Review of fullerene toxicity and exposure – Appraisal of a human health risk assessment, based on open literature

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1. Introduction

Fullerenes, first discovered by Kroto et al. (1985), are carbon allotropes similar in structure to graphene but rolled up to form closed-cage, hollow spheres. The C_{60} fullerene is a remarkably stable compound consisting of 60 carbon atoms with a diameter of approximately 0.7 nm and a molecular weight of 720 g/mol. Thirty carbon double bonds are present in the structure, to which free radicals can easily be added. C_{60} fullerene is therefore also described as a “radical sponge” (for recent reviews, see Nielsen et al., 2008; Johnston et al., 2010).

Fullerenes, like other nanoparticles have properties, which make them interesting for exploitation within technological and medical applications, including their small size, large surface area and high reactivity. However, some of them (with surface modification) may also have increased biological activity due to the favourable presentation of active moieties on the surface of the particle.

Fullerenes often aggregate into larger particles, so in reality they may exist as crystals much larger than 100 nm. A number of different fullerene derivates are available, resulting from the different number of carbon atoms (e.g. C_{70}, C_{80}, C_{90}) used to generate the fullerenes. The diverse array of moieties that can be attached to the fullerene surface, and the various preparation processes which can be utilised. Surface modifications are often used to make fullerenes dispersible in water, allowing their use in, for example, pharmaceutical applications (e.g. fullerol is polyhydroxylated fullerene C_{60}(OH)_n) or in cosmetics (e.g. fullerenes wrapped by polyvinylpyrrolidone). The different physico-chemical properties of different fullerene types influence their biological activity and
consequently their potential toxicity. Fullerenes are generally pro-
duced as functionalised forms, and the functional groups are the
key determinants of their properties.

The anticipated market growth of fullerenes, in combination
with the potential for direct human exposure via several applica-
tions, such as creams used on the skin, or for drug delivery, has
led to widespread concerns about their potential to cause adverse
effects to human health.

In 2008 the EU funded the 12 month project ENRHES (Engi-
neered Nanoparticles: Review of Health and Environmental Safety;
<http://nmi.jrc.ec.europa.eu/project/ENRHES.htm>) in order to as-
sess the current status of hazard, exposure and risk information
pertaining to different nanomaterials. ENRHES involved a compre-
hensive and critical scientific review of the health and environ-
mental safety data of different classes of nanomaterials, as
published in the open literature. Drawn upon the ENRHES report,
a review on “The biological mechanisms and physiochemical char-
acteristics responsible for driving fullerene toxicity” has recently
been published by Johnston et al. (2010). In the current publication
we selected suitable hazard and exposure data complemented
with recent findings in order to investigate the extent to which
occupational, environmental and consumer risks of fullerenes can
be assessed. Where insufficient data was available, we provide rec-
ommendations for further data generation. This article is one in a
series of articles with the same purpose, but addressing different
nanomaterials, please see Aschberger et al. (2010) and Christensen
et al. (2010a,b).

1.1. Scope and approach

This paper gives an up to date review on publicly available ful-
lerene hazard and exposure data (as of May 2010), relevant for
conducting a human health risk assessment. The risk assessment
appraisal presented herein focuses on human exposure to full-
nerenes in the occupational setting, as consumers or exposure to ful-
nerenes via the environment. Exposure through medical
applications is considered to be outside the scope of the current
assessment. The risk assessment appraisal follows the general ap-
proach specified in the REACH Guidance on Information Require-
ments and Chemicals Safety Assessment (ECHA 2008) and the
structure is similar to the format for preparing a chemical safety
report under REACH. However, due to the restricted availability of
information (specifically, a significant lack of knowledge on fuller-
ene use and exposure, as well as data on inherent properties),
the detailed assessments have been adapted to consider the avail-
able data. Thus, the analyses conducted are more similar to the risk
assessment as carried out under the “Existing Substances Regula-
tion” (European Commission, 2003), which was the European
Chemicals Regulation before REACH entered into force.

In this manuscript we first describe details of uses and exposure
of fullerenes, followed by a review of the human hazard data.
Within the limitations of the available data, we attempted to iden-
tify suitable dose descriptors from hazard studies in order to deter-
mine human no-effect-levels. To achieve that, we applied assessment factors in accordance with the methodology suggested in
the REACH guidance (Chapter R.8 in ECHA, 2008) being aware
however that this guidance was not developed to address specific characteristics of nanomaterials. Finally we carry out a risk charac-
terisation by comparing these human no-effect-levels to relevant
exposure values. Where data did not allow for a (semi-) quantita-
tive risk characterisation to be undertaken, we discuss possible
risks qualitatively and finally we provide recommendations for fu-
ture work.

The authors acknowledge the fact that data used in this study
relate to different types of fullerenes (which may differ in agglom-
erations state and thus size distribution, surface modification, etc.).

Moreover, while, in some cases, these differences can be tracked
down to the original papers, in other cases, the characterisation of
the materials was unclear or incomplete. Accordingly care
should be taken with drawing firm conclusions across these types.

2. Uses and exposure

Current applications of fullerenes are focused on targeted drug
delivery systems, lubricants, energy applications (such as fuel cells,
solar cells, and batteries), catalyst, and polymer modifications.
Applications in the consumer market include surfaces for anti-
wear applications, cosmetics and sporting goods. The production
and use of fullerenes in the market is limited at present, but ex-
pected to grow significantly over the next decade. A large scale
production plant for fullerenes has been opened in Japan some
years ago, allowing production of a significant tonnage per year
(production capacity of 40 tons/year) (Aitken et al., 2006; Fujitani
et al., 2008).

2.1. Occupational exposure assessment

In an occupational setting, exposure to fullerenes could in prin-
ciple occur for workers at all phases of the material life cycle.
During the development stage it is probable that the material will be
produced under tightly controlled conditions, typically in small
quantities (few kilograms). Incidental releases due to spills and
accidents are possible.

In commercial production, exposure could potentially occur
during synthesis of the material or in downstream activities. Hu-
man exposure is usually not expected during production because
this process is performed in a closed reaction chamber (Fujitani
et al., 2008). However exposure is more likely to occur after the
manufacturing process, when a reaction chamber is opened or
the product is dried or during the handling of products after their
manufacture.

Various downstream applications of fullerenes have been re-
ported, which can also have the potential to result in exposure to
the workers involved in them. Suggested applications include cat-
alysts for use in the chemical industry, lubricants, chemical feed-
stocks or precursors for drug delivery. Inclusion of fullerenes in
composites may lead to exposure when the material is machined,
cut or drilled, during wear and tear and during disposal. The use
of fullerenes in drug delivery systems may potentially also give rise
to occupational exposure of those who manufacture or administer
them.

The main exposure routes in the occupational setting are con-
sidered to be inhalation and dermal contact. Ingestion can also oc-
cur as a consequence of swallowing inhaled material following
mucociliary clearance or as a result of hand-to-mouth contact for
an individual with exposure on their hands.

There are currently no exposure limits specific to engineered
nanomaterials nor any national or international consensus stan-
dards on measurement techniques for nanomaterials in the
workplace.

2.1.1. Inhalation exposure

Evidence of elevated occupational inhalation exposure to fuller-
ene has been reported during various activities at three manufac-
turing sites and two research laboratories. Table 1 gives an over-
view of all identified occupational exposure values.

In a fullerene factory in Japan (Fujitani et al., 2008) manufactur-
ing a mixture of C60, C70 and other higher fullerenes measurements
were carried out on one day outside the facility to determine the
environmental conditions and on a second day within the facility.
Particle size ranges were classified into four ranges, ranging from a
and 3000 particles/cm$^3$.

Table 1

<table>
<thead>
<tr>
<th>Fullerene type</th>
<th>Process/activity</th>
<th>Maximum number concentration (number/cm$^3$)</th>
<th>Mass concentration (µg/m$^3$)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mixture of C$<em>{60}$, C$</em>{70}$ and other higher fullerenes</td>
<td>Fullerene factory: recovery, bagging, vacuuming, outdoor air</td>
<td>15,000 (10–50 nm)&lt;br&gt;7000 (50–100 nm)&lt;br&gt;3000 (100–200 nm)&lt;br&gt;25,000 (10–50 nm)&lt;br&gt;10,000 (50–100 nm)&lt;br&gt;5000 (100–200 nm)</td>
<td>50–125 (PM$_{2.5}$)</td>
<td>Yeganeh et al. (2008)</td>
</tr>
<tr>
<td>Fullerenes and other carbonaceous nanomaterial (carbon nanotubes)</td>
<td>Production (arc reaction): physical handling of nanomaterials (drilling, cutting)</td>
<td>30–50,000 (14–673 nm)</td>
<td>50–125 (PM$_{2.5}$)</td>
<td>Yeganeh et al. (2008)</td>
</tr>
<tr>
<td></td>
<td>Fume hood (carbonaceous particles)</td>
<td>5350–105,856 (14–673 nm)</td>
<td>52–150 (PM$_{2.5}$)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Work zone (non specific carbonaceous particles)</td>
<td>4761–63,130 (14–673 nm)</td>
<td>36–123 (PM$_{2.5}$)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Background (non specific carbonaceous particles)</td>
<td>4617–63,145 (14–673 nm)</td>
<td>57–128 (PM$_{2.5}$)</td>
<td></td>
</tr>
<tr>
<td>C$_{60}$</td>
<td>Environmental laboratory; weighing of raw MWCNT in hood</td>
<td>1476 (10–1000 nm)$^a$&lt;br&gt;53.1 to &gt;123.4 (300–500 nm)$^a$&lt;br&gt;3.9–34.4 (500–1000 nm)$^a$&lt;br&gt;2176 (10–1000 nm)$^b$&lt;br&gt;23.9–42.8 (300–500 nm)$^b$&lt;br&gt;6.5–23.8 (500–1000 nm)$^b$&lt;br&gt;700 (10–1000 nm)$^b$&lt;br&gt;13.7 (300–500 nm)$^b$&lt;br&gt;1 (500–1000 nm)$^b$</td>
<td>Johnson et al. (2010)&lt;br&gt;Methner et al. (2010b)</td>
<td></td>
</tr>
<tr>
<td>C$_{60}$</td>
<td>Manufacture of metal-containing fullerenes: handling beside synthesis device and beside weighing equipment</td>
<td>0.004 (10–50 nm)&lt;br&gt;2 (2000–10,000 nm)$^b$</td>
<td>NEDO project,&lt;br&gt;Shinohara et al. (2009a)</td>
<td></td>
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<tr>
<td></td>
<td>Manufacture of fullerene secondary products (sporting goods)</td>
<td>1.2 × 10$^{-8}$ (10–100 nm)&lt;br&gt;2.5 × 10$^{-8}$ (100–1000 nm)&lt;br&gt;0.54 (1000–10,000)</td>
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</table>

$^a$ Adjusted for background.
$^b$ Highest possible concentration in the exposure assessment.

Particle diameter ($D_p$) = 10 nm to >5 μm results and were presented as number and volume distributions during a series of different activities and non-activities. The diameter of the mixed fullerene particles, as produced is about 20 μm. The reported measurements suggested particle concentrations in the air during manufacturing related activities of between 10,000 and 15,000 particles/cm$^3$ of the size 10–50 nm (nanoparticle), 7000 particles/cm$^3$ of the size 50–100 nm (nanoparticle or ultrafine particle) and 3000 particles/cm$^3$ of the size 100–200 nm (fine particle). The calculated volume concentrations for these particle sizes are 0.1–0.2, 2, and 10 nm$^3$/cm$^3$ ($1×10^5$), respectively. Outdoor air concentrations were determined to be 25,000, 10,000 and 5000 particles/cm$^3$ for the three sizes, respectively. This study revealed that the concentration of particles increased during certain activities (removal of fullerene from a storage tank and/or weighing) and that the fullerene existed mainly as aggregates/agglomerates in the air. However this study has limitations, as the origin of particles <50 nm is unclear. Outdoor measured particles, which were sometimes even higher than those measured during activities indoors, are expected to originate from urban atmosphere (traffic exhaust and stationary combustion particles).

Yeganeh et al. (2008) conducted measurements in a commercial nanotechnology facility in the US that produces fullerenes and other carbonaceous nanomaterials (carbon nanotubes). Particles were measured during the manufacturing of carbonaceous nanomaterials (1) inside a fume hood, where nanomaterials were produced, (2) just outside the fume hood and (3) in the background. The measurements were not selective for engineered nanomaterials and may have included both engineered nanomaterials and naturally occurring or incidental particles.

Concentrations did not appear to vary much between activities nor between inside the facility and outdoors. However large, short-term increases in PM$_{2.5}$ (aerodynamic diameter <2.5 μm) and particle number concentrations were associated with physical handling of nanomaterials and other production activities (such as drilling and cutting of graphite and metal, which took place in the laboratory within 5 m of the measurement sites). Particle number concentrations (14–673 nm) were observed to be 30–50,000 particles/cm$^3$ in the work area during periods of arc reaction. Measured PM$_{2.5}$ mass concentrations ranged from 50 to 125 µg/m$^3$. Photomission results indicated that the particles suspended during nanomaterial handling inside the fume hood were carbonaceous and therefore likely to include engineered nanoparticles. Particle concentrations in the background were influenced by activities related to nanomaterial production, although it was considered that these particles were not likely to include carbonaceous nanomaterials. Outdoor particle concentrations appeared to influence the indoor measurements indicating that small particles easily penetrated from outdoors to indoors. Based on the measurements in this study, the engineering controls at the facility appear to be effective at limiting exposure to the produced nanomaterials.

The National Institute for Occupational Safety and Health (NIOSH; Methner et al., 2010a,b) has developed a nanoparticle emission assessment technique (NEAT), which is a combination of measurement techniques and instruments to assess potential inhalation exposures in facilities that handle or produce engineered nanomaterials. The NEAT utilises portable direct-reading instrumentation (condensation particle counter and optical particle counter) to detect releases of airborne nanomaterials, supplemented by filter-based air sampling and subsequent chemical and microscopic analysis for particle identification and chemical speciation.

Particle identification (via transmission electron microscopy) is crucial to detect particle sources and to differentiate between different types of particles, e.g. process-related and incidental nanomaterials. This analysis also shows evidence in which forms nanomaterials are emitted, like agglomerates, clusters, bundles or rather individual fibres or spherical particles.
NIOSH conducted field studies at 12 sites in research and development laboratories, pilot plants, and manufacturing facilities handling nanomaterials, using the NEAT. These studies included also one research and development laboratory working with fullerenes and multiple walled carbon nanotubes.

Part of the NEAT project was published by Johnson et al. (2010), who described potential occupational exposure to engineered carbon-based nanomaterials in environmental laboratories. Nanomaterials often agglomerate in aqueous suspensions, requiring continuous mixing or sonication to de-agglomerate. This common laboratory process can result in the release and dispersion of nanomaterials into the air via small water droplets. This may be an overlooked risk for scientists and environmental engineers working with nanomaterials in simulated natural waters. Carbonaceous nanomaterials (CNMs)-containing water droplets have the potential to deposit on the surfaces within the sonication cabinet and in the laboratory. These nanomaterials may be available for dermal deposition if laboratory workers unknowingly contact contaminated surfaces with unprotected skin (e.g. hand/forearms).

The highest airborne particle number concentrations were seen during handling of hydrophobic C60 and raw multiwalled carbon nanotubes (MWCNTs), at the 300 nm size, followed by the 500 nm size. Sonication increased airborne C60 particle number concentrations in the 10–1000 nm size range to 2176 and 2776 particles/cm³ during weighing and sonication, respectively, compared with weighing and handling dry CNMs.

Air concentrations of C60 were monitored within the NEDO project at a metal-containing fullerene manufacturing site in Japan and in a manufacturing site of fullerene secondary products, like sporting goods (Shinohara et al., 2009a).

No emissions from the devices used for synthesising fullerenes were reported. However, particle concentrations increased occupational exposure to fullerenes was possible during the process of collecting the synthesized products from the devices, product packaging and cleaning of the equipment. As fullerenes particles were rarely found in the nanoscale, it was considered that they exist in the form of micron-size aggregates/agglomerates.

The highest concentration of micron sized particles (>2000 nm) in the occupational environment was determined to be 2 μg/m³ during handling operation beside the synthesis device. The highest nanoparticle concentration (<50 nm) was 0.004 μg/m³ during handling operation beside the weighing equipment. Workers are expected to use respiratory protective equipment, which reduces the exposure by a factor of 10–0.2 and 0.0004 μg/m³, respectively.

An estimation for occupational exposure was also conducted for a manufacturing site of sporting goods, producing 10,000 units per year (Shinohara et al., 2009a). It was expected that particles in the range of 1000–10,000 nm diameter contributed the most to the exposure and the highest estimated exposure level was 0.54 μg/m³ for a person handling 1.5 g fullerene for one minute, once per hour, eight times per day. These exposure estimations did not consider the use of engineering measures or respiratory protective equipment, which could reduce the exposure to 1/10 for each case, leading to an estimated exposure of 0.0054 μg/m³.

From the limited available data on fullerene occupational exposure the values from two studies were selected for a risk characterisation appraisal, being aware of the limitations of using these values. From these studies metrics are available in mass/volume, which are the metrics known from available inhalation toxicity studies (for discussion of the most appropriate metric, please refer also to Section 4.2).

The values of PM2.5 of 50–125 μg/m³ (Yeganeh et al., 2008), which were not specific for fullerenes are suggested as a high exposure value, not specific for fullerenes and the values of 0.004–2 μg/m³ for manufacture and 0.54 for production of consumer goods (Shinohara et al., 2009a) can be used as specific values for fullerenes.

### 2.1.2. Dermal and oral exposure

No quantitative dermal exposure data has been identified for fullerenes. Occupational dermal exposure to single walled carbon nanotubes (SWCNTs) between 217 and 6020 μg (0.2–6 mg) per hand has been reported by Maynard et al. (2004). These data could be consulted as indication of possible occupational dermal exposure to fullerenes. No studies have attempted to quantify ingestion exposure to fullerenes resulting from dermal exposure.

### 2.2. Consumer exposure

Fullerenes are used in few consumer products (inventory of nanotechnology-based consumer products, [http://www.nanotechproject.org/inventories/consumer/](http://www.nanotechproject.org/inventories/consumer/)) and the main source of consumer exposure might be expected from cosmetics (Lens 2009). Fullerenes are used in several different cosmetic products, like anti-aging products or sun creams, mainly because of their anti-oxidant properties (see Table 2 for more information). Fullerenes used in cosmetics have been described as fullerene-containing vegetable squalane or polyvinylpyrrolidone wrapped fullerenes (information from the webpage of a producer of fullerene used in cosmetics: Vitamin C60 BioResearch Corporation [http://www.vc60.com/english/index.html](http://www.vc60.com/english/index.html)). In Europe, the European Commission database ‘Cosing’ provides information on cosmetic ingredients contained in the “Cosmetics Directive” ([76/768/EEC](http://www.ec.europa.eu/enterprise/cosmetics/cosing/index.html)) and lists fullerenes and hydroxyfullerenes to be used in cosmetic products.

<table>
<thead>
<tr>
<th>Application</th>
<th>Function</th>
<th>Fulleren type</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>anti-wrinkle) skin care products</td>
<td>free radical scavenger</td>
<td>fullerene-containing vegetable squalane&lt;br&gt;polyvinylpyrrolidone wrapped fullerenes&lt;br&gt;hydroxyfullerenes</td>
<td></td>
</tr>
<tr>
<td>Skin whitening agent</td>
<td>Inhibition of melanogenesis</td>
<td>Radical sponge®&lt;br&gt;(polyvinylpyrrolidone wrapped fullerene)</td>
<td></td>
</tr>
<tr>
<td>Make up</td>
<td>Fillers and pigments</td>
<td>Not specified&lt;br&gt;Fullerene derivatives&lt;br&gt;Fullerenes and hydroxylated derivatives</td>
<td></td>
</tr>
<tr>
<td>Hair care products</td>
<td>Laboratory process can retard hair growth</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Table 2**

Fullerene use and their function in cosmetic products.
in cosmetic products as antimicrobials and for skin conditioning (<http://ec.europa.eu/enterprise/cosmetics/cosing/>).

Fullerenes have also attracted considerable attention over recent years in relation to the treatment of diseases where the underlying mechanism involves reactive oxygen species (ROS) or radical formations, such as neurodegenerative disorders or cancer (Dugan et al., 2001; Chen et al., 2005; Markovic and Trajkovic, 2008).

In addition, fullerenes are used in sporting goods, like tennis rackets to which they provide high stability, high repulsion and low weight. In such products fullerenes are expected to be embedded into a matrix and there should be minimal (e.g. wear and tear) or no exposure due to such use. No quantitative information on exposure from such consumer products has been identified.

Thus, the skin is considered the main exposure route for consumers and dermal exposure even over a longer period could be expected from the use in cosmetics. The quantities of fullerenes in cosmetic products are not usually clearly identified. In such products, fullerenes are usually incorporated into liposomes at concentrations of 0.2–0.5% fullerenes (Lens, 2009; Kato et al. 2010). The liposomes content in a skin cream can probably vary extensively, however it can be estimated that the fullerene concentration in a cream would be lower than 0.5%.

2.3. Exposure of humans via the environment

At the current time, there is no evidence to suggest that manufactured fullerenes are entering the environment at levels which would cause detectable exposure to humans although this could not be discounted in the future as production levels increase.

Beyond engineered C_60 fullerenes, combustion-derived C_60, as identified in particulate matter emitted from coal-fired power plants (Utsunomiya et al., 2002), are being dispersed into the environment. In Russia, naturally occurring fullerenes have also been found in a carbon-rich rock (Buseck et al., 1992).

Within the NEDO project (Shinohara et al., 2009a) fullerene exposure levels near a factory (within 500 m) manufacturing 40 tons of fullerenes per year were estimated. Assuming a scenario of unfiltered emission released from the factory due to malfunction of the emission system, the atmospheric concentrations of fullerenes of all particle sizes (10–10,000 nm) were estimated to be 0.0018 μg/m^3. Estimated fullerene concentrations under normal function of the emission system would be much lower (5.51 × 10^{-3} μg/m^3 for all particle sizes, and 2.2 × 10^{-8} μg/m^3 for particles <1000 nm). Atmospheric concentrations of fullerenes of natural origins and in areas other than near the manufacturing sites, as well as during the use of fullerene containing products, were considered extremely low.

3. Human health hazard

The toxicity and toxicokinetics of fullerenes were extensively reviewed in Johnston et al. (2010). This manuscript includes additional recent information and focuses on describing and extracting data relevant for risk assessment. Table 3 summarises the most important findings from toxicokinetics and toxicity studies.

3.1. Toxicokinetics (absorption, metabolism, distribution and elimination)

Toxicokinetic investigations are considered important to identify possible target organs for fullerenes toxicity following exposure via inhalation, oral or dermal exposure. A number of barriers are in place to prevent absorption from the exposure site and distribution to secondary targets. However if these barriers are overcome by fullerenes, they can become systemically available and exert their toxic effects throughout the body.

3.1.1. Absorption

Table 3

<table>
<thead>
<tr>
<th>Exposure route</th>
<th>Results</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Absorption</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Inhalation</td>
<td>No/limited systemic absorption from lung, fullerenes are phagocytosed by macrophages</td>
<td>Baker et al. (2008)</td>
</tr>
<tr>
<td>Oral</td>
<td>Limited absorption (&lt;3%)</td>
<td>Shinohara et al. (2009)</td>
</tr>
<tr>
<td>Dermal</td>
<td>No penetration into dermis; solvents can modulate fullerene penetration deep into the stratum corneum</td>
<td>Xia et al. (2010)</td>
</tr>
<tr>
<td><strong>Distribution</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pulmonary exposure</td>
<td>Limited/no translocation to liver, brain, kidney, spleen</td>
<td>Kato et al. (2009)</td>
</tr>
<tr>
<td>Intraperitoneal/intravenous</td>
<td>Transport to and accumulation mainly in liver, but also in kidney, lung, spleen, heart and brain</td>
<td>Yamago et al. (1995); Bullard-Dillard et al. (1996); Ghari et al. (2005)</td>
</tr>
<tr>
<td>Oral, intraperitoneal</td>
<td>Via faeces and urine, probably depending on water solubility</td>
<td>Mori et al. (2006)</td>
</tr>
<tr>
<td>Acute/repeated dose toxicity</td>
<td>Very low acute toxicity; no data on repeated exposure</td>
<td>(see table 4)</td>
</tr>
<tr>
<td>Inhalation</td>
<td>Low acute and sub-chronic toxicity</td>
<td>Aoshima et al. (2009b), Ito et al. (2010)</td>
</tr>
<tr>
<td>Dermal exposure</td>
<td>Low acute toxicity, no data on chronic exposure</td>
<td>Aoshima et al. (2009b); Huczko et al. (1999)</td>
</tr>
<tr>
<td><strong>Irritation</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Highly purified fullerenes and fullerene soot were not irritating to skin and eye in animals and in human studies</td>
<td>(see Table 5)</td>
<td></td>
</tr>
<tr>
<td><strong>Sensitisation</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Highly purified fullerenes and fullerene soot were not sensitising in animals and in human studies</td>
<td>Aoshima et al. (2009b); Huczko et al. (1999)</td>
<td></td>
</tr>
<tr>
<td><strong>Mutagenicity</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Highly purified fullerenes and fullerene soot were not genotoxic in animals and in human studies</td>
<td>(see Table 5)</td>
<td></td>
</tr>
<tr>
<td><strong>Carcinogenicity</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anti-inflammatory and anti-tumour effects of certain fullerene types reported; not carcinogenic after intraperitoneal exposure for 25 weeks; no cancer assay via physiologically relevant routes available</td>
<td>Takagi et al. (2008)</td>
<td></td>
</tr>
<tr>
<td><strong>Reproductive/ Developmental toxicity</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intraperitoneal exposure</td>
<td>Developmental effects at high doses in mice; relevance for human exposure situations is questionable</td>
<td>Tsuchiya et al. (1996)</td>
</tr>
</tbody>
</table>
urine, therefore implying that some fullerenes were able to pass through the gut wall (Yamago et al., 1995). Absorption of fullerenes following oral exposure has also been suggested by Folkmann et al. (2009), as oxidative damage was observed in a dose dependent manner in liver and lungs following oral exposure via gavage. The relevance of these findings is not clear, as also the solvent corn oil showed the same effect and the presence of fullerenes in these organs was not demonstrated.

3.1.1.2. Inhalation. In a study by Baker et al. (2008) fullerenes (in a nano, and microparticle form) were not detected in blood following inhalation by rats, suggesting that they do not translocate from their respiratory exposure site. The calculated pulmonary deposition fractions were 50% greater for the C₆₀ fullerene nanoparticle (55 nm, 2.22 mg/m²) exposed group (14.1%) than for the microparticle (0.93 μm, 2.35 mg/m³) group (9.3%). The half life however of 26 days for fullerene nanoparticles was similar to that of fullerene microparticles (29 days), suggesting that similar elimination processes, such as mucociliary clearance and macrophage uptake, are involved during removal from the lungs. Steady-state lung burdens were not expected to be reached until rats are exposed over approximately 5 half-lives, which in the study were 130 and 145 days of exposure for nano and microparticles respectively.

Certain nanoparticles have been shown to be translocated via the olfactory nerve to regions of the brain (see Oberdörster et al., 2004). In fish, fullerenes were observed to induce oxidative stress in the brain following exposure, which could suggest a translocation to the brain (Oberdörster, 2004). However, it is not clear whether these findings are relevant for human exposure situations, especially as translocation of fullerenes has not been reported from studies with mammalian species.

Shinohara et al. (2009a) reported no transfer of C₆₀ particles to organs other than the lung following intratracheal instillation (3.3 mg/kg) and inhalation exposure (0.12 mg/ m³) in rats, supporting that there is negligible absorption of fullerenes following inhalation.

In contrast to that Naota et al. (2009) suggested that fullerene nanoparticles may actually pass through the air-blood barrier (ABB) by both diffusion and caveolae-mediated pinocytosis, resulting in immediate translocation into the systemic circulation and distribution to other organs and tissues in the body. However it is not clear if the observed translocation of particles was not at least partly influenced by the route of administration (instillation), as the suspension induced oedema in the lung may have influenced the physiological transportation system in the lung into the blood circulation. Consequently the results under such conditions may not be relevant for inhalation exposure.

3.1.1.3. Dermal. Xia et al. (2010) have shown in vitro (tape stripping and skin tissue biopsies in weanling pigs) and in vitro (stratum corneum absorption and flow through diffusion cell experiments) that pristine fullerene nanoparticles can penetrate deep into the stratum corneum and be modulated by solvent, in which fullerenes are dispersed. A 500 μL solution containing 200 μg/mL C₆₀ in different solvents was applied with Hill Top Chambers on designated skin sites for 24 h and re-dosed with freshly prepared solutions daily for a total of 4 days. C₆₀ was readily absorbed into the stratum corneum from toluene, cyclohexane and chloroform, but very little from mineral oil. The initial rapid absorption by loosely packed superficial layers (stratum disjunctum) of the stratum corneum was slowed down after 1 h, probably due to a slow transport process through the lipid bilayers. The authors concluded that solvent effects are crucial in the skin penetration of fullerenes and must be considered in the risk assessment of C₆₀ in industrial organic solvents.

Kato et al. (2009) tested C₆₀ dissolved in squalane (Lipo-Fullerene, LF-SQ) for their skin permeability in a modified Bronaugh’s diffusion chamber for 24 h and showed that C₆₀ was not detected on both the epidermis and dermis for administration at low concentrations (2.23 and 22.3 ppm C₆₀ in LF-SQ). However, upon administration at concentrations as high as 223 ppm C₆₀ was detected to permeate into the epidermis but was not detected in the dermis of human skin biopsy. The authors therefore concluded that for C₆₀ dissolved in LF-SQ there is no necessity for considering toxicity due to systemic circulation.

Rouse et al. (2007) showed that a fullerene-substituted peptide can penetrate through the epidermal layers via passive diffusion. Mechanical stressors, such as those associated with a repetitive flexing motion (e.g. when walking barefoot), increase the rate at which these particles traverse into the dermis.

3.1.2. Distribution, metabolism and elimination

Little information on the distribution of fullerenes following inhalation, oral or dermal absorption has been identified, probably because there is limited absorption. For some nanoparticles translocation to secondary organs from the respiratory tract has been shown via uptake into pulmonary lymphatics and blood circulation under conditions of lung particle overload and associated inflammatory state (Oberdörster et al., 2005).

A recent study within the NEDO project (Shinohara et al., 2009a) indicated negligible systemic translocation of fullerenes to the brain (0.17% of lung concentration) and other organs following intratracheal instillation (3.3 mg/kg) and inhalation exposure (0.12 mg/ m³) in rats. Gao et al. (2009) reported that C₆₀ (agglomerated to approximately 1 micron) did not distribute from lung to other tissues up to 168 h post-dosing of 1 or 5 mg/kg via intratracheal instillation. Following inhalation of 1 mg/m³ fullerenes (20 nm) for 6 h, no distribution to liver or spleen and only trace amounts in the kidneys were found. C₆₀ showed poor pulmonary clearance and correlation between C₆₀ and ¹³C disposition suggests that C₆₀ is not being metabolised.

Within the lungs, fullerenes have been demonstrated to be phagocytosed by alveolar macrophages (Fujita et al., 2009; Xia et al., 2006). Macrophages thereby fulfil their role within host defence, however following uptake of fullerenes oxidative or inflammatory events may be stimulated (which requires consideration). Electron microscopic observation showed that phagocytosed fullerenes became finely dispersed granules in alveolar macrophages, but were not transferred to organelles or nuclei (Morimoto et al., 2010).

Following intraperitoneal injection to rats water-soluble polyallylsulfonated fullerenes (500, 750 or 1000 mg/kg) were transported via blood and accumulated in liver, kidney and spleen, with evidence of toxicity manifesting at sites of accumulation (Chen et al., 1998b). Following intravenous injection ¹⁴C labelled fullerenes were rapidly removed from the blood and accumulated primarily in liver (Bullard-Dillard et al., 1996); trimethylenemethane derivatized fullerenes (200–500 mg/kg) were distributed following intravenous injection from the liver to kidney, lung, spleen, heart and brain (Yamago et al., 1995). Nikolić et al. (2009) demonstrated the distribution of intravenously injected radiolabeled 125-I-nanoC₆₀ to liver and spleen, while accumulation in thyroid, stomach, lungs and intestines was significantly lower.

Following intraperitoneal injection Gharbi et al. (2005) observed accumulation of fullerenes (2.5–5 g/kg C₆₀) within Kupffer cells in the liver, with strong macrophagic activity.

In vitro, a number of other cell types have also been demonstrated to internalise fullerenes, such as keratinocytes (Rouse et al., 2006), epithelial cells (Rouse et al., 2009) and eye lens cells (Roberts et al., 2008), often with oxidative and lethal consequences.
The elimination of fullerenes from the body within faeces has been demonstrated for both, trimethylene methane derivatised fullerenes (Yamago et al., 1995) and a mixture of C_{60} and C_{70} fullerenes (Mori et al., 2006) following intravenous and oral exposure in rats. Intravenous administration of the derivatised fullerenes lead to extremely slow excretion; after 160 h, only 5.4% was eliminated into the faeces and the remainder stayed in the body. Only very little was found in the urine, probably due to the high lipophilicity of the molecule (Yamago et al., 1995). Injected water-soluble polyalkylsulfonated fullerenes were rapidly eliminated via urine (Chen et al., 1998).

It can be concluded that there is probably limited absorption of fullerenes following exposure via a physiologically relevant routes. Fullerenes tend to remain at the deposition site, specifically within the lungs and gut, from where they can be eliminated either through alveolar macrophages and mucociliary escalator or faeces and urine. There is probably no or little absorption of fullerenes through skin, dependent on fullerene type (functionalisation), the solvent used and on skin properties. At present it is not possible to make general conclusions on the ADME profile of fullerenes and their derivatives due to the limited amount of information available.

Further investigations are needed to clarify and confirm whether fullerenes can be absorbed following relevant exposure routes, and reach organs distant from the deposition site. It should be kept in mind that the toxicokinetics may be influenced by surface modifications. Specifically, surface coatings may be intentionally introduced during fullerene manufacture, or such modifications to the fullerene surface may be secondary in nature, e.g. due to interaction of the fullerene with biological media in the organism (protein corona) which could then significantly impact the biokinetics of the fullerene. Toxicokinetic information is important in order to interpret the effects observed following fullerene exposure, especially those observed subsequent to intraperitoneal and intravenous application and those seen in vitro. The use of radioisotope or fluorescent labelling is recommended to allow for the better detection of fullerenes.

3.2. Acute and repeated dose toxicity

There are only few standard acute or repeated dose toxicity tests for fullerenes available, and therefore all available and relevant studies are described together in this chapter.

3.2.1. Oral exposure

No lethality, or other signs of toxicity in terms of behaviour or body weight were evident in rats after oral exposure to a single dose of 2000 mg/kg fullerite (a mixture of C_{60} and C_{70}), during an observation period of up to 14 days (Mori et al., 2006). Chen et al. (1998b) demonstrated that 2500 mg/kg polyalkylsulfonated (water soluble) C_{60} showed no effects subsequent to single oral exposure of rats, and as a consequence it was considered to be not acutely toxic. Based on these two studies acute NO-observed-adverse-effect-levels (NOAEL) of 2000 mg/kg bw for fullerite (mixture of C_{60} and C_{70}) and 2500 mg/kg for polyalkylsufonated (water soluble) C_{60} are suggested. However, as in both studies only one dose was tested where no effects were reported, it is likely that the true acute oral NOAEL is higher. It can be concluded that fullerenes have very low acute oral toxicity, but no information following repeated oral exposure is available.

3.2.2. Pulmonary/Inhalation exposure

Inhalation is considered the route of exposure contributing most to concern about (airborne) nanoparticles. The particle size (aerodynamic diameter) determines the deposition of discrete NP in the respiratory tract where nanoparticles can have local effects. Ultrafine, nanometric particles have been shown to induce more inflammation and were more tumourigenic than an equal mass of larger particles. Some nanoparticles in the lungs have also been shown to be transported to the other organs in the body, however no generalisations can be made (Oberdörster et al., 2005). Many studies have demonstrated that a range of nanoparticles induce pro-inflammatory effects in the lung (for a review see e.g. Donaldson and Stone, 2003), however this has not always been seen with fullerenes. The results from inhalation and intratracheal instillation studies are summarised in Table 4.

3.2.2.1. Inhalation. No inflammatory potential or toxicity in the lung was observed in Fischer 344 rats exposed to fullerenes following nasal inhalation at concentrations of 2.22 mg/m\(^3\) (nanoparticle, 55 nm diameter) and 2.35 mg/m\(^3\) (microparticle, 0.93 μm diameter) for 3 h/day for 10 consecutive days (Baker et al., 2008) with toxicological assessments conducted up to 7 days post exposure. C_{60} lung particle burdens were greater in nanoparticle-exposed rats than in microparticle-exposed rats. A significant increase in protein concentrations was identified in the bronchoalveolar lavage fluid (BALF) of nanoparticle-exposed rats. No gross or microscopic lesions were observed at necropsy (e.g. no lesions in liver or hearts). Minimal haematology serum chemistry changes were found. Within the lung, no cellular infiltration (indicative of an inflammatory response) was observed, although C_{60} was internalised by alveolar macrophages. No steady-state lung burdens, which were calculated to be 130 and 145 days for nano and micro-particles, respectively, were reached during this study. Therefore the authors concluded that it is possible that exposure related toxicological findings could be found in longer duration studies.

In a sub-acute inhalation study male Wistar rats were exposed to 0.12 mg/m\(^3\) fullerenes (4.1 × 10\(^4\) particles/cm\(^3\), 96 nm diameter, specific surface area 0.92 m\(^2\)/g) for 6 h a day, 5 days a week, for 4 weeks (Fujita et al., 2009). There was no significant inflammation and tissue injury during the inhalation exposure period (28 days) and a subsequent observation period of up to 3 months (Morimoto et al., 2010). There was no foreign body granuloma in the histopathological findings of the inhalation and the intratracheal instillation study.

The study authors suggested that the NOAEC for lung inflammation might be even higher than the tested dose. Gene expression profiles revealed that few genes involved in the inflammatory response, oxidative stress, apoptosis and metalloendopeptidase activity as well as some genes associated with the immune system process, including major histocompatibility complex (MHC)-mediated immunity were up-regulated at both 3 days and 1 month post exposure. These results however were significantly different from those of ultrafine (Uf)-NiO particles (positive control) which induced high expression of these genes. The induction of gene expression was probably not an adverse but rather an adaptive physiologic response.

The National Toxicology Programme (NTP), based at the National Institute of Environmental Health Sciences (NIEHS; Walker, 2009) recently evaluated the effects of sub-chronic inhalation exposure of Wistar-Han rats and B6C3F1 mice to two different sized particles of C_{60} (0.05 or 1 μm). The animals were exposed by nose-only inhalation for 3 h per day, 5 days per week for 90 days to concentrations of 0.2, 15 and 30 mg/m\(^3\). The pathological effects observed were increased lung pigmentation (attributable to lung deposition of C_{60}) and histiocyte infiltration (compensatory response to clear C_{60} from the lung). There was a shift in inflammatory cell populations in lung lavage fluid. These findings confirm the low toxicity of C_{60} following inhalation exposure, even over a sub-chronic exposure period and further indicate that the decrease in particle size to the nanoscale did not appear to exacerbate toxicity. However no NOAEC or other details are
Table 4
Inhalation and intratracheal instillation studies with fullerenes.

<table>
<thead>
<tr>
<th>Fullerene type</th>
<th>Administration</th>
<th>Dose</th>
<th>Observation period (post exposure)</th>
<th>Results</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Inhalation studies</strong></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>C_{60} (diameter: 55 nm)</td>
<td>Nose-only inhalation; Fischer 344 rat; 10 days; 3 h/day</td>
<td>2.22 mg/m³</td>
<td>1, 5, 7 days</td>
<td>Increased protein levels in BALF; no changes in cell count and cytokines; NOAEC: 2.22 mg/m³</td>
<td>Baker et al. (2008)</td>
</tr>
<tr>
<td>C_{60} (diameter: 0.93 μm)</td>
<td>No intratracheal instillation studies</td>
<td>2.35 mg/m³</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C_{60} (diameter 96 nm)</td>
<td>Whole body inhalation in male Wistar rat; 28 days; 6 h/day; 5 days/week</td>
<td>0.12 mg/m³</td>
<td>3 days, 1 month</td>
<td>No increase of total cell and neutrophil count in BALF or expression of CINC-1,-2,-3; NOAEC: &gt;0.12 mg/m³</td>
<td>Fujita et al. (2009),</td>
</tr>
<tr>
<td>C_{60} (diameter 96 nm)</td>
<td>Intratracheal instillation; male Wistar rat; 28 days; 6 h/day; 5 days/week</td>
<td>0.12 mg/m³</td>
<td>3 days, 1 and 3 months</td>
<td>No increase of total cell and neutrophil count in BALF or expression of CINC-1,-2,-3; NOAEC: &gt;0.12 mg/m³</td>
<td>Morimoto et al. (2010)</td>
</tr>
<tr>
<td>C_{60} (diameter: 50 nm)</td>
<td>Intratracheal instillation; Wistar Han rat; B6C3F1 mice 90 days; 3 h/day; 5 days/week</td>
<td>2, 15, 30 mg/m³</td>
<td>1, 5, 7 days</td>
<td>Increased lung pigmentation (lung deposition) and histiocyte infiltration; increase in inflammatory cells in BALF; → negligible toxicity</td>
<td>Walker 2009</td>
</tr>
<tr>
<td>C_{60} (diameter: 1 μm)</td>
<td>Intratracheal instillation; SD CD rats</td>
<td>0, 0.02, 0.2, 2, 20, 200 μg/mouse</td>
<td>24 h</td>
<td>Increase in lipid peroxidation in BALF at 1.5 and 3 mg/kg; no changes in other markers in BALF or persistent inflammation of lung tissue observed</td>
<td>Sayes et al. (2007)</td>
</tr>
<tr>
<td><strong>Intratracheal instillation studies</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C_{60} (diameter 160 nm); aqueous dispersion C_{60}(OH)_{24} aqueous dispersion</td>
<td>Intratracheal instillation; SD CD rats</td>
<td>0.2, 0.4, 1.5, 3 mg/kg</td>
<td>1 day, 1 and 3 months</td>
<td>Low concentrations (&lt;20 μg) attenuate α-quartz induced neutrophilic inflammation; no changes in cell count and cytokines;</td>
<td>Roursgaard et al. (2008)</td>
</tr>
<tr>
<td>Fullerenol (polyhydroxylated fullerenes)</td>
<td>Intratracheal instillation; SD CD rats</td>
<td>0, 0.02, 0.2, 2, 20, 200 μg/mouse fullerol 2 min prior to α-quartz (50 μg)</td>
<td>1 day, 1 and 3 months</td>
<td>Transient significant increase in neutrophils in BALF and in expression of CINC-1,-2,-3 in lung only at 1 mg, while no significant changes at lower doses.</td>
<td>Morimoto et al. (2010)</td>
</tr>
<tr>
<td>C_{60} (diameter 33 nm); specific surface area: 0.92 m²/g</td>
<td>Intratracheal instillation; male Wistar rat;</td>
<td>0.1 mg (0.33 mg/kg), 0.2 mg (0.66 mg/kg) or 1 mg (3.3 mg/kg)</td>
<td>3 days, 1 week, 1, 3 and 6 months</td>
<td>Cell injury, oxidative/nitrosative stress and inflammation at 5 and 10 mg only</td>
<td>Xu et al. (2009)</td>
</tr>
<tr>
<td>C_{60}(OH)_{24} aqueous dispersion</td>
<td>Intratracheal instillation; Sprague–Dawley rats</td>
<td>1, 5, 10 mg per rat</td>
<td>3 days</td>
<td>Inflammatory protein-2 cytokines (IL-1, TNF-α and IL-6) and Th1 cytokines (IL-12, IFN-γ)</td>
<td>Park et al. (2010)</td>
</tr>
<tr>
<td>C_{60}</td>
<td>Intratracheal instillation; ICR male mice</td>
<td>0.5, 1, 2 mg/kg</td>
<td>1, 7, 14, 28 days</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Notes:

- BALF: bronchoalveolar fluid.
- CINC: cytokine-induced neutrophil chemoattractant.

*available from this study as it is not published yet and only the abstract is available (Walker 2009).

3.2.2.2. Intratracheal instillation. Sayes et al. (2007) intratracheally instilled rats, at concentrations between 0.2 and 3 mg/kg, with undervatised C_{60} (160 nm) and a highly water soluble derivative C_{60}(OH)_{24}. During the observation period of up to 3 months they reported little differences in markers of oxidative stress (lipid peroxidation products in bronchoalveolar lavage) between C_{60} groups and no differences in lung toxicity for C_{60}(OH)_{24} when compared to controls. This was in contrast to the response induced by quartz, which was pro-inflammatory and pro-fibrotic in nature. The highest applied dose of 3 mg/kg would correspond to an inhalation concentration of 0.16 mg/m³ air, assuming an inhalation volume of 8 L/day and an exposure for 6 h/day, 5 days/week for 13 weeks. This concentration is slightly higher than the NOAEC of 0.12 from the 28 days study (Fujita et al., 2009) and the 2.22 mg/m³ from the 10 days study (Baker et al., 2008), if extrapolated to 6 h/day and 90 days (i.e. 0.12 mg/m³).

Roursgaard et al. (2008) exposed mice via intratracheal instillation to doses of 0.02–200 μg/mouse for 24 h, and showed that at low concentrations (<20 μg/mouse), fullerols (i.e. polyhydroxylated fullerenes) may have protective, anti-inflammatory properties probably due to the ability of fullerols to reduce ROS-mediated inflammation, as they have shown to attenuate α-quartz induced neutrophilic inflammation. At higher concentrations (200 μg/mouse) fulleroles exhibited a pro-inflammatory response.

Morimoto et al. (2010) exposed Wistar male rats intratracheally to fullerenes (33 nm) at doses of 0.1, 0.2 or 1 mg per animal and examined the neutrophil infiltration and the expression of the chemokine cytokine-induced neutrophil chemoattractant (CINC) in the lung after 3 days, 1 week, 1, 3 and 6 months. Both, the 0.1
and 0.2 mg fullerene group did not show a significant increase of the total cell and neutrophil count in BALF and CINC expression, while the high dose group (1 mg) only showed a transient significant increase of neutrophils and CINC expression. No persistent inflammation by fullerenes was observed during the period of up to 6 months after exposure.

Xu et al. (2009) reported that intratracheal instillation of 1 mg polyhydroxylated fullerenols C60(OH)5 per rat did not induce adverse pulmonary toxicity as investigated by bronchoalveolar lavage fluid biomarkers and pathological evaluation of lung tissue following 3-day exposure. The two higher doses of 5 or 10 mg/rat induced cell injury effects, oxidative/nitrosative stress and inflammation, showing that these responses are dose dependent, probably depending on the higher retention dose in the lung and the ensuing aggregation of the fullerenols.

In contrast to the above mentioned studies showing rather low toxicity of fullerenes, Park et al. (2010) showed a significant, dose dependent increase of pro-inflammatory cytokines, including IL-1, TNF-α, and IL-6, and an increase of Th1 cytokines such as IL-12 and IFN-γ in the BAL fluid 1 day after instillation of 0.5, 1 and 2 mg/kg, suggesting inflammatory responses of C60 in the lung of mice. The cytokine levels remained elevated during the rest of the experimental period (7, 14 and 28 days). In addition, IgE reached the maximum at 1 day after the treatment in both, BALF and the blood and decreased in a time dependent manner. The authors concluded that C60 remaining in lung tissue can induce continuous tissue damage.

From the small number of available studies it can be concluded that, following pulmonary exposure, fullerenes have shown no or low activity in inducing inflammation in the lung. Even anti-inflammatory responses have been reported, probably depending on the applied dose and the fullerene under investigation. No other than lung effects were reported following pulmonary exposure to fullerenes.

Pulmonary effects of fullerenes have been investigated in intratracheal instillation and inhalation studies. Inhalation studies represent the more realistic scenario of human exposure, however there are only few testing facilities capable of conducting such studies, as expensive equipment and a large amount of test substance are required. A single application via intratracheal instillation mimics the accumulated higher exposure over a longer period, however the mechanisms underlying effects induced by a high dose rate (bolus delivery) are likely very different from those induced when the same dose is delivered by inhalation over a longer period (months). Therefore results from bolus type dose delivery should not be used for purposes of risk assessment (see e.g. Oberdörster, 2010) and the results of the inhalation studies are preferred for deriving an INEL (Section 3.8.1). The results from the intratracheal instillation studies however were regarded relevant in supporting the conclusions of low toxicity and the non progressive course of inflammatory effects.

So far only one sub-chronic, but no chronic study have been reported for fullerenes. No definite results from the sub-chronic study (Walker 2009) are available, except that it confirms the low pulmonary toxicity of fullerenes. In the sub-acute studies (28 days, Fujita et al. 2009; Morimoto et al., 2010) only one relatively low dose (0.12 mg/m³) was tested and the NOAEC is expected to be much higher; Based on a weight of evidence, it is suggested to use the NOAEC of 2.22 mg/m³ from the 10 day study (Baker et al., 2008) for the risk assessment for short and long term exposure (an estimation from the 10 day study NOAEC applying Haber’s law, would be 0.4 mg/m³ for 28 days every day exposure or 0.55 if exposed 5 days a week for 6 h, which is considerable higher than the 0.12 mg/m³). For deriving a human no-effect-level, the short duration of the study is compensated by applying assessment factors for the duration (Section 3.8.1). This seems justified and still conservative enough, as the sub-chronic inhalation study describes negligible effects at much higher doses (up to 30 mg/m³) and studies including a post-observational period of 3 months following inhalation (although lower doses of 0.12 mg/m³ fullerenes) and of 6 months following intratracheal instillation of up to 1 mg/animal did not show a persistent inflammation. This NOAEC seems appropriate for pristine fullerenes of different aggregate sizes within the nano and low microrange, as no significant differences in toxicity were reported. However no generalisation to other forms of fullerenes, with different properties can be made.

For a regulatory risk assessment relevant information from sub-chronic and/or chronic exposure for the fullerene type of interest would be required.

3.2.3. Dermal exposure

3.2.3.1. In vitro. Fullerene effects on skin cells have been investigated in several in vitro dermal models. Fullerenes were shown to be internalised by keratinocytes, however the reactions observed were different in nature. No effects on cell proliferation at concentrations between 20 nM and 2 μM were observed by Sirevans et al. (1994), whereas Bullard-Dillard et al. (1996) observed that C60 elicited a decrease in cell proliferation that was evident at high concentrations (2 μM) and over an extended period of time (8 days). Increased production of pro-inflammatory mediators, such as IL-8, IL-6 and IL-1 and a dose-dependent cytotoxicity via a necrotic mechanism after exposure to phenylalanine derivatised C60 (up to 0.4 mg/mL) was observed in HEK keratinocytes (Rouse et al., 2006). It was observed in one study that penetration of the particles did not occur via direct transport through cells, but indirectly between skin cells via intercellular spaces (Rouse et al., 2007). Sayes et al. (2004) found that the cytotoxic potential (mediated by lipid peroxidation) of different forms of derivatised fullerenes to human dermal fibroblasts (HDF), HepG2 hepatocytes and normal human astrocytes (NHA), was dependent on the type and level of functionalisation.

Kato et al. (2009) showed that fullerenes dissolved in squalane (as might be used in skin creams) did not induce phototoxic effects at doses 0.49–1000 μg/mL under UVA-irradiation in fibroblasts. They suggested that C60 dissolved in squalane may not exert a UVA-catalytic activity resulting in reactive oxygen species production.

3.2.3.2. In vivo. Aoshima et al. (2009b) investigated the toxic potential of highly purified fullerenes (HPF: mixture of C60 and C70, fullerite, 99.5% purity) to skin and eye in various tests. In a contact-phototoxicity test, 25% HPFs applied on clipped free skin of guinea pigs and exposed to long-wavelength UV irradiation (11.2 J/cm²) for 50 min did not reveal any phototoxic potential, up to 72 h after UV radiation. HPFs (0.01 g on a Finn Chamber) showed no skin reaction at 1 and 24 h after exposure (occlusive conditions) in a human patch test (Aoshima et al. 2009b).

Xia et al. (2010) suggested that fullerenes may not cause acute systemic toxicological effects due to their slow systemic absorption (no absorption within 24 h), however, depending on the mode of application (solvent), fullerenes have been found to penetrate deep into the stratum corneum and also reached the viable epidermis. Ito et al. (2010) reported that fullerene has a ROS-reducing effect and confirmed that the co-application of ascorbate with fullerene protected mouse skin from UV radiation in vivo, and the application of 1 w/w% fullerene reduced UV-B-induced erythema (redness of skin caused by capillary congestion) in vivo. With the application of fullerene to UV-irradiated live mouse skin, no toxicity was recognised in comparison with the control and erythema, the ROS index, and the apoptosis index decreased.

From the results of the studies described above it can be concluded that fullerenes do not induce acute toxic effects to the skin.
No long term studies are available – and more information is needed, especially as absorption (of certain fullerene types and/or in certain solvents) cannot be excluded and the skin has to be considered an important exposure route.

3.2.4. Other routes: intraperitoneal exposure

A LD₉₀ of 600 mg/kg was determined via intraperitoneal injection to rats with water soluble, polyalkylsulfonated C₆₀₀ in an acute (up to 1000 mg/kg, for 24 h) or sub-acute setting (up to 60 mg/kg, with daily exposures for 12 consecutive days). The kidney was recognised as a primary site of fullerene elimination and toxicity (nephropathy) (Chen et al., 1998b). The relevance of the findings following intraperitoneal injection for primary routes of exposure (inhalation, dermal and oral) has to be further examined in light of the questionable uptake via physiologically relevant exposure routes (see Section 3.1).

3.2.5. Biological mechanisms and target organ toxicity

3.2.5.1. Inflammation, cytotoxicity, pro and anti-oxidant properties. After inhalation exposure nanoparticles can be translocated from the lung and reach pulmonary alveolus, where they can persist for a long period. There they may induce oxidative stress with production of reactive oxygen species (ROS), persistent or progressive inflammation and causes irreversible chronic lesions such as fibrosis and even tumours. Some of these biological effects may be relevant for fullerenes, however many studies have also shown opposite results. Johnston et al. (2010) has recently reviewed the biological mechanisms driving fullerene toxicity and therefore this will not be discussed in detail and only a brief overview is given below.

In vitro investigations indicate that an inflammatory response may be instrumental to the toxicity of fullerenes, as demonstrated by the enhanced production of pro-inflammatory mediators such as interleukin 8 (IL-8) and tumour necrosis factor α (TNF-α) (e.g. Rouse et al., 2006; Park et al., 2010). A concentration-dependent effect is likely as Roursgaard et al. (2008) demonstrated that fullerols (hydroxylated fullerenes) have an anti-inflammatory effect within the mouse lung at lower doses, but a pro-inflammatory effect at higher concentrations, following intratracheal instillation.

Sayes et al. (2005) demonstrated cytotoxicity mediated through enhanced ROS production, lipid peroxidation and membrane damage for nano-C₆₀₀ (0.24–2400 ppb) in a variety of cell lines (dermal fibroblasts, hepatocytes and astrocytes). Similar observations were made for C₆₀₀ and C₆₀₀(OH)₁₈ eliciting membrane damage under photosensitive conditions, which was accounted for by the appearance of lipid peroxidation within isolated rat liver microsomes (Kamat et al., 2000). C₆₀₀(OH)₁₈ showed greater toxicity than C₆₀₀. No stimulation of ROS production, depletion of glutathione (GSH) or stimulation of haem oxygenase-1 (HO-1) expression and TNF-α production was associated with fullerol within RAW 264.7 macrophages (Xia et al., 2006).

A number of studies relating to anti-oxidant properties of fullerenes suggest that contrary to the suggestion that they might be toxic, C₆₀₀ and its derivatives could actually exhibit beneficial health effects through their potential free radical-scavenging activity (see for example Xiao et al., 2006; Wang et al., 1999; Gharbi et al., 2005; Yin et al., 2009). However, it appears that the anti-oxidant properties exhibited by fullerenes are restricted to particular fullerene forms and depend on parameters such as water solubility and the concentration administered. Fullerenes derivatised for their water solubility are more likely to interact to form larger structures, which can detrimentally impact on the anti-oxidant behaviour (for pro- and anti-oxidant behaviour, see also Johnston et al., 2010).

3.2.5.2. Cardiovascular effects. A positive association between adverse cardiovascular effects and exposure to ultrafine particles (especially from air pollution) have been described in several epidemiological and experimental studies (Simeonova, 2007) and such effects are also discussed for engineered nanoparticles.

In vitro investigations of endothelial cells following acute exposure to C₆₀₀(OH)₂₄ (1–100 μg/mL) resulted in internalisation by cells, and a dose dependent decrease in cell viability (Yamawaki and Iwai, 2006). Subsequent to a prolonged exposure (10 days), fullerenes detrimentally affected cell attachment and slowed cell growth. It was therefore speculated (by the authors) that exposure to fullerenes could be a potential risk for cardiovascular disease ignition or progression. Radomski et al. (2005) showed that fullerenes are less effective than other nanoparticles in eliciting the aggregation of platelets, suggesting that they are less thrombogenic. Injac et al. (2009) reported that C₆₀₀(OH)₂₄ had protective effects against doxorubicin-induced chronic cardiotoxicity and hepatotoxicity in rats with colorectal cancer.

Available information on cardiovascular effects from in vitro studies could be relevant if fullerenes have the potential to translocate from the site of exposure into the circulation, however there is no current evidence for this, and thus exposure of the vasculature to fullerenes is currently expected only after direct administration into the blood through injection. Though, cardiovascular effects may also be induced by inflammatory mediators released from the lung, as has been shown for example with carbon nanotubes (Simeonova and Erdely, 2009).

3.2.5.3. Immune effects. Exposure to ultrafine particles or nanoparticles can be associated with immunological effects (Chang, 2010). It has been shown for example with carbon nanotubes, that lung inflammation can induce the release of inflammatory mediators affecting also the immune system (Mitchell et al., 2009). However, fullerenes have shown to be (also) anti-inflammatory, due to their function as radical scavengers. Ryan et al. (2007) reported an unanticipated role of polyhydroxy C₆₀₀ or N-ethyl C₆₀₀ as a negative regulator of allergic responses. Antigen challenged cells (mast cells and peripheral blood basophils) showed in the presence of fullerene particles a significant inhibition of mast cell and basophil mediator release and an inhibition of IgE mediated cytoplasmatic ROS levels. These fullerene types also prevented the release of histamine and a fall in body temperature.

Liu et al. (2009) observed that water-soluble C₆₀₀(OH)₂₀ showed specific immunomodulatory effects to the immune cells, such as T cells and macrophages, both in vivo and in vitro. While they had almost no adverse effect to the viability of the immune cells, they stimulated the release of more cytokines, in particular TNF-α, which plays a key role in the cellular immune process to help eliminate abnormal cells. In vivo C₆₀₀(OH)₂₀ suppressed the growth of Lewis lung carcinoma, which is probably associated with an increased CD₄/CD₈ lymphocyte ratio.

Cai et al. (2010) observed that the fullerene derivative C₆₀₀(OH)₂₄ protected mice from ionising-radiation-induced mortality and suggested that this was due to enhanced immune function, decreased oxidative damage and improved mitochondrial function.

Following inhalation of 0.12 mg/m³ C₆₀₀ for 28 days, some genes associated with the immune system process, including major histocompatibility complex-mediated immunity were slightly upregulated (Fujita et al., 2009; see also Section 3.2.2).

In conclusion, there is little indication that exposure to fullerenes would adversely affect the immune system. On the contrary, polyhydroxylated fullerenes have shown beneficial effects, by inhibiting allergic or inflammatory responses and as a result they
are investigated for treatment of diseases involving such reactions (e.g. asthma, cancer).

From the above described studies it can be concluded, that fullerenes have a lower potential than other nanoparticles to induce inflammations and no systemic adverse effects are known so far. In any case adverse effects induced by fullerenes are expected to have a threshold and under this assumption the risk assessment will be performed (see also Section 3.8).

3.3. Irritation/corrosivity

3.3.1. Skin

3.3.1.1. In vivo animal tests. Aoshima et al. (2009b) investigated the toxicity potential of highly purified fullerenes (HPFs: mixture of C$_{60}$ and C$_{70}$, fullerite, 99.5% purity) in primary and cumulative skin irritation, skin sensitisation, skin photosensitisation, contact-phototoxicity, clinical patch and eye-irritation test (see below). In a Draize test (GLP) 0.5 g HPFs (in 0.3 mL propylene glycol (PG)) did not induce primary irritation to rabbit skin following 24 h of exposure and during evaluation at 0–48 h after removal of the patches. In a cumulative skin-irritation test 20 mg HPFs in 0.2 mL PG were applied repeatedly and clinical signs observed for 8 and 15 days. HPFs were considered not to induce cumulative skin irritation to rabbits. In a contact-phototoxicity test 25% HPFs applied on clipped free skin of guinea pigs and exposed to long-wavelength UV irradiation (11.2 J/cm$^2$) for 30 min did not reveal any phototoxic potential for up to 72 h after UV radiation.

3.3.1.2. Human information. Huczko et al. (1999) found no detrimental outcome in a patch test model that was used to assess the skin irritant potential of fullerene soot within 30 volunteers (who reported irritation and allergic susceptibilities) following a 96 h exposure time.

3.3.2. Eye

A Draize rabbit eye-irritation test was performed to reveal the potential toxicity of highly purified fullerenes (HPFs: mixture of C$_{60}$ and C$_{70}$, fullerite, 99.5% purity) to the eye (Aoshima et al., 2009b). Instillation of 0.1 g HPFs into the eyes of rabbits did not induce a positive response during the observation period of up to 4 days, although the eye washed group exhibited temporary mild irritation (after 24 h). Huczko et al. (1999) observed no toxicity of a fullerene soot suspension within the eye for up to 72 h.

From the available studies it can be concluded that the fullerene types tested did not show irritating effect to the skin or eye.

3.4. Sensitisation

3.4.1. In vivo animal tests

In a skin sensitisation test (as described by the author Aoshima et al., 2009b in accordance with Guidelines for Toxicity Studies of Drugs) HPFs were injected intra-dermally at concentrations of 50% (w/v) during the induction phase and 25% during the challenge phase. The same concentrations of HPFs were applied in a skin-photosensitisation test where application areas were additionally irradiated with a 10 J/cm$^2$ long-wavelength UV lamp for 30–31 min. No skin sensitising potential and no skin photosensitisation potential (up to 48 h after challenge and UV radiation, respectively) were found in guinea pigs. In the same publication a local lymph-node assay (LLNA) with HPFs reported no sensitivity (Aoshima et al., 2009b).

Following intraperitoneal injection in mice, a C$_{60}$ fullerene derivative conjugated to bovine thyroglobulin (in Freund’s adjuvant) induced antigenic behaviour by stimulating the generation of fullerene specific antibodies of the IgG isotype (Chen et al., 1998a). The findings were expanded upon by Erlanger et al. (2001) who demonstrated that anti-C$_{60}$ antibodies were able to interact with single walled carbon nanotubes, which was imaged using atomic force microscopy. The findings insinuated that C$_{60}$ derivatives may act as sensitising agents and thus have the potential to modulate immune responses.

3.4.2. Human information

HPFs (0.01 g on a Finn Chamber) showed no skin reaction at 1 and 24 h after exposure (occlusive conditions) in a human patch test (Aoshima et al., 2009b). Huczko et al. (1999) found no detrimental outcome in a patch test model with fullerene soot within 30 volunteers (who reported irritation and allergic susceptibilities) for a 96 h exposure time.

Available studies show that the fullerene types tested were not sensitising to the skin. Potential sensitising properties of specific fullerene derivatives following internal exposure are probably not relevant for natural exposure routes of humans.

3.5. Mutagenicity

Several genotoxicity tests have been conducted, mainly in vitro, in order to investigate the damage to DNA elicited by fullerene exposure. The results of these studies are summarised in Table 5. Bacterial reverse mutagenicity studies were negative for all tested fullerene types (C$_{60}$, fullerite, fullerene derivatives, lipo-fullerene), except in one study, where the mutagenic effects were seen under visible light (Sera et al., 1996). In this context it should be noted, that bacterial mutagenicity based assays may not be suitable for detecting genotoxicity induced by nanomaterials because prokaryotes lack the ability to perform endocytosis and the nanomaterials may not be able to diffuse across the bacterial cell wall; consequently this lack of uptake could potentially lead to false negative results (Singh et al., 2009).

Totsuka et al. (2009) showed a dose dependent increase of micronuclei in vitro and DNA damage and mutations in the lung (alkaline come assay and gpt mutations) following intratracheal instillation of C$_{60}$. The authors concluded that oxidative DNA damage might be commonly involved in the mutagenicity of small particles but that the genotoxic potency was not necessarily related to size, as also microsized Kaolin (4.8 um) induced the same effects. The applied particle doses of in this study were extremely high (0.2 mg/mouse) compared to expected human exposure at the workplace.

C$_{60}$ was negative in a comet assays (Jacobsen et al., 2008) and a chromosomal aberration test in vitro and in a micronucleus test in vivo (Shinohara et al. 2009b). Aqueous solution of colloidal C$_{60}$ were genotoxic (Dhawan et al., 2006); whereas fullerite, a mixture of C$_{60}$ and C$_{70}$ showed no chromosomal aberration up to high doses (Mori et al., 2006). Fullerolen (C$_{60}$(OH)$_5$)$_{1-3}$ which exert a strong anti-oxidative activity, may even protect against genotoxic effects. In in vitro assays they were shown to decrease the frequency of micronuclei and chromosomal aberrations in mitomycin C damaged cells (Mrdanovic et al., 2009). The protective effect was more effective at lower doses, which suggests a correlation with anti-oxidative effects at low fullerene doses, which however, can turn into a pro-oxidative response at higher doses.

The above mentioned studies do not allow drawing definitive conclusions on the presence or absence of genotoxic effects of the different types of fullerenes. The contradicting test results, indicating DNA damage in some cases but also the absence of such effects in others, and thus such effects are likely to be influenced by the dose, fullerene type and preparation, exposure time, cell type, model and endpoint measured. Fullerenes have been shown to both quench and, conversely, generate ROS, with evidence demonstrating that fullerenes are able to damage DNA potentially as a result of oxidative stress-based mechanisms. The photoactive
properties of fullerenes are anticipated to be an important component in initiating the genotoxic response. Fullerenes most likely do not have direct DNA reactive effects, with ROS formation playing a major role in mediating the damage to DNA that is observed.

### Table 5

In vitro and in vivo genotoxicity studies with fullerenes.

<table>
<thead>
<tr>
<th>Fullerene type</th>
<th>Concentration /duration</th>
<th>Genotoxicity assay</th>
<th>Results</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>In vitro</strong></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>(C_{60})</td>
<td>1.5–30 µg/plate; visible light irradiation (100 W, 20 min)</td>
<td>Ames test S. typhimurium (TA 102, TA104, YG 3003)</td>
<td>mutagenicity of (C_{60}) dependent on dose and duration of irradiation to visible light; ROS generated by irradiation of (C_{60}) by visible light; Addition of phospholipase A2 (release of unsaturated fatty acid) increased mutagenicity of (8\text{-OH-}dG) formation increased with increasing doses of (C_{60}); anti-oxidants (β-carotene) reduced mutation frequency</td>
<td>Sera et al. (1996)</td>
</tr>
<tr>
<td>(C_{60})</td>
<td>100 µg/mL (3 h)</td>
<td>Comet assay in A549 lung epithelial cells</td>
<td>No DNA damage; generation of FPG (formamidopyrimidin-glycosylase)-sensitive sites indicates oxidation of purines; no CII mutant frequency observed</td>
<td>Jacobsen et al. (2008)</td>
</tr>
<tr>
<td>(C_{60})</td>
<td>0.02–200 µg/mL</td>
<td>Micronucleus test in human lung carcinoma cells (A549)</td>
<td>Dose dependent increase of micronuclei &gt;0.02 µg/mL.</td>
<td>Totsuka et al. (2009)</td>
</tr>
<tr>
<td>(C_{60})</td>
<td>50–1000 µg/plate</td>
<td>Ames test S. typhimurium (TA 98, TA 100, TA1535, TA 1537); E. Coli (WP2uvrA/pKM101)</td>
<td>No mutagenic response up to 1000 µg/plate, regardless of metabolic activation and irradiation</td>
<td>Shinohara et al. (2009)</td>
</tr>
<tr>
<td>(C_{60})</td>
<td>12.5–200 µg/mL</td>
<td>Chromosomal aberration (CHL/IU hamster lung cells)</td>
<td>No increase in chromosomal aberration up to 100 and 200 µg/mL, regardless of metabolic activation and irradiation</td>
<td>Shinohara et al. (2009)</td>
</tr>
<tr>
<td>Aqueous suspensions of colloidal (C_{60}) fullerenes; (n_{C_{60}}: 0.022 µg/L–110 µg/L); EthOH(n_{C_{60}}: 0.42 µg/L–2100 µg/L)</td>
<td>Comet assay in human lymphocytes</td>
<td>Genotoxicity observed at: 2.2 µg/L (aqueous) and 4.2 µg/L (EthOH/(n_{C_{60}}))</td>
<td>Dhawan et al. (2006)</td>
<td></td>
</tr>
<tr>
<td>(C_{60}) derivatives</td>
<td>0.4–1.2 µg/mL</td>
<td>Ames test S. typhimurium (BA13 strain)</td>
<td>Exposure to visible light showed no effects for fullerenes, and antimutagenic effects for the derivative; [61]dimethoxyphosphoryl[61]carbethoxy-fullerenes, and antimutagenic effects for the derivative;</td>
<td>Babynin et al. (2002)</td>
</tr>
<tr>
<td>Mixture of (C_{60}) and (C_{60}) fullerite</td>
<td>39.1–5000 µg/plate; +/- S9 mix</td>
<td>Ames test S. typhimurium (TA 100, TA1535, TA 98, TA 1537); E. Coli (WP2uvrA/pKM101)</td>
<td>No mutagenic response detected up to 5000 µg/plate</td>
<td>Mori et al. (2006)</td>
</tr>
<tr>
<td>Mixture of (C_{60}) and (C_{60}) fullerite</td>
<td>625–5000 µg/mL; +/- S9 mix</td>
<td>Chromosomal aberration test (CHL/IU hamster lung cells)</td>
<td>No chromosomal aberration detected up to 5000 µg/mL; Dose dependent decrease in MN frequency; reduction of MN frequency in mitomycin C (MMC)-damaged cells (lower doses being more effective than higher doses)</td>
<td>Mori et al. (2006)</td>
</tr>
<tr>
<td>Fullerenol (C_{60}(OH)_{24})</td>
<td>55.4–221.6 µM (3 h); 25.7–110.8 µM (24 h)</td>
<td>Micronucleus (MN) in CHO K1 cells</td>
<td>No increase in chromosome frequency and reduction of CA frequency in MMC-damaged cells, higher fullerenol concentrations (44.3 µM) increased CA frequency, but was still lower than control</td>
<td>Mrdanović et al. (2009)</td>
</tr>
<tr>
<td>Fullerenol (C_{60}(OH)_{24})</td>
<td>11–44.3 µM (3, 24 h)</td>
<td>Chromosome aberration (CA) test in CHO K1 cells</td>
<td>Decrease in CA frequency and reduction of CA frequency in MMC-damaged cells, higher fullerenol concentrations (44.3 µM) increased CA frequency, but was still lower than control</td>
<td>Mrdanović et al. (2009)</td>
</tr>
<tr>
<td>Lipo-Fullerene (squalane and (C_{60}))</td>
<td>313–5000 µg/plate</td>
<td>Ames test, S. typhimurium (TA 98, TA 100, TA1535, TA 1537); E. Coli (WP2uvrA/pKM101)</td>
<td>No increase in mutagenicity at any of the tested doses</td>
<td>Kato et al. (2009)</td>
</tr>
<tr>
<td><strong>In vivo</strong></td>
<td></td>
<td></td>
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<tr>
<td>(C_{60})</td>
<td>0.064, 0.64 mg/kg bw in saline or corn oil</td>
<td>Oxidatively damaged DNA (8-oxodG) in colon, liver and lung following intragastric dose (oral gavage)</td>
<td>Elevated levels of 8-oxodG in liver and lung but not in colon mucosa; no increase in repair activity in liver; (the solvent corn oil showed the same effects).</td>
<td>Folkmann et al. (2009)</td>
</tr>
<tr>
<td>(C_{60}) (0.05% Tween 80 in saline)</td>
<td>0.05, 0.2 mg/animal (intratracheal instillation); 3 h</td>
<td>Alkaline comet assay in C57BL/6 mouse lung</td>
<td>Increase of DNA damage after 3 h, decrease after 24 h; (activation of DNA damage repair enzymes) intensity: (C_{60}) &gt; carbon black &gt; kaolin</td>
<td>Totsuka et al. (2009)</td>
</tr>
<tr>
<td>(C_{60})</td>
<td>Single and multiple (4x) doses of 0.2 mg/animal; examined 8 and 12 weeks, respectively after intratracheal instillation;</td>
<td>gpt(^4) mutations in lungs and kidneys of gpt transgenic mice</td>
<td>Lung: increase of gpt mutant frequencies; 2 (single dose) to 3-fold (multiple dose); no increase in Spi mutant frequencies; point mutations and deletions in the lung, but not in the kidneys</td>
<td>Totsuka et al. (2009)</td>
</tr>
<tr>
<td>(C_{60})</td>
<td>22, 45, 88 mg/kg in 20 mL/kg; two doses at 24 h intervals</td>
<td>Micronucleus test (OECD 474); oral gavage in ICR mice</td>
<td>No formation of micronuclei in bone marrow cells of mice</td>
<td>Shinohara et al. (2009)</td>
</tr>
</tbody>
</table>

\(^{4}\) gpt: guanine phosphoribosyltransferase.
However, appropriate in vitro and in vivo studies should deliver more information on mutagenic effects and the conditions influencing the test results.

3.6. Carcinogenicity

Fullerenes (derivatives) may have potential therapeutic properties for the treatment of cancer. Some studies have reported anti-tumour effects of fullerenes in vitro and in vivo, depending on derivatisation, dispersion, and light irradiation (Chen et al., 2005; Tabata et al., 1997; Chaudhuri et al., 2009). It would appear that fullerene can accumulate in tumours due to hyperpermeability of tumour vasculature with very low toxicity to other organs. Light irradiation seems to intensify a tumour destructive effect (Tabata et al., 1997).

Yin et al. (2008) demonstrated the ROS scavenging properties of three different functionalised fullerene materials (Gd@C(60)(OH)22, C60(OH)22 and C60(COOH)22) and concluded that these fullerene derivatives may be valuable in vitro cytotoxicity and therapeutic agents. Following intraperitoneal administration, Gd@C(60)(OH)22 a gadolinium based metallofullerol, with potential use in chemotherapy, has been demonstrated to inhibit the growth of malignant tumours within mice, and that this was due to its ROS scavenging activity (Chen et al., 2005). Liu et al. (2009) demonstrated that water-soluble C60(OH)20 nanoparticles can stimulate immune cells to release more cytokines, in particular TNF-α, which plays a key role in the cellular immune process to help eliminate abnormal cells. They observed that C60(OH)20 was able to inhibit tumour growth in mice efficiently with almost no adverse effects (low cytotoxicity inducing neither cell death nor affecting the viability of lymphocytes and macrophages).

There is one carcinogenicity study available with fullerenes (C60), long multivalved carbon nanotubes (MWCNTs) or crocidolite asbestos administered via a single intraperitoneal injection in p53(+/-) mice (Takagi et al., 2008), p53 deficient mice are not only exquisitely sensitive to (genotoxic) carcinogens because of their inability to repair genotoxic damage and/or to clear damaged cells through apoptosis but also to reactive oxygen species (ROS)-related carcinogenesis. The fullerene group showed no peritoneal adhesion, fibrous thickening nor tumour induction. Only small black plaques were scattered on the serosal surface. Long MWCNT and asbestos had the greatest carcinogenic potential and induced mesotheliomas. A weakness of the study which impacts on the ability to draw a conclusion regarding the absence of carcinogenic effects of fullerenes, is the relatively short exposure time. It is important to note that excessively high concentrations (3 mg/animal in 1 ml suspension) were used and the study was terminated at week 25 for all treatment groups, due to the high mortality with MWCNT treated animals.

Zagovic et al. (2009) compared the effects of nanocrystalline fullerene (nannoC(60)) on tumour cell growth in vitro and in vivo. In vitro, nannoC(60) caused oxidative stress, mitochondrial depolarisation and caspase activation, leading to apoptotic and necrotic death in mouse B16 melanoma cells. In vivo, following intraperitoneal administration, nannoC(60) over the course of two weeks starting from melanoma cell implantation not only failed to reduce, but significantly augmented tumour growth. These data demonstrate that nannoC(60), in contrast to its potent anticancer activity in vitro, can potentiate tumour growth in vivo, possible by causing NO-dependent suppression of anticancer immune response.

Despite potential applications of fullerenes (more specifically polyhydroxylated fullerenes) as anti-tumour agents, the evidence is not sufficient to draw firm conclusions that fullerenes are not carcinogenic. No chronic/carcinogenicity studies using physiologically relevant exposure routes of exposure are available. The lungs are the most probably target organ following inhalation and possible accumulation of fullerenes. ROS formation may occur due to secondary messengers and DNA-damaging effects, causing mutations and thereby inducing cancer of the lungs. Water-soluble fullerols have been shown to cause anti-inflammatory effects, but could promote lung inflammation at high concentrations. Thus the potential mutagenic and/or carcinogenic effects of fullerenes appear highly dependent on the applied dose and the fullerene type.

More information is needed to conclude on the absence or presence of possible carcinogenic effects of fullerenes and the conditions (properties, doses) driving the effects. Any conclusion or strategy for further testing for carcinogenicity is dependent on more information on toxicokinetics and mutagenicity and on the nature of effects seen in sub-chronic/chronic studies.

3.7. Toxicity for reproduction

3.7.1. Effects on fertility

No information on the impacts of fullerenes on the male reproductive system has been identified. The only identified information on the female reproductive system is a cytotoxicity test of fullerene C60 particles (10 mg, dissolved in 250 mL tetrahydrofuran (THF)) in Chinese hamster ovary mammalian cell line (CHO) which showed a dose and time dependent potential toxicity with an LD10 of 33 mg/L determined within 24 h (Han and Karim, 2009). The use of THF as a solvent in the study and the formation of highly reactive side products during THF C60 suspension procedure (Spohn et al. 2009) renders this evidence of little use for risk assessment purposes. Furthermore, the CHO cell line is used only as a model, not specifically to investigate reproductive effects.

3.7.2. Developmental toxicity

One in vivo mammalian study on the effects of fullerenes on the developing embryo has been identified (Tsuchiya et al., 1996). Following intraperitoneal administration of polyvinylpyrrolidone (PVP) solubilised C60 (up to 137 mg/kg, in distilled water) to pregnant mice, on gestation day 10 effects such as abnormal enlargement of the head and tail abnormalities were seen from 50 mg/kg onwards. At the highest dose (137 mg/kg) dead embryos were also found. At 25 mg/kg, one embryo had abnormal enlargement of the head, whereas all other embryos appeared normal. Embryos might have been insufficiently supplied by blood, as the yolk sack appeared with shrunken membrane and narrow blood vessels. The NOAEL was determined to be 16.7 mg/kg. The relevance of the results of this study for risk assessment is questionable due to the limitations of the study (i.e. low number of animals per exposure group) and the unusual route of administration, using a relatively high exposure dose and covering only a small part of the pregnancy period. Further it has to be considered that PVP forms a charge transfer complex with fullerenes which can cross the placental barrier (while the toxic PVP alone cannot) and therefore the observed toxicity is most probably to be attributed to the PVP and not the C60 (Kolosnjaj et al., 2007).

Two studies were identified which assessed effects on embryonic development using the zebra fish model. Zhu et al. (2008) demonstrated that fullerene (C60(OH)16–18) had no adverse effects on newly fertilised eggs whereas a C60 suspension had a conspicuous adverse effect on all parameters (survival, hatching rate, heart beat rate and pericardial oedema) that was lessened by the addition of the anti-oxidant GSH (glutathione). This suggests that the adverse effects of C60 were due, at least in part, to a free radical-induced mechanism or another form of oxidative stress. Usenko et al. (2008) showed that conditions of reduced light (and therefore reduced photocatalytic activity) and co-exposure to the GSH precursor, N-acetylcysteine (NAC), reduced the toxic effects of C60 on zebra fish embryos (e.g. reduced mortality and
pericardial oedema). Fin malformations were only reduced with reduced light but not in the presence of NAC.

In summary, the identified information indicates that fullerenes can have effects on the developing embryo as shown in mice and zebra fish. However, the mammalian developmental toxicity study has limitations and used a non physiologically relevant route of exposure. In any case it is questionable if indirect effects due to insufficient supply of the embryo via the placenta following internal exposure to high doses can be considered relevant to human exposure situations. No studies have been identified that focused on other organs or cell types in the female and male reproductive system.

More information is needed to show if the identified results are relevant for reproductive organs and embryos via primary routes of exposure and for humans at relevant exposure concentrations. In addition, more information on adsorption and distribution of fullerenes in the body would be required to decide whether reproductive organs would be potential target organs of fullerene exposure.

3.8. Establishing human no-effect levels

Based on the information identified from toxicity studies, it can be assumed that the effects caused by fullerenes, such as inflammation and oxidative responses, have a threshold. It has even been suggested that at lower concentrations, some forms of fullerenes may have an anti-inflammatory and anti-oxidative effect. There is not sufficient information available to conclude on non-threshold genotoxicity or other possible non-threshold effects. However, it seems appropriate to derive human indicative no-effect levels (INELs) based on the assumption of threshold effects of fullerenes. This INEL is derived for the purpose of this study only and can be used as reference point for future studies. The term DNEL (derived no-effect level) as used under REACH is avoided to not give the impression that the values suggested in this manuscript could be used for a regulatory risk assessment.

The INELs presented in this paper are derived from the dose descriptor of one selected key study with modifications to the starting point and by applying assessment factors, as described in the REACH guidance on information requirements and chemical safety assessment (see Chapter R.8 in ECHA, 2008 for details), being aware however that this guidance was not developed to address specific characteristics of nanomaterials.

INELs are only derived for inhalation, as there was no appropriate data for the dermal and oral route. The selected studies were mainly looking at endpoints like inflammation and no chronic and carcinogenicity studies have been identified. Therefore it is not known, whether under different test conditions and with other fullerene types different INELs would be derived. It is important to note that different fullerene types should be evaluated separately and thus the INELs below are suggested for the specific fullerene types tested and cannot be used for fullerenes in general. Results from this risk characterisation should not be used for any regulatory decision making.

3.8.1. Inhalation

For inhalation a NOAEC of 2.22 mg/m³ C₆₀ (3 h/day in rats for 10 days; Baker et al., 2008) has been identified, based on the absence of inflammatory effects. As discussed in the hazard (Section 3.2.2) it seems justified using this NOAEC based on a weight of evidence in a risk assessment appraisal for short term and for chronic exposure.

The selected study is not a guideline study and was not performed for the purpose of a risk assessment. However, considering the amount and quality of information the study provides and the fact that other similar studies support the results by showing low toxicity the NOAEC of this study can be considered useful for a risk characterisation appraisal.

3.8.1.1. Modification of the starting point. The inhalation toxicity study was conducted for a duration of 3 h/day. Workers are assumed to be exposed for 8 h per day at light activity (i.e. a slightly higher breathing rate as compared to rest in the experiment). The NOAEC is modified as follows (see Chapter R.8 in ECHA, 2008 for details).

Corrected NOAECₜₚ for 8 working hours

\[ \text{NOAEC}_{\text{8h}} = \frac{\text{NOAEC}}{8} \]

For the general public, continuous exposure

\[ \text{NOAEC}_{\text{8h}} = \frac{\text{NOAEC}_{\text{3h}}}{8} \]

Correction for the difference in respiratory volume between animals at rest and workers at light activity (not applied for the general public) = 0.83 mg/m³ × 6 m³/10 m³ = 0.55 mg/m³

3.8.1.2. Assessment factors. For interspecies variation allometric scaling is not applicable, as the effects (local) do not depend on metabolic rate or systemic absorption and thus only the default factor of 2.5 for other interspecies variation is applied.

For intraspecies variation the default factor of 5 for workers and 10 for the general public is applied.

The default value of 6 for extrapolation of the duration from sub-acute to chronic is suggested. Even though the exposure period of 10 days in the current study is shorter than a sub-acute study, it is still considered a conservative approach as no exacerbation of effects over time has been reported from other studies.

3.8.1.3. Other factors. No further assessment factor is applied for the severity of the effect, as this is not considered appropriate. An assessment factor for confidence in the database due to the limited data available could be discussed, however as the selected NOAEC and the above mentioned assessment factors are considered rather conservative, no such factor is suggested.

Altogether an overall assessment factor of 12.5 (2.5 × 5) for acute and of 75 (2.5 × 5 × 6) for chronic exposure of workers are calculated. The overall assessment factor for chronic inhalation of the general public is 150 (2.5 × 10 × 6).

The INELshort term for worker inhalation is 0.55 mg/m³/12.5 = 0.044 mg/m³ or 44.4 μg/m³.

The INELchronic for worker inhalation is 0.55 mg/m³/75 = 0.0074 mg/m³ or 7.4 μg/m³.

The INELchronic for the general public is 0.28 mg/m³/150 = 0.0019 mg/m³ or 1.9 μg/m³.

4. Discussion and risk characterisation

4.1. Risk characterisation for different exposure routes

4.1.1. Risk following oral exposure

Fullerenes showed very low toxicity after oral exposure and an acute NOAEL of >2000 mg/kg bw for fullerites and of >2500 mg/kg bw for polylkylsulfonated C₆₀ are suggested. There is limited absorption of pristine fullerenes from the gut, indicating that after repeated exposure no toxicity may be expected. However functionalised fullerenes with a higher solubility may behave differently.
Currently no oral exposure estimations are known and it is expected that exposure via the oral route is low. In conclusion, no quantitative risk characterisation for this exposure route will be performed, but qualitatively no or very low risk via this route is expected.

4.1.2. Risk following inhalation

The respiratory tract is considered to be a major portal of nanoparticle entry, because of the likelihood that NPs will become airborne during handling. Exposure to fullerenes via inhalation is mainly expected to occur within the workplace. Environmental exposure of humans to manufactured fullerenes is not assumed to play a role yet, but might become more important once fullerenes are produced in higher volumes. No fullerene containing consumer products were identified, where inhalation could be a relevant exposure route.

Following inhalation, fullerenes are probably not systemically absorbed and remain within the lungs, from where they can be cleared or biotransformed. The effects seen in the lung were primarily the induction of pro- but also anti-inflammatory responses. Human no-effect levels (INEL) were derived from a 10 day inhalation study (see Section 3.8.1).

For worker the INEL_{short term} was determined to be 44.4 \mu g/m^3, and the INEL_{chronic}, 7.4 \mu g/m^3; for the general public the INEL_{chronic} was determined to be 1.9 \mu g/m^3.

Depending on the exposure data to which these INELs are compared, a risk or no risk can be identified. The short term INEL for workers is in the same order of magnitude as the lower range of the unspecific workplace exposure for carbonaceous particles with a high background level (50–125 \mu g/m^3) and the chronic INELs are much lower, which points to a possible risk. However the INELs are much above the more specific fullerenes exposure levels (0.004 to <2 \mu g/m^3), generated under good occupational hygiene conditions (without use of personal protective equipment) and therefore under such conditions no risk for inhalation exposure to fullerenes is expected. It should be noted that the higher value of 2 \mu g/m^3 was the highest possible concentration, including all particles sizes and is therefore also likely an overestimation of possible fullerene exposure. The exposure values from the manufacture of secondary products of 0.54 \mu g/m^3 (without consideration of engineering control and respiratory protective equipment) are lower than the acute and chronic INEL, indicating that under these conditions no (elevated) risk is expected. However there might be downstream handling of fullerenes including other activities and less risk management activities, which could lead to higher exposure levels.

The estimated environmental exposure near a fullerene factory (2.2 \times 10^{-4} \mu g/m^3) is by a factor of 10^3 lower than the estimated human no-effect level and therefore no risk is expected.

There are substantial uncertainties in this risk characterisation in terms of both exposure and effects components and therefore these results should not be used for any regulatory decision. See also discussion below (Section 4.2).

4.1.3. Risk following dermal exposure

Exposure to fullerenes via the dermal route may occur within the workplace from contaminated surfaces or handling of fullerenes in organic solvents. A workplace exposure based on a read across to SWCNTs gives a rough estimation of 12 mg/person/day (reduced by the use of gloves to 1.2 mg/person/day). No toxicity data on dermal effects has been identified that would allow a derived human no-effect level to be compared to exposure data. Therefore no quantitative risk assessment is possible, however based on the currently available information and on the argumentation below, the risk is expected to be rather low.

Adsorption/penetration of fullerenes into the epidermis, but not dermis has been shown in some studies, depending on the fullerene type (functionalisation), solvent in which fullerenes are suspended and on the skin properties. Depending on these factors, absorption can probably be close to zero (or excluded) but can also be considered to be relevant. Currently there is a project ongoing to establish a structure permeability relationship for skin absorption of manufactured nanomaterials for safety evaluation and risk assessment (<www.nanotechproject.org/inventories/ehs/brows/projects/6342>) which could also provide useful information on fullerenes.

The possibility of skin absorption seems highly relevant for the use of fullerene in cosmetics, and in particular their exploitation within skin creams. Fullerenes types used in skin creams have been described to be highly purified fullerene, polyvinylpyrrolidone wrapped fullerenes or fullerenes in vegetable squalene. Studies have shown that fullerenes in mineral oil and Squalane (lipo-fullerenes) do not penetrate to deeper skin layers and therefore absorption via the skin route is considered unlikely. So far, in vivo tests have shown very low toxicity of fullerenes to the skin. Highly purified fullerenes and polyvinylpyrrolidone wrapped fullerenes were not irritating or sensitising to the skin with and without UV radiation at concentrations most likely higher than those used in cosmetics.

It is not known if other fullerene types are used in cosmetics and therefore conclusions on fullerenes in general cannot be made. In addition, no studies have been identified that investigated prolonged exposure and therefore no conclusions on chronic dermal effects of fullerenes can be drawn.

Consumer exposure from other consumer products may also occur through abrasion, but no quantitative data are available. However, such exposure is not expected to be very high.

4.2. Considerations on the risk assessment and the methodology

The risk assessment appraisal is drawn upon publicly available data at the time of drafting the manuscript (May 2010) and has therefore several limitations. This risk assessment appraisal followed the methodology as suggested in REACH, which is however not specific for nanoparticles. Some considerations of the different factors contributing to the limitations are discussed below.

4.2.1. Exposure

Currently available information on occupational exposure gives information mainly on release and potential exposure to fullerenes in the workplace. Available techniques do also allow determining whether engineering controls are effective in preventing exposure in occupational settings. However it is difficult to retrieve specific information on fullerenes exposure, which can be used for a risk assessment.

Measurements of particle concentrations or mass are sometimes not specific for the nanomaterial of interest and the background levels are sometimes quite high. Measurements near the emission source give indications of the increase of particles due to certain activities, but the concentration might be different in the breathing zone of the worker, and this would be the more relevant concentration to be used for the risk assessment.

Discussions are ongoing regarding what the best metrics measured for a correlation with observed toxic effect are. Some authors suggested that the surface area of the particles appears to be better suited as a dose parameter (see e.g. Oberdörster 1996; Tran et al., 2000; Rushton et al., 2010), whereas others (e.g. Pauluhn 2009) conclude that for nanosized particles the key metric is particle mass. A unifying, most appropriate metric of nanoparticles conferring pulmonary biopersistence and toxicity has probably not been demonstrated yet. As from the inhalation toxicity studies only concentrations in mass/volume were available, it was the most practical way forward to use them in the risk assessment.
For a risk assessment it is also important to measure to which particle size humans are most likely exposed to. Nanoparticles can agglomerate and form particles of much bigger size. Depending on their size the particles can reach different areas of the lung where they can be either retained or more easily be removed. The deposition site and the persistency of the particle in the lung would then influence the nature and the severity of potential effects.

For a first tier it seems appropriate to consider the exposure to all particle sizes, as this is considered to represent a worst case. However if such comparison leads to a conclusion of a risk, a refinement with better defined exposure data needs to be made.

4.2.2. Assessment factors in the risk assessment

The above described methodology for deriving a human indicative no-effect levels, applied default modification and assessment factors to the NOAEC by following the REACH guidance on information requirements and chemical safety assessment (see Chapter R.8 in ECHA, 2008 for details).

For the modification to a human situation the different respiratory volumes between rest and light activity (for workers) were taken into consideration. For “other” interspecies differences the default factor of 2.5 was applied. These factors might not be specific enough to consider the nanoparticle specific properties, as the behaviour in the respiratory tract (deposition, persistence) and the nature of toxic effects. Factors that probably have to be considered to account for the differences between laboratory animals and humans include pulmonary deposition, retention half time, alveolar deposition, alveolar macrophage volume or surface area of pulmonary alveoli (see e.g. derivation of occupational exposure limit by Pauluhn 2010, and NEDO risk assessment by Shinohara et al., 2009a).

Without going into details, how these different factors were calculated and used for accounting the differences between rodents and humans, the final factor was in one case 2 (calculated for carbon nanotubes; Pauluhn 2010) and in one case 0.01 (mainly triggered by a bigger surface area of pulmonary alveolus in humans, Shinohara et al., 2009a). This comparison shows, that the default factor of 2.5 in addition to the correction for the respiratory volume is probably to be considered conservative and a lower factor might be suggested, if such a simplification of the different factors is justified.

The above mentioned available risk assessment (e.g. NEDO project, Shinohara et al., 2009a) or proposal for deriving an occupational exposure limit (Pauluhn, 2010) have suggested a factor of 1 for inter-individual differences between workers, as they are not considered a sensitive population. This view is not supported, as some workers may have a predeposition to react more sensitive to particle exposure, e.g. asthma. Therefore a factor of >1 seems justified, but a lower value of 5 might be discussed, if the differences between individuals to show adverse effects following nanoparticle exposures are shown to be smaller than those following chemicals exposure. For the general public a factor of 10 was suggested and this should not be lowered as it includes all sensitive subpopulations.

In the risk assessment appraisal a factor of 6 was used for extrapolation of the duration from the sub-acute to the chronic duration. This factor would change when more information from a longer duration study (sub-chronic or chronic) and/or on the progression of the effects with dose and time becomes available.

In conclusion, the current methodology as in the Technical Guidance Document for performing a risk assessment should be evaluated for its applicability for nanomaterials. There are currently two projects ongoing (REACH Implementation Projects RIP-oN 2 and 3) which aim at developing advice on how guidance on information requirement and chemical safety assessment could be updated to address the specific properties of nanoparticles.

5. Overall conclusions and recommendations

Available information from open literature has been evaluated with an attempt to characterise possible risks for humans exposed to fullerenes at the workplace, as consumers or indirectly via the environment. Recommendations for generating a better dataset are reflected below.

This risk characterisation appraisal has shown that, based on the available information the determination of a possible risk depends primarily on type and quality of information used for the risk assessment. A risk can principally not be excluded especially from chronic inhalation exposure to fullerenes at the workplace, however under good occupational hygiene conditions there is probably low concern for a risk. No appropriate quantitative data for dermal exposure were available to make any definite conclusions, but qualitatively it can be concluded that based on the available information a risk would probably not be expected. In any case it is important to prevent exposure by appropriate precautionary measures and by practising best industrial hygiene.

In terms of consumers, a potential risk could arise from dermal exposure to skin creams due to their direct application and potential widespread use. Available information has shown that tested fullerene types are probably not absorbed, are non-irritating and non-sensitising and may have low dermal toxicity. However, there is no experience from chronic exposure and it is not possible to make generalisations across all fullerene types.

Environmental exposure of humans is not considered to be an exposure route of concern at present, but might become more important once fullerenes are produced in higher volumes.

The conclusions of this risk characterisation appraisal are based on the limited amount of identified information and include many uncertainties with respect to the quality and representativeness of the data for both toxicity and exposure. The derived human no-effect levels cover only inhalation exposure. No suitable data has been identified for chronic exposure and for endpoints such as genotoxicity, carcinogenicity and reproductive toxicity. The risk characterisation would normally be conducted on the leading effect or the lowest human no-effect level for a given exposure pattern, but this not yet known. Thus, the results of this risk characterisation should not be used for any decision making or regulatory risk assessment.

The following priority activities are recommended in order to support an effective risk assessment:

- Generation of reliable exposure data (measurements or models) for fullerenes, enabling the identification and characterisation of fullerenes and their distinction from background particles within workplaces. It should also carefully consider agglomeration effects. For consumer exposure, dermal exposure data is of highest priority;
- Further investigations into fullerene toxicokinetics, particularly the generation of data to provide clarity on which fullerenes can become systemically available following inhalation, dermal and oral exposure and possibly induce systemic toxicity. The sensitivity of detection methods and their influence on the results should be carefully taken into account;
- Conducting repeated dose toxicity studies via inhalation at workplace-relevant concentrations to detect local and possible systemic effects;
- Generation of more data on hazards of fullerenes via the dermal route at concentrations and conditions (i.e. solvents) relevant for consumer
Further in vitro and in vivo investigations to determine primary and/or secondary genotoxic effects; Depending on results from genotoxicity and sub-acute/sub-chronic studies, a testing strategy for carcinogenicity/chronic toxicity should potentially be developed; Depending on results from absorption studies (systemic availability) and indications of effects on reproductive organs/hormones from a repeat dose toxicity study, a testing strategy for reproductive toxicity should potentially be developed.

In any case, all future studies should carefully characterise the materials tested. Fullerenes exist in a variety of forms, varying in terms of carbon number, surface modifications, aggregation states etc. It is therefore problematic to make generalisations about their toxicity and risk, and it is also difficult to devise a testing strategy that could be applied to all fullerene types. In addition, currently available toxicity tests were not designed specifically for nanoparticles. Therefore new testing paradigms need to be invented for the evaluation and assessment especially of the inhalation toxicity of nanoparticles. Also the risk assessment methodology as currently used for the evaluation of chemicals needs adaptation to account for the specific properties of nanoparticles.

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