From INSTITUTE OF ENVIRONMENTAL MEDICINE
Karolinska Institutet, Stockholm, Sweden

NANOTOXICOLOGY ON THE RIGHT TRACK: FOCUS ON METAL AND METAL OXIDE NANOPARTICLES

Anda Roxana Gliga

Karolinska Institutet

Stockholm 2016

Published by Karolinska Institutet.
Printed by AJ E-print AB

ISBN 978-91-7676-452-7

Nanotoxicology on the Right Track:
Focus on Metal and Metal Oxide Nanoparticles

THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

Anda Roxana Gliga

Pharm., MSc.

Principal Supervisor:
Associate professor Hanna Karlsson
Karolinska Institutet
Institute of Environmental Medicine
Division of Biochemical Toxicology

Co-supervisors:
Professor Bengt Fadeel
Karolinska Institutet
Institute of Environmental Medicine
Division of Molecular Toxicology

Jessica Lindvall, PhD
Stockholm University
Department of Biochemistry and Biophysics

Professor Inger Odnevall-Wallinder
Royal Institute of Technology
School of Chemical Science and Engineering,
Division of Surface and Corrosion Science

Opponent:
Professor Herman Autrup
Aarhus University
Department of Public Health

Examination Board:
Professor Peter Møller
University of Copenhagen
Department of Public Health

Professor Karin Broberg
Karolinska Institutet
Institute of Environmental Medicine
Division of Metals and Health

Docent Mikael Huss
Stockholm University
Department of Biochemistry and Biophysics

“We no longer think of chairs as technology; we just think of them as chairs. But there was a time when we hadn't worked out how many legs chairs should have, how tall they should be, and they would often 'crash' when we tried to use them.”

Douglas Adams

The Sunday Times, August 29th 1999

ABSTRACT

The last decade has seen a rapid increase in the manufacture and use of nanomaterials, a development which should be met with appropriate safety assessment strategies in order to ensure the sustainable development of nanotechnology. With decreasing size, the percentage of atoms found at the surface of a given material increases substantially, leading to an increase in surface phenomena and acquisition of novel properties. These new traits can be appealing for industrial purposes, however, they can also enhance the intrinsic toxicity of the materials as compared to their bulk counterparts. Currently, nanotoxicology faces several challenges related to the multitude of materials that need to be tested, the possible interactions of the nanomaterials with the conventional toxicology assays and the potential emergence of novel nano-specific properties. Despite numerous research efforts being made in the last decade to evaluate the toxicity of nanomaterials, most of these studies fall short of several aspects, such as appropriate particle characterization, cellular uptake, relevant doses and exposure duration. The aim of this thesis was to use *in vitro* models to address some of the challenges in nanotoxicology in order to improve our understanding of the interactions between nanomaterials and biological systems. In Paper I we demonstrated that we can use the ToxTracker assay, which consists of reporter stem cells, to screen and predict the genotoxicity of metal oxide nanoparticles and at the same time obtain information about their mechanism of toxicity. In Paper II we used a panel of thoroughly characterized silver nanoparticles to address the issue of size-dependent toxicity in human lung cells. Our results showed that small (10 nm) particles were more cytotoxic than larger particles (>40 nm) after acute exposure (24 hours), and that could be related to a 'Trojan horse' effect by which the particulate form facilitates the cellular uptake of metal, with subsequent release of toxic metal ions. In Paper III we selected two of the silver nanoparticles tested in Paper II and evaluated the effects following low-dose, long-term (6 week) exposure to human lung cells. By using both conventional assays and systems toxicology approaches (RNA-sequencing, genome wide DNA-methylation) we identified that chronic exposure to low doses of silver nanoparticles induced a cancer-like phenotype and had immunosuppressive effects in human lung cells. In Paper IV we explored the effects of antioxidant cerium oxide nanoparticles, which allegedly have promising therapeutic potential, in neural stem cells. On one hand, we showed that pretreatment with cerium oxide nanoparticles provided a temporary neuroprotective effect when cells were challenged with an oxidative stress inducer. On the other hand, by using both immunofluorescence and RNA-sequencing we revealed that the same antioxidant properties can have detrimental effects by suppressing neuronal differentiation, in which reactive oxygen species play an important role as signaling molecules. In all, our studies show that by using well-characterized nanomaterials together with appropriate experimental setups, and a combination of traditional toxicological assays with novel tools such as 'omics', we can improve our understanding of the toxicity of nanomaterials and by these means contribute to the sustainable development of nanotechnology.

LIST OF SCIENTIFIC PAPERS

I. Hanna L. Karlsson, Anda R Gliga, Fabienne MGR Calléja, Cátia SAG Gonçalves, Inger Odnevall Wallinder, Harry Vrieling, Bengt Fadeel, Giel Hendriks. Mechanism-based genotoxicity screening of metal oxide nanoparticles using the ToxTracker panel of reporter cell line. Particle and Fibre Toxicology, 2014, 11:41.

II. Anda R Gliga, Sara Skoglund, Inger Odnevall Wallinder, Bengt Fadeel, Hanna L. Karlsson. Size-dependent cytotoxicity of silver nanoparticles in human lung cells: the role of cellular uptake, agglomeration and Ag release. Particle and Fibre Toxicology, 2014, 11:11.

III. Anda R Gliga, Sebastiano Di Bucchianico, Jessica Lindvall, Bengt Fadeel, Hanna L. Karlsson. Long-term low-dose exposure to silver nanoparticles induces epithelial-mesenchymal transition and cell transformation in human lung cells. [manuscript, submitted 2016]

IV. Anda R Gliga, Karin Edoff, Fanny Caputo, Thomas Källman, Hans Blom, Hanna L. Karlsson, Lina Ghibelli, Enrico Traversa, Sandra Ceccatelli, Bengt Fadeel. Double-edged sword: antioxidant cerium oxide nanoparticles protect against oxidative insult and inhibit neuronal stem cell differentiation. [manuscript, submitted 2016]

CONTENTS

LIST OF ABBREVIATIONS

AAS	atomic absorption spectroscopy
ARE	antioxidant response element
CNTs	carbon nanotubes
DEG	differentially expressed genes
DMNQ	2,3-dimethoxy-1,4-naphthoquinone
ds	double strand
FPG	formamidopyrimidine DNA glycosylase
GF	graphite furnace
GFP	green fluorescent protein
GO	gene ontology
HMT	hexamethylenetetamine
hNPC	human neural progenitor cells
IARC	International Agency for Research on Cancer
ICP-MS	inductively coupled plasma mass spectrometry
ISDD	in vitro sedimentation, diffusion and dosimetry
LDH	lactate dehydrogenase
LPS	lipopolysaccharides
MAPK	mitogen-activated protein kinase
mES	mouse embryonic stem cells
MWCNTs	multi-walled carbon nanotubes
NAC	N-acetyl-cysteine
nanoceria	cerium oxide nanoparticles
NOAEL	no-observed-adverse-effect-level
Nrf2	nuclear factor-erythroid 2-related factor 2
OECD	Organisation for Economic Co-operation and Development
PCCS	photon cross correlation spectroscopy
PVP	polyvinylpyrrolidone
RNA-Seq	RNA sequencing
ROS	reactive oxygen species
SIM	structured illumination microscopy
Sm-CeO_2	samarium-doped cerium oxide nanoparticles
STED	stimulated emission depletion
SWCNTs	single-walled carbon nanotubes
TEM	transmission electron microscopy

1 INTRODUCTION

1.1 PARTICLE TOXICOLOGY *VERSUS* NANOTOXICOLOGY

1.1.1 Lessons learnt from the past

Exposure to (nano)particles has occurred in parallel with human evolution and as a result the lungs have developed mechanisms to cope with particle exposure, *e.g.* phagocytosis followed by mucociliary clearance (Oberdörster *et al.*, 2005). However, since the dawn of the industrial revolution the anthropogenic exposure to particles has increased drastically (Oberdörster *et al.*, 2005). The relationship between exposure to particles and lung diseases has been described as early as the 15th century, when workers in metal mines were 'reported' to have 'breathing problems', which are now believed to have been early accounts of silicosis (Donaldson and Seaton, 2012). From a historical perspective, there are three major culprits for the pathologies related to occupational exposure to particles, namely crystalline silica (quartz), asbestos and coal.

Quartz dust is highly reactive, induces inflammation, genotoxicity and has been found to be carcinogenic in humans following inhalational exposure, hence classified by the International Agency for Research on Cancer (IARC) as a Class 1 carcinogen (IARC, 2012, 100C). **Asbestos** is a composite silicate fiber also classified as a Class 1 carcinogen (IARC, 2012, 100C). Exposure to asbestos has been correlated to a specific type of cancer of the pleura, namely mesothelioma. Research on asbestos laid the foundation for the fibre pathogenicity paradigm which states that long, thin and biopersistent fibers are highly pathogenic and induce chronic inflammation due to, among other factors, 'frustrated phagocytosis'. Basically, macrophages are unable to completely engulf the long thin fibers which results in inflammatory processes (Donaldson and Poland, 2012). **Coal dust** consists of a mixture of carbon, quartz and silicates that upon inhalation can lead to pneumoconicosis, a risk factor for lung fibrosis (Donaldson and Seaton, 2012). However, the relationship between coal dust and lung cancer is unclear and coal dust has yet to be classified as carcinogenic to humans (Class 3) (IARC, 1997, 68).

Apart from occupational exposure, humans are exposed to particles derived from anthropogenic sources such as diesel and engine exhaust particles as well as outdoor and indoor air particles. Diesel exhaust particles consist of a carbonaceous particle core on which a variety of substances are adsorbed, such as gases, poly-aromatic hydrocarbons and metals (Wichmann, 2007), and have been classified as carcinogenic to human (Class 1) (IARC, 2013, 105). In addition, outdoor air pollution and particulate matter in outdoor air pollution have been recently classified as carcinogens (Class 1) (IARC, 2016, 109). Besides the carcinogenic effect, air pollution is correlated with cardiovascular effects such as atherosclerosis and stroke (Brook, 2008).

The steep development of nanotechnology is deemed to increase the exposure to (nano) particles even more (Oberdörster *et al.*, 2005). In a visionary editorial published in 2004 and entitled 'Nanotoxicology: A new frontier in particle toxicology relevant to both the workplace

and general environment and to consumer safety' Ken Donaldson and colleagues addressed for the first time the potential toxicological implications of nanotechnology (Donaldson *et al.*, 2004). The authors mentioned issues such as size, surface reactivity and biodistribution, that could be of potential concern for the toxicity of nanoparticles, and postulated that nanotoxicology would be critical for the sustainable development of nanotechnology (Donaldson *et al.*, 2004). It should be noted, however, that nanotoxicology is not a new discipline but is rather an emerging field grounded in particle and ultrafine particle toxicology (Oberdörster *et al.*, 2005).

Well-established paradigms and previous studies on particles and fibers should and are indeed revisited now in nanotoxicology research. As an example, among all nanomaterials, carbon nanotubes (CNTs) raised a great deal of concern due to their likelihood of fitting in the fiber pathogenicity paradigm. Indeed, some CNTs, *i.e.* the long, and stiff CNTs share similar properties and *in vivo* outcomes with asbestos fibers (Donaldson *et al.*, 2013). A type of multi-walled carbon nanotubes (MWCNTs), namely Mitsui MWCNT-7 was classified as Class 2B (possibly carcinogenic to humans) in absence of human cancer data (Grosse *et al.*, 2014). This is an instance where lessons have been learnt from the past in the sense that now the aim is to predict the human toxicity of engineered nanomaterials before they are being produced in large enough quantities, and before considerable human exposure and subsequent health effects occur (Donaldson and Seaton, 2012).

1.1.2 Definitions

From a regulatory perspective, the current definition of nanomaterials at the European Union level is based on the EU Commission recommendation 2011/696/EU and is expected to be reviewed by the end of 2016.

> "*A natural, incidental or manufactured material containing particles, in an unbound state or as an aggregate or as an agglomerate and where, for 50 % or more of the particles in the number size distribution, one or more external dimensions is in the size range 1 nm - 100 nm.*
>
> *In specific cases and where warranted by concerns for the environment, health, safety or competitiveness the number size distribution threshold of 50 % may be replaced by a threshold between 1 and 50 %.*
>
> *By derogation from the above, fullerenes, graphene flakes and single wall carbon nanotubes with one or more external dimensions below 1 nm should be considered as nanomaterials*" (EU commission, 2011)

As discussed in the Joint Research Center follow-up documents, the size range together with the percentage threshold were bound to aid regulatory processes and were not set out of scientific reasons (Hubert, 2015). Indeed, there is no biologically/toxicologically sound reason for a rigid 1 – 100 nm threshold.

1.1.3 Novel (nano-specific) effects

The issue of size was predicted to be of concern even from the first account of nanotoxicology (Donaldson *et al.*, 2004). For nanomaterials the surface to volume ratio as well as the percentage of atoms found at the surface are significantly higher as compared to the bulk form (Figure 1, left) leading to an increase in surface phenomena (Figure 1, right). This is in turn correlated with an increased reactivity and a potentially enhanced toxicity (Auffan *et al.*, 2009). However, it is not to be generalized that all nano-sized particles imply an increased toxicity compared to the bulk form. (Auffan *et al.*, 2009).

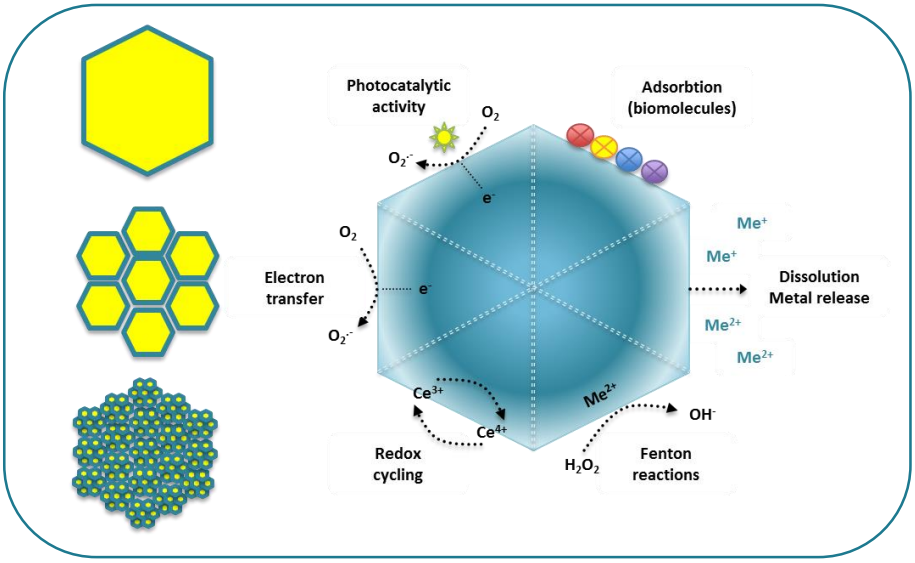

Figure 1. Surface to volume ratio and surface phenomena at the nanolevel

The question of nano-specific modes of toxicity has long been under scrutiny. In a recent review, Donaldson and Poland put forward a sound argument that nanomaterials have no new modes of action compared to conventional particles, but rather bear a gradual magnification of the intrinsic hazard (Donaldson and Poland, 2013). Despite proximal events such as particle uptake and biodistribution being to some extent novel, the final pathways of toxicity, *i.e.* oxidative stress, inflammation and genotoxicity, overlap between nanoparticles and conventional particles (Donaldson and Poland, 2013).

Some accounts of mechanisms of toxicity for nanoparticles have been published but it is unclear how nano-specific they are. For example, the proton sponge effect is elicited by cationic particles that upon entry to the lysosomal compartment sequester protons from the proton pumps, ultimately leading to lysosomal swelling and rupture as a result of the accumulation of Cl^- and H_2O molecules (Nel *et al.*, 2009). Another example is the 'Trojan horse' mechanism by which partially soluble metal nanoparticles are taken up via endocytosis, followed by the release of metal ions inside the cells, thereby increasing intracellular bioavailability of toxic metals (Limbach *et al.*, 2007).

Finally, there are formulated concerns that nanoparticles are in the size range of sub-cellular structures and therefore the 'matching of scales' could imply novel interactions (Hubbs *et al.*, 2013, Maynard *et al.*, 2011). Several studies have indicated that nanomaterials can interact with

cytoskeletal structures. For instance, carbon nanotubes induce actin reorganization (Holt *et al.*, 2010) and disrupt the mitotic spindle with subsequent aneuploidy (Sargent *et al.*, 2012).

1.1.4 Challenges in nanotoxicology

An important challenge in nanotoxicology is represented by the immense diversity of nanomaterials that are produced via different methods, with various levels of residual impurities, and with different shapes and sizes (Johnston *et al.*, 2013). This makes the selection of nanomaterials to be tested challenging if they are to be representative for human exposure. Moreover, this advocates the need for increasing the throughput of our current techniques allowing for fast screening and hazard ranking of nanomaterials (Nel *et al.*, 2013). In line with the 'Toxicity Testing in the 21st Century' paradigm, the high-throughput techniques should also provide mechanistic insight and allow for pathway-based toxicity testing (Nel *et al.*, 2013), a point which was addressed in Paper I.

In addition, thorough characterization in the relevant medium is mandatory in order to correlate certain physico-chemical properties with toxicological outcomes. In most of the cases a primary particle characterization is provided by the manufacturer, but this has to be complemented with characterization in the relevant physiological fluid, where particles acquire their biological identity by *e.g.* formation of the bio-corona.

Due to their intrinsic properties, nanomaterials can interfere with conventional toxicological assays and detection methods, thereby skewing the results. Nanomaterials can have intrinsic fluorescence/absorbance, can adsorb assay reagents or catalyze enzymatic reactions, potentially leading to false results. Interactions between nanoparticles and test systems have been reported for both carbon-based materials (Monteiro-Riviere *et al.*, 2009) and metal-based materials (Kroll *et al.*, 2012). Assessment of interference of nanomaterials with assays should be performed on a routine basis for every tested nanomaterial as results cannot be generalized. In addition, the use of two or more assays to address similar endpoints could increase the reliability of the results.

A common problem in nanotoxicology studies is the use of very high, unrealistic doses (Krug and Wick, 2011). This is partially fueled by the editorial bias towards publishing positive results and is detrimental for achieving scientific progress in the field of nanotoxicology (Krug and Wick, 2011). If we are to make meaningful progress in understanding the toxicity of nanomaterials, relevant doses should be used in both *in vivo* and *in vitro* studies. In addition, most of the work so far reports on the short-term effects of nanomaterials and more chronic, ideally low-dose studies are critical to aid risk assessments endeavors. The use of low-dose chronic-exposure to nanoparticles was evaluated in Paper III.

A review by Krug H. in 2014 entitled "Nanosafety Research – Are We on the Right Track?" paints a fairly pessimistic picture of the progress nanosafety research has seen in the previous 15 years (Krug, 2014). Basically, despite increasing number of publications (over 10 000), major knowledge gaps there still exist, which makes it difficult to draw sound conclusions on the safety of nanomaterials. Again, a major problem is the use of unrealistic doses that provide

mechanistic information but are of questionable use for toxicological assessment. The lack of reference materials and appropriate controls are other factors that pose difficulties for reliable comparison between studies for risk assessment purposes (Krug, 2014).

1.2 INHALATIONAL EXPOSURE TO NANOMATERIALS

Exposure to engineered nanomaterials occurs via inhalation, ingestion and contact with the skin. One of the first lines of exposure occurs in occupational settings via inhalation, which makes the lung an important target organ. Due to their small size, nanoparticles can penetrate and deposit deeper into the lungs, in the alveolar region (Oberdörster *et al.*, 2005) which determines the magnitude of the toxic effect.

Figure 2 depicts the predicted deposition of particles depending on their size according to the International Commission on Radiological Protection. According to the model, both large (1 – 10 µm) and very small (1 nm) particles are mainly deposited in the upper nasal airways, pharynx and larynx, whereas *e.g.* 20 nm particles have the highest deposition in the alveolar area (Oberdörster *et al.*, 2005). *In vivo* studies in rats showed that 10 nm Ag nanoparticles had a higher total lung deposition, a higher predicted alveolar deposition and induced more lung inflammation compared to 410 nm Ag nanoparticles (Braakhuis *et al.*, 2014a).

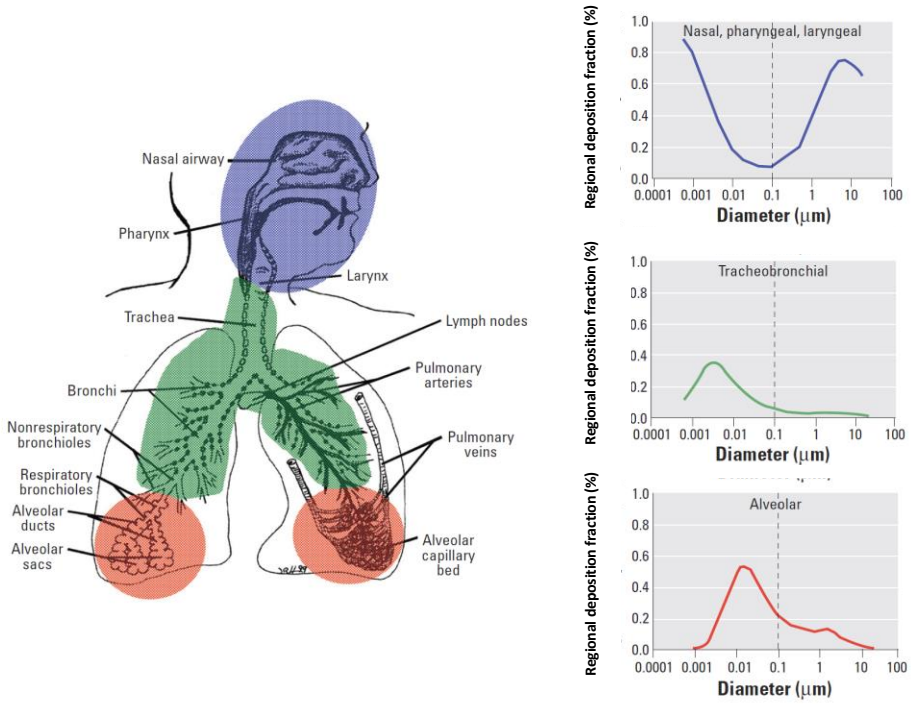

Figure 2. Predicted deposition of inhaled particles in the respiratory tract. Reproduced from Environmental Health Perspectives, Oberdörster et al., 2005, with modifications.

The mechanisms of nanoparticle deposition are governed by diffusion whereas for larger particles (or agglomerates) deposition is mediated by inertial impaction, gravitational settling and interception (Oberdörster *et al.*, 2005). Depending on the deposition site, particles are cleared by different mechanisms. For particles deposited in the alveolar area, macrophage clearance is the main mechanism and is followed by gradual movement towards the mucociliary escalator, with an estimated retention half-time of 700 days in humans

(Oberdörster *et al.*, 2005). The total lung macrophage clearance is very efficient for microparticles but for nanoparticles only 20% of the particles are cleared this way, which allows for interaction of nanoparticles with the epithelial cells and interstitial sites (Oberdörster *et al.*, 2005).

Translocation across the lung-blood barrier for nanoparticles is considered to be in general very low but could be relevant considering accumulation during a life-long exposure scenario (Krug, 2014). Moreover, translocation from the lungs to the secondary organs was reported to be size-dependent; higher for 15 nm *versus* 80 nm iridium nanoparticles (Kreyling *et al.*, 2009) and higher for 2 nm compared to 40 and 80 nm gold nanoparticles (Sadauskas *et al.*, 2009).

In addition, studies have shown that particles can translocate from the nose to the brain via the olfactory bulb, making the brain an additional target organ following inhalation exposure (Oberdörster *et al.*, 2004). For example, Ag nanoparticles were shown to translocate to the brain via the olfactory bulb after inhalational exposure in rats (Patchin *et al.*, 2016). This can pose toxicological concerns considering that Ag nanoparticles were shown to alter cytoskeletal organization in neurons *in vitro* (Cooper and Spitzer, 2015). Moreover, ultrafine carbon particles were reported to reach the brain to a significant extent via sensory nerve endings in the respiratory tract (Oberdörster *et al.*, 2004) and MnO nanoparticles were shown to translocate to the brain of rats via similar routes and induce inflammatory changes (Elder *et al.*, 2006).

A recent study identified combustion-derived magnetite nanoparticles in human brain samples, which were believed to originate from olfactory bulb transport (Figure 3), and postulated a connection with Alzheimer disease (Maher *et al.*, 2016). Other routes of nanoparticle access to the central nervous system apply to nanoparticles that are in the blood stream and imply the crossing of the blood-brain barrier which can occur through endothelial tight junctions for particles smaller than 6 nm or through transcytosis for larger particles (Cupaioli *et al.*, 2014). These routes are, however, more relevant in the light of brain delivery of nanomaterials intended for biomedical applications.

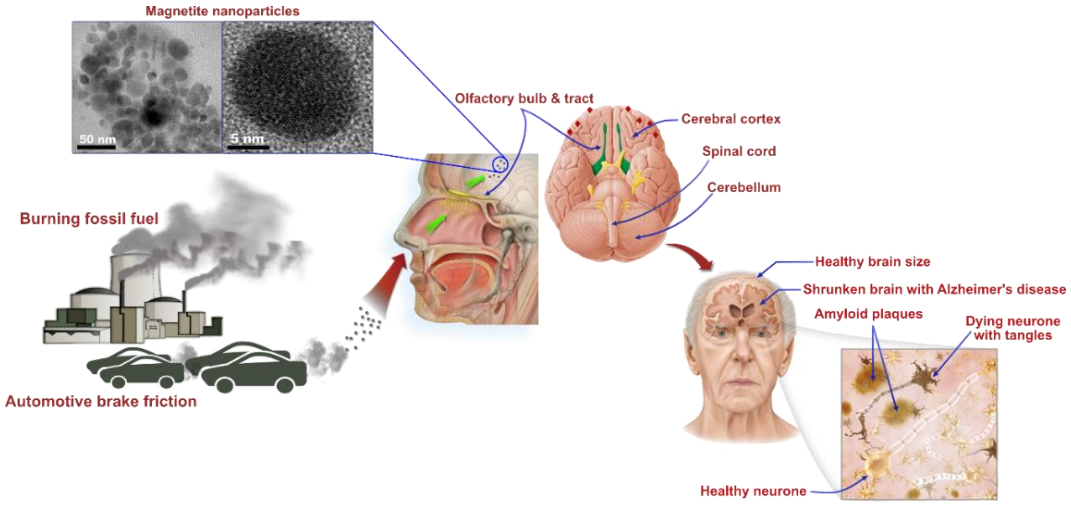

Figure 3. Brain translocation of nanoparticles following inhalation exposure. Picture courtesy of Dr. Imad Ahmed in relation to the reference Maher et al., 2016.

1.3 HAZARD ASSESSMENT OF NANOMATERIALS

1.3.1 Physico-chemical properties

Interaction of nanoparticles with biological systems and subsequent toxicity is closely dependent on physico-chemical properties of nanomaterials such as particle, size, shape, coating, surface area, crystalline structure and composition, some of which will be introduced and discussed below. Considering that even small changes in these properties could result in alteration of biological responses, it is crucial to perform a thorough particle characterization in parallel with the toxicity assessment (Fadeel *et al.*, 2015).

1.3.1.1 *Size*

Size is an aspect that was previously mentioned and that plays an important role for the reactivity of the nanomaterials because with decreasing size there is an increase in the percentage of atoms found at the surface, which are more reactive than the atoms found inside (Auffan *et al.*, 2009). Surface area is also strictly related to the size and increases proportional to the decrease in size, for the same mass (Hubbs *et al.*, 2013). Size-dependent toxicity has been reported for Ag nanoparticles (Braakhuis *et al.*, 2014a, Wang *et al.*, 2014) and was investigated in this thesis (Paper II and III). Since nanoparticles have variable stability in the dispersion medium, it is important to distinguish between the primary particle size and the size of the particle agglomerates and aggregates in the relevant biological environment. In addition, particle agglomeration and sedimentation can influence the uptake (Cho *et al.*, 2011) and consequently the toxicity of nanoparticles.

Size also dictates the uptake mechanisms (Figure 4). When it comes to active uptake pathways, particles up to 100 nm can be taken up by pinocytosis, clathrin and caveolin pathways whereas larger particles are taken up by phagocytosis and macropinocytosis (Krug and Wick, 2011, Kuhn *et al.*, 2014).

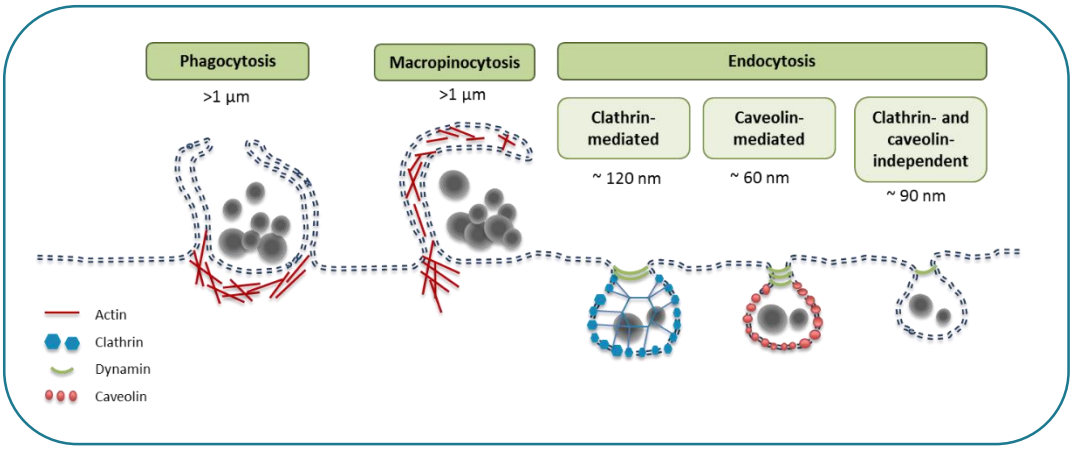

Figure 4. Active uptake mechanisms for (nano)particles

In addition to active mechanisms, diffusion is a passive process which was reported *e.g.* for quantum dots (Wang *et al.*, 2012) and gold nanoparticles (Lin *et al.*, 2010). In general, nanoparticles are taken up by a combination of mechanisms, also shown in this thesis (Paper II). It is worth noting that the nanoparticle uptake is less well regulated compared to the uptake

of metal ions, which is problematic for particles with intrinsic toxicity as it can lead to an increased bioavailability for toxic metals (Krug and Wick, 2011).

1.3.1.2 *Shape*

Nanomaterials can be distinguished into low- aspect-ratio nanoparticles (LARN) comprised of spherical, cubic, prismatic, helical or pillar shaped materials, and high-aspect-ratio nanomaterials (HARN) comprised of nanotubes and nanowires (Colognato, 2012). The similarity between HARN and asbestos raised well-grounded concerns about the potential toxicity of HARN. Tran *et al.* established a hypothetical model to predict HARN toxicity which was based on the pathogenic fiber paradigm and that covers three main aspects: (i) the HARN dimension should be thin enough to allow for deposition in the lower airways; (ii) a high enough deposition of HARN is achieved; (iii) biopersistency (Tran *et al.*, 2011). In contrast to asbestos fibers, carboxylated single-walled carbon nanotubes (SWCNTs) were reported to undergo enzymatic degradation with subsequent reduction of lung inflammation (Kagan *et al.*, 2010), whereas SWCNTs conjugated with polyethylene glycol were shown to be enzymatically degraded in the presence of myeloperoxidase (Bhattacharya *et al.*, 2014). Shape-dependent toxicity has been reported for metal nanoparticles such as Ag for which nanowires (1.5 and 8 µm) were more toxic than nanospheres in A549 cells (Stoehr *et al.*, 2011). In the case of TiO_2 nanomaterials, long (> 15 µm) nanobelts but not short (< 5 µm) nanobelts or nanospheres induced inflammasome activation in alveolar macrophages (Hamilton *et al.*, 2009).

1.3.1.3 *Surface charge*

The surface charge of a nanoparticle is defined by the zeta potential and is determined by the electric potential created between the surface of the particle and the dispersion medium (Cho *et al.*, 2012). Considering the electric potential of cellular membranes, the surface charge of nanoparticles can influence the interactions between nanomaterials and biological systems and by these means modulate the toxicity profile. Fröhlich E. reviewed the issue of surface charge and cellular uptake and reported that cationic particles are more likely to disrupt the cell membrane and induce toxicity as compared to anionic particles which are more prone to induce apoptosis (Fröhlich, 2012). In addition, cationic nanoparticles could induce lysosomal damage via the proton sponge effect (Nel *et al.*, 2009) discussed in the previous section (1.1.3). For low-soluble metal and metal oxide nanoparticles the zeta potential in acidic conditions was correlated with lung inflammation and the authors speculated that a high zeta potential in the acidic lysosomal environment could disrupt the lysosomal membrane and lead to inflammation (Cho *et al.*, 2012). In biological environments the surface charge of nanomaterials changes due to the adsobtion of biomolecules and formation of the bio-corona (Monopoli *et al.*, 2012).

1.3.1.4 *Composition*

While size, shape and surface charge play an important role in determining the magnitude of the toxic outcome, the chemical composition of the nanomaterials is equally important as it defines the intrinsic hazard. According to their composition, nanomaterials can be briefly classified in metal based (*e.g.* Ag, Au, Ni, NiO, SiO_2, CeO_2), carbon based (*e.g.* carbon

nanotubes, graphene) and polymeric nanoparticles (*e.g.* dendrimers), all of which can bear different coatings and functionalisations. Studies revealed that Cu and Zn based materials have the highest acute toxicity both *in vitro* and *in vivo* when compared to *e.g.* Ti and Ce based materials (Cho *et al.*, 2010, Lanone *et al.*, 2009). Purity of the nanomaterials can influence toxicity and is often overlooked in toxicity studies. It has been reported that nanomaterials can be contaminated with endotoxins or organic residues (Crist *et al.*, 2013).

1.3.1.5 *The concept of 'bio-corona'*

When introduced into biological environments, nanoparticles gain their biological identity by adsorbing biomolecules onto their surface with the formation of the bio-corona, a phenomena related to the high free energy at the surface (Monopoli *et al.*, 2012). Depending on the type of molecules adsorbed, the nanoparticles can acquire a protein corona, a lipid corona *etc.* The protein corona is a dynamic entity that consists of a so-called 'hard' corona (comprised of tightly bound molecules) and a 'soft' corona (comprised of loosely associated molecules) (Figure 5, left) (Docter *et al.*, 2015). The formation of the protein corona is a fast and dynamic process; in terms of the protein composition the protein corona undergoes quantitative but not qualitative changes over time (Figure 5, right) (Docter *et al.*, 2015, Tenzer *et al.*, 2013). The composition of the bio-corona depends on the nanoparticle material, surface properties, size, 'exposure' duration as well as type of biological environment and is reported to differ qualitatively and quantitatively from the composition of the biological environment (Westmeier *et al.*, 2016).

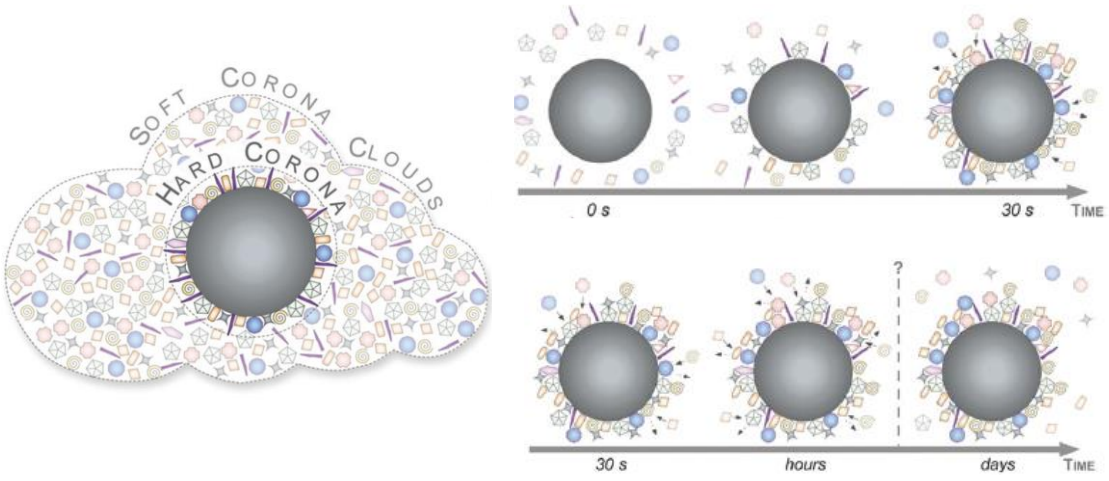

Figure 5. The structure and dynamics of the protein corona. Reproduced from Docter et al., 2015, Chemical Society Reviews, with modifications.

The bio-corona has implications for the physico-chemical properties of the nanoparticles, by influencing particle colloidal stability, as well as for the toxicological outcome. For example, the formation of the protein corona was reported to influence the uptake of Ag nanoparticles in mouse macrophages (Shannahan *et al.*, 2015) and human embryonic kidney cells (Monteiro-Riviere *et al.*, 2013) which can in turn modulate their toxicity. The formation of bio-corona

was not studied in this thesis, however, it would be of interest to address the effects of *e.g.* lung surfactant corona on the toxicity of nanoparticles in lung cells.

1.3.2 Conventional endpoints of toxicity

1.3.2.1 *Cytotoxicity*

Cytotoxicity is commonly one of the first assessments when performing *in vitro* toxicity testing of compounds of interest, including nanoparticles. There are several cytotoxicity tests available and the choice of assay should be made depending on:

(i) type of endpoint of interest *e.g*. membrane integrity (LDH assay), mitochondrial activity (Alamar Blue assay)
(ii) type of cell death of interest *e.g.* necrosis (LDH assay, Trypan blue), apoptosis (Annexin V), autophagic cell death
(iii) possible interference between nanoparticles and the assay, which should be tested on a case by case scenario (Kong *et al.*, 2011a)

A common critique of nanotoxicology studies is the use of very high unrealistic doses that are of no relevance for human exposure (Krug, 2014). However, for an initial evaluation of cytotoxicity one might need to go up to quite high doses in order to observe cell death and get an indication of the cytotoxicity profile. In addition, cytotoxicity assessment is a very crude measurement of nanoparticle toxicity but can be useful for ranking purposes or for establishing doses for other endpoints.

1.3.2.2 *Oxidative stress*

The oxidative stress paradigm is a well-established model for explaining the toxic effects of inhaled particles and its suitability can be extended to nanoparticles as well (Nel *et al.*, 2006). Nanomaterials can induce oxidative stress via several mechanisms:

(i) directly, as a result of the presence of reactive groups at the surface (*e.g.* transition metal-based nanoparticles, transition metal catalysts as residues from the synthesis or free radical intermediates at the surface) that can transfer electrons to oxygen molecules resulting in superoxide radicals that in turn can enter Fenton reaction or undergo dismutation with formation of additional reactive oxygen species (ROS) (Nel *et al.*, 2006, Shvedova *et al.*, 2012)
(ii) following dissolution (in the case of metal nanoparticles) with subsequent release of metal ions that can catalyze Fenton and/or Haber-Weiss reactions (Manke *et al.*, 2013)
(iii) indirectly, following particle interaction with cellular components such an phagosomes, lysosomes and mitochondria (Xia *et al.*, 2006)
(iv) indirectly, as a result of antioxidant depletion (Manke *et al.*, 2013)

According to the hierarchical oxidative stress model depicted in Figure 6, a low increase in ROS activates the nuclear factor-erythroid 2-related factor 2 (Nrf2) pathway (by inhibiting the suppressor activity of Keap1) which leads to the activation of the antioxidant response element (ARE) that in turn upregulates expression of Phase II genes such as glutathione-S-transferases and NADPH:quinone oxido-reductase 1 (Nguyen *et al.*, 2009, Nel *et al.*, 2006). This is a protective mechanism that can be overwhelmed at higher ROS levels when inflammation and eventually cell death occur. Inflammation is believed to be mediated by mitogen-activated protein kinase (MAPK) and NF-κB pathways and results in the secretion of cytokines and chemokines (Nel *et al.*, 2006). In addition, ROS can directly bind to DNA and induce genotoxicity, as discussed below, as well as trigger protein or lipid oxidation with subsequent altered cellular functionality (Manke *et al.*, 2013).

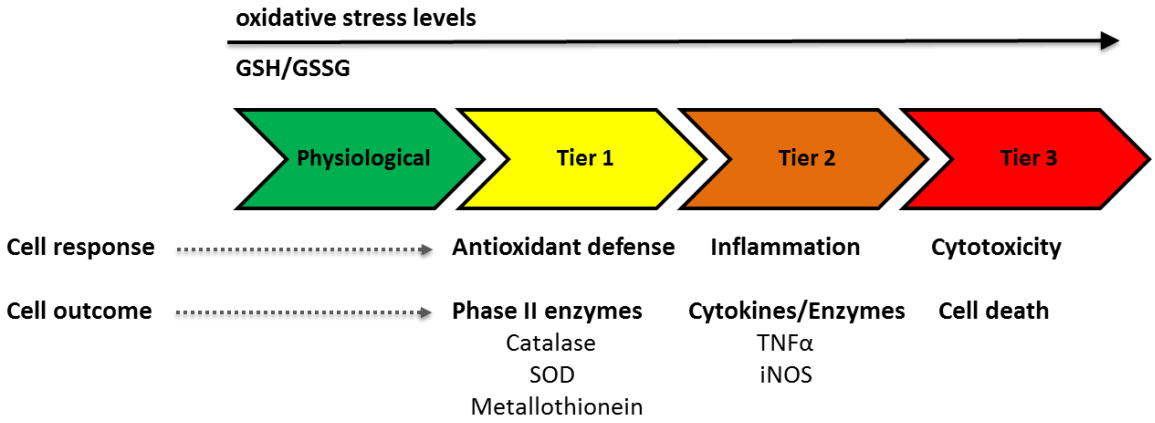

Figure 6. Hierarchical oxidative stress response model

On the other hand, ROS acts as a cellular messenger regulating processes such as cell proliferation and differentiation (Sauer *et al.*, 2001). It is therefore conceivable that some nanoparticles with antioxidant properties such as, *e.g.* CeO_2 could impair the normal ROS balance and by these means alter cellular functions. This is investigated in Paper IV of this thesis.

1.3.2.3 *Inflammation*

Lung inflammation was closely correlated with oxidative stress and has been reported for a wide range of nanomaterials (Braakhuis *et al.*, 2014b). Properties such as particle size, shape, crystallinity and composition are important factors for the outcome of lung inflammation. In general, HARN, particles with a highly reactive surface and/or positively charged showed a higher induction of lung inflammation (Braakhuis *et al.*, 2014b). Lung inflammation can be investigated *in vivo* by performing a cytological analysis (total cell count, neutrophils, eosinophils and lymphocytes) and by evaluating the cytokine/chemokine levels in bronchoalveolar lavage fluid (Cho *et al.*, 2010). It was reported that intratracheal instillation of metal oxide nanoparticles in mice lead to distinct inflammatory patterns; exposure to NiO nanoparticles induced a mild lung inflammation 24-hours post-exposure, which was amplified after 4 weeks; CuO nanoparticles induced a severe lung inflammation 24-hours post-exposure that resolved almost completely after 4 weeks (Cho *et al.*, 2010).

1.3.2.4 *Genotoxicity*

Nanoparticles have the potential to induce DNA damage via primary and/or secondary genotoxic mechanisms (Magdolenova *et al.*, 2014), summarized in Figure 7. If unrepaired or mis-repaired DNA damage can lead to mutations that in turn can promote cancer development. Primary genotoxicity can be the result of either direct or indirect mechanisms and is much easier to assess *in vitro* due to technical and biological considerations.

Direct primary genotoxicity could occur by close interaction of nanoparticles with the DNA and can take place following entry to the nucleus (for small particles that can pass through the nuclear pore) or during cell division when the nuclear envelope is disassembled (Magdolenova *et al.*, 2014). Theoretically, entities with a size of ~5 nm could diffuse through the nuclear pore while larger cargos, up to 40 nm, could be shuttled to the nucleus via *e.g.* interaction with the nuclear pore complex (Wente and Rout, 2010). Once in the nucleus and depending on the cell cycle phase nanoparticles could interact with the DNA and induce genotoxicity. For example, during mitosis particles might induce breaks in the chromosomes (clastogenic effect) or loss of chromosomes (aneuploidy) by *e.g.* direct interaction with centromeric regions, whereas during interphase particles could alter DNA replication and transcription (Magdolenova *et al.*, 2014). Aneuploidy can in turn increase the genomic instability which is a precipitating factor for cancer development (Giam and Rancati, 2015).

In addition, nanoparticles were shown to induce indirect primary genotoxicity via several mechanisms (Magdolenova *et al.*, 2014):

(i) interaction with DNA repair proteins (Jugan *et al.*, 2012)
(ii) interference with the mitotic spindle and cell cycle control checkpoints with subsequent aneuploidy (Huang *et al.*, 2009)
(iii) ROS generation from the surface of the nanoparticles (or from the corresponding released ions in the case of metal based materials) can induce oxidative DNA damage and DNA strand breaks
(iv) depletion of antioxidants such as glutathione, superoxide dismutase and catalase (Sharma *et al.*, 2009)

Another type of indirect genotoxicity which can also be regarded as secondary genotoxicity was reported by Bhabra and colleagues; cobalt-chromium nanoparticles induced both chromosomal and DNA damage to fibroblasts across an intact cellular barrier of BeWo placental cells via ATP-mediated activation of the purinergic receptor P2 on the surface of the fibroblasts (Bhabra *et al.*, 2009).

Conventionally, secondary genotoxicity is triggered during inflammatory responses ('oxidative burst') and is mediated by ROS released from activated immune cells (Magdolenova *et al.*, 2014). This type of genotoxicity is mostly studied *in vivo* due to the biological limitations of the *in vitro* systems. A typical case of secondary genotoxicity can be the 'frustrated phagocytosis' and subsequent mesothelioma induction, which is relevant for HARN (Donaldson *et al.*, 2010). In addition, the general understanding for particle induced

carcinogenesis is that it involves the classic oxidative stress/inflammation pathway (Donaldson and Poland, 2012). In this thesis, mechanisms underlying genotoxicity of nanoparticles were studied in Paper I.

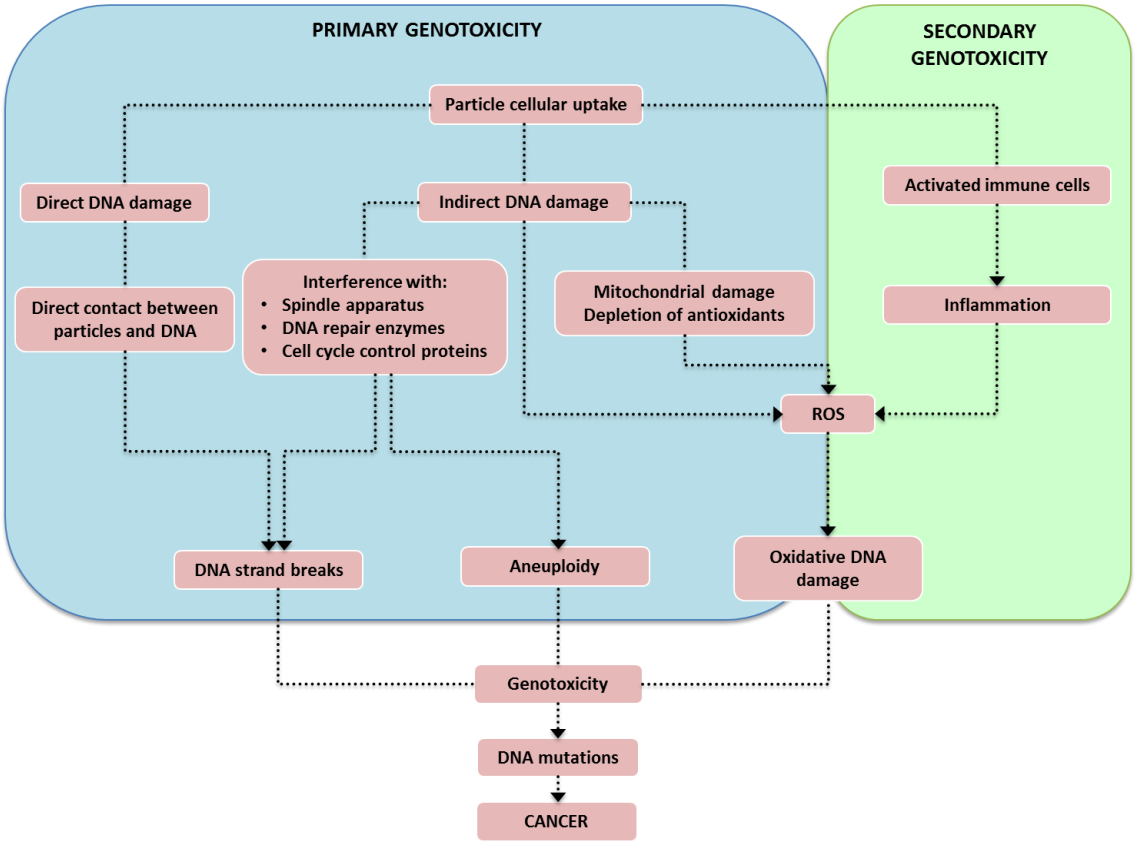

Figure 7. Mechanisms of nanoparticle-induced genotoxicity

1.3.3 Considerations on dosimetry

Despite over a decade of nanotoxicology research there is still a lack of consensus as regards dosimetry issues, which play a crucial role in interpreting and comparing toxicological data (Hussain *et al.*, 2015). Most of the *in vitro* studies describe the dose as mass per volume (µg/mL) but that can introduce confounders when comparing studies with each other, as the exposure volume can differ between experimental setups. Alternative metrics that are used in some studies include mass per surface area (µg/cm^2) or particle number per surface area. In addition, there are some clear distinctions that should be made between the **nominal dose** *i.e.* the theoretical mass that is administered, the **delivered dose** *i.e.* the dose that mechanically reaches the desired target and the **cellular dose** *i.e.* the internalized mass (Kong *et al.*, 2011a). The delivered dose is more relevant to what the particles 'see' than the nominal dose and is related among others to the nanoparticle colloidal stability in the biological environment and the viscosity of the dispersion medium. Since most of the *in vitro* systems use an upright setup for cell culture, nanoparticle sedimentation plays an important role in determining the delivered dose. Cho and colleagues reported that the uptake of gold nanoparticles was higher under upright *versus* inverted cell culture conditions for particles with high sedimentation velocity as compared to diffusion velocity (Cho *et al.*, 2011).

Computational approaches such as the *In vitro* Sedimentation, Diffusion and Dosimetry (ISDD) model, which take into account the kinetics of nanoparticles in the dispersion medium, have been established for estimating the delivered dose *in vitro* (Hinderliter *et al.*, 2010). The ISDD model predicts the delivered dose (particle number, mass) in a time-dependent manner, it can be applied to spherical nanoparticles and takes into account parameters such as hydrodynamic particle size, agglomeration state, particle density, temperature, medium height, medium viscosity and density (Hinderliter *et al.*, 2010). The Multiple Pathway Particle Dosimetry (MPPD) model is another approach used to estimate the *in vivo* lung distribution and deposition, which has been successfully applied to *e.g.* predict total lung burden and alveolar distribution of Ag nanoparticles (Braakhuis *et al.*, 2014a). Both the ISDD and the MPPD models are valuable for predicting the delivered dose and can aid *in vitro-in vivo* correlations.

In general, the cellular dose is believed to be closely correlated with the toxic outcome, however some nanoparticles (or released metal ions) could exert effects from outside the cellular compartment. While modelling the delivered dose is certainly informative, the best practice would be to quantify the actual cellular uptake, when possible. The cellular uptake can be quantified for metal and metal nanoparticles by using techniques such as inductively coupled plasma mass spectrometry (ICP-MS) or atomic absorption spectroscopy (AAS) that will be further described and discussed in Section 3.3-3.4 of this thesis. Even flow-cytometry can be used to get an estimation of cellular uptake for nanomaterials due to the increase in intracellular granularity which results in a side scatter shift (Suzuki *et al.*, 2007). Cellular uptake of nanoparticles was quantified in Paper II-IV and estimated by flow cytometry in Paper I.

1.3.4 *In vitro versus in vivo* models and correlations

With the increase in the number of nanomaterials that are in need of toxicological testing, fast, reasonably priced and ethically sound models are required *i.e. in vitro* models. Efforts have been made to increase the complexity of *in vitro* models for lung exposure to nanoparticles in an attempt to better resemble the *in vivo* scenario and better translate the results to real life exposure. For example, the lung-on-a-chip device mimics the breathing pattern and reported an increased inflammatory response to silica nanoparticles as compared to static conditions (Huh *et al.*, 2010). However, more complex models do not always have a better predictive power. It was reported that in the case of Ag nanoparticles, simple mono-layer cultures better predicted the *in vivo* toxic outcome following acute inhalational exposure as compared to more complex co-culture models (Braakhuis *et al.*, 2016). In this regard, I believe that the quote of George E.P. Box which was mentioned in the context of statistics can be extrapolated to biological models.

"Essentially, all models are wrong, but some are useful." George E. P. Box

When adding an additional level of complexity to a model one also adds an additional level of uncertainty which in the end could defeat the purpose of the model. On the other hand, living

organisms are complex and the development of intelligent models that can encompass that complexity and have predictive power for subtle endpoints is one of the future challenges.

Using *in vitro* models is advantageous for deriving mechanistic information on the toxicity of nanomaterials that is more difficult to obtain from *in vivo* studies. However, to which extent this mechanistic information can be translated to real-life exposure is yet to be established. One issue that proved critical in translating *in vitro* to *in vivo* data is dosimetry. Several studies report that surface area rather than mass is a better metric for correlating *in vitro* with *in vivo* data (Braakhuis *et al.*, 2016, Han *et al.*, 2012).

Teeguarden *et al.* evaluated the *in vitro – in vivo* correlation by addressing the target tissue dosimetry and using superparamagnetic iron oxide nanoparticles as model particle (Teeguarden *et al.*, 2014). The authors used the same dose scale and reported that target tissue doses of 0.009 – 0.4 µg/cm^2 in the alveolar region *in vivo* corresponded to 1.2 – 4 µg/cm^2 *in vitro* in lung epithelial cells as regards the induction of inflammatory markers (that were previously identified following gene expression profiling *in vivo*) (Teeguarden *et al.*, 2014). In addition, the study found a good correlation regarding inflammation between the nanoparticle cellular dose estimated in alveolar macrophages (1-100 pg/cell) and evaluated *in vitro* in bone marrow derived macrophages (8-35 pg/cell) (Teeguarden *et al.*, 2014).

Finally, it was reported that the predictive value of the *in vitro* assays revolves around the mechanism of toxicity; the majority of *in vitro* assays that were tested (cytotoxicity, cytokine secretion) identified toxicity of highly soluble nanoparticles but had a high degree of failure when it came to toxicity mediated by surface reactivity (Cho *et al.*, 2013). The same study reported that only the hemolysis assay was appropriate for predicting *in vivo* lung inflammation for insoluble particles such as CeO_2 for which it is believed that surface reactivity mediates toxicity (Cho *et al.*, 2013).

1.4 SYSTEMS TOXICOLOGY

With the emergence of increasing manufacture and use of nanoparticles, new approaches are needed in order to aid the risk assessment processes and enable sustainable development of nanotechnology. On one hand, fast, high-throughput technologies are required for screening and predictive purposes (Nel *et al.*, 2013). On the other hand, in line with 'toxicity testing in the 21st century', emerging technologies such as 'omics' would provide a mechanistic insight into the pathways of toxicity with the ultimate goal of establishing the human 'toxome' (Hartung *et al.*, 2012, Hartung and McBride, 2011).

Systems toxicology approaches have developed from integrating systems biology and toxicology and are envisioned to provide a holistic and mechanistic understanding of the interactions between xenobiotics and biological systems at different levels of organization (Costa and Fadeel, 2016). The ultimate purpose of these approaches is to help establish adverse outcome pathways, derive predictive models of biological interactions and lay a solid foundation for risk assessment (Costa and Fadeel, 2016, Sturla *et al.*, 2014). In addition, 'omics' technologies can open up the field of toxicology for hypothesis-free research, however,

during the downstream data analysis and interpretation previous knowledge can be used to narrow-down the results (Costa and Fadeel, 2016). The systems toxicology framework in the context of nanosafety research is illustrated in Figure 8.

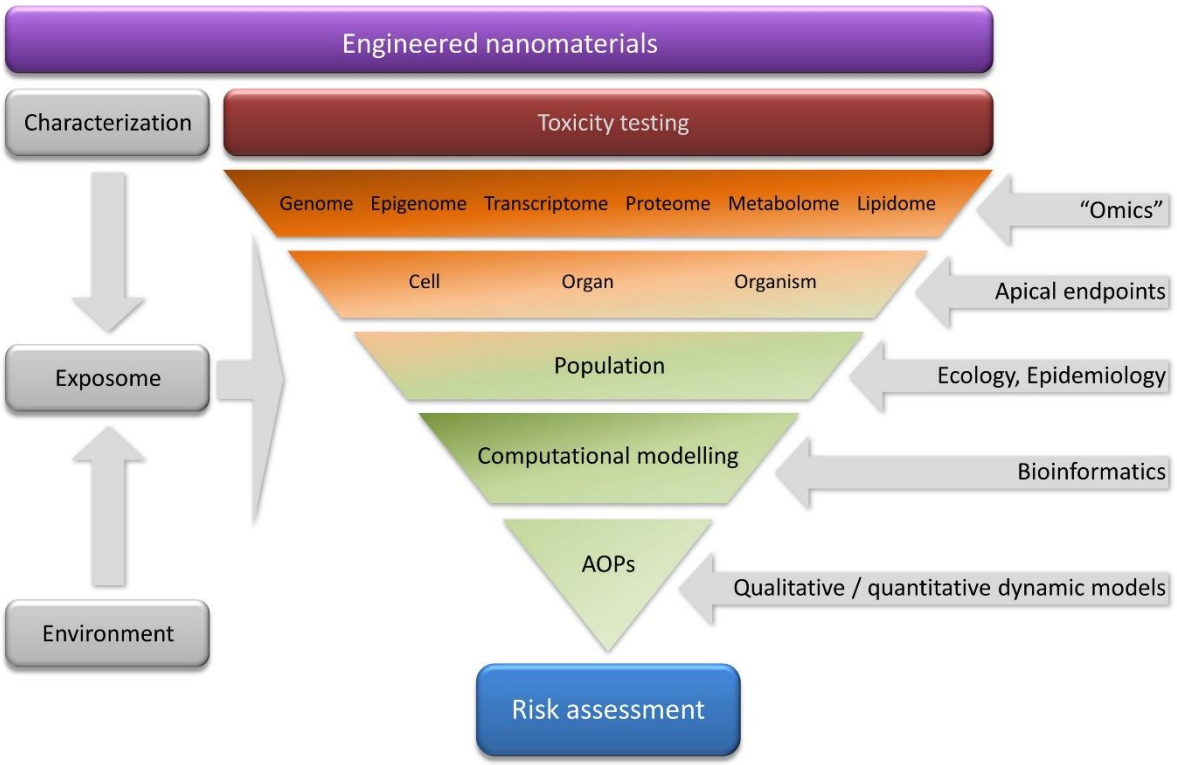

Figure 8. The systems toxicology framework for risk assessment of nanomaterials. Reproduced from Costa and Fadeel, 2016, with permission from Elsevier.

Systems toxicology integrates different 'omics' technologies to globally assess gene expression (transcriptomics), proteins (proteomics), lipids (lipidomics), metabolites (metabolomics) or epigenetic traits (epigenomics). Two of these approaches, namely transcriptomics and epigenomics will be further discussed below.

Transcriptomic approaches are used to quantify genome-wide mRNA levels and comprise of oligonucleotide hybridization techniques (microarray) or newer sequencing techniques such as RNA sequencing (RNA-Seq). Toxicogenomics (mostly hybridization technologies) have been used in toxicological research for over a decade and are considered a powerful approach for identifying perturbed biological pathways, novel toxicological mechanisms as well as biomarkers of toxicity (Chen *et al.*, 2012). One important advantage of using toxicogenomics is the holistic approach that allows for understanding of gene changes in the context of altered pathways and networks thus providing a better understanding of both the mechanisms of toxicity and the toxic response. RNA-seq is a novel technology that allows for robust measurements of RNA transcripts on a genome-wide level (Wang *et al.*, 2009). In contrast to microarray techniques, RNA-Seq is more accurate, has a higher dynamic range, allows for detection of alternative splicing and can be used without preexisting knowledge of the genomic sequence (Wang *et al.*, 2009). Recently, RNA-Seq has emerged as a tool in (nano)toxicology, bound to supersede microarrays in the toxicogenomics field (Costa and Fadeel, 2016). Thus far, RNA-Seq has been employed in nanotoxicology to *e.g.* unravel the low-dose effects of

dendrimers on human lung cells (Feliu *et al.*, 2015) and to identify the effects of exposure to metal nanoparticles in green alga, *Chlamydomonas reinhardtii* (Simon *et al.*, 2013).

Epigenomics approaches aim to understand the genome wide changes in the epigenetic patterns of cells. Epigenetic phenomena such as DNA-methylation, histone modifications and non-coding RNAs are involved in modulating genome-environment interactions without involving changes in the DNA sequence (Mensaert *et al.*, 2014). Epigenomic technologies are relatively new and include both microarray and sequencing technologies for the assessment of DNA methylation and microRNAs (Mensaert *et al.*, 2014). It is currently believed that nanoparticles have the potential to induce epigenetic changes (Shyamasundar *et al.*, 2015) and epigenomics could help unravel some of those effects. DNA methylation arrays have been widely used for epigenome-wide association studies (Morris and Beck, 2015) but to a lesser extent in nanotoxicology studies. There are, however, accounts of micro-RNA studies which addressed the effects of MWCNTs in human lung cells (Nymark *et al.*, 2015) and the effects of Ag nanoparticles in Jurkat cells (Eom *et al.*, 2014).

With time, the cost of 'omics' technologies is bound to decline and the flow of data analysis will become more and more standardized and fast, which will ultimately enable the switch from traditional toxicological approaches to comprehensive 'omics' approaches on a routine basis.

1.5 METAL AND METAL OXIDE NANOPARTICLES

Metal and metal oxide nanoparticles are a heterogenous group of particles important from an occupational and environmental toxicological perspective (Karlsson, 2015a). Metal oxide nanoparticles have semiconductive and catalytical properties and are being manufactured in large quantities for industrial purposes (Zhang *et al.*, 2012). The same traits that are appealing from a technical point of view can, however, imply a propensity for ROS generation and can thus lead to toxicological effects such as *e.g.* lung inflammation that was reported upon inhalation of welding fumes (Antonini *et al.*, 2004). There are currently two theories that aim to predict the lung inflammation potential of metal and metal oxide nanoparticles: the band gap theory and the zeta potential theory.

The band gap theory is based on the likelihood of electron transfer between the valence band of metal oxide nanoparticles and cellular redox couples (Zhang *et al.*, 2012). If valence band energy levels overlap with the biological redox potential, electron transfer should occur more easily with the formation of ROS and oxidized biomolecules, that was in turn correlated with cytotoxicity and lung inflammation (Zhang *et al.*, 2012). From all the tested metal nanoparticles, CuO and ZnO did not fit the prediction and that was believed to be related to their high dissolution and release of toxic ions (Zhang *et al.*, 2012).

The zeta potential theory (Figure 9) postulates that metal and metal oxide nanoparticles with low solubility and high zeta potential in acidic conditions (ζP acid) are more likely to inflict damage on the lysosomal membrane thereby inducing lung inflammation (Cho *et al.*, 2012).

For highly soluble particles, the inflammation is triggered by the release of toxic ions that destabilize the lysosomal membrane (Cho *et al.*, 2012).

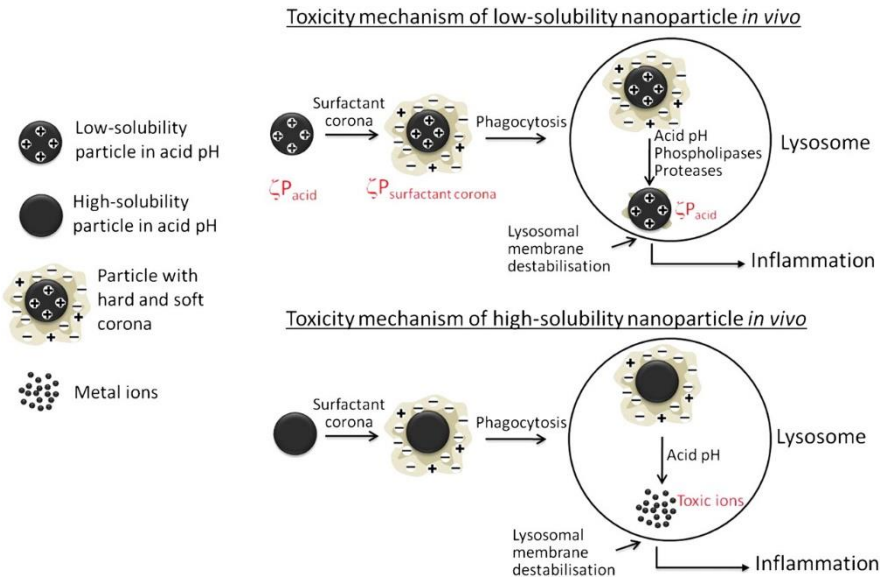

Figure 9. Depiction of the hypothetical 'zeta potential theory' by which metal and metal oxide nanoparticles induce lung inflammation. Reproduced from Cho et al., 2012, with permission from Oxford University Press.

In Paper I several metal and metal oxide nanoparticles (CuO, Fe_3O_4, ZnO, TiO_2, NiO, CeO_2 and Ag) were screened, Paper II-III was focused on Ag nanoparticles while Paper IV was focused on CeO_2 nanoparticles. The particular interest placed on the Ag nanoparticles is related to it having the highest manufacture among nanoparticles as well as incidence in consumer products, whereas the focus on CeO_2 nanoparticles stemmed from its outstanding antioxidant properties and promising industrial as well as biomedical applications. All nanoparticles will be briefly introduced below with a more extensive discussion on Ag and CeO_2 nanoparticles.

1.5.1 Copper oxide nanoparticles

Copper oxide (CuO) nanoparticles have semi-conductive as well as catalytic properties and have multiple industrial applications such as *e.g.* sensors, batteries, solar energy conversion (Karlsson, 2015a). Karlsson and colleagues found that CuO nanoparticles were more toxic than other metal oxide nanoparticles and were able to induce DNA damage (Karlsson *et al.*, 2008). In addition, CuO nanoparticles generated oxidative stress and induced lung inflammation, effects that were correlated with their high dissolution (Zhang *et al.*, 2012, Cho *et al.*, 2013). Another study revealed that the acute lung inflammation following CuO exposure was resolved with time (4 weeks) leaving behind signs of lung fibrosis (Cho *et al.*, 2010). There are accounts of a 'Trojan horse' effect for CuO nanoparticles by which the particulate form increases bioavailability of Cu ions, that in turn induce oxidative stress, disrupt the metal homeostasis and upregulate the expression of metallothioneins (Cuillel *et al.*, 2014) as depicted in Figure 10.

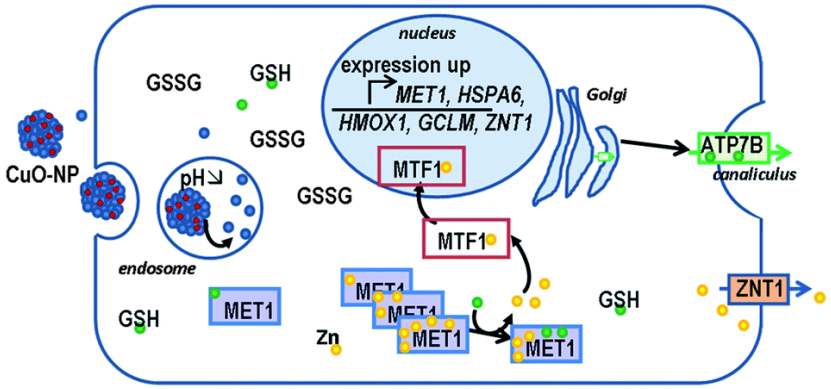

Figure 10. CuO nanoparticles disturb metal homeostasis in hepatocytes. Reproduced from Cuillel et al., 2014, with permission from The Royal Society of Chemistry. (MET1: metallothionein 1, MFT1: metal regulatory transcription factor 1, ZNT1: Zn transporter 1, HMOX1: Heme Oxygenase 1)

1.5.2 Zinc oxide nanoparticles

Zinc oxide (ZnO) nanoparticles bear properties that are appealing for industrial applications *i.e.* high chemical- and photo- stability as well as broad spectra of radiation absorbtion (Kołodziejczak-Radzimska and Jesionowski, 2014). As a result, ZnO nanoparticles are used in the electronic industry, in photocatalysis, as well as in the pharmaceutical and cosmetic industries (Kołodziejczak-Radzimska and Jesionowski, 2014). George and colleagues reported that the toxicity of ZnO nanoparticles is mediated by the dissolved ions that trigger ROS generation, intracellular calcium flux, mitochondrial depolarization, and plasma membrane leakage; effects that were reduced by iron doping and consequent reduction in solubility (George *et al.*, 2010). There are contradicting studies regarding the 'Trojan horse' effect of ZnO nanoparticles. One *in vitro* study in Jurkat cells showed that the extracellular release of Zn ions elicited a similar cytotoxic effect as the ZnO nanoparticles (Buerki-Thurnherr *et al.*, 2013), whereas another study reported that the effects of ZnO nanoparticles occur following particle uptake by BEAS-2B cells and subsequent dissolution (Gilbert *et al.*, 2012). These inconsistencies could be explained by the use of different particles as well as cell systems that could also imply different cell media. Inhalation of ZnO nanoparticles was correlated with lung inflammation *in vivo*, that was again related to the dissolution and release of Zn ions (Cho *et al.*, 2013, Cho *et al.*, 2012, Zhang *et al.*, 2012). ZnO nanoparticles are currently used as sunscreens with a reported low dermal penetration and toxicity when applied in form of a cream, however, there are potential hazards related to spray formulations that could result in inhalation of ZnO nanoparticles (Karlsson, 2015a).

1.5.3 Titanium oxide nanoparticles

Titanium oxide (TiO_2) nanoparticles are currently used in food products and paints as pigments, as well as in sunscreen products for their UV reflective properties (Weir *et al.*, 2012). The cytotoxicity of TiO_2 nanoparticles is considered to be modest and was reported to occur only at high doses (Karlsson, 2015a). However, low-dose, long-term exposure to anatase TiO_2 nanoparticles induced cell transformation in BEAS-2B cells (Vales *et al.*, 2015). In addition,

TiO_2 nanoparticles were found to inhibit DNA repair activity in A549 cells (Jugan *et al.*, 2012). Rutile TiO_2 nanoparticles have been shown to induce IL-1β secretion *in vitro* but did not result in lung inflammation following *in vivo* exposure in rats (Cho *et al.*, 2013). The crystalline structure was found to be important for the photocatalytic properties of TiO_2; anatase TiO_2 induced more DNA damage under light conditions as compared to the rutile form (Karlsson *et al.*, 2015b, Di Bucchianico *et al.*, 2016). Similar to ZnO nanoparticles, European Commission regards TiO_2 nanoparticles as being safe when present in sunscreen products, however, it states that there is not sufficient data to establish safety recommendations for spray products (Karlsson, 2015a).

1.5.4 Nickel oxide nanoparticles

Nickel oxide (NiO) nanoparticles are used for various industrial applications such as catalysis, gas sensors, battery cathodes (El-Kemary *et al.*, 2013) and exposure is likely to occur in occupational settings. Metallic nickel and nickel compounds are classified as carcinogenic to humans (Class 1A), however the IARC report identified differences between different forms of nickel, and stated that there is limited evidence for carcinogenicity for soluble nickel forms such as *e.g.* nickel sulfate and nickel chloride (IARC, 2012, 100C). According to the nickel ion bioavailability theory, the carcinogenicity of nickel compounds is related to the cellular uptake, subsequent dissolution and nuclear availability of nickel ions (Goodman *et al.*, 2011). It is still unclear to which extent this theory applies to nickel nanoparticles. NiO nanoparticles have been reported to induce DNA damage following short-term exposure in A549 and BEAS-2B cells (Latvala *et al.*, 2016, Kain *et al.*, 2012). Exposure to NiO nanoparticles *in vivo* induced a distinct lung inflammation profile characterized by mild lung toxicity 24-hours post-exposure and severe toxicity accompanied by lymphocyte infiltration at 4-weeks post-exposure (Cho *et al.*, 2010).

1.5.5 Iron oxide nanoparticles

Iron oxide (Fe_3O_4, Fe_2O_3) nanoparticles have a wide spectrum of applications, from catalysts, pigments and sensors to biomedical applications for diagnostic purposes (Karlsson, 2015a). Superparamagnetic iron oxide nanoparticles have been approved by the US Food and Drug Administration and are now in clinical use as contrast agents for magnetic resonance imaging (Li *et al.*, 2013), which implies they are safe for systemic administration, however, the effects following inhalational exposure are less clear. *In vitro* studies report in general a low cytotoxicity with no DNA damage or ROS generation in A549 cells (Karlsson *et al.*, 2008). *In vivo* studies are inconsistent and show either no lung inflammation following oropharyngeal aspiration in mice (Zhang *et al.*, 2012) or inflammation in the alveolar region associated with macrophage infiltration following inhalational exposure in mice (Teeguarden *et al.*, 2014).

1.5.6 Silver nanoparticles

1.5.6.1 *Uses and exposure*

Ag nanoparticles are currently one of the most manufactured and used nanomaterials in consumer products (Vance *et al.*, 2015), with an estimated production of 320 tons per year (Nowack *et al.*, 2011). As a result of their antimicrobial properties, Ag nanoparticles are used in textiles, food industry, household products, paints, cosmetics and medical devices. Recently, Ag nanoparticles have gained attention for their bioimaging and allegedly chemotherapeutic properties (Sotiriou and Pratsinis, 2011, Wei *et al.*, 2015). However, exposure to nanoscale Ag is not new as it has over a century of use in pigments, wound dressings and photographics, under the form/name colloidal Ag (Nowack *et al.*, 2011). Despite this historical use of Ag, it is nevertheless expected that the occupational and environmental exposure to Ag nanoparticles will increase, in line with the increase production and use. In addition, new types of Ag nanoparticles are being manufactured, such as Ag nanowires that might pose new toxicological hazards (Stoehr *et al.*, 2011).

Exposure to large amounts of Ag in humans has been reported to induce argyria (discoloration of the skin) and argyrosis (discoloration of the ocular globe due to deposition of silver) but without associated clinical implications (Rosenman *et al.*, 1979). The current occupational exposure limit (OEL) to airborne Ag is 0.01 mg/m^3 for soluble Ag, and 0.1 mg/m^3 for metallic Ag dust and fumes, and is expected to prevent argyria (Weldon *et al.*, 2016). There are no official OELs for Ag nanoparticles but a recent study by Weldon *et al.* derived a OEL of 0.19 µg/m^3 based on sub-chronic inhalational exposure in rats (Weldon *et al.*, 2016). As regards consumer exposure, the inhalational exposure is expected to be low as Ag nanoparticles are often found embedded in various matrices. However, there are specifically formulated spray products such as anti-odour and throat sprays for which inhalational exposure is of particular concern (Quadros and Marr, 2011).

1.5.6.2 *In vitro studies*

The *in vitro* effects of Ag nanoparticles have been investigated in a large amount of studies that report on cytotoxicity, oxidative stress and genotoxicity, among others. Some of the common pitfalls of these studies is the use of poorly characterized materials as well as exposure to high doses of nanomaterials, sometimes deriving mechanistic information under cytotoxic conditions. In addition, considering the wide array of Ag nanoparticles tested with different sizes and coatings and the different cell types, it can be difficult to relate the results with each-other.

The size-dependent cytotoxicity of Ag nanoparticles, with smaller particles being more potent, has been well-established in cell models such as BEAS-2B (Wang *et al.*, 2014), HepG2, HL-60 (Avalos *et al.*, 2014) and BALB/3T3 (Onodera *et al.*, 2015) as well as in Paper II of this thesis. In Paper II we addressed some of the knowledge gaps at that time by using well-characterized Ag nanoparticles. Our results showed that small, 10 nm Ag nanoparticles were more cytotoxic than larger nanoparticles, independent of the coating, polyvinylpyrrolidone

(PVP) or citrate, and that was likely related to the intracellular release of Ag. Similar effects were reported by Wang *et al.* who, in addition, also found coating dependent cytotoxicity with citrate coated 110 nm particles being more toxic than same-sized PVP particles (Wang *et al.*, 2014). This was explained by the inability of the citrate surface to coordinate the released Ag^+ whereas the PVP coating allowed the formation of N-Ag^+ and O-Ag^+ complexes that reduced the Ag bioavailability and cytotoxicity (Wang *et al.*, 2014). In the same study they correlated the Ag cytotoxicity with generation of ROS (Wang *et al.*, 2014).

Additional studies also involved ROS in the toxic effects of Ag nanoparticles. For example, Carlson *et al.* showed a size-dependent induction of ROS in rat alveolar macrophages with 15 nm particles being more potent than 30 and 55 nm ones (Carlson *et al.*, 2008). Some of these studies (Carlson *et al.*, 2008, Wang *et al.*, 2014) determined ROS under cytotoxic conditions which has questionable relevance, as it cannot be causally correlated with the induction of cell death. On the other hand, determining the ROS levels prior to cell death, under non-cytotoxic conditions could indeed unravel potential implications of ROS for the cytotoxic endpoint. Onodera *et al.* reported ROS generation as early as 5 min following exposure of BALB/3T3 cells to 1 nm Ag nanoparticles (Onodera *et al.*, 2015), however the observation was not quantitative. In another study Avalos *et al.* showed ROS generation, glutathione depletion but no alteration of the superoxide dismutase activity following exposure of HepG2, HL-60 cells to Ag nanoparticles (4.7 nm and 42 nm) (Avalos *et al.*, 2014). In addition, the same study reported that the pre-treatment with N-acetyl-cysteine (NAC) rescued the cells from dying, allegedly linking oxidative stress induction by Ag nanoparticles to cytotoxicity (Avalos *et al.*, 2014). ROS generation by Ag nanoparticles was evaluated in Paper II.

As regards *in vitro* genotoxicity, Ag nanoparticles have been reported to induce DNA damage observed by the comet assay in BEAS-2B cells (Nymark *et al.*, 2013), human lung fibroblast cells (IMR-90) and human glioblastoma cells (U251) (AshaRani *et al.*, 2009) as well as micronucleus in IMR-90 and U251 cells (AshaRani *et al.*, 2009). The doses used by AshaRani and colleagues were, however, extremely high (up to 200 µg/mL). Another study by Foldbjerg *et al.* reported the formation of DNA adducts following Ag nanoparticle exposure in A549 cells, which was correlated with increase in ROS levels, and was inhibited by pretreatment with NAC (Foldbjerg *et al.*, 2011). Recently, Guo *et al.* performed an extensive cytotoxicity and genotoxicity assessment for a panel of well-characterized particles as well as ionic Ag using OECD tests (Guo *et al.*, 2016). The results showed a size- and coating-dependent cytotoxicity and genotoxicity in the mouse lymphoma assay and the micronucleus test, with the smaller particles and the citrate coated particles being more potent (Guo *et al.*, 2016). Genotoxicity of Ag nanoparticles has been tested in Paper I-III.

There is increasing amount of evidence that the toxicity of Ag nanoparticles occurs via a 'Trojan horse' mechanism that mediates Ag bioavailability with subsequent intracellular release of toxic ions (Park *et al.*, 2010, Hsiao *et al.*, 2015). In a recent review entitled 'Silver nanoparticles – Wolves in sheep's clothing?' Foldbjerg *et al.* further looked into this theory (Foldbjerg *et al.*, 2015) which is depicted in Figure 11. Upon cellular uptake, Ag nanoparticles

are reported to undergo fast dissolution with release of Ag^+ that is first oxidized to Ag-O- followed by binding to thiol groups with the formation of Ag-S- (Jiang *et al.*, 2015). The intracellular interaction of Ag with the thiol groups from proteins and peptides could result in a change of protein structure and functionality together with depletion of glutathione (as a result of binding the cysteine residue) (Foldbjerg *et al.*, 2015). The high affinity of Ag towards thiol groups has been explored in the past, when Ag-based compounds were used to stain proteins (Merril, 1990).

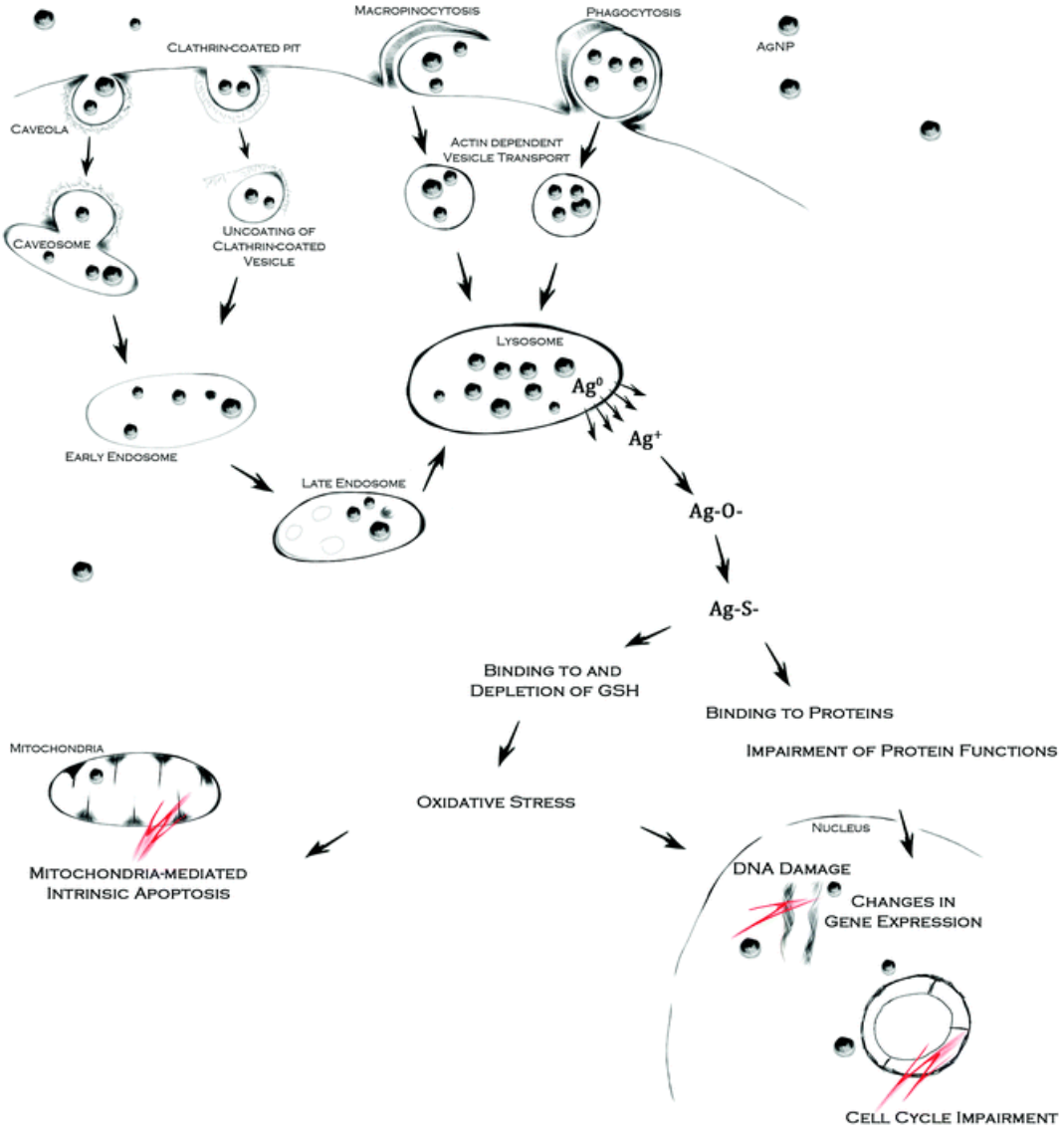

Figure 11. The 'Trojan horse' mechanism of toxicity for Ag nanoparticles. Reproduced from Foldbjerg et al., 2015, with permission from The Royal Society of Chemistry

Despite a lot of research performed on Ag nanoparticles there are still many unknowns as regards their chronic effects. Thus far there is only one study that addressed the chronic effect of long-term (3 months), low-dose (pg/mL) exposure of human keratinocytes to Ag nanoparticles (50 nm), which reported induction of sustained cellular stress (activation of p38, increased Ki67 expression, and altered expression of stress related genes) (Comfort *et al.*, 2014).

1.5.6.3 *In vivo studies*

In general, *in vivo* studies report size-dependent lung inflammation following acute inhalation of Ag nanoparticles. For example, rats exposed nose-only to 15 nm and 410 nm Ag nanoparticles for 6 hours a day for 4 days, showed a significant lung inflammation for the 15 nm particles 24-hours post-exposure, which resolved after 7 days (Braakhuis *et al.*, 2014a). The reasons behind the size-dependent toxicity were attributed to the increased alveolar deposition (Braakhuis *et al.*, 2014a). In a similar study, rats were exposed nose-only to 20 nm or 110 nm Ag nanoparticles for 6 hours and the results showed an inflammatory peak response 7-days post-exposure which was more pronounced for the smaller particles, and that resolved with time (21- and 56-days post-exposure) (Silva *et al.*, 2016). A similar pattern was observed after oropharyngeal aspiration in rats; 20 nm and 110 nm Ag nanoparticles induced acute lung inflammation at 40-hours post-exposure, with more potent effects for the 20 nm particles which was correlated to higher Ag^+ release (Wang *et al.*, 2014). In addition, the same study showed an increase in neutrophil count and collagen content, indicative of early fibrosis for the 110 nm particles at 3-weeks post-exposure (Wang *et al.*, 2014).

A 28-day inhalation study on rats (6 hours/day, 5 days/week) at levels close to the Ag dust limit (0.1 mg/m^3) reported no significant health effects of Ag nanoparticles sized 10-15 nm in terms of hematological and histopathological changes, despite significant Ag distribution in the liver, brain as well as olfactory bulb (Ji *et al.*, 2007). A follow-up study by the same group reported that 90-day inhalational exposure (6 hours/day) performed according to the OECD guidelines to 18 nm Ag nanoparticles resulted in chronic alveolar inflammation and reduction of lung function (Sung *et al.*, 2008) together with dose dependent bile-duct hyperplasia from which they derived a no-observed-adverse-effect-level (NOAEL) of 100 $\mu g/m^3$ (Sung *et al.*, 2009). Moreover, it was concluded that there was no micronuclei induction in the bone marrow of exposed animals (Kim *et al.*, 2011), in the absence of data on the genotoxicity in the target organs, lung or liver. In addition, at the respective NOAEL, lung physiological alterations were still present and were found to be gender-dependent, the males being more sensitive. In an additional follow-up study using the same experimental setup, the authors found a persistent lung inflammation in male rats throughout a 12-week period of recovery post-exposure in the high exposure group (at doses above the NOAEL) (Song *et al.*, 2013). These studies point out the importance of the duration of exposure but, thus far, there are no available *in vivo* studies on the chronic effects of Ag nanoparticles.

It has recently been reported that following inhalational exposure, Ag nanoparticles can be transported to the brain, presumably via the olfactory bulb which could represent additional health hazards (Patchin *et al.*, 2016). This is of particular relevance as non-cytotoxic concentrations of Ag nanoparticles could disrupt cytoskeletal organization and alter neurite extension in rat-derived cultured adult neural stem cells (Cooper and Spitzer, 2015).

1.5.7 Cerium oxide nanoparticles

1.5.7.1 *Uses and exposure*

Cerium oxide (CeO_2) nanoparticles (nanoceria) bear outstanding physico-chemical properties such as catalytic, oxidant/antioxidant as well as spectroscopic traits that make them appealing for industrial purposes such as *e.g.* polishing and catalytical agents, environmental remediation (Andreescu *et al.*, 2014). In addition, nanoceria is used as a combustion catalyst in diesel fuel where it acts by increasing the combustion efficiency and consequently decreasing the emission of soot (Cassee *et al.*, 2011). Apart from the industrial applications, nanoceria has gained interest for biomedical applications which are mainly driven by the broad range of antioxidant properties: superoxide dismutase mimetic, catalase mimetic, nitric oxide radical scavenger, peroxynitrite scavenger (Walkey *et al.*, 2015). Exposure to nanoceria can be industrial (during synthesis and product manufacture), environmental (as a result of its use as a diesel fuel additive) and intentional (for potential biomedical applications). As regards intentional exposure, to date, no clinical trials have been approved with nanoceria.

Environmental exposure to nanoceria, particularly via inhalation can occur as a result of its use as diesel additive (Cassee *et al.*, 2011). Following nanoceria inhalation, the lung is the main target organ, however, translocation to other sites, and even brain (via the olfactory bulb) could occur. This latter mechanism has been confirmed for other nanoparticles such as Ag (Patchin *et al.*, 2016), MnO (Elder *et al.*, 2006) and ZnO (Kao *et al.*, 2012). It was postulated that the addition of nanoceria as a diesel additive would reduce the CO_2 emissions, the total particulate mass, aldehydes and several polycyclic aromatic hydrocarbons, however, these changes were correlated with an increase in ultrafine particle emission, nitric oxide species and benzo[a]pyrene (Zhang *et al.*, 2013). On the other hand, inhalation studies on atherosclerosis-prone mice indicated that exposure to exhaust fumes from fuel with nanoceria, actually had a protective effect against atherosclerosis induction, but lead to mild inflammation in the brain (Cassee *et al.*, 2012). The same study noted a reduction both in the number and surface area of the diesel exhaust particles following addition of nanoceria (Cassee *et al.*, 2012). Moreover, it was revealed that addition of nanoceria reversed the effects of exposure to diesel exhaust *i.e.* decreased the stress-responsive transcription factor AP-1 (Lung *et al.*, 2014).

1.5.7.2 *In vitro studies*

Nanoceria has been shown to have an antioxidant and anti-apoptotic effect in endothelial cells (Chen *et al.*, 2013), in isolated rat pancreatic cells (Hosseini *et al.*, 2013) and in U937 monocytes and Jurkat cell lymphocytes (Celardo *et al.*, 2011). The latter study revealed that the protective antioxidants effects were correlated with the presence of Ce^{3+}/Ce^{4+} redox couple at the surface of the particles (Celardo *et al.*, 2011). In addition, nanoceria provided antioxidant protection up to 7-days post-exposure in cardiac progenitor cells, without interfering with the cellular differentiation (Pagliari *et al.*, 2012) and prevented UV-induced mutagenesis in Jurkat cells (Caputo *et al.*, 2015). Nanoceria was also shown to prevent ROS generation and cell death

induced by cigarette smoke extract in rat embryonic myocytes by suppressing the NF-κB pathway activation (Niu *et al.*, 2011).

Nanoceria was reported to have neuroprotective effects *in vitro* by increasing neuronal survival in a human Alzheimer Disease model via modulation of the brain-derived neurotrophic factor pathway (D'Angelo *et al.*, 2009) and by reducing ischemic cell death in a hippocampal brain slice model of ischemia (Estevez *et al.*, 2011). In addition, nanoceria was shown to promote neuronal differentiation and dopamine secretion (Ciofani *et al.*, 2013) along with alterations in the expression profile of genes involved in antioxidant defense (Ciofani *et al.*, 2014).

On the other hand, several studies have identified potential toxicological implications of nanoceria. For example, nanoceria was reported to induce apoptosis and oxidative stress in BEAS-2B cells (Park *et al.*, 2008), which was later correlated with an induction of heme oxygenase-1 allegedly via the p38-Nrf2 signaling pathway (Eom and Choi, 2009). Similar effects were observed in human peripheral blood monocytes where nanoceria induced apoptosis and autophagy (Hussain *et al.*, 2012) and in A549 cells where nanoceria lead to ROS mediated DNA damage and apoptosis, effects that were attenuated by treatment with the antioxidant NAC (Mittal and Pandey, 2014).

1.5.7.3 *In vivo studies*

Some of the beneficial *in vitro* effects of nanoceria were also corroborated *in vivo* in a series of disease models. Nanoceria administered via peritoneal injections reduced the oxidative stress levels, had beneficial anti-angiogenic effects and reduced the size of the endometrial lesions in mice with endometriosis (Chaudhury *et al.*, 2013). Nanoceria was reported to have similar anti-angiogenic effects and restricted tumor growth in a mouse model of ovarian cancer (Giri *et al.*, 2013). Moreover, topical applications of nanoceria accelerated wound healing in mice by increasing cellular proliferation and migration (Chigurupati *et al.*, 2013).

In addition, several studies report neuroprotective effects following nanoceria treatment. Intravenous injections of nanoceria reduced ROS levels as well as apoptosis and decreased infarct volume in a rat brain ischemic stroke model (Kim *et al.*, 2012). Following intravenous administration nanoceria crossed the blood-brain barrier, reduced ROS levels and diminished motor symptoms in a mouse multiple sclerosis model (Heckman *et al.*, 2013). Moreover, nanoceria in combination with lenalidome, an anti-inflammatory drug, reduced demyelination and clinical symptoms in the same mouse multiple sclerosis model (Eitan *et al.*, 2015). In addition, intravitreal administration of nanoceria had an anti-inflammatory and anti-angiogenic effect in a mouse model of age-related macular degeneration (Kyosseva *et al.*, 2013), whereas systemic injection improved retinal response to light, slowed down the photoreceptor degeneration and reduced the retinal ROS levels in a mouse model of retinal degeneration (Kong *et al.*, 2011b).

On the other hand, Hardas *et al.* reported that a single intravenous administration of nanoceria (5 nm) induced pro-oxidant effects in the brain 30-days post-exposure in the absence of brain

translocation and that those changes were similar to the age- or Alzheimer disease-related effects (Hardas *et al.*, 2012). In another study the same group revealed that a single intravenous administration of nanoceria (approx. 30 nm) elicited a hierarchical oxidative stress response in the rat hippocampus with a peak at day 30 and resolution at day 90 post-exposure (Hardas *et al.*, 2014). The same study noted that the levels of nanoceria in the brain were very low and much of it could be attributed to the levels in the blood vessels perfusing the brain (Hardas *et al.*, 2014). Moreover, in a similar experimental setup nanoceria was found to induce liver injury at day 30 and day 90 post-exposure in the form of granulomas (nanoceria loaded Kupffer cells and mononuclear cells) and increase blood levels of alanine aminotransferase (Tseng *et al.*, 2014).

Apart from the potential toxicity, biodistribution is another problem that might stand in the way of nanoceria being used for clinical applications in neurodegenerative diseases. Yokel *et al.* investigated the biodistribution of different sized nanoceria (5, 15, 30, 55 nm) following intravenous administration and found that particles concentrated in the liver and the spleen with little amounts in the brain parenchyma and with minor differences between the sizes (Yokel *et al.*, 2013).

The reported discrepancies on the effects of nanoceria could be related to the different models and endpoints investigated, different range of doses as well as different physico-chemical properties of nanoceria used in these studies *e.g.* size, shape, surface charge and surface valence. It is known that the ratio Ce^{3+}/Ce^{4+}, which is responsible for some of the antioxidant properties, is negatively correlated with the size or the nanoparticles (Deshpande *et al.*, 2005), and so is their hydroxyl radical scavenging activity (Xue *et al.*, 2011). The percentage of Ce^{3+} at the particle surface was correlated with toxic outcome in *Pseudokirchneriella subcapitata* (aquatic organism model); particles with a higher (58%, 40%) percentage were toxic whereas particles with lower (36%, 38% and 26%) percentage of Ce^{3+} were not (Pulido-Reyes *et al.*, 2015). In addition, long-aspect ratio nanorods were more toxic than spherical nanoceria in a mouse lung model and gastrointestinal tract of zebrafish larvae (Lin *et al.*, 2014). Surface chemistry was also found to be important and studies showed that coating of nanoceria with a layer of amorphous silica reduces lung inflammation and fibrosis in rats (Ma *et al.*, 2015). Also, the reagents used during the manufacturing process can have an influence on the surface reactivity, catalytic properties and toxicity; hexamethylenetetamine (HMT) nanoceria was taken up more and was more toxic to HUVEC cells than H_2O_2 nanoceria or NH_4OH nanoceria (Dowding *et al.*, 2013). The same study indicated that nanoceria with high levels of Ce^{4+} at the surface (HMT and NH_4OH nanoceria) exhibit phosphatase and ATPase activity (Dowding *et al.*, 2013).

2 AIM

With the high increase in the manufacturing and use of nanomaterials, toxicological sciences need to evolve in order to address relevant safety concerns and to ensure the sustainable development of nanotechnology. The overall aim of this thesis was to address some of the challenges as well as knowledge gaps in nanotoxicology using *in vitro* models. To this end we explored a new method for mechanism-based screening (Paper I), we used well-characterized Ag nanoparticles in both short and long-term/low-dose exposure scenarios (Paper II and III) and we applied next generation sequencing for in-depth understanding of nanoparticle-cell interactions (Paper III and IV).

The specific aims for each included project were:

- to evaluate the suitability of new toxicological approaches *i.e.* the ToxTracker reporter stem cells lines, for rapid mechanism-based genotoxicity screening of a panel of metal oxide nanoparticles (Paper I);
- to investigate the size- and coating-dependent acute toxicity of a panel of well-characterized Ag nanoparticles in a human lung cell model (BEAS-2B) and to correlate their toxicity with parameters such as nanoparticle uptake, nanoparticle agglomeration/sedimentation as well as Ag release (Paper II);
- to investigate the low-dose effects following chronic exposure of human lung cells (BEAS-2B) to Ag nanoparticles using conventional as well as systems toxicology approaches (Paper III);
- to explore the effects of antioxidant CeO_2 nanoparticles on neural stem cells with a focus on neuroprotection and neuronal differentiation using traditional toxicological assays combined with next-generation sequencing (Paper IV);

3 METHODOLOGICAL CONSIDERATIONS

In this thesis several methods and *in vitro* models were used in order to investigate the toxicity of nanoparticles as well as to correlate some of the observed effects with their physico-chemical properties. This section provides an overview of these methods and models with an emphasis on their advantages as well as their limitations. Detailed technical information is presented in the Materials and Methods section of the appended articles.

3.1 NANOMATERIALS

The metal and metal oxide nanomaterials used in this thesis are summarized in Table 1.

Table 1. Overview of the metal and metal oxide nanoparticles investigated throughout this thesis

Nanoparticle	Primary particle size (nm)	Coating	Form	Study	References
CuO	20 – 40	-	powder	I	(Karlsson *et al.*, 2008)
Fe_3O_4	20 – 40	-	powder	I	
ZnO	20 – 200	-	powder	I	
TiO_2	20 – 100	-	powder	I	
NiO	2 – 70	-	powder	I	(Kain *et al.*, 2012)
Ag	10	citrate	1 mg/mL dispersion	I, II, III	(Gliga *et al.*, 2014)
	10 *	PVP	1 mg/mL dispersion	II	
	40	citrate	1 mg/mL dispersion	I, II	
	75 *	citrate	1 mg/mL dispersion	II, III	
	40 – 50	-	powder	II	
CeO_2	4 – 30	-	powder	I	(Kain *et al.*, 2012)
	6	-	powder	IV	(Celardo *et al.*, 2011)
Sm- CeO_2	13	-	powder	IV	

* OECD reference material; primary particle size was estimated by transmission electron microscopy in the corresponding references.

3.2 CELL MODELS

Mouse embryonic reporter stem cells were used as a cell model in Paper I. Using mouse embryonic stem cells (mES) is advantageous as they are untransformed, have an indefinite potential for cell division, are proficient in relevant DNA damage response pathways and sensitive to DNA damage as well as oxidative stress (Hendriks *et al.*, 2012). The reporter stem cells were developed for selected biomarker genes using green fluorescent protein (GFP)-

tagged fusion proteins located on bacterial artificial chromosomes, in this way preserving the physiological gene promoter and most of the regulatory elements (Hendriks *et al.*, 2012). Three reporter stem cells were used: Bscl2-GFP which reports on direct DNA damage associated with stalled replication forks, Srxn1-GFP which reports on Nrf2 dependent oxidative stress and Btg2-GFP which is activated by p53 dependent cellular stress. These reporters were validated for a panel of genotoxic chemicals (Hendriks *et al.*, 2012).

The *human lung bronchial cell line,* BEAS-2B, was used in Paper II and III. BEAS-2B are bronchial epithelial cells isolated upon autopsy from healthy individual and immortalized by infection with the adenovirus 12-SV40 (Reddel *et al.*, 1989). BEAS-2B are a transformed but non-tumorigenic cell line that upon injection into nude mice does not form tumors (Reddel *et al.*, 1989). The cells are recommended to be cultured in serum free medium enriched with growth factors, because the presence of serum can induce squamous cell differentiation. BEAS-2B cells are suitable for cell transformation studies induced by heavy metals (Park *et al.*, 2015, Sun *et al*., 2011). In addition, BEAS-2B cells express the CD14 receptor and respond to lipopolysaccharides (LPS) by secreting cytokines (Verspohl and Podlogar, 2012), thus they can be useful for studying the innate immunity of bronchial cells. The cell culture media was shown to influence the cytokine secretion in response to LPS, metals and soil particles (Veranth *et al.*, 2008). In addition, cells grown in 5% serum conditions had a dramatically altered phenotype and were more sensitive to arsenic induced cytotoxicity as compared to serum free conditions (Zhao and Klimecki, 2015). A possible explanation could be given by the large number of common genes affected by both arsenic and FBS, which could result in a synergistic effect (Zhao and Klimecki, 2015). For particle research, the presence of serum in the culture media can additionally alter the toxic response due to the formation of the protein corona that in turn can affect the particle stability in cell media as well as the particle uptake. In the current projects BEAS-2B cells were grown on pre-coated dishes (collagen, fibronectin, albumin), in serum free conditions, supplemented with growth factors, as recommended by the manufacturer.

In Paper IV we used the *mouse embryonic stem cell line, C17.2,* as a cell model. C17.2 cells are a multipotent neuronal progenitor cell line initially isolated from mouse cerebellum and immortalized by transfection with avian myelocytomatosis viral-related oncogene (v-myc) (Snyder *et al.*, 1992). C17.2 cells can differentiate into a mixed culture of neurons and astrocytes and are suggested to be a good model for neurotoxicity testing (Lundqvist *et al.*, 2013). C17.2 cells can be grown in a proliferating state or they can be induced to differentiate by serum deprivation and N2 supplementation, with or without the addition of neuronal growth factors. In addition to C17.2 cells, some of the results were corroborated in human neural stem cells (hNPC). Neural stem cells are present during neuronal development but are also found in adult brains in stem cells niches, therefore, this model is relevant both from a developmental toxicology perspective as well as for neurotoxicity targeting the adult brain (Tofighi *et al.*, 2011).

3.3 NANOMATERIAL CHARACTERIZATION

A thorough nanomaterial characterization is critical for nanotoxicology studies and it can allow for correlations to be made between properties such as shape, size, agglomeration, purity and toxicological outcome. Unfortunately, not all studies report on particle properties, which makes it difficult to relate studies with each other. In this thesis an emphasis was placed on particle characterization as well as particle cellular uptake. The following section will shed light on some of the methods used to assess primary particle size distribution, agglomeration and stability in biological media as well as particle uptake.

3.3.1 Particle size distribution

Primary particle size is the size of the nanomaterials following their synthesis and before they are introduced in biological media. Some particles come in powder form or in concentrated stock dispersions. One of the methods used to estimate particle size in this thesis was by transmission electron microscopy (TEM). TEM is a two-dimensional high-resolution technique that can give reliable information of the particle size, shape and surface characteristics (Powers *et al.*, 2012). A determining factor for this analysis is making sure that the sample is representative and that enough particles have been considered for the analysis. In Paper II we used TEM to confirm the primary size of Ag nanoparticles.

Particle size distribution in cell media gives indications on particle agglomeration/aggregation as well as sedimentation and is commonly evaluated by dynamic light scattering (DLS) techniques. DLS is based on photon correlation spectroscopy and implies illuminating the sample with a laser and measuring the intensity of the scattered light, which is proportional to the particle diameter, that in turn is correlated with the hydrodynamic size of the particles (Fissan *et al.*, 2014). DLS is a straight-forward measurement to perform but has limitations when it comes to poly-dispersed suspensions where larger particles screen out smaller ones, and is recommended to be accompanied by other techniques such as TEM, which are more reliable (Tomaszewska *et al.*, 2013). In addition, in order to avoid multiple scattering, the DLS work should be performed on diluted samples. Some of these limitations (working with highly diluted and monodispersed suspensions) are overcome by the use of photon cross correlation spectroscopy (PCCS). PCCS employs a split in the incident laser beam into two beams that hit the sample at different angles, with the subsequent collection of the scattered lights by two detectors, followed by cross-correlation of the light intensities, and with the exclusion of the multiple scatter that does not cross-correlate (Xu, 2008). In addition to DLS techniques, one can use UV-Vis spectroscopy to characterize particle size distribution. Nanoparticles have distinct optical properties that change with *e.g.* size, shape, agglomeration and sedimentation, that can be easily acquired by UV-Vis spectroscopy (Tomaszewska *et al.*, 2013). The UV-Vis technique has however similar limitations with the DLS technique in the sense that it is difficult to pick apart small particles from larger particles in poly-disperse suspensions (Tomaszewska *et al.*, 2013). In Paper I and II we used a PCCS technique to characterize the particle size distribution and sedimentation over time in cell media whereas in Paper IV we used the

classical DLS approach. In addition, in Paper II the PCCS technique was complemented with UV-Vis spectroscopy.

3.3.2 Particle dissolution in cell media

The quantification of metal release from metal and metal oxide nanoparticles in cell media is important as it gives indications about the particle stability and the potential contribution of the released ions to the biological effects. In addition to cell media, particle dissolution can be tested in other physiologically relevant environments *e.g.* artificial lysosomal fluid (Paper II). Several methods were employed for this purpose: atomic absorption spectroscopy (AAS), inductively coupled plasma mass spectrometry (ICP-MS), inductively coupled plasma with optical emission spectroscopy (ICP-OES).

The graphite furnace (GF)-AAS is technically superior than the classical flame AAS and it has improved sensitivity, however, it has a limited dynamic range and potential matrix effects from *e.g.* analyte retention on the graphite tube (Tyler, 2005). ICP-OES has better detection limits than GF-AAS but it is prone to spectral interferences, whereas ICP-MS has the best detection limits, a high linear dynamic range and allows for analysis of isotopes (Tyler, 2005). ICP-MS has known spectral interferences than can be accounted for, and, improved technologies using collision with *e.g*. He, can further minimize these interferences (Koppenaal *et al.*, 2004). ICP-MS techniques are prone to matrix effects that can be evaluated and overcome by matrix matching (preparation of the calibration standards in the same solution as the samples) and addition of an internal standard (*e.g.* In, Rh). Prior to the analysis, sample digestion is required for all mentioned techniques. Sample digestion is usually performed in acids (*e.g.* HNO_3) and can be complemented with microwave or UV treatments. The ultimate goal is to ensure that all the organic molecules are mineralized and all the metal nanoparticles are dissolved. Sample digestion should be optimized for each type of nanoparticle and matrix. Ag might pose problems as an analyte due to the presence of Cl^- in the cell media matrix that enables the formation of insoluble AgCl that can precipitate and bind to the plastic tubes. For Ag analysis, addition of HCl will ensure the formation of soluble $AgCl_2^-$ complexes that allow for a good analyte recovery. The same techniques can be used for analysis of cellular uptake that will be discussed below.

In Paper I we used both AAS and ICP-OES techniques to determine the metal release in cell medium. In Paper II we used GF-AAS to determine the Ag release in cell medium over time and the cellular uptake. In Paper III and IV we used ICP-MS to evaluate the cellular uptake of nanoparticles.

3.4 CELLULAR UPTAKE OF NANOMATERIALS

Transmission electron microscopy (TEM) is a well-established technique widely used to visualize the intracellular localization of nanomaterials. When using metal and metal oxide nanoparticles which have a high electronic density there is no need for heavy metal stains as the contrast is high enough between the nanoparticles and the cellular structures (Brown *et al.*, 2014). This approach allows for qualitative evaluation of the localization of the nanoparticles

inside the cells as well as intracellular particle agglomeration/aggregation, and to some extent can be used for quantitative purposes (Belade *et al.*, 2012). It should be noted that the TEM sections have a width of 60 – 80 nm which implies that for larger particles artefacts can form during the cutting procedure, *e.g.* particles can smear and slide to other parts of the section. Intracellular uptake by TEM was evaluated in all papers appended to this thesis.

Quantification of the cellular uptake was performed by the GF-AAS (Paper I, II) and by ICP-MS (Paper III and IV). Cell washing prior to harvesting is a critical factor which ensures that most of the nanoparticles attached to the surface of the cells are washed away and will not skew the analysis. Another important aspect that has to be optimized is sample digestion. To note that following cell mineralization, the analysis provides information on the total metal content and cannot distinguish between metal nanoparticle and metal ions. Several techniques have been developed to separate Ag nanoparticles and ions that use Triton-X 114-based cloud point extraction (Yu *et al.*, 2013) or magnetic separation (Mwilu *et al.*, 2014).

3.5 CELL VIABILITY ASSAYS

Lactate-dehydrogenase (LDH) assay detects the presence of LDH in the extracellular environment that leaks upon membrane damage that occurs in necrotic and late apoptotic cells. LDH is a cytosolic enzyme that is kept inside the cells when the cell membrane is intact. The enzymatic activity of LDH can be detected via a colorimetric assay in which the enzyme catalyzes an oxidative reaction that results in the formation of a red formazan compound; the half-life of the enzyme in the extracellular environment is estimated to be approx. 9 hours (Promega, 2016). The enzyme activity is usually determined in the supernatant but can be determined in the cells as well (upon lysis) to account for the total enzyme. Since it involves an enzymatic reaction this assay is prone to interference with the tested nanomaterials by oxidation or adsorbtion of the substrate, inactivation of the enzyme activity, nanomaterial absorbance in a similar wavelength (Han *et al.*, 2011). The LDH assay was used in Paper II to assess the toxicity of Ag nanoparticles.

Alamar blue assay is a cytotoxicity method that detects the metabolic activity of cells (Lancaster, 1996). Healthy cells maintain a reducing environment that can convert resorufin (blue, no fluorescence) to resazurin (red, fluorescent); the detection can be either by fluorescence or absorbtion, with a higher sensitivity for the fluorescence detection (Lancaster, 1996). The metabolic activity of the cells is proportional to the health status as well as to the cell number, therefore Alamar blue can also be used as a proliferation assay. The principle is similar to the traditional tetrazolium dye (MTT) assay but it requires fewer steps and it maintains cell viability as it does not require cell lysis. Nanomaterials can interfere with the detection method and evaluation of this interference should be performed on a case by case scenario. For Ag nanoparticles this method was deemed to have low interference rates (Samberg *et al.*, 2010). The Alamar blue assay was used in all studies from this thesis.

Cell-IQ assay. The Cell-IQ is an automated live cell imaging platform (Chip-man Technologies) that can be used to acquire images over a wide time-frame, while the cells are

placed in an incubator with temperature and CO_2 control; the images are then scored based on their morphology. The Cell-IQ assay was found suitable to assess the toxicity of carbon nanotubes (Meindl *et al.*, 2013). In Paper IV we scored the cells in terms of dead cells, flat cells as well as mitotic cells and cell viability/cell death was deducted. This assay has the advantage of allowing for a time-dependent cell viability assessment. In Paper IV we used the Cell-IQ assay to evaluate the neuroprotective effects of nanoceria in the presence of an oxidative stress insult.

3.6 EVALUATION OF ROS GENERATION

For the evaluation of intracellular ROS generation the dichloro-dihydro-fluorescein diacetate (DCFH-DA) assay was used. DCFH-DA is a lipophilic cell permeable compound that is de-acetylated to $DCFH_2$ and then converted to DCFH anion that can further be oxidized by ROS to DCF which is a fluorescent molecule that can be detected via fluorimetric techniques (flow, cytometry, microscopy, plate reader). DCFH reacts with hydroxyl, peroxyl, alkoxyl and carbonate radicals but to a lower extent with hydrogen peroxide (Kalyanaraman *et al.*, 2012). Karlsson and colleagues argue that the DCFH-DA assay can reflect the lysosomal and mitochondrial membrane integrity since the dye is not able to diffuse through the organelle membrane; upon rupture/permeabilization, redox-active ions and cytochrome *c* are released into the cytoplasm and are available to interact with the DCFH probe (Karlsson *et al.*, 2010). In addition, during apoptosis, cytochrome *c* is released from the mitochondria (Karlsson *et al.*, 2010) and might interfere with the assay if nanoparticles are tested at cytotoxic concentrations. The intracellular ROS generation was evaluated by DCFH-DA assay in Paper II and IV.

In addition to the intracellular evaluation of ROS, this assay can be adjusted to detect ROS production under acellular conditions. As a result of their intrinsic surface reactivity, nanoparticles can oxidize the $DCFH_2$ substrate (following chemical de-acetylation of the DCFH-DA probe). Acellular generation of ROS was evaluated in Paper I.

3.7 GENOTOXICITY ASSESSMENT

3.7.1 The comet assay

The comet assay, or single cell gel electrophoresis, is a well-established method for quantification of DNA damage at a single cell level. Single cells are embedded in agarose and lysed, followed by DNA denaturation and electrophoresis. DNA fragments (negatively charged) from individual cells will migrate upon electrophoresis towards the positively charged anode and will subsequently appear as 'comets' upon visualization with fluorescent dyes. The quantification of the comets is commonly performed using a specific software by scoring at least 50 cells per gel. The results can be expressed using the tail lengths, % DNA in tail as well as the tail moment. Depending of the working pH, different classes of DNA damage can be detected as follows: in neutral conditions mainly double strand breaks, whereas under alkaline (pH>13) the comet assay can detect double- and single-strand breaks as well as alkali labile sites (oxidized bases, alkylated sites, intermediates in base excision repair) (Tice *et al.*, 2000). A variant of the comet assay, employing formamidopyrimidine DNA glycosylase (FPG) can

be used to detect 8oxo guanine, a marker of oxidative DNA damage (Collins, 2004). The sensitivity of the comet assay is very high, and it can detect as low as 50 strand breaks per diploid mammalian cell (Olive and Banath, 2006). It should be noted that under cytotoxic conditions, apoptotic and necrotic cells can appear as 'comets' and confound the results (Tice *et al.*, 2000). In addition, nanoparticles might interfere with the comet assay, for example by interacting with the FPG enzyme (Kain *et al.*, 2012). The *in vivo* mammalian comet assay is part of the OECD guidelines (Test No. 489) for testing chemicals since 2014. The alkaline comet assay was used in Paper I – III, while the neutral and the FPG comet assay were additionally employed in Paper I.

3.7.2 The micronucleus assay

The micronucleus assay is a well-established assay to evaluate cytogenetic damage upon exposure to toxicants. Micronuclei are formed when whole chromosomes or acentric chromosomes fail to be included in the daughter nuclei, because they are unable to attach to the mitotic spindle and migrate towards the poles in anaphase (Fenech *et al.*, 2011). The mechanisms behind micronuclei formation can consist of mis-repaired or unrepaired double strand breaks, defects in the kinetochore assembly, dysfunctional spindle or unresolved replication stress (Fenech *et al.*, 2011). The cytokinesis-block micronucleus assay uses cytochalasin-B that inhibits cytokinesis and makes it possible to distinguish the cells that have undergone cell division as they will appear binucleated (Fenech, 2007). In this way the scoring can be performed only on binucleated cells and reduce the confounding effects of impaired kinetics of the cell division, thus restricting the scoring to the cells that were damaged during the cell exposure (Fenech, 2007). The micronucleus assay is part of the OECD guidelines for *in vivo* setups, Test No. 474: Mammalian Erythrocyte Micronucleus Test, and for *in vitro,* Test No. 487: *In Vitro* Mammalian Cell Micronucleus Test. Gonzalez and colleagues have reviewed the literature on the use of micronucleus assay for evaluating the genotoxicity of nanoparticles and have put forward some critical aspects such as: (i) cytochalasin treatment might interfere with the nanoparticle uptake and (ii) the necessity for cells to undergo mitosis, that would allow for closer contact between nanoparticles and chromatin, especially for the particles that do not cross the nuclear membrane (Gonzalez *et al.*, 2011). In Paper III we used the flow cytometry version of the micronucleus assay which was previously validated against the 'cytome' microscopy version for genotoxicity testing of TiO_2 nanoparticles (Di Bucchianico *et al.*, 2016). This flow cytometry version of the micronucleus assay allows for concomitant determination of cell cycle and cell viability.

3.7.3 γH$_2$AX and RAD51 foci formation

Double strand breaks are the most deleterious type of DNA damage that if not repaired properly or in time can lead to genomic instability (Mah *et al.*, 2010). Upon DNA double strand break induction, the histone variant H_2AX is phosphorylated at the Ser-139 residue to form γH_2AX foci that are involved in signaling and initiation of DNA repair (Mah *et al.*, 2010). In addition, in mES, γH_2AX can also occur at sites of single strand breaks as well as sites of chromatin relaxation (Banath *et al.*, 2009). γH_2AX was assessed in Paper I and II by fluorescence

microscopy. RAD51, another marker of double strand breaks, is a protein which has a critical role in DNA repair by homologous recombination (Daboussi *et al.*, 2002). The formation of RAD51 foci was visualized by fluorescence microscopy in Paper I.

3.7.4 The ToxTracker assay

The ToxTracker assay (see Section 3.2 for details on the cell model) is a reporter cell system that was initially developed to screen and give mechanistic insight into the genotoxicity of chemicals (Hendriks *et al.*, 2012). The assay can distinguish between different mechanisms of DNA damage *i.e.* stalled replication forks, oxidative stress and p53 dependent cellular stress, and allows for concurrent assessment of cytotoxicity. Another advantage is the increased throughput that allows for fast screening of several compounds at the same time. In Paper I we evaluated the suitability of the ToxTracker assay to test the genotoxicity of metal and metal oxide nanoparticles.

3.8 FLOW CYTOMETRY

Flow cytometry allows for performing multiple measurements at a single cell levels by using a fluidics system that collects and evaluates single cells. Flow cytometry employs a laser beam that collides with the cells followed by light scattering that is collected along the same axis (forward scatter, gives information on the circumference of the cells) or at a 90° angle (side scatter, gives information on the intracellular structure of the cells) (Bakke, 2001). If fluorescent probes are used, they will be excited by the laser light and will give off a fluorescent signal that is recorded by a detector (Bakke, 2001).

In nanotoxicology, flow cytometry can give indications on the nanoparticle uptake, which is correlated with an increase in intracellular granularity, that in turn results in shift in the side scattered light (Suzuki *et al.*, 2007). In Paper I, flow cytometry was used to evaluate the GFP expression from the reporter stem cells; the evaluation was performed only on the gate corresponding to viable cells. In Paper III we used flow cytometry to evaluate the surface expression of E-cadherin and N-cadherin. The advantage of using flow cytometry in this context was the possibility to identify the percentage of cells which expressed these surface markers, thereby identifying distinct phenotypes. Moreover, in Paper III we used the flow cytometry version of the micronucleus assay to identify the potential of Ag nanoparticles to induce micronuclei or hypodiploid nuclei after long-term exposure. The flow cytometry version of the micronucleus assay has the advantages of being fast and medium throughput, however, as compared to the 'cytome' version it cannot identify the formation of nuclear buds and nucleoplasmic bridges, which are a measure of DNA amplification and chromosomal rearrangements, respectively (Nelson *et al.*, 2016, Fenech *et al.*, 2011).

3.9 ASSESSMENT OF CANCER-LIKE PHENOTYPES

In Paper III we used cell migration, cell invasion and soft agar cell transformation assay in order to validate the RNA-Seq findings. For the migration and invasion assays we used a transwell approach. Briefly, cells are seeded on top of transwells with 8 µm pores, which are

either coated with the relevant coating for BEAS-2B cells (for the migration assay) or covered with a layer of matrigel (invasion assay). Cells are seeded in supplement free medium on the apical side of the transwell, while medium with supplements is added to the basal side, thus creating a nutrient/growth factor gradient. Cells with cancer-like phenotype will migrate and invade the transwell and attach to its bottom side. The migrating/invading cells can be stained and counted at the end of the experiment. The results can be expressed as the number of invading and migrating cells or by calculating the 'invasion index' (ratio invading/migrating cells) that indicates the specific contribution of cell invasion which is a relevant marker of metastasis (Albini and Benelli, 2007).

The soft agar cell transformation is a well-established assay used to test the potential of cells to escape anoikis and form colonies in a 3D environment which is informative of carcinogenic cell transformation (Borowicz *et al.*, 2014). Single cells are seeded in 0.3-0.5%, agar (37°C) and kept in the incubator for 2-3 weeks followed by evaluation of the number (and/or size) of the colonies. In addition, colonies can be recovered in order to establish treatment-related transformed cell lines (Sun *et al.*, 2011) in this way performing a phenotypic selection and reducing the effect of cell culture heterogeneity. In Paper III we resumed at counting the transformed colonies.

Cell migration, cell invasion and soft agar cell transformation assay have been previously used to assess cancer-like phenotype induced by nanomaterials *e.g.* SWCNTs (Luanpitpong *et al.*, 2014), MWCNTs (Vales *et al.*, 2016) and TiO_2 nanoparticles (Vales *et al.*, 2015).

3.10 EVALUATION OF NEURONAL DIFFERENTIATION

In Paper IV we investigated the neuronal differentiation of C17.2 cells and human neural progenitor cells (hNPC). Both C17.2 and hNPC cells undergo differentiation following serum deprivation and addition of N2 supplement (transferrin, insulin, progesterone, putrescine, selenite). The evaluation of neuronal differentiation was performed after 7 days for C17.2 cells and after 4 days for hNPC and was based on a conventional immunofluorescence technique using β3-tubulin as a neuronal marker. Fluorescence images were taken using at least 6 fields per slide and scored thereafter. The scoring was based on staining intensity as well as neuronal morphology and the results were expressed as percentage neuronal differentiation as compared to the total number of cells (evident from the 4',6-diamidino-2-phenylindole - DAPI nuclear staining).

3.11 CYTOKINE ANALYSIS

In Paper III, the cytokine secretion was evaluated using a multiplex assay (Luminex – BioRad) that allows for detection of multiple analytes in one sample, thus providing a comprehensive picture of the cytokine profile. The assay is based on a capture sandwich immunoassay with capture antibodies coupled with fluorescently dyed magnetic beads and biotinylated detection antibodies, which can allow for detection by streptavidin-phycoerythrin (de Jager *et al.*, 2003). The detection is performed with a flow-cytometer which can quantify the fluorescence

corresponding to each bead color, which is in turn specific for each analyte. The following cytokines were analyzed: IL-1β, IL-1ra, IL-6, IL-8, IL-10, TNF-α and MIP-1α.

3.12 OMICS APPROACHES

3.12.1 RNA-Sequencing

RNA-seq is a novel technology that allows for identification and robust quantification of RNA transcripts in biological samples. In contrast to previous transcriptomic techniques such as microarray, RNA-Seq provides a resolution at single base level, low background noise, high dynamic range (>8000 fold) and is able to distinguish different isoforms and allelic expression (Wang *et al.*, 2009). The main steps in an RNA-Seq experiment are discussed below and illustrated in Figure 12 (Illumina, 2016).

A. cDNA library construction
B. cluster amplification
C. sequencing by synthesis (for the Illumina platform)
D. mapping and estimation of the abundance for each gene

Briefly, the library construction starts with the reversed transcription of the RNAs to cDNA and subsequent double strand (ds) cDNA synthesis; next, adaptors (oligonucleotide sequences) specific for each library will be ligated to both ends of the ds cDNA, followed by polymerase chain reaction amplification of the library using adaptor sequences as primers; the final step is the quality control, normalization and pooling of the libraries (Korpelainen, 2014).

After library construction, the ds cDNA is hybridized on a flow cell based on the complementarity with adaptor sequences resulting in the formation of so-called ‘bridges’ that will be further amplified to generate clusters. Next, one strand from the ds cDNA clusters is removed to enable the sequencing *per se*. The Illumina platform employs the sequencing by synthesis approach which implies a series of additions of fluorescently labelled nucleotides and imaging of the fluorescence signal (Illumina, 2016). The sequencing can be performed either at one end of the cDNA (single read mode) or from both ends (paired-end read mode). Using paired-end read modes can make the analysis more robust and reduce potential biases from the library preparation process (Korpelainen, 2014). Following sequencing, the transcripts are mapped to a reference genome (if that is available) and the expression level of a gene is determined by the number of reads that are mapped to it by alignment (which are named ‘counts’). RNA-Seq was performed in Paper III and IV.

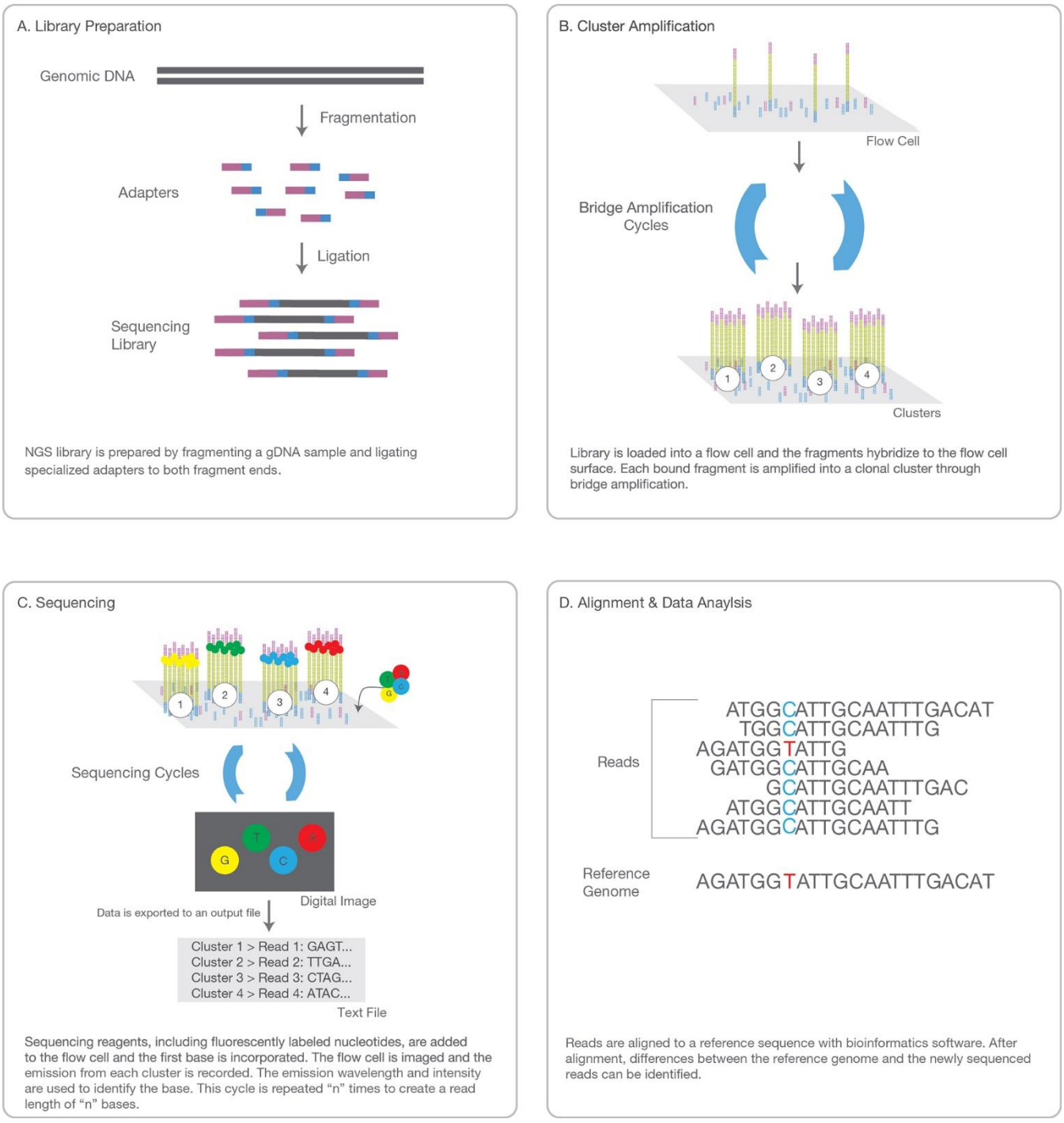

Figure 12. Next-generation sequencing overview. Reproduced from (Illumina, 2016) with permission from Illumina.

3.12.2 DNA methylation array

In Paper III we investigated the genome-wide changes in DNA methylation array using the Infinium Human Methylation450 Bead chip. The Illumina Infinium assay is a multiplex technology based on bisulfite conversion of genomic DNA; upon bisulfite treatment unmethylated cytosines are converted to uracil, which changes their base-pair binding, whereas methylated cytosines are protected from conversion (Illumina, 2012). Following conversion, each locus is tested by using two probes, one against methylated and the other against unmethylated cytosines; the relative methylation (β value) is calculated as the ratio of signal for the methylated probe against the total signal intensity for that locus (Illumina, 2012). The Illumina Human Methylation450 is a widely used assay for epigenome-wide association studies with probes covering 99% of the reference sequence genes (Morris and Beck, 2015). Some of the issues with this technology could be the batch effect and the incomplete bisulfite conversion, which is common to all bisulfite-based methods (Dedeurwaerder *et al.*, 2011). The differential methylation analysis was performed using RnBeads package on Bioconductor, which is a well-established tool for DNA methylation analysis (Assenov *et al.*, 2014).

3.12.3 Bioinformatics analysis of 'omics' data

'Omics' experiments generate immense amounts of data that require further analyses to extract significant differences between the experimental conditions, as well as to put those observations into a biological context. For the RNA-Seq experiments, after identification of the abundance of gene expression, the next step is to identify the genes that are significantly differentially expressed between the samples. In toxicology, a common comparison is done between the treated and untreated samples. There are several methods to perform the differential gene expression analysis, most of which are available as packages in the Bioconductor project (Gentleman *et al.*, 2004). In this thesis we used the DESeq2 package for Paper III and the limma package for Paper IV. The DESeq2 package uses counts as input data and a negative binomial approach (Love *et al.*, 2014), whereas limma uses a linear model for which the count data needs to be transformed to continuous values (Ritchie *et al.*, 2015).

Next, the differentially expressed genes need to be put into the biological context by performing a set of downstream analyses. In Paper III and IV we used Ingenuity Pathway analysis software to perform canonical pathway analysis as well as network analysis to identify the biological pathways and networks that were significantly altered in the dataset. For certain pathways, activity scores are available that indicate whether the pathway is activated or inhibited by the treatment of interest. Gene ontology (GO) enrichment is another type of downstream analysis that identifies significantly enriched ontologies for three domains, namely, biological process, cellular component and molecular function. In Paper IV we used the online tool GOEast which employs a Fischer exact test and an improved weighted scoring algorithm (Zheng and Wang, 2008) and has the advantage that it enriches the lower and most biologically specific hierarchical levels of the GO tree, thereby aiding interpretations of the results. These analyses are valuable for describing the data in an unbiased way and set the stage for hypothesis generation which can take into account previous knowledge on the topic. However, generating

a new hypothesis is merely the first step, and experimental validation of the RNA-Seq data critical for drawing sound conclusions. Experimental validation can be performed either at the protein level or at the functional level. The correlation between mRNA and protein levels was reported to be as low as 40% (Vogel and Marcotte, 2012). A suggested explanation is that transcription acts more like an on-off switch, whereas post-transcriptional and translational events together with the regulation of protein degradation finely-tune the process, thereby playing an important role in controlling the protein levels (Vogel and Marcotte, 2012). These new hypotheses should be further tested experimentally. In Paper III our focus was carcinogenicity and we therefore selected relevant pathways such as *Hepatic fibrosis* and *Regulation of epithelial-mesenchymal transition pathway*. In addition, the *Acute phase response signaling pathway* was also selected for further experimental validation. In Paper IV, the RNA-Seq data confirmed the reduction of β3-tubulin that was previously observed experimentally, but also revealed gene expression alteration of additional genes from the neuronal differentiation network. Moreover, the RNA-Seq aided in the generation of a new hypothesis based on the interaction with the cytoskeletal organization, that was further validated experimentally.

3.13 SUPER-RESOLUTION MICROSCOPY TECHNIQUES

In Paper IV we used two super-resolution microscopy techniques, namely, structured illumination microscopy (SIM) and stimulated emission depletion (STED) microscopy to visualize the structure of the neuronal growth cones. STED microscopy uses selected stimulated depletion of fluorescence which allows the fluorescence to take place within a nanoscale area of the sample, resulting in much sharper images with a maximum resolution of 20 to 50 nm (Blom and Brismar, 2014). STED is a useful tool in cellular imaging and its uses are expected to increase in the future (Blom and Widengren, 2014). An advantage of STED imaging is that there is no need for image processing, even though that can be done to enhance the contrast (Blom and Brismar, 2014). In SIM microscopy the sample is illuminated in a series of sinusoidal stripped patterns which when encounter fine structures in the sample, lead to the formation of interference patterns that are acquired and further mathematically processed to give a high-resolution image (Schermelleh *et al.*, 2010). In contrast to STED, which can require special dyes, in SIM microscopy conventional dyes can be used (Schermelleh *et al.*, 2010).

4 RESULTS

4.1 PAPER I: REPORTER STEM CELLS CAN PROVIDE RAPID MECHANISTIC INSIGHT INTO THE TOXICITY OF METAL OXIDE NANOPARTICLES

The rapid increase in the manufacture and use of nanomaterials stimulates the development of tools for rapid toxicity screening. ToxTracker is one such tool, that consists of a panel of GFP-tagged mouse embryonic stem cells that report on different pathways of toxicity and that was previously developed to screen for the toxicity of chemicals (Hendriks *et al.*, 2012). The panel consists of three cell lines: Bscl2-GFP (reports on direct DNA damage associated with stalled replication forks), Srxn1-GFP (reports on Nrf2 dependent oxidative stress) and Btg2-GFP (activated by p53 dependent cellular stress). The different GFP reporters are combined in one assay and the readout is performed by flow cytometry (Figure 13).

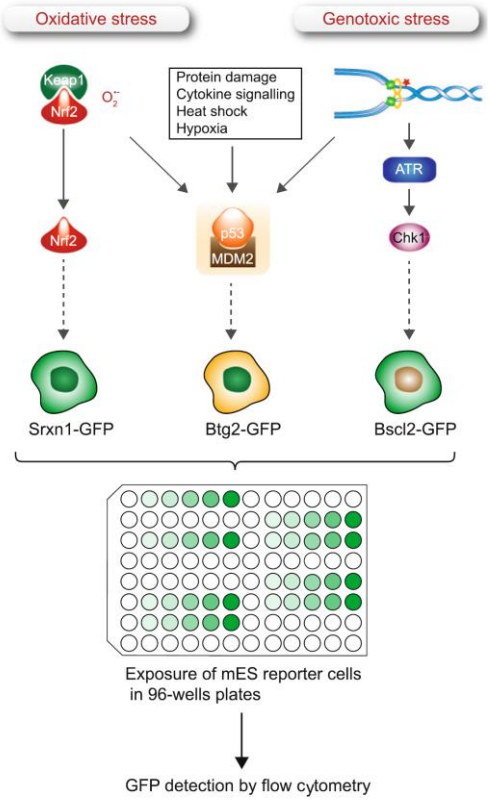

Figure 13. The ToxTracker tool for rapid genotoxicity screening of chemicals and nanomaterials. Reproduced from Karlsson et al., 2014 (Paper I).

The aim of this study was to test whether ToxTracker assay can be used to screen for genotoxicity of a panel of well-characterized metal (Ag) and metal oxide (CuO, ZnO, NiO, CeO_2, Fe_3O_4, TiO_2) nanoparticles. In addition, we used quartz material DQ12 as an insoluble benchmark particle, diesel particles (standard reference material SRM1650b) and MWCNTs.

First we performed a comprehensive characterization of the nanomaterials which included size distribution measurements, metal release in cell medium, acellular ROS and particle cellular uptake. Our results indicated that all particles were taken up by the mouse stem cells and

dissolved to various extents in cell medium. ZnO and CuO dissolved to the highest extent in cell medium whereas NiO and CuO nanoparticles were the most efficient in generating ROS under acellular conditions.

Next we used the ToxTracker panel to test a range of doses for each nanoparticle following 24 hours of exposure. Our results indicated that Srxn1, the oxidative stress reporter, was activated by CuO and NiO nanoparticles whereas the Btg2, the p53 reporter, was activated only by NiO. None of the nanoparticles activated the Bscl2, the DNA replication stress reporter. For ZnO nanoparticles the activation of Srxn1 occurred at highly toxic doses which deemed the results inconclusive. We then proceeded to validate the ToxTracker response with conventional DNA damage assays (FPG-comet assay, RAD51 and γH_2AX foci induction). The results indicated that CuO nanoparticles predominantly induced DNA damage in the form of single strand breaks and oxidative DNA lesions, whereas NiO nanoparticles predominantly induced single strand breaks, in line with the ToxTracker observations.

Our follow-up aim was to test to which extent the released metal ions played a role in the observed ToxTracker effects. We therefore tested a series of metal salts corresponding to the nanoparticles that had a positive effect (CuO, NiO, ZnO). The results indicated that, for CuO nanoparticles the activation of Srxn1 was mainly an ionic effect (due to the extracellular release of toxic Cu ions), whereas for NiO nanoparticles the Srxn1 and Btg2 activation was a particle effect. The results for ZnO were again inconclusive due to the high toxicity.

Finally, in order to benchmark the ToxTracker response we investigated the effect of quartz particles, diesel reference material and MWCNTs. Exposure to quartz activated the oxidative stress reporter, Srxn1, in line with previous findings (Schins *et al.*, 2002). However, neither diesel particles nor MWCNTs activated the ToxTracker reporters.

In summary, we demonstrated that the ToxTracker assay is a suitable medium/high-throughput tool for genotoxicity screening of metal oxide nanoparticles. In addition, the ToxTracker assay provided mechanistic information on the modes of genotoxicity: the DNA damage induced by CuO and NiO was mainly related to oxidative stress and not direct DNA interaction. Moreover, NiO also induced p53 activation suggesting additional reactivity and cellular stress. The response to CuO nanoparticles was mediated by the extracellularly released ions, whereas for the NiO nanoparticles the toxic effect was particle-mediated. Finally, the suitability of the ToxTracker assay for carbon-based nanomaterials requires further investigation.

4.2 PAPER II. SILVER NANOPARTICLES INDUCE A SIZE-DEPENDENT CYTOTOXICITY FOLLOWING SHORT-TERM EXPOSURE OF BRONCHIAL EPITHELIAL CELLS

Ag nanoparticles are currently one of the most manufactured and used nanomaterials. At the time this study was conducted (2013-2014) there were already a multitude of toxicological studies on Ag nanoparticles. However, these studies reported contradicting results and the issue of size-dependent toxicity hadn't been resolved. The reasons behind the lack of consistency between the studies could be attributed to the different cell models and nanoparticles (size, shape, coating) used, different particle purity, absence of reference materials and in general a lack of thorough particle characterization in cell medium.

In this study the aim was to address the size-dependent toxicity of thoroughly characterized Ag nanoparticles in human lung cells (BEAS-2B). To this end we selected a panel of five Ag nanoparticles with different sizes and coatings out of which two were OECD reference materials: 10 nm OECD PVP-coated, 10 nm citrate-coated, 40 nm citrate-coated, 75 nm OECD citrate-coated and 40-50 nm uncoated.

First we performed a thorough characterization of the nanoparticles both in water and in cell medium in terms of primary size, agglomeration as well as sedimentation by means of TEM, PCCS and UV-Vis spectroscopy. The results indicated that all particles agglomerated with time in cell medium and that the PVP-coated particles were more stable than the citrate-coated particles. Next we investigated acute cytotoxicity following short-term exposure (4 and 24 hours) using two assays, Alamar blue and LDH assay. After 24 hours, only the 10 nm Ag nanoparticles (PVP- and citrate-coated) were cytotoxic in both assays, indicating a clear size-dependent toxicity.

The following endpoint of interest was DNA damage and for that we used non-cytotoxic doses of Ag nanoparticles and performed comet assay as well as imaged γH_2AX foci induction. Our results indicated that all Ag nanoparticles induced DNA damage in the comet assay after 24 hours, independent of size or coating and in the absence of γH_2AX foci. This suggested that that DNA damage was more likely to be in the form of single strand breaks and/or oxidized bases. In addition, the induction of DNA damage was not size-dependent suggesting distinct mechanisms for cytotoxicity and genotoxicity. To test whether oxidative stress could be involved in the observed cytotoxicity and DNA damaged, we performed the DCFH-DA assay and we found that neither of the Ag nanoparticles increased the generation of intracellular ROS.

Next we examined additional possible mechanisms for the observed size-dependent toxicity and proceeded to quantify cellular uptake together with the uptake mechanisms (by AAS), image intracellular localization (by TEM) and Ag released in cell medium (by AAS). Our results indicated that all Ag nanoparticles were taken up to a similar extent by a combination of active uptake mechanisms (clathrin- and caveolin-mediated endocytosis, macropinocytosis) with localization in membrane-bound structures inside the cytoplasm. Moreover, all tested Ag nanoparticles released Ag in the cell medium in a time-dependent way, with the 10 nm citrate- and PVP-coated particles having the highest release after 24 hours. Our next question

was whether this high (approx. 20%) extracellular release of Ag from the 10 nm Ag nanoparticles could explain the observed cytotoxicity. To this end we tested the toxicity of the released fraction and found that the extracellular release of Ag did not play a role in the cytotoxicity of the 10 nm Ag nanoparticles.

In summary, in this study we used a panel of well-characterized Ag nanoparticles to address their size-dependent toxicity in BEAS-2B cells. Our results indicated that the 10 nm Ag nanoparticles were more toxic than their larger counterparts, independent of the coating and at similar intracellular concentrations. We also found evidence that the 10 nm Ag nanoparticles released considerably more Ag in cell medium and we speculated that this pattern could follow inside the cytoplasm as well. Finally, our results are in line with the 'Trojan horse' hypothesis by which the particle form promotes the uptake thereby increasing the intracellular bioavailability of toxic metals.

4.3 PAPER III. LOW-DOSE, LONG-TERM EXPOSURE TO SILVER NANOPARTICLES INDUCES A CANCER-LIKE PHENOTYPE IN BRONCHIAL EPITHELIAL CELLS

While there are a multitude of studies on the acute effects of Ag nanoparticles, there is currently scarce data on their long-term effects both *in vitro* and *in vivo*. Nevertheless, long-term studies are critical for evaluating complex processes such as carcinogenesis which develops over time. To our knowledge, only one *in vitro* study addressed the chronic effects of Ag nanoparticles and found that Ag nanoparticles in the pg/mL range induced a sustained stress response and modified cell functionality following 14-week exposure of the HaCaT keratinocyte cell line (Comfort *et al.*, 2014).

In this study the aim was to address the knowledge gaps related to long-term exposure to Ag nanoparticles, from an inhalational exposure perspective. To this end, we designed a long-term, low-dose, *in vitro* experimental setup where BEAS-2B cells were exposed for 6 weeks to low-doses (1 µg/mL) of well-characterized Ag nanoparticles (10 and 75 nm) (Figure 14). We used a combination of conventional toxicology assays to address endpoints such as *e.g.* cell proliferation, genotoxicity, cell transformation together with 'omics' approaches such as RNA-Seq and genome-wide DNA methylation.

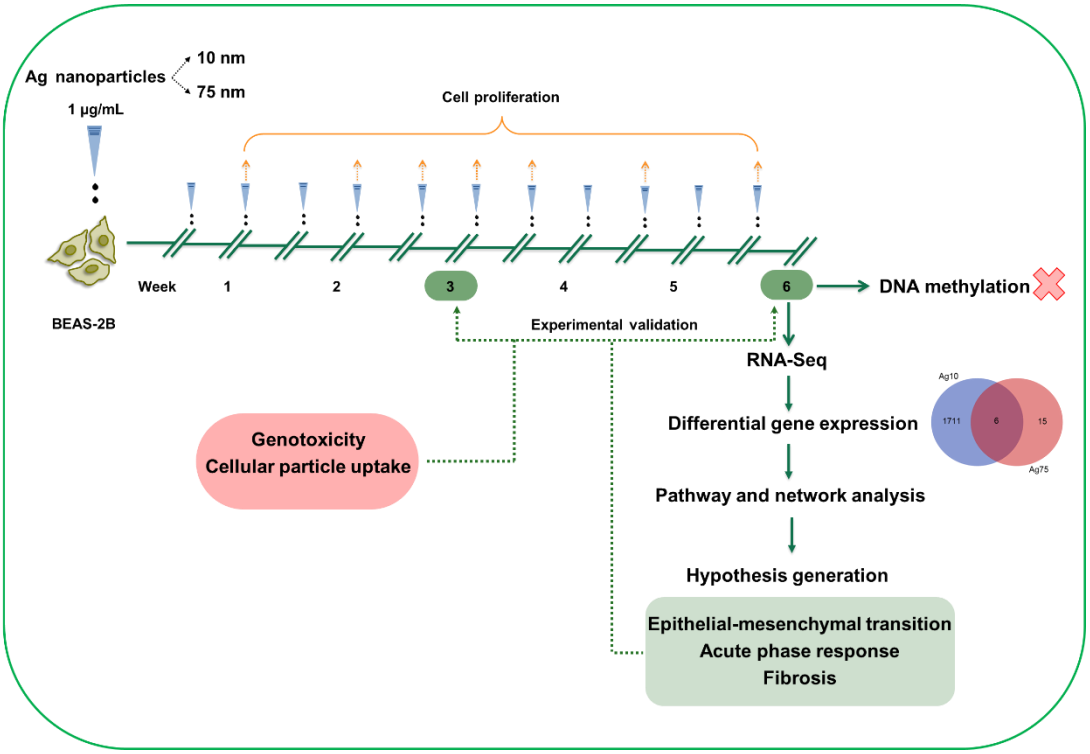

Figure 14. Experimental setup of study III.

The results indicated that Ag nanoparticles altered cell proliferation in a time- and size-dependent way, with the 10 nm Ag nanoparticles being more potent than the 75 nm particles. In addition, only the 10 nm Ag nanoparticles induced DNA damage measured by the alkaline version of the comet assay after 3 and 6 weeks of exposure, but none of the particles had clastogenic or aneuploidogenic effects as indicated by the micronucleus assay. Moreover, there was a clear size-dependent effect in terms of the number of differentially expressed genes

(DEGs) following RNA-Seq after 6 weeks of exposure (1717 DEGs for the 10 nm Ag *versus* 21 DEGs for the 75 nm Ag). The low number of DEGs for the 75 nm Ag nanoparticles precluded further analysis. Next we performed pathway analysis of the RNA-Seq data for 10 nm Ag nanoparticles using the Ingenuity Pathway Analysis tool and identified a series of altered pathways, out of which three pathways were considered for further validation: *Hepatic fibrosis*, *Regulation of epithelial-mesenchymal transition* (EMT) pathway and *Acute phase response signaling* pathway. The initial focus of the study was to identify cancer related alterations induced by chronic exposure to Ag nanoparticles, which was important criteria for the pathway selection.

The *Hepatic fibrosis* pathway was altered in a way consistent with pathway activation evident from the up-regulation the gene expression of 7 out of 8 collagen related genes as well as of *TGFβ1,* an important pro-fibrotic factor, among others. For the experimental validation of this pathway we determined the soluble collagen secreted in the cell medium as well as the collagen deposited on the well plates after 6 weeks of exposure (5 days from the last re-seeding). Our results showed that both Ag nanoparticles altered the collagen kinetics by increasing the collagen deposition, which is an indication of pro-fibrotic potential.

Next, the RNA-Seq data revealed that the *Regulation of EMT* pathway was also activated as defined by cadherin switching (down-regulation of *CDH1* and up-regulation of *CDH12*) as well as up-regulation of genes correlated to EMT induction (*TGFβ1, NOTCH3, MMP2, MRAS*). The experimental validation of this pathway consisted of determination of soft agar cell transformation, cell migration and invasion as well as E- and N-cadherin surface markers. The results indicated that the 10 nm Ag nanoparticles induced cell transformation already after 3 weeks and that both nanoparticles had this effect after 6 weeks. In addition, both particles increased the invasion index (albeit only significant for the 75 nm particles) and induced cadherin switching.

Finally, the *Acute phase response signaling pathway* had an activity pattern consistent with pathway inhibition characterized by down-regulation of the gene expression of *IL-1α, IL-1β, IL-18, MYD88* and *SAA2*, among others. For the experimental validation of this pathway we determined the cytokine secretion in response to bacterial LPS using a multiplex assay. Results indicated that exposure to Ag nanoparticles reduced the secretion of pro-inflammatory cytokines (IL-1β, IL-6, IL-8 and TNF-α) following LPS treatment, which is in line with the RNA-Seq data.

In summary, our results indicate that low-dose chronic exposure to Ag nanoparticles can induce a cancer-like phenotype in BEAS-2B cells, characterized by cell transformation, EMT and fibrosis and that these effects occur independent of alterations in the DNA methylation pattern. In addition, we show that Ag nanoparticles can have immunosuppressive effects by reducing the cytokine secretion in response to LPS, which is of concern considering the biomedical applications of Ag nanoparticles. Ultimately, we show that traditional toxicological assays can be complemented with 'omics' techniques to provide a comprehensive understanding of how nanoparticles perturb cellular functions.

4.4 PAPER IV. ANTIOXIDANT CERIUM OXIDE NANOPARTICLES SUPPRESS DIFFERENTIATION OF NEURAL STEM CELLS

CeO_2 nanoparticles display catalytic antioxidant activity that makes them appealing for both industrial and biomedical applications (Andreescu *et al.*, 2014, Walkey *et al.*, 2015). CeO_2 nanoparticles have been shown to have neuroprotective effects *in vitro* (D'Angelo *et al.*, 2009, Estevez *et al.*, 2011) and beneficial effects in animal models of neurodegenerative diseases (Kim *et al.*, 2012, Heckman *et al.*, 2013). Since oxidative stress has been associated with a series of neurodegenerative diseases (Andersen, 2004), antioxidant therapies have been considered as treatment options (Uttara *et al.*, 2009). On the other hand, reactive oxidative species play an important role as mediators during neuronal development (Kennedy *et al.*, 2012), which raises concern over the potential neurotoxic effects of antioxidants.

With this is mind, our aim was to investigate the effects of antioxidant CeO_2 nanoparticles on neural stem cells (C17.2). Our initial goal was to assess the antioxidant, neuroprotective effect of CeO_2 nanoparticles in this model using traditional assays. The next goal was to evaluate the effects of CeO_2 nanoparticles on the neuronal differentiation of C17.2 cells using both traditional and 'omics' technologies, namely RNA-Seq. Samarium -doped CeO_2 (Sm-CeO_2) nanoparticles, which bear a reduced antioxidant activity, were used as a particle control. In addition, NAC was used as a conventional antioxidant control.

First, by using TEM and ICP-MS we confirmed that both CeO_2 and Sm-CeO_2 nanoparticles were taken up by proliferating C17.2 cells without cytotoxicity. Then, we used 2,3-dimethoxy-1,4-naphthoquinone (DMNQ) as an oxidative stress challenge and evaluated the ROS generation (DCFH-DA assay) as well as cell viability (automated microscopic morphological assessment). Our results showed that CeO_2, but not Sm-CeO_2 reduced the ROS generation following DMNQ exposure, suggestive of an antioxidant affect. In addition, CeO_2, but not Sm-CeO_2 reduced the cell death induced by DMNQ after an early time-point (8 hours), again suggestive of an antioxidant effect. However, at longer time-points (12 hours) none of the particles had any effect on reducing DMNQ induced cell death which indicates that the neuroprotective effect was in essence a delay of cell death and was not correlated with cell recovery.

Next we focused on the effects of CeO_2 nanoparticles on the differentiation of C17.2 cells, which was induced by serum deprivation and addition of N2 supplement. We evaluated neuronal differentiation after 7 days using immunofluorescence staining of β3-tubulin (TuJ1) as an early neuronal marker. Our results showed that that CeO_2 reduced neuronal differentiation at all tested doses (10 – 50 µg/mL) whereas Sm-CeO_2 reduced neuronal differentiation only at the highest tested dose (50 µg/mL). This again is suggestive of an antioxidant effect since Sm-CeO_2 retains a small antioxidant effect that could be enough to inhibit neuronal differentiation at higher doses. However, we cannot exclude the contribution of a particle, antioxidant-independent effect. NAC, the conventional antioxidant, suppressed neuronal differentiation and was proven to be a good control for this endpoint. In addition, we corroborated our results on neuronal differentiation in human progenitor stem cells.

In order to further explore these observations, we decided to perform RNA-Seq at different time-points during differentiation (undifferentiated – day 0, day 1 and day 7) (Figure 15).

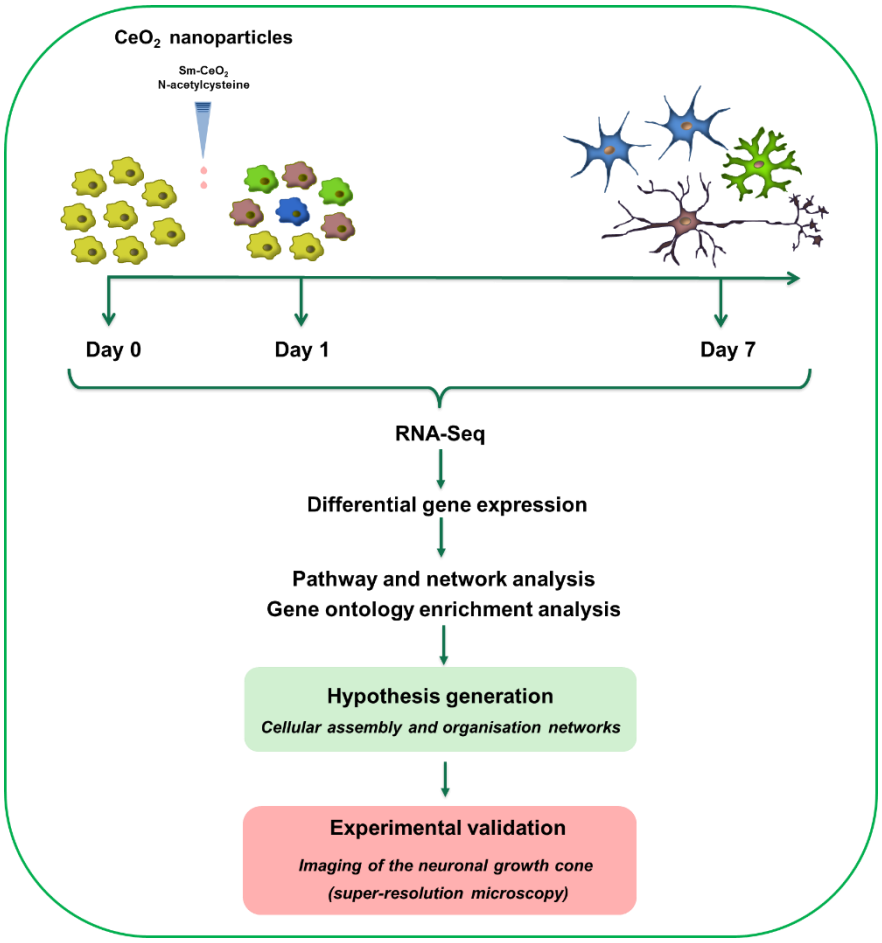

Figure 15. Experimental design of the RNA-Seq evaluation in study IV

The RNA-Seq downstream analysis using Ingenuity Pathway Analysis tool revealed that both CeO_2 and NAC, but not Sm-CeO_2 altered the neuronal differentiation network in line with the immunofluorescence data. In addition, CeO_2 and NAC interfered with axonal guidance signaling as well as neuroglial differentiation in a way consistent with pathway inhibition. All these observations suggest that antioxidant properties play a role in the observed effects. On the other hand, both nanoparticles but to a lesser extent NAC interfered with networks related to 'Cellular assembly and organization' suggestive of a particle effect. To further validate these findings, we used super-resolution microscopy (SIM and STED) to visualize the cytoskeletal structure of the neuronal growth cones, which are essential for axonal guidance and pathfinding. The results confirmed the particle effect; the growth cones were smaller and less likely to have the typical triangular morphology following nanoparticle treatment.

In summary, in this study we combined conventional assays with 'omics' as well as super-resolution imaging to reveal the dual effects of CeO_2 nanoparticles. On one hand, CeO_2 nanoparticles had a temporary protective effect in neural stem cells as a results of its antioxidant properties. On the other hand, CeO_2 nanoparticles inhibited neuronal differentiation and altered the structure of the growth cone which imply potential developmental neurotoxicity.

5 GENERAL DISCUSSION

5.1 *IN VITRO* ASSESSMENT OF NANOMATERIALS USING A COMBINATION OF CONVENTIONAL AND NOVEL OMICS-BASED APPROACHES

In this thesis we used *in vitro* models and a combination of traditional toxicological assays and novel systems toxicology approaches to improve our understanding of the interactions between nanoparticles and cellular systems. Omics technologies provide an unprecedentedly detailed view into the pathways of toxicity and are bound to increase our understanding of how toxicants, including nanoparticles interfere with biological systems. Like in many other techniques the quality of the input parameters is crucial to the quality of the output data. Careful experimental design such as cell system, dose and time-point selection are, therefore, of utmost importance. Pilot studies can be useful to identify dose-response, and time-response trends using conventional toxicological assays.

In Paper III we combined two 'omics' techniques, RNA-Seq and genome-wide DNA methylation array to explore the effects of low-dose, long-term exposure of human bronchial cells to silver nanoparticles. We started off with the observation that low-doses of Ag nanoparticles over long time can reduce cell proliferation for the smaller 10 nm Ag nanoparticles but not for the larger, 75 nm Ag nanoparticles, with subsequent recovery. Next we used an RNA-Seq approach to elucidate these changes and genome-wide DNA methylation to identify potential epigenetic effects of Ag nanoparticles. 'Omics' allows for a hypothesis-free unbiased exploration of the data, but it is often helpful if there is an *a priori* scientific question or if previous knowledge on the matter is taken into account. In this case, we designed the long-term exposure setup to particularly study the potential carcinogenic effects of Ag nanoparticles. We performed a series of comprehensive downstream pathway and network analyses and generated two carcinogenesis-related hypotheses *i.e.* Ag nanoparticles can induce (i) fibrosis and (ii) epithelial-mesenchymal transition. These hypotheses based on RNA-Seq data were validated experimentally using conventional toxicology assays. Indeed, there is a 'long way' from RNA transcription to functional proteins, with RNA-protein correlation being as low as 40% in some cases (Vogel and Marcotte, 2012). In addition to answering our questions related to cell transformation and carcinogenesis, RNA-Seq provided insight into new 'territory' related to the effects of Ag nanoparticles on the innate immune system.

An interesting finding in this study was the potential of 75 nm Ag nanoparticles to induce phenotypical changes despite minimal alterations at the gene expression level. It seems that the cell phenotypes of cells treated with 10 and 75 nm Ag nanoparticles were much more similar than it would follow from the RNA-Seq data. One possible explanation is that individual gene expression changes were too small to pass the significance level following differential gene expression for the 75 nm Ag nanoparticles, but overall could still be enough to induce a change in phenotype. Another possible explanation, was that the RNA-Seq and the functional validation experiments were performed in two different experimental sets, at different time-points and using different batches of nanoparticles. However, considering the high purity and quality of the nanoparticles used (the 75 nm Ag nanoparticles being an OECD reference

standard material) it is unlikely that there was a significant particle batch effect. Nevertheless, in some functional assays, such as DNA damage and cell proliferation only 10 nm Ag nanoparticles elicited an effect, clearly indicating size-dependent toxicity, in line with the RNA-Seq data. In addition, 10 nm Ag nanoparticles were more potent in inducing G1 arrest and it is conceivable that cell cycle alterations will result in more 'severe' effects that could possibly lead to a higher number of differentially expressed genes.

The genome wide DNA methylation did not indicate any relevant significant changes that could explain the RNA-Seq results. Interestingly, even for genes such as E-cadherin, in which promoter methylation plays an important role in controlling gene expression (Reinhold *et al.*, 2010) there was no significant differential methylation (even when disregarding the false discovery rate correction). Despite no observed effect at the DNA methylation level, Ag nanoparticles could still bear potential epigenetic effects via other mechanisms such as histone modifications or micro-RNAs, the latter being found to play a role in the up-regulation of metallothioneins by Ag nanoparticles in Jurkat cells (Eom *et al.*, 2014).

In Paper IV we used RNA-Seq to explore the effect of nanoceria on neuronal differentiation. In this study we already had experimental indications of alterations in neuronal differentiation and we wanted to further investigate these effects. RNA-Seq indicated that nanoceria not only reduced the expression of β3-tubulin as a marker of neuronal differentiation but interfered with the whole network of neuronal differentiation. By using appropriate controls, such as Sm-doped nanoceria (reduced antioxidant effect as a particle control) and NAC (antioxidant control), we could also distinguish between antioxidant related effects and potential particle effects. This experimental setup allowed us to conclude that the effects on the neuronal differentiation overlapped between nanoceria and NAC and were probably related to their antioxidant function. On the other hand, both particles, but not NAC, shared effects related to interference with cellular assembly and filopodia formation. This lead to the generation of a new hypothesis, namely that the particle could interfere with the neuronal growth cone structure, which was validated experimentally by super-resolution microscopy.

On the whole 'omics' approaches are excellent tools for obtaining mechanistic insight into the mechanism of toxicity and for generating new hypotheses. However, in order to close the 'loop' these hypotheses require experimental validation using conventional assays to confirm changes in cellular phenotypes. In addition, 'omics' assay could help identify biomarkers of toxicity that could then be used in conventional or high-throughput assays for screening purposes.

One criticism brought up by Krug H. is the use of high doses which offer pure mechanistic information are of no toxicological relevance (Krug, 2014). While it is true that 'toxicological' studies are required for hands-on risk assessment purposes, mechanistic studies that identify new pathways of action and/or new toxic endpoints are equally valuable. Omics-derived approaches such as the one comprising this thesis are therefore particularly useful for understanding the complexity of nanoparticle – cell interactions and for generating new hypotheses.

5.2 INCREASING THE THROUGHPUT OF ASSAYS TO SCREEN AND PREDICT TOXICITY OF NANOPARTICLES

The increasing number of nanomaterials that are being produced and used in various applications requires appropriate screening tools to enable hazard identification and risk assessment endeavors. In a seminal article published in 2013, Nel and colleagues put forward some guiding directions for the toxicity testing of nanomaterials in which they emphasize the use of mechanism-based toxicity screening tools along with high-throughput technologies (Nel *et al.*, 2013). Enacting these guidelines would increase the predictive power of the assays, allow for hazard ranking and prioritization, and ultimately enable safety by design strategies (Nel *et al.*, 2013). In addition, the use of *in vitro* high-throughput technologies that have predictive power would reduce unnecessary animal testing which require a lot of resources and raise ethical concerns (Nelson *et al.*, 2016). Compared to high-content approaches such as 'omics', that aid in the generation of new hypotheses and in the identification of new important endpoints for toxicity testing, high-throughput technologies enable the testing of numerous endpoints and materials at the same time.

In Paper I we investigated the use of a moderate/high-throughput assay, ToxTracker, for mechanism-based genotoxicity screening of metal and metal oxide nanoparticles. The ToxTracker assay has been developed and validated for screening the genotoxic potential of chemicals (Hendriks *et al.*, 2012), however, assays developed for chemicals cannot be directly translated to nanoparticle research due to potential interferences of the particles with reagents from the assay or the assay readout (Nelson *et al.*, 2016). Since the ToxTracker assay is based on flow cytometry measurements, the presence of particles in the intracellular compartment could lead to side-scatter shifts of the cell populations but this issue can be overcome by adjusting the data analysis.

Following validation with conventional genotoxicity assays, we showed that the ToxTracker was able to identify that the genotoxicity of CuO and NiO nanoparticles was correlated with oxidative stress and dependent on Nrf2 activation. For ZnO nanoparticles, the reporter was activated only at highly cytotoxic doses which could be interpreted as a secondary effect and not the main mechanism of toxicity. An advantage of the assay is the ability to gate the viable cell population and diminish the artefacts related to cell death. On the other hand, when reporter activation occurs only at highly cytotoxic doses it makes it difficult to draw any sound conclusions and questions the sensitivity of the assay. ZnO nanoparticles induced the Srxn1 reporter, only at highly cytotoxic doses, however, other studies showed that ZnO nanoparticles can induce oxidative stress in a tiered manner, in line with the hierarchical oxidative stress paradigm (Xia *et al.*, 2008). In addition, ZnO nanoparticles did not induce genotoxicity after short-term exposure using OECD *in vitro* and *in vivo* assays (Kwon *et al.*, 2014) or after long-term exposure *in vitro* (Annangi *et al.*, 2016).

Another interesting observation was the lack of reporter activation or cytotoxicity for the two Ag nanoparticles tested. In Paper II we showed that the same Ag nanoparticles (10 and 40 nm) can induce DNA damage, as measured by the comet assay, but in the absence of oxidative

stress following acute exposure of BEAS-2B cells. One explanation is that the ToxTracker assay identifies genotoxicity mediated by Nrf2 dependent oxidative stress, stalled replication forks or p53-dependent cellular stress. Despite these being common pathways involved in genotoxicity, other unrelated mechanisms are not considered. Also, it is worth noting that the comet assay is a highly sensitive assay and measures an endpoint, whereas the ToxTracker assay measures activation of a pathway and could therefore have different sensitivity. The lack of cytotoxicity of Ag nanoparticles in the mES as compared to the BEAS-2B cells could be related to aspects such as different intrinsic cellular sensitivity or different agglomeration/sedimentation of the particles in serum containing medium (mES) compared to serum free media (BEAS-2B), which can influence the particle deposition and cellular uptake.

Reporter cells were previously used to address the correlation between physico-chemical properties of Ag nanoparticles and induction of inflammation (Prasad *et al.*, 2013). Prasad and colleagues used stable NF-κB, Nrf2/ARE and AP1 luciferase reporters in HepG2 cells and found that Ag nanoparticles induced all three reporters, with Nrf2/ARE exhibiting the highest activation. In addition, smaller (10 nm) particles were more potent than larger (75 nm) particles and the effects were similar to those of $AgNO_3$ (Prasad *et al.*, 2013). It should be noted that the particles used by Prasad and colleagues had the same source and size (10 nm) as the Ag nanoparticles used in Paper I, and it is therefore intriguing that the ToxTracker assay did not indicate any activation of Nrf2 dependent pathways. A difference between these two cell reporters is their different origin, mouse embryonic stem cells *versus* hepatocytes, which could lead to different sensitivity to nanoparticles. Another explanation for the inconsistent results could be the use of cell culture media with different percentages of fetal bovine serum: 1% in the study by Prasad *et al.* and 10% in the ToxTracker assay. This is likely to impact particle stability, sedimentation, cellular uptake, Ag speciation and toxicity (Kittler *et al.*, 2010). In another study, Stoehr and colleagues showed that a series of lung alveolar cells reporter systems, reporting on IL-8 promoter activation can be used to identify the pro-inflammatory response of ZnO nanoparticles in submerged as well as in air-liquid interface cultures (Stoehr *et al.*, 2015).

In addition to the ToxTracker assay, in this thesis we used the flow cytometry version of the micronucleus assay in Paper III, as a fast tool for genotoxicity assessment, as well as a multiplex assay for cytokine secretion. The flow cytometry version of the micronucleus test is faster, has a higher throughput and considers more events compared with the 'cytome' assay, where all observations are done using microscopy. In addition, we could concurrently analyze the cell cycle progression, and get indications on cell viability. The flow cytometry version of the micronucleus assay has limitations in respect to the identification of nuclear anomalies such as budding and nucleoplasmic bridges, it cannot distinguish between mononucleated and binucleated cells, it scores multiple micronuclei in one cell as separate events, and there are possible interferences of particle agglomerates with the measurement (Nelson *et al.*, 2016).

5.3 HIGH-DOSE ACUTE *VERSUS* LOW-DOSE CHRONIC EXPOSURE TO NANOPARTICLES

The large majority of studies in nanotoxicology have focused on the acute effects of nanoparticles using short-term exposure scenarios and there is a current demand for more chronic studies to address the long-term effects of exposure to nanomaterials (Johnston *et al.*, 2013). Short-term studies can be useful for crude toxicity assessment, screening and prioritization purposes but they cannot give comprehensive information on *e.g.* the carcinogenic potential of nanoparticles. Other drawbacks of nanotoxicology studies are the use of poorly characterized materials, exposure to high doses together with the lack of reference materials (Krug, 2014).

Moreover, it should not be taken for granted that the mechanistic profile observed at high doses can be translated to low-doses; dose does not only make the poison but also has a bearing on the toxic mechanism.

In Paper II and III we addressed some of the challenges in nanotoxicology studies using BEAS-2B as a cell model. To this end we used two distinct experimental setups to test the toxicity of a panel of well-characterized Ag nanoparticles:

(i) Paper II - moderate/high dose (5-50 µg/mL) acute exposure (4 and 24 hours) to 10 nm citrate-coated, 10 nm OECD PVP-coated, 40 nm citrate-coated, 50 nm uncoated and 75 nm OECD citrate-coated Ag nanoparticles;

(ii) Paper III - low-dose (1 µg/mL) chronic exposure (3 and 6 weeks) to 10 nm citrate-coated and 75 nm OECD citrate-coated Ag nanoparticles;

In Paper II we observed a size-dependent cytotoxicity with the small (10 nm) particles being more toxic than the larger particles, independent of their surface coating. This was correlated with an increased release of Ag in cell medium, while the extracellular fraction was not cytotoxic. For the long-term, low-dose exposure in Paper III, we observed a similar pattern, with the 10 nm particles reducing cell proliferation in a time-dependent manner as compared to 75 nm particles which had no effect. In the chronic exposure setup, we observed effects on cell proliferation at much lower doses compared to the short-term exposure. This indicates that similar doses given in an acute or chronic regimen have different outcomes in respect to cell death and cell proliferation. In addition, it is likely that under chronic, low-dose exposure the cells develop mechanisms to cope with the toxic insult correlated with a phenotypical selection.

In both Paper II and III we visualized as well as quantified the cellular uptake. The TEM pictures revealed that after both acute and chronic exposure, Ag nanoparticles were localized in endo-lysosomal compartments, with no indications of nuclear localization. However, the cellular uptake was significantly different for the two exposure scenarios. Following 4-hour exposure, there was no difference in cellular metal content between the 10 nm and the 75 nm particles (approx. 3 pg/cell at doses of 10 µg/mL). After long-term exposure, the metal content for the 10 nm Ag nanoparticles as compared to the 75 nm Ag nanoparticles was lower at both 3 and 6 weeks. In addition, while the uptake of 75 nm Ag nanoparticles was relatively constant

(approx. 1 pg/cell) between 3 and 6 weeks, the uptake of 10 nm Ag nanoparticles decreased significantly from week 3 (approx. 0.7 pg/cell) to week 6 (approx. 0.25 pg/cell). This could be an adaptive mechanism related to a down-regulation of cell uptake mechanisms and/or upregulation of exocytosis pathways. Exocytosis of nanoparticles has not received much interest but it is an important mechanism that can modulate the toxicity of nanoparticles which should be investigated further. However, despite a reduction in Ag content there were no major changes in molecular endpoints (DNA damage) or phenotype (cell transformation, response to LPS) from week 3 to week 6.

Genotoxicity is another end-point studied in both Paper II and III. In Paper II, all tested nanoparticles induced genotoxicity after 24-hour exposure to non-cytotoxic doses (10 µg/mL) and there was no size-dependent difference as observed in the alkaline comet assay. However, following chronic exposure there was a size-dependent genotoxicity measured by the same alkaline comet assay, with the 10 nm particles inducing more DNA damage than the 75 nm particles at both investigated time-points (3 and 6 weeks). Moreover, there was no micronuclei or hypodiploid nuclei induction. Genotoxicity induction provided an indication for carcinogenic potential that was further evaluated after long-term exposure as discussed below.

In Paper III we generated a hypothesis based on the next-generation sequencing data, namely that Ag nanoparticles are able to induce EMT. This hypothesis was experimentally validated and we showed that Ag nanoparticles can induce a cancer-like phenotype in BEAS-2B cells after long-term exposure. Several *in vitro* studies have revealed that long-term exposure of cells to nanomaterials such as carbon nanotubes (Luanpitpong *et al.*, 2014, Wang *et al.*, 2011, Vales *et al.*, 2016), titanium dioxide (Vales *et al.*, 2015) and cobalt nanoparticles (Annangi *et al.*, 2015) can induce cell transformation. For single-walled carbon nanotubes, the *in vitro* cell transformation potential in BEAS-2B cells was corroborated with *in vivo* tumorigenicity (Wang *et al.*, 2011, Luanpitpong *et al.*, 2014). This provides proof that long-term exposure in cells such as BEAS-2B can be a useful model for identifying the carcinogenic potential of nanomaterials. In addition, some of the changes observed after long-term exposure to single-walled carbon nanotubes (Luanpitpong *et al.*, 2014, Wang *et al.*, 2011) were similar to the changes induced by Ag nanoparticles in Paper III: reduction in E-cadherin expression, increased cell invasion and migration as well as increased soft-agar cell transformation.

BEAS-2B cells are considered a good model for long-term studies on carcinogenesis induced by heavy metals and nanoparticles (Park *et al.*, 2015, Wang *et al.*, 2011). However, during long-term exposure, a change in cell phenotype and even cell transformation can occur as a result of cell culture *per se*. Indeed, in Paper III we observed that parameters such as anchorage independent cell growth, N-cadherin expression and the length of G1 cell cycle phase increased at week 6 as compared to week 3, indicating cell transformation. On the other hand, the background level of DNA damage as measured by the comet assay did not change, neither was there an increase in micronuclei, suggesting genomic stability. The phenotypic changes with cell culture should be therefore addressed in *in vitro* studies and the duration of the exposure/cell culture should be optimized.

5.4 REACTIVE OXYGEN SPECIES: TOXIC INSULT *VERSUS* SIGNALLING MOLECULE

It is well established that one mechanism by which (nano)particles can exert toxic effects lies in their ability to elicit oxidative stress in a hierarchical manner in line with the oxidative stress paradigm (Nel *et al.*, 2006, Manke *et al.*, 2013). If out of balance, oxidative stress can induce cellular injuries such as DNA damage, lipid peroxidation, protein denaturation, which in turn can lead to cell death, inflammation, mutations, cancer and fibrosis (Manke *et al.*, 2013). Several pathologies relevant for inhalation of particles such as silicosis following inhalation of quartz (Vallyathan *et al.*, 1997) and pulmonary as well as cardiovascular effects following exposure to air pollution derived particulate matter (Møller *et al.* 2010) have been associated with increased oxidative stress.

In Paper I we showed that both NiO and CuO nanoparticles induced DNA damage via oxidative stress as indicated by the activation of the Srxn1 reporter which accounts for Nrf2 dependent signaling. In the case of CuO nanoparticles, the oxidative stress was related to the dissolution of ions in the extracellular medium, whereas for NiO nanoparticles the Srxn1 activation occurred following particle uptake. Cu ions can induce oxidative stress either by entering Fenton as well as Haber-Weiss reactions or by depleting glutathione levels (Jomova and Valko, 2011). It should be noted that both NiO and CuO nanoparticles were potent in generating ROS under acellular conditions. In Paper II, all tested Ag nanoparticles induced DNA damage following 24-hour exposure in human lung cells, but in this case, there was no correlation with ROS generation, suggesting additional mechanisms of DNA damage.

On the other hand, ROS function as secondary messengers and are involved in regulating processes such as cell proliferation and differentiation (Sauer *et al.*, 2001). Endogenous ROS production occurs following electron leaking from the respiratory chain, electron release from the NADPH cytochrome P450 reductase and NADPH oxidase systems with the subsequent activation signaling cascades (*e.g.* ERK1/2, JNK and p38 MAPK pathways) or direct regulation of transcription factors (*e.g.* NF-κB, AP-1, SP-1, HIF-1α, p53) (Sauer *et al.*, 2001).

Ultimately, according to the 'free radical theory of development' put forward in 1989 by Allen and Balin, ROS influence cellular antioxidant defense mechanisms and modulate gene expression during development of organisms (Allen and Balin, 1989). This raises the question whether antioxidant nanoparticles, could act like a double-edged sword depending on the physiological context, similar to other exogenous antioxidants (Bouayed and Bohn, 2010).

In Paper IV we investigated the effects of antioxidant nanoceria on neural stem cells. Our results showed that pre-incubation of cells with nanoceria delayed cell death induced by oxidative stress, suggesting neuroprotective effects, which have been previously reported for nanoceria (D'Angelo *et al.*, 2009, Estevez *et al.*, 2011). However, nanoceria also reduced the ROS levels in the absence of an oxidative stress inducer indicating that it can interfere with the physiological ROS balance. Next, we reported that nanoceria reduced expression of β3 tubulin, which is a marker of neuronal differentiation, in a way similar to NAC, a conventional antioxidant. The RNA-Seq experiments further confirmed this observation and revealed that

both nanoceria and NAC alter the neuronal differentiation as well as the neuroglial differentiation network in similar ways, however, the effects of nanoceria were more extensive. We therefore showed that the same antioxidant effects which are considered promising for therapeutical applications can lead to detrimental neurotoxic effects *i.e*. interference with neuronal development and differentiation.

6 CONCLUDING REMARKS

This thesis shows that challenges in nanotoxicology can be addressed and overcome using *in vitro* models and a combination of thoroughly characterized nanoparticles, suitable experimental setups, novel screening tools as well as systems toxicology approaches.

In Paper I we demonstrated that the ToxTracker reporter assay is a suitable tool for rapid medium-throughput screening of genotoxicity of metal oxide nanoparticles. In addition, the assay gave insight into the mechanisms of genotoxicity which occurred mainly by oxidative stress (for NiO and CuO nanoparticles) and not by binding to DNA with subsequent stalled replication forks. After further analysis of the corresponding ion salts, we concluded that the effects of CuO were mediated by the ions released in the cell medium, whereas for NiO the effects were related to the particle form.

In Paper II we showed that the acute cytotoxicity of Ag nanoparticles in human lung cells is size-dependent, with smaller particles being more toxic than larger ones. All particles were taken up to similar extents and all particles induced comparable genotoxicity. In addition, the smaller particles had a higher release of Ag in cell medium, which, provided that the same pattern of dissolution follows inside the cells, could explain the observed differences in toxicity.

In Paper III we combined 'omics' and conventional assays to explore the low-dose, long-term effects of Ag nanoparticles in human lung cells. We showed that Ag nanoparticles induced a cancer-like phenotype evidenced by cell transformation, induction of fibrosis markers and epithelial-mesenchymal transition. In addition, both particles had immunosuppressive effects by reducing cytokine secretion following challenge with LPS.

In Paper IV we showed that, on one hand, CeO_2 nanoparticles are antioxidant and have temporary neuroprotective effects in neural stem cells. On the other hand, due to the antioxidant properties, CeO_2 nanoparticles reduced neuronal differentiation which was first evidenced by immunofluorescence and then confirmed by RNA-Seq. In addition, RNA-Seq revealed an antioxidant-independent particle effect *i.e.* interference with the neuronal growth cone that was validated by super-resolution microscopy.

This thesis sheds light on several critical aspects raised by Harald Krug in his review article from 2014 (Krug, 2014) such as: particle characterization, cellular particle uptake, low-dose chronic exposure setups and use of appropriate controls. I hope the work described in this thesis will guide future toxicological endeavors and ultimately aid risk assessment of nanomaterials. I would like to particularly emphasize the importance of particle characterization and quantification of cellular uptake that would facilitate comparisons between studies as well as *in vitro – in vivo* correlations. Next, I hope this thesis will prompt a shift from high-dose, acute exposure experimental setups to low-dose, long-term exposure scenarios that can unravel new and subtle mechanisms of toxicity. Moreover, I believe this thesis could guide future work using systems toxicology approaches to gain more in-depth understanding of the interaction between nanomaterials and biological systems that will be of great benefit in both toxicology and nanomedicine.

7 FUTURE OUTLOOK - PERSONAL REFLECTIONS

The virtuous cycle of science. On one hand, good research answers questions, on the other hand it gives rise to new questions that in turn stimulate the development of novel approaches to answer those questions. Below are a few personal scientific ruminations that emerged while working on this thesis. I regard the first point as a general issue in nanotoxicology, while the last two items are potential follow-up studies to this thesis.

- How does the cell culture heterogeneity influence the toxicity of nanoparticles?

Exposure to nanoparticles (as opposed to conventional chemicals) in cell culture is not homogenous and some cells will 'see' and/or take up more particles than others. It would be valuable to investigate whether there are correlations between toxicological endpoints and particle uptake at a single cell level. On one hand, it might seem obvious that these correlations exist but they have yet to be unraveled. Do cells with higher particle uptake exhibit more DNA damage than cells with lower particle uptake from the same dish? On the other hand, depending on the endpoint of interest, the cellular response might be homogenous and orchestrated. Is the gene expression of cytokines following exposure to nanoparticles correlated with the metal uptake or is this a more orchestrated, homogenous effect at cell culture level? In addition, it is conceivable that these correlations are very much dependent on the nanoparticle *per se*. Are there differences between highly soluble nanoparticles (CuO, ZnO) and less soluble particles (TiO_2, CeO_2) for which the particulate form is more important for the toxic outcome? In addition, cells during cell culture are present in various stages of the cell cycle that could result in different outcomes following exposure to nanoparticles.

By working with pooled samples, it is impossible to address the cell heterogeneity, and some significant effects could be diluted and less likely to be discovered. It is biologically relevant *e.g.* if a few percentage of cells acquire a cancer-like phenotype, however when working with pooled samples these effects could become diluted and have no statistical significance.

I believe that emerging high resolution technologies, such as mass cytometry and single cell RNA-Seq techniques could enable us to answer these fundamental questions. Ultimately this would advance not only our understanding of the cell-nanoparticle interaction but also our understanding of *in vitro* cell culture, and allow us to improve our experimental design.

- How does the formation of lung surfactant bio-corona affect the toxicity of Ag nanoparticles?

In the current thesis we evaluated the effects of Ag nanoparticles on human lung cells, BEAS-2B. These cells grow in serum free medium with a low amount of proteins (less than 0.5%). However, these working conditions do not reflect the physiology of the lung. It would therefore be of relevance to study how the presence of lung surfactant affects both Ag nanoparticle stability and uptake, as well as the toxic outcome.

- Could antioxidant nanoparticles induce genomic instability in neuronal stem cells?

It was reported that physiological levels of reactive oxidative species play an important role in preserving genomic stability in stem cells by activating DNA repair complexes (Li and Marbán, 2010). It is therefore conceivable that antioxidant nanoparticles such as CeO_2 could increase the background DNA damage in neuronal stem cells, and by these means induce neurodevelopmental effects and/or carcinogenesis. I would argue that there are good grounds for further investigation into this hypothesis.

Finally, I hope to explore some of the questions raised within these pages (and many more that will emerge on the way), and to continue contributing to the fascinating field of (nano)particle toxicology.

8 ACKNOWLEDGEMENTS

The studies described in this thesis were carried out at the Division of Molecular Toxicology and Division of Biochemical Toxicology, Institute of Environmental Medicine, Karolinska Institute and were supported by grants from the Swedish Research Council for Health, Working Life and Welfare (FORTE), Swedish Research Council (VR), Swedish Cancer and Allergy Foundation, EU Framework 7 Programme (NANOREG) and KI faculty grant for PhD students (KID). Firstly, I wish to express my deepest gratitude to my main supervisor, Assoc. Prof. Hanna Karlsson for all her support and unfailing positivity. I am also grateful to my co-supervisor Prof. Bengt Fadeel for his invaluable feedback, inspiring scientific expertise and continuous encouragements. I would also like to acknowledge my co-supervisor Jessica Lindvall for generously sharing her knowledge in bioinformatics, my co-supervisor Prof. Inger Odnevall-Wallinder for providing competent advice on the characterization of nanoparticles and my external mentor Klas Udekwu for his friendly advice. Special thanks are due to Sebastiano Di Bucchianico for all his guidance and contagious passion for science, to Pekka Kohonen who was my true mentor during the 'omics' part of this thesis, and to Karin Edoff who guided me through the world of neuroscience. I would like to particularly thank Sara Skoglund, my collaborator and dear friend throughout the years, and Jessica De Loma, for giving me the opportunity to supervise her master thesis work which was a truly enriching experience. In addition, I would like to thank Annika Hanberg, Ralf Morgenstern, Kristian Dreij and Penny Nymark for allowing me to be involved in the education of master students in toxicology. I would also like to thank current and former members of the Molecular Toxicology and Biochemical Toxicology units for creating an enjoyable working environment. Finally, I would like to acknowledge all of my collaborators and co-authors for all their work and for sharing their expertise in their respective fields, which contributed to the interdisciplinary character of this thesis.

9 REFERENCES

Albini, A. and R. Benelli (2007). "The chemoinvasion assay: a method to assess tumor and endothelial cell invasion and its modulation." *Nature Protocols* **2**(3): 504-511.

Allen, R. G. and A. K. Balin (1989). "Oxidative influence on development and differentiation: an overview of a free radical theory of development." *Free radical biology & medicine* **6**(6): 631-661.

Andersen, J. K. (2004). "Oxidative stress in neurodegeneration: cause or consequence?" *Nature Medicine* **10**(25): S18-25.

Andreescu, D., G. Bulbul, R. E. Ozel, A. Hayat, N. Sardesai and S. Andreescu (2014). "Applications and implications of nanoceria reactivity: measurement tools and environmental impact." *Environmental Science: Nano* **1**(5): 445-458.

Annangi, B., J. Bach, G. Vales, L. Rubio, R. Marcos and A. Hernandez (2015). "Long-term exposures to low doses of cobalt nanoparticles induce cell transformation enhanced by oxidative damage." *Nanotoxicology* **9**(2): 138-147.

Annangi, B., L. Rubio, M. Alaraby, J. Bach, R. Marcos and A. Hernandez (2016). "Acute and long-term in vitro effects of zinc oxide nanoparticles." *Archives of toxicology* **90**(9): 2201-2213.

Antonini, J. M., M. D. Taylor, A. T. Zimmer and J. R. Roberts (2004). "Pulmonary responses to welding fumes: role of metal constituents." *Journal of Toxicology and Environmental Health, Part A* **67**(3): 233-249.

AshaRani, P. V., M. G. Low Kah, M. P. Hande and S. Valiyaveettil (2009). "Cytotoxicity and genotoxicity of silver nanoparticles in human cells." *ACS Nano* **3**(2):279–290.

Assenov, Y., F. Muller, P. Lutsik, J. Walter, T. Lengauer and C. Bock (2014). "Comprehensive analysis of DNA methylation data with RnBeads." *Nature Methods* **11**(11): 1138-1140.

Auffan, M., J. Rose, J.-Y. Bottero, G. V. Lowry, J.-P. Jolivet and M. R. Wiesner (2009). "Towards a definition of inorganic nanoparticles from an environmental, health and safety perspective." *Nature Nanotechnology* **4**(10): 634-641.

Avalos, A., A. I. Haza, D. Mateo and P. Morales (2014). "Cytotoxicity and ROS production of manufactured silver nanoparticles of different sizes in hepatoma and leukemia cells." *Journal of Applied Toxicology* **34**(4): 413-423.

Bakke, A. C. (2001). "The Principles of Flow Cytometry." *Laboratory Medicine* **32**(4): 207-211.

Banath, J. P., C. A. Banuelos, D. Klokov, S. M. MacPhail, P. M. Lansdorp and P. L. Olive (2009). "Explanation for excessive DNA single-strand breaks and endogenous repair foci in pluripotent mouse embryonic stem cells." *Experimental Cell Research* **315**(8): 1505-1520.

Belade, E., L. Armand, L. Martinon, L. Kheuang, J. Fleury-Feith, A. Baeza-Squiban, S. Lanone, M.-A. Billon-Galland, J.C. Pairon and J. Boczkowski (2012). "A comparative transmission electron microscopy study of titanium dioxide and carbon black nanoparticles uptake in human lung epithelial and fibroblast cell lines." *Toxicology in Vitro* **26**(1): 57-66.

Bhabra, G., A. Sood, B. Fisher, L. Cartwright, M. Saunders, W. H. Evans, A. Surprenant, G. Lopez-Castejon, S. Mann, S. A. Davis, L. A. Hails, E. Ingham, P. Verkade, J. Lane, K. Heesom, R. Newson and C. P. Case (2009). "Nanoparticles can cause DNA damage across a cellular barrier." *Nature Nanotechnology* **4**(12): 876-883.

Bhattacharya, K., C. Sacchetti, R. El-Sayed, A. Fornara, G. P. Kotchey, J. A. Gaugler, A. Star, M. Bottini and B. Fadeel (2014). "Enzymatic 'stripping' and degradation of PEGylated carbon nanotubes." *Nanoscale* **6**(24): 14686-14690.

Blom, H. and H. Brismar (2014). "STED microscopy: increased resolution for medical research?" *Journal of Internal Medicine* **276**(6): 560-578.

Blom, H. and J. Widengren (2014). "STED microscopy - towards broadened use and scope of applications." *Current Opinion in Chemical Biology* **20**: 127-133.

Borowicz, S., M. Van Scoyk, S. Avasarala, M. K. Karuppusamy Rathinam, J. Tauler, R. K. Bikkavilli and R. A. Winn (2014). "The soft agar solony formation assay." *Journal of Visualized Experiments* (92): e51998.

Bouayed, J. and T. Bohn (2010). "Exogenous antioxidants—Double-edged swords in cellular redox state: Health beneficial effects at physiologic doses versus deleterious effects at high doses." *Oxidative Medicine and Cellular Longevity* **3**(4): 228-237.

Braakhuis, H. M., C. Giannakou, W. J. Peijnenburg, J. Vermeulen, H. van Loveren and M. V. Park (2016). "Simple in vitro models can predict pulmonary toxicity of silver nanoparticles." *Nanotoxicology* **10**(6): 770-779.

Braakhuis, H. M., I. Gosens, P. Krystek, J. A. Boere, F. R. Cassee, P. H. Fokkens, J. A. Post, H. van Loveren and M. V. Park (2014a). "Particle size dependent deposition and pulmonary inflammation after short-term inhalation of silver nanoparticles." *Particle and Fibre Toxicology* **11**(49): 014-0049.

Braakhuis, H. M., M. V. Park, I. Gosens, W. H. De Jong and F. R. Cassee (2014b). "Physicochemical characteristics of nanomaterials that affect pulmonary inflammation." *Particle and Fibre Toxicology* **11**(1): 1-25.

Brook, R. D. (2008). "Cardiovascular effects of air pollution." *Clinical science (London, England: 1979)* **115**(6): 175-187.

Brown, A. P., R. M. D. Brydson and N. S. Hondow (2014). "Measuring in vitro cellular uptake of nanoparticles by transmission electron microscopy." *Journal of Physics: Conference Series* **522**(1): 012058.

Buerki-Thurnherr, T., L. Xiao, L. Diener, O. Arslan, C. Hirsch, X. Maeder-Althaus, K. Grieder, B. Wampfler, S. Mathur, P. Wick and H. F. Krug (2013). "In vitro mechanistic study towards a better understanding of ZnO nanoparticle toxicity." *Nanotoxicology* **7**(4): 402-416.

Caputo, F., M. De Nicola, A. Sienkiewicz, A. Giovanetti, I. Bejarano, S. Licoccia, E. Traversa and L. Ghibelli (2015). "Cerium oxide nanoparticles, combining antioxidant and UV shielding properties, prevent UV-induced cell damage and mutagenesis." *Nanoscale* **7**(38): 15643-15656.

Carlson, C., S. M. Hussain, A. M. Schrand, L. K. Braydich-Stolle, K. L. Hess, R. L. Jones and J. J. Schlager (2008). "Unique cellular interaction of silver nanoparticles: size-dependent generation of reactive oxygen species." *The Journal of Physical Chemistry B* **112**(43): 13608-13619.

Cassee, F. R., A. Campbell, A. J. Boere, S. G. McLean, R. Duffin, P. Krystek, I. Gosens and M. R. Miller (2012). "The biological effects of subacute inhalation of diesel exhaust following addition of cerium oxide nanoparticles in atherosclerosis-prone mice." *Environmental Research* **115**: 1-10.

Cassee, F. R., E. C. van Balen, C. Singh, D. Green, H. Muijser, J. Weinstein and K. Dreher (2011). "Exposure, health and ecological effects review of engineered nanoscale cerium and cerium oxide associated with its use as a fuel additive." *Critical Reviews in Toxicology* **41**(3): 213-229.

Celardo, I., M. De Nicola, C. Mandoli, J. Z. Pedersen, E. Traversa and L. Ghibelli (2011). "Ce(3)+ ions determine redox-dependent anti-apoptotic effect of cerium oxide nanoparticles." *ACS Nano* **5**(6): 4537-4549.

Chaudhury, K., K. N. Babu, A. K. Singh, S. Das, A. Kumar and S. Seal (2013). "Mitigation of endometriosis using regenerative cerium oxide nanoparticles." *Nanomedicine* **9**(3): 439-448.

Chen, M., M. Zhang, J. Borlak and W. Tong (2012). "A decade of toxicogenomic research and its contribution to toxicological science." *Toxicological Sciences* **130**(2): 217-228.

Chen, S., Y. Hou, G. Cheng, C. Zhang, S. Wang and J. Zhang (2013). "Cerium oxide nanoparticles protect endothelial cells from apoptosis induced by oxidative stress." *Biological Trace Element Research* **154**(1): 156-166.

Chigurupati, S., M. R. Mughal, E. Okun, S. Das, A. Kumar, M. McCaffery, S. Seal and M. P. Mattson (2013). "Effects of cerium oxide nanoparticles on the growth of keratinocytes, fibroblasts and vascular endothelial cells in cutaneous wound healing." *Biomaterials* **34**(9): 2194-2201.

Cho, E. C., Q. Zhang and Y. Xia (2011). "The effect of sedimentation and diffusion on cellular uptake of gold nanoparticles." *Nature Nanotechnology* **6**(6): 385-391.

Cho, W.S., R. Duffin, C. A. Poland, S. E. M. Howie, W. MacNee, M. Bradley, I. L. Megson and K. Donaldson (2010). "Metal oxide nanoparticles induce unique inflammatory footprints in the lung: important implications for nanoparticle testing." *Environmental Health Perspectives* **118**(12): 1699-1706.

Cho, W.S., R. Duffin, F. Thielbeer, M. Bradley, I. L. Megson, W. MacNee, C. A. Poland, C. L. Tran and K. Donaldson (2012). "Zeta potential and solubility to toxic ions as mechanisms of lung inflammation caused by metal/metal-oxide nanoparticles." *Toxicological Sciences* **126**(2):469-477

Cho, W.S., R. Duffin, M. Bradley, I. L. Megson, W. MacNee, J. K. Lee, J. Jeong and K. Donaldson (2013). "Predictive value of in vitro assays depends on the mechanism of toxicity of metal oxide nanoparticles." *Particle and Fibre Toxicology* **10**(1): 55.

Ciofani, G., G. G. Genchi, I. Liakos, V. Cappello, M. Gemmi, A. Athanassiou, B. Mazzolai and V. Mattoli (2013). "Effects of cerium oxide nanoparticles on PC12 neuronal-like cells: proliferation, differentiation, and dopamine secretion." *Pharmaceutical Research* **30**(8): 2133-2145.

Ciofani, G., G. G. Genchi, B. Mazzolai and V. Mattoli (2014). "Transcriptional profile of genes involved in oxidative stress and antioxidant defense in PC12 cells following treatment with cerium oxide nanoparticles." *Biochimica et Biophysica Acta* **1**: 495-506.

Collins, A. R. (2004). "The comet assay for DNA damage and repair." *Molecular Biotechnology* **26**(3): 249-261.

Colognato, R., Park, M.V.D.Z., Wick, P., De Jong, W.H. (2012). Chapter 1. Interactions with the human body. In: "Adverse effects of engineered nanomaterials: Exposure, Toxicology, and Impact on Human Health" (Fadeel, B., Pietroiusti, A., Shvedova, A.A. Eds), pp 3-24. Academic Press-Elsevier, San Diego.

Comfort, K. K., L. K. Braydich-Stolle, E. I. Maurer and S. M. Hussain (2014). "Less is more: long-term in vitro exposure to low levels of silver nanoparticles provides new insights for nanomaterial evaluation." *ACS Nano* **8**(4): 3260-3271.

Cooper, R. J. and N. Spitzer (2015). "Silver nanoparticles at sublethal concentrations disrupt cytoskeleton and neurite dynamics in cultured adult neural stem cells." *Neurotoxicology* **48**: 231-238.

Costa, P. M. and B. Fadeel (2016). "Emerging systems biology approaches in nanotoxicology: Towards a mechanism-based understanding of nanomaterial hazard and risk." *Toxicology and Applied Pharmacology* **299**: 101-111.

Crist, R. M., J. H. Grossman, A. K. Patri, S. T. Stern, M. A. Dobrovolskaia, P. P. Adiseshaiah, J. D. Clogston and S. E. McNeil (2013). "Common pitfalls in nanotechnology: lessons learned from NCI's Nanotechnology Characterization Laboratory." *Integrative Biology* **5**(1): 66-73.

Cuillel, M., M. Chevallet, P. Charbonnier, C. Fauquant, I. Pignot-Paintrand, J. Arnaud, D. Cassio, I. Michaud-Soret and E. Mintz (2014). "Interference of CuO nanoparticles with metal homeostasis in hepatocytes under sub-toxic conditions." *Nanoscale* **6**(3): 1707-1715.

Cupaioli, F. A., F. A. Zucca, D. Boraschi and L. Zecca (2014). "Engineered nanoparticles. How brain friendly is this new guest?" *Progress in Neurobiology* **119-120**: 20-38.

D'Angelo, B., S. Santucci, E. Benedetti, S. Di Loreto, R. Phani, S. Falone, F. Amicarelli, M. P. Ceru and A. Cimini (2009). "Cerium oxide nanoparticles trigger neuronal survival in a human Alzheimer disease model by modulating BDNF pathway." *Current Nanoscience* **5**(2): 167-176.

Daboussi, F., A. Dumay, F. Delacote and B. S. Lopez (2002). "DNA double-strand break repair signalling: the case of RAD51 post-translational regulation." *Cellular Signaling* **14**(12): 969-975.

de Jager, W., H. te Velthuis, B. J. Prakken, W. Kuis and G. T. Rijkers (2003). "Simultaneous detection of 15 human cytokines in a single sample of stimulated peripheral blood mononuclear cells." *Clinical and Diagnostic Laboratory Immunology* **10**(1): 133-139.

Dedeurwaerder, S., M. Defrance, E. Calonne, H. Denis, C. Sotiriou and F. Fuks (2011). "Evaluation of the Infinium Methylation 450K technology." *Epigenomics* **3**(6): 771-784.

Deshpande, S., S. Patil, S. V. Kuchibhatla and S. Seal (2005). "Size dependency variation in lattice parameter and valency states in nanocrystalline cerium oxide." *Applied Physics Letters* **87**(13): 133113.

Di Bucchianico, S., F. Cappellini, F. Le Bihanic, Y. Zhang, K. Dreij and H. L. Karlsson (2016). "Genotoxicity of TiO2 nanoparticles assessed by mini-gel comet assay and micronucleus scoring with flow cytometry." *Mutagenesis*. Published online: July 5, 2016 [Epub ahead of print].

Docter, D., D. Westmeier, M. Markiewicz, S. Stolte, S. K. Knauer and R. H. Stauber (2015). "The nanoparticle biomolecule corona: lessons learned - challenge accepted?" *Chemical Society Reviews* **44**(17): 6094-6121.

Donaldson, K. and C. A. Poland (2012). "Inhaled nanoparticles and lung cancer - what we can learn from conventional particle toxicology." *Swiss Medical Weekly* **142**: w13547.

Donaldson, K. and C. A. Poland (2013). "Nanotoxicity: challenging the myth of nano-specific toxicity." *Current Opinion in Biotechnology* **24**(4): 724-734.

Donaldson, K., C. A. Poland, F. A. Murphy, M. MacFarlane, T. Chernova and A. Schinwald (2013). "Pulmonary toxicity of carbon nanotubes and asbestos - similarities and differences." *Advanced Drug Delivery Reviews* **65**(15): 2078-2086.

Donaldson, K., C. A. Poland and R. P. F. Schins (2010). "Possible genotoxic mechanisms of nanoparticles: Criteria for improved test strategies." *Nanotoxicology* **4**(4): 414-420.

Donaldson, K. and A. Seaton (2012). "A short history of the toxicology of inhaled particles." *Particle and Fibre Toxicology* **9**: 13.

Donaldson, K., V. Stone, C. L. Tran, W. Kreyling and P. J. Borm (2004). "Nanotoxicology." *Occupational and Environmental Medicine* **61**(9): 727-728.

Dowding, J. M., S. Das, A. Kumar, T. Dosani, R. McCormack, A. Gupta, T. X. T. Sayle, D. C. Sayle, L. von Kalm, S. Seal and W. T. Self (2013). "Cellular Interaction and Toxicity Depends on Physiochemical Properties and Surface Modification of Redox Active Nanomaterials." *ACS Nano* **7**(6): 4855-4868

Korpelainen, E., J. Tuimala, P. Somervuo, M. Huss, G. Wong (2014). Chapter 1. Introduction to RNA-Sequencing. In: "RNA-Seq data analysis:a practical approach", pp: 1-26, CRC Press, Taylor and Francis Group.

Eitan, E., E. R. Hutchison, N. H. Greig, D. Tweedie, H. Celik, S. Ghosh, K. W. Fishbein, R. G. Spencer, C. Y. Sasaki, P. Ghosh, S. Das, S. Chigurapati, J. Raymick, S. Sarkar, S. Chigurupati, S. Seal and M. P. Mattson (2015). "Combination therapy with lenalidomide and nanoceria ameliorates CNS autoimmunity." *Experimental Neurology* **273**: 151-160.

El-Kemary, M., N. Nagy and I. El-Mehasseb (2013). "Nickel oxide nanoparticles: Synthesis and spectral studies of interactions with glucose." *Materials Science in Semiconductor Processing* **16**(6): 1747-1752.

Elder, A., R. Gelein, V. Silva, T. Feikert, L. Opanashuk, J. Carter, R. Potter, A. Maynard, Y. Ito, J. Finkelstein and G. Oberdörster (2006). "Translocation of inhaled ultrafine manganese oxide particles to the central nervous system." *Environmental Health Perspectives* **114**(8): 1172-1178.

Eom, H.J., N. Chatterjee, J. Lee and J. Choi (2014). "Integrated mRNA and micro RNA profiling reveals epigenetic mechanism of differential sensitivity of Jurkat T cells to AgNPs and Ag ions." *Toxicology Letters* **229**(1): 311-318.

Eom, H.J. and J. Choi (2009). "Oxidative stress of CeO_2 nanoparticles via p38-Nrf-2 signaling pathway in human bronchial epithelial cell, Beas-2B." *Toxicology Letters* **187**(2): 77-83.

Estevez, A. Y., S. Pritchard, K. Harper, J. W. Aston, A. Lynch, J. J. Lucky, J. S. Ludington, P. Chatani, W. P. Mosenthal, J. C. Leiter, S. Andreescu and J. S. Erlichman (2011). "Neuroprotective mechanisms of cerium oxide nanoparticles in a mouse hippocampal brain slice model of ischemia." *Free Radical Biology and Medicine* **51**(6): 1155-1163.

EU commission (2011). Commission Recommendation on the definition of nanomaterial. Official Journal of the European Union 2011/696/EU.

Fadeel, B., A. Fornara, M. S. Toprak and K. Bhattacharya (2015). "Keeping it real: The importance of material characterization in nanotoxicology." *Biochemical and Biophysical Research Communications* **468**(3): 498-503.

Feliu, N., P. Kohonen, J. Ji, Y. Zhang, H. L. Karlsson, L. Palmberg, A. Nystrom and B. Fadeel (2015). "Next-generation sequencing reveals low-dose effects of cationic dendrimers in primary human bronchial epithelial cells." *ACS Nano* **9**(1): 146-163.

Fenech, M. (2007). "Cytokinesis-block micronucleus cytome assay." *Nature Protocols* **2**(5): 1084-1104.

Fenech, M., M. Kirsch-Volders, A. T. Natarajan, J. Surralles, J. W. Crott, J. Parry, H. Norppa, D. A. Eastmond, J. D. Tucker and P. Thomas (2011). "Molecular mechanisms of micronucleus, nucleoplasmic bridge and nuclear bud formation in mammalian and human cells." *Mutagenesis* **26**(1): 125-132.

Fissan, H., S. Ristig, H. Kaminski, C. Asbach and M. Epple (2014). "Comparison of different characterization methods for nanoparticle dispersions before and after aerosolization." *Analytical Methods* **6**(18): 7324-7334.

Foldbjerg, R., D. A. Dang and H. Autrup (2011). "Cytotoxicity and genotoxicity of silver nanoparticles in the human lung cancer cell line, A549." *Archives of Toxicology* **85**(7): 743-750.

Foldbjerg, R., X. Jiang, T. Miclaus, C. Chen, H. Autrup and C. Beer (2015). "Silver nanoparticles - wolves in sheep's clothing?" *Toxicology Research* **4**(3): 563-575.

Fröhlich, E. (2012). "The role of surface charge in cellular uptake and cytotoxicity of medical nanoparticles." *International Journal of Nanomedicine* **7**: 5577-5591.

Gentleman, R. C., V. J. Carey, D. M. Bates, B. Bolstad, M. Dettling, S. Dudoit, B. Ellis, L. Gautier, Y. Ge, J. Gentry, K. Hornik, T. Hothorn, W. Huber, S. Iacus, R. Irizarry, F. Leisch, C. Li, M. Maechler, A. J. Rossini, G. Sawitzki, C. Smith, G. Smyth, L. Tierney, J. Y. Yang and J. Zhang (2004). "Bioconductor: open software development for computational biology and bioinformatics." *Genome Biology* **5**(10): R80.

George, S., S. Pokhrel, T. Xia, B. Gilbert, Z. Ji, M. Schowalter, A. Rosenauer, R. Damoiseaux, K. A. Bradley, L. Madler and A. E. Nel (2010). "Use of a rapid cytotoxicity screening approach to engineer a safer zinc oxide nanoparticle through iron doping." *ACS Nano* **4**(1): 15-29.

Giam, M. and G. Rancati (2015). "Aneuploidy and chromosomal instability in cancer: a jackpot to chaos." *Cell Division* **10**(1): 1-12.

Gilbert, B., S. C. Fakra, T. Xia, S. Pokhrel, L. Mädler and A. E. Nel (2012). "The fate of ZnO nanoparticles administered to human bronchial epithelial cells." *ACS Nano* **6**(6): 4921-4930.

Giri, S., A. Karakoti, R. P. Graham, J. L. Maguire, C. M. Reilly, S. Seal, R. Rattan and V. Shridhar (2013). "Nanoceria: a rare-earth nanoparticle as a novel anti-angiogenic therapeutic agent in ovarian cancer." *PLoS One* **8**(1): e54578.

Gliga, A. R., S. Skoglund, I. Odnevall Wallinder, B. Fadeel and H. L. Karlsson (2014). "Size-dependent cytotoxicity of silver nanoparticles in human lung cells: the role of celbalklular uptake, agglomeration and Ag release." *Particle and Fibre Toxicology* **11**(1): 1-17.

Gonzalez, L., B. J. Sanderson and M. Kirsch-Volders (2011). "Adaptations of the in vitro MN assay for the genotoxicity assessment of nanomaterials." *Mutagenesis* **26**(1): 185-191.

Goodman, J. E., R. L. Prueitt, S. Thakali and A. R. Oller (2011). "The nickel ion bioavailability model of the carcinogenic potential of nickel-containing substances in the lung." *Critical Reviews in Toxicology* **41**(2): 142-174.

Grosse, Y., D. Loomis, K. Z. Guyton, B. Lauby-Secretan, F. El Ghissassi, V. Bouvard, L. Benbrahim-Tallaa, N. Guha, C. Scoccianti, H. Mattock and K. Straif (2014). "Carcinogenicity of fluoro-edenite, silicon carbide fibres and whiskers, and carbon nanotubes." *The Lancet Oncology* **15**(13): 1427-1428.

Guo, X., Y. Li, J. Yan, T. Ingle, M. Y. Jones, N. Mei, M. D. Boudreau, C. K. Cunningham, M. Abbas, A. M. Paredes, T. Zhou, M. M. Moore, P. C. Howard and T. Chen (2016). "Size- and coating-dependent cytotoxicity and genotoxicity of silver nanoparticles evaluated using in vitro standard assays." *Nanotoxicology* **10**(9): 1373-1384.

Hamilton, R. F., N. Wu, D. Porter, M. Buford, M. Wolfarth and A. Holian (2009). "Particle length-dependent titanium dioxide nanomaterials toxicity and bioactivity." *Particle and Fibre Toxicology* **6**(1): 1-11.

Han, X., N. Corson, P. Wade-Mercer, R. Gelein, J. Jiang, M. Sahu, P. Biswas, J. N. Finkelstein, A. Elder and G. Oberdörster (2012). "Assessing the relevance of in vitro studies in nanotoxicology by examining correlations between in vitro and in vivo data." *Toxicology* **297**(1-3): 1-9.

Han, X., R. Gelein, N. Corson, P. Wade-Mercer, J. Jiang, P. Biswas, J. N. Finkelstein, A. Elder and G. Oberdörster (2011). "Validation of an LDH Assay for Assessing Nanoparticle Toxicity." *Toxicology* **287**(1-3): 99-104.

Hardas, S. S., R. Sultana, G. Warrier, M. Dan, R. L. Florence, P. Wu, E. A. Grulke, M. T. Tseng, J. M. Unrine, U. M. Graham, R. A. Yokel and D. A. Butterfield (2012). "Rat brain pro-oxidant effects of peripherally administered 5 nm ceria 30 days after exposure." *Neurotoxicology* **33**(5): 1147-1155.

Hardas, S. S., R. Sultana, G. Warrier, M. Dan, P. Wu, E. A. Grulke, M. T. Tseng, J. M. Unrine, U. M. Graham, R. A. Yokel and D. A. Butterfield (2014). "Rat hippocampal responses up to 90 days after a single nanoceria dose extends a hierarchical oxidative stress model for nanoparticle toxicity." *Nanotoxicology* **1**: 155-166.

Hartung, T. and M. McBride (2011). "Food for Thought ... on mapping the human toxome." *Alternatives to Animal Experimentation* **28**(2): 83-93.

Hartung, T., E. van Vliet, J. Jaworska, L. Bonilla, N. Skinner and R. Thomas (2012). "Systems toxicology." *Alternatives to Animal Experimentation* **29**(2): 119-128.

Heckman, K. L., W. DeCoteau, A. Estevez, K. J. Reed, W. Costanzo, D. Sanford, J. C. Leiter, J. Clauss, K. Knapp, C. Gomez, P. Mullen, E. Rathbun, K. Prime, J. Marini, J. Patchefsky, A. S. Patchefsky, R. K. Hailstone and J. S. Erlichman (2013). "Custom cerium oxide nanoparticles protect against a free radical mediated autoimmune degenerative disease in the brain." *ACS Nano* **7**(12): 10582-10596.

Hendriks, G., M. Atallah, B. Morolli, F. Calleja, N. Ras-Verloop, I. Huijskens, M. Raamsman, B. van de Water and H. Vrieling (2012). "The ToxTracker assay: novel GFP reporter systems that provide mechanistic insight into the genotoxic properties of chemicals." *Toxicological Sciences* **125**(1): 285-298.

Hinderliter, P. M., K. R. Minard, G. Orr, W. B. Chrisler, B. D. Thrall, J. G. Pounds and J. G. Teeguarden (2010). "ISDD: A computational model of particle sedimentation, diffusion and target cell dosimetry for in vitro toxicity studies." *Particle and Fibre Toxicology* **7**(1): 36.

Holt, B. D., P. A. Short, A. D. Rape, Y. L. Wang, M. F. Islam and K. N. Dahl (2010). "Carbon nanotubes reorganize actin structures in cells and ex vivo." *ACS Nano* **4**(8): 4872-4878.

Hosseini, A., M. Baeeri, M. Rahimifard, M. Navaei-Nigjeh, A. Mohammadirad, N. Pourkhalili, S. Hassani, M. Kamali and M. Abdollahi (2013). "Antiapoptotic effects of cerium oxide and yttrium oxide nanoparticles in isolated rat pancreatic islets." *Human and Experimental Toxicology* **32**(5): 544-553.

Hsiao, I. L., Y. K. Hsieh, C. F. Wang, I. C. Chen and Y. J. Huang (2015). "Trojan-horse mechanism in the cellular uptake of silver nanoparticles verified by direct intra- and extracellular silver speciation analysis." *Environmental Science & Technology* **49**(6): 3813-3821.

Huang, S., P. J. Chueh, Y. W. Lin, T. S. Shih and S. M. Chuang (2009). "Disturbed mitotic progression and genome segregation are involved in cell transformation mediated by nano-TiO2 long-term exposure." *Toxicology and Applied Pharmacology* **241**(2): 182-194.

Hubbs, A. F., L. M. Sargent, D. W. Porter, T. M. Sager, B. T. Chen, D. G. Frazer, V. Castranova, K. Sriram, T. R. Nurkiewicz, S. H. Reynolds, L. A. Battelli, D. Schwegler-Berry, W. McKinney, K. L. Fluharty and R. R. Mercer (2013). "Nanotechnology: Toxicologic Pathology." *Toxicologic pathology* **41**(2): 395-409.

Hubert R., G. Roebben, A. Boix Sanfeliu, H. Emons, P. Gibson, R. Koeber, T. Linsinger, K. Rasmussen, J. Riego Sintes, B. Sokull-Kluettgen, H. Stamm (2015). Towards a review of the EC Recommendation for a definition of the term "nanomaterial": Part 3: Scientific-technical evaluation of options to clarify the definition and to facilitate its implementation, Publications Office of the European Union.

Huh, D., B. D. Matthews, A. Mammoto, M. Montoya-Zavala, H. Y. Hsin and D. E. Ingber (2010). "Reconstituting Organ-Level Lung Functions on a Chip." *Science* **328**(5986): 1662-1668.

Hussain, S., F. Al-Nsour, A. B. Rice, J. Marshburn, B. Yingling, Z. Ji, J. I. Zink, N. J. Walker and S. Garantziotis (2012). "Cerium dioxide nanoparticles induce apoptosis and autophagy in human peripheral blood monocytes." *ACS Nano* **6**(7): 5820-5829.

Hussain, S. M., D. B. Warheit, S. P. Ng, K. K. Comfort, C. M. Grabinski and L. K. Braydich-Stolle (2015). "At the crossroads of nanotoxicology in vitro: past achievements and current challenges." *Toxicological Sciences* **147**(1): 5-16.

IARC (1997, 68). "IARC monographs on the evaluation of carcinogenic risks to humans. Silica, Some Silicates, Coal Dust and para-Aramid Fibrils."

IARC (2012, 100C). "IARC monographs on the evaluation of carcinogenic risks to humans. Arsenic, metals, fibres and dusts."

IARC (2013, 105). "IARC monographs on the evaluation of carcinogenic risks to humans. Diesel and Gasoline Engine Exhausts and Some Nitroarenes."

IARC (2016, 109). "IARC monographs on the evaluation of carcinogenic risks to humans. Outdoor Air Pollution."

Illumina. (2012). "DNA Methylation Analysis." Pub. No.270-2008-001, current as of 6 April 2012, accessed Sept 2016 from: http://www.illumina.com/Documents/products/datasheets/datasheet_dna_methylation_analysis.pdf.

Illumina. (2016). "An Introduction to Next-Generation Sequencing Technology." Pub. No. 770-2012-008 current as of 25 May 2016, accessed Sept 2016 from: http://www.illumina.com/content/dam/illumina-marketing/documents/products/illumina_sequencing_introduction.pdf.

Ji, J. H., J. H. Jung, S. S. Kim, J. U. Yoon, J. D. Park, B. S. Choi, Y. H. Chung, I. H. Kwon, J. Jeong, B. S. Han, J. H. Shin, J. H. Sung, K. S. Song and I. J. Yu (2007). "Twenty-eight-day inhalation toxicity study of silver nanoparticles in Sprague-Dawley rats." *Inhalation Toxicology* **19**(10): 857-871.

Jiang, X., T. Miclaus, L. Wang, R. Foldbjerg, D. S. Sutherland, H. Autrup, C. Chen and C. Beer (2015). "Fast intracellular dissolution and persistent cellular uptake of silver nanoparticles in CHO-K1 cells: implication for cytotoxicity." *Nanotoxicology* **9**(2): 181-189.

Johnston, H., G. Pojana, S. Zuin, N. R. Jacobsen, P. Moller, S. Loft, M. Semmler-Behnke, C. McGuiness, D. Balharry, A. Marcomini, H. Wallin, W. Kreyling, K. Donaldson, L. Tran and V. Stone (2013). "Engineered nanomaterial risk. Lessons learnt from completed nanotoxicology studies: potential solutions to current and future challenges." *Critical Reviews in Toxicology* **43**(1): 1-20.

Jomova, K. and M. Valko (2011). "Advances in metal-induced oxidative stress and human disease." *Toxicology* **283**(2–3): 65-87.

Jugan, M.-L., S. Barillet, A. Simon-Deckers, N. Herlin-Boime, S. Sauvaigo, T. Douki and M. Carriere (2012). "Titanium dioxide nanoparticles exhibit genotoxicity and impair DNA repair activity in A549 cells." *Nanotoxicology* **6**(5): 501-513.

Kagan, V. E., N. V. Konduru, W. Feng, B. L. Allen, J. Conroy, Y. Volkov, I. I. Vlasova, N. A. Belikova, N. Yanamala, A. Kapralov, Y. Y. Tyurina, J. Shi, E. R. Kisin, A. R. Murray, J. Franks, D. Stolz, P. Gou, J. Klein-Seetharaman, B. Fadeel, A. Star and A. A. Shvedova (2010). "Carbon nanotubes degraded by neutrophil myeloperoxidase induce less pulmonary inflammation." *Nature Nanotechnology* **5**(5): 354-359.

Kain, J., H. L. Karlsson and L. Moller (2012). "DNA damage induced by micro- and nanoparticles--interaction with FPG influences the detection of DNA oxidation in the comet assay." *Mutagenesis* **27**(4): 491-500.

Kalyanaraman, B., V. Darley-Usmar, K. J. A. Davies, P. A. Dennery, H. J. Forman, M. B. Grisham, G. E. Mann, K. Moore, L. J. Roberts and H. Ischiropoulos (2012). "Measuring reactive oxygen and nitrogen species with fluorescent probes: challenges and limitations." *Free radical biology & medicine* **52**(1): 1-6.

Kao, Y. Y., T. J. Cheng, D. M. Yang, C. T. Wang, Y. M. Chiung and P. S. Liu (2012). "Demonstration of an olfactory bulb-brain translocation pathway for ZnO nanoparticles in rodent cells in vitro and in vivo." *Journal of Molecular Neuroscience* **48**(2): 464-471.

Karlsson, H. L., P. Cronholm, J. Gustafsson and L. Möller (2008). "Copper Oxide Nanoparticles Are Highly Toxic: A Comparison between Metal Oxide Nanoparticles and Carbon Nanotubes." *Chemical Research in Toxicology* **21**(9): 1726-1732.

Karlsson, H. L., A. R. Gliga, F. M. Calleja, C. S. Goncalves, I. O. Wallinder, H. Vrieling, B. Fadeel and G. Hendriks (2014). "Mechanism-based genotoxicity screening of metal oxide nanoparticles using the ToxTracker panel of reporter cell lines." *Particle and Fibre Toxicology* **11**: 41.

Karlsson, H. L., Toprak M.S., Fadeel, B. (2015a). Chapter 4. Toxicity of Metal and Metal Oxide Nanoparticles. In: "Handbook on the Toxicology of Metals (4th Edition)". (Nordberg G.F., Fowler B.A., Nordberg M. Eds), pp 75-112. Academic Press-Elsevier, Amsterdam.

Karlsson, H. L., S. Di Bucchianico, A. R. Collins and M. Dusinska (2015b). "Can the comet assay be used reliably to detect nanoparticle-induced genotoxicity?" *Environmental and Molecular Mutagenesis* **56**(2): 82-96.

Karlsson, M., T. Kurz, U. T. Brunk, S. E. Nilsson and C. I. Frennesson (2010). "What does the commonly used DCF test for oxidative stress really show?" *Biochemical Journal* **428**(2): 183-190.

Kennedy, K. A., S. D. Sandiford, I. S. Skerjanc and S. S. Li (2012). "Reactive oxygen species and the neuronal fate." *Cellular and Molecular Life Sciences* **69**(2): 215-221.

Kim, C. K., T. Kim, I. Y. Choi, M. Soh, D. Kim, Y. J. Kim, H. Jang, H. S. Yang, J. Y. Kim, H. K. Park, S. P. Park, S. Park, T. Yu, B. W. Yoon, S. H. Lee and T. Hyeon (2012). "Ceria nanoparticles that can protect against ischemic stroke." *Angewandte Chemie International Edition* **51**(44): 11039-11043.

Kim, J. S., J. H. Sung, J. H. Ji, K. S. Song, J. H. Lee, C. S. Kang and I. J. Yu (2011). "In vivo Genotoxicity of Silver Nanoparticles after 90-day Silver Nanoparticle Inhalation Exposure." *Safety and Health at Work* **2**(1): 34-38.

Kittler, S., C. Greulich, J. S. Gebauer, J. Diendorf, L. Treuel, L. Ruiz, J. M. Gonzalez-Calbet, M. Vallet-Regi, R. Zellner, M. Koller and M. Epple (2010). "The influence of proteins on the dispersability and cell-biological activity of silver nanoparticles." *Journal of Materials Chemistry* **20**(3): 512-518.

Kołodziejczak-Radzimska, A. and T. Jesionowski (2014). "Zinc Oxide—From Synthesis to Application: A Review." *Materials* **7**(4): 2833.

Kong, B., J. H. Seog, L. M. Graham and S. B. Lee (2011a). "Experimental considerations on the cytotoxicity of nanoparticles." *Nanomedicine (Lond)* **6**(5): 929-941.

Kong, L., X. Cai, X. Zhou, L. L. Wong, A. S. Karakoti, S. Seal and J. F. McGinnis (2011b). "Nanoceria extend photoreceptor cell lifespan in tubby mice by modulation of apoptosis/survival signaling pathways." *Neurobiology of Disease* **42**(3): 514-523.

Koppenaal, D. W., G. C. Eiden and C. J. Barinaga (2004). "Collision and reaction cells in atomic mass spectrometry: development, status, and applications." *Journal of Analytical Atomic Spectrometry* **19**(5): 561-570.

Kreyling, W. G., M. Semmler-Behnke, J. Seitz, W. Scymczak, A. Wenk, P. Mayer, S. Takenaka and G. Oberdörster (2009). "Size dependence of the translocation of inhaled iridium and carbon nanoparticle aggregates from the lung of rats to the blood and secondary target organs." *Inhalation Toxicology* **21**(Suppl 1): 55-60.

Kroll, A., M. H. Pillukat, D. Hahn and J. Schnekenburger (2012). "Interference of engineered nanoparticles with in vitro toxicity assays." *Archives of Toxicology* **86**(7): 1123-1136.

Krug, H. F. (2014). "Nanosafety Research—Are We on the Right Track?" *Angewandte Chemie International Edition* **53**(46): 12304-12319.

Krug, H. F. and P. Wick (2011). "Nanotoxicology: An Interdisciplinary Challenge." *Angewandte Chemie International Edition* **50**(6): 1260-1278.

Kuhn, D. A., D. Vanhecke, B. Michen, F. Blank, P. Gehr, A. Petri-Fink and B. Rothen-Rutishauser (2014). "Different endocytotic uptake mechanisms for nanoparticles in epithelial cells and macrophages." *Beilstein Journal of Nanotechnology* **5**: 1625-1636.

Kwon, J. Y., S. Y. Lee, P. Koedrith, J. Y. Lee, K.-M. Kim, J.-M. Oh, S. I. Yang, M.-K. Kim, J. K. Lee, J. Jeong, E. H. Maeng, B. J. Lee and Y. R. Seo (2014). "Lack of genotoxic potential of ZnO nanoparticles in in vitro and in vivo tests." *Mutation Research/Genetic Toxicology and Environmental Mutagenesis* **761**: 1-9.

Kyosseva, S. V., L. Chen, S. Seal and J. F. McGinnis (2013). "Nanoceria inhibit expression of genes associated with inflammation and angiogenesis in the retina of Vldlr null mice." *Experimental Eye Research* **116**: 63-74.

Lancaster, M.V. and Fields, R.D (1996). Antibiotic and Cytotoxic Drug Susceptibility Assays using Resazurin and Poising Agents. US. [Patent no. 5,501,959]

Lanone, S., F. Rogerieux, J. Geys, A. Dupont, E. Maillot-Marechal, J. Boczkowski, G. Lacroix and P. Hoet (2009). "Comparative toxicity of 24 manufactured nanoparticles in human alveolar epithelial and macrophage cell lines." *Particle and Fibre Toxicology* **6**(1): 1-12.

Latvala, S., J. Hedberg, S. Di Bucchianico, L. Möller, I. Odnevall Wallinder, K. Elihn and H. L. Karlsson (2016). "Nickel release, ROS generation and toxicity of Ni and NiO micro- and nanoparticles." *PLoS ONE* **11**(7): e0159684.

Li, L., W. Jiang, K. Luo, H. Song, F. Lan, Y. Wu and Z. Gu (2013). "Superparamagnetic iron oxide nanoparticles as MRI contrast agents for non-invasive stem cell labeling and tracking." *Theranostics* **3**(8): 595-615.

Li, T.-S. and E. Marbán (2010). "Physiological levels of reactive oxygen species are required to maintain genomic stability in stem cells." *Stem cells* **28**(7): 1178-1185.

Limbach, L. K., P. Wick, P. Manser, R. N. Grass, A. Bruinink and W. J. Stark (2007). "Exposure of engineered nanoparticles to human lung epithelial cells: influence of chemical composition and catalytic activity on oxidative stress." *Environmental Science & Technology* **41**(11): 4158-4163.

Lin, J., H. Zhang, Z. Chen and Y. Zheng (2010). "Penetration of lipid membranes by gold nanoparticles: insights into cellular uptake, cytotoxicity, and their relationship." *ACS Nano* **4**(9): 5421-5429.

Lin, S., X. Wang, Z. Ji, C. H. Chang, Y. Dong, H. Meng, Y. P. Liao, M. Wang, T. B. Song, S. Kohan, T. Xia, J. I. Zink and A. E. Nel (2014). "Aspect ratio plays a role in the hazard potential of CeO2 nanoparticles in mouse lung and zebrafish gastrointestinal tract." *ACS Nano* **8**(5): 4450-4464.

Love, M. I., W. Huber and S. Anders (2014). "Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2." *Genome Biology* **15**(12): 1-21.

Luanpitpong, S., L. Wang, V. Castranova and Y. Rojanasakul (2014). "Induction of stem-like cells with malignant properties by chronic exposure of human lung epithelial cells to single-walled carbon nanotubes." *Particle and Fibre Toxicology* **11**: 22.

Lundqvist, J., J. El Andaloussi-Lilja, C. Svensson, H. Gustafsson Dorfh and A. Forsby (2013). "Optimisation of culture conditions for differentiation of C17.2 neural stem cells to be used for in vitro toxicity tests." *Toxicology In Vitro* **27**(5): 1565-1569.

Lung, S., F. R. Cassee, I. Gosens and A. Campbell (2014). "Brain suppression of AP-1 by inhaled diesel exhaust and reversal by cerium oxide nanoparticles." *Inhalation Toxicology* **26**(10): 636-641.

Ma, J., R. R. Mercer, M. Barger, D. Schwegler-Berry, J. M. Cohen, P. Demokritou and V. Castranova (2015). "Effects of amorphous silica coating on cerium oxide nanoparticles induced pulmonary responses." *Toxicology and Applied Pharmacology* **288**(1): 63-73.

Magdolenova, Z., A. Collins, A. Kumar, A. Dhawan, V. Stone and M. Dusinska (2014). "Mechanisms of genotoxicity. A review of in vitro and in vivo studies with engineered nanoparticles." *Nanotoxicology* **8**(3): 233-278.

Mah, L. J., A. El-Osta and T. C. Karagiannis (2010). "gammaH2AX: a sensitive molecular marker of DNA damage and repair." *Leukemia* **24**(4): 679-686.

Maher, B. A., I. A. M. Ahmed, V. Karloukovski, D. A. MacLaren, P. G. Foulds, D. Allsop, D. M. A. Mann, R. Torres-Jardón and L. Calderon-Garciduenas (2016). "Magnetite pollution nanoparticles in the human brain." *Proceedings of the National Academy of Sciences*. **113**(39): 10797–10801

Manke, A., L. Wang and Y. Rojanasakul (2013). "Mechanisms of nanoparticle-induced oxidative stress and toxicity." *BioMed Research International* Article ID **942916**

Maynard, A. D., D. B. Warheit and M. A. Philbert (2011). "The new toxicology of sophisticated materials: nanotoxicology and beyond." *Toxicological Sciences* **120** (Suppl 1): S109-129.

Meindl, C., M. Absenger, E. Roblegg and E. Fröhlich (2013). "Suitability of cell-based label-free detection for cytotoxicity screening of carbon nanotubes." *BioMed Research International* **2013**: 564804.

Mensaert, K., S. Denil, G. Trooskens, W. Van Criekinge, O. Thas and T. De Meyer (2014). "Next-generation technologies and data analytical approaches for epigenomics." *Environmental and Molecular Mutagenesis* **55**(3): 155-170.

Merril, C. R. (1990). "Silver staining of proteins and DNA." *Nature* **343**(6260): 779-780.

Mittal, S. and A. K. Pandey (2014). "Cerium oxide nanoparticles induced toxicity in human lung cells: role of ROS mediated DNA damage and apoptosis." *BioMed Research International* **2014**: 891934.

Monopoli, M. P., C. Aberg, A. Salvati and K. A. Dawson (2012). "Biomolecular coronas provide the biological identity of nanosized materials." *Nature Nanotechnology* **7**(12): 779-786.

Monteiro-Riviere, N. A., A. O. Inman and L. W. Zhang (2009). "Limitations and relative utility of screening assays to assess engineered nanoparticle toxicity in a human cell line." *Toxicology and Applied Pharmacology* **234**(2): 222-235.

Monteiro-Riviere, N. A., M. E. Samberg, S. J. Oldenburg and J. E. Riviere (2013). "Protein binding modulates the cellular uptake of silver nanoparticles into human cells: Implications for in vitro to in vivo extrapolations?" *Toxicology Letters* **220**(3): 286-293.

Morris, T. J. and S. Beck (2015). "Analysis pipelines and packages for Infinium HumanMethylation450 BeadChip (450k) data." *Methods* **72**: 3-8.

Mwilu, S. K., E. Siska, R. B. N. Baig, R. S. Varma, E. Heithmar and K. R. Rogers (2014). "Separation and measurement of silver nanoparticles and silver ions using magnetic particles." *Science of The Total Environment* **472**: 316-323.

Møller, P., N. R. Jacobsen, J. K. Folkmann, P. H. Danielsen, L. Mikkelsen, J. G. Hemmingsen, L. K. Vesterdal, L. Forchhammer, H. Wallin and S. Loft (2010). "Role of oxidative damage in toxicity of particulates." *Free Radical Research* **44**(1): 1-46.

Nel, A., T. Xia, L. Madler and N. Li (2006). "Toxic potential of materials at the nanolevel." *Science* **311**(5761): 622-627.

Nel, A., T. Xia, H. Meng, X. Wang, S. Lin, Z. Ji and H. Zhang (2013). "Nanomaterial toxicity testing in the 21st century: use of a predictive toxicological approach and high-throughput screening." *Accounts of Chemical Research* **46**(3): 607-621.

Nel, A. E., L. Madler, D. Velegol, T. Xia, E. M. V. Hoek, P. Somasundaran, F. Klaessig, V. Castranova and M. Thompson (2009). "Understanding biophysicochemical interactions at the nano-bio interface." *Nature Materials* **8**(7): 543-557.

Nelson, B. C., C. W. Wright, Y. Ibuki, M. Moreno-Villanueva, H. L. Karlsson, G. Hendriks, C. M. Sims, N. Singh and S. H. Doak (2016). "Emerging metrology for high-throughput nanomaterial genotoxicology." *Mutagenesis*. Published online: August 26, 2016 [Epub ahead of print]

Nguyen, T., P. Nioi and C. B. Pickett (2009). "The Nrf2-antioxidant response element signaling pathway and its activation by oxidative stress." *Journal of Biological Chemistry* **284**(20): 13291-13295.

Niu, J., K. Wang and P. E. Kolattukudy (2011). "Cerium oxide nanoparticles inhibit oxidative stress and nuclear factor-kappaB activation in H9c2 cardiomyocytes exposed to cigarette smoke extract." *Journal of Pharmacology and Experimental Therapeutics* **338**(1): 53-61.

Nowack, B., H. F. Krug and M. Height (2011). "120 years of nanosilver history: implications for policy makers." *Environmental Science & Technology* **45**(4): 1177-1183.

Nymark, P., J. Catalan, S. Suhonen, H. Jarventaus, R. Birkedal, P. A. Clausen, K. A. Jensen, M. Vippola, K. Savolainen and H. Norppa (2013). "Genotoxicity of polyvinylpyrrolidone-coated silver nanoparticles in BEAS 2B cells." *Toxicology* **313**(1): 38-48.

Nymark, P., P. Wijshoff, R. Cavill, M. van Herwijnen, M. L. Coonen, S. Claessen, J. Catalan, H. Norppa, J. C. Kleinjans and J. J. Briede (2015). "Extensive temporal transcriptome and microRNA analyses identify molecular mechanisms underlying mitochondrial dysfunction induced by multi-walled carbon nanotubes in human lung cells." *Nanotoxicology* **9**(5): 624-635.

Oberdörster, G., E. Oberdörster and J. Oberdörster (2005). "Nanotoxicology: an emerging discipline evolving from studies of ultrafine particles." *Environmental Health Perspectives* **113**(7): 823-839.

Oberdörster, G., Z. Sharp, V. Atudorei, A. Elder, R. Gelein, W. Kreyling and C. Cox (2004). "Translocation of Inhaled Ultrafine Particles to the Brain." *Inhalation Toxicology* **16**(6-7): 437-445.

Olive, P. L. and J. P. Banath (2006). "The comet assay: a method to measure DNA damage in individual cells." *Nature Protocols* **1**(1): 23-29.

Onodera, A., F. Nishiumi, K. Kakiguchi, A. Tanaka, N. Tanabe, A. Honma, K. Yayama, Y. Yoshioka, K. Nakahira, S. Yonemura, I. Yanagihara, Y. Tsutsumi and Y. Kawai (2015). "Short-term changes in intracellular ROS localisation after the silver nanoparticles exposure depending on particle size." *Toxicology Reports* **2**: 574-579.

Pagliari, F., C. Mandoli, G. Forte, E. Magnani, S. Pagliari, G. Nardone, S. Licoccia, M. Minieri, P. Di Nardo and E. Traversa (2012). "Cerium oxide nanoparticles protect cardiac progenitor cells from oxidative stress." *ACS Nano* **6**(5): 3767-3775.

Park, E.-J., J. Yi, Y. Kim, K. Choi and K. Park (2010). "Silver nanoparticles induce cytotoxicity by a Trojan-horse type mechanism." *Toxicology in Vitro* **24**(3): 872-878.

Park, E. J., J. Choi, Y. K. Park and K. Park (2008). "Oxidative stress induced by cerium oxide nanoparticles in cultured BEAS-2B cells." *Toxicology* **245**(1-2): 90-100.

Park, Y. H., D. Kim, J. Dai and Z. Zhang (2015). "Human bronchial epithelial BEAS-2B cells, an appropriate in vitro model to study heavy metals induced carcinogenesis." *Toxicology and Applied Pharmacology* **287**(3): 240-245.

Patchin, E. S., D. S. Anderson, R. M. Silva, D. L. Uyeminami, G. M. Scott, T. Guo, L. S. Van Winkle and K. E. Pinkerton (2016). "Size-dependent deposition, translocation, and microglial activation of inhaled silver nanoparticles in the rodent nose and brain." *Environmental Health Perspectives* Published online: May 6, 2016 [Epub ahead of print]

Powers, K. W., P. L. Carpinone and K. N. Siebein (2012). "Characterization of nanomaterials for toxicological studies." *Methods in Molecular Biology* **926**: 13-32.

Prasad, R. Y., J. K. McGee, M. G. Killius, D. A. Suarez, C. F. Blackman, D. M. DeMarini and S. O. Simmons (2013). "Investigating oxidative stress and inflammatory responses elicited by silver nanoparticles using high-throughput reporter genes in HepG2 cells: effect of size, surface coating, and intracellular uptake." *Toxicology In Vitro* **27**(6): 2013-2021.

Promega (2016). "Technical Bulletin CytoTox 96® Non-Radioactive, Cytotoxicity Assay." Accessed September 2016, from: https://se.promega.com/-/media/files/resources/protocols/technical-bulletins/0/cytotox-96-nonradioactive-cytotoxicity-assay-protocol.pdf.

Pulido-Reyes, G., I. Rodea-Palomares, S. Das, T. S. Sakthivel, F. Leganes, R. Rosal, S. Seal and F. Fernández-Piñas (2015). "Untangling the biological effects of cerium oxide nanoparticles: the role of surface valence states." *Scientific Reports* **5**: 15613.

Quadros, M. E. and L. C. Marr (2011). "Silver nanoparticles and total aerosols emitted by nanotechnology-related consumer spray products." *Environmental Science & Technology* **45**(24): 10713-10719.

Reddel, R. R., K. Yang, J. S. Rhim, D. Brash, R. T. Su, J. F. Lechner, B. I. Gerwin, C. C. Harris and P. Amstad (1989). Immortalized human bronchial epitherial mesothelial cell lines [Patent no US4885238]

Reinhold, W. C., M. A. Reimers, P. Lorenzi, J. Ho, U. T. Shankavaram, M. S. Ziegler, K. J. Bussey, S. Nishizuka, O. Ikediobi, Y. G. Pommier and J. N. Weinstein (2010). "Multifactorial regulation of E-cadherin expression: an integrative study." *Molecular Cancer Therapeutics* **9**(1): 1-16.

Ritchie, M. E., B. Phipson, D. Wu, Y. Hu, C. W. Law, W. Shi and G. K. Smyth (2015). "limma powers differential expression analyses for RNA-sequencing and microarray studies." *Nucleic Acids Research* **43**(7): e47.

Rosenman, K. D., A. Moss and S. Kon (1979). "Argyria: clinical implications of exposure to silver nitrate and silver oxide." *Journal of Occupational Medicine* **21**(6): 430-435.

Sadauskas, E., N. R. Jacobsen, G. Danscher, M. Stoltenberg, U. Vogel, A. Larsen, W. Kreyling and H. Wallin (2009). "Biodistribution of gold nanoparticles in mouse lung following intratracheal instillation." *Chemistry Central Journal* **3**(1): 1-7.

Samberg, M. E., S. J. Oldenburg and N. A. Monteiro-Riviere (2010). "Evaluation of silver nanoparticle toxicity in skin in vivo and keratinocytes in vitro." *Environmental Health Perspectives* **118**(3): 407-413.

Sargent, L. M., A. F. Hubbs, S. H. Young, M. L. Kashon, C. Z. Dinu, J. L. Salisbury, S. A. Benkovic, D. T. Lowry, A. R. Murray, E. R. Kisin, K. J. Siegrist, L. Battelli, J. Mastovich, J. L. Sturgeon, K. L. Bunker, A. A. Shvedova and S. H. Reynolds (2012). "Single-walled carbon nanotube-induced mitotic disruption." *Mutation Research* **745**(1-2): 28-37.

Sauer, H., M. Wartenberg and J. Hescheler (2001). "Reactive oxygen species as intracellular messengers during cell growth and differentiation." *Cellular Physiology and Biochemistry* **11**(4): 173-186.

Schermelleh, L., R. Heintzmann and H. Leonhardt (2010). "A guide to super-resolution fluorescence microscopy." *Journal of Cell Biology* **190**(2): 165-175.

Schins, R. P., A. M. Knaapen, G. D. Cakmak, T. Shi, C. Weishaupt and P. J. Borm (2002). "Oxidant-induced DNA damage by quartz in alveolar epithelial cells." *Mutation Research* **517**(1-2): 77-86.

Shannahan, J. H., R. Podila and J. M. Brown (2015). "A hyperspectral and toxicological analysis of protein corona impact on silver nanoparticle properties, intracellular modifications, and macrophage activation." *International Journal of Nanomedicine* **10**: 6509-6521.

Sharma, V., R. K. Shukla, N. Saxena, D. Parmar, M. Das and A. Dhawan (2009). "DNA damaging potential of zinc oxide nanoparticles in human epidermal cells." *Toxicology Letters* **185**(3): 211-218.

Shvedova, A. A., A. Pietroiusti, B. Fadeel and V. E. Kagan (2012). "Mechanisms of carbon nanotube-induced toxicity: Focus on oxidative stress." *Toxicology and applied pharmacology* **261**(2): 121-133.

Shyamasundar, S., C. T. Ng, L. Y. Lanry Yung, S. T. Dheen and B. H. Bay (2015). "Epigenetic mechanisms in nanomaterial-induced toxicity." *Epigenomics* **7**(3): 395-411.

Silva, R. M., D. S. Anderson, J. Peake, P. C. Edwards, E. S. Patchin, T. Guo, T. Gordon, L. C. Chen, X. Sun, L. S. Van Winkle and K. E. Pinkerton (2016). "Aerosolized Silver Nanoparticles in the Rat Lung and Pulmonary Responses over Time." *Toxicologic Pathology* **44**(5): 673-686.

Simon, D. F., R. F. Domingos, C. Hauser, C. M. Hutchins, W. Zerges and K. J. Wilkinson (2013). "Transcriptome sequencing (RNA-seq) analysis of the effects of metal nanoparticle exposure on the transcriptome of Chlamydomonas reinhardtii." *Applied and Environmental Microbiology* **79**(16): 4774-4785.

Snyder, E. Y., D. L. Deitcher, C. Walsh, S. Arnold-Aldea, E. A. Hartwieg and C. L. Cepko (1992). "Multipotent neural cell lines can engraft and participate in development of mouse cerebellum." *Cell* **68**(1): 33-51.

Song, K. S., J. H. Sung, J. H. Ji, J. H. Lee, J. S. Lee, H. R. Ryu, J. K. Lee, Y. H. Chung, H. M. Park, B. S. Shin, H. K. Chang, B. Kelman and I. J. Yu (2013). "Recovery from silver-nanoparticle-exposure-induced lung inflammation and lung function changes in Sprague Dawley rats." *Nanotoxicology* **7**(2): 169-180.

Sotiriou, G. A. and S. E. Pratsinis (2011). "Engineering nanosilver as an antibacterial, biosensor and bioimaging material." *Current opinion in chemical engineering* **1**(1): 3-10.

Stoehr, L. C., C. Endes, I. Radauer-Preiml, M. S. Boyles, E. Casals, S. Balog, M. Pesch, A. Petri-Fink, B. Rothen-Rutishauser, M. Himly, M. J. Clift and A. Duschl (2015). "Assessment of a panel of interleukin-8 reporter lung epithelial cell lines to monitor the pro-inflammatory response following zinc oxide nanoparticle exposure under different cell culture conditions." *Particle and Fibre Toxicology* **12**: 29.

Stoehr, L. C., E. Gonzalez, A. Stampfl, E. Casals, A. Duschl, V. Puntes and G. J. Oostingh (2011). "Shape matters: effects of silver nanospheres and wires on human alveolar epithelial cells." *Particle and Fibre Toxicology* **8**(1): 1-15.

Sturla, S. J., A. R. Boobis, R. E. FitzGerald, J. Hoeng, R. J. Kavlock, K. Schirmer, M. Whelan, M. F. Wilks and M. C. Peitsch (2014). "Systems toxicology: from basic research to risk assessment." *Chemical Research in Toxicology* **27**(3): 314-329.

Sun, H., H. A. Clancy, T. Kluz, J. Zavadil and M. Costa (2011). "Comparison of Gene Expression Profiles in Chromate Transformed BEAS-2B Cells." *PLoS ONE* **6**(3): e17982.

Sung, J. H., J. H. Ji, J. D. Park, J. U. Yoon, D. S. Kim, K. S. Jeon, M. Y. Song, J. Jeong, B. S. Han, J. H. Han, Y. H. Chung, H. K. Chang, J. H. Lee, M. H. Cho, B. J. Kelman and I. J. Yu (2009). "Subchronic Inhalation Toxicity of Silver Nanoparticles." *Toxicological Sciences* **108**(2): 452-461.

Sung, J. H., J. H. Ji, J. U. Yoon, D. S. Kim, M. Y. Song, J. Jeong, B. S. Han, J. H. Han, Y. H. Chung, J. Kim, T. S. Kim, H. K. Chang, E. J. Lee, J. H. Lee and I. J. Yu (2008). "Lung function changes in Sprague-Dawley rats after prolonged inhalation exposure to silver nanoparticles." *Inhalation Toxicology* **20**(6): 567-574.

Suzuki, H., T. Toyooka and Y. Ibuki (2007). "Simple and easy method to evaluate uptake potential of nanoparticles in mammalian cells using a flow cytometric light scatter analysis." *Environmental Science & Technology* **41**(8): 3018-3024.

Teeguarden, J. G., V. B. Mikheev, K. R. Minard, W. C. Forsythe, W. Wang, G. Sharma, N. Karin, S. C. Tilton, K. M. Waters, B. Asgharian, O. R. Price, J. G. Pounds and B. D. Thrall (2014). "Comparative iron oxide nanoparticle cellular dosimetry and response in mice by the inhalation and liquid cell culture exposure routes." *Particle and Fibre Toxicology* **11**(1): 1-18.

Tenzer, S., D. Docter, J. Kuharev, A. Musyanovych, V. Fetz, R. Hecht, F. Schlenk, D. Fischer, K. Kiouptsi, C. Reinhardt, K. Landfester, H. Schild, M. Maskos, S. K. Knauer and R. H. Stauber (2013). "Rapid formation of plasma protein corona critically affects nanoparticle pathophysiology." *Nature Nanotechnology* **8**(10): 772-781.

Tice, R. R., E. Agurell, D. Anderson, B. Burlinson, A. Hartmann, H. Kobayashi, Y. Miyamae, E. Rojas, J. C. Ryu and Y. F. Sasaki (2000). "Single cell gel/comet assay: guidelines for in vitro and in vivo genetic toxicology testing." *Environmental and Molecular Mutagenesis* **35**(3): 206-221.

Tofighi, R., M. Moors, R. Bose, W. N. W. Ibrahim and S. Ceccatelli (2011). Chapter 5. Neural Stem Cells for Developmental Neurotoxicity Studies. In: "In Vitro Neurotoxicology: Methods and Protocols". (G. L. Costa, G. Giordano and M. Guizzetti Eds.) pp 67-80, Humana Press, New Jersey.

Tomaszewska, E., K. Soliwoda, K. Kadziola, B. Tkacz-Szczesna, G. Celichowski, M. Cichomski, W. Szmaja and J. Grobelny (2013). "Detection Limits of DLS and UV-Vis Spectroscopy in Characterization of Polydisperse Nanoparticles Colloids." *Journal of Nanomaterials* Article ID 313081.

Tran, C. L., R. Tantra, K. Donaldson, V. Stone, S. M. Hankin, B. Ross, R. J. Aitken and A. D. Jones (2011). "A hypothetical model for predicting the toxicity of high aspect ratio nanoparticles (HARN)." *Journal of Nanoparticle Research* ***13***(12): 6683-6698.

Tseng, M. T., Q. Fu, K. Lor, G. R. Fernandez-Botran, Z. B. Deng, U. Graham, D. A. Butterfield, E. A. Grulke and R. A. Yokel (2014). "Persistent hepatic structural alterations following nanoceria vascular infusion in the rat." *Toxicologic Pathology* **42**(6): 984-996.

Tyler, G., Yvon, J. (2005). "ICP-OES, ICP-MS and AAS techniques compared. Jobin Yvon Horiba ICP Optical Emission Spectroscopy Technical Note 05." Accessed Sept 2016, from: http://www.horiba.com/fileadmin/uploads/Scientific/Downloads/OpticalSchool_CN/TN/ICP/ICP-OES__ICP-MS_and_AAS_Techniques_Compared.pdf

Uttara, B., A. V. Singh, P. Zamboni and R. T. Mahajan (2009). "Oxidative Stress and Neurodegenerative Diseases: A Review of Upstream and Downstream Antioxidant Therapeutic Options." *Current Neuropharmacology* **7**(1): 65-74.

Vales, G., L. Rubio and R. Marcos (2015). "Long-term exposures to low doses of titanium dioxide nanoparticles induce cell transformation, but not genotoxic damage in BEAS-2B cells." *Nanotoxicology* **9**(5): 568-578.

Vales, G., L. Rubio and R. Marcos (2016). "Genotoxic and cell-transformation effects of multi-walled carbon nanotubes (MWCNT) following in vitro sub-chronic exposures." *Journal of Hazardous Materials* **306**: 193-202.

Walkey, C., S. Das, S. Seal, J. Erlichman, K. Heckman, L. Ghibelli, E. Traversa, J. F. McGinnis and W. T. Self (2015). "Catalytic Properties and Biomedical Applications of Cerium Oxide Nanoparticles." *Environmental Science: Nano* **2**(1): 33-53.

Vallyathan, V., S. Leonard, P. Kuppusamy, D. Pack, M. Chzhan, S. P. Sanders and J. L. Zweir (1997). "Oxidative stress in silicosis: evidence for the enhanced clearance of free radicals from whole lungs." *Molecular and Cellular Biochemistry* **168**(1-2): 125-132.

Vance, M. E., T. Kuiken, E. P. Vejerano, S. P. McGinnis, M. F. Hochella, Jr., D. Rejeski and M. S. Hull (2015). "Nanotechnology in the real world: Redeveloping the nanomaterial consumer products inventory." *Beilstein Journal of Nanotechnology* **6**: 1769-1780.

Wang, L., S. Luanpitpong, V. Castranova, W. Tse, Y. Lu, V. Pongrakhananon and Y. Rojanasakul (2011). "Carbon nanotubes induce malignant transformation and tumorigenesis of human lung epithelial cells." *Nano Letters* **11**(7): 2796-2803.

Wang, T., J. Bai, X. Jiang and G. U. Nienhaus (2012). "Cellular uptake of nanoparticles by membrane penetration: a study combining confocal microscopy with FTIR spectroelectrochemistry." *ACS Nano* **6**(2): 1251-1259.

Wang, X., Z. Ji, C. H. Chang, H. Zhang, M. Wang, Y.-P. Liao, S. Lin, H. Meng, R. Li, B. Sun, L. V. Winkle, K. E. Pinkerton, J. I. Zink, T. Xia and A. E. Nel (2014). "Use of coated silver nanoparticles to understand the relationship of particle dissolution and bioavailability to cell and lung toxicological potential." *Small* **10**(2): 385-398.

Wang, Z., M. Gerstein and M. Snyder (2009). "RNA-Seq: a revolutionary tool for transcriptomics." *Nature Reviews Genetics* **10**(1): 57-63.

Wei, L., J. Lu, H. Xu, A. Patel, Z.-S. Chen and G. Chen (2015). "Silver nanoparticles: synthesis, properties, and therapeutic applications." *Drug Discovery Today* **20**(5): 595-601.

Weir, A., P. Westerhoff, L. Fabricius and N. von Goetz (2012). "Titanium dioxide nanoparticles in food and personal care products." *Environmental science & technology* **46**(4): 2242-2250.

Weldon, B. A., M. F. E, G. Oberdörster, T. Workman, W. C. Griffith, C. Kneuer and I. J. Yu (2016). "Occupational exposure limit for silver nanoparticles: considerations on the derivation of a general health-based value." Nanotoxicology **10**(7): 945-956.

Wente, S. R. and M. P. Rout (2010). "The Nuclear Pore Complex and Nuclear Transport." *Cold Spring Harbor Perspectives in Biology* **2**(10): a000562.

Veranth, J. M., N. S. Cutler, E. G. Kaser, C. A. Reilly and G. S. Yost (2008). "Effects of cell type and culture media on Interleukin-6 secretion in response to environmental particles." *Toxicology in Vitro* **22**(2): 498-509.

Verspohl, E. J. and J. Podlogar (2012). "LPS-Induced Proliferation and Chemokine Secretion from BEAS-2B Cells,." *Pharmacology & Pharmacy* **3**(2): 166-177.

Westmeier, D., R. H. Stauber and D. Docter (2016). "The concept of bio-corona in modulating the toxicity of engineered nanomaterials (ENM)." *Toxicology and Applied Pharmacology* **299**: 53-57.

Wichmann, H. E. (2007). "Diesel exhaust particles." *Inhalation Toxicol* **19** (Suppl 1):241-244.

Vogel, C. and E. M. Marcotte (2012). "Insights into the regulation of protein abundance from proteomic and transcriptomic analyses." *Nature Reviews Genetics* **13**(4): 227-232.

Xia, T., M. Kovochich, J. Brant, M. Hotze, J. Sempf, T. Oberley, C. Sioutas, J. I. Yeh, M. R. Wiesner and A. E. Nel (2006). "Comparison of the abilities of ambient and manufactured nanoparticles to induce cellular toxicity according to an oxidative stress paradigm." *Nano Letters* **6**(8): 1794-1807.

Xia, T., M. Kovochich, M. Liong, L. Mädler, B. Gilbert, H. Shi, J. I. Yeh, J. I. Zink and A. E. Nel (2008). "Comparison of the mechanism of toxicity of zinc oxide and cerium oxide nanoparticles based on dissolution and oxidative stress properties." *ACS Nano* **2**(10): 2121-2134.

Xu, R. (2008). "Progress in nanoparticles characterization: Sizing and zeta potential measurement." *Particuology* **6**(2): 112-115.

Xue, Y., Q. Luan, D. Yang, X. Yao and K. Zhou (2011). "Direct evidence for hydroxyl radical scavenging activity of cerium oxide nanoparticles." *The Journal of Physical Chemistry C* **115**(11): 4433-4438.

Yokel, R. A., M. T. Tseng, M. Dan, J. M. Unrine, U. M. Graham, P. Wu and E. A. Grulke (2013). "Biodistribution and biopersistence of ceria engineered nanomaterials: size dependence." *Nanomedicine* **9**(3): 398-407.

Yu, S. J., J. B. Chao, J. Sun, Y. G. Yin, J. F. Liu and G. B. Jiang (2013). "Quantification of the uptake of silver nanoparticles and ions to HepG2 cells." *Environmental Science & Technology* **47**(7): 3268-3274.

Zhang, H., Z. Ji, T. Xia, H. Meng, C. Low-Kam, R. Liu, S. Pokhrel, S. Lin, X. Wang, Y.-P. Liao, M. Wang, L. Li, R. Rallo, R. Damoiseaux, D. Telesca, L. Mädler, Y. Cohen, J. I. Zink and A. E. Nel (2012). "Use of metal oxide nanoparticle band gap to develop a predictive paradigm for oxidative stress and acute pulmonary inflammation." *ACS Nano* **6**(5): 4349-4368.

Zhang, J., Y. Nazarenko, L. Zhang, L. Calderon, K.-B. Lee, E. Garfunkel, S. Schwander, T. D. Tetley, K. F. Chung, A. E. Porter, M. Ryan, H. Kipen, P. J. Lioy and G. Mainelis (2013). "Impacts of a nanosized ceria additive on diesel engine emissions of particulate and gaseous." *Environmental science & technology* **47**(22): 13077-13085.

Zhao, F. and W. T. Klimecki (2015). "Culture conditions profoundly impact phenotype in BEAS-2B, a human pulmonary epithelial model." *Journal of Applied Toxicology* **35**(8): 945-951.

Zheng, Q. and X. J. Wang (2008). "GOEAST: a web-based software toolkit for Gene Ontology enrichment analysis." *Nucleic Acids Research* **36**(Web Server issue): 358-363.