Systemic Absorption of Nanomaterials by Oral Exposure

Part of the ”Better control of nano” initiative 2012-2015

Binderup, Mona-Lise; Bredsdorff, Lea; Beltoft, Vibe Meister; Mortensen, Alicja; Löschner, Katrin; Löschner, Katrin; Larsen, Erik Huusfeldt; Eriksen, Folmer Damsted

Publication date:
2013

Document Version
Publisher's PDF, also known as Version of record

Danish Ministry of the Environment
Environmental Protection Agency

Systemic Absorption of Nanomaterials by Oral Exposure

Part of the ”Better control of nano” initiative 2012-2015

Environmental Project No. 1505, 2013

Title:

Systemic Absorption of Nanomaterials by Oral Exposure

Editing:

DTU Food, National Food Institute
Mona-Lise Binderup (project coordinator)
Lea Bredsdorff
Vibe Meister Beltoft
Alicja Mortensen
Katrin Löschner
Erik Huusfeldt Larsen
Folmer D. Erikse

Published by:

The Danish Environmental Protection Agency
Strandgade 29
1401 Copenhagen K
Denmark
www.mst.dk/english

Year:

2013

ISBN no.

978-87-93026-51-3

Contents

Foreword

The project “Systemic absorption of nanomaterials by oral exposure” was carried out during the period January to August 2013.

This report and the accompanying database (see appendix 7) are intended to provide a comprehensive evaluation of the knowledge base regarding the systemic absorption of nanomaterials by oral exposure based on the currently available scientific literature.

These results regarding the oral exposure route for nanomaterials are part of the “Better control of nano” initiative conducted by the Danish EPA with the aim of further clarifying possible risks to consumers and the environment.

The project was carried out by the National Food Institute, DTU.

The project group consisted of the following members:

Mona-Lise Binderup (project coordinator)

Lea Bredsdorff

Vibe Meister Beltoft

Alicja Mortensen

Katrin Löschner

Erik Huusfeldt Larsen

Folmer D. Eriksen

Christina Ihlemann, Cand. Scient., Danish Environmental Protection Agency

Gregory Moore, Cand. Scient., Ph.D., Danish Environmental Protection Agency

Anne Mette Boisen, Cand. Scient., Ph.D., Danish Environmental Protection Agency

Main authors:

Mona-Lise Binderup and Erik Huusfeldt Larsen

The project was financed by the National Budget Agreement 2012 on Better Control of Nanomaterials and their Safety (“Bedre styr på nano”).

Danish EPA, September 2013

Dansk resumé

I projektet "Systemisk absorption af nanomaterialer ved oral eksponering" er den udvalgte tilgængelige viden fra den videnskabelige litteratur om systemisk absorption af nanomaterialer efter oral eksponering vurderet. Projektet er en del af den danske Miljøstyrelses initiativ "Bedre kontrol af nano", der skal præcisere den mulige risiko for forbrugerne og miljøet ved udsættelse for nanomaterialer.

Det overordnede formål med projektet var at indsamle og vurdere eksisterende viden på området. De mere specifikke mål var:

1. At udføre en omfattende litteratursøgning og vurdering af pålideligheden og relevansen af studier med systemisk absorption af nanomaterialer ved oral eksponering (fase I).

2. At evaluere de faktorer, der påvirker den systemiske absorption af nanomaterialer ved oral eksponering (fase II), herunder:

    a. En vurdering af de fysiske og kemiske egenskaber af nanomaterialer der er beskrevet i litteraturen, og som forventes at påvirke den systemiske absorption af nanomaterialer efter oral eksponering.

    b. En vurdering af hvilke testmetode(r) der bedst simulerer systemisk absorption af nanomaterialer ved oral eksponering under hensyntagen til kompleksiteten af fordøjelsessystemet og de faktorer, der kan have en indflydelse på den mulige systemiske absorption af nanomaterialer.

3. At identificere manglende viden og forskningsbehov og anbefale, hvilke modeller og målemetoder der er mest velegnet til at simulere systemisk absorption af nanomaterialer ved oral eksponering hos mennesker. Endelig at anbefale relevante nanomaterialer som kandidater til eksperimentel testning i fremtiden (fase III).

En trinvis litteratursøgningsprofil blev anvendt til at identificere relevante videnskabelige dokumenter, som blev screenet for deres relevans med hensyn til absorption (og dermed potentielle sundhedsskadelige effekter på mennesker via oral eksponering (lægemidler undtaget). I alt 64 videnskabelige artikler blev udvalgt til nærmere vurdering af disses videnskabelige kvalitet baseret på de såkaldte Klimisch kriterier (om udførelsen af toksikologiske/biologiske eksperimenter), og detaljerne i karakteriseringen af nanopartiklerne blev noteret. Det endelige antal på 47 artikler med en Klimisch score på 1 eller 2 (nogle få artikler med en score på 3, blev også udvalgt baseret på en ekspert vurdering) blev udvalgt til yderligere vurdering. De typer af nanopartikler, der indgik i den valgte litteratur omfattede: kulstofnanorør, cerium dioxid, fullerener, guld, jernoxid, selen, silicium dioxid, sølv, titandioxid og zinkoxid.

Evalueringen af fysiske og kemiske egenskaber, der forventes at påvirke absorptionen af nanopartikler viste, at få af disse parametre var undersøgt eller dokumenteret. For nogle nanomaterialer kunne en evaluering af de faktorer, der påvirker deres systemiske absorption efter oral eksponering ikke gives på grund af manglende data. Dette gælder for kulstofnanorør, cerium dioxid, fullerener, silicium dioxid og selen. Det blev påvist, at tarmens optagelse af jernoxid, guld og sølvnanopartikler var højere for de mindre partikelstørrelser end for de større partikler.

Indflydelsen på absorptionshastigheden af polymerbelægninger på sølv nanopartikler blev undersøgt, men ingen klar tendens blev observeret. Opløseligheden af sølv og zinkoxid nanopartikler viste sig at være en uventet faktor i studiet af absorptionen på grund af de opløste, ioniske former af disse partikeltyper. For titandioxid nanopartikler var der ingen klare oplysninger om forholdet mellem størrelse og absorption, men der var tegn på, at agglomereringen af anatase krystalformen kunne forklare den lave absorption heraf og dermed indirekte forklare, hvorfor krystalformen rutil blev bedre absorberet end anataseformen.

De metoder, der var mest lovende til vurdering af systemisk absorption af nanomaterialer blev evalueret. Metoder baseret på *in vitro* test eksisterer og er i øjeblikket under udvikling. Der er imidlertid behov for yderligere forfining før disse metoder kan anvendes til vurdering af absorption af nanopartikler efter oral eksponering. Den mest lovende modeltype bør omfatte et syntetisk system under brug af fysiologisk relevante betingelser (enzymer, pH, salte og temperatur) alene eller bør anvendes i kombination med en *in vitro* absorptionsmodel baseret på f.eks. humant tarmepitel eller Caco-2celler. Sølv og siliciumdioxid nanopartikler er blevet testet under sådanne syntetiske betingelser, og tilstedeværelsen af nanopartikulært materiale i kunstige tarmsaft uden eller med tilstedeværelsen af en fødevare matrix blev demonstreret. En *in vitro*-model baseret på humant follikelstimulerende epithel blev udviklet, men der var en tendens til at overvurdere transporten af nanopartikler over denne cellebarriere. Dette viste, at resultaterne fra *in vitro* modeller på absorption af nanopartikler bør fortolkes med forsigtighed.

Det sidste kapitel i rapporten er afsat til identifikation af huller i vores viden og til anbefalinger vedrørende fremtidig testning af nanomaterialer. Generelt er meget få robuste og validerede *in vivo* absorptionsstudier blevet identificeret. Den kombinerede Klimisch og nanomateriale-karakteriseringsscore tyder på, at især fremskridtet i arbejdet med karakterisering af nanopartikler er begrænset. Dette er sandsynligvis forbundet med tekniske og videnskabelige udfordringer forbundet med påvisning af nanopartikler i biologiske matricer. Detaljerede undersøgelser af indflydelsen af fysisk-kemiske egenskaber på absorption er nødvendige, og kunne i første omgang udføres *in vitro* for at spare tid samt minimere anvendelsen af forsøgsdyr. Når flere data er blevet tilgængelige, kan det blive muligt at overføre viden eller at udvikle "*in situ*" modeller for absorption af "næsten ens" nanomaterialer.

Analytiske metoder til karakterisering af nanomaterialer er under intens udvikling og omfatter våd-kemiske teknikker, der ofte er baseret på atomspektroskopi og på billeddiagnostiske metoder, f.eks elektronmikroskopi. Der er behov for yderligere udvikling af følsomme og pålidelige metoder til påvisning og karakterisering *in situ* og til kvantificering af masse og partikelantal især i fødevarer/foder og i væv/celler indsamlet fra *in vivo* eller *in vitro* modeller. Fra sådanne undersøgelser, er det vigtigt at indsamle oplysninger om, hvorvidt stofferne optages som partikler, ioner eller en kombination af begge. I projekter, hvor forskerne har adgang til en moderne "analytisk værktøjskasse", kan den selektive påvisning af ioner vs partikler af et givet nanomateriale opnås på en række måder. I almindelighed kan metoder, som bygger på kombinationen af en separationsteknik og en selektiv detektor (ICP-MS) være en måde at opnå mere relevante karakteriseringsoplysninger i fremtidige projekter.

Baseret på den nærværende vurdering af nanomaterialer, er materialer med et højt oralt eksponeringsniveau de mest relevante kandidater til fremtidige projekter vedrørende systemisk absorption af nanomaterialer efter oral eksponering. Disse materialer omfatter sølv, siliciumdioxid (E 551), titandioxid (E 171) og zinkoxid.

Summary

In the project “Systemic absorption of nanomaterials by oral exposure” the available knowledge selected from the scientific literature on systemic absorption of nanomaterials following oral exposure is evaluated. The project is part of the “Better control of nano” initiative by the Danish EPA aiming at clarifying possible risks to consumers and the environment upon exposure to nanomaterials.

The overall aim of the project was to gather and evaluate existing knowledge in the area. More specifically the objectives were:

1. To perform an extensive literature search and assessment of the reliability and relevance of studies involving systemic absorption of nanomaterials by oral exposure (Phase I).

2. To evaluate the factors influencing systemic absorption of nanomaterials by oral exposure (Phase II) including:

    a. An evaluation of the physical and chemical properties of nanomaterials described in the literature, which are expected to affect the systemic absorption of nanomaterials following oral exposure.

    b. An evaluation of which test method(s) would most closely simulate systemic absorption of nanomaterials by oral exposure taking into account the complexity of the digestive system and the factors that may have an influence on the possible systemic absorption of nanomaterials.

3. To identify knowledge gaps and research needs and to recommend which models and measurement methods are most suited for simulating systemic absorption of nanomaterials by oral exposure in humans. Finally, relevant nanomaterials should be recommended as candidates for experimental testing in the future (Phase III).

A multi-stage literature search profile was applied to identify relevant scientific papers, which were screened for their relevance regarding potential adverse effects to humans of nanomaterials via oral exposure (pharmaceutica excluded). A total of 64 scientific papers were selected for further evaluation of scientific quality based on the so-called Klimisch criteria (about the conduction of toxicological/biological experiments) and the detail of nanoparticles characterisation was listed. A final number of 47 papers with a Klimisch score of at least 2 (a few papers with a score of 3 were also included based on expert judgement) were considered for further evaluation. The types of nanoparticles that were included in the selected literature included: carbon nanotubes, cerium dioxide, fullerenes, gold, iron oxide, selenium, silicium dioxide, silver, titanium dioxide and zinc oxide.

The evaluation of the physical and chemical properties that were expected to influence absorption of nanoparticles showed that generally few of these parameters were investigated or documented. For some substances an evaluation of factors influencing their systemic absorption following oral exposure cannot be given due to lack of data. This includes carbon nanotubes, cerium dioxide, fullerenes, silicium dioxide and selenium. It was demonstrated that the intestinal uptake of iron oxide, gold and silver nanoparticles was higher for smaller the for larger particle sizes. The

influence on absorption rate of polymeric coatings of silver nanoparticles was investigated, but no clear trend was observed. The solubility of silver and zinc oxide nanoparticles proved to be an unexpected factor in the absorption of the dissolved, ionic forms of these particle types. For titanium dioxide nanoparticles there was no clear information on relationship between size and absorption, but there was some indication that agglomeration of the anatase crystal form could explain the low absorption of this form and indirectly explain why the rutile form of TiO2 was better absorbed than the anatase form.

The methods that were most promising for assessment of systemic absorption of nanomaterials were evaluated. Methods based on *in vitro* testing indeed exist and are currently under development. Further refinement is however needed before they may be applied to assessment of hazard or to absorption of nanoparticles after oral exposure. The most promising type of model should include a synthetic set-up using physiologically relevant conditions (enzymes, pH, salts and temperature) alone or should be used in combination with an *in vitro* absorption model based on e.g. human intestinal epithelium or Caco-2 cells. Silver and silicium dioxide nanoparticles were tested under synthetic conditions, and the existence of nanoparticulate matter of these substances in artificial intestinal juice without or with the presence of a food matrix, respectively, was demonstrated. An *in vitro* model based on human follicle epithelium was developed but tended to overestimate the transport of nanoparticles across the cell barrier. This demonstrated that results from *in vitro* models on absorption of nanoparticles should be interpreted with caution.

The final chapter of the report is devoted to the identification of knowledge gaps and to recommendations for future testing of nanomaterials. Generally, very few robust and validated *in vivo* absorption studies have been identified. The combined Klimisch and nanomaterial characterisation scores suggest that especially the advancement of the characterisation work is limited. This is likely because of the technical and scientific challenges associated with detection of nanomaterials in biological matrices. Detailed studies on the influence of physical chemical characteristics on absorption are needed and could be performed initially in the *in vitro* models in order to save time and animals' lives. When more data has been created, it may be possible to "read across" or to develop "*in situ*" models for absorption of "nearly similar" nanomaterials.

Analytical methods for nanomaterial characterisation are under intense development and comprise wet chemical techniques, often based on atomic spectroscopy, and on imaging methods, e.g. electron microscopy techniques. There is a need for further development of sensitive and reliable methods for the detection and characterization in situ and for quantification of mass and number of NPs especially in food/feed and tissues collected from *in vivo* or *in vitro* study models. From such studies it is important to collect information on whether substances are absorbed as particles, ions or a combination of both. In research projects, where the investigators have access to a modern "analytical toolbox", the selective detection of ions vs. NPs of a given nanomaterial can be achieved in a number of ways. In general, methodologies which rely on a combination of a size separation technique with a selective detector (ICP-MS) may be a way to achieve more relevant characterisation information in future projects.

Based on the present evaluation of different nanomaterials, substances with a high exposure level via the oral route are the likely candidates for future projects on systemic absorption of nanomaterials following oral exposure. Such substances include silver, silicium dioxide (E 551), titanium dioxide (E 171) and zinc oxide.

1. Introduction

1.1 Danish initiative for "Better control of nano"

The Danish government and the Red-Green Alliance (a.k.a. Enhedslisten) have signed an agreement called "Bedre styr på nano" ("Better control of nano") 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 and accompanying literature database is part of this series.

1.2 Project outline

As part of this series of projects on key issues regarding nanomaterials in Denmark, such as occurrence, extent of consumer and environmental exposure and assessment of potential risk, the Danish EPA commissioned the present literature study on the systemic absorption of nanomaterials by oral exposure. The overall aim of the project was to gather and evaluate the existing knowledge in the area and assess the need to generate new knowledge, as well as to develop recommendations for the most suitable models of systemic absorption by oral exposure, measurement methods and suggest relevant candidate nanomaterials for experimental testing in the future.

1.3 Background

Nanomaterials have attracted strong interest in various fields of industry and research, since their small size offers new features and enhanced reactivity in comparison to larger particles of the same chemical composition. Their use can already be found in a broad field of applications, for instance as pigments and resins, as UV-filters in cosmetics, in drug delivery systems, or for applications in medical diagnostics. The food industry is starting to use various nanoparticles as food additives or to improve food packaging in an attempt to optimize their products. While microparticles, such as titanium dioxide (TiO2) or silicium dioxide (SiO2) already have a long standing use as food additives, for example as whiteners (e.g. TiO2), enhancers of viscosity, and fluxing agents (e.g. SiO2) (Chaudhry et al. 2008); (Schmid and Riediker 2008) as cited in (Gerloff et al. 2009), nanoparticles (NPs) may also be used as components of novel food packaging materials in the future due to their gas barrier properties, gas exchange and their antimicrobial properties (e.g. ZnO and MgO). More advanced approaches in the food industry include nanoparticle-based sensors to monitor food edibility or nano-encapsulation applications to improve nutrient stability and targeted delivery (Taylor et al. 2005); (Asuri et al. 2007); (Kaittanis et al. 2007) as cited in (Gerloff et al. 2009).

The human body may be intentionally or unintentionally exposed to nanomaterials via several possible routes, including oral ingestion, inhalation, intravenous injection, and dermal absorption. The behaviour of nanomaterials within the gastro-intestinal tract have been poorly investigated and therefore the fate and effects of nanoparticles ingested via food, water or swallowed following cilia-transport from the lungs, remains essentially unknown.

To date, almost all *in vivo* studies on nanomaterials have focused on the evaluation of acute toxicity and repeated-dose toxicity via different exposure routes. Only few studies have investigated

nanomaterials in animals after oral administration. Such studies include pharmacokinetic studies on their absorption, distribution, metabolism, and excretion (ADME) patterns at the systemic level. Pharmacokinetic studies on nanomaterials is of paramount importance in the context of understanding the amount absorbed via the gastro-intestinal tract and how it enters the systemic circulation as well as the kinetic profile of its clearance by the excretory systems. This will provide clues for the underlying debate on the safety of nanomaterials. In recognising the paucity of knowledge on absorption of nanomaterials in humans after oral exposure the Danish Environmental Protection Agency (DEPA) commissioned this project to review the available, relevant literature on oral absorption of nanomaterials *in vivo* and *in vitro*.

Oral exposure to nanomaterials mainly occurs via ingestion of food e.g. as food additives or due to migration from food contact materials (FCM). Risk assessments of such substances are evaluated by the European Food Safety Authority (EFSA). EFSA has published two Scientific Opinions on nanomaterials in food and feed. In these opinions the term “engineered nanomaterial (ENM)” refers to a nanomaterial produced either intentionally or unintentionally (due to the production process) to be used in the food and feed area.

In the present report we will use the term nanoparticles (NPs) when we are talking about specific particles (e.g. Ag-NPs) and nanomaterials (which includes NPs) as a broader and more general term instead of ENM, because it is used in most of the reviewed articles. Only in this chapter citing the EFSAs guidance document we will use ENM.

The first Scientific Opinion: “The potential risks arising from nanoscience and nanotechnology on food and feed safety EFSA (EFSA 2009)” is generic in nature and is not in itself a risk assessment of nanotechnologies used for food and feed.

In the second Scientific Opinion: “Guidance on the risk assessment of the application of nanoscience and nanotechnology on food and feed chain” (EFSA 2011), which is a follow up of the first report from 2009, guidance is provided on: (i) the physico-chemical characterisation requirements of engineered nanomaterials used e.g. as food additives, enzymes, flavourings, food contact materials, novel foods, feed additives and pesticides and; (ii) testing approaches to identify and characterise hazards arising from nano-specific properties which, in general, should include information from *in vitro* genotoxicity studies, studies on absorption, distribution, metabolism and excretion, and studies on repeated- dose 90-day oral toxicity studies in rodents. The guidance allows for reduced information to be provided when no exposure to the engineered nanomaterial is verified by data indicating no migration from food contact materials or when complete degradation/dissolution is demonstrated with no absorption of engineered nanomaterials as such.

In EFSAs “Guidance on the risk assessment of the application of nanoscience and nanotechnologies in the food and feed chain” (2011) it is emphasized that adequate physical/chemical characterisation of nanomaterialss is essential for risk assessment of the nanomaterialss. The characterization should ideally be determined in five stages, i.e. as manufactured (pristine state), as delivered for use in food/feed products, as present in the food/feed matrix, as used in toxicity testing, and as present in biological fluids and tissues.

The ADME of a nanomaterial are likely to be influenced both by the chemical composition of the ENM as well as its physico-chemical properties (e.g. size, shape, solubility, surface charge and surface reactivity).

When it can be demonstrated that an ENM completely dissolves/degrades in then gastro-intestinal tract without absorption of the ENM, the hazard identification and hazard characterisation can rely on data for the non-nanoform substance (if available).

When information on a non-nanoform of the same substance is available and where some or all of the ENM persists in the food/feed matrix and in gastro-intestinal fluids, a testing approach is recommended, which is based on comparing information on ADME, toxicity and genotoxicity of the non-nanoform with ADME, repeated-dose 90-day oral toxicity study and genotoxicity information of the ENM. When information on a non-nanoform is not available and where some or all of the ENM persists in the food/feed matrix and in gastro-intestinal fluids, the approach for toxicity tests on the ENM should follow the relevant EFSA guidance for the intended use with the modifications in the present ENM Guidance to take into account the nano-specific properties.

At present very limited information is available that EFSAs theoretical approach on risk assessment of nanomaterials in food has been used in practice. One study has been identified in the open literature where EFSAs approach has been used for risk assessment of nanosized silicium dioxide in food (Dekkers et al. 2012). The result of this exercise as regards the intestinal absorption of nanosized silicium dioxide is shortly described in chapter 3.12.

1.4 Current understanding and models of systemic absorption of nanoparticles by oral exposure

The mechanism of translocation (absorption) of nanoparticles over the gastro-intestinal (GI) wall appears to be complex and is poorly understood. For the entire GI tract, composed of the oral cavity, the esophagus, the stomach and the small and large intestines, mucus represents an efficient acellular barrier. The pH varies in the different compartments of the GI tract. The pH of the mucus in the oral cavity is estimated to be around pH 6.6. The gastric mucus shows a wide pH range from 1 to 2 (luminal) to ~7 (epithelial surface) (Frölich and Roblegg 2012) and in the intestines the pH is around 8 (Peters et al. 2012).

The epithelium generally represents the highest resistance against the passage of chemical compounds and NMs. The intestinal epithelium consists of a monolayer of predominantly enterocytes, and in certain regions specialized cells called M-cells (Microfold cells). M-cells are preferentially located in the epithelium overlying the Peyer's Patches (located in the lowest portion of the small intestine, the ileum, in humans) which is also called Follicle Associated Epithelium (FAE). M cells differ from normal enterocytes in that they lack microvilli on their apical surface, but instead possess broader microfolds that give the cell its name. They transport organisms and particles from the gut lumen to immune cells across the epithelial barrier via endocytosis or phagocytosis (transcytosis), and deliver foreign substances to the underlying tissues (mucosa lymphoid) to induce immune responses. However M-cells, are also a potential portal for systemic entry of NPs.

Translocation of NPs through the epithelium is a multistep process, involving diffusion through the mucus lining of the gut wall, contact with enterocytes and particularly the M-cells, and finally uptake via either paracellular (passage through the cells) or transcellular (passage between the cells) transport (Bouwmeester et al. 2011).

Translocation of NPs through the epithelium depends on their physic-chemical properties such as size, surface charge, lipophilicity/hydrophilicity, presence/absence of a ligand (Bouwmeester et al. 2011).

Since the plasma membrane of the cells forming the epithelial barrier is lipophilic, lipophilic substances are taken up passively by the transcellular route whereas hydrophilic substances use the paracellular route. The penetration area of the paracellular route is extremely small compared to the transcellular route and restricted to polar substances below 1000 Dalton. Paracellular transport of chemicals is only passive and NPs are not expected to be able to use the paracellular route, because they are considerably larger than 1000 Dalton.

Several *in vitro* models for translocation of chemicals over the intestinal epithelium have been developed. Generally monolayers of Caco-2 cells are preferred as models (Bouwmeester et al. 2011). However, more recently a more sophisticated *in vitro* model of human Follicle Associated Epithelium (FAE) has been developed by des Rieux and co-workers. This model was developed to study NP transport mechanisms by M-cells (des Rieux et al. 2007). The model is based on a co-culture of inverted transwell inserts of Caco-2 cells and human Raij B lymphocytes. The set-up of the system allows a close contact between the two cell types to trigger the conversion of Caco-2 cells into M-cells. The authors showed an M-cell conversion rate of 15-30% by means of scanning electron microscopy. This *in vitro* model seems to have a great potential in the study of both translocation and toxicity of nanomaterials.

1.5 Aim of the project

The aims of the project are:

Phase I: To perform an extensive literature search and assessment of the reliability and relevance of studies involving systemic absorption of nanomaterials by oral exposure.

Phase II: To evaluate the factors influencing systemic absorption of nanomaterials by oral exposure including:

1) An evaluation of the physical and chemical properties of nanomaterials, described in the literature, which are expected to affect the systemic absorption of nanomaterials by oral exposure such as size, shape, electrical charge, surface coating, leaching of chemicals from nanomaterials, agglomeration etc.

2) An evaluation of which test method(s) would most closely simulate systemic absorption of nanomaterials by oral exposure taking into account the complexity of the digestive system and the factors that may have an influence on the possible systemic absorption of nanomaterials

Phase III: To identify knowledge gaps and research needs and to recommend which models and measurement methods are most suited for simulating systemic absorption of nanomaterials by oral exposure. Finally, relevant nanomaterials should be recommended as candidates for experimental testing in the future.

These assessments will be based on the currently available scientific literature in this area including relevant *in vivo* and *in vitro* data on oral exposure.

2. Phase I: Establishing a database based on a survey of relevant literature

2.1 Literature search

2.1.1 Description of the search strategy

The literature search was performed by using SciFinder encompassing Medline and ChemAbs (for further explanation see Appendix 1, introduction).

Step 1:

In our search strategy we used a stepwise approach starting with the search terms listed in table 1.

FIGURE 1 KEY SEARCH TERMS USED IN STEP 1
This figure shows the seven search terms describing various nanomaterials, which were combined with a total of 23 keywords deemed relevant for the purpose of identifying relevant papers concerning the systemic absorption of nanomaterials by oral exposure.

Nanomaterials	Keywords
Nano	Oral, Gavage, Food, Absorption, Gastrointestinal, GI tract, Digestive tract , Gastric, Saliva, Intestine, Stomach, Duodenum, Jejunum, Ileum, Peyer's patch, Cecum, Colon, Feces, Epithelial barrier, Cell line, Caco-2, Enterocyte, Consumer products.
Microparticle	
Particle	
Particulate	
Colloid	
Quantum dot	
Fullerene	

The results of the searches are shown in Appendix 2.

It was agreed at this step to ignore patents as it was foreseen that most of the patents would relate to medicine science and were thus considered of limited relevance for this project aimed at consumer protection.

Step 2:

The number of hits found in step 1 was too many to continue the process and a step was made to identify commonly used terms, not relevant for the purpose of this project, starting with "nano-" and to see if exclusion of these could diminish the number of hits. The terms were:

Nanogram, nanosecond and nanomet* (meter, metre etc).

The results of the searches are shown in Appendix 3.

It was concluded to exclude these terms in all following searches in order to reach a manageable number of relevant papers to be considered in this first part of the project.

Step 3:

The result obtained in step 2 indicated that an additional step was needed to refine the search further. Based on decisions made by the chemistry and toxicology experts the following combinations were used: The number of hits using the search term “nano” were reduced by refining the search. “Nano” hits also containing the word: “oral”, were identified and all reviews and patents were excluded
The search was then further refined by identifying the previous hits containing the words “uptake” followed by “absorption”. From this result, hits containing the following terms were excluded: “nanogram”, “dermal”, “inhalation”. Thereafter all duplicates of papers were removed. This search was performed 25 January 2013.

Based on this result, the search was further refined by excluding terms: [language: Chinese], drug, insulin, nanomolar, nanoemulsion. This search was performed 30 January 2013.The results of the searches are shown in Appendix 4.

Step 4:

In step 4 the searches exclude the following terms to further reduce the vast amount of hits:

Drug, insulin, cancer, delivery, nanomolar, nanoemulsion and nanogram.

This exclusion was based on expert judgement and found to be acceptable because with these search terms, a lot of papers were identified with no relevance for the project. Typically they were primarily related to health effect and drug delivery systems (e.g. for treatment of cancer). There could be a theoretical risk that cancer studies describing absorption would be excluded. However, it is not expected that oral long term studies have been performed on nanomaterials. Papers in Chinese as well as patent information were also excluded.

This search with “nano” as search topic and the above mentioned exclusion resulted in 836,397 hits (search 207, see Appendix 5.)

These “hits” were used for the following search by combining search 207 with each of the following search terms (a-j)

a)	Oral	(search 208)
b)	Oral + absorption	(search 209)
c)	Oral + uptake	(search 210)
d)	Gavage	(search 211)
e)	Gavage - oral	(search 212)
f)	Gastro-intestinal	(search 215)
g)	Gastro-intestinal - oral	(search 216)
h)	Gastro-intestinal - oral - uptake	(search 217)
i)	Caco-2	(search218)
j)	Caco-2 + uptake	(search 219)

The results of the searches 209 (44 hits), 210 (26 hits), 212 (15 hits), 217 (401 hits) and 219 (33 hits), in total 519 hits are shown in Appendix 6. The abstracts from these searches were saved as .pdf files and .ris files (for later import to the Reference Manager database).

Step 5:

In Step 5 a chemist and a toxicologist evaluated the 519 abstracts for relevance in relation to the project. The inclusion was based on “expert judgement”. As a default, the criteria used for inclusion were:

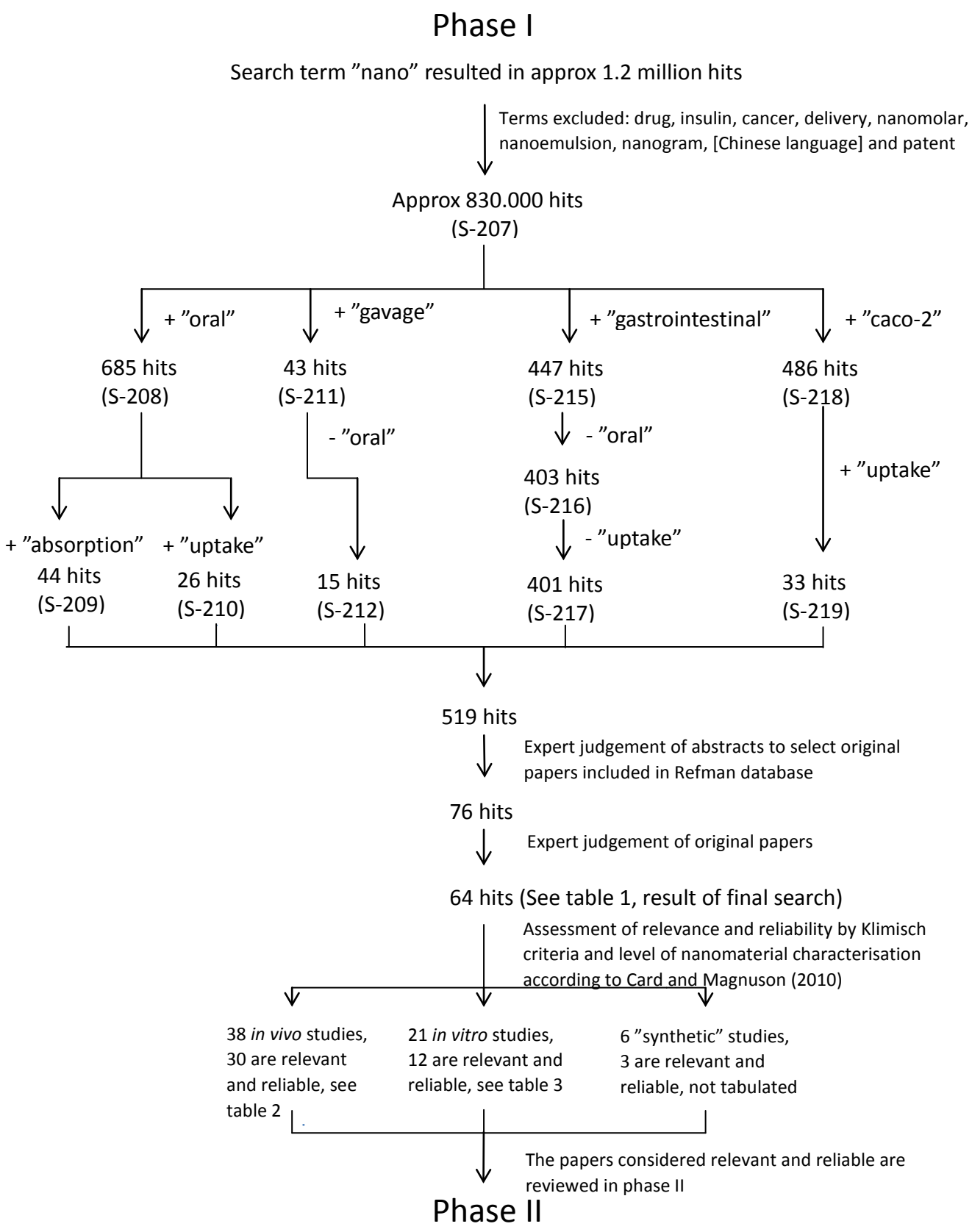
- Only papers describing oral absorption (i.v. and i.p. injection were not included)
- Only papers with relevance for humans i.e. animal studies in mammals/rodents. Studies in snails, fish or insects were not included
- Only studies on nanomaterials used in consumer products or food. Drugs or drug delivery systems were not included

Possible relevant abstracts were included in a Reference Manager database and the original papers downloaded as pdf files. In total, 76 original papers were included in the database.

After a critical review of the 76 papers 64 were found relevant for further evaluation (table 1) and were evaluated in a two-step procedure described by Card et al. 2010 (Card and Magnuson 2010). With a few exceptions only papers with a Klimisch score of 1 or 2 (Klimisch et al. 1997) were described and evaluated in phase II of the project.

Figure 2 summarises phase I of the project “Systemic absorption of nanomaterials by oral exposure”. It concerns the identification of relevant papers in a literature survey performed in 2013 and described in step 1 to step 5, followed by assessment of relevance and reliability by the use of the Klimisch criteria (Klimisch et al. 1997) and nanomaterial characterisation (Card and Magnuson 2010). These papers were further scrutinised in phase II of the project and factors influencing the systemic absorption of nanomaterials after oral exposure were identified. In phase III data gaps and future research needs were identified and discussed.

FIGURE 2 **OVERVIEW OF THE LITERATURE SEARCH AND VALIDATION STRATEGY**



2.1.2 Result of the search

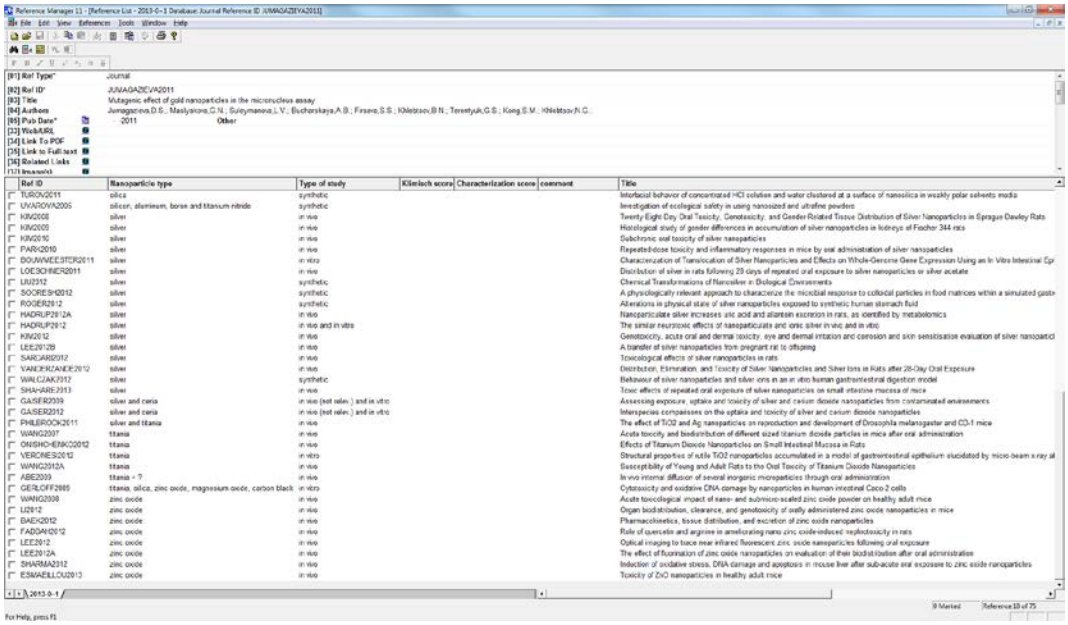
The types of nanomaterials identified, using the described search strategy were:

- Metal oxides: zinc oxide, titanium dioxide, cerium dioxide, silicium dioxide, magnesium oxide, iron oxide
- Metals and metalloids: silver, gold, selenium
- Carbon based: carbon nanotubes, fullerenes
- Quantum dots
- Polymers (PMMA, PLGA, polystyrene)
- Polysaccharides
- Metallo-proteins: ferritin

In the Reference Manager database the inserted user defined keywords by type of particle and study (i.e. *in vivo*, *in vitro* and synthetic) were used to identify original papers describing studies that are either "*in vivo*", "*in vitro*" or "synthetic" and also which naniomaterial type was studied. In this context synthetic means a version of an *in vitro* study in which synthetic experimental conditions have been used aiming at mimicking a set of natural conditions.

This information can be seen as extra columns in the reference list display (see screenshot below).

FIGURE 3 SCREENSHOT FROM REFERENCE MANAGER DATABASE



Using the search term "NP type" (= user Def1) in the Reference Manager database listed abstracts corresponding to the different nanomaterials, which were sub-divided into "*in vivo*", "*in vitro*" and "synthetic". During review of these abstract lists, 9 papers were regarded as irrelevant for this project or not of sufficient quality. Three papers on quantum dots were also not included as this nanomaterial is not considered relevant for consumer products. This resulted in a total of 64 original papers Table 1 below shows the result of the search with the number of papers for the different type of nanomaterials distributed in "*in vivo*", "*in vitro*" and "synthetic" investigations.

TABLE 1 RESULT OF THE FINAL SEARCH WITH THE NUMBER OF PAPERS FOR THE DIFFERENT TYPE OF NANOMATERIALS SUB-DIVIDED BY "*IN VIVO*", "*IN VITRO*" AND "SYNTHETIC" INVESTIGATIONS.

Nanomaterials	*In vivo*	*In vitro*	Synthetic	Total number of original papers
Carbon nanotubes	6	4	1	10
Cerium dioxide	1	1	0	2
Fullerene	1	0	0	1
Gold	2	0	0	2
Iron, Iron oxide, Iron hydroxide and ferritin	2	3	0	5
Polymers (Latex, PLGA, PMMA, polystyrene, polysaccharide)	4	7	0	11
Selenium	0	1	0	1
Silicium dioxide, Silicium aluminium hydroxide	0	0	2	2
Silver	11	3	3	17
Titanium dioxide, silicium dioxide or zinc oxide	4	1	0	5
Zinc oxide	7	0	0	7
	38	**21**[1]	**6**	**64**

[1] One of the *in vitro* studies for carbon nanotubes also contains *in vivo* data, and is consequently reported twice.

2.1.3 Evaluation of the original papers using Klimisch criteria and nanomaterial characterisation

All the papers in the Reference Manager database have been downloaded from the DTU or KU library. The papers were distributed between the participants in the project and evaluated for relevance and reliability using a two-step procedure described in Card (Card and Magnuson 2010 (Card and Magnuson 2010) and Magnuson et al. 2011 (Magnuson et al. 2011). The two-step procedure consisted of an initial “study score” using the ToxRTool (ToxRTool 2013) with Klimisch criteria (Klimisch et al. 1997) followed by an assessment of the level of nanomaterial characterisation.

For this purpose a spreadsheet was made for each nanomaterial and includes in each file evaluation of "*in vivo*", "*in vitro*" and "synthetic" studies (see chapter 2.2.2). In principle only studies with a Klimisch score of 1 or 2 were regarded valid for evaluation and were further assessed for nanomaterial characterisation. However, a few studies with a Klimisch score of 3 were also considered of relevance for this project.

These spreadsheets are submitted together with this report.

Those of the *in vivo* and *in vitro* studies listed in table 1 that were considered relevant and reliable based on this two-step procedure are tabulated in tables 2 and 3 here below. Table 2 list 31 papers covering *in vivo* studies and table 3 lists 10 papers covering *in vitro* studies. The "synthetic" studies are not tabulated, but of the six papers identified three were considered to be of sufficient quality. These are one for nanotubes (Wang et al. 2011) and two papers for silver (Roger et al. 2012 and Walczak et al. 2012).

These papers are used in phase II of the project.

TABLE 2 EVALUATION OF *IN VIVO* STUDIES

Nanomaterials studied *in vivo*	Publication	Species studied	Oral dosing route[1]	Nanostudy score
Carbon nanotubes	Lim et al. 2011a	Rat	Gavage	K1-N0
Carbon nanotubes	Lim et al. 2011b	Rat	Gavage	K1-N0
Carbon nanotubes	Matsumoto et al. 2012	Rat	Gavage	K1-N1
Carbon nanotubes	Awasti et al. 2013	Mice	Gavage	K2-N0
Carbon nanotubes	Sachar and Saxena 2011	Mice	Gavage	K1/3-N0
Cerium dioxide	Park et al. 2009	Rat	Oral	K1-N1
Fullerene	Yamago et al. 1995	Rat	Oral	K2-N3
Fullerene	Yamashita et al. 2013	Mice	Oral	K1-N2
Gold	Zhang et al. 2010	Mice	Oral	K1-N3
Gold	Jumagazieva et al. 2011	Rat	Oral	K2/3-N1
Iron oxide	Singh et al. 2013	Rat	Gavage	K1-N5
Iron oxide	McCullough et al. 1995	Rat	Oral	K2-N0
Silver	Loeschner et al. 2011	Rat	Gavage	K1-N5
Silver	Sardari et al. 2012	Rat	Gavage	K1-N1
Silver	Van der Zande et al. 2012	Rat	Oral	K1-N3
Silver	Kim et al. 2009	Rat	Oral	K2/3-N0
Silver	Kim et al. 2010	Rat	Oral	K1-N1

Silver	Park et al. 2010	Mice	Oral	K1-N1
Silver	Shahare et al. 2013	Mice	Oral	K1-N3
Silver	Hadrup et al. 2012a[2]	Rat	Gavage	K1-N4
Silver	Hadrup et al. 2012b[2]	Rat	Gavage	K1-N4
Silver	Kim et al. 2008[2]	Rat	Oral	K1/3-N0
Titanium dioxide	Wang et al. 2012	Rat	Gavage	K1-N10
Titanium dioxide	Onischenko et al. 2012	Rat	Gavage	K3-N1
Titanium dioxide	Wang et al. 2007	Mice	Oral	K1-N1
Titanium dioxide	Jani et al. 1994	Rat	Gavage	K3-N3
Zinc oxide	Baek et al. 2012	Rat	Gavage	K1-N4
Zinc oxide	Li et al. 2012	Mice	i.p. injection	K1-N4
Zinc oxide	Wang et al. 2008	Mice	Gavage	K1-N5
Zinc oxide	Lee et al. 2012a	Rat	Gavage	K2/3-N2
Zinc oxide	Lee et al. 2012b	Mice	Oral	K1/3-N2

[1] When it is not clear whether the dosing route was via gavage or dietary the term “oral” is used.
[2] These articles were not deemed relevant for oral absorption when reviewed in phase II.

TABLE 3 EVALUATION OF *IN VITRO* STUDIES

Nanomaterials studied *in vitro*	Publication	Cell type	Nanostudy score
Carbon nanotubes	Jos et al. 2009	Caco2	K1/3-N0
Carbon nanotubes	Szendi and Varga 2008	Human lymphocytes S. typhimurium	K1/3-N0
Carbon nanotubes	Cicchetti et al. 2011	Human gingival fibroblasts	K1/3-N0
Carbon nanotubes	Sachar and Saxena 2011	Erythrocytes	K1/3-N0
Cerium dioxide	Gaiser et al. 2009	C3A human hepatocyte Caco2	K1-N4
Selenium	Wang, Fu 2012	Caco2	K1-N2
Silicium dioxide	Peters et al. 2012	Model of human digestion	
Silver	Gaiser et al. 2009	C3A human hepatocyte Caco2	K1-N3
Silver	Bouwmeester et al. 2011	Caco2 cells and human Raij B lymphocytes	K3-N6

Titanium dioxide	Koeneman et al. 2010	Caco2	K1-N0

Three additional articles were identified during the review of the identified articles in the literature search. These were a paper by Kolashnjajtabi et al. 2010 concerning carbon nanotubes, a paper by So et al. 2008 concerning silicium dioxide and a paper by Hillyer and Albrecht 2001 concerning gold. These papers were considered of sufficient quality to be included in the phase II of this project.

2.2 Description of the database

2.2.1 Reference Manager database

As already described in section 2.1.2 condensed information about all original peer-reviewed papers has been downloaded to Reference Manager 11 software (network version). The inserted user defined keywords allowed for sorting the papers not only by the available database information (such as author, journal, publication year etc.), but also by type of particle and study (i.e. *in vivo*, *in vitro* and synthetic).

2.2.2 Extraction of information into spreadsheet format

Information contained in each paper in the Reference Manager database was extracted, evaluated by the experts of the project team and appropriate scores allocated. The spreadsheet contains 21 criteria (Klimisch criteria) and 10 criteria describing the characterisation of the investigated NPs. A score "1" or "0" means that the criterion has been complied with or not complied with, respectively. A subset of the Klimisch criteria was deemed of particular importance for the overall evaluation and is marked in red (figure 4). Failing to comply with these criteria may have led to a down-adjustment of the initially assigned category.

FIGURE 4 EXAMPLE OF ALLOCATION OF KLIMISCH SCORE AND CHARACTERISATION SCORE.

Article ID (e.g. Kong2004a)	**Loeschner2011**
nominal particle type (e.g. silver, gold, fullerene)	Silver
Coatning	PVP
Other phys Chem parameters e.g. chrystalinity	-
nominal particle diameter (in nm)	14
Mechanistic absorption study (no tox, no Klimisch eval.)	
Evaluation of the study according to Klimisch - Answer "0" (no) or "1" (yes):	
1. Was the test substance identified?	1*
2. Is the purity of the substance given?	1
3. Is information on the source/origin of the substance given?	1
4. Is all information on the nature and/or physico-chemical properties of the test item given, which you deem indispensable for judging the data (see explanation for examples)?	1
5. Is the species given?	1*
6. Is the sex of the test organism given?	1

7. Is information given on the strain of test animals plus, if considered necessary to judge the study, other specifications (see explanation for examples)?	1
8. Is age or body weight of the test organisms at the start of the study given?	1
9. For repeated dose toxicity studies only (give point for other study types): Is information given on the housing or feeding conditions?	1*
10. Is the administration route given?	1*
11. Are doses administered or concentrations in application media given?	1
12. Are frequency and duration of exposure as well as time-points of observations explained?	1
13. Were negative (where required) and positive controls (where required) included (give point also, when absent but not required, see explanations for study types and their respective requirements on controls)?	1
14. Is the number of animals (in case of experimental human studies: number of test persons) per group given?	1*
15 .Are sufficient details of the administration scheme given to judge the study (see explanation for examples)?	1*
16. For inhalation studies and repeated dose toxicity studies only (give point for other study types): 17. Were achieved concentrations analytically verified or was stability of the test substance otherwise ensured or made plausible?	1
17. Are the study endpoint(s) and their method(s) of determination clearly described?	1*
18. Is the description of the study results for all endpoints investigated transparent and complete?	1*
19. Are the statistical methods applied for data analysis given and applied in a transparent manner (give also point, if not necessary/applicable, see explanations)?	1
20. Is the study design chosen appropriate for obtaining the substance-specific data aimed at (see explanations for details)?	1*
21. Are the quantitative study results reliable (see explanations for arguments)?	1*
sum	**21**
Numerical result leads to initial Category:	1
Checking * scores leads to revised Category:	1

Evaluation of the NP characterization according to Card et al - Answer "0" (no) or "1" (yes)	
1. Agglomeration and/or aggregation	1
2. Chemical composition	0
3. Crystal structure/crystallinity	0
4. Particle size/size distribution	1
5. Purity	0
6. Shape	1

7. Surface area	0
8. Surface charge	1
9. Surface chemistry (including composition and reactivity)	0
10. Whether any characterization was conducted in the relevant experimental media.	1
score	**5**

Evaluation of the information regarding absorption of NPs - Answer with text	-
Was the absorption process itself studied?	No
Which method was used to detect the NPs?	ICP-MS
Did the method allow to prove that the NPs in tissues were the pristine NPs?	Yes
Was the absorption rate determined?	No
Further results / conclusions	-

The resulting score of the example paper in figure 4 was K1-N5.

2.3 Quality assurance of the search strategy

The quality assurance auditor observed that the documentation of the selection process was following the recommendations in EFSA guidance for those carrying out systematic reviews (EFSA 2010). It was also observed that the acceptance criteria for the search terms were based on an expert evaluation.

To validate the selected references the project coordinator made a list of relevant references based on earlier studies (ENRHES 2009), (Mikkelsen et al. 2011) and relevant original papers already known to the DTU project group considered pertinent to retrieve by the chosen search strategy before the search procedure with selection of references took place. This list was used as a comparator. Of these references, one was missing and one was an old reference without the key selection word “nano-”. Retrospectively, the missing reference was reassessed by the project coordinator and found to be less relevant for absorption after oral exposure (measurement of oxidative damages in different organs).

3. Phase II: Review of current knowledge on absorption of different nanomaterials after oral exposure

In this chapter the literature of acceptable quality (see chapter 2.1.3) is summarized. The usage of each nanomaterial is shortly described. The "key study" for each nanomaterial is summarized and a short description with the most important data is given for "supplementary studies". A final conclusion on each nanomaterial includes 1) identified data gaps in relation to the requirements in the EFSA guidance on risk assessment of nanomaterials 2) future research needs and 3) evaluation of factors influencing systemic absorption of nanomaterials after oral exposure. These items are based on *in vivo* studies.

The *in vitro* studies included in the database are evaluated for relevance for human absorption.

3.1 Carbon nanotubes

3.1.1 Usage

Carbon nanotubes (CNTs) are seamless cylindrical structures comprising single or multiple concentric graphene sheets. Single-wall CNTs (SWCNTs) have a diameter of 1–2 nm and a length of up to 100 µm. Multi-wall CNTs (MWCNTs) consist of several layers of carbon cylinders, which increase the diameter to 10–30 nm. They possess unique electrical, mechanical, and thermal properties, with a potential for a wide range of applications in electronics, computer, aerospace, architecture, and other industries. CNTs have the strongest tensile strength of any synthetic fibre. A composite material containing CNTs may have great strength, potentially sufficient to allow the building of spacecraft structures, space elevators, artificial muscles, combat jackets, membranes for gas separation and land and sea vehicles (Lam et al. 2006). In addition, CNTs are of special interest as potential tools for biomedical applications (Kolasnjajtabi et al. 2010), in which suitable modified CNTs can serve as drug delivery systems (Bianco et al. 2005).

3.1.2 *In vivo* studies

Absorption following oral exposure to CNTs has not been studied in experimental animals. Five papers that deal with oral toxicity of CNTs in laboratory animals did not measure levels of CNTs in blood and/or tissues, thus providing only indirect evidence for absorption of CNT related materials. These studies are summarized in the following.

(Kolasnjajtabi et al. 2010) investigated granuloma formation and the toxicity after large doses of ultra-short and full-length SWCNTs in Swiss mice. Three different SWCNTs, were investigated.

Suspended by ultrasound in 0.9% aqueous NaCl solution containing 0.1% Tween 60: raw SWCNTs (R-SWCNTs), purified SWCNTs (P-SWCNTs) and ultrashort SWCNTs (US-SWCNTs) were characterized with respect to a number of metrics, including diameter, length, iron content, and surface area. The US-SWCNTs had a diameter of 1 nm and were 20-80 nm in length whereas the length of the two other CNTs was in the micrometer size range. Transmission electron microscopy (TEM) was used to image the CNTs in suspension and in tissues, urine and faeces following administration. TEM micrographs showed that the R- and P-SWCNT suspensions used were mainly composed of tangled flexible bundles of nanotubes, while the US-SWCNT suspensions were mainly composed of short, compact bundles of aggregated US-SWCNT.

In an acute oral toxicity test, Swiss mice (10 per group) received a single oral bolus dose of 1000 mg/kg bw of either R-SWCNTs, P-SWCNTs or US-SWCNTs and were sacrificed after 14 days. Irrespective of length, surface area, surface interaction, or iron content of the CNTs, no granuloma formation or acute oral toxicity was observed after a single bolus administration of up to 1000 mg/kg bw in mice. It was not shown in this study, whether the CNTs was absorbed.

In order to ensure full systemic bioavailability, the CNTs were administered to the mice by intra-peritoneal injection (i.p.). Groups of six mice were administered the three different SWNTs at single, increasing i.p. doses (50, 300, and 1000 mg/kg bw for the US-SWCNTs and 50, 300, and 500 mg/kg bw for the R- and P-SWCNTs). The animals were kept under observation until day14 when sacrificed.

Granulomas loaded with large CNT aggregates (mostly >10 µm in length) exhibiting fibre-like structures, were mainly formed by phagocytic cells and foreign body giant cells on the organ surfaces. However, in contrast to the short, compact bundles of US-SWCNTs, the large SWCNT bundles did not diffuse inside the organs. This explains the scarcity of granulomas inside the organs in the case of the large SWCNTs. Smaller aggregates did not induce granuloma formation, but they persisted inside cells. Short (<300 nm) well-individualized SWCNTs could escape the reticuloendothelial system to be excreted through the kidneys and bile ducts. In parallel with granuloma formation, numerous SWCNT aggregates smaller than 10 µm in length were observed within phagocytic cells of liver and spleen without subsequent granulomatous reaction. In some cases, small CNT aggregates were observed inside Ito cells similar to what occurs for fullerene. In very few cases, small US-tube aggregates were observed inside renal tubule epithelium cells. Microscopic examinations of all other organs (heart, lungs, and brain) did not reveal any deposit of the injected material, indicating that under these conditions most of the injected material was captured by the liver and spleen.

Conclusion: No granuloma formation or acute oral toxicity was observed after single oral bolus doses of up to 1000 mg/kg bw to mice of either R-SWCNTs, P-SWCNTs or US-SWCNTs, irrespective of length, surface area, surface interaction, or iron content of the SWCNTs. Because after intraperitoneal administration, SWNTs, irrespective of their length or dose (50-1000 mg/kg bw), could coalesce inside the body to form fiber-like structures, that when structure lengths exceeded 10 µm, they irremediably induced granuloma formation. Smaller aggregates did not induce granuloma formation, but they persisted inside cells for up to 5 months after administration this could indicate no or low oral absorption of the SWNTs.

Lim and co-workers (Lim et al. 2011a and Lim et al. 2011b) administered 0 (control), 40, 200 or 1000 mg multi-wall CNTs (MWCNTs)/kg bw/day orally by gavage to pregnant Sprague-Dawley rats (N=12/group) from gestation days 6 through 19. The MWCNTs used were commercially available with a nominal diameter of 10-15 nm and length around 20 µm. The purity was stated to be 95% carbon and approximately 5% iron. The authors did not embark on any physico-chemical characterization and did not determine if aggregation of the CNTs occurred following the only 3 minutes ultrasound treatment in 0.1% carboxymethylcellulose (stabilizer) solution in water.

According to the authors the no-observed–adverse-effect-level (NOAEL) was 200 mg MWCNTs/kg bw/day for maternal toxicity and 200 mg MWCNs/kg bw/day for developmental toxicity.

Conclusion: This study was not designed to be an absorption study, but, the toxic effects seen at the highest oral dose (1000 mg/kg bw/day), might give some indirect indication that material related to the MWCNTs was absorbed.

Matsumoto and co-workers (Matsumoto et al. 2012) studied the acute and 28-days subchronic toxicity of single wall SWCNTs or MWCNTs following administration of the test materials by gavage to Crl:CD (SD) rats. No acute toxicity was seen after SWCNTs and MWCNTs doses up to 50 mg/kg bw and 200 mg/kg bw, respectively, and no subchronic toxicity was seen after doses up to 12.5 mg/kg bw/day of tSWCNTs (N= 5 or 10/sex/group), or 50 mg/kg bw/day for MWCNTs (N=6 or 12/sex/group) the highest doses tested

The purity of the SWCNTs and MWCNTs were >95% and they were principal samples in the OECD Sponsorship Programme on the Testing of Manufactured Nanomaterials. The structure of SWCNTs was described as honeycomb carbon lattice rolled into cylinder with diameter of around 2 nm mixed with bundles with diameters of several tens of nanometres. The structure of MWCNTs was carbon lattices rolled into a multi-layer tubular shape with diameter of around 30 nm. None of the materials were coated or modified. The vehicle used for stabilization of the ultra-sound suspended test suspensions was 5% gum acacia in aqueous solution. The homogeneity of test suspensions was confirmed by light microscopy.

Conclusion: The authors suggested that SWCNTs and MWCNTs dosed by gavage reached the gastro-intestinal tract as agglomerates and were mostly excreted via faeces but no investigations and results to support this suggestion were presented.

Awasthi and co-workers (Awasthi et al. 2013) administered male Swiss albino mice (N=6/group) single doses of 0 (vehicle control, distilled water), 60, or 100 mg/kg bw) of MWCNTs and studied hepatotoxicity on post dosing days 7, 14, 21 and 28 using liver SOD and CAT activity and microscopic examination as end-points. The tested MWCNTs, which were synthesised by chemical vapour deposition (CVD) technique, were purified and washed to remove metallic and carbonaceous impurities. Their size range was determined by SEM as 20–30 nm and length of 5–50 µm. The testing suspensions were made by physical mixing and ultrasonication of surface-oxidised material, but any further data on characterization or aggregation was missing.

Slight hepatotoxicity was reported at both dose levels, however, no incidences of the lesions were presented to enable comparison with the control group and support their relation to the treatment.

Conclusion: The study does not support that any oral absorption of the test material occurred in mice.

Sachar and Saxena (Sachar and Saxena 2011) administered single doses (100 µg/animal) of either SWCNTs or acid functionalized SWCNTs (AF-SWCNTs) to inbred Swiss and C57BL76 female mice (6–12 week old, weighing 20-25 g; number per group not reported) by either intratracheal instillation, intravenous (i.v.) or intra-peritoneal (i.p.) injections, or orally by gavage. The acid functionalized (AF)-SWCNTs were surface oxidized by a mixture of nitric and sulphuric acid under pressure at elevated temperature. The carboxylic acid moieties formed were derivatised by a fluorophor for imaging purposes, and were intensively purified to remove excess fluorescent dye. The particle size distribution and surface charge was not indicated.

A transient decrease was observed in the number of erythrocytes and levels of blood haemoglobin (from 3 to 48 hours but not after 72 hours) after i.v. injection and to a lesser extent after i.p. injections of AF-SWCNTs as compared to SWCNTs. Administration of AF-SWCNTs through oral

gavage and the i.p. route did not reduce erythrocyte count (haemoglobin was apparently not measured for these routes of as no information is given in the paper).

Conclusion: The study did not give any indication that the SWCNTs were absorbed in mice via the oral route of exposure.

3.1.3 *In vitro* studies

Four *in vitro* studies concerning toxicity of CNTs were identified. These gave indirect information on absorption. Jos and co-workers (Jos et al. 2009) exposed differentiated and non-differentiated Caco-2 cells (a cell line established from a human colon carcinoma, used as an enterocytic model) to carboxylic acid functionalized SWCNTs (COOH-SWCNTs) to concentrations between 5 and 1000 µg COOH-SWCNTs/ml for 24 hours. The average diameter of individual SWCNT was 1.4 ± 0.1 nm, and bundle dimensions were 4-5 nm x 0.5–1.5 µm (according to the provider). The COOH-SWCNTs had a content of nickel around 5-10%. Test suspensions were prepared in serum-free medium. After 24 hours of exposure a concentration dependent trend in cytotoxicity (based on neutral red uptake, tetrazolium salt metabolisation, LDH leakage, viability, and histopathology) was seen, becoming clear at a concentration of 100 µg/ml COOH-SWCNTs. Agglomerates observed in some of the cells exposed to higher concentrations of the test material were considered by the authors of the study to be agglomerates of non-dispersed COOH-SWCNTs.

Conclusion: This *in vitro* study may suggest that COOH-SWCNTs were absorbed or could enter the cells under the conditions of this assay, but only gave limited information on the potential absorption in humans after oral exposure.

Szendi and Varga (Szendi and Varga 2008) studied the possible genotoxicity of SWCNTs (<2nm x 4–15 µm, purity: 90%) and MWCNTs (10-30 nm x 1-2 µm; purity: 95% - 98%) dispersed in carbopol-based semiliquid gel. Urine samples obtained 24 hours after treatment by oral gavage of Fischer-344 male rats (N=3/group) with single doses of 0 (vehicle) or 50 mg/kg bw of SWCNTs or MWCNTs, were 10x concentrated and were tested in bacterial mutation assay (Ames test) in Salmonella typhimurium TA98 and TA100 strains with and without metabolic activation. Oral exposure to the nanotubes did not increase urinary mutagenicity under the conditions of the assay. In addition, no genotoxic effects of SWCNTs or MWCNTs were found in the *in vitro* micronucleus and sister chromatid exchange assays using human lymphocytes.

Conclusion: The negative results of these assays neither proved nor exclude the absorption of NPs into the test cells.

Cicchetti and co-workers (Cicchetti et al. 2011) exposed human gingival fibroblasts in semiconfluent cultures to SWCNT concentrations between 50 and 150 µg SWCNTs/ml for 24 hours. The SWCNTs used were oxidized by treatment with a mixture of nitric and sulphuric acids. The surface area of was 407 m^2/g, and the average external diameter was 1.58 nm ± 0.20 nm and the average length was 0.76 µm ± 0.70 µm. The SWCNTs were reported by the authors to have “a relatively high degree of crystallinity”. The authors reported a genotoxic effect ([DNA damage by the alkaline comet assay (from 75 µg/ml) and increase (at concentrations up to 100 µg/ml) or decrease (125 and 150 µg/ml) in the frequency of micronuclei]), decrease in cell proliferation and survival (125 and 150 µg/ml), increase in reactive oxygen species production (at all concentrations) and Hsp70 induction (at all concentrations).

Conclusion: The effects seen *in vitro* indicated that SWCNT related material was absorbed into the cells, but did not prove the absorption of any intact nanomaterial.

Sachar and Saxena (Sachar and Saxena 2011) investigated the uptake of either SWCNTs or acid functionalized SWCNTs (AF-SWCNTs) in erythrocytes isolated from Swiss or C57BL76 female mice.

The acid functionalized (AF)-SWCNTs were surface oxidized by a mixture of nitric and sulphuric acid under pressure at elevated temperature. The carboxylic acid moieties formed were derivatised by a fluorophor for imaging purposes, and were intensively purified to remove excess fluorescent dye. The particle size distribution and surface charge was not indicated. Particle size distribution and surface charge on AF-SWCNTs were reported before (Saxena et al. 2007 as cited in (Sachar and Saxena 2011).

A dose and time dependent decline (70 to 90%) in erythrocyte recovery was recorded in cultures treated with AF-SWCNTs (concentrations of 10, 25 or 50 µg/ml), while treatment with SWCNTs (50 µg/ml) had no effect on erythrocyte recovery as compared to the untreated control groups. Furthermore, the authors reported an increase in the binding of 8-anilino naphthalene sulfonic acid to erythrocytes treated with AF-SWCNTs, which according to the authors indicated a significant damage of the erythrocyte membrane after exposure to the AF-SWCNT NPs. When erythrocytes freshly derived from female C57BL/6 mice were incubated with an AF-SWCNT preparation (50 µg/ml) tagged with a fluorescence probe, 69% of the erythrocytes were positive for fluorescence indicating an uptake or association with AF-SWCNT. After washing 18.40% of the erythrocytes were still positive for fluorescence. This suggested that AF-SWCNTs could associate with erythrocytes in a loose as well as a relatively stronger manner. Examination by confocal microscopy of erythrocytes incubated with fluorescence tagged AF-SWCNTs showed the localization of fluorescence in erythrocytes.

Conclusion: This study suggested that some fluorescence related to exposure to fluorescence tagged AF-SWCNTs could enter erythrocytes, but no clear evidence about absorption of the intact NPs after oral exposure to SWCNT was provided.

3.1.4 Synthetic set-ups

One study was identified, which investigated the adsorption of phenanthrene on CNTs and the bioavailability of adsorbed phenanthrene in simulated gastric fluids (a pepsin solution (a simulation of a gastric environment) and a bile salt fluid (a simulation of intestinal environment) (Wang et al. 2011). CNTs can be carriers of hydrophobic organic compounds (HOCs) because of their high adsorption affinity and capacity. Polycyclic aromatic hydrocarbons (PAHs) are used in the synthesis of CNTs and they can potentially be adsorbed on any raw CNTs. Sorption by CNTs may therefore alter the fate, motility and bioavailability of PAHs.

Adsorption of phenanthrene on CNTs was suppressed in simulated gastric fluids. Pepsin and bile salts increased the desorbing of the phenanthrene fraction from CNTs (in less than 1 h). Forty three to 69% of phenanthrene was released from CNTs after desorption in the simulated gastric fluid and intestinal fluid with low bile salt concentration. Higher release of phenanthrene was recorded at high bile salt concentration (53-86%).

Conclusion: The possible transmission of HOC contaminants to the human gastro-intestinal system along with CNTs should be considered when performing risk assessments of CNTs.

3.1.5 Conclusion on the studies concerning systemic absorption of CNTs following 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 ob-served in animal treated with oral doses of CNS 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.

One of the reviewed *in vitro* studies reported presence of agglomerates, which were considered by the authors to be non-dispersed COOH-SWCNTs in Caco-2 cells following the exposure via addition of these nanotubes to the culture media, and when examined by light microscopy (Jos et al. 2009). Corresponding examination by confocal microscopy of erythrocytes exposed to AF-SWCNTs suggested that some AF-SWCNTs could enter erythrocytes (Sachar and Saxena 2011). Whether this only occurred at cytotoxic concentration is not clear. These *in vitro* observations solely suggest that the CNTs may be able to enter the cells, but do not provide any basis to conclude that CNTs can cross the intestinal wall and enter into circulation.

3.1.6 Evaluation of factors influencing systemic absorption of CNTs following oral exposure

In light of the occurrence of mainly negative data on absorption of CNT following oral exposure no evaluation of factors influencing their systemic absorption can be given.

3.1.7 Identification of gaps in current knowledge and future research needs in relation to CNTs

Carbon is present in every living cell. Thus lack of techniques which can discriminate the con-tent of the exogenous carbon from CNTs from an abundant background of the endogenous carbon in the blood and other tissues may be a reason for lack of studies of absorption, organ distribution and excretion of CNTs following oral exposure. Despite its high price level, a possible future way to study CNTs (and other carbonaceous materials) would be to synthesize CNTs on the basis of enriched stable ^{13}C sources or by irradiation of naturally abundant carbon isotopes to generate *in situ* the isotopically enriched nanomaterial. In principle, a similar approach could be taken using non-stable (radioactive) ^{14}C, but this would be detrimental due to possible toxic effects. Selective detection of enriched ^{13}C could be carried out by isotope ratio mass spectrometry against the natural abundance of this isotope. This however, requires covalent binding of the enriched stable ^{13}C. A different strategy would be to insert markers (rare earth elements; fluorescent markers) and then detect the CNTs by way of such markers. Again, the stability of the marker is crucial for its practical use. Furthermore, because CNTs often are being used as their surface-derivatives (carboxy-, hydroxyl-, ammonium-, or possibly apolar moieties) a study of the influence of such derivatisations on intestinal uptake and translocation is missing. Hereby, more knowledge about the influence of surface charge on the possible intestinal uptake can be derived. The absorption, organ distribution and excretion of CNTs following oral administration remain to be established.

The study by Sachar and Saxena (Sachar and Saxena 2011) demonstrated that AF-SWCNTs were more toxic to murine erythrocytes both *in vivo* and *in vitro*. This may suggest that the acid functionalisation of CNTs may affect their toxicity as compared to parent CNTs. Studies comparing toxicity of modified CNTs and non-modified ones may be needed in order to perform hazard identification and hazard characterization of the modified material. It cannot be ruled out that reading-across from toxicity data for non-modified CNTs may underestimate the toxic potential of modified CNTs

3.2 Cerium dioxide

3.2.1 Usage

Cerium dioxide NPs (CeO2-NPs) have been developed as a fuel additive to reduce particulate matter emissions from diesel engines in an attempt to reduce the adverse health effects ordinarily associated with particulate exposure (Fall et al. 2007). Other potential industrial uses of CeO2-NPs

may be applications for solar cells, gas sensors, oxygen pumps, and glass/ceramic applications (Park et al. 2009).

3.2.2 *In vivo* studies

One relevant *in vivo* study on organ distribution after oral exposure was identified and is summarised below.

Park and co-workers (Park et al. 2009) studied organ distribution of CeO2-NPs in a toxicity study where male SD rats received orally by gavage a single dose of 0 (control, distilled water, N=12), 100 mg/kg bw (N=18) or 5 g/kg bw (N=18) of CeO2-NPs. The CeO2-NPs used in the study had crystallite size of 30 nm and were suspended in distilled water and was sonicated for 30 min before their administration to the animals. Scanning electron microscope (SEM) images of the CeO2-NPs justified that the material consisted of nanosized, but aggregated structures. The rats were sacrificed on days 1, 7 and 14 after treatment [N= 4 (control) or 6 (treated) per sacrifice]. Determination of CeO2-NPs in blood and tissues was not attempted. Ce concentrations were analysed in tissues from liver, kidney, spleen, lung, testis and brain using ICP-MS.

There is not sufficient evidence for the statement that the crystallites indeed were 30 nm in size, and it therefore remains unclear to which size of CeO2-NPs that the animals were actually exposed. Increased levels of Ce, as measured by inductively coupled plasma mass spectrometry (ICP-MS), were found in all examined tissues of the animals treated with the highest dose while this only was seen in the lungs from animals given the low dose, when compared to the control values. This indicates a low degree of absorption of cerium following exposure to CeO2-NPs. No results were presented to demonstrate that Ce-containing NPs were detected in the tissues.

No mortality occurred and no changes in clinical appearance, haematology, serum clinical chemistry parameters, and microscopically examination of liver, lung and kidney from animals sacrificed on day 14 after treatment. According to the authors of the study the reason for the lack of toxicity could be low absorption of the test material from the gastro-intestinal tract as indicated by a white colour of the faeces.

Conclusion: The results of the study by Park and co-workers indicate low bioavailability of cerium after oral administration of CeO2-NPs. However, the study cannot clarify whether the absorption took place in the form of CeO2-NPs or as Ce ions, or a mixture of both.

3.2.3 *In vitro* studies

One relevant *in vitro* study was identified.

Gaiser and co-workers (Gaiser et al. 2009) investigated the uptake of CeO2-NPs by C3A cells, a human hepatocyte cell line, and Caco-2 cells, a human intestinal epithelial cell line. The CeO2-NPs were <25 nm (CeO2-NPs) and <5 µm (bulk CeO2) in nominal size. The particles were dispersed by sonication at 1 mg/ml in culture medium for 15 minutes.

To assess particle uptake C3A cells plated on glass cover-slips and Caco-2 cells plated on Transwell membranes were incubated with media only (negative controls) or particles suspensions at 3.125 and 31.25 µg/cm2 of CeO2 of both sizes for 2 hours (C3A) or 24 hours (Caco-2). According to the authors of the study both sizes of CeO2 were taken up into both C3A and Caco-2 cells but data to document this statement were not given in the paper.

Conclusion: This *in vitro* study was not adequately reported to judge on the potential uptake of CeO2-NPs in human hepatocytes or enterocytes.

3.2.4 Synthetic set-ups

No relevant studies were identified.

3.2.5 Conclusion on the studies concerning systemic absorption of CeO2-NPs following oral exposure

The only *in vivo* study available measured the organ distribution of Ce after oral administration of CeO2-NPs (Park et al. 2009) to rats and found low levels in major organs which indicated low absorption. Whether the absorption was in the form of Ce ions or as CeO2-NPs, or both, was not shown. The available *in vitro* study was not adequately reported to judge on the potential uptake of CeO2-NPs in human hepatocytes or enterocytes.

3.2.6 Evaluation of factors influencing systemic absorption of CeO2-NPs following oral exposure

The available literature do not provided data for an evaluation to be performed.

3.2.7 Identification of gaps in current knowledge and future research needs in relation to CeO2-NPs

Studies investigating the blood levels, faeces content and urine concentration of intact CeO2-NPs following oral exposure are necessary in future research to assess absorption and fate of the NPs in experimental animals. Studies comparing absorption, distribution and excretion of the same doses of CeO2-NPs and bulk CeO2 are needed to elucidate potential differences or similarities of fate in the body in order to decide whether read–across from toxicity data of the bulk material to nanoparticulate material would be justified for purpose of hazard identification and hazard characterization. Lastly, the possible dissolution behaviour of CeO2-NPs is not reported following per oral administration and should be investigated to allow evaluation of the nanoparticulate material vs. possible dissolved Ce species.

3.3 Fullerenes

3.3.1 Usage

Several areas of use have been proposed for fullerenes such as targeted drug delivery (Vogelson 2001), as 'molecular ball bearings', allowing surfaces to glide over one another, acting as a lubricant (Holister et al. 2003), as reinforcement of a polymer matrix which can lower the density of the resulting material.

Fullerene C60, which is a ball-shaped molecule containing 60 carbon atoms, is used in cosmetics to reduce oxidative stress in the skin. Water soluble fullerene C60 may be used as whitening agent. Water soluble fullerene C60 derivatives were reported to show promise for the treatment of various inflammatory diseases (Yamashita et al. 2013).

3.3.2 *In vivo* studies

Two *in vivo* studies were identified; one of them investigated the oral absorption of a radio-labelled, water soluble fullerene in rats. The other study was an oral toxicity study, but absorption as such was not investigated.

Yamago and co-workers (Yamago et al. 1995) administered a single dose of ^{14}C labelled water-soluble C60 fullerene either orally (presumably by oral gavage, but this information was not given) (18 kBq) or by intravenous injection (i.v.) (9 kBq) to Fischer rats (N=3 males/group; body weight of 85–95 g). Radioactivity was measured in the liver, spleen, lungs, kidneys, heart, brain, testes and blood for up to 160 hours after treatment, in faeces up to 2 days and in urine up to 30 hours.

Following i.v. administration the radioactivity in the blood decreased rapidly and was distributed to various tissues, in particular the liver. In the liver, 73% of the total radioactivity was found after 1 hour, the maximal amount of 92% was recorded after 16 hours, and after 30 hours 80% of the total radioactivity still pertained in the liver.

Conclusion: Following oral administration, absorption of the water soluble fullerene was poor: Ninety-seven per cent of radioactivity was excreted in faeces within 48 hours and only trace radioactivity was found in the liver (and other tissues) after the 3 and 6 hour periods.

Yamashita and co-workers (Yamashita et al. 2013) administered 6 weeks old female C57BL/6 mice (N=6-7/group) 0 (controls) or 50 mg/animal/day (equivalent to 2000 mg/kg bw/day) of polyvinylpyrrolidone (PVP)-wrapped fullerene C60 (PVP-fullerene C60) orally by gavage for 7 days. The PVP-fullerene C60 was used after 5 minutes of sonication and 1 minute of vortexing. The mean size and size distribution were measured by using dynamic light scattering; zeta potential was measured by using laser doppler electrophoresis. The particle size of PVP-fullerene C60 in distilled water was 127 nm, and its zeta potential was -2.2 mV. No effects were seen on body weights, hematological, and clinical chemistry parameters, organ weights and histology.

Conclusion: No toxicity could be demonstrated in this oral toxicity study in mice given large doses of PVP-fullerene C60, which could indicate a low or absent oral absorption.

3.3.3 *In vitro* studies

No *in vitro* studies were identified for fullerenes.

3.3.4 Synthetic set-ups

No relevant studies were identified.

3.3.5 Conclusion on the studies concerning systemic absorption of fullerenes following oral exposure

The only study identified, which compared the distribution of radioactivity after a single dose of 14C labelled water-soluble C60 fullerene following oral and intravenous administration indicates very limited, if any, absorption after oral exposure (Yamago et al. 1995).

In the toxicity study by Yamashita and co-workers (2013) large oral doses of PVP-fullerene C60 did not induce toxic effects which could point to a low, or absent absorption. However, the absorption in terms of blood or liver levels of PVP-fullerene C60 was not measured.

The presented results did not permit any conclusions concerning oral absorption of PVP-fullerene C60 and fullerenes.

3.3.6 Evaluation of factors influencing systemic absorption of fullerenes following oral exposure

In light of the lack of data on absorption of fullerenes following oral exposure no evaluation of factors influencing fullerenes absorption can be performed. No conclusion on the effect of water solubility of fullerenes on absorption from gastro-intestinal tract can be drawn.

3.3.7 Identification of gaps in current knowledge and future research needs in relation to fullerenes.

Fullerenes are compounds consisting of carbon atoms. Fullerene C60 is a compound consisting of 60 carbon atoms, with a diameter of approximately 0.7 nm while PVP-fullerene C60 is one of its water soluble derivative. Thus lack of techniques which can discriminate the content of the exogenous carbon from fullerenes from an abundant background of the endogenous carbon cells in the blood and other tissues may be a reason for lack of studies of absorption, organ distribution and excretion of fullerenes following oral exposure. Knowledge about any possible absorption, organ distribution and excretion of fullerenes following oral administration remain to be established.

3.4 Gold

3.4.1 Usage

Gold NPs (Au-NPs) have been used for various biomedical applications due to their unique surface, electronic and optical properties. Because of their physico-chemical properties, including surface plasmon resonance, fluorescence, and easy surface functionalisation, Au-NPs have been widely used in biosensors, used for cancer cell imaging, photothermal therapy and for drug delivery. Furthermore, because of their rather high stability in liquid suspension, Au-NPs are often used for research applications as model NPs to study processes where a rather low degree of solubility of a NP is desirable.

3.4.2 *In vivo* studies

Three studies were identified.

In a key study, (Hillyer and Albrecht 2001) investigated the gastro-intestinal uptake of metallic colloidal gold particles (Au-NPs) in mice. Instrumental neutron activation analysis (INAA) and transmission electron microscopy (TEM) were used to quantitatively and qualitatively study the gastro-intestinal uptake and subsequent tissue/organ distribution of 4, 10, 28, and 58 nm diameter unconjugated Au-NPs following oral administration to BALB/c mice (sex and animals/group not given) in the drinking water (the concentration was 0.2 mg/ml) for 7 days. 4 nm Au-NPs were identified at low levels in the following organs/tissues in descending concentrations: kidney, small intestine, lung, stomach, spleen, liver, heart, blood, and brain. In the quantitative studies, it was found that Au-NP uptake is dependent on particle size: smaller particles (4 and 10 nm) cross the gastro-intestinal tract more readily than larger ones (than 28 and 58 nm). Electron microscopic studies showed that particle uptake occurred in the small intestine by persorption (the paracellular up-take of particles from the digestive tract into the body) through single, degrading enterocytes in the process of being extruded from a villus.

Conclusion: This study demonstrated that Au-NPs were absorbed from the gastro-intestinal tract at low levels by uptake of particulates by persorption through holes created by extruding enterocytes in the small intestine. Au-NP uptake was dependent on particle size: smaller particles (4 and 10 nm) crossed the gastro-intestinal tract more readily (than 28 and 58 nm).

In a 14-day toxicity study Zhang and co-workers treated groups of male ICR mice (11 weeks old, six per group) with 13.5 nm citrate-stabilized Au-NPs by oral gavage at doses from 137.5-2200 μg/kg

bw/day (Zhang et al. 2010).Transmission electron microscopy (TEM) images were used for imaging of the Au-NPs in red blood cells and bone marrow cells from mice receiving 2200 μg/kg bw/day for 14 days. Multiple vesicles containing Au-NPs were readily observed in the cells, and within the vesicles the Au-NPs appeared to be monodisperse. Many Au-NPs were observed outside of the cell membranes, and their average size was about 10–15 nm.

Conclusion: The study showed that Au-NP related materials were absorbed following oral administration to mice and could be deposited in a "deep" pool such as the bone marrow.

Jumagazieva and co-workers administered Au-NPs of three different sizes to deliver 0.25 mg Au/kg bw/day to groups of six male rats by oral gavage with the purpose of screening its *in vivo* genotoxicity (Jumagazieva et al. 2011). The rats received Au-NPs of 16 or 55 nm in diameter or gold nanoshells of 160 nm in diameter (consisting of a core of 120 nm silicium dioxide and a shell of 20 nm Au) once a day for 7 days. To ensure stability and biocompatibility, the surface of NPs was functionalized with polyethylene glycol (PEG) molecules.

Conclusion: Because no genotoxicity was seen and no attempts were made to detect Au-NPs in tissues the study cannot be used to make a judgement on intestinal absorption following oral exposure.

3.4.3 *In vitro* studies

No *in vitro* studies were identified for gold.

3.4.4 Synthetic set-ups

No relevant studies were identified.

3.4.5 Conclusion on the studies concerning systemic absorption of Au-NPs following oral exposure

Au-NPs were absorbed at low levels following oral administration, and were distributed to a number of organs in experimental animals by uptake of particulates by persorption through holes created by extruding enterocytes from the villi in the small intestine.

3.4.6 Evaluation of factors influencing systemic absorption of Au-NPs following oral exposure

Au-NP uptake was dependent on particle size: smaller particles (4 and 10 nm) crossed the gastro-intestinal tract more readily than 28 and 58 nm particles.

3.4.7 Identification of gaps in current knowledge and future research needs in relation to Au-NPs

It is well-know that Au-NPs attract a corona of available proteins and other biomolecules. Therefore, a detailed study of the fate and formation of aggregates/corona in the GI tract is needed to improve understanding of the fate of particles in the gastro-intestinal environment and their absorption from the gastro-intestinal system in order to evaluate whether read across of data on other gold compounds/metallic gold to nanoparticulate material could be justified when assessing toxicity of Au-NPs after oral administration.

3.5 Iron oxide

3.5.1 Usage

Iron oxide nanoparticles (Fe2O3-NPs) are applied in magnetic resonance imaging (MRI) for contrast enhancement to identify metastases from inflammatory lymph nodes, to provide information on tumour angiogenesis, to differentiate dangerous atherosclerotic plaques, and to differentiate healthy and pathological tissues. Other applications are to induce magnetic hyperthermia (one of the therapies for cancer treatment) or as carriers for targeted drug delivery in treatment of various forms of cancer (Singh et al. 2013).

3.5.2 *In vivo* studies

Two relevant *in vivo* studies on absorption of Fe2O3-NPs after oral exposure were identified and are summarised below.

The key study is a study by Singh and co-workers (Singh et al. 2013).The tissue distribution (and genotoxicity) of nano and micro sized (bulk) Fe2O3 in rats were compared after acute oral gavage administration. Female Wistar rats (N=5/group/treatment/sampling time), 6-8 weeks old and weighing 80-120 g, were given single doses of vehicle (Milli Q water), and 500, 1000 or 2000 mg/kg bw of Fe2O3-NP (30 nm) or Fe2O3- bulk suspended in Milli Q water. The Fe2O3- bulk material had size of <5 µm and purity ≥ 99%. The size of the Fe2O3-NPs was < 50 nm (data from manufacturer). The mean size distributions of Fe2O3-NPs and Fe2O3-bulk were 29.75 nm and 2.15 µm respectively. The hydrodynamic diameter for Fe2O3-NPs in Milli Q water suspension was 363 nm. The results of DLS showed larger values than when measured by TEM, indicating that Fe2O3-30 nm particles formed larger agglomerates in suspension. Zeta potential and electrophoretic mobility measurements were -18.6 mV and -1.47 µ (µm cm/V/s) respectively at pH 7.0. The specific surface area of Fe2O3-NPs and Fe2O3-bulk was 38.02 and 5.67 (m2/g), respectively.

Blood and bone marrow samples were taken for genotoxicity testing (comet assay in peripheral blood lymphocytes, blood micronucleus test, and bone marrow micronucleus test and chromosomal aberration assay) and samples of whole blood, liver, kidneys, heart, brain, spleen and bone as well as pooled samples of urine and faeces were taken at fixed time points up to 48 hours after dosing for studies of iron absorption and organ distribution.

Neither Fe2O3-NPs nor Fe2O3-bulk produced genotoxicity under the conditions of the bioassays.

In the animals treated with Fe2O3-NPs significantly increased concentrations of iron were measured in the blood and all examined tissues, except the brain, when compared to the controls. At all time points and for all doses the highest iron concentrations were recorded in descending order for blood, spleen, liver, kidney, heart, bone marrow. No significant increase of iron was detected in the brain. The incorporation of iron from Fe2O3-NPs in the various tissues was in the range of 0.2 - 9.4%.

In animals treated with Fe2O3- bulk no significant increase in iron concentration was recorded in kidneys, heart, blood and urine when compared to the controls, while significantly increased concentrations of iron were detected in the liver and spleen. The incorporation of iron in the various tissues was in the range of 0.01 – 2.3% in the Fe2O3- bulk treated groups.

In the rats treated with Fe2O3- bulk the urinary concentrations of iron were not significantly different from the control values and were much lower than in the rats treated with the nanoparticulate material.

Based on the results on the distribution and levels of iron in organs of rats it can be concluded that iron was more efficiently absorbed from the gastro-intestinal tract following administration of Fe2O3-NPs than of the bulk material.

Conclusion: The bioavailability of iron from Fe2O3-NPs was significantly higher than from Fe2O3- bulk when administered orally to rats. However, the study was not able to clarify whether the iron is absorbed in the form of nanomaterial or as ions liberated in the gut, or both.

McCullough and co-workers (McCullough et al. 1995) studied uptake and translocation of particulate iron across the gastro-intestinal mucosa. Male Sprague-Dawley rats (6-8 weeks old, N=6/group), fasted for 24 hours, were fed ad libitum either milk (control) or a suspension of iron powder (metallic iron, particles ranging in size from 6-9 µm down to 5-30 nm) suspended in milk (5 g powder/100 ml milk). The size of the iron particles was only given in the abstract, while in Materials and Methods the iron powder was referred to as “iron powder 6-9 micron”. Samples from duodenum were taken from control and treated rats 24 hours after the dosing. The samples were processed and subjected to examination by light microscopy, scanning electron microscopy (SEM), transmission electron microscopy (TEM) and X-ray microanalysis (XRMA).

Iron deposits were observed within the tissue of the duodenum by light microscopy. SEM revealed a selective localization of iron in the villi with variation in levels of iron uptake by the mucosal cells. Ultrastructural and XRMA analysis of duodenum samples established the presence of metallic iron NPs within the brush border, lateral intercellular spaces of the mucosal cells, mitochondrial cristae and cytoplasm of both mucosal and stromal cells.

Conclusion: According to the authors their results indicated that metallic iron particles, in the nano-size range may be taken up by the gastro-intestinal mucosa and that the passage of such particles across the epithelium barrier may take place through both a paracellular as well as transcytotic process.

3.5.3 *In vitro* studies

No *in vitro* studies were identified for iron.

3.5.4 Synthetic set-ups

No relevant studies were identified.

3.5.5 Conclusion on the studies concerning systemic absorption of iron oxide following oral exposure

One study (Singh et al. 2013) investigated the levels of iron in blood and major organs of rats following administration by gavage of nano-sized and bulk Fe2O3. It can be concluded that iron was more efficiently available to organs and tissues from Fe2O3-NPs than from the bulk material. However, this study was not able to clarify whether the iron was absorbed in the form of the nanomaterial or as Fe-ions liberated in the gut, or both. In another study it was shown, that metallic iron NPs can be taken up by the gastro-intestinal mucosa and that the passage of such particles across the epithelia barrier may take place through both a paracellular as well as a transcytotic process.

3.5.6 Evaluation of factors influencing systemic absorption of iron oxide following oral exposure

The results by Singh and co-workers (2013) indicate that the oral absorption of iron was higher for Fe2O3-NPs compared to the bulk material. Thus the size of the particles may influence absorption.

This is supported by the findings by McCullough and co-workers that indicated that metallic iron particles in the nano-size range was taken up by the gastro-intestinal mucosa and crossed the epithelia barrier through both paracellular as well as transcytotic processes

3.5.7 Identification of gaps in current knowledge and future research needs in relation to iron oxide

Although the study by Singh and co-workers (2013) demonstrated that iron is better absorbed from Fe2O3-NPs than from the bulk material, it is not clear whether the iron was absorbed as NPs or in ionized form, or both. Studies to clarify this issue are missing for Fe2O3.

3.6 Selenium

3.6.1 Usage

Selenium NPs (Se-NPs) have found use in food supplements and nutraceutical products as an alternative source of the essential trace element Se. The background for its use is that the acute toxicity is lower than that of sodium selenite, which is commonly used in food supplements and nutraceutical products.

3.6.2 *In vivo* studies

No *in vivo* studies were identified for selenium.

3.6.3 *In vitro* studies

Only one study was identified.

Wang et al. (Wang and Fu 2012) investigated the uptake of selenium (Se) into Caco-2 cells from sodium selenite, Se-NP and selenomethionine by measuring the activity of the selenium containing antioxidant enzyme glutathione peroxidase. The sizes of red elemental Se-NPs were 60–80 nm as determined by dynamic light scattering with the average size of 69 nm. The transport and uptake of the three different forms of Se (all at 0.1 μmol l^{-1}) across a Caco-2 cell monolayer were carried out for 2 hours and the uptake efficiencies were calculated. The highest uptake efficiency was observed in cells treated with Se-NPs. Because the Se content of all samples was determined by atomic absorption spectrometry following dissolution of the specimens in acid, no information was obtained about any intact Se-NPs in the samples.

Conclusion: The study demonstrated that the transport and uptake of Se from Se-NPs were time-dependent and the epithelial transport across Caco-2 cell monolayers mainly occurred via passive transport pathways. In addition, the results strongly indicated that Se-NPs existed as dissolved species in order to make the incorporation into the bioactive enzyme possible. The membrane passage of Se dosed as Se-NPs was superior to that provided by the two other tested molecular Se species.

3.6.4 Synthetic set-ups

No relevant studies were identified.

3.6.5 Evaluation of factors influencing systemic absorption of Selenium following oral exposure

The membrane passage of Se dosed as Se-NPs was time-dependent and mainly via passive transport. Further, was the membrane passage of Se-NPs superior to that provided by the two other tested molecular Se species.

3.6.6 Identification of gaps in current knowledge and future research needs in relation to selenium

The biological effects and efficacy of Se-NPs need further elucidation. The possible uptake of pristine Se-NPs by cells, or the possible deposition of elemental Se^0 following high doses of soluble salts, is an area of future research by EM-based techniques (imaging in cells) and by combined methods involving size-separation coupled with atomic spectrometric detection of Se.

3.7 Silicium dioxide

3.7.1 Usage

Silicium dioxide nanoparticles (SiO2-NPs) can be divided into two major varieties; crystalline and amorphous SiO2-NPs. Crystalline SiO2-NPs may occur in several different forms, including quartz, cristobalite and tridymite. Amophous SiO2-NPs can occur as mesoporous and amorphous SiO2-NPs. SiO2-NPs are used for numerous applications such as ingredients in cement, paints, solid lubricants, shampoos, cosmetics and facial creams. Further potential applications are in bio-electrochemistry, curing, oil recovering applications and formulation of other particles (Mikkelsen et al. 2011).

Synthetic amorphous SiO2-NPs (SAS) have been used for many years in food applications, such as for clearing of beers and wines, as anticaking agent to maintain flow properties in powder products and to thicken pastes. The conventional form of amorphous SiO2-NPs is known as the food additive E 551 (EFSA 2009a). Synthetic amorphous SiO2-NPs in principle do not contain crystalline SiO2-NPs. Although synthetic amorphous SiO2-NPs is permitted for application in food, it probably has not been tested with a focus on the nanometre sizes of SiO2-NPs (Dekkers et al. 2012). According to (ECETOC et al. 2006) commercial SAS does not fall into the class of NPs. However, Dekkers et al, 2011 have measured the particle size of SiO2-NPs in several food products to be in the range of 50 to 200 nm. In a processed food product (hot coffee with creamer) it was between 30 and 120 nm (Dekkers et al. 2011).

3.7.2 *In vivo* studies

Only one study was identified.

Limited information was available on the absorption of SiO2-NPs after oral ingestion. In a limited study, (So et al. 2008) So et al. reports an oral repeated-dose toxicity study with nanosized SiO2-NPs (30–90 nm, obtained from rice husk, not further specified). Groups of Balb/c mice (5 males and 5 females per group) were fed either normal diet (control), 1% nanosized SiO2-NPs diet or 1% microsized SiO2-NPs (0.5–30 nm) diet for 10 weeks. The silicium content was measured in lung and liver by ICP-AES. The exposure resulted in higher serum ALT (alanine aminotransferase) levels in mice dosed with SiO2-NPs compared to mice dosed with micrometre-sized (0.5–30 mm) SiO2 and the control mice (So et al. 2008). However, the level of SiO2-NPs in the liver (and lung) of the nanoparticle dosed mice was lower than that of the micrometre-sized (0.5–30 mm) SiO2-NPs dosed mice and the control group.

Conclusion: The results suggest that SiO2-NPs administered to mice via the diet (1%) during 10 weeks can, to some degree, reach the systemic circulation. However, SiO2-NPs levels were lower in

these mice than in the control mice and the mice offered micrometre-sized (0.5–30 mm) SiO2-NPs. The study is not well reported and lacks some important details on, among others, the characterization of the SiO2-NPs both in the feed and in the organs. It is also remarkable that the effect increased ALT was only found for Balb/c mice and not for C57BL/6 mice.

3.7.3 *In vitro* studies

No relevant *in vitro* studies were identified on the absorption of nano-SiO2-NPs. However in a recent study by Peters et al, (Peters et al. 2012) the presence of SiO2-NPs during *in vitro* digestion of foods was investigated. In this study an *in vitro* model to mimic the human digestion was used.

Different nanomaterials were investigated: 1) A commercially available food additive synthetic amorphous silica (SAS) with a primary particle size of 7 nm and a specific surface area of 388 m2/g. 2) A 32 nm colloidal SiO2-NPs material, stabilized at pH 8.6, 3) a set of SiO2-NP suspensions with particle diameters of 100 and 500 nm. Different food products containing nano-SiO2-NPs was subjected to *in vitro* digestion included (i) hot water, (ii) coffee with powdered creamer, (iii) instant soup, and (iv) pancake which either contained SiO2-NPs as the food additive E551, or to which a form of synthetic amorphous SiO2-NPs or 32 nm SiO2 particles were added.

The results showed that nano-sized (5-200 nm) SiO2-NPs is present in the saliva digestion stage in a relative amount of 5 to almost 40% when products containing E551 were tested. However, during the successive gastric digestion stage, the SiO2-NPs disappeared in the case of coffee and instant soup, while low amounts were found for pancakes in the gastric stage. The absence of nano-sized SiO2 is an effect of the low pH combined with high electrolyte concentrations in the gastric digestion stage. Large SiO2-NPs agglomerates are formed under this condition which is supported by DLS and SEM data. However, when the pH was increased to neutral pH in the intestinal stage, nano-sized SiO2-NPs reappears in even higher amounts than in the saliva stage. The results for coffee with E551 suggest that after complete digestion around 80% of SiO2-NPs are in the nanometre size range. For soup and pancake with E551, that is products containing more particulate and fibrous matter, the amount of SiO2-NPs after complete digestion is around 15%.

Conclusion: These findings suggest that, upon consumption of foods containing E551, the gut epithelium is most likely exposed to nanometre-sized SiO2-NPs. This is important because single SiO2-NPs may be more easily absorbed from the human intestine than agglomerates (Dekkers et al. 2012).

3.7.4 Synthetic set-ups

No relevant studies were identified.

3.7.5 Conclusion on the studies concerning systemic absorption of SiO2-NPs following oral exposure

Only one limited *in vivo* study was identified, and therefore no firm conclusion can be drawn. However, in a recent *in vitro* study it was shown that after complete digestion of foods containing SiO2-NPs (E551) a high amount of SiO2-NPs is present in the gut, which may increase the bio-availability of the substance.

3.7.6 Evaluation of factors influencing systemic absorption of SiO2-NPs following oral exposure

Due to lack of relevant data no conclusion can be drawn.

3.7.7 Identification of gaps in current knowledge and future research needs in relation to SiO2-NPs

Although it has been estimated that the intake via food of SiO2 (nano + non-nano) is 9.5 mg/kg bw/day only very limited data on oral exposure are available. Dekkers et al. (Dekkers et al. 2012), have performed a very comprehensive review of the literature on different forms of SiO2-NPs. They identified knowledge gaps and gave some recommendations for future research needs, which are quoted below:

The evaluation of the most recent information on SiO2-NPs as well as information on SAS leads to the conclusion that more information on the oral absorption and possible accumulation of SiO2-NPs and SAS is needed.

First, indications could be obtained by investigating the *in vitro* absorption/transport of SiO2-NPs with intestinal and dermal cell lines. Ideally, absorption, distribution and elimination could be studied by investigating blood kinetics and tissue distribution of SAS and nanoSiO2-NPs (as processed in food products) after single and preferably repeated oral administration in a rodent model. Special attention should be given to the possibility of accumulation of nano-SiO2-NPs in different tissues. If accumulation occurs, the extent and effects of this accumulation should be investigated. Depending on the tissues in which accumulation is expected to occur, the effects of the accumulation can be studied in a repeated-dose oral toxicity, reprotoxicity and/ or neurotoxicity study. These studies should be conducted with SiO2-NPs (comparable with the SiO2-NPs found in food products) and dose levels ranging from realistic exposure levels to toxic dose levels.

3.8 Silver

3.8.1 Usage

Historically, silver salts have been used in pharmaceuticals because of its bacteriostatic effects, and the forms of administration were on skin as ointments or per orally as solutions. Nowadays, silver NPs (Ag-NPs) are the single most widespread type of NPs used in consumer products, and the number of products available on the market steeply increased in the late 2000s. Similarly to silver salts, Ag-NPs are used because of their bacteriostatic effects in e.g. clothing, household water filters and water bottles. The mechanism of their bacteriostatic effect is not completely clear, but it has been assumed that the effects of silver are linked to silver ions released from the large specific surface of Ag-NPs. If correct, then these NPs constitute a reservoir of the biologically active silver ions, which may become progressively released in concert with the existence of ligands with affinity to silver ions in the surroundings of the Ag-NPs.

3.8.2 *In vivo* studies

Seven studies were identified.

The key *in vivo* study on intestinal uptake of Ag-NPs was authored by Loeschner et al. (Loeschner et al. 2011). This study investigated the distribution of silver in rats after 28 days repeated oral administration (gavage) of 9 mg Ag/kg bw/day as Ag-NPs or silver acetate (AgAc). The Ag-NPs had a size distribution of 14 ± 4 nm in diameter (90% of the NP volume) and were stabilized in aqueous suspension by polyvinylpyrrolidone (PVP). Roughly 10% of the total silver content in the Ag-NPs suspension was present as silver ions or silver atom clusters. In consequence, the Ag-NPs dosed animals were exposed to predominantly Ag-NPs but also in the same dose to a minor fraction of silver as ions or clusters. The PVP-stabilised Ag-NPs remained stable throughout the duration of the 28-day oral toxicity study, which was controlled by repeatedly measuring the distribution of the

hydrodynamic size by dynamic light scattering (DLS) over the dosing period. The results showed that the organ distribution pattern of silver administered as Ag-NPs or as Ag-acetate (AgAc) were similar. The absolute silver concentrations in tissues were however, lower following oral exposure to Ag-NPs. This was in agreement with an indication of a higher faecal excretion following administration of Ag-NPs. The largest silver concentrations were detected in the intestinal system followed by the liver and kidneys. Silver was also found in the lungs and brain. Autometallographic (AMG) staining revealed a similar cellular localization of silver in ileum, liver, and kidney tissue in rats exposed to Ag-NPs or to AgAc. Using transmission electron microscopy (TEM), nanosized granules were detected in the ileum of animals exposed to Ag-NPs or AgAc and were mainly located in the basal lamina of the ileal epithelium and in lysosomes of macrophages within the lamina propria. Using energy dispersive x-ray spectroscopy (EDX) it was shown that the granules in lysosomes of macrophages from rats exposed to Ag-NPs or to AgAc consisted of silver, selenium and sulphur. The diameter of the deposited granules was in the same size range as that of the perorally administered Ag-NPs. No silver granules were detected by TEM in the liver.

Conclusion: The results of the study demonstrated absorption of silver from Ag-NPs after oral administration to rats. The organ distribution of silver was similar when Ag-NPs or AgAc were administered orally but the absolute silver concentrations in the organs were generally lower after administration of Ag-NPs than after administration of AgAc in agreement with an indication of a higher faecal excretion of silver after administration of Ag-NPs. The presence of granules containing silver, selenium and sulphur in the intestinal wall of rats exposed to either of the silver forms suggested a common mechanism of their formation. Additional studies are needed to clarify whether Ag-NPs dissolve in the gastro-intestinal system and/or become absorbed and translocate as intact NPs to organs and tissues.

In a toxicity study, Sardari 2012 (Sardari et al. 2012) administered suspensions of Ag-NPs of 70 nm in diameter in sodium chloride solution using doses of 0, 0.25, 0.5, 1 and 2 mg/kg bw/day by gavage to rats (N=10/group) for 30 consecutive days. Following termination, macroscopically and histologically changes in the kidneys, liver and spleen from treated rats provided an indirect indication of internal exposure to silver following oral dosage with Ag-NP.

van der Zande et al (van der Zande et al. 2012) reported the results of a 28-day oral exposure study in rats exposed to <20 nm non-coated, or <15 nm PVP-coated silver NPs at a dose of 90 mg/kg bw/day, or to Ag-ions as silver nitrate (AgNO3) at 9 mg Ag/kg bw/day, or to carrier solution only (vehicle control). Sacrifice was performed on day 29. Silver was present in all examined organs and its concentration in the tissues was highly correlated to the amount of Ag-ions in the Ag-NP suspension. This indicated that mainly Ag-ions, and to much lesser extent AgNPs, passed the intestines in the Ag-NP exposed rats. Using single particle ICP-MS, which was used for counting the number of NPs, Ag-NPs were detected in silver NP exposed rats, but, remarkably also in AgNO3 exposed rats. This demonstrated that the formation of NPs from Ag-ions occurred *in vivo* which was in accordance with the findings by Loeschner et al (Loeschner et al. 2011).

Conclusion: The main target organs for silver distribution upon oral exposure to Ag-NPs or to AgNO3 are the liver and spleen, followed by the testis, kidney, brain, and lungs, without any difference in the distribution pattern between the PVP-coated and the uncoated Ag-NPs, or the AgNO3 exposed animals. When taking only the soluble silver fraction into account, the proportions of silver taken up of the Ag < 20 nm and AgNO3 were rather similar. This could indicate that silver is mainly absorbed in the ionic form and not in the particulate form after Ag-NP exposure.

Kim et al (Kim et al. 2009) studied the silver tissue distribution in rats following repeated oral administration by gavage of three doses of 60 nm Ag-NPs stabilized with carboxymethylcellulose (CMC) against CMC as the vehicle control for 90 days. The doses of Ag-NPs were not reported, but were probably 0 (vehicle control), 30, 125 and 500 mg/kg bw/day (see below Kim et al. 2010). The tissue distribution of Ag-NPs was investigated using the silver-staining method, which however, is

equally reactive to silver as Ag-NPs and as Ag-ions. Therefore, this method did not provide information about which form of silver was detected in tissues and organs. Examination of slides showed a dose-dependent accumulation of Ag in all the tissues examined, including testes, kidneys, liver, brain, lungs, and blood thus providing indirect evidence that silver was absorbed following oral administration of Ag-NPs to rats.

Conclusion: Although the work by Kim et al. suffers from the use of rather non-specific methods for detection of Ag and Ag-NPs in the rat tissues, the results demonstrated that silver was detected in tissues and organs and thus provided indirect evidence that silver was absorbed following oral administration of Ag-NPs to rats. However, it does not contribute to the discussion about the chemical form (dissolved or as NPs) in which silver crossed the intestinal wall to enter the blood circulation.

Kim et al. (Kim et al. 2010) published a 90-day study in rats which investigated oral toxicity of 56 nm Ag-NPs stabilized by carboxymethylcellulose in doses of 0 (vehicle control), 30, 125 and 500 mg/kg bw/day). Clinical chemistry, hematology, histopathology, and silver distribution were studied after the end of clinical phase of the study. Compared to the controls there was a dose-dependent significant increase in the silver concentration in all tissues from treated rats. The results published by Kim et al. in 2010 are apparently the same as published by Kim et al., 2009 (see above).

Groups of mice were exposed orally by gavage to 1 mg/kg bw/day of un-coated Ag-NPs of three sizes: 22 nm, 42 nm and 71 nm or to large Ag-NPs (323 nm) for 14 days (Park et al. 2010). Tissues for study of the Ag-NPs' distribution were sampled and digested in HNO3 and H2O2 followed by analysis by ICP-MS. Following dosage of the three small sizes of Ag-NPs, silver was distributed to brain, lung, liver, kidney, and testis while dosage of the large-sized Ag-NPs (323 nm) did not lead to elevated silver concentrations in those tissues. Dose-dependent organ toxicity and inflammatory responses correlated with the silver distribution.

Based on the toxicological findings, the authors concluded that repeated oral administration of Ag-NPs caused organ toxicity and inflammatory responses in mice and that these effects were size-dependent.

Conclusion: The authors of this report concluded that silver from small-sized Ag-NPs (22 nm to 71 nm) could be more easily absorbed from the gastro-intestinal tract and translocated to the organs and exert adverse effects in comparison with their larger counterparts. However, the study does not contribute to the discussion about the chemical form (dissolved or as NPs) in which silver crossed the intestinal wall to enter the blood circulation.

Local toxicity of Ag-NPs towards the small intestine was studied in groups of male Swice albino mice (Shahare et al. 2013). Ag-NPs ranging from 3-20 nm in diameter were administered orally by gavage at a dose of 0 (control) 5, 10, 15, and 20 mg/kg bw/day for 21 days. The small intestine was examined by light microscopy and by transmission electron microscopy. Ag-NPs damaged the microvilli of the epithelial cells as well as the intestinal glands.

Conclusion: The authors hypothesized that the local effects of Ag-NPs caused a loss of microvilli, which reduced the absorptive capacity of the intestinal epithelium and hence caused body weight loss of treated animals. The authors concluded that Ag-NPs destroy the mucosa of small intestine and impede its function. However, in what way this affects the absorption of silver from Ag-NPs was not investigated.

3.8.3 *In vitro* studies

Two studies were identified.

In the only identified study, which employed Caco-2 and M-cells in a co-culture as an *in vitro* model for the human intestinal epithelium, Bouwmeester et al. (Bouwmeester et al. 2011) studied the passage of Ag-NPs and Ag^+ ions across the cell membrane. The co-cultures were exposed for 4 hours to four different Ag-NPs which were 20, 34, 61 and 113 nm in diameter and detection was carried out by TEM imaging. Exposure to silver ions was included as a control since 6-17% of the silver NPs were dissociated to silver ions. The translocation experiments showed that silver dosed as Ag-NP suspensions or as AgNO3 (determined by AAS or ICP-MS) can pass the co-culture cell barrier and no significant translocation differences between the two types of dosage forms were found. An important question was if the nature of the translocated silver was as NPs or as Ag^+ ions. Because of the procedures used, the analyses did not directly provide information about the form of silver in the samples. The amount of silver however, that translocated through the Caco-2 cell barrier was equal for the silver ion and NP exposures. The silver ion solution and Ag-NP suspensions contained about the same free silver ion concentration and the results showed that the amount of silver ions that passed the Caco-2 cell barrier was equal for the silver ion and NP exposures.

Conclusion: This *in vitro* study indicates that the translocation of silver across the cell membrane in this model is likely to occur as Ag^+-ions and not as Ag-NPs.

Gaiser and co-workers (Gaiser et al. 2009) investigated the uptake of Ag-NPs by C3A cells, a human hepatocyte cell line, and Caco-2 cells, a human intestinal epithelial cell line. The Ag-NPs were 35 nm (Ag-NPs) and 0.6-1.6 µm (bulk Ag) in nominal size. The particles were dispersed by sonication at 1 mg/ml in culture medium for 15 minutes.

To assess particle uptake C3A cells plated on glass cover-slips and Caco-2 cells plated on Transwell membranes were incubated with media only (negative controls) or particles suspensions at 3.125 and 31.25 µg/cm2 of Ag of both sizes for 2 hours (C3A) or 24 hours (Caco-2). According to the authors of the study both sizes of Ag were taken up into both C3A and Caco-2 cells but data to document this statement were not given in the paper.

Conclusion: This *in vitro* study was not adequately reported to judge on the potential uptake of Ag-NPs in human hepatocytes or enterocytes.

3.8.4 Synthetic set-ups

Two studies were identified.

The fate of Ag-NPs in an *in vitro* digestion model was studied by Walczak et al (Walczak et al. 2012) using 60 nm citrate-stabilized Ag-NPs and silver nitrate as control. The studies were con-ducted in two matrices, one without and one in the presence of proteins. The digestive model involved consecutive treatments first with artificial saliva as medium for Ag-NPs or AgNO3 as source of Ag^+ ions. The mixtures were then treated with artificial gastric juice, duodenal juice and finally bile juice at 37 ^oC using mimicked peristaltic movements during incubation. Samples after saliva, gastric and intestinal digestion were analysed with single particle-ICPMS (detection of Ag-NPs), DLS and SEM with EDX for imaging and elemental analysis and mapping. In presence of proteins and after gastric digestion the number of particles dropped significantly, and rose back to original values after the intestinal digestion. SEM-EDX revealed that reduction in number of particles was caused by their clustering. These NP clusters were composed of silver and chlorine. During intestinal digestion, these clusters disintegrated back into single 60 nm Ag-NPs. The authors concluded that these Ag-NPs under physiological conditions can reach the intestinal wall in their initial size and composition. Importantly, intestinal digestion of AgNO3 in presence of proteins also resulted in particle formation. These NPs (20–30 nm) were composed of silver, sulphur and chlorine as demonstrated by SEM-EDX. The authors concluded that gastro-intestinal digestion impacts Ag-NPs and Ag+ ions so that they undergo changes during gastro-intestinal digestion.

Conclusion: Orally ingested 60 nm Ag-NPs, digested under physiologically relevant conditions (i.e. in the presence of proteins), ultimately can reach the intestinal wall in their size and dispersion. Therefore, ingestion of both Ag-NPs and Ag+ ions ultimately leads to intestinal exposure to NPs, albeit with a different chemical composition.

In a similar investigation Rogers et al. (Roger et al. 2012) studied the physico-chemical changes of Ag-NPs when exposed to synthetic human stomach fluid (SSF) at pH 1.5 and changes in size, shape, zeta potential, hydrodynamic diameter and chemical composition were reported following a 1 hour exposure period. The NPs examined included citrate-stabilized Ag-NPs (40 nm nominal diameter) and polyacrylate-stabilized Ag-NPs with a 1–10 nm nominal diameter range. Surface plasmon resonance (SPR) absorbance, high resolution transmission electron microscopy with energy dispersive X-ray spectroscopy (TEM/EDS), dynamic light scattering (DLS), and X-ray powder diffraction (XRD) were used to describe the possible changes in Ag-NP characteristics. Exposure of Ag-NPs to SSF produced a rapid decrease in the SPR peak at 414 nm characteristic for Ag-NPs in the size range of the tested NPs. During exposure to SSF, changes in zeta potential, aggregation and morphology of the particles were also observed as well as production of silver chloride which appeared physically associated with particle aggregates. The study showed that citrate-stabilized Ag-NPs agglomerated and partially reacted to form AgCl during exposure to SSF. Aggregation of Ag-NPs seen in this study may influence the production of AgCl.

Conclusion: Ingested Ag-NPs may be converted to a variety of aggregated and chemically modified particles, and in the stomach, the absorption of Ag from the studied Ag-containing materials will likely depend on the interactions between this mixture of resulting Ag-containing species or particles and the absorptive surfaces of the gastro-intestinal tract.

3.8.5 Conclusion on the studies concerning systemic absorption of Ag-NPs following oral exposure

There is strong evidence that silver from Ag-NPs become absorbed from the GI tract of experimental animals. Several studies describe that following oral exposure by gavage; silver can be detected in the blood and is further distributed to organs. Some excretion of silver occurs via faeces. The levels in blood and organs are dose-dependent and also depends on the size of the Ag-NPs used (i.e. absorption occurs for NPs not larger than 100 nm in diameter). Larger particles however seem not to be absorbed to any significant extent. Although distribution studies were meticulously made, only few studies touch upon the possible mechanism of the uptake of silver from Ag-NPs and addressed the question whether the Ag-NPs were absorbed as the pristine NPs or become absorbed following dissolution to low-molecular silver species. A few studies however demonstrated that silver-containing NPs were detected by EM imaging in various animal tissues following oral dosage with Ag-NPs or following dosage of Ag+ ions. These findings, aided by elemental imaging methods, revealed that the silver NPs co-existed with other elements such as selenium and sulphur, or with chloride and sulphur for both forms of silver administered via the GI tract. This strongly suggested that Ag-NPs were first dissolved and then deposited as insoluble salts in tissues and organelles. These findings are further supported by research *in vitro* using cell lines or synthetic set-ups, which generally conclude that the trans-membrane passage or release into synthetic digestive solutions occurs equally efficient for Ag-NPs as for Ag^+ ions. This suggests that Ag-NPs dissolve in the GI tract prior to absorption from the small intestine, to enter circulation and subsequently reach primarily the liver and spleen and to a lesser degree other organs. A few papers investigated and compared Ag-NPs with different polymeric coatings/stabilizers, but were unable to find any difference in their fate in the GI tract or in their deposition in the animals' organs.

3.8.6 Identification of gaps in current knowledge and future research needs in relation to Ag-NPs

In order to better predict the possible oral absorption of Ag-NPs, more knowledge is needed about the interaction between Ag-NPs and various co-digested food items or types of food. Information about the influence of food components on the absorption rate of small, intact Ag-NPs (1-20 nm) or following dissolution as Ag^+ ions is missing. Furthermore, future research using Ag-NPs that have been specifically synthesized with a covalently bound outer layer of negatively or positively charged or electrically neutral moieties will be useful for improving our understanding about absorption of Ag-NPs with a well-defined chemical "interface" to the biological surroundings. As far as analytical methodology is concerned, development of easy-to-use methods for determining the NPs' number-size distribution is important bearing in mind the European Commission's recommended definition from 2011 of NPs, i.e. a distribution of particles, for which at least 50% of the number of particles is below 100 nm in diameter.

3.9 Titanium dioxide

3.9.1 Usage

Titanium dioxide (TiO2), is a high-production volume chemical (i.e. produced in more than 1000 tons/year) mainly used as a pigment in its bulk form. It is the most widely used white pigment in paints, paper, plastic, coatings, ceramics, inks, pharmaceuticals, cosmetics, toothpastes and food. While TiO2 rank as one of the most used chemicals world-wide (mainly as a pigment), the tonnages of nanometre sized TiO2 used nationally, in the EU or worldwide can at present not be estimated. This is due to the fact that no specific inventories for nanomaterials exist. However, it should be mentioned, that in some consumer products, e.g. sunscreens, the percentage of nanoparticulate TiO2 (TiO2-NPs) may constitute several per cent of the product (Hansen et al 2008). TiO2 used as food additive (E 171), may also contain a fraction of TiO2-NPs, and it is used for whitening and brightening of food, especially for confectionary, white sauces and dressings and certain powdered foods. It has been estimated, that in UK the dietary intake of TiO2 (non-nano + nano) is 5 mg/person per day (Powell et al, 2010). Given the range of possible applications of TiO2-NPs especially within photo-catalysis (e.g. for self-cleaning surfaces and water treatment), the use of TiO2-NPs is anticipated to increase significantly in the near future. It should also be noted that the food products with the highest content of TiO2 are sweets including chewing gums and it has been estimated that children may have the highest exposure to TiO2 (Weir as cited in (Wang et al. 2012)). A number of different crystal structures of TiO_2 exist of which the rutile and anatase forms of TiO_2 are the most important in relation to the use of TiO_2 in consumer products. Both crystal structures are approved food colours (EFSA 2004).

3.9.2 *In vivo* studies

Four studies were identified.

In a study by Wang et al 2012 (Wang and Fu 2012) the distribution of TiO2-NPs was investigated in young (3 weeks old) and adult (8 weeks old) male Sprague-Dawley rats. The TiO2-NPs were dispersed in ultrapure water and ultrasonicated and stirred before use. The intragastric doses were selected based on the estimated dietary intake of TiO2 particles in the UK which has been estimated to be about 5 mg/person/day, equivalent to approximately 0.1 mg/kg bw/day. In this study, a dose 100 times higher (10 mg/kg bw/day) than the potential human exposure, was used as the low-dose TiO2-NP exposure in rats. The young and adult rats (7 per group) were administrated 0, 10, 50 or 200 mg/kg bw/day of TiO2-NP by gavage, once a day for 30 consecutive days.

The nanomaterial was well characterized in its solid form (i.e. as the powder). It was shown by TEM analysis that the TiO2-NPs were nearly spherical anatase crystals with hydroxyl groups on the

surface. The purity was 99.9%. The average size of the TiO2-NPs was 75 ± 15 nm and the measured Brunauer–Emmett–Teller (BET) specific surface area of the TiO2-NPs was 63.95 m^2/g.

In order to better understand the characteristics of NPs in the exposure medium and the rat gastro-intestinal tract, the hydrodynamic diameter and Zeta potential of TiO2-NPs in ultrapure water, artificial gastric juice (AGJ) and artificial intestinal juice (AIJ) were tested. It was clear that the TiO2-NPs were converted into larger particles in water, AGJ and AIJ, which is likely due to particle aggregation. The largest particles (hydrodynamic diameter) were found in AIJ (2081 nm) followed by AGJ (1702 nm) and water (474 nm).

Blood, liver, kidney and spleen from the highest dose group were analyzed for elemental titanium (Ti) content by ICP-MS. The liver and the mucosa of the stomach and small intestine were dissected into small pieces and analyzed by TEM.

The TEM images clearly showed that some particles with a diameter of approximately 60–200 nm adhered to the intestinal villi and absorbed in the stomach and small intestine tissue. However, no particles were found in the liver of young and adult rats. The contents of titanium in the blood, liver, kidney and spleen of young and adult rats in the TiO2-NPs treated groups were not significantly different from the control group after oral exposure. This indicated a very low absorption of TiO2-NPs from the GI tract and that the NPs located in the mucosa of the stomach and small intestine did not translocate into the systemic circulation.

It is generally agreed that absorption of NPs by the oral route increases with decreasing particle diameter. Based on the large hydrodynamic sizes of TiO2-NPs in artificial gastric juice (AGJ) and artificial intestinal juice (AIJ), the TiO2-NPs quickly aggregated into larger particles (1702 ~ 2081 nm) in the gastro-intestinal tract, which may contribute to the low absorption and translocation rates.

Conclusion: Based on this 30 days study it can be concluded that the absorption of anataseTiO2-NPs at a dose up to 200 mg/kg bw seems to be low, which may be due to aggregation of the NPs to larger particles in the GI tract. The highest dose in this experiment was 2000 times higher than the potential human exposure by the oral route (0.1 mg/kg bw/day).

Onischenko 2012 (Onischenko et al. 2012) investigated the penetration of titanium dioxide NPs into enterocytes after their administration into isolated loops of rat small intestine in an in situ experiment. In a separate 28 days study they investigated the absorption and distribution of TiO2-NPs in rats.

Four male Wistar rats were used for the in situ experiment. The ileac loop (4-5 cm long) was exposed under aseptic conditions through a small incision in the abdominal wall. The loop was carefully isolated with silk ligatures without impairing the mesenteric vessels, and rutile TiO2-NP dispersion (50 mg/cm3) in isotonic salt solution was injected into the intestinal loop distally from the lower ligature. The ligatures were tightened, the abdominal wall and the skin were sutured, and the rats were placed in cages for 3hours until time of sacrifice.

In the 28 days study 4 male rats per group received a low dose (1 mg/kg bw/day) and a high dose (100 mg/kg bw/day) of water suspensions of either anatase TiO2-NPs, rutile TiO2-NPs or micron size TiO2 particles (the food additive E 171). The rats were dosed daily for 28 days by gavage.

The particle size and shape was measured by TEM. The anatase particles were spherical or elliptic with a diameter of 20-25 nm and the rutile form had a diameter of 5-10 nm and a length of 40-50 nm.

The physico-chemical nature of NP detected in the intestinal epithelium was studied also by spectroscopy of characteristic loss of electron energy (SCLEE). The authors emphasized that this method could be properly used only for analysis of NP aggregates, but this finding is not explained in any further detail.

In the in situ study penetration of TiO2-NPs into enterocytes after their administration into the isolated loop of rat's small intestine was shown *in vivo* by transmission electron microscopy. Using electron diffraction, TiO2-NPs were identified in the apical regions of the cells under the plasma membranes and in the deeper parts of the cytoplasm as solitary objects or small aggregations (100-150 nm). In this study it was demonstrated, that at least part of TiO2-NPs present in the intestinal lumen and located on the surfaces of microvilli and individual TiO2-NPs present in the enterocyte apical part and in the cytoplasm had crystal structure. Water dispersions of NPs (3 hour exposure to high concentrations) caused no appreciable morphological changes in enterocyte ultrastructure.

In the 28 days study, the content of titanium was measured in the liver. After 28 days administration of water dispersions of TiO2-NPs with anatase structure and micron sized particles no increase in the base level of titanium in the liver was observed either after low dose (1 mg/kg bw/day) or high dose (100 mg/kg bw/day) exposure. A similar result was observed in animals treated with rutile TiO2-NPs in the low dose group (1 mg/kg). However, titanium concentrations in the liver, determined by ICPMS increased by about a factor of two in rats given rutile TiO2-NPs water dispersion at the high dose (100 mg/kg). This could indicate the penetration of TiO2-NPs through the intestinal barrier, which might also be indicated by the in situ study described above. However, it is not known whether the systemic exposure to titanium occurs in the form of NPs or as dissociated titanium, or both.

Conclusion: This study, which was performed under "near physiological conditions" indicated that TiO2-NPs are more readily absorbed than microsized particles (E 171), and the absorption is also dependent of the crystalinity of the particles, with the rutile form being the most well absorbed. However, titanium could only be detected in the liver after exposure to doses 1000 times higher than the expected human exposure.

The distribution of TiO2-NPs (25 and 80 nm) and fine TiO2 (155 nm) particles in CD-1 mice was investigated by Wang et al. (Wang et al. 2007). The particles were suspended in 0.5% hydroxypropylmethylcellulose (0.5% HPMC). The suspensions were ultrasonicated and mechanically vibrated before administration to the animals. Due to its low acute toxicity a single large dose of 5 g/kg bw of TiO2 particle suspensions was administered by a single oral gavage. Ten males and 10 females pr. group were dosed with 0.5% HPMC (control), 25 and 80 nm TiO2-NPs or large ("fine") TiO2-NPs (155 nm). Histopatological tests were performed using standard test procedures. Two weeks after dosing the animals were sacrificed and blood and tissue samples were taken from heart, liver, spleen, kidneys, lung, brain testis or ovary.

The particle size (at pristine stage and in suspension) was measured by TEM. The tissues were measured for total titanium content by ICP-MS, but not for the presence of particles.

Two weeks after exposure to an extremely high dose of TiO2-NPs no obvious acute toxicity was observed. Accumulation of titanium was observed in female animals in their kidneys, spleen, and lung after exposure to all three types of particles. For the 80 nm TiO2-NPs group, high concentration of titanium was measured in the liver tissue (3970.4±1670.1 ng/g), whereas the titanium content was 106.3±7.8 ng/g in the 25 nm TiO2-NP group and 106.7±25.1 ng/g in the fine TiO2 group, which was not significantly different from the control group. In the kidneys, the titanium concentrations in the 80 and 25 nm TiO2-NPs groups were significantly higher than those in the control and fine TiO2 groups ($p < 0.05$).

Conclusion: This study gives some indication that TiO2-NPs are absorbed from the gastro-intestinal tract after oral exposure to extremely high doses. From this study it is not clear in which form TiO2 is absorbed (ions, particles or both). Furthermore, it is not clear whether absorption is size dependent because the tissue concentrations resulting from dosage of the two nano-sized suspensions appeared to be contradictory (high tissue Ti for larger TiO2-NPs). The authors do not provide any detailed information about the size distribution of the TiO2-NPs in the two suspensions immediately prior to administration. A hypothetical explanation could be that aggregation of the pristine NPs has occurred and therefore influence the intestinal uptake. Two weeks after exposure accumulation was observed in the liver, but it is unclear why only the 80 nm and not the 25 nm and fine particles accumulate. Accumulation was also observed in the kidneys of 25 and 80 nm particles but not “fine” particles (155 nm). It is not mentioned which crystal structure was investigated.

In a study by Jani et al. (Jani et al. 1994) the uptake of rutile TiO2 particles from the rat’s gastro-intestinal tract and translocation to systemic organs after oral administration were investigated. Although the size of TiO2 particles were not strictly in the nanometre sized, but in the size range of 475 ± 24 nm, this study was considered because it was well performed and gave some important information on the absorption and distribution of TiO2 rutile particles to different tissues.

Rutile TiO2 particles of nominal size 500 nm suspended in water were administered at a dose of 12.5 mg/kg bw to female Sprague Dawley rats, by oral gavage daily for 10 days. Size analysis by dynamic light scattering confirmed that the hydrodynamic size (in aqueous suspension) was close to the nominal size of 500 nm in diameter. Organs and tissues such as Peyer's patches, small intestine, colon, mesentery network and nodes, peritoneal tissue, liver, spleen, heart and kidney were removed for histology, scanning electron microscopy, and spectroscopic analysis for titanium, using inductively coupled plasma atomic emission spectroscopy. Histological examination showed the presence of titanium dioxide particles in all the major lymphoid tissue associated with the gut (GALT), and demonstrated that 500 nm titanium dioxide particles were translocated to systemic organs mainly the liver and to a lesser extent the spleen. It was demonstrated, that particle uptake in the gastro-intestinal tract takes place principally via Peyer’s patches, rich in lymphatic supply and phagocytic cells; the particles were then translocated to the mesentery network, and accumulate in the mesentery nodes. Some particles may then enter the general circulation and were taken up by the liver and the spleen. Four per cent of the administered dose was found in the colon, 2.86% in Peyer’s patches the mesentery network and nodes and 1.4% in the liver. It was calculated that absorption of 6.5% of the total dose of titanium dioxide particles in the 500 nm size range administered orally over 10 days takes place.

Conclusion: In this study, there are indications that a minor part of the rutile form of titanium dioxide was absorbed and distributed to some organs in its particulate form after oral exposure even though the administered TiO2 particles were not in the nanometre size range.

3.9.3 *In vitro* studies

One study was identified. Koeneman et al. (Koeneman et al. 2010) studied three different pathways for uptake of TiO2-NPs in an *in vitro* model using the human derived Caco-2 cell line, which in culture can mimic the epithelial cell layer of the gastro-intestinal tract. A commercially available suspension of TiO2-NPs in water was used. The particle diameter as provided by the manufacturer was < 40 nm. The mean particle sizes and size distributions of nanomaterials suspended in water and DMEM medium (Dulbecco’s Modification of Eagle’s Medium) were analysed by dynamic light scattering DLS by the authors to be 220 ± 20 nm in water and > 500 nm in DMEM medium.

Z-average diameters of the nanomaterials in nanopure water (220 nm) were much larger than the values provided by the manufacturer. From particle size distributions, it was observed that the TiO2-NPs were within the size range of 80 to 350 nm. However, compared with nanopure water, DMEM medium led to the further aggregation of nanomaterials up to 500 nm. The Z-average

diameters of nanomaterials also increased rapidly with time. TiO2 was imaged inside the cells by measuring reflection of a HeNe laser by scanning confocal microscopy.

The study provided evidence that TiO2-NPs cross the epithelial lining of the intestinal model at low levels after concentrations at 10 µg/ml and above. Three possible mechanisms by which the nanomaterials could potentially cross an epithelial layer were investigated: (1) by disrupting the junctional complexes without killing cells in the epithelial sheet, (2) by killing cells within the epithelial sheet thereby forming holes in the epithelial sheet and (3) by using the transport function of epithelial cells, used by nutrient, and pass through individual epithelial cells by transcytosis. After investigation into these three pathways, pathway 1 was not considered likely because after exposure to TiO2 the junctional complexes remained intact, except at 1000 µg/mL (a concentration not likely to be encountered). Pathway 2 was also considered unlikely based on the transepithelial electrical resistance (TEER) measurements, the undisturbed pattern of junctional proteins, and the results of the live/dead assays which indicated that the cells remain alive after both acute and chronic exposure to TiO2. Pathway 3 appeared to occur because TiO2-NPs were found both in the cells and underneath the cells without disrupting the junctional complexes or killing the cells.

Conclusion: This study indicated that TiO2-NPs can cross the epithelial lining of the gastro-intestinal tract at low levels by transcytosis after exposure to concentrations of 10 µg/ml and above.

3.9.4 Synthetic set-ups

No relevant studies were identified.

3.9.5 Conclusion on the studies concerning systemic absorption of titanium dioxide following oral exposure

Although TiO2 is one of the most widely used chemicals in the world only few studies exists on the absorption after oral exposure.

An *in vitro* study indicates that TiO2-NPs as such can cross the epithelial lining of the gastro-intestinal tract at low levels by transcytosis after exposure to concentrations of 10 µg/ml and above.

Three *in vivo* studies on absorption of the nanoform were identified. For comparison also one study on absorption of the bulk form is described in this report. Based on these studies it can be concluded that the absorption of TiO2 particles is low and only around 6% for the bulk form. It is not possible to estimate the absorbed amount of TiO2-NPs, but there is some indication that at "near physicological" doses of the anatase form of TiO2-NP (up to 200 mg/kg bw/day) no TiO2-NPs could be detected in blood or liver, kidney and spleen (in general the most relevant organs for accumulation of NPs). Only at extremely high exposure (5 g/kg bw) accumulation was observed in liver, kidneys, spleen and lung. However, it is not possible to evaluate the degree of absorption based on this study, and it is also not possible to conclude whether the absorbed TiO2 occurred as ions, particles or a combination. Finally, in most of the studies, TiO2 agglomeration was often seen in the suspensions administered to the animals. It therefore remains a question which size distribution the experimental animals were actually exposed to.

3.9.6 Evaluation of factors influencing systemic absorption of titanium dioxide following oral exposure

It is generally agreed that absorption of nanomaterials increases with decreasing particle size. However, this is not entirely clear from the available studies on TiO2; but there is some indication that agglomeration of the anatase form could explain the low absorption of these particles. Only one study addressed the influence of crystal structure of NPs on absorption after oral exposure. The result from this study indicated that the rutile form is better absorbed than the anatase form. This is

remarkable bearing in mind that the anatase form is regarded as the most toxic (Mikkelsen et al. 2011).

3.9.7 Identification of gaps in current knowledge and future research needs in relation to titanium dioxide

There are very few well performed *in vivo* studies on the absorption of TiO2-NPs. There remains a need for studies, which can explain whether these NPs are absorbed as ions, particles or a combination of both. There is also a need for studies showing the amount and rate of absorption at physiological conditions and the influence of size, crystal structure and surface treatment.

3.10 Zinc oxide

3.10.1 Usage

Zinc oxide (ZnO) is a very commonly used metal oxide and has traditionally been used in paints, pharmaceuticals and ceramics. During recent years engineered ZnO nanoparticles (ZnO-NPs) have been used in sunscreen cosmetics, dental composites, and dermal ointments. ZnO-NPs have antibacterial activity, which is size dependent and might involve reactive oxygen species (ROS) (Li et al. 2008). Due to its good absorptive and photo catalytic properties ZnO-NPs can also be used in environmental remediation for elimination or degradation of pollutants in water or air (Wang et al. 2008).

3.10.2 *In vivo* studies

Five relevant *in vivo* studies on absorption after oral exposure were identified and are summarized below.

The key study was a study by Baek et al. (Baek et al. 2012). This study explored the pharmacokinetics of ZnO-NPs of two different sizes (20 and 70 nm) after oral administration to male and female rats. The nano-particles were coated with citrate by suspension in 20 mM HEPES buffer containing 1% sodium citrate and dispersed by vortexing for 1 minute. Each type of ZnO-NPs was given to three groups of male and female rats (6 per group) as a single oral dose of 50, 300 or 2000 mg/kg bw by gavage. The control group (3♀ and 3♂) received an equivalent volume of citrate/HEPES buffer. Blood samples were taken from the tail vein at the following time points: time zero and 0.5, 1, 2, 4, 6, 10, 24, 48, 72 and 96 hours after the administration. Samples of the brain, heart, kidney, lung and testis or ovary were taken 1 and 6 hours and 1, 2, 3 and 7 days after the administration.

The citrate modified NPs had a negative surface charge (-28.1 mV for 20 nm and -33.3 mv for the 70 nm size). The average diameters were measured by SEM and TEM to be 21 ± 6 nm and 71 ± 19 nm, respectively. The total zinc content was measured in the organs by ICP-AES.

In addition, liver, kidney and spleen samples were collected from 3 male rats 24 hours after administration of 2000 mg/kg bw of the two different sizes of ZnO-NPs. Three male rats treated with citrate/HEPES buffer were used as controls. TEM and XAS analysis were performed on the organs.

The plasma zinc concentration increased during the first 24 hours after administration of both sizes of ZnO-NPs in a dose dependent manner. Zinc was mainly distributed to liver, lung and kidney within 72 hours. No significant differences were observed related to size of particles or gender of rats.

The estimated rate of absorption was dose dependent (5-17% at the low dose and 28-33% at the high dose). The absorption was slightly higher for the small particles than the larger ones. According to the TEM and XAS studies of the tissues, ZnO-NPs appeared to be distributed into organs in the form of zinc ions rather than as ZnO particles as demonstrated by the observation of zinc sulphide bonds in the tissues. After 7 days, less than 0.1% of the ingested Zn was retained in the analyzed organs.

Only a minor amount of the ZnO was cleared via the urine (1.32 – 1.47% of the administered dose), while most was excreted via the faeces. The excretion rate via urine decreased when the dose was increased, while the excretion via the faeces increases in a dose dependent manner.

Conclusion: Only a minor amount of orally administered ZnO-NPs is absorbed and there is strong indication that the distribution to different organs occurs as ions. Zinc in mainly distributed to the liver, lung and kidney, and most of the administered dose is cleared within 7 days mainly in the faeces, indicating that ZnO-NPs do not accumulate. The uptake of zinc following dosage of smaller ZnO-NPs is slightly higher than from dosage of larger ones.

Li et al. (Li et al. 2012) investigated the distribution and elimination of zinc from ZnO-NPs following a single oral gavage dose of ZnO-NPs with a particle diameter of approximately 50 nm and ZnO micro particles (ZnO-MPs) with at least one external dimension > 100 nm as a reference control. Male ICR mice (10-12 per group) were administered a single oral dose of either vehicle (control group), 2.5 g/kg bw ZnO-NPs suspension or 2.5 g/kg bw ZnO-MP suspension. The NP's morphology, size and agglomeration were characterized by transmission electron microscopy (TEM), and dynamic light scattering (DLS) was used for particle size analysis. Some agglomeration was observed in the dosing suspension of ZnO-NPs but the primary particles were dominant.

DLS showed an average hydrodynamic diameter of the ZnO-NPs of 93.35 ± 14.53 nm and for ZnO-MPs 1226.2 ± 120.4 nm. The ZnO particles were suspended in DMSO, diluted by sterile water and 1% hydroxypropyl methyl cellulose (HPMC) was used as stabilizing agent. The Zn-NPs or Zn-MPs were administered to the animals immediately after preparation. Zinc levels in the serum were measured by ICP-MS.

Blood samples were collected from at least three mice from each group at 0.5, 1, 2, 4, 6, 24, 48 and 72 hours post dosing. Test mice were sacrificed 24, 48 and 72 hours post dosing. Gross necropsy and tissue sampling were performed (heart, kidney, liver, lung, spleen testes and brain).

This study showed that the maximum absorption occurred at 2 hours for both NPs and MPs of ZnO. Within the first 2-6 hours of the testing period the mean serum zinc levels were significantly higher in the ZnO-NPs dosed groups than in the ZnO-MP groups. These data indicate that ZnO-NPs are more efficient absorbed than ZnO-MPs.

The investigation of the distribution showed, that there was no increase in the zinc levels com-pared to the controls in the lung, brain and testes. However, in the liver, spleen and kidney the zinc levels were increased over the period from 24 to 72 hours and more obviously for ZnO-NPs than for ZnO-MPs suggesting a more efficient particle distribution concentration in these organs for NPs than for microparticles, or a higher blood concentration of ZnO-NPs (or dissolved Zn) than of ZnO-MPs.

In order to investigate whether the higher degree of absorption of ZnO-NPs were due to dissolution to zinc ions a dissolution study was performed with NPs, MPs and ZnCl2. The degree of zinc ion release was not significantly different between ZnO-NPs and ZnO-MPs which led the authors to suggest that the observed difference in absorption and tissue levels of zinc in this study was due to particle size.

Histopatological changes in the liver were observed 48 hours post gavage in ZnO-NPs treated mice (affected group(s) not stated) but not in ZnO-MP treated and control mice.

Conclusion: It is likely that absorption, distribution and toxicity to target organs of ZnO depended on the size of the particles and that ZnO-NPs were more efficiently absorbed than the larger ones. The distribution occurred mainly in the liver, spleen and kidney. The very high, but unrealistic, doses and the lack of attempt to characterize the presence of NPs in the tissues or blood render this conclusion of questionable value, and more detailed studies are warranted.

In a study by Wang et al. (Wang et al. 2008) the acute oral toxicity of 20 and 120 nm ZnO NPs was investigated. This dose and size effect study was performed in CD-ICR mice, (5 males and five females/ group). The ZnO particles were suspended in 1% sodium carboxy methyl cellulose used as stabilizer. The mice were administered a single dose of 1, 2, 3, 4 and 5 g/kg bw by gavage of either 20 nm or 120 nm ZnO particles in suspension. The control group received 1% sodium carboxy methyl cellulose. The animals we sacrificed 2 weeks after the start of the experiment. Blood was obtained from optical veins, and the following organs were collected: liver, heart, spleen, stomach, kidneys, pancreas, testis or uterus and brain.

The size distribution in the administered ZnO NP suspension was determined by light scattering. The particle sizes of 20 and 120 nm ZnO in the administration solutions were about 44.8 ± 16 nm and 187 ± 1.3 nm, respectively. The dissolution rate of ZnO NPs in artificial gastric fluid was determined and was slightly higher (0.021%/min) for 20 nm than for 120 nm (0.016%/min) ZnO NPs.

The concentration of Zn in serum, organs and tissues of mice exposed to ZnO-NPs at the highest dose (5 g/kg bw) was measured, and compared to controls. A significantly higher concentration was found in the kidney, pancreas and bone ($p<0.05$) and a slight increase was found in the liver and heart of ZnO treated mice ($p>0.05$). The Zn concentrations in the liver, kidney and pancreas were slightly higher for mice administered the 20 nm than the 120 nm ZnO-NPs, indicating that more Zn was absorbed and distributed to these organs from 20 nm ZnO than from 120 nm ZnO-NPs. The highest concentration was found in bone and the 120 nm dosed mice retained significantly higher Zn levels in the bone than the 20 nm dosed mice. This is in accordance with the observation, that bone is a “reservoir” for dietary Zn (Sandoval et al. 1999 in Wang 2008).

Conclusion: The target organs for distribution of 20 and 120 nm ZnO NPs were liver, heart, spleen, pancreas and bone. From this study it is not possible to conclude whether ZnO-NPs were absorbed as ions or as particles, or both.

In a study by Lee et al. (Lee et al. 2012a) the behaviour and accumulation of nanoscale and submicron-scale zinc oxide (ZnO) particles in the body was investigated using real-time optical imaging based on fluorophor-labelled ZnO-NPs with a nominal size of 20 nm and 100 nm. The size distribution determined by DLS was 25.4 ± 3.9 nm for the 20 nm size and 78.9 ± 13.4 for the 100 nm size, respectively.

Sprague-Dawley rats (sex and number per group not given) were given by gavage 250 mg/kg bw of the labelled ZnO-NPs (Cy5.5 conjugated ZnO-NPs), either 20 nm or 100 nm in diameter, and the control group received 0.5 mg/kg bw of the fluorescent dye Cy5.5NHS. The labelled ZnO-NPs were stable in simulated gastric fluid for 7 hours. Blood samples were obtained at preselected time points after the administration for optical image studies and *in vivo* optical images of rats from the 2 dosage groups and the control group were performed after 1, 2, 3, 5 and 7 hours. The animals were sacrificed 23 hours after the administration and ex vivo optical images of the heart, lung, liver, spleen, pancreas, kidney, muscle and bone were performed. The fluorescent dye Cy5.5NHS showed the strongest optical signal intensity in the blood samples at 3 hours after oral administration, and the signal intensity decreased after that time. The highest signal intensity in rat blood was shown 5-

7 hours after the administration of the labelled ZnO-NPs, showing that ZnO-NPs of both sizes were absorbed with a higher absorption rate for the smaller particles compared to the bigger ones.

Whole rat body optical fluorescence images were also performed showing that nanoscaled ZnO particles moved more rapidly into the gastro-intestinal tract from the stomach than the submicron-scaled ZnO, which remained for a longer time in the stomach. *Ex vivo* images of different organs demonstrated that ZnO-NPs were distributed mainly to the liver and kidney, with the highest signals in the kidneys. There were no significant differences in the signals in the other organs between nano- and submicron-scaled NPs.

Conclusion: Optical imaging can be used to follow the movement of NPs after oral absorption, but cannot be used to investigate the amount of absorbed material or in which form it is absorbed.

In a similar study Lee et al. (Lee et al. 2012b) investigated the behaviour and accumulation of nano-scaled ZnO (20 nm) and submicro-scaled ZnO (100 nm) particles in organic tissues after oral administration using PET imaging in female BALB/c mice after administration of a single oral gavage dose of 100 mg/kg bw. Both types of ZnO NP (20 or 100 nm) were labelled with the radionuclide 18F in high yield via 'click reaction'. ^{18}F labelling on the ZnO-NPs was shown to be maintained stably in simulated gastric fluid (pH 1.2) for 7 hours. Three hours after the administration, PET images indicated that the radioactivity for 18F-labeled ZnO NPs was seen only in the gastro-intestinal (GI) tract. At 5 hours post-administration, the brain, heart, lung, liver, pancreas, spleen, kidney, bone, and muscle, were removed from the mice and *ex vivo* PET/CT imaging was performed. The study using ^{18}F-labeled ZnO-NPs showed radioactivity in the lung, liver and kidney including the GI tract. Submicro-scaled ^{18}F-labeled ZnO-NPs (100 nm) showed stronger radioactivity in the liver and kidney compared to the nano-scaled ^{18}F-labeled ZnO-NPs (20 nm).

Conclusion: PET imaging has the potential to monitor and evaluate the behaviour of ZnO-NPs absorbed in organic tissues following oral exposures, but cannot be used to investigate the amount of absorbed material or in which form it was absorbed.

3.10.3 *In vitro* studies

No relevant *in vitro* studies were identified for ZnO-NPs.

3.10.4 Synthetic set-ups

No relevant studies were identified.

3.10.5 Conclusion on the studies concerning systemic absorption of zinc oxide following oral exposure

The absorption rate of zinc from ZnO NPs is dose dependent (5-33% depending on the dose), and to a lesser extent also size dependent. The absorption is slightly higher for the small particles compared to the larger ones. In most studies zinc was measured and was mainly distributed to liver and kidney but was also measured in lung, pancreas and bone in other studies. From one well performed study there is strong indications that zinc from ZnO-NPs were distributed to the organs in the form of Zn-ions rather than as ZnO-NPs because Zn-S bonds and not Zn-Zn bonds were observed in the tissues where Zn was deposited. ZnO-NPs were mainly excreted via the faeces. Only a minor amount of zinc was retained in the analysed organs indicating that zinc and ZnO-NPs do not accumulate.

3.10.6 Evaluation of factors influencing systemic absorption of zinc oxide following oral exposure

In the available studies on ZnO-NPs the only physical chemical characteristic that was investigated was a difference in size. As described above the absorption of zinc is slightly higher for 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.

3.10.7 Identification of gaps in current knowledge and future research needs in relation to zinc oxide

Only one study was identified where the requirements in EFSAs guideline on characterization of the ENMs in biological fluids and tissues. This study indicates that absorbed ZnO-NPs dissolves/degrades in the gastro-intestinal tract without absorption of the pristine NPs. The hazard identification and hazard characterisation can therefore rely on data for the non-nanoform substance (if available). However, more studies are needed to confirm whether the intact ZnO-NPs indeed are taken up only in the dissolved state or whether a fraction of ZnO (defined by size, surface coating and stabiliser) may reach the target tissues as the intact NPs.

If ZnO-NPs are used in food or consumer products with other modifications than those reported (e.g. different coatings, or surface charges), studies on these modified particles need to be performed for acquisition of information on the absorption rate of such a broader range of ZnO-NPs modifications.

3.11 Phase II.1 Evaluation of physical and chemical properties expected to influence absorption of nanomaterials

It is generally agreed that the nanomaterials' physico-chemical characteristics like size, shape, solubility, crystallinity (crystal structure), coatings/stabilizers, surface charge and surface reactivity can influence their absorption. However, in the studies presented in the present review very few of these parameters were thoroughly investigated or documented. In each of the chapters on the different nanomaterials there is a summary of which physical and chemical properties that had been investigated and which may influence the absorption of that substance. A short summary for all materials is given below.

For the following substances no evaluation of factors influencing their systemic absorption following oral exposure can be given due to lack of data: CNTs, CeO2, fullerenes, SiO2-NPs and Se-NPs.

It is well known that the size of nanomaterials has an influence on their absorption rate, and that absorption of nanomaterials increases with decreasing particle size. This trend was indeed demonstrated for iron oxide, where the results by Singh and co-workers (Singh et al. 2013) indicated that the oral absorption of iron was higher for nanoparticulate material compared to bulk material.

The uptake of Au-NPs from the gastro-intestinal tract was also size dependent. Smaller particles (4 and 10 nm) crossed the gastro-intestinal tract more readily than did the tested 28 and 58 nm particles.

For Ag-NPs the absorption also depends of the size, and it was demonstrated that absorption occurs for NPs up to 100 nm in diameter. It should be noted however, that the apparent higher absorption rate for the smaller Ag-NPs may be under the influence of higher degree of dissolution as Ag-ions or

as Ag atomic clusters causing a higher Ag concentration in tissues and organs. Larger particles however seem not to be absorbed to any significant extent.

For TiO2-NPs is not so clear from the available studies whether the absorption from the gastro-intestinal tract is size dependent. There is however, some indication that agglomeration of the anatase crystal form could explain the low absorption of nanoparticles based on this crystal form

The absorption of ZnO-NPs is slightly higher for 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.

A few papers investigated and compared Ag-NPs with different polymeric coatings used as stabilizers, but any difference in their fate in the gastro-intestinal tract was not demonstrated.

Only one study addresses the influence of crystal structure of NPs on absorption after oral exposure. The result from this study indicates that the rutile form of TiO2 is better absorbed than the anatase form.

Table4 gives an overview of the different physical and chemical properties which may influence the absorption of nanoparticles.

TABLE 4 OVERVIEW OF IDENTIFIED PHYSICAL AND CHEMICAL PROPERTIES WHICH INFLUENCE THE ABSORPTION OF NANOMATERIALS

Nanomaterial	Absorption	Absorption as ions or as NPs	Investigated factors	Influence of factor
Carbon nanotubes	No relevant oral absorption studies. Indication of very low absorption	Not investigated	None	Not investigated
Cerium	Yes		None	Not investigated
Fullerene	Limited absorption		None	Not investigated
Gold	Yes		Size	Higher absorption of smaller particles
Iron	Yes		Size	Higher absorption of NPs than of bulk
Selenium	Too few data to conclude		None	Not investigated
Silicium dioxide	Too few data to conclude		None	Not investigated
Silver	Yes up to 100 nm		Size Polymeric coatings/stabilizers	Higher absorption of smaller particles Influence on absorption not demonstrated

Titanium dioxide	Yes	Agglomeration Chrystal structure	Agglomeration decrease absorption of the anatase form Rutile form better absorbed than the anatase form
Zinc oxide	Yes	Size	Higher absorption of smaller particles

3.12 Phase II.2 Identification of most relevant test method(s) for systemic absorption of nanomaterials following oral exposure

General principles on absorption and distribution, metabolism and excretion (ADME) of chemical substances are described in OECD test guideline 417 on toxicokinetics.

ADME studies are essential for the safety evaluation of nanomaterials because the chemical and physical characteristics of the nanomaterials may result in substantial different absorption and other toxicokinetic parameters than those seen for the non-nanoform of the same material. If there is any potential exposure *e.g.* via the oral route it is recommended by EFSA (EFSA, 2011) to perform an ADME study as one of the initial test. However, there may be difficulties in measuring NPs in blood, tissues and excreta and to establish the form in which they are present in the body. As mentioned in 3.12 Phase II.2 it is important that techniques are available either to detect the nanomaterial as particles or as their dissolved components in tissues and excreta. Alternatively, labelling may be used either directly, as radioactive isotopes, or indirectly using fluorescent dyes or a radiolabel. This is discussed in section 3.12. Analyses by ICP-MS, which have been extensively used has the limitation that only the element is determined but not its presence as an NP. However, if the method is combined with a suitable separation technique this problem could be overcome. Labelling however, has the disadvantage that the label may be released and analytically determined separate from the NPs. This situation would obviously lead to erroneous results for absorption and distribution of the NPs. In addition, the impact on the label itself on the ADME properties and activities of the NPs should be considered.

There are on-going developments of *in vitro* methods, but currently there are no *in vitro* methods validated to be used for hazard assessment or absorption of nanomaterials (or other chemicals) following oral exposure to NPs.

Although some *in vitro* studies related to absorption of nanomaterials after oral exposure have been published during recent years, and have been discussed in this report, only very few relevant and robust studies have been identified.

The most relevant model seems to be a model comprising 1) a digestive study (synthetic setup) under physiologically relevant conditions combined with 2) an *in vitro* absorption model

1) A few digestive studies were identified for Ag-NPs. Walczak et al (Walczak et al. 2012) described a model using three consecutive treatments: 1) artificial saliva as medium for Ag-NPs or AgNO3 as source of Ag-ions. The mixtures were then treated with 2) artificial gastric juice, 3) duodenal juice and finally bile juice at 37 ^{o}C using mimicked peristaltic movements during incubation. In this model it was demonstrated that gastro-intestinal digestion has an impact on the Ag-NPs and Ag-ions so that they undergo changes during gastro-intestinal digestion. Using this model there is indication that ingestion of both Ag-

NPs and Ag^+ ions ultimately leads to intestinal exposure to NPs, albeit with a different chemical composition, such as silver chloride or sulphide.

A similar *in vitro* model was used to mimic human digestion of nano-SiO2-NPs in different food items. The results from this study (Peters et al. 2012) indicated that the intestine is most likely exposed to nanosized SiO2-NPs.

2) An *in vitro* model of human follicle epitelium (FAE) was developed and characterized by des Rieux and co-workers (des Rieux et al. 2007) for the purpose to study transport of NPs by M cells. This method is shortly described in the introduction of this report (section 1.4). The model was well characterized by des Rieux et al. (des Rieux et al. 2007). Mono and co-cultures were analysed by TEM and SEM and the analysis indicated that at least some of the Caco-2 cells were converted to M cells. SEM analysis has also been used to identify and localize M cells in Payer's Patch biopsies.

   The percentage of M cells in co-cultures was evaluated to range between 15-30%. This information is crucial to interpret results and to compare them with *in vivo* data. It should be noted that the percentage of M cells of the total FAE in the Payer's Patches is lower in rodents (10%) and in humans (< 10%). Therefore, this model may overestimate the transport across the intestinal barrier of NPs in humans. However, this inverted co-culture model seems to be a useful tool to study the influence of M cells on NP transport. Des Rieux and co-workers (des Rieux et al. 2007) validated this model using model NPs (e.g. polystyrene model NPs). This model has been used by Bouwmeester et al. (Bouwmeester et al. 2011) to study the translocation of Ag-NPs. This study was described in section 3.8.3.

3.13 Phase II.3 Overall conclusion

In the present report we have evaluated the identified literature on absorption of nanomaterials after oral exposure. The identified literature was based on a comprehensive literature search, followed by an evaluation of the relevance and reliability of the initially identified papers. This resulted in 47 papers, which are discussed in the report and cover the following nanomaterials: Carbon nanotubes (CNTs), cerium dioxide (CeO_2), fullerenes, gold (Au), iron oxide (Fe_2O_3), selenium, silicium dioxide (SiO_2), titanium dioxide (TiO_2) and zinc oxide (ZnO). Both *in vivo* and *in vitro* studies are included as well as a few synthetic set-ups. Although the number of published oral exposure studies has increased during the recent years, only very few well performed studies on intestinal absorption are available, and more studies are needed to provide a sound science basis for risk assessment of exposure of humans to nanoparticles. The conclusions on each of the above mentioned nanomaterials are summarized below.

Carbon nanotubes

None of the *in vivo* studies identified in the open literature for 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 gastrointestinal tract following oral administration. However, the lack of toxicity observed in animal treated with oral doses of CNS compared with the toxicity seen in animals after i.v. or i.p. administration indirectly indicated either no or very low absorption after oral exposure.

One of the reviewed *in vitro* studies reported the presence of agglomerates, which were considered by the authors to be non-dispersed carboxylic acid functionalized single walled-CNTs (COOH-SWCNTs) in Caco-2 cells following the exposure via addition of these nanotubes to the culture media as examined by light microscopy. In another study examination by confocal microscopy of

erythrocytes exposed to acid functionalized SWCNTs (AF-SWCNTs) suggested that some were available to erythrocytes. Whether this only occurred at cytotoxic concentration is not clear. These *in vitro* observations suggest that the CNTs may be able to enter the cells, but do not provide any basis to conclude that CNTs can cross the intestinal wall and enter blood circulation.

Cerium dioxide

The only *in vivo* study available measured the organ distribution of Ce after oral administration of CeO_2-NPs to rats and found low levels in major organs which indicated low absorption. Whether the absorption occurred as Ce-ions or as CeO_2-NPs, or both, is not shown. The available *in vitro* study is not adequately reported to judge on the potential uptake of CeO_2-NPs in human hepatocytes or enterocytes.

Fullerenes

The only study identified, which compared the distribution of radioactivity after oral and intravenous administration of a single dose of ^{14}C labelled water-soluble C_{60} fullerene indicated very limited, if any, absorption after oral exposure. In a toxicity study large oral doses of PVP-coated fullerene C_{60} did not induce toxic effects which could point to a low, or no absorption. However, the absorption in terms of blood or liver levels of PVP-fullerene C_{60} was not measured. The presented results did not permit to draw any conclusions concerning oral absorption of PVP-fullerene C_{60} and other fullerenes.

Gold

Au-NPs were absorbed at low levels following oral administration by uptake of particulates by persorption through holes created by extruding enterocytes from the villi in the small intestine, and were distributed to a number of organs in experimental animals. Smaller Au-NPs were absorbed to a higher degree than larger ones.

Iron oxide

One study investigated the levels of iron in blood and major organs of rats following administration by gavage of nano-sized and bulk Fe_2O_3. It is concluded that iron was more efficiently available to organs and tissues from Fe_2O_3-NPs than from the corresponding bulk material. However, this study was not able to clarify whether the iron was absorbed in the form of the nanomaterial or as Fe-ions liberated in the gut, or both. In another study it was shown, that metallic iron NPs can be taken up by the gastrointestinal mucosa and that the passage of such particles across the epithelia barrier may take place through both a paracellular as well as a transcytotic process.

Selenium

Only one study was identified. It investigated the transport and uptake of Se from Se-NPs. It was concluded that the transport and uptake of Se from Se-NPs were time-dependent and the epithelial transport across Caco-2 cell monolayers mainly took place *via* passive transport pathways. In addition, the results strongly indicated that Se-NPs existed as dissolved species in order to make the incorporation into the bioactive enzyme possible. The membrane passage of Se dosed as Se-NPs was superior to that provided by the two other tested molecular non-nano Se species.

Silicium dioxide

Only one *in vivo* study of limited quality was identified on silicium dioxide, and therefore no firm conclusion can be drawn on intestinal absorption of SiO_2-NPs. However, in a recent *in vitro* study it was shown that after complete digestion of foods containing SiO_2-NPs (E551) a high amount of

nano-SiO_2-NPs was present in the gut, which may potentially favour the bio-availability of the substance.

Silver

There is strong evidence that silver from Ag-NPs become absorbed from the GI tract of experimental animals. Several studies describe that following oral exposure by gavage silver can be detected in the blood and is further distributed to organs. The levels in blood and organs are dose-dependent and also depends on the size of the Ag-NPs used (i.e. absorption occurs for NPs not larger than 100 nm in diameter). Larger particles however seem not to be absorbed to any significant extent. Although the reviewed distribution studies were meticulously planned and conducted, only few studies touched upon the possible mechanism of the uptake of silver from Ag-NPs and addressed the question whether the Ag-NPs were absorbed as the pristine NPs or became absorbed following dissolution as low-molecular silver species. A few studies however demonstrated that silver-containing NPs were detected by electron microscopic (EM) imaging in various animal tissues following oral dosage with Ag-NPs but also following dosage of soluble Ag-ions. These findings, supported by elemental imaging methods, revealed that the silver NPs co-existed with other elements such as selenium and sulphur, or with chloride and sulphur for both forms of silver administered via the GI tract. This strongly suggested that Ag-NPs were first dissolved and then re-deposited as insoluble salts in tissues and organelles. These findings are further supported by research *in vitro* using cell lines or synthetic set-ups, which generally conclude that the trans-membrane passage or release into synthetic digestive solutions occur equally efficient for Ag-NPs as for Ag^-ions. This suggests that Ag-NPs can dissolve in the GI tract prior to absorption from the small intestine, to enter circulation and subsequently reach primarily the liver and spleen and to a lesser degree other organs. A few papers investigated and compared Ag-NPs with different polymeric coatings/stabilizers, but were unable to find any difference in their fate in the GI tract or in their deposition in the animals' organs.

Titanium dioxide

Although TiO_2 is one of the most widely used chemicals in the world, only few studies exist on the absorption after oral exposure.

An *in vitro* study indicated that TiO_2-NPs as such can cross the epithelial lining of the gastrointestinal tract at low levels by transcytosis after exposure to concentrations of 10 µg/ml and above.

Three *in vivo* studies on absorption of the nanoform were identified. For comparison a study on absorption of the bulk form is also described in this report. Based on these studies it can be concluded that the absorption of TiO_2 particles is low and only around 6% for the bulk form. It is not possible to estimate the absorbed amount of TiO_2-NPs from the available literature, but there is some indication that at "near physiological" doses of the anatase form of TiO_2-NP (up to 200 mg/kg bw/day) no TiO_2-NPs could be detected in blood or liver, kidney and spleen (in general the most relevant organs for accumulation of NPs). Only at extremely high exposure (5 g/kg bw) accumulation was observed in liver, kidneys, spleen and lung. However, it is not possible to evaluate the degree of absorption based on this study, and it is also not possible to conclude whether the absorbed TiO_2 occurred as ions, particles or both. Finally, in most of the evaluated studies, TiO_2 agglomeration was often seen in the suspensions administered to the animals. It therefore remains a question to which size distribution of TiO_2 the experimental animals were actually exposed.

Zinc oxide

The absorption rate of zinc from ZnO-NPs is dose dependent (5-33% depending on the dose), and to a lesser extent also size dependent. The absorption was slightly higher for the small particles

compared to the larger ones. In most studies zinc was measured and was mainly distributed to liver and kidney but was also measured in lung, pancreas and bone in other studies. From one well performed study there is strong indications that zinc from ZnO-NPs was distributed to the organs in the form of Zn-ions rather than as NPs because Zn-S bonds and not Zn-Zn bonds were observed in the tissues where Zn was deposited. ZnO-NPs were mainly excreted *via* the faeces. Only a minor amount of zinc was retained in the analysed organs indicating that zinc and ZnO-NPs did not accumulate.

4. Phase III: Identification of knowledge gaps and research needs

Very few *in vivo* absorption studies have been identified as robust and valid for nanoparticle studies. Therefore, studies with an indirect indication of absorption (e.g. distribution to different organs), have also been included in the evaluation presented in this report.

In general more absorption studies are needed on different NPs. In such studies the nanomaterials should be well characterized as described by EFSA (EFSA 2011).

Studies on the influence of physico-chemical characteristics are needed as described in Phase II.1. Such studies could initially be performed in the *in vitro* models described above (Phase II.2), in order to save time and animals and get an idea about the mechanisms behind translocation of NPs, and the influence of physico-chemical parameters. When more data has been created, it may be possible to "read across" or to develop "*in situ*" models for absorption of "nearly similar" nanomaterials.

Analytical methods for nanomaterial characterisation are under enormous development and comprise wet chemical techniques, often based on atomic spectroscopy, and on imaging methods, e.g. electron microscopy techniques. There is a need for further development of sensitive and reliable methods for the detection, characterization and quantification of NPs especially in food/feed and tissues collected from *in vivo* or *in vitro* study models. From such studies it is important to collect information of whether substances are absorbed as particles, ions or a combination of both.

The state-of-the art of analytical characterisation actually allows for characterisation of especially metal-containing intact nanomaterials in food and tissues etc., but relevant data is most likely not reported owing to the fact that the investigators do not realise that a nanomaterial can become dissolved upon interaction with a biological system.

In research projects, where the investigators have access to a modern "analytical toolbox", the selective detection of ions vs. NPs of a given nanomaterial can be achieved in a number of ways. In general, methodologies which first make use of a separation technique such as field flow fractionation or size exclusion chromatography followed by detectors selective to NPs (light scattering or UV spectroscopy) or selective to both the dissolved and the nanoparticle state of a metallic element (ICP-MS) may be a way to achieve more relevant characterisation information in future projects. Finally it should be mentioned that single particle ICP-MS is a particularly promising analytical technique as it is not only highly sensitive (physiological relevant concentrations), but may also at the same time provide information on dissolved ionic matter and number and size of NPs in the same analytical procedure. Such techniques should be further investigated in future research on NPs in a biological or environmental context. Furthermore, such an analytical technique is well-suited to address the question of the number-based size distribution of a metallic NP suspension.

In analogy with the selective chemical characterisation of dissolved and nanoparticulate matter, emphasis should also be placed on NPs labelled with fluorophors. Because the fluorophor is used as a marker for the presence of the NPs to which they were attached, it is crucial that this binding is stable. Otherwise, the characterisation work may erroneously provide information on the detached label and not on the intact fluorophor-NP assembly.

4.1 Recommendations on which nanomaterials could be candidates for future experimental testing

Based on the evaluation of different nanomaterials in the present report substances known to have a high exposure level via the oral route could be candidates in a potential future project on systemic absorption of nanomaterials by oral exposure. Such substances are: silver, SiO2-NPs (E 551), TiO2 (E 171) and ZnO. Based on current knowledge there is strong indications that silver is absorbed partially as ions and therefore the hazard identification and hazard characterization may rely on data for the non-nanoform (EFSA 2011).

References

Asuri P, Karajanagi SS, Vertegel AA, Dordick JS, Kane Rs. 2007. Enhanced stability of enzymes adsorbed onto nanoparticles. J Nanosci nanotech 7:1675-1678.

Awasthi KK, John PJ, Awasthi A, Awasthi K. 2013. Multi walled carbon nano tubes induced hepatotoxicity in Swiss albino mice. Micron 44:359-364.

Baek M, Chung HE, Yu J, Lee JA, Kim TH, Oh JM, Lee WJ, Paek SM, Lee JK, Jeong J, Choy JH, Choi SJ. 2012. Pharmacokinetics, tissue distribution, and excretion of zinc oxide nanoparticles. Int J Nanomedicine 7:3081-3097.

Bianco A, Kostarelos K, Prato M. 2005. Applications of carbon nanotubes in drug delivery. Current opinion in Chemical Biology 9:674-679.

Bouwmeester H, Poortman J, Peters RJ, Wijma E, Kramer E, Makama S, Puspitaninganindita K, Marvin HJP, Peijnenburg AACM, Hendriksen PJM. 2011. Characterization of Translocation of Silver Nanoparticles and Effects on Whole-Genome Gene Expression Using an In Vitro Intestinal Epithelium Coculture Model. ACS Nano 5:4091-4103.

Card JW, Magnuson BA. 2010. A method to assess the quality of studies that examine the toxicity of engineered nanomaterials. Int J. Tox 29(4):402-410.

Chaudhry Q, scotter M, Blackburn J, Ross B, Boxall A, Castle L, Aitken R, Watkin R. 2008. Application and implications of nanotechnologies for the food sector. Food Addit Contam 25(3):241-258.

Cicchetti R, Divizia M, Valentini F, Argentin G. 2011. Effects of single-wall carbon nanotubes in human cells of the oral cavity: Geno-cytotoxic risk. Toxicol in Vitro 25:1811-1819.

Dekkers S, Krystek P, Peters RJB, Lankveld DPK, Bokkers BGH, van Hoeven-Arentzen PH, Bouwmeester H, Oomen AG. 2011. Presence and risks of nanosilica in food products. Nanotoxicology 5(3):393-405.

Dekkers S, Bouwmeester H, Bos PMJ, Rietveld AG, Oomen AG. 2012. Knowledge gaps in risk assessmen of nanosilica in food: evaluation of the dissolution and toxicity of different forms of silica. Natotoxicology Early online, 1-11.

Des Rieux A, Fievez V, Theate I, Mast J, Preat V, Schneider Y-J. 2007. An improved in vitro model of human intestinal follicle-associated epithelium to study nanoparticle transport by M cells. Eur J Pharm Sci 30:380-391.

ECETOC. 2006. Synthetic amorphous silica (CAS No. 7631-86-9). JACC No. 51. ISSN-0773-6339-51. Brussels, September 2006.

EFSA. 2004. Opinion of the Scientific Panel on Food Additives, Flavourings, Processing Aids and materials in Contact with Food on a request from the Commission related to the safety in use of rutile titanium dioxide as an alternative to the presently permitted anatase form. Question N° EFSA-Q-2004-103. Adopted on 7 December 2004. The EFSA Journal (2004) 163:1-12.

EFSA. 2009. The potential risks arising from nanoscience and nanotechnologies on food and feed safety. Scientific opinion of the scientific committee. Question No EFSA-Q-2007-124a. Adopted on 10 February 2009. The EFSA Journal (2009) 958:1-39.

EFSA. 2009a. Calcium silicate and silicon dioxide/silicic acid gel added for nutritional purposes to food supplements. Scientific opinion of the Panel on food additives and nutrient sources added to food. Questions No EFSA-Q-2005-140, EFSA-Q-2006-220, EFSA-Q-2005-098, EFSA-Q-2005-099. Adopted on 5 June 2009. The EFSA Journal (2009) 1132:1-24.

EFSA. 2010. Applications of systematic review methodology to food and feed safety assessments to support decision making. EFSA J. 8(6):1637 [90 pp.]. doi:10.2903/j.efsa.2010.1637.

EFSA. 2011. Guidance on the risk assessment of the application of nanoscience and nanotechnologies in the food and feed chain. EFSA Scientific Committee. EFSA Journal 2011;9(5):2140.

ENRHES. 2009. Engineered Nanoparticles: Review of Health and Environmental Safety. The project was funded under grant 218433 of the Seventh Framework Programme of the European Commission.

Fall M, Guerbet M, Park B, Gouriou F, Dionnet F, Morin J-P. 2007. Evaluation of cerium oxide and cerium oxide based fuel additive safety on organotypic cultures of lung slices. Nanotoxicology 1:226-233.

Fröhlich E., Roblegg E. 2012. Models for oral uptake of nanoparticles in consumer products. Toxicology 291:10–17.

Gaiser BK, Fernandes TF, Jepson M, Lead JR, Tyler CR, Stone V. 2009. Assessing exposure, uptake and toxicity of silver and cerium dioxide nanoparticles from contaminated environments. Environ Health (London, U K) 8:No.

Gerloff K, Albrecht C, Boots A.W, Förster J, Schins R.P.F. 2009. Cytotoxicity and oxidative DNA damage by nanoparticles in human intestinal Caco-2 cells. Nanotoxicology 3(4): 355–364.

Hadrup N, Lam HR, Loeschner K, Mortensen A, Larsen EH, Frandsen H. 2012a. Nanoparticulate silver increases uric acid and allantoin excretion in rats, as identified by metabolomics. J Appl Toxicol 32:929-933.

Hadrup N, Loeschner K, Mortensen A, Sharma AK, Qvortrup K, Larsen EH, Lam HR. 2012b. The similar neurotoxic effects of nanoparticulate and ionic silver in vivo and in vitro. NeuroToxicology 33:416-423.

Hansen SF, Michelson ES, Kamper A, Borling P, Stuer-Lauridsen F, Baun A. 2008. Categorization framework to aid exposure assessment of nanomaterials in consumer products. Ecotoxicology 17(5):438-477.

Hillyer JF, Albrecht RM. 2001. Gastrointestinal persorption and tissue distribution of differently sized colloidal gold nanoparticles. J Pharm Sci 90:1927-1936.

Holister P, Vas CR, Harper T. 2003. Fullerenes. Technology White papers nr 7. Cientifica 2-12.

Jani PU, McCarthy DE, Florence AT. 1994. Titanium dioxide (rutile) particle uptake from the rat GI tract and translocation to systemic organs after oral administration. Int J Pharm 105:157-168.

Jos A, Pichardo S, Puerto M, Sanchez E, Grilo A, Camean AM. 2009. Cytotoxicity of carboxylic acid functionalized single wall carbon nanotubes on the human intestinal cell line Caco-2. Toxicol in Vitro 23:1491-1496.

Jumagazieva DS, Maslyakova GN, Suleymanova LV, Bucharskaya AB, Firsova SS, Khlebtsov BN, Terentyuk GS, Kong SM, Khlebtsov NG. 2011. Mutagenic effect of gold nanoparticles in the micronucleus assay. Bull Exp Biol Med 151:731-733.

Kaittanis C, Nase SA, Perez JM. 2007. One-Step, Nanoparticle-Mediated Bacterial Detection with Magnetic Relaxation. Nanoletters 7(2):308-383.

Kim YS, Kim JS, Cho HS, Rha DS, Kim JM, Park JD, Choi BS, Lim R, Chang HK, Chung JH, Kwon IH, Jeong J, Han BS, Yu IJ. 2008. twenty-eight-day oral toxicity, genotoxicity, and gender-related tissue distribution of silver nanoparticles in Sprague-Dawley rats. Inhalation Toxicology 20:575–583.

Kim WY, Kim J, Park JD, Ryu HY, Yu IJ. 2009. Histological study of gender differences in accumulation of silver nanoparticles in kidneys of Fischer 344 rats. J Toxicol Environ Health, Part A 72:1279-1284.

Kim YS, Song MY, Park JD, Song KS, Ryu HR, Chung YH, Chang HK, Lee JH, Oh KH, Kelman BJ, Hwang IK, Yu IJ. 2010. Subchronic oral toxicity of silver nanoparticles. Part Fibre Toxicol 7:No.

Klimisch H-J, Andrea M, Tillmann U. 1997. A systematic approach for evaluation the quality of experimental toxicological and ecotoxicological data. Reg. Tox. Pharma 25:1-5.

Koeneman BA, Zhang Y, Westerhoff P, Chen Y, Crittenden JC, Capco DG. 2010. Toxicity and cellular responses of intestinal cells exposed to titanium dioxide. Cell Biol Toxicol 26:225-238.

Kolosnjajtabi J,Hartman KB, Boudjemaa S, Ananta JS, Morgant G, Szwarc H, Wilson LJ, Moussa F. 2010. In vivo behavior of large doses of ultrashort and full-length single-walled carbon nanotubes after oral and intraperitoneal administration to Swiss mice. ASCNANO 4(3):1481–1492.

Lam C-W, James JT, McCluskey R, Arepalli S, Hunter RL. 2006. A Review of Carbon Nanotube Toxicity and Assessment of Potential Occupational and Environmental Health Risks. Critical Reviews in Toxicology, 36:189–217.

Lee CM, Jeong HJ, Kim DW, Sohn MH, Lim ST. 2012a. The effect of fluorination of zinc oxide nanoparticles on evaluation of their biodistribution after oral administration. Nanotechnology 23:205102.

Lee CM, Jeong HJ, Yun KN, Kim DW, Sohn MH, Lee JK, Jeong J, Lim ST. 2012b. Optical imaging to trace near infrared fluorescent zinc oxide nanoparticles following oral exposure. Int J Nanomed 7:3203-3209.

Li CH, Shen CC, Cheng YW, Huang SH, Wu CC, Kao CC, Liao JW, Kang JJ. 2012. Organ biodistribution, clearance, and genotoxicity of orally administered zinc oxide nanoparticles in mice. Nanotoxicology 6:746-756.

Lim JH, Kim SH, Lee IC, Moon C, Kim SH, Shin DH, Kim HC, Kim JC. 2011a. Evaluation of Maternal Toxicity in Rats Exposed to Multi-Wall Carbon Nanotubes during Pregnancy. Environ Health Toxicol 26:e2011006.

Lim JH, Kim SH, Shin IS, Park NH, Moon C, Kang SS, Kim SH, Park SC, Kim JC. 2011b. Maternal exposure to multi-wall carbon nanotubes does not induce embryo-fetal developmental toxicity in rats. Birth Defects Res , Part B 92:69-76.

Loeschner K, Hadrup N, Qvortrup K, Larsen A, Gao X, Vogel U, Mortensen A, Lam HR, Larsen EH. 2011. Distribution of silver in rats following 28 days of repeated oral exposure to silver nanoparticles or silver acetate. Part Fibre Toxicol 8:18.

Magnuson BA, Jonaitis TS, Card JW. 2011. A brief review of the occurrence, use, and safety of food-related nanomaterials. J Food Sci 76(6). R126-33. doi: 10.1111/j.1750-3841.2011.02170.x.

Matsumoto M, Serizawa H, Sunaga M, Kato H, Takahashi M, Hirata-Koizumi M, Ono A, Kamata E, Hirose A. 2012. No toxicological effects on acute and repeated oral gavage doses of single-wall or multi-wall carbon nanotube in rats. J Toxicol Sci 37:463-474.

McCullough JS, Hodges GM, Dickson GR, Yarwood A, Carr KE. 1995. A morphological and microanalytical investigation into the uptake of particulate iron across the gastrointestinal tract of rats. J Submicrosc Cytol Pathol 27:119-124.

Mikkelsen SH, Hansen E, Christensen TB, Baun A, Hansen SF, Binderup M-L. 2011. Survey on basic knowledge about exposure and potential environmental and health risks for selected nanomaterials. MST. Environmental Project No. 1370 2011.

Onishchenko GE, Erokhina MV, Abramchuk SS, Shaitan KV, Raspopov RV, Smirnova VV, Vasilevskaya LS, Gmoshinski IV, Kirpichnikov MP, Tutelyan VA. 2012. Effects of titanium dioxide nanoparticles on small intestinal mucosa in rats. Bulletin of Experimental Biology and Medicine 154(2): 265-270.

Park EJ, Bae E, Yi J, Kim Y, Choi K, Lee SH, Yoon J, Lee BC, Park K. 2010. Repeated-dose toxicity and inflammatory responses in mice by oral administration of silver nanoparticles. Environ Toxicol Pharmacol 30:162-168.

Park EJ, Park YK, Park K. 2009. Acute toxicity and tissue distribution of cerium oxide nanoparticles by a single oral administration in rats. Toxicol Res (Seoul, Repub Korea) 25:79-84.

Peters R, Kramer E, Oomen AG, Herrera Rivera ZE, Oegema G, Tromp PC, Fokkink R, Rietveld A, Marvin HJP, Weigel S, Peijnenburg AACM, Bouwmeester H. 2012. Presence of nano-sized silica during in vitro digestion of foods containing silica as a food additive. Ascnano 6(3):2441–2451.

Powell JJ, Faria N, Thomas-McKay E, Pele LC. 2010. Origin and fate of dietary nanoparticles and microparticles in the gastrointestinal tract. J Autoimmun 34(3):J226-33. Epub 2010 Jan 21.

Roger KR, Bradham K, Tolaymat T, Thomas DJ, Hartmann T, Ma L, Williams A. 2012. Alterations in physical state of silver nanoparticles exposed to synthetic human stomach fluid. Sci Total Environ 420:334-339.

Sachar S, Saxena RK. 2011. Cytotoxic effect of poly-dispersed Single walled Carbon Nanotubes on erythrocytes in vitro and in vivo. PLoS One 6:e22032.

Sardari RRR, Zarchi SR, Talebi A, Nasri S, Imani S, Khoradmehr A, Sheshde SAR. 2012. Toxicological effects of silver nanoparticles in rats. Afr J Microbiol Res 6:5587-5593.

Saxena RK, Williams W, McGee JK, Daniels MJ, Boykin E, Gilmour MI. 2007. Enhanced in vitro and in vivo toxicity of poly-dispersed acid-functionalized single-wall carbon nanotubes. Nanotoxicology 1(4):291-300.

Shahare B, Yashpal M, Singh G. 2013. Toxic effects of repeated oral exposure of silver nanoparticles on small intestine mucosa of mice. Toxicol Mech Methods 23(3):161-7.

Singh SP, Rahman MF, Murty USN, Mahboob M, Grover P. 2013. Comparative study of genotoxicity and tissue distribution of nano and micron sized iron oxide in rats after acute oral treatment. Toxicol Appl Pharmacol 266:56-66.

Schmid K, Riediker M. 2008. Use of Nanoparticles in Swiss Industry: A Targeted Survey. Environ Sci Tech 42(7):2253-2260.

So SJ, Jang IS, Han CS. 2008. Effect of Micro/Nano Silica Particle Feeding for Mice. J Nanosci nanotech 8:5367-5371.

Szendi K, Varga C. 2008. Lack of genotoxicity of carbon nanotubes in a pilot study. Anticancer Res 28:349-352.

Taylor TM, Weiss J, Michael Davidson P, Bruce BD. 2005. Liposomal Nanocapsules in Food Science and Agriculture. Food Sci Nutri 45(7-8):587-605.

ToxRTool. Spreadsheets for in vivo and in vitro studies, available from download at http://ecva.jrc.it/; “Publications “ section.

van der Zande M, Vandebriel RJ, Van D, Kramer E, Herrera R, Serrano-Rojero CS, Gremmer ER, Mast J, Peters RJB, Hollman PCH, Hendriksen PJM, Marvin HJP, Peijnenburg AACM, Bouwmeester H. 2012. Distribution, Elimination, and Toxicity of Silver Nanoparticles and Silver Ions in Rats after 28-Day Oral Exposure. ACS Nano 6:7427-7442.

Vogelson CT. 2001. Advances in drug delivery systems. Drug Delivery Discovery 4:49-50, 52.

Walczak AP, Fokkink R, Peters R, Tromp P, Herrera RZ, Rietjens IMCM, Hendriksen PJM, Bouwmeester H. 2012. Behaviour of silver nanoparticles and silver ions in an in vitro human gastrointestinal digestion model. Nanotoxicology Early Online, 1–13.

Wang B, Feng W, Wang M, Wang T, Gu Y, Zhu M, Ouyang H, Shi J, Zhang F, Zhao Y, Chai Z, Wang H, Wang J. 2008. Acute toxicological impact of nano- and submicro-scaled zinc oxide powder on healthy adult mice. J Nanopart Res 10:263-276.

Wang J, Zhou G, Chen C, Yu H, Wang T, Ma Y, Jia G, Gao Y, Li B, Sun J, Li Y, Jiao F, Zhao Y, Chai Z. 2007. Acute toxicity and biodistribution of different sized titanium dioxide particles in mice after oral administration. Toxicol Lett 168:176-185.

Wang Y, Fu L. 2012. Forms of Selenium Affect its Transport, Uptake and Glutathione Peroxidase Activity in the Caco-2 Cell Model. Biol Trace Elem Res 149:110-116.

Wang Y, Chen Z, Ba T, Pu J, Chen T, Song Y, Gu Y, Qian Q, Xu Y, Xiang K, Wang H, Jia G. 2012. Susceptibility of Young and Adult Rats to the Oral Toxicity of Titanium Dioxide Nanoparticles. SmallAhead 2013 May 27;9(9-10):1742-52.

Wang Z, Zhao J, Song L, Mashayekhi H, Chefetz B, Xing B. 2011. Adsorption and Desorption of Phenanthrene on Carbon Nanotubes in Simulated Gastrointestinal Fluids. Environ Sci Technol 45:6018-6024.

Yamago S, Tokuyama H, nakamura E, Kikuchi K, Kananishi S, Sueki K, Nakahara H, Enomoto S, Ambe F. 1995. In vivo biological behavior of a water-miscible fullerene: 14C labeling, absorption, distribution, excretion and acute toxicity. Chem Biol 2:385-389.

Yamashita K, Yoshioka Y, Pan H, Taira M, Ogura T, Nagano T, Aoyama M, Nagano K, Abe Y, Kamada H, Tsunoda SI, Aoshima H, Nabeshi H, Yoshikawa T, Tsutsumi Y. 2013. Biochemical and hematologic effects of polyvinylpyrrolidone-wrapped fullerene C60 after oral administration. Pharmazie 68:54-57.

Zhang XD, Wu HY, Wu D, Wang YY, Chang JH, Zhai ZB, Meng AM, Liu PX, Zhang LA, Fan FY. 2010. Toxicologic effects of gold nanoparticles in vivo by different administration routes. Int J Nanomed 5:771-781.

Appendix 1: Literature search

As the reference research tool SciFinder has been used. SciFinder is a bibliographic and reference research tool that provides access to the Main Chemical Abstracts Service (CAS) literature database of over 36 million references (CAPLUS) back to 1900. SciFinder also provides access to the entire MEDLINE database (1946+), journal tables of contents, and citation linking. In the below reported literature search no limitation was made on period.

Terms used in the following Appendixes:

Nano: Term used to identify words starting with “nano-“.

Refine: means to *identify and include* hits within a number of hits (e.g.: “refine: oral”: gives the hits containing the word oral *within* the group of hits of nano-).

Exclude: means to *identify and exclude* hits within a number of hits (e.g.: “exclude patents” will exclude all patents from the search).

Appendix 2: Available hits based on “nano” and other search terms

Appendix 2 contains a table with the first search made with the search term “nano” and other with various limitations applied to the search. The result revealed that the number of hits was too many to process by retrieving relevant papers based on this search.

To continue a first step was made to identify commonly used terms, not relevant for the purpose of this project, starting with “nano-“ – see Appendix 3.

Date of search: 20/1/2013

SEARCH-number	Search terms (e.g. substance name, CAS No and combinations.etc)	Limitations applied to search	No of 'hits'	Comments & follow-up actions
SEARCH-1	Nano		1203755	
SEARCH-2	Nano	Exclude Patents	1045829	
SEARCH-3	Nano	Exclude Patents Refine: oral	4760	
SEARCH-4	Nano	Exclude Patents Refine: oral Refine: uptake	537	
SEARCH-5	Nano	Exclude Patents Refine: oral Refine: absorption	777	
SEARCH-6	Nano	Exclude Patents Refine: gavage	123	
SEARCH-7	Nano	Exclude Patents Refine: food	15430	Includes “feed”
SEARCH-8	Nano	Exclude Patents Refine: absorption	70970	
SEARCH-9	Nano	Exclude Patents Refine: gastro-intestinal	1559	
SEARCH-10	Nano	Exclude Patents Refine: gi tract	1041	
SEARCH-11	Nano	Exclude Patents Refine: digestive tract	1041	
SEARCH-12	Nano	Exclude Patents Refine: gastric	60435	
SEARCH-13	Nano	Exclude Patents Refine: saliva	641	
SEARCH-14	Nano	Exclude Patents	2554	

SEARCH-number	Search terms (e.g. substance name, CAS No and combinations.etc)	Limitations applied to search	No of 'hits'	Comments & follow-up actions
		Refine: intestine		
SEARCH-15	Nano	Exclude Patents Refine: stomach	1101	
SEARCH-16	Nano	Exclude Patents Refine: duodenum	117	
SEARCH-17	Nano	Exclude Patents Refine: jejunum	114	
SEARCH-18	Nano	Exclude Patents Refine: ileum	226	
SEARCH-19	Nano	Exclude Patents Refine: peyer's patch	106	
SEARCH-20	Nano	Exclude Patents Refine: cecum	65	
SEARCH-21	Nano	Exclude Patents Refine: colon	1432	
SEARCH-22	Nano	Exclude Patents Refine: feces	389	Includes "faeces"
SEARCH-23	Nano	Exclude Patents Refine: epithelial barrier	156	
SEARCH-24	Nano	Exclude Patents Refine: cell line	22213	
SEARCH-25	Nano	Exclude Patents Refine: caco-2	1367	
SEARCH-26	Nano	Exclude Patents Refine: enterocyte	354	
SEARCH-27	Nano	Exclude Patents Refine: consumer products	1354	
SEARCH-28	Microparticle		50383	
SEARCH-29	Microparticle	Exclude Patents	28666	
SEARCH-30	Microparticle	Exclude Patents Refine: oral	962	
SEARCH-31	Microparticle	Exclude Patents Refine: uptake	677	
SEARCH-32	Microparticle	Exclude Patents Refine: gavage	20	
SEARCH-33	Microparticle	Exclude Patents Refine: food	621	Includes "feed"

SEARCH-number	Search terms (e.g. substance name, CAS No and combinations.etc)	Limitations applied to search	No of 'hits'	Comments & follow-up actions
SEARCH-34	Microparticle	Exclude Patents Refine: absorption	960	
SEARCH-35	Microparticle	Exclude Patents Refine: gastro-intestinal	296	
SEARCH-36	Microparticle	Exclude Patents Refine: gi tract	218	Includes “digestive tract”
SEARCH-37	Microparticle	Exclude Patents Refine: gastric	1405	
SEARCH-38	Microparticle	Exclude Patents Refine: saliva	47	
SEARCH-39	Microparticle	Exclude Patents Refine: intestine	518	
SEARCH-40	Microparticle	Exclude Patents Refine: stomach	130	
SEARCH-41	Microparticle	Exclude Patents Refine: duodenum	8	
SEARCH-42	Microparticle	Exclude Patents Refine: jejunum	16	
SEARCH-43	Microparticle	Exclude Patents Refine: ileum	17	
SEARCH-44	Microparticle	Exclude Patents Refine: peyer’s patch	101	
SEARCH-45	Microparticle	Exclude Patents Refine: cecum	3	
SEARCH-46	Microparticle	Exclude Patents Refine: colon	134	
SEARCH-47	Microparticle	Exclude Patents Refine: feces	41	Includes “faeces”
SEARCH-48	Microparticle	Exclude Patents Refine: epithelial barrier	31	
SEARCH-49	Microparticle	Exclude Patents Refine: cell line	684	
SEARCH-50	Microparticle	Exclude Patents Refine: caco-2	115	
SEARCH-51	Microparticle	Exclude Patents Refine: enterocyte	30	
SEARCH-52	Microparticle	Exclude Patents	25	

SEARCH-number	Search terms (e.g. substance name, CAS No and combinations.etc)	Limitations applied to search	No of 'hits'	Comments & follow-up actions
		Refine: consumer product		
SEARCH-53	Particle		2035968	
SEARCH-54	Particle	Exclude Patents	1569025	
SEARCH-55	Particle	Exclude Patents Refine: oral	10051	
SEARCH-56	Particle	Exclude Patents Refine: uptake	25407	
SEARCH-57	Particle	Exclude Patents Refine: gavage	181	
SEARCH-58	Particle	Exclude Patents Refine: food	35903	Includes “feed”
SEARCH-59	Particle	Exclude Patents Refine: absorption	64704	
SEARCH-60	Particle	Exclude Patents Refine: gastro-intestinal	3932	
SEARCH-61	Particle	Exclude Patents Refine: gi tract	2622	Includes “digestive tract”
SEARCH-62	Particle	Exclude Patents Refine: gastric	152641	
SEARCH-63	Particle	Exclude Patents Refine: saliva	1332	
SEARCH-64	Particle	Exclude Patents Refine: intestine	6213	
SEARCH-65	Particle	Exclude Patents Refine: stomach	2383	
SEARCH-66	Particle	Exclude Patents Refine: duodenum	473	
SEARCH-67	Particle	Exclude Patents Refine: jejunum	360	
SEARCH-68	Particle	Exclude Patents Refine: ileum	369	
SEARCH-69	Particle	Exclude Patents Refine: peyer’s patch	289	
SEARCH-70	Particle	Exclude Patents Refine: cecum	202	
SEARCH-71	Particle	Exclude Patents Refine: colon	1659	

SEARCH-number	Search terms (e.g. substance name, CAS No and combinations.etc)	Limitations applied to search	No of 'hits'	Comments & follow-up actions
SEARCH-72	Particle	Exclude Patents Refine: feces	2630	Includes “faeces”
SEARCH-73	Particle	Exclude Patents Refine: epithelial barrier	258	
SEARCH-74	Particle	Exclude Patents Refine: cell line	28248	
SEARCH-75	Particle	Exclude Patents Refine: caco-2	2249	
SEARCH-76	Particle	Exclude Patents Refine: enterocyte	701	
SEARCH-77	Particle	Exclude Patents Refine: consumer product	2207	
SEARCH-78	Particulate		197692	
SEARCH-79	Particulate	Exclude Patents	155207	
SEARCH-80	Particulate	Exclude Patents Refine: oral	727	
SEARCH-81	Particulate	Exclude Patents Refine: uptake	3871	
SEARCH-82	Particulate	Exclude Patents Refine: gavage	26	
SEARCH-83	Particulate	Exclude Patents Refine: food	5164	Includes “feed”
SEARCH-84	Particulate	Exclude Patents Refine: absorption	5285	
SEARCH-85	Particulate	Exclude Patents Refine: gastro-intestinal	724	
SEARCH-86	Particulate	Exclude Patents Refine: gi tract	459	Includes “digestive tract”
SEARCH-87	Particulate	Exclude Patents Refine: gastric	32440	
SEARCH-88	Particulate	Exclude Patents Refine: saliva	229	
SEARCH-89	Particulate	Exclude Patents Refine: intestine	1084	
SEARCH-90	Particulate	Exclude Patents	384	

SEARCH-number	Search terms (e.g. substance name, CAS No and combinations.etc)	Limitations applied to search	No of 'hits'	Comments & follow-up actions
		Refine: stomach		
SEARCH-91	Particulate	Exclude Patents Refine: duodenum	121	
SEARCH-92	Particulate	Exclude Patents Refine: jejunum	72	
SEARCH-93	Particulate	Exclude Patents Refine: ileum	110	
SEARCH-94	Particulate	Exclude Patents Refine: peyer's patch	82	
SEARCH-95	Particulate	Exclude Patents Refine: cecum	48	
SEARCH-96	Particulate	Exclude Patents Refine: colon	263	
SEARCH-97	Particulate	Exclude Patents Refine: feces	652	Includes "faeces"
SEARCH-98	Particulate	Exclude Patents Refine: epithelial barrier	65	
SEARCH-99	Particulate	Exclude Patents Refine: cell line	2117	
SEARCH-100	Particulate	Exclude Patents Refine: caco-2	183	
SEARCH-101	Particulate	Exclude Patents Refine: enterocyte	134	
SEARCH-102	Particulate	Exclude Patents Refine: consumer product	640	
SEARCH-103	Colloid		276502	
SEARCH-104	Colloid	Exclude Patents	204649	
SEARCH-105	Colloid	Exclude Patents Refine: oral	1523	
SEARCH-106	Colloid	Exclude Patents Refine: uptake	3440	
SEARCH-107	Colloid	Exclude Patents Refine: gavage	37	
SEARCH-108	Colloid	Exclude Patents Refine: food	4373	Includes "feed"

SEARCH-number	Search terms (e.g. substance name, CAS No and combinations.etc)	Limitations applied to search	No of 'hits'	Comments & follow-up actions
SEARCH-109	Colloid	Exclude Patents Refine: absorption	12361	
SEARCH-110	Colloid	Exclude Patents Refine: gastro-intestinal	1149	
SEARCH-111	Colloid	Exclude Patents Refine: gi tract	494	Includes “digestive tract”
SEARCH-112	Colloid	Exclude Patents Refine: gastric	8258	
SEARCH-113	Colloid	Exclude Patents Refine: saliva	326	
SEARCH-114	Colloid	Exclude Patents Refine: intestine	1486	
SEARCH-115	Colloid	Exclude Patents Refine: stomach	951	
SEARCH-116	Colloid	Exclude Patents Refine: duodenum	149	
SEARCH-117	Colloid	Exclude Patents Refine: jejunum	86	
SEARCH-118	Colloid	Exclude Patents Refine: ileum	80	
SEARCH-119	Colloid	Exclude Patents Refine: peyer’s patch	27	
SEARCH-120	Colloid	Exclude Patents Refine: cecum	69	
SEARCH-121	Colloid	Exclude Patents Refine: colon	440	
SEARCH-122	Colloid	Exclude Patents Refine: feces	294	Includes “faeces”
SEARCH-123	Colloid	Exclude Patents Refine: epithelial barrier	36	
SEARCH-124	Colloid	Exclude Patents Refine: cell line	1383	
SEARCH-125	Colloid	Exclude Patents Refine: caco-2	348	
SEARCH-126	Colloid	Exclude Patents Refine: enterocyte	101	
SEARCH-127	Colloid	Exclude Patents	264	

SEARCH-number	Search terms (e.g. substance name, CAS No and combinations.etc)	Limitations applied to search	No of 'hits'	Comments & follow-up actions
		Refine: consumer product		
SEARCH-128	Quantum dot		68889	
SEARCH-129	Quantum dot	Exclude Patents	63329	
SEARCH-130	Quantum dot	Exclude Patents Refine: oral	45	
SEARCH-131	Quantum dot	Exclude Patents Refine: uptake	560	
SEARCH-132	Quantum dot	Exclude Patents Refine: gavage	0	
SEARCH-133	Quantum dot	Exclude Patents Refine: food	240	Includes “feed”
SEARCH-134	Quantum dot	Exclude Patents Refine: absorption	6110	
SEARCH-135	Quantum dot	Exclude Patents Refine: gastro-intestinal	35	
SEARCH-136	Quantum dot	Exclude Patents Refine: gi tract	23	Includes “digestive tract”
SEARCH-137	Quantum dot	Exclude Patents Refine: gastric	2126	
SEARCH-138	Quantum dot	Exclude Patents Refine: saliva	12	
SEARCH-139	Quantum dot	Exclude Patents Refine: intestine	47	
SEARCH-140	Quantum dot	Exclude Patents Refine: stomach	35	
SEARCH-141	Quantum dot	Exclude Patents Refine: duodenum	3	
SEARCH-142	Quantum dot	Exclude Patents Refine: jejunum	1	
SEARCH-143	Quantum dot	Exclude Patents Refine: ileum	2	
SEARCH-144	Quantum dot	Exclude Patents Refine: peyer's patch	2	
SEARCH-145	Quantum dot	Exclude Patents Refine: cecum	0	

SEARCH-number	Search terms (e.g. substance name, CAS No and combinations.etc)	Limitations applied to search	No of 'hits'	Comments & follow-up actions
SEARCH-146	Quantum dot	Exclude Patents Refine: colon	51	
SEARCH-147	Quantum dot	Exclude Patents Refine: feces	24	Includes “faeces”
SEARCH-148	Quantum dot	Exclude Patents Refine: epithelial barrier	7	
SEARCH-149	Quantum dot	Exclude Patents Refine: cell line	1028	
SEARCH-150	Quantum dot	Exclude Patents Refine: caco-2	14	
SEARCH-151	Quantum dot	Exclude Patents Refine: enterocyte	5	
SEARCH-152	Quantum dot	Exclude Patents Refine: consumer product	31	
SEARCH-153	Fullerene		58538	
SEARCH-154	Fullerene	Exclude Patents	52673	
SEARCH-155	Fullerene	Exclude Patents Refine: oral	26	
SEARCH-156	Fullerene	Exclude Patents Refine: uptake	225	
SEARCH-157	Fullerene	Exclude Patents Refine: gavage	2	
SEARCH-158	Fullerene	Exclude Patents Refine: food	157	Includes “feed”
SEARCH-159	Fullerene	Exclude Patents Refine: absorption	5485	
SEARCH-160	Fullerene	Exclude Patents Refine: gastro-intestinal	21	
SEARCH-161	Fullerene	Exclude Patents Refine: gi tract	16	Includes “digestive tract”
SEARCH-162	Fullerene	Exclude Patents Refine: gastric	2694	
SEARCH-163	Fullerene	Exclude Patents Refine: saliva	3	
SEARCH-164	Fullerene	Exclude Patents Refine: intestine	25	

SEARCH-number	Search terms (e.g. substance name, CAS No and combinations.etc)	Limitations applied to search	No of 'hits'	Comments & follow-up actions
SEARCH-165	Fullerene	Exclude Patents Refine: stomach	11	
SEARCH-166	Fullerene	Exclude Patents Refine: duodenum	0	
SEARCH-167	Fullerene	Exclude Patents Refine: jejunum	2	
SEARCH-168	Fullerene	Exclude Patents Refine: ileum	2	
SEARCH-169	Fullerene	Exclude Patents Refine: peyer's patch	0	
SEARCH-170	Fullerene	Exclude Patents Refine: cecum	0	
SEARCH-171	Fullerene	Exclude Patents Refine: colon	8	
SEARCH-172	Fullerene	Exclude Patents Refine: feces	8	Includes “faeces”
SEARCH-173	Fullerene	Exclude Patents Refine: epithelial barrier	3	
SEARCH-174	Fullerene	Exclude Patents Refine: cell line	210	
SEARCH-175	Fullerene	Exclude Patents Refine: caco-2	3	
SEARCH-176	Fullerene	Exclude Patents Refine: enterocyte	4	
SEARCH-177	Fullerene	Exclude Patents Refine: consumer product	54	

Appendix 3: List of non-relevant "nano-terms"

Appendix 3 contains a table showing the results of a step in the literature search made to identify commonly used terms, *not* relevant for the purpose of this project, starting with "nano-". This was done to see if exclusion of these search terms could diminish the number of hits.

Two approaches were used: first approach identified the number of hits containing the commonly used terms. Second approach identified the number of hits containing nano* *but not* commonly used terms deemed not relevant.

Date of search: 23/1/2013

SEARCH-number	Search term	Number of hits	Comments
SEARCH-178	Nano*	1205965	1205965 references were found containing the concept* "nano" (i.e. all words containing "nano" were identified in this search)
SEARCH-179	Nano	172260	172260 references were found containing "nano" as entered
SEARCH-180	Nanogram	8055	8055 references were found containing the concept "nanogram"
SEARCH-181	Nanosecond	24432	24432 references were found containing the concept "nanosecond"
SEARCH-182	Nanomet* (meter, metres, metre etc)	79032	79032 references were found containing the concept "nanomet"
SEARCH-183	Nano*	1205965	
SEARCH-184	Refine: NOT nanogram	1197910	The number of hits for nano* excluding all hits containing nanogram
SEARCH-185	Refine: NOT nanosecond	1173478	The number of hits for nano* excluding all hits containing nanosecond
SEARCH-186	Refine: NOT nanometer	1094819	The number of hits for nano* excluding all hits containing nanometer

Appendix 4: First screening for relevant original papers

Appendix 4 contains a table showing the results of literature searches based on decisions made by the chemistry and toxicology experts that the following combination shown in the table should be applied. Reviews were excluded as it was suggested that a search could be made at a later stage on specifically on reviews.

25/1/2013: Search performed for original papers considered relevant for this evaluation with the number of hits shown.

30/1/2013: Search performed for original papers considered relevant for this evaluation and further refined by excluding terms considered *not* relevant for the purpose of this project.

Date of search: 25 & 30/1/2013

SEARCH-number	Search terms (e.g. substance name, CAS No and combinations etc)	Limitations applied to search	No of 'hits' 25/1	No of 'hits' 30/1	Comments & follow-up actions
SEARCH-187	Nano		1207566	945516*	*All reports in Chinese have been excluded as have reviews and patents before search on Nano was initiated
SEARCH-188	Nano	Refine: oral	9058	6444	
SEARCH-189	Nano	Refine: oral Exclude Patents and review	6866	--*	
SEARCH-190	Nano	Refine: oral Exclude Patents and review Refine: uptake	732	723	
SEARCH-191	Nano	Refine: oral Exclude Patents and review Refine: uptake Refine: absorption	280	276	
SEARCH-192	Nano	Refine: oral Exclude Patents and review Refine: uptake Refine: absorption excluding “nanogram”	277	273	
SEARCH-193	Nano	Refine: oral Exclude Patents and	275	271	

SEARCH-number	Search terms (e.g. substance name, CAS No and combinations etc)	Limitations applied to search	No of 'hits' 25/1	No of 'hits' 30/1	Comments & follow-up actions
		review Refine: uptake Refine: absorption excluding “nanogram” excluding “dermal”			
SEARCH-194	Nano	Refine: oral Exclude Patents and review Refine: uptake Refine: absorption excluding “nanogram” excluding “dermal” excluding “inhalation”	269	266	
SEARCH-195	Nano	Refine: oral Exclude Patents and review Refine: uptake Refine: absorption excluding “nanogram” excluding “dermal” excluding “inhalation” excluding duplicates of papers	226	223	25/1 Abstracts in pdf: Nano uptake abs 250113
SEARCH-196	Nano	Refine: oral Exclude Patents and review Refine: uptake Refine: absorption excluding “nanogram” excluding “dermal” excluding “inhalation” excluding duplicates of papers excluding “drug”		14	
SEARCH-197	Nano	Refine: oral Exclude Patents and review Refine: uptake Refine: absorption excluding “nanogram” excluding “dermal” excluding “inhalation” excluding duplicates of papers excluding “drug” excluding “insulin”		12	

SEARCH-number	Search terms (e.g. substance name, CAS No and combinations etc)	Limitations applied to search	No of 'hits' 25/1	No of 'hits' 30/1	Comments & follow-up actions
SEARCH-198	Nano	Refine: oral Exclude Patents and review Refine: uptake Refine: absorption excluding “nanogram” excluding “dermal” excluding “inhalation” excluding duplicates of papers excluding “drug” excluding “insulin” excluding “nanomolar”		12	
SEARCH-199	Nano	Refine: oral Exclude Patents and review Refine: uptake Refine: absorption excluding “nanogram” excluding “dermal” excluding “inhalation” excluding duplicates of papers excluding “drug” excluding “insulin” excluding “nanomolar” excluding “nanoemulsion”		12	30/1 Abstracts in pdf: Nano uptake abs 300113

Appendix 5: Second screening for relevant original papers

Appendix 5 contains a table showing the results of literature searches based on decisions made on the previous results with the exception that reviews were included in the below search, however, they have not been scrutinised at this stage.

It was concluded that the search should exclude the following terms: drug, insulin, cancer, delivery, nanomolar, nanoemulsion, nanogram, [language: Chinese], patent – and the search was subsequently refined with each of the following terms: Oral, Oral + Absorption, Oral + Uptake, Gavage, Gavage not oral, Gavage + Absorption, Gavage + Uptake, Gastro-intestinal, Gastro-intestinal not oral, Gastro-intestinal not oral and not uptake, Caco-2 + Uptake.

Abstracts of the relevant results were printed out and chemistry and toxicology experts went through the abstracts. See Appendix 5 for a list of these relevant results.

.Ris files were saved for import to the Reference Manager database.

Date for search: 3/2/2013

SEARCH-number	Search terms (e.g. substance name, CAS No and combinations.etc)	Limitations applied to search	No of 'hits'	List of relevant items	Comments & follow-up actions
SEARCH-200	Nano	Exclude patents and language Chinese	965715		
SEARCH-201	Nano	Exclude patents and language Chinese excluding “drug”	882784		
SEARCH-202	Nano	Exclude patents and language Chinese excluding “drug” excluding “insulin”	881248		
SEARCH-203	Nano	Exclude patents and language Chinese excluding “drug” excluding “insulin” excluding “cancer”	865907		
SEARCH-204	Nano	Exclude patents and language Chinese excluding “drug” excluding “insulin” excluding “cancer” excluding “delivery”	856567		
SEARCH-205	Nano	Exclude patents and language Chinese	843101		

SEARCH-number	Search terms (e.g. substance name, CAS No and combinations.etc)	Limitations applied to search	No of 'hits'	List of relevant items	Comments & follow-up actions
		excluding “drug” excluding “insulin” excluding “cancer” excluding “delivery” excluding “nanomolar”			
SEARCH-206	Nano	Exclude patents and language Chinese excluding “drug” excluding “insulin” excluding “cancer” excluding “delivery” excluding “nanomolar” excluding “nanoemulsion”	842472		
SEARCH-207	Nano	Exclude patents and language Chinese excluding “drug” excluding “insulin” excluding “cancer” excluding “delivery” excluding “nanomolar” excluding “nanoemulsion” excluding “nanogram”	836397		The following limitations are applied to this figure Doublets are automatically removed
SEARCH-208	Nano	The search result (836397 hits) Refine: “Oral”	685		
SEARCH-209	Nano	The above search result (685) Refine: “oral” + “absorption”	44		Hits saved as .ris and .pdf (abstracts)
SEARCH-210	Nano	The above search result (685) Refine: “oral” + “uptake”	26		Hits saved as .ris and .pdf (abstracts)
SEARCH-211	Nano	The search result (836397 hits)	43		

SEARCH-number	Search terms (e.g. substance name, CAS No and combinations.etc)	Limitations applied to search	No of 'hits'	List of relevant items	Comments & follow-up actions
		Refine: "Gavage"			
SEARCH-212	Nano	The above search result (43) excluding "Oral"	15		Hits saved as .ris and .pdf (abstracts)
SEARCH-213	Nano	The above search result (15) Refine: "Absorption"	1	Not relevant – concerns $NaNO_2$	
SEARCH-214	Nano	The above search result (15) Refine "Uptake"	1		Hits saved as .ris and .pdf (abstracts)
SEARCH-215	Nano	The search result (836397 hits) Refine: "Gastro-intestinal"	447		
SEARCH-216	Nano	The above search result (447) excluding "oral"	403		
SEARCH-217	Nano	The above search result (403) excluding "uptake"	401		Hits saved as .ris and .pdf (abstracts)
SEARCH-218	Nano	The search result (836397 hits) Refine "Caco-2"	486		
SEARCH-219	Nano	The above search result (486) Refine "Caco-2" + "uptake"	33		Hits saved as .ris and .pdf (abstracts)

Appendix 6: List of abstracts from second screening

Appendix 6 contains a list of abstracts based on the literature search described in Appendix 5.

A rough sorting based on the abstracts in these lists was made by at least one toxicologist and one chemist. Possible relevant papers were marked and included in a Reference Manager database and the original papers obtained. The Reference Manager database contains one database with original papers.

SEARCH-number	name of the .pdf containing the result of limitation applied to the search	number of abstracts in the .pdf
SEARCH-209	Nano_oral-abs_030213	44
SEARCH-210	Nano_oral_uptake_030213	26
SEARCH-212	Nano_gavage_not oral_030213	15
SEARCH-217	Nano_gastro_not oral uptake_030213	401
SEARCH-219	Nano_caco2_uptake_030213	33
	Total	519

Appendix 7: List of all references included in the Reference Manager database

Abe, S., Koyama, C., Esaki, M., Akasaka, T., Uo, M., Kuboki, Y., Morita, M., & Watari, F. 2009. In vivo internal diffusion of several inorganic microparticles through oral administration. *Biomedical Materials and Engineering*, 19, (2-3) 221-229

Asuri, P., Karajanagi, S.S., Vertegel, A.A., Dordick, J.S., & Kane, R.S. 2007. Enhanced stability of enzymes adsorbed onto nanoparticles. *Journal of Nanoscience and Nanotechnology*, 7, (4/5) 1675-1678

Awasthi, K.K., John, P.J., Awasthi, A., & Awasthi, K. 2013. Multi walled carbon nano tubes induced hepatotoxicity in Swiss albino mice. *Micron*, 44, 359-364

Baek, M., Chung, H.E., Yu, J., Lee, J.A., Kim, T.H., Oh, J.M., Lee, W.J., Paek, S.M., Lee, J.K., Jeong, J., Choy, J.H., & Choi, S.J. 2012. Pharmacokinetics, tissue distribution, and excretion of zinc oxide nanoparticles. *International journal of nanomedicine*, 7, 3081-3097

Behrens, I., Pena, A.I.V., Alonso, M.J., & Kissel, T. 2002. Comparative uptake studies of bioadhesive and non-bioadhesive nanoparticles in human intestinal cell lines and rats: the effect of mucus on particle adsorption and transport. *Pharmaceutical research*, 19, (8) 1185-1193

Bianco, A., Kostarelos, K., & Prato, M. 2005. Applications of carbon nanotubes in drug delivery. *Current Opinion in Chemical Biology*, 9, (6) 674-679

Bouwmeester, H., Poortman, J., Peters, R.J., Wijma, E., Kramer, E., Makama, S., Puspitaninganindita, K., Marvin, H.J.P., Peijnenburg, A.A.C.M., & Hendriksen, P.J.M. 2011. Characterization of Translocation of Silver Nanoparticles and Effects on Whole-Genome Gene Expression Using an In Vitro Intestinal Epithelium Coculture Model. *ACS Nano*, 5, (5) 4091-4103

Card, J.W. & Magnuson, B.A. 2010. A method to assess the quality of studies that examine the toxicity of engineered nanomaterials. *International Journal of Toxicology*, 29, (4) 402-410

Chaudhry, Q., Scotter, M., Blackburn, J., Ross, B., Boxall, A., Castle, L., Aitken, R., & Watkins, R. 2008. Applications and implications of nanotechnologies for the food sector. *Food Additives & Contaminants, Part A: Chemistry, Analysis, Control, Exposure & Risk Assessment*, 25, (3) 241-258

Cicchetti, R., Divizia, M., Valentini, F., & Argentin, G. 2011. Effects of single-wall carbon nanotubes in human cells of the oral cavity: Geno-cytotoxic risk. *Toxicology in Vitro*, 25, (8) 1811-1819

Dekkers, S., Krystek, P., Peters, R.J.B., Lankveld, D.P.K., Bokkers, B.G.H., Van, H.A., Bouwmeester, H., & Oomen, A.G. 2011. Presence and risks of nanosilica in food products. *Nanotoxicology*, 5, (3) 393-405

Dekkers, S., Bouwmeester, H., Bos, P., Peters, R., Rietveld, A., & Oomen, A. 2012. Knowledge gaps in risk assessment of nanosilica in food: evaluation of the dissolution and toxicity of different forms of silica. *Nanotoxicology*

des Rieux, A., Fievez, V., Theate, I., Mast, J., Preat, V., & Schneider, Y.J. 2007. An improved in vitro model of human intestinal follicle-associated epithelium to study nanoparticle transport by M cells. *European Journal of Pharmaceutical Sciences*, 30, (5) 380-391

ECETOC, Bosch, A., Heinemann, M., Hendrickx, B., Maier, M., Reteuan, C., Bars, R., Calow, P., de, W., Doe, J., Douben, P., Fluckiger, A., Greim, H., Hutchinson, T., Money, C., Owen, D., Swaen, G., van, R., & Wieand, H. J. 2006, *Synthetic amorphous silica (CAS No. 7631-86-9)*, Degussa.

EFSA 2004, *Opinion of the Scientific Panel on Food Additives, Flavourings, Processing Aids and materials in Contact with Food on a request from the Commission related to the safety in use of rutile titanium dioxide as an alternative to the presently permitted anatase form. Question N° EFSA-Q-2004-103.* 163.

EFSA 2009, *The potential risks arising from nanoscience and nanotechnologies on food and feed safety. Scientific opinion of the scientific committee. Question No EFSA-Q-2007-124a.* 958.

EFSA 2009, *Calcium silicate and silicon dioxide/silicic acid gel added for nutritional purposes to food supplements. Scientific opinion of the Panel on food additives and nutrient sources added to food. Questions No EFSA-Q-2005-140, EFSA-Q-2006-220, EFSA-Q-2005-098, EFSA-Q-2005-099.* 1132.

EFSA 2010, *Applications of systematic review methodology to food and feed safety assessments to support decision making. Question No EFSA-Q-2008-717.* 8 (6).

EFSA 2011, *Guidance on the risk assessment of the application of nanoscience and nanotechnologies in the food and feed chain. Question No EFSA-Q-2009-00942.* 9 (5).

ENRHES 2009, *Engineered Nanoparticles: Review of Health and Environmental Safety (ENRHES). The project was funded under grant 218433 of the Seventh Framework Programme of the European Commission.* Project Final Report.

Faddah, L.M., Abdel, B., Al-Rasheed, N.M., Al-Rasheed, N.M., Fatani, A.J., & Atteya, M. 2012. Role of quercetin and arginine in ameliorating nano zinc oxide-induced nephrotoxicity in rats. *BMC Complementary and Alternative Medicine*, 12, 60

Fall, M., Guerbet, M., Park, B., Gouriou, F., Dionnet, F., & Morin, J.P. 2007. Evaluation of cerium oxide and cerium oxide based fuel additive safety on organotypic cultures of lung slices. *Nanotoxicology* , 1, (3) 227-234 available from: http://www.informaworld.com/smpp/content-í+æ+ócontent=a787296599-í+æ+ódb=all-í+æ+óorder=page

Folkmann, J.K., Risom, L., Jacobsen, N.R., Wallin, H., Loft, S., & Moller, P. 2009. Oxidatively Damaged DNA in Rats Exposed by Oral Gavage to C-60 Fullerenes and Single-Walled Carbon Nanotubes. *Environmental Health Perspectives*, 117, (5) 703-708

Gaiser, B.K., Fernandes, T.F., Jepson, M., Lead, J.R., Tyler, C.R., & Stone, V. 2009. Assessing exposure, uptake and toxicity of silver and cerium dioxide nanoparticles from contaminated environments. *Environmental Health (London, United Kingdom)*, 8, (Suppl. 1) No available from: http://www.ehjournal.net/content/pdf/1476-069X-8-S1-S2.pdf

Gaiser, B.K., Fernandes, T.F., Jepson, M.A., Lead, J.R., Tyler, C.R., Baalousha, M., Biswas, A., Britton, G.J., Cole, P.A., Johnston, B.D., Ju-Nam, Y., Rosenkranz, P., Scown, T.M., & Stone, V. 2012. Interspecies comparisons on the uptake and toxicity of silver and cerium dioxide nanoparticles. *Environmental Toxicology and Chemistry*, 31, (1) 144-154

Gao, B., Wu, S.h., Zhang, H.l., Tao, Y., & Su, Z.q. 2011. Study on the lipid-lowering effect of water-soluble chitosan nanoparticles and microspheres in vitro. *Advanced Materials Research (Zuerich, Switzerland)*, 217-218, (Pt. 1, High Performance Structures and Materials Engineering) 306-310

Gaumet, M., Gurny, R., & Delie, F. 2010. Interaction of biodegradable nanoparticles with intestinal cells: the effect of surface hydrophilicity. *Int.J Pharm.*, 390, (1) 45-52

Gerloff, K., Albrecht, C., Boots, A.W., Foerster, I., & Schins, R.P.F. 2009. Cytotoxicity and oxidative DNA damage by nanoparticles in human intestinal Caco-2 cells. *Nanotoxicology*, 3, (4) 355-364 available from: http://informahealthcare.com/loi/nan

Hadrup, N., Lam, H.R., Loeschner, K., Mortensen, A., Larsen, E.H., & Frandsen, H. 2012. Nanoparticulate silver increases uric acid and allantoin excretion in rats, as identified by metabolomics. *Journal of Applied Toxicology*, 32, (11) 929-933

Hadrup, N., Loeschner, K., Mortensen, A., Sharma, A.K., Qvortrup, K., Larsen, E.H., & Lam, H.R. 2012. The similar neurotoxic effects of nanoparticulate and ionic silver in vivo and in vitro. *NeuroToxicology*, 33, (3) 416-423

Hillyer, J.F. & Albrecht, R.M. 2001. Gastrointestinal persorption and tissue distribution of differently sized colloidal gold nanoparticles. *Journal of Pharmaceutical Sciences*, 90, (12) 1927-1936

Holister, P., Román, C., & Harper, T. 3 A.D., *Fullerenes*, Científica, 7.

Hussain, N., Jani, P.U., & Florence, A.T. 1997. Enhanced oral uptake of tomato lectin-conjugated nanoparticles in the rat. *Pharmaceutical research*, 14, (5) 613-618

Jahn, M.R., Nawroth, T., Fuetterer, S., Wolfrum, U., Kolb, U., & Langguth, P. 2012. Iron Oxide/Hydroxide Nanoparticles with Negatively Charged Shells Show Increased Uptake in Caco-2 Cells. *Molecular Pharmaceutics*, 9, (6) 1628-1637

Jahn, M.R., Shukoor, I., Tremel, W., Wolfrum, U., Kolb, U., Nawroth, T., & Langguth, P. 2011. Hemin-coupled iron(III)-hydroxide nanoparticles show increased uptake in Caco-2 cells. *The Journal of pharmacy and pharmacology*, 63, (12) 1522-1530

Jani, P., Halbert, G.W., Langridge, J., & Florence, A.T. 1990. Nanoparticle uptake by the rat gastrointestinal mucosa: quantitation and particle size dependency. *The Journal of pharmacy and pharmacology*, 42, (12) 821-826

Jani, P.U., McCarthy, D.E., & Florence, A.T. 1994. Titanium dioxide (rutile) particle uptake from the rat GI tract and translocation to systemic organs after oral administration. *International Journal of Pharmaceutics*, 105, (2) 157-168

Jos, A., Pichardo, S., Puerto, M., Sanchez, E., Grilo, A., & Camean, A.M. 2009. Cytotoxicity of carboxylic acid functionalized single wall carbon nanotubes on the human intestinal cell line Caco-2. *Toxicology in Vitro*, 23, (8) 1491-1496

Jumagazieva, D.S., Maslyakova, G.N., Suleymanova, L.V., Bucharskaya, A.B., Firsova, S.S., Khlebtsov, B.N., Terentyuk, G.S., Kong, S.M., & Khlebtsov, N.G. 2011. Mutagenic effect of gold nanoparticles in the micronucleus assay. *Bulletin of Experimental Biology and Medicine*, 151, (6) 731-733

Kaittanis, C., Naser, S.A., & Perez, J.M. 2007. One-Step, Nanoparticle-Mediated Bacterial Detection with Magnetic Relaxation. *Nano letters*, 7, (2) 380-383

Kim, W.Y., Kim, J., Park, J.D., Ryu, H.Y., & Yu, I.J. 2009. Histological study of gender differences in accumulation of silver nanoparticles in kidneys of Fischer 344 rats. *Journal of Toxicology and Environmental Health, Part A: Current Issues*, 72, (21 & 22) 1279-1284

Kim, Y.S., Kim, J.S., Cho, H.S., Rha, D.S., Kim, J.M., Park, J.D., Choi, B.S., Lim, R., Chang, H.K., Chung, Y.H., Kwon, I.H., Jeong, J., Han, B.S., & Yu, I.J. 2008. Twenty-Eight-Day Oral Toxicity, Genotoxicity, and Gender-Related Tissue Distribution of Silver Nanoparticles in Sprague-Dawley Rats. *Inhalation Toxicology*, 20, (6) 575-583

Kim, Y.S., Song, M.Y., Park, J.D., Song, K.S., Ryu, H.R., Chung, Y.H., Chang, H.K., Lee, J.H., Oh, K.H., Kelman, B.J., Hwang, I.K., & Yu, I.J. 2010. Subchronic oral toxicity of silver nanoparticles. *Particle and Fibre Toxicology*, 7, No available from: http://www.particleandfibretoxicology.com/content/pdf/1743-8977-7-20.pdf

Klimisch, H.J., Andreae, M., & Tillmann, U. 1997. A systematic approach for evaluating the quality of experimental toxicological and ecotoxicological data. *Regulatory Toxicology and Pharmacology*, 25, (1) 1-5

Koeneman, B.A., Zhang, Y., Westerhoff, P., Chen, Y., Crittenden, J.C., & Capco, D.G. 2010. Toxicity and cellular responses of intestinal cells exposed to titanium dioxide. *Cell Biology and Toxicology*, 26, (3) 225-238

Kolosnjaj-Tabi, J., Hartman, K.B., Boudjemaa, S., Ananta, J.S., Morgant, G., Szwarc, H., Wilson, L.J., & Moussa, F. 2010. In Vivo Behavior of Large Doses of Ultrashort and Full-Length Single-Walled Carbon Nanotubes after Oral and Intraperitoneal Administration to Swiss Mice. *ACS Nano* , 4, (3) 1481-1492

Lam, C.w., James, J.T., McCluskey, R., Arepalli, S., & Hunter, R.L. 2006. A review of carbon nanotube toxicity and assessment of potential occupational and environmental health risks. *Critical Reviews in Toxicology*, 36, (3) 189-217

Lee, C.M., Jeong, H.J., Yun, K.N., Kim, D.W., Sohn, M.H., Lee, J.K., Jeong, J., & Lim, S.T. 2012. Optical imaging to trace near infrared fluorescent zinc oxide nanoparticles following oral exposure. *Int.J.Nanomed.*, 7, 3203-3209

Lee, C.M., Jeong, H.J., Kim, D.W., Sohn, M.H., & Lim, S.T. 2012. The effect of fluorination of zinc oxide nanoparticles on evaluation of their biodistribution after oral administration. *Nanotechnology*, 23, (20) 205102

Lee, Y., Choi, J., Kim, P., Choi, K., Kim, S., Shon, W., & Park, K. 2012. A transfer of silver nanoparticles from pregnant rat to offspring. *Toxicological Research (Seoul, Republic of Korea)*, 28, (3) 139-141

Li, C.H., Shen, C.C., Cheng, Y.W., Huang, S.H., Wu, C.C., Kao, C.C., Liao, J.W., & Kang, J.J. 2012. Organ biodistribution, clearance, and genotoxicity of orally administered zinc oxide nanoparticles in mice. *Nanotoxicology*, 6, (7) 746-756

Lim, J.H., Kim, S.H., Shin, I.S., Park, N.H., Moon, C., Kang, S.S., Kim, S.H., Park, S.C., & Kim, J.C. 2011. Maternal exposure to multi-wall carbon nanotubes does not induce embryo-fetal developmental toxicity in rats. *Birth Defects Research, Part B: Developmental and Reproductive Toxicology*, 92, (1) 69-76

Lim, J.H., Kim, S.H., Lee, I.C., Moon, C., Kim, S.H., Shin, D.H., Kim, H.C., & Kim, J.C. 2011. Evaluation of Maternal Toxicity in Rats Exposed to Multi-Wall Carbon Nanotubes during Pregnancy. *Environmental health and toxicology*, 26, e2011006

Liu, J., Wang, Z., Liu, F.D., Kane, A.B., & Hurt, R.H. 2012. Chemical Transformations of Nanosilver in Biological Environments. *ACS Nano*, 6, (11) 9887-9899

Loeschner, K., Hadrup, N., Qvortrup, K., Larsen, A., Gao, X., Vogel, U., Mortensen, A., Lam, H.R., & Larsen, E.H. 2011. Distribution of silver in rats following 28 days of repeated oral exposure to silver nanoparticles or

silver acetate. *Particle and Fibre Toxicology*, 8, 18 available from: http://www.particleandfibretoxicology.com/content/pdf/1743-8977-8-18.pdf

Magnuson, B.A., Jonaitis, T.S., & Card, J.W. 2011. A Brief review of the occurrence, use, and safety of food-related nanomaterials. *J.Food Sci.*, 76, (6) R126-R133

Matsumoto, M., Serizawa, H., Sunaga, M., Kato, H., Takahashi, M., Hirata-Koizumi, M., Ono, A., Kamata, E., & Hirose, A. 2012. No toxicological effects on acute and repeated oral gavage doses of single-wall or multi-wall carbon nanotube in rats. *Journal of Toxicological Sciences*, 37, (3) 463-474

McCullough, J.S., Hodges, G.M., Dickson, G.R., Yarwood, A., & Carr, K.E. 1995. A morphological and microanalytical investigation into the uptake of particulate iron across the gastrointestinal tract of rats. *Journal of submicroscopic cytology and pathology*, 27, (1) 119-124

MST, Mikkelsen, S. H., Hansen, E., Christensen, T. B., Baun, A., Hansen, S. F., & Binderup, M.-L. 2011, *Survey on basic knowledge about exposure and potential environmental and health risks for selected nanomaterials. Environmental Project No. 1370 2011.* Danish Environmental Protection Agency.

Nefzger, M., Kreuter, J., Voges, R., Liehl, E., & Czok, R. 1984. Distribution and elimination of polymethyl methacrylate nanoparticles after peroral administration to rats. *Journal of Pharmaceutical Sciences*, 73, (9) 1309-1311

Onishchenko, G.E., Erokhina, M.V., Abramchuk, S.S., Shaitan, K.V., Raspopov, R.V., Smirnova, V.V., Vasilevskaya, L.S., Gmoshinski, I.V., Kirpichnikov, M.P., & Tutelyan, V.A. 2012. Effects of Titanium Dioxide Nanoparticles on Small Intestinal Mucosa in Rats. *Bulletin of Experimental Biology and Medicine*, 154, (2) 265-270

Park, E.J., Park, Y.K., & Park, K. 2009. Acute toxicity and tissue distribution of cerium oxide nanoparticles by a single oral administration in rats. *Toxicological Research (Seoul, Republic of Korea)*, 25, (2) 79-84

Park, E.J., Bae, E., Yi, J., Kim, Y., Choi, K., Lee, S.H., Yoon, J., Lee, B.C., & Park, K. 2010. Repeated-dose toxicity and inflammatory responses in mice by oral administration of silver nanoparticles. *Environmental Toxicology and Pharmacology*, 30, (2) 162-168

Peters, R., Kramer, E., Oomen, A.G., Herrera Rivera, Z.E., Oegema, G., Tromp, P.C., Fokkink, R., Rietveld, A., Marvin, H.J.P., Weigel, S., Peijnenburg, A.A.C.M., & Bouwmeester, H. 2012. Presence of Nano-Sized Silica during In Vitro Digestion of Foods Containing Silica as a Food Additive. *ACS Nano*, 6, (3) 2441-2451 available from: http://dx.doi.org/10.1021/nn204728k Accessed 31 May 2013.

Pietzonka, P., Rothen-Rutishauser, B., Langguth, P., Wunderli-Allenspach, H., Walter, E., & Merkle, H.P. 2002. Transfer of lipophilic markers from PLGA and polystyrene nanoparticles to caco-2 monolayers mimics particle uptake. *Pharmaceutical research*, 19, (5) 595-601

Roblegg, E., Froehlich, E., Meindl, C., Teubl, B., Zaversky, M., & Zimmer, A. 2012. Evaluation of a physiological in vitro system to study the transport of nanoparticles through the buccal mucosa. *Nanotoxicology*, 6, (4) 399-413

Rogers, K.R., Bradham, K., Tolaymat, T., Thomas, D.J., Hartmann, T., Ma, L., & Williams, A. 2012. Alterations in physical state of silver nanoparticles exposed to synthetic human stomach fluid. *Science of the Total Environment*, 420, 334-339

Sachar, S. & Saxena, R.K. 2011. Cytotoxic effect of poly-dispersed Single walled Carbon Nanotubes on erythrocytes in vitro and in vivo. *PLoS One*, 6, (7) e22032 available from: http://www.plosone.org/article/fetchObjectAttachment.action?uri=info%3Adoi%2F10.1371%2Fjournal.pone.0022032&representation=PDF

Sakuma, S., Sudo, R., Suzuki, N., Kikuchi, H., Akashi, M., & Hayashi, M. 1999. Mucoadhesion of polystyrene nanoparticles having surface hydrophilic polymeric chains in the gastrointestinal tract. *International Journal of Pharmaceutics*, 177, (2) 161-172

San, M., Garri, C., Pizarro, F., Walter, T., Theil, E.C., & Nunez, M.T. 2008. Caco-2 intestinal epithelial cells absorb soybean ferritin by ++2 (AP2)-dependent endocytosis. *Journal of Nutrition*, 138, (4) 659-666

Sardari, R.R.R., Zarchi, S.R., Talebi, A., Nasri, S., Imani, S., Khoradmehr, A., & Sheshde, S.A.R. 2012. Toxicological effects of silver nanoparticles in rats. *African Journal of Microbiology Research*, 6, (27) 5587-5593

Saxena, R.K., Williams, W., McGee, J.K., Daniels, M.J., Boykin, E., & Gilmour, M.I. 2007. Enhanced in vitro and in vivo toxicity of poly-dispersed acid-functionalized single-wall carbon nanotubes. *Nanotoxicology*, 1, (4) 291-300 available from: http://www.informaworld.com/smpp/content-í+æ+ócontent=a788629584-í+æ+ódb=all-í+æ+óorder=page

Schmid, K. & Riediker, M. 2008. Use of Nanoparticles in Swiss Industry: A Targeted Survey. *Environmental Science & Technology*, 42, (7) 2253-2260

Shahare, B., Yashpal, M., & Singh, G. 2013. Toxic effects of repeated oral exposure of silver nanoparticles on small intestine mucosa of mice. *Toxicology mechanisms and methods*

Sharma, V., Singh, P., Pandey, A.K., & Dhawan, A. 2012. Induction of oxidative stress, DNA damage and apoptosis in mouse liver after sub-acute oral exposure to zinc oxide nanoparticles. *Mutation Research, Genetic Toxicology and Environmental Mutagenesis*, 745, (1-2) 84-91

Singh, S.P., Rahman, M.F., Murty, U.S.N., Mahboob, M., & Grover, P. 2013. Comparative study of genotoxicity and tissue distribution of nano and micron sized iron oxide in rats after acute oral treatment. *Toxicology and Applied Pharmacology*, 266, (1) 56-66

So, S.J., Jang, I.S., & Han, C.S. 2008. Effect of micro/nano silica particle feeding for mice. *Journal of Nanoscience and Nanotechnology*, 8, (10) 5367-5371

Sooresh, A., Zeng, Z., Chandrasekharan, J., Pillai, S.D., & Sayes, C.M. 2012. A physiologically relevant approach to characterize the microbial response to colloidal particles in food matrices within a simulated gastrointestinal tract. *Food and Chemical Toxicology*, 50, (9) 2971-2977

Szendi, K. & Varga, C. 2008. Lack of genotoxicity of carbon nanotubes in a pilot study. *Anticancer Research*, 28, (1A) 349-352

Taylor, T.M., Davidson, P.M., Bruce, B.D., & Weiss, J. 2005. Liposomal nanocapsules in food science and agriculture. *Critical Reviews in Food Science and Nutrition*, 45, (7-8) 587-605

ToxRTool. ToxRTool - Toxicological data Reliability Assessment Tool. http://ihcp.jrc.ec.europa.eu/our_labs/eurl-ecvam/archive-publications/toxrtool . 31-5-2013. Ref Type: Electronic Citation

Turov, V.V., Gun'ko, V.M., Turova, A.A., Morozova, L.P., & Voronin, E.F. 2011. Interfacial behavior of concentrated HCl solution and water clustered at a surface of nanosilica in weakly polar solvents media. *Colloids and Surfaces, A: Physicochemical and Engineering Aspects*, 390, (1-3) 48-55

Uvarova, I., Boshitskaya, N., Lavrenko, V., & Makarenko, G. 2005. Investigation of ecological safety in using nanosized and ultrafine powders. *Ceramic Transactions*, 159, (Ceramic Nanomaterials and Nanotechnology III) 265-271

van der Zande, M., Vandebriel, R.J., Van, D., Kramer, E., Herrera, R., Serrano-Rojero, C.S., Gremmer, E.R., Mast, J., Peters, R.J.B., Hollman, P.C.H., Hendriksen, P.J.M., Marvin, H.J.P., Peijnenburg, A.A.C.M., & Bouwmeester, H. 2012. Distribution, Elimination, and Toxicity of Silver Nanoparticles and Silver Ions in Rats after 28-Day Oral Exposure. *ACS Nano*, 6, (8) 7427-7442

Vogelson, C.T. 2001. Advances in drug delivery systems. *Modern Drug Discovery*, 4, (4) 49-50, 52

Walczak, A.P., Fokkink, R., Peters, R., Tromp, P., Herrera, R.Z., Rietjens, I.M.C.M., Hendriksen, P.J.M., & Bouwmeester, H. 2012. Behaviour of silver nanoparticles and silver ions in an in vitro human gastrointestinal digestion model. *Nanotoxicology*

Wang, B., Feng, W., Wang, M., Wang, T., Gu, Y., Zhu, M., Ouyang, H., Shi, J., Zhang, F., Zhao, Y., Chai, Z., Wang, H., & Wang, J. 2008. Acute toxicological impact of nano- and submicro-scaled zinc oxide powder on healthy adult mice. *Journal of Nanoparticle Research*, 10, (2) 263-276

Wang, J., Zhou, G., Chen, C., Yu, H., Wang, T., Ma, Y., Jia, G., Gao, Y., Li, B., Sun, J., Li, Y., Jiao, F., Zhao, Y., & Chai, Z. 2007. Acute toxicity and biodistribution of different sized titanium dioxide particles in mice after oral administration. *Toxicology Letters*, 168, (2) 176-185

Wang, Y. & Fu, L. 2012. Forms of Selenium Affect its Transport, Uptake and Glutathione Peroxidase Activity in the Caco-2 Cell Model. *Biological Trace Element Research*, 149, (1) 110-116

Wang, Y., Chen, Z., Ba, T., Pu, J., Chen, T., Song, Y., Gu, Y., Qian, Q., Xu, Y., Xiang, K., Wang, H., & Jia, G. 2012. Susceptibility of Young and Adult Rats to the Oral Toxicity of Titanium Dioxide Nanoparticles. *Small (Weinheim an der Bergstrasse, Germany)* Ahead

Wang, Z., Zhao, J., Song, L., Mashayekhi, H., Chefetz, B., & Xing, B. 2011. Adsorption and Desorption of Phenanthrene on Carbon Nanotubes in Simulated Gastrointestinal Fluids. *Environmental Science & Technology*, 45, (14) 6018-6024

Xu, A., Yao, M., Xu, G., Ying, J., Ma, W., Li, B., & Jin, Y. 2012. A physical model for the size-dependent cellular uptake of nanoparticles modified with cationic surfactants. *Int.J.Nanomed.*, 7, 3547-3554

Yamago, S., Tokuyama, H., Nakamura, E., Kikuchi, K., Kananishi, S., Sueki, K., Nakahara, H., Enomoto, S., & Ambe, F. 1995. In vivo biological behavior of a water-miscible fullerene: 14C labeling, absorption, distribution, excretion and acute toxicity. *Chemistry & Biology*, 2, (6) 385-389

Yamashita, K., Yoshioka, Y., Pan, H., Taira, M., Ogura, T., Nagano, T., Aoyama, M., Nagano, K., Abe, Y., Kamada, H., Tsunoda, S.I., Aoshima, H., Nabeshi, H., Yoshikawa, T., & Tsutsumi, Y. 2013. Biochemical and hematologic effects of polyvinylpyrrolidone-wrapped fullerene C60 after oral administration. *Die Pharmazie*, 68, (1) 54-57

Zhang, L., Laug, L., Munchgesang, W., Pippel, E., Gosele, U., Brandsch, M., & Knez, M. 2010. Reducing stress on cells with apoferritin-encapsulated platinum nanoparticles. *Nano letters*, 10, (1) 219-223

Zhang, X.D., Wu, H.Y., Wu, D., Wang, Y.Y., Chang, J.H., Zhai, Z.B., Meng, A.M., Liu, P.X., Zhang, L.A., & Fan, F.Y. 2010. Toxicologic effects of gold nanoparticles in vivo by different administration routes. *Int.J.Nanomed.*, 5, 771-781 available from: http://www.dovepress.com/getfile.php?fileID=7781

Systemic Absorption of Nanomaterials by Oral Exposure
This report and accompanying database systematically evaluates the reliability and relevance of the existing scientific literature regarding systemic absorption of nanomaterials by oral exposure and makes specific recommendations for future testing approaches.

Rapporten og den tilhørende database gennemgår systematisk pålideligheden og relevansen af den eksisterende videnskabelige litteratur vedrørende optag af nanomaterialer over mavetarmkanalen og kommer med konkrete anbefalinger til fremtidige testmetoder.

Danish Ministry of the Environment
Environmental Protection Agency

Strandgade 29
1401 Copenhagen K, Denmark
Tel.: (+45) 72 54 40 00

www.mst.dk