Exposure of zinc oxide nano-particles on lung epithelium

Julia Dobric

DIVISION OF ERGONOMICS AND AEROSOL TECHNOLOGY | DEPARTMENT OF DESIGN SCIENCES
FACULTY OF ENGINEERING LTH | LUND UNIVERSITY
2019

MASTER'S THESIS

Exposure of zinc oxide nanoparticles on lung epithelium

A comparison between submerged and air-liquid interface cultures

Published by

Department of Design Sciences
Faculty of Engineering LTH, Lund University
P.O. Box 118, SE-221 00 Lund, Sweden

Subject: Aerosol Technology (MAMM05)
Division: Ergonomics and Aerosol Technology
Supervisor: Karin Lovén
Co-supervisor: Jenny Rissler
Examiner: Anders Gudmundsson

Abstract

Zinc oxide nanoparticles (ZnO NPs) are often used in sunscreen and other cosmetic products with sun protection. They are also candidate to be used for various biological applications such as drug delivery, or in material science in for example solar cells. ZnO NPs are produced as liquid suspensions but also as dry powders, which increases the risk of exposure via the air. In this master's thesis, the effects of aerosolized ZnO NP exposure on lung epithelial cells were examined and compared to the effects of exposure to particles suspended in liquid. Immortalized human alveolar type II-like A549 cells were used as a model for the lung.

Cell cultures were cultured on insert membranes in 24- or 6-well plates, either submerged (SUB (24) and SUB (6), respectively) in growth media or at the air-liquid interface (ALI) with air on one side. The cells were exposed to suspended particles in isotonic solution (containing 9 mg/ml sodium chloride) or growth media and to aerosol particles in the NACIVT system (nano aerosol chamber for *in vitro* toxicity). ALI and SUB (24) cultures showed similar responses for the lower doses used, while SUB (24) cultures seemed more affected than ALI cultures by the higher doses. This was probably due to that larger particles were present in the isotonic solution used to expose ALI cultures. Generally, SUB (6) cultures showed greater responses than both ALI and SUB (24) cultures. Cultures exposed to aerosol particle doses comparable to the two lowest doses in exposures to particles suspended in liquid, had a significantly higher metabolic activity than the untreated negative controls 3 hours after the exposure. They also showed a lower metabolic activity 24 hours after the exposure. This indicates that aerosolized ZnO NPs affect the cellular metabolism in low doses.

Keywords: lung toxicology, nanotoxicology, aerosol, air-liquid interface, ALI, NACIVT, nanoparticles, zinc oxide

Sammanfattning

Nanopartiklar av zinkoxid (ZnO NPs) används ofta i solkräm och andra kosmetiska produkter med solskydd. De skulle även kunna användas i olika biologiska applikationer, så som läkemedelstransport i kroppen, eller inom materialvetenskap i till exempel solceller. ZnO NPs produceras som lösningar men kan även produceras i pulverform, vilket ökar risken för exponering via luften. I denna masteruppsats undersöktes och jämfördes effekterna av ZnO NPs-aerosolexponering av lungepitelceller med effekterna av exponering för partiklar i lösning. Den immortaliserade mänskliga typ II alveolcellinjen A549 användes som modell för lungorna.

Cellkulturerna växtes på insatsmembran i 24- eller 6-brunnarsplattor, antingen nedsänkta i tillväxtmedium (SUB (24) och SUB (6), respektive) eller vid det så kallade air-liquid interfacet (ALI) med luft på ena sidan. Cellerna exponerades för partiklar i en isotonisk lösning (som innehöll 9 mg/ml natriumklorid) eller i tillväxtmedium och för luftburna aerosolpartiklar i NACIVT-systemet (nano aerosol chamber for *in vitro* toxicity). ALI- och SUB (24)-celler visade liknande respons av exponering för de lägre doserna, medan SUB (24)-cellerna verkade mer påverkade av de högre doserna än ALI-cellerna. Detta berodde troligtvis på att partiklarna var större i den isotoniska lösningen som användes för att exponera ALI-cellerna. Generellt verkade SUB (6)-cellerna visa större effekter än både ALI- och SUB (24)-celler. De celler som blev exponerade för aerosolpartiklarna, med doser som var jämförbara med de två lägsta doserna i exponeringarna med partiklar i vätska, visade signifikant högre metabolisk aktivitet jämfört med de negativa kontrollcellerna 3 timmar efter exponeringen. De visade också en lägre metabolisk aktivitet 24 timmar efter exponeringen. Detta indikerar att de aerosoliserade ZnO NPs påverkar den cellulära metabolismen i låga doser.

Nyckelord: lungtoxikologi, nanotoxikologi, aerosol, air-liquid interface, ALI, NACIVT, nanopartiklar, zinkoxid

Acknowledgments

This master's thesis was performed during the spring of 2019 at the department of Design Sciences, division of Ergonomics and Aerosol Technology at the Faculty of Engineering, Lund University as a part of the program Engineering Nanoscience - Nanobiomedicine.

The master's thesis has been a part of a research project, funded by AFA Insurance, concerning toxicological effects from exposure to particles present in different occupational environments. Some of the results obtained within this thesis will be included in a manuscript to be enclosed in Karin Lovén's PhD thesis.

Without the help from some other persons, this master's thesis would not have been possible. Thank you to:

Karin Lovén, for being my supervisor and for all your time, help and guidance in and outside the lab during the whole process.

Jenny Rissler, for being my co-supervisor and for valuable input, advises and discussions.

Christina Isaxon, for letting me work in your project.

Deniz Bölükbas, Lung Bioengineering and Regeneration, for your time in the lab, help with the cells and discussions about the results.

Darcy Wagner and the entire Lung Bioengineering and Regeneration Lab at Lund University for letting me work in your lab.

Tommy Cedervall, Biochemistry and Structural Biology, for help with the DLS-measurements.

The aerosol group, for inspiration and help whenever needed. A special thanks to *Jonas Jakobsson*, for preparing the cell lab for the experiments.

Lund, June 2019

Julia Dobric

Table of contents

List of acronyms and abbreviations

A549	immortalized human alveolar type II-like cell line
ALI	air-liquid interface
APS	aerodynamic particle sizer
BSI	British Standard Institute
CPC	condensation particle counter
DLS	dynamic light scatter
DMA	differential mobility analyser
DMEM	Dulbecco's modified Eagle's medium
DMSAE	Dutch Minister of Social Affairs and Employment
F12	Ham's nutrient mixture F12
FBS	foetal bovine serum
IFA	German Social Accident Insurance
IL-8 (6)	interleukin 8 (6)
LDH	lactate dehydrogenase
LDH assay	assay to detect lactate dehydrogenase in cell culture media
mRNA	messenger ribonucleic acid
NACIVT	nano aerosol chamber for *in vitro* toxicity
NaCl	sodium chloride
NPs	nanoparticles
SA:*V*	surface area to volume ratio
SD	standard deviation
SMPS	scanning mobility particle sizer
SUB (6/24)	submerged condition for 6- or 24-well plates
Zn	zinc
ZnO	zinc oxide

$ZnSO_4$	zinc sulphate
v/v	volume to volume ratio
WEL	work exposure limit of coarse material
WST-1	water soluble tetrazolium salt 1
WST-1 assay	cellular assay to evaluate metabolic activity in cell cultures
wt %	weight percent

1 Introduction

Nanoparticles (NPs) have always been a part of the environment. They are produced in natural processes such as forest fires and volcanic eruptions. Human inventions, like engines and power plants, also release NPs to the environment. Nowadays, NPs are intentionally integrated into many products used daily in our lives, for example sunscreen and cosmetic products, electronics and fabrics in our clothes [1]. Also, based on the qualities of NPs, nanomedicines are being developed to get drugs with increased bioavailability as well as higher metabolic stability [2]. With integration into many products, the availability of and exposure to NPs increases and questions about safety arise. To answer these questions, systems to evaluate the effects of the particles have to be developed and tested.

One of the routes for unintentional exposure of NPs is via the lungs. NPs can reach the alveolar parts of the lungs, where the gas-exchange to oxygenize the blood takes place. Epithelial cells in the alveoli are in contact with the outside air and live in an air-liquid interface (ALI). When evaluating toxicity of airborne particles, classical submerged exposures with particles suspended in liquid might not be enough since the cellular environments are different and nanoparticles have different properties in liquid and in air. As a consequence, results gathered from such tests might not reflect the reality of cells exposed to airborne particles. Therefore, it is important to find and use new ways of exposing cell cultures, to test and compare them with conventional tests.

One type of NP that poses a possible risk of human exposure via the lungs is zinc oxide (ZnO) NPs, which is used in for example sunscreen. These NPs can be produced as powders, which makes employees working with the production and packaging vulnerable to unintentional occupational exposure via the air they are breathing in [3].

1.1 Objectives

In this master's thesis, the cellular responses to ZnO NPs were studied in submerged and ALI cultures. The cultures were exposed to particles suspended in liquid on classical cell culture plates with inserts and aerosol particles in the NACIVT system (nano aerosol chamber for *in vitro* toxicity). In total, eleven doses were used to expose the immortalized human alveolar type II-like cell line

A549 to particles suspended in liquid. These were estimated from limit values for coarse ZnO in Sweden [4], from suggested limit values for ZnO NPs in three European countries [5-7] with parameters for the NACIVT system. The lowest ZnO NP doses found to induce pro-inflammatory and cytotoxic responses in previous studies [8-10] were also used. Three doses were used in the aerosol exposures and the responses from those were compared. The number of workdays needed to work in an environment with one of the three limit values to reach the dose in the aerosol exposures was estimated to conclude if the doses were realistic.

There are many interesting aspects of this project whereof some were addressed in this master's thesis. First, both submerged (SUB) and ALI cultures were exposed to ZnO NPs to see whether the ALI state affected the cells notably. Different well sizes were used to compare the responses from cell cultures grown on a small surface to those grown on a larger surface. The effects of the exposures with ZnO NPs suspended in liquid were compared to the exposures with aerosol ZnO NPs in the NACIVT system. These comparisons were made to see if there were any differences in the cellular responses and if the cell cultures were more sensitive to ZnO NPs suspended in liquid or to aerosol ZnO NPs.

2 Background

2.1 Nanoparticles

Man-made nanoparticles (NPs) are becoming more and more common today. These can be classified in two different groups; unintentional and intentional. Unintentional NPs does not have a certain purpose, whilst intentional or engineered NPs are designed to fit a certain need. With the advances in material- and nanotechnology, engineered NPs has become more common in the last decades. Nowadays NPs can be found in many consumer products used daily in our lives [1]. This makes it necessary to evaluate and rule out toxicity of the particles for both consumers and producers.

According to the ISO-standard for nanotechnology vocabulary from 2015 [11], a nanoparticle is a nano-object with the length, height and width in the scale from 1 nm to 100 nm, with the longest dimension no more than three times larger than the shortest. This means that spherical particles, with a diameter between 1 nm and 100 nm, are nanoparticles, whereas fibre-like objects with a length of more than three times the diameter are not. Nanoparticles can also be referred to as ultrafine particles.

2.1.1 **The properties of nanoparticles affect their toxicity**

NPs have some special qualities often leading to different effects in biological systems compared to bulk materials. Some characteristics that influence the behaviour of NPs in biological systems are their size, shape, surface charge and specific surface area [12], which is the total surface area per mass or volume of a material.

The relevance of NP size has been demonstrated in many studies [13-15]. These suggest that smaller particles generally induce greater responses. Many NP shapes have also been tested to see if any differences in response occur. In one previous study, Hsiao and Huang [14] investigated the size and shape dependent toxicity of zinc oxide (ZnO) NPs. In their experiments, agglomerated spherical and rod-like primary NPs were tested. They concluded that both the size and the shape of the primary NPs affect the cellular response. Interestingly, exposure to rod-like NPs resulted in more interleukin 8 (IL-8) release and a higher cytotoxicity compared to

spherical NPs. In a similar experiment, Heng *et al.* [16] tested spherical and sheet-shaped NPs with approximately the same specific surface area. Their results suggested that the spherical NPs were more toxic. Baek *et al.* [15] tested the influence of the size and surface charge of ZnO NPs on cellular toxicity. They used positively and negatively charged particles with a primary diameter of 20 nm and 70 nm and found that the small, positively charged particles had a greater influence on cytotoxicity and reactive oxygen species generation. This observation was probably due to the higher cellular uptake of these particles [17].

For large objects, most of the material content is in the bulk and only a small fraction make up the surface. For NPs, most of the material instead make up the surface giving them a large surface area to volume ratio, *SA*:*V*, compared to other particles or materials. Also, with the same mass concentration, NPs have a larger total surface area, *SA*, than larger particles (Figure 2.1).

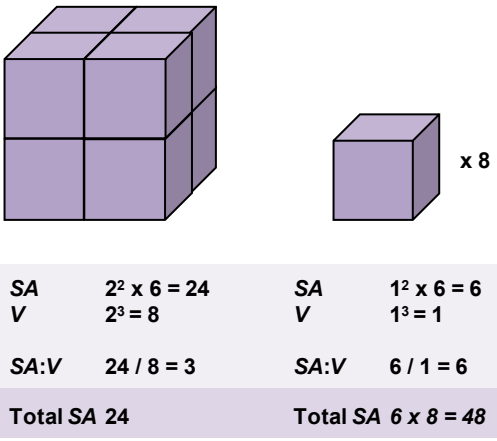

Figure 2.1. A large object (left) has a larger surface area, *SA*, and volume, *V*, than a smaller object (right), but the surface area to volume ratio, *SA:V*, is smaller for the large object. At the same mass concentration, a smaller object has a larger total surface area, total *SA*.

Results from some studies have shown that the surface area of the tested NPs plays an important role for pro-inflammatory [14, 18, 19] and inflammatory [20, 21] responses in cells and the body. For example, in one study the instilled surface area of particles was directly proportional to inflammation in the lung, measured by the amount of granulocytes in broncho-alveolar lavage fluid [20]. Two other studies showed the same relationship of particle surface area to IL-8 release [18] and viability [14] in A549 cells. This supports that a high response evoked by NPs is partly due to their large surface area [1]. However, there have been studies where the surface area and cytotoxicity could not be correlated for certain experimental conditions [22].

2.1.2 **Different ways to report dose**

There are three different ways to report the dose for NP exposures; administered dose, deposited or delivered dose and cellular dose. The administered dose is the least specific but the most common. It specifies how much particles there are in the volume that is added to the cells. In exposure situations with NPs all particles might not reach the cells, which means there will only be a fraction of the administered particles that exert an effect on the cells. To correct for this, the deposited dose can be reported. The deposited dose is the amount of particles that actually gets in contact with the cells. It is useful in both exposures to particles suspended in liquid, where all particles might not sediment onto the cells, and aerosol exposures, where particles might be lost in the system. The deposited dose can be calculated by for example using particle size and sedimentation or by techniques that measure how much mass is deposited on a surface. Another way to report dose is to report the amount of particles that end up inside the cells and most certainly affect them, this is called the cellular dose. It is a specific way of reporting dose but it does not take into account that particles can be taken up and then transported out of the cell [8].

Administrated doses are often quantified as mass per volume liquid or air, while deposited doses can be given in mass per cell area. However, particles with the same mass concentration can have different sizes and since the size of a NP is important for its effects this expression does not say enough about the particle properties. Another way of expressing deposited dose is number of particles per cell area. This expression will separate doses with the same mass based on particle size, for example a higher particle dose will be achieved for smaller particles compared to large particles.

The surface area of a particle is one of the features believed to have a large impact on its effects. Because of this it has been suggested that the dose should be given in particle surface area per cell area (expressed as cm^2/cm^2) [1, 19]. Still, there are some things worth thinking about. In serum-free media studies have shown that the toxicity is dependent on the particle surface area [19, 22]. Particles suspended in media supplemented with serum will get a protein corona, which means that proteins attach to the particle surface. This possibly makes the surface unreactive and changes the particle properties. Also, soluble particles will have different sizes, thus different surface areas, at different time points. This will change the dose over time [8].

2.1.3 **Zinc oxide nanoparticles**

ZnO NPs can be found in products used widely by many consumers [23]. Their ability to absorb UV radiation and at the same time appear transparent on skin [3] have made them an important component of sunscreens and other cosmetic

products with sun protection. ZnO NPs are also a component in some paints [24] and has potential to be used in many future applications, for example solar cells or biological applications such as drug delivery and biological labelling [25]. ZnO NPs are manufactured as suspensions but also as dry powders, meaning that unintentional inhalation during manufacturing is a possible risk for employees working with the production and packaging [3]. In 2012, Piccinno *et al.* [24] performed a study of the worldwide production of nanomaterial (nanoparticles included). The study resulted in an estimation of the production volume to 550 tonnes ZnO/year worldwide, which was among the highest estimation of the nanomaterials investigated.

ZnO NPs are stable at neutral pH, but have been shown to dissolve in acidic pH [26]. Schaudien *et al.* [27] conducted a study in which the size of ZnO NPs, among other NPs, was measured before and after internalization into different cell types. The size of the ZnO NPs was significantly lower after 24 h exposure, indicating that the particles were dissolved inside the cells.

As for other NPs, the size of ZnO NPs influence their uptake [17] and toxicity [13, 15]. The toxicity of ZnO NPs has been thoroughly investigated. ZnO NPs have been shown to affect the cell viability [28], be cytotoxic [9, 29], induce the release of pro-inflammatory mediators [16] and up-regulate their messenger ribonucleic acid (mRNA) expression [30, 31].

Cho *et al.* [26] showed that lysosomal destabilization could be coupled to cytotoxicity after treatment with ZnO NPs and suggested that the destabilization is due to leaked zinc (Zn) ions. However, also the ZnO NP core has been found to contribute to the induced toxicity in submerged cultures [32] and extracellular Zn ions released from the particle core does not seem to affect the cytotoxicity [22]. This is supported in Mihai *et al.* [9], where exposure of submerged cultures to ZnO NPs and zinc sulphate, $ZnSO_4$, and exposure of air-liquid interface (ALI) cultures to ZnO NPs lead to approximately the same cytotoxicity but different intracellular levels of Zn ions. The highest Zn ion levels were found in the $ZnSO_4$ treated cultures and the lowest in the ALI cultures treated with ZnO NPs. By comparing the location of the Zn ions the authors suggest that the toxicity of ZnO NPs is induced by a low local release of Zn ions from ZnO NPs in the endosomes and lysosomes. Recently, Wang *et al.* [13] demonstrated that ZnO NPs could cause lysosomal dysfunction, which further confirms earlier results. In their review, Liu *et al.* [33] stresses that the exposure route, cell lines and doses or concentrations used can affect the outcome of whether it is the Zn ions or the ZnO NPs that plays the largest role in inducing cytotoxicity and other cellular responses.

2.1.4 **Exposure limits and regulations**

The integration of nanoparticles into products in many cases makes them better; materials become stronger, fabrics can become water-resistant and the sunscreens absorbs the UV-light more efficient. But it may also pose a larger risk for employees working with the production of the particles and products, for the environment in the case of pollution from production and possibly also for the consumer when using the product. With the integration into a lot of products, the safety of these particles has to be considered.

In Sweden (2019) no occupational exposure limits exist for nanoparticles. Since the size [14, 20], surface area [18-20, 34] and shape [14, 16] of the particles have been shown to influence the effects, the exposure limit values for coarse materials might not be enough for nanoparticles. Some countries have tried to address this and have proposed exposure limits in mass [5] and in number of particles [5-7] based on the NP material. With more information on the toxicity of NPs consisting of different materials, hopefully these limit values can be adjusted to safe values for both toxic and non-toxic particles.

Even though no rules on exposure limit values have been set yet, new regulations regarding the NP content in products are soon to come. In 2018, the European Commission decided to revise the REACH information requirements to also apply to nanomaterial. This means that in January 2020, all companies intentionally producing nanomaterial have to register known effects of these to the authorities [35]. In Sweden, companies producing chemical products containing NPs are since February 2019 obliged to report their function, particle size and surface properties among others to the product registry [36].

2.2 The respiratory system

The respiratory system includes the airways, the lungs and the muscles that facilitate respiration. The airways have a conducting part, which leads the air to the respiratory zone where carbon dioxide is exchanged for oxygen. The lungs have several different functions; they regulate the blood pH, defend the body against pathogens, filter out some substances from the blood and produce others that may reach the entire body. As commonly known, they also handle the gas exchange between inhaled air and the blood, which takes place in the alveoli [37].

2.2.1 **The alveoli**

The gas exchange occurs at the blood/gas barrier in the alveolar walls. Here, endothelial cells, in the capillaries, are in close contact with alveolar epithelial

type I cells. Due to that the basement membrane of the two cell types are fused, the alveolar walls are very thin so the gases can easily diffuse over them. The alveolar epithelial cells have direct contact with the inhaled air on one side [37]. Most of the surface in the alveoli are lined with thin type I alveolar cells. In between the type I cells there are type II alveolar cells, which are thicker. These cells produce surfactants that are important for the surface tension and stability of the alveoli [38]. Furthermore, alveolar macrophages reside in the alveolar lumen and play an important role in clearing the alveoli from particles and pathogens entering via the inhaled air [39]. Thus, the epithelial cells and macrophages are among the first cells to come in contact with particles in the inhaled air.

The alveoli are not static structures but instead very dynamic. During ventilation the alveoli are stretched and expanded, meaning that these cells live in a very dynamic environment with mechanical forces constantly present. As concluded in Doryab *et al.* (in review) [40] mechanical forces play a key role in for example lung cell proliferation and metabolism.

2.2.2 **Particle deposition**

Particles in the inhaled air can deposit in different parts of the respiratory tract and by different mechanisms. Generally, particles larger than about 300-400 nm mainly deposit in the head airways while NPs between 10 nm and 100 nm deposit in the alveoli and below 10 nm deposit in the trachea-bronchial region and head airways (Figure 2.2).

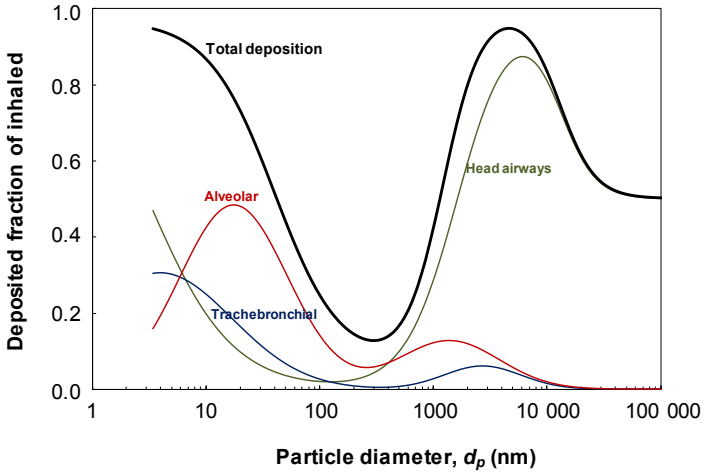

Figure 2.2. Deposition efficiency for differently sized particles in three regions of the respiratory tract. The curves are generated for a sedentary person from values suggested in ICRP publication 66 [41].

Spherical particles deposit in the lungs by three primary mechanisms; settling, inertial impaction and diffusion (Figure 2.3). Large particles settle due to gravitational forces and if they reach the lung surface they deposit there. Large particles are also affected by inertial impaction; they do not follow the airstream when it changes direction, but instead continue in the original direction due to their inertia. If they hit a surface they deposit there. Diffusional deposition means that the particles diffuse in the airstream and eventually reach a surface to deposit on [33]. This often applies to NPs deposited in the alveoli [42, 43]. Larger particles may also deposit in the alveoli, but then by sedimentation. Although the particle characteristics to some extent determine the deposition mechanism, the respiratory breathing pattern also plays an important role [42], this is however not discussed here.

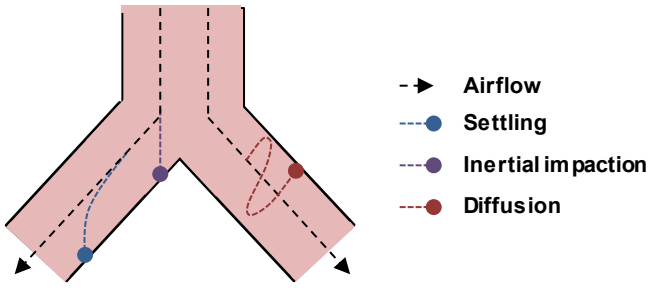

Figure 2.3. The three main mechanisms for particle deposition in the respiratory tract.

The efficiency of diffusional deposition depends on the diffusion coefficient, D_C, and the residence time, τ, in the lung [44]. The diffusion coefficient is inversely proportional to the particle diameter, as shown in Equation 2.1. Consequently, it is higher for particles with a smaller diameter.

$$D_C = \frac{kTC}{3\pi\eta d_p} \tag{2.1}$$

where k is the Boltzmann constant, T is the temperature, η is the gas viscosity, d_p is the particle diameter and C is the slip correction factor [45]. The residence time increases further down in the lung, where the flow velocity decreases. Thus, the alveoli have a long residence time [44] and the diffusional deposition efficiency, DE, there is high (Equation 2.2).

$$DE \propto D_C \propto \frac{1}{d_p}$$
$$DE \propto \tau \tag{2.2}$$

2.2.3 **Clearing mechanisms**

To keep particles out of the lungs, the airways have developed clearing mechanisms, some of these are: transportation into blood, the mucous escalator, macrophages and clearance by the lymphatic system [8]. Specialized cells in the upper parts of the airways produce and secrete mucus that trap particles from the inhaled air. The mucous is then transported out of the airways to the throat by the mucous escalator. The concept of the escalator arises from epithelial cells with cilia, which beats and moves the mucous up the airways [37]. Another feature that keeps the lungs healthy are macrophages, which can be found in the alveoli as well as other parts of the lungs. The alveolar macrophages are responsible for the main clearing of particles deposited in the alveoli. By engulfing microbes and particles and alarming other parts of the immune system, macrophages have a key role in the defence and are essential for inhibiting pulmonary infection [39].

2.2.4 **Inflammatory responses**

Organisms or foreign objects entering the body will first come in contact with the epithelial cells and some innate immune cells. If these first barriers are not enough to clear objects from the body, these cells will start to recruit help. To attract more immune cells they release chemokines, proteins that lead these cells to the infected area. One chemokine recruiting help is IL-8 (also known as CXCL8). Other proteins, such as interleukin 6 (IL-6), can be released for a more local communication between cells that are already close to each other. These proteins are involved in for example activation of immune cells.

Several cell types including macrophages, endothelial- and epithelial cells release IL-8. Whilst in the blood, IL-8 will attract and mobilize immune cells, for example T-cells and neutrophils, to the site of infection. This is done by up-regulating attachment proteins, integrins, on the cellular surface. Due to this, these cells can migrate through tissues to the site of infection. For the same reason, IL-8 can also promote the formation of new blood vessels in the infected tissue. IL-6 is mainly released by immune cells, such as macrophages, but can also be released by other cell types. For example, innate immune cells release it to stimulate naïve T-cell differentiation. Further, active T-cells can release it to activate B-cells for antibody production. This can lead to fever and acute-phase protein production, which can for example help the immune cells to kill invading organisms [46]. Thus, both IL-8 and IL-6 promote inflammation through recruitment and activation of the adaptive immune system.

These immune responses are necessary to keep invading organisms away and to keep the body healthy. However, there are not only organisms that can trigger them. NPs can induce similar effects when they come into contact with cells. It has for example been shown that IL-8 release increase with exposure time and dose of

ZnO NPs in ALI cultures [31]. Also, effects have been seen on mRNA expression, which was up-regulated for both IL-8 and IL-6 after ZnO NP exposure [30, 31].

2.3 Toxicity tests

Historically, *in vivo* (in life) studies, commonly referred to as animal studies, have been performed to evaluate effects of new drugs or other substances. The main advantage with these studies is that the whole organism is exposed to the substance, which means that the body's own defence systems is intact and that the exposure can reveal systemic effects. While this knowledge is of great importance for a toxicological study, there are some disadvantages when using animals in toxicity studies. First, often the study has to involve many animals in order to get significant results; this is not only an ethical but also an economic concern. Second, different organisms have different genes and express different proteins, which means that results from animal studies may not always be relevant for humans [8].

The alternatives for *in vivo* studies are *in vitro* (in glass) studies, which are performed with cells outside an organism. These are often cheaper to perform and the ethical concerns are fewer. However, also in *in vitro* tests animal based products and cells are frequently used. Another advantage of *in vitro* tests is that they are fast, which enables high-throughput screening. While *in vitro* studies often give answers to questions about how cells respond to the substance and give clues to what happens inside the cell they are presently unable to state anything about what will happen on an organ- or organism-level [8]. However, the design of the tests is often flexible and the possibilities of developing new systems that better match the *in vivo* environment and give results closer to the reality are plentiful. Thus, by modifying conventional *in vitro* tests to fit the purpose of the study, they can be used to draw more realistic conclusions about the complex reality than conventional *in vitro* tests.

2.3.1 ***In vitro*** **lung toxicity tests**

Conventional *in vitro* tests are done in submerged conditions, meaning that the cells are covered in growth media. As mentioned before, lung epithelial cells have contact with the inhaled air, making the submerged conditions non-realistic for testing lung toxicity. Despite this, most of the published studies on pulmonary toxicity by 2012 were performed in this way [47]. For these tests the cell environment should be adjusted to better reflect the reality. One way to do this is to expose lung epithelia to airborne particles instead of particles suspended in liquid. This means that the cells must have air on one side and growth media on the other to provide them with nutrients (Figure 2.4). This will reveal the ALI

present in lungs. In recent years, ALI studies have become more common and many groups have compared the classical submerged culture exposure to exposure of ALI cultures [9, 29-31, 47, 48]. Some of these have reported greater effects in ALI cultures [9, 30, 31, 48].

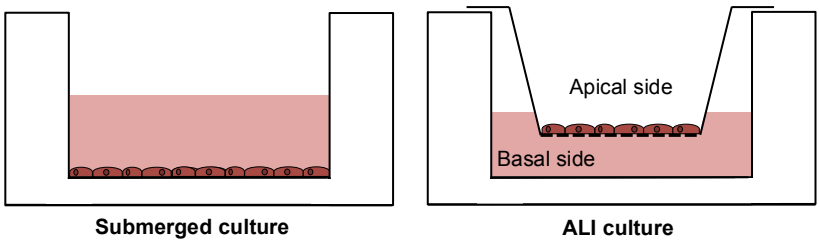

Figure 2.4. The difference between classical submerged cultures (left) and ALI cultures (right). ALI cultures get nutrients from the basal media through the porous membrane on which they grow. Growth media is shown in light red and cells in darker red.

Although ALI cultures provide a more realistic exposure situation, there are still many things to be improved. For example, there are no clearing mechanisms, such as the mucous escalator, which can hinder particles from reaching the alveoli. Also, there is no clearance of particles that reach these parts, as there would be in a real situation, in which the alveoli contain macrophages. Different parts of the lungs also contain many different cell types. For a more realistic model of the human epithelial airway barrier Raemy *et al.* [29] used a triple co-culture with epithelial cells, macrophages and dendritic cells. This model enables clearing of the particles by macrophages and crosstalk between the different cell types.

Another important aspect to consider, as is discussed in Doryab *et al.* (in review) [40], is the membrane the cells are cultured on. For ALI cultures, static two-dimensional membranes are commonly used. However, to resemble the *in vivo* environment the membranes should for example be elastic and stretchable, contain small pores (< 2.5 nm) and not be too thick. With these types of membranes cells would have access to nutrients through the pores. Also, ventilation could be simulated by stretching the membranes, which would enable an *in vivo*-like environment.

2.3.1.1 The NACIVT system

One system developed to improve the testing of lung-based models is the nano aerosol chamber for *in-vitro* toxicity (NACIVT) [49-51]. In this system, ALI cells are cultured and aerosol particles are deposited from a perpendicular flow (Figure 2.5). Prior to the exposure, the particles are charged and the deposition is aided by an electric field, this makes the deposition efficiency of the particles high. The developers have concluded that the electric field alone does not affect the cells in the wells [51]. The NACIVT system has previously been used with polystyrene latex [51], carbon [52], silver [52, 53], palladium and copper [53] particles to expose different cell cultures.

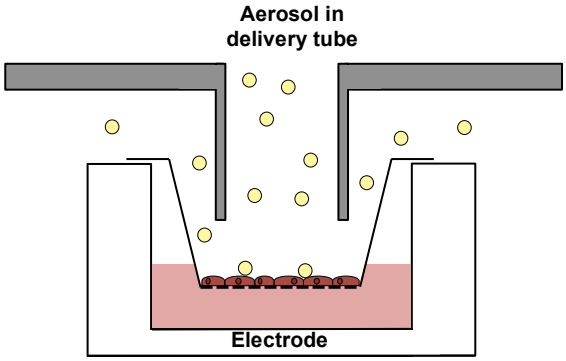

Figure 2.5. A schematic image of one well inside the NACIVT system. The aerosol particles (yellow) are positively charged and deposited onto the cell culture at ALI by electrostatic deposition. Growth media is shown in light red and cells in darker red.

Since not all aerosol particles are deposited onto the cell cultures, the deposition of the particles and deposition efficiency of the system have been examined. It has been shown that deposition of particles decreases with increasing distance from the centre of the inserts and that smaller particles have higher deposition efficiency than larger. This is seen in experimentally determined deposition efficiencies in Jeannet *et al*. [51], where they used 200 nm polystyrene latex and 20 nm silver NPs and found that the small particles had more than twice as high deposition efficiency. Also, Svensson *et al.* [53] determined a three well average deposition efficiency, which was in correspondence to the other values, for polydisperse spherical silver nanoparticles. The experimentally determined values are shown in Table 2.1. The deposition efficiency has also been determined theoretically for particles with more sizes (Figure 2.6) [51].

Table 2.1. Experimentally determined deposition efficiencies for different particles and particle sizes in the NACIVT system.

Particles	*Size*	*Deposition efficiency*	*Reference*
Silver	20 nm	40 %	[51]
Polystyrene latex	200 nm	15 %	[51]
Silver	20-80 nm	36 %	[53]

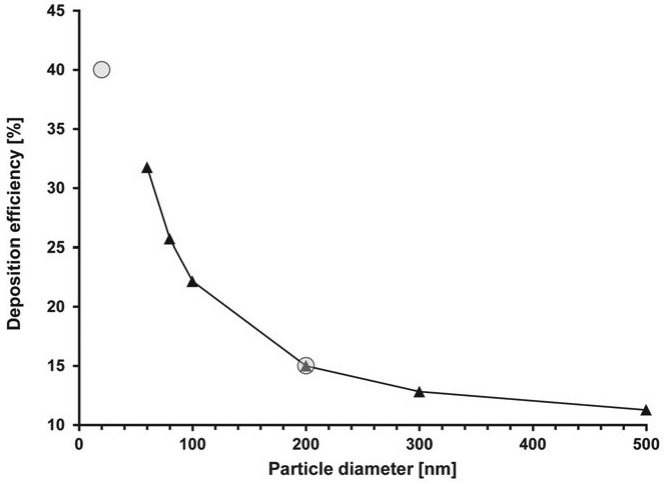

Figure 2.6. Theoretically (triangles) and experimentally (circles) determined values for the deposition efficiency in the NACIVT system. Figure from Jeannet *et al.* [51].

2.3.2 Challenges when using ZnO NPs *in vitro*

When performing *in vitro* tests with NPs, there are a lot of aspects to consider that may affect the cellular responses and results of the study. These include the properties of the suspension media and the interference of the NPs with cellular assays.

2.3.2.1 Effects from the suspension media

The particle concentration strongly affects the aggregation as shown in Zhou and Keller [54] who observed a faster aggregation in suspensions containing a higher ZnO NP concentration. This means that when testing different particle concentrations, the aggregate sizes may vary as well. Additionally, particles in liquid suspensions are highly affected by the surrounding liquid. Properties such as pH [55] and ionic strength (amount of salt) [55-57] of the liquid have been shown to affect the aggregation of different NPs. When the pH changes it affects the NP surface electrical charge density, and if the electrical charge density is zero, the repulsion forces between the NPs disappear and they start to aggregate. In the same way, ions in the solution makes the NPs form aggregates, which have shown to become larger in an ion concentration dependent manner [55].

A common liquid used when testing NPs *in vitro* is culture media. To supply nutrients to the cells, the culture media is often supplemented with serum proteins. These proteins can adhere to the NP surface making the interactions between the NPs smaller and the NPs less prone to aggregate. Hsiao and Huang [22] performed a study with ZnO NPs, comparing the results in media with and without foetal bovine serum (FBS). They found that NPs dispersed in serum-free media formed

larger aggregates than NPs dispersed in FBS-containing media. FBS-containing media also made the suspensions more stable. Studies on NP toxicity have been performed with both serum-free media [19, 30, 48] and media containing FBS [9, 15, 32, 47]. A consequence of using serum-free media is that the cells will not get the nutrients and proteins they need and will thus not grow as they usually do. The lack of nutrients can also make the cells stressed and more prone to take up particles, which will have an effect on the resulting toxicity of the particles [22].

It is not only the particle aggregation that changes based on the properties of the liquid they are suspended in. Hsiao and Huang [22] also investigated other effects such as ion release and sedimentation rate (Table 2.2) in three differently composed culture media. In addition to that NPs aggregate more in serum-free media, they found that the ion release is lower in such media and that the sedimentation rate is faster. All together, many different things affect the NP properties in suspension. The properties of the NPs will in turn affect the cell cultures studied, which means that it is important to consider these aspects and keep them in mind when the results are analysed.

Table 2.2. Differences in ZnO NP behaviour and effects in media with and without serum. The table is from Hsiao and Huang [22].

	DMEM Serum-free	***DMEM 5 % FBS***	***DMEM 10 % FBS***
Secondary size	Large	Small	Small
Sedimentation rate	Fast	Slow	Slow
Zn ion release	Low	-	High
Protein protection	No	Yes	Yes
Cell growth rate	Slow	Medium	Fast
Resultant toxicity	High	Medium	Low

DMEM: Dulbecco's Modified Eagle's Medium
FBS: foetal bovine serum
-: not determined

2.3.2.2 Interference with cellular assays

Another factor is the interference of NPs with cellular assays. NPs have been shown to interfere with cytotoxicity assays in a concentration-, particle-, and assay-specific manner [58].

One assay that has been tested is the LDH assay [58, 59], which measures the level of the enzyme lactate dehydrogenase (LDH) in the media of exposed cell cultures. When the cell membrane is damaged, LDH leaks out of the cell and into the media. Thus, LDH in the media is an indicator of cytotoxicity. Kroll *et al.* [58] showed that samples containing ZnO NPs showed lower LDH activity compared to control and Da Silva *et al.* [59] reported that absorbance readings of LDH mixed with different concentrations of ZnO NPs gave different results regarding the LDH concentration in the samples. This indicates that ZnO NPs affect the readout of LDH levels in a concentration-dependent manner.

Another assay that is used in evaluating possible effects after exposure to different substances is the WST-1 assay. The metabolic activity in the cell cultures is proportional to the colour change of the WST-1 reagent. Guadagnini *et al.* [60] tested interference of several different NPs with the WST-1 assay and found that for some NPs the cellular effects could be underestimated if the NPs were present in the samples. Wilhelmi *et al.* [61] found that ZnO NPs had minor effects on the measured absorbance in the range of 350 nm to 650 nm. These were slightly larger for the shorter wavelengths. When the WST-1 assay was tested, no artefacts in the results could be seen in cell free-samples. However, it could be a good idea to eliminate NPs in the samples before reading the results from the WST-1 assay.

Together, this shows that care should be taken when designing experiments with NP exposures as well as when drawing conclusions based on results from *in vitro* assays. Preferably, the behaviour of the tested NPs in solution should be assessed, assays should be tested for interactions and results validated with other assays if necessary.

3 Methodology

Four different exposure conditions, shown in Figure 3.1 were used and the responses from the cell cultures compared. Submerged (SUB) cultures were exposed to ZnO NPs suspended in growth media, ALI cultures were exposed to ZnO NPs suspended in a sodium chloride (NaCl) solution (9 mg/ml), referred to as isotonic solution, and NACIVT cultures in the NACIVT system were exposed to aerosolized ZnO NPs. SUB cultures were grown in two different plate formats, 6-well plates, SUB (6), and 24-well plates, SUB (24), with a growth area of 4.67 cm^2 and 0.33 cm^2 respectively. This was done to investigate if there was a difference in response due to the size of the wells the cells were grown in. ALI and NACIVT cultures were grown in 24-well plates.

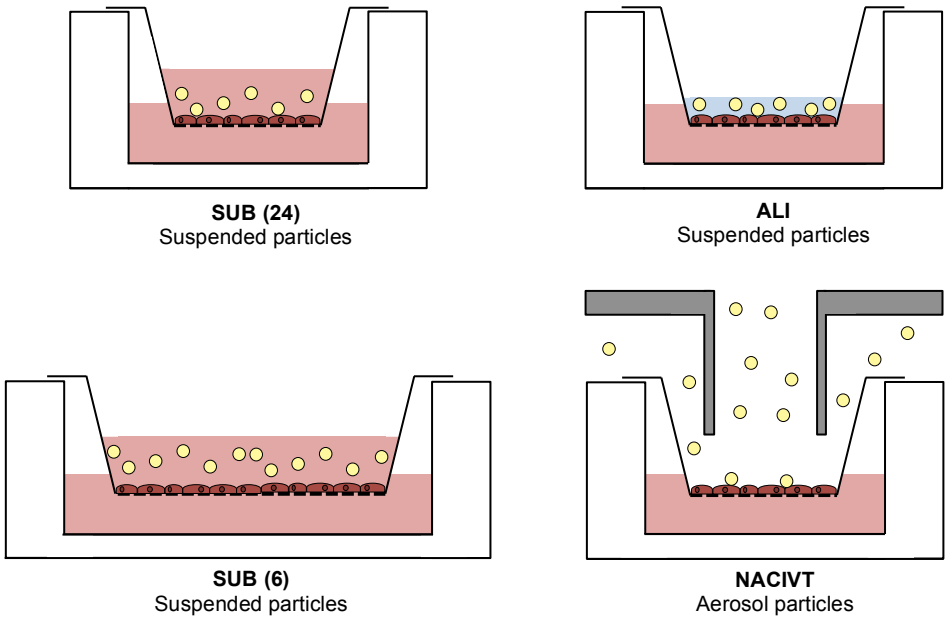

Figure 3.1. Schematic figures of the four different exposure conditions. The drawings show one well with an insert on which the cells (dark red) grow. Cells were exposed to ZnO NPs (yellow) on the apical side in three different ways; in growth media (light red), in isotonic solution (light blue) or through the air.

The four-day experiments involved different procedures, all further described in the following sections. A summary of an experiment, with particles suspended in

liquid and aerosol particles, is shown in Table 3.1. For each exposure condition, three separate experiments were performed.

Table 3.1. Summary of what was done each day in an experiment with either SUB and ALI cultures exposed to particles suspended in liquid or NACIVT cultures exposed to aerosol particles.

	Experimental procedure	
	Particles suspended in liquid *SUB (24), ALI, SUB (6)*	***Aerosol particles*** *NACIVT*
Day 1	Cells are seeded in 24- and 6-well plates	Cells are seeded in 24-well plates
Day 2	Apical media in the wells on the ALI plate is removed	Apical media in the wells is removed
Day 3	Cells are exposed to ZnO NPs	Cells are exposed to ZnO NPs Triton X-100 is added as a positive control Basal media is collected (1 h and 3 h) for LDH assay WST-1 assay is performed
Day 4	Triton X-100 is added as positive control Basal media is collected (24 h) for LDH assay WST-1 and LDH assays are performed	Triton X-100 is added as a positive control Basal media is collected (24 h) for LDH assay WST-1 and LDH assays are performed

3.1 Doses

A total of eleven doses were used in experiments with particles suspended in liquid and three with aerosol particles, all are shown in section 4.2. Some of the doses used in exposures to ZnO NPs suspended in liquid were based on the existing limit value for coarse ZnO in Sweden [4], from suggested limit values for ZnO NPs from the German Social Accident Insurance (IFA) [6], the Dutch Minister of Social Affairs and Employment (DMSAE) [7] and British Standard Institute (BSI) [5] calculated with parameters from the NACIVT system. Some doses were also based on previous studies where the lowest dose of ZnO NPs to induce pro-inflammatory or cytotoxic responses has been reported [8-10]. The origin of these doses can be found in Table 3.2.

Table 3.2. Origin of some of the doses that were used in the experiments.

Origin	*Value*	*Reference*
Occupational exposure limit value for ZnO in Sweden	5 mg/m^3	[4]
Lowest dose for observed cytotoxicity in ALI cultures exposed to aerosolized ZnO NPs	4.13 µg/cm^2	[8, 9]
Lowest dose for significant release of pro-inflammatory mediators in ALI cultures exposed to aerosolized ZnO NPs	1.0 µg/cm^2	[8, 10]
Proposed exposure limit value for ZnO NPs (BSI)	0.066 · WEL 0.33 mg/m^3 (for WEL in Sweden)	[5]
Proposed exposure limit value for ZnO NPs (IFA, DMSAE)	$4 \cdot 10^{10}$ particles/m^3	[6, 7]

WEL: work exposure limit of coarse material

3.1.1 **Dose calculations**

Doses corresponding to the limit values listed in Table 3.2 were calculated according to Equation 3.1 with parameters for the NACIVT system. This equation was also used to calculate the deposited doses in the experiments with aerosol particles.

$$D = \frac{C_M \cdot Q \cdot t \cdot DE}{A} \tag{3.1}$$

where D is the calculated mass dose (µg/cm^2), C_M is the mass concentration in air (mg/m^3), Q is the air flow, t is the exposure time, DE is the deposition efficiency and A is the area on which the cells grow. The particle concentration in air can also be expressed as number of particles per air volume, C_N. Equation 3.2 can be used to calculate this from C_M.

$$C_N = \frac{C_M \cdot 10^{-9}}{\rho \cdot v} \tag{3.2}$$

where C_N is the particle concentration in air (cm^{-3}), ρ is the density of ZnO (5.61 g/cm^3) and v is the volume of one primary particle (cm^3).

For experiments with particles suspended in liquid, the particle concentration was calculated from D according to Equation 3.3.

$$C = \frac{D \cdot A}{V} \tag{3.3}$$

where C is the concentration of particles in the liquid (µg/ml), D is the dose (µg/cm^2), A is the area on which the cells grow and V is the volume of the liquid (ml).

The doses for both particles suspended in liquid and aerosol particles were expressed in two more units, number of particles per area, D_N (cm^{-2}), and particle surface area per area, D_{SA} (cm^2/cm^2), see Equations 3.4 and 3.5 respectively.

$$D_N = \frac{C \cdot V}{\rho \cdot v \cdot A} = \frac{D}{\rho \cdot v} \tag{3.4}$$

$$D_{SA} = \frac{C \cdot V}{\rho \cdot v \cdot A} \cdot SA = D_N \cdot SA \tag{3.5}$$

where SA is the surface area for one particle.

3.2 Cell cultures

As a model for the alveoli, the immortalized alveolar type II-like cell line A549 was used. A549 cells were cultured in DMEM/F12 (Gibco™) media supplemented with 10 % v/v FBS (Sigma-Aldrich) and 1 % v/v penicillin/streptomycin (10 000 units/ml and 10 mg/ml respectively, Gibco™), from now on referred to as growth media, at 5 % CO_2, 95 % humidity and 37 °C. The cells were split (1:10) two times a week and cells used in the experiments were seeded from cultures having a passage number of maximum 16.

When confluent, the cells were seeded in Transwell® inserts (polyester membrane, pore size: 0.4 µm, pore density: $4 \cdot 10^6$, Corning®) in 6- or 24-well plates. For 6-well plates 250 000 cells were seeded in each well and for 24-well plates 10 000 cells were seeded in each well. After 20-30 min incubation, the cells had briefly attached to the membrane and fresh growth media was added below the inserts, on the basal side, in each well. Table 3.3 shows the volumes and cell densities seeded in the different plate formats.

Table 3.3. Insert surface area, cell density and growth media volumes directly after seeding for the two different plate formats used in the experiments.

	24-well plates	***6-well plates***
Basal media (ml)	1	2
Apical media (ml)	0.1	0.4
Cell density (cells/ml)	100 000	625 000
Total amount of seeded cells in one well	10 000	250 000
Insert surface area (cm^2)	0.33	4.67

24-well plates: SUB (24) and ALI with particles suspended in liquid, NACIVT with aerosol particles
6-well plates: SUB (6)

Submerged cultures, SUB (24) and SUB (6), were grown for 48 h before exposure. ALI and NACIVT cultures were grown for 24 h, and then the apical media was

removed by flipping the inserts. The cells were grown for another 24 h in ALI-state before exposure.

3.3 Exposure to particles suspended in liquid

Cell cultures in three exposure conditions were exposed to particles suspended in liquid, SUB (24), ALI and SUB (6) cultures. For each exposure condition three separate experiments were performed.

3.3.1 **Particle suspensions and doses**

ZnO NPs were bought in liquid suspension (20 wt % in H_20, < 100 nm, 0.34 g/ml, mean size: 40 nm, Sigma-Aldrich) and were diluted to different concentrations before each experiment. In the first experiment, 45 µl of the NP stock was pipetted to 5.955 ml growth media or isotonic solution (made from water produced with reversed osmosis and capacitive deionization, conductivity: 0.8 µS/cm, VWR), for a ZnO NP concentration of 2550 µg/ml. Isotonic solution is used instead of water to avoid cell bursting due to osmosis. Since aggregation of the particles was suspected in the isotonic solution based on dynamic light scattering (DLS) measurements (data not shown) and previous studies [54, 55, 57], the protocol was changed for the second and third experiments. In these experiments, a concentration of 2550 µg/ml was prepared in water instead of the isotonic solution.

All suspensions were bath-sonicated in an Elmasonic S30H (220-240 V and 37 kHz, Elma Schmidbauer GmbH) for 5 min and then directly further diluted to the final concentrations. To avoid aggregation, in the second and third experiments, NaCl (45 mg/ml) was added to the water suspensions in the very last step, after diluting the suspensions in water (100 µl NaCl solution to 400 µl particle suspension). For the second and third experiments, one concentration was replaced with a higher. The particle dilution series for each of the three experiments are shown in Appendix A.

3.3.1.1 Particle measurements – DLS

Size of the NPs in suspension was measured with DLS (DynaPro Plate Reader-II, WYATT Technology, Software: Dynamics v7) for particles in the liquids used in the exposures as well as water. Three stocks with 2550 µg/ml ZnO NPs were prepared in growth media, isotonic solution and water. The tubes were bath-sonicated for 5 min (Elmasonic S30H) and then diluted in growth media (Table A.4), isotonic solution (Table A.1), water and water with 45 mg NaCl/ml added in the last step to the concentrations used in the exposures (Table A.3).

DLS-measurements were done with 100 µl of each suspension in a 96-well plate. Measurements started 1 h after sonication and took just over 3 h to complete.

3.3.2 **Cell exposures**

For SUB (24) and SUB (6) cultures, the apical media was removed by flipping the inserts and each concentration of the particle suspensions was added on the apical side of the cells in two wells. The particle sedimentation in these two exposure conditions should be the same. To achieve this, 700 µl particle suspension was added to SUB (6) cultures and 50 µl to SUB (24) cultures, to get the same liquid pillar heights above the cells. For ALI cultures, 20 µl of each particle concentration was added to the apical side of the cells in two wells. Since media was observed on the apical side of ALI cultures in the second experiment, inserts with ALI cultures were also flipped before exposure in the third experiment to make sure no media was present when the particles were added. Three separate experiments were performed with each dose in duplicates.

For all exposure conditions a negative and two positive controls were included. Growth media was used as a negative control for submerged conditions (700 µl or 50 µl) and isotonic solution (20 µl) was used for ALI conditions. As positive control, Triton X-100 (1 % in growth media), a surfactant that lyse the cells, was used in the same volumes as the negative controls. Negative control was added on the exposure day and Triton X-100 was added one day after, on the analysis day, 15 to 30 min before the analysis.

3.4 Aerosol exposures

A schematic drawing of the exposure set-up can be seen in Figure 3.2, an image of the set-up and the NACIVT system are shown in Appendix B. Particles were generated with an atomizer and dried before entering a mixing volume and then continuing to either the NACIVT, to expose the cells, to the SMPS (scanning mobility particle sizer) system or to the APS (aerodynamic particle sizer) where the particle size and number concentrations were measured. The different parts of the set-up are described further in the following sections.

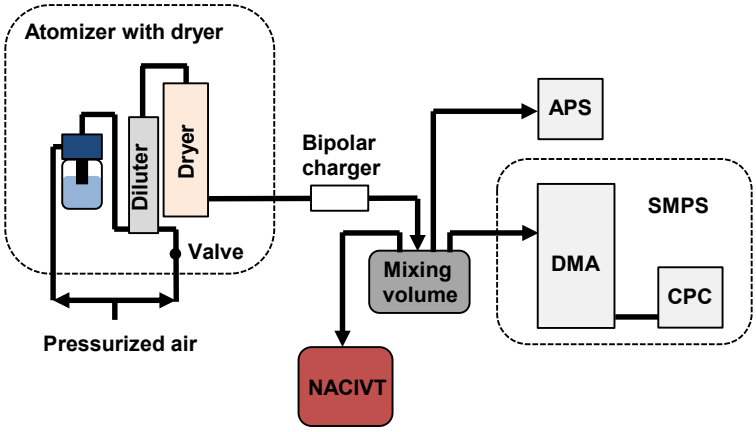

Figure 3.2. The set-up used in the experiments with aerosol particles. The ZnO NPs are generated in the atomizer and dried in the dryer. To get rid off any charges from the generation the aerosol goes through a bipolar charger. The particle size and number concentration are measured in the SMPS system and APS. Cell cultures are placed in the NACIVT exposure chamber where they are exposed to the ZnO NPs.

3.4.1 **Particle generation – Atomizer**

The atomizer (from a Condensation Aerosol Generator SLG270, TOPAS GmbH) generates particles by spraying a liquid into the air to form droplets with particles inside. These droplets are then mixed with a regulated amount of air in the dilutor and dried in the dryer to get rid of the water. The total number concentration of the aerosol could be regulated by turning a needle valve placed before the diluter in the atomizer (Figure 3.2) to dilute the generated aerosol with more or less air.

To test the generation system before the cell exposures, an experiment with different particle concentrations (0, 100, 150 and 200 µg/ml) in water was performed. A volume to get the desired concentration of ZnO NPs was added to 150 ml water (conductivity: 0.8 µS/cm, produced with reversed osmosis and capacitive deionization, VWR). The solution was bath sonicated for 5 min (Elmasonic S30H). Afterwards, it was connected to the atomizer and an aerosol was generated when pressurized air was turned on. For these experiments, the bipolar charger before the mixing volume was not present and the aerosol was only measured by the SMPS system.

Based on the results from these experiments, a particle concentration of 150 µg/ml was chosen for the cell exposures. The solution was prepared in the same way for the exposures by adding 66 µl of the stock ZnO NPs to 150 ml water.

3.4.1.1 Particle measurements – SMPS and APS

A SMPS system was built by coupling a charger and a DMA (differential mobility analyser, TSI, model 3071) with a CPC (condensation particle counter, TSI,

model 3775) in series. The DMA is a long cylinder, containing a centre rod and an outer shell as electrodes. When particles enter the SMPS, they are charged according to a bipolar charge distribution. In the DMA the particles are separated according to their electrical mobility, which is highly dependent on the size. Differently sized particles will get different electrical mobilities and trajectories in the electric field. At a given voltage over the DMA, particles of one electrical mobility reach the DMA sample extraction port whilst the others end up on the electrodes or in the excess air outlet [62]. To determine the particle size distribution, the voltage of the electric field in the DMA is changed stepwise.

From the DMA sample extraction port, the particles enter the CPC, where they are counted continuously. Vaporizing butanol condenses onto a particle creating a droplet, which is large enough for the detector to count [63]. In this way, the different sizes of the NPs are separated in the DMA and counted one by one in the CPC.

In the experiments, the sheath flow through the DMA was 6 lpm and the aerosol sampling flow was 1.5 lpm to the CPC. On these settings, the CPC counted particles with a diameter of 9.65-421.7 nm. The scan up time was 120 s and the retrace time 15 s.

An APS (TSI, model 3321) was used to estimate the particle size of particles larger than those counted by the SMPS. At the inlet of the APS, the airflow and the particles are accelerated and passed through two laser beams. The time it takes for a particle to go from one beam to the other (time-of-flight) is measured and the particle velocity is calculated. Large particles are less accelerated than smaller, thus their time-of-flight becomes longer and their velocity smaller. The velocity of the particle is then related to a particle size by a calibration curve [64].

For the APS, the sample time was 5 s. It counted particles with a diameter of 0.542-19.81 µm.

3.4.2 **The NACIVT system**

The NACIVT system was used to expose the cells with aerosol particles generated with the atomizer. It has several different parts, first the aerosol is lead through a unipolar charger, which charges the particles positively. The flow is split; one part of the flow goes through an electrometer to control the stability of the aerosol concentration, the other part, a flow of 0.6 l/min, passes through a humidifier (85 %) and heater (37 °C) before it is split into 24-subflows (0.025 l/min) that reach the exposure chamber. The humidity (85 %) and temperature (37 °C) of the aerosol are set to resemble physiological conditions and are monitored continuously. Inside the exposure chamber there are 24 wells, in which Transwell® inserts are placed. The inserts have semi-permeable membranes onto which ALI cells are grown. The system uses a perpendicular flow to deposit the

particles onto the cells. The deposition is aided by a unipolar electric field, which drags the charged particles towards the cell cultures. The electric field does not affect the cells [51].

The NACIVT was started and run for about 1 h to stabilize the temperature and humidity before the cells were placed in the exposure chamber.

3.4.3 **Cell exposures**

Inserts with cells were moved from the 24-well plates into a corresponding well in the chamber well plate. 400 µl basal growth media was pipetted from each well in the 24-well plate to the corresponding well in the chamber well plate. The chamber well plate was then put inside the NACIVT exposure chamber (Figure 3.3).

The aerosol generated with the atomizer was coupled to the NACIVT and the exposure continued for 1 h. Afterwards, filtered room air was run through the system for 5 min. The chamber well plate was then taken out of the exposure chamber and the inserts were moved back to the 24-well plate. For control cultures, filtered room air was run through the system for 1 h and 5 min. Triton X-100 was added to one control well for each time point analysed (1 h, 3 h and 24 h) 0.5-2 h before analysis. Three separate experiments were performed.

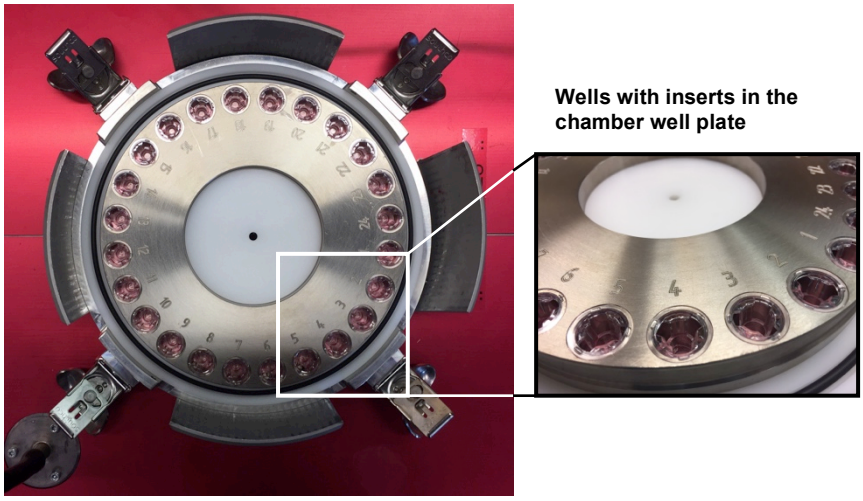

Figure 3.3. Inserts are put into the chamber well plate (silver), which is placed in the NACIVT exposure chamber. Each of the 24 wells has an aerosol tube from which the ZnO particles reach the cells.

3.5 Endpoint measurements

3.5.1 **Metabolic activity: WST-1 assay**

WST-1 is an assay that uses a tetrazolium salt, which can be reduced to a water-soluble formazan product. This reaction happens at the cell surface but is dependent on the production of NAD(P)H in the citric acid cycle [65]. Therefore the reaction only happens on metabolically active cells. The WST-1 assay was used to investigate the metabolic activity, as an indicator of cell viability, of the ZnO NP exposed cells.

The WST-1 assay (Cell proliferation reagent WST-1, Roche Diagnostics GmbH) was performed 24 h after exposure to ZnO NPs suspended in liquid and after a 1 h, 3 h and 24 h incubation after exposure to aerosolized ZnO NPs. WST-1 reagent was added to growth media in a 1:10 dilution to get WST-1 solution, as suggested by the manufacturer. For experiments with particles suspended in liquid the apical NP solution was removed before addition of WST-1 solution. For 24- and 6-wells, 110 µl or 200 µl WST-1 solution was added to each well respectively. The plates were incubated for 30 min (5 % CO_2, 37 °C) and then 100 µl of the supernatant was pipetted into a well on a 96-well plate for analysis. The absorbance in the wells was read with a plate reader (for experiments with particles suspended in liquid: PHERAstar FS, BMG Labtech, for experiments with aerosol particles: Multiskan GO, Thermo Scientific) at 420 nm, 450 nm and 480 nm. Data from 450 nm was used for analysis.

3.5.2 **Cytotoxicity: LDH assay**

LDH, lactate dehydrogenase, is an intracellular enzyme, which leak out to the growth media from cells with damaged cell membranes. This is utilized in the LDH assay, where LDH in the growth media from damaged cells is detected [66].

Basal growth media was collected 24 h after exposure to ZnO NPs suspended in liquid and after a 1 h, 3 h or 24 h incubation after exposure to aerosolized ZnO NPs. 50 µl of the basal growth media was pipetted into one well in a 96-well plate. LDH (Cytotoxicity Detection Kit Plus, Roche Diagnostics GmbH) solution was prepared by adding 1 part of the catalyst to 45 parts of the dye solution. The LDH solution was vortexed and 50 µl of it was added to each well in the 96-well plate. The plate was incubated in room temperature in the dark for 30-40 min, depending on the colour in the NP-treated wells compared to Triton X-100 treated wells, which should be darker. The absorbance in the wells was read with a plate reader (for experiments with particles suspended in liquid: PHERAstar FS, BMG Labtech, for experiments with aerosol particles: Multiskan GO, Thermo Scientific) at 490 nm and 600 nm. Data from 490 nm was used for the analysis.

3.6 Statistics

Unpaired two-tailed T-tests, for data with different variances, were performed to evaluate significant differences between control cells and cells exposed to ZnO NPs, between the different exposure conditions and between aerosol generations with different particle concentrations in the liquid. A p-value below 0.05 was assumed to show significance.

4 Results

4.1 Particle parameters

4.1.1 **Particles suspended in liquid**

Measurements of the particle size in different liquids with DLS indicated that aggregation occurred in both water and NaCl suspensions. However, since many measurements were incomplete (probably due to sedimentation during the measurement), the data could not be used as it was intended – to see variation in aggregate size in different media and at different particle concentrations. Thus, the particle aggregate size might have differed between the different exposure systems and doses. Optimally, this should have been determined by for example the DLS-measurements.

4.1.2 **Aerosol particles**

4.1.2.1 Aerosol generation without exposing cell cultures

The generated aerosol from liquid suspensions with 100 µg/ml, 150 µg/ml and 200 µg/ml had broad size distributions ranging from a few nanometres to over 100 nm. In Appendix C, the particle number concentration and mean sizes over the entire aerosol generation can be seen. A clear difference in the particle number concentration (Figure C.1.1) was observed when comparing the aerosols generated from three different particle suspensions. Both 150 µg/ml and 200 µg/ml resulted in a significantly higher number concentration compared to 100 µg/ml, no difference could be found between 200 µg/ml and 150 µg/ml. This is also indicated in Figure 4.1, which shows the size distributions of the aerosol ZnO NPs.

When comparing the number particle mean size (Figure C.1.2) between the liquid suspensions, it was shown that 200 µg/ml resulted in significantly larger particles than 100 µg/ml and 150 µg/ml. There was no significant difference between 100 µg/ml and 150 µg/ml. The average number particle mean size and the average number concentrations for each suspension are presented in Table 4.1.

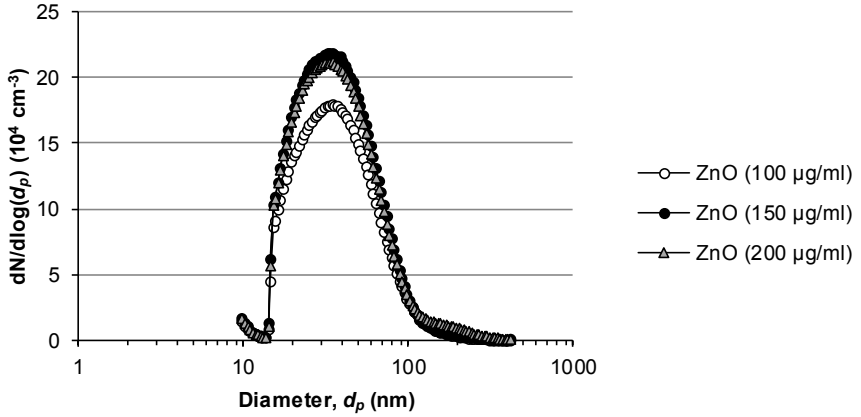

Figure 4.1. Size distribution for aerosol generated with three different concentrations of ZnO NPs in liquid suspensions. The data is from SMPS measurements and is shown as an average of all samples.

Table 4.1. Mean sizes and number concentrations of the generated ZnO NP aerosol with three different liquid suspension concentrations. Values are presented as mean ± SD.

	Number concentration (10^3 cm^{-3})	*Number particle mean size (nm)*
100 µg/ml	98.0 ± 25.6	41.4 ± 1.33
150 µg/ml	120.2 ± 11.2	41.2 ± 0.52
200 µg/ml	119.6 ± 13.7	43.7 ± 0.54

Since a high number concentration was needed and a small mean size desired, a liquid concentration of 150 µg/ml was chosen for particle generation in the exposure experiments.

4.1.2.2 Aerosol generation for cell exposures

Three experiments were performed with the same concentration of particles in the liquid suspensions (150 µg/ml). The resulting sizes and concentrations of ZnO NPs in the aerosols were measured with both an SMPS system and an APS. Size distributions for all three experiments are shown in Figure 4.2.

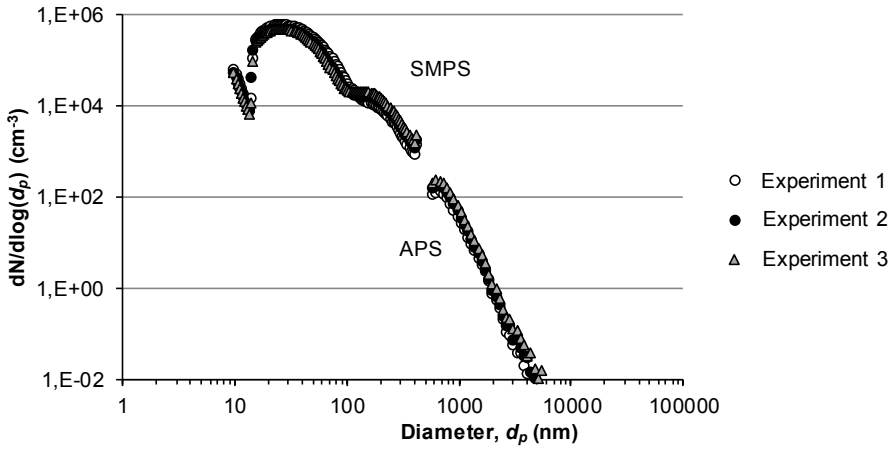

Figure 4.2. Size distributions of the ZnO NP generated aerosols, measured with an SMPS (10 nm-500 nm) and an APS (0.5 µm – 20 µm) for all three experiments.

In Appendix C, the aerosol number concentrations and the number particle mean sizes over the entire generation and exposures can be seen. The number concentration continued to rise during all experiments with a total increase of around 35 000 cm^{-3} in one hour (Figure C.2.2). The number particle mean sizes remained on about the same level for the whole exposures (Figure C.2.4).

The resulting average number and mass concentrations as well as the number particle mean sizes of ZnO NPs in the aerosol experiments are shown in Table 4.2. The mass concentrations were calculated from the number concentrations by using Equation 3.2 with all the different particle diameters. For these calculations, the particles were assumed to be spherical.

Table 4.2. The average concentrations and the number particle mean sizes for each exposure with aerosol particles in the NACIVT system. Values are calculated from the SMPS only, and values in brackets from both the SMPS system and the APS.

	Number concentration (10^3 cm^{-3})	*Mass concentration (mg/m^3)*	*Number particle mean size (nm)*
Experiment 1	322.26 [322.28]	0.17 [0.20]	35.5 ± 0.18
Experiment 2	264.42 [264.46]	0.18 [0.23]	35.5 ± 0.24
Experiment 3	254.22 [254.27]	0.22 [0.28]	36.5 ± 0.09

4.2 Dose

4.2.1 **Experiments with particles suspended in liquid**

By applying Equation 3.1 and 3.2 the doses for the existing and proposed limit values for coarse ZnO and ZnO NPs, corresponding to exposures in the NACIVT system were calculated (t = 60 min, Q = 0.025 l/min, A = 0.33 cm^2). Since no results were obtained from the DLS-measurements, the particle mean size could not be determined in the liquid suspensions. Also, the size distribution was unknown. Therefore, all particles were assumed to have the mean size reported by the supplier, which was 40 nm. Also, the ZnO NPs were assumed to be spherical. A deposition efficiency of 36 %, based on previously published data (Figure 2.6), was used for the calculations. The doses corresponding to the air concentrations of the different limit values can be seen in Table 4.3. It is important to note that these doses would not be the same if parameters for the lung had been used instead. More doses were added for a total of eleven reference doses for the experiments with particles suspended in liquid, these are shown in the reference column in Table 4.4.

Table 4.3. Doses corresponding to the existing limit value for coarse ZnO and proposed limit value for ZnO NPs, calculated with parameters for the NACIVT system. These doses, amongst others, were used in the experiments with ZnO NPs suspended in liquid.

Value	*Corresponding dose (µg/cm^2)*
5 mg/m^3	8.2
0.066 · WEL 0.33 mg/m^3 (for WEL in Sweden)	0.54
$4 \cdot 10^{10}$ particles/m^3	0.012

WEL: work exposure limit of coarse material

For experiments with particles suspended in liquid, particle concentrations in liquid corresponding to the eleven reference doses were calculated with Equation 3.3. When doing the dilution series, the volumes were rounded to the nearest integer, which changed the concentrations in the suspensions slightly. Therefore, actual doses based on the concentrations in the particle suspensions were calculated for each exposure condition, these are compared with the reference doses in Table 4.4. With the assumption that all particles sediment within 24 h, the actual doses presented are the deposited doses for exposures to ZnO NPs suspended in liquid.

In Table 4.5 the doses are expressed as the number particle and surface area dose, D_N and D_{SA} as calculated with Equation 3.4 and 3.5. The volume of the assumed spherical 40 nm ZnO NPs was $3.3 \cdot 10^{-17}$ cm^3 and the surface area was $5.0 \cdot 10^{-11}$ cm^2. It is important to note that the assumptions introduce errors in the

calculations and ideally the doses would have been calculated with a size distribution for each of the liquid suspension used.

Table 4.4. The administered and actual doses for each exposure condition in the experiments with particles suspended in liquid. For ALI cultures, some administered doses differed in experiment 1 compared to experiment 2 and 3 due to changes in the dilutions series, these doses are shown as: experiment 1 / experiment 2 and 3.

Administered dose (µg/ml)		*Reference (µg/cm³)*	*Actual dose,* **D** *(µg/cm²)*		
ALI	*SUB*		*ALI*	*SUB (24)*	*SUB (6)*
- / 1120.0	- / 450.5	*68.0*	- / 67.9	- / 68.3	- / 67.5
559.3 / 560.0	221.0	*34.0*	33.9	33.5	33.1
399.3 / 400.0	158.7	*25.0*	24.2	24.0	23.8
279.5 / 280.0	99.2	*16.4*	16.9 / 17.0	15.0	14.9
199.8 / 200.1	79.3	*12.0*	12.1	12.0	11.9
139.8 / 140.1	51.0	*8.2*	8.5	7.7	7.6
99.8 / -	39.7	*6.0*	6.1 / -	6.0 / -	6.0 / -
69.9 / 70.1	29.8	*4.1*	4.2 / 4.3	4.5	4.5
17.5 / 17.6	5.7	*1.0*	1.1	0.9	0.9
10.0	3.3	*0.54*	0.61	0.50	0.50
0.2	0.08	*0.012*	0.012	0.012	0.012

Table 4.5. The doses for each exposure condition in the experiments with particles suspended in liquid expressed as number of particles per cell area, D_N, and surface area per cell area, D_{SA}.

Number particle dose, D_N *(10^{10} cm^{-2})*			*Surface area dose,* D_{SA} *(cm²/cm²)*		
ALI	*SUB (24)*	*SUB (6)*	*ALI*	*SUB (24)*	*SUB (6)*
- / 36.67	- / 36.89	36.46	- / 18.34	- / 18.45	- / 18.23
18.31	18.10	17.88	9.16	9.05	8.94
13.07	13.00	12.86	6.54	6.50	6.43
9.13 / 9.18	8.10	8.05	4.57 / 4.59	4.05	4.03
6.54	6.48	6.43	3.27	3.24	3.22
4.59	4.16	4.11	2.30	2.08	2.06
3.29 / -	3.24 /-	3.24 / -	1.65 /-	1.62 /-	1.62 /-
2.27 / 2.32	2.43	2.43	1.14 / 1.16	1.22	1.22
0.59	0.49	0.49	0.30	0.25	0.25
0.33	0.27	0.27	0.17	0.14	0.14
0.0065	0.0065	0.0065	0.003	0.003	0.003

4.2.2 **Aerosol exposures in the NACIVT system**

Since the size distribution in the aerosol was broad and the deposition efficiency in the NACIVT system is dependent on particles size, the deposition efficiency was estimated for each size. A curve was extrapolated (Figure 4.3) from the theoretical

values estimated by Jeannet *et al.* [51] shown in Figure 2.6. From this curve, the deposited mass of particles with each size was calculated for the three experiments (Figure 4.4).

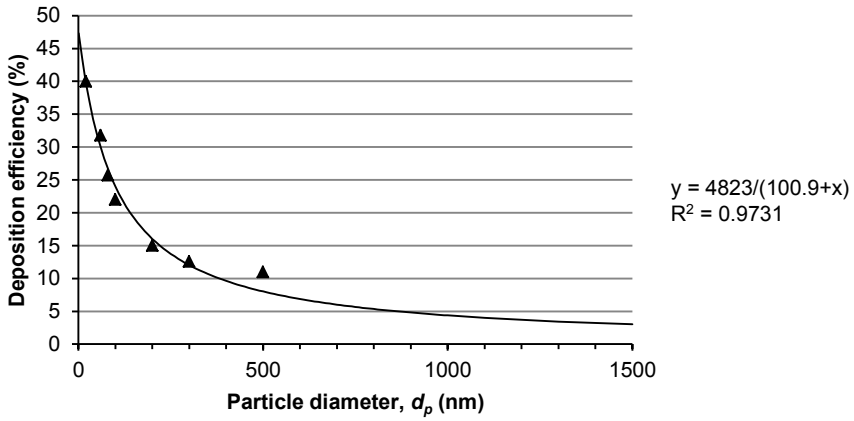

Figure 4.3. An extrapolated curve for the deposition efficiency in the NACIVT system. Extrapolated from values published in Jeannet *et al.* [51].

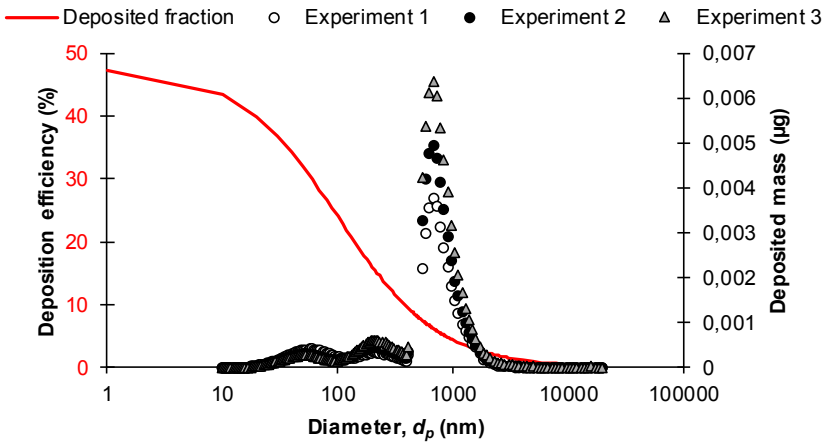

Figure 4.4. Deposition efficiency in the NACIVT (primary axis) and the resulting deposited masses for each particle size in the three experiments (secondary axis).

The deposited doses for the three experiments in the NACIVT system were calculated with Equation 3.1 and 3.2, assuming spherical particles. The deposition efficiencies in the calculations were estimated with the curve equation, for the different particle sizes. From the deposited mass doses the number particle and surface area doses were calculated with Equations 3.4 and 3.5 for each size and

then added to a total. In Table 4.6 these values are shown for SMPS data only as well as for SMPS data and APS data. By comparing the values it is obvious that particles larger than 500 nm (measured by the APS) mainly affect the mass doses.

Table 4.6. Doses for experiments with aerosol particles in the NACIVT system expressed in three different units. Values are calculated from the SMPS only, and values in brackets from both the SMPS and the APS.

	Deposited dose, **D** *(μg/cm²)*	*Number particle dose,* D_N *(10^{10} cm^{-2})*	*Surface area dose,* D_{SA} *(cm²/cm²)*
Experiment 1	0.070 [0.172]	0.024	0.011 [0.012]
Experiment 2	0.069 [0.203]	0.019	0.009 [0.011]
Experiment 3	0.078 [0.253]	0.019	0.009 [0.012]

4.2.2.1 Correspondence to the reality

Even if most experiments performed *in vitro* accelerates a response by introducing higher doses than expected in real exposure situations, it is relevant to relate the doses used *in vitro* with the corresponding real exposure levels. For the present experiments, the deposited doses in the lungs in a real situation are not the same as the deposited doses in the NACIVT system. Therefore, the number of workdays (8 h) a person needs to be in an environment with a certain concentration of ZnO NPs to reach the deposited doses in each of the aerosol experiments was calculated.

The concentrations used were 1) the existing occupational limit value for coarse ZnO in Sweden, the proposed limit value for ZnO NPs from 2) BSI and 3) IFA and DMSAE and 4) the mass concentration used in the present aerosol experiment. Table 4.7 shows the values of these for each experiment. Since Limit value 3 is given as a number of particles, Equation 3.2 was used to convert it to a corresponding mass dose. For these calculations the particle mean sizes in each experiment, seen in Table 4.2, were used.

Table 4.7. Mass concentrations used to calculate the number of workdays corresponding to the doses for each exposure with aerosol particles. Value 4 is calculated from the SMPS only, and values in brackets from both the SMPS system and the APS.

	Experiment 1	***Experiment 2***	***Experiment 3***
Limit value 1 (mg/m³)	5	5	5
Limit value 2 (mg/m³)	0.33	0.33	0.33
Limit value 3 (mg/m³)	0.0053	0.0053	0.0057
Value 4 (mg/m³)	0.17 [0.20]	0.18 [0.23]	0.22 [0.28]

The lungs were assumed to have a total surface area of 60 m^2. A ventilation volume of 13.5 m^3/workday for an adult male heavy worker, recommended for dosimetric modelling by ICRP [41], was used to estimate the number of workdays. A deposition efficiency of 40 %, based on Figure 2.2 for particles with a diameter of 40 nm, was used for all calculations. Values were inserted in Equation 3.1 and *t*

was calculated as the number of workdays. The calculated numbers of workdays are shown in Table 4.8.

Table 4.8. Number of workdays for a heavy worker corresponding to the deposited doses calculated and four different mass concentrations. Values are calculated from doses calculated with the SMPS only, and in brackets from both the SMPS and the APS.

	Experiment 1 (workdays)	*Experiment 2 (workdays)*	*Experiment 3 (workdays)*
Limit value 1	1.6 [3.8]	1.5 [4.5]	1.7 [5.6]
Limit value 2	24 [58]	23 [68]	26 [85]
Limit value 3	1474 [3602]	1444 [4254]	1520 [4932]
Value 4	46 [95]	43 [98]	39 [100]

If constantly working in an environment with the present Swedish occupational exposure limit value (Limit value 1), the doses tested in the NACIVT system will be reached after a few workdays to just over a workweek. The proposed limit value for ZnO NPs from BSI (Limit value 2) is very similar to the mass concentrations (Value 4) used in the aerosol experiments. With these mass concentrations a work period of 1 - 4 months would be required to reach the doses. The mass concentration proposed from IFA and DMSAE (Limit value 3) is much lower than the others and would require a work period of many years to even come close to the doses used in the present experiments.

4.3 ZnO NP effects on A549 cells

SUB (6) cultures exposed to the highest doses (25.0, 34.0 and 68.0 $\mu g/cm^2$) of ZnO NPs suspended in growth media were visibly affected by the particles. As seen under light microscope, the morphology of these cells was different from the negative control cells. Also, cell debris was observed in the media. ALI and SUB (24) cultures were only affected in this way by the highest dose (68.0 $\mu g/cm^2$). Cells treated with Triton X-100 were completely lysed and negative control cells as well as those treated with the lower doses of ZnO NPs looked healthy. In NACIVT experiments with aerosol particles, the ZnO NPs did not visibly affect cells.

ALI and SUB (24) control cells showed similar raw data values for both assays. SUB (6) cultures generally showed higher raw data values since they consisted of more cells.

4.3.1 **Metabolic activity**

The metabolic activity was clearly disrupted by 24 h exposure to the highest dose of ZnO NPs (25.0, 34.0 and 68.0 µg/cm^2) in experiments with particles suspended in liquid, as shown in Figure 4.5. SUB (6) cultures showed the largest effects after the exposures, whilst SUB (24) and ALI cultures exposed to the lower doses of ZnO NPs seemed to show more metabolic activity compared to control (> 100 % of control).

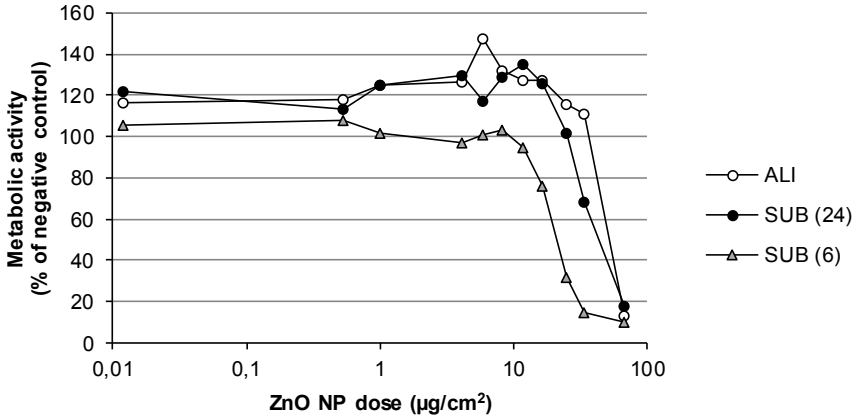

Figure 4.5. Dose-response curve for metabolic activity from WST-1 assay for A549 cells treated with different concentrations of ZnO NPs for 24 h in three different exposure conditions: submerged in 24- or 6-well plates with particles suspended in growth media and in ALI with particles suspended in isotonic solution. n = 6 for all doses except for 6.0 µg/cm^2 (n = 2) and 68.0 µg/cm^2 (n = 4).

Some responses were not significantly different compared to control and the standard deviations were quite large for some doses (Figure 4.6). For SUB (6), doses below 25.0 µg/cm^2 did not change the metabolic activity significant compared to control, except 0.54 µg/cm^2, which resulted in a metabolic activity significantly higher than control. However, doses from 25.0 µg/cm^2 and higher resulted in significantly reduced metabolic activity. For ALI and SUB (24), only the highest dose (68.0 µg/cm^2) was significantly lower than negative control cells. The lower doses were significantly higher than negative control cells or did not differ from negative control cells. The higher metabolic activity of exposed cells could be due to that the cells are trying to clear away the NPs to get rid of them. These results are in accordance with the visible effects on the cells in all three systems. For the experiments with aerosol particles in the NACIVT system, no cellular responses were significantly different from control 24 h after the exposure.

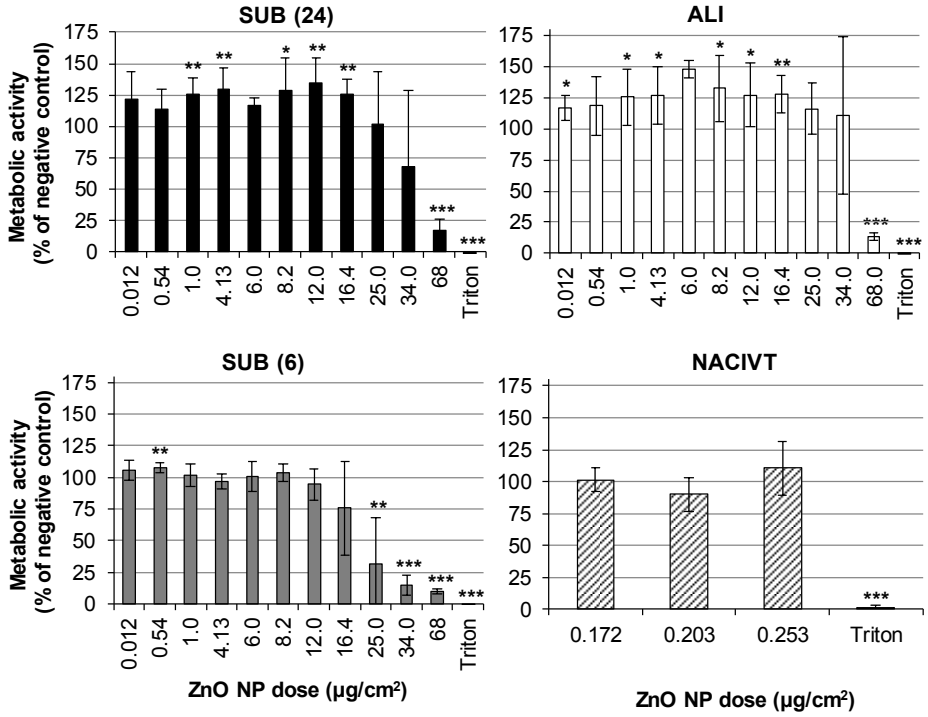

Figure 4.6. The metabolic activity differed significantly from control for some doses and exposure conditions. ALI, SUB (24) and SUB (6) cultures were exposed to ZnO NPs suspended in different liquids for 24 h, NACIVT cultures were exposed to aerosol particles for 1 h and the results were analysed after a 24 h incubation. Bars show mean value in % of control for a total of six replicates (n = 6), except for 6.0 µg/cm^2 (n = 2) and 68.0 µg/cm^2 (n = 4) for experiments with particles suspended in liquid and eight replicates for experiments in the NACIVT system (n = 8). *p < 0.05, ** p < 0.01, *** p < 0.001.

Effects on the metabolic activity were also evaluated 1 h and 3 h after NACIVT culture exposure to ZnO aerosol particles. Figure 4.7 shows the results for all three doses analysed 1 h, 3 h and 24 h after the exposures.

For 0.172 µg/cm^2 and 0.253 µg/cm^2 the cells showed significantly higher metabolic activity compared to control after 3 h incubation. This is not true for 0.203 µg/cm^2, where the responses seemed to be generally lower compared to the other doses. For all three doses, the metabolic activity was lower 24 h after exposure compared to 3 h after exposure. This difference was significant for 0.172 µg/cm^2 and 0.203 µg/cm^2. For 0.253 µg/cm^2 responses 1 h compared with 3 h after exposure instead differed significantly.

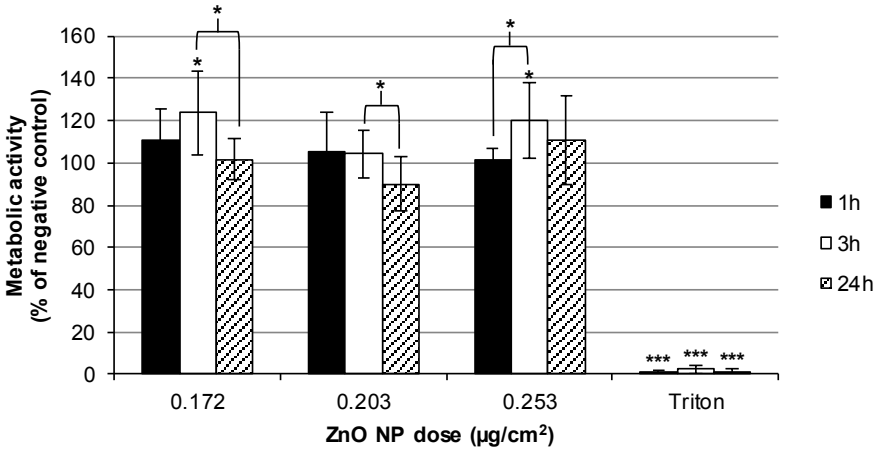

Figure 4.7. Metabolic activity of NACIVT cultures, as a result of a 1 h ZnO NP aerosol exposure, 1 h, 3 h and 24 h after exposure. A star over an error bar marks significance compared to control. A star over a bracket shows significance between the two time points in the bracket. Each time point had eight replicates and only one experiment was performed with each dose (n = 8). *p < 0.05, ** p < 0.01, *** p < 0.001.

4.3.2 **Cytotoxicity**

Unlike the metabolic activity, SUB (6) cells seemed to release less LDH compared to the positive control than SUB (24) cells (Figure 4.8). However, for all exposure conditions the results agree with the results from the WST-1 assay, if the irregular increases and decreases in the curves are overlooked for now. The metabolic activity starts decreasing at 16.4 µg/cm^2 or 25.0 µg/cm^2 and the LDH release increases at 25.0 µg/cm^2 (although not significant) for SUB (6) and SUB (24) cultures respectively. For ALI cultures, both assays indicate that the cells are only affected after exposure to the highest dose, 68.0 µg/cm^2.

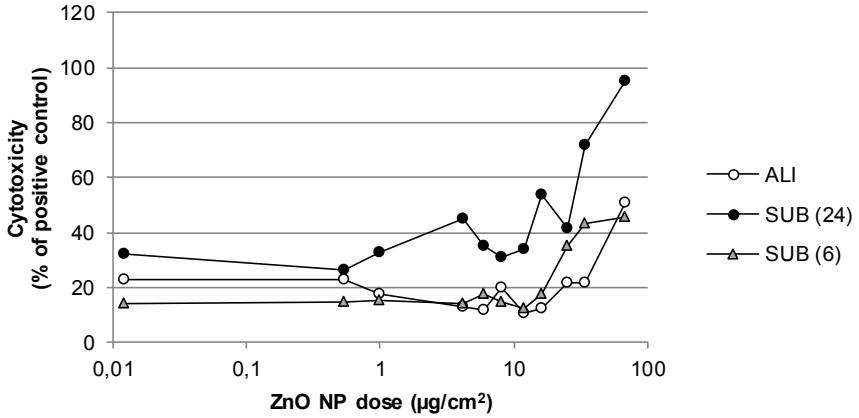

Figure 4.8. Dose-response curve for cytotoxicity from LDH analysis for A549 cells treated with different concentrations of ZnO NPs for 24 h in three different exposure conditions: submerged in 24- or 6-well plates, SUB (6) and SUB (24), with particles suspended in growth media and in ALI with particles suspended in isotonic solution. Six replicates (n = 6) were used for all doses except for 6.0 µg/cm^2 (n = 2) and 68.0 µg/cm^2 (n = 4).

For ALI and SUB (24) cultures data for LDH release was not very consistent over the different doses. Also, it often differed between the experiments, which resulted in high standard deviations for experiments with ZnO NPs suspended in liquid, especially for SUB (24) cultures. As a consequence, not many responses differed significantly from the negative controls (untreated), except for SUB (24) cultures where the highest dose (68.0 µg/cm^2) and SUB (6) cultures where the three highest doses (25.0, 34.0 and 68.0 µg/cm^2) resulted in significantly more LDH in the basal growth media compared to control. For experiments in the NACIVT system no results were significantly different from the respective negative control 24 h after the aerosol exposures (Figure 4.9), indicating that the ZnO NPs did not cause membrane damage in the doses used in these experiments.

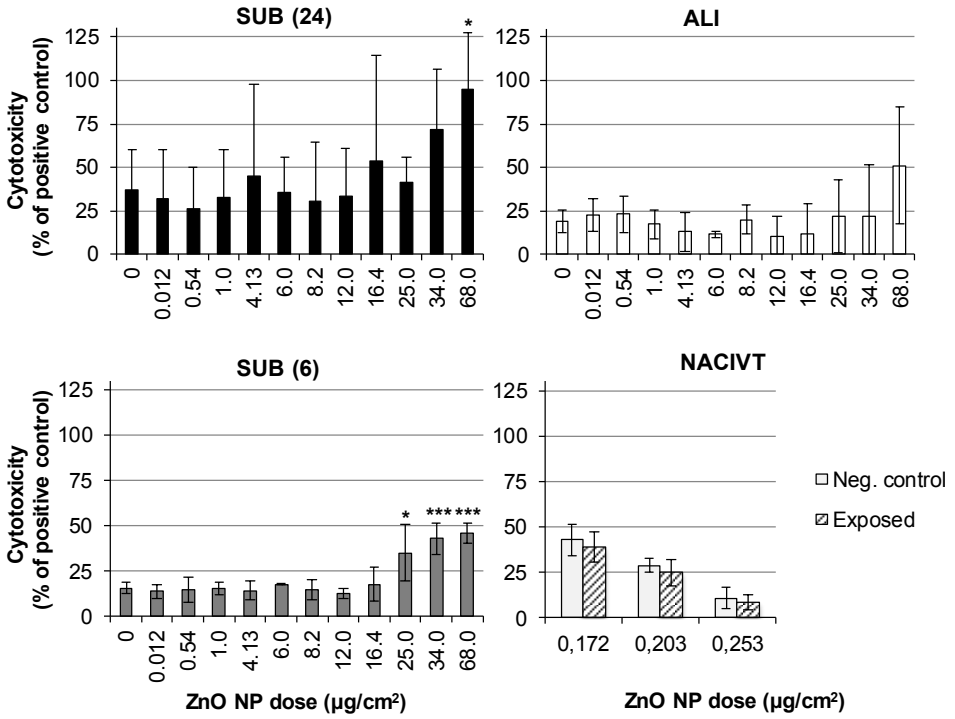

Figure 4.9. LDH in basal media differed significantly from the negative control (0) for a few doses and exposure conditions. ALI, SUB (24) and SUB (6) cultures were exposed to ZnO NPs suspended in different liquids for 24 h, NACIVT cultures were exposed to aerosol particles for 1 h and the results were analysed after a 24 h incubation. Bars show mean value in % of positive control for a total of six replicates (n = 6), except for 6.0 µg/cm^2 (n = 2) and 68.0 µg/cm^2 (n = 4) for experiments with particles suspended in liquid and six replicates for experiments in the NACIVT system (n = 6). *p < 0.05, ** p < 0.01, *** p < 0.001.

The LDH assay showed no indications of membrane damage for ZnO NP aerosol exposed cultures in the NACIVT system 1 h, 3 h or 24 h after exposure (Figure 4.10). One significantly different response was between 1 h and 24 h after exposure to 0.172 µg/cm^2, where the cells seemed to have released more LDH 24 h after exposure. Also, for 0.203 µg/cm^2 the response increased significantly between 1 h and 3 h as well as between 1 h and 24 h. In NACIVT cultures the results from the WST-1 and LDH assays agree, no decrease in metabolic activity was seen and no increase in basal media LDH compared to the negative controls could be detected.

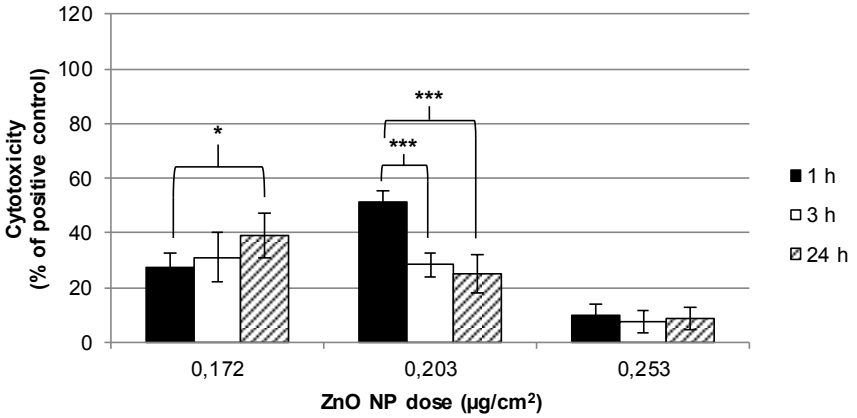

Figure 4.10. LDH release of NACIVT cultures after a 1 h exposure to ZnO NP aerosol, 1 h, 3 h and 24 h after exposure. A star over an error bar marks significance compared to the negative control. A star over a bracket shows significance between the two time points in the bracket. Experiments resulting in each dose were performed once and six replicates from each experiment were used (n = 6). *p < 0.05, ** p < 0.01, *** p < 0.001.

4.3.3 **Comparisons between the exposure conditions**

Most doses gave similar responses, which were not significantly different between the different exposure conditions with particles suspended in liquid. The effects on both metabolic activity, analysed with the WST-1 assay (W), and cytotoxicity, analysed with the LDH assay (L), in the different exposure conditions with particles suspended in liquid were also compared to each other (Table 4.9). For ALI and SUB (24) cultures the metabolic activity was overall similar and only cells treated with 6.0 µg/cm^2 responded significantly different, however the responses from exposures with this dose does not fit nicely into the dose-response curves for either ALI or SUB (24) cultures (Figure 4.5). Also, the number of samples was only two for this dose since it was taken away in the second and third experiments. Many doses, six for ALI and five for SUB (24) cultures, gave significantly different results when compared with responses in SUB (6) cultures. This suggests that SUB (6) cultures were more sensitive to ZnO NP exposure. For the cytotoxic responses, only one significantly different response was found between SUB (24) and ALI cultures exposed to 34.0 µg/cm^2. This was probably due to high standard deviations.

Table 4.9. Comparison between some of the exposure conditions. A non-significant difference ($p > 0.05$) in response between two exposure conditions is marked by a letter. W is for WST-1 assay, which measure metabolic activity and L is for LDH assay, which evaluate cytotoxicity.

Dose ($\mu g/cm^2$)	*0.012*	*0.54*	*1.0*	*4.13*	*6.0*	*8.2*	*12.0*	*16.4*	*25.0*	*34.0*	*68.0*
ALI SUB (24)	W, L	W, L	W, L	W, L	L	W, L	W, L	W, L	W, L	W	W, L
SUB (24) SUB (6)	W, L	W, L	L	L	W, L	W, L	L	L	L	W, L	W, L
SUB (6) ALI	W, L	W, L	W, L	L	W, L	L	L	L	L	L	W, L

Responses from NACIVT cultures, 24 h after exposure, were compared to those from ALI and SUB (24) cultures exposed to the two lowest doses, 0.012 $\mu g/cm^2$ and 0.54 $\mu g/cm^2$ (Table 4.10). For Experiment 2, the metabolic activity was significantly different from ALI and SUB (24) cultures exposed to both doses. Also, metabolic activity in Experiment 1 was significantly different from ALI cultures exposed to 0.012 $\mu g/cm^2$. For all significant differences NACIVT cultures showed lower metabolic activity than ALI and SUB (24) cultures. Significant differences could also be seen in cytotoxicity between NACIVT cultures in Experiment 1 or 3 compared to both of the lowest doses (0.012 and 0.54 $\mu g/cm^2$) in ALI.

Table 4.10. Some responses differed significantly between ALI or SUB (24) cultures exposed to 0.012 $\mu g/cm^2$ and 0.54 $\mu g/cm^2$ and NACIVT cultures after 24 h incubation. A non-significant difference ($p > 0.05$) in response between two exposures is marked by a letter. W is for WST-1 assay, which measured metabolic activity and L is for LDH assay, which evaluated cytotoxicity.

NACIVT	*ALI*		*SUB (24)*	
	0.012 $\mu g/cm^2$	*0.54 $\mu g/cm^2$*	*0.012 $\mu g/cm^2$*	*0.54 $\mu g/cm^2$*
Experiment 1		W	W, L	W, L
Experiment 2	L	L	L	L
Experiment 3	W	W	W, L	W, L

5 Discussion

5.1 Particles suspended in liquid

To compare the responses from cell cultures in the different exposure conditions, the particle and aggregate size in the different liquids was examined with DLS-measurements. These were performed with particles suspended in isotonic solution (prepared in two different ways), growth media and water. No results were gained from these measurements. However, based on previously published results some suggestions on the particle sizes can be presented. Zhou and Keller [54] tested aggregation of ZnO NPs in different concentrations and found that aggregation was faster in solutions with high particle concentrations. Since the concentrations in the present experiments had a large range (a factor of 14 000 between the highest concentration in isotonic solution and the lowest concentration in growth medium), it is probable that the particle sizes differed between the exposure conditions and doses used for the experiments with particles suspended in liquid.

Also, a higher concentration of NaCl has been shown to increase aggregation [54] while a higher concentration of serum in growth media decreases the aggregation and particle size [22]. This directly affects the particles that the cultures in the different exposure conditions are exposed to and it indirectly affects the deposited doses in the experiments since particles with different sizes have different sedimentation rates.

Because of the problems when suspending high concentrations of particles in a NaCl solution, in the second and third experiments the particles were first suspended in water and NaCl was added in the last step. Since the particles were more diluted than in the stock concentrations prepared this would probably decrease the aggregation in the isotonic solution. Changing the suspension protocol may have affected the cellular responses between the first and second or third experiments. However, more aggregation is still expected in the isotonic solution compared to the growth media.

The doses used in the experiments with ZnO NPs suspended in liquid were very high and it is not expected that such doses are reached in a realistic exposure. However, since the thought was to compare exposures in different exposure conditions, a response was needed. Also, the responses to the highest doses confirmed that the particle suspensions were done properly, and that the particles had the ability to affect the cell cultures.

5.2 Aerosol particles

The particle concentrations differed between the different experiments (Table 4.2). Experiment 1 had the highest number particle concentration while Experiment 3 had the highest mass concentrations, with data from both SMPS and APS and only SMPS. This indicates that the aerosol in Experiment 1 contained more small particles or aggregates and that more aggregates were present in the aerosol used in Experiment 3. The values were slightly different when estimated from SMPS and APS data compared to only SMPS data. The mass concentration was more affected than the number concentration, which is due to the fewer particles with much larger mass measured with the APS.

As expected, the larger particles have a large effect on the mass doses, no effect on the number particle dose and only a small effect on the surface area dose (Table 4.6). This even though the deposition efficiency in the NACIVT system was considered. Two aspects argue that doses should only be calculated with the SMPS data. Very large particles will probably not follow the airstream all the way from the generation to reach the cell cultures in the NACIVT system, instead they might deposit in the tubing. One could speculate that if these particles manage to reach the cultures it is not certain that they will affect the cells notably. This since it is believed that the toxic effects from ZnO NPs come from particles in the endosomes and lysosomes [9, 13], and particles on the micrometre scale can be too large for the cells to engulf. However, it might be so that the primary particles contained in the large aggregates may be suspended into the primary particles after deposition on the cells, and again acting as small nanosized particles. Therefore, it is not possible at this point to say which of the doses is most accurate.

5.2.1 **Are the doses realistic?**

The doses used in the aerosol experiments correspond to a few workdays up to about a weeks work in an environment with the Swedish occupational limit value for coarse ZnO. For this limit value, the doses used in the aerosol experiments are highly relevant to evaluate effects from ZnO NP exposure in a real environment.

For the proposed limit value for ZnO NPs from BSI, the doses correspond to 1-3 months work. This might be harder to justify since the longer time period might cause chronic effects, which would not be visible in a one-time exposure. Alternatively, the one-time exposure of the cell cultures might give rise to effects that would not be visible after exposure to a much lower dose for a longer time. Thus, it is harder to compare the exposures and to draw conclusions about the reality. This applies even more to the proposed number concentration from IFA and DMSAE, since the dose would not be reached until after multiple years in an environment of constant exposure to very low numbers of particles.

5.3 Cell responses

5.3.1 **Exposure to particles suspended in liquid**

The cell line A549 used in the experiments is derived from a lung tumour, which means that the cell characteristics can differ from normal cells present in a healthy lung. Often, cell lines derived from cancer tissue are tougher than normal or primary cells, which has to be considered when looking at the results. Another aspect to consider, as mentioned earlier, is the lack of clearing and defence mechanisms in *in vitro* cell experiments. Thus, the results from the exposures in the present experiments can not be directly transferred to normal lung cells in a realistic exposure.

The different cultures exposed to ZnO NPs suspended in liquid; ALI, SUB (24) and SUB (6), showed different responses. SUB (6) cultures seemed to be more sensitive than both ALI and SUB (24) cultures. At the same time, both SUB (24) and ALI cultures showed significantly more metabolic activity than control for some doses (Figure 4.5), which indicates that their metabolic activity increased after ZnO NP exposure. It could be possible that the NP exposed cells increase their metabolic activity as an acute response in order to recover from the external stress they were subjected to. Another possible explanation to the observed effects is that the control cells could have been overgrown. All cultures were confluent on day three, when the cells were exposed to ZnO NPs. While the particle exposure might have inhibited growth in the exposed cultures, the control cells could continue growing until they reached their maximum confluency and started apoptosis.

The responses from the present experiments are comparable to but larger than responses seen in Thongkam *et al.* [28]. In A549 cells exposed to uncoated 100 nm ZnO NPs, they observed 60 % viability in cultures exposed to 20 $\mu g/cm^2$ and 20 % viability in cultures exposed to 80 $\mu g/cm^2$. In the present experiments the viability was 30 % in SUB (6) cultures exposed to 25.0 $\mu g/cm^2$ and around 10 % in cultures exposed to 68.0 $\mu g/cm^2$. These differences might be due to the larger particles used in their experiments (100 nm compared to on average 40 nm in the present experiment). Even though the wells used in Thongkam *et al.* has the same growth area as the inserts in 24-well plates (used for ALI and SUB (24) cultures), the responses in ALI and SUB (24) cultures in the present experiments differ more compared to their results.

The LDH release corresponds well to the decreases in metabolic activity, especially for SUB (6) cultures where a response can be seen after exposure to 16.4 $\mu g/cm^2$ and 25.0 $\mu g/cm^2$ for WST-1 and LDH assays respectively. It is hard to say anything about the LDH release from SUB (24) cultures since the standard deviations were very high. No doses resulted in significantly increased LDH

release compared to the negative control cells in ALI cultures, and in SUB (24) cultures only the highest dose (68.0 μg/cm^2) resulted in a significantly increased LDH release.

As investigated by Da Silva *et al.* [59], ZnO NPs appear to affect the results of the LDH assay. In the present experiments such interference is not expected since particles are added to the apical side and the LDH assay is performed on the basal growth media.

5.3.1.1 Comparison between ALI and SUB (24) cultures

A significant difference could only be detected between the metabolic activity of ALI and SUB (24) cultures exposed to one dose (6.0 μg/cm^2). However, ALI cultures seemed to be less affected by the higher doses of particles (Figure 4.5). According to the LDH-assay significance could only be shown for the cultures exposed to 34.0 μg/cm^2. This is probably due to that responses from SUB (24) cultures differed between the experiments and doses resulting in high standard deviations and irregularity over the doses.

The similar raw data values from ALI and SUB (24) control cells show that the ALI cultures could survive without being submerged in growth media. However, it is worth to mention that media was found on the apical side of the cells the day after they were brought to the ALI-state, which could affect the results from the ALI cultures. The reason is that the cells were not completely confluent the day they were brought to the ALI-state. As a consequence, basal growth media could have leaked through the insert membrane to the cellular compartment. How long time it took before the cells were completely submerged in media is not known. To account for this problem, cells could have been brought to the ALI-state when they were confluent. This would mean to grow them for one extra day or to seed more cells, which would compromise the time they can live in the ALI-state before they are exposed since control cells may be overgrown.

Despite the leaked growth media, two important differences still remains between the exposure conditions, cells were living in the ALI-state for some time and they were exposed to the particles in isotonic solution since the media was removed before exposure. Since previously published studies [54] suggest that ZnO NPs aggregate fast in NaCl solutions, the difference in responses of ALI and SUB (24) cultures to the highest doses could be due to much larger particles in the isotonic solution with high particle concentrations compared to the growth media. This could have been confirmed if the particle and aggregate sizes in the different liquids was determined.

5.3.1.2 Comparison between the two well sizes

For SUB (6) cultures the three highest doses (25.0, 34.0 and 68.0 μg/cm^2) resulted in metabolic activities significantly lower than control as well as more LDH release or cytotoxicity. Also, for 16.4 μg/cm^2 and 25.0 μg/cm^2 SUB (6) cultures

showed significantly lower metabolic activity compared to SUB (24) as for 16.4 µg/cm^2, 25.0 µg/cm^2 and 34.0 µg/cm^2 compared to ALI cultures. The other significant differences were due to ALI and SUB (24) cultures showing higher metabolic activity than control. No dose resulted in significant differences in LDH release between SUB (6) and ALI or SUB (24) cultures. Based on this, the responses to the ZnO NPs were larger in SUB (6) cultures compared to both ALI and SUB (24) cultures.

As for the comparison between ALI and SUB (24) cultures, the differences of SUB (6) and ALI cultures are believed to be due to different ZnO NP sizes in the particle suspensions. Since the suspensions used to expose SUB (24) and SUB (6) cultures were the same, there must be another reason for why the responses are different between these cultures. The experiments were designed so that the liquid pillar heights in both SUB (6) and SUB (24) wells were the same height, so the sedimentation of the particles could be assumed to be the same in these wells. Since 24-wells are smaller the risk of particles attaching to the well walls is larger, which suggests that not as many ZnO NPs reach the cells as in the 6-wells. This could explain the different responses.

Another reason could be the fact that there were more cells in the SUB (6) cultures. This means that a changed metabolic activity in some cells will not affect the result as much as in SUB (24) cultures, which contained fewer cells. Thus, results from SUB (6) cultures were not as affected by the possibly overgrown control cells. However, since cells in SUB (6) cultures were visibly affected by the three highest doses (25.0, 34.0 and 68.0 µg/cm^2) and SUB (24) and ALI cultures only by the highest dose, it is possible to argue that the deposited doses in the different well formats were different.

5.3.2 **Exposure to aerosol particles**

No significant effects were visible on the LDH assay compared to the negative control for NACIVT cultures. Only two responses were significantly different from control in the WST-1 assay, both indicated higher metabolic activity 3 h after exposure. This means that no impairment of cellular metabolism or membrane damage could be detected with the doses used in the aerosol experiments. However, since the negative control cells might have a decreased metabolic activity on the last analysis day, the cells analysed 24 h after exposure might have an even lower metabolic activity than what is shown by the WST-1 assay (Figure 4.7).

The results from the WST-1 assay indicate that the cellular metabolism is slightly increased 1 h after exposure (all responses higher than 100 % although not significant), significantly increased 3 h after exposure and has gone back to normal or decreased 24 h after exposure. Since it is assumed that the cultures were not overgrown on the third day of experiments, the increase in metabolic activity 3 h

after exposure is not expected to be due to less metabolic activity in control cells. This is interesting since it can be argued that the increased metabolic activity in NACIVT cultures is an effect of the exposure to low doses of ZnO NPs.

5.3.3 **Comparison between particles suspended in liquid and aerosol particles**

Responses from NACIVT cultures 24 h after exposure were compared to responses from ALI and SUB (24) cultures exposed to 0.012 μg/cm^2 and 0.54 μg/cm^2 ZnO NPs (Table 4.10). The cytotoxicity seemed to be higher in NACIVT cultures in Experiment 1 (0.172 μg/cm^2) and lower in NACIVT cultures in Experiment 3 (0.253 μg/cm^2) compared to ALI cultures exposed to both 0.012 μg/cm^2 and 0.54 μg/cm^2. A significant difference in metabolic activity was also observed between ALI cultures exposed to 0.012 μg/cm^2 and NACIVT cultures in Experiment 1 (0.172 μg/cm^2), where the aerosol exposed cultures showed lower metabolic activity. Also, for NACIVT cultures in Experiment 2 (0.203 μg/cm^2) the metabolic activity was significantly lower than both doses in ALI and SUB (24) cultures. The NACIVT cultures did not show a significantly lower metabolism than NACIVT control cells. However, NACIVT control cells are expected to have decreased metabolic activity on the fourth day. Therefore it can be argued that the NACIVT cultures indeed were affected by the ZnO NP exposure 24 h after the exposure as opposed to the ALI and SUB (24) cultures.

Conclusions

The doses used in the experiments with ZnO NPs suspended in liquid were very high, and exposure to such high doses is not expected in real occupational environments. However, doses in the aerosol experiments can be correlated to a few days of work in an environment with the Swedish occupational exposure limit for coarse ZnO (5 mg/m^3). Thus these doses are considered realistic.

Different responses could be seen between ALI and SUB (24) cultures for the highest doses, but not for the lowest. These effects were probably due to larger particles in the isotonic solution used to expose ALI cultures.

It was clearly shown that cultures in the larger well size showed a greater response to the ZnO NPs, the cause could be either that there were more cells in the SUB (6) cultures or that the deposited doses were higher. This has to be further investigated.

The results indicate that the metabolic activity in NACIVT cultures changes at different time points after the exposure, and that it has decreased 24 h after the exposure. On the contrary, for experiments with particles suspended in liquid the metabolic activity seems to have increased 24 h after exposure to 0.012 $\mu g/cm^2$ and 0.54 $\mu g/cm^2$ for both ALI and SUB (24) cultures.

To investigate more differences in responses and responses to the lower doses, further analysis is needed.

Outlook

This project has many different aspects whereof only a few of them could be addressed in this master's thesis. Thus, more research is needed to draw conclusions on the cellular responses and the particle properties.

The particle size distribution and shape need to be further investigated. This could be addressed by first looking at the particles in an electron microscope and then redoing the DLS-measurements. With this knowledge, the suspensions could be optimized to get little aggregation and the conversion between number and mass could be done in a more accurate way. Knowing the aggregate size in suspension, the sedimentation of the particles can be modelled, which means that deposited doses could be estimated more accurately for all different particle suspensions and concentrations.

Some optimization is also required for the aerosol generation to get higher mass concentrations, which could better be compared to different limit values. This would provide another possibility to discuss the cellular responses. The doses for the aerosol experiments could also be improved by investigating the deposition efficiency in the NACIVT system for ZnO NPs, or by directly measure the deposited doses.

Further optimization on the cell culture studies can be done. For ALI cultures, media was found on the apical side. This problem needs to be addressed without compromising the health of the control cells. To do this, experiments without NP exposures can be performed to see how the cells grow and when their condition starts to deteriorate. Also, to examine the effects of the lower doses protein analysis could be performed for example on IL-6 and IL-8. This would provide complementary information about the toxicity of the particles and how the cells respond to the particles. It could also reveal effects in cultures exposed to the lowest doses of ZnO NPs.

As for the NACIVT system, the possibilities are endless and many more experiments can be performed. For example, co-cultures could be used to for example investigate the role of macrophages, and primary cell lines could be used to see how other, more sensitive cells respond to ZnO NP exposures. Moreover,

different membranes, more closely resembling the *in vivo* environment, could be evaluated and possibly used in this system. To further evaluate the effects of ZnO NPs and try to resemble real exposure situations longer exposures with lower aerosol concentrations could be tested. Also, repeated exposures could be performed and for example compared to a longer single exposure.

References

1. Oberdorster, G., Oberdorster, E. and Oberdorster, J., 2005. Nanotoxicology: an emerging discipline evolving from studies of ultrafine particles. *Environmental Health Perspectives*, **113**(7): p. 823-839.
2. Onoue, S., Yamada, S. and Chan, H.K., 2014. Nanodrugs: pharmacokinetics and safety. *International Journal of Nanomedicine*, **9**: p. 1025-1037.
3. Osmond, M.J. and Mccall, M.J., 2010. Zinc oxide nanoparticles in modern sunscreens: An analysis of potential exposure and hazard. *Nanotoxicology*, **4**(1): p. 15-41.
4. Arbetsmiljöverket. AFS 2015:7; Hygieniska gränsvärden, Sweden. 2015.
5. BSI (British Standards Institution). PD 6699-1 : 2007 Nanotechnologies: Good practice guide for specifying manufactured nanomaterials. 2011.
6. Institut für Arbeitsschutz (IFA) der Deutschen Gesetzlichen Unfallversicherung. Criteria for assessment of the effectiveness of protective measures. [online] Accessed March 12, 2019. Available from: https://www.dguv.de/ifa/fachinfos/nanopartikel-am-arbeitsplatz/beurteilung-von-schutzmassnahmen/index-2.jsp.
7. van Broekhuizen, P*., et al.*, 2012. Exposure limits for nanoparticles: report of an international workshop on nano reference values. *Annals of Occupational Hygiene*, **56**(5): p. 515-524.
8. Secondo, L.E., Liu, N.J. and Lewinski, N.A., 2017. Methodological considerations when conducting in vitro, air-liquid interface exposures to engineered nanoparticle aerosols. *Critical Reviews in Toxicology*, **47**(3): p. 225-262.
9. Mihai, C*., et al.*, 2015. Intracellular accumulation dynamics and fate of zinc ions in alveolar epithelial cells exposed to airborne ZnO nanoparticles at the air-liquid interface. *Nanotoxicology*, **9**(1): p. 9-22.
10. Lenz, A.G*., et al.*, 2009. Comparison of the Inflammatory Resonse of Cells Exposed to Zinc Oxide Nano-Particles at the Air-Liquid Interface and under Submerged Conditons. *American Journal of Respiratory and Critical Care Medicine*, **179**.
11. International Organization for Standardization. ISO/TS 80004-2:2015; Nanotechnologies-Vocabulary-Part 2: Nano-objects. 2015.
12. Nel, A.E*., et al.*, 2009. Understanding biophysicochemical interactions at the nano-bio interface. *Nature Materials*, **8**(7): p. 543-557.

13. Wang, B.*, et al.*, 2018. The size of zinc oxide nanoparticles controls its toxicity through impairing autophagic flux in A549 lung epithelial cells. *Toxicology Letters*, **285**: p. 51-59.
14. Hsiao, I.L. and Huang, Y.J., 2011. Effects of various physicochemical characteristics on the toxicities of ZnO and TiO2 nanoparticles toward human lung epithelial cells. *Science of the Total Environment*, **409**(7): p. 1219-1228.
15. Baek, M.*, et al.*, 2011. Factors influencing the cytotoxicity of zinc oxide nanoparticles: particle size and surface charge. *Journal of Physics: Conference Series*, **304**.
16. Heng, B.C.*, et al.*, 2011. Evaluation of the cytotoxic and inflammatory potential of differentially shaped zinc oxide nanoparticles. *Archives of Toxicology*, **85**(12): p. 1517-1528.
17. Yu, J.*, et al.*, 2011. Effects of physicochemical properties of zinc oxide nanoparticles on cellular uptake. *Journal of Physics: Conference Series*, **304**.
18. Duffin, R.*, et al.*, 2007. Proinflammogenic effects of low-toxicity and metal nanoparticles in vivo and in vitro: Highlighting the role of particle surface area and surface reactivity. *Inhalation Toxicology*, **19**(10): p. 849-856.
19. Monteiller, C.*, et al.*, 2007. The pro-inflammatory effects of low-toxicity low-solubility particles, nanoparticles and fine particles, on epithelial cells in vitro: the role of surface area. *Occupational and Environmental Medicine*, **64**(9): p. 609-615.
20. Brown, D.M.*, et al.*, 2001. Size-dependent proinflammatory effects of ultrafine polystyrene particles: A role for surface area and oxidative stress in the enhanced activity of ultrafines. *Toxicology and Applied Pharmacology*, **175**(3): p. 191-199.
21. Duffin, R., Tran, C.L., Clouter, A., Brown, D.M., MacNEE, W., Stone, V. and Donaldson, K., 2002. The Importance of Surface Area and Specific Reactivity in the Acute Pulmonary Inflammatory Response to Particles. *Annals of Occupational Hygiene*, **46**: p. 242-245.
22. Hsiao, I.L. and Huang, Y.J., 2013. Effects of serum on cytotoxicity of nano- and micro-sized ZnO particles. *Journal of Nanoparticle Research*, **15**: p. 1829.
23. The Nanodatabase. [online] Accessed May 13, 2019. Available from: http://nanodb.dk/en/.
24. Piccinno, F.*, et al.*, 2012. Industrial production quantities and uses of ten engineered nanomaterials in Europe and the world. *Journal of Nanoparticle Research*, **14**(9).
25. Vaseem, M., Umar, A. and Hanhn, Y.-B., *ZnO Nanoparticles: Growth, Properties, and Applications*, in *Metal Oxide Nanostructures and Their Applications*, Y.-B. Hahn and A. Umar, Editors. 2010, American Scientific Publishers. p. 1-36.

26. Cho, W.S., *et al.*, 2011. Progressive severe lung injury by zinc oxide nanoparticles; the role of Zn2+ dissolution inside lysosomes. *Particle and Fibre Toxicology*, **8**: p. 27.
27. Schaudien, D., Knebel, J. and Creutzenberg, O., 2012. In vitro study revealed different size behavior of different nanoparticles. *Journal of Nanoparticle Research*, **14**(8).
28. Thongkam, W., *et al.*, 2017. Oxidant generation, DNA damage and cytotoxicity by a panel of engineered nanomaterials in three different human epithelial cell lines. *Mutagenesis*, **32**(1): p. 105-115.
29. Raemy, D.O., *et al.*, 2012. Effects of flame made zinc oxide particles in human lung cells - a comparison of aerosol and suspension exposures. *Particle and Fibre Toxicology*, **9**.
30. Lenz, A.G., *et al.*, 2013. Inflammatory and Oxidative Stress Responses of an Alveolar Epithelial Cell Line to Airborne Zinc Oxide Nanoparticles at the Air-Liquid Interface: A Comparison with Conventional, Submerged Cell-Culture Conditions. *Biomed Research International*.
31. Stoehr, L.C., *et al.*, 2015. Assessment of a panel of interleukin-8 reporter lung epithelial cell lines to monitor the pro-inflammatory response following zinc oxide nanoparticle exposure under different cell culture conditions. *Particle and Fibre Toxicology*, **12**: p. 29.
32. Cho, W.S., *et al.*, 2012. Differential pro-inflammatory effects of metal oxide nanoparticles and their soluble ions in vitro and in vivo; zinc and copper nanoparticles, but not their ions, recruit eosinophils to the lungs. *Nanotoxicology*, **6**(1): p. 22-35.
33. Liu, J., *et al.*, 2016. The toxicology of ion-shedding zinc oxide nanoparticles. *Critical Reviews in Toxicology*, **46**(4): p. 348-384.
34. Duffin, R., *et al.*, 2002. The Importance of Surface Area and Specific Reactivity in the Acute Pulmonary Inflammatory Response to Particles. *Annals of Occupational Hygiene*, **46**: p. 242-245.
35. Eurpoean chemical agency (ECHA). Companies to provide more information on nanomaterials. [online] Accessed February 25, 2019. Available from: https://echa.europa.eu/sv/-/companies-to-provide-more-information-on-nanomaterials.
36. Kemikalieinspektionen. Anmälningsplikt för nanomaterial. [online] Accessed February 25, 2019. Available from: https://www.kemi.se/produktregistret/anmalningsplikt-for-nanomaterial.
37. Widmaier, E.P., Raff, H. and Strang, K.T., *Vander's Human Physiology: The mechanisms of body function*. Thirteenth ed. 2014, New York: McGraw-Hill.
38. Knudsen, L. and Ochs, M., 2018. The micromechanics of lung alveoli: structure and function of surfactant and tissue components. *Histochemistry and Cell Biology*, **150**(6): p. 661-676.

39. Kopf, M., Schneider, C. and Nobs, S.P., 2015. The development and function of lung-resident macrophages and dendritic cells. *Nature Immunology*, **16**(1): p. 36-44.
40. Doryab, A.*, et al.* Evolution of Bioengineered Lung Models: Recent Advances and Challenges in Tissue Mimicry for Studying the Role of Mechanical Forces in Cell Biology.
41. ICRP, 1994. Human Respiratory Tract Model for Radiological Protection. ICRP publication 66. *Annals of the ICRP*, **24**(1-3).
42. Foster, M.W., *Deposition and Clearance of Inhaled Particles*, in *Air Pollution and Health*, S.T. Holgate, et al., Editors. 1999, Academic Press. p. 295-324.
43. Geiser, M. and Kreyling, W.G., 2010. Deposition and biokinetics of inhaled nanoparticles. *Particle and Fibre Toxicology*, **7**: p. 2.
44. Geiser, M.*, et al.*, 2017. Evaluating Adverse Effects of Inhaled Nanoparticles by Realistic In Vitro Technology. *Nanomaterials (Basel)*, **7**(2).
45. Baron, P.A. and Willeke, K., *Gas and Particle Motion*, in *Aerosol Measurement: Principles, Techniques, and Applications*, P.A. Baron and K. Willeke, Editors. 2001, Wiley: New York. p. 61-82.
46. Murphy, K. and Weaver, C., *Janeway's immunobiology*. 9th ed. 2017, New York, New York: Garland Science, Taylor & Francis Group, LLC.
47. Xie, Y.M.*, et al.*, 2012. Aerosolized ZnO Nanoparticles Induce Toxicity in Alveolar Type II Epithelial Cells at the Air-Liquid Interface. *Toxicological Sciences*, **125**(2): p. 450-461.
48. Lenz, A.G.*, et al.*, 2009. A dose-controlled system for air-liquid interface cell exposure and application to zinc oxide nanoparticles. *Particle and Fibre Toxicology*, **6**.
49. Savi, M.*, et al.*, 2008. A novel exposure system for the efficient and controlled deposition of aerosol particles onto cell cultures. *Environmental Science & Technology*, **42**(15): p. 5667-5674.
50. Mertes, P.*, et al.*, 2013. A compact and portable deposition chamber to study nanoparticles in air-exposed tissue. *Journal of Aerosol Medicine and Pulmonary Drug Delivery*, **26**(4): p. 228-235.
51. Jeannet, N.*, et al.*, 2015. Nano aerosol chamber for in-vitro toxicity (NACIVT) studies. *Nanotoxicology*, **9**(1): p. 34-42.
52. Jeannet, N.*, et al.*, 2016. Acute toxicity of silver and carbon nanoaerosols to normal and cystic fibrosis human bronchial epithelial cells. *Nanotoxicology*, **10**(3): p. 279-291.
53. Svensson, C.R.*, et al.*, 2016. Validation of an air-liquid interface toxicological set-up using Cu, Pd, and Ag well-characterized nanostructured aggregates and spheres. *Journal of Nanoparticle Research*, **18**: p. 86.

54. Zhou, D.X. and Keller, A.A., 2010. Role of morphology in the aggregation kinetics of ZnO nanoparticles. *Water Research*, **44**(9): p. 2948-2956.
55. Peng, Y.H.*, et al.*, 2017. Influence of water chemistry on the environmental behaviors of commercial ZnO nanoparticles in various water and wastewater samples. *Journal of Hazardous Materials*, **322**: p. 348-356.
56. Peng, Y.H.*, et al.*, 2015. The effect of electrolytes on the aggregation kinetics of three different ZnO nanoparticles in water. *Science of the Total Environment*, **530**: p. 183-190.
57. Han, Y.*, et al.*, 2014. Aggregation and dissolution of ZnO nanoparticles synthesized by different methods: Influence of ionic strength and humic acid. *Colloids and Surfaces A: Physicochemical and Engineering Aspects*, **451**: p. 7-15.
58. Kroll, A.*, et al.*, 2012. Interference of engineered nanoparticles with in vitro toxicity assays. *Archives of Toxicology*, **86**(7): p. 1123-1136.
59. Da Silva, E.*, et al.*, 2019. Interaction of biologically relevant proteins with ZnO nanomaterials: A confounding factor for in vitro toxicity endpoints. *Toxicology In Vitro*, **56**: p. 41-51.
60. Guadagnini, R.*, et al.*, 2015. Toxicity screenings of nanomaterials: challenges due to interference with assay processes and components of classic in vitro tests. *Nanotoxicology*, **9 Suppl 1**: p. 13-24.
61. Wilhelmi, V.*, et al.*, 2012. Evaluation of apoptosis induced by nanoparticles and fine particles in RAW 264.7 macrophages: facts and artefacts. *Toxicol In Vitro*, **26**(2): p. 323-334.
62. Flagan, R.C., *Electrical Mobility Methods for Submicrometer Particle Characterization*, in *Aerosol Measurement: Principles, Techniques, and Applications*, P.A. Baron, K. Willeke, and P. Kulkarni, Editors. 2011, John Wiley & Sons, Inc.: Hoboken, New Jersey. p. 339-364.
63. Cheng, Y.-S., *Condensation particle counters*, in *Aerosol Measurement: Principles, Techniques, and Applications*, P.A. Baron, K. Willeke, and P. Kulkarni, Editors. 2011, John Wiley & Sons, Inc.: Hoboken, New Jersey. p. 381-392.
64. Baron, P.A.*, et al.*, *Real-time techniques for aerodynamic size measurements*, in *Aerosol Measurement: Principles, Techniques, and Applications*, P.A. Baron, K. Willeke, and P. Kulkarni, Editors. 2011, John Wiley & Sons, Inc.: Hoboken, New Jersey. p. 313-338.
65. Merck KGaA. Cell Proliferation Reagent WST-1. [online] Accessed March 12, 2019. Available from: https://www.sigmaaldrich.com/catalog/product/roche/cellproro?lang=en&region=SE.
66. Merck KGaA. Cytotoxicity Detection KitPLUS (LDH). [online] Accessed March 12, 2019. Available from:

https://www.sigmaaldrich.com/catalog/product/roche/cytodetro?lang=en&region=SE.

Appendix A Dilution series for particle suspensions

Table A.1. Dilution series for particle suspensions used to expose ALI cultures on 24-well plates in experiment 1. Dose 1 is made from the stock concentration, which is 2550 µg/ml in isotonic solution.

Reference dose (µg/cm²)	*Conc. in particle suspension (µg/ml)*	*Suspension volume (ml)*	*Take from previous (µl)*	*Add isotonic solution (ml)*
34	559.3	1.5	329	1.171
25	399.3	1.5	1071	0.429
16.4	279.5	1.5	1050	0.450
12	199.8	1.3	929	0.371
8.2	139.8	1.2	840	0.360
6	99.8	1	714	0.286
4.13	69.9	0.7	490	0.210
1	17.5	0.8	200	0.600
0.54	10.0	0.5	286	0.214
0.012	0.2	0.5	10	0.490

Table A.2. Dilution series for particle suspensions used to expose submerged cultures in 24- and 6-well plates in experiment 1. Stock concentration is 2550 µg/ml in growth media.

	Reference dose (µg/cm²)	*Conc. in particle suspension (µg/ml)*	*Suspension volume (ml)*	*Take from stock (µl)*	*Add medium (ml)*
	34	221.0	1.6	138	1.462
	25	158.7	1.6	100	1.500
	16.4	99.2	1.6	63	1.537
	12	79.3	1.6	50	1.550
	8.2	51.0	1.6	31	1.569
	6	39.7	1.6	25	1.575
2x	4.13	29.8	1.2	14	1.186
				Take from previous (µl)	
2x	1	5.7	1.5	300	1.213
2x	0.54	3.3	1	583	0.417
2x	0.012	0.08	0.9	21	0.879

Table A.3. Dilution series for particle suspensions used to expose ALI cultures on 24-well plates in experiment 2 and 3. Dose 1 is made from the stock concentration, which is 2550 µg/ml in H_2O. The particles were first suspended in H_2O, when all dilutions were made, 0.1 ml NaCl (45 mg/ml in H_2O) was added to each suspension to get an isotonic solution.

Reference dose (µg/cm²)	*Conc. in isotonic solution (µg/ml)*	*Conc. in water (µg/ml)*	*Suspension volume (ml)*	*Take from previous (µl)*	*Add water (ml)*
68	1120.0	1400.0	1	549	0.451
34	560.0	700.0	1.2	600	0.600
25	400.0	500.0	1.12	800	0.320
16.4	280.0	350.0	1.03	721	0.309
12	200.1	250.2	0.88	629	0.251
8.2	140.1	175.1	0.68	476	0.204
4.13	70.1	87.6	0.56	280	0.280
1	17.6	22.0	0.63	158	0.472
0.54	10.0	12.5	0.41	234	0.176
0.012	0.2	0.3	0.4	8	0.392

Table A.4. Dilution series for particle suspensions used to expose submerged cultures in 24- and 6-well plates in experiment 2 and 3. Stock concentration is 2550 µg/ml in growth media.

	Reference dose (µg/cm²)	*Conc. in particle suspension (µg/ml)*	*Suspension volume (ml)*	*Take from stock (µl)*	*Add medium (ml)*
2x	68	450.5	0.9	159	0.741
2x	34	221.0	0.9	78	0.822
2x	25	158.7	0.9	56	0.844
2x	16.4	99.1	0.9	35	0.865
2x	12	79.3	0.9	28	0.872
2x	8.2	51.0	0.9	18	0.882
2x	4.13	29.8	1.2	14	1.186
				Take from previous (µl)	
2x	1	5.7	1.5	287	1.213
2x	0.54	3.3	1	583	0.417
2x	0.012	0.08	0.9	21	0.879

Appendix B The set-up for exposures in the NACIVT system

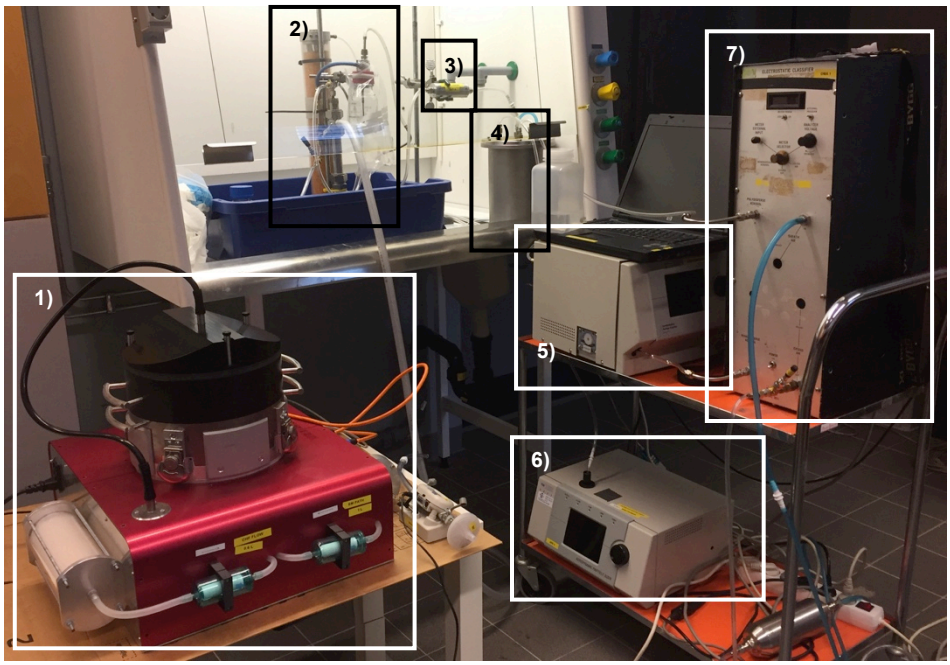

Figure B.1. The set-up used for the experiments with aerosol particles. The components are 1) the NACIVT system, 2) the atomizer (with dryer), 3) a bipolar charger, 4) the mixing volume, 5) the CPC, 6) the APS and 7) the DMA.

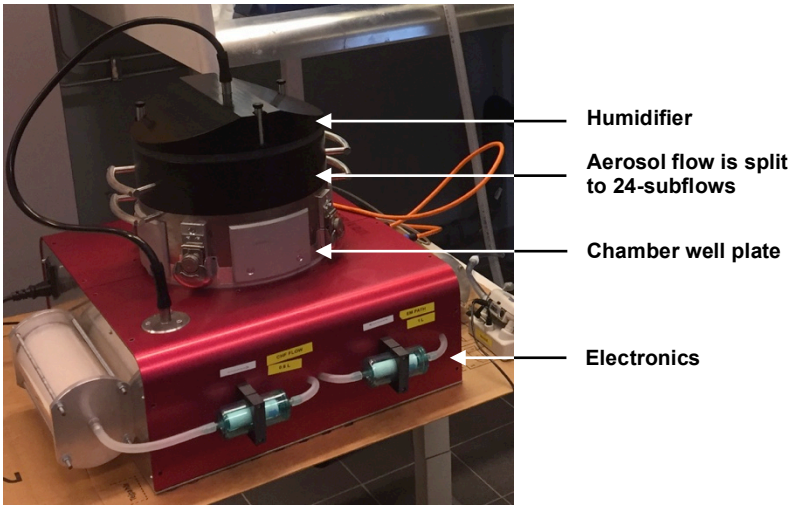

Figure B.2. The NACIVT system.

Appendix C Aerosol total concentrations and mean sizes

C.1 Aerosol generation without cell exposures

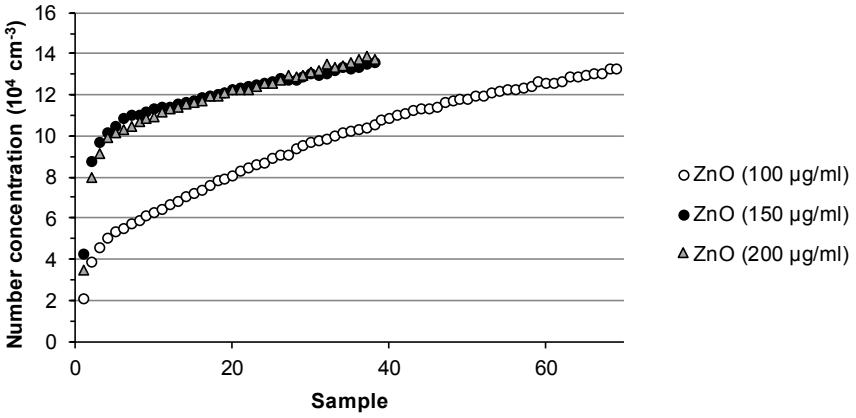

Figure C.1.1. Number concentration for each sample and ZnO NP concentration in the solution.

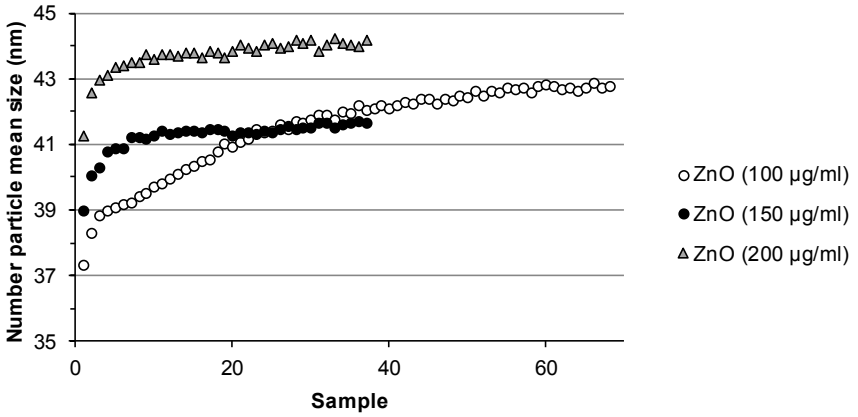

Figure C.1.2. Number particle mean size for each sample and ZnO NP concentration in the solution.

C.2 Aerosol generation for the cell exposures

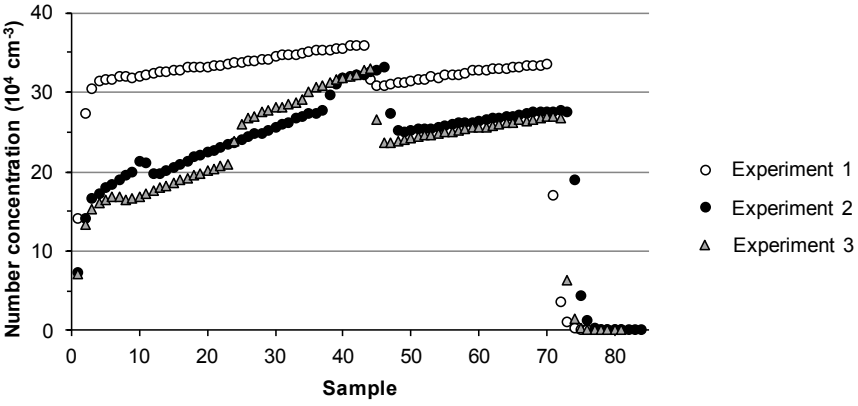

Figure C.2.1. Number concentration for the entire particle generation in each experiment with aerosol particles. When the NACIVT system is coupled to the mixing volume the particle concentration decreases (just after sample 40 for each experiment).

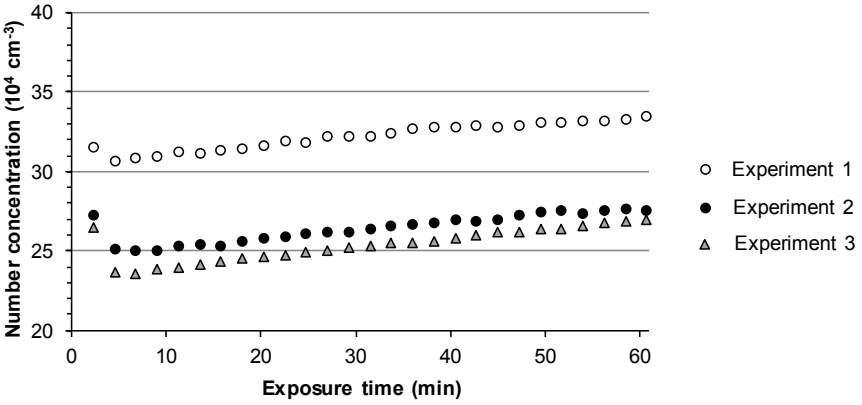

Figure C.2.2. Number concentration for the three experiments with aerosol particles. The number concentration of particles continued to rise during the entire exposures.

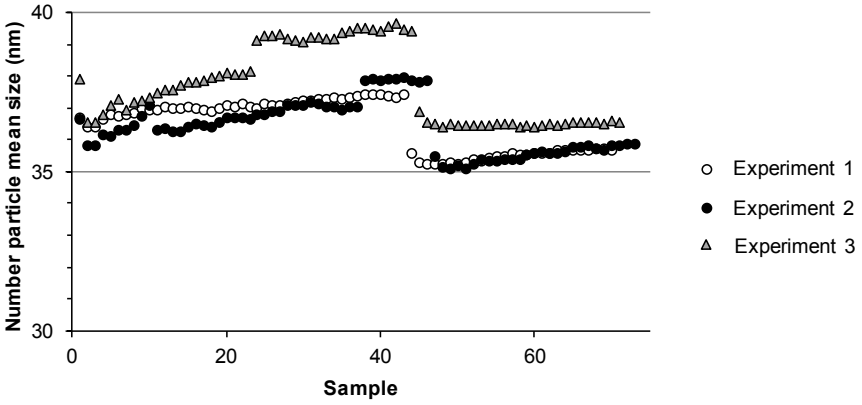

Figure C.2.3. Number particle mean size for all samples in the particle generation in each experiment with aerosol particles. When the NACIVT system is coupled to the mixing volume the particle concentration decreases, which seems to result in smaller particle sizes in all experiments (just after sample 40 for each experiment).

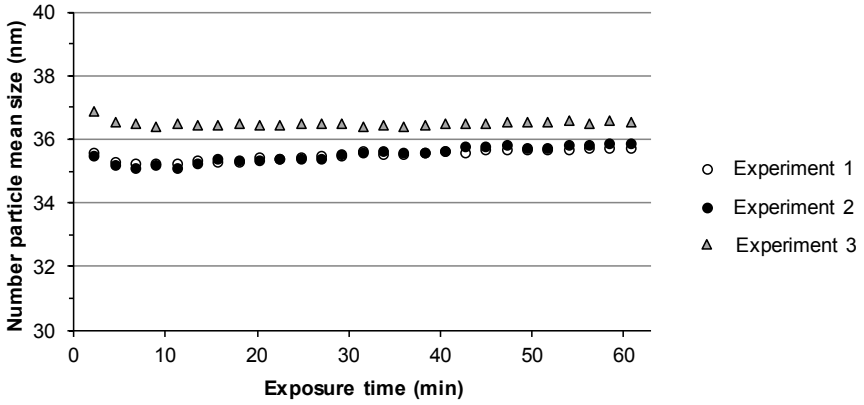

Figure C.2.4. Number particle mean sizes for the three experiments with aerosol particles. The particles remained to be about the same size during the entire exposures.