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

Anda Roxana Gliga

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

THESIS FOR DOCTORAL DEGREE (Ph.D.)

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“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


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]

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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-CeO₂ 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 pneumoconiosis, 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](image)

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 H₂O 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).

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.

![Figure 2. Predicted deposition of inhaled particles in the respiratory tract. Reproduced from *Environmental Health Perspectives*, Oberdörster et al., 2005, with modifications.](image-url)
(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).

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

![Figure 4. Active uptake mechanisms for (nano)particles](image-url)
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 TiO₂ 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 adsorption 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, SiO₂, CeO₂), 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 as 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).

![Hierarchical oxidative stress response model](image)

**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.

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/cm²) 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).

Figure 7. Mechanisms of nanoparticle-induced genotoxicity
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/cm² in the alveolar region *in vivo* corresponded to 1.2 – 4 µg/cm² *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 CeO₂ 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.](image)

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 (\(\zeta P\) 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).

![Diagram of toxicity mechanisms]

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, Fe₃O₄, ZnO, TiO₂, NiO, CeO₂ and Ag) were screened, Paper II-III was focused on Ag nanoparticles while Paper IV was focused on CeO₂ 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 CeO₂ 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 CeO₂ 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.
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 absorption (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 (TiO₂) 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 TiO₂ 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 TiO₂ 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$_3$O$_4$, Fe$_2$O$_3$) 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 photography, 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 $0.19 \mu g/m^3$ 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).
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 µg/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 catalytic 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$_2$O$_2$ nanoceria or NH$_4$OH nanoceria (Dowding et al., 2013). The same study indicated that nanoceria with high levels of Ce$^{4+}$ at the surface (HMT and NH$_4$OH 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

<table>
<thead>
<tr>
<th>Nanoparticle</th>
<th>Primary particle size (nm)</th>
<th>Coating</th>
<th>Form</th>
<th>Study</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>CuO</td>
<td>20 – 40</td>
<td>-</td>
<td>powder</td>
<td>I</td>
<td>(Karlsson et al., 2008)</td>
</tr>
<tr>
<td>Fe₂O₄</td>
<td>20 – 40</td>
<td>-</td>
<td>powder</td>
<td>I</td>
<td></td>
</tr>
<tr>
<td>ZnO</td>
<td>20 – 200</td>
<td>-</td>
<td>powder</td>
<td>I</td>
<td></td>
</tr>
<tr>
<td>TiO₂</td>
<td>20 – 100</td>
<td>-</td>
<td>powder</td>
<td>I</td>
<td></td>
</tr>
<tr>
<td>NiO</td>
<td>2 – 70</td>
<td>-</td>
<td>powder</td>
<td>I</td>
<td>(Kain et al., 2012)</td>
</tr>
<tr>
<td>Ag</td>
<td>10</td>
<td>citrate</td>
<td>1 mg/mL dispersion</td>
<td>I, II, III</td>
<td>(Gliga et al., 2014)</td>
</tr>
<tr>
<td></td>
<td>10 *</td>
<td>PVP</td>
<td>1 mg/mL dispersion</td>
<td>II</td>
<td></td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>citrate</td>
<td>1 mg/mL dispersion</td>
<td>I, II</td>
<td></td>
</tr>
<tr>
<td></td>
<td>75 *</td>
<td>citrate</td>
<td>1 mg/mL dispersion</td>
<td>II, III</td>
<td></td>
</tr>
<tr>
<td></td>
<td>40 – 50</td>
<td>-</td>
<td>powder</td>
<td>II</td>
<td></td>
</tr>
<tr>
<td>CeO₂</td>
<td>4 – 30</td>
<td>-</td>
<td>powder</td>
<td>I</td>
<td>(Kain et al., 2012)</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>-</td>
<td>powder</td>
<td>IV</td>
<td>(Celardo et al., 2011)</td>
</tr>
<tr>
<td>Sm- CeO₂</td>
<td>13</td>
<td>-</td>
<td>powder</td>
<td>IV</td>
<td></td>
</tr>
</tbody>
</table>

* 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: Bsc12-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. HNO₃) 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 AgCl₂⁻ 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 adsorption 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 absorbion, 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 deacetylated 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 TiO₂ 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 γH₂AX 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 H₂AX is phosphorylated at the Ser-139 residue to form γH₂AX foci that are involved in signaling and initiation of DNA repair (Mah et al., 2010). In addition, in mES, γH₂AX can also occur at sites of single strand breaks as well as sites of chromatin relaxation (Banath et al., 2009). γH₂AX 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 TiO₂ 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 ($\beta$ 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 NANO PARTICLES

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: Bsc12-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).

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, CeO₂, Fe₂O₃, TiO₂) 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

![Figure 13. The ToxTracker tool for rapid genotoxicity screening of chemicals and nanomaterials. Reproduced from Karlsson et al., 2014 (Paper I).](image-url)
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 Bsc1, 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 γH2AX 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 γH2AX 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 γH2AX 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.

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 aneuploidiogenic effects as indicated by the micronucleus assay. Moreover, there was a clear size-dependent effect in terms of the number of differentially expressed genes.

Figure 14. Experimental setup of study III.
(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 in 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 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).

The RNA-Seq downstream analysis using Ingenuity Pathway Analysis tool revealed that both CeO₂ and NAC, but not Sm-CeO₂ altered the neuronal differentiation network in line with the immunofluorescence data. In addition, CeO₂ 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 CeO₂ nanoparticles. On one hand, CeO₂ nanoparticles had a temporary protective effect in neural stem cells as a result of its antioxidant properties. On the other hand, CeO₂ 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 NANOPIERCICLES

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 (Anangi 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 \textit{i.e.} interference with neuronal development and differentiation.
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, CeO₂ nanoparticles are antioxidant and have temporary neuroprotective effects in neural stem cells. On the other hand, due to the antioxidant properties, CeO₂ 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 (TiO₂, CeO₂) 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 CeO₂ 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.
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