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Hazard assessment of nanomaterials in consumer products

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Hazard assessment of nanomaterials in consumer products

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Preface

Nanomaterials are applied in a wide range of consumer products, and the commercial use of nanomaterials in both amounts and diversity is anticipated to increase rapidly in the near future. It is increasingly recognised that nanomaterials can have unique properties as compared to their bulk substances favouring the use of nanomaterials in products, articles and technologies. At the same time concerns in relation to the possible health and environmental properties and impacts of nanomaterials have surfaced.

On this background, the Danish government and the Red-Green Alliance (a.k.a. Enhedslisten) have signed an agreement for four years (2012-2015) that focuses on the use of nanomaterials in products on the Danish market and their consequences on consumers and the environment. The Danish Environmental Protection Agency (EPA) has initiated a series of projects with the aim of further clarifying possible risks to consumers and the environment.

The current project addresses consumer exposure and risk assessment of nanomaterials in products on the Danish market. It runs from third quarter 2013 through second quarter 2015.

The project is foreseen to result in four reports:

- Occurrence and exposure assessment of nanomaterials in consumer products and review of available risk assessment tools
- Hazard assessment of nanomaterials in consumer products (the current report)
- Human exposure to nanomaterials in the environment – as a reference to nanomaterials exposure from consumer products
- Consumer risk assessment and overall conclusions (final report)

The first three reports will be finalised during 2014, whereas the final report with the consumer risk assessment and overall conclusions will be finalised during the second quarter of 2015.

The project has been implemented with support from a reference group:

- Susan Dekker, National Institute for Public Health and the Environment (RIVM), The Netherlands
- Andrea Haase, Bundesinstitut für Risikobewertung (BfR), Germany
- Gregory Moore, Swedish Chemicals Agency (KEMI), Sweden
- Derk Brouwer, Netherlands Organisation for Applied Scientific Research (TNO), The Netherlands
- Lena Høglund (The Danish EPA), Denmark
- Katrine Bom (The Danish EPA), Denmark
- Anne Mette Boisen (The Danish EPA), Denmark
- Kim Petersen (The Danish EPA), Denmark

The reference group has assisted with comments and ideas but is not responsible for the content of the project reports.

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Abbreviations and acronyms

3T3	NIH 3T3 rodent embryonic fibroblasts
5-HT	5-hydroxy-tryptamine / Serotonin (neurotransmitter)
ACGIH	American Conference of Governmental Industrial Hygienists
ADME	Absorption-Distribution-Metabolism-Excretion
BAL	BronchoAlveolar Lavage
BALB	Bagg Albino (inbread mouse strain)
BALF	BronchoAlveolar Lavage Fluid
BBB	Blood-Brain Barrier
bw	Body Weight
CB	Carbon Black
CNS	Central Nervous System
CNT	Carbon NanoTubes
CRP	C-Reactive Protein (acute phase protein)
DA	DopAmine (neurotransmitter)
DNEL	Derived No Effect Level
DNT	Developmental NeuroToxicity
ECETOC	European Centre for Ecotoxicology and Toxicology Of Chemicals
ECHA	European CHemicals Agency
EFSA	European Food Safety Agency
ENP	Engineered NanoParticles
EPA	Environmental Protection Agency
GI	Gastro Intestinal
HARN	High Aspect Ratio Nanomaterials
IARC	International Agency for Research on Cancer
IL	InterLeukin
INEL	Indicative No-Effect Level
ICP-MS	Inductively Coupled Plasma Mass Spectrometry
LD_{50}	Lethal Dose, 50 %
LDH	Lactic Acid Dehydrogenase
LOAEC	Lowest Observed Adverse Effect Concentration
LOAEL	Lowest Observed Adverse Effect Level

MCMC	Mean Cell Haemoglobin Concentration
Mg-PSZ	Magnesia Partially Stabilized Zirconia
MNC	Montmorillonite NanoComposite
MnO	Manganese Oxide
MRC-5	Medical Research Council cell strain 5
MRI	Magnetic Resonance Imaging
MTT assay	Monocyte mediated cytotoxicity assay
MWCNT	MultiWall Carbon Nanotubes
NA	NorAdrenaline (neurotransmitter)
Nano-	Abbreviation used in words such as nano-sized or nano-Ag
NICNAS	National Industrial Chemicals Notification and Assessment Scheme
NIOSH	National Institute for Occupational Safety and Health
NIST	National Institute of Standards and Technology
NOAEC	No Observed Adverse Effect Concentration
NOAEL	No Observed Adverse Effect Level
OECD	Organisation for Economic Co-operation and Development
OEL	Occupational Exposure Limit
OSHA	Occupational Safety and Health Administration
PBS	Phosphate Buffered Saline
PEG	PolyEthyleneGlycol
REACH	Registration, Evaluation, Authorisation and Restriction of Chemicals
ROS	Reactive Oxygen Species
SAS	Synthetic Amorphous Silica
SCCS	Scientific Committee on Consumer Safety
SCENIHR	Scientific Committee on Emerging and Newly Identified Health Risks
SWCNT	SingleWalled Carbon NanoTubes
TEM	Transmission Electron Microscopy
TGF-β	Transforming Growth Factor beta
TODA	3,6,9-TriOxaDecanoic Acid
US EPA	United States Environmental Protection Agency
UV	Ultraviolet
WHO	World Health Organization
Y-TZP	Yttrium-stabilized Tetragonal Zirconia Polycrystals

Conclusion and Summary

Background
Under the Agreement "Better Control of Nanomaterials" (“Bedre styr på nanomaterialer”), the Danish Environmental Protection Agency (Danish EPA) has commissioned a number of projects aiming to investigate and generate new knowledge on the presence of nanomaterials in products on the Danish market and to assess the possible associated risks to consumers and the environment. This report is part of a series of four from a project which addresses consumer exposure and risk assessment of nanomaterials in products on the Danish market.

The aim of this report is to perform a hazard assessment of nanomaterials in consumer products. The consumer is potentially exposed to nanomaterials in their final, intended use, i.e. when the nanomaterials are part of a matrix, and therefore, this report focuses on the hazard of nanomaterials when part of a consumer matrix. However, free nanomaterials may be liberated during the use phase and therefore the hazard of pristine nanomaterials is also described.

The aim of the report is to refer consensus rather than isolated findings. Since the focus of the hazard evaluation is on nanomaterials during their intended use, the referred literature concerning pristine nanomaterials consists primarily of reviews, while all identified original studies of hazard related to nanomaterials as part of a product are described.

The structure of the report is that we review the data relevant to assessing the hazard of nanomaterials in consumer products, i.e. biokinetics of nanomaterials (Chapter 2), adverse effects of pristine nanomaterials (Chapter 3), adverse effects of nanomaterials when part of a matrix (Chapter 4), physico-chemical factors of importance for toxicity (Chapter 5), perform a hazard assessment of a selection of nanomaterials (Chapter 6), discuss how to bridge between hazard and risk assessment (Capter 7) and finally we summarise and conclude (Chapter 8).

Biokinetics
For the consumer, the lungs, gastrointestinal tract and the skin are considered to be the primary absorption routes for nanomaterials. In addition, uptake may occur through the eyes and the olfactory system. We did not identify any studies covering how biokinetics are modified when the nanomaterial is part of a matrix.

Inhalation of particles results in deposition of particles in the respiratory tract (nasopharyngeal, tracheobronchial and alveolar regions). The fate of the particles after deposition depends on a combination of physico-chemical properties of the particles and responses both locally in the lung and in other parts of the body. The larger particles are trapped by the mucociliary system in the upper airways and are removed relatively fast (hours to days). In contrast, the smaller particles deposit in the alveolar region where they can stay for years in humans. A low degree of translocation of particles from the lung to the circulation has been described.

In 2013 the Danish EPA published in 2013 a report on systemic absorption of nanomaterials by oral exposure which gives an overview of the existing literature (Danish EPA, 2013c). In general, the reviewed literature indicates that the gastrointestinal absorption is low. It is concluded that physico-chemical characteristics such as size, agglomeration and crystal structure may affect absorption. The report concludes that the number of publications on gastrointestinal absorption is increasing, but only a few of these have been well performed. In particular it is of concern that only

a few of the physico-chemical parameters that are expected to influence absorption of nanoparticles were reported.

In 2013 the Danish EPA has published a report on dermal absorption of nanomaterials which provides an overview and evaluation of the knowledge base regarding dermal absorption of nanomaterials (Danish EPA, 2013b). The overall conclusion in the report is that absorption of particles in the nano-range through the skin is possible although it seems to occur to a very low degree. However, the level of penetration, depending on chemistry and experimental conditions, may be greater for particles in the nano-range than for larger particles.

The present knowledge about absorption of nanomaterials into the eye and the eye toxicity is too limited to be assessed and evaluated in general for the hazard assessment.

Olfactory absorption of nanomaterials has been demonstrated in laboratory animals and may also be relevant for humans (Elder et al., 2006). At present, consistent knowledge is too limited for hazard assessment in general or specifically.

Only a limited number of studies have investigated the potential metabolism of nanomaterials. Where such information exists, most of the studies show that the nanomaterials, and in particular insoluble nanomaterials, are not metabolised (Landsiedel et al., 2012).

Upon absorption following pulmonary, dermal, eye or gastrointestinal exposure, nanomaterials may reach the blood circulation and/or the lymphatic system. The liver is the major distribution organ and 40 nm Au particles have been shown to accumulate in the Kupffer cells of the liver rather than being excreted (Sadauskas et al., 2009a). The placenta constitutes the nutritional interface between mother and fetus. Studies of the passage of engineered nanoparticles (ENP) across the placenta are still few in number, but rodent studies describe transplacental transport of nano-sized particles, with rates ranging from almost negligible to high (several percent of the administered dose).

Adverse effects of pristine nanomaterials

Pulmonary exposure to nanomaterials may cause pulmonary inflammation, fibrosis, DNA damage and cancer. Concern has been raised that pulmonary exposure may also result in adverse cardiovascular effects as seen for human exposure to particles from the ambient air. The mechanisms behind the cardiovascular effects are suggested to be a systemic inflammatory and acute phase response, particle translocation or respiratory reflexes.

The knowledge on the toxicity of orally administered nanomaterials in the gastrointestinal tract is limited and it is therefore not possible to identify any overall conclusions regarding the toxicity of nanomaterials for intended use in food and food-related products (Card et al., 2011). However, the toxicity of the nanoformulation of a specific ingredient or material was not always consistently increased as compared to the non-nanoformulation.

Overall, there is little evidence of dermal toxicity following topical application of nanomaterials. In general, the uptake through intact healthy skin is very low. However, if nanomaterials are able to penetrate the skin and enter the bloodstream, they may exert a number of adverse effects.

Adverse effects of nanomaterials when part of a matrix

Only a very limited number of studies have focused on the hazard of nanocomposites. Most of these have focused on the hazard following pulmonary deposition of dust obtained by sanding different types of nanocomposites (paint, cement and thermoplastic). The published studies on the toxicological effects of sanding dusts from nanocomposites are in good agreement with each other. No additional toxicity (inflammation and genotoxicity) have been detected for any of the nano-

composites compared to the corresponding products without nanomaterials. A few studies on the toxicological effects of sprays containing nanomaterials have been published. A rat inhalation study of a commercial spray containing nano titanium dioxide (nano-TiO_2) particles indicated similar toxicity of nano-TiO_2 when part of the product and when nano-TiO_2 was tested alone. Testing of a commercial Ag spray product in rats resulted in moderate cardiovascular effects that were not observed in rats exposed to a standard Ag reference product. A third study showed no increased toxicity of a polymer dispersion compared to the non-nano product. Thus, based on the very few available studies it seems that the toxicity of nanomaterials following pulmonary exposure are masked in solid matrices while the toxicity of nanomaterials in some cases remains in spray products.

A few studies have investigated penetration of nanomaterials in sunscreen products. The penetration of nanomaterial containing cosmetics through skin was considered minimal, and there was no evidence that nano-sized or submicronised TiO_2 penetrated the intact epidermis to any significant extent or evidence of systemic absorption.

The oral safety of food-related nanomaterials that have potential use from *in vitro* and *in vivo* studies in laboratory was reviewed by Card et al. No adverse toxic effects were revealed in any of the very few studies with well performed physico-chemical characterisation of the nanomaterials. None of the studies characterised the nanomaterials in the diet or in any organ (Card et al., 2011).

No Derived No Effect Levels (DNELs) on hazard of nanomaterials as part of a matrix have been identified. More studies are needed to make conclusions within this area.

Physico-chemical factors of importance for toxicity

A number of physico-chemical properties (size, shape, surface properties, composition, solubility, aggregation/agglomeration, nanomaterial uptake, presence of mutagens and transition metals associated with the nanomaterials etc.) have been suggested to be important for toxicity of nano-materials. For insoluble so-called inert particles such as TiO_2 and carbon black (CB), the specific surface area of the particles has been shown to be correlated to the inflammatory response. The fact that multiwall carbon nanotubes (MWCNT) and asbestos fibres have been shown to induce similar genotoxic effects in rodents is an example of the effect of particle shape for genotoxity. For soluble nanomaterials, such as for example zinc oxide (ZnO), the liberation of Zn ions is important for the potential toxicity. The importance of physico-chemical factors for toxicity stresses the need for toxicological studies using well-characterised nanomaterials.

Hazard assessment of seven nanomaterials

A specific hazard assessment has been performed for seven nanomaterials with relevance for consumer exposure to serve as input for the consumer risk assessment of 20 scenarios. The consumer risk assessment will be published in the final report. The nanomaterials have been chosen to represent a diverse group of nanomaterials, i.e. 1) “inert” insoluble particles such as TiO_2 and CB, 2) soluble nanoparticles such as ZnO and Ag and 3) high aspect ratio particles such as carbon nanotubes (CNT) since nanomaterials constitute a very broad group which differs with respect to chemistry, solubility, coating etc. The main conclusions on the hazard assessment of the seven nanomaterials are inserted below.

Carbon black (CB)

The hazard assessment of CB in a matrix is primarily based on the recent Scientific Committee on Consumer Safety (SCCS) opinion on CB (SCCS, 2014c). The aim of this SCCS opinion on CB was specifically to decide if CB is safe for use as a colorant with a concentration up to 10 % in cosmetic products, and is therefore considered highly relevant for the present purpose, namely to serve as an input for the hazard part of the risk assessment of CB in mascara. The focus in the present assessment was on hazard related to skin and eye exposure because these routes of exposure are

considered to be the most relevant in relation to consumer use of mascara. Three studies on skin absorption of cosmetic formulations containing CB (all 20-30 nm in size) were evaluated by the SCCS and did not indicate any skin absorption. As emphasized by the SCCS, the conclusion on no risk of adverse effects of up to 10 % as CB as a colorant in cosmetic products is only valid when the skin is intact and the CB particles are 20 nm or larger. No studies on eye absorption of CB were evaluated by the SCCS. Therefore, hazard associated with eye absorption cannot be evaluated even though it is highly relevant for the hazard assessment of consumer use of mascara containing CB. The risk of eye irritation of CB cannot be excluded (SCCS, 2014c). We agree with the final concluding remarks of the SCCS opinion stressing that the skin absorption studies have only been done for CB sizes above 20 nm and that it is therefore not possible to conclude on cosmetic products containing smaller sized CB.

Carbon nanotubes (CNT)

The hazard assessment of CNT will serve as background documentation for the risk assessment of use (wear and tear) and sanding of a golf club containing CNT. With regard to the intended use of a golf club, dermal exposure is considered to be the only relevant exposure route. No studies were identified on the dermal toxicity of CNT incorporated into a solid matrix. If the CNT containing golf club is sanded, sanding dust containing CNT and potentially free CNT may be liberated and exert toxicity primarily by the pulmonary and dermal routes. The hazard assessment of free CNT is based on a recently published report on risk assessment of CNT by the Danish EPA (Danish EPA, 2015a). Animal studies have shown that pulmonary exposure to CNT consistently give asbestos-like toxicological response characterised by persistent inflammation, granulomas and fibrosis with low no-effect levels. Chronic human indicative no-effect levels (INELs) for the general public have been suggested to be 0.25 µg/m^3 (inhalation) and 0.78 and 2.3 mg/person (dermal) for two different scenarios (Aschberger et al., 2010). Two studies were identified on the toxicity of sanding dusts from different types of CNT composites (Wohlleben et al., 2011; Wohlleben et al., 2013). None of the studies showed increased toxicity of sanding dust from the CNT materials compared to the conventional products without CNT. However, it has not yet been tested if sanding dust from ultraviolet (UV)-exposed or otherwise weathered materials have a different toxicity profile due to a potentially increased liberation of free CNT.

Nano-sized synthetic amorphous silica (nano-SAS)

The hazard assessment of synthetic amorphous silica (SAS) as part of a matrix is primarily based on recent reviews by Dekkers et al (Dekkers et al., 2011; Dekkers et al., 2013), a review of the hazard of SAS by (Fruijtier-Pôlloth, 2012), and reports on SAS by the International Agency for Research on Cancer (IARC) (IARC Monographs on the evaluation of carcinogenic risks to humans, 1997) and by the European Centre for Ecotoxicology and Toxicology of Chemicals (ECETOC) through the Joint Assessment of Commodity Chemicals (JACC) program (European Centre for Ecotoxicology and Toxicology of Chemicals (ECETOC), 2006). The purpose of a hazard assessment is to serve as an input for the hazard part of the risk assessment of SAS in 1) food items and food containers, 2) face powders, and 3) “easy to clean” impregnation. Thus, the focus is put on the potential hazards associated with exposure by the gastrointestinal route (relevant for the food items and food container scenarios) and exposure by the dermal and the inhalation routes (relevant for the face powder and the “easy to clean” impregnation product). The critical effect following oral exposure is assessed as the hepatic effect. The No Observed Adverse Effect Level (NOAEL) has been suggested to be 1,500 mg/kg Body Weight (bw)/day (Dekkers et al., 2011). The critical effect following pulmonary exposure is pulmonary inflammation. Based on the evaluation by ECETOC, the Lowest Observed Adverse Effect Levels (LOAELs) and NOAELs were typically 1-50 mg/m^3 and 0.5-10 mg/m^3, respectively (European Centre for Ecotoxicology and Toxicology of Chemicals (ECETOC), 2006). These differences were by ECETOC evaluated as particle size-dependent: i.e. in general the NOAEL/LOAEL decreased by particle size. Our literature search identified several recent studies showing that the NOAEL/LOAEL was affected by size and surface modification, highlighting that the physico-chemical properties have to be taken into account. No studies were identified by

ECETOC on the dermal or oral absorption of SAS (European Centre for Ecotoxicology and Toxicology of Chemicals (ECETOC), 2006). For that reason no NOAEL/LOAEL has been suggested. Thus, the overall conclusion by ECETOC is that "*SAS is essentially non-toxic in humans via the oral, dermal/ocular and inhalation routes of exposure and no data exist on systemic effects in humans*".

Nano-sized silver (Nano-Ag)

The purpose of this hazard assessment is to serve as an input for the hazard part of the risk assessment of Ag when used in food supplements, paints for spraying, nano-filtering, disinfectant pump and propellant sprays, textiles and wound dressings. Thus, pulmonary, gastrointestinal and dermal exposures are all relevant exposure routes for the chosen risk scenarios.

The hazard assessment is mainly based on recent reports and references therein on nano-Ag by The Scientific Committee on Emerging and Newly Identified Health Risks (SCENIHR) and The Danish EPA (Scientific Committee on Emerging and Newly Identified Health Risks (SCENIHR), 2014a; Danish EPA, 2011) and recent reviews (Hadrup & Lam, 2014; Johnston et al., 2010a). Ag has no known essential function in man. The daily human intake has been estimated to be 0.007-0.5 µg/kg bw/day as the sum from all routes of exposure. Nano-Ag dissolves in solution and releases Ag^+. There is substantial evidence suggesting that the released Ag^+ are responsible for toxicological effects (Hadrup & Lam, 2014). The best described adverse effects in humans caused by long-term exposure to Ag is a permanent bluish-grey discoloration (argyria or agyrosis) of the skin and/or eyes (Scientific Committee on Emerging and Newly Identified Health Risks (SCENIHR), 2014a). Human risk assessment of Ag is most often based on epidemiological studies showing development of argyria. World Health Organisation (WHO) has set a NOAEL of 5 µg/kg bw/day as the sum of all routes of exposure (WHO, 2003). This NOAEL has been adopted by The European Food Safety Agency (EFSA) (EFSA Panel on Food Contact Materials, 2011). Likewise, The United States Environmental Protection Agency (US EPA) has published an oral reference dose of 5 µg Ag/kg bw/day in relation to a life-time exposure to Ag (U.S.Environmental Protection Agency, 1996). Dermal absorption through damaged skin has been reported in humans applying wound dressings containing nano-Ag. No toxic effects were reported in these test persons and less than <0.1 % of dose was estimated to be absorbed (Vlachou et al., 2007; Danish EPA, 2011; Moiemen et al., 2011; Walker & Parsons, 2014). The DNEL, as set by the REACH registrant, is 0.1 mg Ag/m^3 and 0.04 mg Ag/m^3 in the air for workers and the general population, respectively (European Chemicals Agency (ECHA), 2014). SCENIHR concluded that the toxicity of Ag, including nanoparticles of Ag, to humans is generally low. Futhermore, it is concluded that more data is needed to understand 1) the bacterial response to ionic Ag as well as Ag nanoparticles and 2) the hazard associated with the dissemination of resistance (Scientific Committee on Emerging and Newly Identified Health Risks (SCENIHR), 2014a). Overall, we agree in these thresholds and consider them applicable for the risk assessment.

Nano-sized titanium dioxide (Nano-TiO_2)

The hazard assessment of nano-sized TiO_2 is intended to serve as background documentation for the risk assessment of TiO_2 used in chewing gum, sunscreen, sunscreen lipstick, paint and cement and in relation to sanding a surface painted with nano-TiO_2-containing paint. The different applications of TiO_2 involve exposure by the oral, dermal, eye and pulmonary route and focus is therefore on the hazards associated with these exposure routes.

The SCCS has concluded that nano-TiO_2 at concentrations up to 25 % (wt/wt) and containing maximum 5 % anatase in sunscreens for dermal application are considered safe for the consumers. Use of TiO_2 nanoparticles in sprayable products is not recommended (SCCS, 2014d). Based on the available information, lung toxicity following inhalation appears to be the most critical effect in relation to long-term exposure to nano-TiO_2. Deposition in the lung depends on the dose and particles may be retained in the lung for a long time. Inflammation appears to be determined by the surface area of the particles. A NOAEL of 62.5 mg/kg bw/day was established based on a 30 days

oral (gavage) study in mice exposed to anatase TiO_2 nanomaterials with a primary particle size of 5 nm. A LOAEL of 5 mg/kg bw/day was derived based on impaired neurofunction and behaviour from a 60 days oral gavage study in mice exposed to anatase TiO_2 nanomaterials with primary particle size 5 nm. The occupational exposure limit (8-hour average) for TiO_2 in all forms (calculated as Ti) is 6 mg/m^3 in Denmark corresponding to 10 mg/m^3 TiO_2. The National Institute for Occupational Safety and Health (NIOSH) has recommended a threshold of 2.5 mg/m^3 for fine TiO_2 and 0.3 mg/m^3 for ultrafine TiO_2 (< 100 nm) for up to 10 hours/day during a 40-hour work week (NIOSH, 2011).

Nano-sized zinc oxide (Nano-ZnO)
The purpose of this hazard assessment of ZnO in nanoform (nano-ZnO) is to serve as background documentation for the risk assessment of sunscreen pump sprays containing nano-ZnO. The most relevant exposure route associated with consumer use of nano-ZnO sunscreen pump spray is dermal exposure. However, oral and pulmonary exposure may also occur but to a lesser extent. Nano-ZnO dissolves in biological fluids including artificial gastrointestinal fluid and lung fluid to form Zn^{2+} that seems to be distributed systematically to organs. This hazard assessment of nano-ZnO in a matrix is primarily based on the recent SCCS opinion on ZnO (SCCS, 2012) and two addendums related to this opinion (SCCS, 2014a; SCCS, 2014b). The aim of these SCCS opinions on ZnO was specifically to decide if ZnO in nanoform is safe for use as an UV-filter with a concentration up to 25 % in cosmetic products and is therefore considered highly relevant for the present purpose. It is concluded by the SCCS that the use of the different forms of ZnO nanoparticles as specified in the opinions at a concentration up to 25% as a UV-filter in sunscreens can be considered not to pose a risk of adverse effects in humans after dermal application. However, this does not apply to other applications that might lead to inhalation exposure to ZnO nanoparticles (such as sprayable products) because pulmonary exposure induces serious pulmonary effects. Therefore, the SCCS concludes that the use of ZnO nanoparticles in spray products that could lead to exposure of the consumer's lungs to nano-ZnO by inhalation cannot be considered safe.

Nano-sized zirconia (Nano-ZrO_2)
The hazard assessment of nano-sized zirconium dioxide (ZrO_2) is intended to serve as background documentation for the risk assessment of ZrO_2 used in dental fillings (implants). This particular use involves exposures of the consumer related to the application process (with spatula), to sanding and polishing, and to contact with the material migrating from the dental filling to the oral mucosa and saliva. Focus will be on the hazards associated with exposure by the inhalation route and exposure by the gastrointestinal route. In addition, exposure to the eye will be covered. ZrO_2 is generally described as a substance of low toxicity. There are very few *in vivo* studies available investigating the toxicity of ZrO_2. ZrO_2 has extremely low solubility in water and absorption is expected to be low from all exposure routes. When deposited in the alveolar region, particles are expected to be engulfed by alveolar macrophages and only a small amount to end up in the blood via the lymphatic system (European Chemicals Agency (ECHA), 2014). No specific adverse effects have been identified for the substance in the more recent literature. In older literature, immunostimulating effects are reported following injections in the thorax cavity and the peritoneum of mice. In addition, an ability to cause axilliary granulomas when applied in deoderants is described (Health Council of the Netherlands, 2002). The general occupational exposure limit (8-hour average) for Zr compounds (calculated as Zr) is 5 mg/m^3 in Denmark. The Occupational Safety and Health Administration (OSHA) in the US have applied the same value.

Bridging between hazard and risk assessment
Only a few studies on the hazard of nanomaterials as part of a matrix have been published and no DNELs for nanomaterials when part of matrix have been identified. Although consumers are not assumed to be exposed to free nanomaterials to the same extent as workers, the requirements for sufficient hazard data are relevant in relation to setting DNELs for consumers as well. There are a

number of important barriers for the establishment of health-based exposure limits for nanomaterials: 1) There is a lack of hazard data, 2) there is a limited understanding of how the physicochemical properties affect the toxicity of a nanomaterial, 3) there is an uncertainty regarding the most appropriate dose and exposure metric, and 4) there is still a lack of standardised and validated methods for measuring air concentrations of nanomaterials. As described above there are several barriers for setting exposure threshold limits for nanomaterials and to our knowledge presently no legally binding specific exposure limits for nanomaterials exist. However, some initiatives have been taken. For example, NIOSH has proposed specific Occupational Exposure Limits (OELs) for 1) all CNT, and 2) for nano-sized TiO_2 compared to larger sized TiO_2.

Overall, the main uncertainties related to hazard assessment and derivation of DNELs for nanomaterials in consumers products for further risk assessment are:

- Hazard data for pristine nanomaterials are applied in absence of hazard data for the nanomaterials in matrix.
- Results are not always available for the most relevant exposure routes to be addressed.
- As detailed characteristics of the nanomaterials are often not available, the information used for evaluation of nanomaterials today may be based on data generated for different forms of the particles with varying surface area, coating, and size distribution. There is little data on how the physicochemical parameters influence toxicity.
- The quality of data varies significantly and test results with insufficient characterisation of the nanoform may be included in the evaluations but may be less useful.
- It is not known if the generally applied assessment factors are valid for nanomaterials.

Conclusion

The aim of this report is to perform a hazard assessment of nanomaterials in consumer products. The consumer is potentially exposed to nanomaterials in their final, intended use, i.e. when the nanomaterials are part of a matrix, and therefore, this report focuses on the hazard of nanomaterials when part of a consumer matrix. Only a few studies on the hazard of nanomaterials as part of a matrix have been published and no DNELs for nanomaterials when part of matrix have been identified. A few studies on pulmonary exposure to sanding dust from solid matrices and spray products containing nanomaterials have been published. No additional hazard was observed when nanomaterials were part of a solid matrix, and the matrix was most important for the toxicity. A few studies indicate that the hazard of nanomaterials may not always be masked when part of a liquid matrix (e.g. spray products). Dermal application of CB containing mascara and nano-ZnO containing sun screen resulted in no to very moderate dermal absorption. The studies on gastrointestinal absorption and toxicity are too few for conclusion. More research is needed to characterise the hazard to consumers exposed to nanomaterials.

Due to the lack of information on hazard of nanomaterials when part of a consumer product, the hazard of free nanomaterials can be used to predict the hazard in a worst-case scenario. However, use of hazard data for free nanomaterials may also be challenging because of knowledge gaps. In general, there is a lack of long-term toxicity studies. The continuous introduction of new nanomaterials (with new surface modifications etc) makes it impossible to test all nanomaterials. Therefore recent research strategies have emphasised that grouping and ranking are necessary tools to predict hazard.

Dansk sammenfatning

Baggrund
Under overskriften "Bedre styr på nanomaterialer" har den danske Miljøstyrelse iværksat en række projekter, der sigter på at undersøge og generere ny viden om forekomsten af nanomaterialer i produkter på det danske marked og vurdere potentielle risici for forbrugerne og miljøet. Denne rapport er en del af en serie på fire i et projekt, som omhandler forbrugereksponering og risikovurdering af nanomaterialer i produkter på det danske marked.

Formålet med denne rapport er at foretage en farevurdering af nanomaterialer i forbrugerprodukter. Forbrugeren er potentielt eksponeret for nanomaterialer når disse er en del af en matrice. Derfor fokuserer denne rapport på faren forbundet med nanomaterialer som en del af et forbrugerprodukt. Eftersom frie nanomaterialer kan frigøres i forbindelse med brug af produktet, bliver faren af frie nanomaterialer også beskrevet.

Formålet med denne rapport er at referere konsensus frem for enkeltstående fund. Eftersom fokus for farevurderingen er på nanomaterialer i brugsfasen, består den refererede litteratur vedrørende pristine[1] nanomaterialer primært af reviews, hvorimod alle identificerede originale farestudier af nanomaterialer, som indgår i et produkt, er beskrevet.

Rapporten er struktureret således, at data, som er relevante for at vurdere faren af nanomaterialer i forbrugerprodukter, gennemgås, herunder nanomaterialers biokinetik (Kapitel 2), nanomaterialers sundhedsskadelige effekter (Kapitel 3), de sundhedsskadelige effekter af nanomaterialer i matricer (Kapitel 4), de fysisk-kemiske faktorer af betydning for nanomaterialers toksicitet (Kapitel 5) samt farevurderinger af udvalgte nanomaterialer (Kapitel 6). Endvidere præsenteres en diskussion af, hvordan man bygger bro mellem fare og risikovurdering (Kapitel 7) afsluttende med en opsummering og konklusion (Kapitel 8).

Biokinetik
For forbrugeren anses lungerne, mave-tarm-kanalen og huden for at være de primære absorptionsveje for nanomaterialer. Desuden kan nanomaterialer optages via øjnene og det olfaktoriske system. Vi identificerede ikke nogen studier, som dækker, hvordan biokinetikken modificeres, når nanomaterialet indgår i en matrice.

Indånding af partikler fører til partikeldeponering i luftvejene (næse-svælg, luftrør, bronkier og alveoler). Hvad der sker med partiklerne efter deponering, afhænger af en kombination af partiklernes fysiske-kemiske egenskaber og responset både lokalt i lungerne og i andre dele af kroppen. De større partikler opfanges af det mukociliære system i de øvre luftveje og fjernes relativt hurtigt (timer til dage). I modsætning hertil deponeres mindre partikler i den alveolære region, hvor partiklerne hos mennesker kan blive i årevis. En begrænset translokation af partikler fra lunger til kredsløb er blevet beskrevet.

I 2013 publicerede den danske Miljøstyrelse en rapport om absorption af nanomaterialer ved oral eksponering, som giver et overblik over den eksisterende litteratur. Generelt indikerer den

[1] Ved pristine nanomaterialer forstås i denne rapport nanomaterialer, som endnu ikke er en del af et produkt (komposit, spray eller lignende).

gennemgåede litteratur, at absorption fra mave-tarm-kanalen er lille. Rapporten konkluderer, at fysiske-kemiske egenskaber som størrelse, agglomerering og krystalstruktur kan påvirke absorption. Det konkluderes endvidere, at antallet af publikationer om absorption fra mave-tarm-kanalen er stigende, men at kun få af disse studier er veludførte. Det vakte især bekymring, at kun få af de fysike-kemiske parametre, som forventes at påvirke absorptionen, blev rapporteret (Danish EPA, 2013c).

I 2013 publicerede den danske Miljøstyrelse en rapport om absorption af nanomaterialer gennem huden, som giver et overblik og en vurdering af den eksisterende viden om optagelse af nanomaterialer gennem huden. Rapportens overordnede konklusion er, at hudabsorption af partikler i nanostørrelse er muligt, omend det ser ud til at forekomme i et meget lille omfang. Omfanget af penetration, afhængigt af kemi og eksperimentelle forhold, kan være større for partikler i nanostørrelse end for større partikler (Danish EPA, 2013b).

Den eksisterende viden om øjenabsorption af nanomaterialer og øjentoksicitet er for lille til at blive vurderet og generelt evalueret til en farevurdering.

Olfaktorisk absorption af nanomaterialer er blevet vist i laboratoriedyr og kan også være relevant for mennesker (Elder et al., 2006). Den nuværende viden er for begrænset til at foretage en farevurdering.

Den potentielle metabolisme af nanomaterialer er kun undersøgt i et begrænset antal studier. Hvor sådan information findes, viser de fleste studier, at nanomaterialer, og i særdeleshed uopløselige nanomaterialer, ikke metaboliseres (Landsiedel et al., 2012).

Efter absorption fra lunger, hud, øjne eller mave-tarm-kanal, kan nanomaterialer nå blodcirkulationen og/eller det lymfatiske system. Leveren er det primære distribueringsorgan, og det har vist sig, at 40 nm Au nanopartikler snarere akkumulerer i leverens Kupffer celler end bliver udskilt (Sadauskas et al., 2009a). Placenta udgør den ernæringsmæssige grænseflade mellem mor og foster. Der er stadig kun få studier af passagen af nanopartikler på tværs af placentaen, men studier i gnavere beskriver transplacential transport af partikler i nanostørrelse, som spænder fra næsten negligerbart til højt (adskillige procent af den administrerede dosis).

Pristine nanomaterialers skadelige effekter

Eksponering af lunger for nanomaterialer kan medføre lungeinflammation, fibrose, DNA skade og kræft. Der er blevet rejst bekymring for, at eksponering af lungerne også kan resultere i hjertekareffekter, ligesom det er set ved human eksponering for partikler fra den omgivende luft. Det er blevet foreslået, at mekanismerne bag hjertekareffekter er systemisk inflammation og akutfaserespons, translokation af partikler og respiratoriske reflekser.

Der er begrænset viden om toksiciteten af oralt givne nanomaterialer i mave-tarm-kanalen, og det er derfor ikke muligt at give nogle generelle konklusioner vedrørende toksiciteten af nanomaterialer beregnet til anvendelse i fødevarer eller fødevare-relaterede produkter (Card et al., 2011). Dog var toksiciteten af nanoformuleringen af en specifik ingrediens eller et specifikt nanomateriale ikke altid forhøjet i sammenligning med en ingrediens eller et nanomateriale, der ikke var nanoformuleret.

Samlet set er der kun lille evidens for dermal toksicitet ved hudpåførsel af nanomaterialer. Generelt er optaget af nanomaterialer gennem intakt sund hud meget lille. Hvis nanomaterialer er i stand til at penetrere huden og få adgang til blodbanen, vil de imidlertid kunne forårsage forskellige skadelige effekter.

Skadelige effekter af nanomaterialer i en matrice
Kun et meget begrænset antal studier har fokuseret på faren ved nanokompositter, dvs. kompositter som indeholder nanomaterialer. De fleste af disse studier har fokuseret på faren efter lungedeponering af støv fremkommet ved slibning af forskellige typer nanokompositter (maling, cement og termoplastik). De publicerede studier er i god overensstemmelse med hinanden. Der blev ikke detekteret nogen yderligere toksicitet (inflammation og genotoksicitet) for nanokompositterne sammenlignet med tilsvarende kompositmaterialer uden nanomaterialer. Et rotteinhalationsstudie af en kommerciel spray indeholdende nano titaniumdioxid (nano-TiO2) partikler indikerede tilsvarende toksicitet, når nano-TiO2 indgik i et produkt, som når nano-TiO2 blev testet alene. Testning af en kommerciel Ag spray i rotter resulterede i moderate hjertekareffekter, som ikke blev observeret i rotter eksponeret for et standard Ag reference produkt. Et tredje studie viste ikke øget toksicitet af en polymerdispergering sammenlignet med det tilsvarende "non-nano" produkt. De meget få tilgængelige studier tyder altså på, at toksiciteten ved lungeeksponering maskeres i faste matricer, mens toksiciteten af nanomaterialer i visse tilfælde bevares i sprayprodukter.

Enkelte studier har undersøgt hudpenetrationen af nanomaterialer i solcremer. Penetration af huden af solcreme indeholdende nanomateriale blev antaget at være minimal, og der var ikke nogen evidens for at TiO_2 i nano-størrelse eller submikroniseret TiO_2 penetrerede intakt epidermis i noget betydeligt omfang. Der var heller ikke evidens for større systemisk absorption.

In vivo og *in vitro* studier af nanomaterialer med potentiel anvendelse i fødevarer blev gennemgået i et review af Card et al. Der blev ikke fundet nogen skadelige effekter i de få studier, som indeholdt veludført karakterisering af nanomaterialer. Ingen af studierne karakteriserede nanomaterialer i fødevarer eller i organer (Card et al., 2011).

Der blev ikke identificeret "Derived No Effect Levels" (DNELs) for faren for nanomaterialer som del af en matrice. Det er nødvendigt med flere studier for at foretage konklusioner på dette område.

Fysisk-kemiske faktorer af betydning for toksicitet
En række fysisk-kemiske egenskaber såsom størrelse, form, overfladeegenskaber, sammensætning, opløselighed, aggregering/agglomerering, nanomaterialeoptag, tilstedeværelse af mutagener og overgangsmetaller associeret med nanomaterialerne osv. kan være vigtige for toksiciteten. For uopløselige, såkaldte inerte, partikler som TiO_2 og carbon black (CB), har partiklernes specifikke overfladeareal vist sig at være korreleret til det inflammatoriske respons. Det faktum, at flervæggede kulstofnanorør (MWCNT[2]) og asbestfibre har vist sig at inducere sammenlignelige genotoksiske effekter i gnavere, er et eksempel på partikelformens betydning for genotoksicitet. For opløselige nanomaterialer som f.eks. zinkoxid (ZnO) er frigørelsen af Zn ioner vigtig for den potentielle toksicitet. Betydningen af de fysiske-kemiske faktorer for toksiciteten understreger behovet for, at de undersøgte nanomaterialer i toksikologiske studier er velkarakteriserede.

Farevurdering af syv nanomaterialer
Der er blevet foretaget en specifik farevurdering af syv nanomaterialer, som har relevans for forbrugereksponering, og denne skal fungere som baggrundsdata ved risikovurderingen af 20 forbrugerscenarier. Forbrugerrisikovurderingen vil blive publiceret i den endelige rapport. Eftersom nanomaterialerne udgør en meget bred gruppe, som adskiller sig med hensyn til kemi, opløselighed, coating m.m., er nanomaterialerne udvalgt, så de repræsenterer denne forskelligartethed, dvs. 1) inerte. uopløselige partikler som TiO_2 og CB, 2) opløselige partikler som ZnO og Ag, og 3) "high aspect ratio"[3] partikler som kulstofnanorør (CNT).

[2] MWCNT (multiwalled carbon nanotubes)
[3] High aspect ratio (stort længdebreddeforhold)

Carbon black (CB)

Farevurderingen af CB i en matrice er primært baseret på EUs videnskabelige komité for forbrugersikkerheds (SCCS) "Opinion[4] on Carbon Black (nano form)" (SCCS, 2014c). Formålet med denne SCCS holdning var helt specifikt at afgøre, om det er sikkert at anvende CB som farvestof med et indhold på op til 10 % i kosmetiske produkter. Denne holdning antages derfor at være særdeles relevant for det nærværende formål, nemlig at fungere som baggrundsviden for faredelen af risikovurderingen af CB i mascara. I denne vurdering er fokus på hud- og øjeneksponering, eftersom disse eksponeringsveje anses at være de mest relevante i relation til forbrugeranvendelse af mascara. SCCS vurderede tre studier med hudabsorption af CB fra kosmetiske formuleringer (CB størrelse: 20-30 nm) og ingen af disse indikerede hudabsorption. Som det understreges af SCCS, er konklusionen, om at der ikke er nogen risiko forbundet med brugen af kosmetiske produkter med op til 10 % CB som farve, kun gyldig, når huden er intakt, og CB partiklerne er 20 nm eller større. SCCS vurderede ikke nogen øjenabsorptionsstudier af CB. Derfor kan faren forbundet med øjeneksponering ikke vurderes, selvom det er særdeles relevant for farevurderingen af forbrugeres anvendelse af mascara indeholdende CB. Risikoen for øjenirritation kan ikke udelukkes (SCCS, 2014c). Vi er enige i de endelige konkluderende bemærkninger i SCCSs holdning, som understreger, at hudabsorptionsstudierne kun er blevet udført for CB størrelser over 20 nm, og at det derfor ikke er muligt at konkludere på kosmetiske produkter med CB i mindre partikelstørrelse.

Kulstofnanorør (CNT)

Farevurderingen af CNT vil fungere som baggrundsdokumentation for risikovurderingen af brug (slitage) og slibning af en golfkølle indeholdende CNT. Hudeksponering antages at være den eneste relevante eksponeringsvej i forbindelse med den sædvanlige brug af en golfkølle. Der blev ikke identificeret nogen studier af den dermale toksicitet af CNT indbygget i en fast matrice. Ved slibning af en golfkølle indeholdende CNT vil der potentielt kunne frigives både slibestøv indeholdende CNT og frie CNT, som vil kunne medføre toksicitet via lunge- og hudeksponering. Farevurderingen af frie CNT er baseret på en rapport om risikovurdering af CNT, som er under udarbejdelse ved den danske Miljøstyrelse (Danish EPA, 2015a). Dyrestudier har vist, at eksponering af lunger for CNT giver et konsistent asbestose-lignende toksikologisk respons, som er karakteriseret ved vedvarende inflammation, granulomer og fibrose med lave "no-effect" niveauer. Det er blevet foreslået, at kroniske humane "Indicative No-Effect Levels" (INELs) for den almindelige befolkning er 0,25 μg/m^3 (indånding) og 0,78 mg/person (hud) (Aschberger et al., 2010). Der blev identificeret to studier omhandlende toksiciteten af slibestøv fra forskellige typer CNT kompositter (Wohlleben et al., 2013; Wohlleben et al., 2011). Ingen af disse studier viste øget toksicitet af slibestøv fra CNT materialer sammenlignet med de konventionelle produkter uden CNT. Det er dog ikke blevet testet, om slibestøv fra materiale, som er blevet ultraviolet (UV)-eksponeret eller forvitret på anden måde, har en anden toksicitetsprofil pga. potentiel frigørelse af frie CNT.

Syntetisk amorft silica i nanostørrelse (nano-SAS)

Farevurderingen af syntetisk amorft silica (SAS) som del af en matrice er primært baseret på nylige reviews af Dekkers et al (Dekkers et al., 2013; Dekkers et al., 2011), et review af faren forbundet med SAS (Fruijtier-Pôlloth, 2012) og rapporter om SAS skrevet af WHOs canceragentur IARC (IARC Monographs on the evaluation of carcinogenic risks to humans, 1997) og ECETOC[5] (European Centre for Ecotoxicology and Toxicology of Chemicals (ECETOC), 2006). Formålet med farevurderingen er at udgøre baggrunden for faredelen af risikovurderingen af SAS i 1) fødevarer og fødevareemballage, 2) ansigtspudder og 3) "easy to clean" imprægnering. Således er fokus på den potentielle fare forbundet med eksponering via mave-tarm-kanalen (relevant for fødevarer og fødevareemballage) og eksponering via hud og indånding (relevant for ansigtspudder og "easy to clean" imprægneringsprodukter). Den kritiske effekt ved oral eksponering er vurderet til at være effekt på leveren. 'No Observed Adverse Effect Level' (NOAEL) er blevet foreslået at være 1,500

[4] "Opinion" omtales herefter som holdning i denne rapport
[5] European Centre for Ecotoxicology and Toxicology of Chemicals

mg/kg kropsvægt/dag (Dekkers et al., 2011). Den kritiske effekt ved lungeeksponering er lunge inflammation. Baseret på ECETOCs vurdering er 'Lowest Observed Adverse Effect Levels (LOAELs) og NOAELs typisk henholdsvis 1-50 mg/m^3 og 0,5 mg/m^3 (European Centre for Ecotoxicology and Toxicology of Chemicals (ECETOC), 2006). ECETOC vurderede disse forskelligheder som partikelstørrelsesafhængige idet NOAEL/LOAEL generelt faldt med partikelstørrelse. Vores litteratursøgning identificerede adskillige nyere studier, som viste, at NOAEL/LOAEL var påvirket af størrelse og overflademodifikation, hvilket understreger, at de fysisk-kemiske egenskaber skal tages i betragtning. ECETOC identificerede ingen studier om dermal og oral absorption (European Centre for Ecotoxicology and Toxicology of Chemicals (ECETOC), 2006) og der er på denne baggrund ikke foreslået nogen NOAEL/LOAEL. ECETOCs overordnede konklusion er, at SAS i al væsentlighed ikke er toksisk for mennesker hverken via mave-tarm-kanalen, hud, øjne eller via inhalation, og der eksisterer ingen data på systemiske effekter i mennesker.

Sølv i nanoform (nano-Ag)

Farevurderingen af nano-Ag har til formål at danne baggrund for risikovurderingen af nano-Ag ved anvendelse i kosttilskud, i sprøjtemaling, ved nano-filtrering, som desinfektionsmiddel i pumpe- og drivmiddel-sprays, i tekstiler og i sårbandager. Eksponering via lunger, mave-tarm-kanal og hud er alle relevante eksponeringsveje for de nævnte risikoscenarier.

Nærværende farevurdering er hovedsageligt baseret på rapporter fra SCENIHR[6] og den danske Miljøstyrelse (Scientific Committee on Emerging and Newly Identified Health Risks (SCENIHR), 2014a; Danish EPA, 2011) og på referencer heri samt på nyere review-artikler (Hadrup & Lam, 2014; Johnston et al., 2010a). Ag har ingen kendt livsvigtig funktion i mennesket. Daglig indtagelse er estimeret til 0,007 - 0,5 µg/kg legemsvægt/dag. Nano-Ag opløses i vandigt miljø og frigiver herved Ag^+ ioner. Der er overvejende sandsynlighed for, at disse frigivne Ag^+ ioner er ansvarlige for sølvs toksikologiske virkninger (Hadrup & Lam, 2014). De bedst beskrevne alvorlige effekter i mennesker som følge af langvarig udsættelse for Ag er permanent blålig-grå misfarvning (argyri eller agyrose) af hud og/eller øjne (Scientific Committee on Emerging and Newly Identified Health Risks (SCENIHR), 2014a). Risikovurdering baseres oftest på sådanne undersøgelser i mennesker. Verdenssundhedsorganisationen WHO har fastsat en NOAEL på 5 µg/kg legemsvægt/dag som summen af alle eksponeringsveje (WHO, 2003). Denne NOAEL er blevet tilsluttet af Den Europæiske Fødevaresikkerhedsautoritet (EFSA) (EFSA Panel on Food Contact Materials, 2011). Ligeledes har USAs miljøstyrelse (US EPA), anvist en oral referencedosis på 5 µg Ag/kg legemsvægt/dag ved livsvarig udsættelse for Ag (U.S.Environmental Protection Agency, 1996). Absorption gennem beskadiget hud er i forsøg blevet set hos mennesker, der påsattes sårbandager indeholdende nano-Ag. Ingen giftvirkninger blev rapporteret hos disse testpersoner, og mindre end 0,1 % af den påsatte dosis blev anslået at blive absorberet (Walker & Parsons, 2014; Moiemen et al., 2011; Danish EPA, 2011; Vlachou et al., 2007). Af en REACH-registrant er DNEL i indåndingsluft sat til 0,1 mg Ag/m^3 for arbejdere og 0,04 mg Ag/m^3 for den almindelige befolkning (European Chemicals Agency (ECHA), 2014). SCENIHR har konkluderet, at toksiciteten af Ag, herunder nano-Ag, i mennesker generelt er lav. Herudover konkluderer SCENIHR, at der er behov for flere data for at kunne forstå 1) det bakterielle respons på ionisk Ag og nano- Ag og 2) faren i forbindelse med udbredelsen af bakteriel resistens (Scientific Committee on Emerging and Newly Identified Health Risks (SCENIHR), 2014a).

Vi er enige i ovenfor nævnte tærskelværdier og anser dem for brugbare for vores risikovurdering ved de nævnte scenarier.

TiO_2 i nanostørrelse (nano-TiO_2)

Farevurderingen af nano-TiO_2 har til formål at danne baggrund for risikovurderingen af nano-TiO_2 ved anvendelse i tyggegummi, solcreme, læbepomade med solbeskyttelse, maling og cement, samt i relation til slibning af en overflade malet med nano-TiO_2-holdig maling. De forskellige anvendelser

[6] EUs videnskabelige komité vedrørende fremtidige og nyligt identificerede farer

af TiO_2 involverer eksponering via henholdsvis indtagelse, i hud, i øjne og i lunger, og fokus er derfor på farer forbundet med disse eksponeringsveje.

EUs videnskabelige komité for forbrugersikkerhed (SCCS) har konkluderet, at nano-TiO_2 i koncentrationer på op til 25 % (v/v) og med indhold af højst 5 % anatase[7] i solcremer til påføring på huden kan anses for sikre for forbrugerne. Anvendelse af TiO_2 nanopartikler i produkter til spray-påføring anbefales ikke (SCCS, 2014d). På baggrund af den tilgængelige viden synes lungetoksicitet efter inhalation at være den kritiske effekt i relation til langvarig udsættelse for nano-TiO_2. Aflejring i lungerne afhænger af dosis, og partiklerne kan tilbageholdes i lungerne i lang tid. Inflammationen ser ud til at være bestemt af overfladearealet af partiklerne. En NOAEL på 62,5 mg/kg kropsvægt/dag er fastlagt baseret på et 30 dages oralt (sonde) studie i mus eksponeret for nano-anatase TiO_2 med en primær partikelstørrelse på 5 nm. En LOAEL på 5 mg/kg kropsvægt/dag blev afledt, baseret på nedsat funktion af nervesystemet og adfærdspåvirkning fra et 60 dages oralt studie i mus udsat for nano-anatase TiO_2 med primær partikelstørrelse 5 nm i føden. Grænseværdien (8 timers gennemsnit) i arbejdsmiljøet for TiO_2 i alle former (beregnet som Ti) er 6 mg/m^3 i Danmark svarende til 10 mg/m^3 TiO_2. National Institute for Occupational Safety and Health (NIOSH) har anbefalet en grænseværdi på 2,5 mg/m^3 for fine partikler af TiO_2 og 0,3 mg/m^3 for ultrafine TiO_2-partikler (<100 nm) i op til 10 timer/dag ved en arbejdsuge på 40 timer (NIOSH, 2011).

ZnO i nanostørrelse (nano-ZnO)
Farevurdering af nano-ZnO har til formål at kunne bruges som baggrund for risikovurderingen af nano-ZnO ved anvendelse af solcreme pumpe sprays som indeholder nano-ZnO. Her er den mest relevante eksponeringsvej eksponering af hud. Herudover kan oral eksponering og eksponering ved indånding også forekomme, omend i mindre omfang. Nano-ZnO opløses i biologiske væsker, herunder kunstig mave- og lungevæske, hvorved der dannes Zn^{2+} ioner, som efterfølgende fordeles til organismens forskellige organer. Farevurdering af nano-ZnO i denne matrix er primært baseret på en nylig fremkommet holdning fra EUs videnskabelige komité for forbrugersikkerhed (SCCS) (SCCS, 2012) og to tilføjelser hertil (SCCS, 2014a; SCCS, 2014b). Formålet med disse SCCS holdninger var netop at vurdere, om nano-ZnO er sikkert til brug som UV-filter ved en koncentration op til 25 % i kosmetiske produkter, og kan således betragtes som yderst relevante til nærværende formål. SCCS konkluderer, at anvendelsen af nano-ZnO som UV-filter i solcremer i en koncentration på op til 25 % af de forskellige former for nano-ZnO (nærmere specificeret i SCCSs holdninger), ikke anses at kunne udgøre en risiko for alvorlige effekter i mennesker efter påsmøring af hud. Denne vurdering gælder ikke for andre anvendelser, som kan føre til eksponering ved indånding (f.eks. spraybare produkter), fordi en sådan eksponering kan inducere alvorlige effekter i lungene. Derfor konkluderer SCCS, at brugen af nano-ZnO i produkter, som kan føre til eksponering af lungerne ved indånding, ikke kan betragtes som sikre.

ZrO_2 i nanostørrelse (nano- ZrO_2)
Farevurderingen af ZrO_2 i nanostørrelse har til formål at danne baggrund for risikovurderingen af ZrO_2 brugt i tandfyldninger (implantater). Denne anvendelse involverer eksponering af forbrugeren i forbindelse med påføringsprocessen (med spatel), ved slibning og polering og ved kontakt med stof, der migrerer ud af tandfyldningsmaterialet til mundslimhinden og spyt. Fokus er derfor på farer forbundet med eksponering ved indånding og ved indtagelse. Desuden vil eksponering af øjene blive dækket. ZrO_2 beskrives generelt som et stof med lav toksicitet. Der er meget få *in vivo* studier tilgængelige til belysning af toksiciteten af ZrO_2. ZrO_2 har meget lav opløselighed i vand, og det forventes at absorptionen er lav ved alle eksponeringsveje. Når partiklerne aflejres i den alveolære region, forventes de at blive fagocyteret af alveolære makrofager, og kun en lille mængde vil ende i blodet via lymfesystemet (European Chemicals Agency (ECHA), 2014). Der er ikke identificeret specifikke negative effekter af stoffet i den nyere litteratur. Ældre litteratur har

[7] En naturlig form af TiO_2

rapporteret om immunostimulerende effekter efter injektion i brystkassen (thorax) og bughinden (peritoneum) af mus. Desuden har stoffet forårsaget axillære granulomer i forbindelse med tidligere anvendelse i deoderanter (Health Council of the Netherlands, 2002). Den generelle grænseværdi (8 timers gennemsnit) for Zr-forbindelser (beregnet som Zr) er 5 mg/m^3 i Danmark. Arbejdstilsynet i USA (OSHA) har anvendt samme værdi.

Brobygning mellem fare og risikovurdering
Der er kun blevet publiceret få studier om faren af nanomaterialer som del af en matrice, og der er ikke blevet identificeret nogen DNELs for disse. Selvom det ikke antages, at forbrugere bliver eksponeret for nanomaterialer i samme omfang som arbejdere, er det alligevel relevant at have tilstrækkelige faredata i relation til fastsættelse af DNELs for forbrugere. Der er en stribe afgørende barrierer i forhold til at etablere helbredsbaserede eksponeringsgrænser for nanomaterialer: 1) Der er mangel på faredata, 2) der er begrænset forståelse for, hvordan de fysiske-kemiske egenskaber påvirker nanomaterialets toksicitet, 3) der er usikkerhed mht. den mest passende dosis og eksponeringsmål og 4) der er stadig en mangel på standardiserede og validerede metoder til måling af luftkoncentrationer af nanomaterialer. Som beskrevet er der adskillige barrierer for at sætte grænseværdier for nanomaterialer. Dog er der taget nogle initiativer, idet fx NIOSH har foreslået specifikke erhvervsmæssige grænseværdier for CNT og for nano-TiO_2 sammenlignet med større TiO_2.

Samlet set er de største usikkerheder for risikovurdering, som er relateret til farevurdering og udledning af DNELs for nanomaterialer i forbrugerprodukter, følgende:

- Faredata for pristine nanomaterialer anvendes i mangel på faredata for nanomaterialer i kompositmaterialer
- Der er ikke altid tilgængelige resultater for den mest relevante eksponeringsvej
- Eftersom der sjældent foreligger detaljerede karakteriseringer af nanomaterialer, anvendes der i dag ofte data genereret fra forskellige partikelformer med varierende overfladeareal, coating og størrelsesfordeling. Der er få data på, hvordan fysiske-kemiske parametre påvirker toksiciteten
- Kvaliteten af data varierer betydeligt, og testresultater med utilstrækkelig karakterisering af nanoformen kan blive inkluderet i vurderingerne, men kan være mindre brugbare
- Det vides ikke, om de generelt brugte sikkerhedsfaktorer er gyldige for nanomaterialer

Konklusion
Formålet med rapporten er at foretage en farevurdering af nanomaterialer i forbrugerprodukter. Forbrugeren er potentielt eksponeret for nanomaterialer i brugsfasen, dvs. når nanomaterialerne indgår i et forbrugerprodukt, og derfor fokuserer denne rapport på faren af nanomaterialer, når disse indgår i en forbrugermatrice. Der er kun publiceret få studier om faren af nanomaterialer som del af en matrice, og ingen DNELs for disse er blevet identificeret. Der er blevet publiceret få studier om lungeeksponering for slibestøv fra henholdsvis faste matricer og sprayprodukter med nanomaterialer. Der blev ikke observeret yderligere toksicitet, når nanomaterialer indgik i en fast matrice, og matricen var af størst betydning for toksiciteten. Få studier indikerer, at nanomaterialers fare ikke altid maskeres, når nanomaterialer er del af en væskematrice (f.eks. sprayprodukter). Hudpåførsel af mascara indeholdende CB og solcreme indeholdende nano-ZnO resulterede i ingen til meget moderat hudabsorption. Der er for få studier omhandlende optag af nanomaterialer og toksicitet i mave-tarm-kanalen til, at man kan konkludere noget herpå. Der er behov for mere forskning til at være i stand til at karakterisere forbrugeres fare ved eksponering for nanomaterialer.

I mangel på information på faren af nanomaterialer, når disse indgår i et forbrugerprodukt, kan faren for de frie nanomaterialer anvendes til at prædiktere faren i et "worst-case scenario". Dog kan anvendelsen af faredata for frie nanomaterialer også være udfordrende pga. huller i den tilgængelige viden. Der er generelt en mangel på langtidstoksicitetsstudier. Den fortsatte

introduktion af nye nanomaterialer (med nye overflademodifikationer osv.) gør det umuligt at teste alle nanomaterialer. Derfor har nyere forskningsstrategier understreget behovet for gruppering og rangordning som nødvendige værktøjer til at kunne prædiktere fare.

1. Introduction

1.1 Background

There is an increasing use of nanomaterial-containing products. Nanomaterials are added to very diverse product groups to achieve improved properties. These product groups include skin care products, food packaging, paints and other chemical products. Therefore more and more consumers are at potential risk of nanomaterial exposure.

1.2 Objective

The overall aim of this report is to perform a hazard assessment of nanomaterials in consumer products. The consumer is potentially exposed to nanomaterials in their final, intended use, i.e. when the nanomaterials are part of a matrix. Thus, the report is focusing on the following:

1. Identification of the hazard of free/pristine nanomaterials, including comparison with the macro counterpart where relevant,
2. Identification of the hazard of nanomaterials when part of a consumer matrix rather than the hazard of the pristine nanoparticle, where possible, and
3. Identification of the influence on hazard of physical chemical characteristics such as size, surface coating, shape etc.

The aim of the report is to refer consensus rather than isolated findings. Since the focus of the hazard evaluation is on nanomaterials during their intended use, the referred literature concerning pristine nanomaterials consists primarily of reviews, while all identified original studies of hazard related to nanomaterials as part of a product are described.

In contrast to the pristine nanomaterials, the number of publications regarding the toxicological effects of nanomaterials when part of consumer products is very limited. Therefore all such studies identified in the literature search have been included.

For seven nanomaterials with relevance for consumer exposure, a specific hazard assessment has been performed. Because nanomaterials constitute a very broad group which differs with respect to chemistry, solubility, coating etc., the nanomaterials have been chosen to represent a diverse group of nanomaterials, i.e. 1) “inert” insoluble particles such as TiO_2 and CB, 2) soluble nanoparticles such as ZnO and silver (Ag) and 3) high aspect ratio particles such as CNT.

Nanomaterials as pharmaceutical drug-carriers and tattoo colours are outside the scope of this report.

2. Biokinetics

The term biokinetics covers the fate of a foreign material in the body. It includes the following sequence of processes: absorption, distribution, metabolism and excretion (ADME). In this chapter these different parts of the biokinetics for nanomaterials are discussed. No studies covering how biokinetics is modified when the nanomaterial is part of a matrix were identified.

2.1 Absorption of nanomaterials

For the consumer, the lungs, gastrointestinal tract and the skin are considered to be the primary absorption routes of nanomaterials. In addition, uptake may occur through the eyes and the olfactory system. The different absorption routes will be dealt with in the following paragraphs.

2.1.1 Pulmonary absorption

Inhalation of particles results in deposition of particles in the respiratory tract (nasopharyngeal, tracheobronchial and alveolar regions). The fate of the particles after deposition depends on a combination of physico-chemical properties of the particles and responses locally in the lung and in other parts of the body. This paragraph is based on three recent reviews on the biokinetics of inhaled particles (Geiser & Kreyling, 2010; Kreyling et al., 2013; Landsiedel et al., 2012) and a review covering most parts of nanotoxicology (Oberdörster et al., 2005 and references therein).

The deposition pattern of particles in the different parts of the respiratory tract is strongly dependent on size of the aerosolised particle agglomerate – that it the size of the inhaled agglomerate of particles. The fractional deposition of inhaled particles has been provided by results from The Human Respiratory Tract Model of the International Commission of Radiological Protection. As can be seen from Figure 1, the particles reaching the alveolar region consist primarily of nano-sized particles and to a smaller extent on particles in sizes up to 10 μm. However, it is also evident that inhaled nanoparticles deposit in the entire respiratory tract. In contrast, most of the larger particles (> 1-2 μm) deposit in the upper airways.

Particles may be removed from the respiratory tract by several different clearance mechanisms. Basically, the clearance of particles will either take place by chemical clearance or by physical translocation of particles. Chemical clearance of particles or particle compounds occurs when the particles or the coating of the particles are dissolved in body fluids. Chemical clearance may occur in all parts of the respiratory tract and is followed by absorption of solutes by a process similar to soluble chemicals. In contrast, the efficiency of physical translocation differs between the different regions of the respiratory tract. The bronchi, bronchioles and nose are covered by a blanket of mucus overlying the cilia. In these regions of the respiratory tract, deposited particles are removed by the mucociliary escalator. Deposited particles are moved upwards by the mucociliary escalator and are subsequently swallowed. This process usually takes hours to days. In the alveoli, the primary mechanism for particle clearance is macrophage mediated phagocytosis of particles. Hereafter the particle-loaded macrophages are moved toward the mucociliary escalator. This clearance mechanism is very slow and the half-time of particles in the alveolar region is months in rats and years in humans.

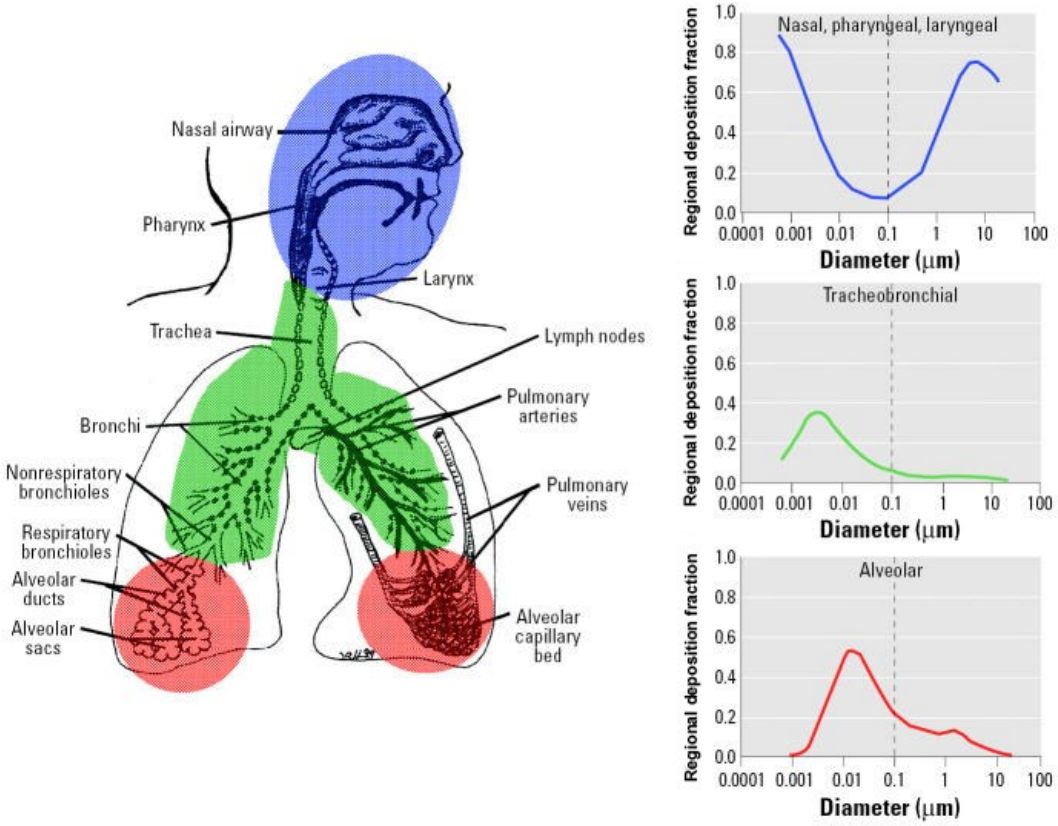

FIGURE 1
PREDICTED FRACTIONAL DEPOSITION OF INHALED PARTICLES IN THE NASOPHARYNGEAL, TRACHEOBRONCHIAL AND ALVEOLAR REGION OF THE HUMAN RESPIRATORY TRACT DURING NOSE BREATHING. REPRODUCED WITH PERMISSION FROM (OBERDÖRSTER ET AL., 2005) AND ACCORDING TO THE PUBLISHED ADDENDUM ((OBERDÖRSTER ET AL., 2010)).

The very slow removal of particles from the alveolar region increases retention time and the possibility for uptake of particles in alveolar cells and systemic translocation. Translocation of ultrafine particles from the lung into the blood circulation has been described in humans as well as in animals. Nemmar et al. reported from a study in humans, that particles could be detected in the blood circulation shortly after inhalation of 99m Tc-labeled ultrafine carbon particles (Technegas) (Nemmar et al., 2002). This indicates that translocation of particles from the lungs into the cardiovascular system takes place. Similar findings have been reported for other species. In rats exposed to ultrafine carbon, particles accumulated in the liver after 24 hours, indicating translocation from the respiratory system to the blood circulation (Oberdörster et al., 2002). Similar findings have been reported in hamsters (Nemmar et al., 2002).In contrast, other studies in humans with 99mTc-labeled carbon particles (Technegas) by Brown et al. (Brown et al., 2002) and by Mills et al. (Mills et al., 2006) did not confirm such uptake into the liver. In summary, the literature, especially in humans, on the translocation of small particles from the lungs into the blood circulation is limited and still conflicting. However, summarising the existing evidence from animal and human studies, this suggests that particle translocation from lung to circulation also exists in humans. As reviewed by Geiser & Kreyling, human studies have shown that the translocated nanoparticle mass fraction is less than 1 % of the dose delivered to the lungs (Geiser & Kreyling, 2010). Furthermore, particle size and surface characteristics/chemistry of the particles seem to be important determinants for the degree of extrapulmonary translocation.

2.1.2 Gastrointestinal absorption

Gastrointestinal exposure may occur, both directly by intentional intake of food or beverages containing nanomaterials, or indirectly by e.g. leakage of nanomaterials from food packaging to food. In addition, gastrointestinal exposure may occur following translocation of inhaled particles through e.g. the mucociliary escalator which are subsequently swallowed.

The gastrointestinal tract is composed of the oral cavity, the esophagus, the stomach, and the small and large intestine. To be absorbed from the gastrointestinal tract particles have to 1) diffuse through the mucus covering the surface of the gastrointestinal tract and 2) either be taken up by enterocytes or M-cells (phagocytizing enterocytes), or be absorbed by paracellular transport (Bergin & Witzmann, 2013).

As recently reviewed by Landsiedel et al., some studies report no absorption or only limited absorption while others report quite high levels of gastrointestinal absorption (Landsiedel et al., 2012). For example, no or minimal (~0.2 %) absorption was detected in rodents exposed orally to ^{192}Ir, ^{14}C-taurine-MWCNT or fullerene (C60). In contrast, when rats were exposed to large (130 nm) and small (48 nm) polystyrene particles by oral gavage, respectively, 26 % and 34 % of the doses were absorbed. A recent study on gastrointestinal uptake of different TiO_2 nanoparticles in rats showed that gastrointestinal absorption occurs to a very limited extent (Geraets et al., 2014).

The Danish EPA published in 2013 a report on systemic absorption of nanomaterials by oral exposure which gives an overview of the existing literature. In general, it was concluded that only a very limited number of well performed studies on gastrointestinal absorption exists and that more studies are needed to be able to perform a risk assessment of exposure of humans to nanomaterials (Danish EPA, 2013c).

The report gives an overview of physicochemical properties which were identified to affect the absorption of nanomaterials.

Size
Size may affect the absorption of nanomaterials. For example, a higher absorption of smaller particles compared to the absorption of larger sized particles of same chemical composition has been documented for Au, Ag, Fe and ZnO particles. It should be noted that the higher absorption of small Ag and ZnO particles may be due to higher dissolution of small particles compared to larger particles.

Agglomeration
Agglomeration of anatase nano-TiO_2 has in one study been shown to decrease absorption.

Crystal structure
A single study on the effect of crystal structure on the gastrointestinal absorption was identified. The study showed that rutile TiO_2 was better absorbed than anatase TiO_2.

In addition to size, agglomeration and crystal structure, the following physicochemical characteristics are mentioned as having a possible effect on absorption: Coatings/stabilisers, surface charge and surface reactivity. However, no documentation for these parameters was identified.

In general it is recommended by EFSA to perform an ADME study in animals if there is any potential exposure via the oral route (Danish EPA, 2013c and references therein). Currently, no *in vitro* methods for the hazard assessment of gastrointestinal absorption of nanomaterials have been validated (Danish EPA, 2013c). Most of the referred studies have been conducted in rodents. Important interspecies differences exist between rodents and humans. An example is that the rodent gastrointestinal tract is characterised by a much higher proportion of M-cells in the Peyer's Patches compared to humans (Landsiedel et al., 2012 and references therein). Other differences are that rats do not have a gall bladder (Fröhlich & Roblegg, 2012 and refererences therein), and in the human stomach the pH is about 1-2, whereas it is 3-4 in the rat stomach (McConnell et al., 2008; Fröhlich & Roblegg, 2012). These inter-species differences should be taken into account when extrapolating from rat to humans, especially when considering soluble nanomaterials. In addition,

it is stressed that it is important that techniques for determination of nanomaterials in tissues are available (Danish EPA, 2013c).

In general, the report concludes that the number of publications on gastrointestinal absorption is increasing, but only a few of these have been well performed. The gastrointestinal uptake of nanomaterials is dependent on physico-chemical properties such as particle size and surface chemistry. A recent study on gastrointestinal uptake of different TiO_2 nanomaterials in rats showed very limited absorption. Following absorption from the gastrointestinal tract, nanomaterials may reach the systemic circulation and be distributed to the rest of the body.

2.1.3 Dermal absorption

Dermal exposure can occur in all phases of the life cycle of a nanomaterial and the products containing the nanomaterial. Some application areas such as cosmetics result in very direct dermal exposure and often daily applications whereas exposure to nanomaterials from other application areas, e.g. where the materials are bound in a solid matrix/polymeric composite material as in the case of a tennis racket, is expected to be more limited. Following dermal exposure, the possible distribution of nanoparticles in the skin and the extent to which the particles reach a site of action or the systemic circulation depend on specific properties of the nanomaterials.

Topically applied substances may penetrate the skin through different pathways: the intercellular lipid pathway, by transcellular permeation or through hair follicles and sweat glands. Although hair follicles contribute to less than 0.1 % of the total skin surface area it has been suggested that dermal uptake of nanoparticles primarily occurs through the follicles. There is, however, no evidence to confirm that this pathway offers a viable mechanism of entry into systemic circulation (Choksi et al., 2010).

The Danish EPA published a report in 2013 on dermal absorption of nanomaterials which offers a comprehensive overview and evaluation of the knowledge base regarding dermal absorption of nanomaterials. The report is accompanied by a literature database with a systematic evaluation of the reliability and relevance of the existing scientific literature (Danish EPA, 2013a).

This existing literature has been used to evaluate the role of various physico-chemical properties on nanoparticle absorption into the skin as summarised under the following broad physicochemical properties, concerning the role of:

- Size
- Composition
- Surface chemistry
- Shape

The report also presents an evaluation of which test methods that are most appropriate in order to simulate the transport of nanomaterials over the skin and it identifies gaps in the current knowledge base where further research and development or validation is required.

Dermal absorption is defined as the situation where a substance has penetrated through the layers of skin and reached the site of action or the systemic circulation. Skin permeation is the diffusion of a substance *into* a certain skin layer, and the subsequent diffusion *through* that layer represents skin penetration as illustrated in (Danish EPA, 2013b).

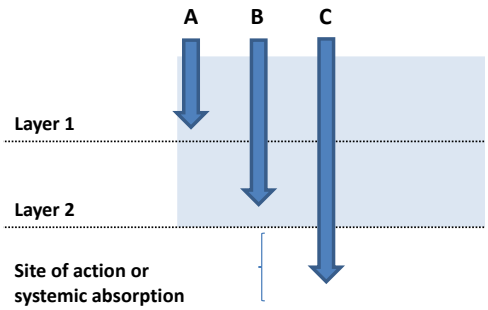

Figure 2 REPRESENTATION OF SKIN PERMEATION (A), SKIN PENETRATION (B) AND SKIN ABSORPTION (C).

In general, the report emphasises that there is a vast amount of literature available discussing dermal penetration and absorption, but limited possibility to compare across the many information sources due to differences and lack of consistency in experimental parameters and conditions.

Size

Particle size is part of the European Union (EU) definition of a nanomaterial and it is generally considered that on the same mass basis nanomaterials exhibit greater toxicity compared to larger particles. This also applies to the dermal exposure route. The report concludes that there are relatively few studies that include a broad size comparison, and comparison among different studies is difficult due to numerous confounding factors such as experimental model, species, doses, duration and particles with different physico-chemical characteristics.

Based on the available literature, the overall conclusion in the report is that absorption of particles in the nano-range through the skin is possible although it seems to occur to a very low degree and that the level of penetration, depending on chemistry and experimental conditions, may be greater than for larger particles.

In vivo absorption studies are available for some nanomaterials, in particular those available in sunscreen products.

Results from blood and urine measurements from a pilot study, where a sunscreen formulation containing approximately 18 % ZnO nanoparticles enriched with a Zn-isotope (^{68}Zn) was applied to the back of 3 human subjects, indicated very low levels of absorption through the skin, corresponding to less than 0.001% of the applied dose. The essentially phytochemical-based formulation was applied to the back of the 3 human subjects twice daily for five days during the Southern Hemisphere winter (Gulson et al., 2012).

Sadrieh et al. has made an attempt to quantify the amounts of coated nano-sized, uncoated nano-sized and uncoated submicron sized TiO_2 in sunscreens that penetrated into the skin of minipigs. Estimations based on electron microscopy-energy dispersive x-ray analysis (EDX) and transmission electron microscopy (TEM) showed that all three types of particles were most concentrated in the stratum corneum. A small number of scattered isolated particles were seen in the dermis of pigs treated with sunscreen formulations containing nanoparticles. These particles were however considered a possible result of contamination. However, quantification of the the level of TiO_2 that was confirmed to be present in the electron micrographs was made based on a number of assumptions regading the exposed area, the particle size and density. Based on the findings and assumptions it was estimated that the 10 confirmed particles of uncoated nano-TiO_2 would equate to $8 \cdot 10^{-5}$ percent of the total applied dose, the one partilcle of coated nano-scale TiO_2 would equate to $5.7 \cdot 10^{-6}$ percent of the total applied dose, and the one submicron TiO_2 in the dermis would equate to $2.3 \cdot 10^{-4}$ percent of the total applied dose. The organ-based Ti-analyses (lymph nodes, liver) confirmed the observations in the skin, suggesting “minimal” to “no penetration (Sadrieh et al., 2010).

Composition

Composition is considered in two ways in the report: 1) The primary macro composition of the particle (e.g. carbon for CNT) or, 2) if the nanoparticle contains a minor constituent or contaminant

that exerts a biological effect. Within each compositional group, particle characteristics such as size, crystallinity, charge, coating etc. differs considerably. For a substance like TiO_2, the crystalline structure is also a compositional issue which needs to be considered.

Another aspect which needs to be considered in relation to composition is solubility of the nano-particle. This is because the soluble fraction of a nanoparticle may penetrate deeper into the layers of the skin compared to the insoluble particle and potentially become systemically available. In the case of soluble metal (oxide) nanoparticles such as Ag and ZnO, it is generally not known whether only the metal ions are taken up through the skin or if dermal uptake of the nanoparticles also occurs.

The overall conclusion regarding the role of composition is that it appears to have little effect on dermal penetration of the nanomaterial (Danish EPA, 2013b).

Surface chemistry
The term surface chemistry is a relatively broad and non-specific term which is closely linked with other surface properties such as solubility equilibrium, catalytic properties, surface charge, and surface adsorption and desorption of molecules from solution. In addition surface chemistry is influenced by chemical purity, functionalization and surface coating, and the extent of coverage of the applied or acquired coating (Danish EPA, 2013b).

The Danish EPA (Danish EPA, 2013b) conclude that information from available studies indicates that surface chemistry is an important factor with influence on the ability of nanoparticles to penetrate into the skin, with surface charge (through the modification of surface coating/functionalisation) being the most investigated aspect. However, as results are inconclusive and characterisation often insufficient there are currently no firm conclusions regarding the role of surface chemistry. In addition, other factors such as the nature of the vehicle and its pH, particle colloidal stability and aggregation potential are mentioned as influencing the interaction of the charged particles with the skin.

Shape
The Danish EPA (Danish EPA, 2013b) conclude that it appears that shape has minimal impact on the penetration efficiency of nanoparticles. It is mentioned though that there is limited peer-reviewed information available on fibrous-shaped particles within the WHO-definition[8] of a fibre. Fibrous materials such as CNT have in several publications been suggested to be able to pierce cell membranes and the ability to influence skin penetration needs further investigation. It should however be mentioned that available information on CNT does not support this hypothesis, as observes skin irritation mainly was attributed to Fe-contamination.

Other factors
Other factors which may influence the dermal uptake due to disruption of the skin barrier integrity include skin diseases like psoriasis, allergic and irritant contact dermatitis and atopic eczema. Furthermore, mechanical flexions, irritant detergents, and the presence of other chemicals may also have an impact on the skin absorption.

Conclusion
As concluded by The Danish EPA (Danish EPA, 2013b), limitations in characterisation of nanomaterials and reporting of physico-chemical data and/or the alteration of multiple experimental parameters in a non-systematic way make the drawing of firm conclusions regarding the influence on dermal penetration very challenging.

[8] Particle with a length greater than 5 μm, a diameter of less than 3 μm and a length to width ratio (its aspect ratio) greater than 3:1.

The following is concluded:

- Size: Indication that penetration of nanoparticles into skin is possible but occurs to a low degree and is greater than for large particles.
- Composition: Macro composition seems to have little effect on dermal penetration/absorption. Ion absorption may occur rather than particle absorption due to solubilitsation of some particles.
- Surface chemistry: Seems to play an important role although results are not conclusive. A tendency towards greater uptake of positively charged particles is indicated.
- Shape: Seems to have little effect on penetration/absorption, although the ability of fibrous-shaped particles to influence skin penetration needs further investigation.

2.1.4 Eye absorption

A typical way of exposure of the eye to nanomaterials is by using cosmetics (mascara, eyeliner and possibly disposable lenses), sprays generating aerosols, by splashes and as dust and drops containing nanomaterials (Kuo et al., 2011).

The corneal and conjungtival epithelium offers protection against absorption of nanomaterials to the vitreous humour in the anterior and posterior chamber of the eye. This liquid is in direct contact with the retina, optic nerve and macula (Zhou et al., 2013).

Exposure of the eye may induce effects on and in the cornea or the conjunctiva localised inside the eyelids and on the sclera (white part of the eye). The outer eye is drained from liquid via the lacrimal channel and cleared into the gastrointestinal tract in a degree that is not known for certain but assumed to be low.

If absorbed into the eye, effects may be induced in structures within the eye (e.g. lens and its ligaments, the iris) or the inner surface of the retina, optic nerve and macula.

In theory, absorbed nanomaterials from the vitreous humour may be distributed into the blood or translocated by the optic nerve to its first synapse. The inner eye is drained via the Schlemm's channel that by a valve-like mechanism collects excess aqueous humor from the anterior chamber and drains it into the bloodstream. However, no knowledge exists regarding the effects of exposure to nanomaterials on these phenomena.

There are many different ocular diseases potentially treatable by nanomaterials and nanomaterial applied as drug carriers (Prow et al., 2008). These diseases include glaucoma, corneal diseases, wound on corneal epithelium, uveitis and retinal diseases (Zhou et al., 2013). This is an active area of nanomaterials drug development (Hainfeld & Powell, 2000), and knowledge is gained about specific nanomaterials with potential application as drugs and drug carriers. It is not known if such knowledge can be extrapolated to nanomaterials relevant for this report.

The database search did not reveal any information on eye absorption of relevant nanomaterials and the possible consequences.

In conclusion, available data are too limited to evaluate the potential eye absorption of nano-materials and to perform a hazard assessment.

2.1.5 Olfactory absorption

When exposed to the respiratory tract, nanomaterials may be taken up via general epithelial transfer or by specific translocation in sensory nerves.

General epithelial transfer: After crossing a single epithelial cell layer, nanomaterials may be transferred directly into systemic circulation without any first-pass hepatic metabolism. However,

the nanomaterials need to cross the blood-brain barrier (BBB) to gain access to the brain (Illum, 2000). In studies on intranasal installation and inhalation this route may participate in nasal absorption, but to what extend and relevance is at present not known.

Transfer via sensory nerves: In theory, transfer may take place along all the sensory nerves. However, only two routes may be important; the olfactory and the trigeminal nerve translocation. The olfactory nerves extend from the nose cavity, the trigeminus extends from various places including the eye lids and the conjunctiva and they both project in the central nervous system (CNS). No data exist on the trigeminal translocation (Aschner, 2009; Oberdörster et al., 2005; Illum, 2000). No more recent relevant information has been identified.

In humans, the nasal cavity covers approximately 150 cm^2. In the upper posterior part, approximately 10 cm^2 is covered with olfactory epithelium containing the olfactory nerves. Their axons bundle together and create the two olfactory nerves that lead to the two olfactory lobes which are projections of each cerebral hemisphere (Borm et al., 2006). Because these nerves project without any synapse directly into the brain's olfactory bulb, they offer direct access of nanomaterials from the nose to the brain by circumventing the protecting BBB. The transport mechanism is either a slow transport into the nerve cell axoplasm or a faster movement in the surrounding cerebro-spinal fluid. The velocity in axoplasm was determined to be about 2.4 mm/hour (Oberdörster et al., 2005). In rats and humans the axons of the olfactory neurons have a diameter around 200 nm and they are tightly packed when passing through the cribriform plate pores into the brain (De Lorenzo, 1970; Plattig, 1989). Consequently in order to pass through the axons, the diameter of the nano-material agglomerate should be less than 200 nm.

From the olfactory bulbs, chemicals including nanomaterials may have further access to the lower parts of the brain from where they may distribute to higher parts (Oberdörster et al., 2005; Aschner, 2009; Illum, 2000).

In fact, olfactory nerves have been reported to be portals for entry into CNS as examplified by intranasally instilled 30 nm polio and 50 nm herpes virus particles in chimpanzee and rhesus monkeys, 50 nm Ag-coated Au nanomaterial in squirrel monkeys, 35 nm carbon nanomaterials in rats, and soluble manganese (Mn) and inhaled 30 nm manganese oxide (MnO) nanomaterial in rats (For review see (Oberdörster et al., 2005; Aschner, 2009). Only the almost insoluble MnO nano-material has been shown to distribute to deeper parts of the brain presumably in the particle form (Aschner, 2009).

Besides the size, other important parameters for absorption and translocation are chemistry, charge, shape, aggregation and agglutination (Oberdörster et al., 2005). However, at present no coherent knowledge exists.

Relevance for humans
Olfactory absorption has been demonstrated in various laboratory animals and may also be relevant to humans (Elder et al., 2006). However, there are anatomical and functional interspecies differences.

Owing to these interspecies differences it is likely that olfactory absorption of a compound is more pronounced in rats than in humans (Oberdörster et al., 2005; Illum, 2000) and consequently implies an overestimation of the hazard when extrapolating to humans if this is not taken into account.

TABLE 1
INTERSPECIES DIFFERENCES BETWEEN RAT AND MAN OF RELEVANCE FOR OLFACTORY ABSORPTION*.

	Rat	Human
Breathing mode	Olfactory nose	Nasal and oronasal
Area of nasal mucosa	~ 16 cm^2	~ 105 cm^2
Area of olfactory mucosa (%total mucosa)	~ 8 cm^2 (50 %)	~ 5.25 cm^2 (5 %)
Percent nasal airflow going to olfactory mucosa	~ 15 %	~ 10 %
Weight of olfactory bulb	~ 85 ng	~ 168 ng
Body weight	400 g	60 kg

*Modified from (Oberdörster et al., 2005)

At present, no study has systematically investigated interspecies absorption variation so the relevance to human health is difficult to assess. This needs further systematic research on specific absorption and translocation and studies which demonstrate secondary CNS effects, e.g. affected neurotransmitter concentrations or sensory functions need to be preceded by proven olfactory absorption and translocation.

2.2 Distribution of nanomaterials

Upon absorption following pulmonary, dermal, eye or gastrointestinal exposure, nanomaterials may reach the blood circulation and/or the lymphatic system.

2.2.1 General (blood/organs)

The blood is central for organ distribution of absorbed nanomaterials and their excretion. If absorbed, nanomaterials following dermal, respiratory, and gastrointestinal exposure will reach the blood either directly and/or via the lymph although additional minor routes (eye, olfactory absorption) may also participate (Oberdörster et al., 2005; Oberdörster et al., 2009).

It is supposed that nanomaterials owing to their high surface energy will become coated or otherwise react endogenously very fast *in vivo* (e.g. with blood proteins or primary albumin) and consequently not remain as pristine nanomaterial. These reactions change their toxicokinetics (Borm et al., 2006).

Generally, regarding absorbed and endogenously derivatised nanomaterials, the half-life in blood seems primary to depend on uptake by the mononuclear phagocyte system rather than body clearance (Landsiedel et al., 2012). Blood circulation time depends on size, charge and water affinity. In general, hydrophilic nanomaterial, nanomaterial <100 nm and uncharged nanomaterial remains for a longer time in blood circulation (Borel & Sabliov, 2014).

In brief, most systematic knowledge about distribution to blood and organs, primarily the liver, originates from administration of very high doses for a short duration of time. Often blood is not analysed itself but indirect evidence originates from distribution to organs and excretion. The relevance of these settings for humans cannot be stated at present. Furthermore, nanomaterials constitute a heterogeneous group, and most knowledge exists on metals that can easily be detected

and quantified as elements, whereas knowledge about their nanoform is very limited. At present, extrapolation to other nanomaterials for hazard assessment is not possible.

2.2.2 CNS

Access of nanomaterials to the CNS may take place via olfactory absorption or after oral, dermal and inhalation exposure via the blood. However, an important anatomical and functional barrier protects the brain from entry of chemicals present in the blood, namely the BBB. Generally, crossing the BBB is restricted to molecules which are lipophilic, actively transported or small soluble molecules < 500 Da (Hagens et al., 2007).

However, owing to their special characteristics, nanomaterials have been hypothesised to be able to cross the BBB. Thereby, some nanomaterials, including Ir, Zn, Ag, Au, carbon particles, MnO_2, TiO_2, Al_2O_3, and ZnO may distribute to the brain and induce toxic effects to the CNS (Boyes et al., 2012; Hu & Gao, 2010; Sharma & Sharma, 2010; Simkó & Mattsson, 2010; Yang et al., 2010; Oberdörster et al., 2009). No interspecies studies are conducted, thus the relevance to humans cannot be stated.

2.2.3 Fetus

The placenta constitutes the nutritional interface between mother and fetus. As such it does not represent a complete barrier between the maternal and fetal compartments. Studies of the passage of ENP across the placenta are still few in number, but rodent studies describe transplacental transport of nano-sized particles, with rates ranging from almost negligible to high (several percent of the administered dose). At present, placental transfer has been studied and rendered possible for particles of Au, polystyrene, nano-sized TiO_2, and CNT (Huang et al., 2014; Hougaard et al., 2011a; Menezes et al., 2011). Exposure to nanoparticles of silica and Ag or quantum dots (with a core of cadmium) led to increased metallic content in fetal tissues in rats. Whether this was caused by particulate or ionic transfer is unclear (Melnik et al., 2013; Buerki-Thurnherr et al., 2012; Menezes et al., 2011). Studies in the human placenta model confirm that placental transfer and uptake may also take place in humans (Hougaard et al., 2011a). Additional transfer of particles from mother to offspring could potentially take place during lactation (Melnik et al., 2013).

The capability for transplacental transfer depends highly on the physico-chemical properties of the particles. This is well illustrated in a study of 13 nm Au nanoparticles administered intravenously to pregnant mice. Early after establishment of pregnancy, close to 1 % of the maternally administered dose was found in the fetus and 1 % in the immediate surrounding (extraembryonic) tissues. Later, when the placental barrier function was just established, fetal uptake was down to 0.1 %. Of note, extraembryonic tissues now held 10 % of the maternal dose, approximately the same percentage as was recovered from maternal liver. The pattern was similar for two types of biocompatible Au particles, whereas accumulation was drastically reduced for the negatively charged particle (Yang et al., 2012).

The finding has several other important implications. Timing of exposure during pregnancy is of importance. Transfer may be higher early in pregnancy and decrease in the immediate period after establishment of the placental barrier. In humans, transfer may increase late in pregnancy, where also transfer of large antibody molecules takes place (Hougaard et al., 2011a). Furthermore, particles may be taken up in extraembryonic tissues. Several studies find nanoparticles to be internalised in placental cells at the maternal-fetal interface, consistent with the placenta acting as a sequestration organ (Hougaard et al., 2011a; Menezes et al., 2011; Wick et al., 2010).

Size presents an important factor in placental transfer. In the human placenta model, close to 30 % of 50 nm and 80 nm polystyrene beads transferred from maternal to fetal circulation within hours of exposure. For 240 nm and 500 nm particles, 9 % and 1 % crossed the placenta, respectively (Wick

et al., 2010). Finally, transfer of particles across the placenta has been proposed to increase under some conditions, for example inflammation (Hougaard et al., 2011a).
It does therefore not seem a question whether nano-sized particles are able to reach the fetus, but rather to which degree it takes place and which characteristics govern particle transfer across the placenta. Overall, nanoparticle type, size and surface composition as well as stage of embryonic development determine the extent to which particles transfer from mother to fetus.

2.3 Metabolism

Only a limited number of studies have investigated the potential metabolism of nanomaterials. Where such information exists, most of the studies show that the nanomaterials are not metabolised (Landsiedel et al., 2012).

It is supposed that nanomaterials due to high surface energy will be coated or otherwise non-enzymatically quickly react with e.g. proteins, agglomerate or agglutinate *in vivo* and consequently not remain as pristine and discrete particles if absorbed.

The liver is a target organ for many nanomaterials in laboratory animals. Traditionally, first-pass metabolism is considered to play an important role for detoxifying or activating xenobiotics in the liver. However, the relevance of this phenomenon for nanomaterials is not known. At present it is very limited what is known about the metabolism of nanomaterials and the underlying processes (Borel & Sabliov, 2014; Card et al., 2011). Some general principles include:

- Inorganic nanomaterials are very stable (e.g. TiO_2, Au, Pt, silica) and are impossible to metabolise endogenously by enzymes (Allen et al., 2008). Generally, metabolism does not seem likely for inorganic nanomaterials as such, whereas any organic group attached to the surface could be modified (Hagens et al., 2007). Breakdown of soluble nanomaterials such as Ag or ZnO nanomaterials caused by low pH or chemical non-enzymatic oxidation/modification are not regarded as metabolism.

- Organic nanomaterials, especially those with branched side chains or hydrophilic groups, could be metabolised e.g.by human oxidative enzymes (Hagens et al., 2007). However, these nanomaterials are outside the scope of this report.

- It has been reported that single-walled CNT can be degraded *in vitro* by horseradish peroxidase (Allen et al., 2008). It is not known whether other peroxidases are able to degrade these nanomaterials *in vivo*.

The database search did not reveal further relevant information on the metabolism of relevant nanomaterial - free or bound in a matrix - and its potential consequences. In conclusion, available data are too limited to be extrapolated to other nanomaterials in humans. A case-by-case strategy should be applied for hazard assessment.

2.4 Excretion/accumulation

2.4.1 Excretion

The blood is central for the distribution and excretion of absorbed nanomaterials which may be removed by renal excretion and/or in feces via bile excreted from the liver. A minor potential excretion route may be via breast milk. However, the extent is not known (Landsiedel et al., 2012; Oberdörster et al., 2009; Oberdörster et al., 2005).

Some nanomaterials are excreted directly from the port of entry. For example, the majority of orally dosed nanomaterials are almost completely excreted via the feces without or with minimal absorption into the body. Rats orally exposed to ^{14}C-labelled C-60 fullerenes eliminated 97 % of the dose in the feces within 48 hours (Yamago et al., 1995).

As reviewed by Landsiedel et al. (Landsiedel et al., 2012), two functionalised CNT, one single walled carbon nanotubes (SWCNT) and one MWCNT are examples of nanomaterials for which an efficiently excretion via the kidneys are seen in rats dosed by intravenous injection. Hepatic excretion has been observed in rats exposed to polystyrene nanoparticles. Of the administered dose 4 % was excreted via the bile within the first 24 hours.

Characteristics of importance for kidney and liver excretion are size, shape, surface charge, attached functional groups, endogenous coating, aspect ratio and internal agglomeration and/or aggregation (Borel & Sabliov, 2014; Landsiedel et al., 2012).
Generally, kidneys excrete nanomaterial < 5 nm whereas renal clearance seems to be minimal for nanomaterial > 5 nm. Uncharged and negatively charged particle are eliminated from the kidneys, whereas positively charged particles may accumulate in the kidneys. Only hydrophilic particles are eliminated via urine.

Orally dosed nanomaterial 2-200 nm in size are excreted from the liver via bile whereas particles < 500 nm in size are are not taken up via the gastrointestinal tract (GI-tract) and are excreted directly by feces. The relevance of charge for fecal excretion is not clear.

Because of the very little translocation of nanomaterials following exposure via the main exposure ways (lungs, skin and gastrointestinal tract) most excretion studies inclusive the above mentioned have been performed in rodents dosed by intravenous injection. Intravenous exposure is not the way a consumer will be exposed but this exposure way is appropriate for mechanistic studies and simulates a worst-case scenario in which 100 % of the administered dose is absorbed and reaches the circulation. More knowledge on excretion following oral, dermal and inhalation exposure is urgently needed.

2.4.2 Accumulation

It seems that nanomaterials in the sizes 20-100/200 nm can remain in the blood for a longer time and in that way add to the organ distribution (Borel & Sabliov, 2014; Wang et al., 2013b).

Studies have shown that nanomaterial in systemic circulation can accumulate in the body rather than being excreted. The liver has been shown to accumulate more than 90 % of the translocated nanomaterials (compared to the other organs) (Kermanizadeh et al., 2014). For example, no excretion from rodents exposed by intravenous injection of different kinds of quantum dots could be detected during the follow-up time of the experiment (between 10 and 28 days). Another example is a study showing protracted elimination of 40 nm Au nanoparticles from mouse liver following intravenous injection. Au particles were accumulated in Kupffer cells in the liver one day after exposure. The Au content of liver from exposed mice was only decreased slightly after 6 months (Sadauskas et al., 2009a). The size of nanomaterials is important for the proportion of nanomaterials that reaches the liver. A mouse intratracheal instillation study showed that 2 nm Au particles translocated to the systemic circulation and was detected in the liver to a greater extent than 40 nm and 100 nm sized Au particles (Sadauskas et al., 2009b). The smallest Au particles (2 nm) seemed to be removed not only by endocytosis by macrophages, but also to be excreted via the urine (Sadauskas et al., 2007).
In general, distribution to organs besides liver and kidneys includes spleen, heart, muscle, placenta, bone marrow, CNS and the lungs and most other organs if analysed (Wang et al., 2013a; Oberdörster et al., 2009; Oberdörster et al., 2005). The liver is the major distribution organ followed by the spleen, the lymph nodes and the bone marrow. These organs may constitute the

prime target organs for accumulation and for toxicity. However, most knowledge about distribution and excretion originates from studies applying intravenous injections and studies applying high-level exposure for a short time. No relevant study has been identified on long-term bioaccumulation.

In conclusion, at present consistent knowledge about organ accumulation cannot be concluded from available data. Systematic long-term, low-dose studies of bioaccumulation and persistence in the body are needed to assist hazard assessment. Furthermore, nanomaterials constitute a heterogeneous group and most knowledge exits on metals as they can easily be detected and quantified as elements, whereas knowledge about their nanoforms is very limited. At present, extrapolation from ADME and organ accumulation of bulk material and other nanomaterials is not possible.

2.5 Summary

It is difficult to make general conclusions on the biokinetics on nanomaterials due to the very diverse physico-chemical properties. The present knowledge on toxicokinetics of nanomaterials is summarised below.

Absorption: For the consumer, the lungs, the gastrointestinal tract and the skin are considered to be the primary absorption routes of nanomaterials. In addition, uptake may occur through the eyes and the olfactory system. Pulmonary exposure to nanomaterials is the exposure route of highest concern. Inhaled nanomaterials deposit in the alveolar region of the lungs from where they are removed very slowly and consequently particles may stay for years. A small amount of the particles may translocate from the lungs to the systemic circulation. Most studies on dermal absorption have shown no to very moderate absorption of nanomaterials following application on healthy skin. Similarly, most gastrointestinal studies have shown that many nanomaterials are almost completely excreted by faeces although a few studies report high uptake of specific nanomaterials.

Distribution: The blood is central for organ distribution of absorbed nanomaterials and their excretion. If absorbed, nanomaterials following dermal, respiratory and gastrointestinal exposure will reach the blood either directly and/or via the lymph although additional minor routes (eye, olfactory absorption) may also participate.

Metabolism: In general, nanomaterials are not metabolised.

Excretion/accumulation: Once in systemic circulation, small nanoparticles (less than 6 nm) may be excreted in urine via renal excretion. Larger particles and other nanomaterials accumulate in various tissues including spleen, lymph nodes and bone marrow, and liver accumulation seems to be most dominant. The larger nanoparticles may be removed into feces via bile excreted from the liver although the rate is very low. A minor potential excretion route may be via breast milk. However, the extent is not known.

3. Adverse effects of pristine nanomaterials

3.1 Respiratory system

Pulmonary effects associated with ambient particle exposure include asthma (D'Amato et al., 2005), chronic obstructive pulmonary disease and lung cancer (Cohen & Pope, III, 1995; Vineis & Husgafvel-Pursiainen, 2005). However, so far most of the information on the pulmonary effects of nanomaterials has been derived from studies with rodents.

The processes and mechanisms behind particle-induced effects in the lungs have recently been presented by (Donaldson & Poland, 2012) and are summarised below and a few additional references have been included where relevant.

Cellular stress

Uptake of nanomaterials in pulmonary cells (epithelial cells and macrophages) may induce cellular stress and this determines if the nanomaterial has the potential for causing inflammation. Several potential pathways and mechanisms for particle-induced cellular stress have been proposed: 1) The main hypothesis for the adverse effects of nanomaterials is oxidative stress. Oxidative stress is defined as an imbalance between oxidants and antioxidants. Nanomaterials may generate reactive oxygen species (ROS) either directly by physicochemical properties, by soluble compounds or transition metals, altered function of mitochondria or cellular calcium homeostasis, or indirectly through inflammatory processes (Li et al., 2008), 2) Following uptake of positively charged nano-materials, the positively-charged surface may interact with and destabilise the lysosomal membrane, 3) Lysosomes may get destabilised if an acid-soluble nanomaterial dissolves within the lysosome resulting in release of ions which may destabilise the lysosome, and 4) Frustrated phagocytosiscytosis may be induced by high aspect ratio nanomaterials (HARN) because the macrophages are not capable of complete enclosure of the long fibers. This results in destabilisation of the phagolysosomal process leading to oxidative stress.

Inflammation

As already described in Paragraph 2.1.1, the primary mechanism for particle clearance in the alveoli is macrophage-mediated phagocytosis of particles. As long as the capacity of the macrophages to engulf the particles is not exceeded and the particles do not induce stress in the macrophages, this process only has minor impact on macrophages and the particle-loaded macrophages are cleared by the mucociliary escalator. However, if the macrophages are stressed they induce an inflammatory response. The inflammatory response is characterised by macrophage secretion of cytokines, chemokines and other signaling molecules mediating recruitment of granulocytes (especially neutrophils but also eosinophils) and monocytes from the blood circulation into the lung lumen. Exposure studies have shown that nanoparticles cause more inflammation in the lungs of rodents than exposure to the same mass concentration of fine particles. There is much evidence that the inflammatory response induced by low-toxicity low-solubility particles correlates well with the total surface area of the pulmonary deposited particles (Saber et al., 2012b; Jacobsen et al., 2009; Tran et al., 2000). For HARN, inflammation is induced by frustrated phagocytosis.

Fibrosis
Fibrosis is characterised by the accumulation of a matrix of collagen and fibronectin, proliferation of fibroblasts and transformation of myofibroblasts. In general, persistent inflammation is suggested to result in fibrosis.

Genotoxicity and cancer
Nanomaterials may induce genotoxicity by primary or secondary mechanisms (further described in Paragraph 3.9).

3.2 Cardiovascular system

Human exposure to particles from the ambient air has been associated with a number of cardiovascular conditions. These include myocardial infarction, hypertension, atherosclerosis, heart rate variability, thrombosis, and coronary heart disease (Nelin et al., 2012). Concern has been raised that engineered nanomaterials may have similar effects on the cardiovascular system (Saber et al., 2014). The mechanisms behind particle-induced effects on the cardiovascular system are still not well understood. However, there are three major theories (Schulz et al., 2005): 1) Particles translocating from the lungs to the circulation may have a direct effect on the blood vessels and the blood cells, and, 2) Particles may exert an indirect effect due to particle-induced pulmonary inflammation which leads to release of signaling molecules into the bloodstream, thereby inducing systemic inflammation, and 3) Particle-induced effects on the autonomous nervous system may result in heart rate variability. Recently it was proposed that inhalation of (nano)particles induce pulmonary inflammation and acute phase response and that the pulmonary acute phase response via systemic circulation promotes atherosclerosis and cardiovascular disease (Saber et al., 2014).

Particle translocation
As already described in Paragraph 2.1.1., the degree of translocation of particles from lungs to circulation differs considerably between studies. An example is that one study of humans exposed to ^{99}Tc-labelled 20 nm carbon nanoparticles showed translocation from lungs to blood circulation and accumulation of particles in the liver, while another study of the same particles showed no translocation (Landsiedel et al., 2012 and references therein). Translocated particles reaching the blood circulation may interact and cause adverse effects in the blood vessels. The effect of exposure to particles on vasomotor function has been investigated by exposing vessel segments *ex vivo* (reviewed by Møller et al., 2011). Such *ex vivo* studies enable assessment of the direct particle effect without the effects of the accompanying inflammation. However, these *ex vivo* studies assess the effects of a much larger particle concentration than present in the *in vivo* situation, where only a very limited number of particles translocate to the systemic circulation and where the circulation time seems to be relatively short. Therefore cautions should be taken before extrapolating the results to the *in vivo* situation.

Systemic inflammation
A chronically elevated acute phase response is one of the most important known risk factors for cardiovascular disease. Epidemiological studies have shown a link between air pollution and the contents of the acute phase protein C-reactive protein (CRP) in the blood (Ridker et al., 2000). An elevated level of an acute phase protein called serum Amyloid A (SAA) is also causally linked to the development of atherosclerosis in mice (Dong et al., 2011). Recently, it has been shown that exposure to a number of very diverse nanomaterials (nano-TiO_2, Nano-CB, MWCNT) and other particles result in acute phase response in the lungs of mice (Saber et al., 2013). Other studies have shown that the acute phase response is causally related to increased risk of atherosclerosis and cardiovascular diseases (reviewed by Saber et al., 2014). This suggests that the acute phase response in the lungs may be a possible mechanistic explanation for particle-induced cardiovascular disease. The extent of the particle-induced acute phase response was shown to depend on the total surface area of pulmonary deposited particles (Saber et al., 2014).

Autonomic nervous system
The third theory concerns the adverse effects of particles on the autonomous nerve system, which can either be a direct particle effect or an inflammation-mediated effect on the autonomous nervous system.

The hypotheses described above, whereby the systemic effects of inhaled nanomaterials are caused by translocation of particles, pulmonary inflammation and respiratory reflexes, are not mutually excluding but assumed to interact. For instance, lung inflammation has been shown to increase the extrapulmonary translocation of particles (Chen et al., 2006).

3.3 Gastrointestinal tract

Recent reviews have indicated that the knowledge on the toxicity of orally administered nanomaterials in the gastrointestinal tract is limited (Bergin & Witzmann, 2013; Jepson, 2012; Card et al., 2011). As described previously in Paragraph 2.1.2, the uptake of nanomaterials from the gastrointestinal tract differs a lot between different types of nanomaterials. If absorbed from the gastrointestinal tract, nanomaterials can reach the systemic circulation and accumulate in various tissues or interact with blood components. The review by Card et al identified 30 studies on the safety of oral exposure to food-related nanomaterials (Card et al., 2011). Of these, only few were considered reliable because of insufficient characterisation of the tested nanomaterials in most of the studies. Only two *in vivo* studies were regarded as having sufficiently described the studied nanomaterials. One of these studies tested the oxidative damage in rats following a single oral gavage to C60 fullerenes and SWCNT ((Folkmann et al., 2009) as cited by (Card et al., 2011)). Both C60 and SWCNT exposure increased the hepatic and pulmonary levels of 8-oxo-7,8-dihydro-2-deoxyguanosine, while no effects were seen in the colon. It was concluded by the authors that the systemic effects were caused by direct genotoxicity. The other study was a 15-day repeated dose dietary study of different types of nano-sized $FePO_4$ and $FeSO_4$. No increase in liver damage, inflammatory changes or any other adverse effects were detected in the iron-supplemented rats ((Rohner et al., 2007) as cited by (Card et al., 2011)). Based on only these two *in vivo* studies and a number of *in vitro* studies the authors concluded that the data set is too limited "*to derive any overall conclusions regarding the toxicity of nanomaterials for intended use in food and food-related products*" (Card et al., 2011). However, the authors conclude that "*the current review does provide sufficient evidence to dismiss or refute some purported generalizations. For example, the toxicity of the nanoformulation of a certain ingredient or material was not consistently increased as compared to the non-nanoformulation. In some cases, there were no differences in the biological endpoints measured between the nanoformulation and non-nanoformulations and, in the case of nanoselenium, evidence for reduced toxicity of the nanoformulation as compared to other forms of selenium was found in several studies. Secondly, several studies demonstrated enhanced beneficial effects of orally administered nanomaterials, with no evidence of adverse effects. Clearly it is possible to develop engineered nanomaterials that present no safety concerns due to oral exposure at specific dose levels*" (Card et al., 2011).

Since the review by Card et al., a review on the toxicity of Ag nanoparticles has been published by Hadrup & Lam. Ag induces a blue-grey discoloration in the skin (argyria). The review concludes that the effects caused by Ag nanoparticles are mediated via Ag ions released from the particle surface (Hadrup & Lam, 2014). The toxicity of nano-Ag is further discussed in Chapter 6.4.

To summarise, more studies on oral toxicity of well-characterised nanomaterials are needed to perform a clear risk assessment of oral exposure to nanomaterials.

3.4 CNS

The CNS consists of the brain and the spinal cord. At present there is no internationally accepted definition of chemically induced neurotoxicity but it is generally agreed that CNS-neurotoxicity is any adverse effect on biochemistry, structure and/or function of the CNS during development or at maturity induced by a chemical or physical agent. Adversity is any decreased ability to function fully or to function based to compensatory mechanisms.

Some nanomaterials, including Ir, Zn, Ag, Au, carbon particles, MnO_2, TiO_2 and ZnO seem to distribute to the brain and may induce effects on CNS (Boyes et al., 2012; Hu & Gao, 2010; Sharma & Sharma, 2010; Simkó & Mattsson, 2010; Oberdörster et al., 2009; Yang et al., 2010) as based on elemental analyses and thus not necessary as nanomaterials.

Human epidemiological studies, controlled animal studies and mechanistic *in vitro* studies have shown that exposure to air pollution can lead to neurotoxicity (Costa et al., 2014).

Especially, much relevant knowledge on effects of nanomaterials on the brain originates from animal studies.

Rats were dosed orally by gavage with 2.25, 4.5 or 9 mg/kg bw/day with 14 nm nano-Ag. Dosing 2.25 and 4.5 mg/kg bw/day for 14 days reduced brain dopamine (DA) neurotransmitter concentration, whereas noradrenaline (NA) and 5-hydroxy-tryptamine (5-HT) neurotransmitter concentrations were not affected. Dosing 2.25 and 4.5 nano-Ag mg/kg bw/day for 28 days increased the DA concentration. Dosing 9.0 mg nano-Ag/kg bw/day for 28 increased DA and 5-HT brain concentrations (Hadrup et al., 2012c).

Nano-ZnO, when administered intraperitoneal in doses of 4 mg/kg bw biweekly (20-80 nm) for 8 weeks to rats and in doses of 5.6 mg/kg bw every other day (8 doses in total) (20-80 nm) to mice, have been shown to affect spatial learning and memory functions (Xie et al., 2012; Han et al., 2011).

Wang demonstrated increased brain NA and 5-HT brain neurotransmitter concentrations in mice 10 days after intranasal installation of nano-TiO_2 (25-155 nm) in a dose of 50 mg/kg bw (25-125 nm) whereas the brain DA concentration was decreased (Wang et al., 2007b). Mice were instilled 250 µg CB (14 nm) (CB, Printex 90) intranasally. CB increased the extracellular concentrations in bulbus olfactorius of glutamate and glycine neurotransmitters, whereas taurine and gamma-aminobutyric acid concentrations were not affected (Win-Shwe et al., 2008).

ZnO is soluble whereas both nano-TiO_2 and CB are insoluble. Zn may reach the brain as Zn^{2+} whereas TiO_2 and CB both seem to reach the brain as particles.

The presence of the administered nanomaterials in nanoform in the brain has not been proven but only evidenced by elemental analyses, thus the mechanisms underlying nanomaterials crossing of the intact BBB remain elusive and there are still open questions as to the long-term consequences of nanomaterials CNS-accumulation and fate (Simkó & Mattsson, 2010; Oberdörster et al., 2009).

Any possible functional consequences of the above changes in brain neurotransmitter concentrations as induced by Ag, TiO_2 and CB are not known whereas the behavioral changes as induced by ZnO are functional changes that could be a manifestation of effects on neurotransmitter concentrations that were not measured.

CNS-effects induced in rats and mice after olfactory exposure to ZnO, CB and TiO_2 are analysed in a number of studies. A recent key study (Gao et al., 2013) investigates the effects of nano-ZnO (30 nm) when instilled intra-nasally as a single dose in rats. Magnetic Resonance Imaging (MRI) scanning was performed 1, 2, 3 and 7 days after instillation. The scans revealed that ZnO caused

significant damage to the olfactory epithelium including edema, inflammation and disruption of epithelial structures, and mitochondrial destruction. These effects were accompanied by disturbed sniffing behavior. At the end of the study (7 days post instillation) TEM revealed no indication of cellular uptake of nano-ZnO as such. There is a slightly acidic milieu in the nasal mucosa (Mei et al., 2008) so the nano- ZnO might be dissolved and taken up as Zn^{2+}.

In a second study rats were exposed 1 day for 6 hours to 2.1 x 10^6 particles/cm^3 of nano- ZnO (38 nm) for preparation of olfactory synaptosomes (isolated presynaptic nerve endings) or 3 days for 4 hrs to 2.0, 3.4 and 6.6 x 10^6 particles/cm^3 nano-ZnO (12-14 nm) for TEM. The demonstrated increased olfactory synaptosomal Zn concentration proved olfactory-brain absorption and translocation and the TEM showed numerous black spots (of not investigated composition) in olfactory bulbs and brains of the exposed animals (Kao et al., 2012).

Furthermore, nano-ZnO was reported to induce degeneration of olfactory sensory neurons (Takeda et al., 1997; Burd, 1993). Short-term inhalation exposure of rats to nano-ZnO at a concentration of 0.5 mg/m^3, 6hrs/day for 5 days induced nasal necrosis (Landsiedel et al., 2012). Pico molar concentrations of nano-Zn have been reported to strongly enhance the odorant responses of olfactory sensory neurons (Aschner, 2009). This indicates that Zn nanoparticles interact with olfactory receptor neurons (Vodyanoy, 2010).

Mice were instilled 250 µg CB (14 nm) (Printex 90) intra-nasally. Microdialysis in the olfactory bulb revealed increased extracellular concentrations of the CNS neurotransmitters glutamate and glycine whereas the taurine and gamma-aminobutyric acid concentrations were not affected (Win-Shwe et al., 2008).

Ten days after intranasal installation of TiO_2 in mice, affected brain neurotransmitter concentrations were demonstrated as increased NA and 5-HTconcentrations and decreased DA concentration (Wang et al., 2007b).
Overall, at present it is not possible to predict the toxicological consequences of the above findings, if any. Normally, functional and structural findings are considered more relevant than biochemical changes in the CNS. Most often brain biochemistry is investigated because it is more economic with respect to time and costs than studies of functional and structural changes.

Based on *ex vivo* experiments with perfusion of human placenta with fluorescence labelled polystyrene nanomaterials (50, 80, 240, 500 nm), fluorescence was demonstrated to pass the human placenta barrier (Wick et al., 2010). Other nanomaterials (TiO_2, 70nm, 10-100nm; SiO_2, 35 nm) have been shown to accumulate as elements in fetal organs, including the brain of rats and mice and induce effects on CNS (Gao et al., 2011; Yamashita et al., 2011; Hougaard et al., 2010). Therefore, a recent US-EPA review raises concern that nanomaterials may induce developmental neurotoxicity (DNT) following prenatal or early postnatal exposure of mothers and pup, respectively. The review concludes "*The evidence for nanomaterials DNT to date is not strong enough to reach any conclusions about potential risk; however, it indicates that a closer look is warranted*" (Powers et al., 2013). Thus, this is still an important issue that should be further studied.

3.5 Other organs

Effects of nanomaterials on organs distant from the route of exposure (e.g. lung, gastrointestinal tract, skin) may be caused either by direct effects of translocated particles or by systemic inflammation. Different kinds of nanomaterials have been shown to be able to translocate from site of exposure (e.g. gastronintestinal tract, lungs) to organs (e.g. liver, kidney). The liver has been shown to accumulate more than 90 % of the translocated nanomaterials (compared to the other organs) (Kermanizadeh et al., 2014). The toxic effects of nanomaterials on the liver have been

summarised in a recent review (Kermanizadeh et al., 2014). Intravenous injection of nanomaterials into rats and mice have resulted in different adverse effects in the liver such as changes in the hepatic gene expression of genes related to detoxification, lipid metabolism and the cell cycle in rats following injection of 20 nm Au particles ((Balasubramanian et al., 2010) as cited by (Kermanizadeh et al., 2014)) and changes in the hepatic gene expression of genes related to apoptosis, cell cycle, inflammation and metabolic processes following intravenous exposure of Bagg Albino (BALB/c) mice to 4 and 100 nm polyethyleneglycol (PEG) Au coated nanomaterials ((Cho et al., 2009) as cited by (Kermanizadeh et al., 2014)). Kermanizadeh et al. mention a number of factors of importance for the outcome of liver-related effects in *in vivo* experiments: 1) the route of exposure is very important for the fraction of dosed nanomaterials that accumulates in the liver. Intravenous injection results in accumulation of a much larger fraction of nanomaterials in the liver compared to other exposure routes (e.g. lung, gastrointestinal tract), 2) the size of nanomaterials is important for the proportion of nanomaterials that reaches the liver. For example, a mouse intratracheal instillation study showed that 2 nm Au particles translocated to the systemic circulation and was detected in the liver to a greater extent than 40 and 100 nm sized Au particles (Sadauskas et al., 2009b). The smallest Au particles (2 nanometer) seemed to be removed not only by endocytosis by macrophages but also to be excreted via the urine (Sadauskas et al., 2007), and 3) The coating of nanomaterials by proteins may also affect the liver toxicity. The coating depends on the route of exposure.

3.6 Skin

As discussed in Paragraph 2.1.3 there is little evidence of dermal absorption of nanoparticles through healthy skin as well as through skin damaged by UV radiation or psoriasis. Consequently, there is also little evidence of dermal toxicity. Discrepancies in the literature are likely to be related to differences in techniques and methods employed, laboratory conditions and absence of standardised evaluation protocols (Crosera et al., 2009).

In a review article on nanoparticle dermal absorption and toxicity, Crosera et al. refer to a number of studies reporting cytotoxic effects on dermal cells exposed to different nanomaterials (Crosera et al., 2009). No details are available on the individual studies. Examples of observed effects are summarised in Table 2 based on information from the review article.

Based on the experimental findings reported by Crosera et al. (Crosera et al., 2009), the authors conclude that the results are contradictory and no specific conclusions regarding dermal toxicity are drawn. Overall it is concluded that more information in needed in order to understand under which conditions dermal absorption may occur and what adverse effects can be expected.

Conclusion

Overall there is little evidence of dermal toxicity following typical application of nanomaterials. However, if nanoparticles are able to penetrate the skin and enter the bloodstream, they may exert a number of adverse effects. As demonstrated in numerous *in vitro* studies, these effects include generation of reactive oxygen species, oxidative stress, protein, DNA and membrane injury, mitochondrial damage and inflammation resulting in e.g. tissue infiltration, fibrosis, and granuloma formation. Other proposed effects of nanoparticles entering the systemic circulation include adverse effects on cells of e.g. the immune systems and passage into the CNS (Choksi et al., 2010).

3.7 Eyes

The eyes may potentially be exposed to nanomaterials during manufacturing, use and disposal of the nanomaterials (Ema et al., 2013a). This may give rise to irritation due to surface contact or toxicity if absorbed into the eye.

TABLE 2
EXAMPLES OF EFFECTS OF DIFFERENT NANOPARTICLES ON DERMAL CELLS*

Compound	Type of cells	Outcome
Iron oxide (10 nm)	Human dermal fibroblast	· Disruption to cell cytoskeleton · Reduced proliferation.
SWCNT	Human epidermal keratinocytes	· Increased oxidative stress · Inhibited cell proliferation · Increased expression of stress responsive genes · Cellular toxicity with formation of free radicals · Accumulation of peroxidative products · Antioxidant depletion · Loss of cell viability · Ultrastructural and morphological changes · Dose-dependent irritation response with increase in IL-8 and decrease in cell viability
MWCNT	Human epidermal keratinocytes	· Irritation response · Induction of the release of proinflammatory cytokine · Alteration of protein expression (metabolism, cell signaling, stress, cytoskeletal elements, vesicular trafficking)
Ag	Human epidermal keratinocytes	· Inhibited proliferation · Affected cell morphology
Cobalt chrome alloy (30 nm)	Human dermal fibroblast	· DNA damage, aneuploidy and cytotoxicity · Disintegration within the cells with the creation of electron dense deposits enriched in cobalt
TiO_2	Human epidermal keratinocytes Human dermal fibroblasts Primary human melanocytes Human immortalized sebaceous gland cells	· Significant and cell-type dependent effects on cellular functions, such as viability, proliferation, apoptosis and differentiation · Inflammation · Cytotoxicity related to phase composition
Quantum dots	Human epidermal keratinocytes	· Increased cytokine production and quantum dot uptake · Irritation and decreased cell viability · Release of IL-6 and IL-8
Fullerenes	Human dermal fibroblast	· Oxidative damage to cell membranes and cell death · Disruption of normal cellular function through lipid peroxidation

*SUMMARISED FROM (CROSERA ET AL., 2009)

There are a few studies on eye irritation of nanomaterials. In one study performed according to the Organisation for Economic Co-operation and Development (OECD) test guideline 405, 100 mg C-60-fullerene and CNT were applied to rabbit eye showing a reversible but minimal potential for irritation (Ema et al., 2013a; Ema et al., 2011). Two different products of SWCNT and one of two MWCNT were not eye irritants whereas one other MWCNT product was a very weak irritant (Ema et al., 2011). Weak eye irritation was confirmed for MWCNT by others (Kishore et al., 2009). Ag nanomaterials 10 nm did not induce irritation in rabbits when studied according to OECD test guideline 405 (Kim et al., 2013). In a study, ultrafine TiO_2 particles induced reversible ocular conjunctival redness in rabbits (Warheit et al., 2007). In conclusion, C(60) fullerene, SWCNT, MWCNT, Ag nanomaterials and ultrafine TiO_2 seem not to induce adverse irritation of the eye.

Concerning toxicity inside the eye, one study in rabbits investigates the toxicity of intravitreal injected (i.e. into the eye humor) 67 or 670 µmole Au nanomaterials (<220 nm). By light microscopy, one month post injection, neither 67 nor 670 µmole Au nanomaterials showed any toxicity in terms of ocular inflammation, or cellular atrophy and/or disorganisation in retina or in the optic nerve by this very special administration route (Bakri et al., 2008). This shows that if absorbed from the environment, nano-Au <220 nm is not likely to induce any of these effects inthe eye.

In conclusion, present knowledge about eye absorption and toxicity into the eye is too limited to be stated and evaluated in general for hazard assessment.

3.8 Developmental and reproductive system

No epidemiological studies have been published in this area of nanotoxicology. Overall, data from experimental studies suggest that ENP of various types possess the potential to influence male fertility as well as fetal development, with very little knowledge with regards to female fertility. Mechanistically, ENP may potentially affect reproduction and development due to toxicological properties related to specific chemical constituents of the particles (e.g., Cd in quantum dots) or properties related to the particles *per se*. The rationale from the latter comes from the high potential of particles to induce oxidative stress and inflammation in biological tissues. This may have several implications in both maternal and fetal tissues. Specifically for inhalation exposure, particles may deposit in the airways and lungs, where oxidative stress and inflammation may arise in response. Neither particles nor the inflammatory condition need to be confined to the lungs. Particles may translocate and inflammatory mediators may be released to the bloodstream and transported to organs of importance for reproduction, pregnancy and fetal development, such as neuro-endocrine circuits, the placenta, the fetus, testicles and ovaries (reviewed by Hougaard et al., 2011a; Iavicoli et al., 2013). Particulates may confer their oxidative and inflammatory action directly in the tissues, and inflammatory mediators may have consequences in several tissues that in turn may interfere with reproduction and pregnancy. Some CNT have also been proposed to interfere directly with cellular and extracellular constituents and thereby alter vital cellular processes, a mechanism that may be especially relevant in the early phases of embryo development (Shvedova et al., 2012).

Maternal gestational exposure to nanomaterials has been associated with effects in the offspring that include changes in function of the CNS, immune system, male reproductive system as well as differential expression of genes (Hougaard & Campagnolo, 2012; Hougaard et al., 2011a). These findings are somewhat supported by epidemiological and animal studies of ultrafine particles (e.g., in ambient air and diesel exhaust) (Ema et al., 2013b).

Most research within this field must be categorised as hypothesis generating. Published reports present a great diversity of study designs, with respect to kind and size of ENP, route of exposure, model systems and species, dose levels and endpoints. Furthermore, the methodology does often not always reflect state-of-the-art, and even if easily available, most mammalian studies do not provide data on gestational and lactational endpoints (e.g. maternal weight gain, gestation length, litter size etc.) (Hougaard & Campagnolo, 2012).

Developmental toxicity

Developmental toxicity embraces any effect interfering with normal development during pregnancy as well as after birth. As exposure route probably plays a major role for induction of adverse effects, findings are grouped by route of exposure.

Airway exposure during pregnancy (by inhalation, instillation or intranasal insufflation)

Human exposure to particles from ambient air has been associated with low birth weight, preterm birth and being born small for gestational age (Stieb et al., 2012; Shah & Balkhair, 2011). Rodent

studies of gestational exposure to diesel exhaust and particles in rodents have revealed changes in offspring growth, sexual development, male fertility, behavior, susceptibility to develop allergy and induction of mutations in the DNA of the male fetuses that were inherited by male offspring in the next generation (Ema et al., 2013b).

So far nano-sized particles of Cd and TiO_2, CB and CNT have been studied after airway exposure. Inhalation of cadmium oxide nanoparticles was overtly toxic to the uterine contents, probably due to Cd affecting the placenta (Blum et al., 2012). For CB and TiO_2, maternal gestational airway exposure to any of the particles did not seem to interfere with traditional pregnancy and lactational measures, even if airway exposure was associated with overt lung inflammation in the mother (Jackson et al., 2012a; Hougaard et al., 2010). Gene expression in the offspring was, however, found to differ significantly between control and exposed offspring, with female offspring being apparently more sensitive than males (Jackson et al., 2013; Jackson et al., 2012b). CB also induced strand breaks in liver from mothers and offspring when assessed after birth (Jackson et al., 2012a), and has been found to adversely interfere with fertility in the male offspring (Kyjovska et al., 2013; Yoshida et al., 2010). Maternal intranasal insufflation of both TiO_2 and CB altered offspring phenotype indicative of increased propensity to develop allergy (Fedulov et al., 2008). Exposure to both particle types caused behavioral alterations in the offspring (Jackson et al., 2011; Hougaard et al., 2010). A single dose of MWCNT (67 μg/kg) instilled to female mice the day before cohabitation with male was not associated with changes in gestational and developmental measures. Malformations have however been observed after a single intratracheal instillation of a very high dose of MWCNT (>4 mg/kg) concomitant with significant increases in leucocyte counts in maternal peripheral blood. At 3 mg/kg, neither malformations nor maternal leukocyte counts was significantly changed (Hougaard et al., 2013; Fujitani et al., 2012; Ema et al., 2014).

Intravenous exposure during pregnancy
SWCNT, silica and TiO_2 nanoparticles interfered adversely with mouse embryonic development (fetal death, malformations) and placental vascularisation when administered intravenously to the mother early in gestation (Pietroiusti et al., 2011).

Subcutaneous exposure during pregnancy
Subcutaneous injection of TiO_2 to pregnant mice have been associated with significant alterations in offspring brain; in gene expression during lactation (only assessed in male offspring), in DA levels in some brain areas and in increased numbers of caspase-3-positive cells in the olfactory bulb (the only investigated structure). Furthermore, TiO_2 exposure interfered adversely with fertility in the male offspring (reviewed by Hougaard & Campagnolo, 2012; Powers et al., 2013; Ema et al., 2014).

Gavage exposure
Gavage administration of multi walled carbon nanotubes (MWCNT) to pregnant rats was not associated with treatment-related differences in pregnancy measures including body and placental weights, but SWCNT appear embryolethal and teratogenic in mice when given by oral gavage. Gavage exposure of pregnant or lactating mice to TiO_2 interfered with function of the CNS (Gao et al., 2011). Ag nanoparticles were without effects on classical pregnancy and birth parameters at dose levels of up to 1,000 mg Ag/day in some but not all studies (Hong et al., 2014; Philbrook et al., 2011). Ag has, however, shown toxic to fetal development after exposure in ionic form (malformations and very high postnatal lethality in rodent studies) (Shavlovski et al., 1995).

Fertility
Fertility includes the reproductive processes up to and including implantation of the fertilised ovum.
Whether administration is by inhalation or by the intravenous route, particles seem to distribute to organs with relevance for male and female fertility, i.e. testes, the uterus and ovaries (although the amount may vary with route of exposure) (Hougaard & Campagnolo, 2012).

Male fertility
A few epidemiological studies indicate that heavy road traffic might affect reproductive parameters in men. This is corroborated by findings of decreased semen quality and hormonal changes in male rodents exposed to diesel exhaust and particles (reviewed by Ema et al., 2013b).

The sensitivity of male fertility to nanomaterial exposure has been investigated in several rodent studies. Most tested particles were associated with adverse effects on spermatogenesis, although a few are without adverse effects. Both exposure through the airways and by subcutaneous injection affected male reproductive parameters, e.g. sperm counts and male reproductive hormones (reviewed by Lan & Yang, 2012; Ema et al., 2014).

Female fertility
Female fertility has been studied very little. Some i*n vitro* studies indicate that nanomaterials can be toxic to ovarian cell (reviewed by Iavicoli et al., 2013). *Ex vivo* studies of, e.g., pre-implantation mouse embryos have been performed of Ag and polystyrene nanoparticles and indicate that Ag particles are toxic during this stage of development whereas polystyrene nanoparticles are not, even if the particles were observed to enter and distribute inside the embryo (summarised in Hougaard & Campagnolo, 2012). Lung exposure of female mice to MWCNT the day before cohabitation with a male introduced a 5 day delay in time to delivery of the first litter (Hougaard et al., 2013). Additional evidence comes from a study in mice, where female mice were housed in heavy traffic pollution. The female reproductive cycle was disturbed and becoming pregnant also took longer for mouse couples breeding under polluted conditions (Veras et al., 2009).

3.9 Genotoxicity and cancer

A number of reviews on nanomaterial-induced genotoxicity have been published (e.g. (Magdolenova et al., 2014; Kumar & Dhawan, 2013; Møller et al., 2013; Oesch & Landsiedel, 2012; Xie et al., 2011; Singh et al., 2009)). Despite the magnitude of literature within this area, it is difficult to draw general conclusions due to study limitations such as lack of standardised test methods, incomplete particle characterisation and the diversity in the used test systems. So far, most genotoxicity studies have been performed *in vitro*. A recent review by Magdolena et al. reports that out of 112 identified genotoxicity studies only 22 are *in vivo* studies while the rest are performed *in vitro* (Magdolenova et al., 2014).

The mechanisms behind nanomaterial-induced genotoxicity are still not well understood. It is well agreed that these mechanisms can be divided into primary and secondary genotoxicity. Primary genotoxicity can be further subdivided into 1) Direct primary genotoxicity which is the result of direct interaction of the nanomaterial with the DNA, and 2) Indirect primary genotoxicity which is the result of e.g. nanomaterial-induced reactive oxygen species, or of the release of toxic ions from the nanomaterial. Secondary genotoxicity is caused by nanomaterial-induced inflammation resulting in release of reactive oxygen species from activated phagocytes (Magdolenova et al., 2014).

A number of physicochemical properties (size, shape, surface properties, composition, solubility, aggregation/agglomeration, nanomaterial uptake, presence of mutagens and transition metals affiliated with the nanomaterials etc.) have been suggested to be important for toxicity. However, it is not known which of these properties that is most important for genotoxicity (Magdolenova et al., 2014). For insoluble so-called inert particles such as TiO_2 and CB, the specific surface area of the particles has been shown to be correlated to the inflammatory response (please refer to Paragraph 3.1 for more information). The fact that MWCNT and asbestos fibres of same shape and size have been shown to induce similar genotoxic effects in rodents is an example of the effect of particle shape for genotoxity (Poland et al., 2008). The importance of physicochemical properties for toxicity is further discussed in Chapter 5.

If DNA damage is not repaired or wrongly repaired, it may lead to mutations. Cancer is the result of an accumulation of mutations in cells. Therefore, nanomaterials capable of inducing genotoxicity may also have the capacity to induce cancer.

It is well documented that rodents pulmonary exposed to a variety of nanomaterials (nano-TiO_2, nano-CB, SWCNT, MWCNT etc.) develop pulmonary inflammation which may result in secondary genotoxicity and thereby also cancer. Studies in rodents have demonstrated the carcinogenic potential of a number of nanomaterials. Instillation and inhalation studies with rodents have shown that insoluble particles such as TiO_2 and CB induce lung cancer (IARC, 2010). Another example of the carcinogenic effect of nanomaterials is the induction of mesothelioma in animals following intraperitoneal and intrascrotal administration of CNT (The Nordic Expert Group for Criteria Documentation of Health Risks from Chemicals, 2013). CB and TiO_2 (as groups with no size-specifications) are both categorised by IARC as possibly carcinogenic to humans (Group 2B) (IARC, 2010).

It is not possible to give an overall conclusion on the genotoxic and carcinogenic potential of nanomaterials as a group because of the diversity within the group of nanomaterials. However, based on the recent reviews it can be concluded that the physicochemical characterisation of the nanomaterials is very important for the prediction of the genotoxic potential (Kumar & Dhawan, 2013). Some of the nanomaterials are considered potential carcinogens. For example, nano-TiO_2 is considered a potential carcinogen by NIOSH. This is based on the pattern of inflammation and a chronic animal inhalation study showing increase in adenocarcinomas in animals exposed to nano-TiO_2 (NIOSH, 2011). NIOSH concludes "*that TiO_2 is not a direct-acting carcinogen, but acts through a secondary genotoxicity mechanism that is not specific to TiO_2 but primarily related to particle size and surface area*."

3.10 Immunotoxicity

Nanoparticles may interact with the immune system and result in immunosuppression or immunostimulation. Properties such as size, charge, hydrophobicity, solubility, and presence of targeting moieties and not least surface area determine the interaction with biological tissues and components of the immune system, and thereby the immunotoxicity. Based on available literature, nanoparticles do not seem to introduce new immunotoxic responses specific to nanomaterials and traditional testing regimes are applied to investigate nanoparticle immunotoxicity. It has, however, been mentioned that *in vitro* assays may need modifications because nanoparticles may interphere with the reagents and detection methods and thereby produce unreliable results (Dobrovolskaia et al., 2009).

Different responses are reported in the literature depending on the characteristics of the nano-particles. Examples include elicitation of autoimmunity and involvement in allergic sensitisation. It is, however, mentioned that it is unlikely that nanoparticles can act as haptenes and induce specific imunoglobulin E production, and it is suggested that the mechanism more likely is based on nanoparticles acting as adjuvants e.g. inducing specific patterns of cytokines and antibodies (Gioacchino et al., 2011, abstract only).

Several nanoparticles have, based on animal studies, been shown to induce pro-inflammatory effects in the lung with changes in cytokine profile. Di Gioacchino et al. concludes that "*all considered, the available data suggest that through the elicitation of an oxidative stress mechanism, engineered NPs may contribute to pro-inflammatory disease processes in the lung, particularly allergy*" (Di et al., 2011).

4. Adverse effects of nanomaterials when part of a matrix

The use of nanomaterials in different kinds of materials is increasing due to product advantages compared to the non-nano-containing conventional products. While there is increasing knowledge on the hazard of free nanomaterials, only a limited number of studies have focused on the hazard of nanocomposites.

4.1 Composites (solid)

Paints and lacquers are examples of a product group where nanomaterials are used in relatively large quantities to provide advantages such as self-cleaning properties or greater scratch resistance. A few studies have focused on the hazard of sanding dust from nanomaterial-containing paints compared with conventional products without nanomaterials.

In a Danish project, NanoKem, the purpose was to examine how substitution of larger particles in paints, lacquers and fillers with nanoparticles of the same chemical affects risk of exposure and adverse health effects. The adverse health effects of sanding dust from a number of otherwise identical paints with and without addition of nanomaterials were tested. The tested products were 8 different nanomaterials and 13 dust samples obtained by sanding different painted boards (painted with 5 conventional products without nanoparticles and 8 versions with nanoparticles).

The products were selected in collaboration with the Danish Coatings and Adhesives Association and therefore had industrial relevance. Sanding of some of the nanomaterial-containing paints led to increased formation of nano-sized particles compared to the reference paint, but there was no consistent pattern in which paints gave rise to increased formation of nano-sized sanding particles, nor the amount (Koponen et al., 2011). Sanding dust from paints and lacquers with and without nanomaterials were tested in mice 24 hours after a single intratracheal instillation of 54 μg test material (Saber et al., 2012c). Sanding dust from nanoparticle-containing paints or lacquers did not result in statistically significant increased inflammation or DNA damage as compared to dust from conventional products even though some of the added nanomaterials induced inflammatory responses in mice when dosed as pristine nanomaterial (nano-TiO_2, photocat-TiO_2, Axilate, Kaolin) and some induced DNA damage (nano-TiO_2, fine-TiO_2) (Saber et al., 2012b). However, when comparing the different paint and lacquer matrices, the genotoxic response differed between the paint and lacquer types. Dusts from the two lacquers (one with nano-SiO_2 and one without) and the outdoor acrylic based reference paint resulted in statistically significantly increased level of DNA damage, while dust from PVA-based paint, filler, and binders did not result in increased level of DNA damage compared to vehicle exposed mice.

Based on the initial screening, one nanomaterial, nano-TiO_2, and sanding dusts from the corresponding paints with (Indoor-nano-TiO_2) and without nano-TiO_2 (Indoor-R) were tested for dose-response relationship at different times because nano-TiO_2 was inflammogenic and genotoxic

(Saber et al., 2012a). The tested nano-paint contained 10 % nano-TiO_2. To be able to compare the same amount of nano-TiO_2 in paint, two different dose ranges for the pure nano-TiO_2 (18, 54 and 162 µg) and the sanding dusts (54, 162 and 486 µg) were chosen. The 18 µg nano-TiO_2 dose approximately corresponded to the nano-TiO_2 content in 162 µg of sanding dust and similarly for the 54 µg dose of nano-TiO_2 and 486 µg dose of sanding dust (indoor-nano-TiO_2). There was no additive effect of adding nano-TiO_2 to the paint compared to the reference paint for any of the measured toxicological endpoints (Saber et al., 2012a).

The same sanding dusts from paints containing nanomaterials and the added nanomaterials as tested in the screening experiment were also tested by measuring cell surface expressions of vascular cell adhesion molecule-1 (VCAM-1) and intracellular adhesion molecule-1 (ICAM-1) in primary human umbilical vein endothelial cells (HUVEC). The results were in agreement with the *in vivo* results. The sanding dusts with and without nanomaterials had similar toxicity and on a mass basis the nanomaterials had a larger effect than the sanding dusts (Mikkelsen et al., 2013).

The same overall conclusion was reached in a recent study on the toxicity of nanoparticles (TiO_2, Ag and SiO_2) embedded in a paint matrix compared to control paints without nanoparticles and the pristine nanoparticles (Smulders et al., 2014). Pulmonary inflammation and systemic effects were evaluated two and 28 days after the last of five oropharyngeal aspirations of 20 µg of particles or milled paint exposed to UV-A as an ageing process (day 0, 7, 14, 21 and 28, total dose 100 µg). The pristine nanomaterials induced some inflammation while little to no adverse effects were seen in mice exposed to nanomaterials incorporated in an aged paint matrix. Of importance for the interpretation of the study is that the content of nanomaterials in the paints as measured by Inductively Coupled Plasma Mass Spectrometry (ICP-MS) was reduced significantly after milling and ageing.

To the best of our knowledge, the above mentioned publications are the only ones testing the toxic effects of sanding dusts from nanoparticle doped paints and lacquers. However, Wohlleben et al. have published two other studies on dust obtained by sanding other types of nanocomposites than paints and lacquers: 1) A study in rats testing toxic properties of sanding dusts from cement and plastic with and without CNT (Wohlleben et al., 2011), and 2) an *in vitro* study using 'Precision Cut Lung Slices' exposed to sanding dust from thermoplastic polyurethane with and without CNT (Wohlleben et al., 2013).et al. 2013).

In the *in vivo* study toxicological endpoints were evaluated 3 days and 3 weeks after instillation of 0.36 mg of sanding dust from the composite cement and plastic materials and 0.09 mg CNT (Nanocyl NC7000) in rats (Wohlleben et al., 2011). The following endpoints were analysed: Pulmonary histology, inflammation (differential cell counts on broncho alveolar lavage (BAL) cells and protein concentrations in bronchoalveolar lavage fluid (BALF)), genotoxicity (Comet assay in lung tissue and micronuclei in bone marrow) and blood were analysed for hematology and acute phase proteins. The physicochemical characterisation showed similar shape and size of the sanding dusts with and without CNT. Overall, there were no differences between the toxicity of sanding dust from the composites with and without CNT. The low content of CNT in the products (2 wt % in cement and less than 5 wt % in polyoxymethylene plastic) did not allow a study design to determine if the composite-embedded CNT resulted in a reduced toxicity compared to the free CNT at the same CNT mass.

The cytotoxicity of sanding dust from elastic CNT-polyurethane nanocomposite was evaluated in comparison with sanding dust from the corresponding product without CNT by use of 'Precison Cut Lung Slices' (Wohlleben et al., 2013). This *in vitro* method assesses lung functions under cell culture conditions. Cytotoxicity was evaluated after 24 hours by measuring membrane leakage of lactate dehydrogenase and by measuring mitochondrial activity. No cytotoxicity was detected at the tested doses. CNT was tested at 1 mg/ml while the sanding dusts were tested up to 20 mg/ml. As no

cytotoxicity was detected in this set-up, a possible difference between sanding dust with and without CNT could not be evaluated.

To summarise, the very limited number of published studies on the toxicological effects of sanding dusts from nanocomposites are in good agreement with each other. No additional toxicity have been detected for any of the nanocomposites compared to the corresponding products without nanomaterials. More studies are needed to make conclusions within this area.

4.2 Sprays

The attention towards possible adverse health effects of "nano" sprays aroused when a number of cases of pulmonary health problems were reported among German consumers using different kinds of "nano" sprays ("Magic Nano Glass & Ceramic" spray and "Magic Nano Bath" spray) (Hahn et al., 2008; Hahn, 2007). However, analysis of the products documented that solid nano-sized particles were not generated during use and therefore the adverse effects, which were also demonstrated by rat inhalation studies (Pauluhn et al., 2008), were not caused by nanomaterials. Instead, the name "nano" referred to the thickness of the coating generated on the surface upon spraying.

In the present report we were asked by the Danish EPA only to cover spray products containing solid nanomaterials and therefore the above and similar studies are not further discussed. The literature search identified only a few studies on the toxicity following inhalation of liquid aerosols of nanocomposites.

In two of these studies, commercial (solid) nanomaterial-containing sprays were examined (McKinney et al., 2012; Roberts et al., 2013). Both studied the pulmonary and cardiovascular effects of antimicrobial spray products in rats.

One of the examined sprays was a spray can product marketed as containing "nano" TiO_2 particles and was intended to be used as a surface antimicrobial product, such as a bathroom sanitiser (McKinney et al., 2012). Further information on the content was not available from the manufacturer. Simulated consumer use of the spray resulted in 3.4 mg/m^3 75 nm mean diameter TiO_2 particles and the particle concentration was 1.2 x 10^5 particles/cm^3 (Chen et al., 2010). The rats were exposed by inhalation under the following conditions: 2.62 mg/m^3 for 2 hours (low dose), 1.72 mg/m^3 4hours/day for 2 days (medium dose) and 3.79 mg/m^3 4hours/day for 4 days (high dose), respectively. This resulted in three doses; 314 mg/m^3 min (low dose), 826 mg/m^3 min (medium dose) and 3,638 mg/m^3 min (high dose), respectively. The alveolar deposition per rat was estimated to 4 (low dose), 10 (medium dose) and 43 µg (high dose). Twenty-four hours after end of exposure, pulmonary (breathing rate, specific airway resistance, inflammation and lung damage) and cardiovascular endpoints (the responsiveness of the tail artery to constrictor or dilatory agents) were assessed. Only the high dose resulted in significant effects. These included pulmonary inflammation, increases in breathing rate and lung cell damage. The specific airway resistance and the cardiovascular endpoints (systemic vascular responsiveness: vasoconstriction and redilation of ventral tail arteries) were unaffected even at the high dose. The study design did not include a comparison of the spray product with nano-sized TiO_2 powder. However, the authors compared the response with one of their previously performed rat instillation studies in which rats were instilled with 260 µg TiO_2 and responded with an 11-fold increase in neutrophils in BAL 1 day after instillation (Sager et al., 2008). In the present study, the neutrophil influx was doubled at the high dose (43 µg). Extrapolation from the 43 µg to the 260 µg dose shows that a 12-fold increase in neutrophils would have been expected in the present study. This indicates a similar response of TiO_2 as powder and when TiO_2 is part of the tested spray product.

A similar study was performed to characterise the hazard of an antifungal spray containing nano-sized Ag particles (MesoSilver, Purest Colloids, Inc.). Rats were exposed for 5 hours by inhalation to

100 μg/m³ MesoSilver (low dose). This was compared to the effects in rats exposed for the same period of time to 1,000 μg/m³ Ag spray from National Institute of Standards and Technology, US (NIST) or a spray consisting of sterile, deionised water (control mice). The alveolar deposition per rat was estimated to 0 (control spray), 1.4 (MesoSilver) and 14 μg (NIST Ag), respectively. The choice of the low dose (100 μg/m³) was selected to be the same as the threshold limit value time-weighted average (TLW-TWA) set by the American Conference of Governmental Industrial Hygienists (ACGIH) for particulate Ag. For comparison, the Danish OEL for particulate (and soluble) Ag is 1/10 of the limit set by ACGIH, namely 0.01 mg/m³ (~10 μg/m³). The manufacturer of the MesoSilver spray indicated the total concentration of Ag to be 20 mg/L in deionised water and the primary nanoparticle size to be 0.65 nm. The content of Ag particles and free Ag ions was given to be 75 % and 25 %, respectively (Roberts et al., 2013). One and 7 days after the end of exposure the same endpoints for inflammatory and cardiovascular effects as in the above study by McKinney et al was measured (McKinney et al., 2012). In addition, measurement of alveolar macrophage activity and heart rate and blood pressure in response to isoproterenol and noradrenaline, respectively, was included in the present study. Only a few pulmonary and cardiovascular changes were reported. One day after exposure to the commercial spray product MesoSilver (low dose), rats responded with elevated heart rate in response to isoproterenol, and one day after exposure to the NIST silver (high dose) standard, the rats responded with an modest increase in the number of blood monocytes (1.7 fold) and decreased dilation of tail artery after acethylcholine stimuli (Roberts et al., 2013). The authors discuss that the difference in potency regarding the moderate cardiovascular changes observed in rats exposed to the commercial product at low dose, which was not seen in rats exposed to the high dose NIST silver sample, could be the higher content of Ag ions in the commercial product. Another plausible reason for this slight change could also be adverse effects of unknown contents of the commercial spray product.

A third study has been performed in which the toxicity of an acrylic ester-based polymer dispersion containing nanomaterials was compared to a reference product without nanomaterials (Ma-Hock et al., 2012). In contrast to the two previously referred studies, the products tested in this study were not spray products but dispersions that were aerosolised in the test system. Despite this, the study has been included because of the very limited number of studies in this field and because the study contributes to the overall picture of the toxicological impact of inhalation of nanomaterials in liquid products. The two products, which were tested in rats exposed by inhalation, were identical except for the content of nanomaterials (approximately 11 % (w/w) polymer particles in the “nano” version). Rats were exposed by nose-only exposure for 6 hours/day for 5 consecutive days at target concentrations 3 and 10 mg/m³. Compared to the reference product without nanomaterials, the toxicity of the nanomaterial-containing version of the products was not changed. When evaluating the present study it should be noted that none of the products resulted in any adverse effects at the tested concentrations (3 and 10 mg/m³) and time-points for evaluation after exposure (3 and 23 days). Thus, the NOAELs for both dispersions were >10 mg/m³ and were independent on the content of nanomaterials.

To summarise, a very limited number of studies on the toxicological effects of sprays containing nanomaterials have been published. A rat inhalation study of a commercial spray containing “nano” TiO_2 particles indicated similar toxicity of nano-TiO_2 when part of the product and when nano-TiO_2 was tested alone. Testing of a commercial Ag spray product in rats resulted in moderate cardio-vascular effects that were not observed in rats exposed to a standard Ag reference product. The third study evaluated in the above paragraph showed no increased toxicity of a polymer dispersion compared to the non-nano product. More studies are needed to make conclusions within this area.

4.3 Liquids

The most commonly investigated nanomaterials in liquid matrices are TiO_2 and ZnO used in cosmetics. The SCCS has adopted opinions on both substances in the nano form in 2013 and 2012,

respectively. The overall conclusion in the two opinions is that the use of TiO_2 and ZnO nano-materials with specified characteristics as indicated in the opinions, at a concentration up to 25 % as a UV-filter in sunscreens, can be considered to not pose any risk of adverse effects in humans after application. This does, however, not apply to other applications that might lead to inhalation exposure of the nanoparticles (such as sprayable products) (SCCS, 2014d; SCCS, 2012).

Most of the documentation is based on studies with the pristine nanomaterial, as only few relevant studies investigating the effects of dermal application of sunscreen products are available. However, none of these studies provide evidence of dermal absorption or other effects on the skin.

A few studies have investigated penetration of the nanomaterials in sunscreen products. Sadrieh et al. studied dermal penetration of TiO_2 particles applied in a sunscreen product to the skin from weanling Yorkshire pigs. When exposed to UV-B radiation (sunburn simulation), the study demonstrated that UV-B-sunburned skin slightly had enhanced *in vitro* or *in vivo* penetration of rutile TiO_2 (uncoated mixture of anatase and rutile and dimethicone/methicone copolymer-coated rutile TiO_2) present in the sunscreen formulations into the stratum corneum. The penetration was, however, considered minimal, and there was no evidence that nano-sized or submicronised TiO_2 penetrated the intact epidermis to any significant extent and there was no evidence of systemic absorption (Sadrieh et al., 2010).

Based on a review of a number of skin penetration studies investigating penetration of solid nanoparticles in cosmetics, Nohynek et al conclude that there is little evidence suggesting that slightly compromised skin in general has greater susceptibility to skin penetration by small particles. It is however concluded that some pathological skin conditions may affect skin penetration of topically applied substances (Nohynek & Dufour, 2012).

Gulson et al have investigated dermal uptake of ZnO from sunscreen applied to human skin *in vivo* over five days and found that small amounts of elemental Zn could be detected in blood and urine. This is taken as an indication of possible passage through the skin, although at a very low level (Gulson et al., 2012; Gulson et al., 2010). However, in the SCCS opinion on ZnO in the nano form it is concluded that measured elemental Zn is not a result of dermal penetration of nanoparticles but rather a result of Zn-ions released from the ZnO nanoparticles (SCCS, 2012).

4.4 Food

The application of nanomaterials in food, beverages and food contact materials in the EU is still rare (Wilson Center, 2014) and has been hampered by concern about safety, ethical, political and regulatory issues which all are caused by lack of knowledge (Borel & Sabliov, 2014).

In the EU, three types of nanomaterial are authorised to be used in food packaging materials:

- Titanium nitride was evaluated by EFSA in 2008 and has been approved as an additive for use in some food contact plastics since 1 May 2011. Use is limited to PET (polyethylene terephthalate) bottles and refrigerators in a concentration up to 20 mg/kg. The Agency is not aware of any commercial use of this additive. There is no migration from this material into food (European Commission, 2014).
- CB can be used in rubber, silicones and printing inks for e.g. meat (Additive, primary particles 10-300 nm which are aggregated and may form agglomerates, 2.5 % w/w) (European Commission, 2014).
- Silicone dioxide can be used in printing inks, paper, rubbers and silicones (Antislip-agent, primary particles 1-100 nm which are aggregated and may form agglomerates) (European Commission, 2014).

Four nanomaterials not authorised in the EU can be used in food contact materials in the US according to the US Food and Drug Administration. They all have the status of 'Generally recognised as safe' implying that they can be applied in the US in concentrations limited to fulfill technical demands (Wagner, 2013):

- Al. Applications: Filler in polymers, scratch- and abrasion-resistance in coatings, improvement of barrier properties and UV-filter,
- Ag. Applications: Antimicrobial, antibiotic and antistatic agent. Ag-nanomaterials are used in food packaging materials,
- Nanoclay (bentonite). Applications: Improvement of barrier properties) and
- ZnO. Applications: UV-filter antimicrobial.

The use of these products in EU and specifically in Denmark is not known, but it is assumed to be very limited.

Gastrointestinal exposure may occur either directly by intentional intake of food or beverages containing nanomaterials, or indirectly by e.g. leakage from dental implants or from nanomaterials from food packaging. In addition, gastrointestinal exposure may occur following translocation of inhaled particles through the mucociliary escalator which are subsequently swallowed. Oral absorption of nanomaterial from nanomaterial-containing medicine is also a potential exposure scenario. However, in the present report we were asked by the Danish-EPA not to cover nanomedicine and therefore such studies are not discussed.

A very limited number of studies on the adverse effects of nanomaterials in a food matrix or a food related item such as food packaging have been identified.

Toxicity of nanomaterials when part of food
Chewing gum is an example of a food item for which there is an extensive use of nano-TiO_2 as an additive. This is, however, not approved in EU. A Chinese study was designed to characterise and perform a preliminary assessment of TiO_2 as an additive in chewing gum (Chen et al., 2013b). The toxicity of nano-TiO_2 isolated from 6 different brands of commercially available chewing gum was evaluated by exposure of human gastric epithelial cells and human epithelial colorectal adenocarcinoma cells. Viability, lactic acid dehydrogenase release and ROS generation was evaluated after 24 hours exposure to 0, 10, 25, 50, 100 and 200 µg/ml nano-TiO_2. Neither viability nor membrane leakage of lactic acid dehydrogenase was affected at the tested doses, while cell viability was slightly reduced in P25 TiO_2 exposed cells included as positive control. ROS generation for the isolated TiO_2 was similar for all brands and P25 TiO_2. Estimation of the amount of TiO_2 in gum before and after chewing indicated that approximately 95 % of the chewing gum content of nano-TiO_2 was swallowed during chewing. The fraction of TiO_2 particles below 200 nm in the chewing gum was more than 93 % and 18-44 % of the TiO_2 particles were below 100 nm in size. The hydrodynamic size distribution of TiO_2 in one of the chewing gum brands was determined in a simulated digestive system consisting of three different compartments and showed that the number percentages of nano-TiO_2 was lowered to 34, 13 and 22 % in 1) artificial fluid, 2) gastric fluid and 3) intestinal fluid, respectively. Under the given experimental conditions this *in vitro* study does not indicate high toxicity. However, further studies and especially *in vivo* studies are needed to evaluate the potential toxicity associated with the chewing of nano-TiO_2 containing chewing gum.

In vivo studies in laboratory animals dosed with nanomaterials via the diet
A thorough review evaluates the oral safety of food-related nanomaterials that have potential use from *in vitro* and *in vivo* studies in laboratory. Only the *in vivo* studies are considered here (Card et al., 2011).

No human studies were identified. In laboratory animals, 21 studies were identified on oral administration (17 by gavage, 4 by diet) (Card et al., 2011).

Rohner et al. dosed rats with ferric phosphate (used for iron fortification) *ad libitum* in their diet at concentrations at 10 and 20 mg/kg diet (10.7, 30.5, or 64.2 nm) for 15 days. Histological examinations and measurement of thiobarbituric acid reactive substances as indication of oxidative stress showed no toxicity (Rohner et al., 2007).

Jia et al. dosed female and male rats with Se-nanomaterials (20-60 nm) at 4 different concentrations in the diet for 13 weeks corresponding to doses of 0.19/ 0.12, 0.33/0.22, 0.44/0.31, and 0.50/0.42 mg Se/kg bw/day (female/male) . The authors concluded a NOAEL at 0.33 and 0.22 mg Se/kg bw/day for female and male rats, respectively (Jia et al., 2005).

Chan et al. dosed mice with nano-sized Black Soybeans (52 % <100 nm, 42 % 100-200 nm) in the diet at a concentration of 10 % for 12 weeks. The Black Soybeans were grounded to nano-size by a company in Taiwan (details not specified). The study indicated enhanced immune system responsiveness (Chan et al., 2009).

Pigs were orally dosed with 0.5 % montmorillonite nanocomposite (MNC) in the diet for 83 days. This corresponded to an oral intake of 11 mg MNC/animal/day. The size was not specified (Xu et al., 2004).

No adverse toxic effects were reported in any of the above studies. None of the studies characterised the nanomaterials in the diet or in any organ (Card et al., 2011).

In conclusion, at present there is no clear overview of food items and contact materials actually containing nanomaterials in EU, but present and especially future consumer exposure to nanomaterials via food seems likely. However, initiatives regarding analytical tools need to be developed for verification of data from industry, for product control, for labeling information and to validate database information. Such knowledge is, bearing in mind the European Commission's recommended definition from 2011 of nanomaterials, mandatory (Danish EPA, 2013c). Therefore, intensive method development and validation are in progress, some of which are funded by the EU (Linsinger et al., 2013; Loeschner et al., 2013a; Loeschner et al., 2013b). Here, a recent study investigates and evaluates the liberation of spiked nanomaterials from a food matrix (chicken meat) and their characterisation afterwards by asymmetric field flow fractionation in combination with on-line optical detection and mass spectrometry (Loeschner et al., 2013b).

Assessment of exposure is hampered by gap of knowledge on all important aspects including detection of nanomaterials in food, consumption, exposure, toxicokinetics, gastrointestinal absorption, distribution, metabolism, absorption and all endpoints of toxicity. Free, insoluble nanomaterials including agglomerates are considered of highest concern to the consumers (Bouwmeester et al., 2009).

It was overall concluded that there are not sufficient reliable data for relevant assessment of the safety of oral exposure to food-related nanomaterials (Card et al., 2011). Furthermore, in a complex matrix such as food, more knowledge is needed about the interaction between nanomaterials and various food components, the interplay between nanomaterials and the microbial flora in the gut and ADME of the nanomaterial form.

Potential carry-over from meat and liver
Three studies have been carried out in pigs and chicken administered nano-Ag through their diet or in drinking water. In the first study, pigs administered 20 or 40 ppm nano-Ag in their feed for 5 weeks showed no Ag retention in muscle and only minimal retention in the liver; 0.435 and 0.837

μg/g wet tissue, respectively (Fondevila et al., 2009). In the second study, poultry administered 25 ppm nano-Ag in drinking water had very low concentrations in muscle and liver (Ahmadi & Kordestany, 2011). In the third study, chicken were administered up to 15 ppm nano-Ag in their feed up to 42 days. Ag was distributed to various organs including eatable tissues. The highest concentrations were demonstrated in breast, femur muscles and the liver; 13.5, 14.2 and 6.8 ppm, respectively (Ahmadi & Kordestany, 2011). In neither of these studies were the size of the nanomaterials specified, and it was not demonstrated that the nanomaterials were on nano-form in the diet, drinking water or in the tissue. However, Ag should not to be present in the nanoform in the meat and after eating it. The studies suggest that animals fed with nanomaterial-containing food represent a potential for carry-over if the meat is used for consumption. Furthermore, these are academic studies, and it is not likely that such meat or liver are on the market in EU.

Study in humans via Ag nanomaterial solution
In a very recent study, healthy humans were orally dosed with 10 ppm Ag nanomaterials (5-10 nm) in an experimental aqueous solution for 3, 7 and 14-days or to 32 ppm (32.8 nm) for 14 days corresponding to 100 and 480 μg/person/day. Persons underwent thorough metabolic analyses (all common clinical biochemical analyses in plasma), complete blood counts, urine analyses, and chest and abdominal MRI. The authors concluded that no important changes on any of these parameters at any time or dose were detected (Munger et al., 2014).

Nanocomposites used as dental fillings
Nanocomposites have the potential, when used as dental fillings, to restore affected teeth following dental caries. There is a long lasting exposure because the nanocomposite will be in contact with the oral tissue for many years. The cytotoxicity and genotoxicity of an extract from a dental nano-composite containing 35 wt % nanosilica filler was evaluated *in vitro* by exposure to human lung fibroblast cells (Medical Research Council cell strain 5 (MRC-5 cells)) (Musa et al., 2013). The cells were exposed to cell medium containing an extract from the nanocomposite. The extract was prepared in two steps: 1) 24 hours incubation of a suspension of 0.2 g/mL granulated cured nanocomposite in cell media at 37^0C and 2) filtration of the suspension through a 0.45 μm filter. The cytotoxicity was evaluated by the monocyte mediated cytotoxicity assay (MTT assay) following 72 hours exposure to various concentrations of nanocomposite extract. The genotoxicity was determined at doses resulting in less than 50 % cell death. Genotoxicity was assessed by the comet assay and chromosome aberration tests (6, 24 and 48 hours exposure) with or without addition of a metabolic activation system. No genotoxic effects were detected at the used test conditions. The value of the study is limited in this context due to shortcomings in relation to the assessment of the toxicity of nanomaterial-containing composites. No characterisation of the nanocomposite extract was performed. Therefore it cannot be assessed if any of the nano-content of the suspension was liberated to the cell media. In addition, the choice of a lung fibroblast cell line is not an optimal choice for the assessment of oral toxicity.

Summary
Three types of nanomaterial (TiN, CB, SiO_2) are authorised by EU to be used in food packaging materials. In addition, four nanomaterials (Al, Ag, nanoclay, ZnO) may be used in food contact materials in the US. All such products may be available in Denmark. Their production and use is not known but is assumed to be limited. However, future consumer exposure seems likely.
Studies suggest that farming animals fed with nanomaterial-containing food may represent a potential for carry-over if the meat is used for consumption. However, this was no longer in the nanoform.

To gain knowledge, analytical means need to be developed for verification of data from industry, product control, labeling information, to validate database information and to quantify and verify nanomaterials in diet, drinking water and organs from toxicological studies.

In laboratory animals, four studies dosing nanomaterials via diet were identified. No adverse toxic effects were reported. It is stressed that none of the studies characterised the nanomaterials in the diet, drinking water or in any organ owing to lack of appropriate methods.
In a study, healthy humans were orally dosed via drinking fluid with nano-Ag for 14 days. No adverse effects were detected.

In a complex matrix such as food, more knowledge is needed about the interaction between nanomaterials and various food components, the interplay between nanomaterials and the microbial flora in the gut, ADME and toxicity of the nanomaterial form.
At present, free, insoluble nanomaterials including agglomerates are considered of highest concern to the consumers.

Any hazard assessment of nanomaterials in food is hampered by a deep gap of knowledge on all important aspects including detection and quantification of nanomaterials, consumption, toxicokinetics, and by lack of sufficient and consistent data on toxicity.
At present, any evaluation needs to be on a case-by-case basis and to be performed by skilled experts.

5. Physico-chemical factors of importance for toxicity

A number of different physico-chemical properties have been shown to be important for nano-material-induced toxicity. This chapter summarises the present knowledge based on examples within this area. For more information on specific types of nanomaterials, please refer to Chapter 6.

5.1 Size

Regarding pulmonary exposure, the size of the nanomaterial is an important determinant for lung deposition (further described in Paragraph 2.1.1). In brief, nanoparticles with aerosolised or aggregate/agglomerate size in the size range from 10-100 nm will deposit in the alveoli to a greater degree than larger particles. The mucociliary system removes particles deposited in the upper airways very efficiently. However, the mucociliary system is not present in the alveoli resulting in much longer clearance time (half-time of particles in the alveolar region is months in rats and years in humans) (Paragraph 2.1.1 and (Braakhuis et al., 2014)). Size is also an important factor regarding translocation. In general, when comparing the translocation of a particle of the same chemical composition but with different sizes, the translocation has been shown to be higher for smaller particles compared to the larger. For example, 2 nm Au particles translocated, following intratracheal instillation in mice, from the lungs to the liver, while the 40 and 100 nm sized Au particles did not translocate (Sadauskas et al., 2009b).

5.2 Agglomeration/aggregation

Aerosolised nanomaterials aggregate or agglomerate into larger sized particles over time and therefore aerosol exposure will often consist of aggregates or agglomerates rather than of single nanomaterials. Agglomeration of nanomaterials is caused by adhesion of particles to each other by weak forces. These larger agglomorates can rather easily be disrupted. Aggregates of nanomaterials are connected with covalent or metallic bonds which are difficult to disrupt. It is not known if agglomerates are broken up to single particles in the compartments of the body. However, several studies have shown that single particles form agglomerates in biological fluids (further described in Paragraph 5.10 *Behavior in biological media*). The toxicity of aggregates/agglomerates of nanomaterials may be different than the toxicity of the individual particles (Landsiedel et al., 2012). As described in Paragraph 2.1.1, the deposition pattern of particles in the different parts of the respiratory tract is strongly dependent on the size of the aerosolised particle agglomerate/aggregate and this affects toxicity. On the other hand, pulmonary inflammation, in terms of neutrophil influx and pulmonary acute phase response, are proportional to BET surface area that is the total surface area of the primary particles irrespectively of the aggregation state.

5.3 Specific surface area

The smaller the size of the particles, the larger are their specific surface area. It is well established that the inflammatory response of low toxicity-low solubility particles is proportional to the total surface area of the instilled primary particles rather than the mass (e.g. by (Saber et al., 2012b;

Stoeger et al., 2006; Jacobsen et al., 2009). Chronic inflammation has been associated with respiratory and cardiovascular diseases, as previously described in Chapter 3.

5.4 Shape

The shape of nanomaterials has been shown to affect toxicity. For example, fibre-shaped nanomaterials are more toxic compared to spherical-shaped nanomaterials of the same chemical composition (Braakhuis et al., 2014). Fibres are cleared much slower from the lungs compared to spherical particles, and short fibres have been shown to be cleared faster than longer fibres. Fibres longer than 15-20 µm cannot be phagocytised by macrophages and induce frustrated phagocytosis (Braakhuis et al., 2014; Donaldson et al., 2010).

5.5 Crystallinity

SiO_2 is an example of a substance for which the crystal phase is important for toxicity. SiO_2 is available in multiple crystalline and amorphous phases. While inhalation of crystalline SiO_2 (quartz) can lead to the development of silicosis, bronchitis and lung cancer, these adverse health effects have not been observed following exposure to amorphous SiO_2 (IARC Monographs on the evaluation of carcinogenic risks to humans, 1997). Another example is TiO_2 which exists in two main crystal phases, rutile and anatase, and where anatase TiO_2 has been shown to be more toxic than the rutile form (Johnston et al., 2009).

5.6 Dissolution rate

Some nanomaterials are insoluble in the biological environment (e.g. lung lining fluid) while others may dissolve at the portal of entry or after being phagocytised leading to formation of ions. The insoluble particles may lead to chronic inflammation in the lungs. Inflammation causes release of ROS which can result in DNA damage and lead to the development of cancer. An example of a soluble and toxic nanomaterial is ZnO. When testing the solubility of nano-ZnO in different media, it was shown that 90 % of the nano-ZnO was dissolved within a day when incubated with artificial lysosomal fluid (pH = 4.5) while nano-ZnO did not dissolve in artificial intestinal fluid (pH =7.4) (Cho et al., 2011). Nano-Ag particles are another example of a soluble nanomaterial. In contrast to nano-ZnO, nano-Ag particles are rather non-toxic (Hadrup & Lam, 2014). As illustrated in the example given above, pH differences may affect dissolution rate and therefore result in different exposure to ions in different compartments of the body. Overall, the dissolution rate of nano-materials depends on particle size, coating, stability, manufacturing process and biological environment (Braakhuis et al., 2014).

5.7 Chemical composition

The chemical composition of the nanomaterial is a major determinant of the toxicity. For example, nano-ZnO is a highly toxic nanomaterial following pulmonary deposition. The high toxicity of nano-ZnO has been suggested to be a combination of the large alveolar deposition of nano-ZnO and the following release of toxic Zn ions in the lyzosomes (Cho et al., 2011). Nanomaterials often contain more than one chemical. The particles may be surface modified to provide the nanomaterial with certain properties (as described in Paragraph 5.8) or nanomaterials may contain impurities introduced during the production or in the environment. As an example, CNT and nano-sized CeO_2 have been shown to absorp certain toxic chemicals (Feng et al., 2012; Fang et al., 2008; Yan et al., 2008). Moreover, CNT often contain different metal oxides in various amounts. The metal oxides are impurities from the production process (Danish EPA, 2015a). Therefore, the toxicity of a certain nanomaterial is a combination of the toxicity of the nanomaterial per see and the toxicity of the absorped contaminants.

5.8 Surface modifications

The surface of nanomaterials can be extensively modified by surface functionalisation, a process by which surface moieties are attached to the surface. Surface modifications may reduce or increase the toxicity of nanomaterials. As an example, surface modifications of SAS have been show to modify toxicity. A recent study investigated the toxicological effects of one uncoated SAS and four surface-modified SAS (surface modifications: polyacrylate, PEG, phosphate amino). Rats were exposed by inhalation for 5 consecutive days with 14- or 21-day post-exposure observation. Uncoated SAS induced moderate pulmonary inflammation but no systemic effects. Because no adverse effects were observed in rats exposed to SAS coated with PEG, phosphate or amino, the results suggest that some types of surface modification masks the toxicity of SAS. However, in contrast to the uncoated SAS the acrylated SAS induced effects in the spleen while no pulmonary effects were seen. This indicates that surface coating with acrylate increases the systemic but not the pulmonary toxicity (Landsiedel et al., 2014). In another recent study, the toxicity of COOH-modified SAS was compared to uncoated SAS following intraperitoneally injection in mice The inflammatory response was evaluated by measuring cytokines in the peritoneal cavity lavage fluid and found to be significantly reduced in mice exposed to the surface modified SAS compared to the uncoated SAS (Morishige et al., 2012).

5.9 Surface charge

A review by Braakhuis et al. concluded that positively charged nanomaterials are taken up by cells more easily than neutral and negatively charged nanomaterials which resulted in a higher degree of inflammation and cell death (Braakhuis et al., 2014). The surface charge can be measured by the zeta-potential, which is the electric potential created between the charged groups associated with the surface of a particle and the suspension medium.

5.10 Behavior in biological media

Nanomaterials behavior in biological media has been reviewed recently (Landsiedel et al., 2012). As soon as a nanomaterial is taken up by an organism its surface usually becomes coated by proteins. This spontaneous coating by proteins is called the protein corona. The composition of the corona depends on the exposure route. If the nanomaterials are inhaled, the corona will be composed of proteins present in the lung lining fluid, while nanomaterials entering the blood stream will become coated with plasma proteins. Certain types of absorbed proteins such as e.g. albumin can counteract agglomeration of nanomaterials. This protection against agglomeration is exploited in studies in which a well dispersed particle suspension is needed (e.g. when preparing particle suspensions for intratracheal instillation).

To summarise, the hazard of a specific nanomaterial is not a result of a single physico-chemical property but is rather the result of a combination of the properties.

6. Hazard evaluation of a selection of nanomaterials

6.1 Carbon black (CB)

6.1.1 Introduction

The purpose of this chapter on hazard assessment of CB is to serve as background documentation for the risk assessment of mascara containing CB. Thus, the main focus will be the hazards associated with dermal and eye exposure to CB because those are the most relevant exposure routes associated with consumer use of mascara. The assessment will primarily be based on the recent opinion on CB adopted by the SCCS (SCCS, 2014c). The aim of this SCCS opinion on CB was specifically to decide if CB is safe for use as a colorant with a concentration up to 10 % in cosmetic products and is therefore considered highly relevant for the present purpose. The text is partly condensed from this SCCS opinion. To some extent, key references of importance for the chosen risk scenario are referred based on the description in the SCCS evaluation.

CB is a low-solubility particle which is primarily manufactured from hydrocarbon sources although CB used as food colorant is manufactured from vegetable sources. CBs produced from hydrocarbon sources are nanomaterials used as reinforcement in rubber and as black pigment in plastics, paints, coatings and inks (IARC, 2010). Furthermore, CB is approved for use as a cosmetic colorant (e.g. mascaras and eyeliners) (SCCS, 2014c). The yearly production of CB was approximately 10 million tonnes in 2005. CB consists of spherical primary particles (10-500 nm in diameter) that form aggregates (50-600 nm in diameter) and agglomerates (many micrometers in diameter). The content of extractable adsorbents is less than 1 % (IARC, 2010). CB has been used as a model particle within the area of particle toxicology. Therefore, there is a rather large database on the toxicity of CB.

Vegetable carbon is approved as a food additive (E 153) in Europe and consists of finely divided carbon manufactured by steam activation of carbonised raw material of vegetable origin (EFSA Panel on Food Additives and Nutrient Sources added to food (ANS), 2012). EFSA concluded in their evaluation that the currently marketed vegetable carbon products do not contain nanoparticles. Because of this and the chosen risk scenario of mascara containing CB, vegetable carbon will not be further dealt with in the present report.

6.1.2 Biokinetics

The text is partly condensed from the SCCS (SCCS, 2014c). To some extent, the text also refers to the more detailed description of general particle attributes in the chapter on biokinetics in the present report (Chapter 2).

Absorption

The pulmonary retention of inhaled CB particles has been studied in experimental animals (rats, mice and hamsters). CB is a typical insoluble particle and the deposition, clearance and translocation of CB is therefore already described in the general chapter on pulmonary absorption (Paragraph 2.1.1). CB particles have been found to translocate from the lungs to other organs (e.g. liver, spleen and brain). It has been shown in experimental animal studies that CB can act as a

carrier of adsorbed material such as polyaromatic hydrocarbons. This results in a slower clearance from the lungs compared to the clearance following exposure to the material alone (SCCS, 2014c).

After oral administration uptake and distribution from the gut to gut-associated lymphoid tissue has been described (SCCS, 2014c).

The SCCS concludes that: "*…the available data show that there is no indication of CB particles (>20 nm) being absorbed through the intact skin*" (SCCS, 2014c). This is based on the results from three *in vitro* studies using human skin. In one study, absorption of CB, from a typical eyeliner formulation containing 6 % 20-30 nm CB applied on the surface of human skin *in vitro* and left for 24 hours, was evaluated. No absorption of CB was detected by TEM ((Hallegot & Grégoire, 2011) as cited by (SCCS, 2014c)). However, as the SCCS noted that no accepted guideline was used. Another limitation of the study is that only a single skin sample was used. In two other studies by the same group ((Johnson, 2013a; Johnson, 2013b) as cited by (SCCS, 2014c)), the *in vitro* absorption of CB through dermatomed human skin was analysed by TEM following 24 hours exposure to a 12 % CB formulation. In the first study tape stripping was performed ((Johnson, 2013a) as cited by (SCCS, 2014c)) while the skin surface was only washed-off while no tape stripping was performed in the second study ((Johnson, 2013b) as cited by (SCCS, 2014c)). No CB particles were detected in any of the tape strips beyond the second tape strip (T2 level). The authors concluded that "*…the absence of CB particles in the lower tape strips, epidermis and dermis indicates that CB does not penetrate into or beyond the Stratum Corneum following application of the cosmetic formulation*." ((Johnson, 2013a; Johnson, 2013b) as cited by (SCCS, 2014c)). SSCS notes that the available studies on skin absorption of CB have several shortcomings, such as: 1) TEM imaging was not considered to be sufficiently quantitative, 2) The smallest particles tested were 20 nm and therefore the available data do not give any information regarding possible uptake of particles smaller than 20 nm and 3) The CB samples are not characterised with regard to purity and the amounts of aggregates in the formulation before and after exposure (SCCS, 2014c).

The SCCS did not report any studies on absorption of CB into the eyes (SCCS, 2014c).

Distribution
The SCCS concludes that inhaled CB may be retained in the lung for a considerable time period. However, translocation from the lungs to the circulation may result in accumulation in other organs such as liver, spleen and brain. The SCCS concludes that translocation of CB nanomaterial through the skin following dermal application is unlikely. The SCCS concludes that some reports indicate distribution of CB particles from the gut to lymphoid tissue following oral administration (SCCS, 2014c). If particles are intravenous injected in mice, particles accumulates in the liver (Kermanizadeh et al., 2014).

Metabolism
The SCCS does not report any studies on metabolism of CB (SCCS, 2014c). In general, no information indicates any metabolism of insoluble particles including CB.

Excretion/accumulation
The SCCS does not report any studies on the excretion/metabolism of CB (SCCS, 2014c). If particles reach the circulation in mice, particles accumulate in the liver (Kermanizadeh et al., 2014).

6.1.3 Adverse effects of CB

Respiratory system
Several studies have reported that nano-sized CB induced pulmonary inflammation in mice and rats following exposure by inhalation (e.g. (Jacobsen et al., 2009; Saber et al., 2005; Driscoll et al., 1996)) and intratracheal instillation (Bourdon et al., 2012; Gilmour et al., 2004). As reported in Paragraph 1.5, it is generally acknowledged that the specific surface area of insoluble particles

including CB is proportional to the induced inflammation. As reported in Paragraph XXX on Genotoxicity and cancer, CB is categorised as possibly carcinogenic to humans by the WHOs IARC (IARC, 2010). Systemic effects following inhalation of CB has been reported and will be dealt with in the relevant paragraphs below. Systemic effects may be caused by particles translocating from the lung into the circulation or be caused by systemic inflammation/acute phase response. Subchronic studies were summarised by the SCCS and showed that rats, mice and hamsters responded similarly following inhalation of CB at 1 and 7 mg/m^3. The NOAEL for inhalation of CB nanomaterials for all three species was 1 mg/m^3. In contrast, inhalation of 7 mg/m^3 and 50 mg/m^3 CB induced pathological lung effects in mice and rats that were irreversible in the recovery period for up to 11 months after exposure (SCCS, 2014c).

Cardiovascular system
Effects on the cardiovascular system are not addressed specifically by the SCCS. However, the SCCS concludes that absorption of CB nanomaterials through intact skin is unlikely (SCCS, 2014c) and therefore we conclude that the risk of cardiovascular effects following dermal exposure is considered to be negligible. In contrast by pulmonary exposure, CB has been shown to generate vasomotor dysfunction and to exacerbate the progression of atherosclerosis (Møller et al., 2011). CB inhalation/instillation has recently been shown to induce a pulmonary acute phase in mice (Saber et al., 2013) and has been suggested to be the causal link between particle inhalation and cardiovascular disease (Saber et al., 2014).

Gastrointestinal tract
SCCS concludes that, the maximal non-lethal dose of CB after single administration by the oral route in rats is higher than 10 000 mg/kg bw, and CB is therefore considered to have no acute toxicity potential by the oral route. Repeated dose toxicity of 20-30 nm CB by oral gavage was tested in a 13 week study in rats following the OECD guidelines. Rats were dosed by gavage (0, 100, 300, and 1,000 mg/kg/day) for 90 consecutive days. The NOAEL of CB in the evaluated study was 1,000 mg/kg/day (SCCS, 2014c).

CNS
CB-induced effects on CNS are not addressed by the SCCS and are not supposed to be of any concern following dermal/eye exposure (SCCS, 2014c). General information on particle-induced CNS effects is addressed in Paragraph 3.4.

Other organs
Hepatic DNA damage was observed in mice intratracheally instilled with CB (Bourdon et al., 2012).

Skin
Application of CB on intact and abraded skin from rabbits under occlusion for up to 24 hours did not result in any cutaneous signs of oedema or erythema. Neither did the testing of up to 10 % CB in sunflower oil result in any irritation when tested in an *in vitro* model of reconstructed human epidermis (SCCS, 2014c).

The SCCS concludes that no carcinogenicity was observed following dermal exposure to CB (SCCS, 2014c). More details in Paragraph on genotoxicity and cancer.

Eyes
The SCCS refers a study on eye irritation: "*The acute ocular irritation potential of undiluted CB (furnace blacks Printex G: Degussa AG, 1977d; Spezialschwarz 4: Degussa AG, 1977e; Printex-140: Degussa AG, 1978c) was evaluated following a single instillation to rabbit eyes. No irritant effects were found in any of the animals at any observation time. The study authors concluded "that under the conditions used in those studies, CB was considered to be non-irritating to rabbit eyes*"" (SCCS, 2014c).

Developmental and reproductive system
The SCCS identified five studies, four with mice and one with rats, on the reproductive toxicity of CB. The SCCS concludes "*…that oral and dermal exposure to carbon black is of little concern in relation to reproductive toxicity, however, inhalation exposure should be avoided*"(SCCS, 2014c).

Genotoxicity and cancer
CB has been categorised as possibly carcinogenic to humans (Group 2B). This is based on sufficient evidence for the carcinogenicity of CB in experimental animals and inadequate evidence in humans for the carcinogenicity of CB (IARC, 2010). The carcinogenicity of CB was tested in three skin painting studies from the 1950s. The SCCS evaluates that the carcinogenicity of CB by topical application cannot be evaluated based on these old studies because important information is lacking such as the doses and type of CB used. In addition, the evaluation is hampered by the fact that 1 % benzene was used as a vehicle for some extracts (SCCS, 2014c). The SCCS concludes "*…that carbon black can induce malignant tumors in female rats after inhalation exposure or intratracheal instillations. The potency of carbon black particles with a diameter of 14 nm was higher than the potency of carbon black particle with diameter of 95 nm. Thus, the evidence presented indicates that smaller nanoparticles have a higher potency of causing tumors in lung than relatively larger nanoparticles. There is no empirical support for a dose threshold from the animal carcinogenicity studies*" (SCCS, 2014c).

With regard to genotoxicity the SCCS concludes: "*Carbon black nano particles have been shown to induce single strand breaks both in cell-free studies as well as in mammalian cells. In addition, carbon black has been shown to induce mutations in an alveolar epithelial cell line. The genotoxic effects of nano carbon black in vitro are probably at least in part caused by primary genotoxicity. Conceptually, primary genotoxicity might operate via various mechanisms, such as the actions of ROS (e.g., as generated from reactive particle surfaces), or DNA–adduct formation by reactive metabolites of particle associated organic compounds (e.g., polycyclic aromatic hydrocarbons)*" (SCCS, 2014c).

6.1.4 Adverse effect of carbon black when part of a matrix

Only two studies (one *in vivo* and one *in vitro*) on the toxic effect of CB when part of a solid matrix were identified. Both studies compared the toxicity of the same sample of sanding dust from paint with and without nano-sized CB in. The *in vivo* study tested pulmonary inflammation and DNA damage in mice following pulmonary deposition. The *in vitro* study tested the expression of markers of importance for cardiovascular disease. None of the studies detected any additional effects for the nanopaint. Neither the SCCS opinion nor our own literature search reported/identified any studies on adverse effects of CB when part of solid matrix following dermal/eye application.

In details, in the *in vivo* study, sanding dust from indoor acrylic paint with and without 2.5 % 95 nm CB particles was tested by intratracheal instillation of 54 µg in mice. Pulmonary inflammation, oxidative stress and DNA damage was assessed after 24 hours (Saber et al., 2012c). The same CB as used in the sanding dust study was tested in another study with an identical set-up (Saber et al., 2012b). Neither the CB alone nor the sanding dusts with and without CB induced any significant effects. In addition to the indoor acrylic paint with and without CB, the testing included sanding dust from 11 other different paints (in total 8 with and 5 without nanomaterials). For none of the sanding dusts additional toxicity was detected for the nanoparticle-containing dusts compared with the dusts obtained by sanding traditional products without nanoparticles. However, the paint/lacquer types differed in toxicity. Thus, sanding dust from the tested lacquer was found to be genotoxic. However, the genotoxic effect did not depend on the addition of nanomaterial in the lacquer. The study is further described in Paragraph 4.1.

The same sanding dusts and CB as described above were tested *in vitro* by exposure of primary human umbilical vein endothelial cells by measuring cell surface expression of vascular cell adhesion and intracellular adhesion molecules (Mikkelsen et al., 2013). The results were similar to the *in vivo* studies in that no additional toxicity was detected for the nanomaterial containing dusts compared to the dusts from the conventional paints. The study is further described in Paragraph 4.1.

6.1.5 Physico-chemical properties of importance for toxicity

For insoluble particles including CB specific particle characteristics such as size, specific surface area and carcinogenic compounds adhered to the particle core and ROS generating capability have been suggested to be important for particle-induced toxicity. For example chemically identical CB particles induced different systemic and pulmonary responses depending upon the particle size (Gilmour et al., 2004). There is large evidence that that the specific surface area of insoluble particles in general is proportional to the induced inflammation (Jacobsen et al., 2009; Donaldson et al., 2002; Tran et al., 2000). In a recent study, inflammation measured as neutrophil influx in mice instilled with five different types of particles, of which two were CBs, correlated strongly with the specific surface area (Saber et al., 2012b). Due to the large specific surface areas of CB particles, various chemicals and metals may be adsorbed to the carbon core. Polyaromatic hydrocarbons are some of the most commonly identified chemicals in CB extract (IARC, 2010). The presence of carcinogenic polyaromatic hydrocarbons may affect the toxicity of the particles. Particles may lead to the formation of ROS by different mechanisms. ROS may react with DNA and cause DNA damage.

6.1.6 Summary

This hazard assessment of CB in a matrix is primarily based on the recent SCCS opinion on CB (SCCS, 2014c). The aim of this SCCS opinion on CB was specifically to decide if CB is safe for use as a colorant with a concentration up to 10 % in cosmetic products and is therefore considered highly relevant for the present purpose, namely to serve as an input for the hazard part of the risk assessment of CB in mascara. The focus in the present assessment was on hazard related to skin and eye exposure because these routes of exposure are considered to be the most relevant in relation to consumer use of mascara. Three studies on skin absorption of cosmetic formulations containing CB (all 20-30 nm in size) were evaluated by the SCCS and did not indicate any skin absorption. As emphasised by the SCCS, the conclusion on no risk of adverse effects of up to 10 % as CB as a colorant in cosmetic products is only valid when the skin is intact and when the CB particles are 20 nm or larger. No studies on eye absorption of CB were evaluated by the SCCS. Therefore, hazard associated with eye absorption cannot be evaluated even though it is highly relevant for the hazard assessment of consumer's use of mascara containing CB. The risk of eye irritation of CB cannot be excluded (SCCS, 2014c). We agree with the final concluding remarks of the SCCS opinion stressing that the skin absorption studies have only been done for CB sizes above 20 nm and that it is therefore not possible to conclude on cosmetic products containing smaller sized CB.

The conclusions by the SCCS, IARC and EFSA on CB are inserted below.

The Scientific Committee on Consumer safety (SCCS)

On December 2013, the SCCS adopted a draft opinion on CB on nanoform. March 2014 a revised and final opinion was published following a commenting period (SCCS, 2014c). On the basis of the available evidence, the SCCS concluded: "*...that the use of carbon black CI 77266 in its nano-structured form with a size of 20 nm or larger at a concentration up to 10 % as a colorant in cosmetic products, is considered to not pose any risk of adverse effects in humans after application on healthy, intact skin. However, on the basis of the evidence provided, an eye irritation potential of carbon black cannot be excluded. This opinion does not apply to applications that might lead to inhalation exposure to carbon black nanoparticles, where the preparation might lead to inhalable particles*". Furthermore, the SCCS states that the purity of CB

nanomaterials used in cosmetic products should be above 97 % and that the impurity profile of CB should be comparable with those nanomaterials tested for toxicity in the submission and should also comply with the US Food and Drug Administration specifications with respect to CB produced by furnace method.

In their safety evaluation the SCCS concludes:
"*The calculation of margin of safety (MoS) is not relevant for this assessment given the very low, if any, dermal penetration of nano-carbon black when applied on skin, and in consideration of the low toxicity (NOAEL for oral administration of carbon black to rats as 1000 mg/kg bw/d) observed. The NOAEL for inhalation of carbon black nanomaterials for rats, mice and hamsters is reported to be 1 mg/m³. In view of the potential for persistent lung inflammation, similar to other nanomaterials evaluated so far, applications that might lead to inhalation exposure of the consumer to carbon black nanoparticles (such as powders or sprayable products) are not recommended*" (SCCS, 2014c).

This hazard assessment of CB does not deal with vegetable carbon because the vegetable carbon particles on the market today are not nano-sized (EFSA Panel on Food Additives and Nutrient Sources added to food (ANS), 2012). However, for completeness the recent assessment of vegetable carbon by EFSA is inserted below.

The European Food Safety Authority (EFSA)
EFSA recently considered the available toxicological data on vegetable carbon (E153) (EFSA Panel on Food Additives and Nutrient Sources added to food (ANS), 2012). EFSA concluded that the toxicological data base was too limited to establish an Acceptable Daily Intake for vegetable carbon. Based on the reported use levels, vegetable carbon containing less than 1.0 μg/kg of residual carcinogenic polyaromatic hydrocarbons expressed as benzo[*a*]pyrene was concluded not to be of safety concern. This conclusion was also stated to be based on the long historical use of CB for medicinal purposes, the inertness of vegetable carbon and for essentially no absorption following oral intake.

The International Agency for Research on Cancer (IARC)
IARC has categorised CB as possibly carcinogenic to humans (Group 2B). This is based on sufficient evidence for the carcinogenicity of CB in experimental animals and inadequate evidence in humans for the carcinogenicity of CB (IARC, 2010).

6.1.7 Input to risk assessment

Table 3 summarises key hazard data to be used for the risk assessment of CB.

TABLE 3
SUMMARY TABLE FOR CB

Substance	Exposure routes	Critical effect	N(L)OAEL / N(L)OAEC	DNEL / Assessment factors (AF)	Source
Pristine	Inhalation		NOAEL for pulmonary inflammation: 1 mg/m³ (rats, mice, hamsters)	Irritation (respiratory tract): 2 mg/m³ (long term exposure[4])	(SCCS, 2014c)
	Oral		NOAEL (rats): 1000 mg/kg bw/day		(SCCS, 2014c)
	Dermal				
	Eye				
Matrix bound	Inhalation				
	Oral				
	Dermal			No absorption, no risk[1]	
	Eye			No data[2]	
Occupational exposure limit	1. DK 3.5 mg/m³ (bulk form)[3]				
Comments / uncertainties	1 No risk of adverse effects following dermal application of up to 10 % CB (above 20 nm) as colorant in cosmetic products (SCCS, 2014c). No knowledge on uptake from damaged skin or uptake of particles smaller than 20 nm. 2 No studies on eye absorption were evaluated by the SCCS and therefore no conclusion on hazard following eye exposure can be taken (SCCS, 2014c). 3 (Danish Working Environment Authority, 2007) 4 (European Chemicals Agency (ECHA), 2014)				
Conclusion	No risk of adverse effects following dermal application of up to 10 % CB (above 20 nm) as colorant in cosmetic products. No knowledge on uptake from damaged skin or uptake of particles smaller than 20 nm (SCCS, 2014c).				

6.2 Carbon nanotubes (CNT)

6.2.1 Introduction

CNT are incorporated into different kinds of matrices such as plastics and cement to increase strength or reduce weight. Concern has been raised that CNT due to their physical similarity to asbestos may induce similar adverse effects in humans. CNT constitute a very broad group of chemicals varying with regard to wall number (single- and multiwalled), length and composition. The purpose of this chapter on hazard assessment of CNT is to serve as background documentation for the risk assessment of use (wear and tear) and sanding of a golf club containing CNT. The assessment will also address the potential hazards associated with the impact of UV-exposure that may result in increased liberation of free CNT over time. Thus, the main focus will be the hazards associated with 1) exposure via the dermal route, because this is the most likely exposure from the

intended consumer use of a golf club, and 2) pulmonary exposure to sanding dust from a CNT containing golf club, because this the most relevant exposure route associated with this scenario, respectively. Part of this paragraph is literally identical to the summary part on human toxicology of CNT from the just published report on carbon nanotubes from the Danish EPA (Danish EPA, 2015a). No reviews have been identified regarding the hazard of CNT when part of a matrix. Therefore, the hazard assessment of CNT when part of a matrix is based on original scientific publications.

6.2.2 Biokinetics

This paragraph is literally identical to the summary part on human toxicology of CNT from the just published report on carbon nanotubes from the Danish EPA (Danish EPA, 2015a).

Absorption and distribution

Regarding absorption from the gastrointestinal tract, the Danish EPA report concludes: "*For assessment of oral uptake, a recent well-performed study with a low detection limit shows no ingested CNT beyond the GI tract. Thus, there is no evidence to suggest that CNT are taken up from the GI tract"* (Danish EPA, 2015a). The same conclusion is reached by another recent review report from the Danish EPA specifically focusing on the systemic absorption of nanomaterials by oral exposure: "*None of the in vivo studies identified in the open literature for the purpose of this review measured absorption of CNTs after oral exposure of laboratory animals in terms of blood and tissue levels. Thus, the reviewed in vivo studies neither prove nor rule out any absorption of CNTs from the gastro-intestinal tract following oral administration. However, the lack of toxicity observed in animals treated with oral doses of CNT compared with the toxicity seen in animals after i.v. or i.p. administration indicated that either no or a very low absorption occurred after oral exposure* " (Danish EPA, 2015a). Thus, both these recent reviews come up with the same conclusion that there is no evidence that CNT are absorbed from the gastrointestinal tract.

Regarding absorption from the lungs, the Danish EPA report concludes: "*After lung exposure, pulmonary dosed CNT on the other hand is slowly cleared from the lungs after being phagocytised by macrophages. Phagocytosis seems to occur relatively fast. The macrophages also transport the CNT into the subplural regions of the lungs, which is a prerequisite if CNT are to cause mesotheliomas. Half-lives of up to 300 days have been reported. There is evidence that CNT translocate from the lungs via the blood vessels to secondary organs. Thus, 1 % of inhaled CNTs were found in the tracheobronchial lymph nodes in murine lungs after 1 day and 7 % after 336 days, whereas 0.01 % and 0.04 % were localised in extra-pulmonary tissues after 1 day and after 336 days, respectively. After lung exposure, pulmonary dosed CNT on the other hand is slowly cleared from the lungs after being phagocytosed by macrophages. Phagocytosis seems to occur relatively fast. The macrophages also transport the CNT into the subplural regions of the lungs, which is a prerequisite if CNT are to cause mesotheliomas. Half-lives of up to 300 days have been reported* " (Danish EPA, 2015a).

No studies on translocation of CNT to the blood from the skin were identified in a review on CNT (Johnston et al., 2010b). The recent EPA report on dermal absorption of nanomaterials came to the same conclusion: "*...all of these* [studies on dermal application of CNT] *have focused on the toxicity of CNT towards the skin and not on penetration*" and "*Therefore, no definitive conclusions as to the penetration efficiency of CNTs can be drawn*" (Danish EPA, 2013b). Neither did our literature search identify any studies addressing the absorption of CNT through the skin. It can therefore be concluded that so far there are no studies available to evaluate if skin absorption of CNT is possible. However, as described in Paragraph 2.1.3, in general the absorption of insoluble nanomaterials has been shown to be very low.

Metabolism

No data so far indicates that CNT are metabolised.

Excretion
Regarding excretion/accumulation the Danish EPA report concludes: *"There is evidence that CNT translocate from the lungs via the blood vessels to secondary organs. Thus, 1 % of inhaled CNTs were found in the tracheobronchial lymph nodes in murine lungs after 1 day and 7 % after 336 days, whereas 0.01 % and 0.04 % were localised in extra-pulmonary tissues after 1 day and after 336 days, respectively. Once CNT reach the blood vessels, there is amble evidence that they will accumulate in Kupffer cells in the liver with a very low rate of elimination. Several different kinds of CNT have been observed in liver cells up to 1 year after exposure. In a quantitative study, 0.03 % of the inhaled MWCNT localised to the liver after 336 days. In another quantitative study, 0.75 % of radioactively labelled MWCNT dosed by aspiration localised to the liver and 0.2 % localised to the spleen after 1 year"* (Danish EPA, 2015a).

6.2.3 Adverse effects of carbon nanotubes

This chapter is almost literally identical to the summary and conclusion part on human toxicology of CNT from the just published report on carbon nanotubes (Danish EPA, 2015a). However, a few adjustments and additions have been made to follow the outline of the present report.

Respiratory system
Based on OECD guideline, subchronic (90 days) inhalation studies, a No Observed Adverse Effect Concentration (NOAEC) (Pauluhn, 2010) and a Lowest Observed Adverse Effect Concentration (LOAEC) (Ma-Hock et al., 2009) of 0.1 mg/m^3 MWCNT were identified for pulmonary inflammation. Based on these studies, Aschberger et al propose 0.25 µg/m^3 as the chronic human INEL for inhalation for the general public, using overall assessment factors of 100 (details specified in the (Aschberger et al., 2010)).

Regarding respiratory effects the Danish EPA report concludes: "*Numerous studies have investigated the pulmonary toxicity of CNT and found negative toxicological effects at reasonable doses. We have put emphasis on the two recently published long-term inhalation studies following OECD guidelines. In both studies, rats were exposed to two different commercially available MWCNT and subclinical symptoms of inflammation was observed at a concentration of 0.1 mg/m^3, which was the lowest tested dose and therefore yielding a Lowest Observed Effect Level. Both studies used aggregated or agglomerated MWCNT rather than single CNT fibres. It is not known whether inhalation of MWCNT that do not form aggregates will have effects at lower concentrations*" (Danish EPA, 2015a).

Cardiovascular system
Regarding CNT-induced cardiovascular effects the Danish EPA report concludes: "*There is evidence that pulmonary exposure to SWCNT in combination with a high-fat diet leads to plaque progression, and there is evidence that CNT present in the blood in high concentrations will promote platelet aggregation. Since the publication of the Danish EPA report on CNTs it has been shown that pulmonary exposure to CNTs result in an induction of the acute phase response (Saber et al., 2014). This is interesting because acute phase proteins in blood are predictors of risk of cardiovascular disease in prospective epidemiological studies*" (Danish EPA, 2015a).

Gastrointestinal tract
Regarding oral uptake of CNT the Danish EPA report concludes: "*For assessment of oral uptake, there is very little literature, but no ingested CNT has been detected beyond the GI tract. Thus, there is no evidence to suggest that CNT are taken up from the GI tract*" (Danish EPA, 2015a).

Skin
Based on a dermal exposure study, a NOAEL for inflammation of 40 µg of free CNT/mouse and a LOAEL of 80 µg free CNT/mouse for 5 days was identified (Murray et al., 2009). The study was not a guideline study but was used by Aschberger et al due to the lack of better studies. This limitation was accounted for by applying an extra assessment factor. Based on this study, Ascberger et al propose the following INEL's: $INEL_{acute}$ 4.7 mg/person and $INEL_{chronic}$ 0.78 mg/person (details specified in (Aschberger et al., 2010)).

Regarding CNT-induced dermal effects the Danish EPA report concludes: "*Dermal inflammation was observed after exposure to unpurified SWCNT, but not after exposure to purified SWCNT and commercially available MWCNT. This might be caused by the high levels of impurities in the unpurified SWCNT rather than the CNT*" (Danish EPA, 2015a).

Eyes
Eye toxicity was not addressed in the report on CNT by the Danish EPA (Danish EPA, 2015a) or in the other recent reviews addressed for this report (NIOSH, 2013; Johnston et al., 2010b). Our literature search identified a study summarising a few studies on CNT-induced eye toxicity (Ema et al., 2011). Acute eye irritation of two SWCNT and two MWCNT was tested in rabbits. One of the MWCNT was a very weak acute irritant to the eyes one hour after eye application, while the other tested CNT were nontoxic under the test conditions. The effects were reversible since no effects were observed 24 hours after eye application.

Developmental and reproductive system
Regarding CNT induced developmental and reproductive effects the Danish EPA report concludes: "*In reprotoxicological tests, CNTs were found not to accumulate in testis cells after IP[9] injection of CNTs, and injection of CNTs had no effects on male fertility. The significance of the found results is highly questionable, since there is little evidence that CNT will enter circulation at all. However, the results indicate that even if CNT would enter the body, there are no indications of direct effects of CNT on male fertility. However, reprotoxicological effects have been found for other NMs[10] and ascribed to be most likely caused by indirect effects after pulmonary CNT exposure*" (Danish EPA, 2015a; Hougaard et al., 2013).

Genotoxicity and cancer
Regarding CNT-induced genotoxicity and cancer the Danish EPA report concludes: "*Instillation of SWCNT induced single strand breaks in DNA in BAL cells after 24 hours. Oral dosing of the same SWCNT induced single strand breaks in DNA in liver cells. Mitsui MWCNT-7 MWCNT has caused mesotheliomas in rat and in a susceptible mouse model in a dose-dependent manner. Mitsui MWCNT-7 MWCNT are long MWCNT with a low Fe content. In contrast, SWCNT and a shorter MWCNT did not cause mesothelioma in rats. Thus, this evidence suggests that long and straight MWCNT like Mitsui MWCNT-7 may be carcinogenic*" (Danish EPA, 2015a).

Immunotoxicity
Regarding CNT-induced immunotoxicity the Danish EPA report concludes: "*Suppressed T-cell response was found by two independent groups using inhalation or instillation, respectively, of two different MWCNT. Moreover, increased expression of TGF-β which initiates the immune-supression response was found both after inhalation of MWCNT and SWCNT*" (Danish EPA, 2015a).

[9] Intraperitoneal
[10] Nanomaterials

6.2.4 Adverse effect of CNT when part of a matrix

The use of CNT in different kinds of materials is increasing due to product advantages compared to conventional products. While there is increasing knowledge on the hazard of free CNT, only a limited number of studies have focused on the hazard of CNT when part of a matrix. Wohlleben et al. have published two studies on dust obtained by sanding different kinds of nanocomposites: 1) a study in rats testing toxic properties of sanding dusts from cement and plastic with and without carbon nanotubes (Wohlleben et al., 2011) and 2) an *in vitro* study using 'Precision Cut Lung Slices' exposed to sanding dust from thermoplastic polyurethane with and without CNT (Wohlleben et al., 2013).

In the *in vivo* study, toxicological endpoints were evaluated 3 days and 3 weeks after instillation of 0.36 mg of sanding dust from the composite cement and plastic materials and 0.09 mg CNT (Nanocyl NC7000) (Wohlleben et al., 2011). The following endpoints were analysed: pulmonary histology, inflammation (differential cell counts on BAL cells and protein concentrations in BALF) and genotoxicity (Comet assay in lung tissue and micronuclei in bone marrow), and blood were analysed for hematology and acute phase proteins. The physicochemical characterisation showed similar shape and size of the sanding dusts with and without CNT. Overall, there were no differences between the toxicity of sanding dust from the composites with and without CNT. The low content of CNT in the products (2 wt % in cement and less than 5 wt % in plastic) (polyoxymethylene)) did not allow for a study design to determine if the composite-embedded CNT resulted in a reduced toxicity compared to the free CNT at the same CNT mass.

The cytotoxicity of sanding dust from elastic CNT-polyurethane nanocomposite was evaluated *in vitro* in comparison with sanding dust from the corresponding product without CNT by use of 'Precison Cut Lung Slices" (Wohlleben et al., 2013). No cytotoxicity was detected at the tested doses (CNT at 1000 μg/ml; sanding dusts up to 20 mg/ml), hence a possible difference between sanding dust with and without CNT could not be evaluated.

To summarise, the two published studies on the toxicological effects of sanding dusts from nanocomposites containing CNT are in good agreement with each other: No additional toxicity was detected for the nanocomposites compared to the corresponding products without nanomaterials. More studies are needed to make conclusions within this area.

6.2.5 Physico-chemical properties of importance for toxicity

CNT constitute a very broad group of chemicals varying with regard to wall number (single- or multi walled), length and composition (e.g. metal content). The physico-chemical attributes are suspected to affect the toxicity. A recent review concluded that the most important drivers of the toxicity of CNT were: 1) CNT length, 2) metal content, 3) tendency to aggregate/agglomerate and 4) surface chemistry (Johnston et al., 2010b). The relationship between the physico-chemical characteristics of CNT and toxicity is summarised by (Johnston et al., 2010b): " *There has been a focus on the properties of CNTs that might account for toxicity, with SWCNTs often being shown to be more toxic than MWCNTs, although this is difficult to confirm due to other parameters that differ between CNT samples such as length. In fact longer length (> 15 mm) has been demonstrated to result in greater pathogenicity in some in vivo models and frustrated phagocytosis in vitro. Increased functionalization of the surface chemistry and reduced metallic impurities have both been associated with a relative decrease in toxicity, while the consequences of aggregation/agglomeration are highly dependent upon the model used. While physico-chemical characterization of CNT samples is clearly important for such studies, evaluating the attributes of CNTs that are responsible for driving CNT toxicity is also complicated by the experimental design. While some studies indicate an ability of CNT to elicit oxidative stress and inflammation, which ultimately culminate in cytotoxicity in vitro or disease in vivo, more work is required to establish whether this is applicable for all routes of exposure and target organs*," (Johnston et al., 2010b).

6.2.6 Summary

The purpose of this chapter on hazard assessment of CNT is to serve as background documentation for the risk assessment of use (wear and tear) and sanding of a golf club containing CNT. With regard to the intended use of a golf club, dermal exposure is considered to be the only relevant exposure route. No studies were identified on the dermal toxicity of CNT incorporated in a solid matrix. If the CNT-containing gulf club is sanded, sanding dust-containing CNT and potentially free CNT may be liberated and exert toxicity primarily by the pulmonary and dermal route. Two studies were identified on the toxicity of sanding dusts from different kinds of CNT composites (Wohlleben et al., 2013; Wohlleben et al., 2011). None of the studies showed increased toxicity of sanding dust from the CNT materials compared to the conventional products without CNT. However, it has not yet been tested if sanding dust from UV-exposed materials has a different toxicity profile that may be due to an increased liberation of free CNT.

Based on the OECD guideline, subchronic (90 days) inhalation studies, a No Observed Adverse Effect Concentration (NOAEC) (Pauluhn, 2010) and a Lowest Observed Adverse Effect Concentration (LOAEC) (Ma-Hock et al., 2009) of 0.1 mg/m^3 MWCNT were identified for pulmonary inflammation. Based on these studies, Aschberger et al propose 0.25 µg/m^3 as the chronic human INEL for inhalation for the general public, using overall assessment factors of 100 (details specified in the (Aschberger et al., 2010)).

Based on a dermal exposure study, a NOAEL for inflammation of 40 µg of free CNT/mouse and a LOAEL of 80 µg free CNT/mouse for 5 days was identified (Murray et al., 2009). The study was not a guideline study but was used by Aschberger et al due to the lack of better studies. This limitation was accounted for by applying an extra assessment factor. Based on this study, Ascberger et al propose the following INEL's :NEL$_{acute}$ 4.7 mg/person and INEL$_{chronic}$ 0.78 mg/person (details specified in (Aschberger et al., 2010)).

Because no relevant oral exposure scenarios of CNT are known, no INEL was proposed in the risk assessment of CNT by Aschberger et al. (Aschberger et al., 2010).

As reviewed in the Danish EPA report on CNT, occupational exposure limits for CNT exposure has been suggested by a number of scientists and is summarised as follows (Danish EPA, 2015a):

"Based on scientifically derived occupational exposure limits and results from reviews, the proposed inhalation exposure limits are one to three orders of magnitude lower than the currently regulatory enforced exposure limits for CNT. The highest exposure limit was derived by Kobayashi et al who suggested that an exposure of 210 µg/m^3 was acceptable for working 8 hours/day 5 days a week. Pauluhn has suggested an occupational exposure limit of 50 µg/m^3 based on his own OECD guideline inhalation experiment. In 2010, NIOSH proposed a recommended exposure limit (REL) of 7 µg/m^3 of CNT or carbon nanofibers in air as an eight-hour, time-weighted average, respirable mass concentration (www.cdc.gov/niosh/docket/review/docket161A.html). Just recently, NIOSH revised this REL exposure limit to be 1 µg carbon/m^3 in April, 2013 (http://www.cdc.gov/niosh/docs/2013-145). Previously, Aschberger proposed a comparable OEL (1 µg/m^3) and an exposure limit of 0.25 µg/m^3 for the general public (including consumers) (Aschberger et al., 2010). The earliest exposure limits were based on a smaller data set, and also reflect results from tests on different CNT. It is unclear whether the differences are fully related to different procedures for establishment of exposure limits or differences in materials. From a precautionary principle, we favour the lowest derived exposure limits due to the long pulmonary retention times of CNT and the fact that none of the risk assessments are based on the most critical endpoint, mesothelial cancer" (Danish EPA, 2015a).

We agree with the conclusions from the recent Danish EPA report on CNT.

6.2.7 Input to risk assessment

Table 4 summarises key hazard data to be used for the risk assessment of CNT.

TABLE 4
SUMMARY TABLE FOR CNT

Substance	Exposure routes	Critical effect	N(L)OAEL / N(L)OAEC	DNEL / Assesment factors (AF)	Source
Pristine	Inhalation	Pulmonary inflammation	0.1 mg/m³ (rats)	INEL: 0.25 µg/m³ / 100	NOAEL: (Pauluhn, 2010) (Ma-Hock et al., 2009) INEL: (Aschberger et al., 2010)
	Oral				
	Dermal	Inflammation	NOAEL: 40 µg/mouse LOAEL: 80 µg/mouse	INEL (acute): 4.7 mg/person / INEL (chronic): 0.78 mg/person	(Aschberger et al., 2010)
	Eye				
Matrix bound	Inhalation				
	Oral				
	Dermal				
	Eye				
Occupational exposure limit	1. To our knowledge, no regulatory or legal binding OELs for CNT exist.				
	2. NIOSH has suggested 1 µg/m³				(NIOSH, 2013)
Comments / uncertainties	The authors stress that the term INELs is used instead of DNELs because the authors do not want to give the impression that these values could be used for regulatory risk assessment: The INELs were derived from studies on certain types of CNT and the evaluated endpoint was inflammation and not long-term effects such as carcinogenicity.				
Conclusion	NIOSH has suggested 1 µg CNT/m³as OEL. The toxicity of CNT-containing sanding dust was not increased compared to sanding dust from the conventional product in a mouse study.				

6.3 Amorphous silica (nano-SAS)

6.3.1 Introduction

The purpose of this chapter on hazard assessment of nano-SAS is to serve as background documentation for the risk assessment of the following consumer products containing nano-SAS: 1) food items and food containers, 2) face powder and 3) “easy to clean” surface impregnation. Thus, the focus will be the hazards associated with exposure by the gastrointestinal route (relevant for the food items and food container scenarios) and exposure by the dermal and the inhalation route (relevant for the face powder and the “easy to clean” surface impregnation product).

There are three main types of silica: 1) crystalline silica, 2) naturally occurring amorphous silica, and 3) SAS. SAS is the primary form used in consumer products and for that reason, only a hazard assessment of SAS will be performed in this report. SASs can be further categorised into two groups based on the manufacturing method: 1) manufacturing by the thermal route (pyrogenic silica), and

2) manufacturing by the wet route (precipitated silica or silica gel) (European Centre for Ecotoxicology and Toxicology of Chemicals (ECETOC), 2006). SAS is a group of materials consisting of a mixture of primary nanoparticles, nano- or micrometer-sized aggregates and micrometer-sized agglomerates (Fruijtier-Pôlloth, 2012). SASs are hydrophilic and are sometimes surface-modified to increase hydrophobicity (Fruijtier-Pôlloth, 2012).

Industrial production of SAS was initiated in the 1950s and the annual world production exceeded 1 million ton in 2006 (European Centre for Ecotoxicology and Toxicology of Chemicals (ECETOC), 2006). SAS has been used for decades in a number of different consumer products such as as food additive (E 551) without limitations regarding particle size (Michel et al., 2013) and cosmetics, as filler in the rubber industry or as anticaking agent for drug preparations (Michel et al., 2013; Dekkers et al., 2011). Recently, also newer engineered nano-scaled SAS have been put into production (Jensen & Saber, 2014).

It has recently been debated in the scientific literature whether or not the available hazard data for the traditional commercial SAS products can be extrapolated to be valid for the new engineered SAS (Bergin & Witzmann, 2013; Dekkers et al., 2013; Bosch et al., 2012). The main difference between the traditional and the new engineered SAS is that the former usually consists of a mixture of larger aggregates/agglomerates and only of a limited amount of primary nano-scaled particles while the latter potentially can be produced as well-dispersed homogeneous nano-scaled particles. In addition, the engineered SAS may be surface modified and this may also affect the toxicity. The argument for using the original hazard data is that primary particles of SAS have been in the nano-scale already in the past, and even though the primary particles aggregate/agglomerate it is likely that nano-scaled particles have been part of SAS types tested in the older studies (Michel et al., 2013; Bosch et al., 2012). The counterargument has been presented by Dekkers et al., who argues that the amount of nano-scaled SAS in the studies of bulk SAS is unknown and that the toxicity profile may be different for the intentionally nano-scaled SAS (Dekkers et al., 2013; Dekkers et al., 2011).

Because of this uncertainty regarding the hazard of engineered nano-scaled SAS, this assessment tries to summarise both the conclusions of the hazard assessment of the “traditional” SAS presented in the ECETOC report (European Centre for Ecotoxicology and Toxicology of Chemicals (ECETOC), 2006) and from Fruijtier-Pölloth et al. (Fruijtier-Pôlloth, 2012) and the available information from recent reviews on the newer single dispersed nano-scaled SAS (Dekkers et al., 2013; Dekkers et al., 2011). To some extent a few key references of importance for the chosen risk scenarios are also referred.

6.3.2 Biokinetics

Absorption

ECETOC concludes that there is limited accumulation of SAS in body tissues after ingestion and that SAS is rapidly eliminated (European Centre for Ecotoxicology and Toxicology of Chemicals (ECETOC), 2006).

Based on the limited literature concerning absorption of nano-SiO_2 from the gastrointestinal tract, it is concluded by Dekkers et al that nano-SiO_2 can be assumed to be absorbed from the gastro-intestinal tract. No studies were available to quantify the absorption of SAS or nano-SAS from the gastrointestinal tract (Dekkers et al., 2013). A recent study indicates translocation from the gastrointestinal tract and accumulation of a 7 nm sized SAS following 84 days of oral exposure to SAS mixed with food (van der Zande et al., 2014). The study is further described in Paragraph 1.4.

ECETOC reports a study in which rats were intratracheally instilled with 2 mg pyrogenic SAS, where 82 % and 18 % of the dose was retained in the lungs after 6 hours and 2 days, respectively. After 2 days the half-life was approximately 11 days ((Ernst et al., 2002) as cited by (European

Centre for Ecotoxicology and Toxicology of Chemicals (ECETOC), 2006)). ECETOC concludes that SAS is rapidly eliminated from the lungs after end of exposure.

ECETOC does not report any studies on dermal absorption of SAS. However, two studies on intradermal injection of pyrogenic SAS showed that SAS was removed quickly from the site of injection, as after both 2 months ((Klosterkötter, 1969) as cited by (European Centre for Ecotoxicology and Toxicology of Chemicals (ECETOC), 2006)) and 6 weeks ((Degussa, 1964) as cited by (European Centre for Ecotoxicology and Toxicology of Chemicals (ECETOC), 2006)) more than 95 % of the dose was eliminated.

Distribution
Silica nanoparticles (synthesised by precipitation) administered systemically has been shown to distribute to the liver and kidney (summarised by van der Zande et al., 2014). This is similar to what have been shown for other particles.

Metabolism
No data on the metabolism of SAS was identified by ECETOC. It is concluded by ECETOC that "*SAS is generally considered to undergo no metabolism except perhaps some conjugation*" ECETOC (European Centre for Ecotoxicology and Toxicology of Chemicals (ECETOC), 2006).

Excretion/accumulation
ECETOC concludes that SAS is soluble in physiological media and soluble chemical compounds are formed. These are excreted via the urine without modification after intestinal resorption (European Centre for Ecotoxicology and Toxicology of Chemicals (ECETOC), 2006).

6.3.3 Adverse effects of SAS

The overall conclusion by ECETOC is that "*SAS is essentially non-toxic in humans via the oral, dermal/ocular and inhalation routes of exposure and no data exist on systemic effects in humans*" (European Centre for Ecotoxicology and Toxicology of Chemicals (ECETOC), 2006). The background for these conclusions from ECETOC supplemented with knowledge from more recent papers on toxicity of engineered nano-SAS is summarised below.

Respiratory system
In epidemiological studies, occupational exposure to SAS did not result in adverse pulmonary effects (European Centre for Ecotoxicology and Toxicology of Chemicals (ECETOC), 2006). A large number of repeated dose, subchronic and chronic inhalation studies in rodents with SAS in concentrations from 0.5 mg/m^3 to 150 mg/m^3 have been evaluated by ECETOC: The LOAELs and NOAELs obtained from studies with rodents were typically 1-50 mg/m^3 and 0.5-10 mg/m^3, respectively. These differences were by ECETOC evaluated as particle size-dependent, i.e. in general the NOAEL/LOAEL decreased by particle size (European Centre for Ecotoxicology and Toxicology of Chemicals (ECETOC), 2006).

The acute pulmonary toxicity of 14 and 213 nm sized colloidal silica particles following intratracheal instillation was evaluated in mice ((Kaewamatawong et al., 2006; Kaewamatawong et al., 2005) both as cited by (Fruijtier-Pôlloth, 2012)). The smaller particles induced more lung inflammation and tissue damage than the larger particles.

Rats exposed for one- or three-days to *de novo* synthesised, aerosolised amorphous silica nanoparticles (1.8 or 86 mg/m^3 corresponding to 3.7 x 10^7 or 1.8 x 10^8 particles/m^3) did not result in pulmonary inflammation, genotoxic- or adverse lung histopathological effects ((Sayes et al., 2010) as cited by (Fruijtier-Pôlloth, 2012)).

A recent study investigated the toxicological effects of one uncoated nano-SAS (precipitated amorphous silica) and four surface-modifications thereof (surface modifications: polyacylate, PEG, phosphate, amino). Rats were exposed by inhalation for 5 consecutive days with 14- or 21-day post-exposure observation (Landsiedel et al., 2014). The NOAEC for uncoated nano-SAS (15 nm) was assessed to be 2.5 mg/m^3 based on pulmonary inflammation, while the NOAEC for pulmonary inflammation was assessed to be at least 50 mg/m^3 for the PEG, phosphate and amino-coated nano-SAS (all 15 nm). The NOAEC for the acrylate coated nano-SAS (20 nm) differed regarding the local and systemic effects as the NOAEC for pulmonary effects was at least 50 mg/m^3 while the NOAEC for systemic effects was 0.5 mg/m^3.

In a sub-chronic study, rats were exposed to three different types of nano-SAS (precipitated, gel and pyrogenic) by inhalation for 6 hours a day on 5 consecutive days (1, 5 or 25 mg/m^3) and pulmonary effects were evaluated 1 day, 1 or 3 months after last exposure (Arts et al., 2007). The effects were compared with the effects in rats exposed to 25 mg/m^3 crystalline silica. Pulmonary inflammation (measured as influx of neutrophils) was induced both in rats exposed to SAS and crystalline silica. At day 1, the inflammatory response was greater in rats exposed to 25 mg/m^3 SAS than in rats exposed to crystalline silica. However, after 1 and 3 months the neutrophil influx in rats exposed to SAS was almost reversed while the neutrophil influx in rats exposed to crystalline silica was increased. For all types of SAS the NOAEL was evaluated as 1 mg/m^3.

To summarise, the NOAEL of different types of SAS seems to depend on particle size and surface modifications.

Cardiovascular system
As already referred in the introduction to Paragraph 1.3, ECETOC evaluated that no data exists on systemic effects in humans (European Centre for Ecotoxicology and Toxicology of Chemicals (ECETOC), 2006). However, our own literature search identified 3 recent studies on the cardiovascular effects of SAS (1 *in vivo* and 2 *in vitro*).

The cardiovascular toxicity of different sizes (30, 60, 90 and 600 nm) and different doses (2, 5 and 10 mg/kg bw) of amorphous silica particles was investigated after intratracheal instillation in Wistar rats. Silica was detected in the heart which suggested translocation of silica from the lungs to the circulation. The translocation was dependent on the size of silica. The serum concentration of the acute phase protein CRP and several cytokines was increased in rats exposed to 10 mg/kg bw for three of the particles. The authors concluded that the cardiovascular toxicity of silica nanoparticles was related to particle size and dose. Oxidative stress could be involved in inflammatory reaction and endothelial dysfunction, all of which could aggravate cardiovascular toxicology. In addition, endothelial nitric oxide/nitric oxide synthase system disorder caused by nanoparticles could be one of the mechanisms for endothelial dysfunction (Du et al., 2013).

Two recent *in vitro* studies have shown that SAS may affect platelet aggregation (Corbalan et al., 2012) and the vasodilator function of the aortic vessels (Akbar et al., 2011).

Gastrointestinal tract
In general, reviews on the toxicity of SAS suggest safety for consumers when exposed to SAS by food intake (Fruijtier-Pôlloth, 2012; European Centre for Ecotoxicology and Toxicology of Chemicals (ECETOC), 2006). Based on liver toxicity in rats, LOAEL has been determined to 1,500 mg/kg bw/day (Dekkers et al., 2011). These evaluations are based on rather old studies from the 1980s and may not, as previously discussed, represent the hazard of the new engineered nano-SAS. No *in vivo* studies on the toxicity of ingested nano-scaled SAS were identified by the recent review on nano-particle toxicity by the oral route (Bergin & Witzmann, 2013).

However, recently concern has been expressed by Dekkers et al. (Dekkers et al., 2013) because silica nanoparticles have been detected in human consumption products and because nano-sized silica particles are still present in the intestinal content of an *in vitro* digestion model which simulates the conditions of the human gastrointestinal tract (Peters et al., 2012).

CNS
No data were found concerning neurotoxicity in humans following exposure to SAS (European Centre for Ecotoxicology and Toxicology of Chemicals (ECETOC), 2006). No studies reporting effects or no-effects on CNS are mentioned in the recent review by Fruijtier-Pölloth (Fruijtier-Pôlloth, 2012).

Other organs
As summarised by van der Zande et al, several recent studies demonstrate particle-, size, and dose-related liver toxicity following intravenous or intraperitoneal exposure to silica nanoparticles. Fibrosis was observed in the liver from rats following oral exposure to one type of SAS (NM-202) while the other tested SASs did not. These effects were described as much lower in both severity and incidence as observed in studies in which silica have been systemically administered (van der Zande et al., 2014).

Skin
ECETOC concludes that SAS is neither a skin irritant nor a sensitiser. Repeated skin exposure does not result in any significant toxicity. However, dryness and cracking may be the result of repeated skin exposure (European Centre for Ecotoxicology and Toxicology of Chemicals (ECETOC), 2006). A literature search of more recent studies on the toxicity of nano-SAS following dermal application identified a single study (Nabeshi et al., 2011): The transdermal penetration and biodistribution was evaluated by TEM after application of 70 nm sized SAS particles on the skin on the inner side of both ears of mice. After 28 days SAS particles were detected in the skin, the regional lymph nodes, the liver, the cerebral cortex and the hippocampus. The study suggests that SAS particles may translocate through the skin and be systemically distributed. However, the results from this study needs to be confirmed by other studies before a final conclusion on skin uptake can be made.

Eyes
ECETOC concludes that SAS is essentially non-toxic in humans via the ocular route of exposure (European Centre for Ecotoxicology and Toxicology of Chemicals (ECETOC), 2006). Our own literature search did not identify any recent studies on eye effects of SAS or nano-SAS.

Developmental and reproductive system
ECETOC concludes that no developmental toxicity was induced by SAS tested in several species (European Centre for Ecotoxicology and Toxicology of Chemicals (ECETOC), 2006).

Genotoxicity and cancer
In 1997 IARC reviewed the carcinogenicity of SAS and concluded that amorphous silica is not classifiable to its carcinogenicity to humans (Group 3) (IARC Monographs on the evaluation of carcinogenic risks to humans, 1997). ECETOC concluded in 2006 that the reviewed literature indicates that SAS is non genotoxic in *in vivo* assays (European Centre for Ecotoxicology and Toxicology of Chemicals (ECETOC), 2006).
These conclusions are in line with the recent review by Fruijtier-Pölloth which is also based on earlier reports including IARC and ECETOC but is updated with the more recent relevant literature. Fruijtier-Pölloth concludes that there is no evidence for SAS-induced mutagenicity or SAS-induced cancer in animals or humans (European Centre for Ecotoxicology and Toxicology of Chemicals (ECETOC), 2006).

Immuntoxicity
As reviewed by ECETOC, contact allergy has never been observed in workers at SAS production plants despite that SAS production plants have existed for more than 50 years (European Centre for Ecotoxicology and Toxicology of Chemicals (ECETOC), 2006). In a recent study, rats orally exposed to two different types of SAS (NM-202 (100, 500 or 1,000 mg/kg bw/day) and a commercially available food grade SAS (100, 1000 or 2,500 mg/kg bw/day)) did not result in any immunotoxic effects after 28- and 84 days of exposure (van der Zande et al., 2014).

6.3.4 Adverse effect of SAS when part of a matrix

Only a few studies on the toxic effect of nano-SAS when part of a matrix were identified.

Two studies compared the toxicity of the same sample of sanding dust from paint/lacquer with and without different types of nano-sized SAS in an *in vivo* and an *in vitro* study, respectively (reviewed by Jensen & Saber, 2014). The *in vivo* study tested pulmonary inflammation and DNA damage in mice following pulmonary deposition. The *in vitro* study tested the expression of markers of importance for cardiovascular disease. None of the studies detected any additive effects for the nanoparticle containing paint/lacquer. The studies are further described below.

The toxicological effects of three different nano-SAS particles, sanding dusts from reference paints and lacquer with and without the same nano-SAS particles were tested in mice by intratracheal instillation. One of the testet nano-SAS products (Axilat) induced inflammation in mice 24 hours after intratracheal instillation of 54 µg/mouse. The toxicity was masked when Axilat was part of a binder matrix. These data indicate that the toxicity of sanding dust of these paints and lacquer types depends more on the paint matrix than the added fillers and nanomaterials (Jensen & Saber, 2014; Saber et al., 2012c). The studies are further described in Paragraph 4.1.

The same sanding dusts and nano-SASs as described above were tested *in vitro* by exposure of primary human umbilical vein endothelial cells by measuring cell surface expression of vascular cell adhesion molecules and intracellular adhesion molecules (Mikkelsen et al., 2013). The results were in agreement withthe *in vivo* studies as no additional toxicity was detected for the nanomaterial-containing dusts compared to the dusts from the conventional paints. The study is further described in Paragraph 4.1.

Nanocomposites have the potential for use as dental fillings to restore affected teeth following dental caries. There is a long lasting exposure because the nanocomposite will be in contact with the oral tissue for many years. The cytotoxicity and genotoxicity of an extract from a dental nano-composite containing 35 wt % nano-SiO2 filler was evaluated *in vitro* by exposure of MRC-5 cells (Musa et al., 2013). The cells were exposed to cell medium containing an extract from the nano-composite. The extract was prepared in two steps: 1) 24 hours incubation of a suspension of 0.2 g/mL granulated cured nanocomposite in cell media at 37^0C and 2) filtration of the suspension through a 0.45 µm filter. The cytotoxicity was evaluated by the MTT assay following 72 hours exposure to various concentrations of nanocomposite extract. The genotoxicity was determined at doses resulting in less than 50 % cell death. Genotoxicity was assessed by the comet assay and chromosome aberration tests (6, 24 and 48 hours exposure) with or without addition of a metabolic activation system. No genotoxic effects were detected at the used test conditions. The value of the study is limited in this context due to shortcomings in relation to the assessment of the toxicity of nanomaterial-containing composites.No characterisation of the nanocomposite extract was performed: Therefore it cannot be assessed if any of the nano-content of the suspension was liberated to the cell media. In addition, the choice of a lung fibroblast cell line is not an optimal choice for the assessment of oral toxicity.

The effects of two different types of hydrophilic, pyrogenic nano-SAS (SAS1: 7 nm and SAS2: NM-202, 10-25 nm) mixed with a food matrix were tested in rats by oral exposure (van der Zande et al.,

2014). Nano-SAS was mixed with standard feed and chocolate milk to make the combination more palatable and rats were orally exposed to 100, 1,000 or 2,500 mg/kg bw/d of SAS1 and 100, 500 or 1,000 mg of SAS2 (NM-202) for 28 days, or to the highest dose of SAS1 or SAS2 for 84 days. None of the exposure to the SASs resulted in detectable tissue accumulation after 28 days after exposure. However, 84 days of exposure to the smallest of the SAS types (SAS1) resulted in detectable accumulation of silica in the spleen. Histopathology showed increased incidence of liver fibrosis in rats exposed to SAS2 for 84 days. The SASs were characterised both in the feed matrix and in the intestinal content by an *in vitro* digestion model. The nano-size fraction of the total SAS content was 40 w/w % in the SAS1 feed mixture while the SAS2 feed mixture contained 100 w/w % SAS in nano-size. In the intestinal content, more than 50 % of the SAS1 and 80 % of the SAS2 was present as 5-200 nm sized particles. An examination of the intestinal content by the *in vitro* digestion model indicated that the two highest tested doses resulted in more gelation than the low dose. Lower gelation is suggested to result in higher bioaccesability. On that background, the authors concluded that it would be relevant to test the long-term effects with lower and more realistic consumer exposure levels.

Two of the identified studies on the toxicity of SAS when part of a matrix was studied on the effects of adding nano-SAS to paints and lacquers. The addition of nano-SAS did not result in any increased adverse effect and the effects were more dependent on the paint/lacquer matrix than the added nano-SAS particles (further described above in Paragraph 2.2). However, this has only been tested for a few types of paints/lacquers and the same result may not be true for other types of matrices that may be less durable (mechanically and/or water-soluble) or for aged matrices following years of UV-exposure and humidity. In the latter case, free particles may be released over time as it has been shown for other types of paints containing nano-Ag and nano-TiO_2 (Kaegi et al., 2010; Kaegi et al., 2008).

6.3.5 Physico-chemical properties of importance for toxicity

If free SAS particles are liberated from the matrix, several physicochemical properties have been shown to affect the toxicity.

The physicochemical properties, in particularly solubility and particle size, affect the toxicity of SAS. SASs have a rather low solubility in water (1.9-2.5 mmol/L~114-150 mg/L (European Centre for Ecotoxicology and Toxicology of Chemicals (ECETOC), 2006). Surface treatments result in lower water solubility because the hydrophobicity increases (Dekkers et al., 2013). Dekkers et al. raise the concern that for nano-SAS used in many studies on kinetics and toxicity, the water solubility is not given (Dekkers et al., 2013). The importance of size has been demonstrated in a study in which mice were intratracheally instilled with 14 and 213 nm sized silica. The inflammatory response was greater in mice exposed to the smaller particles compared to the larger particles ((Kaewamatawong et al., 2006; Kaewamatawong et al., 2005) as cited by (Fruijtier-Pôlloth, 2012)). The hepatic toxicity was also dependent on particle size in mice intravenously injected with 70 nm, 300 nm or 800 nm sized silica particles. The 70 nm sized silica induced liver injury while the larger particles did not ((Nishimori et al., 2009) as cited by (Fruijtier-Pôlloth, 2012)). In addition to particle size and solubility, also surface modifications of SAS have been shown to modify the toxicity A recent study investigated the toxicological effects of one uncoated and four surface-modifications thereof (surface modifications: polyacylate, PEG, phosphate amino). Rats were exposed by inhalation for 5 consecutive days with 14- or 21-day post-exposure observation (Landsiedel et al., 2014). Uncoated SAS induced moderate pulmonary inflammation but no systemic effects. Because no adverse effects were observed in rats exposed to SAS coated with PEG, phosphate or amino, the results suggest that the some types of surface modification masks the toxicity of SAS. However, in contrast to the uncoated SAS, the acrylated SAS induced effects in the spleen while no pulmonary effects were seen. This indicates that surface coating of acrylate increases the systemic but not the pulmonary toxicity. In another recent study, the toxicity of COOH-modified SAS was compared to uncoated SAS following intraperitoneal injection in mice (Morishige et al., 2012). The inflammatory response

was evaluated by measuring cytokines in the peritoneal cavity lavage fluid and found to be significantly reduced in mice exposed to the surface-modified SAS compared to the uncoated SAS. In summary, the above studies on the effects of surface modification of SAS suggest that some types of surface modification may reduce the toxicity of SAS while other modifications may increase the toxicity. SAS forms aggregates and agglomarates, and a recent study suggest that larger aggregates and agglomerates of SAS can break up into smaller aggregates in conditions similar to the gastrointestinal fluid (van der Zande et al., 2014).

To summarise, the toxicity of SAS is dependent on the physicochemical properties (e.g. size, surface modifications, solubility etc). It is therefore a general problem that many studies on the toxicity of SAS only include limited characterisation of SAS and the amount of nano-sized silica in the SAS products (Dekkers et al., 2013). These modified SAS particles may therefore have a different toxicological profile than the corresponding uncoated SAS product and will need to be assessed on a case-by-case basis (Fruijtier-Pôlloth, 2012).

6.3.6 Summary

This hazard assessment of SAS as part of a matrix is primarily based on the recent reviews by Dekkers et al (Dekkers et al., 2013; Dekkers et al., 2011), a review of the hazard of SAS (Fruijtier-Pôlloth, 2012),, an IARC monograph (IARC Monographs on the evaluation of carcinogenic risks to humans, 1997) and the ECETOC report on SAS (European Centre for Ecotoxicology and Toxicology of Chemicals (ECETOC), 2006). The purpose of the present paragraph is to serve as an input for the hazard part of the risk assessment of SAS in 1) food items and food containers, 2) face powder and 3) “easy to clean” impregnation. Thus, the focus will be the hazards associated with exposure by the gastrointestinal route (relevant for the food items and food container scenarios) and exposure by the dermal and the inhalation route (relevant for the face powder and the “easy to clean” impregnation product).

The critical effect following oral exposure is assessed as the hepatic effect. The NOAEL has been suggested to be 1,500 mg/kg bw/day (Dekkers et al., 2011).

The critical effect following pulmonary exposure is pulmonary inflammation. Based on the evaluation by ECETOC, the LOAELs and NOAELs were typically 1-50 mg/m^3 and 0.5-10 mg/m^3, respectively (European Centre for Ecotoxicology and Toxicology of Chemicals (ECETOC), 2006). These differences were by ECETOC evaluated as, i.e. in general the NOAEL/LOAEL decreased by particle size. Our literature search identified several recent studies showing that the NOAEL/LOAEL were affected by size and surface modification, highlighting that the physico-chemical properties have to be taken into account.

No studies were identified by ECETOC on the dermal or oral absorption of SAS (European Centre for Ecotoxicology and Toxicology of Chemicals (ECETOC), 2006). For that reason no NOAEL/LOAEL has been suggested. However, the overall conclusion by ECETOC is that “*SAS is essentially non-toxic in humans via the oral, dermal/ocular and inhalation routes of exposure and no data exist on systemic effects in humans*”. We identified a single recent study showing dermal uptake of 70 nm sized SAS (Nabeshi et al., 2011). After 28 days, SAS particles were detected in the skin, the regional lymph nodes, the liver, the cerebral cortex and the hippocampus. The study suggests that SAS particles may translocate through the skin and be systemically distributed. However, the results from this study needs to be confirmed by other studies before a final conclusion on skin uptake can be made.

The conclusions by ECETOC and EFSA on SiO_2 are inserted below.

ECETOC, 2006

"This report has been produced as part of the ECETOC Joint Assessment of Commodity Chemicals (JACC) programme. It presents a critical evaluation of the physico-chemical properties, toxicology, ecotoxicology and environmental fate and impact of (non-crystalline) synthetic amorphous silica (SAS, plural: SASs). SASs are white, fluffy powders or milky-white dispersions of these powders (usually in water). SASs are hydrophilic, but can be made hydrophobic by surface treatment. SASs are produced by the wet route (precipitated silica, silica gel) or the thermal route (pyrogenic silica). SASs are used in various industrial applications (e.g. thickening of elastomers) and in consumer products (e.g. cosmetics and pharmaceuticals). Crystalline and/or amorphous silicas are ubiquitous on the earth in soils and sediments, and in living organisms (e.g. diatoms), but only the dissolved form is bioavailable. On a global scale, the level of man-made SAS represents up to 2.4 % of the dissolved silica naturally present in the aquatic environment. The rate of SAS released into the environment during the product life cycle is negligible in comparison with the natural flux of silica in the environment. Based on available data, SAS is not toxic to environmental organisms (apart from physical desiccation). In conclusion, SAS presents a low risk for adverse effects to the environment.

When experimental animals inhale SAS dust, it dissolves in the lung fluid and is rapidly eliminated. If swallowed, the vast majority of SAS is excreted in the faeces and there is little accumulation in the body. Following absorption across the gut, SAS is eliminated via urine without modification in animals and humans. SAS is not expected to be broken down (metabolised) in mammals. Both the mammalian and environmental toxicology of SASs are significantly influenced by the physical and chemical properties, particularly those of solubility and particle size. SAS has no acute intrinsic toxicity by inhalation. Adverse effects, including suffocation, that have been reported were caused by the presence of high numbers of respirable particles generated to meet the required test atmosphere. These results are not representative of exposure to commercial SASs and should not be used for human risk assessment. Though repeated exposure of the skin may cause dryness and cracking, SAS is not a skin or eye irritant, and it is not a sensitiser.

Repeated-dose and chronic toxicity studies confirm the absence of toxicity when SAS is swallowed or upon skin contact. Long-term inhalation of SAS caused some adverse effects in animals (increases in lung inflammation, cell injury and lung collagen content), all of which subsided after exposure. Neither inhalation nor oral administration caused neoplasms (tumours). SAS is not mutagenic in vitro. No genotoxicity was detected in in vivo assays. SAS does not impair development of the foetus. Fertility was not specifically studied, but the reproductive organs in long-term studies were not affected.

In humans, SAS is essentially non-toxic by mouth, skin or eyes, and by inhalation. Epidemiology studies show little evidence of adverse health effects due to SAS. Repeated exposure (without personal protection) may cause mechanical irritation of the eye and drying/cracking of the skin" (European Centre for Ecotoxicology and Toxicology of Chemicals (ECETOC), 2006).

EFSA (2009)

"The present opinion deals only with the safety of calcium silicate, silicon dioxide/silicic acid gel, as sources of silicon (Si) and with the bioavailability of silicon from these sources. The Panel notes that one petitioner also applied for the use of calcium silicate as source of Calcium silicate and silicon dioxide/silicic acid gel added to food supplements The EFSA Journal (2009) 1132, 18-24 calcium. The safety of silicon and calcium itself, in terms of amounts that may be consumed and the consideration of silicon as a nutrient, are outside the remit of this Panel.

The Panel notes the low solubility of calcium silicate in hydrochloric acid and its practical insolubility in water, but in the absence of specific data, the Panel cannot conclude on the

bioavailability of either calcium or silicon from the source. No data have been submitted on the bioavailability of silicon from either silicon dioxide or silicic acid gel. However, several studies have shown that silicon present in a similar form was readily available from foods and in many cases showed absorption similar to that of silicon from liquids. Furthermore, given the conversion of silicon dioxide/silicic acid to orthosilicic acid upon hydration and the bioavailability of silicon from orthosilicic acid, the Panel considers that silicon from silicon dioxide/ silicic acid gel is bioavailable.

The Panel concludes that, in view of the Safe Upper Level for silicon of 700 mg silicon/day established by the EVM[11] for supplemental use and of 2500 mg calcium/day for adults established by the SCF[12], and given the exposure to calcium and to silicon resulting from the proposed uses of calcium silicate as a source of respectively silicon and calcium in food supplements, the use of calcium silicate in food supplements at the proposed use levels is of no safety concern, provided that it complies with the specifications for its use as a food additive. The Panel also concludes that the use of silicon dioxide up to 1500 mg SiO2/day (equal to 700 mg of silicon/day) and of silicic acid gel to supply up to 200 mg silicon/day added to food supplements is of no safety concern" (EFSA Panel on Food Additives and Nutrient Sources added to food (ANS), 2009).

[11] Expert group on Vitamins and Minerals
[12] Scientific Committee on Food

6.3.7 Input to risk assessment

Table 5 summarises key hazard data to be used for the risk assessment of nano-SAS.

TABLE 5

SUMMARY TABLE FOR NANO-SAS

Substance	Exposure routes	Critical effect	N(L)OAEL / N(L)OAEC	DNEL / Assesment factors (AF)	Source
Pristine	Inhalation	Pulmonary inflammation	$LOAELs_{rodent}$[1]: 1-50 mg/m³ $NOAELs_{rodent}$[1]: 0.5-10 mg/m³	4 mg/m³ [4]	(European Centre for Ecotoxicology and Toxicology of Chemicals (ECETOC), 2006)
	Oral	Hepatic effects Liver fibrosis	$LOAEL_{rat}$[2] : 1,500 mg/kg bw/day $LOAEL_{rat}$[3]: 1,000 mg/kg bw/day		(Dekkers et al., 2011) (van der Zande et al., 2014)
	Dermal	Neither a skin irritant nor a sentitiser Dryness and cracking may be the result of repeated exposure			(European Centre for Ecotoxicology and Toxicology of Chemicals (ECETOC), 2006)
	Eye	Non-toxic in humans via the ocular route of exposure			(European Centre for Ecotoxicology and Toxicology of Chemicals (ECETOC), 2006)
Matrix bound	Inhalation				
	Oral				
	Dermal				
	Eye				
Occupational exposure limit	1. DK 2.0 mg/m³(bulk form) [5]				
Comments / uncertainties	1 In general, the NOAEL/LOAEL decreased by particle size (European Centre for Ecotoxicology and Toxicology of Chemicals (ECETOC), 2006) 2 This evaluation is based on rather old studies from the 1980s and may not represent the hazard of the new engineered nano-SAS (Dekkers et al., 2011) 3 Only one of two tested nano-SAS in the study by (van der Zande et al., 2014) reached statistical significance. Only one dose was tested and the response was evaluated after 84 days of exposure 4 Workers, hazard via the inhalation route (bulk form) (European Chemicals Agency (ECHA), 2014) 5 (Danish Working Environment Authority, 2007)				
Conclusion	No studies have been identified concerning the adverse effects of silica when part of the chosen consumer products for the risk assessment scenarios. Therefore no NOAELs or LOAELs for silica as part these matrices can be given. A description of hazard related to a few other consumer products is found in Paragraph 6.3.4.				

6.4 Nano silver (nano-Ag)

6.4.1 Introduction

The main purpose of this chapter on hazard assessment of Ag in its nanoform (nano-Ag) is to serve as background documentation for the risk assessment when used in food supplements, paints for spraying, nano-filtering, disinfectant pump and propellant sprays, textiles and wound dressing.

There exist recent relevant reviews (Scientific Committee on Emerging and Newly Identified Health Risks (SCENIHR), 2014a; Hadrup & Lam, 2014; Johnston et al., 2010a) and a report on nano-Ag (Danish EPA, 2011). In addition, some relevant, recent research, papers as identified in the open scientific literature, are included.

Several investigations have demonstrated continuous release of Ag ions from the surface of nano-Ag particles in solution (Danish EPA, 2011). In biological systems Ag becomes oxidized to the monovalent cation, Ag^+. Ag^+ reacts with important biologically very abundant ions such as Cl^-, S^{--}, and Se to form complexes with very low solubility. In addition, Ag^+ forms complexes with proteins. Thereby the chemical and toxicological available Ag^+ concentration in biological fluids and organs is very low. Ag has no known essential function in man. The daily human intake has been estimated to bo 0.007-0.5 µg/kg bw/day as the sum from all routes of exposure (Hadrup & Lam, 2014).

Ionic Ag has been used for centuries as an antimicrobial agent. Today, nano-Ag is widely applied in different kinds of products including food packaging materials (the EU migration limit of Ag into food is 0.05 mg/kg food(Scientific Committee on Emerging and Newly Identified Health Risks (SCENIHR), 2014a)), food supplements (only to be used if allowed by EU), textiles, paints, electronics, household products, cosmetics, medical devices including wound dressings, water disinfectant and sprays for rooms and cloths. The oral and dermal routes of exposure are regarded as being the most relevant for man although inhalation following use in sprays may occur (Scientific Committee on Emerging and Newly Identified Health Risks (SCENIHR), 2014a; Hadrup & Lam, 2014).

In order to describe the potential toxicity of nano-Ag, it is considered relevant to take into account both data on nano-Ag and conventional forms (metal, salts, colloids) of Ag.

At present, human risk assessment of Ag is most often based on epidemiological studies showing development of argyria in humans. Here an oral NOAEL of 5 µg/kg bw/day can be calculated from a lifetime oral NOAEL of 10 g Ag/life by assuming a person of 70 kg is living 75 years (WHO, 2003). Argyria was the critical effect. SCENIHR has concluded that Ag and nano-Ag may have low toxic potential following ingestion, dermal exposure and inhalation (Scientific Committee on Emerging and Newly Identified Health Risks (SCENIHR), 2014a).

6.4.2 Biokinetics

Absorption

Oral exposure: Animal studies strongly suggest that nano-Ag was dissolved by release of ionic Ag that afterwards was deposited as insoluble salts in tissues and organelles (Hadrup & Lam, 2014; Danish EPA, 2013c).

After oral exposure to nano-Ag, Ag absorbed in the gastro-intestinal tract can be detected in the blood from where it is further distributed to organs (Danish EPA, 2013c). Several examples of argyria show oral absorption in man. In a rat study it was suggested that 1-4 % of the oral dose of nano-Ag is taken up systemically whereas the absorption is about 18 % in man after exposure to ionic Ag (Hadrup & Lam, 2014).

Inhalation exposure: After inhalation exposure of laboratory animals, Ag has been detected primarily in lungs but also in secondary organs such as liver, spleen, kidney, heart, olfactory bulb, brain and kidneys (Smulders et al., 2014; Danish EPA, 2011). This might be direct uptake via the lungs (Smulders et al., 2014) or due to uptake via the gastrointestinal-tract after clearance from the lungs (Scientific Committee on Emerging and Newly Identified Health Risks (SCENIHR), 2014a).

In humans, absorption of Ag following pulmonary administration of nano-Ag has been shown in the blood but the organ distribution was not investigated (Scientific Committee on Emerging and Newly Identified Health Risks (SCENIHR), 2014a).

Dermal exposure: Dermal absorption to serum through damaged skin has been reported in humans applying wound dressings containing nano-Ag. No toxic effects were reported due to nano-Ag. Less than <0.1 % of the dose was estimated to be absorbed (Danish EPA, 2011; Moiemen et al., 2011; Vlachou et al., 2007).The absorption through intact and deceased human skin, if any, is uncertain, perhaps due to strong bindings to proteins and cell surface structures in the skin, wound and plasma (Walker & Parsons, 2014).

None of the available studies clarified whether oral, inhalation or dermal absorption is as nano-Ag, Ag ions or a combination. If nano-Ag is absorbed, nanomaterial may continuously release Ag ions into the tissue and may therefore be more toxic than non-nanoforms (Danish EPA, 2011).

In conclusion, Ag from nano-Ag can especially be absorbed via oral exposure and after inhalation exposure. Minimal dermal absorption can also take place through damaged human skin.

Distribution
Ag seems to be distributed to all of the organs investigated (Hadrup & Lam, 2014). The main organs for distribution are spleen, liver, kidney and sometimes the testes (Scientific Committee on Emerging and Newly Identified Health Risks (SCENIHR), 2014a) whereas a lesser distribution is reported to other organs including the brain and skin epidermis (Hadrup & Lam, 2014; Danish EPA, 2011). Ag from 8 nm nano-Ag seems to be able to pass the placental barrier (Lee et al., 2012).

None of the available studies clarify whether distribution following oral, inhalation and dermal absorption occurs as nano-Ag-, Ag ions or a combination thereof (Danish EPA, 2011). It should be stressed that in one rat study, dosing nano-Ag (14 nm) and soluble Ag-acetate to rats for 28 days, nano-scale structures of Ag also containing chloride, sulfur and selenium were shown indicating formation of heavy-soluble Ag complexes (Loeschner et al., 2011).

Metabolism
Ag as an element cannot be metabolised by endogenous enzymes and there is no overall data suggesting how Ag is further transformed once in the body (Danish EPA, 2011).

Excretion
Excretion of Ag takes place by feces via the bile and by the urine. Most orally administered Ag from Ag nitrate (90-99.6 %) is excreted via feces in mice, rats, dogs and monkeys indicating low levels of absorption. Urinary excretion is very low. In humans, retention of orally administered Ag was determined to be 18 % in a woman already suffering from argyria (Hadrup & Lam, 2014).

6.4.3 Adverse effects of nano-Ag

Animal studies
Acute studies:
The acute toxicity of Ag is low. In rats an LD_{50} (lethal dose, 50 %) was found to be 280 mg/kg bw and in rabbits it was found to be 800 mg/kg bw (Tamimi et al., 1998).Orally administered nano-Ag

was not toxic to guinea pigs in oral single doses up to 5,000 mg/kg bw/day (Maneewattanapinyo et al., 2011).

Repeated-dose toxicity studies
Subacute studies:
In a 28-day OECD guideline repeated oral dose toxicity study on nano-Ag in mice, dose-dependent liver toxicity was reported at relative high doses. No oral NOAEL could be established owing to limited histopathological information (Danish EPA, 2011).

In a key series of comprehensive toxicological studies (Hadrup et al., 2012b; Loeschner et al., 2011) rats were dosed orally by gavage for 28 days with well-characterised and stable nano-Ag (14 ± 2 nm for 90 %) in doses of 2.25, 4.5 or 9 mg/kg bw/day. The organ distribution of Ag in decreasing order was: small intestine (bound to the surface), stomach, kidney as observed in glomeruli and proximal tubules, renal papilla and liver as observed around the central vein and portal tract. Ag was also detected in lung, muscle, brain and plasma in lower concentrations. There was low 24-hour urinary excretion (0.005 % of daily dose) but a much higher excretion via feces (63 % of daily dose). Spherical, strongly aggregated granules were found in lysosomes of macrophages localised in lamina propria and in individual granula in the basal lamina of the epithelium at about the same size as the nanomaterial, i.e. approximately 12 nm or smaller. In the lysosomes the granula were composed of Ag, Se and S after dosing with nano-Ag (Loeschner et al., 2011). The authors conclude that the toxicity of nano-Ag seems to be very low.

One study applying the doses as specified above placed focus on the brain. Animals were not affected by dosing (general appearance, behavior) but doses at 2.25 and 4.5 mg/kg bw/day for 14 days reduced the brain's DA neurotransmitter concentration (no dose-relation), whereas NA and 5-HT neurotransmitter concentrations were not affected. The doses of 2.25 and 4.5 nano-Ag mg/kg bw/day for 28 days increased DA concentration (not dose related). Dosing 9.0 mg/kg increased the 5-HT concentration in the brain (Hadrup et al., 2012c).

In a second study applying the doses as specified above there were no toxicological findings in animals dosed with nano-Ag (clinical appearance, feed intake, body weight or pathological changes). Dosing changed haematological parameters (haematocrit increased at the dose of 9 mg/kg bw/day; mean corpuscular haemoglobin decreased at all three doses, not dose-related; and the mean cell hemoglobin concentration decreased at the doses of 4.5 and 9 mg/kg bw/day, not dose-related). Clinical biochemical parameters and relative organ weight (in adrenals, brain, heart, kidney, liver, lymphnode mesentarialis, ovaries, spleen, testes and thymus) were not affected. This study suggests a NOAEL >9 mg/kg bw/day (highest tested dose) for 28 days to 14 nm nano-Ag (Hadrup et al., 2012b).

In a third study, metabolomic differences in the composition of urine were found in females, not in males. In females nano-Ag increased the excretion of uric acid and allantoin (its metabolite) (Hadrup et al., 2012a).

Subchronic studies:
In a 90 day OECD guideline study with daily oral doses of 0, 30, 125 and 500 mg/kg bw/day of 56 nm nano-Ag the liver was found to be the target organ. Dose-related effects on activity of liver enzymes and cholesterol was reported at and above 125 mg/kg bw/day. This may reflect liver toxicity which was also indicated by histopathological findings (bile duct hyperplasia, fibrosis and pigmentation) and dose-dependent accumulation of Ag in the liver. A NOAEL of 30 mg/kg bw/day and a LOAEL of 125 mg/kg bw/day were established based on the effects on the liver (Kim et al., 2010). The relevance of these findings and at which exposure levels this might be relevant to humans needs to be further investigated.

Long-term studies:
No relevant study exist on the induction of long-term effects was found.

Respiratory system
Two 28 day studies on inhalation of nano-Ag in rats showed no consistent toxic effect whereas a 90 day rat inhalation study on 18-19 nm nano-Ag showed accumulation in lungs and liver accompanied by inflammatory responses in the lungs and altered lung function indicating a LOAEC at the lowest dose corresponding to 49 μg/m^3. Ag was also found in liver, olfactory bulb, brain and kidneys (Danish EPA, 2011).

A 28 day study in mice ((Smulders et al., 2014), for details please refer to Paragraph 4.1) investigated the organ distribution and toxicity of pristine nano-Ag (average size 25-28 nm) in mice exposed to nano-Ag by oropharyngeal aspiration at days 0, 7, 14, 21 and 28. The doses corresponded to 20 μg/dose or 100 μg in total. Some mice were sacrificed at day 30 or after a recovery period at day 56. At day 30, nano-Ag induced increased neutrophils in BAL and a two-fold increase in keratinocyte chemoattractant and interleukin-1β (IL-1β) concentrations in lung tissue. Disposition of Ag, as an element measured by ICP-MS, was found in lung, liver, spleen and kidney. At day 56 after the recovery period from the last dosing at day 28, no effects were identified showing that the acute effects were reversible.

In a 90 day inhalation study of 18 nm nano-Ag in rats at concentrations of 0.7, 1.4 and 2.9 x 10^6 particles/cm^3 for 6 hours/day indicated dose-dependent inflammatory responses in the lungs for all doses. However, these findings were not statistically significant (Sung et al., 2008).
In another study with 18-19 nm nano-Ag (Sung et al., 2009) male and female rats were dosed as follows: control (fresh-air), low dose (0.6 x 10^6 particle/cm^3, 49 μg/m^3), middle dose (1.4 x 10^6 particle/cm^3, 133 μg/m^3) and high dose (3.0 x 10^6 particle/cm^3, 515 μg/m^3). The animals were exposed 6 h/day, 5 days/week for 13 weeks in whole-body inhalation chambers. In addition to mortality and clinical observations, body weight, food consumption and pulmonary function tests were recorded weekly. At the end of the study, the rats were subjected to a full necropsy, blood samples were collected for hematology and clinical chemistry tests, and the organ weights were measured. Bile-duct hyperplasia in the liver increased dose dependently in both the male and female rats. Histopathological examinations indicated dose-dependent increases in lesions related to Ag nanoparticle exposure, including mixed inflammatory cell infiltrate, chronic alveolar inflammation and small granulomatous lesions. Target organs for Ag nanoparticles were considered to be the lungs and liver in both the male and female rats. No adverse effect was noted at the dose level of 1.4 x 10^6 particle/cm^3 (133 μg/m^3) and overall a NOAEL of 100 μg/m^3 was suggested by the authors (Sung et al., 2009).
However, when measuring the lung function, a decrease in lung fuction was noted at all dose-levels (Sung et al., 2008) suggesting that the low dose of 49 μg/m^3 should be considered as an LOAEL for subchronic inhalation exposure to nano-Ag (Christensen et al., 2010).

The available studies, applying respiratory exposure, demonstrate translocation of Ag to liver, spleen and brain. However, it is not possible to state if Ag is particulate or ionic due to lack of appropriate techniques (Johnston et al., 2010a).

Cardiovascular system
Ionic Ag in drinking water (doses not specified) was reported to induce reversible heart hypertrophy in rats and birds and different effects on the hematocrit value whereas nano-Ag increased the hematocrit value. However, no study has shown any nano-Ag induced histopathological changes in the heart (Hadrup & Lam, 2014).

Gastrointestinal tract
In two laboratory animal oral studies of acute toxicity the studies have shown evidence of gastrointestinal-inflammation due to excessive doses of nano-Ag. However, the relevance of these findings is dubious (Danish EPA, 2011).
Dosing nano-Ag (4.5 and 9 mg/kg bw/day) has resulted in high deposition of Ag in the gastrointestinal tract and three investigations have reported pathological findings including damages in the epithelium and intestinal glands, increased goblet cell that have released their mucus granules and abnormal ileum pigmentation due to excessive doses. A NOAEL could not be set (Hadrup & Lam, 2014).
At present, it is not possible to state the relevance, if any, of effects on the gastrointestinal tract induced by these excessive doses of nano-Ag.

CNS
It still remains controversial if Ag crosses the BBB (Hadrup & Lam, 2014). However, 14 nm nano-Ag orally administered for 28 days affected brain NA, DA and 5-HT neurotransmitter concentrations in rat brain, but no behavioral effects were registered (Hadrup et al., 2012c). These effects cannot be taken as evidence for Ag crossing the BBB since they may be indirect effects secondary to effects induced elsewhere in the body. The relevance of these effects needs to be further investigated.
A case study reported induction of myoclonic status epilepticus in man after oral ingestion of excessive amounts of colloidal Ag (Hadrup & Lam, 2014).

Other organs
Ionic Ag and nano-Ag have shown effects on liver enzyme activities in plasma, increased serum cholesterol and increased urinary excretion of uric acid and its metabolite allantoin. No hepatotoxicity due to ionic Ag or nano-Ag was observed 28 days of dosing (Hadrup & Lam, 2014).

Immunotoxicity
Nano-Ag and ionic Ag induced decreased weight of thymus after 28 days of administration (Hadrup & Lam, 2014). Nano-Ag has also been shown to increase plasma and lung concentrations of different interleukins in mice (Hadrup & Lam, 2014; Smulders et al., 2014).
Nano-Ag (15 nm) was orally administered to mice at a dose of 2.5 mg. Three days post exposure evidence of inflammation was shown including changes in four genes used as markers for inflammation. The concentration was not investigated in any organ (Johnston et al., 2010a). The toxicological outcome of these findings needs to be evaluated.

Skin
No information on repeated dermal application was identified (Danish EPA, 2011).

Ag uptake from wound dressing over burned skin has resulted in significant serum concentrations whereas any uptake over intact skin cannot be quantified (Scientific Committee on Emerging and Newly Identified Health Risks (SCENIHR), 2014a).
In a study on nano-Ag, dermal irritation and corrosion test in rabbits were negative (Kim et al., 2013).

Eyes
No consistent adverse effects have been documented.
In a study on nano-Ag, an acute eye irritation test in rabbits was negative (Kim et al., 2013).

Developmental and reproductive system
A recent study conducted according to OECD test guideline 422, where 8 nm citrate capped nano-Ag was administered by oral gavage, did not reveal any treatment-related toxic maternal effects or effects on development or reproduction. Two other studies in mice and two studies in rats

confirmed these negative results (Scientific Committee on Emerging and Newly Identified Health Risks (SCENIHR), 2014a).

Genotoxicity.
In vitro, controversial results on genotoxicity have been reported by SCENIHR. Genotoxicity could not be confirmed by the few existing *in vivo* studies. It was concluded that further studies are necessary for a final conclusion on genotoxicity to be drawn (Scientific Committee on Emerging and Newly Identified Health Risks (SCENIHR), 2014a).

In a recent review it is concluded that Ag has only limited genotoxic effects, if any at all (Hadrup & Lam, 2014).

Irritation, corrosion and sensitisation
No studies have been identified on the irritational effects to eyes, lungs or skin of nano-Ag and nano-Ag is not expected to be corrosive. No studies have been identified on the sensitising effects of nano-Ag, and based on no reported effects when used in wound dressings, nano-Ag is not expected to be a dermal sensitiser (Danish EPA, 2011).

Studies in humans
In an expert assessment of Ag, WHO (WHO, 2003) indicated that, as shown in several cases, the only known clinical picture of chronic Ag intoxication is that of argyria, following chronic exposure to Ag including colloidal Ag and manifested as permanent discoloration of skin and eyes (Wadhera & Fung, 2005; Kim et al., 2009; Chang et al., 2006). Other toxic effects in humans have only been observed after very high concentrations (Danish EPA, 2011).
For many years $AgNO_3$ 1 %, has been used prophylactic in newborns against gonococcocal ophthalmia neonatorum (Scientific Committee on Emerging and Newly Identified Health Risks (SCENIHR), 2014a).

In a recent study, healthy humans were orally dosed with 10 ppm nano-Ag (5-10 nm) for 3, 7 and 14-days or to 32 ppm nano-Ag (32.8 nm) for 14 days corresponding to 100 and 480 µg/person/day, respectively. Persons underwent metabolic studies, blood cell counts, urine analyses, sputum induction, and chest and abdomen MRI. The authors concluded that no important changes on any of these parameters at any time or dose were detected (Munger et al., 2014). This is in accordance with WHO (WHO, 2003).

Underlying mechanisms
The toxicity of nano-Ag appears to be mediated by the induction of oxidative stress (ROS induction) that might stimulate inflammation and genotoxic events, apoptosis and necrosis in different organs (Danish EPA, 2011).

Intestinal bacteria flora and resistance
Due to its bacteriostatic effect, nano-Ag can induce changes in the composition of the bacterial flora, and there is an, until now, ongoing inconclusive debate on the development of resistance induced by nano-Ag (Scientific Committee on Emerging and Newly Identified Health Risks (SCENIHR), 2014a). Moreover, one (Gunawan et al., 2013) of two recent studies that were not considered/available to SCENIHR showed that *Bacillus sp* adapt to nano-Ag cytotoxicity under controlled experimental conditions *in vitro*. In the other study, 28 days oral dosing of rats with nano-Ag did not show any *in vivo* changes in the caecal bacterial flora or any induction of resistance because the amount of ceacal firmicutes bacteria or bacteroidetes bacteria and the expression of three Ag resistance genes (*sil*RS, *sil*P, *sil*CBA) were not affected (Hadrup et al., 2012b). Being an *in vivo* study, the second study is considered more relevant than the *in vitro* study. However, these two studies do not change the relevance of the opinion of SCENIHR that no consistent

documentation is available at this moment and that this represents a serious gap of knowledge (Scientific Committee on Emerging and Newly Identified Health Risks (SCENIHR), 2014a).

6.4.4 Adverse effect of nano-Ag when part of a matrix

Only a few available studies address adverse effect of nano-Ag when part of composites.

Food

Three studies have been carried out in pigs and chicken administered nano-Ag through their diet or drinking water. Pigs were administered 20 or 40 ppm nano-Ag in their feed for 5 weeks. They showed no Ag retention in muscle and only minimal retention in the liver; 0.435 and 0.837 μg/g wet tissue, respectively, thus indicating dose-response relationship (Fondevila et al., 2009). Poultry administered 25 ppm nano-Ag in their drinking water had very low concentrations in muscle and liver (Ahmadi & Kordestany, 2011). When chickens were administered up to 15 ppm nano-Ag in their feed for up to 42 days, Ag was distributed to various organs including eatable tissues. The highest concentrations were demonstrated in breast, femur muscles and the liver; 13.5, 14.2, and 6.8 ppm, respectively (Ahmadi & Kordestany, 2011). In none of these studies was the nanomaterial size specified, and it was not demonstrated that the nanomaterials were on nano-form in the diet, drinking water or tissue.

The studies suggest that animals fed with nanomaterial-containing food and drinking water represent a potential for carry-over if the meat is used for consumption. However, these are academic studies, and it is not likely that such meat or liver are on the market in EU.
In order to better predict the possible oral absorption of nano-Ag, more knowledge is needed about the interaction between nano-Ag and various co-digested food items or types of food, and food supplements (Danish EPA, 2013c).

Wound dressings

In wound dressings, Ag^+-ions are liberated from different Ag-formulations and constitute the active principle. Released Ag rapidly interacts with proteins that may be absorbed and translocated systematically. A recent review considers risk of clinical application of Ag-containing wound dressings (nano-form not specified in any) on damaged human skin for weeks. It is concluded that the risk of absorbed/protein bound Ag^+ to induce short- or long-term local or systematic toxicity is "*considered to be low to negligible*" (Walker & Parsons, 2014).

SCENIHR has recently prepared a preliminary opinion "*Guidance on the Determination of Potential Health Effects of Nanomaterials Used in Medical Devices*" to be implemented. It recommends a four- phased approach based on potential release and characteristic of nanomaterials (Scientific Committee on Emerging and Newly Identified Health Risks (SCENIHR), 2014b).

Paints

A 28 day comparative study in mice by Smulders et al. investigated the organ distribution and toxicity of prestine nano-Ag (average size 25-28 nm) and paint powder containing nano-Ag as obtained after painting, removing the 24 hours dried paint using a spartula and milling to end up with a powder containing larger (>10 μm) and smaller particles (<1μm). Particles were likewise obtained from the same paint without nano-Ag. The mice were exposed to these Ag-particles and the control particles by oropharyngeal aspiration of 25 μL suspensions at days 0, 7, 14, 21 and 28. This corresponded to 20 μg/dose or 100 μg in total. Some mice were sacrificed at day 30 or after a recovery period at day 56. At day 30, prestine nano-Ag induced increased neutrophils in BAL and a two-fold increase in IL-1β and keratinocyte chemoattractant concentrations in lung tissue. Disposition of Ag, as an element, was measured by ICP-MS, and was found in lung, liver, spleen and kidney. At day 56 after the recovery period from last dosing at day 28, no effects of prestine nano-Ag were identified, showing that the acute effects were reversible. There was no significant organ

distribution or toxic effect of the tested paint powder at day 30, therefore the effects after recovery was not tested at day 56. The authors concluded "*that even though direct exposure to nano-Ag induced some toxic effects, once they were incorporated in a matrix little to no adverse toxicological effects were identified*"(Smulders et al., 2014).

Spray: As described in details in Paragraph 4.2, a recent study has been performed to characterise the hazard of exposure to Ag-containing aerosols as generated during use of spray products (Roberts et al., 2013).

In brief, rats were single-exposed for 5 hours by inhalation to 100 µg Ag/m^3 MesoSilver (low dose), 1,000 µg Ag/m^3 from a spray from NIST (high dose) or to a spray consisting sterile, deionized water (control). The mean aerodynamic diameters of the aerosol were 33 nm (low dose) and 39 nm (high dose). No nano-Ag diameter was specified in the aerosols, but nano-sized particles were demonstrated in the exposure chambers after drying of the aerosols.

The alveolar deposition of Ag per rat was estimated to 0 (control spray), 1.4 (MesoSilver) and 14 µg (NIST silver), respectively. Only a few pulmonary and cardiovascular changes were registered: One day after the exposure to the spray product from MesoSilver (low dose), rats responded with elevated heart rate when stimulated with isoproterenol, and one day after exposure to the NIST silver (higher dose), the rats responded with a modest increase in the number of blood monocytes (1.7 fold) and decreased dilation of tail artery following stimulation with acethylcholine.
The authors conclude that "*short-term inhalation of nano-Ag did not produce apparent marked acute toxicity in this animal model"* (Roberts et al., 2013).

It can be concluded, that no adverse effects have been identified when nano-Ag is part of food, wound dressing, or paints. Modest effects were observed in rats exposed to a spray product containing nano-Ag.

6.4.5 Physico-chemical properties of importance for toxicity

There exists no consistent information on the importance of form, shape or surface chemistry. However, binding in compounds with low solubility, aggregation and especially agglomeration seem to reduce toxicity (Scientific Committee on Emerging and Newly Identified Health Risks (SCENIHR), 2014a).
Elementary Ag is rapidly oxidised to Ag^+ in biological fluids, especially at low pH, i.e. in the stomach and in lysosomes following uptake in cells. Therefore, Ag nanoparticles are dissolved after oral or pulmonary uptake, and the Ag^+ are distributed throughout the body. The concentration of free Ag^+ in biologic fluids is low due to formation of low-solubility complexes with Cl^-, selenium and sulfide. Due to the oxidation of nano-Ag, the toxicity of nano-Ag will be very similar to the toxicity of Ag^+.

6.4.6 Summary and conclusions

Key properties
Nano-Ag dissolves in solution and releases Ag^+ that seems to bind to proteins in serum and organs. The released Ag^+ could be responsible for the described biological/toxicological effects (Hadrup & Lam, 2014).

Critical effects
Critical effects are based on oral exposure and comprise development of argyria in humans, liver toxicity in rats and increased serum concentration of transforming growth factor beta (TGF-β) and increased lung cytokine concentrations in mice, as specified below.

At present, human risk assessment of Ag is most often based on accumulation of silver and the development of argyria as seen in epidemiological studies and human case studies. Taking into account the pharmakokinetic aspects of oral silver exposure and accumulation, an oral NOAEL of

10 g Ag for life-time exposure was estimated by WHO. This corresponds to an oral NOAEL of 5 µg/kg bw/day for every-day exposure during lifetime (WHO, 2003).

Recently, in a rat 90-day oral study of nano-Ag, a NOAEL of 30 mg/kg bw/day for nano-Ag was set based on indications of liver toxicity (Scientific Committee on Emerging and Newly Identified Health Risks (SCENIHR), 2014a).

Increased serum concentration of TGF-β and lung cytokine concentrations was shown in orally dosed mice (Smulders et al., 2014; Park et al., 2010) and a NOAEL of 0.25 mg/kg bw/day was set (Park et al., 2010).

No study applying dermal exposure identifies any relevant critical effects. When part of a complex matrix (food, wound dressing, paints), no adverse effects of nano-Ag could be identified.

Thresholds
Oral exposure: Based on the development of argyria in humans, an oral NOAEL of 5 µg Ag/kg bw/day can be set. This value was calculated on a life dose of 10 g during 75 years for a person of 70 kg bw. Underlying data originated from epidemiological studies and case studies. This NOAEL has been adopted by EFSA when assessing the safety in humans of silver zenolite A used for food contact materials (EFSA Panel on Food Contact Materials, 2011). The US EPA has published an oral reference dose of 5 µg Ag/kg bw/day in relation to life-time exposure to Ag (U.S.Environmental Protection Agency, 1996).

Recently, in a rat 90-day oral study a NOAEL of 30 mg/kg bw/day for nano-Ag was set based on indications of liver toxicity (Scientific Committee on Emerging and Newly Identified Health Risks (SCENIHR), 2014a). However, it has to be further elucidated whether the liver toxicity as found in experimental animal studies is to be considered as a further critical effect and at which exposure levels this would be relevant in humans.

In the general population, a daily human intake of Ag of 0.007-0.5 µg/kg bw/day has been estimated, which is sufficiently below the NOAEL values.

Inhalation exposure: In connection with a REACH registration of silver DNELs for inhalation exposure of 0.1 mg Ag/m^3 for workers and 0.04 mg Ag/m^3 for the general population have been set by the REACH registrant (European Chemicals Agency (ECHA), 2014). The Danish Working Environment Authority has set an occupational limit value of 0.01 mg/m^3 for silver (Danish Working Environment Authority, 2007).

From subchronic inhalation exposure to rats a LOAEL of 49 µg/m^3 (90 days exposure 6 h/day, 5 days/week) can be derived based on impairment of lung function at this dose level (Sung et al., 2008; Christensen et al., 2010).

Dermal exposure: No data in relation to dermal exposure allow for identification of a specific N(L)OAEL value.

Summary and conclusion
Nano-Ag is oxidised to Ag^+ in biological fluids. Released Ag^+ quickly reacts with highly abundant anions such as e.g. Cl^- (present at 0.9 % wt/v of biological fluids) to form almost insoluble AgCl. Ag has also been shown to be bound to proteins and in the previously mentioned low-solubility complexes with chloride, sulfur and selenium. Such binding of Ag^+ results in low availability of Ag+ in biological fluids and thus minimises toxicity. Hazard assessment of nano-Ag based on Ag^+ concentration resulting from total solubilisation of Ag to Ag^+ seems to represent a wost-case scenario.

The above studies in laboratory animals and the epidemiological studies in humans show low toxicity and no consistent adverse effects have been reported. In its overall conclusion, SCENIHR concluded that the toxicity of Ag in its different forms is considered to be low following ingestion, dermal exposure and inhalation (Scientific Committee on Emerging and Newly Identified Health Risks (SCENIHR), 2014a).

6.4.7 Input to risk assessment

Table 6 summarises key hazard data to be used for the risk assessment of Ag.

TABLE 6
SUMMARY TABLE FOR Ag

Substance	Exposure routes	Critical effect	N (L)OAEL / N(L)OAEC	DNEL / Assessment factors (AF)	Source
Pristine	Inhalation	Lesions in the lungs Impairment of lung function	NOAEL 100 µg/m³ LOAEL 49 µg/m³		Sung et al., 2009 Sung et al., 2008; Christensen et al., 2010
	Oral	Argyria in humans	NOAEL: 5 µg/kg bw/day[1,2,3]		(Scientific Committee on Emerging and Newly Identified Health Risks (SCENIHR), 2014a; U.S.Environmental Protection Agency, 1996; EFSA Panel on Food Contact Materials, 2011; WHO, 2003)
	Dermal	Low toxicity if any[1]			(Scientific Committee on Emerging and Newly Identified Health Risks (SCENIHR), 2014a)
	Eye	No relevant study			
Matrix bound	Inhalation	Low acute toxicity when part of spray			(Roberts et al., 2013)
	Oral	No adverse effect[3,4]			(EFSA Panel on Food Contact Materials, 2011; WHO, 2003; U.S.Environmental Protection Agency, 1996)
	Dermal	Less than 0.1 % of dose was absorbed in humans when applied as wound dressings. No toxic effects have been reported			(Walker & Parsons, 2014; Moiemen et al., 2011; Vlachou et al., 2007)
	Eye	No relevant study			
Occupational exposure limit	DK: OEL: 0.01 mg/m³ (bulk form)				(Danish Working Environment Authority, 2011)
	REACH registration: DNEL for workers 0.1 mg/m³(bulk form) DNEL for general population 0.04 mg/m³ (bulk form)				Set by the REACH registrant (European Chemicals Agency (ECHA), 2014)
Comments / uncertainties	1 Studies in laboratory animals and the epidemiological studies in humans show low toxicity and no consistent adverse effects. In its overall conclusion SCENIHR concluded that the toxicity of Ag in its different forms is considered to be low following ingestion, dermal exposure, and inhalation (Scientific Committee on Emerging and Newly Identified Health Risks (SCENIHR), 2014a). 2 As the sum of human life-time from all exposure routes 3 There is documentation of uptake of Ag after oral exposure, however the form, including the nano-form, is not documented 4 A concentration in food supplements corresponding to an oral uptake of up to 5 µg Ag/kg bw/day is considered without adverse effects in humans. This should apply to other matrices				
Conclusion	The concentration of free Ag^+ will be very low in serum and organs due to immediate binding in insoluble compounds. Overall, the toxicity of Ag in its different forms is considered to be low following ingestion, dermal exposure and inhalation. Any hazard assessment on nano-Ag based on the free Ag^+ concentration assumed or resulting from total solubilisation of the dose of nano-Ag will represent a worst-case scenario.				

6.5 Nano titanium dioxide (nano-TiO_2)

6.5.1 Introduction

The purpose of the chapter on hazard assessment of nano-sized TiO_2 is to serve as background documentation for the risk assessment of TiO_2 used in chewing gum, sunscreen, sunscreen lipstick, paint and cement and in relation to sanding of a surface painted with nano-TiO_2-containing paint. The different applications of TiO_2 involve exposure by the oral, dermal, eye and pulmonary route and focus will therefore be on the hazards associated with these exposure routes.

TiO_2 occurs in nature as rutile, anatase or brookite crystals and in two high-pressure forms, a monoclinic baddeleyite-like form and an orthorhombic α-PbO_2-like form. The rutile form is the most common in nature. In commercial products, rutile and anatase TiO_2 are the preferred forms as brookite is more difficult to obtain as a pure phase. The difference in the anatase and the rutile forms lie in the arrangement of the titanium and oxygen atoms in the unit cell. Both the rutile and anatase forms exhibit photocatalytic reactivity, with the anatase form being the most reactive.

TiO_2 is widely used as a pigment to increase whiteness or opacity in industrial and consumer products such as paints, coatings, adhesives, paper and paperboard, plastics and rubber, printing inks, coated fabrics and textiles, catalyst systems, ceramics, floor coverings, roofing materials, cosmetics and pharmaceutical products, food colorants etc. In the nano-form, TiO_2 has proven useful as a UV-filter in e.g. sunscreen products, because it, unlike the larger macro form, scatters very little visible light and thereby appears transparent on the skin rather than opaque white.

Rutile TiO_2 and anatase TiO_2 are assigned with CAS Registry Numbers 1317–70–0 (EC No.: 215-280-1) and 1317–80–2 (EC No.: 1317-80-2), respectively. In addition, the CAS Registry number 13463–67–7 (EC No.: 236-675-5) is used as a general term for TiO_2 (both anatase and rutile). TiO_2 (CAS Registry No.: 13463–67–7) is registered under REACH[13] (Registration, Evaluation, Authorisation and Restriction of Chemicals), and the majority of registrations derive from a lead dossier of a REACH registration joint submission.

The SCCS has evaluated the safety of TiO_2 in the nano form when used as a UV-filter in cosmetics with focus on both rutile and anatase forms (SCCS, 2014d). Several other reviews are available as well as a vast amount of literature describing the toxicity of nano-TiO_2 in the pure form and as part of a matrix. Due to the widespread use in cosmetics and sunscreen products, much of the literature base has considered toxicity from exposure via the dermal route. In the following, focus will be on information generated from *in vivo* studies, where relevant.

A recent survey from the Danish EPA on "*Occurrence and effects of nano-sized anatase titanium dioxide in consumer products*" (Danish EPA, 2015b) also summarises the toxicity, exposure and risk of nano- TiO_2 with focus on the anatase form based on both reviews and background papers. Information from this survey will also be included in the following.

6.5.2 Biokinetics

Absorption

A recent report from the Danish EPA provides an overview of systemic absorption of nanoparticles by oral exposure (Danish EPA, 2013c). Four *in vivo* studies on the absorption of TiO_2 nanoparticles have been identified, three studies in rats and one in mice.

[13] Regulation (EC) No 1907/2006 of the European Parliament and of the Council of 18 December 2006 concerning the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH), establishing a European Chemicals Agency, amending Directive 1999/45/EC and repealing Council Regulation (EEC) No 793/93 and Commission Regulation (EC) No 1488/94 as well as Council Directive 76/769/EEC and Commission Directives 91/155/EEC, 93/67/EEC, 93/105/EC and 2000/21/EC

Results of an investigation of the distribution of TiO_2 nanoparticles (anatase crystals with hydroxyl groups on the surface) following administration of doses up to 200 mg/kg bw by gavage in young and adult rats indicated a very low absorption from the gastro intestinal tract. No translocation of the nanoparticles, located in the mucosa of the stomach and in the small intestine, into systemic circulation was observed (Wang et al., 2013b). Geraets et al. confirmed these observations in a study investigating tissue distribution and blood kinetics following oral and intravenous administration of one single or five repeated doses of TiO_2 nanoparticles with a mean particle size in the range of 38 - 267 nm (primary particle size 6 – 90 nm). The results demonstrated a very limited bioavailablility after oral exposure, mainly distribution to the liver, spleen and lung, and slow tissue elimination. The latter may indicate a potential accumulation following long-term frequent exposure. Only minor differences in kinetic profile were observed between the different nanoparticles (Geraets et al., 2014).

In a 28-day study in male Wistar rats receiving a low dose (1 mg/kg bw/day) and a high dose (100 mg/kg bw/day) of water suspensions of anatase, rutile or micro-sized TiO_2 particles (the food additive E 171) by gavage, the absorption and distribution of TiO_2 nanoparticles was investigated. The content of titanium was measured in the liver, and the results indicated that TiO_2 nanoparticles are more readily absorbed than micro-sized particles (E 171), with the rutile form being the best absorbed. There was no major difference in measured levels of titanium in tissues between micron-sized particles at the low dose and at the high dose, whereas the titanium concentration in the liver increased significantly in rats receiving the high dose of rutile TiO_2 nanopaticles intragastrically. Levels in the liver were measured at 0.500±0.036 mg/kg following doses of 1 mg/kg and 0.370 ±0.267 mg/kg following the high dose of 100 mg/kg (Onishchenko et al., 2012).

Another study investigated the distribution of TiO_2 nanoparticles (25 and 80 nm, crystal form not specified) and fine TiO_2 (155 nm) particles in CD-1 mice administered a single large dose of 5 mg/kg bw by gavage. The results indicated that TiO_2 nanoparticles were absorbed from the gastrointestinal tract after oral exposure to extremely high doses and distributed to liver, spleen, lung and kidneys (Wang et al., 2007a).

The uptake of rutile TiO_2 particles (nominal size 500 nm) was investigated in female rats receiving doses of 12.5 mg/kg bw by oral gavage for 10 days. Histological examination demonstrated that 500 nm TiO_2 particles were absorbed and translocated to systemic organs; mainly the liver and to a lesser extent the spleen. Absorption of 6.5 % of the total dose of TiO_2 particles in the 500 nm size range administered orally over 10 days was calculated based on the results. The study results indicated that a minor part of the rutile form of TiO_2 with a nominal size of 500 nm was absorbed and translocated to mainly the liver and the spleen after oral exposure (Jani et al., 1994).

Agglomeration of TiO_2 was seen in the suspensions administered to the animals in most studies and that raises the question about which size distribution and form the experimental animals were actually exposed to. The overall conclusions regarding the influence of physical and chemical properties on the absorption of nano-sized TiO_2 are that agglomeration decreases the absorption of the anatase form and that the rutile form is better absorbed than the anatase form following oral intake/administration (Danish EPA, 2013c).

Brun et al. demonstrated *in vivo* and *ex vivo* that agglomerates of TiO_2 nanoparticles cross both the regular ileum epithelium lining and through Peyer's patches. Transepithelial passage was, however, shown to be low (Brun et al., 2014).

Another recent report from The Danish EPA provides a comprehensive overview of dermal absorption of nanomaterials (Danish EPA, 2013b).
According to (Danish EPA, 2013b), TiO_2 nanoparticles are generally reported to penetrate no further than the stratum corneum. However, deeper penetration into the basal cell layer and even

dermis has been reported. Actual penetration is, however, often reported as being a very small fraction or infrequent.

Results from a study with skin from weanling Yorkshire pigs exposed to UV-B radiation (sunburn simulation) demonstrated that UV-B-sunburned skin slightly enhanced the *in vitro* or *in vivo* penetration of rutile TiO_2 present in the sunscreen formulations into the stratum corneum. The penetration was, however, considered minimal and there was no evidence of any significant penetration of the intact epidermis or evidence of systemic absorption (Sadrieh et al., 2010).

No conclusive evidence to indicate penetration of TiO_2 nanoparticles through the skin to viable cells of the epidermis has been presented. The SCCS opinion highlights that a number of studies have shown that nano-sized TiO_2 particles can penetrate into the outer layers of the stratum corneum, and enter hair follicles and sweat glands, and therefore recommends not to use TiO_2 with high photocatalytic activity in sunscreen formulations in order to avoid deposition of photocatalytic active TiO_2 in these areas and possible generation of ROS (SCCS, 2014d).

Reports from the Danish EPA (Danish EPA, 2013b) and the SCCS (SCCS, 2014d) emphasise that limited studies are available studying the influence of damaged and flexed skin on the penetration/absorption of nanomaterials.

Few studies regarding pulmonary absorption and translocation are available. Li et al. studied intratracheal instillation of 3 nm TiO_2 in mice. The results showed that instilled nano-sized TiO_2 could induce lung damage and change the permeability of alveolar-capillary barrier, and also indicated that TiO_2 nanoparticles may pass through the BBB and induce brain injury through oxidative stress response (Li et al., 2010).

Hougaard et al. studied inhalation exposure of time-mated mice (C57BL/6BomTac) exposed 1 hour/day to 40 mg/m^3 aerosolised powder (1.7·106 n/cm^3; peak-size: 97 nm) on gestation days 8-18 and effects on maternal lung inflammation, gestational and litter parameters, offspring neurofunction and fertility. The study results did not demonstrate translocation of inhaled nanoparticles to offspring liver tissue where the titanium content was found to be below the detection limit. However, the detection limit was relatively high (Hougaard et al., 2010; Hougaard et al., 2011b).

In a review of current toxicological data on TiO_2 nanoparticles, Shi et al. conclude that TiO_2 nanoparticles can translocate, although at a low rate, from the lung into the circulatory system to systemic tissues and from the nasal cavity into sensory nerves to the nervous system. Available evidence does not allow distinguishing between the anatase and rutile forms with or without coating (Shi et al., 2013).

Distribution

In the SCCS opinion on TiO_2 in the nano form it is concluded that the limited available evidence suggest that if TiO_2 nanoparticles become systemically available, they may accumulate mainly in the liver with a very slow clearance (SCCS, 2014d).

Chen et al. examined particle distribution in mice administered doses between 0 and 2,593 mg/kg bw of 80 nm and 100 nm anatase TiO_2 by intraperitoneal injection in a two week acute toxicity study. The results demonstrated that 1, 2, 7 and 14 days post-exposure, accumulation of TiO_2 nanoparticles (80 nm, 100 nm, anatase) was highest in the spleen, followed by the liver, the kidneys and the lungs (Chen et al., 2009).

In the lungs most particles in the size range of 1–5 nm distribute throughout the nasopharyngeal, tracheobronchial and alveolar regions. Particles of 1 nm and 20 nm are mostly distributed in the

nasopharyngeal region and alveolar regions, respectively. Particles of 0.5-10 μm remain on the epithelial surface of the airways and alveoli (Shi et al., 2013).

Husein et al. investigated gene expression, protein synthesis and particle retention in mouse lungs following intratracheal instillation of rutile, nano-sized TiO_2 in female C57BL/6-mice at doses of 18, 54 and 162 μg/mouse. Mice were sampled 1, 3 and 28 days post-exposure. Results demonstrated dose-dependent deposition and sustained retention of nano-TiO_2 over 28 days following exposure for both the lowest and the highest dose (Husain et al., 2013).

Hougaard et al. (Hougaard et al., 2010; Hougaard et al., 2011b) found that following eleven days of inhalation of TiO_2 nanoparticles in mice, high amounts (21 %) of the titanium deposited in the lungs still retained in the lungs 26-27 days following the last exposure. Animals were exposed 1 hour/day at 40 mg TiO_2/m^3 corresponding to half the 8-hour time weighted average OEL according to Danish Regulations (Danish Working Environment Authority, 2007).

Metabolism and excretion
No specific literature was identified regarding metabolism and excretion of nano-sized TiO_2. Clearance of nano-sized TiO_2 distributed to the liver does however seem to be slow.

6.5.3 Adverse effects of nano-TiO_2

Several reviews are available including information on the toxicity of TiO_2 and the nano form. The summary of adverse effects of nano-TiO_2 presented in this Paragraph is based on available reviews which have also provided background for the survey on anatase TiO_2 (Danish EPA, 2015b) together with information from selected, more recent studies.

Acute toxicity
Oral toxicity studies in rats with anatase/rutile mixtures of TiO_2 (85 % anatase and 15 % rutile) coated with trimethoxy-caprylylsilane or trimethoxy-n-octyl-silane show low acute oral toxicity with LD_{50} values above 2,150 mg/kg (single dose study) (SCCS, 2014d). Results from other studies suggest that different ages may require different biomarkers for identifying and monitoring oral toxicity of nanoparticles (Wang et al., 2013b).

An LD50 value of > 2,000 mg/kg bw from an older study on dermal toxicity of ultrafine TiO_2 have been reported by the applicant in the SCCS opinion (SCCS, 2014d). Based on the results from absorption/penetration studies with TiO_2 nanoparticles, no systemic toxicity is expected when nanoparticles are applied to healthy or UV-damaged skin.

In vivo inhalation studies in mice and rats have shown that there is clear evidence that inhalation of TiO_2 nanoparticles is more toxic than inhalation of micro-sized TiO_2, and that the effect is dose-dependent (Grassian & Adamcakova-Dodd, 2007).

Subchronic, repated dose toxicity
Based on a 60 days oral gavage study in mice exposed to anatase TiO_2 nanomaterials (primary particle size 5 nm), the SCCS concludes that a LOAEL of 5 mg/kg bw/day may be derived based on impaired neurofunction and behaviour at all dose levels (SCCS, 2014d). Results from a 30 days oral (gavage) study in mice exposed to anatase TiO_2 nanomaterials with a primary particle size of 5 nm, a NOAEL of 62.5 mg/kg bw/day was suggested, based on body weight reduction, increased coefficients of the liver, kidney, spleen and thymus and serious damage to liver function at doses ≥ 125 mg/kg bw/day (SCCS, 2014d).

Sub-chronic inhalation studies have demonstrated inflammatory responses, epithelial hypertrophy and hyperplasia in the lungs at high exposure doses. The response appears to be influenced by particle size and crystal form of the nanoparticle resulting in differences in pulmonary clearance

and inflammatory response, and rats seems to be more sensitive than mice and hamsters, possibly due to lung overload leading to progression of histopathological lesions (Bermudez et al., 2004; Bermudez et al., 2002).

Respiratory system
Effects on the respiratory system has been the focus of several studies investigating the toxicity of nano-TiO_2 following inhalation, intratracheal instillation and intranasal (oro-pharyngial) exposure.

TiO_2 nanoparticles administered through the lung can produce inflammatory responses in the form of infiltration of inflammatory cells and interstitial thickening. Effects may be of a transient nature. Particle size/surface area and exposure dose of TiO_2 nanoparticles have an important impact on the pulmonary toxicity. Liu et al. demonstrated that intratracheal instillation of 5 and 21 nm TiO_2 particles in rat lungs at a dose level of 50.0 mg/kg TiO_2 can induce pulmonary lesions whereas 50 nm TiO_2 could not. When exposure dose was 0.5 mg/kg, lesions in lungs treated with 50 nm TiO_2 primary particles were significantly more severe than those treated with 5 and 21 nm TiO_2 primary particles. The authors concluded "*that low doses of small-size nanoparticles could be transported to other organs through the circulation which, to a certain extent, may reduce the burden to lung tissue. Due to their large size, 50 nm TiO_2 particles cannot enter the circulation through pulmonary alveoli, but they can deposit in the alveolar wall, which promotes damage to lung tissue*". Pulmonary toxicity caused by 5 nm TiO_2 particles was found to be more severe than toxicity caused by 21 and 50 nm TiO_2 particles. Primary particles of 5 nm nanoparticles may suppress the phagocytotic ability of alveolar macrophages if the exposure dose is ⩾50 mg/kg, (Liu et al., 2009).

NIOSH has referred to a study by Tran et al. (Health and Safety Executive (HSE) Great Britain, 1999) who estimated NOAELs for fine-sized TiO_2 particles based on the relationship between the particle surface area dose, overloading of lung clearance and neutrophilic inflammation in male Wistar rats exposed by whole body inhalation (7 hours/day, 5 days/week) to either 25 mg/m_3 for 7.5 months (209 calendar days) or to 50 mg/m^3 for 4 months (118 calendar days). Findings demonstrated that retardation of alveolar macrophage-mediated clearance, particle transfer to the lung-associated lymph nodes and influx of polymorphonuclear leukocytes were related to the lung burden as particle surface area dose. NIOSH refers that "*a mean airborne concentration of 3 mg/m^3 fine-sized TiO_2 was estimated as the NOAEL, which was defined as a 95 % probability that the lung responses would be below those predicted using the "no overload level" for the average animal*"(NIOSH, 2011).

Cardiovascular system
Studies are available linking exposure to nano TiO_2 with cardiovascular disease. Chen et al. examined the inhalation toxicology of nano-TiO_2 in an atherosclerosis susceptible animal model (ApoE knockout mice; ApoE-/- mice). ApoE-/- mice received tracheal instillation of anatase nano-TiO_2 particles with a diameter ranging from 5 to 10 nm at doses of 100 microgram, 50 microgram and 10 microgram and phosphate buffered saline (PBS) solution per week respectively, totally for six weeks. Indicators of inflammation such as endothelial dysfunction and lipid metabolism in serum were measured, and plaque formation on the aorta was determined. After six weeks of treatment, there was significant difference between the high dose group and PBS control group in terms of CRP, nitric oxide, endothelial nitric oxide synthases, total cholesterol and high density lipoprotein cholesterol in serum. The results showed that tracheal instillation of nano-TiO_2 particles induced considerable systemic inflammation, endothelial dysfunction and lipid metabolism dysfunction, contributing to the progression of atherosclerosis (Chen et al., 2013a). Modestly increased plaque progression was also found in ApoE-/- mice following intra-tracheal instillation of nano-TiO_2 (Mikkelsen et al., 2011).

In the 3-week oral toxicity study conducted by Wang et al., heart injury was observed in young rats as well as slight liver and kidney injury in adult rat was observed following oral exposure at doses of 0, 10, 50, 200 mg/kg bw/day for 30 days (Wang et al., 2013b).

Pulmonary exposure to nano-TiO_2 was shown to induce a pulmonary acute phase response in a time- and dose-dependent manner (Saber et al., 2013; Halappanavar et al., 2011), leading to systemic circulation of acute phase proteins. Acute phase proteins are risk factors for cardiovascular disease (Saber et al., 2014).

Gastrointestinal tract
In the study by Brun et al., it was demonstrated that the agglomerates of TiO_2 also induce epithelium impairment and persist in gut cells where they can possibly induce chronic damage. Transepithelial passage is, however, low (Brun et al., 2014).

CNS
In a 30-days study involving nasal instillation of 500 μg/mouse every other day for a total of 30 days, it was shown that both rutile and anatase TiO_2 nanoparticles bypass the BBB and translocate via the olfactory nerve to the brain, where they accumulate within the cerebral cortex, thalamus and hippocampus (main target). Exposure to what must be considered a very high dose resulted in morphological alterations and loss of neurones in the hippocampus, induction of oxidative stress and initiation of inflammation (Danish EPA, 2011) referring (Wang et al., 2008).

Other organs
In the 3-week oral toxicity study conducted by Wang et al., liver and heart injury in young rats as well as slight liver and kidney injury in adult rats was observed following oral exposure at doses of 0, 10, 50 and 200 mg/kg bw/day for 30 days (Wang et al., 2013b).

Skin
Based on two unpublished studies involving 85 % anatase and 15 % rutile TiO_2, coated with trimethoxy-caprylylsilane or trimethoxy-n-octyl-silane, the SCCS have concluded that TiO_2 nanomaterials appear to be either mild or non-irritant to skin. There is no information available to further characterise the test substances. The primary irritation index was estimated to be zero and 0.3, and the materials were regarded as non-irritant on rabbit skin (SCCS, 2014d).

Eyes
The SCCS have concluded that nano-sized TiO_2 can be regarded as having a low eye irritation potential. This conclusion is based on two unpublished studies involving 85 % anatase and 15 % rutile TiO_2, coated with trimethoxy-caprylylsilane or trimethoxy-n-octyl-silane. There was no information to further characterise the test substances (SCCS, 2014d).

Developmental and reproductive system
In the SCCS opinion it is stated that no conclusive evidence is available regarding reproductive and developmental toxicity of nano-TiO_2 particles (SCCS, 2014d). Shi et al. concluded that although experimental evidence shows that absorbed TiO_2 particles may be able to move across the placenta into fetal tissue, it has not yet been established whether human exposure to TiO_2 particles causes reproductive and developmental toxicities (Shi et al., 2013). Available studies on exposure of pregnant mice showed no evidence of particle translocation, no effect on classical reproductive parameters, no effect on DNA strand break levels in liver in offspring, but found changes in hepatic gene expression in newborn offspring (Jackson et al., 2013; Hougaard et al., 2010). No effects on DNA stability (assessed as microsattelite instability) were detected in the germline cells of F1 females exposed *in utero* to nano-TiO_2 from gestation day 8-18 relative to control females (Boisen et al., 2012). In contrast, maternal airway exposure to nano-TiO_2 tended to reduce sperm counts in the

F1 generation male offspring, although the effect was not statistically significant (Kyjovska et al., 2013).

Genotoxicity and cancer
In a study in mice, Trouiller et al. investigated the genotoxicity, oxidative DNA damage and inflammation of nano-sized TiO_2. A mixture of 75 % anatase and 25 % rutile TiO_2 with a particle size of 21 nm was administered to male mice for 5 days in the drinking water with doses corresponding to 0, 50, 100, 250 and 500 mg/kg bw. Pregnant dams were dosed with 500 mg/kg bw for 10 days at gestation days 8.5 to 18.5 post-coitum in drinking water. DNA single strand breaks were measured in male mice by the comet assay at the highest dose tested (500 mg/kg bw). Furthermore, nanoparticles were shown to induce detectable clastogenicity in mice peripheral blood. DNA double strand breaks were measured by λλ-H2AX immunostaining assay in bone marrow, showing an increase in double strand breaks in a dose-dependent manner. Oxidative DNA damage (8-hydroxy-2' -deoxyguanosine) was measured in the liver at the highest dose tested and a pro-inflammatory response, measured as changes in cytokine expression, was seen in peripheral blood. The study showed that the mixture of anatase and rutile nano-sized TiO_2 administrated orally is systemically distributed to different tissues such as blood, bone marrow and liver, where it can induce genotoxicity at relatively high exposure levels. The inflammatory response and oxidative damage in liver indicate that the mechanism behind the observed genotoxicity may be due to a secondary response following inflammation and oxidative stress (Trouiller et al., 2009). The same study also demonstrated that maternal exposure to 500 mg/kg TiO_2 nanoparticles during gestation resulted in significantly elevated frequencies of DNA deletions in the offspring.

Several *in vitro* studies are also available with both negative and positive results. The Australian National Industrial Chemicals Notification and Assessment Scheme (NICNAS) concludes in a review from 2014 that the effects observed in both *in vitro* and *in vivo* studies are considered secondary to an inflammatory response and/or oxidative stress with no direct correlation to physicochemical properties of TiO_2 nanoparticles (Australian NICNAS, 2014).

IARC has concluded that there is inadequate evidence in humans for the carcinogenicity of TiO_2 but sufficient evidence in experimental animals. Titanium was categorised as a group 2B carcinogen (possibly carcinogenic to humans) (IARC, 2010). NIOSH has concluded that "*titanium dioxide is not a direct-acting carcinogen, but acts through a secondary genotoxicity mechanism that is not specific to titanium dioxide but primarily related to particle size and surface area*". It is expected that carcinogenicity occurs following pulmonary particle overload and thus has a threshold, and that the effects are considered to be caused by the particle exposure rather than the specific chemical substance (NIOSH, 2011).

As summaried by Shi et al., "*the mechanisms of metal-induced carcinogenesis are not well understood. Both genetic and non-genetic factors elicited by TiO_2 nanopaticles in cells may predispose to carcinogenicity*". Shi et al. also suggest that ROS formation, induction of inflammation and alterations in cell signal transduction induced by TiO_2 nanoparticles may play an important role in the etiology of their carcinogenesis (Shi et al., 2013).

Immunotoxicity
In a study by Fu et al., an evaluation of the systemic immune effects of nano-TiO_2 was investigated in Sprague Dawley rats treated by intratracheal instillation with nano-TiO_2 at doses of 0.5, 4 and 32 mg/kg bw, with micro-TiO_2 at 32 mg/kg bw and with 0.9 % NaCl, respectively. The study was conducted to improve the overall understanding of the immune response associated with inhalable nano-TiO_2. The animals were exposed twice a week for four consecutive weeks. In summary, it was demonstrated that nano-TiO_2 induced pathological changes in the spleen and cervical and axillary lymph nodes including slight congestion and brown particulate accumulation. Furthermore, increased proliferation of spleen-derived T cells and B cells following mitogen stimulation and

enhanced natural killer cell activity was observed by repeated instillation of nano-TiO_2. The number of B cells was also increased in the blood. The results suggested that nano-TiO_2 exposure may modify the systemic immune response (Fu et al., 2014).

6.5.4 Adverse effect of TiO_2 when part of a matrix

Food
TiO_2 is a common additive in many food products including chewing gum. It is registered as a food additive in the EU as E 171. Results of a study by Chen et al. found that over 93 % of TiO_2 in gum is nano-TiO_2, and that the nano-TiO_2 was easily liberated from the chewing gum and could be swallowed by a person who chews gum. They also conclude based on preliminary cytotoxicity assays that the gum nano-TiO_2 particles in sugar-coated chewing gum are relatively safe for gastro-intestinal cells within 24 hours even at a concentration of 200 μg/mL (Chen et al., 2013b). Weir et al. have estimated that a child in general consumes 2-4 times as much TiO_2 per kg bw as an adult. Children consume relatively more sweet products, which have a high content of TiO_2, than adults (Weir et al., 2012).

Paints
Saber et al. investigated dose-response relations of inflammation and DNA damage in mice exposed to a single intratracheal instillation of 18, 54 and 162 μg of Nano-TiO_2 or 54, 162 and 486 μg of sanding dust from paint containing Nano-TiO_2 and paint without Nano-TiO_2. There was was no effect of adding Nano-TiO_2 to the paint compared to the reference paint for any of the measured toxicological endpoints. Endpoints included presence of inflammatory cells in lung fluids (inflammatory response), mRNA expression of TGF-β (fibrotic response) and DNA strand breaks in broncheoalveolar lavage cells and liver tissue by the Comet assay as a sensitive assay for genotoxicity (DNA damage). Nano-TiO_2 particles were shown to be inflammogenic and to induce hepatic DNA-damage at the highest dose, but did not induce DNA damage in lung lining cells. Increased pulmonary inflammation was observed in mice exposed to sanding dusts from paints with and without TiO_2 independent of TiO_2-content. No DNA damage was observed in BAL cells or liver tissue (Saber et al., 2012a). In a screening study, Saber et al. concluded that addition of nanoparticles to paint or lacquers did not increase the potential of sanding dust for causing inflammation, oxidative stress or DNA damage in mice exposed by intratracheal instillation to a single dose of 54 μg of sanding dust from boards painted with or without nanoparticles. The results suggested that the paint/lacquer matrix is more important as determinant of DNA damage than the nanomaterial (Saber et al., 2012c).

Smulders et al. investigated exposure to pristine TiO_2 nanoparticles and powder from aged paints containing TiO_2 particles in male BALB/c OlaHsd mice through oropharyngeal aspiration once a week for five weeks. A limited but significant increase of neutrophils in BAL fluid was observed in mice exposed to pristine TiO_2 nanoparticles at day 30. No increase was observed in mice exposed to powder from aged paints. Overall it was concluded that nano-TiO_2 particles incorporated in a complex paint matrix had little or no adverse toxicological effects on the exposed mice (Smulders et al., 2014).

Cosmetic/Sunscreen
Sadrieh et al. demonstrated that UV-B-sunburned skin from weanling Yorkshire pigs slightly enhanced the *in vitro* or *in vivo* penetration of TiO_2 (uncoated mixture of anatase and rutile and dimethicone/methicone copolymer-coated rutile TiO_2) present in sunscreen formulations into the *stratum corneum*. The penetration was considered minimal, and there was no evidence that nano-sized or submicronised TiO_2 penetrated the intact epidermis to any significant extent and no evidence of systemic absorption (Sadrieh et al., 2010).

The overall conclusion from the SCCS based on the available evidence for TiO_2 in the nano-form is, that the use of TiO_2 nanomaterials at a concentration up to 25 % as a UV-filter in sunscreens, can be considered not to pose any risk of adverse effects in humans after application on healthy, intact or sunburnt skin. The required characteristics include: i) a purity of ≥99.5 %, ii) a median primary particle size based on the number-based size distribution of 30 to 100 nm or larger, iii) TiO_2 particles that are composed of mainly the rutile form, or rutile with up to 15 % anatase, iv) photostability in the final formulation, and v) a coating with a coating material considered safe and which is stable in the final formulation and during use. The conclusion from the SCCS does however not apply to applications that might lead to inhalation exposure to TiO_2 nanoparticles (such as powders or sprayable products) (SCCS, 2014d).

Dust from sanding of cement
No studies have been identified investigating inhalation exposure to sanding dust from cement containing nano-TiO_2.

Few *in vivo* studies are available studying inhalation exposure to different spray products. McKinney et al. studied rats exposed to an antimicrobial spray (whole body) at 314 mg/m³ min (low dose), 826 mg/m³ min (medium dose) and 3,638 mg/m³ min (high dose) of TiO_2 under the following conditions: 2.62 mg/m³ for 2 hours, 1.72 mg/m³ 4 hours/day for 2 days and 3.79 mg/m³ 4 hours/day for 4 days, respectively. Pulmonary and cardiovascular effects were monitored 24 hours post-exposure. The authors found no significant pulmonary or cardiovascular changes at low and middle dose levels. Significant increases in breathing rate, pulmonary inflammation and lung cell injury were observed at the high dose level. Based on the results it was concluded that occasional consumer exposure to this spray product should not be a hazard whereas extended exposure of workers routinely applying this product to surfaces should be avoided (McKinney et al., 2012).

NIOSH has, in the background document for the recommendation of a threshold of 3.4 mg/m³ for fine TiO_2 and 0.3 mg/m³ for ultrafine TiO_2 (< 100 nm) for up to 10 hours/day during a 40-hour work week based on the an evaluation of the carcinogenic potential of TiO_2, also evaluated the potential for coatings to modify the toxicity of TiO_2, as many industrial processes apply coatings to TiO_2 particles. Based on the available scientific literature, NIOSH conclude that TiO_2 toxicity has been shown to increase after coating with various substances (NIOSH, 2011).

6.5.5 Physico-chemical properties of importance for toxicity

Nano-TiO_2 is insoluble in water (SCCS, 2014d). For pulmonary exposure, the size of the aerosolised particle-aggregate will determine the pulmonary deposition pattern, and thereby indirectly the rate of clearance, since clearance from the alveolar region is much slower than clearance from the upper airways. The total surface area of the primary particles will predict the inflammatory and acute phase response.

The chemical compositions of industrially relevant TiO_2 particles, which will appear in consumer products, have been shown to vary considerably although this is seldom declared by the suppliers (Saber et al., 2012b).

The size of the primary particle and of aggregates and surface chemisty are also important determinant of oral uptake.

6.5.6 Summary

Key properties
TiO_2 has low solubility in water. Absorption through skin to viable cells of the epidermis has not been demonstrated and absorption from the gastrointestinal tract after oral exposure has only been observed at very high doses. TiO_2 nanoparticles have been shown to translocate at a low rate from

the lung into the circulatory system to systemic tissue and from the nasal cavity into sensory nerves to the nervous system.

Mixtures of anatase/rutile TiO_2 with and without coating show low acute oral toxicity with LD_{50} values above 2,150 mg/kg (single dose tested). No *in vivo* tests investigating dermal toxicity have been identified and systemic toxicity is not expected based on results from absorption/penetration studies showing that nano-TiO_2 is not likely to pass the stratum corneum.

Acute and subchronic inhalation studies have demonstrated a pulmonary inflammation response (neutrophil and macrophage infiltration) at occupationally relevant doses.

Available studies indicate either mild or non-irritating effects to skin and eyes and nano-TiO_2 is considered to have low or no sensitising potential.

Liver, kidney and heart injury has been observed following oral exposure of rats. TiO_2 nanoparticles can bypass the BBB and translocate via the olfactory nerve to the brain. Effects on the nervous system have been observed following nasal instillation in mice.

Nano-TiO_2 has demonstrated the potential to cause DNA damage, although documentation is not considered conclusive. NIOSH has concluded that TiO_2 is not a direct acting carcinogen, but acts through a secondary genotoxicity mechanism which is related to particle size and surface area more than the specific material.

No conclusive evidence is available regarding reproductive and developmental toxicity of nano-TiO_2 particles. Available studies on exposure of pregnant mice showed no evidence of particle translocation, no effect on classical reproductive parameters but found changes in hepatic gene expression in newborn offspring and marginal effects on sperm counts.

Lung tumours are observed in rats exposed to a mixture of nano-sized anatase and rutile TiO_2 but are considered a result of particle overload and of little relevance for man. So far the mechanisms behind a possible carcinogenic potential are not fully understood. Several factors such as ROS formation may play a role. IARC has categorised TiO_2 as a Group 2B carcinogen (possibly carcinogenic to humans) (IARC, 2010).

A study evaluating the systemic immune effects following intratracheal instillation of nano-TiO_2 in rats demonstrated that nano-TiO_2 may induce a systemic immune response.

Critical effects
Based on the available information on lung toxicity following inhalation, inflammation appears to be the most critical effect in relation to long-term exposure to nano-TiO_2. Inflammation is determined by the total surface area of the deposited particles.

Key studies
A NOAEL of 62.5 mg/kg bw/day was established based on a 30 days oral (gavage) study in mice exposed to anatase TiO_2 nanomaterials with a primary particle size of 5 nm.

A LOAEL of 5 mg/kg bw/day was derived based on impaired neurofunction and behaviour based on a 60 days oral gavage study in mice exposed to anatase TiO_2 nanomaterials with primary particle size 5 nm.

Thresholds
The OEL (8-hour average) for TiO_2 in all forms (calculated as Ti) is 6 mg/m^3 in Denmark, corresponding to 10 mg/m^3 TiO_2.

NIOSH has recommended a threshold of 3.4 mg/m³ for fine TiO_2 and 0.3 mg/m³ for ultrafine TiO_2 (< 100 nm) for up to 10 hours/day during a 40-hour work week (NIOSH, 2011).

6.5.7 Input to risk assessment

Table 7 summarises key hazard data to be used for the risk assessment of TiO_2.

TABLE 7
SUMMARY TABLE FOR TiO_2

Substance	Exposure routes	Critical effect	N(L)OAEL / N(L)OAEC	DNEL / Assessment factors (AF)	Source
Pristine	Inhalation	Pulmonary inflammation Secondary genotoxic mechanism involving chronic inflammation and cell proliferation	0.004 mg/m³ (ultrafine TiO_2)[1] 0.3 mg/m³ [2]	-	(NIOSH, 2011) (NIOSH, 2011)
	Oral	Impaired neurofunction and behaviour	5 mg/kg bw/day		(SCCS, 2014d)
	Dermal	No indication of absorption	-		(SCCS, 2014d)
	Eye	Low eye irritation potential No information on absorption	-		(SCCS, 2012)
Matrix bound	Inhalation[3]	Nanoparticles in matrix (paint) had little or no adverse effect on mice			(Saber et al., 2012c)
	Oral		Use level confectionary: 0.068 %		(EFSA Panel on Food additives, 2005)
	Dermal		Safe up to 25 % in cosmetic with maximum 5 % anatase TiO_2		(SCCS, 2014d)
	Eye	No data			
Occupational exposure limit	1. DK: 6 mg/m³ (as Ti) corresponding to 10 mg/m³ TiO_2 (bulk form)				(Danish Working Environment Authority, 2007)
	2. US: NIOSH REL: 0.3 mg/m³ (nano-TiO_2)				(NIOSH, 2011)
Comments / uncertainties	1 Occupational exposure concentrations designed to prevent pulmonary inflammation and thus prevent the development of secondary toxicity 2 Occupational exposure that would allow reduction of the risk of lung tumors to a 1/1,000 lifetime excess risk level 3 (SCCS, 2014b) recommends not to use nano-TiO_2 in sprayable products due to demonstrated carcinogenic activity				(NIOSH, 2011)
Conclusions	Nano-TiO_2 has low acute toxicity from the oral route No penetration to viable epidermis is demonstrated Inhalation toxicity and inflammatory effects are the critical effects Inflammatory response may promote genotoxicity and possible carcinogenicity in experimental animals				(NIOSH, 2011; IARC, 2010)

6.6 Nano zink oxide (nano-ZnO)

6.6.1 Introduction

The main purpose of this chapter on hazard assessment of ZnO in nanoform (nano-ZnO) is to serve as background documentation for the risk assessment of sun screen pump sprays containing nano-ZnO. Thus, the main focus will be the hazards associated with dermal exposure because this is the most relevant exposure route associated with consumer use of sun screen pump spray containing nano-ZnO. However, oral and pulmonary exposure may occur but to a lesser extent. The assessment will primarily be based on the opinion on ZnO adopted by the SCCS (SCCS, 2012), an addendum for this opinion adopted by the SCCS (SCCS, 2014a) and an opinion by the SCCS specifying the meaning of the term "sprayable applications/products" (SCCS, 2014b). The aim of the first SCCS opinion on ZnO was specifically to decide if ZnO in nanoform is safe for use as an UV-filter with a concentration up to 25 % in cosmetic products and is therefore considered highly relevant for the present purpose. The text is partly condensed from these three SCCS opinions (SCCS, 2012; SCCS, 2014a; SCCS, 2014b). To some extent, key references of importance for the chosen risk scenario are referred.

The SCCS assessment applies to nano-ZnO with similar characteristics of the nano-ZnO submitted by manufacturers to the dossier (for detailed information, please refer to Paragraph 6.6.6).

A number of studies have demonstrated that nano-ZnO releases Zn^{2+} in aqueous solution (SCCS, 2012; Massalski, 1990) including artificial body fluids. Thus, in order to describe the potential toxicity of nano-ZnO both data on nano-ZnO and conventional forms (metal, salts and colloids) can be applied.

As an essential trace element and part of human food, at present and during evolution, Zn is regarded to be of low toxicity to man in relation to oral toxicity (Baek et al., 2012). Furthermore, the amount of absorbed Zn is likely to be insignificant compared to the large amount of Zn already present in the body (SCCS, 2012).

6.6.2 Biokinetics

This text is partly condensed from opions on ZnO from the Scientific Commity on Consumer Safety (SCCS, 2012).

Absorption/distribution

Regarding dermal absorption, the SCCS concludes: *"None of the studies or projects yielded any evidence that nano-sized ZnO particles are able to cross the skin barrier in intact or compromised skin. The literature data and the data which were provided for this submission suggest that there is only minimal absorption and resulting systemic availability of zinc from application of ZnO nanoparticles containing sunscreens on the skin. Whether this zinc is available as zinc oxide nanoparticles or zinc ions has not been determined. These studies include numerous in vitro (using human, porcine and nude mice skin) and in vivo human volunteer studies. In studies that analysed particles, no penetration beyond the stratum corneum was seen. In studies that analysed Zn, small amounts were detected in deeper skin layers and receptor fluid/blood. Zn could only be detected in one out of seven of the in vitro studies evaluated, and was detected in the receptor liquid by elemental analysis with ICP-MS indicating some passage (maximally 0.03 % of the applied dose) of the skin barrier. It was shown that some Zn may pass the skin barrier in a human volunteer study in which a small fraction of the blood Zn-pool was demonstrated to originate from a dermally applied sunscreen preparation. No major differences were seen between coated/uncoated ZnO nanomaterials, and no significant differences were observed between damaged skin versus normal healthy skin"* (SCCS, 2012).

In view of the discussion above, it is assumed that penetration of the skin, if any, is caused by Zn ions released from ZnO nanoparticles. Therefore the solubility of ZnO is one of the critical parameters that should be considered in the characterisation of ZnO used for sunscreen formulations.

Regarding oral absorption/distribution, the SCCS concludes: *"After oral exposure there is some uptake of Zn in the systemic circulation. ZnO nanoparticles of approximately 50 nm in size (TEM evaluation) were compared to ZnO microparticles showing at least one diameter >100 nm (TEM evaluation). After oral and intraperitoneal administration for both ZnO nanoparticles and microparticles, Zn could be observed in serum indicating uptake from the GI–tract, either as particulate materials or as dissolved Zn ions. For ZnO nanoparticles the systemic availability was somewhat higher compared to that of ZnO microparticles as indicated by Zn measurements by ICP-MS. Zn showed a higher distribution in the liver, spleen and lung after treatment with ZnO nanoparticles compared to treatment with ZnO microparticles"* (SCCS, 2012).

In the safety evaluation of a sunscreen containing 25% ZnO, the SCCS evaluation used an oral absorption rate for ZnO of 20% (based on read-across) and a dermal skin penetration rate of 0.03% (based on *in vitro* skin permeation data for nano-ZnO using human skin samples) (SCCS, 2012).

In a report from the Danish EPA it is overall concluded that the oral absorption rate of Zn from nano-ZnO is dose-dependent (5-17 % at the low dose and 28-33 % at the high dose), and to a lesser extent also size dependent (Danish EPA, 2013c). Zn absorption is slightly higher from the small particles compared to the larger ones, which could be due to a higher dissolution rate of smaller particles compared to their larger counterparts (Danish EPA, 2013c).

Regarding pulmonary absorption, the SCCS concludes: *"After inhalation exposure elevated zinc levels were detected in various organs, most likely due to zinc ions dissolved from the ZnO particles*" (SCCS, 2012).

Metabolism
Being an element Zn cannot be metabolised by endogenous enzymes.

Excretion
When orally administered as nano-ZnO, Zn was mainly excreted via the feces and only minimally via urine (Danish EPA, 2013c). This was confirmed by Baek et al. showing that 0.32-1.47 % of a dose of nano-ZnO was excreted via urine, while most was excreted via feces. The percentage excreted via urine decreased with increasing dose, while percentage excretion via feces increased in a dose-related manner (probably due to limited absorption) (Baek et al., 2012). The 70 nm nanomaterial seems to be cleared via urine and feces a bit slower than to 20 nm nanomaterials, but not statistically significant.

In a 13-week oral study the excretion with feces was dose-related. There was a dose-response related increase in Zn concentration in urine (Cho et al., 2013).

6.6.3 Adverse effects of nano-ZnO

Respiratory system
SCCS conclude that *"Upon inhalation of ZnO nanoparticles, serious local effects in the lung were observed. Even if this may be due to the solubilized Zn ions, the effects are a direct result of the exposure to the ZnO nanoparticles. Therefore, the SCCS is of the opinion that, on the basis of available information, the use of ZnO nanoparticles in spray products that could lead to exposure of the consumer's lungs to nano ZnO by inhalation cannot be considered safe",* and furthermore state: *"In view of the lung inflammation induced by ZnO particles after inhalation, the use of ZnO*

in cosmetic products that may result in exposure of the consumer's lungs by inhalation is of concern" (SCCS, 2014b).

The SCCS conclusion was based on a few relevant *in vivo* inhalation toxicity studies identified by the SCCS:

A 5-day study applying head-nose inhalation exposure of triethoxycaprylsiloxane coated nano-ZnO in rats (0, 0.5, 2.5 or 12.5 mg/m^3), including a 14-day recovery period, resulted in local concentration-related inflammation at all doses in the lungs as indicated by changed parameters in BALF and histological examinations. No systemic effects were shown and no NOAEL was established (SCCS, 2012).

A recent repeated-dose 90-days OECD guideline 413 inhalation study on nano-ZnO including a 28-days recovey period in male rats exists. Rats were nose-only exposed for 0, 0.3, 1.5 or 4.5 mg/m^3 6 hours/day, 5 days/week. The particle size was not specified. No persistent toxicity was found. Transient (recovered within the 28-day recovery period) local effects on the respiratory tract were only observed in the highest dosed group. The authors concluded a NOAEL of 1.5 mg/m^3 based on observations in BAL and lung histopathology but, in contrast to the SCCS, for this study concluded a NOAEL of 0.3 mg/m^3 based on activation of lung macrophages and lung draining lymph nodes (SCCS, 2014a).

No signs of clinical symptoms were found in humans following inhalation of nano-ZnO for 2 hours/day for three days by mouth piece at a concentration of 500 µg/m^3 (SCCS, 2012).

Our own literature search identified a recent study on the pulmonary toxicity of nano-ZnO. In a recent subacute and subchronic inhalation toxicity study mice were whole body exposed to 3.6±0.5 (subacute) or 3.3±0.6 (subchronic) mg/m^3 of nano-ZnO (average size 40 nm) 4 hours/day for 2 or 13 weeks, respectively (Adamcakova-Dodd et al., 2014). Subacute exposure resulted in minimal pulmonary inflammation (elevated IL-12) and macrophage inflammatory protein 1α in BAL) that was normalised after 3 weeks. No other cytokines/chemokines were affected in BAL. There was no effect after 13 weeks of exposure. There was no cytotoxicity or histopathological changes. In the 13-weeks study, the total dose was 10.9 mg/kg bw corresponding to a daily dose of 121 µg/kg bw/day. No adverse effects were reported and the authors concluded that the subchronic inhalation toxicity of nano-ZnO was low.

Cardiovascular system
Effects on the cardiovascular effects of nano-ZnO are not addressed specifically in the SCCS opinion from 2012 (SCCS, 2012). However in general, cardiovascular effects due to dermal exposure of nano-Zn are considered to be of limited risk for the consumer. A recent study investigated the toxicity to the heart of rats orally exposed for 600 and 1,000 mg/kg bw/day of nano-ZnO (50 nm) for 5 days by oral exposure. This is the first and only identified study on heart toxicity. It showed dose-related decreased weight of heart and increased concentrations of several cardiac injury markers as well as the cardiac Ca^{2+} concentration (Baky et al., 2013). Further studies are necessary to determine the importance of this findning.

Gatrointestinal tract
Regarding oral toxicity, the SCCS concludes the following: *"In one exploratory study in mice, systemic availability of Zn was indicated after a single oral exposure. However, no differences were observed between ZnO administered as nanoscale or microscale particles. It is likely that absorbed Zn in the GI-tract was in the dissolved ionic form. In view of the data provided, oral exposure of nano-ZnO via applications of nano-ZnO as a cosmetic ingredient in sunscreens should*

be considered to be of a similar risk to micron-sized ZnO as previously evaluated in the RAR[14]*. The NOAEL for oral intake of ZnO is* 50 mg/bw day[15] *(Reference 44, sub III). The oral exposure to ZnO nanoparticles as cosmetic ingredient in sunscreens is limited to accidental ingestion of small fractions of lip products and sun protection products and can be considered to be low"* (SCCS, 2012).

In a recent 13-week subchronic study by Seok et al., where rats were administered nano-ZnO in doses of 67.1, 134.2, 268.4 or 536.8 mg/kg bw/day. There was no effect on food or water consumption or on organ weight for any sex. Body weight gain was lower in male rats. The highest dose female and male rats at the 536.8 mg/kg bw/day dose showed significant effects on anemia-related hematological parameters and development of moderate pancreatitis with focal lymphocyte infiltration and mild acinar apoptosis. There were no pathological changes in any other organs including the liver and the brain of either sex. The authors conclude a NOAEL for nano-ZnO in rats corresponding to 268.4 mg/kg bw/day in rats (Seok et al., 2013).

CNS
The SCCS opinion does not address nano-ZnO-induced effects (SCCS, 2012). Our literature search did not identify any studies on CNS effects of dermal application of nano-ZnO, but our literature search identified a single study on CNS effects following oral exposure: Mice were administered nano-ZnO (size <100 nm) in a dose of 500 mg/kg bw/day for 21 days. The results indicated that statistically significant oxidative stress was induced in the brain. The DA and NA neurotransmitter concentrations were increased in the brain cerebral cortex (Shrivastava et al., 2014). In addition, we have identified a few studies on different endpoints for CNS effects following other exposure routes of little importance for the present risk scenario (intraperitoneal exposure (Han et al., 2011), intranasal (Gao et al., 2013) or pulmonary exposure (Kao et al., 2012). It is difficult to interpret the consistency and toxicological relevance of these isolated findings from non-guideline studies and therefore it is uncertain if or how these data can be used in a risk assessment context.

Immunotoxicity
No studies on immunotoxicity were presented in the SCCS opinion (SCCS, 2012).

Other organs
A few intravenous toxicity studies of nano-ZnO are discussed by the SCCS. These studies show that, if absorbed and thereby reaching the systemic circulation, nano-ZnO has the potential to induce hepatic toxicity. The SCCS concludes the following: *"In general it can be concluded that based on the observations on serum liver enzyme levels and histopathology, the systemic availability of either ZnO nanoparticles or Zn ions has the potential to induce liver toxicity"* (SCCS, 2012).

Skin
The SCCS opinion concludes on skin irritation: *"A skin irritation study (not according to a guideline) was performed in which male Guinea pigs were exposed to 25 % and 40 % of 20 nm ZnO dispersed in ethanol. No effects were observed at any time during the administration and observation periods in the 25 % test substance group. Slight erythema was observed in one of three animals in the 40 % test substance group on day 3 of administration"* (SCCS, 2012).
As further discussed in Paragraph 6.6.2 regarding absorption/distribution, the SCCS opinion concludes the following on dermal absorption: *"From the available information, there is no indication for penetration of ZnO nanoparticles through the skin. In one study it was shown that Zn from ZnO nanoparticles in a tested sunscreen formulation made a minor contribution to the blood Zn pool of human volunteers. This shows that some Zn was absorbed from the sunscreen,*

[14] *Risk assessment report*
[15] SCCS, 2012 concludes an oral NOAEL of "50 mg/bw day". Correctly is should have been 50 mg/day (corresponding to 0.833 mg/kg bw/day).

although it was not known whether this was absorbed in nanoparticulate form or as solubilized Zn ions. Considering the dissolution of ZnO, it is most likely that the zinc was absorbed in ionic form. The overall weight of evidence therefore suggests that a very small proportion of Zn ions released from the ZnO nanoparticles may be available for systemic exposure when applied dermally" (SCCS, 2012).

SCCS noted that: *"The sensitization assay was not performed according to a recognized OECD guideline. Furthermore, a concurrent positive control with a well known weak sensitizer was not included in this assay, so there is no certainty as to whether the test system used was able to identify weak sensitizers. However, for eight contact sensitizers, similar responses were found in the GPMT (Guinea pig maximisation test) performed according to the OECD Guideline 406, and in a shortened test. The validity of this (or any) test for demonstrating sensitization potency of nanomaterials has not yet been demonstrated. The inclusion of a positive particle control might overcome this problem. However, no positive particle control has been identified thus far" (SCCS, 2012).*

Eyes
The SCCS refers a single study on eye irritation from a ZnO nanomaterial: *"The ZnO nanomaterial, both as a neat dispersion and 25 % solution as used in sunscreen, was slightly and transiently irritating to the eyes when tested in rabbits.*

Developmental and reproductive system
SCCS concluded that *"given the data available, it is concluded that zinc oxide is of no concern for reproductive toxicity"*. In a recent OECD Guideline 421-like study, nano-ZnO <100 nm at a dose of 500 mg/kg bw/day was given to female rats from 2 weeks before mating and until postnatal day 4 (Jo et al., 2013). Males were also exposed 2 weeks before mating. There was no effect on male and female fertility or the number of implants but a reduced number of newborn and live pubs induced by increased fetal resorption. There was decreased pup bodyweight. Zn was distributed to the pups.

Genotoxcity and cancer
Regarding genotoxcicity the SCCS concludes: *"Based on the available database and additional in-depth evaluation of the studies, and in view of the uncertainties over whether or not nanoparticles reached the target cells/DNA in the tests, there is no conclusive evidence to conclude whether or not micro-or nano-sized ZnO particles pose a mutagenic/genotoxic, photo-toxic or photo-mutagenic/genotoxic risk to humans. However, where ZnO nanoparticles are applied on the skin in a sunscreen formulation, there is sufficient evidence to conclude that due to the very low if any systemic exposure, the risk to the consumer is negligible. The evidence from in vitro and in vivo studies presented in this dossier (reference to SCCS), and other studies on different metal/metal oxide nanoparticles (e.g. titanium dioxide – Nanoderm Project1) shows that penetration of nano or larger particles is generally limited to the upper few layers of the stratum corneum and there is no significant dermal penetration of the particles to systemic circulation. Whilst this leaves the possibility for nanoparticle mediated local effects, it diminishes the possibility for any harmful effects at the systemic level"* (SCCS, 2012).

Regarding carcinogenicity the SCCS concludes: "*Specific data on carcinogenicity studies of ZnO nanomaterials are not available. In view of the occurring dissolution of the ZnO nanoparticles it can be assumed that the carcinogenic risk is similar to the conventionally manufactured ZnO preparations. According to the EU Risk assessment report (Reference 44, submission III), there is no clear experimental or epidemiological evidence for a direct carcinogenic action of zinc or its compounds"* (SCCS, 2012).

6.6.4 Adverse effect of nano-ZnO when part of a matrix

Two *in vivo* studies in humans have been conducted to study the uptake of nano-ZnO when nano-ZnO containing sunscreen was applied on human volunteers using real-life conditions of use. In the first study, sunscreen containing 20 % (wt/wt) of nano-ZnO (19 nm) or larger sized ZnO (120 nm) was applied (Gulson et al., 2010). The stable ^{68}Zn-isotope was spiked to distinguish uptake from endogenous Zn. Sunscreen application was performed over 5 days. Blood and urine samples were analysed by ICP-MS. The authors concluded that small amount of Zn, less than 0.001 % of the applied dose, passed through the skin as detected as elemental Zn. Whether it was as Zn-ions or particulate Zn could not be specified by the applied analysis. There was a gender difference between the absorption of Zn as blood and urine levels of ^{68}Zn were higher in women treated with nano-sunscreen compared to men.

In the second study, applying the same experimental procedures, 30 nm nano-ZnO particles were used (Gulson et al., 2012). This study also showed very low levels of dermal absorption (<0.01 %) of the applied dose. Whether the 10-fold higher dermal absorption of the 30 nm particles can be explained by different particle size, experimental or individual variation is not known. Taken together, the reported levels of absorption were very low both when considered as absorption and when compared to endogenous Zn levels. No adverse effects were reported in any of the two studies.

The SCCS assumes that the skin penetration *"if any, is caused by Zn-ions released from the ZnO nanoparticles"* and that there is no evidence for dermal absorption of nano-ZnO particles (SCCS, 2012).

It is stressed by the SCCS that use of nano-ZnO containing cosmetics that may result in pulmonary exposure is of concern:*"In view of the lung inflammation induced by ZnO particles after inhalation, the use of ZnO in cosmetic products that may result in exposure of the consumer's lungs by inhalation is of concern"* (SCCS, 2014b).

No toxicological studies on oral or pulmonary exposure to nano-ZnO when part of any product/matrix have been identified.

6.6.5 Physico-chemical properties of importance for toxicity

Nano-ZnO dissolves in solution including artificial gastrointestinal fluid and lung fluids and releases Zn^{2+} that could be responsible for the potential biological and toxicological effects.

The SCCS evaluates the physico-chemical characteristics that are important for the hazard of nano-ZnO (SCCS, 2014a).

"• When compared in terms of solubility, micro-sized ZnO has been shown to be less soluble (in water), and equally soluble (in tissue culture medium) compared to nano-ZnO.

• Experimental evidence shows that both nano and non-nano particulate forms of ZnO are not absorbed through the skin. Also on a theoretical ground, larger sized (nonnano) particles of ZnO are less likely to be absorbed through the skin than the nanoforms.

• As far as evaluated in the toxicity testing, micro-sized ZnO has been shown to induce either similar toxic effects (in terms of general toxicity, lung toxicity after inhalation, uptake from gastrointestinal-tract, serum liver enzyme presence) or lower toxic effects (in terms of genotoxicity, liver histopathology) when compared to nano-sized ZnO. No toxic effects were observed at similar doses in a human volunteer inhalation study for both nano-sized ZnO (40 nm) and fine ZnO (291 nm).

Thus the calculation of MoS[16] for nano-sized ZnO for use as a cosmetic ingredient in sunscreen formulations can also be used for the non-nano form of ZnO.

The SCCS would like to point out that a re-evaluation may be needed in the case of use of other specific coatings or specific absorption enhancers in the formulation, which can promote the dermal penetration of ZnO particles (nano or non-nano), or indications of dermal absorption after long-term use of nano ZnO containing formulations."

The SCCS concludes that the SCCS opinion is valid for nano-ZnO nanomaterials with the following characteristics (SCCS, 2014a):

1. ZnO nanoparticles of purity ≥96%, with wurtzite crystalline structure and physical appearance as clusters that are rod-like, star-like and/or isometric shapes, with impurities consisting only of carbon dioxide and water, whilst any other impurities are less than 1% in total.

2. ZnO nanoparticles with a median diameter (D50: 50% of the number below this diameter) of the particle number size distribution above 30 nm, and the D1 (1% below this size) above 20nm.

3. ZnO nanoparticles that are either uncoated or coated with triethoxycaprylylsilane, dimethicone, dimethoxydiphenylsilanetriethoxycaprylylsilane cross-polymer, or octyl triethoxy silane. Other cosmetic ingredients can be used as coatings as long as they are demonstrated to the SCCS to be safe and do not affect the particle properties related to behaviour and/or effects, compared to the nanomaterials covered in the current opinion.

4. ZnO nanoparticles that have a comparable solubility to that reported in the dossier, i.e. below 50 mg/L (approximately the maximum solubility of the ZnO nanomaterials for which data are provided in the dossier).

Smaller nano-ZnO have been shown to dissolve/dissociate faster and release Zn^{2+} in biological fluids faster than larger ZnO particles. This may be a consequence of the larger specific surface area of nanomaterials (Danish EPA, 2011).

6.6.6 Summary and conclusions

Nano-ZnO dissolves in biological fluids including artificial gastrointestinal fluid and lung fluid to form Zn^{2+} that seems to be distributed systematically to organs as Zn^{2+}. This hazard assessment of nano-ZnO in a matrix is primarily based on the recent SCCS opinion on ZnO (SCCS, 2012) and two addendums related to this opinion (SCCS, 2014a; SCCS, 2014b).The aims of these SCCS opinions on ZnO was specifically to decide if ZnO in nanoform is safe for use as an UV-filter with a concentration up to 25 % in cosmetic products and is therefore considered highly relevant for the present purpose, namely to serve as input for for the hazard part of the risk assessment of nano-ZnO in a pump spray sunscreen.

The SCCS has the the following overall conclusion:
"In summary, it is concluded on the basis of available evidence that the use of ZnO nanoparticles with the characteristics as indicated below, at a concentration up to 25% as a UV-filter in sunscreens, can be considered not to pose a risk of adverse effects in humans after dermal application. This does not apply to other applications that might lead to inhalation exposure to ZnO nanoparticles (such as sprayable products). Also, this assessment only applies to ZnO nanoparticles that are included in this dossier, or are similar materials that have the following characteristics" (SCCS, 2012) (for more on the characteristics, please refer to Papragraph 6.6.5).

[16] Margin of Safety

The SCCS concludes based on the following background information:

•" There is no evidence for the absorption of ZnO nanoparticles through skin and via the oral route. Even if there was any dermal and/or oral absorption of ZnO nanoparticles, continuous dissolution of zinc ions would lead to complete solubilization of the particles in the biological environment. In the MoS calculation, the calculation of the exposure to ZnO nanoparticles assuming Zn^{2+} uptake results in acceptable MoSs for both the oral and dermal routes.

• Nano ZnO-containing cosmetic formulations are likely to contain a small proportion of solubilized zinc, a further small proportion of which may be absorbed through skin and other routes. The rate and amount of the absorbed zinc is, however, likely to be insignificantly small compared to the large zinc pool already present in the body.

• Although the current evidence in relation to potenial genotoxicity of ZnO is not conclusive, the use of nano ZnO in cosmetic products should not pose a risk to the consumer in the absence of a significant systemic exposure.

• Based on the parameters described in the dossier, the different particle sizes, surface modifications, and crystalline structures and morphologies investigated do not significantly alter the uptake, bioavailability and overall safety profile.

• The different typical formulations as described in this submission also do not change the overall safety profile of the tested ZnO nanoparticles" (SCCS, 2012).

The key studies on toxicity following exposure by different exposure routes are summarised below:

Inhalation exposure has shown effects in the lungs and a NOAEL of 0.3 mg/m^3 has been established (SCCS, 2014a). In 2014, the SCCS concluded that *"in view of the lung inflammation induced by ZnO particles after inhalation, the use of ZnO in cosmetic products that may result in exposure of the consumer's lungs by inhalation is of concern"* (SCCS, 2014b). In the 90-days inhalation key study described in Paragraph 6.6.3 regarding the respiratory system, SCCS concluded a NOAEL of 0.3 mg/m^3 based on activation of lung macrophages and lung draining lymph nodes (SCCS, 2014a).

Toxic effects have been shown in liver of mice and pancreas of rats orally exposed to nano-ZnO. Generally the oral toxicity is considered to be low in laboratory animals. The NOAEL of 50 mg/day has been set in a 90-days study in humans (SCCS, 2012). The relevance and applicability of this NOAEL can be questioned as the real no effect level may be considerable higher. Without specifying the reference, the SCCS in 2012 refers *"An NOAEL of 50 mg/day"* corresponding to 0.83 mg Zn^{2+}/day was derived from a 10-week oral study with human volunteers. The applicability of this NOAEL can be questioned as the real no effect level may be considerable higher (SCCS, 2012).

The dermal absorption, if any, is very low and when used in sunscreens up to a concentration of 25 % that are applied dermally as creams, the SCCS concluded that this does not pose any risk (SCCS, 2012). We agree with the final concluding remarks of the SCCS opinion stressing that inhalation exposure to ZnO nanoparticles by using e.g. sprayable products is of concern.

6.6.7 Input to risk assessment

Table 8 summarises key hazard data to be used for the risk assessment of ZnO.

TABLE 8
INPUT PARAMETERS FOR ZnO

Substance	Exposure routes	Critical effect	N(L)OAEL / N(L)OAEC	DNEL / Assessment factors (AF)	Source
Pristine	Inhalation	Effects in mice lungs Activation in rats of lung macrophages and lung draining lymph nodes	 NOAEL: 0.3 mg/m³		(Adamcakova-Dodd et al., 2014) (SCCS, 2014a)
	Oral	Rat: Pancreatitis Humans: Adverse effects not reported	NOAEL: 268.4 mg/kg bw/day NOAEL: 50 mg/day or 0.833 mg/kg bw/day[2] LOAEL: not given[2]		(Seok et al., 2013) (SCCS, 2012)
	Dermal	Not specified[3]			(SCCS, 2012)
	Eye	No data			
Matrix bound	Inhalation	Not specified Nano-ZnO in sprays cannot be considered safe			(SCCS, 2012)
	Oral	No indication of absorption as nano-ZnO			(SCCS, 2012)
	Dermal	Safe up to 25 % in sunscreen creams[3]			(SCCS, 2012)
	Eye	No data			
Occupational exposure limit	DK: OEL: 4 mg ZnO/m³ for inhalable particles and fumes				(Danish Working Environment Authority, 2007)
	REACH registration : DNEL: 2.5 mg ZnO/m³ for inhalable particles for the general population as set by the REACH registrant				(European Chemicals Agency (ECHA), 2014)
Comments / uncertainties	1 Based on a repeated dose, 90-days inhalation study in rats. Particle size was not specified 2 The evaluation was based on human Zn supplement studies where no adverse effect traditionally is wanted. NOAEL was estimated for a 60-kg person. No LOAEL was reported or propably even could be set. The real no effect level may be considerably higher. The relevance of this NOAEL is questioned 3 A dermal absorption rate of 0.03 % for nano-Zn is used in the safety evaluation				
Conclusion	See above for the pristine nanomaterial and when bound in matrix				

6.7 Nano zirconium dioxide (nano-ZrO2)

6.7.1 Introduction

Zirconium (Zr) does not exist as a free metal in nature. Zirconium dioxide (ZrO_2), which is also referred to as zirconium oxide or zirconia, is an inorganic metal oxide that is mainly used in ceramic materials. ZrO_2 succeeds zirconium as the compound of the element Zr that most frequently occurs in nature. Zr is a heavy metal of which 0.016 % is found in the earth crust and which, thus, occurs more frequently than the elements Cl and Cu. Its great hardness, low reactivity and high melting point have made it the oldest mineral that can be found on the earth (Data and knowledge on nanomaterials - DaNa 2.0, 2011).

Zr does not occur massively but is bound in minerals, mainly in zirconate (ZrO_2-SiO_2, $ZrSiO_4$) and baddelyite (ZrO_2), and most of the material used is chemically extracted from these two minerals. In nature, ZrO2 occurs in the mineral form as baddelyite, a modification in monoclinic crystal lattices (which is often found as weathered grit in gravel). ZrO_2 resulting from baddelyite, which is also known as zirconia, is a course oxide that presents a monoclinic crystal structure at room temperature. The monoclinic structure of pure ziconia is stable up to 1,170°C. Between this temperature and 2,370°C tetragonal zirconia is formed, while cubic zirconia is formed at temperatures above 2,370°C. After processing, and depending on the cooling process, the tetragonal phase becomes monoclinic at about 970°C. Due to polymorphism, pure zirconia cannot be used at elevated temperatures due to a large volume change (3-5 %) which occurs during cooling to the monoclinic phase. This change is sufficient to exceed the elastic and fracture limits, resulting in cracks and flaws in ceramics (Volpato et al., 2011).

Different oxides, such as yttrium oxide (Y_2O_3), calcium oxide (CaO) or magnesium oxide (MgO), can be added to ZrO_2 to stabilise it, allowing the tetragonal form to exist at room temperature after sintering. The addition of stabilisers is used in the production of e.g. dental ceramics with properties such as high flexural strength and fracture toughness, high hardness, excellent chemical and thermal resistance and good conductivity ions (Volpato et al., 2011).

ZrO_2 often contains high amounts of natural radioactive impurities of long half-life, such as thorium (Th) and uranium (U).

ZrO_2 is widely used in the ceramics industry. ZrO_2, at both nano- and micro-scale, is also a widely used metal oxide used for ceramic implants and in densitry. Implants based on ZrO_2 ceramics (yttrium-stabilised tetragonal polycrystals) were introduced into dental implantology as an alternative to titanium implants because of the tooth-like colour, the mechanical strength and the biocompatibility (Depprich et al., 2008). Biocompatibility does, however, depend on the purification of the radioactive contents (Volpato et al., 2011). Yttrium-stabilised tetragonal polycrystals (Y-TZP) are generally used when there is a need for a material which is exceptionally strong, wear-resistant, chemically inert and which has a high fracture toughness. Combined with a more elastic behavior with a bending strength and high hardness this makes Y-TZP ceramics useful for dental implants as well as aerospace applications. Y-TZP components can be produced with extremely fine surface finishes. This comes primarily from the sub-micron crystal size that is always associated with this family of stabilised zirconias (Superior Technical Ceramics, 2013).

The preparation of Y-TZP zirconia ceramic has been described in a dissertation including a literature review of ZrO_2in densitry and fracture resistance. The preparation begins with the incorporation of Y_2O_3 (yttria). Yttria as stabilising component is critical for the material quality in the final product. The incorporation is done by the so-called sol-gel process in which the mixing at the atomic level occurs. Starting from the zirconium sand, zirconium silicate ($ZrSiO_4$) is obtained by chemical release of $ZrOCl_2$, which together with YCl3 is used as starting material for the sol-gel process. The sol-gel process is the same as hydrolysis. After the dispersion of Y_2O_3, a ceramic nano-

powder with an average primary grain diameter of less than 100 nanometers is produced by multi-stage washing, drying and calcination processes in conjunction with a final milling step (Katz, 2007).

Other types of zirconia used in densitry include 1) ceramics based on zirconia combined with a matrix of alumina (Al_2O_3), forming a structure known as 'zirconia toughened alumina' (alumina reinforced with zirconia grains), and 2) 'magnesia partially stabilised zirconia' (Mg-PSZ) where the microstructure of Mg-PSZ consists of an array of cubic zirconia partially stabilised by 8 to 10 mol % of magnesium oxide (Volpato et al., 2011).

Only limited information referring to the hazards of the ZrO_2 and in particular the nano-form has been identified and no specific reviews of the compound were identified.

ZrO_2 is registered under REACH with CAS No. 1314-23-4 in the tonnage band 10,000 – 100,000 tonnes per annum, as a reaction mass of CeO_2 and ZrO_2 (1,000-10,000 tonnes per annum) and as a mass of SiO_2 and ZrO_2 (1-10 tonnes per annum). Information on manufacture and use of ZrO_2 include manufacture of ceramics. In the following, reference will be made to the information on the macro chemical where no information is available for the nano-form of ZrO_2, which, according to the four key particle size distribution studies, has a mean diameter (D50 percentile[17]) of 3.347 – 107.387 μm (European Chemicals Agency (ECHA), 2014).

The purpose of the chapter on hazard assessment of nano-sized ZrO_2 is to serve as background documentation for the risk assessment of ZrO_2 used in dental fillings (implants). This particular use involves exposures of the consumer related to the application process (with spatula), to sanding and polishing, and to contact with the material migrating from the dental filling to the oral mucosa and saliva. Focus will on be the hazards associated with exposure by the inhalation route and exposure by the gastrointestinal route. In addition, exposure to the eye will be covered. Inhalation may in reality be less relevant, as sanding and polishing usually takes place with suction and application of water. It is also mentioned in relation to Y-TZP that sandblasting of this material should be eliminated to maintain the surface integrity and prevent transformation from the tetragonal to the monoclinic form (Volpato et al., 2011).

6.7.2 Biokinetics

Absorption

No information on absorption and distribution of nano-ZrO_2 has been identified.

Oral: A qualitative assessment of the toxicokinetic behaviour is presented in the REACH registration dossier based on physico-chemical properties as well as on toxicological data available for both macro-ZrO_2 and other Zr compounds. According to this assessment, and due to the extremely low solubility of ZrO_2, significant oral absorption via passive absorption is not expected. It is, however, noted that it may be possible for small particles to be taken up by pinocytosis[18]. Based on this assumption, and in the absence of reliable experimental data, a worst-case oral absorption factor of 10 % is proposed in the dossier (European Chemicals Agency (ECHA), 2014).

Inhalation: In the registration dossier it is noted that the particle size distribution of macro-ZrO_2 is dependent on the production process of the material as well as on the anticipated use, and that particle size distributions vary widely with D50 values roughly between 3 and 100 μm. It is therefore concluded that at least some ZrO_2 materials contain particles that can reach the alveolar region of the respiratory tract (50 % of the particles with an aerodynamic diameter of 4 μm are

17 50 % percentile

18 The uptake of fluid and dissolved substances by a cell by invagination of the cell membrane followed by formation of vesicles within the cell.

assumed to belong to the respirable fraction, i.e., the fraction that reaches the alveoli). Because of the extremely low water solubility, particles depositing in the alveolar region would mainly be engulfed by alveolar macrophages. The macrophages will then either translocate particles to the ciliated airways or carry particles into the pulmonary interstitium and lymphoid tissues. Particles which settle in the tracheo-bronchial region would mainly be cleared from the lungs by the mucociliary mechanism and swallowed. A small amount may, however, be taken up by phagocytosis and transported to the blood via the lymphatic system. Based on this, and in the absence of reliable experimental data, a worst-case inhalation absorption factor of 10 % is proposed in the dossier (European Chemicals Agency (ECHA), 2014).

Dermal: Because of the very low water solubility there is little potential for dermal absorption and also no indication of adverse effects. Based on this, and in the absence of reliable experimental data, a worst case dermal absorption factor of 10 % is proposed. It is noted in the registration dossier, that the actual absorption factors for zirconium dioxide will be much lower and there is reference to data on zirconium dichloride oxide in mouse and rat showing oral absorption to be at levels of 0.01 to 0.05 % of the administered dose (European Chemicals Agency (ECHA), 2014).

It is mentioned that this 'water soluble' zirconium compound (zirconium dichloride) could be regarded as a reference for ZrO_2 as it will instantaneously be converted to ZrO_2 in aqueous solutions at physiologically relevant pH levels. However, in the absence of reliable experimental data, the registrant suggested that the worst-case absorption factor of 10 % would not be lowered (European Chemicals Agency (ECHA), 2014).

Distribution
No information on distribution of nano-ZrO_2 has been identified.

No relevant data are presented for the macro chemical or other Zr-compounds in the registration dossier.

Metabolism
No information on metabolism of nano-ZrO_2 has been identified.

No relevant data are presented for the macro chemical or other Zr-compounds in the registration dossier.

Excretion
No information on excretion of nano-ZrO_2 has been identified.

In the REACH registration dossier it is concluded that, since no effects were observed in rats after oral exposure to (single) high doses of ZrO_2 and absorption via the gastrointestinal tract is expected to be extremely limited, elimination can be expected to occur mainly via the faeces (European Chemicals Agency (ECHA), 2014).

6.7.3 Adverse effects of nano- ZrO_2

ZrO_2 is generally described as a substance of low toxicity.

In a comparative study, the cytotoxicity of 24 manufactured nanoparticles of similar equivalent spherical diameter and various elemental compositions was investigated in human alveolar epithelial and macrophage cell lines; the A549 cell line, representative of alveolar type II cells and the THP-1 cell line (Phorbol Myristate Acetate-differentiated monocytes to macrophages). These two cell types were chosen because they are potential targets of nanomaterials *in vivo* after inhalation. In the test titania, alumina, ceria, Ag, Ni and zirconia-based nanomaterials showed low

to moderate toxicity. No correlation between cytotoxicity and equivalent spherical diameter or specific surface area was found (Lanone et al., 2009).

In vitro toxicity, antioxidant potential and bioactivity of nano- and micro-ZrO_2 and -TiO_2 particles was evaluated using NIH 3T3 rodent embryonic fibroblasts (3T3), di(phenyl)-(2,4,6-trinitrophenyl) iminoazanium and simulated body fluid, respectively. The cell line viability % indicated that both ZrO_2 and TiO_2 nanoparticles were less toxic than the micro-particles up to 200 mg/ml. Higher antioxidant activity was seen in the nano form compared to the micron counterparts. The simulated body fluid study revealed that nanoparticles possessed higher bioactivity as they contained higher percentage of calcium deposition compared with the microparticles. Overall, it was concluded that cytotoxicity alone was not a sufficient evaluation method for toxicity (Karunakaran et al., 2013).

Brunner et al. studied the effect of solubility on cytotoxicity of oxide nanoparticles including ZrO_2 in human mesothelioma and 3T3 cell lines. The study was developed as a pre-screening method to provide a relative measure for cytotoxicity of nanomaterials. Cytotoxicity of insoluble nanoparticles, including $ZrO,_2$ was relatively low up to 30 ppm. A rough comparison of the investigated nanomaterials showed the following order of cytotoxicity in human mesothelioma cells after 3 days of treatment: Fe_2O_3 ≈ asbestos > ZnO > CeO_2 ≈ ZrO_2 ≈ TiO_2 ≈ $Ca_3(PO_4)_2$. This remained consistent for 6 days of treatment. For 3T3 cells, reduced proliferation, expressed as lower DNA content, followed the order: ZnO > asbestos ≈ ZrO_2 > $Ca_3(PO_4)_2$ ≈ Fe_2O_3 ≈ CeO_2 ≈ TiO_2 and showed an unexpected strong response of zirconia after 3 days of exposure. The overall cell culture activity (MTT assay) was drastically reduced for ZnO and asbestos while ZrO_2, $Ca_3(PO_4)_2$, Fe_2O_3 and CeO_2 were not much affected. After 6 days both cytotoxicity parameters in 3T3 cells recovered for zirconia while cultures exposed to Zn and asbestos were irreversibly affected (Brunner et al., 2006).

In the REACH registration dossier, a reliable acute oral toxicity study according to OECD guideline 423 and according to Good Laboratory Practice is presented. Six female rats were administered 2,000 mg/kg bw CC10 zirconium oxide by gavage. No clinical signs, effects on body weight or other treatment-related effects were observed.

Respiratory system
Acute pulmonary effects of ZrO_2 with primary particle size 40 nm (particle size range 10-130 nm) were investigated in male Wistar rats. Dust aerosols were generated from ZrO_2 with a target concentration of 0.5, 2.5 and 10 mg/m^3. Groups of 14 animals were head-nose exposed to the dust aerosols for 6 hours a day for five consecutive days. The respiratory tract was evaluated by light microscopy in groups of six animals, either immediately after the last exposure or 3 weeks thereafter (study days 5 and 26), as well as the content of the test material in the lung and in the mediastinal lymph nodes. BALF and histopathological sections of the entire respiratory tract were examined. BAL was performed in satellite animals (five animals per group and time point) 3 days after the exposure and 3 weeks thereafter (study days 8 and 29) (Landsiedel et al., 2010a). Landsiedel et al. elaborates on the results of what is believed to be the same study as just mentioned (Landsiedel et al., 2009). Several biochemical and cytological parameters as well as a large panel of cytokines/chemokines were measured in the in BALF. Pulmonary deposition and clearance and test material translocation into extra-pulmonary organs were also assessed. It was concluded that nano-ZrO_2 did not induce any treatment-related effects in cytological, protein, enzyme, cytokine or chemokine levels in the BALF or in cytokine levels in the lung tissue, even though a comprehensive panel of 68 cell mediators was assessed both in the BALF and lung tissue. Likewise, the hematological parameters and acute phase protein levels in the blood remained unchanged. There were no histopathological changes of the respiratory tract, and cell proliferation rates and apoptotic reactions in lung cells were comparable to those from the control groups. Thus, ZrO_2 did not show any adverse effects at the highest tested aerosol concentration of 10 mg/m^3 and a NOAEC of 10 mg/m^3 was established based on the study results. ZrO_2 surface-coated with either 3,6,9-

trioxadecanoic acid (TODA) or acrylate was also tested without observation of any adverse effects (Landsiedel et al., 2014).

Klein et al. refers to two reports investigating the effects of zirconium compounds on the lung health of workers. In one report from 1981, 32 manual finishers of zirconium metal were exposed to 5.75–14.7 mg/m^3 of dust (25 % zirconium). No exposure-related symptoms were observed. Another report on ZrO_2-exposed workers showed that even under long-term exposure conditions of up to 20 years and peak concentrations up to 30 mg/m^3, zirconium exposure elicited neither abnormal chest radiographs, for example granuloma formation, nor did it impair lung function parameters such as Forced Expiratory Volume1/Forced Vital Capacity (FEV_1/FVC) (Klein et al., 2012).

Supplier information regarding health and safety from two different suppliers of nano-ZrO_2 (American Elements® and Sigma Aldrich®) about nano-ZrO_2 powder indicates that the substance should be classified as a respiratory irritant. No studies have been identified to support this conclusion.

Cardiovascular system
No studies resulting in systemic toxicity of nano- or macro-ZrO_2 have been identified.

Gastrointestinal tract
No studies resulting in systemic toxicity of nano- or macro-ZrO_2 have been identified.

CNS
No studies resulting in CNS toxicity of nano- or macro-ZrO_2 have been identified.

Other organs
No studies resulting in systemic toxicity of nano- or macro-ZrO_2 have been identified.

Skin
No studies investigating the effects of skin exposure have been identified.

Zirconium is one of four granuloma-forming chemicals (beryllium, zirconium, silica and talc) (James, 2000). Earlier uses of zirconia have included cosmetic (dermatological) applications such as deodorants. However, due to reports of zirconia as the cause of deodorant axillary granulomas in sensitised individuals this use was stopped (James, 2000; Ganrot, 1986).

Supplier information regarding health and safety from the two different suppliers of nano-ZrO_2 powder indicates that the substance should be classified as a skin irritant. However, no studies have been identified to support this conclusion.

Study results for the macro-substance reported in the REACH registration dossier show that macro-ZrO_2 was not considered irritating based on a dermal irritation/corrosion study according to OECD guideline 404 and Good Laboratory Practice (European Chemicals Agency (ECHA), 2014).

Eyes
No studies resulting in eye irritation of nano- or macro-ZrO_2 have been identified.

Supplier information regarding health and safety from the two different suppliers of nano-ZrO_2 powder indicates that the substance should be classified as an eye irritant. However, no studies have been identified to support this conclusion.

Developmental and reproductive system
No studies resulting in developmental and reproductive effects of nano- or macro-ZrO_2 have been identified.

Genotoxicity and cancer
No studies resulting in genotoxic or carcinogenic effects of nano- or macro-ZrO_2 have been identified.

Immunotoxicity
As mentioned in relation to effects on the skin, zirconium compounds have been shown to be involved in production of axillary granulomas when used in deodorants, as it was the case for both zirconium lactate and zirconium aluminium glysate. This effect is sometimes considered an allergic reaction (Health Council of the Netherlands, 2002). The Health Council of the Netherlands further refers to an abstract describing immunostimulating effects reported for zirconium oxide after single or repeated injections (every other day for 2, 4 or 6 months) into the thorax cavity or the peritoneum of mice (Health Council of the Netherlands, 2002).

No further information on ZrO_2 (nano or macro) immunotoxicity has been identified.

6.7.4 Adverse effect of ZrO_2 when part of a matrix

Composites
Adverse effects related to ZrO_2 nanoparticles derived from ceramic materials have also been investigated in a few studies.

In a study by Lucarelli et al., the interaction of nanoparticles derived from ceramic objects and handicrafts with the human body was investigated with emphasis on the defense mechanisms. ZrO_2 nanoparticles were examined for the ability to modulate the innate defense functions of human macrophages (U937 myelomonocytic cell line) and epithelial cells (liver-derived cell line HepG2), which are two cell types involved in the response to mineral dust. ZrO_2 did not induce necrosis of the cultured cells. The proliferation rate and cell survival was also unaffected up to 400 µg nanoparticles/10^6 cells for up to 96 hours. The production of cytokines involved in inflammatory response was also investigated and ZrO_2 nanoparticles selectively increased IL-1β levels without effect on tumor necrosis factor-α levels, whereas TiO_2 nanoparticles did not show any significant effect and SiO_2 dramatically induced production and release of both cytokines (Lucarelli et al., 2004).
Covacci et al. studied the *in vitro* mutagenic and oncogenic effects of a new Y-TZP. This ceramic was sintered from high purity powders. For comparison, ceramics made from unpurified zirconia powder were also tested. Fibroblasts irradiated by a linear accelerator were used as positive control. The results obtained showed that Y-TZP ceramic did not elicit either mutagenic or transforming effect on C3H/10T(1/2) (10T(1/2)) cells and it was concluded that ceramic from high purity powders can be considered suitable for biomedical applications regarding the effects of its content of radioactive impurities (Covacci et al., 1999).

Olmedo et al. performed a long-term evaluation of the distribution, destination and potential risk of TiO_2 and ZrO_2 micro-particles that might result from the corrosion process of biomaterials used in implants. Wistar rats were injected intraperitoneal with an equal dose of either TiO_2 or ZrO_2 suspension. The following end-points were evaluated at 3, 6 and 18 months: (a) the presence of particles in blood cells and liver and lung tissue, (b) Ti and Zr deposit quantitation, (c) oxidant–antioxidant balance in tissues and (d) $O2^-$ generation in alveolar macrophages. Results from the study were presented as follows: "*Ti and Zr particles were detected in blood mononuclear cells and in organ parenchyma. At equal doses and times post-administration, Ti content in organs was consistently higher than Zr content. Ti elicited a significant increase in $O2^-$ generation in the lung compared to Zr. The consumption of antioxidant enzymes was greater in the Ti than in the Zr*

group. The present study shows that the biokinetics of TiO2 and ZrO2 depends on particle size, shape, and/or crystal structure" (Olmedo et al., 2011).

Depprich et al. introduced 24 screw-type zirconia implants (yttrium-stabilised tetragonal polycrystals) with roughened surfaces produced by acid etching and 24 implants of commercially pure titanium with acid etched surfaces, all of similar shape and surface structure, into the tibia of 12 Göttinger minipigs. Block biopsies were harvested 1 week, 4 weeks or 12 weeks (four animals each) after surgery. Scanning electron microscopy analysis was performed at the bone implant interface. Results showed remarkable bone attachment already after 1 week which increased further to intimate bone contact after 4 weeks, observed on both zirconia and titanium implant surfaces. After 12 weeks, osseointegration without interposition of an interfacial layer was detected. At the ultrastructural level, there was no obvious difference between the osseointegration of zirconia implants with modified ablative surfaces and titanium implants with a similar surface topography (Depprich et al., 2008).

6.7.5 Physico-chemical properties of importance for toxicity

Nano-ZrO_2 is an insoluble white powder. The solubility has been found reported in the range of 4-190 ppm. Water solubility of the macro-ZrO_2, reported in the REACH registration dossier, is < 55 µg/L.

Form

Nano- ZrO_2 is available in a monoclinic (<1170°C), tetragonal (1170-2370°C) or cubic (>2370°C) form.

Shape

Particle morphology is generally described as irregular/rounded and spherical. Particle size range is 10-130 nm (Landsiedel et al., 2010b).

Metal content

Metal content in ZrO_2 is approximately 74 %. Impurities may be present, e.g. Al.

Tendency to aggregate/agglomerate

No specific information has been identified. In relation to a particular toxicity study, agglomerate size of uncoated ZrO_2 as test material was reported at 1,000-5,000 nm (scanning electron microscopy) (Landsiedel et al., 2014).

Surface chemistry

In the same study as mentioned above, surface chemistry was reported as follows (Landsiedel et al., 2014):

Zr: 24 %; O: 53 %; C: 19 % (C-C, C-O, O-C=O); N: 3 %, Al: 1 %

6.7.6 Summary

ZrO_2 is generally described as a substance of low toxicity. There are very few *in vivo* studies available investigating the toxicity of ZrO_2.

ZrO_2 has extremely low solubility in water and absorption is expected to be low from all exposure routes. When deposited in the alveolar region, particles are expected to be engulfed by alveolar macrophages and only a small amount to end up in the blood via the lymphatic system.

No specific adverse effects have been identified for the substance in the more recent literature. In older literature immunostimulating effects are reported following injections in the thorax cavity and

the peritoneum of mice. In addition, an ability to cause axilliary granulomas when applied in deoderants is described.

The OEL (8-hour average) for zirconium compounds (calculated as Zr) is 5 mg/m³ in Denmark (Danish Working Environment Authority, 2007). OSHA in the US has applied the same value.

6.7.7 Input to risk assessment

Table 9 summarises key hazard data to be used for the risk assessment of ZrO_2.

TABLE 9
SUMMARY TABLE FOR ZrO_2

Substance	Exposure routes	Critical effect	N(L)OAEL / N(L)OAEC	DNEL / Assessment factors (AF)	Source
Pristine Dental filling	Inhalation	NA	10 mg/m³		
	Oral	No data			
	Dermal	No data			
	Eye	No data			
Matrix bound	Inhalation	No data			
	Oral	No data			
	Dermal	No data			
	Eye	No data			
Occupational exposure limit	1. DK: 5 mg/m³ (as Zr) corresponding to 6.75 mg/m³ ZrO_2 (bulk form)				
	2. OSHA (US): 5 mg/m³ (as Zr)				
Comments / uncertainties	1 No effects observed at highest dose				
Conclusion	Low toxicity, all exposure routes Some evidence of immunotoxic potential Cytotoxicity of the nano-form seems to be comparable with nano-TiO_2				

7. Bridging between hazard and risk assessment

The aim of this chapter is to serve as bridging between the hazard as presented in this report and the risk assessment to be carried out as the next step in the main project. Focus will be on the 20 exposure scenarios chosen to be addressed specifically in this project as well as general considerations for assessing risks associated with consumer product containing nanomaterials. The chapter will also outline methodological considerations and uncertainties associated with bridging between hazards and risks.

Examples of the occurrence and exposure of nanomaterials in consumer products is published in a separate report from the main project *("Exposure assessment of nanomaterials in consumer products"*). In brief, consumer products may contain nanomaterials in liquid suspension (e.g. cosmetics and paints) or products where nanomaterials are incorporated in a solid matrix (e.g. cured paint and sports equipment). Therefore, in most cases consumers are only exposed to free nanomaterials to a limited extent. However, free nanomaterials may be released from solid matrices containing nanomaterials during the use phase, such as for example when sanding a nanomaterial-containing product. Release may be increased from spray products or by degradation of the matrix caused by wear and tear.

Hazard assessment for consumers when using nanomaterial-containing products is challenging because very little is known regarding adverse effects of nanomaterials when part of a matrix. A limited number of animal studies on how toxicity is affected when nanomaterials are part of a matrix have been published. Most of these have focused on the effects following pulmonary exposure of nanomaterial-containing spray products (three studies) or dusts obtained by sanding of nanomaterial-containing paints, cement or thermoplastic (three studies). Other studies have focused on the uptake of ZnO from sunscreen. In some of the studies described in this report, the toxicity of the nanomaterial-containing material was compared to the conventional product without nanomaterial. None of these studies showed increased toxicity of the nanomaterial-containing product compared to the conventional product. However, the studies only assessed acute toxicity up to 1 month after the last exposure. No DNELs for nanomaterials when part of matrix was identified in our literature search. This problem is in principle the same for the assessment of "traditional" chemicals in matrices. However, they may be more pronounced for nanomaterials where the physical entity and characteristics may be especially important and affect the properties of the material.

Due to lack of information on hazards of nanomaterials when part of a consumer product, the hazard data for the free/pristine nanomaterials may be used to predict the hazard in a worst-case scenario where it is assumed that the pristine nanomaterial is released from the product or that the consumer is in direct contact with the nanomaterial in the product. DNELs for materials such as for example CB, TiO_2 and Ag, can be found on the ECHA dissemination site as extracted from REACH registrations by importers and manufacturers. However, these DNELs are usually representative for the macro-form of the material rather than for the nano-sized particles. Furthermore, it has to be noted that derivation of the DNEL-values is the responsibility of the registrants and is the outcome of the registrant's interpretation of the data and use of assessment factors. Thus, these DNEL-values cannot be regarded as generally accepted values. For some of the materials, DNELs are given both

for consumer and occupational exposure. In general, the assessment factors recommended to be used for deriving consumer DNELs are a factor 2 larger than the assessment factors used for deriving occupational DNELs. Thus, the consumer DNELs are generally lower than occupational DNELs (European Chemicals Agency (ECHA), 2012). In the ECHA guidance on information requirements and chemical safety assessment and the Appendix R8-15 on recommendations for nanomaterials it is mentioned in relation to evaluation of the quality of the whole database used to calculate the DNEL that application of an extra assessment factor to account for deficiencies within the data set can be particularly relevant for nanomaterials.

As described above, consumers are only exposed to free nanomaterials to a limited extent. This is in contrast to workers who potentially are at higher risk of exposure to free nanomaterials. Therefore, research has primarily focused on the challenges regarding worker safety. OELs are traditionally based on the toxicity of the macro-material. Therefore, concern has been raised whether the existing OELs are sufficient when it comes to the nanoform. A recent workshop report summarises the challenges regarding setting OELs for engineered nanomaterials (Gordon et al., 2014). Although consumers (see above) are not assumed to be exposed to free nanomaterials to the same extent as workers, the requirements for sufficient hazard data are relevant in relation to setting DNELs for consumers as well. The report lists the following conditions as important barriers for the establishment of health-based exposure limits for nanomaterials:

1. There is a lack of hazard data. This is particularly the case for long-term animal inhalation experiments and epidemiological studies. The continuous introduction of new nanomaterials (inclusive variations of "first generation" nanomaterials such as TiO_2 and CB) with an enormous diversity of physico-chemical attributes makes it unlikely that appropriate hazard data can be generated for each compound.
2. There is a limited understanding of how the physico-chemical properties affect the toxicity of a nanomaterial and it is still not possible to predict the toxicity based on the physico-chemical properties alone.
3. There is an uncertainty regarding the most appropriate dose and exposure metric. Traditionally existing OELs are mass-based (mg/m^3) with the exception of asbestos which is given as the number of fibres in the air ($fibres/m^3$). Animal studies have shown correlations between hazard of nanomaterials and a number of different dose metrics (surface area, number, volume etc). More research is required to clarify if other dose-metrics than mass should be considered when setting exposure limits.
4. There is still a lack of standardised and validated methods for measuring air concentrations of nanomaterials.

Thus, there are several barriers for setting occupational exposure levels for nanomaterials, and to our knowledge there are presently no legally binding exposure limits for nanomaterials. However, some initiatives have been taken. For example, NIOSH has recommended that the airborne exposure limits of TiO_2 should be size-dependent. NIOSH propose an exposure limit of 2.4 mg/m^3 for fine-TiO_2 (0.1 – 4 μm) and 0.3 mg/m^3 for ultrafine-TiO_2 (< 0.1 μm), as time-weighted average concentrations for up to 10 hours/day during a 40-hours work week (NIOSH, 2011). NIOSH has also proposed a recommended exposure limit for CNT at 1 $\mu g/m^3$ (NIOSH, 2013). A draft criteria document for the Scientific Committee on Occupational Exposure Limits (SCOEL) on CNT was submitted in 2013. In addition, some suggestions for exposure limits for nanomaterials have been proposed by manufacturers and researchers.

Hazard assessment of nanomaterials should be done for each form of a nanomaterial with specific characteristics separately unless it is evidenced that different nanomaterial may be grouped. However, for both economical and time reasons it is not possible to test all specific forms of nanomaterials before use. Therefore, a lot of effort is put into intelligent testing strategies and grouping of nanomaterials in order for in the future to be able to predict the adverse effects based on the physico-chemical properties (Stone et al., 2014; Finnish Institute of Occupational Health,

2013). One of the research strategies emphasised that knowledge of the biological mechanism of action would enable grouping and ranking of nanomaterials and allow design of high through-put testing and computer-based hazard testing of nanomaterials (Quantitative nanoStructure-Activity Relationship) (Stone et al., 2014). However, nano(Q)SAR is still in early stages of development and its implementation is far from reality (Tantra et al., 2014).

In Chapter 6 we performed a hazard assessment of seven nanomaterials (CB, CNT, SiO_2, Ag, TiO_2, ZnO and ZrO_2). This aim of this hazard assessment is to serve as input for the risk assessment of 20 scenarios. For each material, specific uncertainties related to the hazard assessment and selection of NOAEL values and derivation of DNELs are highlighted where relevant in the summay tables serving as input for the risk assessment.

The hazard assessments are, to the extent possible, based on data for the nanomaterials although information is limited for some of the substances. However, data for the non-nano form of the substances have been used where data for the nanomaterials were not available. This is also the case in relation to OELs and identified DNEL values referred to in the assessments. Detailed information about the studies behind the DNEL values identified from the REACH registration dossiers is generally not provided with the information available on the ECHA homepage.

The quality of the available hazard data of the nanomaterials vary and often the tested materials are not sufficiently characterised to allow robust evaluation of the mode of action and comparison of the results for different materials. This is also linked to the need for further development of validated test systems and the fact that nanomaterials vary endlessly.

The DNEL values identified in the summary tables are as mentioned primarily derived for the non-nano form of the substances and most of them for the purpose of occupational exposure using assessment factors developed for the non-nano form of the substances. The applicability of these values will be evaluated and discussed against all available data in relation to the risk assessment to be carried out as the next step in the main project. If not considered appropriate, new DNELs may be derived, e.g. by introducing an extra assessment factor as suggested in the ECHA guidance for nanomaterials. However, this may vastly underestimate the real hazard. An example could be extrapolating CNT hazard from CB hazard. If the available DNELs are not considered appropriate for the risk assessment, even when introducing an extra assessment factor, an attempt will be made to suggest alternative specific DNEL values. As a consequence of data limitation, some endpoints relevant for the exposure scenarios may have to be assesses based on other routes of exposure.

Overall, the main uncertainties related to hazard assessment and derivation of DNELs for nanomaterials in consumers products for further risk assessment are:

- Hazard data for pristine nanomaterials are applied in absence of hazard data for the nanomaterials in matrix.
- Results are not always available for the most relevant exposure routes to be addressed.
- As detailed characteristics of the nanomaterials are often not available, the information used for evaluation of nanomaterials today may be based on data generated for different forms of the particles with varying surface area, coating or size distribution. There is little data on how these physico-chemical parameters influence toxicity.The quality of data varies significantly and test results with insufficient characterisation of the nanoform may be included in the evaluations but may be less useful.
- It is not known if the generally applied assessment factors are valid for nanomaterials.

8. Summary

Background
Under the Agreement "Better Control of Nanomaterials" (“Bedre styr på nanomaterialer”), the Danish Environmental Protection Agency (Danish EPA) has commissioned a number of projects aiming to investigate and generate new knowledge on the presence of nanomaterials in products on the Danish market and to assess the possible associated risks to consumers and the environment. This report is part of a series of four from a project which addresses consumer exposure and risk assessment of nanomaterials in products on the Danish market.

The aim of this report is to perform a hazard assessment of nanomaterials in consumer products. The consumer is potentially exposed to nanomaterials in their final, intended use, i.e. when the nanomaterials are part of a matrix, and therefore, this report focuses on the hazard of nanomaterials when part of a consumer matrix. However, free nanomaterials may be liberated during the use phase and therefore the hazard of pristine nanomaterials is also described.

The aim of the report is to refer consensus rather than isolated findings. Since the focus of the hazard evaluation is on nanomaterials during their intended use, the referred literature concerning pristine nanomaterials consists primarily of reviews, while all identified original studies of hazard related to nanomaterials as part of a product are described.

The structure of the report is that we review the data relevant to assessing the hazard of nanomaterials in consumer products, i.e. biokinetics of nanomaterials (Chapter 2), adverse effects of pristine nanomaterials (Chapter 3), adverse effects of nanomaterials when part of a matrix (Chapter 4), physico-chemical factors of importance for toxicity (Chapter 5), perform a hazard assessment of a selection of nanomaterials (Chapter 6), discuss how to bridge between hazard and risk assessment (Capter 7) and finally we summarise and conclude (Chapter 8).

Biokinetics
For the consumer, the lungs, gastrointestinal tract and the skin are considered to be the primary absorption routes for nanomaterials. In addition, uptake may occur through the eyes and the olfactory system. We did not identify any studies covering how biokinetics are modified when the nanomaterial is part of a matrix.

Inhalation of particles results in deposition of particles in the respiratory tract (nasopharyngeal, tracheobronchial and alveolar regions). The fate of the particles after deposition depends on a combination of physico-chemical properties of the particles and responses both locally in the lung and in other parts of the body. The larger particles are trapped by the mucociliary system in the upper airways and are removed relatively fast (hours to days). In contrast, the smaller particles deposit in the alveolar region where they can stay for years in humans. A low degree of translocation of particles from the lung to the circulation has been described.

In 2013 the Danish EPA published in 2013 a report on systemic absorption of nanomaterials by oral exposure which gives an overview of the existing literature (Danish EPA, 2013c). In general, the reviewed literature indicates that the gastrointestinal absorption is low. It is concluded that physico-chemical characteristics such as size, agglomeration and crystal structure may affect absorption. The report concludes that the number of publications on gastrointestinal absorption is

increasing, but only a few of these have been well performed. In particular it is of concern that only a few of the physico-chemical parameters that are expected to influence absorption of nanoparticles were reported.

In 2013 the Danish EPA has published a report on dermal absorption of nanomaterials which provides an overview and evaluation of the knowledge base regarding dermal absorption of nanomaterials (Danish EPA, 2013b). The overall conclusion in the report is that absorption of particles in the nano-range through the skin is possible although it seems to occur to a very low degree. However, the level of penetration, depending on chemistry and experimental conditions, may be greater for particles in the nano-range than for larger particles.

The present knowledge about absorption of nanomaterials into the eye and the eye toxicity is too limited to be assessed and evaluated in general for the hazard assessment.

Olfactory absorption of nanomaterials has been demonstrated in laboratory animals and may also be relevant for humans (Elder et al., 2006). At present, consistent knowledge is too limited for hazard assessment in general or specifically.

Only a limited number of studies have investigated the potential metabolism of nanomaterials. Where such information exists, most of the studies show that the nanomaterials, and in particular insoluble nanomaterials, are not metabolised (Landsiedel et al., 2012).

Upon absorption following pulmonary, dermal, eye or gastrointestinal exposure, nanomaterials may reach the blood circulation and/or the lymphatic system. The liver is the major distribution organ and 40 nm Au particles have been shown to accumulate in the Kupffer cells of the liver rather than being excreted (Sadauskas et al., 2009a). The placenta constitutes the nutritional interface between mother and fetus. Studies of the passage of engineered nanoparticles (ENP) across the placenta are still few in number, but rodent studies describe transplacental transport of nano-sized particles, with rates ranging from almost negligible to high (several percent of the administered dose).

Adverse effects of pristine nanomaterials

Pulmonary exposure to nanomaterials may cause pulmonary inflammation, fibrosis, DNA damage and cancer. Concern has been raised that pulmonary exposure may also result in adverse cardiovascular effects as seen for human exposure to particles from the ambient air. The mechanisms behind the cardiovascular effects are suggested to be a systemic inflammatory and acute phase response, particle translocation or respiratory reflexes.

The knowledge on the toxicity of orally administered nanomaterials in the gastrointestinal tract is limited and it is therefore not possible to identify any overall conclusions regarding the toxicity of nanomaterials for intended use in food and food-related products (Card et al., 2011). However, the toxicity of the nanoformulation of a specific ingredient or material was not always consistently increased as compared to the non-nanoformulation.

Overall, there is little evidence of dermal toxicity following topical application of nanomaterials. In general, the uptake through intact healthy skin is very low. However, if nanomaterials are able to penetrate the skin and enter the bloodstream, they may exert a number of adverse effects.

Adverse effects of nanomaterials when part of a matrix

Only a very limited number of studies have focused on the hazard of nanocomposites. Most of these have focused on the hazard following pulmonary deposition of dust obtained by sanding different types of nanocomposites (paint, cement and thermoplastic). The published studies on the toxicological effects of sanding dusts from nanocomposites are in good agreement with each other.

No additional toxicity (inflammation and genotoxicity) have been detected for any of the nano-composites compared to the corresponding products without nanomaterials. A few studies on the toxicological effects of sprays containing nanomaterials have been published. A rat inhalation study of a commercial spray containing nano titanium dioxide (nano-TiO_2) particles indicated similar toxicity of nano-TiO_2 when part of the product and when nano-TiO_2 was tested alone. Testing of a commercial Ag spray product in rats resulted in moderate cardiovascular effects that were not observed in rats exposed to a standard Ag reference product. A third study showed no increased toxicity of a polymer dispersion compared to the non-nano product. Thus, based on the very few available studies it seems that the toxicity of nanomaterials following pulmonary exposure are masked in solid matrices while the toxicity of nanomaterials in some cases remains in spray products.

A few studies have investigated penetration of nanomaterials in sunscreen products. The penetration of nanomaterial containing cosmetics through skin was considered minimal, and there was no evidence that nano-sized or submicronised TiO_2 penetrated the intact epidermis to any significant extent or evidence of systemic absorption.

The oral safety of food-related nanomaterials that have potential use from *in vitro* and *in vivo* studies in laboratory was reviewed by Card et al. No adverse toxic effects were revealed in any of the very few studies with well performed physico-chemical characterisation of the nanomaterials. None of the studies characterised the nanomaterials in the diet or in any organ (Card et al., 2011).

No Derived No Effect Levels (DNELs) on hazard of nanomaterials as part of a matrix have been identified. More studies are needed to make conclusions within this area.

Physico-chemical factors of importance for toxicity

A number of physico-chemical properties (size, shape, surface properties, composition, solubility, aggregation/agglomeration, nanomaterial uptake, presence of mutagens and transition metals associated with the nanomaterials etc.) have been suggested to be important for toxicity of nano-materials. For insoluble so-called inert particles such as TiO_2 and carbon black (CB), the specific surface area of the particles has been shown to be correlated to the inflammatory response. The fact that multiwall carbon nanotubes (MWCNT) and asbestos fibres have been shown to induce similar genotoxic effects in rodents is an example of the effect of particle shape for genotoxity. For soluble nanomaterials, such as for example zinc oxide (ZnO), the liberation of Zn ions is important for the potential toxicity. The importance of physico-chemical factors for toxicity stresses the need for toxicological studies using well-characterised nanomaterials.

Hazard assessment of seven nanomaterials

A specific hazard assessment has been performed for seven nanomaterials with relevance for consumer exposure to serve as input for the consumer risk assessment of 20 scenarios. The consumer risk assessment will be published in the final report. The nanomaterials have been chosen to represent a diverse group of nanomaterials, i.e. 1) "inert" insoluble particles such as TiO_2 and CB, 2) soluble nanoparticles such as ZnO and Ag and 3) high aspect ratio particles such as carbon nanotubes (CNT) since nanomaterials constitute a very broad group which differs with respect to chemistry, solubility, coating etc. The main conclusions on the hazard assessment of the seven nanomaterials are inserted below.

Carbon black (CB)

The hazard assessment of CB in a matrix is primarily based on the recent Scientific Committee on Consumer Safety (SCCS) opinion on CB (SCCS, 2014c). The aim of this SCCS opinion on CB was specifically to decide if CB is safe for use as a colorant with a concentration up to 10 % in cosmetic products, and is therefore considered highly relevant for the present purpose, namely to serve as an input for the hazard part of the risk assessment of CB in mascara. The focus in the present

assessment was on hazard related to skin and eye exposure because these routes of exposure are considered to be the most relevant in relation to consumer use of mascara. Three studies on skin absorption of cosmetic formulations containing CB (all 20-30 nm in size) were evaluated by the SCCS and did not indicate any skin absorption. As emphasized by the SCCS, the conclusion on no risk of adverse effects of up to 10 % as CB as a colorant in cosmetic products is only valid when the skin is intact and the CB particles are 20 nm or larger. No studies on eye absorption of CB were evaluated by the SCCS. Therefore, hazard associated with eye absorption cannot be evaluated even though it is highly relevant for the hazard assessment of consumer use of mascara containing CB. The risk of eye irritation of CB cannot be excluded (SCCS, 2014c). We agree with the final concluding remarks of the SCCS opinion stressing that the skin absorption studies have only been done for CB sizes above 20 nm and that it is therefore not possible to conclude on cosmetic products containing smaller sized CB.

Carbon nanotubes (CNT)
The hazard assessment of CNT will serve as background documentation for the risk assessment of use (wear and tear) and sanding of a golf club containing CNT. With regard to the intended use of a golf club, dermal exposure is considered to be the only relevant exposure route. No studies were identified on the dermal toxicity of CNT incorporated into a solid matrix. If the CNT containing golf club is sanded, sanding dust containing CNT and potentially free CNT may be liberated and exert toxicity primarily by the pulmonary and dermal routes. The hazard assessment of free CNT is based on a recently published report on risk assessment of CNT by the Danish EPA (Danish EPA, 2015a). Animal studies have shown that pulmonary exposure to CNT consistently give asbestos-like toxicological response characterised by persistent inflammation, granulomas and fibrosis with low no-effect levels. Chronic human indicative no-effect levels (INELs) for the general public have been suggested to be 0.25 μg/m^3 (inhalation) and 0.78 and 2.3 mg/person (dermal) for two different scenarios (Aschberger et al., 2010). Two studies were identified on the toxicity of sanding dusts from different types of CNT composites (Wohlleben et al., 2011; Wohlleben et al., 2013). None of the studies showed increased toxicity of sanding dust from the CNT materials compared to the conventional products without CNT. However, it has not yet been tested if sanding dust from ultraviolet (UV)-exposed or otherwise weathered materials have a different toxicity profile due to a potentially increased liberation of free CNT.

Nano-sized synthetic amorphous silica (nano-SAS)
The hazard assessment of synthetic amorphous silica (SAS) as part of a matrix is primarily based on recent reviews by Dekkers et al (Dekkers et al., 2011; Dekkers et al., 2013), a review of the hazard of SAS by (Fruijtier-Pôlloth, 2012), and reports on SAS by the International Agency for Research on Cancer (IARC) (IARC Monographs on the evaluation of carcinogenic risks to humans, 1997) and by the European Centre for Ecotoxicology and Toxicology of Chemicals (ECETOC) through the Joint Assessment of Commodity Chemicals (JACC) program (European Centre for Ecotoxicology and Toxicology of Chemicals (ECETOC), 2006). The purpose of a hazard assessment is to serve as an input for the hazard part of the risk assessment of SAS in 1) food items and food containers, 2) face powders, and 3) "easy to clean" impregnation. Thus, the focus is put on the potential hazards associated with exposure by the gastrointestinal route (relevant for the food items and food container scenarios) and exposure by the dermal and the inhalation routes (relevant for the face powder and the "easy to clean" impregnation product). The critical effect following oral exposure is assessed as the hepatic effect. The No Observed Adverse Effect Level (NOAEL) has been suggested to be 1,500 mg/kg Body Weight (bw)/day (Dekkers et al., 2011). The critical effect following pulmonary exposure is pulmonary inflammation. Based on the evaluation by ECETOC, the Lowest Observed Adverse Effect Levels (LOAELs) and NOAELs were typically 1-50 mg/m^3 and 0.5-10 mg/m^3, respectively (European Centre for Ecotoxicology and Toxicology of Chemicals (ECETOC), 2006). These differences were by ECETOC evaluated as particle size-dependent: i.e. in general the NOAEL/LOAEL decreased by particle size. Our literature search identified several recent studies showing that the NOAEL/LOAEL was affected by size and surface modification, highlighting that

the physico-chemical properties have to be taken into account. No studies were identified by ECETOC on the dermal or oral absorption of SAS (European Centre for Ecotoxicology and Toxicology of Chemicals (ECETOC), 2006). For that reason no NOAEL/LOAEL has been suggested. Thus, the overall conclusion by ECETOC is that "*SAS is essentially non-toxic in humans via the oral, dermal/ocular and inhalation routes of exposure and no data exist on systemic effects in humans*".

Nano-sized silver (Nano-Ag)
The purpose of this hazard assessment is to serve as an input for the hazard part of the risk assessment of Ag when used in food supplements, paints for spraying, nano-filtering, disinfectant pump and propellant sprays, textiles and wound dressings. Thus, pulmonary, gastrointestinal and dermal exposures are all relevant exposure routes for the chosen risk scenarios.
The hazard assessment is mainly based on recent reports and references therein on nano-Ag by The Scientific Committee on Emerging and Newly Identified Health Risks (SCENIHR) and The Danish EPA (Scientific Committee on Emerging and Newly Identified Health Risks (SCENIHR), 2014a; Danish EPA, 2011) and recent reviews (Hadrup & Lam, 2014; Johnston et al., 2010a). Ag has no known essential function in man. The daily human intake has been estimated to be 0.007-0.5 µg/kg bw/day as the sum from all routes of exposure. Nano-Ag dissolves in solution and releases Ag^+. There is substantial evidence suggesting that the released Ag^+ are responsible for toxicological effects (Hadrup & Lam, 2014). The best described adverse effects in humans caused by long-term exposure to Ag is a permanent bluish-grey discoloration (argyria or agyrosis) of the skin and/or eyes (Scientific Committee on Emerging and Newly Identified Health Risks (SCENIHR), 2014a). Human risk assessment of Ag is most often based on epidemiological studies showing development of argyria. World Health Organisation (WHO) has set a NOAEL of 5 µg/kg bw/day as the sum of all routes of exposure (WHO, 2003). This NOAEL has been adopted by The European Food Safety Agency (EFSA) (EFSA Panel on Food Contact Materials, 2011). Likewise, The United States Environmental Protection Agency (US EPA) has published an oral reference dose of 5 µg Ag/kg bw/day in relation to a life-time exposure to Ag (U.S.Environmental Protection Agency, 1996). Dermal absorption through damaged skin has been reported in humans applying wound dressings containing nano-Ag. No toxic effects were reported in these test persons and less than <0.1 % of dose was estimated to be absorbed (Vlachou et al., 2007; Danish EPA, 2011; Moiemen et al., 2011; Walker & Parsons, 2014). The DNEL, as set by the REACH registrant, is 0.1 mg Ag/m^3 and 0.04 mg Ag/m^3 in the air for workers and the general population, respectively (European Chemicals Agency (ECHA), 2014). SCENIHR concluded that the toxicity of Ag, including nanoparticles of Ag, to humans is generally low. Futhermore, it is concluded that more data is needed to understand 1) the bacterial response to ionic Ag as well as Ag nanoparticles and 2) the hazard associated with the dissemination of resistance (Scientific Committee on Emerging and Newly Identified Health Risks (SCENIHR), 2014a). Overall, we agree in these thresholds and consider them applicable for the risk assessment.

Nano-sized titanium dioxide (Nano-TiO_2)
The hazard assessment of nano-sized TiO_2 is intended to serve as background documentation for the risk assessment of TiO_2 used in chewing gum, sunscreen, sunscreen lipstick, paint and cement and in relation to sanding a surface painted with nano-TiO_2-containing paint. The different applications of TiO_2 involve exposure by the oral, dermal, eye and pulmonary route and focus is therefore on the hazards associated with these exposure routes.

The SCCS has concluded that nano-TiO_2 at concentrations up to 25 % (wt/wt) and containing maximum 5 % anatase in sunscreens for dermal application are considered safe for the consumers. Use of TiO_2 nanoparticles in sprayable products is not recommended (SCCS, 2014d). Based on the available information, lung toxicity following inhalation appears to be the most critical effect in relation to long-term exposure to nano-TiO_2. Deposition in the lung depends on the dose and particles may be retained in the lung for a long time. Inflammation appears to be determined by the

surface area of the particles. A NOAEL of 62.5 mg/kg bw/day was established based on a 30 days oral (gavage) study in mice exposed to anatase TiO_2 nanomaterials with a primary particle size of 5 nm. A LOAEL of 5 mg/kg bw/day was derived based on impaired neurofunction and behaviour from a 60 days oral gavage study in mice exposed to anatase TiO_2 nanomaterials with primary particle size 5 nm. The occupational exposure limit (8-hour average) for TiO_2 in all forms (calculated as Ti) is 6 mg/m^3 in Denmark corresponding to 10 mg/m^3 TiO_2. The National Institute for Occupational Safety and Health (NIOSH) has recommended a threshold of 2.5 mg/m^3 for fine TiO_2 and 0.3 mg/m^3 for ultrafine TiO_2 (< 100 nm) for up to 10 hours/day during a 40-hour work week (NIOSH, 2011).

Nano-sized zinc oxide (Nano-ZnO)

The purpose of this hazard assessment of ZnO in nanoform (nano-ZnO) is to serve as background documentation for the risk assessment of sunscreen pump sprays containing nano-ZnO. The most relevant exposure route associated with consumer use of nano-ZnO sunscreen pump spray is dermal exposure. However, oral and pulmonary exposure may also occur but to a lesser extent. Nano-ZnO dissolves in biological fluids including artificial gastrointestinal fluid and lung fluid to form Zn^{2+} that seems to be distributed systematically to organs. This hazard assessment of nano-ZnO in a matrix is primarily based on the recent SCCS opinion on ZnO (SCCS, 2012) and two addendums related to this opinion (SCCS, 2014a; SCCS, 2014b). The aim of these SCCS opinions on ZnO was specifically to decide if ZnO in nanoform is safe for use as an UV-filter with a concentration up to 25 % in cosmetic products and is therefore considered highly relevant for the present purpose. It is concluded by the SCCS that the use of the different forms of ZnO nanoparticles as specified in the opinions at a concentration up to 25% as a UV-filter in sunscreens can be considered not to pose a risk of adverse effects in humans after dermal application. However, this does not apply to other applications that might lead to inhalation exposure to ZnO nanoparticles (such as sprayable products) because pulmonary exposure induces serious pulmonary effects. Therefore, the SCCS concludes that the use of ZnO nanoparticles in spray products that could lead to exposure of the consumer's lungs to nano-ZnO by inhalation cannot be considered safe.

Nano-sized zirconia (Nano-ZrO_2)

The hazard assessment of nano-sized zirconium dioxide (ZrO_2) is intended to serve as background documentation for the risk assessment of ZrO_2 used in dental fillings (implants). This particular use involves exposures of the consumer related to the application process (with spatula), to sanding and polishing, and to contact with the material migrating from the dental filling to the oral mucosa and saliva. Focus will be on the hazards associated with exposure by the inhalation route and exposure by the gastrointestinal route. In addition, exposure to the eye will be covered. ZrO_2 is generally described as a substance of low toxicity. There are very few *in vivo* studies available investigating the toxicity of ZrO_2. ZrO_2 has extremely low solubility in water and absorption is expected to be low from all exposure routes. When deposited in the alveolar region, particles are expected to be engulfed by alveolar macrophages and only a small amount to end up in the blood via the lymphatic system (European Chemicals Agency (ECHA), 2014). No specific adverse effects have been identified for the substance in the more recent literature. In older literature, immunostimulating effects are reported following injections in the thorax cavity and the peritoneum of mice. In addition, an ability to cause axilliary granulomas when applied in deoderants is described (Health Council of the Netherlands, 2002). The general occupational exposure limit (8-hour average) for Zr compounds (calculated as Zr) is 5 mg/m^3 in Denmark. The Occupational Safety and Health Administration (OSHA) in the US have applied the same value.

Bridging between hazard and risk assessment

Only a few studies on the hazard of nanomaterials as part of a matrix have been published and no DNELs for nanomaterials when part of matrix have been identified. Although consumers are not assumed to be exposed to free nanomaterials to the same extent as workers, the requirements for

sufficient hazard data are relevant in relation to setting DNELs for consumers as well. There are a number of important barriers for the establishment of health-based exposure limits for nanomaterials: 1) There is a lack of hazard data, 2) there is a limited understanding of how the physicochemical properties affect the toxicity of a nanomaterial, 3) there is an uncertainty regarding the most appropriate dose and exposure metric, and 4) there is still a lack of standardised and validated methods for measuring air concentrations of nanomaterials. As described above there are several barriers for setting exposure threshold limits for nanomaterials and to our knowledge presently no legally binding specific exposure limits for nanomaterials exist. However, some initiatives have been taken. For example, NIOSH has proposed specific Occupational Exposure Limits (OELs) for 1) all CNT, and 2) for nano-sized TiO_2 compared to larger sized TiO_2.

Overall, the main uncertainties related to hazard assessment and derivation of DNELs for nanomaterials in consumers products for further risk assessment are:

- Hazard data for pristine nanomaterials are applied in absence of hazard data for the nanomaterials in matrix.
- Results are not always available for the most relevant exposure routes to be addressed.
- As detailed characteristics of the nanomaterials are often not available, the information used for evaluation of nanomaterials today may be based on data generated for different forms of the particles with varying surface area, coating, and size distribution. There is little data on how the physicochemical parameters influence toxicity.
- The quality of data varies significantly and test results with insufficient characterisation of the nanoform may be included in the evaluations but may be less useful.
- It is not known if the generally applied assessment factors are valid for nanomaterials.

Conclusion

The aim of this report is to perform a hazard assessment of nanomaterials in consumer products. The consumer is potentially exposed to nanomaterials in their final, intended use, i.e. when the nanomaterials are part of a matrix, and therefore, this report focuses on the hazard of nanomaterials when part of a consumer matrix. Only a few studies on the hazard of nanomaterials as part of a matrix have been published and no DNELs for nanomaterials when part of matrix have been identified. A few studies on pulmonary exposure to sanding dust from solid matrices and spray products containing nanomaterials have been published. No additional hazard was observed when nanomaterials were part of a solid matrix, and the matrix was most important for the toxicity. A few studies indicate that the hazard of nanomaterials may not always be masked when part of a liquid matrix (e.g. spray products). Dermal application of CB containing mascara and nano-ZnO containing sun screen resulted in no to very moderate dermal absorption. The studies on gastrointestinal absorption and toxicity are too few for conclusion. More research is needed to characterise the hazard to consumers exposed to nanomaterials.

Due to the lack of information on hazard of nanomaterials when part of a consumer product, the hazard of free nanomaterials can be used to predict the hazard in a worst-case scenario. However, use of hazard data for free nanomaterials may also be challenging because of knowledge gaps. In general, there is a lack of long-term toxicity studies. The continuous introduction of new nanomaterials (with new surface modifications etc) makes it impossible to test all nanomaterials. Therefore recent research strategies have emphasised that grouping and ranking are necessary tools to predict hazard.

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Hazard assessment of nanomaterials in consumer products
Under the Agreement "Better Control of Nanomaterials" (“Bedre styr på nanomaterialer”), the Danish EPA has commissioned a number of projects aiming to investigate and generate new knowledge on the presence of nanomaterials in products on the Danish market and assess the possible associated risks to consumers and the environment. This report is part of a series of four from a project which addresses consumer exposure and risk assessment of nanomaterials in products on the Danish market.
The consumer is potentially exposed to nanomaterials in their final, intended use, i.e. when the nanomaterials are part of a matrix.

This report focuses on the hazard of nano¬materials when part of a consumer matrix. However, free nanomaterials may be liberated during the use phase and therefore the hazard of pristine nanomaterials is also described.

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