REVIEW ON THE TOXICITY OF MANUFACTURED NANOMATERIALS APPLIED IN THE CONSTRUCTION SECTOR

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1 EXECUTIVE SUMMARY

This report presents a review of literature data on the substance-specific health effects and dose-response-relationships of the five different nanomaterials included in the Scaffold project. The data presented in this report is applied as a basis for deriving health-based occupational exposure limit values (OELs) for the same materials (Scaffold Public Document SPD7). The nanomaterials selected for Scaffold were silicon dioxide (amorphous silica), titanium dioxide, nanoclays, nanocellulose and carbon nanofibres.

The work included literature searches for collecting and analysing all available relevant toxicological and epidemiological data, and identification of the critical health effects and the critical effect levels. In the assessment, the focus was on the following toxicological end-points: toxicokinetics, acute toxicity, irritation, sensitisation, repeated dose toxicity, mutagenicity, carcinogenicity and reproductive toxicity.

Of the materials being in the focus of the Scaffold project, extensive data is available on the toxic potential of silicon dioxide (amorphous silica), most likely due to the fact that it has already been used for decades. The main concerns related to exposure to amorphous silica particles are their possible lung effects. Repeated dose inhalation toxicity studies with synthetic amorphous silica have been performed, indicating hazardous effects such as chronic inflammation and fibrotic lesions. It should, however, be noted that the inflammatory effects induced by silica seem to be reversible upon the cessation of exposure, and as opposed to the persistent lesions caused by crystalline silica. Limited data are available on the carcinogenicity of amorphous silica. In one study, a statistically significant tumour response was observed after repeated intratracheal administration of amorphous silica particles in rats. Tumour responses correlated with inflammatory responses in lungs and mechanisms related to lung overloading may have played a role in the tumour response. Most of the genotoxicity studies on amorphous silica particles show negative results.

Nano-sized titanium dioxide (TiO$_2$) has during the last ten years been intensively studied, and a lot of research is still going on. There is evidence that nano-sized TiO$_2$ is considerably more toxic than micro-sized TiO$_2$. Among the TiO$_2$ induced adverse effects, respiratory tract is considered the most critical site. The pulmonary responses to TiO$_2$ include inflammation, epithelial damage, increased permeability of the lung epithelium, oxidative stress and cytotoxicity, and morphological alterations within the lungs. Despite of the increased production and use of TiO$_2$ nanoparticles the epidemiological data are lacking. The genotoxicity of TiO$_2$ nanoparticles is thought to be driven by particle mediated reactive oxygen species production. The particles themselves are not thought to be inherently genotoxic, but may trigger genotoxicity via an indirect threshold-driven inflammatory mechanism involving oxidative stress.

Dermal studies have shown little evidence for skin penetration of nano-TiO$_2$. However, there may be a risk associated with nano-TiO$_2$ applied to damaged skin. Long-term TiO$_2$ inhalation studies in rats have reported lung tumours. Oxidative stress, induced by ROS generation, may play an important role in the initiation and progression of carcinogenesis of TiO$_2$ nanoparticles.
The few studies on nanocellulose toxicity conducted so far suggest that the different types of nanocellulose materials can be slightly toxic in vitro but the effect is milder than the one caused by some types of MWCNTs and asbestos fibres. The two in vivo studies on acute toxicity in mice after intraperitoneal injection did not detect adverse effect. Further studies, especially repeated dose inhalation studies, would bring important new information, facilitating conclusion making about the toxic potential of nanocellulose.

So far, there are only very limited data available on the potential toxicity of nanoclays. As many nanoclays are modified to form nanocomposites, it seems important to elucidate whether the modifiers are causing the toxic events or if the nanoclay itself can induce harmful effects. Moreover, the variations in the in vitro testing conditions (cell lines, cell culture conditions) of the currently published studies makes it is impossible to draw conclusion on the cytotoxicity or genotoxicity. In vivo studies intending to investigate pulmonary toxicity of nanoclays are so far limited to one study, in which intratracheal instillation of the material produced transient short-term neutrophilic responses and occasional multinucleate giant cell formation up to 3 months post-exposure.

Information on the potential health hazards of carbon nanofibres (CNFs) have only been published in a few studies. Based on the few published study reports, there are indications that CNFs may be able to induce inflammatory and fibrotic effects in the lungs of exposed animals. There are also indications that some CNFs may cause genotoxicity.

More toxicological data, including also in vivo inhalation studies, is available for different types of carbon nanotubes (CNTs). Carbon nanotubes are being intensively studied for their toxicity, due to the theory that they may behave in an asbestos-like manner. There are, however, a lot of different types of carbon nanotubes at the market, and it appears that the toxic effects may vary a lot depending on which type of material has been tested. Anyhow, it seems to be proven that some carbon nanotubes may induce lung inflammation, genotoxicity and carcinogenicity. In some studies, carbon nanotubes have even been shown as more potent than asbestos. Due to the physico-chemical similarities between CNT and the hazardous effects of some CNTs, toxicological data on CNTs is considered to be relevant for the assessment of the hazards of CNFs.

The assessment of the toxicological potential of nanocellulose, nanoclays and carbon nanofibres is complicated by the fact that these are not single substances, but heterogenous groups of materials. At the moment, it seems like the toxicity studies have to be carried out in a case-by-case manner, since modifications on the material structure and surface may result in drastic changes in biological systems making generalization of toxicity results from one material to another impossible. Sophisticated in vitro models coupled with realistic exposure systems could in the future respond to the vast testing demands of new nanomaterials.
2 OBJECTIVES AND SCOPE

The aim of the present work was to identify and review literature data on the substance-specific health effects and dose-response-relationships of the nanomaterials included in the Scaffold project, to be applied as a basis for deriving health-based occupational exposure limit values for the materials (Scaffold Public Document SPD7). The work included literature searches for collecting and analysing all available relevant toxicological and epidemiological data, and identification of the critical health effects and the critical effect levels. In the assessment, the focus was on the following toxicological end-points: toxicokinetics, acute toxicity, irritation, sensitisation, repeated dose toxicity, mutagenicity, carcinogenicity and reproductive toxicity.
3 INTRODUCTION

The amount of published studies related to nanotoxicology has rapidly increased during the last ten years. For the assessment of risks related to the nanomaterials included in the Scaffold project it was therefore decided to review what toxicological data has been published so far on these nanomaterials.

The main findings of the literature searches are presented in section 6 of this report.
4 METHODOLOGY

The scientific publications describing toxicological (and epidemiological) studies on the nanomaterials included in the Scaffold project – amorphous silicon dioxide (SiO$_2$), titanium dioxide (TiO$_2$), nanocellulose, nanoclays and carbon nanofibres (CNF) – were retrieved from PubMed, ToxSeek and Google Scholar (last searches carried out in January 2014). Some unpublished data from the FP7 HINAMOX project was also included. All relevant toxicological (and epidemiological) information related to toxicokinetics, acute toxicity, irritation, sensitisation, repeated dose toxicity, mutagenicity, carcinogenicity and reproductive toxicity of the material were evaluated.

The following data on each study were retrieved (if mentioned in the study reports): particle size and other physical-chemical information, methods and materials used (cell lines, test animals etc.), exposure routes, exposure duration, test doses applied and data describing the (adverse) effects. Although the validity of current test guidelines for the hazard identification of nanomaterials has been questioned and the best battery of tests is yet under development, special attention was paid to the studies performed using OECD guideline protocols.

From the available data, the critical health effects, i.e. adverse effects appearing at the lowest exposure or dose, and the no-observed adverse effects levels (NOAEL) and the lowest observed adverse effect levels (LOAEL) were identified for each of the materials, where conceivable.
5 RESULTS

5.1 Silicon dioxide (amorphous silica)

5.1.1 Identity of the substance

There are several different forms of silica. A common CAS number for all silicas is 7631-86-9. However, each different polymorph of silica has its own polymorph specific CAS number. Crystalline silica is known from its carcinogenic properties and its ability to cause silicosis. Crystalline silica does not, however, have a nanospecific structure and is, therefore, out of the scope of this review.

Amorphous silica can be divided to synthetic amorphous silica, natural amorphous silica (diatomaceous earth), fused silica (quartz glass) and by-products of metal industry (silica fume) (ECETOC 2006). Of these, synthetic amorphous silica and silica fume contain primary particles in the size range below 100 nm. Based on their production process, synthetic amorphous silica is further divided to pyrogenic silica (also known as fumed silica), produced by flame hydrolysis of (alkyl)chlorosilanes, and precipitated silica, colloidal silica (silica sol) and silica gel, produced by a reaction of alkali metal silicate solution with mineral acid. Monodisperse colloidal silica may also be produced by controlled hydrolysis and condensation of tetraethylorthosilicate in ethanol (Stöber process) (Napierska et al. 2010). Stöber silica is mainly applied for research purposes.

The physico-chemical properties and particle characteristics differ between the different types of amorphous silica (Table 1). The key properties likely to affect the toxicity include particle size, porosity, hydrophilic-hydrophobic properties and solubility. The primary particles of pyrogenic silica, precipitated silica and silica gel are strongly bonded or fused to larger aggregates (ECETOC 2006; Fruijtier-Pölloth 2012). The aggregates represent the smallest stable unit of these materials. In addition, the particles/aggregates of pyrogenic silica, precipitated silica, silica gel and silica fume tend to agglomerate, i.e. form loosely bond assemblies bond together by weak forces (e.g. van der Waal forces). By contrast, colloidal silica is typically composed of dispersed primary particles (Fruijtier-Pölloth 2012).

The number of silanol groups in the surface of amorphous silica particles affects the hydrophilicity of the material (Napierska et al. 2010). Synthetic amorphous silicas can be also surface modified rendering them more hydrophobic (ECETOC 2006). Organic silicon compounds, such as dichloromethylsilane, are usually applied for the surface modification.

Pyrogenic silica typically contains ≥99.8 wt% of amorphous silica (ECETOC 2006). Traces of metal oxides and/or chlorides may be present in the material. Precipitated silica and silica gel contain ≥95% of amorphous silica, together with sulphates, sodium oxide and trace amounts of other metal oxides. Colloidal silica is typically composed of amorphous silica (≥99.5 wt% of dry weight), sodium oxide, aluminium oxide (stabilizer) and traces of sulphates. Silica fume contain ≥85 wt% of amorphous silica (Friede 2006). Typical impurities include carbon, silicon, sulphur, silicon carbide and a variety of metal oxides (e.g. iron, aluminium, magnesium and potassium) (Friede 2006; Siddique & Khan 2011). Silica fume may also contain trace amounts of crystalline silica, mainly quartz particles originating from the raw materials used in the production process.
(Friede 2006). The quartz particles are mainly associated with the coarse (non-respirable) fraction of silica fume.

Since synthetic amorphous silicas have been on the market already for several decades, and they are used for a large number of different applications, including consumer uses, there are several in vivo and in vitro studies available on their health effects. However, a part of the studies are non-published reports owned by the industry. These have been recently reviewed by OECD (2004) and ECETOC (2006).

5.1.2 Toxicokinetics

The data on the kinetics of synthetic amorphous silica in the lungs show rather consistently that, in contrast to crystalline silica, which exhibits a marked tendency to accumulate, amorphous silica reaches a plateau level at which elimination equates with deposition (ECETOC 2006). After the cessation of exposure, synthetic amorphous silica is rapidly eliminated from the lung tissue.

For example, Reuzel et al. (1991) exposed rats to two pyrogenic silicas and one precipitated silica for 6 hours/day, 5 days/week for 13 weeks at a concentration of 31 mg/m$^3$ with recovery period up to 52 weeks. At the end of the exposure period, small amounts of silica could be detected in the lungs in all animals of the high dose group. Some trace amounts of silica were detected also 13 and 26 weeks after the exposure, but at the 39 week time point the levels had decreased below the detection limit. The levels of silicon in the lungs and lymph nodes of the animals exposed to quartz (60 mg/m$^3$) remained high during the whole post-exposure period (52 weeks). This difference between crystalline and amorphous silica can be at least partly explained by the higher solubility of amorphous silica in the lung tissue.

Since silicon in different forms is ubiquitous in the environment, various foods and drinking water contain various amounts of silicon dioxide (Jugdaohsingh 2007; Martin 2007). Our normal dietary intake of silicon is between 20 and 50 mg Si/day (EFSA 2004). The available information suggests that silicon from the diet is fairly well absorbed, which is seen as a high proportion of dietary silicon excreted in the urine. For example, in a study on 8 volunteers, the average of urinary excretion of dietary silicon was 41% (Jugdaohsingh et al. 2002). In blood, silicon exists as a monosilicic acid (Martin 2007).
### Table 1. Properties of synthetic amorphous silicas and silica fume (ECETOC 2006; Friede 2006; Flörke et al. 2012; Diamond et al. 2004).

<table>
<thead>
<tr>
<th></th>
<th>Pyrogenic silica</th>
<th>Precipitated silica</th>
<th>Colloidal silica</th>
<th>Silica gel</th>
<th>Silica fume</th>
</tr>
</thead>
<tbody>
<tr>
<td>CAS No.</td>
<td>112945-52-5</td>
<td>112926-00-8</td>
<td>112926-00-8</td>
<td>112926-00-8</td>
<td>69012-64-2</td>
</tr>
<tr>
<td>EINECS No.</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>273-761-1</td>
</tr>
<tr>
<td>Description</td>
<td>Amorphous silicon dioxide particles from the flame hydrolysis of (alkyl)chlorosilanes (typically SiCl₄).</td>
<td>Amorphous silicon dioxide particles from the reaction of alkali metal silicate solution with mineral acid, followed by precipitation, filtration, drying and grinding.</td>
<td>Stable dispersions of amorphous silicon dioxide particles in a liquid (typically water) produced by partial neutralisation of alkali metal silicate solution or by re-dispersion of other synthetic amorphous silicas.</td>
<td>Interconnected random array of amorphous silicon dioxide particles from the reaction of alkali metal silicate solution with mineral acid, followed by drying.</td>
<td>Amorphous silicon dioxide particles from the volatilization and vaporization of furnace feed materials in the manufacture of silicon and ferrosilicon.</td>
</tr>
<tr>
<td>SiO₂ content (wt%)</td>
<td>≥99.8</td>
<td>≥95</td>
<td>≥99.5</td>
<td>≥95</td>
<td>≥85</td>
</tr>
<tr>
<td>Primary particle size (nm)</td>
<td>5–50</td>
<td>5–100</td>
<td>5–20</td>
<td>1–20</td>
<td>20–1000</td>
</tr>
<tr>
<td>Aggregate size (nm)</td>
<td>100–1000</td>
<td>100–1000</td>
<td>NA</td>
<td>100–20000</td>
<td>NA</td>
</tr>
<tr>
<td>Agglomerate size (µm)</td>
<td>1–250</td>
<td>1–250</td>
<td>NA</td>
<td>NA</td>
<td>from one to several hundred</td>
</tr>
<tr>
<td>Pore size (nm)</td>
<td>nonporous</td>
<td>&gt;30</td>
<td>NA</td>
<td>0.1–1000</td>
<td>nonporous</td>
</tr>
<tr>
<td>Pore size distribution</td>
<td>NA</td>
<td>very wide</td>
<td>wide</td>
<td>narrow</td>
<td>NA</td>
</tr>
<tr>
<td>Specific surface area (m²/g)</td>
<td>50–400</td>
<td>30–500</td>
<td>50–400</td>
<td>250–1000</td>
<td>15–30</td>
</tr>
<tr>
<td>Silanol group density (OH/nm²)</td>
<td>1.3–2.5</td>
<td>5.0–5.7</td>
<td>4.6</td>
<td>4.6–7.9</td>
<td>ND</td>
</tr>
<tr>
<td>pH</td>
<td>3.6–4.5</td>
<td>5–9</td>
<td>3– 5 / 8–11</td>
<td>3–8</td>
<td>ND</td>
</tr>
</tbody>
</table>

NA: Not applicable. ND: No data available.
5.1.3 Acute toxicity

The acute toxicity of synthetic amorphous silicas has been extensively studied. In the acute inhalation toxicity studies with hydrophilic silica reviewed in ECETOC (2006), no mortality or other signs of toxicity were observed in rats exposed to 140 mg/m³ of pyrogenic silica (respirable; Aerosil 200) or 690 mg/m³ of precipitated silica (respirable; Sipernat 22S) for 4 hours. No mortality occurred in rats exposed 4 hours to 2,080 mg/m³ (MMAD 0.76 µm; Cab-O-Sil) or 1 hour to 191,300 or 207,000 mg/m³ (total dust; Cab-O-Sil) of pyrogenic silica. One of 10 animals died after 1 hour inhalation exposure to 2,200 mg/m³ of silica gel (respirable; Syloid 244). Signs of respiratory irritation and dyspnoea were observed in the animals.

In two 4 hours inhalation studies with hydrophobic pyrogenic silicas (MMAD <0.20–7.7 µm; HDK SKS130), no mortality was observed in rats exposed to 770 or 900 mg/m³ (ECETOC 2006). 40% mortality occurred at 2,200 mg/m³ and 100% mortality at doses ≥ 2,530 mg/m³. In rats exposed 4 hours to another type of hydrophobic pyrogenic silica (MMAD 1.2 µm; Cab-O-Sil TS610), the mortality was 0% at 210 mg/m³, 70% at 540 mg/m³ and at 100% at 2,100 mg/m³ (ECETOC 2006). The mortality observed in these studies is associated with suffocation caused by the extremely high number of respirable particles generated to meet the required test atmosphere.

In acute dermal toxicity studies, only slight erythema with intact skin and slight erythema and oedema with abraded skin was observed in rabbits exposed to hydrophilic precipitated silica and silica gel at doses up to 5,000 mg/kg bw for 24 hours (ECETOC 2006). In the acute oral toxicity studies of hydrophilic and hydrophobic synthetic amorphous silicas, no signs of toxicity were observed at single doses of up to 5,000 mg/kg bw (ECETOC 2006).

5.1.4 Irritation and sensitization

In the skin irritation studies, no signs of irritation or, occasionally, very slight erythema was observed in rabbits exposed dermally to hydrophilic pyrogenic or precipitated silica, silica gel or hydrophobic pyrogenic or precipitated silica for 24 hours (ECETOC 2006). In the eye irritation studies, no or very slight reversible irritation was seen in the eyes of rabbits exposed to these silicas (ECETOC 2006).

Amorphous silica has not been tested for its sensitizing properties. However, taking into account the widespread environmental exposure to silicon dioxide this endpoint is not a concern.

5.1.5 Repeated dose toxicity

5.1.5.1 Epidemiological studies

Lung effects after inhalation exposure have been the main concern related to the exposure to amorphous silica. The few available epidemiological studies on workers exposed to synthetic amorphous silica, without apparent co-exposure to crystalline silica, are summarised in Table 2. No indications of pneumoconiosis or other exposure-related pulmonary diseases have been observed in the studies.
Table 2. Epidemiological studies on amorphous silica.

<table>
<thead>
<tr>
<th>Material</th>
<th>Study type</th>
<th>Study population</th>
<th>Exposure duration and characteristics</th>
<th>Findings, comments</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pyrogenic and precipitated silica</td>
<td>Cross-sectional</td>
<td>397 current and 178 former workers; 210 controls</td>
<td>no data</td>
<td>No indications of pneumoconiosis; slightly higher prevalence of chronic bronchitis and minor differences in spirometric parameters among exposed subjects (assumed to be related to smoking habits)</td>
<td>Merget &amp; Kappler 2005 as cited in ECETOC 2006</td>
</tr>
<tr>
<td>Precipitated silica</td>
<td>Cross-sectional</td>
<td>150 workers</td>
<td>12 (&gt;5) years</td>
<td>No indications of pneumoconiosis; no significant exposure related changes in pulmonary function</td>
<td>Garnier 1996 as sited in ECETOC 2006</td>
</tr>
<tr>
<td>Pyrogenic silica</td>
<td>Cross-sectional</td>
<td>41 workers; 90 controls</td>
<td>8 (1–28) years; inhalable dust ≤10.5 mg/m³; respirable dust ≤3.4 mg/m³</td>
<td>No differences in blood gases, chest radiographs or most respiratory symptoms; indications of a synergistic effect between smoking and exposure on decrements in pulmonary function</td>
<td>Choudat et al. 1990</td>
</tr>
<tr>
<td>Pyrogenic silica (Aerosil)</td>
<td>Case series</td>
<td>143 workers</td>
<td>1–34 years</td>
<td>Respiratory symptoms not associated with exposure; no indications of pneumoconiosis</td>
<td>Ferch et al. 1987a as cited in Merget et al. 2002</td>
</tr>
<tr>
<td>Precipitated silica (Hi-Sil; Silene)</td>
<td>Case series</td>
<td>165 workers</td>
<td>8.6 years; total dust &lt;1–10 mg/m³</td>
<td>Respiratory symptoms not associated with exposure; no indications of pneumoconiosis on the 143 workers without previous exposure to limestone</td>
<td>Wilson et al. 1979</td>
</tr>
<tr>
<td>Precipitated silica (Hi-Sil; Silene)</td>
<td>Case series</td>
<td>78 workers</td>
<td>4.7 (1–16) years; 0.3–204 mg SiO₂/m³</td>
<td>Mechanical irritation of skin, eyes, nose and throat; no indications of pneumoconiosis or other pulmonary diseases</td>
<td>Plunkett &amp; De Witt 1962</td>
</tr>
<tr>
<td>Pyrogenic silica (Aerosil)</td>
<td>Case series</td>
<td>215 workers</td>
<td>total dust: filling nozzle 15–100 mg/m³; bagging room 2–6 mg/m³; production room 3–7 mg/m³</td>
<td>No indications of pneumoconiosis</td>
<td>Volk 1960</td>
</tr>
<tr>
<td>Fumes formed in the production of ferrosilicon (containing amorphous silica fume)</td>
<td>Cohort</td>
<td>6494 workers</td>
<td>≥18 months before the year 1970</td>
<td>No increase in mortality or cancer incidence (neither total incidence or specific cancer types assessed separately); no association of cancer incidence with job category or cumulative employment time</td>
<td>Kjuus et al. 1986</td>
</tr>
<tr>
<td>---</td>
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<td>---</td>
<td>---</td>
</tr>
<tr>
<td>Fumes formed in the production of ferrosilicon (containing amorphous silica fume)</td>
<td>Cohort</td>
<td>320 workers</td>
<td>≥12 months</td>
<td>No increase in mortality or cancer incidence (neither total incidence or specific cancer types assessed separately)</td>
<td>Langård et al. 1990</td>
</tr>
</tbody>
</table>
| Fumes formed in the production of ferrosilicon and silicon (containing amorphous silica fume) | Cohort | 8530 workers | ≥6 months; total dust (mean) 3.4-4.8 mg/m³ (~50% of which was amorphous silica) | Increased incidence of lung cancer (SIR 1.57; 95% CI 1.15-2.09) and testicular cancer (SIR 2.30; 1.05-4.37) among the furnace workers (n=2534); no statistically significant association of the cancer incidences with duration of work  
Note: No adjustment with smoking, or with exposure to asbestos or crystalline silica. Proportion of smokers among furnace workers reported to be higher than in general population. | Hobbesland et al. 1999 |
5.1.5.2 Animal studies

Inhalation studies

Repeated dose inhalation studies with synthetic amorphous silicas are summarized in Table 3. Arts et al. (2007) reported transient histopathological changes and changes in broncho-alveolar lavage (BAL) in rats exposed to hydrophilic pyrogenic silica for 5 consecutive days, with the no observed adverse effect concentration (NOAEC) of 1 mg/m$^3$ and the lowest observed adverse effect concentration (LOAEC) of 5 mg/m$^3$. While quartz caused progressive changes in the lungs, synthetic amorphous silica caused only transient changes, which were resolved during the three-month follow-up period.

In the study of Reuzel et al. (1991), lung inflammation, granulomatous lesions and interstitial fibrosis were detected in rats exposed to hydrophilic pyrogenic silica for 13 weeks. However, as opposed to quartz, these changes were mostly reversible after the cessation of the exposure. Although a slight reversible response was detected already at the lowest dose tested (1.3 mg/m$^3$), this was evaluated as a non-pathological change with low-grade severity (OECD 2004). Thereby, the NOAEC of this study was 1.3 mg/m$^3$, and the LOAEC 6 mg/m$^3$. Less severe response was observed with hydrophobic pyrogenic silica and hydrophilic precipitated silica (only one dose tested).

A dose-dependent lung inflammatory response was observed also in rats exposed to dried colloidal silica for 4 weeks, with NOAEC of 10 mg/m$^3$ and LOEAC of 50 mg/m$^3$ (Warheit et al. 1991; Lee & Kelly 1993).

Groth et al. (1981) reported early nodular fibrosis in the lungs and effects on the lung function in monkeys exposed for 13 or 18 months to hydrophilic pyrogenic silica, with a LOAEC of 15 mg/m$^3$, corresponding to approximately 6–9 mg/m$^3$ of respirable particles.

Based on these studies, it may be concluded that inhalation of synthetic amorphous silica produces a dose-related non-progressive lung inflammation in animals, with a lowest reported NOAEC of 1 mg/m$^3$.

Oral studies

In the well documented chronic oral feeding study of Takizawa et al. (1988), no alterations in the body weight, food consumption or physical features were observed in mice or rats exposed to food-grade micronized silica gel with doses up to 5% of the feed for 93 or 104 weeks. No significant dose-related effects were seen at any dose level upon clinical laboratory examinations. The pathological examinations revealed no gross or microscopic changes in the tissues examined. The occasional presence of neoplasms did not reveal any consistent dose-related trends in any dose group. Also, the results of the older non-published feeding studies on hydrophilic and hydrophobic pyrogenic silica or silica gel support the absence of a notable toxicity of synthetic amorphous silicas by the oral route (Table 4).
The European Food Safety Authority (EFSA) reviewed the use of silica in food additives and concluded that an intake of up to 1500 mg/day of silicon dioxide as food supplements is not a safety concern (EFSA 2009).

**Dermal studies**

ECETOC (2006) reviews one non-published repeated dose dermal toxicity study on synthetic amorphous silica. No evidence of systemic toxicity, enhanced skin irritation or gross or microscopic pathology was observed in rabbits (4/dose) exposed dermally, through intact or abraded skin, to hydrophilic pyrogenic silica (Cab-O-Sil) for 3 weeks (18 h/day, 5 days/week) at 5000 or 10000 mg/kg bw/day (Cabot 1958).

5.1.5.3 **In vitro studies**

*In vitro* studies on the cytotoxicity of different amorphous silica particles in different cell culture systems have been recently reviewed by Napierska et al. (2010) and Fruijtier-Pöllooth (2012). In general, the results vary with the particle type and cell type tested. It seems however, that both the particle surface area and the surface properties (especially the number of silanol groups on the surface) play a role in the cytotoxicity of amorphous silica particles (Napierska et al. 2010).
<table>
<thead>
<tr>
<th>Material</th>
<th>Species</th>
<th>Exposure duration and doses</th>
<th>Endpoints</th>
<th>Findings, comments</th>
<th>NOAEC/LOAEC</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pyrogenic silica (Cab-O-Sil M5; selected MMAD 1-4 µm)</td>
<td>Male Wistar rats (10/dose);</td>
<td>5 days (6 h/day); nose-only exposure; 1, 5 or 25 mg/m³ (all SAS); 25 mg/m³ (quartz dust)</td>
<td>Clinical signs, body weight, retention of test material in the lungs and regional lymph nodes, BAL, collagen content of the lungs, gross and microscopic pathology; necropsy at 1 day or 4 or 13 weeks post-exposure</td>
<td>SAS: Dose-dependent increase in inflammatory markers (e.g. neutrophil count) in BAL at 1 day post-exposure at 5 and 25 mg/m³ (reversible by 4 weeks); slight increase in lung and lymph node weight at 25 mg/m³ (reversible by 13 weeks); slight histological changes in lungs at 1 day to 13 weeks at 25 mg/m³ (Cab-O-Sil M5); no changes in body weight or food intake</td>
<td>NOAEC: 1 mg/m³; LOAEC: 5 mg/m³</td>
<td>Arts et al. 2007</td>
</tr>
<tr>
<td>Precipitated silica (Zeosil 45; selected MMAD 1-4 µm)</td>
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<tr>
<td>Micronized silica gel (Syloid 74; selected MMAD 1-4 µm)</td>
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<tr>
<td>Quartz dust as positive control (selected MMAD 1-4 µm)</td>
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<tr>
<td>Pyrogenic silica (primary particle size 14 nm; MMAD 2.1 µm)</td>
<td>Female BN rats (6/group)</td>
<td>6 days (6 h/day); 30 mg/m³</td>
<td>Breathing frequency and pattern during exposure (whole-body plethysmograph), body and organ weights, BAL, airway histopathology</td>
<td>Transient changes in breathing parameters at exposure days 1-2; increase in inflammatory markers (e.g. neutrophil count) in BAL; granulomatous-neutrophilic alveolar inflammation; hyperplasia of nasal septum; increased relative liver weight</td>
<td>LOAEC: 30 mg/m³</td>
<td>Arts et al. 2008</td>
</tr>
<tr>
<td>Colloidal silica, dried (Ludox; primary particle size 22 nm; MMAD 2.9-3.7 µm)</td>
<td>Male CD rats (18/dose)</td>
<td>2 or 4 weeks (6 h/day; 5 days/week); nose-only exposure; 10,</td>
<td>Lung silica content, BAL, pulmonary macrophage morphology and quantification of phagocytosis, cell labelling; necropsy at 1 day or 13 weeks</td>
<td>Dose-dependent increase in lung silica content; dose-dependent increase in BAL cell count and granulocyte count after 4 weeks exposure at 50 or 150 mg/m³ (reversible at 50 mg/m³); increase in LDH and protein concentration in BAL at 150 mg/m³;</td>
<td>NOAEC: 10 mg/m³; LOAEC: 50 mg/m³</td>
<td>Warheit et al. 1991</td>
</tr>
</tbody>
</table>

*Table 3. Repeated dose inhalation toxicity studies on amorphous silica.*
<table>
<thead>
<tr>
<th>Material Description</th>
<th>Animal Subject</th>
<th>Exposure Details</th>
<th>Histopathology / Pathology</th>
<th>NOAEC</th>
<th>LOAEC</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Colloidal silica, dried (Ludox; primary particle size 22 nm; MMAD 2.9-3.7 µm)</td>
<td>Male CD rats</td>
<td>4 weeks (6 h/day; 5 days/week); nose-only exposure; 10, 50 or 150 mg/m³</td>
<td>Lung histopathology; necropsy at 1 day, 10 days or 13 weeks post-exposure; Dose-related increase in lung weight at ≥ 50 mg/m³ (reversible by 13 weeks); particle-laden AM and PML, and foci with hyperplastic histiocytes without signs of inflammation, at ≥ 50 mg/m³ (largely reversible by 13 weeks); silicotic nodules with reticulin fibres in bronchioles and histiocytic granulomas at 150 mg/m³ at 13 weeks post-exposure (collagen fibres not observed)</td>
<td>NOAEC: 10 mg/m³</td>
<td>LOAEC: 50 mg/m³</td>
<td>Lee &amp; Kelly 1993</td>
</tr>
<tr>
<td>Amorphous silica particles (NanoAmor; 10 nm)</td>
<td>Female BALB/c mice (8/group)</td>
<td>4 weeks (2 h/day; 4 days/week); 10 mg/m³</td>
<td>BAL at 24 hours after the last exposure; No indications of pulmonary inflammation (neutrophilia)</td>
<td>NOAEC: 10 mg/m³</td>
<td></td>
<td>Rossi et al. 2010</td>
</tr>
<tr>
<td>Pyrogenic silica (Aerosil 200; mean primary particle size 12 nm)</td>
<td>Male and female Wistar rats (10+10/dose); mg/m³ (quartz dust)</td>
<td>2 weeks (6 h/day; 5 days/week); 17, 44 or 164 mg/m³ (Aerosil 200); 31, 87 or 209 mg/m³ (Aerosil R974); 46, 180 or 668 mg/m³ (Sipernat 22S); 70, 211, 901</td>
<td>Body and organ weights, haematology, gross and microscopic pathology; Aerosil 200: Dose-dependent histological changes in the lungs at ≥17 mg/m³; reduced weight-gain in males at ≥44 mg/m³; one death at 164 mg/m³; Aerosil R974: Dose-dependent increase in lung weight and histological changes in the lungs at ≥31 mg/m³; elevated haemoglobin content, red blood cell count and packed cell volume at ≥87 mg/m³; reduced weight-gain at ≥87 mg/m³; six deaths at 209 mg/m³; Sipernat 22S: Dose-dependent increase in lung weight and histological changes in the lungs at ≥46 mg/m³; elevated haemoglobin content, red blood</td>
<td></td>
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<td>Reuzel et al. 1991</td>
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<tr>
<td>Material Description</td>
<td>Biological Effects</td>
<td>Dose Range</td>
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<tr>
<td>Control (mean primary particle size 8000 nm)</td>
<td>Cell count and packed cell volume in males at ≥170 mg/m³; reduced weight-gain in males at ≥170 mg/m³; one death at 680 mg/m³</td>
<td>≥170 mg/m³</td>
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<tr>
<td><strong>Quartz dust</strong>: Histological changes in the lungs in all groups (no information on dose-response); increased lung weight at 911 mg/m³; reduced weight-gain in males at ≥211 mg/m³</td>
<td></td>
<td>≥211 mg/m³</td>
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<tr>
<td><strong>Pyrogenic silica (Aerosil 200; mean primary particle size 12 nm)</strong></td>
<td>Aerosil 200: Reversible increase in lung weight at ≥6 mg/m³; dose-dependent increase in lung collagen content at ≥1-6 mg/m³ (decreased during the post-exposure period); dose-dependent increase in accumulation of alveolar macrophages, septal cellularity and leucocyte infiltration at ≥1 mg/m³ (only slight changes recovered by 13 weeks observed at 1 mg/m³); focal interstitial fibrosis at 30 mg/m³</td>
<td>≥6 mg/m³</td>
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<tr>
<td>Pyrogenic silica, hydrophobic (Aerosil R974; mean primary particle size 12 nm)</td>
<td>Aerosil R974: Reversible increase in lung weight, accumulation of alveolar macrophages, septal cellularity and granuloma-like lesions; reversible decrease in body weight gain in males; reversible increase in leucocyte count, red blood cell count, haemoglobin content and packed cell volume</td>
<td>≥30 mg/m³</td>
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<tr>
<td>Precipitated silica (Sipernat 22S; mean primary particle size 18 nm)</td>
<td>Sipernat S22: Reversible increase in lung weight and accumulation of alveolar macrophages; reversible decrease in body weight gain in males</td>
<td>≥60 mg/m³</td>
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<tr>
<td><strong>Quartz dust as positive control (mean primary particle size 8000 nm)</strong></td>
<td>Quartz dust: Progressive histological changes (including focal interstitial fibrosis) and increasing lung weight and lung collagen content during the post-exposure period; non-reversible decrease in body weight gain in males; non-reversible increase in</td>
<td>≥60 mg/m³</td>
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<tr>
<td>Male and female Wistar rats (70+70/dose); 1, 6 or 30 mg/m³ (Aerosil 200); 30 mg/m³ (Aerosil R974); 30 mg/m³ (Sipernat 22S); 60 mg/m³ (quartz dust)</td>
<td>13 weeks (6 h/day; 5 days/week) Clinical signs, body weight, haematology, biochemistry, urinalyses, organ weights, retention of test material in the lungs and regional lymph nodes, collagen content of the lungs, gross and microscopic pathology; necropsy at 1 day or 13, 26, 39 or 52 weeks post-exposure</td>
<td>1-6 mg/m³</td>
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<td></td>
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</tr>
<tr>
<td>Reuzel et al. 1991</td>
<td>NOAEC (LOEC): 1 mg/m³ LOAEC: 6 mg/m³</td>
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<tr>
<td>Pyrogenic silica (Aerosol 200; MMAD 0.810 µm)</td>
<td>Male Fisher 244 rats (4/group); 50 mg/m³ (SAS); 3 mg/m³ (cristobalite)</td>
<td>13 weeks (6 h/day; 5 days/week)</td>
<td>Lung burden, BAL, lung histology, lung immunohistochemistry (cell apoptosis/necrosis); post-exposure period of 3 or 8 months</td>
<td>SAS: Increased silica lung burden (significant decrease during the post-exposure period); changes in BAL (reversible by 3-8 months); non-progressive histological changes (including fibrotic lesions); no indications of excessive cell apoptosis/necrosis</td>
<td>Cristobalite: Non-reversible increase in silica lung burden; non-reversible changes in BAL, progressive histological changes, cell apoptosis/necrosis; increased frequency of HPRT mutations in alveolar cells</td>
<td>LOAEC: 50 mg/m³</td>
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<tr>
<td>Pyrogenic silica (Cab-O-Sil) Precipitated silica (Hi-Sil) Silica gel</td>
<td>Male SD rats (80/group); male Hartley guinea pigs (20/group); male cynomolgus monkeys (10/group); 15 mg/m³ (total dust)</td>
<td>13-18 months (6 h/day; 5 days/week)</td>
<td>Gross and microscopic pathology, haematology, pulmonary function test for monkeys at 3, 6, 14 and 14 months; necropsy for monkeys at 10 and 18 months, and at 3, 6 and 12 months for rats and guinea pigs</td>
<td>Impaired lung function, particle depositions in lung macrophages and tracheal lymph nodes, and macrophage and mononuclear cell aggregates containing reticulin fibres in monkeys (collagen fibres observed only with pyrogenic silica); fewer and smaller cell aggregates in the lungs of rats and guinea pigs</td>
<td>LOAEC: 15 mg/m³ (total dust); ~6.9 mg/m³ (respirable)</td>
<td>Groth et al. 1981</td>
</tr>
</tbody>
</table>
Table 4. Repeated dose oral toxicity studies on amorphous silica.

<table>
<thead>
<tr>
<th>Material</th>
<th>Species</th>
<th>Exposure duration and doses</th>
<th>Endpoints</th>
<th>Findings, comments</th>
<th>NOAEL (mg/kg bw/day)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pyrogenic silica (Cab-O-Sil)</td>
<td>Male and female CR rats (30/dose)</td>
<td>13 weeks; 1, 3 or 5% in feed</td>
<td>Clinical signs, body weight, gross and microscopic pathology</td>
<td>No gross signs of systemic toxicity; no effect on growth rate, food consumption or survival; no gross or microscopic pathological changes</td>
<td>~5000</td>
<td>Cabot 1958 as cited in ECETOC 2006</td>
</tr>
<tr>
<td>Precipitated silica (Sipernat 22)</td>
<td>Male and female Wistar rats (20/dose)</td>
<td>13 weeks; 0.5, 2 or 8% in feed</td>
<td>Clinical signs, body weight, gross and microscopic pathology</td>
<td>Increased food intake associated with a decreased food efficiency at 8%; mean absolute and relative weight of the caecum increased at 8%; no treatment-related gross or microscopic pathological changes</td>
<td>~4000</td>
<td>Degussa 1981 as cited in ECETOC 2006</td>
</tr>
<tr>
<td>Micronized silica gel (Syloid 244)</td>
<td>Male and female B6C3F1 mice (40/dose), male and female Fisher rats (40/dose)</td>
<td>93 weeks (mice); 104 weeks (rats); 1.25, 2.5 or 5% in feed</td>
<td>Clinical signs, body weight, haematology, gross and microscopic pathology</td>
<td>No clinical effects, no changes in body weight or food consumption, no gross or microscopic alterations (occasional neoplasms observed did not reveal any consistent dose-related trends)</td>
<td>~5000</td>
<td>Takizawa et al. 1988</td>
</tr>
<tr>
<td>Micronized silica gel (Syloid 244)</td>
<td>Male and female CD rats (10/dose)</td>
<td>26 weeks; 3.4 or 10% in feed</td>
<td>Clinical signs, body weight, haematology, gross and microscopic pathology</td>
<td>No effects on physical appearance, food consumption, growth, survival, haematology or clinical-chemistry; no gross pathological or microscopic findings</td>
<td>~8000</td>
<td>Grace 1975 as cited in ECETOC 2006</td>
</tr>
<tr>
<td>Pyrogenic silica, hydrophobic (Cab-O-Sil, silane-treated)</td>
<td>Male and female CR rats (10/dose)</td>
<td>13 weeks; 1, 2 or 4% in feed</td>
<td>Clinical signs, body weight, gross pathology</td>
<td>No effect on physical appearance, behaviour, growth, survival, clinical studies or gross pathology; minimal changes in the thyroid gland morphology in males at ≥ 2%</td>
<td>~1000</td>
<td>Cabot 1970 as cited in ECETOC</td>
</tr>
<tr>
<td>Study Description</td>
<td>Species and Number per Dose</td>
<td>Duration</td>
<td>Route and Dosage</td>
<td>Endpoints Investigated</td>
<td>Results</td>
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<tr>
<td>Pyrogenic silica, hydrophobic (Aerosil R972)</td>
<td>Male and female Wistar rats (10/dose)</td>
<td>5 weeks; high dose 8 weeks; 500, 1000 or 2000 mg/kg bw/day (stepwise increase to 16000 mg/kg bw day)</td>
<td>Clinical signs, body weight, haematology, gross and microscopic pathology</td>
<td>Reduction of body weight and decreased food intake at ≥2000 mg/kg bw/day; no significant modification of biological parameters or macroscopic findings; microscopic examination revealed severe atrophy in the liver epithelium at ≥2000 mg/kg bw/day</td>
<td>~1000</td>
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</tr>
<tr>
<td>Pyrogenic silica, hydrophobic (Aerosil R972)</td>
<td>Male and female Wistar rats (40/dose)</td>
<td>26 weeks (gavage 5 x weeks); 500 mg/kg bw/day</td>
<td>Clinical signs, body weight, gross pathology</td>
<td>No clinical symptoms; no macroscopic findings</td>
<td>~500</td>
<td></td>
</tr>
</tbody>
</table>
5.1.6 Reproductive and developmental toxicity

There are limited data available on the fertility effects of amorphous silica. However, no systemic toxicity or harmful effects on reproductive organs have been reported in the repeated dose toxicity tests with inhalation, oral or dermal exposure. No effects on pregnancy rate was observed in Wistar rats (10/group) exposed orally to 500 mg/kg bw/day of hydrophobic pyrogenic silica (Aerosil R972) for 8 or 17 weeks before mating with similarly exposed males (Lewinson et al., 1994).

Developmental toxicity of micronized silica gel (Syloid 244) has been studied in rats, mice, rabbits and hamsters exposed orally during gestation (FDA 1973; ECETOC 2006) (Table 5). No embryotoxicity was observed, and the weight and number of external, visceral or skeletal abnormalities in the pups did not differ from controls. No maternal toxicity was seen even at the highest dose of 1600 mg/kg bw/day.

Yamashita et al. (2011) reported structural and functional defects in the placenta, increased resorption rate and decreased size of the foetus in mice exposed intravenously to silicon nanoparticles (70 nm) at two days during the pregnancy at a maternally toxic dose of 40 mg/kg bw/day. Due to the applied exposure route and the observed maternal toxicity (decreased bodyweight of the dams), no firm conclusions can be drawn from this study.

5.1.7 Genotoxicity

Table 6 lists the available in vitro studies on the genotoxicity of amorphous silica on mammalian cell lines. The chromosome aberration tests on hydrophilic and hydrophobic pyrogenic silica show constantly negative results. Also, most of the micronucleus assay studies on amorphous silica nanoparticles show negative results. In one Comet assay study on colloidal amorphous silica particles, a positive response was observed without evident cytotoxicity (Mu et al. 2012).

In addition to the genotoxicity studies on mammalian cell lines, several studies on the mutagenicity of hydrophilic and hydrophobic synthetic amorphous silicas on micro-organisms have been carried out (see OECD 2004 and ECETOC 2006). All of these showed a negative response.

Table 7 summarizes the in vivo genotoxicity studies on amorphous silica. In the 13 weeks inhalation toxicity study of Johnston et al. (2010), the increased mutation frequency in the HPRT gene of isolated alveolar epithelial cells of male rats detected with crystalline silica (3 mg/m^3) was not observed with hydrophilic pyrogenic silica (50 mg/m^3), although a lung inflammatory response occurred with both types of silica.

Downs et al. (2012) observed a small but statistically significant increase in DNA damage in liver and micronuclei in circulating reticulocytes in rats after three consecutive intravenous injections of colloidal silica (particle size 15 nm) at a maximum tolerated dose of 50 mg/kg bw. An increase in micronuclei was also observed with larger particles (55 nm) at the maximum tolerated dose of 125 mg/kg bw. Since neutrophilic infiltration was observed in the liver tissue, together with an increase in plasma inflammatory markers, the genotoxic effects were
suggested to be related to a secondary genotoxic mechanism involving a release of inflammatory cell-derived oxidants.

No induction of micronuclei was observed in rats exposed by inhalation to freshly generated amorphous silica nanoparticles at doses ranging from 1.8 to 86 mg/m$^3$ for one or three days (Sayes et al. 2010).

5.1.8 Carcinogenicity

There is only limited data available related to potential carcinogenicity of amorphous silica. Human epidemiological data are available only from ferrosilicon/silicon metal industry in which there is, among other exposures, exposure to freshly generated ultrafine amorphous silica fumes (Kjuus et al. 1986; Langård et al. 1990; Hobbesland et al. 1999; see Table 2). These studies do not show increased incidence of cancer attributed to ultrafine silica fumes present in furnace work.

Table 8 lists the sparse animal carcinogenicity studies on amorphous silica. No increase in tumour incidence was seen in any dose group in the study of on Tagizawa et al. (1988), where mice and rats were fed with food-grade micronized silica gel (Syloid 244) at dose levels up to 5% of feed for 93 and 103 weeks, respectively. However, as the group size was relatively small (20 animals/sex/dose), the study has limited sensitivity.

One old inhalation carcinogenicity study with amorphous silica in mice reported an increase in lung tumour incidence (Campbell et al. 1940). The study cannot, however, be regarded reliable due to technical deficiencies, and especially due to poor substance identification. Intra-pleural implantation of two different preparations of pyrogenic silica did not increase the incidence of tumours in the study of Stanton and Wrench (1972).

Recently, Kolling et al. (2011) published a study in rats, in which they evaluated the induction of lung tumours after repeated intratracheal instillation of crystalline silica, amorphous silica (pyrogenic Aerosil 150, particle size 14 nm), carbon black and coal dust at the dose levels which were known to induce pulmonary inflammation. The positive control, crystalline silica (1 × 3 mg), elicited the highest inflammatory reactions in lungs, fibrosis, and the highest incidence of primary lung tumours (39.6%). After repeated instillation of soluble, ultrafine amorphous silica (30 × 0.5 mg) a statistically significant tumour response (9.4%) was observed. Tumour responses correlated with inflammatory responses in lungs. Overall, the results showed a relationship between tumour responses and non-cancerous effects (like inflammation and fibrosis) in rats. For assessing these data, the different dose rate and distribution of material delivered by intratracheal instillation versus inhalation have to be taken into consideration. The frequent intratracheal instillation may have added to the development of the neoplasms (Kolling et al. 2011).

In another study applying repeated intratracheal instillation, an increased incidence of lung tumours (7.9%) was observed in rats at a cumulative dose of 30 mg of amorphous silica particles (14 nm) (Morfeld et al. 2006). The tumour response was significantly lower than observed with equal dose of low-solubility particles, carbon black (14 nm; 77%), diesel exhaust particles (41%) or titanium dioxide (30 nm; 62%). No increase of lung tumour incidence was observed with amorphous silica at a cumulative dose of 15 mg.
The International Agency for Research of Cancer concluded in their evaluation (IARC 1997) that amorphous silicas are generally less toxic than crystalline silica and are cleared more rapidly from the lungs. The conclusion of the IARC on amorphous silica was that there is inadequate evidence in humans and animals for the carcinogenicity of amorphous silica and amorphous silica is not classifiable as to its carcinogenicity in humans (Group 3).

5.1.9 Conclusions

The main concerns related to amorphous silica particles are their possible lung effects. In the repeated dose inhalation toxicity studies with synthetic amorphous silica, chronic inflammation and fibrotic lesions have been seen. However, these have been mostly reversible after the cessation of exposure, as opposed to the lesions caused by crystalline silica. Limited data are available on the carcinogenicity of amorphous silica. In one study, a statistically significant tumour response was observed after repeated intratracheal administration of amorphous silica particles in rats. Tumour responses correlated with inflammatory responses in lungs and mechanisms related to lung overloading may have played a role in the tumour response. Most of the genotoxicity studies on amorphous silica particles show negative results.
**Table 5. Developmental toxicity studies on amorphous silica (adapted from ECETOC 2006).**

<table>
<thead>
<tr>
<th>Material</th>
<th>Species</th>
<th>Exposure period, route and doses</th>
<th>Findings, comments</th>
<th>NOAEL, maternal and fetal toxicity (mg/kg bw/day)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Micronized silica gel (Syloid 244)</td>
<td>Wistar rats</td>
<td>6–15 GD; gavage: 13.5, 62.7, 292 or 1350 mg/kg bw/day</td>
<td>No signs of maternal toxicity; no effects on the percentage of implantation or resorption, or on the weight gain of live pups; no difference in the number of external, visceral or skeletal abnormalities in comparison with controls</td>
<td>&gt;1350 (oral)</td>
<td>US FDA 1973a as cited in ECETOC 2006</td>
</tr>
<tr>
<td>Micronized silica gel (Syloid 244)</td>
<td>CD mice</td>
<td>6–15 GD; gavage: 13.5, 62.7, 292 or 1340 mg/kg bw/day</td>
<td>No signs of maternal toxicity; no effects on the percentage of implantation or resorption, or on the weight gain of live pups; no difference in the number of external, visceral or skeletal abnormalities in comparison with controls</td>
<td>&gt;1340 (oral)</td>
<td>US FDA 1973b as cited in ECETOC 2006</td>
</tr>
<tr>
<td>Micronized silica gel (Syloid 244)</td>
<td>Dutch-belted rabbits</td>
<td>6–18 GD; gavage; 16, 74.3, 345 or 1600 mg/kg bw/day</td>
<td>No signs of maternal toxicity; no effects on the number of corpora lutea, percentage of implantation or resorption, or on the weight gain of live pups; no difference in the number of external, visceral or skeletal abnormalities in comparison with controls</td>
<td>&gt;1350 (oral)</td>
<td>US FDA 1973c as cited in ECETOC 2006</td>
</tr>
<tr>
<td>Micronized silica gel (Syloid 244)</td>
<td>Golden hamsters</td>
<td>6–10 GD; gavage; 16, 74.3, 345 or 1600 mg/kg bw/day</td>
<td>No signs of maternal toxicity; no effects on the percentage of implantation or resorption, or on the weight gain of live pups; no difference in the number of external, visceral or skeletal abnormalities in comparison with controls</td>
<td>&gt;1600 (oral)</td>
<td>US FDA 1973d as cited in ECETOC 2006</td>
</tr>
<tr>
<td>Pyrogenic silica, hydrophobic (Aerosil R972)</td>
<td>Wistar rats (10 females/group)</td>
<td>8 or 17 weeks before mating with males exposed for 20 weeks; continued during breeding; diet: 500 mg/kg bw/day</td>
<td>No signs of parental toxicity; no effects on pregnancy rate, litter size or birth weight; no effects on weight gain or gross pathology of the pups (at 4 weeks of age)</td>
<td>&gt;500 (oral)</td>
<td>Lewinson et al. 1994</td>
</tr>
</tbody>
</table>
Amorphous silica particles, DY-676 fluorescent labelled (70 nm (nSP70); 70 nm, surface modified (nSP70-C, nSP70-N); 300 nm (nSP300); 1000 nm (mSP1000)) | BALB/c mice (11-24 females/group) | GD16 and GD17 (necropsy at GD18); intravenous injection (tail vein): 0.8 mg/mouse/day (~40 mg/kg bw / day) | nSP70: Degreased maternal body weight; accumulation of the particles in the placenta; structural and functional defects in the placenta on the maternal side; increased foetal resorption rate; decreased uteri and foetal size; indications of presence of the particles in the foetal liver and brain (observed also with nSP70-C and nSP70-N); SP300 and nSP1000: no accumulation or adverse effects detected | <40 (intravenous) | Yamashita et al. 2011

GD: gestational day.
Table 6. *In vitro* genotoxicity studies on amorphous silica (mammalian cell lines).

<table>
<thead>
<tr>
<th>Material</th>
<th>Endpoint</th>
<th>Cell line</th>
<th>Exposure duration, doses, metabolic activation</th>
<th>Findings, comments</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pyrogenic silica, hydrophobic (Cab-O-Sil EH5)</td>
<td>6-Thioguanine resistance</td>
<td>Chinese hamster ovary cells</td>
<td>5 h; 10-250 µg/ml (-S9); 100-500 µg/ml (+S9)</td>
<td><strong>Negative</strong>; no significant mutagenic activity</td>
<td>Cabot 1990a as cited in ECETOC 2006</td>
</tr>
<tr>
<td>Pyrogenic silica, hydrophobic (Cab-O-Sil EH5)</td>
<td>Unscheduled DNA synthesis</td>
<td>Primary rat hepatocytes</td>
<td>18-20 h; 0.3-1000 µg/ml (+/-S9)</td>
<td><strong>Negative</strong>; no genotoxic activity</td>
<td>Cabot 1989 as cited in ECETOC 2006</td>
</tr>
<tr>
<td>Pyrogenic silica, hydrophobic (Cab-O-Sil EH5)</td>
<td>Chromosome aberration</td>
<td>Chinese hamster ovary cells</td>
<td>16 h; 38-300 µg/ml (-S9); 2 h; 250-1000 µg/ml (+S9)</td>
<td><strong>Negative</strong>; no clastogenic activity</td>
<td>Cabot 1990b as cited in ECETOC 2006</td>
</tr>
<tr>
<td>Pyrogenic silica, hydrophobic (Cab-O-Sil TS500)</td>
<td>Chromosome aberration</td>
<td>Chinese hamster ovary cells</td>
<td>12 h; 63-500 µg/ml (-S9); 4 h; 63-500 µg/ml (+S9)</td>
<td><strong>Negative</strong>; no clastogenic activity</td>
<td>Cabot 1995a as cited in ECETOC 2006</td>
</tr>
<tr>
<td>Pyrogenic silica, hydrophobic (Cab-O-Sil TS530)</td>
<td>Chromosome aberration</td>
<td>Chinese hamster ovary cells</td>
<td>12 h; 63-500 µg/ml (-S9); 4 h; 63-500 µg/ml (+S9)</td>
<td><strong>Negative</strong>; no clastogenic activity</td>
<td>Cabot 1994 as cited in ECETOC 2006</td>
</tr>
<tr>
<td>Pyrogenic silica, hydrophobic (Cab-O-Sil TS610)</td>
<td>Chromosome aberration</td>
<td>Chinese hamster ovary cells</td>
<td>12 h; 63-500 µg/ml (-S9); 4 h; 63-500 µg/ml (+S9)</td>
<td><strong>Negative</strong>; no clastogenic activity</td>
<td>Cabot 1995b as cited in ECETOC 2006</td>
</tr>
<tr>
<td>Pyrogenic silica, hydrophobic (Cab-O-Sil TS720)</td>
<td>Chromosome aberration</td>
<td>Chinese hamster ovary cells</td>
<td>12 h; 42-333 µg/ml (-S9); 4 h; 42-333 µg/ml (+S9)</td>
<td><strong>Negative</strong>; no clastogenic activity</td>
<td>Cabot 1995c as cited in ECETOC 2006</td>
</tr>
<tr>
<td>Micronized silica gel (Syloid 244)</td>
<td>Chromosome aberration</td>
<td>Human embryonic lung cells (Wi-38)</td>
<td>24 h; 1-1000 µg/ml (-S9)</td>
<td><strong>Negative</strong>; no clastogenic activity</td>
<td>US-FDA 1974 as cited in ECETOC 2006</td>
</tr>
<tr>
<td>Amorphous silica particles (commercial and laboratory-synthesized; 20-400 nm)</td>
<td>DNA damage (Comet assay)</td>
<td>Mouse embryonic fibroblasts (3T3-L1)</td>
<td>3, 6 or 24 h; 4 or 40 µg/ml</td>
<td><strong>Negative</strong>; no significant DNA migration</td>
<td>Barnes et al. 2008</td>
</tr>
<tr>
<td>Material Description</td>
<td>Assay Type</td>
<td>Cell Types</td>
<td>Exposure Duration</td>
<td>Concentration</td>
<td>Result Description</td>
</tr>
<tr>
<td>-------------------------------------------------------------------------------------</td>
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<tr>
<td>Amorphous silica particles (70 nm; unmodified (nSP70) or modified with amine (nSP70-N) or carboxyl groups (nSP70-C))</td>
<td>DNA damage (Comet assay)</td>
<td>Human keratinocyte cells (HaCat)</td>
<td>3 h</td>
<td>30 mg/ml</td>
<td>Positive/inconclusive; statistically significant increase in DNA damage; surface modification significantly reduced the DNA damage; no clear data on cytotoxicity; only one dose tested</td>
</tr>
<tr>
<td>Colloidal silica (Ludox SM-30)</td>
<td>DNA damage (Comet assay)</td>
<td>Human lung carcinoma cells (A549); Human colon cancer cells (HT29); Human keratinocyte cells (HaCat)</td>
<td>24 h</td>
<td>0.01-10 μg/ml</td>
<td>Positive; statistically significant increase in DNA damage at ≥ 0.1 μg/ml with all cell types (cytotoxicity at ≥ 1 μg/ml)</td>
</tr>
<tr>
<td>Silica gel (Spherisorb 5 μm)</td>
<td>DNA damage (Comet assay)</td>
<td>Human embryonic lung fibroblasts (Hel 299); Chinese hamster lung fibroblasts (V79)</td>
<td>3 h</td>
<td>17-138 μg/cm²</td>
<td>Positive/inconclusive; statistically significant increase in DNA damage at ≥ 69 μg/cm²; no data on cytotoxicity</td>
</tr>
<tr>
<td>Non-porous and mesoporous amorphous silica particles (250 and 500 nm)</td>
<td>DNA damage (Comet assay)</td>
<td>Human lung carcinoma cells (A549); Murine macrophages (RAW264.7)</td>
<td>4 or 24 h</td>
<td>5-80 μg/cm²</td>
<td>Positive/inconclusive; statistically significant increase in DNA damage at scattered doses without a clear dose-response</td>
</tr>
<tr>
<td>Non-porous and mesoporous amorphous silica particles (250 and 500 nm)</td>
<td>Micronucleus frequency</td>
<td>Human lung carcinoma cells (A549); Murine macrophages (RAW264.7)</td>
<td>72 h</td>
<td>5-80 μg/cm²</td>
<td>Inconclusive: no induction of micronuclei in A549; statistically significant induction of micronuclei in RAW264.7 at ≥ 20 μg/cm²; high cytotoxicity observed at the inducing doses</td>
</tr>
<tr>
<td>Silica gel (Spherisorb 5 µm)</td>
<td>Micronucleus frequency</td>
<td>Chinese hamster lung fibroblasts (V79)</td>
<td>24 h; 20-160 µg/cm²</td>
<td><strong>Inconclusive</strong>; statistically significant induction of micronuclei at ≥ 80 µg/cm²; high cytotoxicity observed at the inducing doses</td>
<td>Liu et al. 1996</td>
</tr>
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<tr>
<td>Colloidal silica (Levasil 200/40% (15 nm); Levasil 50/50% (55 nm))</td>
<td>Micronucleus frequency</td>
<td>Human peripheral blood lymphocytes</td>
<td>24 h; ≤1000 µg/ml</td>
<td><strong>Negative</strong>; no induction of micronuclei</td>
<td>Downs et al. 2012</td>
</tr>
<tr>
<td>Stöber silica (S-16 (16 nm); S-60 (60 nm); S-104 (104 nm))</td>
<td>Micronucleus frequency</td>
<td>Human lung carcinoma cells (A549)</td>
<td>40 h; 10-300 µg/ml</td>
<td><strong>Negative/inconclusive</strong>; slight, not statistically significant increase in micronucleus frequency (particle number and total surface area correlated with the amplitude of the effect)</td>
<td>Gonzalez et al. 2010</td>
</tr>
<tr>
<td>Amorphous silica particles (NM-200 (15-23 nm); NM-201 (13-15 nm); NM-202 (10-14 nm); NM-203 (16-24 nm))</td>
<td>Micronucleus frequency</td>
<td>Human peripheral blood lymphocytes</td>
<td>30 h; ≤256 µg/ml (NM-200/201); ≤1250 µg/ml (NM-202/203)</td>
<td><strong>Negative</strong>; no induction of micronuclei</td>
<td>Tavares et al. 2013</td>
</tr>
<tr>
<td>Amorphous silica particles (NM-202 (10-25 nm); NM-203 (5-30 nm); NRT-808 (35 nm); NRT-817 (15 nm); NRT-820 (90 nm); NRT-944 (80 nm))</td>
<td>Micronucleus frequency</td>
<td>Mouse embryonic fibroblasts (Balb/3T3)</td>
<td>24 h; 100 µg/ml</td>
<td><strong>Negative</strong>; no induction of micronuclei</td>
<td>Uboldi et al. 2012</td>
</tr>
<tr>
<td>Amorphous silica particles (same as above)</td>
<td>Cell transformation</td>
<td>Mouse embryonic fibroblasts (Balb/3T3)</td>
<td>72 h; 100 µg/ml</td>
<td><strong>Negative</strong>; no morphological transformations</td>
<td>Uboldi et al. 2012</td>
</tr>
<tr>
<td>Stöber silica (S-10 (11 nm); S-30 (34 nm); S-80 (34 nm); S-400 (248 nm))</td>
<td>Micronucleus frequency</td>
<td>Mouse embryonic fibroblasts (3T3-L1)</td>
<td>24 h; 4, 40 or 400 µg/ml</td>
<td><strong>Inconclusive</strong>; statistically significant induction of micronuclei at ≥ 40 µg/ml observed only with S-80 (cytotoxicity at 400 µg/ml)</td>
<td>Park et al. 2011</td>
</tr>
<tr>
<td>Stöber silica (same as above)</td>
<td>Mutant frequency</td>
<td>Mouse embryonic fibroblasts (MEF-LacZ)</td>
<td>16 h; 4-400 µg/ml</td>
<td><strong>Inconclusive</strong>; statistically significant increase in mutant frequency at ≥ 40 µg/ml observed only with S-30</td>
<td>Park et al. 2011</td>
</tr>
</tbody>
</table>
Table 7. *In vivo* genotoxicity studies on amorphous silica.

<table>
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<th>Material</th>
<th>Test species</th>
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<th>Methods, endpoints</th>
<th>Findings, comments</th>
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</thead>
<tbody>
<tr>
<td>Pyrogenic silica (Aerosol 200; MMAD 0.810 µm) Cristobalite dust as positive control (MMAD 1.3 µm)</td>
<td>Male Fisher 244 rats (4/group)</td>
<td>Inhalation; 50 mg/m$^3$ (SAS); 3 mg/m$^3$ (cristobalite); 13 weeks (6 h/day; 5 days/week)</td>
<td>HPRT clonal selection assay on isolated alveolar epithelial (type II) cells</td>
<td>Negative; no significant increase in HPRT mutation frequency (as opposed to cristobalite)</td>
<td>Johnston et al. 2010</td>
</tr>
<tr>
<td>Amorphous silica particles (freshly generated by homogeneous nucleation, $d_{50}$ 37 or 83 nm)</td>
<td>Male Crl:CD (SD)IGS BR rats (5/group)</td>
<td>Inhalation (nose only); $3.1 \times 10^7$ or $1.8 \times 10^8 /\text{cm}^3$ (1.8-86 mg/m$^3$); 1 or 3 days (6 h/day)</td>
<td>Micronuclei frequency in peripheral blood at 24 h post exposure</td>
<td>Negative; no significant induction of micronuclei</td>
<td>Sayes et al. 2010</td>
</tr>
<tr>
<td>Colloidal silica (Levasil 200 (15 nm); Levasil 50 (55 nm))</td>
<td>Male Wistar rats</td>
<td>Intravenous injection; 3 x 25–125 mg/kg bw</td>
<td>Micronuclei frequency in peripheral blood at 4 h post exposure</td>
<td>Positive; statistically significant induction of micronuclei at the maximum tolerated dose of 50 mg/kg bw (Levasil 200) or 125 mg/kg bw (Levasil 50)</td>
<td>Downs et al. 2012</td>
</tr>
<tr>
<td>Colloidal silica (Levasil 200 (15 nm); Levasil 50 (55 nm))</td>
<td>Male Wistar rats</td>
<td>Intravenous injection; 3 x 25–125 mg/ml</td>
<td>DNA damage in blood and liver and lung tissues determined by Comet assay</td>
<td>Positive/inconclusive; statistically significant increase of DNA damage in liver tissue at the maximum tolerated dose of 50 mg/kg bw of Levasil 200, accompanied by neutrophilic infiltration and other histopathological changes in the liver tissue; no significant increase of DNA damage with Levasil 50</td>
<td>Downs et al. 2012</td>
</tr>
<tr>
<td>Micronised silica gel (Syloid 244)</td>
<td>Mice (host) + S. typhimurium TA 1530</td>
<td>Gavage; 1 or 5 × 1.4-5000 mg/kg bw</td>
<td>Host mediated gene mutations (S. typhimurium injected i.p. into orally exposed mice)</td>
<td><strong>Negative</strong>: no mutagenic activity</td>
<td>US-FDA 1974 as cited in ECETOC 2006</td>
</tr>
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</tr>
<tr>
<td>Micronised silica gel (Syloid 244)</td>
<td>Mice (host) + S. cerevisiae D3</td>
<td>Gavage; 1 or 5 × 1.4-5000 mg/kg bw</td>
<td>Host mediated gene mutations (S. typhimurium injected i.p. into orally exposed mice)</td>
<td><strong>Negative</strong>: no genotoxic activity</td>
<td>US-FDA 1974 as cited in ECETOC 2006</td>
</tr>
<tr>
<td>Micronised silica gel (Syloid 244)</td>
<td>Male SD rats</td>
<td>Gavage; 1 × 1.4-5000 mg/kg bw</td>
<td>Chromosome aberrations in bone marrow at 6, 24 or 48 h after last administration</td>
<td><strong>Negative</strong>: no significant increases in chromosomal aberrations</td>
<td>US-FDA 1974 as cited in ECETOC 2006</td>
</tr>
<tr>
<td>Micronised silica gel (Syloid 244)</td>
<td>Male SD rats</td>
<td>Gavage; 5 × 1.4-5000 mg/kg bw</td>
<td>Chromosome aberrations in bone marrow at 6 h after last administration</td>
<td><strong>Negative</strong>: no significant increases in chromosomal aberrations</td>
<td>US-FDA 1974 as cited in ECETOC 2006</td>
</tr>
<tr>
<td>Micronised silica gel (Syloid 244)</td>
<td>Male SD rats (8/dose)</td>
<td>Gavage; 1 or 5 × 1.4-5000 mg/kg/bw</td>
<td>Dominant lethal mutation; uterus examination at 14 days after mating</td>
<td><strong>Negative</strong>: no significant effects on fertility index, total number of implantations of corpora lutea, pre-implantation losses or dead implants</td>
<td>US-FDA 1974 as cited in ECETOC 2006</td>
</tr>
</tbody>
</table>
### Table 8. Carcinogenicity studies on amorphous silica.

<table>
<thead>
<tr>
<th>Material</th>
<th>Exposure route</th>
<th>Species</th>
<th>Exposure duration and doses</th>
<th>Methods/endpoints</th>
<th>Findings/comments</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Micronized silica gel (Syloid 244)</td>
<td>Oral</td>
<td>Male and female B6C3F1 mice (40/dose), male and female Fisher rats (40/dose)</td>
<td>93 weeks (mice); 104 weeks (rats); continuous exposure; 1.25, 2.5 or 5% in feed</td>
<td>Clinical signs, body weight, haematology, gross and microscopic pathology</td>
<td>No significant increase in tumour incidence in either species; no clinical effects, no changes in body weight or food consumption</td>
<td>Takizawa et al. 1988</td>
</tr>
<tr>
<td>Precipitated silica</td>
<td>Inhalation</td>
<td>Male and female mice (75/group)</td>
<td>52 weeks (1 hour/day; 5 days/week) + 33 weeks recovery period; visible dust cloud (≥500 mg/day into each cage)</td>
<td>Gross and microscopic pathology of the lungs</td>
<td>Increased incidence of pulmonary tumours: 21 % (13/61) vs. 7.9 % (5/63) in control group; overgrowth and hyperplasia in tracheobronchial lymph nodes and nodular fibrotic overgrowth of mediastinal connective tissue; no evidence of lung fibrosis Note: Study of limited value due to poorly controlled experimental conditions, and deficiencies of experimental techniques, study design and reporting</td>
<td>Campbell 1940</td>
</tr>
<tr>
<td>Pyrogenic silica; (Cab-O-Sil aggregate size 0.05-0.15 μm) Amorphous silica particles (particle size 0.005-0.015 μm)</td>
<td>Intra-pleural implantation</td>
<td>Female rats (30/exposed group; 90 controls)</td>
<td>108 weeks; continuous exposure; 40 mg in gelatine covered pledget</td>
<td>Gross and microscopic pathology of the lungs</td>
<td>No significant increase in tumour incidence: 0/24 animals with tumours in silica particle treated group, 1/18 in the Cab-O-Sil group (mesothelioma) and 0/58 in the control group</td>
<td>Stanton and Wrench 1972</td>
</tr>
<tr>
<td>Pyrogenic silica (Aerosil 150)</td>
<td>Intra-tracheal injection</td>
<td>Female Wistar rats (59/group)</td>
<td>125 weeks; injections at 14 days intervals; cumulative dose 15 mg (30 x 0.5 mg)</td>
<td>Gross and microscopic pathology of the lungs</td>
<td>Statistically significant increase in primary pulmonary tumours: 9.4% (5/53) vs. 0% (0/55) in the control group</td>
<td>Kolling et al. 2011</td>
</tr>
<tr>
<td>Amorphous silica particles (particle size 14 nm)</td>
<td>Intra-tracheal injection</td>
<td>Female Wistar rats (37-38/exposed group; 91 controls)</td>
<td>Injections at 7 days for ≤20 weeks; cumulative dose 15 and 30 mg</td>
<td>Gross and microscopic pathology of the lungs at 125-129 weeks after the first exposure</td>
<td>Increase in pulmonary tumours at 30 mg (3/38; 7.9%); no increase at 15 mg (0/37; 0.0%) (control 0/91; 0.0%)</td>
<td>Morfeld et al. 2006</td>
</tr>
</tbody>
</table>
5.2 Titanium dioxide

5.2.1 Identity of the substance

Titanium dioxide is naturally occurring oxide of titanium. Often distinction is made by TiO$_2$ manufacturers between pigmented and ultrafine grade. The primary crystal size typically ranges from 150 to 300 nm for TiO$_2$ of pigmentary grade and the surface area from 6 to 60 m$^2$/g. The ultrafine grade typically has a primary crystal size from 10 to 150 nm, and surface area between 50 and 200 m$^2$/g. The pigmentary TiO$_2$ has a white colour and is therefore widely used in paints etc. The ultrafine, including nano-sized, TiO$_2$ is transparent. In contrast to the bulk TiO$_2$ (>100 nm) that is considered chemically inert, nano-scale TiO$_2$ can act as a photocatalyst, and can generate reactive oxygen species upon illumination. A wide range of applications exist, exploiting the various properties of TiO$_2$ nanomaterials. In paints and for water treatment nano-sized TiO$_2$ is used as a photocatalyst producing reactive oxygen that may degrade other organics. Adding nano-TiO$_2$ into concrete aims to enhance its’ durability and to maintain whiteness throughout the lifetime of the construct. In glass nano-TiO$_2$ is used for heat and fire protection and for its’ self-clean properties. A number of other very diverse areas of application exist such as catalysts, toothpaste, sunscreens and other cosmetics, air filtration devices, semiconductors, etc. (van Broekhuizen et al. 2011)

5.2.2 Toxicokinetics

5.2.2.1 Absorption

**Oral and gastrointestinal**

Wang et al. (2007a) exposed mice orally to 5 g/kg of 25 and 80 nm rutile forms of TiO$_2$. Two weeks post-exposure, both types of NPs were distributed to liver, spleen, lungs and kidney, indicating systemic uptake of nano-TiO$_2$ via oral route. In a study by Hu et al. (2011), 5 nm anatase form TiO$_2$ was found to accumulate in the mouse hippocampus after intragastric administration. The results suggest that orally administered TiO$_2$ can cross the blood-brain barrier.

**Inhalation**

Ferin et al. (1992) found that in rats after a twelve-week lung exposure, ultrafine NPs (21 nm) had a longer clearance time from the lung than fine particles (250 nm), showing $t_{1/2}$=501 and 174 days, respectively. Wang et al. (2008a,b) have shown that rutile (80 nm) and anatase (155 nm) TiO$_2$ particles following nasal instillation exposure (500 µg, every other day for a total of 30 days) of mice were able to access the brain with accumulation within the cerebral cortex, thalamus and hippocampus (main target), and was postulated to occur by via the olfactory bulb.

**Dermal**

Several studies using different methods have been conducted in order to evaluate the dermal absorption of TiO$_2$ in vivo (humans, porcine and rat) and in vitro (porcine and human skin) (Mavon et al. 2007; Adachi et al. 2010; Gontier et al. 2008; Pflücker et al. 1999; Gamer et al. 2006). The studies report none or negligible absorption through stratum corneum. The
NANODERM European project (2002-2007) focused on skin penetration on nano-sized TiO$_2$ from sunscreen formulations. It was concluded that the penetration was limited to the upper part of stratum corneum. The particle shape, formulation, and exposure time appeared not to have any significant effect.

Wu et al. (2009) have shown contradictory results that do suggest some skin penetration of TiO$_2$. Following 60 days dermal exposure in vivo they reported distribution and toxicity in secondary organs. However, doubts about the methodological deficiencies of the study have been expressed (Jonaitis and Card 2010).

5.2.2.2 Distribution

Once NPs have reached the bloodstream, they will circulate until they are absorbed into tissue or cleared by the immune system or by filtration. Factors that determine in which organs the NPs accumulate or are removed by are influenced by the physico-chemical characteristics of the NPs. NP size is of particular importance in biodistribution as the fenestrae or pores of organ endothelium differ by organ type and function. For example, the liver, spleen and bone marrow have fenestrae sizes between 50-100 nm and particles sized below these may pass through the endothelium and enter the tissue (Alexis et al. 2008).

In orally exposed (5 g/kg) mice, two grades of nano-sized (20 nm and 80 nm) TiO$_2$ were found to be distributed to the liver, spleen, lungs and kidneys two weeks post-exposure (Wang et al. 2007a). Following intravenous injection (rat, 5 mg/kg, 20-30 nm) the NP levels were highest in the liver, but were also detected in the spleen, lung and kidneys (Fabian et al. 2007).

Quantitative biokinetics analysis after administration of radiolabelled NPs in the whole body of rats have been reported (Kreyling et al. 2010). Following two-hour inhalation more than most of the NP remained in the lungs during the whole observation period (28 days). Small fractions of NP translocated in secondary target organs, soft tissue and skeleton. The amount of translocated NP was approximately 2-7% of the amount deposited in the lungs. A prominent number of translocated NPs were found in the remainder. The translocation was accomplished within two to four hours after inhalation followed by retention in all organs and tissues studied without any detectable clearance within 28 days.

Wang et al. (2008a,b) found that instilled NPs (500 µg per mouse, every other day for a total of 30 days) could enter the brain through the olfactory bulb. Particles were accumulated especially in the hippocampus region. Takeda et al. (2009) administered TiO$_2$ (100 µg/day for 3-15 days, anatase, 25-70 nm) subcutaneously to pregnant mice. In six-week-old mice NPs were identified in testis and in the olfactory bulb and cerebral cortex of brain.

After intravenous injection of titanium dioxide (35 nm, rutile) to pregnant mice, NPs were detected in the placenta, fetal liver and fetal brain (Yamashita et al. 2011).

5.2.2.3 Metabolism

No studies were available on possible metabolism of TiO$_2$ NPs.

5.2.2.4 Elimination

No data found in the literature.
5.2.3 Acute toxicity

5.2.3.1 Oral

The distribution and acute toxicity of 25 nm and 80 nm of TiO$_2$ were investigated after a single high dose of NPs (5 g/kg body weight) in male and female CD-1 mice (Wang et al. 2007b). No acute toxicity was observed. Two weeks post-exposure, the NPs were distributed to liver, spleen, lungs and kidneys. Markers of cardiac damage were found to be elevated. Pathological evidence of hepatic injury and kidney damage was also observed. However, the very high dose administered should be taken into account when interpreting the results.

Warheit et al. (2007a) conducted an acute oral toxicity study of P25 TiO$_2$ (21 nm) according to OECD guidelines. A single dose (concentrations up to 5,000 mg/kg) of the TiO$_2$ particles was administered by gavage to rats. No signs of acute toxicity were observed.

5.2.3.2 Inhalation

**Pulmonary toxicity**

Table 9 summarizes the acute and sub-acute inhalation toxicity studies related to pulmonary effects of nano-TiO$_2$. The acute pulmonary responses include inflammation (Ferin et al. 1992; Chen et al. 2006; Warheit et al. 2007b; Renwick et al. 2004; Grassian et al. 2007), epithelial damage, increased permeability of the lung epithelium, oxidative stress, and cytotoxicity (Renwick et al. 2004), and morphological alterations within the lung (Chen et al. 2006).

There is clear evidence that nano-sized TiO$_2$ is considerably more toxic than micro-sized TiO$_2$ (Ferin et al. 1992; Renwick et al. 2004; Chen et al. 2006). In addition, it was found that the crystallinity (or the specific crystal form) of TiO$_2$ NPs is thought to influence the toxicity, with the anatase form expected to be more toxic than the rutile form (Warheit et al. 2007b).

Grassian et al. (2007) exposed mice by whole-body inhalation to TiO$_2$ NPs (5 and 21 nm) for either four hours (acute) or four hours per day for 10 days (subacute). TiO$_2$ concentrations were 0.8 or 7.0 mg/m$^3$ for acute exposure and 9 mg/m$^3$ for the subacute exposures. No adverse effects were observed after the acute exposure. A significant but modest inflammatory response (macrophage infiltration) was observed in the mice at 1 or 2 weeks after subacute exposures, with recovery at the 3rd week post-exposure.

**Cardiovascular toxicity**

Inhalation studies related to cardiovascular toxicity of nano-TiO$_2$ are summarised in Table 10. Systemic microvascular function was characterized after pulmonary exposure to fine and ultrafine TiO$_2$ aerosols (Nurkiewicz et al. 2008). After 24 hours of inhalation in vivo microscopy was performed. Significant microvascular dysfunction was observed in rats exposed to ultrafine particles as compared to microvascular function in control rats and those exposed to similar pulmonary load of fine particles. Cardiovascular effects occurred at particle exposure concentrations below those causing adverse pulmonary effects. In a similar experimental settings NP exposure reduced microvascular nitric oxide bioavailability and altered COX-mediated vasoreactivity (Knuckles et al. 2012) and increased phosphorylation of p38, mitogen-
activated protein kinase and cardiac troponin in the heart and substance P in nodose ganglia (Kan et al. 2012).

Cardiotoxicity of nano-TiO$_2$ has also been studied *in vitro*. Saber et al. (2013) analysed the mRNA expression of Serum Amyloid A (Saa3) in lung tissue from female C57BL/6J mice exposed to nano-TiO$_2$ particles and found that exposure to particles strongly increased Saa3 mRNA levels in lung tissue. Pulmonary expression of Saa3 correlated with the number of neutrophils in BAL. The authors suggest that pulmonary acute phase response may constitute a direct link between particle inhalation and risk of cardiovascular disease.

Helfenstein et al. (2008) demonstrated that TiO$_2$ (up to 2.5 µg/ml) was able to affect cardiomyocyte electrophysiology and enhanced ROS production. In the study of Jawad et al. (2012) contraction amplitude was reduced in conc. >100 µg/ml in rat myocytes. In hESC-cells 10 µg/ml reduced the beating rate significantly.
Table 9. Pulmonary effects of nano-TiO\(_2\) after acute or sub-acute inhalation exposure.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Species and doses</th>
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<th>Methods, endpoints</th>
<th>Findings, comments</th>
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<tbody>
<tr>
<td>Aeroxide(^{TM}), particles were sieved and dried before exposure, 21 nm Degussa</td>
<td>SPF Sprague Dawley rats, 1.5-12 mg/m(^3) corresponding dose of 6-38 µg</td>
<td>240-720 min</td>
<td>Lung histology, In vivo microscopy after 24 postexposure</td>
<td>No significant inflammatory changes in histopathology</td>
<td>Nurkiewicz et al. 2008</td>
</tr>
<tr>
<td>2-5 nm, 210±10 m(^2)/g (dispersed), aggregates in aerosol were 128 nm of size. Nanostructured and Morphous Materials</td>
<td>Male C57Bl/6 mice Concentration of NPs: 0.77-7.22 mg/m(^3) (acute) 8.88 mg/m(^3) (subacute)</td>
<td>4 h (acute), 4h/d for 10 d (subacute)</td>
<td>Inflammation (BALF cells), Cytotoxicity (BALF LDH), Lung histopathology</td>
<td>Acute exposure: no adverse effects Subacute exposure: macrophage infiltration Mice recovered by week 3 postexposure. No changes in LDH and histopathology</td>
<td>Grassian et al. 2007</td>
</tr>
<tr>
<td>1)10-40 nm, 132 m(^2)/g, rutile, SiO(_2) coating (&lt;5%) 2)&lt;25 nm,222 m(^2)/g, anatase 3) 30-40 nm, 23 m(^2)/g/rutile+anatase 4) reactor generated: 21 nm, 61 m(^2)/g, anatase+brookite Sigma Aldrich NanoAmor, Reactor generated/FIOH</td>
<td>BALB/c/Sca mice dose: 10 mg/m(^3)</td>
<td>Groups: once for 2 h, 2h for 4 days, 2h for 4 days for 4 w and sacrificed after 24 h of last exposure</td>
<td>Inflammation (BALF analysis), Chemokine expression Cytokine TNF-alpha protein</td>
<td>Exposure of 2 h for 4 d, and 4h for 4 d for 4 w produced significant number of neutrophils in BAL with nano TiO(_2)+SiO(_2) rutile NPs. Also these NPs induced signific. elevated expression of CXCL1 and TNF-alpha mRNA after exposure for 2 h on 4 consecutive d. Uncoated particles did not induce significant inflammation. No connection between inflammatory potential and particle size, surface area, agglomeration size or the particle induced .OH radical formation capacity</td>
<td>Rossi et al. 2010</td>
</tr>
<tr>
<td>Study</td>
<td>Nanomaterial Details</td>
<td>Route of Exposure</td>
<td>Exposure Details</td>
<td>Endpoints</td>
<td>Outcome</td>
</tr>
<tr>
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</tr>
<tr>
<td>Hougaard et al. 2010</td>
<td>Kemira UV-Titan L181, rutile, 20 nm, 107.7 m$^2$/g, modified with Al, Si and Zr, coated with polyalcohols</td>
<td>i.v.</td>
<td>42 mg/m$^3$ 1h/day, on gestational days 8-18, observations after 5 and 26-27 days</td>
<td>Maternal lung inflammation (BALF analysis)</td>
<td>38 mgTi/kg was detected in the lungs on day 5 Lung inflammation detected in lungs after 5 and 26-27 days</td>
</tr>
<tr>
<td>Halappanavar et al. 2011</td>
<td>Kemira UV-Titan L181, rutile, 20 nm, 107.7 m$^2$/g, modified with Al, Si and Zr, coated with polyalcohols</td>
<td>i.v.</td>
<td>42 mg/m$^3$ 1h/day for 11 days observations after 5</td>
<td>RNA, DNA and miRNA expression profiling PCR</td>
<td>Increased levels of mRNA for acute phase markers serum amyloid A-1, serum amyloid A-3, several chemokines, cytokine tumor necrosis factor genes Overall, changes in the expression of genes associated with acute phase, inflammation and immune response Changes in miRNA expression</td>
</tr>
<tr>
<td>Ma-Hock et al. 2009</td>
<td>From National Toxicology program, NTP, ILSI-HESI Nanomaterial Safety Consortium 25 nm, 14% rutile, 86% anatase surface area 51 m$^2$/g zeta potential 16.5 mV</td>
<td>i.v.</td>
<td>Wistar rats 6h/d for 5 consecutive days 3 and 16 days of recovery before endpoint measurements Doses 2, 10 and 50 mg/m$^3$</td>
<td>BAL fluid analysis Lung burden analysis Cell mediator analysis in BALF and serum (cytokines, chemokines, adhesion molecules, matrix metalloproteinases, acute phase proteins, apoptosis and cell proliferation proteins Hematology and serum troponin I Histopathology</td>
<td>Morphological changes in lungs with 50 mg/m$^3$. Dose-dependent increase in total cell counts and neutrophils in BALF, total protein content, enzyme activities and levels of a number of cell mediator NO indications of systemic effects Some endpoints returned to control levels on day 21s. LOAEC 2 mg/m$^3$.</td>
</tr>
<tr>
<td>NPs generated in a controlled gas-phase generator</td>
<td>Crl:OF1 mice</td>
<td>Doses 8, 20 and 30 mg/m³</td>
<td>Respiratory parameters, tidal volume, inspiration and expiration time, Time of braking, time of pause, respiratory rate, Sensory and pulmonary irritation</td>
<td>Single and repeated exposure caused minor airflow limitation in the conducting airways at each studied exposure concentration. Not dose dependent response. No significant sensory and pulmonary irritation (irritation was observed also in the control group).</td>
<td>Leppänen et al. 2011</td>
</tr>
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<tr>
<td>Mass concentrations 8, 20 and 30 mg/m³ gave average sizes in aerosol (GDM) 91, 113 and 130 nm</td>
<td>Primary particle size 20 nm</td>
<td>30 min exposure (single exposure) 16 h, 1 h/d, 4 d/w, for 4 w (repeated exposure) 30 mg/m³</td>
<td>BAL</td>
<td></td>
<td></td>
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<tr>
<td>Chry stal phase anatase+brookite (3: 1)</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>HINAMOX</th>
<th>BALB/cJ mice</th>
<th>Doses: 271 (acute) and 208 (subchronic) mg/m³</th>
<th>Respiratory parameters: 1) Tidal volume 2) Respiratory rate 3) Time of brake</th>
<th>Time-dependent decrease in tidal volume was seen. However, the response gradually resolved within the recovery period. Also in the breathing frequency a decrease was first detected, but after end of exposure the response gradually resolved. Initial increase in the time of break, but the response disappeared during the recovery period.</th>
<th>HINAMOX (NRCWE) DS.3.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Particle P</td>
<td>60 min exposure 24 h recovery period</td>
<td>in the detection of sensory irritation of upper respiratory tract</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>HINAMOX</th>
<th>BALB/cJ mice</th>
<th>Doses: 271 (acute) and 208 (subchronic) mg/m³</th>
<th>BALF analysis (total cell number, macrophages, neutrophils, lymphocytes and epithelial cells, total protein)</th>
<th>No signs of inflammation after 24 h and 90 days after exposure based on BALF analysis</th>
<th>HINAMOX (NRCWE) D 5.4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Particle P</td>
<td></td>
<td></td>
<td>Histology Oxidative stress</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 10. Cardiovascular effects of nano-TiO$_2$ after acute or sub-acute inhalation exposure.

<table>
<thead>
<tr>
<th>Material</th>
<th>Characteristics</th>
<th>Species, methods, doses</th>
<th>Exposure duration</th>
<th>Findings/comments</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Degussa Aeroxide</td>
<td>21 nm, ssa 44 m$^2$/g, anatase/rutile (80%/20%)</td>
<td>Male Sprague Dawley rats, 6mg/m$^3$, 4h for 1 day (10 µg lung burden); equivalent to the workers exposed at 0.1 mg/m$^3$ for 27 workdays in occupational environment Intravital microscopy</td>
<td>24 h</td>
<td>NP exposure reduced microvascular nitric oxide bioavailability and altered COX-mediated vasoreactivity (microvascular dysfunction)</td>
<td>Knuckles et al. 2011</td>
</tr>
<tr>
<td>Degussa Aeroxide</td>
<td>21 nm</td>
<td>Male Sprague Dawley rats, 6mg/m$^3$, 4h for 1 day (10 µg lung burden)&lt;br&gt;&lt;br&gt;\textit{In vitro}: cardiac myocytes, ROS assay, cardiac proteins by Western blots Substance P immunohistochemistry of nodose ganglia</td>
<td>4 h</td>
<td>Phosphorylation of p38, mitogen-activated protein kinase and cardiac troponin in the heart and substance P in nodose ganglia was increased after pulmonary exposure.&lt;br&gt;&lt;br&gt;\textit{In vitro}, in cardiac myocytes phosphorylation of the same proteins were unaltered with 1 µg/ml.</td>
<td>Kan et al. 2011</td>
</tr>
<tr>
<td>Degussa Aeroxide</td>
<td>Particles were sieved and dried before exposure, 21 nm</td>
<td>SPF Sprague Dawley rats, 1.5-12 mg/m$^3$ corresponding dose of 6-38 µg Intravital microscopy Histopathology</td>
<td>240-720 min</td>
<td>24 h after exposure impaired vasodilator capacity in the systemic microcirculation in a dose-dependent manner. No significant inflammatory changes in histopathology</td>
<td>Nurkiewicz et al. 2008</td>
</tr>
</tbody>
</table>
5.2.3.3 **Intratracheal instillation**

In intratracheal instillation, test materials are dispersed in liquids and then directly instilled into the trachea of the test animal by using syringe or other similar instrument. Subsequently, the biological effects on the lungs and other organs at different time points are investigated. This method has been widely used as an alternative to the inhalation test.

In the study conducted by Ferin et al. (1992), rats were administered TiO$_2$ of various sizes via intratracheal instillation up to 1,000 µg/rat. Examinations after 24 post-exposure indicated that nano-TiO$_2$ induced greater pulmonary inflammation response (characterized by neutrophil infiltration) than its fine counterpart. The clearance of smaller particles was slower.

Also in the studies of Renwick et al. (2004) nano-TiO$_2$ was more damaging in the lungs of rats than larger particles. Twenty-four hours after instillation, rats treated with nano-TiO$_2$ had induced neutrophil infiltration, elevated protein concentration (measure of epithelial permeability), and lactate dehydrogenase (an indicator of cytotoxicity).

Chen et al. (2006) exposed mice with 0.1 and 0.5 mg to ultrafine (19-21 nm) and determined their pulmonary toxicity. Morphology of emphysema-like alterations in the lungs was evident. An inflammatory response, as indicated by the infiltration of macrophages (that were particle laden), and upregulation of cytokines was also observed. Pott and Roller (2005) and Mohr (2006) included two TiO$_2$ NPs (21 nm and 25 nm) in the intratracheal instillation study. The former (dosage 15 x 0.5 mg, 30 x 0.5 mg) and latter (5 x 3 mg, 5 x 6 mg, 10 x 6 mg) induced lung tumours with incidences 0% and 7%, 52%, 67%, 70%, respectively.

In summary, in several studies there is clear evidence that nanosized TiO$_2$ is considerably more toxic than microsized TiO$_2$ (for example Ferin et al. 1992). In addition, it was found that the crystallinity (or the specific crystal form) of TiO$_2$ nanoparticles is thought to influence the toxicity, with the anatase form expected to be more toxic than the rutile form (Warheit et al. 2007b). The pulmonary response to TiO$_2$ is inflammation (Ferin et al.1992; Chen et al. 2006; Warheit et al. 2007b; Renwick et al. 2004; Grassian et al. 2007), epithelial damage, increased permeability of the lung epithelium, oxidative stress and cytotoxicity (Renwick et al., 2004), and morphological alteration within the lung (Chen et al. 2006).

5.2.4 **Irritation and sensitization**

OECD guideline tests for dermal and eye irritation and dermal sensitization have been performed. No dermal irritation or sensitization was observed. Eye irritation test was negative (Warheit et al. 2007a).

5.2.5 **Repeated dose toxicity**

5.2.5.1 **Oral**

Repeated dose toxicity (intragastric administration) studies with 5-7 nm of nano-titanium dioxide have been performed in mice (Cui et al. 2010 and Duan et al. 2010) (Table 11). After administration of 5, 10 and 50 mg/kg bw for every other day for 60 days, hepatocyte apoptosis was observed in 10 and 50 mg/kg bw groups. Moreover, levels of oxidative stress markers were elevated (Cui et al. 2010).
**Table 11.** Subchronic oral toxicity studies on nano-TiO$_2$.

<table>
<thead>
<tr>
<th>Material</th>
<th>Characteristics</th>
<th>Species, doses, methods, endpoints</th>
<th>Exposure duration</th>
<th>Findings/comments</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hangzhou Wanjing New Material Co</td>
<td>7 nm, anatase, surface area 173 m$^2$/g</td>
<td>CD-1 mice Intragastric administration 5, 10, 50 mg/kg sample preparation: sonication and vibration In liver: histology, oxidative stress and gene expression</td>
<td>samples were given for every other day for 60 days</td>
<td>Histology: mitochondria swelling (5mg/kg group, hepatocyte apoptosis (10-50 mg/kg) Levels of oxidative stress markers elevated in 10-50 mg/kg groups SOD, CAT, GSH-Px, MT, GST, HSP70, p53 expression decreased, CYP1A increased in 10-50 mg/kg groups</td>
<td>Cui et al. 2010</td>
</tr>
<tr>
<td>Hydrolysis of titanium tetrabutoxide</td>
<td>5 nm, anatase</td>
<td>CD-1 mice Intragastric administration Ultrasonication and vibration Histopathology of liver and kidney Liver enzymes Lymphocyte subset analysis Hematology Interleukins 62, 3, 124, 250 mg/bw</td>
<td>samples were given for every other day for 30 days</td>
<td>Liver histology: with the highest dose abnormal structure in hepatocyte was observed =&gt; 125 mg/kg groups activities of ALT, ALP, AST LDH ChE and TP were significantly elevated In hematological parameters dose-dependent changes Immunologically competent cells were lower than in control in 250 mg/kg bw group Dose-dependent reduction of IL-2 protein &quot; For humans such intake (several 10 g per person per day) is impossible in the environment and daily living&quot;</td>
<td>Duan et al. 2010</td>
</tr>
</tbody>
</table>
5.2.5.2 Inhalation

Pulmonary effects

Table 12 summarises the sub-chronic and chronic inhalation toxicity studies related to pulmonary effects of nano-TiO$_2$. In the study by Ma-Hock et al. (2009) rats were exposed to 25 nm TiO$_2$ (2, 10 and 50 mg/m$^3$) by inhalation for five days. Morphological changes in the lungs were observed in the 50 mg/m$^3$ group. Dose-dependent increase in total cell counts and neutrophils in BALF, total protein content, enzyme activities and levels of a number of cell mediators were found. A LOAEC of 2 mg/m$^3$ was identified in this study.

In the study of Bermudez et al. (2004), rats, mice, and R hamsters were exposed to P25 particles for 13 weeks at concentrations 0.5, 2, or 10 mg/m$^3$. The P25 particles are TiO$_2$ particles that are manufactured by Degussa and consist of 80% anatase and 20% rutile. The have an average particle diameter of 21 nm. Pulmonary response was assessed up to 52 weeks post-exposure. It was demonstrated that the pulmonary response was stimulated by TiO$_2$ within mice and rats, but was absent in hamsters. At 52 weeks post-exposure, minimal to mild metaplastic changes and minimal to mild particle-induced alveolar septal fibroplasia were seen in rats. The effects observed were dose dependent. High concentrations (10 mg/m$^3$) of particles impaired their clearance from lungs in rats and mice. Pulmonary inflammation was evidenced by increased numbers of macrophages and neutrophils and increased concentrations of soluble markers (total protein and LDH) in BALF in rats and mice exposed to 10 mg/m$^3$. In rats responses were also observed in animals exposed to 2 mg/m$^3$. A no observed adverse effect concentration (NOAEC) of 0.5 mg/m$^3$ was identified.

Systemic microvascular function was characterized after inhalation of fine and ultrafine TiO$_2$ aerosols. After 24 hours of inhalation (concentrations 1.5-12 mg/m$^3$) _in vivo_ microscopy was performed. Significant microvascular dysfunction was observed in rats exposed to ultrafine particles as compared to microvascular function in control rats and those exposed to similar pulmonary load of fine particles (Nurkiewicz et al. 2008).

Lung toxicity and developmental effects in offspring after exposing rats, for 11 days, 42 mg/m$^3$ with surface-coated TiO$_2$ were studied by Hougaard et al. (2010) and Halappanavar et al. (2011). Exposure resulted in changes in neutrophil, macrophage and lymphocyte amounts and increased levels of genes associated with acute-phase, inflammation and immune-response five days post-exposure. Prenatally exposed offspring displayed moderate neurobehavioral alterations.

In a chronic inhalation study of Heinrich et al. (1995), rats were exposed to P25 -particles (manufactured by Degussa) for 2 years. The average exposure concentration was approximately 10 mg/m$^3$, and the cumulative particle exposure (g/m$^3$ x h) calculated by multiplying the particle concentration with the exposure time was 88.1. In comparison with the control groups, there was a significant increase in mortality, decrease in body weight, increase in lung weight, and decrease in clearance after three months of exposure. Incidence of lung tumours was significantly increased after exposure for 18 months or more. The relevance of these data for risk assessment is dubious due to the very high dose used. However, the International Institute for Research of Cancer has assessed TiO$_2$ (even the micro-
sized form, if exposure is high enough) to be a Class 2B carcinogen (possibly carcinogenic to humans) (IACR 2006). The US National Institute of Safety and Health (NIOSH) has determined that inhaled TiO₂ is a potential occupational carcinogen and recommended an exposure limit of 0.3 mg/m³ (NIOSH 2011).

Rats were exposed via inhalation to 23 mg/m³ (6 hours per day for 5 days per week for up to 12 weeks) of TiO₂ (21 nm, manufactured by Degussa) and 250 nm and examination of the consequences were evaluated over a 64-week post-exposure period (Ferin et al. 1992). Ultrafine TiO₂ induced a greater pulmonary inflammatory response (characterised by neutrophil infiltration), than its fine counterpart, which did not elicit any changes in the inflammatory status. Ultrafine TiO₂ particles were also find to remain within the lungs for longer (501 days) periods, following inhalation, than the fine particles (174 days), thus highlighting that the clearance of smaller particles form the lung was slower.

In summary, the pulmonary response of nano-TiO₂ after repeated exposure has been demonstrated to be inflammagenic. Moreover, some studies have demonstrated epithelial damage and cytotoxicity. In addition, chronic exposure to nano-TiO₂ with high doses has the ability to promote tumour development.
Table 12. Sub-chronic and chronic inhalation toxicity studies on nano-TiO₂.

<table>
<thead>
<tr>
<th>Material</th>
<th>Species</th>
<th>Exposure duration and doses</th>
<th>Methods, endpoints</th>
<th>Findings, comments</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Particle size in aerosol as mean mass median aerodynamic diameter, MMAD: 1.37 µm Degussa P25</td>
<td>Female B3C3F1/CrlBr mice, CDF(F344)/CrlB rats, Lak:LVG(SYR)BR hamsters</td>
<td>0.5, 2.0 and 10 mg/m³, for 6h/d, 5d/w, for 13 weeks. Observation periods 4, 13, 26 and 52 weeks postexposure</td>
<td>Inflammation (BALF and histology) Lung particle burden Cytotoxicity (LDH) and permeability (protein)</td>
<td>Pulmonary overload observed in rats and mice after inhalation 10mg/m³ dose for 13 w, but not in hamsters. Persistent increase of number of neutrophils. In rats inhaling 10mg/m³, histopathological findings included epithelial and fibroproliferative changes, interstitial particle accumulation and alveolar septal fibrosis. Animals exposed to 2 mg/m³ showed almost no effect =&gt; NOAEC 0.5 or 2 mg/m³. At 52 weeks postexposure, minimal to mild metaplastic changes and minimal to mild particle-induced alveolar septal fibroplasia were seen in rats. Hamsters were able to most efficiently clear particles and pulmonary responses were absent in them. Increase in LDH and protein levels</td>
<td>Bermudez et al. 2004</td>
</tr>
<tr>
<td>anatase, 21 nm, ssa: 50 m²/g, MMAD in aerosol 1.7 µm Degussa P25 also fine NPs, anatase, 250 of size, MMAD 1.9 µm was tested</td>
<td>Male Fisher 344 rats</td>
<td>23 mg/m³, exposure 6h/d, 5d/w for up to 12 w. Observation period 4, 8, 12, 41 and 64 w after the start of the exposure</td>
<td>Inflammation (neutrophil infiltration, histology) Lung burden and particle clearance</td>
<td>Increasing dose in terms of particle numbers and decreasing particle size promoted particle entry into the pulmonary interstitium. Particle translocation was accompanied with an acute inflammatory response. Clearance t₁/₂ for fineNP was 174 and for ultrafine NP 501 d, due to the high lung burden considered to be particle overload range. No full histopathology performed. The aerosol aggregates from fine and ultrafine samples were almost similar in size. However, the effect of the particles are linked to the particle size and number. &quot;It seems that the aggregation forces for these particles are not strong&quot;.</td>
<td>Ferin et al. 1992</td>
</tr>
<tr>
<td>1) ultrafine: size 20 nm</td>
<td>Male Fisher F344 rats</td>
<td>6h/d, 5 d/w for 12 w, follow-up 64 w</td>
<td>For ultrafine-TiO$_2$ the total retained lung burden at the end of exposure was 6.6 mg and for fine-TiO$_2$ 5.2 mg. Retention half-times were approx. 500 d for ultrafine- and 170 d for fine-TiO$_2$. A significantly larger fraction of ultrafine particles was transferred to the regional lymph nodes, as compared to fine particles, indicating a greater ability of ultrafine particles to enter interstitial spaces after alveolar deposition. Ultrafine particles elicited higher inflammation reaction. The inflammatory response was reversible, since after 6 months, no significant difference was observed in comparison with the controls. Oberdörster et al. 1994 ( basically same study as Ferin et al. 1992)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2) fine: size 200 nm</td>
<td>Degussa P25?</td>
<td>1) Dose: 24 mg/m$^3$ 2) Dose: 22 mg/m$^3$</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Degussa P25</td>
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</table>

| MMAD: 1.5 µm | Female Wistar rats Crl:(WI)BR, female NMRI mice Crl:NMRI BR, female C57BL/6N mice | 2-year exposure: for rats 7.2mg/m$^3$x4 m, 14.8 mg/m$^3$x4m, 9.4 mg/m$^3$x16m  Observation period 6 m postexposure  Mice: on average 10mg/m$^3$ for 13.5 months. Observation period 9.5 months | Cumulative particle exposure (g/m$^3$xs) calculated by multiplying the particle concentration with the exposure time, was 88.1 for rats and 51.5 for mice. At 6 months 99/100 rats developed bronchioloalveolar hyperplasia. Slight to moderate interstitial fibrosis of the lungs were observed in all rats after 2 y of exposure. At 24 m lung tumors were observed in 4 of nine rats (2 scc, 1 adenoca., 2 benign squamous cell tumor). At 30 m, a statistically signif. increase in adenocarcinomas was found (13 adenoca. and 3 scc, 4 adenomas) in 100 rats. In addition, 20 rats had benign keratinizing cystic squamous cell carcinomas. Experiments with mice were not valid because of high mortality rate. Particle overload conditions may have an effect on the findings Heinrich et al. 1995 |
| Degussa P25 | | | |
5.2.6  *In vivo* studies on neurotoxicity

Table 13 summarises the identified studies on neurotoxicity of nano-TiO$_2$. As already described in the toxicokinetics section, Wang et al. (2008a,b) showed that in rats TiO$_2$ particles (80 nm/rutile and 155 nm/anatase) can be translocated from the nasal area to the central nervous system via the olfactory nerve and bulb. Accumulation of TiO$_2$ resulted in morphological alterations and loss of neurons in the hippocampus. In addition it was suggested that TiO$_2$ elicited oxidative stress within the brain due to the elevation of superoxide dismutase (SOD), and catalase activity, and evidence of increased lipid peroxidation and protein oxidation was found. Furthermore, an inflammatory response (indicated by IL-1$\beta$, and TNF$\alpha$) within the brain was stimulated by TiO$_2$ exposure. The adverse effects were more severe with the anatase TiO$_2$ particles.

5.2.7  *In vitro* studies on cytotoxicity and ROS generation

Several studies have shown various degrees of toxicity of nano-TiO$_2$ to epithelial lung cells (for example Gurr et al. 2005; Simon-Deckers et al. 2008). Signs of oxidative stress are often observed. Long et al. (2006) and Lai et al. (2008) have indicated that nano-TiO$_2$ can cause ROS driven toxicity to neural cells *in vitro*. Helfenstein et al. (2008) and Jawad et al. (2011) have observed adverse effects of nano-TiO$_2$ to heart cell function and increase in ROS production. The generation of ROS and induction of inflammation leading to alterations of signalling components due to nano-TiO$_2$ exposures have been reviewed in the paper of Shi et al. (2013). Several studies (for example Renwick et al. 2001; Palomäki et al. 2010) suggest that nano-TiO$_2$ may affect different cell types of the immune system.
### Table 13. *In vivo* studies on neurotoxicity of nano-TiO₂.

<table>
<thead>
<tr>
<th>Material</th>
<th>Characteristics</th>
<th>Species, methods, doses</th>
<th>Exposure duration</th>
<th>Findings, comments</th>
<th>Reference</th>
</tr>
</thead>
</table>
| 1) 80 nm rutile from Hangzhou Dayan Nanotechnology  
2) 155 nm anatase Zhonglian Chemical Medicine | 1) ssa: 22,69 m²/g  
2) ssa: 10,47 m²/g | CD-1(ICR) mice, nasal instillation of 500 µg/ NPs every other day  
Enzyme activity (GSH, catalase, SOD, glutathione peroxidase)  
Lipid peroxidation (MDA)  
Protein oxidation (protein carbonyl formation) | 30 d | NPs were found in brain tissue, morphological changes in hippocampal neurons were found. CFAP-positive astrocytes in the CA4 region were observed.  
Increased MDA  
Increased catalase  
Decreased SOD  
Increased protein oxidation  
The changes were more severe with the anatase Nps. | Wang et al. 2008a |
| 1) 80 nm rutile from Hangzhou Dayan Nanotechnology  
2) 155 nm anatase Zhonglian Chemical Medicine | 1) ssa: 22,69 m²/g  
2) ssa: 10,47 m²/g | CD-1(ICR) mice, nasal instillation of 500 µg/ NPs every other day. TEM and Niss1 staining | 30 d | Instilled NPs were observed in the olfactory bulb and hippocampus after intranasal instillation. Anatase form produced significant elevated interleukin (IL-1B) levels. | Wang et al. 2008b |
<p>| NPs were prepared via hydrolysis of titanium tetrabutoxide | 5 nm, ssa 174,78 m²/g, anatase | CD-1 female mice, intragastric administration, 5, 10 and 50 mg/kg | 60 d | Levels of various enzymes and neurotransmitters were altered. Electrolyte contents of brain were changed. Significant changes were detected with the doses of 10 and 50 mg/kg. In histopathology changes in spingiocyte morphology was observed, suggesting pathological changes of neurons. | Hu et al. 2010 |</p>
<table>
<thead>
<tr>
<th>NPs were prepared via hydrolysis of titanium tetrabutoxide</th>
<th>5 nm, ssa 174,78 m²/g, anatase</th>
<th>CD-1 female mice, intragastric administration, 5, 10 and 50 mg/kg</th>
<th>60 d</th>
<th>NPs were observed to accumulate in mouse hippocampus. Hippocampal apoptosis and impairment in spatial recognition memory was observed. ROS accumulation also detected. Dose dependent effects.</th>
<th>Hu et al. 2011</th>
</tr>
</thead>
<tbody>
<tr>
<td>NPs were prepared via hydrolysis of titanium tetrabutoxide Bulk material</td>
<td>5 nm, ssa 174,78 m²/g, anatase Bulk material</td>
<td>CD-1 female mice Histopathology Titanium content ROS, lipid peroxidation in brain Antioxidant enzyme activities in brain tissue Neurochemicals assay Abdominal injection 5, 10, 50, 100 and 150 mg/kg bw daily</td>
<td>14 d</td>
<td>Histopathological changes in the 100 and 150 mg/kg bw group Titanium accumulation in brain Significant ROS formation with doses ≥ 50 mg/kg bw Activities of antioxidant enzymes increased with doses ≥ 10 mg/kg bw and 150 mg/kg bulk titanium dioxide Significant NOS activity in the 50, 100 and 150 mg/kg bw group</td>
<td>Ma et al. 2010</td>
</tr>
</tbody>
</table>
5.2.8 Reproductive and developmental toxicity

Table 14 summarises identified studies on reproductive and developmental effects of nano-TiO$_2$. After subcutaneous injection of nano-TiO$_2$ to pregnant mice, several adverse effects on offspring have been reported in three publications. Takeda et al. (2009) demonstrated reduced sperm production and abnormal testicular morphology. In 6-week-old mice exposed prenatally to TiO$_2$, NPs were identified in testis and in the olfactory bulb and cerebral cortex of brain. Functional and pathological effects reported were: reduced sperm production, abnormal testicular morphology and reduced number of Sertoli cells. Changes in gene expression related to development and function of central nervous system has been reported by Shimizu et al. (2009). Yamashita et al. (2011) investigated biodistribution and toxicity of titanium dioxide (rutile, 35 nm). Mice treated with NPs had smaller uteri and smaller foetuses. Particles were found in the placenta, foetal liver and foetal brain. LOEC was 0.8 mg/ mouse. In a recent study Zhao et al. (2013) found that nano-TiO$_2$ induced fertility reduction and ovary injury in female mice. Mice were exposed to 2.5, 5, and 10 mg/kg nano-TiO$_2$ by intragastric administration for 90 days. Nano-TiO$_2$ was deposited in the ovary, resulting in significant reduction of body weight and fertility, relative weight of ovary, alterations of haematological and serum parameters and sex hormone levels, atretic follicle increases, inflammation, and necrosis.

Dose-dependent cytotoxicity to testes Leydig cells in vitro has been shown by Komatsu et al. (2008). Takahashi et al. (2010) investigated the impact of prenatal exposure to titanium dioxide NPs on the dopaminergic system. They found increased levels of dopamine and its metabolites in the brain of pups.

In summary, there is some evidence that nano-sized titanium dioxide can cross the placenta and may cause adverse effects in offspring.
Table 14. Reproductive and developmental studies on nano-TiO₂.

<table>
<thead>
<tr>
<th>Material</th>
<th>Species, endpoints</th>
<th>Doses</th>
<th>Exposure route and duration</th>
<th>Findings, comments</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tayca Co. 35 nm, fluorescent labelled, rutile form</td>
<td>BALB/c mice Biodistribution, pregnancy complications and histopathology</td>
<td>0.2, 0.4, 0.8 mg/mice intravenous injection 24 h</td>
<td>NPs were found in the placenta, fetal liver and fetal brain. Mice treated with NPs had smaller uteri and smaller fetuses. LOEC 0.8 mg/mouse.</td>
<td>Yamashita et al. 2011</td>
<td></td>
</tr>
<tr>
<td>Sigma Aldrich 25-70 nm, 20-25 m²/g, anatase form</td>
<td>ICR mice cDNA microarray analysis</td>
<td>100 ug/mouse/day subcutaneous injection gestational days 6,9,12,15</td>
<td>Changes in gene expression related to development and function of central nervous system.</td>
<td>Shimizu et al. 2009</td>
<td></td>
</tr>
<tr>
<td>Sigma Aldrich 25-70 nm, 20-25 m²/g, anatase form</td>
<td>ICR mice Histopathology, testicular funtions, TiO₂ distribution to testis and brain of offspring</td>
<td>100 ug/mouse/day subcutaneous injection gestational days 3,7,10,14</td>
<td>In the offspring: Reduced sperm production, abnormal testicular morphology, aggregates (100-200 nm) in Leydig cells, Sertoli cells and spermatids in the testis. In 6-week- old mice exposed prenatally, NPs were identified by energy dispersive X-ray spectroscopy testis and in the olfactory bulb and cerebral cortex of brain.</td>
<td>Takeda et al. 2009</td>
<td></td>
</tr>
<tr>
<td>Sigma Aldrich 25-70 nm, 20-25 m²/g, anatase form</td>
<td>ICR mice Dopamine levels in several regions of brains detected by HPLC</td>
<td>100 ug/mouse/day subcutaneous injection gestational days 6,9,12,15</td>
<td>Levels of dopamine and its metabolites were increased in the prefrontal cortex and the neostriatum.</td>
<td>Takahashi et al. 2010</td>
<td></td>
</tr>
<tr>
<td>Kemira UV-Titan L181, 20 nm, rutile, 107.7 m²/g, modified with Al, Si and Zr, coated with polyalcohols</td>
<td>pregnant C57BL/6BomTac mice (BALF analysis); offspring organ weight; offspring behaviour and fertility; titanium content of lung and liver tissues and milk</td>
<td>42 mg/m³ inhalation 1h/day, on gestational days 8-18, observations after 5 and 26-27 days</td>
<td>38 mgTi/kg was detected in the lungs on day 5 Offspring displayed moderate neurobehavioral alterations</td>
<td>Hougaard et al. 2010</td>
<td></td>
</tr>
</tbody>
</table>
5.2.9 Genotoxicity

5.2.9.1 Genotoxicity in vitro

Table 15 summarises the identified *in vitro* genotoxicity studies on nano-TiO$_2$.

**Micronuclei studies**

Rahman et al. (2002) studied the potential of nano-TiO$_2$ to elicit chromosomal damage in SHE fibroblasts. The number of micronucleated cells was increased with the doses ≥1 µg/cm$^2$. Moreover, elevated apoptosis within cells was detected. In peripheral lymphocytes and in lymphoblastoid cells, micronuclei formation has shown dose-dependency (Kang et al. 2008, Wang et al. 2007a, Sanderson et al. 2007). Exposing cells to 10 µg/ml was found to induce micronuclei in human bronchial cells (Gurr et al. 2005). In human bronchial epithelial cells, anatase TiO$_2$ induced micronuclei formation at doses 10 and 60 µg/cm$^2$ in the 72-hour treatment. No effect on micronuclei formation with the rutile, SiO$_2$ coated form, was observed (Falck et al. 2009). Two other studies with coated TiO$_2$ nanoparticles were not able to show evidence on micronucleus formation *in vitro* (Linnainmaa et al. 1997, Landsiedel et al. 2010b).

**Chromosomal aberrations**

Testing doses up to 300 µg/ml of TiO$_2$ (<25 nm, anatase form) elicited chromosomal aberrations in peripheral lymphocytes with the highest dose after 48 hours treatment (Catalan et al. 2011). HPRT mutation assay was positive when human lymphoblastoid cells were exposed to 130 µg/ml of TiO$_2$ (Wang et al. 2007b).

Genotoxicity in liver cells has been reported by comet assay (Petkovic et al. 2011; Shukla et al. 2011). Up-regulation of DNA damage responsive genes (p53, mdm2) and induction of reactive oxygen species were also reported. Anatase TiO$_2$ (>25 nm) was more toxic compared to the rutile (<100 nm) form (Petkovic et al. 2011). Apoptosis induction was also detected (Shukla et al. 2011). At 20 µg/ml (six hours), micronuclei induction was observed (Shukla et al. 2011).

**Comet assays**

Both positive and negative results have been obtained in comet assays *in vitro* conducted in various cell lines (several publications).

5.2.9.2 Genotoxicity in vivo

Table 16 summarises the identified *in vivo* genotoxicity studies on nano-TiO$_2$. Trouiller et al. (2009) studied genotoxicity, oxidative DNA damage and inflammation of 21 nm TiO$_2$ (75% anatase, 25% rutile) in mice after oral administration (five days) of 0, 50, 100, 250 and 500 mg/kg body weight. Moreover, for an in utero exposure study, pregnant dams were given NPs in drinking water for ten days during days 8.5-18.5 post coitum. The highest dose tested (500 mg/kg/body weight) induced micronuclei in peripheral blood and DNA-single strand breaks in comet assay. DNA double strand breaks, as measured by the γ-H2AX-assay, were detected in bone marrow in a dose-dependent manner. At the highest dose tested, oxidative DNA damage by 8-OHdG analysis was detected in mouse livers and pro-inflammatory cytokines in peripheral blood were up-regulated. In utero exposure of foetuses via the mother caused an increase in large deletions in offspring.
Genotoxicity of nano-sized (33 nm) and microsized (160 nm) TiO$_2$ was studied in six organs of mice after oral exposure (seven days) (Sycheva et al. 2011). DNA-damage, as detected by comet assay, was positive in bone marrow cells after exposing mice to 40 and 200 mg/kg wt of both particles. Also positive micronucleus tests were reported (33 nm NP, bone marrow cells, liver cells).

Lindberg et al. (2012) performed a short-term inhalation study in mice with TiO$_2$ particles (74-89 nm, 74% anatase, 26% brookite) that were generated by thermal decomposition of titanium tetraisopropoxide. Mice were exposed for five days, four hours/day, with 0.8, 7.2 and 28.5 mg/m$^3$ of test particles, using ethylene oxide as positive control. The highest dose produced an increase of neutrophils in BAL. However, no indication of genotoxicity in comet and micronucleus assays was observed.

In another inhalation study (exposure: 10 mg/m$^3$, five days, NP: rutile, 50 nm) no genotoxic effect in lung cells of rats were detected by the comet assay (Landsiedel et al. 2010a).

Driscoll et al. (1997) investigated mutations at Hprt loci in alveolar epithelial cells after exposing rats to TiO$_2$ particles (180 nm, anatase) using intratracheal instillation. Mutation frequency was increased at 100 mg/kg bw, but not at 10 mg/kg bw. Numbers of inflammatory cells (macrophages, neutrophils and lymphocytes) were significantly elevated after exposure to 100 mg/kg bw, suggesting that an increased mutation rate is associated with inflammation.
Table 15. *In vitro* genotoxicity studies on nano-TiO₂.

<table>
<thead>
<tr>
<th>Material</th>
<th>Characteristics, preparation, doses</th>
<th>Test system</th>
<th>Results</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1) CEA</td>
<td>1) 95% anatase, spherical, 12 nm, ssa 92 m²/g, BET 17 nm, PZC 6.4, DMEM 0</td>
<td>1) Alkaline Comet assay</td>
<td>1) positive with CEA-NPs from ≥100 µg/ml, negative with other TiO₂ NPs</td>
<td>Barillet et al. 2010</td>
</tr>
<tr>
<td>2) Degussa</td>
<td>2) 89% anatase, spherical, 24±4 nm, ssa 44 m²/g, BET 33 nm, 7 PZC, DMEM 1 mV</td>
<td>2) ROS</td>
<td>2) positive with all TiO₂ NPs ≥ 50 µg/ml</td>
<td></td>
</tr>
<tr>
<td>Aeroxide</td>
<td>3) 100% anatase, spherical, 142±36 nm, ssa 10 m²/g, BET 152 nm, PZC, 5.2, DMEM 0 mV</td>
<td>3) gamma-H2AX for monitoring double strand breaks</td>
<td>3) negative</td>
<td></td>
</tr>
<tr>
<td>3) Sigma, T8141</td>
<td>4) 100% rutile, elongated, L: 68 nm, D: 9 nm, SSA 118 m²/g, DMEM -1 mV</td>
<td>NRK-52E rat kidney cells</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4) Sigma, 637262</td>
<td>Doses: ROS: 1-200 µg/ml,</td>
<td></td>
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<tr>
<td>TiO₂ from</td>
<td></td>
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<tr>
<td>sunscreens</td>
<td>Comet assay: 20-200 µg/ml</td>
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<tr>
<td></td>
<td>20-50 nm in diameter with different anatase/rutile ratios were extracted from sunscreens</td>
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<tr>
<td></td>
<td>Cells treated with 0.0125% sunscreen TiO₂</td>
<td></td>
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<tr>
<td></td>
<td>Agarose gel electroforesis of supercoiled plasmid and Comet assay in human MRC-5 fibroblasts ± solar irradiation</td>
<td>Positive in electroforesis and in Comet assay after combined treatment with sunscreen extract±irradiation</td>
<td></td>
<td>Dunford et al. 1997</td>
</tr>
<tr>
<td>Sigma-Aldrich</td>
<td>1) rutile</td>
<td>Trypan blue exclusion test 10-160 µg/cm², 24, 48, and 72 h</td>
<td>rutile: low cytotoxicity, decrease in number of living cells detected only with the highest dose</td>
<td>Falck et al. 2009</td>
</tr>
<tr>
<td></td>
<td>99.5% pure, contained &lt;5% SiO₂ size 10x40 nm, ssa 132 m²/g</td>
<td>Alkaline comet assay 1-100 µg/cm², 24, 48 and 72 h</td>
<td>anatase: cell viability started to decrease in the 24 h treatment at 40 µg/cm², and in 48 and 72 h treatments at 80 µg/cm².</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2) anatase, 99.7% pure size &lt; 25 nm</td>
<td>Micronuclei assay 1-100</td>
<td></td>
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</tr>
<tr>
<td>Supplier</td>
<td>NP Type and Characteristics</td>
<td>Assay Method</td>
<td>Cytoxicity and Photogenotoxicity Details</td>
<td>Reference</td>
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<tr>
<td>1) Aldrich</td>
<td>1) anatase, BET 10 m²/g, size 40-300 nm</td>
<td>µg/cm²</td>
<td>In comet assay dose-dependent effects were seen after 48 and 72 h treatments with anatase NP, LOAL 10 µg/cm². Rutile form induced DNA damage only in the 24 h treatment and 80 and 100 µg/cm² in the 72 h treatment (with a dose-dependent effect). In MN assay: Anatase NPs produced elevated frequency of MNs at 10 and 60 µg/cm² (no dose-dependency. No effect on MN formation with the rutile form.</td>
<td>Gerloff et al. 2009</td>
</tr>
<tr>
<td>2) Degussa</td>
<td>2) 80% ana., BET m²/g, 20-80 nm</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3) Sigma, Hombikat</td>
<td>3) anatase modified, BET &lt;300 m²/g, size &lt; 10 nm</td>
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<tr>
<td></td>
<td>Cells initially grown in 20% FCS, with NPs starvation media was used</td>
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<td></td>
<td>Doses 20 and 80 µg/cm², for cytotoxicity also 1.25 and 5 µg/cm² conc. were used</td>
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<tr>
<td></td>
<td>Incubation 4 and 24 h</td>
<td></td>
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<tr>
<td>Sigma Aldrich</td>
<td>Anatase TiO₂, and ZnO (from Sigma), both 40-70 nm suspended in DMSO, For TiO₂ the doses used were 3.73, 14.92, 29.85 and 59.7 µg/ml</td>
<td>µg/cm²</td>
<td>Positive, significant at doses ≥3.73 µg/ml. Dose-dependent increase in DNA damage with TiO₂ and ZnO in both cell types. Photogenotoxic response was seen only in lymphocytes</td>
<td>Gopalan et al. 2009</td>
</tr>
</tbody>
</table>

### Table Notes:
- **BEAS 2B cells**: In comet assay dose-dependent effects were seen after 48 and 72 h treatments with anatase NP, LOAL 10 µg/cm². Rutile form induced DNA damage only in the 24 h treatment and 80 and 100 µg/cm² in the 72 h treatment (with a dose-dependent effect).
- **In MN assay**: Anatase NPs produced elevated frequency of MNs at 10 and 60 µg/cm² (no dose-dependency. No effect on MN formation with the rutile form.
- **Cell Culture**: Human colon adenocarcinoma cell line, Caco-2
- **Assay Methods**: Fpg-modified comet assay, Cytotoxicity determined by LDH assay and WST assay, Human colon adenocarcinoma cell line, Caco-2
- **Incubation**: 4 and 24 h
For ZnO, the doses used were 11.5, 46.2, 69.4 and 92.3 µg/ml

<table>
<thead>
<tr>
<th>Product/Manufacturer</th>
<th>Formulation Details</th>
<th>Assays Used</th>
<th>Assay Results</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>1) Hombikat UV100</td>
<td>1) Anatase Mean size: &lt;10 nm SSA: &lt;250 m²/g</td>
<td>1) Alkaline Comet assay ± FPG 2) Cytokinesis Block Micronucleus assay</td>
<td>1) Comet positive 2) Micronuclei positive</td>
<td>Gurr et al. 2005</td>
</tr>
<tr>
<td>2) Millenium PC500</td>
<td>2) Anatase Mean size: 5-10 nm SSA: 340 m²/g particles were suspended in phosphate-buffered saline Concentration of NPs: 10 µg/ml</td>
<td>Both assays were performed in darkness</td>
<td></td>
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</tr>
<tr>
<td>Sigma Aldrich</td>
<td>15-30 nm anatase, BSA added as a stabilizer, pH 7.4, In TEM aggregations, mean sized 285±52 nm were observed, 10, 25, 50 and 100 µg/ml conc. were used</td>
<td>Comet assay in human nasal mucosal cells Cytotoxicity was tested with trypan blue exclusion</td>
<td>Negative Intracytoplasmic NPs found in 11% of cells. However no signs of cytotoxicity or DNA strand breaks were seen. Aggregation property possibly had an effect to the negative result.</td>
<td>Hackenberg et al. 2010</td>
</tr>
<tr>
<td>Degussa/ Aerioxide P25</td>
<td>70-85% anatase, 15-30% rutile TiO₂. Ssa: 50 m²/g, particle size 30 nm. Zeta-potential in culture medium was -11.6±1.2 mV. The negative surface charge at physiological pH had an isolectric point 6.4 Concentrations: 20-100 µg/ml, sonication before exposure</td>
<td>Micronucleus assay in lymphocytes Comet-assay p53-protein ROS by DCFDA</td>
<td>CA: positive, clear dose dependent effect on DNA fragmentation, p&lt;0.05, with 20 µg/ml p53 accumulation detected</td>
<td>Kang et al. 2008</td>
</tr>
<tr>
<td>Scaffolds</td>
<td>Properties</td>
<td>Tests</td>
<td>Results</td>
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</tr>
</tbody>
</table>
| Sigma     | anatase, 50 nm, DLS 124 nm, BET 5-10 nm, zeta potential -17.6 mV  
Doses: 0.008 - 8 µg/plate | Ames test with S. typhimurium TA98, TA100, TA1535, TA1537 and E.coli WP2 uvrA | Positive, statistically sign. (two-fold or more as comp. to control) increase in mutant colonies in TA98, TA1537 and E.coli WP2 uvrA strains with and without S9 at con. 0.008-0.8 µg/plate | Kumar et al. 2011 |
| BASF     | 1) rutile, surface-coated, particle size 10x50 nm, acicular-shaped, SSA 100 m²/g, TiO₂ content 79-89%, mean agglomerates 200 nm  
2) rutile, surface-coated, particle size 10x50 nm, SSA 100 m²/g, TiO₂ content 69-73%, mean agglomerates 200 nm  
vehicles: DMSO, FBS  
metabolic activation | a) OECD guideline 471, Ames test  
TA98, TA100, TA1535, TA1537 mutations  
doses: 20-5000 µg/plate  
b) in vitro mammalian cell micronucleus test (OECD draft TG 487) doses: 75, 150, 300 µg/ml  
c) in vivo Comet assay in alveolar cells from Wistar rats exposed to 10 mg/m³, a subacute five-day inhalation test for nanomaterials | a), b), c) negative | Landsiedel et al. 2010 |
<p>| Degussa Aerioxide P25 and UV-TITAN M160 | UV-TITAN M160: average crystal size 20 nm, rutile coated by aluminium hydroxide and stearic acid, before exposure washed with ethanol to remove the stearic acid to make the particles hydrophobic and suspensible | Cytokinesis Block Micronucleus Assay in rat liver epithelial cells. After 1 h of incubation the slides were irradiated with UV | Negative | Linnainmaa et al. 1997 |</p>
<table>
<thead>
<tr>
<th>Pigmentary TiO$_2$ (170 nm, uncoated anatase)</th>
<th>All suspensions were sonicated</th>
<th>Doses: 5, 10 and 20 µg/cm$^2$</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Particles (&lt; 20 nm) were sterilized and suspended in phosphate-buffered saline &quot;Particles were gift from Dr. Obersdörster&quot;</td>
<td>*Concentrations 1.0, 5, 10 µg/cm$^2$  *Treatment: 12-72h.</td>
<td>1) Micronucleus assay in Syrian Hamster Embryo Fibroblasts 2) Agarose gel electrophoresis</td>
<td>1) Increase in micronuclei, p&lt;0.001 with ≥1.0 µg/cm$^2$, no significant increase in kinetochore-positive micronuclei 2) Typical apoptotic structures were revealed by electrophoresis with 10 µg/cm$^2$</td>
</tr>
<tr>
<td>Sample Description</td>
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<tr>
<td>P25 and T805 from Degussa</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>P25 (surface hydrophilic) and T805 (surface made hydrophobic by treatment with trimethoxoctylsilane) were suspended in physiological saline supplemented with 0.25% lecithin. Primary particle diameter 20 nm, but particles were highly aggregated, sonication not leading to primary particles. Intratracheal instillation in rats followed by bronchoalveolar lavage for immunological determination of 8-oxoguanine.</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Negative</td>
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<td></td>
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</tr>
<tr>
<td>Rehn et al. 2003</td>
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</table>

<table>
<thead>
<tr>
<th>Sample Description</th>
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</thead>
<tbody>
<tr>
<td>Samples A-C from Degussa</td>
</tr>
<tr>
<td>A, B, C: crystal anatase 80%, rutile 20%, primary particle size 21 nm; A: coating trimethoxycaprylsilane, B: no coating, doped with 2% diiron trioxide, C: no coating. D: 100% rutile, primary particle size 14 nm, coating 8-11% alumina and 1-3% simethicone, E: 100% anatase, aggregate size 60 nm, coating 37% alumina and 12-18% silica; F-H: 100% rutile, F: primary particle size 20 nm; coating 5-6% alumina and 1-4% dimethicone, H: primary particle size 20-22 nm, coating 10.5-12.5% alumina and 3.5-5% silica, 30-150 nm aggregates are expected in the samples tested. For test, A was dissolved in absolute ethanol, B and C in physiological saline, D-H in DMSO.</td>
</tr>
<tr>
<td>Chromosome aberration test in CHO-WBL cells ± UV irradiation</td>
</tr>
<tr>
<td>Cell culture contained 10% FCS</td>
</tr>
<tr>
<td>NP concentrations: 800-5000 µg/ml</td>
</tr>
<tr>
<td>Negative</td>
</tr>
<tr>
<td>None of the different forms TiO$_2$ induced increases of chromosome aberrations either with or without UV irradiation in Chinese hamster ovary cells.</td>
</tr>
<tr>
<td>Theogaraj et al. 2007</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Sample Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Samples D-H from various companies (manufacturers of sunscreens)</td>
</tr>
<tr>
<td>A, B, C: crystal anatase 80%, rutile 20%, primary particle size 21 nm; A: coating trimethoxycaprylsilane, B: no coating, doped with 2% diiron trioxide, C: no coating. D: 100% rutile, primary particle size 14 nm, coating 8-11% alumina and 1-3% simethicone, E: 100% anatase, aggregate size 60 nm, coating 37% alumina and 12-18% silica; F-H: 100% rutile, F: primary particle size 20 nm; coating 5-6% alumina and 1-4% dimethicone, H: primary particle size 20-22 nm, coating 10.5-12.5% alumina and 3.5-5% silica, 30-150 nm aggregates are expected in the samples tested. For test, A was dissolved in absolute ethanol, B and C in physiological saline, D-H in DMSO.</td>
</tr>
<tr>
<td>Alkaline Comet Assay</td>
</tr>
<tr>
<td>HPRT Mutation Assay</td>
</tr>
<tr>
<td>Cytokinesis Block Micronucleus Assay</td>
</tr>
<tr>
<td>All in cultured WIL2-NS human lymphoblastoid cells</td>
</tr>
<tr>
<td>1) Positive (with 65µg/ml, p&lt;0.05, toxicity in 20% of the cells)</td>
</tr>
<tr>
<td>2) Positive (130 µg/ml, p&lt;0.05)</td>
</tr>
<tr>
<td>3) Positive (with 130 µg/ml, p&lt;0.01)</td>
</tr>
<tr>
<td>Dose-dependent relationship observed.</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Sample Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td>Particles (99% pure, size by volume 6,57 nm:(100%), by intensity 8,2 nm:(80,4%) and 196,5 nm:(19,4%) were sonicated in RPMI 1640 Agglomeration state in medium 19,6% were agglomerates of size 197 in size SSA: 147,9 m$^2$/g</td>
</tr>
<tr>
<td>1) Alkaline Comet Assay</td>
</tr>
<tr>
<td>2) HPRT Mutation Assay</td>
</tr>
<tr>
<td>3) Cytokinesis Block Micronucleus Assay</td>
</tr>
<tr>
<td>All in cultured WIL2-NS human lymphoblastoid cells</td>
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<tr>
<td>1) Positive (with 65µg/ml, p&lt;0.05, toxicity in 20% of the cells)</td>
</tr>
<tr>
<td>2) Positive (130 µg/ml, p&lt;0.05)</td>
</tr>
<tr>
<td>3) Positive (with 130 µg/ml, p&lt;0.01)</td>
</tr>
<tr>
<td>Dose-dependent relationship observed.</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Authors</th>
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<tbody>
<tr>
<td>Rehn et al. 2003</td>
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<tr>
<td>Theogaraj et al. 2007</td>
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<tr>
<td>Wang et al. 2007</td>
</tr>
<tr>
<td>Sanderson et al. 2007</td>
</tr>
</tbody>
</table>
Surface charge 6.88 at pH 6.75
Porosity (4v/a), 86.7889 Å
Dose: 0.26, 65 and 130 µg/ml, incubation for 6, 24 and 48 h

### Ultrafine TiO₂
- **Size**: average 140 nm, fractions also below 100 nm, manufactured by DuPont
- **Particles**: 79% rutile, 21% anatase, median size 140 nm, surface 38.5 m²/g, composition: 90% TiO₂, 7% alumina, 1% amorphous silica. Suspended in water
- **SSA**: 30-60 m²/g
- **Doses**: 100, 333, 1000, 3333, 5000 µg/plate
- **1) Bacterial reverse mutation test with S. typh. TA98, TA100, TA1535, TA1537 and E.coli WP2uvr, according to OECD 471**
- **2) Chromosomal aberration in CHO cells ± Aroclor induced rat liver S9, according to OECD 473**

<table>
<thead>
<tr>
<th>Warheit 2007</th>
</tr>
</thead>
</table>

### Sigma Aldrich
- **Anatase particles with sizes**: 5 nm, 40 nm and at 325 mesh in diameter. BET surface area 114 m²/g, 38 m²/g and 9 m²/g respectively. Purity >99%. NPs were sonicated for 30 min before use. Diluted in cell culture medium containing 15% FBS
- **Doses**: 0.1-100 µg/ml

<table>
<thead>
<tr>
<th>Positive</th>
</tr>
</thead>
</table>

A significant increase in mutation yield at TiO₂ 40 nm was observed at concentrations ≥ 0.1 µg/ml (p<0.05), 5 nm NPs yielded 2.2 fold increase at the same concentration. In contrast the mutation frequency was not much altered by TiO₂ 325 mesh at doses 0.1-30 µg/ml.

Dose dependent formation of ONOO was observed in 5 and 40 nm NPs.

Xu et al. 2009
<table>
<thead>
<tr>
<th><strong>Sigma Chemical Co.</strong></th>
<th><strong>Sigma Aldrich</strong></th>
<th><strong>Sigma Aldrich</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>30-70 nm, anatase, hydrodynamic diameter: 125 nm (in Milli Q), 193 nm (in cell culture media) Doses: 1-80 µg/ml (corresponding 0.31-25 µg/cm²) in CMEM medium</td>
<td>&lt;25 nm, ssa 222 m²/g, anatase Doses: 6.25-300 µg/ml Exposure times: 24, 48, 72 h Also carbon nanotubes studied.</td>
<td>&lt;25 nm anatase, 129,3 m²/g, &lt;100 nm rutile, 116,7 m²/g Doses 1, 10, 100, 250 µg/ml for 2, 4 and 24 h in comet and ROS assays</td>
</tr>
<tr>
<td>human hepatoma, HepG2 cells, fpg-comet assay, micronuclei and ROS formation (fluorescence method) Immunoblot assays for p53 and apoptosis markers</td>
<td>Chromosomal aberrations, OECD 473 test Peripheral lymphocytes from volunteers Mitomycin C as positive control</td>
<td>human hepatoma HepG2 cells comet assay, ROS formation, mRNA expression (DNA damage responsive genes: p53, mdm2, gadd45a, p21)</td>
</tr>
<tr>
<td>Positive</td>
<td>Positive</td>
<td>Positive (anatase form) ROS formation in all doses (TiO₂-Ana) 250 µg/ml (TiO₂-Ru) Induction of fpg-sensitive sites in comet assay (TiO₂, 100, 250 µg/ml, all incub. times), only 250 µg/ml (TiO₂-Ru) 100 µg/ml of both NPs induced activation of DNA damage responsive genes</td>
</tr>
<tr>
<td>Oxidative DNA damage observed at 1 µg/ml by fpg-Comet assay. Positive in micronuclei formation (20µg/ml, 6 h). ROS formation significant at conc. 10µg/ml). Apoptosis induction as detected by immunoblot markers.</td>
<td>50 % cytotoxicity not observed in any of the doses. 48 h treatment with 300 µg/ul showed significantly increase amount of chromatid-type chromosomal aberrations.</td>
<td></td>
</tr>
</tbody>
</table>

*Shukla et al. 2011*

*Catalan et al. 2011*

*Petkovic et al. 2011*
Table 16. *In vivo* genotoxicity studies on nano-TiO$_2$.

<table>
<thead>
<tr>
<th>Material</th>
<th>Characteristics, preparation, doses</th>
<th>Test system</th>
<th>Findings, comments</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Degussa Aerioxide P25</td>
<td>Ultrasonication for 15 min in drinking water at 60, 120, 300 and 600 µg/ml just before use. Water intake was 3-7 ml/ mouse/d. Doses correspond 0, 50, 100, 250 and 500 mg/kg bw. For in utero exposure, pregnant dams were given NPs for 10 d from 8,5-18,5 d post coitum at concentration of 300 µg/ml corresponding 500mg/kg bw.</td>
<td>Oral administration Mouse strain: C57Bl/6jpun/pun 1) <em>In vivo</em> DNA deletion assay 2) Comet assay 3) Micronuclei assay in peripheral blood cells 4) γ-H2AX-assay in bone marrow cells 5) 8-OHdG oxidative damage in liver cell In utero exposure of fetuses analyzed</td>
<td>1-5) positive Genotoxicity was observed in blood bone marrow and embryo. NPs (500 mg/kg) induce DNA-single stranded breaks and chromatosomal aberrations. H2AX formation was the most sensitive test and showed an increase of double strand breaks in a dose dependent manner. In utero exposure of fetuses caused an increase in large deletions. Mechanism of genotoxicity might be via oxidative stress Warheit and Donner, Nanotoxicology, 2010 4: 409 -413 : &quot;shortcomings in the experimental design in this study (lack of positive and negative controls, particle characterization and inadequate documentation of the dose), suggest that standardized models and repeatability of results should be necessary prerequisites for the establishment of verifiable genotoxicity assays for nanomaterials.&quot; &quot;older aged mice than routinely used for conventional <em>in vivo</em> micronucleus testing&quot;</td>
<td>Trouiller et al. 2009</td>
</tr>
</tbody>
</table>
| Particles were generated by thermal decomposition of titanium tetraisopropoxide | size 74-89 nm, dependent on mass concentration, 74% anatase, 26% brookite Doses: 0.8, 7.2, 28.5 mg/m$^3$, respective surface area concentrations 0.049, 0.44 and 1.74 m$^2$/m$^3$ No excessive agglomeration observed with the doses used. | Short-term inhalation study. Male C57BL/6J mice exposed 4 h per day for 5 consecutive days. Ethylene oxide (600 mg/m$^3$) as positive control Inflammatory cells in BAL, Comet assay form alveolar type II/Clara cells, micronucleus assay in peripheral blood erythrocytes | Negative The highest dose produced increase of neutrophils in BAL. However, no indication of genotoxicity in Comet and micronucleus assays. "Possibly the inflammatory response induced in our experiment was not adequately high or did not prevail enough to provoke a genotoxic effect. It may also that there is no simple association between the inflammatory and genotoxic effects of TiO$_2$."

Lindberg et al. 2012 | Fisher Scientific 180 nm, ssa 8.8, m$^2$/g, anatase doses: 10 and 100 mg/kg | F344 Fisher rats intratracheal instillation Alveolar type II cells from BAL fluid were analyzed for Hprt mutations | Positive Mutation frequency was increased at 100 mg/kg but not at 10 mg/kg. Number of inflammation cells (macrophages, neutrophils and lymphocytes) were significantly elevated after 100 mg/kg suggesting that increased mutation rate is associated with lung inflammation. | Driscoll et al. 1997 | Sensient Cosmetic Technologies LCW 33 nm and 160 nm particles, anatase form Doses 40, 200, (1000) mg/kg for seven days oral administration comet assay (brain, liver and bone) micronuclei, atypical nuclei, multinucleated cells, mitotic/apoptotic index (bone marrow, forestomach, colon and testes cells) | Positive Comet assay was positive with both NPs and doses in bone marrow cells Micronucleus assay was positive with 33 nm-NP in bone marrow and liver cells. Mitotic index was elevated in forestomach and epithelial cells with both NPs | Sycheva et al. 2011 |
5.2.10 Carcinogenicity

Only one study on carcinogenicity was indentified (Heinrich et al. 1995). In this chronic inhalation study, rats were exposed to P25-particles (manufactured by Degussa) for 2 years. The average exposure concentration was approximately 10 mg/m$^3$, and the cumulative particle exposure (g/m$^3 \times h$) calculated by multiplying the particle concentration with the exposure time was 88.1. In comparison with the control groups, there was a significant increase in mortality, decrease in body weight, increase in lung weight, and decrease in clearance after three months of exposure. Incidence of lung tumours was significantly increased after exposure for 18 months or more. The relevance of these data for risk assessment is dubious due to the very high dose used.

However, the International Agency for Research on Cancer has assessed TiO$_2$ (even the micro-sized form, if exposure is high enough) to be a Class 2B carcinogen (possibly carcinogenic to humans) (IARC 2010). The US National Institute of Safety and Health (NIOSH) has determined that inhaled TiO$_2$ is a potential occupational carcinogen and recommended an exposure limit of 0.3 mg/m$^3$ (NIOSH 2011).

5.2.11 Conclusions

There is clear evidence that nano-sized TiO$_2$ is considerably more toxic than micro-sized TiO$_2$. Among the TiO$_2$ induced adverse effects, respiratory tract is considered the most critical site. The pulmonary response to TiO$_2$ is inflammation, epithelial damage, increased permeability of the lung epithelium, oxidative stress and cytotoxicity, and morphologic alteration within the lung. Despite of the increased production and use of TiO$_2$ nanoparticles the epidemiological data are lacking.

The genotoxicity of TiO$_2$ nanoparticles is thought to be driven by particle mediated reactive oxygen species production. The particles themselves are not thought to be inherently genotoxic, but may trigger genotoxicity via an indirect threshold driven inflammatory mechanism involving oxidative stress.

In rodents, nano-TiO$_2$ has been shown to be able to translocate into the central nervous system via axons of sensory neurons in the upper respiratory tract. In the human body, the relevance of transfer via this route is however questionable. Some evidence exists of neurotoxic potential of nano-TiO$_2$. A limited amount of data suggest that TiO$_2$ nanoparticles may affect the cardiovascular system.

Dermal studies have shown little evidence for skin penetration after dermal applications of nano-TiO$_2$. However, there may be a risk associated with nano-TiO$_2$ applied to damaged skin.

Long-term inhalation studies in rats have reported lung tumours. Oxidative stress induced by ROS generation may play an important role in the initiation and progression of carcinogenesis of TiO$_2$ nanoparticles.
5.3 Nanocellulose

5.3.1 Identity of the substance

The sources and properties of nanocelluloses are summarized in Table 17. Although the nomenclature of nanocelluloses has not been used in a uniform manner, microfibrillated cellulose (MFC), nanocrystalline cellulose (NCC) and bacterial nanocellulose (BNC) all belong to the family of nanocellulose materials (Klemm et al. 2011).

The recent reviews by Brinchi et al. (2013), Klemm (2011) and Lavoine (2012) summarize the main applications of MFC and NCC as follows: improvement of nanocomposite mechanical, thermal and optical properties, barrier properties for instance in food packing and in toxic metal separations from wastes and fuel cell membranes. The material properties of MFC and NCC, i.e. high strength and stiffness, give rise to potential use of nanocellulose in many applications. BNC has a three-dimensional structure consisting of an ultrafine network of cellulose nanofibres, whose structure has led to number of medical applications such as wound dressings and skin substitutes (Saska et al. 2012).

The size, shape, aggregation properties, degree of branching and specific surface properties, such as chemical surface modifications, among others, still poorly understood factors, may affect the interactions of cellulose nanofibers with cells and living organisms. Moreover, the raw material, the pre-treatment, and the mechanical treatment have to be considered in the toxicity studies of nanocellulose (Lavoine et al. 2012).

Table 17. The family of nanocellulose materials.

<table>
<thead>
<tr>
<th>Type of nanocellulose</th>
<th>Synonyms</th>
<th>Typical sources</th>
<th>Formation and average size</th>
</tr>
</thead>
<tbody>
<tr>
<td>Microfibrillated cellulose</td>
<td>Nanofibrils and microfibrils, nanofibrillated</td>
<td>Wood, sugar beet, potato tuber, hemp, flax</td>
<td>Delamination of wood pulp by mechanical pressure before and/or after chemical or enzymatic treatment</td>
</tr>
<tr>
<td>(MFC)</td>
<td>cellulose</td>
<td></td>
<td>Diameter: 5-60 nm</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Length: several micrometers</td>
</tr>
<tr>
<td>Nanocrystalline cellulose</td>
<td>Cellulose nanocrystallites, whiskers, rodlike</td>
<td>Wood, cotton, hemp, flax, wheat straw, mulberry</td>
<td>Acid hydrolysis of cellulose from many sources</td>
</tr>
<tr>
<td>(NCC)</td>
<td>cellulose microcrystals</td>
<td>bark, ramie, Avicel, tunicin, cellulose from algae</td>
<td>Diameter: 5-20 nm</td>
</tr>
<tr>
<td></td>
<td></td>
<td>and bacteria</td>
<td>Length: 100-250 nm (from plant cellulose)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>100 nm to several micrometers (from celluloses of tunicates, algae, bacteria)</td>
</tr>
<tr>
<td>Bacterial nanocellulose</td>
<td>Bacterial cellulose, microbial cellulose,</td>
<td>Low-molecular weight sugars and alcohols</td>
<td>Bacterial synthesis</td>
</tr>
<tr>
<td>(BNC)</td>
<td>biocellulose</td>
<td></td>
<td>Diameter: 20-100 nm, different types of networks</td>
</tr>
</tbody>
</table>
5.3.2 Acute toxicity

5.3.2.1 Oral and inhalation

No data available in the open literature.

5.3.2.2 Intraperitoneal

Identified studies on intraperitoneal toxicity of nanocelluloses are summarized in Table 18. In the study of Ferraz et al. (2012), extracted PPy-cellulose membranes as test material (concentration 6 cm²/ml) were injected intraperitoneally to mice at a dose of 50 ml/kg. The mice were observed for adverse reactions after dosing and at 4, 24, 48 and 72 h. All animals appeared clinically normal at the beginning and throughout the study. No evidence for mortality or significant systemic toxicity was observed.

In the study of Jeong et al. (2010) toxicity of nanocellulose fibres (NFs) from *Gluconacetobacter xylinus* was determined in C57/B16 mice. The animals were injected intraperitoneally with 0.5 and 5 mg/ml NFs. Serum analyses of albumin, aspartate aminotransferase (AST), alanine transaminase (ALT), creatinine and triglyceride (TG) did not show differences between test and control group.

**Table 18.** Intraperitoneal toxicity studies on nanocelluloces.

<table>
<thead>
<tr>
<th>Material</th>
<th>Methods, endpoints, doses</th>
<th>Findings, comments</th>
<th>Reference</th>
</tr>
</thead>
</table>
| Polymer polypyrrole (PPy) coated nanocellulose | Extracted PPy-cellulose membranes as test material  
Concentration 6 cm²/ml  
Mice were ip. injected at a dose of 50 ml/kg  
Observations for adverse reactions immediately after dosing and at 4, 24, 48 and 72 h  
Five mice per group  
Weighing 24, 48, and 72 h after injection | All animals appeared clinically normal at the beginning and throughout the study  
No mortality  
No evidence for significant systemic toxicity | Ferraz et al. 2012 |
| Bacterial cellulose from *Gluconacetobacter xylinus* | C57/B16 mice  
Mice were ip. injected with 0.5 and 5 mg/ml test material  
Serum albumin, total cholesterol, AST, ALT, creatinine and triglyceride analyses from serum  
Controls were injected with PBS | No differences between control and test animals | Jeong et al. 2010 |
5.3.3 Irritation and sensitization

No data available in the open literature.

5.3.4 Repeated dose toxicity

No data available in the open literature.

5.3.5 In vitro studies on cytotoxicity and inflammatory effects

Table 19 summarises the identified in vitro studies on cytotoxicity and inflammatory effects of nanocelluloses. In the study of Vartiainen et al. (2011), the inflammatory effects caused by microfibrillated cellulose (MFC; also referred to as nanocellulose) in cultured mouse macrophages and human monocyte derived macrophages were evaluated after 6 and 24 h exposures to 30, 100 and 300 μl/ml. Cell death was assayed with Trypan blue staining. Cytokine and chemokine production (18rRNA, IL-1β, TNFα, CXCL-8, IL-6, IL-10, MIP-1a, IL-12p40 and CXCL1) was studied using quantitative real-time PCR and secretion of TNF-α, IL-1β by ELISA. The authors report no significant cytotoxic or inflammatory effects.

The cytotoxic and (pro-)inflammatory responses induced by cotton cellulose nanowhiskers (CCN), multiwalled CNT’s (MWCNT) and asbestos fibres were examined by using a 3D in vitro cell co-culture model of the human epithelial airway barrier (Clift et al. 2011). The model is derived from macrophages, dendritic cells and bronchial cells. CCNs were internalized within vesicles of phagocytic cells. A significant dose dependent correlation in cytotoxicity (LDH assay) was observed at concentrations of 5, 15 and 30 μg/ml on the apical side (consisting of macrophages) of the coculture model. MWCNTs and asbestos fibres elicited a much higher release of LDH at the same concentration.

The effect of nanocellulose-polymer polypyrrole (PPy) composite on cell viability was studied in vitro using fibroblast and monocyte cell lines, and acutely in vivo in mice by intraperitoneal injection (Ferraz et al. 2012). The results indicated that the leachables of the composite did not induce any cytotoxic response in vitro or in vivo, but that the biocompatibility of the PPy composite depends largely on the rinsing and pre-treatment as well as the aging of the material.

Two fluorescence-labelled nanocrystalline celluloses (NCC) were evaluated for cytotoxicity in the study of Mahmoud et al. (2010). The test materials had no effects on human kidney cells in vitro.

Nanofibrillar cellulose (NFC) isolated from bleached birch pulp was tested for cytotoxicity (mitochondrial metabolic activity) in HepaRG and HepG2 liver cell cultures. No cytotoxic effects were observed using 0.1-1wt% NFC hydrogels (Bhattacharya et al. 2012).
<table>
<thead>
<tr>
<th>Material</th>
<th>Methods, endpoints, doses</th>
<th>Findings, comments</th>
<th>Reference</th>
</tr>
</thead>
</table>
| MFC (microfibrillated cellulose) | Mouse macrophage cell line (RAW 264.7)  
Human peripheral lymphocytes (PBM)  
Cell death (trypan blue)  
Production of cytokines and chemokines (18rRNA, IL-18, TNFα, CXCL-8, IL-6, IL-10, MIP-1a, IL-12p40, CXCL1) qPCR assay | No evidence of cytotoxicity or inflammatory effects | Vartiainen et al. 2010 |
| MCC (microcrystal cellulose) used as reference material | | | |
| Polymer polyprrole (PPy) coated nanocellulose | Test material was extracted for 24 h in culture medium. Dose: Surface/volume ratio 6 cm²/ml  
Human dermal fibroblasts:  
1) Trypan blue staining  
2) Alamar blue assay  
Human monocytes TH-1 (Human acute leukemia cell line)  
1) Trypan blue staining  
2) LDH assay | No cytotoxicity detected. Cell proliferation for the composite extract was 83±8% (toxicity limit 70%). | Ferraz et al. 2012 |
| Cellulose nanowhiskers derived from cotton (CCN) | LDH assay  
Proinflammatory cytokine/chemokine stimulation  
TEM  
3D cell culture model  
of the human epithelial airway derived from macrophages, dendritic cells and bronchial cells  
Doses: 0.005, 0.015 and 0.03 mg/ml  
Exposure: 24 h | CCNs were internalized within vesicles of phagocytic cells.  
A significant dose dependent correlation in cytotoxicity was observed at conc. 1.5 and 30 ug/ml on the apical (macrophages) side of the triple cell coculture model. MWCNTs and asbestos elicited a much higher release of LDH at the same concentration | Clift et al. 2011 |
| Asbestos and MWCNTs also studied | | | |
| Bacterial cellulose from *Gluconacetobacter xylinus* | Cell viability by MTT  
Cell morphology using microscope | No toxicity observed  
No effects on apoptosis | Jeong et al. 2010 |
<table>
<thead>
<tr>
<th>Nanofibres were prepared using acid hydrolysis and sonication</th>
<th>Flow cytometric evaluation of apoptosis</th>
<th>Flow cytometric evaluation of apoptosis</th>
<th>No cytotoxicity detected</th>
</tr>
</thead>
<tbody>
<tr>
<td>Length, width not mentioned in the article</td>
<td>Concentrations: 0.1, 0.5 and 1 mg/ml</td>
<td>Exposure: 24 and 48 h</td>
<td>Bhattacharya et al. 2012</td>
</tr>
<tr>
<td>Nanofibrillar cellulose from bleached birch pulp</td>
<td>Cell viability by mitochondrial metabolic activity assay, 4 h exposure</td>
<td>No cytotoxicity detected</td>
<td></td>
</tr>
<tr>
<td>Obtained from UPM Finland</td>
<td>Liver cells: HepaRG and HepG2 cultures</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Width 20-30 nm</td>
<td>10% FBS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Exact length unclear</td>
<td>0-1% nanofibrillar cellulose hydrogel</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cellulose nanocrystals (CNC)</td>
<td>Human embryonic kidney 293 (HEK 293) cells</td>
<td>CNC-RBITC was uptaken by the cells without affecting the membrane integrity</td>
<td></td>
</tr>
<tr>
<td>10-20 nm, 120-300 nm long (pristine)</td>
<td>Cytotoxicity assessed by electric cell-substrate impedance sensing (ECIS)</td>
<td>No cytotoxicity detected</td>
<td></td>
</tr>
<tr>
<td>Fluorescence labeled (FITC- and RBITC)</td>
<td>Dose 0.1 mg/well</td>
<td>No significant internalization of CNC_FITC was noted</td>
<td></td>
</tr>
<tr>
<td>Surface are 150 m2/g</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CNCs studied for cytotoxicity and cellular uptake</td>
<td></td>
<td>No cytotoxicity detected</td>
<td></td>
</tr>
<tr>
<td>The possibly applications of the materials: cell imaging and drug delivery</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1) Nanofibrillar cellulose from pulp by grinding (Masuko Sangyo Ltd)</td>
<td>Cytotoxicity by total protein content and highest tolerated dose method</td>
<td>No cytotoxicity detected</td>
<td></td>
</tr>
<tr>
<td>Diameter 20-60 nm, with large fraction at 100-350 nm</td>
<td>24-72 h exposures</td>
<td>Mahmoud et al. 2010</td>
<td></td>
</tr>
<tr>
<td>Length up to several μm</td>
<td>Human keratinocytes, HaCaT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2) Arbocel MF40 by RettenmaierSöhne GmbH</td>
<td>Human cervix carcinoma cell line (HeLa229)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Whisker-like short fibrils</td>
<td>Mouse hepatoma cell line (Hepa1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Smallest fibrils 20-100 nm but also larger ones</td>
<td>Doses:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Length 400-500 nm</td>
<td>1) Up to 0.24 mg/ml</td>
<td>Pitkänen et al. 2010</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2) Up to 2 mg/ml</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
5.3.6 Reproductive and developmental toxicity
No data available in the open literature.

5.3.7 Genotoxicity

Only a few in vitro studies on the possible genotoxicity of nanocellulose fibres have been published so far (Table 20), and the mechanism of material penetration into the cells is not known. de Lima et al. (2012) examined the genotoxicity of cellulosic fibres derived from white, brown, ruby, green cotton, and curaua in plants cells (Allium cepa roots) and lymphocyte- and fibroblast cell cultures in vitro by using chromosomal aberration and the comet assays, as well as cytogenetic analyses. Comet assay was positive and DNA breaks were detected after exposing cells with 0.1-1\% brown cotton and curaua fibres. Mitotic index was significantly altered with 0.1\% curaua NFs.

Genotoxicity of bacterial cellulose (G. xylinus) NFs was studied by Moreira et al. (2009). The cellulose NFs were isolated from cellulose using combination of acid hydrolysis and sonication. Needle shaped fibres with 50-1500 nm length and 3-5 nm width were obtained. Salmonella reversion assay using suspension containing 0.1, 0.5 and 1.0 mg/ml NFs was negative. Same dosage in the comet assay using Chinese hamster ovary (CHO) cells elicited no DNA breaks after 48 h incubation. The authors report on slight reduction in proliferation assay (72 h) on CHO cells and 3T3 fibroblasts, but no alterations in cell morphology.

Pitkänen et al. (2010) tested the ability of nanocellulose to elicit mutations in vitro by Salmonella typ. Reverse mutation assay (Ames test). No mutations were detected with doses up to 1.2 mg/ml. Two wood based nanocelluloses, 1P Masuko MFC from birch pulp and Arbocel MF40 were used. The 1P Masuko NFC consist of long and curly fibrils with a high aspect ratio (length to diameter ratio). The size range of fibrils is wide: thickness varies from 20 to 1000 nm and fibril length up to several micrometers. The diameter of the smallest fibrils is around 20-60 nm. Arbocel MF40 consists of whisker-like fibrils. Both fibril size and morphology differ clearly from 1P Masuko NFC. The fibrils are shorter and needle like with a low aspect ratio. Most fibres have a diameter in the range of 20-100 nm. There are a lot of small fibrils but also some larger fibre fragments. The thickness of the thinnest fibrils is about 10 nm with a length up to 400-500 nm.

So far, no in vivo studies on genotoxic potential of nanocellulose exist in the open literature.
Table 20. *In vitro* genotoxicity studies on nanocelluloses.

<table>
<thead>
<tr>
<th>Material</th>
<th>Methods, endpoints, doses</th>
<th>Findings, comments</th>
<th>Reference</th>
</tr>
</thead>
</table>
| Average length, diameter, zeta potential, respectively as follows:  
White cotton: 135±50 nm, 14±4 nm, -31 mV; brown cotton: 140±45 nm, 11±3 nm, 26 mV;  
green cotton: 180±45 nm, 11±3 nm, 26 mV;  
ruby cotton: 130±25 nm, 11±3 nm, -25 mV;  
curaua: 80-170 nm, 6-10 nm, -30 mV | Whole blood lymphocytes (from Ficoll-Paque)  
3T3 cell mouse fibroblasts  
Cytogenetics, mitotic index, exposure with 0.1% nanofibres  
DNA analysis by agarose electrophoresis, exposure with 1% nanofibres  
Comet assay (perif. lymphocytes) with 0.1% nanofibres | Mitotic index signif. altered with curaua nanofibres  
DNA breaks with curaua and brown cotton fibres  
Comet assay positive using brown cotton and curaua fibres.  
Toxicity of nanofibres varies depending of the origin of the material | de Lima et al. 2012 |
| Bacterial cellulose (G. xylinus) nanofibres  
Needle shaped  
50-1500 nm length  
3-5 nm width  
Nanofibres were produced from bacterial cellulose by a combination of acid and ultrasonic treatment | Salmonella t. reverse mutation assay (Ames test)  
Proliferation test (MTT) using 3T3 fibroblasts and CHO cells (0.24, 48 and 72 h incubations)  
Comet assay using CHO cells (48 h incubation)  
Doses (applied in all tests): 0.1, 0.5 and 1 mg/ml | No evidence of genotoxicity  
Slight reduction in proliferation assay (72 h), but no alterations in cell morphology | Moreira et al. 2009 |
| 1) Nanofibrillar cellulose from pulp by grinding (Masuko Sangyo Ltd)  
Diameter 20-60 nm, with large fraction at 100-350 nm  
Length up to several μm  
2) Arbocel MF40 by RettenmaierSöhne GmbH  
Whisker-like short fibrils  
Smallest fibrils: 20-100 nm but also larger ones  
Length: 400-500 nm | *Salmonella typ. rev. mutation* assay, Ames test  
TA102 strain  
Doses:  
1) Up to 1.2 mg/ml  
2) Up to 10 mg/ml | No genotoxicity detected | Pitkänen et al. 2010 |
5.3.8 Carcinogenicity

No data available in the open literature.

5.3.9 Toxicological data on cellulose and other organic fibres

Relatively little is known about the hazards of inhaled cellulose and other organic fibres. In the study of Muhle et al. (1997) rats were exposed intratracheally to a single dose (2 mg/animal) of microcrystalline cellulose fibers (4.2 μm x 0.87 μm) or insulation material (7.6 μm x 0.50 μm). Six months later a slight interstitial fibrosis, fibre associated granulomata, alveolar histiocytosis, alveolar lipoproteinosis and alveolar cell hyperplasias were observed. Fibres were detected in the lungs one year after the exposure. The half-time of the crystalline cellulose fibres was estimated to 564 days. No half-time could be calculated for the fibres from the insulation material as the fibres were divided into very thin fibrils.

Milton et al. (1990) instilled cellulose dust in hamsters by a single dose of 0.75 mg/100 g body weight. Hamsters were killed after 8 weeks. Lungs of animals showed a significant number of granulomata and thickened interalveolar septae.

Hadley et al. (1992) studied dust from cellulose insulation material. Rats were exposed by inhalation for 28 days to 100, 500, and 2000 mg/m³ to the aerosol. Pulmonary pathology showing dose-related changes in severity was characterized by diffuse macrophage infiltration, microgranuloma formation, alveolitis and epithelial hyperplasia. Limited collagen deposition was noted in the peribronchiolar interstitium at the high dose level.

Cellulose-based materials have been reported to cause airway diseases. Moreover, there are studies suggesting that cellulose fibres have a long biopersistence (Kobayashi et al. 2004, Muhle et al. 1997).

It has also been shown that intraperitoneal injections (3 injections spaced at weekly intervals) of high doses of cellulose fibres can cause tumours (sarcomas) in the abdominal cavity of male Wistar rats (Cullen et al. 2002).

Dustiness (=tendency of dry materials to liberate dust in the air) of cellulose building insulation materials has been assessed. Cellulosic loose-fill materials were tested as very dusty (Breum et al. 2003).

The few publications of cellulose have reported adverse effects, however studies of chronic effects after exposure to low and realistic doses of inhaled cellulose are not available.

5.3.10 Conclusions

Fibre-shape, high-aspect ratio (length to diameter) and biopersistence are properties, which may play important role in the nanomaterial toxicity. Nanocellulose represents high-aspect nanomaterials, however, studies of the biopersistence of nanocellulose are not available at the moment.

The few studies on nanocellulose toxicity conducted so far suggest that the different types of nanocellulose materials can be slightly toxic in vitro but the effect is milder than the one caused by some MWCNTs and asbestos fibres. The two in vivo studies on acute toxicity in mice
after intraperitoneal injection did not detect adverse effect. Further studies, especially chronic inhalation studies, are necessary in order to make it possible to draw conclusions about the toxic potential of nanocellulose. Moreover, the toxicity studies of fibres should to be carried out in a case-by-case manner, since modifications on the material structure and surface may result in drastic changes in biological systems making generalization of toxicity results from one material to another impossible. Sophisticated in vitro models coupled with realistic exposure systems could in the future respond to the vast testing demands of new nanomaterials.

Toxicity testing is recommended to be applied early in the production process in order to design nanomaterials with low toxicity. An in vitro model in combination with an exposure system in order to mimic exposure to airborne cellulose nanocrystal has been established (Endes et al. 2013). The development and validation of such systems are needed since conducting animal studies may not be practically feasible in responding the testing demands of the new nanomaterials.
5.4 Nanoclays

5.4.1 Substance identification and introduction

Nanoclays are nanoparticles formed of layered mineral silicated and they are commonly blended with polymers to form nanocomposites. Nanoclays and polymer-layered nanocomposites are used in a wide range of applications, e.g., in the production of inks, paints, cosmetics, in water treatment applications, and food packaging products. As with other nanosized materials also nanoclays may have toxic effects which are not apparent in the bulk material. (Lordan et al. 2011)

The health effects of bentonite and kaolin clays, which are widely used in different industrial fields, have been fairly extensively examined. Bentonite is formed of highly colloidal and plastic clays composed mainly of montmorillonite. Kaoline is a mixture of different minerals with the main component being kaolinite. The large variability in composition of clay materials has, however been a challenge for the hazard characterization. The amount of crystalline silica, which is always present in clays, has often been the decisive factor in clay induced toxicity. As a summary, there is still very little information about the possible carcinogenicity or genotoxicity of bentonite and kaolin clays. Based on the available studies it has been shown that long term exposure to kaolin may lead to pneumoconiosis but that the potency is at least one order of magnitude less than quartz. Bentonite is likely to be less dangerous to humans than kaolin. (WHO 2005)

5.4.2 Acute toxicity

An acute oral toxicity test in Sprague-Dawley rats showed a low toxic potential (LD50 >5700 mg/kg bw) of nanosilicate platelets (NSP) (Li et al. 2010) (see Table 21). The NSPs with average dimensions of ca. 80x80x1 nm were derived from natural montmorillonite clay. Furthermore, assays on cytotoxicity and genotoxicity indicated low toxicity (see sections 4 and 7).

Warheit et al. (2010) examined the toxicity of one type of nanoclay (Sepiolite) by intratracheal instillation in male Crl:CD(SD) rats at doses of 1 mg/kg and 5 mg/kg. Neutrophilic inflammation occurred 24 hours post-exposure but the effects decreased in severity over time. Occasional multinucleate giant cell formation at 1 week, 5 weeks, and 3 months post-exposure was observed. No extrapulmonary target organ effects were observed at 3 months postexposure.

A limited number of studies have been reported with regard to toxicological effects related to Sepiolite exposures. In a long-term inhalation study reported by Wagner et al. (1987) groups of male and female rats were exposed by inhalation (10mg/m³) to Sepiolite commercial product dust for 12 months. The dimensions of the fibres were reported to be approximately <6 μm in length and < 0.5 μm in diameter. UICC crocidolite asbestos fibres were used as positive control fibres. Histopathological evaluation of lung tissue in Sepiolite-exposed rats revealed an early interstitial reaction at 12 months post exposure. Only one rat developed a lung tumour, however, a similar finding of one lung carcinoma was reported in the positive control group exposed to crocidolite asbestos fibres.

In a study on Sepiolite workers, McConnochie et al. (1993) evaluated the fibre characteristics, chest radiographs and pulmonary function status in a cross-sectional study of the total
workforce of a large Sepiolite production plan located near Madrid, Spain. Based upon personal monitoring devices, the reported size distribution data indicated the ~88% of measured airborne Sepiolite fibers were less than 3 μm in length; with ~12%>3 μm. In conducting exposure assessments, rather high aerosol concentrations (up to 18.5 mg/m\(^3\)) were measured in the bagging department. However, the authors indicated that respirators were usually worn by the exposed workers. In addition, the investigators reported that Sepiolite-exposed workers had significantly reduced pulmonary function indices, i.e., decreased FEV1 and FVC values, when compared with workers who had had little exposure to dusts. Chest radiograph analysis indicated no clear patterns. It was concluded that decrements in pulmonary function correlated with dust exposures.

Herrera et al. (1995) implanted Sepiolite-collagen complexes into rat osseous tissue in order to stimulate bone growth. The reaction produced by the Sepiolite-collagen complex stimulated a foreign body reaction with abundant macrophages and giant cells. The investigators reported, however, that subsequent to implantation and contact with bone tissue, the Sepiolite complex did not produce any toxic effect or necrosis, thus facilitating bone activity.

According to IARC, there is inadequate evidence in humans for the carcinogenicity of Sepiolite clays (IARC, 1997).
**Table 21. Acute toxicity studies on nanoclays.**

<table>
<thead>
<tr>
<th>Material</th>
<th>Methods / endpoints / doses</th>
<th>Findings / comments</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nanosilicate platelets derived from natural montmorillonite</td>
<td>Fourteen-day oral toxicity study Sprague-Dawley rats Doses: 1500 mg/kg 3000 mg/kg 5700 mg/kg LD$_{50}$ assessed according to the mortality and the toxicity symptoms</td>
<td>No mortalities, clinical signs or macroscopic changes. No changes in the body weight or food consumption. ⇒ LD$_{50}$ acute oral toxicity more than 5700 mg/kg</td>
<td>Li et al. 2010</td>
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<tr>
<td>Sepiolite S-9 nanoclay (natural clay from a mined sample, which processed. Quartz content &lt;1%, 40nm x100-300 nm, Surface area 334 m$^2$/g) Coated and uncoated</td>
<td>Intratracheal instillation Crl:CD(SD) rats Samples: Sepolite S-9 nanoclay, UF TiO$_2$, quartz Doses: 1 mg/kg and 5 mg/kg BALF analysis (LDH, microprotein, ALP) Cell proliferation test 1 w, 1 and 3 months after instillation Morphological/histopathological analysis from alveolar and bronchial cells 24 h 1 w, 1 m and 3 m after instillation Coated form not tested for tissue morphology</td>
<td>Both uncoated and coated nanoclays produced significant neutrophilic response response 24 h (reversible at 1 w). In contrast, quartz particles produced sustained neutrophilic pulmonary inflammatory response. Both types produced enhanced numbers of multinucleated macrophage-agglomerates at 24 h in BALF. Giant cells observed at 1w, 5 w and 3 m Increased LDH in BALF with 5 mg/kg, at 24 h Increased BALF protein values with 5 mg/kg, 24 h</td>
<td>Warheit et al. 2010</td>
</tr>
</tbody>
</table>
5.4.3 Irritation and sensitization
No data available in the open literature.

5.4.4 Repeated dose toxicity
No data available in the open literature.

5.4.5 In vitro studies on cytotoxicity
Table 22 summarises the identified in vitro studies on the cytotoxicity of nanoclays. The toxic effects of unmodified nanoclay (Cloisite Na+®; mainly tactoid structures with lengths between 30–100 µm) and organically modified nanoclay (Cloisite 93A® length 3–35 µm) were studied in human hepatoma HepG2 cells in vitro using doses of 1, 5, 10, 50, 100, 500, 1000 µg/ml. In MTT assay significant decrease in viable cells at conc. ≥1 µg/ml with both clays was observed. Moreover, significant LDH release with ≥ 50 µg/ml (both clays) was reported. Concentrations ≥50 µg/ml of Cloisite Na+ induced ROS formation, while Cloisite 93A had little effect on ROS levels.

Nanoclays have been used in the production of bio-nanocomposites where chitin based polyurethane (PU) bio-nanocomposites are prepared using chitin, Delite HPS bentonite nanoclay enriched in montmorillonite, 4,4’-diphenylmethane disocyanate and polycaprolactone polyl CAPA 231. The effect of nanoclay contents on mechanical properties and in vitro biocompatibility was investigated (Zia et al. 2011). Cytotoxicity evaluation using L-929 fibroblasts revealed that in case of the final PU bio-nanocomposite, having 2% Delite HPS bentonite nanoclay contents, increasing the amount of nanoclay also increased the cytotoxicity in fibroblasts.

Lordan and Higginbotham (2012) reported that addition of 2.5% or 5% foetal calf serum (FCS) in the dispersion medium of unmodified nanoclay (Cloisite Na+) can influence the outcome of toxicity studies. Clay particles in medium without FCS or supplemented with 2.5% FCS significantly inhibited cell growth in human monocytic U937 cells, while medium with 5% FCS had little effect on cell growth. In media supplemented with FCS, the nanoclay agglomerated together to form bundles, whereas they were evenly dispersed throughout the medium in the absence of serum. They conclude that serum supplementation is an important consideration in the toxicological assessments.

Cytotoxicity of nanosilicate platelets (NSP) derived from natural montmorillonite clay was assessed by the standard MTT and LDH assays using doses of 62.5, 125, 250, 500, 1000 µg/ml (Li et al. 2010). The tests showed a low cytotoxicity on CHO cells below 1000 µg/ml after 12 h incubation period and a dose-dependent effect after 24 incubation. The cell culture medium was supplemented with 10% FBS.
Table 22. *In vitro* cytotoxicity studies on nanoclays.

<table>
<thead>
<tr>
<th>Material</th>
<th>Methods, endpoints, doses</th>
<th>Findings, comments</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cloisite® Na⁺</td>
<td>Human monocytes U937 Fluorochrome-mediated viability assay by Strauss (1991) 0, 2, 5, 5% FCS RPMI medium Exposure: 0, 24, 48 h Dose: 1 mg/ml</td>
<td>The article reports the effect of serum on cytotoxicity of nanoclay particles. After 48 h, cell growth in 2.5% FCS was signif. inhibited by test material, while no effect was seen with 5% FCS Without serum cell growth was minimal and exposure to 1 mg/ml nanoclay completely diminished the cells Cell membrane integrity was intact in the presence of 2.5 and 5% FCS</td>
<td>Lordan and Higginbotham 2012</td>
</tr>
<tr>
<td>Cloisite® Na⁺</td>
<td>Human hepatoma HepG2 cells in DMEM 2.5% FBS MTT assay LDH release ROS Caspase 3/7 assay Light microscopy Exposure: 24 h Doses: 1, 5, 10, 50, 100, 500, 1000 μg/ml</td>
<td>MTT: Signif. decrease in viable cells at conc. ≥1 μg/ml with both clays LDH: Signif. LDH release with ≥ 50 μg/ml (both clays) ROS: Concentrations ≥50 ug/ml of Cloisite Na⁺ had effect on ROS production The effect of Cloisite 93A was less prominent</td>
<td>Lordan et al. 2011</td>
</tr>
<tr>
<td>Chitin based polyurethane biobionanocomposites (PUBNC) (bentonite nanoclay 0.0-8.0% by mass)</td>
<td>L-929 fibroblast cells Biocompatibility studied The cytotoxicity of the degradation products were measured by live/dead cell staining</td>
<td>Biocompatibility was best with material with 2% bentonite The material with highest bentonite content showed highest cytotoxicity</td>
<td>Zia et al. 2011</td>
</tr>
<tr>
<td>Nanosilicate platelets derived from natural montmorillonite Size on average: 80x80x1 nm Polygonal shape Cationic exchange capacity 120 mequiv/100g</td>
<td>Chinese hamster ovary (CHO) cells Culture medium supplemented with 10% FBS MTT assay 24 h Doses: 62.5, 125, 250, 500, 1000 μg/ml 3, 12, 24 h LDH assay Doses: 62.5, 125, 250, 500, 1000 μg/ml 24 h</td>
<td>In MTT assay IC₅₀ was larger than 1000 μg/ml LDH assay with highest dose (1000 μg/ml) showed 40% increase in LDH levels</td>
<td>Li et al. (2010)</td>
</tr>
</tbody>
</table>
5.4.6 Reproductive and developmental toxicity

No data available in the open literature.

5.4.7 Genotoxicity

Unmodified nanoclay (Cloisite Na+) and organo-modified nanoclay (Cloisite® 30B) exposure did not induce mutations as measured by the Ames test or ROS production in a cell-free test system (Sharma et al. 2010) (Table 23). However, the unfiltered and filtered (particles above nanometre range removed) Cloisite® 30B particles induced DNA strand breaks in a dose-dependent manner in Caco-2 (human colon cancer) cells after 24 hours of exposure. The conclusion of the study was that the DNA damage observed was caused by the organo-modifier and not by the particles themself.

The genotoxic potential of nanosilicate platelets (NSP) derived from natural montmorillonite clay was addressed in vitro and in vivo (Li et al. 2010). The comet assay showed no DNA damage when Chinese hamster ovary (CHO) cells were exposed to 62.5, 125, 250, 500, 1000 μg/ml of the material. The cell culture medium was supplemented with 10% fetal bovine serum (FBS). No significant micronucleus induction were observed in peripheral blood polychromatic erythrocytes in ICR mice after oral exposure (20, 200, 500 mg/kg). No mutations by the Salmonella gene mutation assay were detected. Based on the cytotoxicity studies of Lordan and Higginbothman (see section 7), the serum content above 2.5% in the cell culture may mask adverse effects. Thus, future studies and recommendations on the in vitro testing conditions are needed before proper interpretation on in vitro genotoxicity studies of nanoclays can be made.

In conclusion, the data on genotoxicity of nanoclay are far too limited to draw conclusions.
Table 23. Genotoxicity studies on nanoclays.

<table>
<thead>
<tr>
<th>Material</th>
<th>Methods, endpoints, doses</th>
<th>Findings, comments</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1) Cloisite® Na (a natural montmorillonite)</td>
<td>Caco-2 cells (a human colon-cancer cell line) DMEM 10% FBS Cytotoxicity by Alamar blue assay Doses: 14, 28, 56, 85, 100, 113, 140, 170, 226 μg/ml Comet assay Doses: 56.5, 85, 113, 170 μg/ml Salmonella/microsome assay (OECD 471) with TA 98 and TA100 strains, with and without a metabolic activation system ROS Doses: 0, 14, 28, 57, 113, 226 μg/ml</td>
<td>Cloisite 30B induced about 40% cytotoxicity at 226 μg/ml 1) Cloisite Na (unfiltered and filtered): negative 2) Cloisite 30B(unfiltered and filtered): concentration dependent production of DNA strand breaks Salmonella/microsome assay and ROS: Both particles, unfiltered and filtered did not cause mutations or ROS formation Cloisite 30B: In the filtered samples quaternary ammonium modifier was detected by no traces of Al (=&gt; no clay), thus the genotoxicity of the material in comet assay is likely to be caused by the modifier</td>
<td>Sharma et al. 2010</td>
</tr>
<tr>
<td>2) Cloisite® 30B (a natural montmorillonite, modified with a quaternary ammonium)</td>
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<tr>
<td>Nanosilicate platelets derived from natural montmorillonite</td>
<td>Chinese hamster ovary (CHO) cells Culture medium supplemented with 10% FBS Comet assay Doses: 62.5, 125, 250, 500, 1000 μg/ml MTT assay 24 h Doses: 62.5, 125, 250, 500, 1000 μg/ml 3, 12, 24 h LDH assay Doses: 62.5, 125, 250, 500, 1000 μg/ml 24 h Ames test using TA98, TA100, TA1535, TA1537, TA102 strains Doses: 62.5, 125, 250, 500, 1000 μg/plate</td>
<td>In vitro and in vivo genotoxicity tests were negative. In MTT assay IC₅₀ was larger than 1000 μg/ml LDH assay with highest dose (1000 μg/ml) showed 40% increase in LDH levels</td>
<td>Li et al. (2010)</td>
</tr>
</tbody>
</table>

Nanosilicate platelets derived from natural montmorillonite Size on average: 80x80x 1 nm Polygonal shape Cationic exchange capacity 120 mequiv/100g

In vivo micronucleus assay ICR mice Oral dose: 20, 200, 500 mg/kg Peripheral-blood cells collected after 24 and 48 h Mitomycin C as positive control
5.4.8 Carcinogenicity

No data available in the open literature.

5.4.9 Conclusions

So far, there are only very limited data available on the potential toxicity of nanoclays. As many nanoclays are modified to form nanocomposites, it seems important to elucidate if the modifiers are causing the toxic events or if the nanoclay itself can induce harmful effects. Moreover, the variations in the in vitro testing conditions (cell lines, cell culture conditions) makes it is impossible to draw conclusion on the cytotoxicity or genotoxicity. The results of Lordan and Higginbotham on the effects of serum concentrations on particle behaviour should be considered in the future in vitro toxicity studies on nanoclays. In vivo studies intending to investigate pulmonary toxicity of nanoclays are so far limited to one study, in which intratracheal instillation of the material produced transient short-term neutrophilic responses and occasional multinucleate giant cell formation up to 3 months post-exposure.
5.5 Carbon nanofibres

5.5.1 Substance identification and introduction

Carbon nanofibres (CNFs) typically have a diameter of 50–200 nm and structurally they resemble multi-walled carbon nanotubes (MWCNTs). The primary characteristic that makes them different from carbon nanotubes (CNTs) is the graphene alignment – if the graphene plane and fibre axis do not align, the structure is defined as a CNF. It is less expensive to produce CNFs as compared to CNTs and they are used, for example, in composite materials to improve strength, stiffness, electrical conductivity, or heat resistance. (Kisin et al. 2011)

The widespread use of synthetic fibrous carbon materials, including single- (SWCNT) and multi-wall carbon nanotubes, has raised safety concerns about their possible health effects. Especially CNTs with a high aspect ratio (length:thickness >3:1), consisting of long fibres (>20 μm) are of particular concern, since they might be able to induce lung cancer and mesothelioma in a manner similar to asbestos fibres (Linton et al. 2012). The mechanism by which carbon nanofibres, including CNTs, could cause inflammation and ultimately cancer is, however, largely unknown. The reason for this is partially the large variability in the physico-chemical characteristics of the materials, including their length, diameter, surface area, density, shape, contaminant metals, and crystallinity. (Nagai and Toyokuni, 2010).

The toxicity of different types of CNTs has been studied fairly extensively during the past few years by using both in vitro cell line and in vivo animal test systems. Although the toxic potential of CNTs has been demonstrated, the results are sometimes conflicting and inconclusive and therefore more relevant data, especially on long-term effects, are still needed for a better hazard and exposure assessment. The main mechanism of CNT toxicity appears to be oxidative stress and inflammation, which can ultimately lead to genotoxic and carcinogenic responses. (Aschberger et al. 2010)

In the Scaffold project, CNTs were not specifically addressed as one of the focus areas. Instead, CNFs were selected as vital for the project. As the border between CNTs and CNFs is not always very clear, and as most toxicological tests have been carried out with CNTs, and not CNFs, section 6.5.9 summarizes very briefly the main observations and trends observed in the large amount of scientific data published on the potential hazards related to exposure to CNTs. The data on CNFs specifically are presented in the present chapter.

5.5.2 Acute toxicity

No data available in the open literature.

5.5.3 Irritation and sensitisation

No data available in the open literature.

5.5.4 Repeated dose toxicity

A subchronic inhalation toxicity study of inhaled vapour grown carbon nanofibres (CNF) (VGCF-H) was conducted by DeLorme et al. (2012). The CNF test sample was composed of >99.5% carbon with virtually no catalyst metals, and BET surface area measurements of 13.8 m²/g and mean lengths 5.8 μm and 158 nm, respectively. The design of the study was based on the
OECD 413 testing guideline. Male and female Sprague Dawley rats were exposed nose-only (6 h/day, for 5 days/week) to 0, 0.54, 2.5, or 25 mg/m$^3$ VGCF-H over a 90-day period and evaluated 1 day later. In addition, groups of 0 and 25 mg/m$^3$ exposed rats were evaluated at 3 months post exposure.

The wet lung weights were significantly elevated compared to controls in male rats at 25 mg/m$^3$ and in female rats at 2.5 and 25 mg CNF at 1-day post-exposure; lung weights remained elevated in each sex in the 25 mg/m$^3$ exposure group, and interstitial thickening with type II pneumocyte proliferation in the 25 mg/m$^3$ exposure group. Histopathologic evidence of inflammation and the presence of fibre-laden macrophages were reported to be reduced but still present in the high dose group after a 3 months recovery period.

Inflammation within the alveolar space (as measured by PMN levels in BALF) was statistically significant only in the rats exposed to 25 mg/m$^3$ CNF. However, the number (%) of PMNs increased in a dose-responsive manner: 1.2 (±0.81), 1.4 (±0.79), 2.7 (±0.67), and 11 (±2.0), respectively in the 0, 0.54, 2.5, and 25 mg/m$^3$ exposure groups. LDH and other BALF markers were elevated at the end of the 13-wk exposure only in the 25 mg/m$^3$ exposure group, and LDH remained elevated at 3 months post-exposure in that group. The aerosol exposures induced concentration-related small, detectable accumulation of extra-pulmonary fibres with no adverse tissue effects. The NOAEL for VGCF-H nanofibres was considered to be 0.54 mg/m$^3$ (4.9 fibres/cc). The lowest observed adverse effect level (LOAEL) was reported to be 2.5 mg/m$^3$ based on small increase in female lung weights (>15% vs. controls), and histopathological assessment of minimal inflammation of the terminal bronchiole and alveolar duct areas (DeLorme et al. 2012).

Murray et al. (2012) performed a comparative study assessing pulmonary inflammation, fibrosis, and systemic immune responses to carbonaceous fibrous nanomaterials and asbestos. C57BL/6 mice were exposed by a single pharyngeal aspiration to respirable CNFs (120 µg/mouse), SWCNTs (40 µg/mouse) or asbestos (120 µg/mouse) and groups of mice were sacrificed on days 1, 7, and 28 post-exposure. Polymorphonuclear lymphocyte (PMN) accumulation in CNF-exposed mice vs. controls was 150-fold on day 1. By day 28 post exposure, PMNs in BALF of CNF exposed mice had decreased to 25-fold vs. controls. Additional lung effects included increased lung permeability (elevated total protein in BALF), cytotoxicity (elevated lactate dehydrogenase, LDH), which remained significantly elevated compared to controls at day 28 post-exposure. Oxidative damage was significantly elevated at days 1 and 7, but not at day 28. Collagen accumulation at day 28 post-exposure was 3-fold higher in CNF-exposed mice vs. controls by biochemical measurements. Consistent with the biochemical changes, morphometric measurement of Sirius red-positive type I and III collagen in alveolar walls (septa) was significantly greater than controls at day 28 post-exposure. In CNF-exposed mice, T cell mitogen (concavalin A) responsiveness indicated decrease of T cell responses in the spleen. Interstitial fibrosis was increased 28 days post SWCNT, CNF or asbestos exposure (mass-based potency SWCNT>CNF>asbestos). The study found a correlation with the calculated effective surface area of particle agglomerates with protein levels in BAL fluids of nanomaterial exposed mice.
In the study of Yokoyama et al. (2005), so called hat-stacked CNFs (length 100 nm-1 μm, diameter 30-100 nm) were implanted in the subcutaneous tissue of Wistar rats. The rats were sacrificed at 1 and 4 weeks after implantation (Yokoyama et al. 2005). At 1 week, clusters of CNFs were surrounded by granulation tissue with a slight inflammatory change. Many mesenchymal cells, macrophages, and foreign body giant cells were observed around CNFs. At 4 weeks, clusters of CNFs were surrounded by fibrous connective tissue. No necrosis, degeneration, or neutrophil infiltration was observed.

Currently, there are no studies reported in the literature on the adverse health effects in workers producing or using CNF. However, because humans can also develop lung inflammation and fibrosis in response to inhaled particles and fibres, it is reasonable to assume that at equivalent exposures (e.g., lung burden/alveolar epithelial cell surface) to CNF, workers may also be at risk of developing these adverse effects (NIOSH 2013).

5.5.5  In vitro studies on cytotoxicity

Limited studies have evaluated the toxicity of different CNFs (Table 24). The cytotoxic potential of carbon-based nanofibres (Pyrograf(R)-III) were examined in lung fibroblast (V79) cells and a comparison was made with asbestos fibres (crocidolite) and SWCNTs (Kisin et al. 2011). A concentration- and time-dependent loss of V79 cell viability after exposure to all tested materials in the following order was seen: asbestos>CNF>SWCNT. Also cellular uptake and generation of oxygen radicals was seen in the murine RAW264.7 macrophages following exposure to CNF or asbestos but not after administration of SWCNT.

A similar finding has been reported by Price et al. (2004) providing evidence of time-and dose-dependent CNF cytotoxicity using human osteoblast CRL-11372 cells. Moreover, CNFs were seen to decrease the viability of H596 lung tumour cells in dose-dependent manner (Margrez et al. 2006). This work indicated a stronger toxicity of CNFs as compared to MWCNTs. In contrast, Grabinski et al. (2007) reported that CNF exposure did not significantly affect cell viability or increase ROS production in mouse keratinocytes (HEL-30) when compared to SWCNTs and MWCNTs.

Two types of CNFs (NF1, platelet and herringbone structure; NF2, platelet structure) did not increase toxicity in human peripheral mononuclear cells isolated from healthy donors as measured by the lactate dehydrogenase (LDH) assay nor did they induce apoptosis or necrosis in THP-1 (human acute monocytic leukemia cell line) cells (Brown et al. 2007). However, the NF1 appeared to increase O-2 production in the mononuclear cells and inhibited the phagocytosis capacity of THP-1 cells suggesting that the toxicity of CNFs may depend on their graphene structure.
Table 24. *In vitro* studies on the cytotoxicity of CNF.

<table>
<thead>
<tr>
<th>Material</th>
<th>Methods, endpoints, doses</th>
<th>Findings, comments</th>
<th>Reference</th>
</tr>
</thead>
</table>
| CNFs, from Pyrograf Products Inc.  
Iron content: 1.4%  
Surface area: 35-45 m$^2$/g  
Diameter: 60-150 nm  
Aspect ratio: 500  
SWNT  
Asbestos (crocidolite) | V79 cells, lung fibroblasts  
Cytotoxicity by Trypan blue staining  
Dosage: 0,3,12, 48 μg/cm$^2$  
3 and 24 h incub. | A concentration- and time-dependent loss of V79 cell viability in the following order: asbestos>CNF>SWCNT | Kisin et al. 2011 |
| Carbon nanofibres from Applied Sciences, Inc.  
Width 60-200 nm  
Lenght (agglomerated particles) 5346-8819 nm | Human osteoblasts, CRL-11372  
10% FBS  
Molecular Probes cell viability assay, fluorescence detection  
Doses 0.000558 -0.00558 g/100 ml, incub. 3, 6, 11 and 24 h | Time and dose-dependent cytotoxicity | Price et al. 2004 |
| Carbon nanofibres from Pyrograf Products Inc.  
Diameter 150 nm  
Aspect ratio 30-40  
Carbon black  
MWCNTs | H596 lung tumor cells  
Doses: 0.002-0.2 μg/ml  
Incubation time: 1-5 days  
MTT assay | Dose-dependent cytotoxicity  
Toxicity order: carbon black>CNFs>MWCNTs | Margez et al. 2006 |
| CNFs produced through the pyrolysis  
Diameter 100 nm  
MWCNTs  
SWCNTs  
CFs | Mouse keratinocytes, HEL-30  
Doses: 5,10,25,50 μg/ml  
Incubations 12-72 h  
MTT assay  
ROS | CNFs and CFs were not cytotoxic  
MWCNTs and SWCNTs reduced cell viability in dose-dependent manner  
CNFs did not increase ROS production | Grabinski et al. 2007 |
| CNFs -NF1, platelet and herringbone | Human mononuclear cells  
THP-1 (human acute monocytic leukemia cell line) | No cytotoxicity  
No apoptosis or necrosis detected | Brown et al. 2007 |
<table>
<thead>
<tr>
<th>structure</th>
<th>Diameter: 50-150 nm</th>
<th>Surface area 144 m²/g</th>
<th>Ni content: 0.3 wt%</th>
</tr>
</thead>
<tbody>
<tr>
<td>-NF2, platelet structure</td>
<td>Diameter: 50-150 nm</td>
<td>Surface area 88 m²/g</td>
<td>Ni content: 0.4 wt%</td>
</tr>
<tr>
<td>LDH assay</td>
<td>Doses: 15, 31, 63, 125 μg/ml</td>
<td>4h incubation</td>
<td>TNF-α</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Apoptosis</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Necrosis</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Phagocytosis</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>O₂ production</td>
</tr>
<tr>
<td>O₂ production in the mononuclear cells detected</td>
<td></td>
<td></td>
<td>Phagocytosis capacity of THP-1 cells detected</td>
</tr>
</tbody>
</table>
5.5.6 Reproductive toxicity
No data available in the open literature.

5.5.7 Genotoxicity
Kisin et al. (2011) studied the genotoxic potential of carbon-based nanofibres (Pyrograf(R)-III) in lung fibroblast (V79) cells and a comparison was made with asbestos fibres (crocidolite) and SWCNTs.

DNA damage was found after exposure to all tested materials. Moreover, exposure with 12 μg/cm² showed significant induction of MN. Fluorescence in situ hybridization analysis in SAECs indicated that MN formed upon CNF exposure comprised both aneugenic and clastogenic events. A generation of oxygen radicals was seen in the murine RAW264.7 macrophages. These results are suggesting that CNFs may cause genotoxicity via two mechanisms: first by production of ROS and second by interfering physically with DNA/chromosomes and/or mitotic apparatus. Different amounts of surface iron on the nanofibres could be correlated with their propensity to trigger ROS production.

5.5.8 Carcinogenicity
No data available in the open literature.

5.5.9 Brief overview of the hazards related to carbon nanotubes
The toxicity of different types of CNTs has been studied fairly extensively during the past few years by using both in vitro cell line and in vivo animal test systems, due to the fact that it has been assumed that they may behave similarly as asbestos fibres. Although the toxic potential of CNTs has been demonstrated, the results are sometimes conflicting and inconclusive and therefore more relevant data, especially on long-term effects, are still needed for a better hazard and exposure assessment. However, there are indeed clear indications that some CNT materials may induce pulmonary inflammation and may be genotoxic and carcinogenic.

Several in vivo studies with rats or mice exposed to MWCNTs and SWCNTs by short- and long-term inhalation, or intratracheal instillation, have shown a significant ability to cause inflammation, fibrosis, granuloma formation, and immunosuppression after acute, sub-acute and sub-chronic exposure (Lam et al. 2004; Muller et al. 2005; Shvedova et al. 2005; Li et al. 2007; Mitchell et al. 2007; Nygaard et al. 2009).

However, there are also numbers of in vivo studies indicating a low toxicity of CNTs (Fiorito et al. 2006; Davoren et al. 2007; Pulska et al. 2007; Muller et al. 2009). The differences in the test results are most likely dependent on variations in the structural properties of the tested CNTs.

For the assessment of the carcinogenic hazard of CNTs, the evaluation of the genotoxic potential of the materials is of crucial importance. The main modes of action of CNTs have been proposed to be related to generation of reactive oxygen species (ROS), generated by the particles themselves upon particle-cell contact, or due to particle-elicited inflammation or mechanical interference with cellular components (Gonzalez et al. 2008; Donaldson et al. 2010).

One important study demonstrating the possible carcinogenic capacity of long MWCNTs (MITSUI MWCNT-7) was carried out by Takagi et al. (2008) showing that a single...
An intraperitoneal injection in p53+/− mice induced mesothelioma even more effectively than crocidolite asbestos. The same type of MWCNTs have also been shown to activate the NLRP3 inflammasome in a similar manner as asbestos (Palomäki et al. 2011) and induce mesothelioma in p53+/− mice in a dose-dependent manner by so-called frustrated phagocytosis (Takagi et al. 2012).

Another study examining the exposure of the mesothelial lining of the body cavity of C57BL/6 mice to long MWCNTs, which contained a substantial proportion of fibres longer than 20 μm, also resulted in an asbestos-like, length-dependent, pathogenic behaviour (Poland et al. 2008). However, no carcinogenic response was detected in rats after a single intraperitoneal injection of another type of MWCNTs (diameter 11.3 nm, length 0.7 μm). The authors suggested that the lack of response could be due to insufficient sustainability of the inflammatory reaction in the peritoneal cavity or that the MWCNTs used did not contain a sufficient number of long nanotubes (Muller et al. 2009).

5.5.10 Conclusions

CNFs have been studied for their potential health hazards only in a limited number of studies. Based on the few published study reports, there are indications that CNFs may be able to induce inflammatory and fibrotic effects in the lungs of exposed animals. There are also indications that some CNFs may cause genotoxicity.

More toxicological data, also in vivo inhalation studies, are available on CNTs. Some types of CNTs are likely to induce inflammatory and fibrotic effects and may even be genotoxic and carcinogenic, whereas other CNTs have not shown such effects. Due to the pulmonary inflammation and interstitial fibrosis observed in animals exposed to CNF, and due to physical-chemical similarities between CNT and CNF, the hazard profiles of CNTs are considered to be relevant also for the risk assessment of CNFs.
6 CONCLUSIONS

Of the materials being in the focus of the Scaffold project, extensive data is available on the toxic potential of silicon dioxide (amorphous silica), most likely due to the fact that it has already been used for decades.

The main concerns related to exposure to amorphous silica particles are their possible lung effects. Repeated dose inhalation toxicity studies with synthetic amorphous silica have been performed, indicating hazardous effects such as chronic inflammation and fibrotic lesions. It should, however, be noted that the inflammatory effects induced by silica seem to be reversible upon the cessation of exposure, and as opposed to the persistent lesions caused by crystalline silica. Limited data are available on the carcinogenicity of amorphous silica. In one study, a statistically significant tumour response was observed after repeated intratracheal administration of amorphous silica particles in rats. Tumour responses correlated with inflammatory responses in lungs and mechanisms related to lung overloading may have played a role in the tumour response. Most of the genotoxicity studies on amorphous silica particles show negative results.

Nano-sized titanium dioxide (TiO$_2$) has during the last ten years been intensively studied, and a lot of research is still going on. There is evidence that nano-sized TiO$_2$ is considerably more toxic than micro-sized TiO$_2$. Among the TiO$_2$ induced adverse effects, respiratory tract is considered the most critical site. The pulmonary responses to TiO$_2$ include inflammation, epithelial damage, increased permeability of the lung epithelium, oxidative stress and cytotoxicity, and morphological alterations within the lungs. Despite the increased production and use of TiO$_2$ nanoparticles the epidemiological data are lacking.

The genotoxicity of TiO$_2$ nanoparticles is thought to be driven by particle mediated reactive oxygen species production. The particles themselves are not thought to be inherently genotoxic, but may trigger genotoxicity via an indirect threshold-driven inflammatory mechanism involving oxidative stress.

Dermal studies have shown little evidence for skin penetration of nano-TiO$_2$. However, there may be a risk associated with nano-TiO$_2$ applied to damaged skin. Long-term TiO$_2$ inhalation studies in rats have reported lung tumours. Oxidative stress, induced by ROS generation, may play an important role in the initiation and progression of carcinogenesis of TiO$_2$ nanoparticles.

The few studies on nanocellulose toxicity conducted so far suggest that the different types of nanocellulose materials can be slightly toxic in vitro but the effect is milder than the one caused by some types of MWCNTs and asbestos fibres. The two in vivo studies on acute toxicity in mice after intraperitoneal injection did not detect adverse effect. Further studies, especially repeated dose inhalation studies, would bring important new information, facilitating conclusion making about the toxic potential of nanocellulose.

So far, there are only very limited data available on the potential toxicity of nanoclays. As many nanoclays are modified to form nanocomposites, it seems important to elucidate whether the modifiers are causing the toxic events or if the nanoclay itself can induce harmful effects. Moreover, the variations in the in vitro testing conditions (cell lines, cell culture
conditions) of the currently published studies makes it is impossible to draw conclusion on the cytotoxicity or genotoxicity. In vivo studies intending to investigate pulmonary toxicity of nanoclays are so far limited to one study, in which intratracheal instillation of the material produced transient short-term neutrophilic responses and occasional multinucleate giant cell formation up to 3 months post-exposure.

Information on the potential health hazards of carbon nanofibres (CNFs) have only been published in a few studies. Based on the few published study reports, there are indications that CNFs may be able to induce inflammatory and fibrotic effects in the lungs of exposed animals. There are also indications that some CNFs may cause genotoxicity.

More toxicological data, including also in vivo inhalation studies, is available for different types of carbon nanotubes (CNTs). Carbon nanotubes are being intensively studied for their toxicity, due to the theory that they may behave in an asbestos-like manner. There are, however, a lot of different types of carbon nanotubes at the market, and it appears that the toxic effects may vary a lot depending on which type of material has been tested. Anyhow, it seems to be proven that some carbon nanotubes may induce lung inflammation, genotoxicity and carcinogenicity. In some studies, carbon nanotubes have even been shown as more potent than asbestos. Due to the physico-chemical similarities between CNFs and CNTs and the hazardous effects of some CNTs, toxicological data on CNTs is considered to be relevant for the assessment of the hazards of CNFs.

The assessment of the toxicological potential of nanocellulose, nanoclays and carbon nanofibres is complicated by the fact that these are not single substances, but heterogenous groups of materials. At the moment, it seems like the toxicity studies have to be carried out in a case-by-case manner, since modifications on the material structure and surface may result in drastic changes in biological systems making generalization of toxicity results from one material to another impossible. Sophisticated in vitro models coupled with realistic exposure systems could in the future respond to the vast testing demands of new nanomaterials.
7 REFERENCES

7.1 Silicon dioxide (amorphous silica)


EFSA (2009) Calcium silicate and silicon dioxide/silicic acid gel added for nutritional purposes to food supplements 1 Scientific Opinion of the Panel on Food Additives and Nutrient Sources added to Food. The EFSA Journal 1132, 1–24.


human lung carcinoma cells and amorphous monodisperse silica nanoparticles as models. Nanotoxicology 4, 382–395.


7.2 Titanium dioxide


IARC (2006). Overall evaluations of carcinogenicity to humans, Group 2B: Possibly carcinogenic to humans. IARC Monogr 93(1-5).


NANODERM Quality of skin as a barrier to ultra-fine particles. QLK4-CT-2002-02678 Final Report.


Yazdi AS, Guarda G, Riteu N, Drexler SK, Tardivel A, Coullin I, Tschopp J (2010) Nanoparticles activate the NLR pyrin domain containing 3 ((Nlrp3) inflammasome and cause pulmonary inflammation through release of IL-1

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