DEVELOPMENT AND COMPARISON OF IN VITRO TOXICITY METHODS FOR NANOPARTICLES - FOCUS ON LUNG CELL EXPOSURE

Siiri Latvala
Development and Comparison of \textit{In Vitro} Toxicity Methods for Nanoparticles

Focus on lung cell exposure

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“It ain’t what you don’t know that gets you into trouble. It’s what you know for sure that just ain’t so.”
- Mark Twain
Abstract

Concerns for the toxic effects of airborne nanoparticles have been raised along with the increasing production of nanostructured materials. However, the health risks of nanoparticle exposure are currently not fully understood. The most commonly used techniques to study nanoparticle toxicity, both in vivo and in vitro, have several limitations. These include for example challenges regarding dosimetry or the lack of similarity of overall exposure conditions and the physico-chemical particle characteristics. Therefore, there is a need for more advanced methods to study the pulmonary toxicity of airborne nanoparticles.

This thesis presents the use of air-liquid interface (ALI) systems as a possible approach to this challenge. While utilizing the direct deposition of airborne nanoparticles on lung cell cultures, the ALI approach can more realistically mimic the characteristics of the human respiratory tract and the interactions of airborne particles with lung cells. This allows for a better understanding of the health risks posed by inhalation exposure to nanoparticles.

Two different ALI systems were investigated and their use was compared to submerged exposure methods. One of the ALI exposure systems utilizes electrostatic force in order to make the deposition of charged airborne nanoparticles more efficient, while the other system operates by the diffusion of airborne nanoparticles. ALI and submerged exposure methods were used for investigating the cytotoxicity and genotoxicity of Ni-containing nanoparticles as well as the cytotoxicity and inflammatory potential of CeO2-nanoparticles. While Ag-nanoparticles were used as a test material during the development of the electrostatic ALI system, their cytotoxicity was investigated in ALI exposure. In conclusion, the ALI exposure methods provide more realistic conditions and make the particle dosimetry more controllable.

Keywords: nanoparticles, exposure, inhalation, ALI, in vitro.
Sammanfattning


Den här avhandlingen beskriver användningen av en metod som kallas för ”ALI” (från orden air-liquid interface). Det betyder i princip att lungceller odlas emellan vätska och luft. Systemet som används efterliknar den verkliga miljön i människans luftvägar, vilket gör att interaktionerna mellan nanopartiklar och lungceller blir lättare att studera. När lungceller odlas i ALI och exponeras för luftburna nanopartiklar, blir det möjligt att på ett bättre sätt förstå vad som händer i lungcellerna när nanopartiklar andas in.
Development and Comparison of In Vitro Toxicity Methods for Nanoparticles

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This doctoral thesis consists of a summary and four papers.

LIST OF PAPERS

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Contributions of Latvala, S.

I Planned and performed the toxicological experiments, performed the data-analysis and wrote the manuscript.

II Performed the optimization of the exposure system, planned and performed the experiments and most of the laboratory work, performed the data-analysis and wrote the manuscript.

III Planned and performed the experiments and all laboratory work, performed the data-analysis and wrote the manuscript.

IV Designed and performed the ICP-MS analysis, performed the data-analysis and took part in writing the manuscript.

Rodhe, Y., Skoglund, S., Latvala, S., Odnevall Wallinder, I., Potáková, Z., Möller, L. Zn nanoparticles induce more DNA damage compared to ZnO nanoparticles in HL60 cells. Manuscript.
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<tr>
<td>A549</td>
<td>Continuous human type II alveolar epithelial cell line</td>
</tr>
<tr>
<td>AAS</td>
<td>Atomic absorption spectroscopy</td>
</tr>
<tr>
<td>ALF</td>
<td>Artificial lysosomal fluid</td>
</tr>
<tr>
<td>BET</td>
<td>Specific surface area measured with the Brunauer–Emmet–Teller method</td>
</tr>
<tr>
<td>CFE</td>
<td>Colony forming efficiency</td>
</tr>
<tr>
<td>DCFH</td>
<td>2’7-dichlorodihydrofluorescin</td>
</tr>
<tr>
<td>DCFH-DA</td>
<td>2’7-dichlorodihydrofluorescin diacetate</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s minimal essential medium</td>
</tr>
<tr>
<td>DMPS</td>
<td>Differential mobility particle sizer</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>HPRT</td>
<td>Hypoxanthine guanine phosphoribosyl transferase</td>
</tr>
<tr>
<td>ICP-MS</td>
<td>Inductively coupled plasma mass spectrometry</td>
</tr>
<tr>
<td>LDH</td>
<td>Lactate dehydrogenase</td>
</tr>
<tr>
<td>MF</td>
<td>Mutation frequency</td>
</tr>
<tr>
<td>NP</td>
<td>Nanoparticle or nano-sized particle (i.e. particle with a size of 1-100 nm)</td>
</tr>
<tr>
<td>p53</td>
<td>Tumor suppressor protein encoded by TP53</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>SAR</td>
<td>Structure-activity relationship</td>
</tr>
<tr>
<td>SB</td>
<td>DNA strand break</td>
</tr>
<tr>
<td>SEM</td>
<td>Scanning electron microscopy</td>
</tr>
<tr>
<td>TEM</td>
<td>Transmission electron microscopy</td>
</tr>
<tr>
<td>THP-1</td>
<td>Peripheral blood monocyte cell line</td>
</tr>
<tr>
<td>V79</td>
<td>Chinese hamster lung fibroblast cell line</td>
</tr>
</tbody>
</table>
Introduction

Nanoparticles
According to a commonly used definition, nanoparticles are objects that have all their three external dimensions between the size range from 1 to 100 nm (1). Although the research on nanoparticles has rapidly advanced during the recent years (2), they are not a new phenomenon (3,4). In addition to the engineered nanoparticles with intentionally designed structures, unintentionally produced nanoparticles occur from natural (5) as well as anthropogenic sources (6).

Nanoparticles, due to their extremely small sizes (large surface-to-mass ratio), have unique properties that can be applied in novel materials and technologies (3,4,7,8). These can for example be utilized in order to make certain products or techniques more sustainable (4,9,10). Those same properties, however, that make nanoparticles technologically exceptional can also affect their toxicological properties (3,8,11). Therefore, there are concerns for potential adverse effects of nanoparticle exposure for humans as well as the environment.

Nanotoxicology
The term “nanotoxicology” was established due to the need for focused efforts to study the possible risks related to the widespread production and use of new types of nanoparticles. The intention of this relatively new discipline is to support the safe and sustainable development of nanotechnology through the understanding of how particle size and surface area affect the deposition, translocation as well as toxicity of NPs (12).

Before the definition of nanotoxicology, the study of adverse health effects of nano-sized particles was addressed within particle toxicology (12). Under this discipline, nanoparticles are commonly called ultrafine particles (4,12). Both disciplines have mostly concentrated on understanding the effects and toxicological mechanisms of particle exposure from a pulmonary exposure
perspective (13). Inhalation is generally considered as the most relevant exposure route for airborne nanoparticles. When nanoparticles are inhaled, they move in the respiratory tract following the airflow. They can travel all the way to the alveolar region, which is the most distant part of the lung. This region is where the O₂-CO₂ gas-exchange takes place and is therefore consisted of a thin single-cell layer between the air and the capillaries. Nanoparticles can be deposited anywhere along their route in the respiratory tract (3,14).

Epidemiological studies have found associations between ultrafine (i.e. nano-sized) particles and respiratory-cardiovascular diseases (15-17). As these effects are potentially important on a global scale, understanding the associated mechanisms in nanotoxicology in closer detail is important for making effective measures to limit human exposure to airborne particles in any environments.

**In vitro studies**

Russel and Burch described a principle emphasizing the need for alternative approaches to toxicological *in vivo* studies already in the 1950’s (18). It defined the term “three R’s” as the replacement, reduction and refinement of animal studies. The principle is still highly relevant in toxicological studies, not only due to ethical aspects, but also due to the potential of more efficiently used resources and more reliable results (19).

*In vitro* methods are common in nanotoxicology. They can offer rapid and effective solutions for determining mechanistic principles or dose-response relationships (20). *In vitro* methods are also particularly useful for conducting high-throughput screening studies or developing different *in silico* models, such as structure-activity relationships (SAR) or read-across approaches (20,21). Due to the vast amount of different nanoparticles with different characteristics and currently unknown toxicological properties, development of well established *in silico* approaches for hazard ranking of nanoparticles is deemed necessary (21).

Despite their potential, *in vitro* methods have certain limitations. For example, *in vitro* studies alone cannot currently give answers to translocation, biodistribution, toxicokinetics or other equally complex processes (22). Technical challenges include the uncertainty of the applicability of different *in vitro* assays on nanoparticles. This includes the possible interference of nanoparticles with the materials of the assay (23). Furthermore, the introduction of standardized methods for toxicological testing of nanoparticles is not comprehensive (20,24,25). The lack of international standards or
quality criteria makes it more challenging to compare the data from different studies and to use the available results in risk assessments.

With regards to pulmonary toxicology, the specific challenges of \textit{in vitro} studies are largely related to the biological and physiological complexity of the respiratory system (26-30). Other major obstacles are the practical considerations when applying methods originally designed for solutions and molecular contaminants on airborne particles. The most commonly used \textit{in vitro} methods in pulmonary nanotoxicology, indeed, use lung cell cultures submerged under a suspension of cell culture medium and nanoparticles. These conditions are highly distinctive from the physiological conditions of the pulmonary tissue. Controlling and understanding the dosimetry in these conditions is problematic as the cellular dose depends on the sedimentation rate of the particles in the liquid (31). In addition to the cellular dose, other particle properties can be changed when particles agglomerate or form complexes with biomolecules contained in the suspension. The complex-formation can further be affected by the properties of the cell culture media (11,32,33).

Despite the clear advantages of \textit{in vivo} studies in regards with whole organism exposure and higher complexity, they have several limitations. For example, the most commonly used laboratory animals have different lung physiology and anatomy than humans (27,34). These differences affect the deposition of airborne particles in the respiratory tract, which can cause inter-species differences. This can lead to results that cannot reliably be extrapolated to humans. Similarly as with \textit{in vitro} studies, also \textit{in vivo} studies are faced with challenges in controlling the dosimetry of nanoparticles and choosing appropriate exposure methods (22).

\textbf{Air-liquid interface exposure}

In studies aimed at investigating the toxicity of airborne nanoparticles with pulmonary exposure scenarios, \textit{in vitro} methods based on aerosol exposure are potentially more relevant than submerged exposure methods (35). Therefore, several air-liquid interface (ALI) exposure systems have been developed (36-38). The basic concept of these methods is that (lung) cells are cultured on thin porous membranes. The cells are fed with cell culture medium kept only on the basal side of the membrane, and therefore, the cells can be exposed directly to airborne particles (Figure 1).
Due to the complexity of the lung tissue structure, several co-culture models consisting of two or more cell types as well as organotypic cell cultures have been developed (39-42). Compared to simple monocultures that consist of one cell type, these are structurally and biologically more alike the human lung tissue. Co-cultures have also been shown to respond differently to particle exposure than monocultures (43). Although the co-culture and organotypic models are generally more challenging to use than monocultures, they are considered potential alternatives for in vivo studies (41).

Yet another challenge for designing appropriate in vitro exposure systems for nanoparticles is their tendency to move along with the airflow (44). Brownian diffusion is the main deposition mechanism of nanoparticles (44), which can be challenging to control. Therefore, in order to enhance the deposition of nanoparticles on cell cultures in ALI, different methods from cloud settling to electrostatic deposition have been applied (36-38).

**Figure 1. A schematic illustration of the main differences between submerged and air-liquid interface exposure methods within in vitro nanotoxicology.**
Thesis objectives

The overall objective of the research presented in this thesis was to improve the understanding of different methods used in nanotoxicology. More specifically, the objective was to compare the more traditional submerged exposure *in vitro* methods to ALI approaches. Particular focus throughout the thesis was placed on considering appropriate exposure quantification. The specific aims of this thesis are detailed below.

Aim I

The first aim of this thesis was to develop and optimize the use of ALI exposure systems for toxicity studies on airborne nanoparticles.

Aim II

The second aim was to investigate the toxicity of nano- and micron-sized particles in lung cell cultures and compare the effects in submerged and ALI exposures.

Aim III

The third aim of this thesis was to critically evaluate how particle doses should be studied both in submerged and ALI exposures.
Study outline

The main objective of **paper I** was to investigate and compare the reactivity and toxicity of four Ni-containing particles. The exposures were conducted using a traditional submerged exposure method. Cytotoxicity, oxidative stress and DNA damage induction as well as cell proliferation in A549 cells were studied. Ni release and particle uptake in cells was also investigated.

**Paper II** was focused on the development and optimization of an electrostatic ALI exposure system. Furthermore, it was investigated how the deposition of nanoparticles is affected by different conditions of the electrostatic ALI system and how these conditions, as well as Ag nanoparticle (Ag-NP) exposure, affect the viability of A549 cells.

**Paper III** was a continuation for both **paper I** and **paper II**. The electrostatic ALI exposure system was used for further investigating the genotoxicity of Ni nanoparticles (Ni-NPs). Cytotoxicity, DNA damage and HPRT mutation induction were studied in V79 cells.

In **paper IV**, another ALI exposure system was applied for studying the cytotoxicity and inflammatory potential of CeO$_2$ nanoparticles (CeO$_2$-NPs). In this study, ALI exposure was compared to submerged exposure of the same particles. A co-culture system of A549 and THP-1 cells was used.
Methodology

Nanoparticle exposure \textit{in vitro}

\textit{Submerged exposure}

In \textbf{paper I}, submerged lung cell cultures were exposed to four different Ni-containing nano- and micron-sized particles dispersed in cell culture medium. In \textbf{paper IV}, submerged exposure to CeO$_2$ nanoparticles was used as a comparison to ALI exposures of the same particles. Unlike in \textbf{paper I}, where a monoculture was grown on tissue culture treated multiwell plates, in \textbf{paper IV} co-cultured cells were grown on cell culture inserts (transwells). This culturing method was chosen in order to allow for thorough comparisons between the submerged and the ALI exposures.

\textit{Air-Liquid Interface exposure}

The development and optimization of an ALI exposure system is presented in \textbf{paper II}. This system comprises the generation of airborne particles, the conditioning of the aerosol with a physiological gas mixture (20vol-\% O$_2$, 5vol-\% CO$_2$) and humidity as well as the exposure of lung cell cultures to this aerosol. During the exposure, the cell culture inserts are kept in 6 separate exposure chambers with cell culture medium on the basal side of the insert membrane. In this ALI exposure system, the deposition of nanoparticles is enhanced by electrostatic force (figure 2). In order to generate particles from diverse materials, the exposure system can be coupled to different aerosol generators. In \textbf{paper II} a high temperature furnace was used to generate silver nanoparticles (Ag-NPs), whereas in \textbf{paper III}, nickel nanoparticles (Ni-NPs) were obtained using a spark discharger.
The ALI exposure system used in paper IV consists of an aerosol generator (PreciseInhale™) and a cell exposure unit (XposeALI). Unlike the particle generators used in paper II and paper IV, the PreciseInhale™ system generates aerosols from dry powders. In the XposeALI system, 3 cell culture inserts are exposed simultaneously (figure 3). The cells are kept in contact with cell culture medium from the basal side of the insert membrane. In paper IV, the use of this commercially available system was tested and optimized for CeO$_2$ nanoparticles (CeO$_2$-NPs).
Particle characterization

The physico-chemical characteristics of nanoparticles, including their size, shape, surface area, solubility and surface chemistry, have a crucial impact on their toxicological properties (23,45). Therefore, unlike with molecular contaminants when the assessment of material purity and exposure concentration may be sufficient, the study of nanoparticles requires focused efforts on material characterization.

Particle size and morphology

In paper I, the size measurements of nanoparticles, dispersed in cell culture medium, were performed by photon cross-correlation spectroscopy (PCCS), while the size of micron-sized particles was determined with correlation spectroscopy. Particle size measurements of the airborne nanoparticles in paper II and paper III were performed with a differential mobility particle sizer (DMPS). In paper IV the size of deposited particles was investigated using Scanning Electron Microscopy (SEM).

Particle morphology, as well as particle agglomeration, was investigated by Transmission Electron Microscopy (TEM) in paper I and paper II. In paper I, the specific surface area of the particles was measured by BET-analysis (Brunauer-Emmet-Teller method) (46).

Particle reactivity

Generation of reactive oxygen species (ROS) is a central paradigm in nanotoxicology (8). The intrinsic reactivity of particles was in this thesis considered as one of their physico-chemical characteristics. Therefore, in paper I, the oxidative reactivity of the nano- and micron-sized particles was studied with the 2’ 7-dichlorodihydrofluorescein diacetate (DCFH-DA) assay (47). The main principle of the acellular DCFH-DA assay is that the DA group is cleaved from the non-fluorescent DCFH-DA prior to exposing it to nanoparticles. Upon the nanoparticle-induced reactions DCFH can then be oxidized forming a fluorescent dichlorofluorescein (DCF). The production of DCF can be determined by measuring the fluorescence in a plate reader. This oxidation can be induced by several oxidative species including RO₂⁻, RO•, OH•, HOCl and ONOO⁻ (48,49).

In this thesis, another important characteristic of particle reactivity was the extent of metal release from the particles in different environments. This was studied in paper I by incubating the Ni-containing particles in cell culture medium and in artificial lysosomal fluid (ALF). After different incubation periods, the particles were
separated from the solution, which was then analyzed by Atomic Absorption Spectroscopy (AAS) to quantify the extent of released Ni. In paper I, the intracellular Ni release was further investigated with TEM. However, this analysis was merely qualitative.

A summary of the particle characterization methods used in this thesis is given in Table 1. The CeO$_2$-NPs that were studied in paper IV are a well-characterized reference material (NM-212), and were therefore not characterized further in this thesis.

Table 1. Summary of the methods used for physico-chemical characterization of particles in this thesis.

<table>
<thead>
<tr>
<th>Particle characteristic</th>
<th>Method</th>
<th>Paper</th>
</tr>
</thead>
<tbody>
<tr>
<td>Morphology</td>
<td>TEM</td>
<td>I, II</td>
</tr>
<tr>
<td>Reactivity</td>
<td>DCF-DAC</td>
<td>I</td>
</tr>
<tr>
<td></td>
<td>Metal release</td>
<td>I</td>
</tr>
<tr>
<td>Size</td>
<td>PCCS</td>
<td>I</td>
</tr>
<tr>
<td></td>
<td>SEM</td>
<td>IV</td>
</tr>
<tr>
<td></td>
<td>DMPS</td>
<td>II, III</td>
</tr>
<tr>
<td>Specific surface area</td>
<td>BET</td>
<td>I</td>
</tr>
</tbody>
</table>

Particle quantification

In paper II and paper III, the particle concentration in the exposure aerosol was analyzed with a DMPS prior to the exposure. In these studies the DMPS was also used for monitoring the particle concentrations during the exposures. In this case, the measurements were performed downstream of the exposure system. In paper IV the particle concentrations were analyzed by estimating the delivered particle mass by a light dispersion instrument. Monitoring the particle concentrations by these two methods could give a direct, however, not precise, estimation of particle deposition in the ALI systems.

In order to determine the particle dose, to which the cells were exposed, the metal concentrations on the exposed cell cultures were quantified with chemical analysis. AAS was used in paper II, whereas Inductively Coupled Plasma Mass Spectrometry (ICP-MS) was utilized in paper III and paper IV. In summary, the cell culture insert membranes, containing the exposed cells, were separated from the insert walls. After this, the membranes were placed in a tube where the metals were digested under an acid treatment. In paper II and paper IV also an additional digestion procedure was performed.
Thereafter the concentrations of the studied metals were quantified in these solutions either with AAS or ICP-MS. The total mass of the deposited particles was then calculated based on the analyzed concentrations. After the submerged exposures in paper I and paper IV, the cellular Ni and CeO2 concentrations were quantified with AAS and ICP-MS, respectively.

In paper II and paper III the chemical methods of quantifying particle deposition were compared to a gravimetric method. In this procedure, the particles were deposited on cell-free cell culture inserts containing an additional membrane. These membranes were weighed both before and after the particle deposition, after which the mass of the deposited particles were directly obtained. A summary of the used quantification methods in papers I-IV is given in Table 2.

### Table 2. Summary of the methods used for particle quantification.

<table>
<thead>
<tr>
<th>Quantification method</th>
<th>Paper</th>
</tr>
</thead>
<tbody>
<tr>
<td>AAS</td>
<td>I, II</td>
</tr>
<tr>
<td>DMPS</td>
<td>I, II, III</td>
</tr>
<tr>
<td>ICP-MS</td>
<td>III, IV</td>
</tr>
<tr>
<td>Light dispersion</td>
<td>IV</td>
</tr>
<tr>
<td>Gravimetric</td>
<td>II, III</td>
</tr>
</tbody>
</table>

**Particle toxicity**

**Cell cultures**

All cell cultures were maintained in serum-supplemented cell culture medium in a humidified (>99 % RH) 5 % CO2 atmosphere at 37 °C. In paper I and paper II, simple monocultures of A549 cells (obtained from the American Type Culture Collection, ATCC, Manassas, USA) were used. These cells are transformed alveolar type II lung cells. In paper IV, the A549 cells (obtained from NANoREG partners, Bundesanstalt Fuer Arbeitsschutz und Arbeitsmedizin, BAuA, Germany) were accompanied by THP-1 cells (ATCC, Manassas, USA) in a co-culture. A wild type Chinese hamster lung fibroblast cell line, called V79 (obtained from Thompson LH), was chosen to paper III due to its suitability for the HPRT mutation assay.
Cytotoxicity and cell viability

For the purpose of this thesis, cell viability is recognized as the proportion of healthy cells in a cell population at a specific time-point (50). Cell viability is closely linked to cytotoxicity, which may proceed via several mechanisms (51). Cell viability in paper I, paper II and paper IV was analyzed by measuring the metabolic activity of the cells. This was done using the alamar blue assay (alamarBlue®, Invitrogen, Life Technologies). In paper I, where multiwell plates were used with the submerged exposures, the procedure of the assay followed the manufacturer’s instruction. Due to the use of cell culture inserts in paper II and paper IV, the procedure was modified for both studies.

Furthermore, in paper I, paper II and paper IV, cell viability was analyzed by measuring the integrity of the cell membrane. In paper I and paper II, trypan blue exclusion assay was used, but in paper IV, the cell membrane integrity was assessed by lactate dehydrogenase (LDH) release assay. Generally, compared to cell membrane integrity, cellular metabolic activity is a more sensitive marker of cell viability. The trypan blue assay, for example, detects necrotic cells and cells in the late stages of apoptosis. Depending on the cell death type, this assay may not detect the first signs of cell death, while the alamar blue assay is more likely to do so (51). However, discrimination between necrosis and apoptosis is not reliable with any of these assays.

Colony forming efficiency was determined in paper III to study cell viability, but in paper I it was specifically used to study cell proliferation. The analysis was performed by seeding a certain amount of cells on tissue culture plates (paper III) or on multiwell plates (paper I). The cells were allowed to grow until they formed visible colonies, which were then dyed and counted. The amount of colonies represents the amount of surviving or proliferating cells. A summary of the used cytotoxicity assays in papers I-IV is given in Table 3.

Table 3. Summary of the cytotoxicity methods used in this thesis.

<table>
<thead>
<tr>
<th>Endpoint</th>
<th>Method</th>
<th>Paper</th>
</tr>
</thead>
<tbody>
<tr>
<td>Metabolic activity</td>
<td>Alamar blue</td>
<td>I, II, IV</td>
</tr>
<tr>
<td>Membrane integrity</td>
<td>LDH release</td>
<td>IV</td>
</tr>
<tr>
<td></td>
<td>Trypan blue</td>
<td>I, II</td>
</tr>
<tr>
<td>Colony forming efficiency</td>
<td>CFE</td>
<td>I, III</td>
</tr>
</tbody>
</table>
Cytokine release
The release of inflammatory cytokines after exposure to CeO\textsubscript{2}-NPs was analyzed in paper IV. The analysis was performed using multiplex bead array technology (Luminex). Soluble cytokines from the cell supernatants were captured on microbeads with distinct spectral addresses. The beads were then labeled with fluorescent markers for detection. Thereafter, the spectral addresses of the microbeads were determined and the signal of the captured cytokines was quantified. In this study, cytokines IL-1\textbeta, IL-6, TNF\textalpha and MCP-1 were analyzed.

Oxidative stress
Besides measuring the intrinsic oxidative reactivity of Ni-containing particles in paper I, the DCFH-DA assay was used to study intracellular ROS production. This was done in order to assess whether the particle exposure induces oxidative stress in the cell cultures. Oxidative stress is broadly defined as the imbalance between antioxidants and ROS in a biological organism (52). This can lead to adaptation, including up-regulation of the antioxidant defense and repair of the acquired damage. If the stress is extensive and/or prolonged it can also lead to cell senescence and cell death (53).

The basic principle of the cellular DCFH-DA assay used in this thesis is similar to the acellular version. The main difference is that DCFH-DA is taken up by cells and is thereafter cleaved by intracellular esterases. DCFH is then trapped inside the cell and can there be oxidized to DCF by intracellular ROS. Thereafter, a plate reader can be used to measure the fluorescence of DCF.

DNA damage
The single cell gel electrophoresis assay is commonly known as the comet assay. It was used in paper I to study DNA damage (DNA strand breaks or alkaline labile sites (54)) after exposure to Ni-containing nano- and micron-sized particles. In the alkaline version of this assay used in this thesis, the studied cells are embedded in agarose gel on microscope glass slides and lysed in an alkaline solution (pH>13), which allows the nuclear DNA to unwind. This creates DNA strands in different lengths depending on the amount of DNA damage acquired by the cell. During electrophoresis, the DNA strands migrate towards the anode. As the shortest strands migrate the fastest, this creates the appearance of a comet in a fluorescence microscope after the cells are stained with a fluorescent DNA binding dye. The amount
of DNA damage can then be analyzed with image analysis. Different units for DNA damage, such as tail length, % DNA in tail or (olive) tail moment, can be used. The unit % DNA in tail was chosen in this thesis. It represents the fraction of DNA contained in the comet tail and is regarded as a robust marker for DNA damage (55).

As an alternative method to the comet assay, alkaline DNA unwinding (ADU) technique was used in paper III to study DNA damage after exposure to Ni-NPs. This method analyzes the number of strand breaks (SB) in DNA (56,57). The principle is that the DNA strands are separated from each other by unwinding them in an alkaline solution. Similar to the comet assay, this creates DNA strands that vary in length based on the extent of DNA damage acquired by the cells. The single stranded and double stranded DNA in the samples are separated using hydroxyapatite chromatography (58). In order to detect the separated DNA strands, the DNA is labeled with tritiated thymidine (3HTdR) before the treatment. The radioactive decay of 3H is then measured in a scintillation counter. The obtained fraction of labeled single stranded DNA is an estimation of the number of SBs, and therefore of DNA damage.

Mutagenicity

The hypoxanthine guanine phosphoribosyl transferase (HPRT) mutation assay was used in paper III to assess the induction of mutations in cells exposed to Ni-NPs. HPRT is an enzyme responsible for the phosphoribosylation of guanine and hypoxanthine (59-61). The enzyme has its function in catalyzing the rescue of these purines by converting them into nucleoside-5-monophosphates (62). The hprt gene is located in the X chromosome in both human and rodent cells (62). A mutation in the hprt gene disrupts the function of the enzyme, which makes the cell also unable to metabolize purine analogs, such as 6-thioguanine (6-TG). The HPRT mutation assay therefore measures the functional loss of the HPRT enzyme caused by a mutation in the hprt gene (59). This is achieved by growing the cells in the presence of 6-TG, which in normal cells is metabolized by the functional HPRT enzyme. This metabolite is toxic and therefore the normal cells do not survive, while the cells with a mutated HPRT phenotype are not affected by 6-TG.
Results and discussion

Aim I

“Development and optimization of the use of ALI exposure systems for toxicity studies on airborne nanoparticles”

Particle deposition

In paper II, it was shown, that particle deposition on cell culture inserts in the electrostatic ALI system increased with increasing aerosol flow rate and electrostatic field strength (Figure 4 and 5). However, a limitation for increasing the electrostatic field strength was observed when the deposition patterns on the cell culture inserts were studied: the distribution of Ag-NPs was relatively uniform on the cell culture inserts when the electrostatic field was ±0.5 or ±1 kV, but when this was increased to ±2 kV, the particles were more frequently deposited on the center of the insert membrane. Therefore, it was concluded that in order to achieve the most equally distributed particle deposition on the cell cultures, the strength of the electrostatic field should be below ±2 kV. In this study, cell doses up to 0.74 μg/cm² of Ag were detected.

The PreciseInhale™/XposeALI system that was used in paper IV to expose co-cultured lung cells to CeO₂-NPs also produced a uniform deposition on the cell culture inserts (Figure 6). Cell doses as high as 5.5 μg/cm² of CeO₂ were measured.

Effects on cell cultures

Cell viability of A549 cells in the electrostatic ALI system in paper II was not affected after exposure to clean air: there were no significant differences in cell viability between cells exposed to clean-filtered air and cells kept in the incubator. The PreciseInhale™/XposeALI system did not induce cytotoxicity in the cell cultures exposed to clean air in paper IV. Therefore, the experimental formats of these ALI systems were considered not to cause adverse effects on the cell cultures.

In conclusion, paper II and paper IV showed that the corresponding ALI exposure systems can be used for exposing lung
cell cultures to airborne nanoparticles in a more realistic environment when compared to traditional in vitro methods. As the ALI systems preserve the intrinsic physico-chemical properties of airborne particles more closely than submerged exposures, it was concluded that they can be used to produce more consistent comparisons between different particles and their effects.

Figure 4. The dependence of Ag-NP deposition of aerosol flow rate (100, 214 and 390 mL/min) and strength of the applied electrostatic field (±1 and ±2k V) in the air-liquid interface exposure system (adapted from Paper II).

Figure 5. Transmission electron micrographs of Ag-NPs deposited on cell culture inserts in the electrostatic air-liquid interface exposure system (adapted from paper II).
Figure 6. Scanning electron micrograph of CeO$_2$-NPs deposited on cell culture inserts in the PreciseInhale$^{\text{TM}}$/XposeALI air-liquid interface system.
Aim II

“Investigating the toxicity of nano- and micron-sized particles in lung cell cultures and comparing the effects in submerged and ALI exposures”

Ni-containing nano- and micron-sized particles

Each of the four Ni-containing particles (Ni-n, NiO-n, Ni-m1 and Ni-m2) tested using submerged exposure in paper I showed increased cytotoxicity in A549 cells at the highest Ni doses (20–40 μg/cm²) when assessed by colony forming efficiency (CFE). Among these particles, a micron-sized Ni particle (Ni-m1) induced the highest cytotoxicity when assessed by alamar blue assay.

At lower Ni doses (0.1 or 1 μg/cm²), however, each of the particles induced an increased CFE (Figure 7). This suggests that these particles in low concentrations induce cell proliferation, which may be an important factor for the carcinogenic effects of Ni. Increased cell proliferation due to Ni exposure has been described previously (63-65). Ni compounds are also confirmed carcinogens (66-68). The increased proliferation is suspected of being a result of a shifted redox-balance in the cells, which in this case would be caused by the redox-active Ni (69).

Furthermore, when Ni-NP exposure was studied at ALI in paper III, an indication of increased CFE (second CFE, used for mutation frequency determination) was observed in V79 cells at Ni doses approximately in the same range as in paper I (0.05–0.32 μg/cm²). However, directly after the 48 h ALI exposure (first CFE, used for viability determination), CFE was significantly (p < 0.05) reduced at Ni concentrations of 0.15 and 0.32 μg/cm².
Although in *paper I*, Ni-m1 and NiO-n induced significant (p < 0.05) acellular ROS production, intracellular ROS in A549 cells was not increased by any of the particles. DNA damage, analyzed by the comet assay, was induced significantly (p < 0.05) by NiO-n (4 and 24 h exposure) and Ni-m1 (24 h exposure) at Ni concentration of 20 μg/cm² (Figure 8). DNA damage after Ni²⁺ and Ni-NP exposure has also been observed before using the comet assay (70,71). Previously, the Ni-induced DNA damage has been proposed to be an effect of Ni²⁺ in the cell nucleus (68,70,72). In *paper III*, Ni-NP exposure did not induce DNA damage, when analyzed by the ADU assay. In a previous study, similar results for Ni²⁺ were obtained with the same assay (73).

Of these two DNA damage assays, the comet assay has been more common in nanotoxicology (74). It is a well-established and internationally validated method (54,75,76). However, there have been concerns for the possibility of false positive results if remaining NPs in the agarose-embedded and lysed cells react with the naked DNA (77). Both assays can be modified to study specific DNA lesion types or DNA damage repair. For instance, the comet assay can be modified to study base excision repair (BER) by using lesion-specific enzymes to cleave off the damaged bases (78).
Ni-NPs in ALI exposure (paper III) did not induce HPRT mutations at the studied Ni concentrations (0.05 – 0.32 μg/cm²) and exposure time (48 h). Ni has been previously described as weakly mutagenic (66,79), and therefore, these results were not unexpected. The carcinogenic effects of Ni are indeed more commonly associated with indirect genotoxic effects, than with direct mutagenicity (66,79). For instance, Ni has been reported to induce intrachromosomal homologous recombination in the hprt gene in V79 cells (80). These effects are further suggested to be due to the disruption of DNA repair (80). Furthermore, it is proposed that DNA repair machinery, including nucleotide excision repair (NER) and BER, is affected by redox-reactions induced by transition metal ions, such as Ni²⁺ (81).

**Figure 8.** DNA damage, studied by the Comet assay, in A549 cells exposed to four Ni-containing particles (Ni-n, NiO-n, Ni-m1 and Ni-m2) for 4 and 24 h (adapted from Paper I).
Importantly, the disruption of DNA repair may enhance the effects of other DNA damaging agents (79,82,83). Furthermore, an increased HPRT mutation frequency may be an effect of both direct mutations in the *hprt* gene or the inhibition of DNA repair (84). Therefore, studying Ni-NP exposure in combination with another genotoxic agent could reveal higher amounts of HPRT mutations than studying them separately. Indeed, the effects of co-exposure to Ni and other contaminants (such as Co, Cr, and polycyclic aromatic hydrocarbons) commonly present in the same occupational settings have been acknowledged (85,86). For thoroughly understanding the carcinogenic effects of Ni-NP exposure, co-exposure studies would therefore be essential.

**Ag nanoparticles**

In **paper II**, the cytotoxic effects of Ag-NPs were studied in ALI exposed A549 cells. When studied with the alamar blue assay, Ag-NP exposure at Ag concentrations of 0.45 and 0.74 μg/cm² reduced the cell viability to 64 and 46 % (p < 0.05), respectively (compared to the corresponding controls). Similar exposures did not, however, induce cytotoxicity, when studied with the trypan blue assay. The endpoint of this assay is more direct cell membrane injury, whereas the alamar blue assay measures metabolic activity, which can be a more sensitive marker. Compared to **paper II**, previous studies with submerged exposures have reported weaker cytotoxicity of Ag-NPs (87), which may suggest a higher sensitivity of ALI exposures. However, negative results for Ag-NP cytotoxicity have also been reported (88). Due to numerous differences between different Ag-NP studies, direct comparisons of the results are difficult to conduct (87,89-91). One additional reason for the varying toxicity of Ag-NPs among different studies may be the strong effects of the extra- and intracellular conditions on the bioavailability of Ag-NPs (92).

**CeO₂ nanoparticles**

In **paper IV**, exposure of co-cultured A549 and THP-1 cells to CeO₂-NPs in ALI did not induce cytotoxicity when studied by alamar blue assay. However, in the highest concentration (5 μg/cm²) of the ALI exposures, LDH release was slightly increased. In the submerged exposures, no effects on cytotoxicity were observed.

Release of inflammatory cytokines IL-1β, IL-6, TNFα and MCP-1 in **paper IV** was not significantly increased by any of the CeO₂-NP exposures. There was, however, a slight increase in TNFα release at the highest submerged exposure concentrations (15 and 22 μg/cm²). In
conclusion, due to the low toxicity of CeO$_2$-NPs, strong comparisons between ALI and submerged exposure were difficult to obtain.

**Summary of the effects**

A summary of the toxic effects of different particles and exposure conditions (ALI or submerged) in papers I-IV is given in Table 4. The differences in Ni-induced cytotoxicity in paper I and paper III can be influenced by several factors. The most apparent difference between these studies is the exposure method: submerged exposure in paper I and ALI exposure in paper III. Additionally, the cell lines used in these studies were different: A549 is a carcinoma cell line from the human lung epithelia (93), whereas V79 cells are derived from the lungs of Chinese hamster (94). A549 cells have certain features of alveolar type II epithelial cells, but due to their transformation, they do not differentiate properly and therefore lack several characteristics of the normal alveolar type II phenotype (42,95,96). Other differences are the cell culture conditions and the administration of the particles in submerged and ALI exposures as well as the particles themselves.

Table 4. Summary of the toxicological endpoints studied in this thesis and the effects observed for each particle. “Sub.” = submerged; “ALI” = air-liquid interface; “+” = statistically significant response; “–” = no response; blanks = not studied.

<table>
<thead>
<tr>
<th>Paper</th>
<th>Particle</th>
<th>Exposure</th>
<th>Cytotoxicity</th>
<th>Cell proliferation</th>
<th>Oxidative stress</th>
<th>Cytokine release</th>
<th>Genotoxicity</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Ni-NP</td>
<td>Sub.</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>NiO-NP</td>
<td>Sub.</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Ni-m1</td>
<td>Sub.</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Ni-m2</td>
<td>Sub.</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>II</td>
<td>Ag-NP</td>
<td>ALI</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>III</td>
<td>Ni-NP</td>
<td>ALI</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>IV</td>
<td>CeO$_2$</td>
<td>Sub.</td>
<td>–</td>
<td></td>
<td>–</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>ALI</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Despite their differences, both A549 and V79 cells are continuous and transformed. Importantly, they both have a mutated p53 (97), which can limit their reliability in genotoxicity studies. The p53 protein is normally elevated in response to DNA damage (98-100) as it functions for example in binding to DNA strand breaks and re-annealing them (101). However, when p53 is non-functional or absent, these processes, including apoptosis, cannot proceed as they do in normal cells (99,100). Therefore, this should be kept in mind when interpreting results obtained with these cell lines.

Although these simplified cell culture systems have their limitations, they were chosen in order to avoid the complexity of highly differentiated cell cultures or co-culture systems in the method development and testing phase of the ALI system. The choice of V79 cells in paper III was additionally due to the suitability of these cells for the HPRT mutation assay (60). They have a rapid growth rate and a short lag period (doubling every 12-16 h), which allows for fast experimentation (62). They also have a high cloning efficiency and a stable karyotype (62), both of which are important for the HPRT assay.
Aim III

“Critical evaluation of how particle doses should be studied both in submerged and ALI exposures”

Cell dose

In paper I and paper II, the cell doses of Ni and Ag, were quantified by AAS, whereas in paper III and paper IV, cell doses of Ni and Ce, were quantified by ICP-MS. These methods are useful for quantifying the total amount of the studied metals, but they do not allow the direct measurement of the particles as such (e.g. particle number concentrations). The protocols used in this thesis were used for quantifying both the fraction of the metal bound to the cell membrane and the fraction that is taken up by the cells. A summary of the quantified exposure doses of each metal is given in Table 5.

Table 5. The quantified cell doses, nominal doses and dry dose (cell-free deposition) of the studied particles in the corresponding exposure methods (“submerged” and “ALI” = air-liquid interface).

<table>
<thead>
<tr>
<th>Paper</th>
<th>Metal</th>
<th>Exposure</th>
<th>Cell dose (μg/cm²)</th>
<th>Nominal dose (μg/cm²)</th>
<th>Dry dose (μg/cm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Ni</td>
<td>Submerged</td>
<td>3.0 – 6.3</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td>II</td>
<td>Ag</td>
<td>ALI</td>
<td>0.45 – 0.74</td>
<td></td>
<td>1.7</td>
</tr>
<tr>
<td>III</td>
<td>Ni</td>
<td>ALI</td>
<td>0.05 – 0.32</td>
<td></td>
<td>1.1 – 6.4</td>
</tr>
<tr>
<td>IV</td>
<td>Ce</td>
<td>Submerged</td>
<td>0.6 – 25</td>
<td>2 – 40</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>ALI</td>
<td>0.3 – 5.5</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*These values were calculated based on the cell uptake data (% uptake) presented in paper I.

*Only the results obtained with chemical quantification methods (AAS and ICP-MS) are listed here.

In the submerged exposures, large variations could be seen between the nominal doses and the actual cell doses (i.e. cell-associated fraction) of Ni in paper I and Ce in paper IV (Table 5). This highlights the importance of quantifying the cell dose even in submerged exposures.

In addition to the cell dose analysis, the deposition of Ag-NPs in paper II and Ni-NPs in paper III in the electrostatic ALI system was quantified from cell-free culture inserts (referred to as “dry dose” in Table 5). However, the measured doses in the cell-containing and cell-
free inserts revealed considerably different values in both studies. In paper II, there was an approximately 56% difference between the cell-containing and cell-free samples for Ag when analyzed by AAS. In paper III the difference was approximately 93%. In this case, however, these measurements were obtained using different methodology: gravimetric quantification for the cell-free inserts and chemical quantification for the cell-containing inserts. Although this could explain a part of the observed difference between the values, it was considered unlikely that the different methods would be the sole explanation. This is because the gravimetric measurements on cell-free inserts in paper II were verified by analyzing the same samples by AAS, and the obtained difference between these measurements (gravimetric vs. chemical) was approximately 26%. Therefore, the cell-free conditions for particle deposition in the electrostatic ALI system are not comparable to the experiments made on cell cultures, and therefore, cannot be used for exposure dose estimations.

The deposition of nanoparticles in both studied ALI systems was approximately in the same scale as in some previously published ALI in vitro studies (88,91). Furthermore, the deposited concentrations of Ag-NPs in paper II were close to concentrations that are estimated to be relevant for humans (102).

**Dose measurement and study design**

In each of the papers (I-IV) included in this thesis, attempts were made to quantify the cellular dose of the studied particles. However, the approaches regarding dose quantification were different between the studies (Table 6). Therefore, observations could be made on the suitability of these approaches.

The accuracy of the dose estimations can be considered to increase from paper I towards paper IV. Using one exposure concentration, out of many that were used in the toxicological assays (paper I), to study the cellular dose, may not give a realistic picture of the cellular dose in the remaining concentrations. Similarly, estimating the cellular dose based on a separate set of experiments, although conducted under similar conditions (paper I and paper II), might not reflect similar doses. In paper III, the estimation of cell dose was obtained from cells exposed at the same time as the cells that were taken further to toxicological assays (CFE and HPRT mutation assay). Due to the nature of these assays, the same cell cultures could not be used for quantifying the metal concentrations. Despite this, the assessment of cell dose in paper III was considered to be more accurate than in paper I and paper II. In paper IV, the cell dose assessment was done using the same cells that were used for the toxicological assays.
Although this restricts the use of those toxicological assays in which the cells are consumed by the assay (such as comet assay and HPRT mutation assay), this approach is considered to be the most accurate.

Therefore, it is concluded that in order to quantify the true exposure dose both in submerged and ALI exposures, the same cells should be used for the quantification as for measuring the toxicological endpoints.

Table 6. Summary of the different approaches to cell dose quantification used in this thesis.

<table>
<thead>
<tr>
<th>Paper</th>
<th>Study design in relation to cell dose quantification</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Cell dose was quantified using one exposure concentration of the five concentrations used in the study. Cell dose was studied from a different set of cell cultures than the toxicological responses (with the same nominal dose).</td>
</tr>
<tr>
<td>II</td>
<td>Cell doses were quantified from a different set of cell cultures than the toxicological responses, but were exposed under similar conditions.</td>
</tr>
<tr>
<td>III</td>
<td>Cell doses were quantified from parallel cell cultures that were exposed during the same experiment as the cells from which (a part of the) toxicological responses were measured.</td>
</tr>
<tr>
<td>IV</td>
<td>Cell doses were quantified from the same cell cultures as the toxicological responses.</td>
</tr>
</tbody>
</table>
Conclusions and future perspectives

From **paper I** it was concluded that the micron-sized Ni-m1 was both intrinsically more reactive and toxic in A549 cells than the nano-sized Ni-n. Therefore, opposing the general assumptions, Ni-NPs are not more toxic *per se* than micron-sized Ni particles. Additionally, although not confirmed in this thesis, it appears that the characteristics of the surface oxide of Ni-containing particles have a strong impact on their toxicity and reactivity. An important conclusion from **paper I** was that each of the studied Ni-containing particles induced cell proliferation at low concentrations. However, this study was conducted with submerged cell cultures and therefore, studying the proliferative effects in ALI could give results that can be more easily extrapolated and compared to *in vivo* effects.

**Paper II** concluded the electrostatic ALI system to be suitable for conducting toxicological studies on airborne nanoparticles. Therefore, the system was further used in **paper III** to study the genotoxic effects of airborne Ni-NPs. The main conclusion from **paper III**, as well as from **paper IV**, was that the rigorous control of nanoparticle dose is a prerequisite for toxicological studies of high quality. Furthermore, it was concluded that the cell-free conditions should not be used for estimating the cell doses.

Compared to submerged *in vitro* methods (**paper I** and **paper IV**), the ALI exposure systems (**paper II-IV**) are considered to more closely resemble the human lung environment. Additionally, the ALI systems, to a higher extent, preserve the inherent particle properties. This makes it easier to compare the physico-chemical characteristics and effects of different particles.

In conclusion, based on the knowledge gained in this thesis, the following aspects are suggested to be important to consider in future nanotoxicology research within pulmonary exposure scenarios:

1. Despite the technical challenges, ALI exposure should be prioritized over submerged exposure methods in order to obtain more representative and comparable results of the responses between nanoparticles and lung cells.
2. In order to increase the biological relevance of the ALI methods, more genuine cell culture systems should be applied. Instead of using transformed cell lines, such as A549, normal human lung cells could constitute cell cultures that have better preserved the characteristics of the lung tissue. Even more so, co-culture systems consisting of alveolar type I and type II cells together with macrophages would allow the incorporation of important cell-cell interactions.

3. Due to the immense amount of different nanoparticles in different sizes with different surface coatings, the testing of each combination of these, and several other, physico-chemical characteristics of nanoparticles is not feasible. Therefore, focus should be placed on creating read-across platforms and structure activity relationships for nanoparticles to allow prioritization of toxicological testing.
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