g/cm^2$	Sayes et al. (2007)
Endothelial cells	SAS (pyrogenic)	14 nm (range 4–40)	Commercial	5, 50 mg/L for 24 h	Internalisation, slight impairment of proliferative activity; IL-8 release, no cytotoxicity	Peters et al. (2004)
Myocardial H9c2(2-1) cells	Colloidal SiO_2	21 and 48 nm	Center of Analysis and Test Research (East China University of Science and Technology, Shanghai, China)	12, 24, 36 and 48 h at 100–1600 mg/L/cytotoxicity (LDH, MTT, H&E staining)	Cytotoxicity dependent on size, dose and time; oxidative stress, induced G1 phase arrest and upregulated levels of p53 and p21	Ye et al. (2010a)
Pulmonary system						
Human A549 lung epithelial cell line	nr	15 and 46 nm (SSA 268 and 53 m^2/g);	Commercial	10–100 mg/L for up to 72 h; hydrodyn diam. in de-ion. water 590 and 617 nm	Dose and time-dependent cytotoxic response; no difference between the two particle sizes; only 15 nm at ≥10 mg/L: ROS generation↑; concomitant GSH depletion, lipid peroxidation↑	Lin et al. (2006)
Human immortalised bronchial epithelial cell line BEAS-2B	Fumed and porous silica	7 nm (SSA 350 m^2/g, fumed) and 5–15 nm (644 m^2/g, porous)	Commercial	1 mg/L dispersed for 20 min in DMEM/F12 (aggregate size 20–400 nm as measured by DLS)/viability (MTT), apoptosis, oxidative stress response	Formation of ROS and induction of antioxidant enzymes; porous SNP showed more response than 7 nm fumed SNPs; viability *ca.* 80%	Eom and Choi (2009)
Human primary pulmonary fibroblasts	Amorphous silica	Not specified	Donated by university of vermont	Baked at 180 °C for 2 h, 1–100 mg/L suspension in MEM, 24, 48 h; IL-6, IL-8, MCP-1,TGF-beta; PGs, immunocyto-chemistry, cytotoxicity (MTT)	COX-2↑ at ≥10 mg/L, PGE synthase and PGE2↑↑, PGF2a↑, IL-8↑; non-toxic	O'Reilly et al. (2005)
Rat L2 lung epithelial cells, primary alveolar macrophages, co-cultures	Precipitated SAS (Zeofree 80, purity 98%)	1–3 μm, SSA 77.7 m^2/g	Commercial	0.0052–520 $\mu g/cm^2$ for 1–48 h, cytotoxicity (MTT, LDH) at several time periods; inflammatory cytokines (MIP-2; TNF-alpha, IL-6) at 24 h	LDH↑ in L2 at ≥5.2 $\mu g/cm^2$ (24, 48 h), ≥52 $\mu g/cm^2$ (4 h). and at 520 $\mu g/cm^2$ (1 h); no increase in alveolar macrophages in co-cultures at ≥5.2 $\mu g/cm^2$ (48 h) and at 520 $\mu g/cm^2$ (24 h); MTT↓ in alveolar macrophages at 5.2 and 52 $\mu g/cm^2$ (4 h), in all cell types at 5.2 and 52 $\mu g/cm^2$ after 24 h; MIP-2↑ (only in co-cultures and alveolar macrophages at 0.52 and 5.2 $\mu g/cm^2$); IL-6↑ (only in co-cultures at ≥0.52 $\mu g/cm^2$); TNF-alpha↑ only in co-cultures at 0.52 and 5.2 $\mu g/cm^2$	Sayes et al. (2007)

Mouse alveolar macrophages; Mouse lung epithelial type II	Spherical; naked or coated with ovalbumin or antibody	1, 3 μm	Commercial	16.5, 25, or 50 μg/cm^2; endotoxin-free; viability (propidium iodide staining at 0–24 h)	48% staining of macrophages after 4 h at 50 μg/cm^2; 100% cell death after 24 h; contact with cell necessary for toxicity; not toxic to other cell types	Costantini et al. (2011)
MLE15 mouse cell line	Aerosil 200	*ca.* 12 nm, SSA 200 ± 25 m^2/g	Commercial	0, 4.7, 9,5, 18.9 μg/cm^2 for 24 h	MIP-2↑ at ≥9.5 μg/cm^2; LDH↑ at ≥4.7 μg/cm^2;activation of AP-1	Singal and Finkelstein (2005); Singal (2010)
Skin						
HEL-30 mouse keratinocytes	Amorphous silica, not specified further	30, 48, 118, 535 nm	Laboratory	10–200 mg/L for 24 h	30, 48 nm: LDH↑ at 100 ppm, cytotoxic (MTT); GSH↓ at ≥50 ppm for 30 nm particles only; no change in ROS	Yu et al. (2009)
HaCaT human skin cell line	Purity > 99.7%, amorphous	15 and 30 nm, and micro-sized particles, zeta potential −14.37, −63.31, and −59.70 mV	Manufacturer not specified	2.5, 5, 10, 15, 20, 40, 60, 80 mg/L for 24 h; 10 mg/L (protein expression)	Cell viability↓ after 24 h at 10 mg/L (morphology); IC_{50} 23.0, 27.3, 34.8 mg/L, for 15-, 30- and micro-sized particles; cell cycle inhibited, but no significant dose- and size relation; dose- and size-related apoptosis; Prx6 and GSTP1↓	Yang et al. (2010)
Nerve system						
SH-SY5Y neuronal cell line	LUDOX® AS-20, CL and AM, polygon	16.9, 13.3, 15.3 nm; charge (pH, stabiliser): neg (9.1, NH_4OH), pos (4.5, NaCl alumina coated, neg (8.9, sodium aluminate)	Commercial	48 h, up to 1000 ppm	Cell viability↓ at ≥100 ppm (AS-20, AM) or >1000 ppm (CL; MTT assay); intracellular ROS↑ at >100 ppm only in AS-20 and AM-treated cells; Comet assay inconclusive	Kim et al. (2010)
Liver						
Human hepatic L-02 cell line	Colloidal SiO_2	21. 48 and 86 nm	Center of Analysis and Test Research (East China University of Science and Technology, Shanghai, China)	200–1000 mg/L for 12, 24, 36 and 48 h; cytotoxicity (LDH release), ROS and ultrastructure; glutathione, lipid peroxidation, apoptosis	Only 21 nm particles were cytotoxic and induced oxidative stress. apoptosis and upregulated levels of p53 and Bax-Bcl-2 ratio	Ye et al. (2010b)
Others						
Human mesothelioma MSTO-211H cells; mouse embryo 3T3 fibroblasts	Food-grade SAS	nr	Commercial	0–15 ppm (6 days); 0–30 ppm (3 days)	No effects on MTT conversion and DNA content	Brunner et al. (2006)
Human skin and lung fibroblasts (WS1; CCD-966sk; MRC-5s); Human A549 lung epithelial tumour, MKN-28 gastric epithelial adenocarcinoma and HT-29 colon adenocarcinoma cell line	Synthesised from silicates or TEOS	21, 80 (TEOS) nm (from TEOS); hydrodynamic size 188.3 and 236.3 (from TEOS)	Laboratory	48 h	Slightly cytotoxic (MTT, LDH) at high concentrations (*ca.* 138 ppm); fibroblast cells with long doubling times more susceptible than tumour cells with short doubling times	Chang et al. (2007)
Mouse peritoneal macrophage cell line RAW 264.7	SAS	14 nm (SSA 77.7 m^2/g); "mild" aggregation (not specified further)	Commercial	0.0052, 0.052, 0.52, 5.2, 52, and 520 μg/cm^2). 24 h/cytotoxicity (MTT, LDH), apoptosis	Dose-dependent cytotoxicity ≥0.0052 μg/cm^2: apoptosis (DNA fragmentation) after treatment with 2 μg/cm × 52 μg/cm for 24 h; annexin V binding after 6 h)	Kim et al. (2009)
Mouse peritoneal macrophage cell line RAW 264.7	SAS, purity 99.8%	12 nm	Commercial	5–40 ppm	ROS↑ intracellular GSH↓ nitric oxide↑	Park and Park (2009)
Mouse 3T3 fibroblasts	Ludox TM50	38 nm (hydrodynamic diameter, DLS)	Commercial	5–100 mg/L for 24 h, XTT viability assay	Viability decreased to about 60% at 100 mg/L; in DMEM with FCS increase in particle size due to agglomeration and reduced toxicity	Drescher et al. (2011)

DLS: Dynamic light scatterin; DMEM/F-12: Dulbecco's modified eagle medium/nutrient mixture F-12; FCS: Foetal calf serum; H&E: Haematoxylin and eosin; MEM: Minimum essential medium; na: Not available; nr: Not reported; RBC: Red blood cell; RT: Room temperature; SAA: Specific surface area; SNP: Silica nanoparticle.

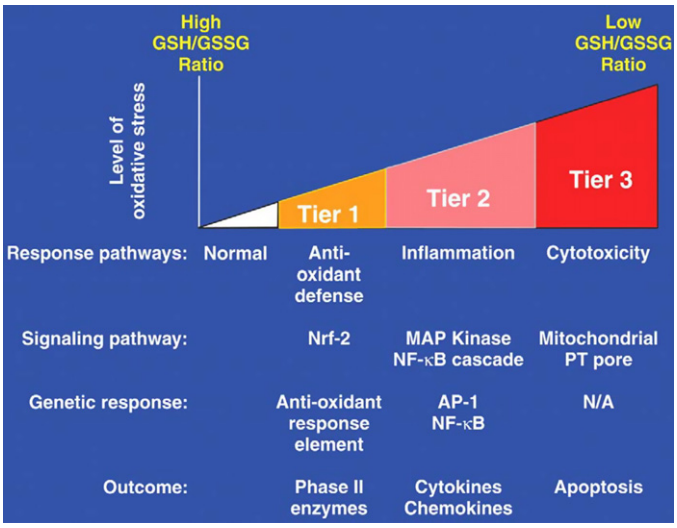

Fig. 4. Response pathways to oxidative stress.

pro-inflammatory cytokine and chemokine production and release. Perturbation of the mitochondrial functions and disruption of electron transfer may result in cellular necrosis or apoptosis. Response pathways to levels of oxidative stress are shown in the following scheme (see Fig. 4, reproduced from Nel et al., 2006 with permission).

After SAS exposure, ROS generation and lipid peroxidation were found in human A549 cells (Lin et al., 2006) and in conjunction with decreased intracellular GSH levels (an indicator that the cellular anti-oxidant system is overloaded) in the RAW 264.7 cell line (derived from a mouse peritoneal macrophage cell line) with silica particles at 5–40 ppm (average primary particle size 12 nm) (Park and Park, 2009). Oxidative stress responses were also seen in a neuronal cell line after *in vitro* exposure to LUDOX® AS-20 and AM at ≥100 ppm, but not after treatment with the positively charged LUDOX® CL up to the highest tested concentration of 500 ppm (Kim et al., 2010). Only with the smallest particles (30 nm) the redox potential of cells (GSH) was reduced significantly at concentrations of 50 ppm or higher. Particles larger than 30 nm showed no changes in GSH levels, nor was there ROS formation (Yu et al., 2009). Ye et al. (2010a) reported that colloidal silica particles (primary particle sizes of 21 and 48 nm, 100–1600 ppm) caused oxidative stress, induced G1 phase arrest and upregulated levels of p53 and p21 in H9c2(2-1) cells.

An increase in IL-8, a key factor in neutrophil chemotaxis was found *in vitro* in primary human lung fibroblasts (O'Reilly et al., 2005) and in endothelial cells by Peters and co-workers (Peters et al., 2004). O'Reilly et al. (2005) found that crystalline and amorphous silica differentially regulated the cyclooxygenase-prostaglandin pathway. In primary human pulmonary fibroblasts, amorphous silica had the ability to directly upregulate the early inflammatory mediator COX-2, the prostaglandin E (PGE) synthase and the downstream antifibrotic mediator PGE2.

Precipitated SAS has been shown to increase the production of macrophage inflammatory protein (MIP)-2 cytokines in primary rat alveolar macrophages (Sayes et al., 2007). Also in the immortalised alveolar type II tumour cell line MLE15, a dose-dependent increased expression of MIP-2 was found after 24 h of incubation with SAS (Aerosil 200) (Singal, 2010; Singal and Finkelstein, 2005). The increase in MIP-2 protein was partly caused by an increase in ROS generation as it was shown that MIP-2 production was inhibited by the addition of antioxidants. The silica particles also induced inflammatory gene expression through the activation of nuclear factor-kappa B (NF-κB) and activator protein 1 (AP-1) *via* the mitogen-activated protein (MAP) kinase pathway. In addition, NF-E2-related factor (Nrf)-2 and HO-1 protein expression were influenced by incubation of MLE15 cells with Aerosil 200. The inflammatory protein expression was delayed as compared to the time course observed with a soluble pro-inflammatory stimulus. The induction of HO-1 *via* NF-κ B and Nrf2, as well as the extracellular signal-related kinase (ERK) MAP kinase signal transduction pathway were also observed by Eom and Choi (2009) in a human bronchial epithelial cell line exposed to pyrogenic and porous silica particles. Cells exposed to porous silica particles showed a more sensitive response than those exposed to pyrogenic silica.

In vivo, cytokine levels (IL-1beta, IL-6, IL-8, and TNF-alpha) as well as those of the chemokines MCP-1 (monocyte chemoattractant protein 1) and MIP-2 were increased in mice after intratracheal SAS instillation (2, 10 or 50 mg/kg of SAS with a primary particle size of 14 nm). The histopathological examination revealed acute inflammation. Complete reversibility of all changes was found one week after exposure (Cho et al., 2007). These cytokines and chemokines can activate NALP3, a member of the cytoplasmic Nod-like receptor family that regulates the activity of Caspase-1 *via* formation of the inflammasome. Activated Caspase-1 triggers the cleavage of pro-inflammatory cytokines (IL-1beta and IL-18) for subsequent activation and secretion, which is likely to be part of the pathway leading to silicosis. However, there is no *in vivo* correlate for this pathway, as SAS is not involved in progressive fibrosis or silicosis of the lung. High doses of SAS may however indeed result in acute pulmonary inflammatory responses. Apoptosis was not found in A549 and rat alveolar cells up to a concentration of 100 ppm SAS.

Treatment of ICR mice by single intraperitoneal injection of 50, 100 or 250 mg/kg of pyrogenic silica (average primary particle size 12 nm) caused increased blood levels of IL-1beta and TNF-alpha, and increased nitric oxide release from peritoneal macrophages. *Ex vivo,* cultured peritoneal macrophages harvested from the treated mice showed the expression of inflammation-related genes (IL-1, IL-6, TNF-alpha, inducible nitric oxide synthase, cyclooxygenase 2). In the spleen, the relative distribution of natural killer cells and T cells was increased 184.8% and 115.1%, respectively, as compared with control animals, and that of B cells was decreased to 87.7% (Park and Park, 2009).

Gene expression profiles after exposure to amorphous silica particles were studied in human epidermal keratinocytes (HaCaT cells) (Yang et al., 2010; see Table 2 for particle characterisation). At 10 mg/L – the only reported, slightly cytotoxic concentration–a downregulation of oxidative-stress associated proteins (Prx1, Prx6, Trx, GSTP1) may indicate a reduced antioxidant capacity following the induction of cytotoxicity by particle exposure. Similarly, changes in molecular chaperones and energy metabolism-associated proteins were indications for silica-induced cytotoxicity. The typical alterations of apoptotic marker proteins were not found. Cytoskeleton-associated proteins (keratin 9, keratin 4) were upregulated and may represent a compensatory stress response.

5.2.4. Key events causing SAS toxicity

The cascade of key events causing toxicity after SAS exposure, *i.e.*, the mode of action (MOA) of SAS and its relevance are summarised in Table 3.

SAS may interact with blood cells. *In vitro*, haemolysis and clotting of cells has been found in the presence of hydrophilic SAS. *In vivo*, intravenous or intraperitoneal injections of mesoporous silica particles caused the death of laboratory animals, probably by pulmonary embolism. Further studies would therefore be necessary to explore dose-response relationships, if those routes are considered, for instance, when using silica particles for the administration of medicinal or diagnostic products.

Table 3
The cascades of key events.

Key event	*In vitro*	*In vivo*	Weight of evidence
Haematotoxicity			
Physical adsorption to RBCs	Demonstrated for hydrophilic SAS	Not studied	Considerable *in vitro*, none *in vivo*
Clotting, coagulation	Demonstrated for hydrophilic SAS and positively charged particles	Not studied	Considerable *in vitro*, none *in vivo*
Thrombosis	Not studied	Found with mesoporous silica particles in animal studies after i.v. and i.p. administration	None *in vitro*, some *in vivo*
Lung toxicity			
Particle uptake by alveolar macrophages	Several mechanisms demonstrated *in vitro* (receptor-mediated, translocation, phagocytosis); overload conditions of phagosomes can generate reactive oxygen species, and stimulate pro-inflammatory gene transcription	Demonstrated in animal studies	Considerable *in vitro* and *in vivo*
Oxidative stress	ROS generation outweighs anti-oxidant capacity in some studies, demonstrated in mouse peritoneal macrophages, alveolar macrophages and keratinocytes as well as neuronal cells	No evidence (not studied)	Limited *in vitro*, none *in vivo*
Inflammation	Induction of pro-inflammatory cytokines and chemokines in alveolar macrophages	Reversible changes of biomarkers in BAL (macrophages, neutrophils, lymphocytes, lactate dehydrogenase, total protein, glutathione reductase) and morphological changes in lung tissue; incidence/severity of inflammation correlates with exposure concentration; no clinical evidence in humans, no fibrosis, no silicosis	Considerable *in vitro* and in animals, none in humans
Genotoxicity	Not mutagenic, not genotoxic	Not mutagenic in rats, even after prolonged inhalation of irritant concentrations	None *in vitro*, none in animals, none in humans
Lung tumours	No cell transformation tests available	No evidence from epidemiology studies	None *in vitro*, none in animals, none in humans

SAS induced pulmonary injury in animals *via* an inflammatory process following high exposure concentrations. Due to fast and complete elimination of SAS from pulmonary tissues and the body, no SAS accumulation occurs. The observed changes in animal experiments are reversible up to very high exposures, which can practically not be obtained under normal conditions of handling and use of these materials by workers and consumers. As non-threshold effects (mutagenicity) are not involved in the cascade of key events, there is no human health risk associated with SAS if current occupational hygiene standards are met.

6. Discussion

The biological activity and toxicity of silica is related to its physical and chemical properties (such as crystallinity, shape, composition and surface reactivity). The specific physical and chemical properties need to be considered in the ecotoxicological or toxicological testing. In particular, SAS materials usually do not exist as single particles (primary particles, nodules) but in the form of micro-metre-sized, firmly bound aggregated and loosely connected agglomerates. However, authors of studies on SAS or "nanosilica" often only report the primary particle size and insufficiently characterise their test material, which makes interpretation and comparison with other test materials and studies difficult. Stabilised colloidal silica with isolated particles in the nano-size range is commercially available, however it usually also quickly polymerizes to bigger aggregates under physiological testing conditions. Aggregation and agglomeration of SAS particles grossly reduces their bioavailability.

In contrast to crystalline silica, SAS slowly dissolves in aqueous environments and body fluids. None of the SAS types was shown to bioaccumulate and all disappear within a few weeks from living organisms by physiological excretion mechanisms. The tendency to supersaturate increases the elimination from body tissues. Any silica absorbed (either as particle or in dissolved form) is excreted by the kidneys without evidence of accumulation in the body. This is very different from crystalline silica forms which exhibit a marked tendency to accumulate and persist in the lung and lymph nodes.

SAS adsorbs to cellular surfaces and can affect membrane structures and integrity. The biological activity and *in vitro* cytotoxicity can be related to the particle surface characteristics interfacing with the biological milieu rather than to particle size. The physical properties and the results from mechanistic studies with other particles suggest that smaller particles, due to their greater surface area per unit of mass, may be more effective in inducing toxic effects. For SAS particles, the abundance of negatively charged groups on the external particle surface and the number of accessible silanol groups mainly determine the extent of the interaction with cell membranes and the probability of coagulation and haemolysis.

Effects on algae and fish were only observed at extremely high SAS concentrations that exceed current cut-off values for classification as hazardous. No effects on growth and reproduction parameters were found in *daphniae* or aquatic midge. Even after direct injection into the yolk of zebrafish embryos, no adverse effects were seen with spherical silica particles, while nanowires caused malformations. Toxicity to bacteria and damage to the cell membrane in yeast were observed only at very high silica concentrations of $\geq$1000 ppm.

In humans, SAS did not induce silicosis, lung cancer or any other form of cancer. There is no evidence that SAS induces mutations either *in vitro* or *in vivo*. Though genotoxicity was observed in a few *in vitro* test systems, this was generally at dose levels and concentrations that also induced cytotoxicity. No genotoxicity was found after *in vivo* exposure of experimental animals. In rats, SAS

produced transient lung inflammation, and reversible increases of pro-inflammatory cytokines and chemokines at exposure levels of 5 mg/m^3 (respirable dust) or higher with 1 mg/m^3 (respirable dust) being the No-observed-effect-level (NOEL). As elimination mechanisms include the clearance of particles by macrophages and since human macrophages have about four times the volume of rat macrophages (Krombach et al., 1997), the rat is assumed to respond with more chronic inflammation and epithelial responses as compared to humans.

Important insight into the mechanisms and modes of action of SAS, including colloidal silica, has been gained from mechanistic studies (*e.g.*, *via* intratracheal instillation in experimental animals) and from *in vitro* models. In this context, it has to be considered that results of studies using a suspension medium to apply silica particles either to animals *via* intratracheal instillation or in *in vitro* studies, are strongly influenced not only by the particle characteristics but also by the protein and lipid content of the suspension medium which may influence the degree of particle aggregation. Furthermore, using intratracheal instillation or pharyngeal aspiration as the delivery route to the respiratory tract of experimental animals involves administration of high doses as a bolus, *i.e.*, within a very short time period whereas it would take much longer (hours, days or even weeks) to deliver the same dose *via* inhalation exposure. This bolus administration implies that many physiological defence mechanisms may be disrupted and artificial health responses be generated that would not occur under physiological *in vivo* conditions. Interestingly, milder effects have been shown after intratracheal instillation of "nano" silica as compared to micrometre-sized silica particles, possibly because of a faster translocation and elimination (Chen et al., 2004). Findings from studies employing the intratracheal route can nevertheless be useful as proof-of-principle studies.

Similarly, a drawback of *in vitro* studies is generally the difficulty to extrapolate the applied dose to the *in vivo* situation, especially in the case of particle suspensions. Overload doses can cause effects that may have little or no relevance under physiological conditions *in vivo* (see *e.g.* Donaldson et al., 2008; Lison et al., 2008; Sayes et al., 2007; Teeguarden et al., 2007). Biological effects were indeed described after *in vitro* exposure of various cells and cell lines to SAS materials. It was shown that silica particles in the nano-, but also micrometre-size range can be taken up into the cytoplasm of different kinds of cells either by internalisation *via* phagocytosis, endocytosis and pinocytosis mechanisms or by a receptor-mediated transport. The particles may enter cells however also after dissolution. Surface charge and reactivity, in particular hydrophilicity of surface silanol groups and their interaction with cell membrane proteins are important in determining biological reactivity and the uptake mechanism(s). Many *in vitro* studies investigated vitality and metabolic capacity. Others reported effects included ROS generation, induction of pro-inflammatory cytokines and chemokines. A comparison of effects from various studies shows that the results are highly dependent on duration of treatment, preparation of test material and the type of cells. It was demonstrated *in vitro*, that the specific surface silanol groups (SiOH) of silica are directly involved in haemolysis of red blood cells *via* membrane interactions (Pandurangi et al., 1990). Surface-treated cationic silica particles, on the other hand, were suggested as potential alternatives for gene transfection because of their low *in vitro* and *in vivo* toxicity (Ravi Kumar et al., 2004).

Often the tested materials were not characterised with regard to their chemical purity, in particular metal impurities introduced through the synthesis of the particles in the laboratory. The importance of adequately characterised materials to interpret potential causes of biological effects can be demonstrated by the fact that metal oxide impurities are known to strongly induce oxidative stress and have catalytic properties. Limbach et al. (2007) exposed human pulmonary epithelial cells *in vitro* to silica nanoparticles and found that traces of iron impurities on the silica surface are implicated in free radical release at the surface and in subsurface layers of particles.

For smaller particles, the surface termination, especially the role of oxygen and silanol groups, becomes more important because the ratio of surface to bulk Si atoms increases (O'Farrell et al., 2006). Unless specifically engineered and stabilised, small silica particles however aggregate and agglomerate rapidly under normal environmental and testing conditions and hence their biological effects become indistinguishable from those of the bulk materials.

Silica particles exist naturally in millions of tons, and have been found in material more than 10,000 years old. However, recent findings of adverse health outcomes after exposure to particular kinds of other nanomaterials and scares relating to nanotechnology-enabled products in general (*e.g.*, Böl et al., 2010) have biased current risk perception and necessitated enormous investment into the assessment of risks by nanomaterials. With regard to SAS, based on the available environmental and mammalian toxicology studies, epidemiology and safety data, there do, however, not appear to be significant differences in the environmental and health effects of nanostructured silica materials and silica nano-objects. Hence, "nanosilica" (in the form of colloidal silicon dioxide) and nanostructured SAS should not be considered new chemicals with unknown properties, but well-studied materials that have been in use for decades.

Nano-forms of silica containing metals, organically modified surfaces or dyes, however, may have altered surface characteristics, altered cellular uptake mechanisms or may release toxicants. Metals and certain organic coating materials, such as those containing quinones, may cause redox cycling and/or catalytic reactions. Such modified, engineered silica nanomaterials may therefore cause toxic effects and will need to be assessed on a case-by-case basis.

7. Conclusions and recommendation

Extensive data exist on the physico-chemical, ecotoxicological and toxicological properties of SAS, including several studies considering colloidal silica, surface-treated silica and nano-sized SAS forms. Primary SAS particles usually form aggregates and agglomerates and are not normally found as discrete particles in air or aqueous environments. Both nanostructured SAS (*i.e.*, the "bulk material") as well as nano-objects of silica dissolve in aqueous environments and body fluids. None of the SAS types was shown to be biopersistent or to bioaccumulate. All types disappear within a short time from living organisms by physiological excretion mechanisms. In animal studies, no relevant differences in the toxicities of the different commercial SAS types were found.

The mode of action of SAS is related to the particle surface characteristics interfacing with the biological milieu rather than to particle size. By physical and chemical interactions, SAS may adsorb to cellular surfaces and can affect membrane structures and integrity. Cellular toxicity is linked to mechanisms of interactions with outer and inner cell membranes, signalling responses, and vesicle trafficking pathways. Interaction with membranes may induce the release of endosomal substances, reactive oxygen species, cytokines and chemokines and thus induce inflammatory responses. While all of these mechanisms have been observed *in vitro*, the only effects demonstrated in animal studies were inflammatory responses after high inhalation, intratracheal, intraperitoneal or subcutaneous SAS doses and lung embolism after intravenous injection of high bolus doses. None of the available *in vitro* and *in vivo* data gives any evidence for a novel, hitherto unknown mode of action.

Commercial SAS types (including colloidal silicon dioxide and surface-treated forms) are well-studied materials that have been in use for decades with significant exposures resulting from their use in oral and topical pharmaceutical and cosmetic products and as an anti-caking agent in food. There were no reports of adverse reactions from these uses.

Based on the available evidence, it is concluded, that despite the new nomenclature designating SAS as a nanomaterial, SAS should not be considered a new chemical with unknown properties. None of the recent available data gives any evidence for a novel, hitherto unknown mechanism of toxicity that may raise concerns with regard to human health or environmental risks.

Conflict of interest

None.

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