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EXPLORING TOXICITY AND FATE OF METAL-BASED PARTICLES IN THE LUNG
– FROM MECHANISTIC SCREENING TO LUNG DEPOSITION MODELLING

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Exploring toxicity and fate of metal-based particles in the lung – from mechanistic screening to lung deposition modelling

THESIS FOR DOCTORAL DEGREE (Ph.D.)

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To my father

POPULAR SCIENCE SUMMARY

Particles are constantly present in the air that we breathe as a result of both natural sources and human activities. In addition, many industries involve activities resulting in workers being exposed to high levels of particles. Yet, many questions remain unanswered about the potential threats these particles pose to us. Particle pollution has been linked with serious health problems including cancer and inflammation. The most dangerous particles are believed to be those that are really small, often referred to as nanoparticles. These particles, indeed too small to be visible to the human eye, are considered to be of greatest concern since they can reach deep into the lung and because nanoparticles have a greater surface area which can make them more reactive.

This thesis investigated harmful effects of metal-based particles in the lung mainly using human cells in culture. In the first study of this thesis, so called reporter cells were used which are designed to signal when certain molecular happenings occur within the cells. By exposing these reporter cells to nanoparticles, we can get insight into what cellular consequences they cause and by that predict if the nanoparticles could potentially result in harmful effects, such as cancer. Our results suggest that these reporter cells can be valuable in evaluating potentially harmful particles and identify particles of specific concern.

The second and third study of this thesis aimed to increase our understanding about the toxicity of particles generated by stainless steel welding. Our results showed that harmful effects varied largely depending on which welding technique that was used. More detailed investigations revealed that it was primarily some of the metals being released from the particles, and not the particles themselves, that seemed to cause most of the toxic effect. This was especially evident in the case of released hexavalent chromium. In collaboration with industrial partners, we further showed that the harmful effects of welding fumes can be reduced by modifying welding equipment (more specifically electrodes) into generating particles that released less hexavalent chromium. Our findings can therefore contribute to improve the health of welders.

A main challenge when using cell models to study toxic effects, as was done in this thesis, is to understand how the results can be interpreted from a human health perspective. In the fourth study, we wanted to know how close our cell experiments were to mimic a human lung being exposed to welding fumes. Therefore, we used a computational model to estimate how much of the welding fumes that could end up in the lung of a welder. The estimated lung doses were compared with the doses we used for our cell experiments and shown to be comparable in many cases. Our results showed that already after one full working shift of welding, the same amount of particles could be found in the upper part of the lung of welders as what we observed to be harmful in our cell experiments.

In summary, these findings contribute to our understanding of how particles end up and behave in the lung as well as the resulting harmful effects. In addition, the studies in this thesis provide insight related to new ways of studying toxic effects of particles in the lung using cultured cell models. Understanding the harmful effects of particles and how we best study them is important to protect human health by ensuring safer work practices and appropriate regulations.

POPULÄRVETENSKAPLIG SAMMANFATTNING

Partiklar är konstant närvarande i luften vi andas till följd av naturliga källor och mänskliga aktiviteter. Dessutom leder specifika industrier till att människor riskerar att bli exponerade för höga nivåer av partiklar i sin arbetsmiljö. Trots det så är det fortfarande mycket vi inte vet om vilka möjliga faror detta innebär, men partiklar i luften har visats kunna leda till olika hälsoeffekter så som inflammation i lungan och cancer. De mest farliga partiklarna tros vara de som är riktigt små och kallas ”nanopartiklar”. Oron för dessa partiklar, så små att de ofta inte syns, beror på att de kan nå djupt ner i lungan men också för att de är väldigt många, har större yta och är mer reaktiva.

Syftet med arbetet i den här avhandlingen har varit att öka kunskapen om vad som händer i lungan när vi andas in partiklar som består av olika metaller. Detta har framförallt gjorts med hjälp av celler från människor som odlas på laboratoriet och som kan användas för att utreda skadliga effekter (toxicitet) med hjälp av olika metoder. I den första studien användes så kallade ”rapportör-celler”, som är celler specifikt designade för att signalera när vissa molekylära händelser sker inuti cellen. Genom att utsätta cellerna för nanopartiklar kan man få inblick i de cellulära konsekvenser som sker. På så sätt kan man exempelvis förutspå skadliga effekter på cellernas arvsmassa som i sin tur kan öka risken för uppkomst av cancer. Resultaten av studien visade att rapportör-cellerna kan vara värdefulla för att utvärdera effekter av partiklar i lungan och för att identifiera de som är mest hälsofarliga.

Studie två och tre fokuserade på att förstå mer om skadliga effekter relaterad till partiklar i svetsrök. Svetsning används för att sammanfoga olika metallstycken och vanligen smälts ett tillsatsmaterial in i svetsfogen från en tråd (rörelektrod). Genom att studera svetsrök genererad från svetsning av rostfritt stål visades att skadliga effekter i lungceller varierade stort beroende på svetsteknik och speciellt vilka trådar som användes. Mer detaljerade undersökningar visade att de skadliga effekterna främst kunde förklaras av frisatta (lösliga) metaller från partiklarna, och inte partiklarna själva. Detta gällde framförallt frisättningen av sexvärt krom. I samarbete med industriella partners visades även att skadliga effekter av svetsrök kan minskas genom att förändra svetsutrustningen (trådarna) till att generera partiklar med mindre halter lösligt sexvärt krom. Resultat kan därför bidra till att förbättra arbetsmiljön och riskerna för svetsare.

En stor utmaning när man använder cellmodeller för att studera toxicitet, så som gjorts i denna avhandling, är att förstå hur resultaten kan tolkas i förhållande till människors hälsa. I den fjärde studien var målet att undersöka hur bra dosen (mängden partiklar) i cell-experiment avspeglar den i lungan hos en svetsare som utsätts för svetsrök. Det är en komplex uppgift eftersom bara en del av alla partiklar vi andas in faktiskt hamnar och stannar kvar i lungan. Därför användes en datorbaserad modell för att uppskatta hur mycket svetspartiklar som kan hamna i lungan när arbetare andas in svetsrök. Därefter jämfördes mängden partiklar i lungan med doserna som använts för cell-experimenten, och de visade sig i många fall var jämförbara. Resultat visade att redan efter ett jobb-skift som svetsare uppnåddes samma mängd partiklar i de övre luftvägarna som vi sett vara skadliga i våra cell-studier.

Sammanfattningsvis bidrar studierna i denna avhandling till vår förståelse för vilken mängd partiklar som hamnar i lungan, hur de beter sig samt vilka skador som kan uppstå. Denna avhandling har också bidragit till kunskap om nya sätt att studera farliga effekter av partiklar i lungan med hjälp av odlade celler. Vår förståelse för skadliga effekter av partiklar och hur vi bäst studerar dem är viktigt för att skydda människors hälsa och kan på sikt även bidra till förbättrad lagstiftning och säkrare arbetsmiljöer.

ABSTRACT

We are all exposed to small particles in the air that we breath and some of them will contain metals in a dose and form that may be harmful. In addition, the field of nanotechnology holds great promises, but an increased production of nanoparticles leads to a higher risk of exposure. Metal-based particles are indeed also present in various traditional occupational settings resulting in an exposure to the workers within this field. Welders are one group at risk for exposure to metal containing particles. Despite this, many knowledge gaps remain regarding the possible risks that particles pose on human health. With the emerging use of nanomaterial and the move away from animal-based experiments, there is currently a need to establish approaches of testing particles in efficient and informative ways using alternative test strategies. This thesis aims to gain a deeper understanding of the toxicity and fate of metal-based particles in the lung by employing experimental approaches ranging from mechanistic screening and established *in vitro* assays to lung deposition modelling.

In **Paper I** and **II**, the toxicity and associated mechanisms for a wide selection of metal-containing nanoparticles were investigated using the reporter cell based ToxTracker assay. Reporters related to oxidative stress were most frequently activated in response to the nanoparticles, whereas fewer nanoparticles activated reporters linked to DNA damage. However, the latter ones were suggested to be considered of particular concern. With the variation in activation of various reporters, this suggests that the ToxTracker can be used as a sensitive tool to gain rapid and efficient mechanistic insight into the toxicity of particles.

In **Paper II**, the toxicity and underlying mechanisms of welding fume particles generated by welding of stainless steel were investigated *in vitro* as a function of welding techniques, settings and materials. Observations revealed a high variation in toxic potential of different welding fumes, primarily depending on choice of welding electrode. Welding fumes generated with flux cored wire (FCW) were most toxic. This was strongly associated with higher metal release, in particular hexavalent chromium (Cr(VI)). In the follow-up **Paper III**, the released metal fraction was shown to induce similar cytotoxicity and DNA damage as the particles, further emphasizing the importance of released metals in acute toxicity induced by welding fumes. Furthermore, **Paper III** demonstrated the potential benefit in substituting standard Cr(VI)-generating FCW electrodes with Cr(VI)-reduced electrodes in order to create less hazardous fume particles and a safer working environment for welders. These studies furthermore highlight the beneficial collaboration between academia and industry to improve occupational environments.

In **Paper IV** we wanted to understand the applied *in vitro* doses of welding fumes in the context of human exposure. Therefore, a review of the literature was performed to obtain information on welding fume exposure at occupational settings. Next, human lung doses were estimated by simulating real-life occupational welding scenarios in the Multiple-Path Particle Dosimetry (MPPD) model. Interestingly, lung doses following both acute and more chronic exposure were found comparable to *in vitro* doses where we observed toxic effects in **Paper III**. The lung dose of the tracheobronchial region was found to exceed a cytotoxic *in vitro* dose already after

one working shift. Moreover, this study demonstrates the significant contribution of dosimetry modelling in order to understand the relation between *in vitro* doses and human exposure, and its potential future importance for risk assessment and study design.

In conclusion, the results of the studies within the framework of this thesis demonstrate a variation in toxic potency and mode of action for metal-based particles. Metal release is shown to be an important factor for metal-particle induced toxicity, with results showing metal release, rather than metal content, to be largely responsible for acute toxicity induced by welding fumes. This thesis especially highlights the use of *in vitro* models for the hazard assessment of particles, identifying both the ToxTracker and lung deposition modelling as important tools for improving the efficiency and regulatory weight of *in vitro* approaches.

LIST OF SCIENTIFIC PAPERS

I. **McCarrick S**, Cappellini F, Kessler A, Moelijker N, Derr R, Hedberg J, Wold S, Blomberg E, Odnevall Wallinder I, Hendriks G, Karlsson HL. ToxTracker Reporter Cell Lines as a Tool for Mechanism-Based (geno)Toxicity Screening of Nanoparticles-Metals, Oxides and Quantum Dots. Nanomaterials. 2020 Jan;10(1):110.

II. **McCarrick S**, Wei Z, Moelijker N, Derr R, Persson KA, Hendriks G, Odnevall Wallinder I, Hedberg Y, Karlsson HL. High variability in toxicity of welding fume nanoparticles from stainless steel in lung cells and reporter cell lines: the role of particle reactivity and solubility. Nanotoxicology. 2019 Dec;13(10):1293-1309.

III. **McCarrick S**, Romanovski V, Wei Z, Westin EM, Persson KA, Trydell K, Wagner R, Odnevall I, Hedberg YS, Karlsson HL. Genotoxicity and inflammatory potential of stainless steel welding fume particles: an *in vitro* study on standard vs Cr(VI)-reduced flux-cored wires and the role of released metals. Archives of Toxicology. 2021 Sep;95(9):2961-2975.

IV. **McCarrick S,** Karlsson HL, Carlander U. Modelled lung deposition and retention of welding fume particles in occupational scenarios: a comparison to doses used *in vitro*. Archives of Toxicology. 2022 Apr;96(4):969-985.

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Westin EM, **McCarrick S**, Laundry-Mottiar L, Wei Z, Wagner R, Persson KA, Trydell K, Odnevall I, Karlsson HL, Hedberg YS. New weldable 316L stainless flux-cored wires with reduced Cr (VI) fume emissions: part 2—round robin creating fume emission data sheets. Welding in the World. 2021 Dec;65(12):2339-48.

CONTENTS

LIST OF ABBREVIATIONS

AAS	Atomic absorption spectroscopy
AOP	Adverse outcome pathway
Cdk4	Cyclin-dependent kinase 4
CdTe QDs	Cadmium telluride quantum dots
Cr	Chromium
Cr(III)	Trivalent chromium
Cr(VI)	Hexavalent chromium
Cu	Copper
DCFH-DA	2′7-dichlorodihydrofluorescein diacetate
DCFH	2′7-dichlorodihydrofluorescein
DCF	2′,7′-dichlorofluorescein
EC50	Effective concentration 50 %
EDS	Energy-dispersive X-ray spectroscopy
ER	Endoplasmic reticulum
FCAW	Flux cored arc welding
FCW	Flux cored wire
Fe	Iron
FPG	Formamidopyrimidine DNA glycosylase
GFP	Green fluorescent protein
GMAW	Gas metal arc welding
HRP	Horseradish peroxidase
hTERT	Human telomerase reverse transcriptase
IARC	International Agency for Research on Cancer
ICP-MS	Inductive coupled mass spectrometry
iNOS	Inducible nitric oxide synthase
IVIVE	*In vitro* to *in vivo* extrapolation
MAG	Metal active gas (welding)
Mn	Manganese
MCW	Metal cored wire
mES	Mouse embryonic stem cells

Mo	Molybdenum
MPPD	Multiple-path particle dosimetry
Ni	Nickel
Nrf2	Nuclear factor-erythroid 2-related factor 2
OECD	Organization for Economic Co-operation and Development
OEL	Occupational exposure limit
PBS	Phosphate buffered saline
PCCS	Photon cross correlation spectroscopy
PM	Particulate matter
PMA	Phorbol 12-myristate 13-acetate
QDs	Quantum dots
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SEM	Scanning electron microscopy
TEM	Transmission electron microscopy
UPR	Unfolded protein response
XPS	X-ray photoelectron spectroscopy
XRD	X-ray diffraction

1 INTRODUCTION

Humans have always been exposed to airborne particles originating from natural sources such as mineral weathering, erosion, and volcanic eruption. The industrial revolution subsequently resulted in a dramatic increase in particle exposure due to major anthropogenic sources including combustion engines and power plants. The more recent, yet rapid development of the field of nanotechnology represents a new source of human exposure to airborne particles, which are often referred to as engineered nanomaterials. Nonetheless, our knowledge on the possible toxic effects particles exert is still limited. The inhalation exposure to particles poses a potential hazard to human health and particle pollution is one of the major contributors to the global burden of disease.

One way to address this issue is to recognize the properties, behavior, fate and underlying mechanisms involved in the toxicity of particles, since this is crucial to understand the link between exposure and health effects. The focus of this thesis is the hazards related to workplace exposure to metal-based particles including those intentionally produced as well as unintentionally produced particles from welding stainless steel with the aim to improve our understanding of their toxicity and fate in the lung. As the title reflects, this thesis also aims to explore and shed some light on different aspects of toxicity testing of particles ranging from high-throughput mechanistic screening to lung deposition modelling as a tool for understanding *in vitro* doses in a human context. By this, I hope to fill some of the knowledge gaps related to the effects and cellular mechanisms of metal-based particle induced toxicity and how we best study them to ultimately promote better and safer occupational environments and improve human health.

2 BACKGROUND

2.1 PARTICLES – PROPERTIES AND EXPOSURE

Particles can be generated both naturally through environmental phenomena and via human activities such as combustion or fabrication processes such as welding. In addition, there are also manufactured particles that are designed and engineered according to societal needs. The use of manufactured nanoparticles has increased immensely in various fields and industrial sectors during the last decades, including everything from clothing and cosmetics to electronics (Stark et al. 2015, Khan et al. 2019). Nanoparticles are currently investigated and used in numerous medical applications such as imaging, therapeutics, and drug delivery (Salata 2004, Daraee et al. 2016, Elahi et al. 2018). There are some apparent differences between intentional and unintentionally formed particles, where unintentionally formed particles generally are polydispersed and chemically complex in contrast to the more monodispersed and precise chemical composition of engineered particles. However, the latter is strongly material specific as for example metal particles readily agglomerate due to strong van der Waal forces.

Particles are often defined and grouped depending on their size. Within aerosol science, this includes the use of the terms coarse (>2.5 um), fine (<2.5 um) and ultrafine (<100 nm) particulate matter (PM) to describe naturally occurring and unintentionally formed particles. Similar to ultrafine particles, nanoparticles are routinely defined as particles below 100 nm and have different properties compared to the bulk version of the same material (Auffan et al. 2009). Many authors restrict the term nanoparticles to particles produced by controlled engineered processes, but since there is no consensus on the definition of nanoparticles it can differ depending on context. The European Commission adopted a regulatory definition of nanomaterials in 2011 (European comission 2011):

"*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 % "

The same unique properties and behaviour, as well as toxicological principles generally apply to all nano-sized particles compared to larger sized particles (Oberdörster et al. 2005). In the context of this thesis, the term ultrafine is interchangeable with nanoparticles. As emphasized in Stone et al. (2017), there is an overlap in the toxicology of ultrafine particles and engineered nanoparticles, and a crosstalk between the areas is important in the understanding of toxicity of nano-sized particles.

In this thesis, several manufactured nanoparticles are studied in **Paper I**, whereas non-intentionally generated particles generated during welding, primarily within the nano-size range, are explored in **Paper II-IV**.

2.1.1 Occupational exposure to metal-based particles

Occupational exposure is of particular concern since workplace conditions may result in much higher exposure levels than is typically found at ambient conditions or in consumer products. Particle aerosols in workplace environments may be derived from a great variety of sources depending on the type of activity and processes taking place. Exposure to metal-based particles is occupationally relevant and includes both the formation and application of engineered metal-based nanoparticles as well as unintentionally produced particles as a consequence of metal work such as welding of stainless steel structures.

Measurements of aerosol mass concentration are standard procedure in both occupational and ambient environments. When quantifying the occupational exposure to particles, the most commonly used metrics are mass or number concentration. Compared to larger sized particles, nano-sized particles have a negligible mass but instead dominate in terms of number of particles. In a study by Zou et al. (2015), number and surface area concentrations were reported to be significantly distinct from mass concentrations when characterizing exposure to airborne nano-sized particles in various workplaces. Occupational exposure limits (OELs) are implemented for monitoring and evaluating levels of workplace exposures and are established to benchmark levels of admissible exposure that is not likely to affect the health of the workers. OELs are primarily based on mass concentrations per cubic meter in air.

2.2 LUNG EXPOSURE

Inhalation is considered the most critical route of exposure for particles, making the lung a major target organ. The respiratory system is organized in a complex structure resembling a branching tree, which can be divided into upper respiratory tract, tracheobronchial region, and alveolar region. Identifying the deposition and fate of particles in the lung is important to understand the relationship between exposure, lung dose and potential hazardous effects. Ultimately, the internal exposure i.e., the lung dose determines the biological effects and consequently also the risk, as illustrated in Figure 1.

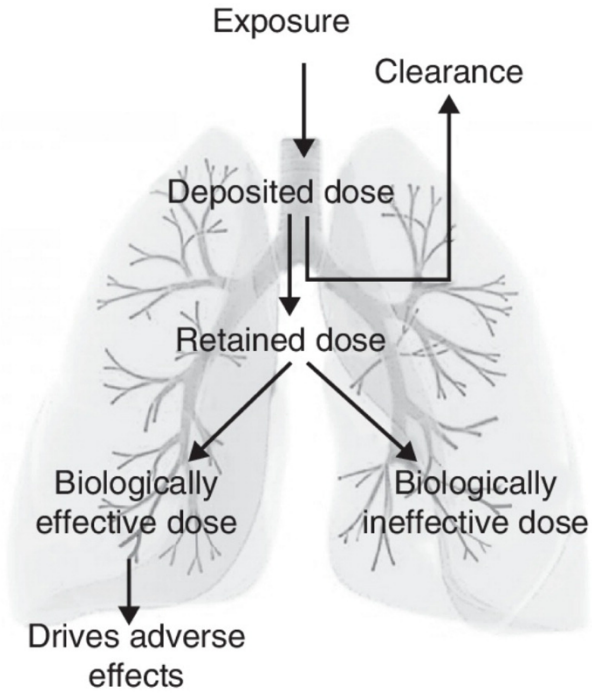


Figure 1. Schematic illustration of the biologically effective dose of particles. Reprinted with permission from Current opinion in Biotechnology, Donaldson and Poland (2013).

2.2.1 Deposition

The location and extent of deposition of particles in the lung is dependent on several factors including particle properties, exposure conditions as well as the physiological and anatomical properties of the exposed species (Oberdörster et al. 2005, Hofmann 2020). The most common mechanisms to describe deposition are impaction, sedimentation and diffusion (Hofmann 2011). The mechanisms, patterns as well as efficiency of particle deposition in the respiratory tract are largely governed by the aerodynamic or thermodynamic diameter of the particles (Geiser and Kreyling 2010). Deposition of nano-sized particles is driven almost exclusively by means of diffusion, where the small size allows them to travel and deposit in the alveolar region with higher efficiency compared to larger sized particles. The mechanisms of impaction in the upper airways and sedimentation in the tracheobronchial region regulates the deposition of larger sized particles (Oberdörster et al. 2005, Hofmann 2011). Therefore, particles of varying size, but at similar mass-based exposure dose, will have different deposition patterns in the regions of the lung, as illustrated in Figure 2. The deposition efficiency in the different region could have consequences on the toxic effects induced by inhaled particles as well as on their fate in the body. As an example, Braakhuis et al. (2014a) estimated the alveolar deposition of 15 nm silver nanoparticles in rats to be 66,000 times higher compared to that 410 nm silver nanoparticles.

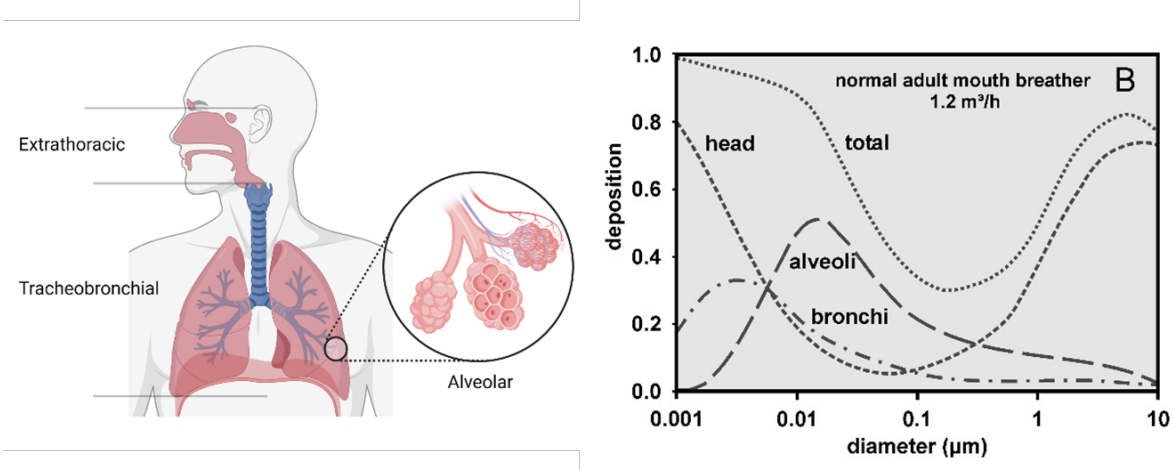


Figure 2. Predicted deposition of inhaled particles in the different regions of the respiratory tract as a function of particle size. Created using biorender.com (left) and reproduced from Particle and Fibre Toxicology, Geiser and Kreyling 2010 (right) under Creative Commons CC-BY license.

The deposition pattern of particles in the lung may be heterogenous and contain sites accumulating large portions of the particles deposited. These sites are often referred to as "hotspots" and are primarily located at tracheobronchial bifurcations. This results in that certain epithelial cells will receive massive particle doses as compared to the surrounding tissue. The particle deposition at tracheobronchial bifurcations has been estimated to be increased more than 100 times for micron-sized particles and 5 to 60 times for nano-sized particles, as compared to the average lung dose (Balashazy et al. 2003).

2.2.2 Retention and clearance

The retention time of particles in the lung is determined by clearance, which in turn depends largely on deposition site, particle characteristics as well as the interaction of particles with the inner lung surface. The classic respiratory clearance mechanisms are grounded on two basic principles: physical translocation of particles and chemical (and electrochemical) dissolution processes.

The physical translocation clearance mechanisms differ between the regions of the lung. From the nasal passage and tracheobronchial region, poorly soluble particles will be cleared by the mucociliary pathway which transports particles to the oropharynx where they are swallowed and excreted. The rate of the mucociliary clearance is dependent on both the cilia and the lung lining layers and is generally considered a rapid clearance mechanism, which gets slower with increasing airway generation (Geiser and Kreyling 2010).

Further down in the unciliated airways and alveoli, the most prevalent clearance is via alveolar macrophages. Alveolar macrophages phagocyte particles rapidly, usually within 6-12 h, which is followed by a slow clearance via movements towards either the ciliated airways and the mucociliary escalator or to lymphatic vessels and the pulmonary lymph nodes. The retention half-time of poorly-soluble particles in the alveolar region of humans has been estimated to be 700 days based on this clearance mechanism (Oberdörster et al. 2005). The efficiency of the alveolar clearance depends on the effectiveness of the macrophages to recognize the particles and to phagocyte them. Studies have proposed that particles within the nano-size range escape phagocytosis to a greater extent compared to larger sized particles (Kreyling et al. 2006, Yang et al. 2008).

Particle dissolution can occur in all three major regions of the respiratory tract when particles come into contact with mucus or lung lining fluids (Oberdörster et al. 2005, Borm et al. 2006). This process results in soluble species and compounds that can undergo absorption and diffusion or bind to proteins and other cellular structures. These are cleared into blood and lymphatic circulation and can be distributed throughout the body and finally excreted via urine or faeces depending on their physical properties.

2.2.3 Extrapulmonary translocation

If particles reach the capillaries and circulating cells, they can be translocated by circulation to other organs of the body where they may be deposited and accumulate. It has been suggested that nano-sized particles may use different transfer routes and translocation mechanisms compared to larger sized particles (Oberdörster et al. 2005, Geiser and Kreyling 2010). Particle size has been concluded to be a strong factor for translocation (Nakane 2012).

A prolonged lung retention results in an increased probability for particles to be taken up by epithelial cells and transported into the systemic circulation. Particles can be phagocytized by macrophages and dendritic cells which may transport the particles to the regional lymph nodes of the lung, enabling the particles to enter the blood circulation via thoracic lymph duct. A

study by Choi et al. (2010) demonstrated a size dependent translocation from the alveolar luminal surface with a size threshold of approximately 34 nm for translocation to regional lymph nodes and into the bloodstream. Even smaller nanoparticles below 6 nm were shown to enter the bloodstream quickly from the alveolar airspace, thus meaning they can potentially be systemically distributed throughout the body. An additional pathway involves particle elimination from the lungs towards the larynx, where they can be swallowed into the gastrointestinal tract and be absorbed across the walls of the gastrointestinal tract (Riediker et al. 2019). It should be noted that particle clearance via translocation has in general been reported to be very low (less than 0.5 %) (Braakhuis et al. 2014b)

The potential translocation of particles to the brain has raised the concern about their connections to neurodegenerative and neurological disorders (Oberdörster et al. 2009, Heusinkveld et al. 2016). Studies have indicated translocation of particles to occur via several pathways including translocation from the nose to the brain via the olfactory nerve, but also reaching the brain via the systemic circulation (Oberdörster et al. 2004, Heusinkveld et al. 2016).

2.3 HAZARD ASSESSMENT OF PARTICLES

The hazard assessment of particles includes two important aspects: physicochemical characterization and toxicity assessment, as discussed below.

2.3.1 Physicochemical properties

The interaction of particles with biological systems and consequent toxic effects are largely dependent on the physicochemical properties of the particles (Nel et al. 2009), see Figure 3. Therefore, a thorough characterization is crucial in the hazard assessments of particles. Biological responses have been shown to be altered due to even very small changes in nanoparticle properties (Fadeel et al. 2015). The inclusion of particle characterization in toxicity studies is also important to enable comparison and discussion of results in relation to other studies, something that was previously largely hindered by the lack of particle characterization within the field of nanotoxicology (Warheit 2008). Properties that are crucial in the uniqueness of particles include for example particle size, shape, and composition, of which some will be introduced and discussed below.

When particles enter a biological environment, proteins and other biomolecules immediately bind to the particle in a process called opsonisation forming a corona (Monopoli et al. 2012, Fadeel et al. 2013). The formation and composition of the corona depends on several parameters such as the physicochemical properties of the particles as well as the complexity of the surrounding media. In turn, the corona can affect the physicochemical properties and biological interaction of the particles. As emphasized in Monopoli et al. (2012), the biomolecular corona is what primarily interacts with biological systems. Therefore, this is a crucial aspect in the biological identity of the particles and can largely affect the biodistribution and uptake and thus toxicological outcome.

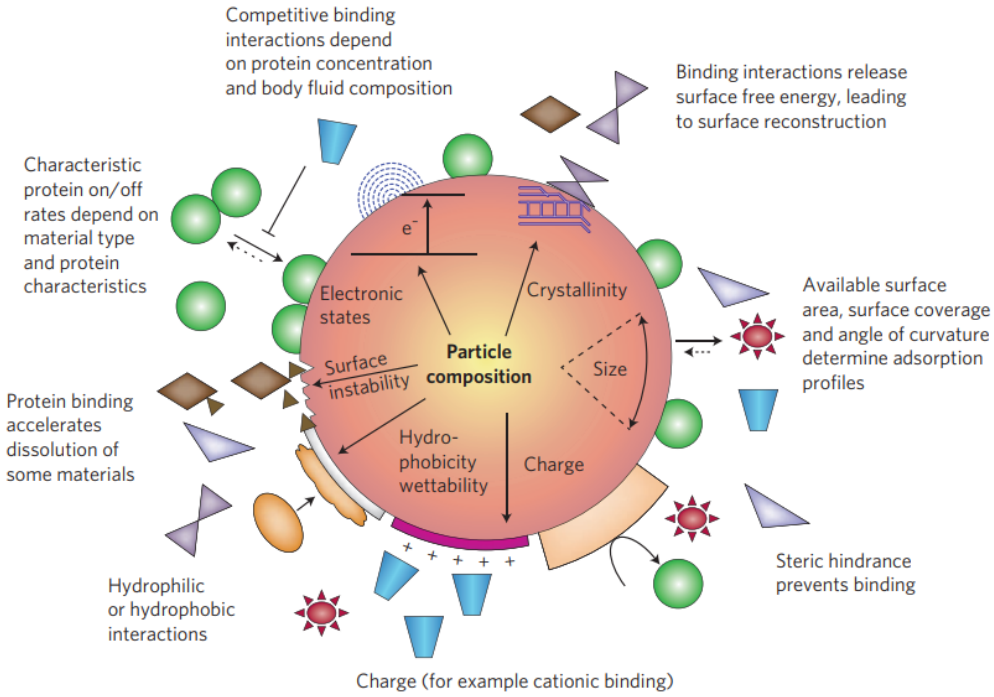


Figure 3. Schematic illustration of the physicochemical properties of particles of importance for their biological interactions. Reproduced with permission from Nature materials, Nel et al. 2009.

2.3.1.1 Particle size

Materials acquired at the nanoscale are attractive from a technical point of view due to their unique size-related properties and characteristics. Nonetheless, the same properties are causing toxicological concern regarding the potential deleterious effects of nano-sized particles for human health. When the size of particles is altered, the surfaces and volumes change in parallel with the diameter. The smaller size of nanoparticles results in a larger surface area to volume ratio, resulting in a significantly higher number of atoms at the particle surface compared to larger particles, see Figure 4. Consequently, nano-sized particles are more likely to interact with the environment and may result in enhanced chemical and biological reactivity including redox reactions and dissolution processes (Auffan et al. 2009), which in turn may affect both particle exposure and hazard.

The size of the particles can also dramatically influence to what extent and in which way particles come into contact and interact with the human body. This includes alterations in deposition, absorption, distribution, metabolism and excretion which can result in differences in critical dose levels and locations of health effects compared to larger sized particles. Particle size has been demonstrated to influence cellular uptake mechanisms (Behzadi et al. 2017, Foroozandeh and Aziz 2018). There are several pathways involved in nanoparticle uptake with specific dynamics and size rules including phagocytosis, micropinocytosis and the endocytic pathways of clathrin mediated, caveolin mediated and clathrin/caveolin-independent endocytosis (Zhu et al. 2013). In addition to these active mechanisms, diffusion is a passive uptake mechanism that has been shown for nanoparticle uptake (Behzadi et al. 2017).

Particle size is also a key factor in determining the efficiency of cellular uptake. Studies have suggested that a size around 50 nm results in the highest cellular uptake, where 50 nm gold nanoparticles have been shown to be taken up both at the highest rate and to the largest extent in numerous mammalian cells, as compared to both smaller- and larger-sized particles (Chithrani et al. 2006, Chithrani and Chan 2007). Huo et al. (2014) demonstrated that nanoparticles smaller than 10 nm (2 and 6 nm) could enter the nucleus, whereas larger sized particles were only found in the cytoplasm of MCF-7 breast cancer cells. It is believed that toxic effect in many cases is dependent on extent of uptake, and size-dependent cellular uptake may therefore contribute to size-dependent toxicity of particles.

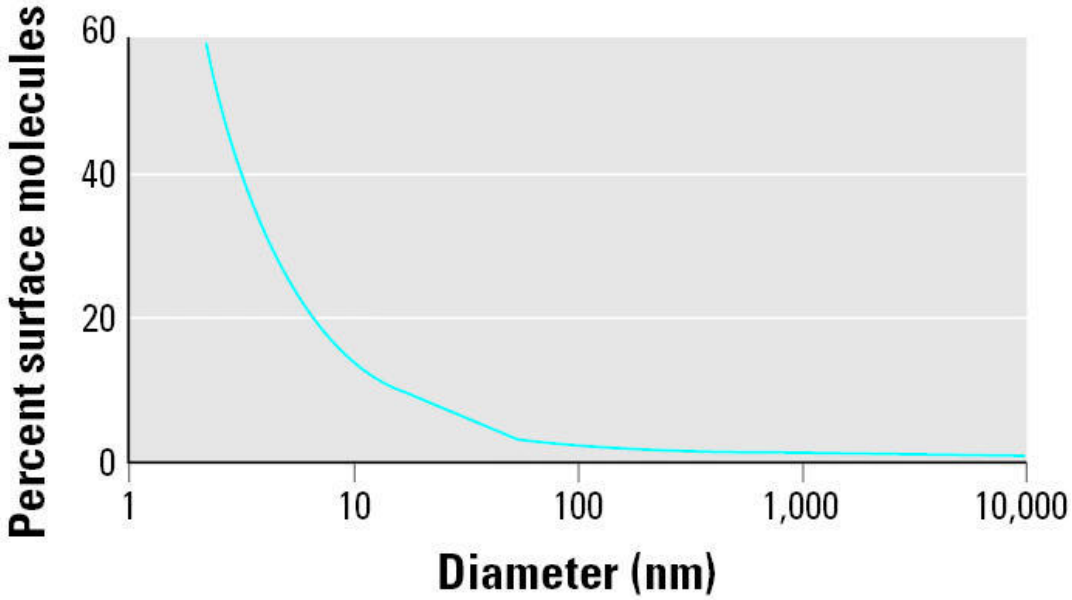


Figure 4. Surface molecules as a function of particle size. Reproduced from Environmental Health Perspectives, Oberdörster et al (2005) with permission from the author.

When considering particle size, it is important to distinguish between primary size and the size (and changes of size) of agglomerates or aggregates formed in a biological environment. Agglomerates typically include dispersed particles held together by weak physical interaction, in contrast to aggregates where the particles typically are strongly bonded with an irreversible process. As a result, agglomerates are generally fairly easy to separate by dispersion or a small amount of energy, while this is unlikely for aggregates (Stone et al. 2009).

2.3.1.2 Composition

Particles and nanomaterials are often divided into subgroups based on their composition, including metal-based, carbon-based or polymeric materials. The focus of this thesis is metal-based particles, which is a large and diverse group of particles including both those engineered with few (or single) components as well as non-engineered including combustion derived or welding fume particles with a complex composition of several metals and other constituents. The metals present in particles can occur in their metallic or oxide form as well as have different valence states. The chemical composition as well as the surface composition of particles are important aspects to consider as it largely defines the intrinsic reactivity and subsequent hazard

of particles, by containing and releasing more or less biologically active substances. As an example, particles may contain transition metals that generally comprise unpaired electrons with the ability to accept and donate single electrons, resulting in them being more prone to undergo redox reaction. If particles contain these soluble transition metals on their surface, this can e.g. generate ROS via Fenton-type reactions in solution which can result in toxic effects (Manke et al. 2013). As an example, Mn is a transition metal, existing in the oxidation states of -3 to +7, where some valence states are consider more stable in contrast to other which easily enters redox reactions (Sobańska et al. 2021). Furthermore, the surface composition, which can be very different from the bulk composition, also influences the properties of the particles including surface charge and dissolution rate, factors shown to be crucial for their toxic potency (Stockmann-Juvala et al. 2013, Hedberg et al. 2016b, Wang et al. 2019).

2.3.1.3 Dissolution and metal release

The process of dissolution is a key element in understanding the fate of particles in the body and the possible effects they can exert. If and how dissolution occurs can change the exposure, dose, behavior, and nature of the toxicant and by including this aspect in the study of particles, one can more correctly interpret biological response observed. As discussed in section 2.2.2, dissolution is a key factor determining bio-persistence and retention of particles in the lung. In nanotoxicological contexts, a great challenge is so evaluate whether the observed toxicity is due to the particle itself, the released ions or perhaps a combination of both (Midander et al. 2009, Misra et al. 2012, Cronholm et al. 2013, Karlsson et al. 2013).

Both solubility and rate of dissolution are greatly dependent on the particles chemical and surface properties. Other factor that may affect the dissolution of particles include the surrounding media and its composition as well as availability of constituents to form complexes with released ions (Misra et al. 2012, Utembe et al. 2015, Hedberg et al. 2016a, Cappellini et al. 2018). Size is another critical factor, where the larger surface area to volume-ratio can increase the ability for dissolution compared to larger sized particles (Utembe et al. 2015). This can be explained by that surface atoms are generally looser compared to bulk atoms and thus size affects the thermodynamically dissolution. Aggregation is also an important parameter, because it will decrease the available external surface area and thus influence the ion release (Utembe et al. 2015).

Dissolution is important for the risk assessment of nanoparticles, where it is considered a crucial parameter in order to identify whether a nanomaterial is stable enough to exert nano-specific behavior resulting in the need for a nano-specific approach (Dekkers et al. 2016). If the nanomaterial immediately dissolves into its molecular or ionic form before reaching its target, no nano-specific behavior is exerted. Dissolution is also considered important for categorization and grouping of nanoparticles (Giusti et al. 2019, Riediker et al. 2019, Braakhuis et al. 2021).

2.3.2 Mechanisms of toxicity

Toxicity of metal and metal oxide nano-sized particles have been studied and confirmed in a wide range of endpoints and some of the paradigms in particle-mediated toxicity involves oxidative stress, inflammation, and genotoxicity (Manke et al. 2013, Sengul and Asmatulu 2020). It has been argued that nano-sized materials do not elicit any unique modes of actions compared to larger sized particles, but instead have a gradual magnification of the intrinsic toxic potency and hazard as a function of decreasing size (Donaldson and Poland 2013).

Whether particle-induced toxicity is related to the particles themselves, released ions or a combination of both is in many cases not known. The uptake of particles is generally less well-regulated as compared to the uptake of metal ions (Krug and Wick 2011). An important aspect in the toxicity of nanoparticles is the "Trojan horse" transport mechanism, a mechanisms for the transport of ions into cells in the form of particles (Limbach et al. 2007). Briefly, metal ions are transported across the cell membrane in a highly regulated manner via ion channels while, in contrast, metal in particulate form is primarily taken up via endocytosis. Once inside the cell, the particles can undergo dissolution resulting in a release of ions potentially resulting in toxic effects. Therefore, the concentration of ions inside the cells can be significantly higher when exposed to metal nanoparticles compared to the corresponding ions. Lysosomal degradation resulting in release of toxic ions due to the acidic conditions has been proposed as a general mechanism for intracellular toxicity of metal-containing nanoparticles (Sabella et al. 2014). The impact of particles vs released ions for toxicity induced by welding fume particles is explored in **Papers II** and **III**.

2.3.2.1 Oxidative stress

The generation of reactive oxygen species (ROS) has been proposed as one of the main mechanisms behind particle induced toxicity and oxidative stress is an established paradigm within nanotoxicology (Donaldson et al. 2005, Shvedova et al. 2012, Manke et al. 2013). Similarly, ambient particulate matter has been shown to exert adverse effects via oxidative stress (Li et al. 2008).

The mechanisms behind particle induced ROS are not fully elucidated and are believed to differ depending on the physicochemical properties of the particles (Manke et al. 2013). Some particles, in particular metal-based ones, can be self-oxidative in nature and induce ROS intrinsically. If pro-oxidant functional groups are present at the surface of the particles, electrons can transfer to oxygen molecules creating superoxide radicals (Mortezaee et al. 2019). Another example is the release of metals ions following dissolution which can catalyse Fenton and/or Haber-Weiss reactions via redox cycling (Lee et al. 2012).

In addition, particles can interact with cells to activate cellular redox systems and induce intracellular generation of ROS. This can e.g. occur via interruption of the mitochondrial respiration resulting in a disrupted redox balance in the cell (Manke et al. 2013). Further, the action of phagocytes is an important defence against invasion of microorganisms and particles in the lung. When professional phagocytes of the immune system internalize particles, this

induces substantial ROS via the NADPH oxidase enzyme system. Due to their inducible nitric oxide synthase (iNOS), also reactive nitrogen species (RNS) including nitric oxide and the highly reactive peroxynitrite can be formed. The production and release of ROS and RNS from inflammatory cells is often referred to as "oxidative burst" (Shvedova et al. 2012).

The intrinsic ROS production of welding fume particles was assessed in **Paper II**. Oxidative stress was investigated as an endpoint in the ToxTracker assay used in **Papers I** and **II**.

2.3.2.2 Genotoxicity

Genotoxicity describes the potential of a substance to induce damage to the DNA and is a crucial part in the risk and safety evaluation of particles. If the DNA damage is left unrepaired or misrepaired, this can result in mutations that in turn can promote carcinogenesis. The mechanisms behind the genotoxicity of particles are still not well understood, but three forms of genotoxic mechanisms have been proposed (Magdolenova et al. 2014, Golbamaki et al. 2015). These are described below as well as illustrated in Figure 5.

i) **Direct primary genotoxicity** where particles interact directly with the genome. Particles that cross cell membranes may reach the nucleus via diffusion or transportation via nuclear pore complexes where they can potentially bind and interfere with processes resulting in DNA damage. Particles can also enter the nucleus during mitosis when the nuclear membrane is dissolved. During mitosis, particles could interact with chromosomes possibly resulting in chromosomal breaks (clastogenic effects) or loss of chromosomes (aneugenic effects). During interphase, particles could instead alter DNA replication and transcription.

ii) **Indirect primary genotoxicity** does not involve direct contact with the DNA molecule, instead the DNA damage could result from the particles interacting or disturbing other parts of the cell including nuclear proteins, mitotic spindle or cell cycle checkpoint functions. The generation of ROS is another example of indirect primary genotoxicity, generated either from the surface of the particle, release of transition metal ions, mitochondrial ROS or inhibition of antioxidant defence. ROS may attack the DNA resulting in oxidative DNA damage.

iii) **Secondary genotoxicity** refers to the genotoxicity generated via particle-elicited inflammation. The presence of particles can trigger inflammatory phagocytes, such as neutrophils and macrophages, to generate ROS as an innate inflammatory response to invading pathogens. However, the generation and release of ROS may have the undesirable effect of inducing genotoxicity in surrounding cells. In cases of chronic inflammation, this can result in persistent oxidative stress and thus repeated DNA insults.

In addition, three major indirect mechanisms have been suggested for carcinogenic metal compounds including oxidative stress, inhibition and interference with DNA repair mechanisms as well as deregulation of cell proliferation (Beyersmann and Hartwig 2008).

The endpoint of genotoxicity in the form of DNA strand breaks was assessed following exposure to welding fumes in **Papers II** and **III**. The more high-throughput screening tool ToxTracker assay was used to gain mechanistic insight into particle induced genotoxicity in **Paper I** and **II**, with reporters targeting pathways associated with DNA-double strand breaks or DNA replication stress.

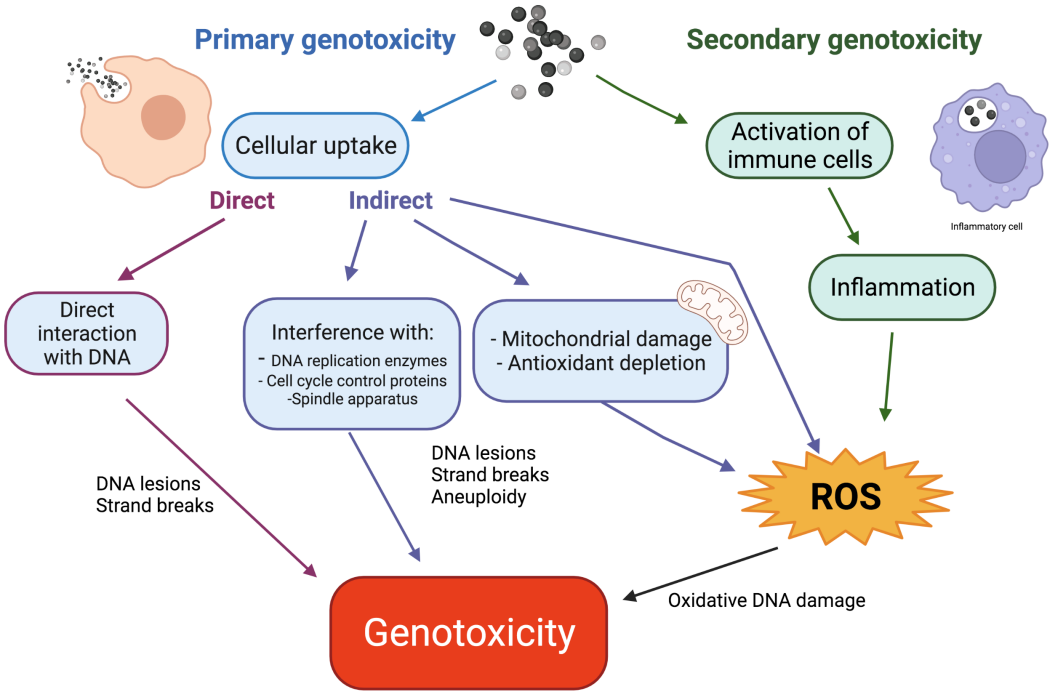


Figure 5. Illustration of the mechanisms of particle-induced genotoxicity. Illustration created using Biorender.com

2.3.2.3 Inflammation

Deposition of particles in the lung will initiate inflammation by the secretion of inflammatory cytokines and chemokines by epithelial cells. This results in the infiltration of macrophages and the recruitment of neutrophils and monocytes from the circulatory system to the site of inflammation (Mortezaee et al. 2019).

The induction of pulmonary inflammation depends largely on site and extent of particle deposition as well as on clearance. It has been proposed that the sooner the particle is cleared from the lung, the smaller the risk of developing inflammation (Braakhuis et al. 2014b). Chronic inflammation with induced ROS production and cytokine release can lead to impairment of normal functions such as fibrosis. Inflammation is directly linked with carcinogenesis and has been suggested to be the major driver of genotoxic effects of nanomaterials (Mortezaee et al. 2019, Kohl et al. 2020).

The inflammatory effects of welding fume particles were assessed by cytokine release *in vitro* in **Paper III**.

2.4 TOWARDS ALTERNATIVE HAZARD TESTING STRATEGIES

The traditional way of studying toxicological effects is by the use of animal models but this is considered time-consuming, costly as well as ethically problematic. The 3R principle (Reduce, Replace, Refine) is more and more becoming a public and legal demand, supporting the replacement of animal models with other human-relevant alternatives (Törnqvist et al. 2014). With the rapid increase of new nanomaterials constantly being put on the market, this emphasizes further that we cannot rely solely on traditional risk assessment strategies with strong dependence on animal data. Faster and more ethically sound models are therefore required, thus alternative models to animal testing are ongoing themes within particle- and nanotoxicology. In line with the paradigm of the Toxicity testing of the 21st century (National Research Council 2007), the field of nanotoxicology is progressing towards combined approaches of mechanistic *in vitro* data with bioinformatics and *in silico* modeling.

2.4.1 *In vitro* models to study particle toxicity

In vitro models provide simple, fast and more cost-efficient evaluations compared to animal testing. Cellular systems offer the possibility to study and explain mechanisms of toxicity, since they can provide information on molecular and cellular level. In addition, *in vitro* models include the possibility to manipulate molecular parameters under well-controlled settings. By providing a deeper insight into mechanisms of particle-induced effects, this can provide valuable input for e.g. ranking and prioritization for further toxicity testing.

Even though the future hazard testing is predicted to depend primarily on cell culture assays, uncertainties remain regarding the power of *in vitro* assays to predict *in vivo* effects and dose-response relationships which hampers their use in regulatory toxicology (Groothuis et al. 2015). From the fact that *in vitro* models are limited to one or a few cell types follows that they are not able to fully replicate the complex interaction occurring between cell types in an intact organism (Stone et al. 2009). As a consequence, *in vitro* responses can better reflect *in vivo* in certain manners including e.g. cell uptake, cell signalling and gene expression, whilst other more complex biological endpoints are limited including toxicokinetics and immune responses. A single *in vitro* assay will likely never be informative enough, but the combination of several assays and approaches may better reflect the hazards *in vivo* (Collins et al. 2017).

Significant efforts have been made to increase the complexity of *in vitro* models for lung exposure by co-culture and 3D cell culture systems, to improve the *in vivo* resemblance and better mimic lung physiology. As an example, Ji et al. (2017) developed a 3D model with a co-culture of primary human bronchial epithelial cells and fibroblasts. However, this is not a straight path since when adding additional levels of complexity this may also result in increased level of uncertainty which could defeat the purpose of the model. As an example, simple mono-layer cell models have been reported to better predict the acute toxicity of Ag nanoparticles compared to more advanced co-culture systems (Braakhuis et al. 2016).

With the increased reliance on *in vitro* models, there is a demand for a higher level of reliability, reproducibility, and predictability. Several essential aspects of the design and implementation of *in vitro* studies play a role in this, of which some are discussed below.

2.4.1.1 Dose metric

In vitro doses are in most cases given as particle mass per volume administered to the cell culture (e.g., μg/mL). Since particles will likely sediment over time, the dose can therefore also be expressed as particle mass per culture surface area (μg/cm^2). There is a lack of consensus regarding in which way the *in vitro* dose should be quantified and reported. Sayes et al. (2007) reported minor correlation between *in vitro* and *in vivo* inflammatory responses to nanomaterials when using the dose metric of mass per lung surface area. However, alternative nanoparticle dose-metric approaches (other than mass) have been observed to show better correlation for inflammatory effects including normalization to the surface area of the nanomaterial (Teeguarden et al. 2007, Groothuis et al. 2015) or cell target dose (Teeguarden et al. 2014). Since the toxicity can be dependent on different physicochemical characteristics of the particles, e.g. surface reactions or ions release, a unifying dose metric for all types of nano-sized materials is unlikely (Braakhuis et al. 2014b). An understanding of the toxic mechanisms of the particular material is likely needed to define the most appropriate dose metric under the given conditions.

To determine the most suitable dose metric for CdTe QDs in the ToxTracker assay, dose metric modelling analysis was performed in **Paper I**.

A clear distinction should in addition be made between nominal dose and delivered dose when using cellular models. In contrast to soluble chemicals, particles in solution sediment, diffuse and aggregate depending on their physicochemical properties as well as those of the surrounding solution. The effective dose *in vitro* is often assumed to be estimated by, or at least proportional to, the nominal dose i.e. the administered dose (Guggenheim et al. 2018). In reality, the delivered dose of particles is much more dynamic and complex and therefore less comparable between particle types and experimental setups as compared to soluble chemicals (Pradhan et al. 2016). It has been argued that nominal particle concentrations should be considered as exposure rather than dose, since nominal exposure does not accurately reflect the delivered dose in contact with the cells (Teeguarden et al. 2007, Groothuis et al. 2015). Cho et al. (2011) demonstrated cellular uptake of gold nanoparticles to be dependent on sedimentation and diffusion velocities of the particles rather than size, density, and initial particle concentration. For more accurate dose-response estimations and comparable interpretations of *in vitro* results, the determination of the local cell dose could be of importance. In addition, the delivered dose has the advantage to be directly comparable to delivered doses *in vivo*, e.g. where the *in vitro* delivered dose per surface area can be compared to the lung dose per surface area. The cellular dose can be determined by either experimental dosimetry involving the direct measurement of cellular uptake, but also by computational dosimetry (Teeguarden et al. 2007). The use of *in vitro* deposited dose has been shown to improve the correlation to *in vivo* data (Pal et al. 2015, Thrall et al. 2019).

The cellular uptake/dose following exposure to welding fume particles was quantified in **Paper III**.

2.4.1.2 In vitro to In vivo extrapolation

The shift from animal-based approaches towards *in vitro*-based mechanistic understanding poses a great challenge for health risk assessment since *in vitro* data cannot directly substitute animal studies. Instead, an extrapolation strategy is required in order to link *in vitro* responses to whole organism (*in vivo*) responses. The concept of *In Vitro* to *In Vivo* Extrapolation (IVIVE) can be generally defined as approaches applying *in vitro* experimental data to predict *in vivo* phenomena (Bell et al. 2018) and is crucial to improve the regulatory weight of *in vitro* data.

In order for *in vitro* results to be useful in a regulatory context, it is important that *in vitro* doses can be transformed to concentrations being relevant in environmental or occupational contexts (Drasler et al. 2017a). The physiological relevance of *in vitro* doses is frequently questioned, suggesting that unrealistic high doses are often used (Oberdörster et al. 2005). The use of more realistic doses should therefore be a high priority for *in vitro* testing. However, it is a tough task to provide human justification of applied particle concentrations *in vitro*, as relating nominal concentration inducing effect *in vitro* to exposure causing adverse effects in humans is not straightforward. The translation of particle dose from *in vitro* systems to relevant human exposure remains a major challenge in assessing the risk of particles.

IVIVE approaches can be used to better understand the relationship between real-world exposure scenarios and *in vitro* test concentrations, where the combination of *in vitro* dosimetry and lung dosimetry has been suggested as a valuable tool in the assessment of the lung toxicity of inhaled particles (Romeo et al. 2020). By using lung dosimetry models, one can simulate the deposition of particles in a human or animal lung which can provide detailed information on the extent of deposition in the regions of the lung. This can be used to both extrapolate deposited lung doses in animals to humans (Oller et al. 2014), or to estimate relevant *in vitro* doses based on human exposure (Gangwal et al. 2011, Smith and Skinner 2021). As emphasized in Romeo et al. (2022), despite the value of dosimetry models, their use for selecting relevant *in vitro* doses and comparing *in vitro* and *in vivo* data is still not general practice.

In order to better understand the physiological relevance of our *in vitro* dose levels and findings in **Papers II** and **III**, lung dosimetry modeling was used to derive human lung doses following short- and long term real-life occupational welding scenarios in **Paper IV**.

2.4.2 Predictive toxicology

A great challenge in both toxicity testing and risk assessment of particles is the enormous diversity in for example core material, surface composition, size, shape and impurities of the nanomaterials being produced. The dynamic transformation of surface corona as well as batch-to-batch variability of engineered nanoparticles impose further challenges (Singh et al. 2019).

With the huge number of nanoparticles, a proper safety evaluation of every single nanoform on a case-to-case basis would be extremely costly and time-consuming. There is therefore an urgent need for testing strategies that can provide a screening approach for evaluating potential hazards of nanomaterial and to enable the prioritization for toxicological testing. Predictive toxicology is thought to be used to rapidly evaluate potential hazards where inconclusive or lacking data exists.

2.4.2.1 Grouping and read-across

In the risk assessment of particles, predictive modeling approaches such as grouping and read-across are growing into important tools (Clark et al. 2011). Read-across is a technique for predicting the fate or hazard for one substance, based on data on the same endpoint from another substance with more available data (European Chemicals Agency 2017). The principle is built on that the substances are similar or follow a certain trend regarding e.g. chemical structure and physicochemical or toxicological properties, allowing them to be considered as a group. This approach aims to increase the efficiency of hazard assessments and can be used to fill data gaps in hazard assessments as well as reduce the necessity for additional *in vivo* or *in vitro* studies.

Currently, no regulatory framework exists but a series of grouping approaches has been developed and proposed for nanomaterials in the last decade as summarized in Giusti et al. (2019). In a recent paper by Visser et al. (2022), six subgroups of engineered nanomaterials were proposed to serve as reference points for deriving health-based occupational limit values. The grouping for nanomaterial is considered much more complex than for non-nanoform material due to the variation in properties related to chemical identification and physical characterization. Some of the current limitations are the lack of knowledge regarding relationships between physiochemical properties and particle behaviour as well as the amount of available data (Koltermann-Jülly et al. 2018).

2.4.2.2 High throughput screening

Conventional *in vitro* toxicity assays are traditionally based on one endpoint and treatment at a time. This results in a mismatch between the high number of nanomaterials being introduced on the market and the current available low-throughput methods for evaluating their toxicity. To meet the demand of the nanotechnological market, more rapid and efficient tools are needed in combination with a move towards predictive toxicology. High-throughput methods involve the use of automated tools to facilitate rapid toxicity testing of a large number of substances simultaneously, providing an efficient way of evaluating unwanted effects of novel compounds (Nel et al. 2013). This is in line with Toxicity testing in the 21st century, promoting the move from descriptive animal testing to quantitative, mechanistic and pathway-based toxicity testing *in vitro* using high throughput approaches (National Research Council 2007). *In vitro* screening methods can facilitate the hazard ranking of nanomaterials and allow for the prioritization for further toxicity testing. Ideally, the screening also allows for mechanistic profiling to get a more

comprehensive understanding of potential outcomes as well as improve risk assessment strategies.

In **Papers I** and **II**, we used the ToxTracker assay as a mechanistic high-throughput screening tool for the genotoxic evaluation of a variety of metal-based manufactured nanoparticles as well as welding fumes particles.

2.5 MANUFACTURED METAL-BASED NANOPARTICLES

Metal- and metal oxide nanoparticles are the most widely used nanoparticles within industry due to their e.g. optical, semi-conductive, photo-thermal and catalytic properties making them appealing from a technical point of view for a wide range of applications (Sengul and Asmatulu 2020, Karlsson et al. 2022). Some examples of applications of metal-based nanoparticles include silver nanoparticles being widely used as antimicrobial agents and gold-based nanoparticles extensively investigated for medical and biomedical applications including diagnostics and drug delivery (Karlsson et al. 2022). Quantum dots (QDs) are used for optical, bioanalytical and bioimaging applications (Reshma and Mohanan 2019). Manganese oxide nanoparticles have been proposed as a novel magnetic resonance imaging contrast agents but also to be used for soil remediation (Sobańska et al. 2021).

There is limited available information about the health risks associated with exposure to engineered nanoparticles and understanding the occupational health and safety aspects is an ongoing process (Pietroiusti et al. 2018). With the immense variation of manufactured nanomaterial, this makes it impossible to evaluate them as a single class of substances which further emphasizes the importance of read-across and prioritization. There is still not enough scientific data to establish OELs for any type of nanomaterial, owing to the large knowledge gaps and lack of robust scientific evidence on nanomaterial hazards, dose-response relationships and also the inconclusiveness regarding which dose metric should be used (Mihalache et al. 2017, Pietroiusti et al. 2018). Nonetheless, a systematic review in 2017 identified 56 proposed OEL values for manufactured nanomaterials, in which the OELs for metal-based nanomaterials varied with a factor from 100 to 300 (Mihalache et al. 2017).

2.6 WELDING FUME PARTICLES

Welding is a broad term for the process used to join metal pieces together by high temperature in which welding fumes are created as an inevitable consequence. Individual fume particles in the nano- and micrometer size range are primarily formed near the arc, but quickly aggregate to form longer chains of primary particles (Berlinger et al. 2011). More than 11 million workers perform welding as their primary occupation worldwide, whereas a total of 110 million people are believed to somehow be exposed to welding fumes in their occupational setting (IARC. 2017). The corresponding numbers of full-time welders in Sweden was estimated to 25 thousand, while approximately 250 thousand people perform welding as part of their job, as stated in a report conducted by the Swedish Work Environment Authority in 2013 (Swedish Work Environment Authority 2013). Thus, welders are a large occupational group at risk of being exposed to high concentrations of metal-based particles present in welding fumes.

There are many different welding techniques routinely employed in occupational settings. In arc welding, an arc is formed between the base metal and an electrode which creates heat to melt and join the metals together. Some of the more common types of arc welding include gas metal arc welding (GMAW) and flux cored arc welding (FCAW), of which GMAW is the most common industrial welding process (IARC. 2017). In both GMAW and FCAW welding, a consumable wire electrode is fed through the welding gun together with a shielding gas in order to protect the weld from contaminants and eliminating slag, schematically illustrated in Figure 6. The shielding gas is typically helium, argon, carbon dioxide or a blend of these gases. The main difference between GMAW and FCAW lies in the consumable electrode, where GMAW uses a solid electrode and FCAW uses a flux cored wire (FCW) consisting of a metal shell with a flux containing core.

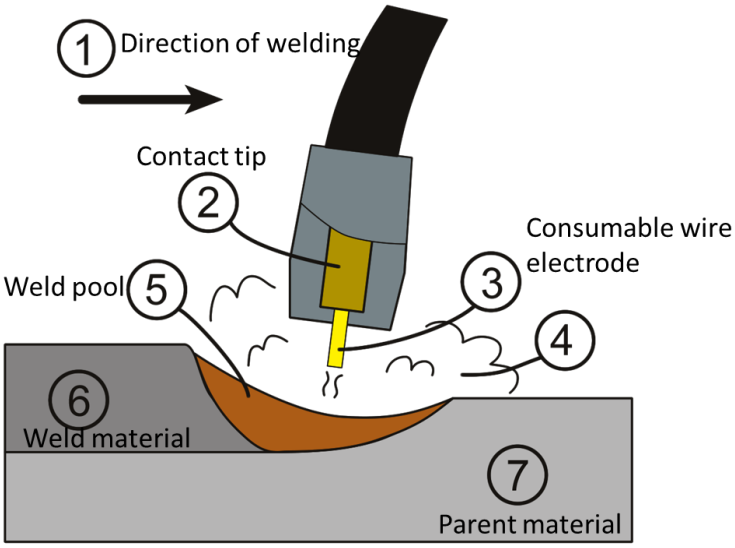


Figure 6. Image of the Gas metal arc (GMAW) welding process. Reprinted and modified under creative commons license from https://commons.wikimedia.org/wiki/File:GMAW_weld_area.png

The welding fumes investigated in **Paper II** and **III** are generated by GMAW or FCAW of stainless steel. All types of welding fumes were considered for deposition modelling in **Paper IV**.

2.6.1 Particle characteristics

Welding fume particles are a heterogenous group of particles of varying properties including size, bulk- and surface composition, and solubility. These properties are largely determined by the welding conditions and materials. The composition of the fume particles depends on several sources including base metal, electrode, shielding gas or surface coating (Mei et al. 2018, Zeidler-Erdely et al. 2019). Welding fume particles consist mainly of oxidized metals from the base or filler metal as well as silicates and fluorides often originating from the coating of electrodes and fluxes used (Floros 2018). All these sources, together with the fact that that welding materials consist of metal alloys with diverse amounts of e.g. Iron (Fe), Manganese (Mn), Chromium (Cr) and Nickel (Ni), results in welding fume particles of rather complex and diverse chemical compositions.

In occupational settings, welding is most commonly used to weld mild steel or stainless steel, with the majority being mild steel welding (approx. 90 %). All types of steel are alloys of Fe and other elements, where mild steel contains predominantly Fe with a small percentage Mn. Stainless steel contains, depending on grade, considerable levels of Cr, Mn and/or Ni and

molybdenum (Mo), in addition to Fe, which make it considerably more corrosion resistant (due to the microstructure and formation of a passive surface oxide) compared to mild steel. This results in fume particles containing Cr, Mn and/or Ni. Generated fume particles upon welding of mild steel have clearly much lower levels (or none) of Cr and Ni.

Several of the metal constituents of welding fumes are established carcinogens or have been linked to other health effects. Cr is often detected in two valence states in welding fumes: trivalent chromium (Cr(III)) and hexavalent chromium (Cr(VI)), where Cr(VI) is a well-recognized carcinogen (IARC. 2012a). Ni compounds have also been classified as group 1 carcinogens by IARC (IARC. 2012b). The presence of Mn in welding fumes is considered of concern mainly due to potential neurotoxic effects (Antonini et al. 2006, Taube 2013, Rana et al. 2019). Fe is the predominant metal in most welding fumes and has been considered a nuisance dust of low probability in causing chronic lung disease. However, more recently also Fe has been suggested to play a large part in the toxicity of welding fumes (Falcone et al. 2018b).

Processes and conditions during the welding process such as welding technique, applied potential and melting rate may also influence the particle properties (Zimmer and Biswas 2001, Yoon et al. 2003, Keane et al. 2009, Antonini et al. 2011a, Brand et al. 2013). As an example, Brand et al. (2013) showed that welding processes with high emission rates mainly resulted in agglomerated particles with diameters exceeding 100 nm, in contrast to welding processes with low emission rate emitting predominantly ultrafine fume particles. This was suggested due to faster agglomeration processes taking place at high mass emission rates. As emphasized in Antonini et al. (2011a), the understanding of how welding conditions affect welding fume aerosol formation as well as associated biological response can contribute to welding process improvement and development of novel technologies to prevent adverse health outcomes.

Papers II and **III** focus on the influence of welding parameters on the particle properties and toxicity of welding fume particles and how these can be modified in order to generate less toxic welding fumes and thereby attain safer occupational environments for welders.

2.6.2 Exposure and regulatory limit values

Occupational exposure to fumes from welding stainless steel and mild steel has been reported to vary with levels up to more than 25 or 50 mg/m^3, respectively (IARC. 2017). The high variability in exposure levels determined can be explained by the different base metals, welding techniques and varying circumstances. Some processes generate inherently low amounts of fume, whilst some methods are associated with higher fume emissions. FCAW has been demonstrated to generate the highest levels of welding fumes among several different welding techniques (Lehnert et al. 2012). In addition, welders may work in various settings at both indoor or outdoor conditions and in spaces ranging from confined to wide-open with more or less sufficient ventilation. Lehnert et al. (2012) measured the exposure at 33 welding worksites and reported concentrations to be mainly predicted by the welding process but significantly higher when the local exhaust ventilation was inefficient or when welding was performed in

confined spaces. It has also been suggested that the quality of the welding performance affects the exposure level, indicating increased exposure concentrations for apprentice welders or welders with little training (Graczyk et al. 2016).

Limit values used in the regulation of occupational exposure to welding fumes are generally set to 5 mg/m^3 (IARC. 2017). Many countries do not have a specific limit value for welding fumes, but instead apply limit values for inhalable/respirable dust. This is the case in Sweden, where the OELs for inorganic dust (5 mg/m^3 inhalable, 2.5 mg/m^3 respirable) is applicable in welding settings (Swedish Work Environment Authority 2018). In a recent editorial by Sjögren et al. (2021), it is argued that due to accumulating evidence for serious health effects at levels below 5 mg/m^3, an OEL specific for welding fumes is urgently needed. It is also emphasized that the OEL should be based on all health effects related to welding fume exposure as well as take the various welding methods into account. Prior to the printing of this thesis, an evaluation of OEL for welding fumes has been initiated by the European Chemicals Agency.

Occupational exposure is also regulated by the level of different metal constituents of welding fumes. Relevant OELs for welding fumes are those for Cr (0.5 mg/m^3), Ni (0.5 mg/m^3) and Mn (0.2 mg/m^3) in Sweden (Swedish Work Environment Authority 2018). Since the involvement of metal constituents in welding fume toxicity still remains to be elucidated, the approach of only applying limit values for individual metals may not be the best practice. This is especially the case for those categorized as less toxic such as Fe. Therefore, it is crucial that we work towards understanding the contribution of individual metals or combinations of metals as well as their associated mechanisms. The combination of a general OELs for welding fumes together with specific OELs for metal constituents would make it easier to ensure safe levels for different types of welding practices (Sjögren et al. 2021).

2.6.3 Health effects related to welding fumes

The exposure to welding fume particles has been linked to several health effects, primarily in the respiratory region. Epidemiological studies have indicated welders to have an increased risk of developing numerous lung and airway conditions including acute respiratory injuries as well as common chronic inflammatory respiratory diseases such as asthma, bronchitis and obstructive pulmonary disease (Antonini 2003, Riccelli et al. 2020). The exposure to welding fumes has also been suggested to be associated with the development of neurological dysfunction, which has been attributed to the presence of Mn in welding fumes (Antonini et al. 2006, Bailey et al. 2018).

The International Agency for Research on Cancer (IARC) recently classified welding fumes as Group 1 carcinogenic to humans, as an upgrade from the Group 2 (possibly carcinogenic to humans) classification that was established in 1989 (IARC. 2017). This was based on the majority of approximately 50 epidemiological studies reporting an increased risk of lung cancer in welding related occupations. Epidemiological studies have indicated the risk of lung cancer to be increased with the exposure duration and total cumulative exposure (Siew et al. 2008, t Mannetje et al. 2012, Matrat et al. 2016).

2.6.3.1 Mechanisms of toxicity

The mechanisms behind welding fume induced toxicity and carcinogenicity are not fully elucidated and as discussed in section 2.6.2, there is limited knowledge on the effect and involvement of the individual metal constituents or their combinations. It has been suggested that welding of mild steel, accounting for the majority of all welding, results in a small risk for developing lung cancer due to the absence of carcinogenic metals including Cr in the form of Cr(VI) and Ni. On the contrary, IARC have concluded that there is no evidence for the increased cancer-risk being limited to fumes formed when welding stainless steel which contain Cr and Ni (IARC. 2017). Instead, an increased risk of lung cancer was concluded for welding fumes regardless of welding process or material used.

The main carcinogenic characteristic of welding fumes has been suggested to be their ability to induce chronic inflammation (IARC. 2017, Guyton et al. 2018). The exposure to welding particles generated with stainless steel welding has been reported to induced mild yet chronic inflammation in tumor-susceptible A/J mice but were not found to initiate tumor formation (Zeidler-Erdely et al. 2008, Zeidler-Erdely et al. 2011). Instead, these welding fume particles were later demonstrated to act as tumor-promoters in A/J mice using a two-stage initiation-promotion model (Zeidler-Erdely et al. 2013, Falcone et al. 2017). Using the same model, fumes generated by mild steel welding were also shown to promote lung tumor *in vivo*, yet in the absence of inflammation (Falcone et al. 2018a). This provides support that welding fumes formed during welding of both stainless steel and mild steel could act as lung tumor promoters rather than initiators. These results align with the epidemiological evidence demonstrating also welders of mild steel to be at increased risk for lung cancer, despite the lack of carcinogenic metal constituents in the fumes. The fact that lung tumor promotion was observed regardless of chronic lung inflammation indicates additional or alternative mechanisms to be involved.

The presence and combinations of metal constituents in welding fumes have been shown to be important determinants for the respiratory toxicity in animals. In general, the pneumotoxic potential of fumes from welding mild steel is suggested to be low (Antonini et al. 1997, Taylor et al. 2003, Antonini et al. 2011b, Shoeb et al. 2017a, Shoeb et al. 2017b). Fumes formed during welding of stainless steel have been demonstrated to result in more pulmonary toxicity *in vivo* compared to mild steel (Taylor et al. 2003, Zeidler-Erdely et al. 2008, Antonini et al. 2010, Antonini et al. 2011b), results suggested to be connected to the presence of more toxic metals. *In vitro* studies have also shown fumes from stainless steel welding to be more reactive and toxic compared to mild steel (Antonini et al. 1999, Antonini 2003, Antonini et al. 2005, Leonard et al. 2010, Cediel-Ulloa et al. 2021).

The generation of ROS and oxidative stress has been proposed to be a main driver of the welding induced toxicity. Several of the metals found in welding fumes are transition metals including Fe, Cr and Mn. The pro-inflammatory effects have been shown to be driven by oxidative stress as a result of soluble transition metal components *in vitro* (McNeilly et al. 2004). The chemical composition is suggested to have a significant impact on the ROS generation capacity, where fumes of stainless steel welding have been reported to induce more

ROS generation and oxidative damage compared to those of mild steel (Antonini et al. 1997, Leonard et al. 2010, Shoeb et al. 2017a, Cediel-Ulloa et al. 2021).

Understanding the contribution of the individual metals in the pneumotoxic effects of welding fumes are important to ensure welder health and safety. A study by Falcone et al. (2018b) investigated the pulmonary toxicity and tumor promoting activity in A/J mice following exposure to either fumes from stainless steel welding or individual surrogate metal oxides including Fe, Ni, Cr(III) and Cr(VI). The results demonstrated particles of iron oxide (Fe_2O_3) to have the highest inflammatory potential of the individual metals tested and to be the only metal oxide which induced persistent pneumotoxic effects and significantly promoted lung tumors. Cr compounds were shown to induce acute but non-persistent pneumotoxic effects. This suggests that Fe may be an important constituent of welding fume toxicity and supports the findings that fumes generated during welding of both mild and stainless steel welding increases the risk of lung cancer.

The mechanisms and role of metal constituents in the acute toxicity of fume particles formed upon stainless steel welding were explored *in vitro* in **Papers II** and **III**.

3 RESEARCH AIMS

The overarching aim of this thesis was to gain a deeper understanding of the toxicity and fate of metal-based particles in the lung and how these aspects can be assessed. This was accomplished by studying toxicity and cellular mechanisms of particles *in vitro* using cell models (**Papers I-III**). In addition, challenges of particle toxicology were explored ranging from high-throughput mechanistic screening (**Paper I**) to relating *in vitro* doses to human exposure by lung deposition modelling (**Paper IV**). The particles investigated include both manufactured nanoparticles (**Paper I**) and non-intentionally generated during welding (**Papers II-IV**). The increased knowledge of hazards related to metal-based particles is believed to contribute to improved regulations as well as the development of better work-related practices, ultimately resulting in a safer occupational environment for workers within this field. More specifically, the objectives of the studies were to:

- Investigate the genotoxicity and underlying mechanisms of a wide battery of metal- and metal oxide nanoparticles, including welding particles, by using the ToxTracker reporter cell assay as a method for mechanistic screening (**Papers I** and **II**)
- Evaluate the influence of welding methods and materials on the *in vitro* toxicity of welding fume particles formed upon welding of stainless steel as well as explore the relation between toxicity, particle characteristics and metal release in a human lung cell model (HBEC-3kt) (**Paper II**)
- Explore the influence of released hexavalent chromium in welding fume induced toxicity in human lung cells (HBEC-3kt) and monocyte derived macrophages (THP-1) and investigate if modified electrodes can aid in the generation of less toxic welding fumes (**Paper III**).
- Estimate the deposition of welding fume particles in the lung based on occupational exposure scenarios in order to correlate human lung doses to doses used for *in vitro* investigations (**Paper IV**).

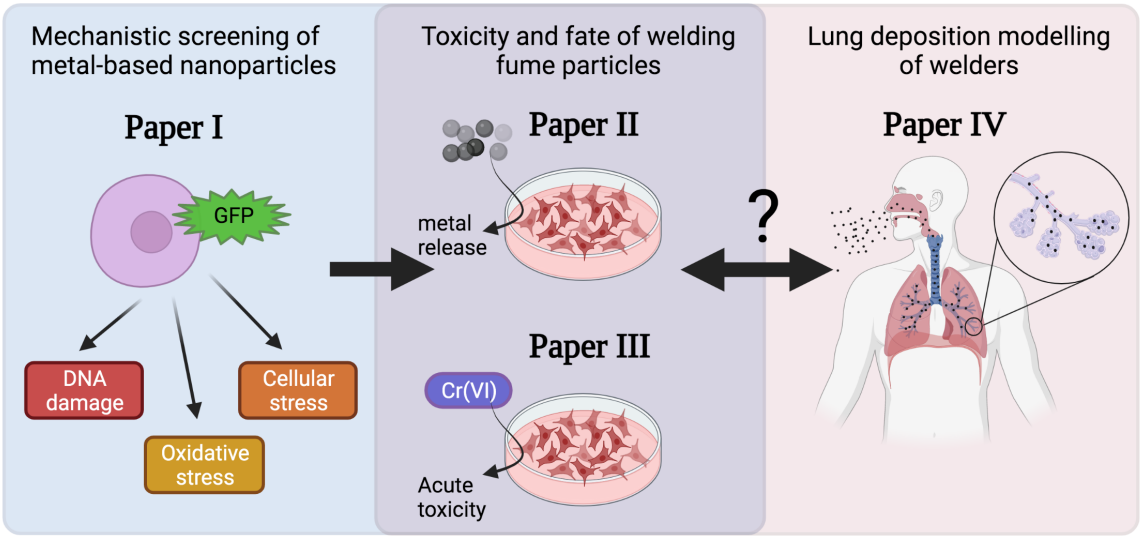


Figure 7. Overview of the studies included in this thesis. Illustration created using Biorender.com

4 METHODOLOGICAL APPROACH

4.1 GENERAL STUDY APPROACH

The general approach of this thesis is based on *in vitro* models (**Paper I-III**), where several cell types, method for particle characterization as well as toxicity endpoints have been used to assess the toxicity of the particles investigated. A different approach was conducted in **Paper IV**, which was instead built on a literature review in combination with deposition modelling. This section of the thesis provides an overview of the models and methods utilized in this thesis with specific emphasis on their benefits as well as their limitations. More detailed technical information is given in the Material and Methods section of the appended **Paper I-IV**.

4.2 MANUFACTURED NANOPARTICLES

Table 1. Manufactured nanoparticles used in this thesis

Particle	Primary size	Form	Paper	Reference
Ag	5 nm	1 mg/mL dispersion (citrate-coated)	**I**	McCarrick et al. 2020
Au	5 nm	1 mg/mL dispersion (milli-Q)	**I**	
CdTe QDs	1.5-8.6 nm	Powder	**I**	
Cr	35-45 nm	Powder	**I**	
Cr_2O_3	10-30 nm	Powder	**I**	
Mn	20-40 nm	Powder	**I**	
Mn_3O_4	<100 nm	Powder	**I**	
Pt	5 nm	1 mg/mL dispersion (citrate)	**I**	
Sb	N/A	Powder	**I**	
Sb_2O_3	150 nm	Powder	**I**	
Sn	10-20 nm	Powder	**I**	
SnO_2	20-40 nm	Powder	**I**	
V	80-100 nm	Powder	**I**	
V_2O_5	80 nm	Powder	**I**	

4.3 WELDING FUME COLLECTION AND PREPARATION

The fume particles were collected on Macherey Nagel MN 640 w (ash content < 0.01 wt%) cellulose filters (Ø 240 mm) using standard particulate fume emission procedures (fume box according to ISO EN 15011-1). The welding process was performed by an experienced operator and performed until a sufficient mass of fumes was collected on the filter. The filter with fume particles were closed immediately after welding and stored in band-heat sealed plastics bags

The welding fume particles where either brushed of the filter and provided as powder or provided as particles on filter. In the latter case, extraction of fume particles for *in vitro* toxicity testing was performed by immersing the filter in ultrapure water and ultrasonicating the sample before the filter piece was removed from the suspension. The particle concentration in the water suspension were based on the difference in filter weight before and after extraction.

Table 2. Welding fume particles investigated within the framework of this thesis

ID	Filler material	Base alloy	Form	Paper	Reference
S1	Solid, 308L	AISI 304L	Powder	**II**	McCarrick et al. 2019
M1	MCW, 308L	AISI 304L	Powder	**II**	
M2	MCW, UNS S32101	UNS S32101	Powder	**II**	
F1 (**Paper II**)	FCW, 308L	AISI 304L	Filter	**II**	
F2 (**Paper II**)	FCW, 308L	AISI 304L	Filter	**II**	
F3	FCW, UNS S32101	UNS S32101	Filter	**II**	
F4	FCW, UNS S32101	UNS S32101	Filter	**II**	
F5	FCW, UNS S32101	UNS S32101	Filter	**II**	
F6	FCW, UNS S32101	UNS S32101	Filter	**II**	
F1 (**Paper III**)	Standard FCW, 2205	UNS S2205	Filter	**III**	McCarrick et al. 2021
F2 (**Paper III**)	Standard FCW, 316 L	AISI 316L	Filter	**III**	
Red1	Cr(VI)-reduced FCW, 316 L	AISI 316L	Filter	**III**	
Red2	Cr(VI)-reduced FCW, 316L	AISI 316L	Filter	**III**	

4.4 PARTICLE CHARACTERIZATION

A thorough characterization is a crucial part in the evaluation of particles and allows the correlation between particle characteristics and toxicological outcome. Therefore, particle characterization was included as key parts in the *in vitro* studies conducted (**Papers I-III**).

The particle characterization was performed under inter-disciplinary collaborations and primarily performed by researchers at the Division of Surface and Corrosion science at the Royal Institute of Technology (KTH) in Stockholm.

4.4.1 Particle size and morphology

Primary particle size is the particle size after generation or synthesis, i.e. before the particles are put in biological solutions. A common method used to estimate primary particle size is by the use of transmission electron microscopy (TEM), which was utilized in **Papers I-III.** TEM is a type of electron microscope which uses a broad beam of electrons that transmit through the object which, at arrival at the detector, creates an image of the internal structure of the sample including morphology and crystal structure (Modena et al. 2019). TEM has a huge magnification capability of up to 10-50 million times, providing details at the atomic level (Lin et al. 2014).

Scanning electron microscopy (SEM) is a different type of electron microscopy, providing information on the samples surface and corresponding composition. SEM was used for surface morphology and compositional analysis of welding fume particles in **Paper III**. The principle is based on a high-energy fine beam of electrons used to scan across the surface of the sample. The microscope captures details about the interaction between the sample and the electrons reflecting the atomic composition and topographic details of the sample surface, creating a magnified image (Lin et al. 2014). SEM yields lower resolution images compared to TEM. In general, SEM is more user-friendly compared to TEM and enables faster measurements (Modena et al. 2019). TEM and SEM are both usually performed in dry state, and sample

destruction and measurement under non-physiological conditions are drawbacks of both methods. Examples of TEM and SEM images are shown in Figure 8.

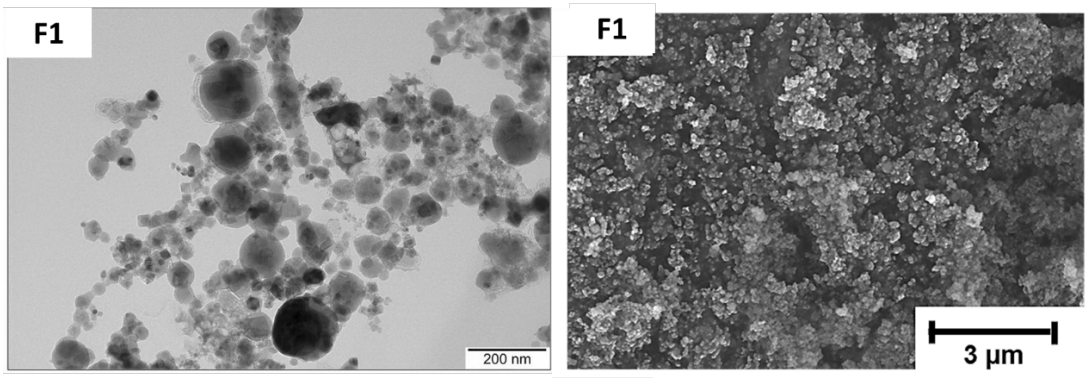


Figure 8. TEM (left) and SEM (right) image of welding fume particles from **Paper III**.

4.4.2 Agglomeration

By measuring particle size distribution in cell media, information on extent of particle agglomeration/aggregation and sedimentation can be attained. In **Paper I**, photon cross correlation spectroscopy (PCCS) was used to study the size distribution of welding fume particles. In PCCS, scattered light from suspended particles under Brownian motion is used to obtain their hydrodynamic size distribution. The principle of PCCS is an optical arrangement which splits the incident beam into two, illuminating the sample at different angels. The scattered light is further collected by two detectors, followed by a cross-correlation of the light intensities (Xu 2008). This technique allows for precise filtering of the single scattered fractions from the scattered intensity, which enables the exclusion of multiple scatter being an advantage when measuring smaller sized particles. The drawback is that the technique assumes spherical particles.

4.4.3 Chemical composition

Compositional analysis of the bulk and surface of the investigated particles can be performed using a variation of different techniques of which several were employed in this thesis.

Atomic Absorption Spectroscopy (AAS) is used for measuring total concentrations of metallic elements in material or in solution. Solid material needs to be digested before metal analysis by AAS which is usually performed in acids (e.g. HNO3 or aqua regia) with the goal to mineralize all organic molecules and dissolve all metal particles. Digestion can also be performed using microwave- or hydrogen peroxide-based techniques. The sample digestion needs to be optimized for each particle type and matrix used. In the AAS analysis, the material is atomized in flame or electrothermal atomizers. Elements can then be identified based on the amount of light absorbed at a defined wavelength corresponding to element specific absorption spectra. AAS is a highly sensitive method providing a high degree of accuracy. It offers high-throughput analysis, but a major drawback is that it can only determine concentration of one element at a time. AAS was used in **Papers II** and **III**.

The phase composition and crystal structure of welding fume particles were characterized by X-ray diffraction (XRD) in **Paper III**. XRD is a tool for resolving the tertiary structures of crystalline materials at the atomic scale. Incident X-rays with a well-known wavelength diffract on the periodically arranged atoms which the material consists of.

The outermost surface (5-10 nm) composition was determined by X-ray photoelectron spectroscopy (XPS). This method uses an x-ray beam to excite the molecules on the surface of a sample resulting in release of photoelectrons. The kinetic energies of the photoelectrons are measured and used to identify elements due to their specific characteristics and disclose chemical state information from the elements in the sample (Stevie and Donley 2020). XPS was used in **Paper II** and **III**.

Energy-dispersive x-ray spectroscopy (EDS) can be combined with SEM analysis. When the sample is excited by the electron beam of an electron microscope, some of the absorbed energy can eject core-shell electrons. An outer-shell electron of higher energy further fills its place, releasing the energy difference as an X-ray. The X-ray has a characteristic spectrum based on the atom of origin allowing for the compositional analysis of a sample. EDS was utilized in **Paper III**.

4.4.4 Redox potential

Redox potential is a measure of a systems affinity for electrons. The movement of electrons between the reduction and oxidation of two compounds results in an electric potential. This potential is determined by the ratio of activities of oxidized and reduced species which can be measured by an oxidation-reduction potential electrode. The redox potential of welding fume particles was measured in **Paper III**.

4.4.5 Metal release

The measurement of metal release from metal-based particles is crucial to understand the stability of the particle as well as the potential toxic contribution of released ionic species. In **Papers II** and **III**, metal release from welding fume particles in phosphate-buffered saline (PBS) was determined. This was done by incubating welding fume particles in PBS for 24 h at 37 °C with agitation. The particles were separated from the dissolved species by an inorganic syringe membrane filter. In **Paper III**, metal release was also determined after incubation in cell medium. The welding fume particles were incubated in cell medium for 24 h at 37 °C, followed by particle separation by means of centrifugation.

Two different methods were used for the quantification of released metals: AAS (as described in section 4.4.3) and inductive coupled plasma mass spectrometry (ICP-MS). ICP-MS is a type of mass spectrometry using an inductively coupled plasma to ionize the sample, where the ions are sorted out according to their corresponding mass (Wilschefski and Baxter 2019). Similar to AAS, ICP-MS analysis requires sample digestion prior to analysis. Measurements are prone to matrix effects, but this can be overcome by matrix matching and the inclusion of an internal standard element. ICP-MS has extremely low detection limits (in some cases lower compared

to AAS), a high linear dynamic range and allows for the analysis of isotopes. ICP-MS has the major advantage of multi-element capacity, allowing multiple elements to be analyzed simultaneously. This is in contrast to AAS, being specific for one element per analysis. Disadvantages are the occurrence of spectral and non-spectral interferences as well as high cost.

4.5 CELL MODELS AND EXPOSURE

In this thesis, several different cell lines have been utilized to investigate toxic effects of the metallic particles.

Mouse embryonic reporter stem (mES) cells are the basis of the ToxTracker reporter assay that was used in **Paper I**. mES cells are a genetically stable and are proficient for the major cellular pathways associated with DNA damage and cellular stress. They divide indefinitely and have a high rate of proliferation resulting in high sensitivity to genotoxicity and oxidative stress (Hendriks et al. 2012, Giachino et al. 2013). This makes them a good model option for developing *in vitro* genotoxicity assays.

The human bronchial epithelial cell line HBEC-3kt was used in **Papers I** and **III**. HBEC-3kt is a cell line immortalized by human telomerase reverse transcriptase (hTERT) and cyclin-dependent kinase (Cdk4) (Ramirez et al. 2004). The cell line has epithelial morphology, expressing epithelial markers and an intact p53 checkpoint pathway. Despite them being immortalized, they do not display any other cancer cell phenotypes such as disrupted p53 pathway and ability to form tumors in immunodeficient mice (Sato et al. 2013). They do not form colonies in soft agar or tumors in nude mice (Ramirez et al. 2004). This makes HBEC-3kt a physiologically appropriate *in vitro* model for studying cancer effects in bronchial epithelial cells with normal phenotype.

The human monocyte cell line THP-1 was used to derive macrophages in **Paper III**. THP-1 cells are cancerous cells originally obtained from a 1 year old infant with acute monocytic leukemia cultured in suspension (Tsuchiya et al. 1980). With the use of Phorbol 12-myristate 13-acetate (PMA), the monocytes can be differentiated into a macrophage-like phenotype with functional characteristics resembling primary macrophages. Following differentiation, the cells change morphology, express macrophage markers, become adherent and stop proliferating. THP-1 cells have become the widely most used cell line for investigating human macrophage response to pro-inflammatory stimuli (Chanput et al. 2014).

For the particle exposures in **Paper I-III**, cells were grown in cell line-specific medium and kept in a humidified environment with 37 °C and 5 % CO2. For exposure, the culture medium was removed, and cells were exposed to particles under submerged conditions.

In **Paper III**, cells were also exposed to the released metal fraction of welding fume particles, in order to elucidate particle and released metal ion effects. The released fraction was prepared by incubating fume particles with fresh cell medium for 24 h at 37 °C. Next, the suspensions were centrifuged creating a pellet of undissolved particles. The supernatant was considered as

the released metal fraction of these specific fumes. The preparation of the released fraction is illustrated in Figure 9.

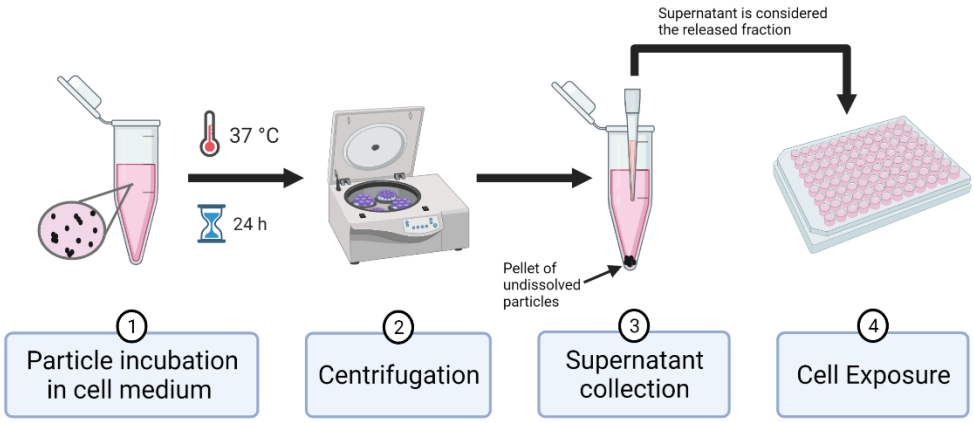


Figure 9. Flow chart of the generation of released fraction. Illustration created using Biorender.com

4.5.1 Cellular dose and intracellular uptake

The use of TEM imaging is a well-established approach to visualize the intracellular localization of particles. The principle of TEM imaging is described in section 4.4.1. TEM imaging provides a qualitative evaluation of the intracellular localization as well as particle agglomeration or aggregation. Intracellular uptake by TEM was evaluated in **Paper III**.

A more quantitative approach to assess the cellular uptake of particles is by quantifying cellular metal content via AAS or ICP-MS. The latter was done in **Paper III**. ICP-MS is described in section 4.4.5. The analysis requires full digestion of the cellular structures and particles by acid digestion, resulting in a complex cellular matrix for the analysis. The ICP-MS analysis then provides quantitative information on total metal content but does not allow differentiation between particles and metal ions, or between internalized particles and extracellular particles attached to the cell surface (Drasler et al. 2017b).

4.6 TOXICITY ASSAYS

4.6.1 Cell viability

The assessment of cytotoxicity is a crucial first step in *in vitro* studies since it allows the identification of sublethal exposure concentrations for the further testing of mechanistic endpoints.

The Alamar Blue assay is a well-established cytotoxicity assay that detects the metabolic activity of cells. The active ingredient is the non-fluorescent dye resazurin which is non-toxic, water soluble and permeable through cell membranes. Resazurin is reduced to the pink-colored, highly fluorescent resorufin by the metabolic activity of live cells. Following exposure and incubation with Alamar blue, fluorescence is quantified at an excitation wavelength at 530-560 nm and emission at 590 nm. Since the results are a reflection of the metabolic activity of cells, it is also an indirect measure of proliferation where alterations in proliferation may lead to false

positive or negative cytotoxicity results (Rampersad 2012). The Alamar blue assay does not require cell lysis, but instead offers the possibility to keep the cells in culture to observe changes over time as well as perform additional analyses of the same sample. The Alamar Blue dye is photosensitive and therefore exposure and incubation should be done in darkness. The assay is prone to false positives/negatives and cross-reactivity of Alamar Blue with the compound should be checked for.

Alamar Blue assay was used to assess cell viability in **Papers II** and **III**.

4.6.2 Intrinsic ROS generation

Oxidative stress is recognized as one of the major pathways in particle induced toxicity. 2′7-dichlorodihydrofluorescein diacetate (DCFH-DA) is the most commonly used probe to detect ROS (Wardman 2007). It is used in both cellular systems and for measuring the intrinsic capacity of particles to generate ROS in acellular systems. In cells, the probe can penetrate through cell membranes where the deacetylation to the non-fluorescent compound DCFH (2′7-dichlorodihydrofluorescein) is facilitated by cellular esterases. For acellular measurements, acetate is removed in alkaline solution before use. DCFH is then oxidized by ROS to produce the fluorescent DCF (2′,7′-dichlorofluorescein) which is used to quantify the ROS level (Chen et al. 2010). DCFH detects a wide range of oxidative species including RO_2, RO, $OH^{\cdot}$, HOCl and ONOO but not $O_2^{\cdot-}$ and H_2O_2. In acellular systems, the enzyme horseradish peroxidase (HRP) can be added as a catalyst to enable the detection of H_2O_2.

The DCF-assay is a simple and inexpensive method that is valuable for unspecific ROS measurements in toxicity screening, but less valuable in mechanistic studies where one desires to measure specific ROS species or identify their origin. An important aspect to consider is that the DCFH probe is sensitive to light and can be oxidized in ambient air. This can potentially result in high background signals as a result of auto-oxidation, suggested to result in false-positive results (Pal et al. 2011, Zhao and Riediker 2014). Kessler et al. (2021) demonstrated that HRP can absorb onto the nanoparticles as well as influence the metal release which in turn could affect the HRP activity effecting the measured ROS responses.

Acellular generation of ROS was evaluated in **Paper II**.

4.6.3 Genotoxicity

Assessing genotoxicity is a vital part in the risk and safety evaluation of particles. The potential of particles to induce DNA damage is critical due to the relationship with mutations and further carcinogenesis.

The comet assay is one of the most commonly used methods within particle toxicology and measures DNA damage at the level of single cells. A neutral version detecting double strand breaks was first described by Ostling and Johanson (1984). Next, Singh et al. (1988) introduced an alkaline version (pH >13) of the method which includes the detection of single strand breaks as well as alkali-labile sites. In short, following exposure the single cells are embedded in

agarose and further lysed followed by DNA denaturation and electrophoresis. During the electrophoresis, the negatively charged DNA fragments will migrate towards the positively charged anode. The DNA from a single cell will appear as comets upon visualization using fluorescent dyes and are often quantified by using specific software. The fluorescence in the comet tail represents the amount of migrated DNA and is directly proportional to the DNA breaks present in the cell. If the comet assay is performed at cytotoxic conditions, both apoptotic and necrotic cells can appear as comets and thus result in false positives. In a review by Karlsson (2010), a strong consistency between the comet assay and the micronucleus test was concluded across a range of nanoparticles. The comet assay can also be modified to detect oxidatively damaged DNA by including bacterial formamidopyrimidine DNA glycosylase (FPG) (Collins et al. 1993).

The comet assay has a high sensitivity. A main limitation is the relatively small sample number that can be run simultaneously, something that is often restricted by the number of microscope slides that fit in the electrophoresis tank. The potential problem of interactions between the nanoparticles and the outcome of the comet assay has been raised (Karlsson 2010). Although additional damage during assay performance cannot be totally excluded, possibilities of interference of nanomaterial with the comet assay was later concluded not to be significant (Karlsson et al. 2015).

The alkaline comet assay was utilized in **Papers II** and **III**.

4.6.4 ToxTracker assay

The ToxTracker assay comprises a battery of mES reporter cell lines modified with green fluorescent protein (GFP) to fluorescence upon activation of specific signaling pathways involved in carcinogenesis (Hendriks et al. 2012, Hendriks et al. 2016), see Table 3. The use of this assay allows a rapid and reliable screening of activated cellular pathways upon exposure and can provide important insight into the primary mode of action of the compound. Two of the GFP reporters target pathways related to DNA damage: Bscl2-GFP is activated upon replication inhibition and is dependent on the ATR-Chk1 signaling pathway, whereas the activation of Rtkn-GFP monitors occurs in response to DNA double strand breaks and NF-kB signaling. The two reporters Srxn1-GFP and Blvrb-GFP target oxidative stress, being Nuclear factor-erythroid 2-related factor 2 (Nrf2)-dependent and Nrf2-independent, respectively. Btg2-GFP is p53-responsive and a marker of more general cellular stress meaning that is activated upon exposure to a broad spectrum of toxic compounds. The final reporter Ddit3-GFP monitors the activation of the unfolded protein response (UPR), where Ddit3 has been associated with cell cycle arrest, apoptosis and endoplasmic reticulum (ER) stress. The ToxTracker assay has been validated using reference chemicals (Hendriks et al. 2012, Hendriks et al. 2016). The assay is performed in a 96-well plate and allows for fast screening of several compounds at the same time.

The ToxTracker assay was used in **Papers I** and **II**

Table 3. Cellular signaling pathways targeted by the ToxTracker assay

Biological damage	Cellular pathway	Biomarker gene
Oxidative stress	Nrf2 antioxidant response	Srxn1
	Nrf2 independent	Blvrb
DNA damage	NF-kB signaling	Rtkn
	ATR/Chk1 DNA damage signaling	Bscl2
Protein damage	Unfolded protein response	Ddit3
Cellular stress	P53 signaling	Btg2

4.6.5 Inflammatory markers

Inflammation *in vivo* is a complex interaction including multiple cell types, which makes it complicated to measure *per se* using *in vitro* models. Nevertheless, it is possible to quantify markers of proinflammatory signaling that are anticipated to drive inflammation *in vivo*. Measuring cellular cytokine production and release is one of the most widespread ways to assess proinflammatory signaling and is generally established as biomarkers of immune-toxicity (Elsabahy and Wooley 2013). Cytokines are bioactive molecules involved in functions to mediate and regulate immune responses, where each individual cytokine plays a specific role in promoting or controlling inflammation. The release of cytokines can be measured in cell culture medium following exposure, where the medium is first centrifuged to remove cellular debris and particles followed by a quantification of cytokine content.

In this thesis, inflammation in the form of cytokine release was evaluated using a multiplex electrochemiluminescence immunoassay. The assay is based on well plates containing high-affinity capture ligands in each well. The detection antibodies are conjugated with electrochemiluminescence labels. The light intensity is then measured to quantify the analytes in the sample (Tighe et al. 2015). In comparison to the more traditionally used single-plex ELISAs, multiplex immunoassays can simultaneously quantify several cytokines at once using a single small volume sample. This results in a more high-throughput method which in addition can provide a wider overview of cytokine release. With this method being more sensitive and precise, samples of different magnitude can be measured without using multiple dilutions.

In **Paper III**, the following cytokines were analyzed: IL-1B, IL-6, IL-8 and TNF-α.

4.7 DEPOSITION MODELLING

Dosimetry models can provide detailed, regional and local deposition patterns in the human lung. Determining particle deposition by experimental means is limited to predominantly total lung deposition. Therefore, dosimetry models can serve as a complement, or alternative, to *in vivo* and inhalation studies since deposition modeling is faster, cheaper and can fill certain gaps that cannot be addressed by experimental studies.

The multiple-path particle dosimetry (MPPD) model is a widely accepted dosimetry model (Anjilvel and Asgharian 1995, Miller et al. 2016). The model is freely available, highly user-

friendly and allows specification of a range of input values including exposure concentration, particle characteristics, individual breathing parameters and lung parameters allowing for modeling variable exposure scenarios. Dose-deposition predictions are further provided as an output where detailed information on the total, regional and lobar deposition fraction per airway is predicted as a function of particle properties and breathing parameters. The model allows for deposition assessments following activity patterns over length of time, as well as the inclusion of lung clearance giving the possibility to assess lung retention following a specified amount of time. With the option to vary several input parameters, one can also investigate the influence of e.g. particle specific aerosol characteristics and respiratory parameters on the deposition and retention.

The MPPD model was used to derive lung doses following occupational exposure to welding fumes in **Paper IV**.

4.8 ETHICAL CONSIDERATIONS

This project relies on toxicity testing using primarily established human cell lines without the need for ethical considerations. The concept of 3R is an ongoing theme throughout this thesis, aiming to establish and validate non-animal-based approaches. One exception is the use of mES cells used in the ToxTracker assay, which required the use of animal models. In addition, experimental components such as fetal bovine serum and certain antibodies are derived from animal tissue and thus the experimental approach cannot be considered totally “animal free”.

5 RESULTS AND DISCUSSION

This thesis addresses the toxicity and fate of metal-based particles in the lung and intends to cover several aspects of particle toxicity testing ranging from mechanistic screening to lung deposition modelling as a tool to understand *in vitro* doses. The following section will summarize and discuss the results of **Papers I-IV** within the framework of this thesis.

5.1 MECHANISTIC SCREENING OF NANOPARTICLES

A main concern of particle exposure is their potential to induce genotoxicity, however the current approaches for genotoxicity testing have the limitations of being low throughput, time consuming and laborious (Nelson et al. 2017). In addition, they usually provide limited mechanistic information. As an alternative, in **Paper I**, we utilized the ToxTracker reporter assay to elucidate the toxicity and associated genotoxic pathways of various metal- or metal oxide nanoparticles as well as QDs of different size. In **Paper II**, we also used the ToxTracker assay to evaluate two types of welding fumes.

The ToxTracker consists of a battery of GFP-tagged mouse embryonic stem cells that are specifically designed to target different mechanistic pathways of toxicity and carcinogenicity (Hendriks et al. 2012, Hendriks et al. 2016). The panel consists of six cell lines, where two target oxidative stress; Srxn1 (Nrf2 dependent) and Bscl2 (Nrf2 independent), two target DNA damage; Rtkn (DNA double strand breaks) and Bscl2 (replicational stress), one target unfolded protein response; Ddit3 (ER-stress) and one general cellular stress; Btg2 (p53). Following exposure of 24 h, the fluorescent readout is measured by flow cytometry. The GFP reporters are combined in one assay and utilizes a 96-well plate format allowing for a high-throughput approach. The workflow of the ToxTracker assay is illustrated in Figure 10.

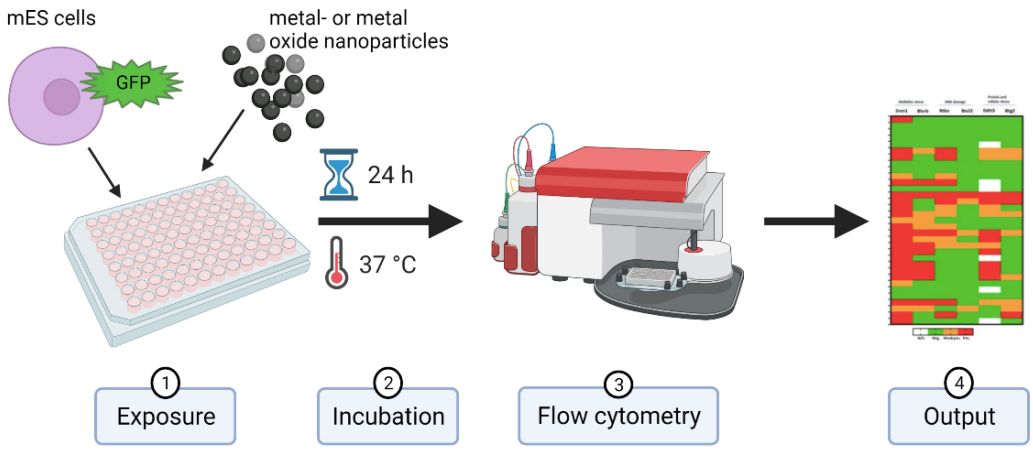


Figure 10. Workflow of the ToxTracker assay utilized in **Papers I** and **II**. Reporter cells are exposed to particles for 24 h, followed by analysis by flow cytometry generating the output. Illustration created using Biorender.com

5.1.1 Mechanisms involved in nanoparticle induced toxicity

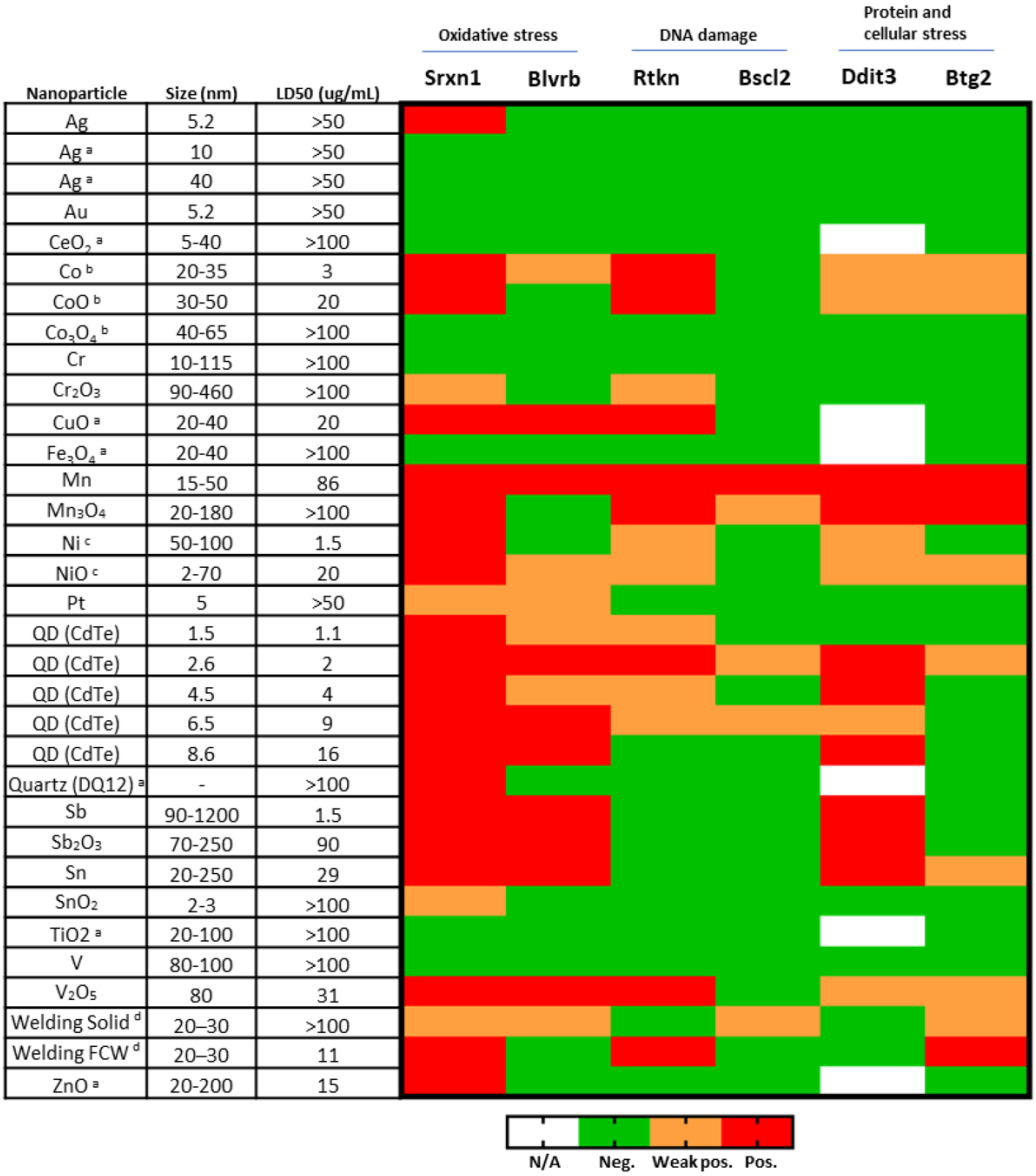
The mapping of activated cellular pathways in response to particles can provide mechanistic insight into both biological reactivity and primary mode of action. A total of 18 nanoparticles were originally tested in **Paper I**, and the results were combined with those of particles previously tested with ToxTracker (32 in total) including the welding fume particles in **Paper II**. This was done to facilitate the aim to summarize and draw general conclusions on nanoparticle-induced effects and underlying pathways. The results are shown in Figure 11.

The oxidative stress marker Srxn1 was by far the most activated reporter (20 out of 32 particles), suggesting Nrf2-dependent oxidative stress. Nrf2 is a redox sensitive transcription factor regulating the expression of a range of antioxidant and detoxifying enzymes, including Srxn1 (Hendriks et al. 2016). The involvement of Nrf2 has been reported for particle-induced toxicity including welding fumes generated by stainless steel welding (Shoeb et al. 2017a) and ambient particulate matter (Pardo et al. 2020). Fewer particles activated the oxidative stress marker Blvrb (9 out of 32), being Nrf2-independent. The results of **Paper I** supports the general notion that ROS generation and oxidative stress are main mechanisms behind particle induced toxicity.

In comparison to oxidative stress, fewer particles activated reporters related to DNA damage. Eight types of particles activated the Rtkn reporter, which is associated with the ATM DNA damage kinase and primarily activated upon DNA double strand breaks (Hendriks et al. 2016). This activation was seen in response to several nanoparticles including established or suspected carcinogens including Co-based nanoparticles (Co, CoO) (Lison et al. 2018) and welding fumes (IARC. 2017). Exposure to Ni, NiO as well as Cr_2O_3 nanoparticles resulted in a weak Rtkn-induction, of which both Nickel-compounds (IARC. 2012b) and hexavalent chromium (IARC. 2012a) are established carcinogens. The fact that many particles activate oxidative stress markers, but few activate Rtkn (including several known or suspected carcinogens) indicates that the latter may be considered of particular concern. This includes Mn, Mn_3O_4, CdTe QDs and CuO nanoparticles, which also resulted in an activation of Rtkn. The lack of activation of Bscl2 suggests that none of the tested nanoparticle (except Mn nanoparticles) or released metal species could bind directly to DNA and cause stalled replication forks. The activation of both DNA damage reporters has in general been observed for directly DNA-reactive chemicals (Brandsma et al. 2020).

Interestingly, all particles activating the Rtkn reporter also resulted in activation of the oxidative stress marker Srxn1. This suggests the DNA damage may be due to oxidative damage. It has been proposed that if the activation of the Srxn1 reporter is observed at lower concentrations compared to the Rtkn reporter, the latter is likely related to oxidative damage (Brandsma et al. 2020). This was the case for V_2O_3 nanoparticles, but not the Co particles or FCW welding fumes for which the Rtkn-reporter was induced prior to the Srxn1 (Paper I). For CoO as well as Mn based nanoparticles (Mn and Mn_3O_4), the Srxn1 and Rtkn reporters were induced at the same exposure concentration. The use of ROS scavengers has been proposed as a tool for

identify the role of oxidative stress in the ToxTracker assay, however this was not utilized in **Papers I** or **II**.



Nanoparticle	Size (nm)	LD50 (ug/mL)
Ag	5.2	>50
Ag [a]	10	>50
Ag [a]	40	>50
Au	5.2	>50
CeO_2 [a]	5-40	>100
Co [b]	20-35	3
CoO [b]	30-50	20
Co_3O_4 [b]	40-65	>100
Cr	10-115	>100
Cr_2O_3	90-460	>100
CuO [a]	20-40	20
Fe_3O_4 [a]	20-40	>100
Mn	15-50	86
Mn_3O_4	20-180	>100
Ni [c]	50-100	1.5
NiO [c]	2-70	20
Pt	5	>50
QD (CdTe)	1.5	1.1
QD (CdTe)	2.6	2
QD (CdTe)	4.5	4
QD (CdTe)	6.5	9
QD (CdTe)	8.6	16
Quartz (DQ12) [a]	-	>100
Sb	90-1200	1.5
Sb_2O_3	70-250	90
Sn	20-250	29
SnO_2	2-3	>100
TiO2 [a]	20-100	>100
V	80-100	>100
V_2O_5	80	31
Welding Solid [d]	20–30	>100
Welding FCW [d]	20–30	11
ZnO [a]	20-200	15

Figure 11. Heatmap of ToxTracker activation in response to all particles tested. Weak positive corresponds to >1.5- but <2-fold increase, while positive corresponds to >2-fold increase at viability levels above 25 %. Figure reproduced from **Paper I.**

The particles with the most noteworthy response in the ToxTracker assay were Mn and Mn_3O_4 nanoparticles resulting in immense induction of oxidative stress markers, induction of DNA damage reporters as well as protein unfolding and p53-related stress. Interestingly, the activation of pathways related to both oxidative stress and DNA damage occurred already at the lowest dose tested and at non-cytotoxic concentrations. This indicates that the oxidative stress and DNA damage are not indirect results of cytotoxicity. Mn is an essential metal for human health and an optimal level of Mn is needed to maintain cellular processes regulating oxidative stress, mitochondrial function and many other (Pfalzer and Bowman 2017). Nonetheless, Mn is also a transition metal of which the higher valence states easily enter redox reactions. The main toxic mechanism of Mn based nanoparticles has been proposed to involve induction of oxidative stress resulting in DNA damage and apoptosis (Sobańska et al. 2021).

The fact that all reporters were activated in response to Mn-based nanoparticles provides new mechanistic insight and warrants further investigations to confirm and elaborate these findings in other cell lines. The results further emphasize the particular concern of Mn as a constituent in non-engineered nanoparticles, such as welding fumes.

5.1.2 ToxTracker as a tool for grouping and risk assessment

The compiled ToxTracker results in **Paper I** show large diversity in activation of GFP-markers as well as magnitude of induction, indicating the ToxTracker assay to be a sensitive tool for screening nanoparticles. There is a great need for the development and establishment of rapid, robust and reproducible assays for particle hazard assessment in order to speed up toxicological testing. By providing detailed mechanistic information, ToxTracker can provide insight into the mode of action of the particles tested and distinguish between DNA damage resulting from DNA interaction, oxidative stress or general cellular stress. The ability to identify genotoxic- and non-genotoxic compounds is considered crucial in the hazard and risk assessment of a compound. An extension of the ToxTracker assay with cell cycle and aneuploidy analysis has been proposed to enable the further distinction between clastogenic or aneugenic mode of action (Brandsma et al. 2020).

The ToxTracker assay was developed and validated for the mechanistic evaluation of chemicals (Hendriks et al. 2012, Hendriks et al. 2016), but **Papers I** and **II** together with other published papers (Karlsson et al. 2014, Akerlund et al. 2018, Cappellini et al. 2018, Cediel-Ulloa et al. 2021) have shown its applicability for testing particles. The ToxTracker assay has also been suggested as a mechanism-based screening assay of particles within food and nutrition (Brown et al. 2019). Cellular uptake in mES cells has been previously shown for nanoparticles (Karlsson et al. 2014) and welding fume particles (Cediel-Ulloa et al. 2021). In Cediel-Ulloa et al. (2021), welding fume particles were tested both in ToxTracker mES cells and human small alveolar epithelial cells. The study reported a considerably lower cellular metal content in mES cells compared to small alveolar epithelial cells tested at the same nominal dose. This was suggested to be due to the properties of the different cell medias as well as the phagocytic capacity of the different cell lines. Possible differences in cellular dose is therefore an important aspect to consider when comparing results in the ToxTracker assay based on mES cells to results in other cell lines.

In order for new high-throughput assays to gain regulatory acceptance, they require validation against more conventional methods. According to the company Toxys behind the ToxTracker assay, the Bscl2-GFP reporter is highly predictive for the results of Ames test, whereas the Rtkn-GFP is predictive of the micronucleus assay as based on the ECVAM-suggested library of mutagenic/non mutagenic and genotoxic/non-genotoxic chemicals (ToxTracker Academy 2022). Dose-response relationships of chemicals from ToxTracker reporters (Bscl2, Btg2 and Rtkn) have been shown to provide quantitative genotoxic potency estimates that correlate with results of the *in vivo* micronucleus assay (Wills et al. 2021). In Karlsson et al. (2014), the result of particles tested by the ToxTracker assay was confirmed in more conventional genotoxicity assays including alkaline and FPG comet assay as well as γH2AX and RAD51 foci. With the

ToxTracker assay being based on rodent stem cells, the results may need to be evaluated for suitability and comparability to more tissue specific cells of relevance for the target organ. In **Paper II**, the genotoxicity of two types of welding fume particles were tested both in the ToxTracker assay based on mES cells as well as by the comet assay in human bronchial epithelial cells. A good agreement was found between the endpoints and cell lines, suggesting genotoxicity and in particular DNA double strand breaks in response to the FCW-generated fume but not the solid wire-generated fume. The study by Brown et al. (2019), showed comparable results to conventional toxicity and oxidative stress assays but reported some discrepancies between ToxTracker and the comet assay in both mES cells and other cell lines (HepG2 and Caco-2). However, it is worth noting that the methods measure different endpoints and could have different sensitivity.

The mechanistic details provided by the ToxTracker can aid in the classification and grouping of nanomaterials/particles initiating adverse outcomes by triggering common early cellular events. This can be used as a valuable tool for both prioritization and read-across approaches. In **Paper I**, some of the tested particles were chosen with the subgoal of comparing the metallic nanoparticles to their corresponding oxides. The Mn-based particles show similar potency and mechanistic pattern, suggesting they may be considered for grouping. This was not the case for the other metallic nanoparticles tested, where differences in potency was observed for metal vs oxides and no conclusions could be drawn. In Cappellini et al. (2018), Co and CoO but not Co_3O_4 nanoparticles showed similar potency and activation pattern in ToxTracker and were suggested to be grouped together for risk assessment. Understanding the mode-of-action of a genotoxic compound can also be valuable in the adverse outcome pathway (AOP) methodology (Sasaki et al. 2020).

As emphasized in several reviews, the establishment of high-throughput methods for the testing of nanomaterials is crucial in order to reduce animal testing (Collins et al. 2017, Nelson et al. 2017, Kohl et al. 2020). Taken together, the ToxTracker shows great potential for rapidly testing the genotoxicity of nanomaterials/particles and offers detailed mechanistic insight. ToxTracker is currently under evaluation for official acceptance and inclusion in the standard regulatory safety testing of chemicals, where an OECD (Organization for Economic Co-operation and Development) validation study has been initiated prior to the printing of this thesis. The ToxTracker assay needs to be further validated for particles, since the study and toxicity of particles is in many ways more complex. With the specific cellular pathways targeted in the ToxTracker assay, this may not reflect the full range of processes involved in the genotoxicity of particles. One example includes the assessment of secondary genotoxicity generated as a consequence of particle-elicited inflammation. This is however not a challenge only for the methodology of ToxTracker assay but *in vitro* assays in general, since secondary genotoxicity is a complex process requiring a larger complexity and interplay between cells than what can be attained from standard *in vitro* approaches (Evans et al. 2017, Kohl et al. 2020).

5.2 WELDING FUME TOXICITY – CAN IT BE REDUCED?

The properties of welding fume particles are largely determined by the welding process and materials used. With the different welding methods, equipment and practices available, this results in an immense variety of generated welding fume particles. The impact of welding parameters and conditions on the particle characteristics, and how this in turn affects the toxicity is not well understood. Nevertheless, this is important in order to identify best and worst choices as well as contribute to the development of safer welding equipment and occupational environments. Therefore, in **Paper II**, we sought to investigate the influence of welding parameters and wire materials on the generated fume particle characteristics and associated toxicity upon welding of stainless steel. This formed the basis for the experimental approach in **Paper III**, where we investigated the role of released Cr(VI) and if modifications of the welding electrode could aid in the generation of less toxic welding fumes.

5.2.1 Solubility, and not composition, is a good predictor of acute welding fume toxicity

In **Paper II**, we investigated human lung cell toxicity welding fume particles generated by means of metal active gas (MAG) welding of stainless steel, varying filler material (solid wire, metal cored wire (MCW) or FCW), shielding gas, base alloy and melting rate, see **Paper II.** The relative compositional fractions of Fe, Cr, Mn, and Ni in the fume particles were shown to primarily depend on the wire filler material and ranged from 12-46 wt%. In general, welding fumes generated by solid wire or MCW contained higher amounts of these metals compared to fumes generated by FCW electrodes.

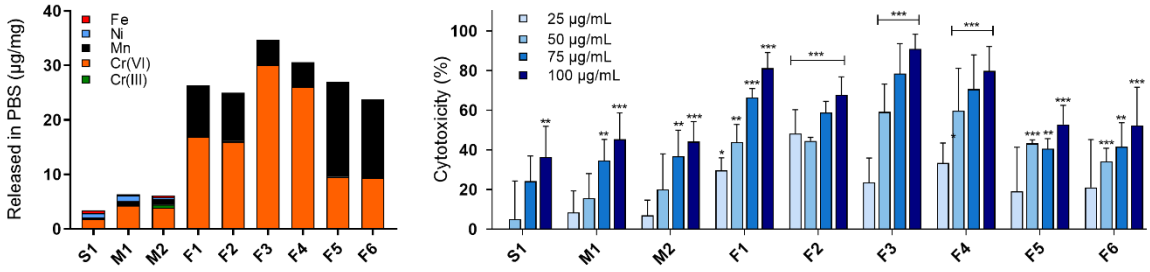


Figure 12. Metal release (iron, nickel, manganese, chromium (III), chromium (IV)) in PBS (left) and cytotoxicity (24 h) of welding fume particles generated by stainless steel welding (right). Figures adapted from **Paper II.**

In agreement with previous studies (Taylor et al. 2003, Antonini et al. 2005, Mei et al. 2018, Hedberg et al. 2021), Cr(VI) was shown to be the main soluble metal species from welding fumes formed upon stainless steel welding. An interesting finding in **Paper II** was that the extent of metal release in PBS was not reflected by the total metal content of the fume particles. Instead, the FCW generated particles, which were shown to contain less amounts of main alloying metals, released considerably higher amounts of Cr(VI) and Mn in PBS as compared to those fumes generated from solid core or MCW welding (Figure 12). Thus, there was a weak correlation between the total metal content of the fume particles and the release of

corresponding metals in PBS. Observed findings are supportive to previously reported data in Mei et al. (2018).

A great variation in acute toxicity was observed *in vitro* in response to the different welding fumes. The FCW generated welding fumes were considerably more acute cytotoxic compared to solid wire or MCW generated welding fumes in HBEC-3kt, Figure 12. In accordance, FCW generated welding fumes were also found to significantly induce acellular ROS, DNA damage and activation of signaling pathways related to DNA double strand breaks and p53 signaling. Such effects were not observed in response to either solid core or MCW generated fumes. One of the most important findings in **Paper II** was that solubility, and not composition, was a good predictor of welding fume induced acute lung toxicity. A correlation between the release of Cr(VI) in PBS and acute cytotoxicity was found, as well as between Mn-release and acellular ROS production, see Figure 13. Several *in vitro* studies have suggested the toxic response of welding fumes from stainless steel welding to be related to the soluble component of the fume, in particular the solubility of Cr (Antonini et al. 1999, McNeilly et al. 2004, Antonini et al. 2005, Shoeb et al. 2017a).

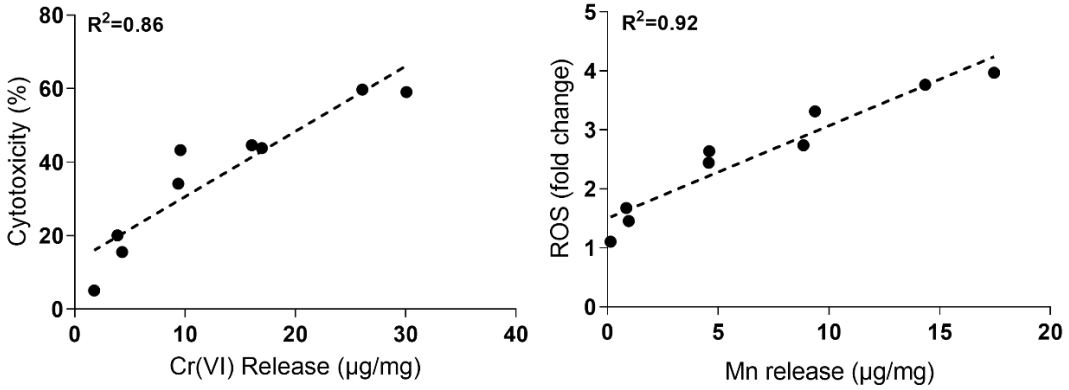


Figure 13. Correlation between the release of chromium (VI) and manganese and *in vitro* toxicity in terms of cytotoxicity or generation of ROS for welding fume particles generated by stainless steel welding. Figures reproduced and adapted from **Paper II**.

5.2.2 Acute welding fume toxicity can largely be explained by the released fraction

Based on the findings in **Paper II**, we wanted to explore the role of released metals more closely and therefore assessed the toxic response to either FCW generated fume particles or solely to their released metal fraction in **Paper III**. The released metal fraction of standard FCW-generated particles, containing large amounts of Cr(VI), were shown to induce similar levels of cytotoxicity and DNA damage as compared to that induced by particles (Figure 14). In contrast, the released metal fraction of fume particles generated with Cr(VI)-reduced wires, containing predominantly Mn, did not induce any cytotoxicity, see **Paper III**. Together with the findings of **Paper II**, this suggests that the released metal fraction, in particular the release of Cr(VI), bares a central role in the acute toxicity observed in response to welding fume particle exposure as a result of stainless steel welding.

The approach of using the released metal fraction of welding fumes to elucidate the role of released metals has previously been done *in vitro*. Antonini et al. (1999) demonstrated that the soluble fraction of welding fume particles generated by stainless steel welding, but not from mild steel, induced comparable or elevated levels of cell death in rat lung macrophages compared to total fume (particles and metal release). McNeilly et al. (2004) demonstrated the soluble fraction of welding fumes to enhance IL-8 expression at equivalent levels as whole fume suspensions in A549 cells. The latter is somewhat in contrast to our findings in **Paper III**, where cytotoxicity and DNA damage, but not inflammatory effects (cytokine release), could be explained by the released metal fraction. The same study showed that the inflammatory effects of the soluble fraction were reduced when adding transition metal chelator, suggesting soluble transition metals to have the main role of pro-inflammatory induction of welding fumes (McNeilly et al. 2004). This was further confirmed *in vivo* in rats (McNeilly et al. 2005).

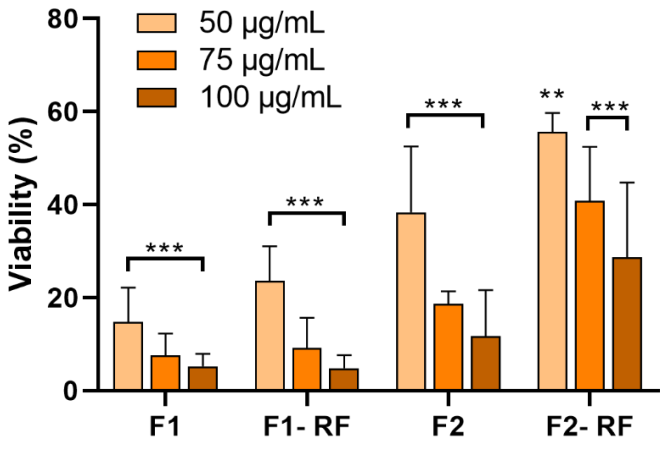


Figure 14. Cell viability following exposure to standard FCW welding fume particles (F1/F2) or its released fraction (RF). Figure adapted from **Paper III**.

Technically, the released fraction represents metals that have been released extracellularly, and effects observed indicates extracellular release to be accountable for the toxicity. In **Papers II** and **III**, we used a timepoint of 24 h for metal release and the generation of released fraction. Other studies primarily measured metal release at a more immediate timepoint without incubation (Antonini et al. 1999, McNeilly et al. 2004). McNeilly et al. (2004) investigated the timescale over which the soluble fraction could exert their inflammatory effects and showed the soluble fraction obtained following 10 minute up to 24 h incubation to induce comparable IL-8 expression. Cediel-Ulloa et al. (2021) measured metal release of welding fume particles (mild and stainless steel) right after sonication as well as after 24 h and found no time-dependent difference between metal release in two different cell medias (except Mn in one of them). As previously been shown for many metals and alloys such as stainless steel (Hedberg and Odnevall Wallinder 2016), the results suggest that most metals are released into solution already during the first few minutes. On the other hand, Berlinger et al. (2008) demonstrated the highest concentration of metal release (Fe, Mn, Cr, Ni) after a 24 h period. However, it is generally difficult to compare dissolution data from different studies, since varying approaches, dissolution medias and analytical methods are used.

In contrast to several studies suggesting the soluble fraction of welding fumes to be responsible for the toxicity, Taylor et al. (2003) demonstrated that both the soluble and insoluble fractions

of particles generated by stainless steel welding were required to result in the maximal response of lung toxicity in rats. The study suggested the soluble and insoluble fraction to be individually responsible for eosinophil and neutrophil recruitment, respectively, indicating a differential inflammatory response. With welding fume particles being highly complex mixtures as a function of welding parameters, the fume composition, solubility, and toxicity are likely to differ between welding fumes investigated in different studies. This could explain some of the discrepancies reported in the literature. It is also important to note that *in vivo* studies generally assess more systemic endpoints at longer timepoints compared to *in vitro*, thus making a direct comparison complex. The exposure to soluble metals alone results in fast clearance from the lung, whereas particles can retain in the lung for longer periods. This is in contrast to static *in vitro* systems with no clearance. As an example, Shinohara et al. (2017) demonstrated that, following intratracheal administration of Ni based nanoparticles in rats, 40-60 % of the Ni-dose remained in the lungs after 90 days for poorly soluble spherical NiO nanoparticles in contrast to <0.3 % in the case of soluble NiO nanowires. The most problematic particles *in vivo* are likely those contain toxic metals and are poorly soluble which result in longer retention and a slow but sustained release of toxic metals.

Another important aspect to consider is that released metals are rarely present as free cations in biological settings, but instead as different complexes which in turn affects the bioaccessibility (Hedberg and Odnevall Wallinder 2016). The metal release in cell media alone followed by incubation prior to cell exposure likely results in a higher degree of complexation and thus a different bioaccessibility compared to metals being released directly at the cell surface (*in vitro* or *in vivo*).

The fact that most dissolution testing is done at static conditions has been criticized for only reflecting equilibrium solubility, and thus not mimicking the dynamic environment in the lung (Klaessig 2018). In agreement, the type of simplified metal release testing performed in this thesis can be argued to primarily mimic the submerged *in vitro* set-up. Efforts have been made to assess acellular dissolution in more dynamic systems to gain a dissolution rate, which can be argued to more closely mimic *in vivo* situations (Oberdörster and Kuhlbusch 2018). Our results suggesting the released metal fraction to be largely responsible for the acute toxicity of welding fumes warrants further investigations using more advanced and physiologically relevant dissolution models to better understand the fate of welding fume particles *in vivo*.

5.2.3 The role of hexavalent chromium

Despite significant advancements in the field of welding fume toxicity research, the contribution of specific metal constituents in welding fume induced toxicity is not well established. The findings of **Papers II** and **III** clearly suggest the release of Cr(VI) to be the main driver behind the acute toxic effects observed *in vitro* in response to fumes formed upon welding of stainless steel. In **Paper III** we further confirmed the hypothesis that a reduction of released Cr(VI) from welding fumes particles result in less acute toxic effects, including DNA damage as shown in Figure 15. This goes well in hand with studies suggesting particles originating from mild steel welding to be less toxic and reactive compared to stainless steel,

both *in vitro* (Antonini et al. 1999, Antonini et al. 2005, Leonard et al. 2010, Cediel-Ulloa et al. 2021) and *in vivo* (Taylor et al. 2003, Zeidler-Erdely et al. 2008, Antonini et al. 2010, Antonini et al. 2011b).

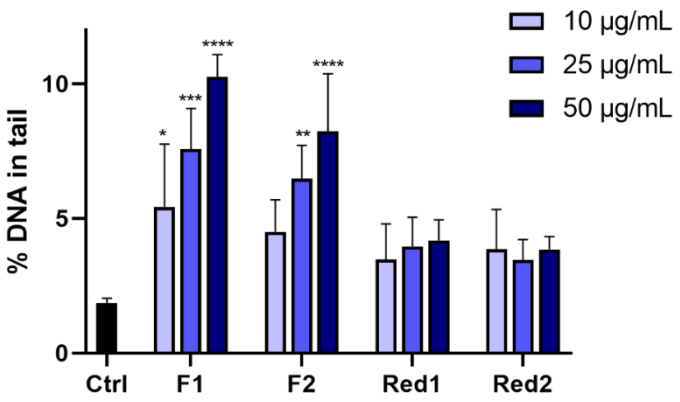


Figure 15. DNA damage following 3 h exposure to welding fume particles generated with FCWs (F1, F2) or Cr(VI)-reduced FCWs (Red1, Red2) electrodes. Reprinted and modified from **Paper III**.

The cellular uptake and toxic potency of Cr depend on the oxidation state. Cr(VI) more readily enters cells via anion transporters in contrast to Cr(III) entering through passive diffusion (Salnikow and Zhitkovich 2008, Proctor et al. 2014, Wang et al. 2017). Cr(VI) is furthermore unreactive towards DNA. However, Cr(VI) is rapidly reduced in multiple steps in biological systems which involves the formation of highly reactive Cr(V) and Cr(IV) intermediates and the final oxidative form of Cr(III). The reduction process can take place both inside and outside the cell by non-enzymatic cellular reductants including ascorbate and glutathione (Zhitkovich 2011). If occurring extracellularly, the reduction serves as a detoxification process resulting in poorly permeable Cr(III) complexes. Conversely, the intracellular reduction of Cr(VI) could in turn result in DNA damage via the direct interaction of Cr(III) with the DNA molecule, resulting in several types of DNA lesions including stable DNA adducts (Salnikow and Zhitkovich 2008). Furthermore, the reduction process generates a spectrum of ROS possibly resulting in oxidative DNA damage. Oxidative stress has been proposed to be a main mechanism of the genotoxicity of Cr(VI) (Proctor et al. 2014, Wang et al. 2017).

In general, Cr(III) is considered less toxic and genotoxic compared to Cr(IV). Nonetheless, the potential hazard of particle bound Cr(III) has been raised (Beyersmann and Hartwig 2008). The particulate Cr(III) could result in the particles entering the cell via endocytosis followed by intracellular Cr(III) release, which would evade the cell membrane barrier otherwise restricting the cellular uptake and toxicity of extracellular Cr(III). In a recent publication by Schumacher et al. (2022), Cr_2O_3 micro- and nanoparticles were demonstrated to not induce cytotoxicity or genotoxicity. Yet, this was only the case for particles not releasing Cr(VI). The cellular damage was concluded to not depend on particle uptake, but instead exclusively on the fact whether or not Cr(VI) was released from the particles. Cr_2O_3 nanoparticles where further investigated by the ToxTracker assay in **Paper I** and demonstrated low cytotoxicity and only a weak induction of oxidative stress and DNA damage markers. The release of Cr(III) or Cr(VI) species was however not assessed, but should be considered for further interpretation.

Papers II and **III** show that Cr from fume particles of welding stainless steel is released primarily in the more toxic form of Cr(VI). An important finding in **Paper III** was that the metal uptake was considerably higher following exposure to particles compared to exposure to the released fraction only. Based on this, the toxicity of welding fume particles is likely attributed to dissolution and consequently the release of metals including Cr(VI) both intra- and extracellularly. There is currently no established method to quantify intracellular metal release, although efforts have been made by us (Carlander et al. 2019, McCarrick et al. 2021) and others (Sabella et al. 2014). The proposed cellular fate and toxicity induced by Cr released from welding fume particles is illustrated in Figure 16.

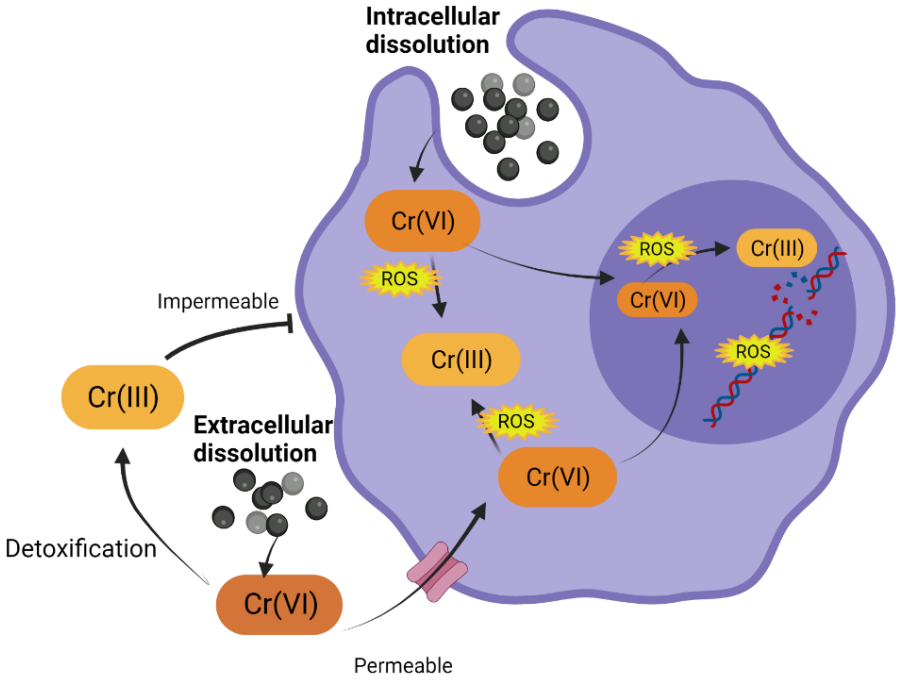


Figure 16. Proposed fate, cellular uptake and toxicity of released chromium(VI) from welding fume particles of stainless steel welding. Illustration created using Biorender.com.

Soluble Cr alone has been shown to be the main acute pneumotoxic component of fume particles formed upon stainless steel welding in mice, inducing similar lung injury and inflammation compared to fume particles in A/J mice but not C57BL/6J mice (Zeidler-Erdely et al. 2008). Falcone et al. (2018b) further exposed mice to metal constituents based on their mass concentration in welding fumes generated upon welding of stainless steel. Slightly soluble forms of Cr(VI) and Cr(III) were found to induce mild acute toxicity and inflammation at 1 and 7 days post exposure but no persistent effects. It has been suggested that the toxic and carcinogenic effects of Cr(VI) is largely determined by the solubility rate, where highly soluble Cr(VI) compounds exhibit less toxicity due to the rapid inactivation via extracellular reduction. In contrast, the low solubility particles can adhere to airway epithelial cells resulting in a slow and continuously release of Cr(VI) at the cell surface, escaping extracellular reduction (Urbano et al. 2012). Welding fume particles generated by welding of stainless steel have been reported to be cleared from the lung *in vivo* at a slower rate compared to fumes from mild steel welding (Antonini et al. 1996, Antonini et al. 2011b, Shoeb et al. 2017b). An increased bio-persistence

of fume particles from welding of stainless steel has been suggested as an explanation for the increased and more persistent penumotoxic effects compared to findings for fume particles formed upon mild steel welding (Antonini et al. 1997, Shoeb et al. 2017b).

By studying redox potential, **Paper III** demonstrated Cr(VI) to be stable in cell medium over a prolonged time. This was suggested to be due to the co-released stabilizing oxidizing species. This is an important finding in the interpretation of the results demonstrating the released metal fraction (primarily Cr(VI)) to explain a large part of the acute toxicity observed. Nonetheless, the fact that Cr(VI) seem to be stable in our model system may be argued to not correctly reflect physiological Cr(VI) metabolism, where a rapid extracellular reduction is expected to occur due to higher and/or alternative presence of cellular reductants *in vivo*. In addition, the mechanisms and steps of reduction depend on the reductant present. The issue has been raised regarding if the ascorbate-reduced *in vitro* circumstances can accurately recapitulate the genotoxicity of Cr(VI) *in vivo* (Reynolds et al. 2012)

Despite the results of this thesis emphasizing the involvement of Cr(VI) in the toxicity of welding fumes, it is important to consider that **Papers II** and **III** only regard the acute aspect of welding fume induced toxicity. Therefore, no conclusions on the involvement of Cr(VI) for long-term (or systemic) effects can be drawn. Proctor et al. (2014) described a hypothesized mode of action (MOA) for Cr(VI)-induced lung cancer. This involved deposition and accumulation of particulate chromium in lung bifurcations (key event 1), dissolved Cr(VI) or chromate particles entering cells (key event 2), reduction of dissolved Cr(VI) resulting in ROS and subsequent protein and DNA damage and further tissue irritation, inflammation and cytotoxicity (key event 3). Together with increased cell proliferation, this result in changes in DNA sequences or methylation status (key event 4), possibly resulting in tumorigenesis. However, as previously discussed, epidemiological evidence does not point towards Cr(VI)-containing welding fumes being more carcinogenic compared to fumes formed upon welding of mild steel. A study by Krawic and Zhitkovich (2018) suggests a weakened carcinogenic potency of Cr(VI) when present in biological solution in a mixture with other metals such as Fe(III). The release of Fe ions are proposed to enhance the detoxification of solubilized Cr(VI) by extracellular reduction. This is proposed to result in the loss of toxic potency of Cr(VI) when co-released with catalytic amounts of other welding fume components. On the other hand, Fe has been shown to be poorly soluble in contrast to the rapid solubility of Cr(VI) both *in vitro* (**Paper II** and **III**) and *in vivo* (Antonini et al. 2011b).

With the complexity of welding fume properties and constituents in combination with the range of associated health effects, one metal constituent may not be entirely responsible for welding fume induced toxicity. In **Paper III**, we observed an inflammatory response (IL-8 release) in THP-1 derived macrophages for all welding particles, independent on the release of Cr(VI). The release of Mn was correlated to acellular ROS production in **Paper II**. In **Paper I**, Mn-based nanoparticles were found to be the most toxic and active nanoparticles. Falcone et al. (2018b) investigated the toxicity of welding fumes generated by stainless steel welding or surrogate metals *in vivo,* with results pointing towards Fe being the primary mediator of

welding fume induced toxicity and carcinogenicity. Nonetheless, although several of the metal constituents resulted in toxicity, the pneumotoxic effects were greatest for the total welding fume (particles and released metals) formed upon welding of stainless steel. This points towards that various combination of metals, valence states and their interplay may largely determine the toxicity in combination with effects caused by particulate bound or released metal species.

5.2.4 The potential of altered welding electrode for a safer occupational environment

The hazards of welding fume exposure emphasize the importance of proper ventilation and personal protection equipment. However, still today, many welders do not have sufficient protection resulting in potentially hazardous exposure. For example, a Canadian cohort reported approximately 50 % of welders to never use respiratory protection when welding (Cherry et al. 2018). A logical approach for improved health and workplace safety is to prevent adverse exposures. Since the quantity and properties of fumes vary greatly with welding practices, conversion to alternative processes may contribute to reduced or less hazardous exposure.

Results of **Paper II** pointed out the potential of welding electrode to be modified in order to reduce the toxicity of the fume generated. In collaboration with industrial partners in **Paper III**, we further investigated welding fumes generated with newly developed FCW electrodes. These were specifically designed to generate fume particles that release less Cr(VI), referred to as Cr(VI)-reduced FCWs. Welding fumes generated with the Cr(VI)-reduced FCWs were substantially less cytotoxic compared to those of standard FCWs and did not induce any DNA damage. The conclusions of **Paper III** therefore suggest a potential benefit in substituting standard FCWs with Cr(VI)-reduced wires to achieve less toxic welding fumes and thus a reduced risk for welders.

Multiple factors are the basis for selecting welding process, parameters and equipment, therefore investigations remain to determine if modifications like those proposed in **Paper III** can be established in practice at industrial settings. Good function and weldability are crucial factors when introducing modified products in order to convince users to replace established and functioning welding practices. The Cr(VI)-reduced FCWs utilized in **Paper III** were developed with the aim to reduce the toxicity of the welding fume without compromising their weldability. However, later investigations demonstrated an unsatisfactory weldability which resulted in further development. Similar to what was demonstrated in **Paper III**, the updated Cr(VI)-reduced FCWs were less cytotoxic compared to standard FCWs, demonstrating the possibility of maintaining a satisfactory weldability despite altered or substituted key components (Westin et al. 2021).

In addition to welding electrodes, other welding parameters and conditions have been suggested to be optimized in order to generate less hazardous fumes. In Sriram et al. (2015), the fume composition was shown to be altered by specifically modulating welding voltage

which in turn resulted in fume particles of different neurotoxic effects. Welding fumes generated with higher voltage resulted in less neurotoxicity, which was suggested to be due to significantly lower amounts of soluble Mn in the fume particles. This is in line with our results of **Paper II**, where the FCW welding fume particles generated with spray arc (high melting rate and voltage) released less Mn per total fume mass as compared to short arc (low melting rate and voltage). Nonetheless, the fact that a high melting rate resulted in higher release of Cr(VI) as well as twice the fume generation rate compared to a low melting rate, poses concerns. Higher voltage has further been associated with higher levels of fine and ultrafine particles (Hovde and Raynor 2007). This emphasizes the complexity of welding fume particles, where beneficial modifications in one aspect might result in other physicochemical properties being altered in an unfavorable way. Similarly, the reduction of a certain metal in the fume may not always improve its safety if the presence of other metal components are maintained, or even increased. One example is the increased release of Mn observed for the Cr(VI)-reduced wires investigated in **Paper III**, where a potential beneficial effect regarding pneumotoxicity has to be weighed against a potential increased risk of neurotoxic effects.

Additional investigations are needed to further determine the influence of welding parameters, conditions and variables on the fume profile and toxicity, in order to identify approaches for the generation of less toxic welding fumes. To better understand the implication of these results, future studies could address more long-term and repeated dose exposures to understand the role of released metals for more chronic effects as well as include the use of more complex cell-models such as co-cultures and exposure under air-liquid interface. Welders often perform multiple types of welding processes, which complicates epidemiological conclusions on their influence. Instead, experimental studies under controlled conditions are better suited to understand the influence of the properties and components of welding fume.

5.3 LUNG DOSIMETRY MODELLING AS A TOOL TO UNDERSTAND *IN VITRO* DOSES

Interpreting particle dose from *in vitro* systems in the context of human exposure remains a huge challenge for establishing *in vitro* models in human health risk assessment. In order to understand our (**Papers II and III**) and others *in vitro* results of welding fume toxicity in the perspective of human exposure, **Paper IV** intended to assess the lung deposition and retention of welding fumes following real-life occupational scenarios. The lung dose was modelled using computer simulations in the software MPPD. Hypothetical exposure scenarios were run over both short- and long term (6 h up to 45 years), based on welding specific exposure parameters from available literature, together with assumptions of respiratory parameters. The workflow of **Paper IV** is illustrated in Figure 17.

The results of **Paper IV** demonstrated the deposition per lung surface area to be generally higher in the tracheobronchial region compared to the alveolar region. Nonetheless, due to their different clearance mechanisms, the retention was found to rapidly decrease in the tracheobronchial region but instead slowly built up in the alveolar region. This emphasizes the

concern of tracheobronchial retention for more short-term exposure, but primarily alveolar retention following long-term and chronic exposure.

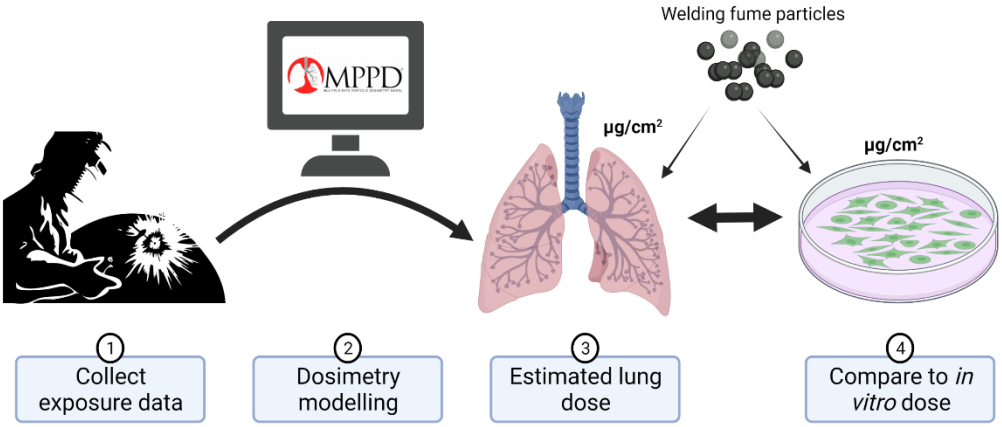


Figure 17. Workflow of **Paper IV**. Exposure data of welding fumes was used for simulating real-life welding exposure scenarios in the MPPD model. This resulted in estimated regional lung doses that could be compared to the dose used *in vitro*. Illustration created using Biorender.com.

5.3.1 Do our *in vitro* doses reflect human exposure?

The derived lung doses were directly compared to the *in vitro* doses used in **Paper III**, corresponding to 0.08-1.57 µg/cm^2 when considering cell dose (assuming 5 % uptake) and the most cytotoxic particle with an effective concentration eliciting 50 % cytotoxicity (EC50) corresponding to 0.125 µg/cm^2. An interesting finding in **Paper IV** was that *in vitro* effective concentrations were to a large extent comparable to or exceeded by human lung doses following real-life occupational exposure scenarios, see Table 4.

Similar to most *in vitro* studies, the experimental approach in **Paper III** represents an acute response. Interestingly, the *in vitro* EC50 was exceeded in the tracheobronchial region after a single work shift (6 h) at the OEL-exposure level, see Figure 18. This timespan can be compared to the acute timescale under which the *in vitro* data was conducted (24 h). Nonetheless, the tracheobronchial retention was found to rapidly decrease to be well below the EC50 until the end of the day (24 h). Important to note is though that the simulations are based on poorly soluble particles whilst the results of **Paper II** and **III** suggest a large part of Cr(VI) to be released within 24 h for the some of the welding fumes investigated. Based on these findings, rapid dissolution is likely to occur in the human lung.

In a study by Gangwal et al. (2011), a similar approach as in **Paper IV** was implemented to estimate lung deposition after occupational exposure to nanomaterials in order to recommend *in vitro* testing concentrations. A critique expressed towards their methodology was that the suggested *in vitro* concentrations reflected the deposited amount of particles following 45 years of exposure (Oberdörster 2012). This was argued to be highly misleading when human doses attained for long-term chronic inhalation were suggested as determinants for *in vitro* approaches reflecting primarily acute or sub-acute responses. Similar to Gangwal et al. (2011), we also simulated a life-time occupational exposure (45 years) to a low-end aerosol

concentration resulting in an alveolar retention comparable to the *in vitro* EC50 dose. Despite the fact that long-term simulations may not be directly applicable for *in vitro* dose selection, they still provide important understanding of *in vitro* doses in the context of human exposure. Chronic simulations could also identify extreme, yet somewhat realistic, doses that can be used as a guidance for upper limit *in vitro* doses.

Table 4. Deposited welding fume particle doses for human inhalation exposure and *in vitro* exposure systems. The modelled lung doses are presented in the tracheobronchial (TB) or alveolar (Alv) region. Table reproduced from **Paper IV**.

Exposure scenario	Exposure concentration	Exposure duration	Dose (µg/cm^2)	
Human inhalation			TB	Alv
OEL exposure	5 mg/m^3	6 h	0.89	0.017
		1 week	0.102	0.083
		1 year	1.15	2.85
Low end exposure	0.05 mg/m^3	Lifetime(45y)	0.023	0.16
High end exposure	45 mg/m^3	1 week	0.92	0.75
***In vitro* (HBEC-3kt)**			Nominal	Cell dose
Dose range	5-100 µg/mL	24 h	1.6 – 31.3	0.08-1.57
EC50[1]	8 µg/mL		2.5	0.125

The advancement of *in vitro* systems towards allowing chronic testing would enhance the predictivity of *in vivo* effects. Cells exposed to a bolus dose, such as those in this thesis, have been demonstrated to not have the same response as compared to repeated exposure over longer periods of time despite the same cumulative dose (Annangi et al. 2016, Mukherjee et al. 2020). A high bolus dose may therefore be more valuable for ranking and not directly extrapolated as occurring under *in vivo* response conditions (Oberdörster and Kuhlbusch 2018). An effort to mimic a more occupationally relevant welding exposure *in vitro* was done by Samulin Erdem et al. (2020), where low concentrations (0.035-4.375 µg/mL) of welding fumes originating from mild steel welding were applied for 6 h exposure per day during 5 consecutive days. Other efforts include six weeks low-dose exposure to Ni and NiO nanoparticles (Gliga et al. 2020) and 14-week long exposure to silver nanoparticles (Comfort et al. 2014). However, there are many technical obstacles to overcome in order to use *in vitro* models for chronic testing including the rapid proliferation of most cell lines and risk for de-differentiation during prolonged time in culture (Drasler et al. 2017a). Nonetheless, by gaining mechanistic insight, it may be possible to identify shorter and simpler *in vitro* tests or batteries of tests that can adequately predict chronic toxicity. This could involve screening tools such as the ToxTracker utilized in **Paper I**.

A main strength of **Paper IV** is the fact that the simulations were based on real-life exposure data. The baseline exposure concentration of the simulations in **Paper IV** was set to the OEL for inhalable inorganic dust (5 mg/m^3), with high and low-end concentrations corresponding to 45 and 0.05 mg/m^3. All of these exposure scenarios can be considered as rather extreme exposure cases and does likely not reflect the extent and pattern of exposure for most welders. The OEL can be considered as a legally bound worst-case scenario and the estimated lung

doses should be considered likewise. Yet, legal limit values may be exceeded due to several reasons including concentrations spikes as well as non-compliance or lack of regulation.

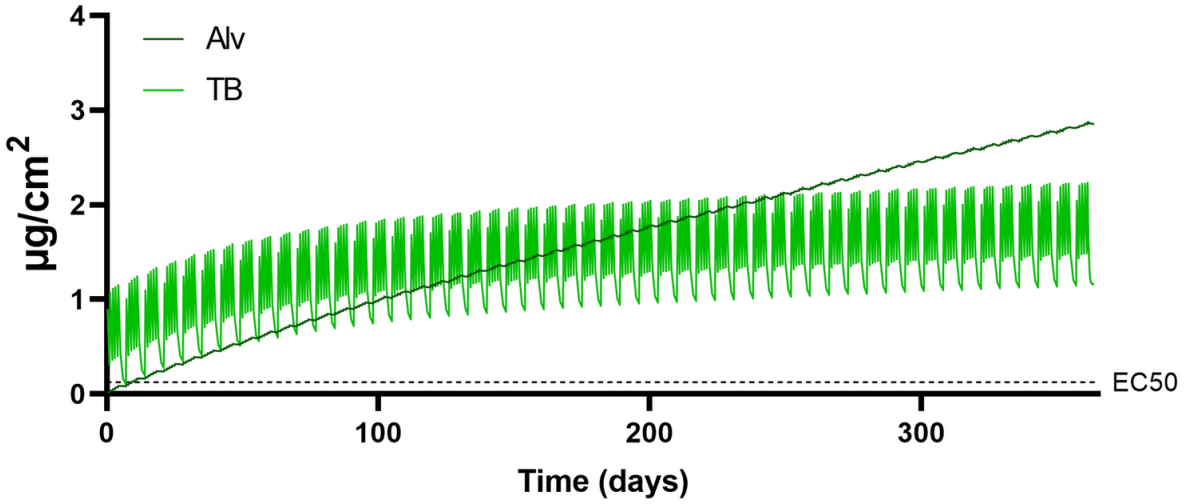


Figure 18. Tracheobronchial and alveolar retention of welding fumes per surface area versus days of exposure at the occupational exposure level of 5 mg/m3. Figure reproduced from **Paper IV**.

Similarly, the *in vitro* effective dose was based on the most cytotoxic welding fume tested thus, also representing a worst-case scenario. Furthermore, the *in vitro* dose was based on the endpoint of cytotoxicity which is primarily used to find appropriate doses for mechanistic endpoints. Future studies should focus on relating *in vitro* doses eliciting other toxic effects such as DNA damage or inflammation, being of more relevance for human health. The welding fume F1 in **Paper III**, which was the basis of the EC50 used in the comparison of **Paper IV**, did induce significant DNA damage following 3 h exposure at a comparable nominal exposure concentration (approximately 2.5 µg/cm^2) as the applied EC50. This suggests that also doses eliciting DNA strand breaks could be attained in the lung following real-life occupational welding scenarios.

5.3.2 On the road to establish *in vitro* models for risk assessment

Gaining regulatory acceptance of *in vitro* studies is key for a successful shift towards alternative models to animal testing. Dose is a fundamental concept within toxicology, since it is ultimately the dose, and not the exposure, determining the outcome. In mechanistic *in vitro* studies, a wide range of concentrations, including unrealistic high doses, might be required in order to determine both effect- and no-effect levels (Drasler et al. 2017a). Nevertheless, in order for the *in vitro* data to be used in a regulatory context, the concentrations should to some extent be selected based on realistic human exposure. However, there is no established approach to enable the link between *in vitro* doses and human exposure. The case of inhalation exposure and lung dose is considered a particularly complex case. The concept of IVIVE is necessary for dose-response assessment of *in vitro* data for risk assessment applications (Pal et al. 2015, Thrall et al. 2019).

As emphasized in Phalen et al. (2021), these types of challenges require the collaboration of several disciplines in order to integrate several methodologies. Based on an evaluation of

available *in vitro* and *in silico* models, Romeo et al. (2020) proposed an integrated pathway for the use of *in vitro* data in risk assessment. The pathway is based on *in vitro* human data supported by *in vitro* dosimetry models which is further coupled with kinetic models such as PBPK and MPPD modelling in order to provide the essential link between *in vitro* response and whole organism exposure, see Figure 19. The final outcome is predictive values for point of departure of relevance for human health risk assessment. This is a similar approach as to what was applied in **Paper IV**, with the exception of using quantitative cell dose measurements instead of computational modelling for the *in vitro* dosimetry.

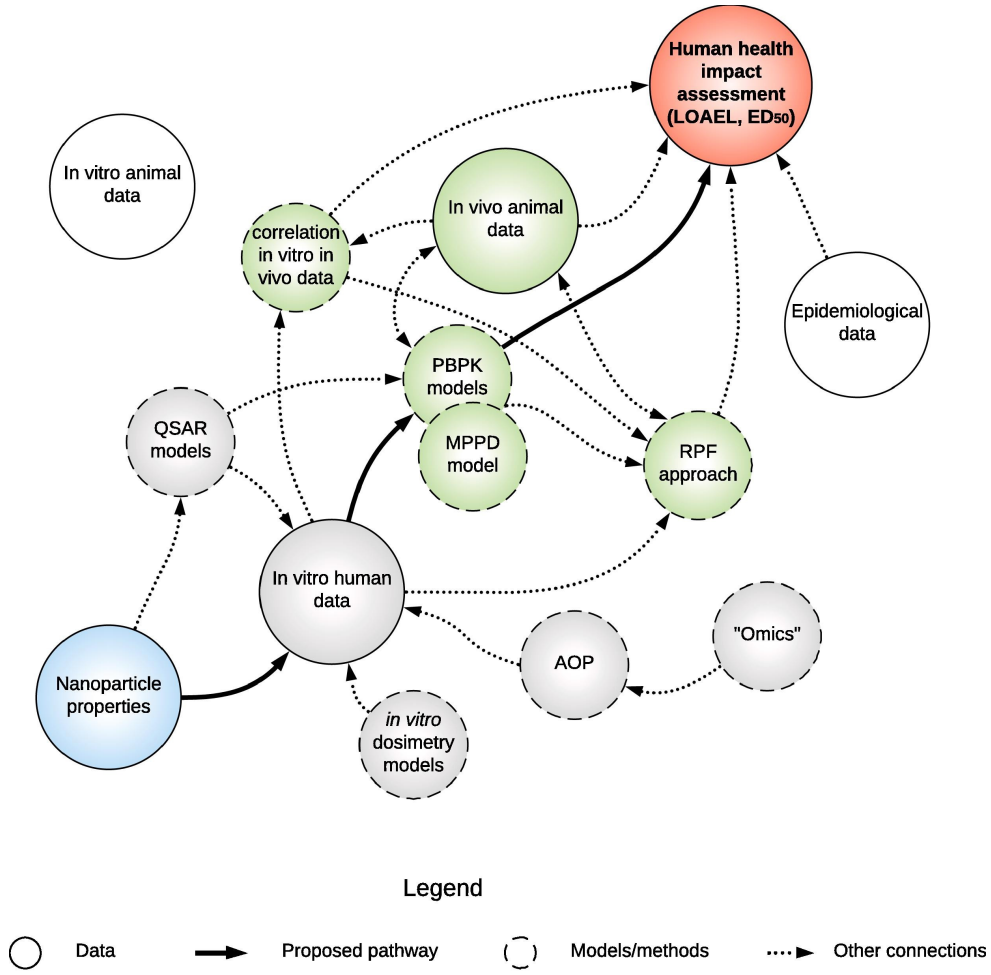


Figure 19. Pathway for future-oriented hazard assessment of manufactured nanomaterials as proposed by Romeo 2020. Figure reprinted from Environmental International, Romeo et al. (2020) under Creative Commons CC-BY license.

To facilitate the implementation of this pathway, a combined dosimetry model estimating air concentrations corresponding to *in vitro* doses was presented in a follow-up paper (Romeo et al. 2022). This approach involved both the one-dimensional Distorted Grid model for *in vitro* doses and the MPPD model for lung dosimetry. Using this model for a case study of TiO_2 nanoparticles, the results demonstrate that most *in vitro* doses were representative for lung doses reached primarily after one year or longer occupational exposure. This can be compared to the results of **Paper IV**.

The combination of *in vitro* assays and lung dosimetry can be used to create specific-use cases, as was done for welding fumes in **Paper IV**. This could promote the use of *in vitro* data in risk assessment. Nonetheless, the employment of dosimetry models requires an estimation of human exposure. A major hindrance towards applying this methodology in the testing of engineered nanomaterials is the scarce or unavailable exposure data for the majority of the materials. **Paper IV** further emphasizes the importance of well characterized aerosols to enable the use of dosimetry modelling. However, it is important to note that both exposure measurement methods as well as in dosimetry models have limitations and does not fully mimic the complexity of an aerosol. Ultimately, the accuracy of dosimetry models relies on how realistic the input parameters are. For example, using the (mass/count) median diameter to describe the particle size distribution is likely a great simplification of the real distribution. Other limitations include the MPPD model assuming poorly soluble particles and not considering uneven distribution throughout the lung. This emphasizes that, despite the great promise of combined dosimetry modelling for understanding *in vitro* doses, some questions remain to be answered before this approach can be used to directly extrapolate *in vitro* results to *in vivo* effects.

6 CONCLUSIONS AND POINTS OF PERSPECTIVE

This thesis aimed to explore aspects of toxicity and fate of metal-based particles in the lung in order to gain a deeper understanding of the associated hazards and how this best can be studied.

In **Papers I** and **II**, we showed the applicability of using the ToxTracker assay for mechanistic screening of genotoxic properties of nano-sized particles. The assay gave insight into the genotoxic mechanisms of the nanoparticles tested, being primarily oxidative stress. The particles activating reporters related to DNA-damage were considered of particular concern. There is a high demand for more efficient and rapid ways for screening the tremendous number of nanoparticles being put on the market. **Paper I** emphasized the potential of ToxTracker as a tool for providing mechanistic insight and potentially contributing to grouping, read-across and prioritization of nanoparticles.

This thesis further attempted to provide knowledge regarding which welding fume particles of stainless steel welding and their metal components are most hazardous to workers health. **Paper II** demonstrated the choice of filler material (electrode) to be a major determinant of *in vitro* toxicity of welding fume particles, where FCW generated particles were found to be most toxic. The predictive value of metal release for the acute toxic potential of welding fumes was highlighted, specifically the release of Cr(VI) and Mn. These findings were elaborated in **Paper III,** demonstrating the acute toxicity of welding fumes formed during welding of stainless steel to be largely explained by the released fraction containing predominantly Cr(VI). The results further confirmed our hypothesis that a reduction of released Cr(VI) results in less toxic fumes, and suggested a potential benefit in substituting standard FCWs with Cr(VI)-reduced wires to achieve less toxic welding fumes and thus a reduced risk for welders.

The results of **Papers II** and **III**, indicate the release of Cr(VI) to be a main driver in the acute lung toxicity of welding fumes generated by stainless steel welding, but also emphasize the complexity of welding fumes and their particle properties and associated toxicity. This implies that pinpointing welding methods, settings, materials or constituents as worst choice is not a straightforward path. Nonetheless, this type of research is important as it can contribute to better worksite regulations but also provide guidance in the development of new welding techniques such as welding consumables with reduced hazardous components. This thesis further highlights the importance of communication and collaboration between academia and industry in the process of ensuring safer occupational environments. Our collaboration with the welding industry provided us a unique chance to contribute to the development of safer welding material which, in turn, can have a direct impact on the occupational environment of welders.

In **Paper IV,** we demonstrated that human lung doses attained following both short and long-term exposure to welding fumes were found comparable to cell doses where toxic effects had been observed in **Paper III.** Importantly, *in vitro* cytotoxic concentrations were attained in the tracheobronchial region already after one working shift of being exposed to the regulatory occupational exposure limit. This somewhat challenges the conventional idea that only unrealistically high doses are being used *in vitro*. **Paper IV** further demonstrated the value of

combining occupational exposure measurements with deposition modelling in order to understand the relationship between human exposure and lung dose, and to further relate this to *in vitro* doses and toxic effects. This approach can be valuable in the interpretation of *in vitro* results for human hazard and risk assessment and in the study design of future *in vitro* studies.

Throughout the years, many lessons have been learnt regarding the most suitable approach to study particles for hazard assessment. Nevertheless, there are still many challenges to overcome and the discipline of (nano)particle toxicology is constantly adapting. This thesis has addressed several important aspects including dose metric calculations, quantification of cellular dose and the need to increase the speed and efficiency of the toxicological testing of nanomaterials, where the ToxTracker can aid as a tool. The move from animal-based models towards mechanistic *in vitro* and *in silico* data for the risk assessment of particles pose a massive challenge where concerted efforts are required to move forward. One important step in increasing the regulatory weight of *in vitro* data remains in answering the billion-dollar question: *How relevant are in vitro doses for human exposure?* I believe that the approach and results of **Paper IV** could guide future studies in this challenge.

On a final note, the findings of this thesis are clearly in line with the consensus that particles cannot be considered a uniform class of materials, not even the group of welding fume particles, and that there is no specific property or mechanism that can be held responsible for the toxicity. Nonetheless, our results especially shed light on the involvement of oxidative stress and metal release in the toxicity of metal-based particles. This thesis has further expanded the knowledge on the fate and toxicity of metal-based particles in the lung and provided important tools and directions for future studies on the lung toxicity related to particles.

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