TOXICITY OF METAL AND METAL OXIDE NANOPARTICLES

The importance of physicochemical properties and cellular uptake

Pontus Cronholm

Stockholm 2012
Cover: TEM image of intracellular Ag nanoparticles.

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To Family and Friends
ABSTRACT

The use of nanoparticles holds great promises in many technical as well as medical applications. However, development of new technologies, such as nanotechnology, is connected with uncertainties and risks. The same properties that from a technical point of view are beneficial may in other aspects be unwanted and harmful for both humans and the environment. In order to avoid unnecessary risks and facilitate the use of safe nanotechnology there is a need for adequate toxicological research, as well as risk assessments of nanoparticles and nanotechnologies. This thesis is mainly focusing on the hazards (toxicity) of nanoparticles, and more specifically metal and metal oxide containing nanoparticles.

In paper I, the ability of different nanoparticles, as well as multi-walled carbon nanotubes (MWCNT), to induce a cellular response based on their material composition, was investigated. A high variation between the different particles to induce cytotoxicity, DNA damage and oxidative DNA lesions was observed, where CuO nanoparticles were the most potent.

In paper II and III, the role of particle-size on cytotoxicity, DNA damage, mitochondrial depolarization and induction of oxidative DNA lesions was studied. Amongst a number of particle types, only Cu and CuO particles displayed clear size-dependent effects where the nanoparticles were more toxic than the micro-sized particles.

In paper IV, the impact of different methodological settings, such as sonication and the use of serum in the cell medium when preparing nanoparticle suspensions, was investigated. Observations revealed that sonication of Cu nanoparticles caused decreased cell viability and increased Cu release compared to non-sonicated particles. Furthermore, serum in the cell medium resulted in less particle agglomeration and increased Cu release compared with medium without serum, but no clear difference in toxicity was detected.

In paper III, IV and V, the degree of metal release from Cu, CuO and Ag nanoparticles and subsequent impact on particle toxicity, was investigated. Even though a high Cu release was observed within hours after suspending the particles in cell medium, a toxic response was dependent on intracellular particle uptake, via a so-called Trojan horse type mechanism. In comparison to the high toxicity observed for Cu and CuO nanoparticles, no DNA damage or cytotoxicity was observed after exposure to the Ag nanoparticles, which is likely to depend on low Ag release from the particles.

In conclusion, a key property of metal and metal oxide nanoparticles is the release of ions facilitating a toxicological response. Via a so-called Trojan horse type mechanism the solid particles can facilitate uptake into cells and subsequently release toxic ionic species.
LIST OF PUBLICATIONS

I. Hanna L. Karlsson, Pontus Cronholm, Johanna Gustafsson, Lennart Möller. Copper oxide nanoparticles are highly toxic: A comparison between metal oxide nanoparticles and carbon nanotubes. Chemical research in toxicology, 2009, 21(9), 1726-1732

II. Hanna L. Karlsson, Johanna Gustafsson, Pontus Cronholm, Lennart Möller. Size-dependent toxicity of metal oxide particles-A comparison between nano- and micrometer size. Toxicology letters, 2009, 188(2), 112-118


Additional publications:


TABLE OF CONTENTS

1 Nanoparticle toxicology ........................................................................................................ 1
  1.1 A perspective .................................................................................................................. 1
    1.1.1 What can we learn from history: Ambient air pollution as well as
      occupational exposure ................................................................................................. 1
    1.1.2 Nanotechnology in the 21th Century: Saviour or risk? .................................. 3
  1.2 No exposure no risk ........................................................................................................ 5
  1.3 Lung deposition and mechanisms of toxicity ................................................................. 6
    1.3.1 Cytotoxicity ........................................................................................................... 8
    1.3.2 DNA damage ......................................................................................................... 9
    1.3.3 Oxidative stress ................................................................................................... 10
    1.3.4 Inflammation ....................................................................................................... 11
  1.4 Definitions ..................................................................................................................... 12

2 Research aims .................................................................................................................... 13

3 General approach and analytical methods ......................................................................... 14
  3.1 Particle Characterisation .............................................................................................. 14
    3.1.1 Particle size and shape ....................................................................................... 14
    3.1.2 Agglomeration .................................................................................................... 15
    3.1.3 Zeta potential ...................................................................................................... 15
    3.1.4 Surface area ......................................................................................................... 15
    3.1.5 Surface chemical composition ............................................................................ 16
  3.2 Metal release .................................................................................................................. 16
  3.3 Toxicity testing .............................................................................................................. 17
    3.3.1 DNA damage ....................................................................................................... 17
    3.3.2 Cytotoxicity ......................................................................................................... 18
    3.3.3 Mitochondrial damage ....................................................................................... 18
    3.3.4 Intracellular ROS ............................................................................................... 18
  3.4 Cellular dose and intracellular uptake ......................................................................... 19
    3.4.1 Cellular dose ....................................................................................................... 19
    3.4.2 Intracellular uptake ............................................................................................ 19

4 Particle properties influencing toxicity – Discussion and Results .................................... 20
  4.1 Material composition ..................................................................................................... 20
  4.2 Size and surface area .................................................................................................... 22
  4.3 Crystal structure and surface reactivity ......................................................................... 25
  4.4 Metal release and particle solubility ............................................................................ 26
  4.5 The Trojan horse type mechanism - Cellular uptake and subsequent
    intracellular metal release ............................................................................................. 29
  4.6 Shape ............................................................................................................................. 33
  4.7 Surface charge ............................................................................................................. 34
  4.8 Methodological aspects and toxicity – Use of serum and sonication ......................... 35
  4.9 Concluding remarks .................................................................................................... 38

5 Acknowledgements ........................................................................................................... 40

6 References ......................................................................................................................... 42
### LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>A549</td>
<td>Human type II alveolar epithelial cell line</td>
</tr>
<tr>
<td>AAS</td>
<td>Atomic absorption spectroscopy</td>
</tr>
<tr>
<td>ALS</td>
<td>Alkali labile site</td>
</tr>
<tr>
<td>BEAS-2B</td>
<td>Human bronchial epithelial cell line</td>
</tr>
<tr>
<td>BET</td>
<td>Brauner-Emmett-Teller</td>
</tr>
<tr>
<td>Caco-2</td>
<td>Epithelial colorectal adenocarcinoma cell line</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
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<tr>
<td>CNT</td>
<td>Carbon nanotubes</td>
</tr>
<tr>
<td>COPD</td>
<td>Chronic obstructive pulmonary disease</td>
</tr>
<tr>
<td>CRM</td>
<td>Confocal Raman Microscopy</td>
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<tr>
<td>DCFH-DA</td>
<td>Dichlorofluorescein diacetate</td>
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<tr>
<td>DDT</td>
<td>Dichlorodiphenyltrichloroeythane</td>
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<tr>
<td>DLS</td>
<td>Dynamic light scattering</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<tr>
<td>ECHA</td>
<td>European Chemical Agency</td>
</tr>
<tr>
<td>FaPyAde</td>
<td>4,6-diamino-5-formamidopyrimidine</td>
</tr>
<tr>
<td>FaPyGua</td>
<td>2,6-diamino-4-hydroxy-5-formamidopyrimidine</td>
</tr>
<tr>
<td>FPG</td>
<td>Formamido pyrimidine DNA glycosylase</td>
</tr>
<tr>
<td>FTIR</td>
<td>Fourier-transform infrared spectroscopy</td>
</tr>
<tr>
<td>GI</td>
<td>Gastrointestinal</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>Granulocyte macrophage stimulating factor</td>
</tr>
<tr>
<td>HaCaT</td>
<td>Human keratinocyte cell line</td>
</tr>
<tr>
<td>HARN</td>
<td>High aspect ration nanomaterials</td>
</tr>
<tr>
<td>LD$_{50}$</td>
<td>Lethal dose for 50% of the population</td>
</tr>
<tr>
<td>LSCM</td>
<td>Laser scanning confocal microscopy</td>
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<tr>
<td>MTT</td>
<td>3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide</td>
</tr>
<tr>
<td>MWCNT</td>
<td>Multi-walled carbon nanotubes</td>
</tr>
<tr>
<td>n</td>
<td>Nano = (10$^{-9}$)</td>
</tr>
<tr>
<td>N7 methylGua</td>
<td>N7-methylguanine</td>
</tr>
<tr>
<td>8-oxoGua</td>
<td>8-oxoguanine</td>
</tr>
<tr>
<td>p</td>
<td>Pico = (10$^{-12}$)</td>
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<tr>
<td>PCB</td>
<td>Polychlorinated biphenyl</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
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<tr>
<td>---------</td>
<td>-------------</td>
</tr>
<tr>
<td>PM0.1</td>
<td>Particulate matter &lt; 0.1 μm</td>
</tr>
<tr>
<td>PM2.5</td>
<td>Particulate matter &lt; 2.5 μm</td>
</tr>
<tr>
<td>PM10</td>
<td>Particulate matter &lt; 10 μm</td>
</tr>
<tr>
<td>PNS</td>
<td>Peripheral nervous system</td>
</tr>
<tr>
<td>REACH</td>
<td>Registration, Evaluation, Authorisation and Restriction of Chemicals</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>SEM</td>
<td>Scanning electron microscopy</td>
</tr>
<tr>
<td>SPG</td>
<td>Stockholm Particle Group</td>
</tr>
<tr>
<td>SSB</td>
<td>Single stand break</td>
</tr>
<tr>
<td>SWCNT</td>
<td>Single-walled carbon nanotubes</td>
</tr>
<tr>
<td>TEM</td>
<td>Transmission electron microscopy</td>
</tr>
<tr>
<td>TMRE</td>
<td>Tetramethylrhodamine ethyl ester</td>
</tr>
<tr>
<td>μ</td>
<td>Micro = (10^-6)</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organisation</td>
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<tr>
<td>XPS</td>
<td>X-ray photoelectron spectroscopy</td>
</tr>
<tr>
<td>XRD</td>
<td>X-ray powder diffraction</td>
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1 NANOPARTICLE TOXICOLOGY

1.1 A PERSPECTIVE

1.1.1 What can we learn from history: Ambient air pollution as well as occupational exposure

The use of nanoparticles holds promises in many technical, as well as medical applications. On the other hand, development of new technologies, such as nanotechnology, is connected with uncertainties and risks with regards to safety and impact on human health. This thesis is mainly focusing on the hazards (toxicity) of nanoparticles, and more specifically, metal and metal oxide containing nanoparticles. Nanoparticles are generally defined as particles smaller than 100 nm in diameter. When generated naturally or incidentally by anthropogenic activity, in contrast to nanoparticles that are purposely engineered, they are commonly also called “ultrafine particles” [2]. When considering an overall exposure to particles, not only to those in the nano-size range, history, as well as present day events, have taught us that airborne particulate matter greatly impacts people’s health [3]. Thus, to assess the hazards of nanoparticles, it is important to embrace a wide spectra of knowledge regarding human health effects of airborne particle matter.

One of the best known historical incidences, which resulted in severe health outcomes due to airborne particulate matter, is the London smog event in December 1952. Apart from industrial use, intensive burning of coal for heating generated high levels (>1000 μg/m$^3$) of airborne particles that remained trapped over the city due to staid atmospheric conditions; foggy weather and very little wind. This episode was followed by a substantial increase in mortality, with around 12,000 deaths. Together with earlier incidences, such as in Meuse Valley in Belgium in 1931 and in Donora in Pennsylvania in 1948, the London smog event contributed to an increased public awareness to the health effects of air pollutants. This led to governmental acts to reduce emissions from industries and homes. In 1956, The British clean air act was introduced as the first action to reduce airborne particle levels. This first act was accompanied by epidemiological studies on the health effects of airborne particle matter and efforts were made to reduce the emissions [4].

Exposure to crystalline silica (quartz) has historically been a cause of severe lung disease. Occupationally related deaths from silicosis (a fibrotic lung disease caused by inhalation of crystalline silicon dioxide or silica) have been described since the 18th century amongst knife grinders and construction workers of millstones. Since quartz is one of the most abundant minerals, miners and construction workers have been exposed since beginning of modern history. During a tunnel construction in West Virginia in the 1920s, 764 people died in acute silicosis and another 1 500 developed the disease, out of a total of 2 500 workers [5]. Even though the cases of silicosis are declining in developed countries, it is a major public health threat even to this day. China has most patients with silicosis, where the situation is most acute for small-scale miners. Annually, about 24,000 deaths are reported due to silicosis in China [6]. The use of asbestos has also been a large contributor to lung disease and death. It has been used in
for example cement, tiles, fillers, brake linings, pipes and insulation [5]. It is the shape and bio-persistent (non-degradable) nature of the material that causes the toxic response. Macrophages, an essential cell type handling clearance of inhaled foreign material, cannot cope with the asbestos fibre, which causes chronic inflammation and leakage of oxidative radicals [7]. A disease intimately associated to asbestos exposure is mesothelioma, a malignant cancer in the plural space of the lung with no known cure. Although asbestos is banned in most western countries it is still causing hundreds of thousands of deaths each year due to its historical use. Taking the United Kingdom as an example, deaths in mesothelioma due to asbestos exposure are predicted to peak between 2011 and 2015, resulting in the loss of about 2000 lives each year. Exposure to asbestos is still occurring to this present day when released during reconstruction/tearing of old buildings, as well as continued usage in many countries [5, 8].

According to air quality data from WHO in 2011, 2 million deaths globally are each year estimated to be caused by airborne pollution and particle matter. The particles are mostly generated from motor vehicles, industries and burning of fossil fuels or biomass for heating and cooking and large scale coal power plants. In 2008, outdoor air pollution in cities was estimated to cause 1.34 million deaths, which is a substantial number considering that if WHO guidelines of 20 μg/m³ annual mean of PM10 would be met, about one million premature deaths could be avoided each year. The average level of PM10 in the world (inhalable particles sized ≤ 10 μm) is 71 μg/m³, spanning from 21 to 142 μg/m³ depending on region. These values are based on observations from monitoring stations in over 1000 cities, representative of human exposure [9].

Exposure to airborne particulate matter is mainly related to cardiovascular and pulmonary disease [10, 11]. Exposure to high concentrations of airborne particles, are in many developing countries increasing the risk for lower respiratory infections, resulting in mortality in young children. It is also a major risk for chronic obstructive pulmonary disease (COPD) and lung cancer in adults [12, 13]. The levels of particles (PM10) in Sweden are in the lower part of the scale, reported to be 25 μg/m³ as mean annual concentration, as population-weighted average in cities with more that 100 000 citizens [14]. However it has been observed that reductions in exposure levels also give positive health effects even with relatively small changes in particle concentrations. Differences in health outcomes are evident even comparing relatively low particle concentrations, where the trend is extending below 15 μg/m³ [11]. In a large study conducted in the US, it was seen that an increase in annual average exposure level of PM2.5 (2.5 μm or less) with 10 μg/m³, increased the incidences of death as a consequence of cardiopulmonary diseases and lung cancer with 9%, and 14%, respectively [10].

There have always been difficulties in understanding the associations between specific properties of airborne particle matter and health effects. This is a result of the complexity of their physicochemical nature and the various sources of particle origin. Particles can be derived from both natural as well as anthropogenic sources and be subcategorised based on size and chemical composition [3]. One important chemical component is transition metal constituents. Transition metals impact on health due to their potential to produce reactive oxygen species (ROS) through Fenton reactions or catalyzed by Harber-Weiss reactions [15]. Such metals are iron, copper, chromium and vanadium, to mention a few. Metals that are leaching, or present on the particle
surface, affect biological systems \[15\]. During inflammation and subsequent release of oxidative species, transition metals can trigger redox-cycling cascades and thereby increase the oxidation potential causing depletion of antioxidants and cell damage \[16\]. In the following equation the net result of iron catalyzed Harber Weiss and Fanton reaction is seen \[15\].

\[
\cdot O_2 + H_2O_2 \xrightarrow{Fe} \cdot OH + OH^- + O_2
\]

Particle size is also an important factor when investigating the health effects of particle exposure \[3\], thus, special awareness has been drawn to the nano-sized particles and their contribution to observed health effects. Toxicological evidence suggest that the nano-sized fraction pose particularly detrimental effects on the cardiovascular system \[11\]. Airborne particle matter (PM) is normally categorized as follows; thoracic particles are less than 10 \(\mu\)m (PM10) and represent the upper limit of respirable particles. Coarse particles span between 2.5 and 10 \(\mu\)m (PM 2.5-10) and fine particles are less than 2.5 \(\mu\)m (PM2.5). Ultrafine particles (PM0.1), interchangeably also termed nanoparticles, are less than 0.1 \(\mu\)m (100 nm), and are deposited farthest into the lung (figure 1). Due to their small size and high abundance, nanoparticles display a large surface-to-weight ratio \[17\], which needs to be considered when determining limit values on particle concentrations in occupational and environmental settings.

With the emergence of industries based on nanotechnology, various nano-products have been presented on the market, calling for more detailed mapping of possible health effects. Thus, alongside this development, a new field of toxicology has appeared – nanotoxicology.

1.1.2 Nanotechnology in the 21th Century: Saviour or risk?

Owing to the small size of nanoparticles, properties that are normally attributed to molecules (movement) and solid states (e.g. optical, magnetic, thermodynamic), can be combined in them \[18\]. As discussed later (section 1.3 and 4.5), these physicochemical properties enable the solid particles to pass barriers, such as cell membranes and thereby affect their uptake and distribution in an organism or single cell. Nanoparticles smaller than about 30 nm have an excess of energy on a thermodynamically unstable surface, which can effect material properties. For example, the melting point of indium and tin decreases with about 100 °C when particle diameter changes from 100 nm to 10 nm, and below 15 nm the melting point decreases exponentially \[19\].

The physical, chemical and biological applications of nanomaterials and nanoparticles have revolutionized the technology within industrial, environmental, communication and medical engineering \[17\]. In medicine, nanoparticles can be used as drug carriers in cancer treatment. Increased cancer cell targeting can be achieved through target-molecules bound to the particle surface, and specific drug delivery by loading the particle core with therapeutic agents, which are then released upon reaching the target. Fluorescent and magnetic nanoparticles are key players in the development of probes for disease diagnosis and imaging. Nanoparticles and nanomaterials also have applications in personalised medicine for targeting treatments suitable for individuals of
diverse genetic backgrounds that affects metabolism and response to environmental factors [20, 21]. Examples of environmental benefits in using nanotechnology include reducing energy consumption, green house gas emissions and pollutions as cleaner and more effective industrial processes can be achieved [22-24].

Nanoparticles and nanomaterials of various properties are frequently used and new applications are found on a daily basis. Silver, gold, titanium, silica/silicon and zinc are, together with carbon, rated as the top six of the most commonly used materials in nanoparticles. Since the beginning of the nano-product inventory started in 2006 the amount of consumer products based on nanotechnology have increased more than five fold. In 2010, the number of products listed to contain nanomaterials had raised over 1000 and around 2000 products are estimated to be on the market by 2012 [25]. In the beginning of the 20th century nanotechnology was thought to be exclusive to researchers only. However, the nanotechnology industry has been estimated to a staggering 2.6 trillion US dollars in manufactured goods by 2014 [26]. As previously mentioned, metal nanoparticles have uses in optical, magnetic, thermal, electrical and sensor devices, as well as cosmetics and biomedicine [27, 28]. For instance, Cu nanoparticles are used in a wide range of products like facial sprays, lubricants, antioxidant and anode materials for lithium ion batteries, as well as fuel additives to reduce friction for a mending effect in engines, [29]. Silver is the most frequently used nanomaterial in consumer products (electronics, paints, cosmetics, household machines, clothes/fabrics, in food technology etc.) and has gained growing interest in the biomedical field due to its potent antibacterial activity [28, 30, 31]. In addition to Ag nanoparticles, the metal oxide CuO also serves as an antimicrobial agent used in wood preservation, paints and antibacterial textiles, and these nanoparticles have also applications as heat transfer agents in thermal fluids [32, 33]. CuO and silver are both effective in killing a range of microorganisms including yeast, algae, bacteria and viruses, rendering them commonly used antimicrobial agents [32].

The applications of nanotechnology are many and the benefits of its use is institutionalised among many stakeholders in society, by the industry, politicians and the scientific community [34].

"By creating jobs, stimulating economic growth, and providing solutions to some of the toughest challenges facing humankind, nanotechnology has great potential to change the world for the better." (President’s Council of Advisors on Science and Technology, March 2010) [35].

"Nanotechnology is an area, which has highly promising prospects for turning fundamental research into successful innovations. Not only to boost the competitiveness of our industry but also to create new products that will make positive changes in the lives of our citizens, be it in medicine, environment, electronics or any other field." (European Commissioner for Science & Research, Janez Potočnik) [36].

The above quotes give clear indications of the belief that nanotechnology has no limits. However, development of new technologies is always associated with uncertainties and risks. Properties that are desirable in technical aspects can prove to be unwanted and harmful for both humans and the environment. Some examples from
the past are asbestos, DDT and PCB, chemicals that were once considered promising, but are banned since decades as knowledge of their detrimental health effects were revealed [37]. We are still in a relatively early stage of nanotechnology development and its impact on our society is likely to grow, warranting increased understanding of nanomaterial toxicity in order to avoid negative health effects and environmental outcomes. On the other hand, research and risk assessment is also important for avoidance of unnecessary prohibition of useful materials and products that could benefit society.

Nanotechnology: Saviour and risk?

In order to navigate in the fast growing era of nanotechnology and create regulations, there is a need for a close collaboration between nanotechnology development, policy makers, and research concerning possible hazards and risks for the overall benefit of society. Furthermore, as stated by Andrew D. Maynard in the report, Nanotechnology: a research strategy for addressing risk 2006 [38].

“With a sound, science-based and sensible research strategy, we can provide nano-businesses –large and small- with the tools they need to identify and reduce or remove possible dangers to health and the environment. But without the right research plan and investments, the safety and sustainability of emerging nanotechnologies is uncertain at best.”

The promises of nanotechnology are many, however without adequate research making it possible to avoid future risk, those promises can suffer serious set backs.

1.2 NO EXPOSURE NO RISK

The risk of drowning is negligible if you are not close to water. In analogy, this is also true for nanoparticles, as risk is the product of hazard and exposure. This thesis is mainly focusing on the hazards (toxicity) of nanoparticles, although it is equally important to estimate exposure and nanoparticle life cycle in order to understand the impact on human health.

Depending on the area of nanotechnology application, the route of exposure and uptake by biological systems are different. The respiratory tract is the most investigated point of entry [38] as it serves as a first, crude target for airborne particles [39]. On a daily basis a person can inhale 20 m³ of air resulting in deposition of airborne particles on the epithelial surface of the lung. Other routes of uptake involve the skin, ingestion and injection. Skin uptake of nanoparticles occurs through consumer products such as cosmetics and cloths. Uptake via the gastro intestinal tract is relevant for nanoparticles added into food and food-packaging. It is also a feasible route for particles that are cleared from the lung by mucociliary clearance and subsequently swallowed. Finally, nanoparticle use in medicine for imaging and drug delivery, is dependent on delivery through injections [40, 41].
In order to assess nanoparticle exposure, a complete view of the nanoparticle/nanoproduct life cycle needs to be addressed. The life cycle can broadly be divided into three main sections, i) production, ii) usage and iii) disposal \[42\]. During production, exposure can occur through handling materials that pose a risk. Exposure during the use of a nanoproducts is highly dependent on the area of application and whether the nanomaterials is used in conjunction with other materials. Exposure to nanomaterials used in cosmetics and clothing, directly on or close to the skin, is more likely to occur compared to exposure to nanomaterials bound inside a material in for example an electronic product. If careful handling of nanomaterial-containing products is needed, it is likely that disposal can be done in a controlled way. To present day, there is no mandatory regulation on labelling goods containing nanomaterials, making it extremely difficult for both consumers and disposal plants to know if products may contain nanomaterials or not. Upon establishing regulation, there might be an impending risk, similarity to other waste products, for these nanomaterials to end up in countries with less strict regulations \[42\].

Apart from engineered nanomaterials, environmental (e.g. combustion) and occupational exposure (e.g. welding and in steelworks) to nanoparticles that are unintentionally produced, may pose a large risk as compared to particles that are produced in a controlled manner.

1.3 LUNG DEPOSITION AND MECHANISM OF TOXICITY

Key parameters to assess the potential health risks from nanoparticles are deposition in the respiratory tract, internal fate and translocation. Ultimately, it is the deposited dose and internal exposure that determines the response and, thereby, also the risk \[3, 43\]. Besides concerns regarding the reactivity of nanoparticles, their size enable deposition deep into the lung with exposure and uptake different to those observed for larger particles. As seen in figure 1, larger micro-sized particles are predominantly deposited in the upper airways, whereas particles in the nano-size range are transported, and to large extent deposited in the alveolus and tracheobronchial region. Particles in the lower nano-size range are, however, mostly deposited before reaching the trachea. It should be noted that the aerodynamic properties and mobility size, determining particle deposition, cannot be predicted from the primary particle dimension if the particles are agglomerated or aggregated \[1\]. For hygroscopic particles, the mobility size will increase due to uptake of water in the humid lung \[44\]. Deposition of nanoparticles is mainly dependent on diffusion, which is the principle deposition mechanism for particles less than 500 nm. In contrast to nanoparticles, deposition of larger particles is dependent of impaction, gravitational settling and interception \[45, 46\].
On inhalation, the fate of the particles depends on their size, solubility and site of deposition. In the ciliated airways, deposited particles are predominately cleared by mucociliary transport. However, the alveoli region lacks such mechanisms and clearance is mediated by alveolar macrophages that internalize the particle by phagocytosis and move toward the mucociliary escalator \cite{40,47}. After reaching the pharynx the particles are swallowed into the GI tract and are eliminated from the respiratory tract \cite{48}. Transport of the macrophages out of the alveolus region is rather slow and can take from weeks to moths, whereas, the clearance in the upper airways occurs within hours post deposition \cite{48}. The optimal particle size for macrophage clearance is estimated to be between 1 and 5 μm and the uptake efficacy of both smaller and larger particles is reduced. Within this size range, phagocytosis is fairly effective and results in uptake of nearly 100 % of the particles. For nanoparticles, however, uptake has been observed \textit{in vivo} to be less effective, suggesting that they can escape phagocytic clearance mechanisms in the alveoli \cite{48}.

A prolonged retention and failure of macrophages to recognize and efficiently remove nanoparticles, results in the possibility of the particles to be internalized by epithelial cells and connective tissue, and further transport into systemic circulation \cite{49}. In a recent review (2010), Geiser and Kreyling reported that there was evidence for translocation of gold, silver, TiO$_2$, polystyrene and carbon nanoparticles across the air-blood barrier in animal models, as particles were found in the blood circulation or secondary organs \cite{48}. The literature on translocation of nanoparticles from the lung in human subjects, however, is rather conflicting and there is no clear evidence for

\textbf{Figure 1.} Predicted regional deposition of inhaled particles of different sizes. Based on data from the International Commission on Radiological Protection (1994). The figure is reproduced from Oberdörster et al \cite{1}, with permission from Environmental Health Perspectives, with slight modifications.
translocation of more than 1% of the mass dose delivered. Methodological difficulties with leakage of radiolabel isotopes, as well as other technical and ethical constraints are making the research difficult \cite{48, 50}. The efficiency of systemic translocation is evidently influenced by the physicochemical properties of the particles. In an \textit{in vivo} study investigating the translocation of a number of differently sized and charged nanoparticles, non-positively charged particles less the 34 nm were most efficient to translocate from the lung into the lymph nodes, where further distribution to the bloodstream and organs can occur \cite{51}. Figure 2 illustrates uptake and translocation pathways following exposure to nanoparticles.

Exposure to airborne nanoparticles is associated to induction of cardiac events such as heart rhythm disturbances and heart infarctions. It is possible that these effects can be explained by inflammation however it may also include effects generated by particles that have been able to pass the pulmonary system and translocate to the blood circulation \cite{17}. The concerns regarding the impact of nanoparticles on human health are both due to the increased ability of nanoparticles to reach deep in to the lungs, with possible translocation to distal organs, as well as an increased reactivity and toxicity of nanoparticles compared to corresponding lager particle of the same material \cite{17}. This thesis is mainly focusing on the latter, e.g how different nanoparticle properties are influencing the toxicological response and the mechanisms by which the toxicity occurs.

![Figure 2. Uptake and translocation of nanoparticles in the body. Although many uptake and translocation routes are established, a substantial number is still hypothetical and requires further investigated. The translocation rates are also largely unknown. CNS and PNS is the peripheral and central nervous system, respectively. The figure is reproduced from Oberdörster et al \cite{1}], with permission from Environmental Health Perspectives.]

1.3.1 Cytotoxicity

Cytotoxicity, synonymous to measurement of cell death (or its opposite, cell viability) is one of the most studied endpoints when conducting \textit{in vitro} toxicological and nanotoxicological research. Cytotoxicity can be seen as a relatively rough measure of toxicity, were cells are counted as either dead or live. However, with the recent
advances in the studies of cell death, 13 different types of cell deaths are listed [52].
Despite the many different mechanisms, cell death has generally been divided and
described as either necrosis or apoptosis. In broader terms, apoptosis is seen as
programmed and cell-regulated induction of cellular destruction/death, whereas
necrosis is defined as uncontrolled. Necrosis results in unrestrained leakage of the
cellular components into the surrounding capable of initiating an inflammatory
response [53, 54]. More recent evidence, however, shows that necrosis can also occur in a
regulated manner called necroptosis [55] illuminating that there is more to the
toxicological significance of cell death compared to previous beliefs. As an example,
impairment of cell death through compromised apoptotic mechanisms contribute to
tumour progression, but induction and activation of these mechanisms are also
responsible for acute toxicity of many investigated toxicants [52].

Because nanoparticles of different origin have diverse physicochemical characteristics,
their biological responses and subsequent mechanisms will produce dissimilar
cytotoxic effects. A thorough characterisation and identification of the important
physicochemical properties on the different mechanisms of cytotoxicity require a broad
range of cytotoxicity assays [56]. This thesis will present two different cytotoxicity
assays; MTT and Trypan blue staining. The MTT assay measures cell viability by
determining mitochondrial function in living cells, whereas Trypan blue staining is
investigating the integrity of the cell membrane. Further details about the cytotoxicity
assays used in this thesis are described in the section covering analytical methods
(3.3.2).

1.3.2 DNA damage

Investigation of DNA damage is important in all fields of toxicology research. If not
repaired correctly it can lead to mutations with subsequent risk for numerous diseases
[57]. DNA damage is considered as an important aspect in cancer development and
assays measuring various gene mutations, chromosomal aberrations and DNA strand
breaks can therefore be used in cancer risk assessments [58]. From of a nanotoxicology
perspective there are a number of mechanisms through which nanoparticles are
believed to cause DNA damages:

(1) Nanoparticles may pass into the cell nuclei, either during mitosis or through
the nuclear pores. In the nuclei, nanoparticles can gain direct contact with the
DNA and cause damage [57].
(2) Generation of ROS on the surface of nanoparticles, or by releasing metal or
organic species from the surface. The ROS generated are subsequently able to
react with the cell and damage the DNA [17].
(3) The nanoparticles may interact to damage or stimulate target cells to produce
ROS by affecting the electron transport in mitochondria, and inducing
cytochrome P450 enzymes or NADPH-oxidase [15, 57].
(4) The nanoparticles may activate the inflammatory system and cause DNA
damage via ROS derived in activated phagocytes, such as neutrophils and
macrophages [57, 59].
As described in (4), DNA damage dependent on nanoparticle dose, which generates an inflammatory response is generally referred to as secondary genotoxicity. The dose must be sufficiently high and surpass a threshold to trigger inflammation [60]. Primary genotoxicity is defined by genetic damage obtained by the particles, either directly as in (1) or indirectly as in (2) and (3). In contrast to secondary genotoxicity, primary genotoxicity has no dose threshold and the DNA damage is linearly related to the dose [61]. DNA damages mainly consist of transient lesions with a balance between damages and repair [62]. Even if there is no threshold, theoretically a single DNA lesion can cause a mutation and lead to increased cancer incidence although the increased cancer risk for each DNA damage is infinitesimally small [61].

This thesis focuses on primary genotoxicity directly caused by particles, ROS, other intermediate species generated by the particles or by target cells. Assessment has been done using the comet assay measuring DNA damages in the form of single strand breaks and alkali labile sites, as well as oxidative lesions (mainly oxidized purines). In figure 3 the listed mechanisms, which induce DNA damage are depicted.

**Figure 3.** Different mechanism of DNA damage in nanotoxicology. 1) Nanoparticles may pass into the cell nuclei to directly cause DNA damage. 2) ROS generated, or metals and organic species released from the particle surface can subsequently interact with cellular components and damage the DNA. 3) The nanoparticles may interact to damage or stimulate target cells to produce ROS by affecting the electron transport in mitochondria or inducing cytochrome P450 enzymes or affecting NADPH-oxidase. 4) Activating the inflammatory system and cause DNA damage by ROS derived in inflammatory cells.

### 1.3.3 Oxidative stress

One key pathological pathway behind health effects related to particle exposure is oxidative stress. Oxidative stress can be defined as an imbalance between pro-oxidants and antioxidants with the former taking over. Under normal conditions ROS are created in low levels and are balanced by the antioxidant defence, but on excess production of ROS the defence system can be overwhelmed and oxidative stress occurs. Oxidative stress as a response to particle exposure can roughly be divided into two different mechanisms where ROS is generated (1) on the particles surfaces, or by metals and organic fractions, and (2) as a consequence of activated immune system or disruption of cellular components, such as the mitochondria in target cells [17]. ROS can, apart from inducing DNA lesions, also damage other macromolecules such as lipids, proteins and...
cellular membranes \[60, 63\]. The hierarchical oxidative stress model, is as follows; Tier 1) at low levels of oxidative stress antioxidant and detoxification enzymes maintain a balance in the cell. Tier 2) at higher levels of oxidative stress the balance cannot be maintained and an inflammatory response occur. Tier 3) oxidative stress progresses and the defence system is overwhelmed with subsequent cytotoxic effects where pro-apoptotic factors are released and cells may undergo apoptosis \[17\]. Oxidative stress caused by ROS is the most important mechanism behind outcomes such as respiratory infections, lung cancer, and chronic cardiopulmonary diseases following exposure to airborne particulate matter \[64\]. Due to this it is essential to evaluate the toxicity of nanomaterials, as well as their ability to generate ROS and induce oxidative stress. In the present thesis, generation of ROS has been investigated using the oxidation-sensitive fluoroprobe 2',7'-dichlorofluorescin diacetate (DCFH-DA). Further, a modified version of the alkaline comet assay, utilising formamidopyrimidine DNA glycosylase (FPG), has been used to measure oxidative lesions (mainly oxidized purines) in DNA.

1.3.4 Inflammation

Inflammation is a natural defence response to various numbers of physiological assaults, which can be pathogenic microorganisms, mechanical injuries, dust, drugs and various chemicals \[65\]. This response is beneficial for our health, however, uncontrolled inflammation can lead to severe disorders and diseases \[65\]. As an example, systemic release of cytokines upon exposure to particles may lead to initiation of heart attacks. This happens as the presence of clotting factors increases in the blood that cause dense clots and ruptured atherosclerotic plaque, which are known hallmarks of heart attacks \[63\]. As discussed in the previous section (1.3.3), failure to maintain a balance between pro-oxidants and antioxidants will lead to oxidative stress. As a response, a pro-inflammatory mechanisms can be provoked with activation of an intracellular cascade that regulate production of cytokines and chemokines \[63\]. In the hierarchical oxidative stress model this is described under Tier 2 \[17\]. Example of cytokines and chemokines connected to inflammation includes tumour necrosis factor alpha (TNF\(\alpha\)), interleukin IL8, IL1\(\alpha\), IL1\(\beta\), IL6 and granulocyte macrophage stimulating factor (GM-CSF). Each of these factors play a specific role in controlling and promoting inflammation \[60\]. During inflammation polymorphonuclear neutrophil cells (PMNs) are the first type of leukocytes to migrate to an inflammatory site, and by producing inflammatory mediators, they recruit more PMNs and other cell types like macrophages and lymphocytes. Macrophages exert a primary response to particle exposure. They can initiate and propagate an inflammatory reaction with their ability to recognise, engulf and digest the particle, which is important for the distribution and potential biodegradation of the particles. Particle interaction with macrophages commonly results in activation of the NADPH-oxidase system leading to production and release of ROS along with oxidative burst \[66\].
1.4 DEFINITIONS

The term nano is derived from the Greek word nano meaning dwarf[^67], and it refers to something being small. The prefix nano refer to a measure of $10^{-9}$ units, consequently 1 nm is $1 \times 10^{-9}$ m. Nanoparticles are often defined as a discrete entity with three dimensions in the order of 100 nm or less. This is consistent with what has been proposed by SCENIHR, 2008[^68]. Further, nanofibers or nanorods are defined to have two dimensions less than 100 nm and nanomaterials or nano-objects to have at least one dimension in the order of 100 nm or less (see figure 4). The nomenclature is, however, not settled and there are different parallel definitions or suggestions existing. In this thesis, nanoparticles are used as a collective term for both engineered and unintentionally produced, or formed nanoparticles, defined to have three dimensions in the order of 100 nm or less. In the scientific field, especially when considering occupational or environmental exposures, the term “ultrafine particles” is also used and then referring to particles smaller than 100 nm. Particles can group together to create larger structures. If these structures are held together by weak forces, such as van der Waals, electrostatic forces and/or surface tension, then they are referred to as agglomerates. If the particles are bound together by stronger forces, such as covalent or metallic bonds, then they are called aggregates[^68] (see figure 4).

![Diagram of nanoparticle, agglomerate, and aggregate](image)

**Figure 4.** Nanoparticles, nanofibers and nano-objects are defined as having three, two or one dimension less than 100 nm. Agglomerates are described as structures bound together by weak forces, whereas aggregates are described as structures bound together by strong forces.

There are several factors other than just the size that modulate toxicity and risk, which makes a definition difficult, especially for regulatory purposes[^69]. Making a definition strictly defined by particle size does not imply that larger or smaller particles are more or less relevant out of health and environmental point of view[^42]. As discussed by Oberdörster, there is no biologically plausible reason for a strict borderline at 100 nm that separate nanoparticles from larger particles[^70], and the definitions are there only as a gross estimate for assessment. For regulatory purposes, on the other hand, it may be better with a list of key attributes based on the latest knowledge. Such a list can for example include a range of attributes, regarding physicochemical characteristics important for particle toxicity (such as size and surface area). If a material fulfils a combination of these attributes, it can be defined as a material falling under certain regulations[^69].
2 RESEARCH AIMS

Increased understanding of nanoparticle toxicity and its impact on human health is essential to enable reliable risk assessments and safe use of nanoparticles in our society.

An effort has therefore been taken to study the toxicity, and the importance of different particle characteristics on the mechanisms of mainly metal and metal oxide nanoparticles.

Specific aims of this thesis include:

a) Assessing the toxicity in terms of cytotoxicity, DNA damage and oxidative stress following nanomaterial exposure as well as the role of material composition for the induction of such effects.

b) Investigating the role of particle size on the toxicity of a number of metal oxide particles.

c) Measuring the degree of metal release and dissolution of Cu and CuO nano- and micro-sized particles, and the impact of released copper fraction on particle toxicity.

d) Delve into the role of a Trojan horse type mechanism on the toxicity of Ag and CuO nanoparticles, i.e. the importance of the solid particle to mediate cellular uptake and subsequent release of metals inside the cell.

e) Evaluate the effect of different methodological settings used in nanotoxicological research. More specially, the impact of sonication and use of serum on the dispersion of the particle suspension, and toxicity of Cu nanoparticles in vitro.
3 GENERAL APPROACH AND ANALYTICAL METHODS

An inter-disciplinary approach has been used in order tackle the demands needed in nanotoxicology. The work summarized in this thesis is the product of a research establishment called Stockholm Particle Group (SPG), started in 2007. SPG combines toxicology, surface and corrosions science, and aerosol science. The different disciplines are represented by researches from the unit of Analytical Toxicology and Molecular Toxicology at Karolinska Institutet, the Divisions for Surface and Corrosion Science at the Royal Institute of Technology, and the group for Workplace Aerosols at Stockholm University. Understanding the interaction between biology, and the physicochemical properties of nanoparticles, is the key essence for understanding nanoparticle toxicity. The combination of expertise and instrument assets in SPG has been fruitful. In the following sections, a presentation of the different analytical techniques and their use is given.

3.1 PARTICLE CHARACTERISATION

Nanoparticle features are heterogeneous. They can come in different forms and with different properties. Fundamental in nanotoxicology is to understand the link between the physicochemical properties and the toxic response.

3.1.1 Particle size and shape

Size and shape of the particles has been studied using scanning and transmission electron microscopy (SEM and TEM). Electron microscopes use electrons instead of photons (normal light) to visualize a specimen. As electrons have a wavelength 100 000 times shorter than normal light electron microscopy gives greater resolution and magnification with the benefit of studying structures on the nano-size level. In all paper (I-V), electron microscopy has been used to visualize the particle features. In figure 5 TEM images of CuO nano- and micrometer particles, as analysed in paper II.
3.1.2 Agglomeration

Particles can group together creating larger structures, agglomerates. Agglomerates are generally defined as assemblage of particles joined together by relatively weak forces. The term aggregate is interchangeably used but is often referred to as an entity held together by stronger forces. When suspending particles in cell media, the nutrient medium used in such in vitro studies, particles will agglomerate to different extent. This process depends on several physicochemical properties of the particle, including its charge, but it is also reliant on particle concentration and the constituents of the surrounding medium. Particle agglomeration is likely to influence the outcome of a study, by affecting biodistribution and the interaction of the nanoparticles with the cell. Therefore, nanoparticle agglomeration is a feature important to monitor when conducting nanotoxicological studies. Particle agglomerate size has been measured in all papers (I-V), using laser diffraction or dynamic light scattering techniques (see figure 6). In paper IV, dynamic light scattering (DLS) was used to investigate the role of serum added to the growth media, on particle agglomeration. By measuring the random particle motion in the suspension (Brownian motion) and using the Stokes-Einstein equation the size of the particles/agglomerates can be calculated.

3.1.3 Zeta potential

Using the same system as for DLS (Zetasizer nano ZS, Malvern, UK), the zeta potential of particles have been measured. The zeta potential is often referred to as the particle charge. However, the zeta potential is sensitive to the constituents in the solution and is affected by adsorption of for example charged species. In paper I-II and V the zeta potential have been assessed. The zeta potential of the particles are not only a determining factor of agglomeration and particle stability in solution, but also impacts the creation of a so-called corona and particle interaction with cellular components. Zeta potential is measured by applying an electrical field through the particle solution. It will force particles with a specific zeta potential to move at different speed toward an oppositely charged electrode. The zeta potential is calculated from the speed, as it is proportional to the zeta potential.

3.1.4 Surface area

The surface area of particles have been analysed by means of Brauner-Emmett-Teller (BET) analysis, either measured within SPG or reported as specified by the manufacturer. The surface area of particles is central in nanotoxicological research. Then the size of particles decreases the relative surface area, per unit mass, increases.

Figure 6. Particle agglomerate size presented as percentage of number for Cu and CuO nanoparticles as studies by laser diffraction technique in paper III.
As a result more atoms are exposed on the surface rather than in the interior of the particle, giving an increased area that can mediate and drive toxic reactions. The BET measurements of specific surface area are based on the adsorption of nitrogen atoms on the particle surface where the adsorption of N$_2$ molecules allow to determine the particle surface area. The BET measurements are done on dry powder and not in particle suspension. Particle agglomerating, bound together by weak forces (as in a particle powder) will not affect the measured surface area, and the measurement is therefore representative for the surface area of the primary particles $^{[71]}$.

3.1.5 Surface chemical composition

Crystalline phase and surface composition of the particles investigated in paper III was analysed by means of XRD (X-ray powder diffraction), FTIR (Fourier-transform infrared spectroscopy), XPS (X-ray photoelectron spectroscopy). The CuO and Cu particles in paper III are of the same particle type as those in paper I, II, IV and V (see figure 7). In paper V the surface composition of the Ag and CuO nanoparticles, after exposure to the cells, was analysed by means of CRM (Confocal Raman Microscopy). Surface composition, as well as crystalline phase of particles, is decisive for the particles’ physicochemical properties and release of metals, and as such, it is important to characterise these factors when conducting nanotoxicological research.

3.2 METAL RELEASE

Measuring metal release and dissolution of particles is vital then studying toxicity of metal and metal oxide particles. Stable particles can accumulate in the body and stay active for a long time, whereas degradable particles may release reactive species causing acute effects. Analysing metal release and particle solubilisation is essential when considering nanoparticle life cycle analysis and persistence in eco-systems. In paper III, IV and V atomic absorption spectroscopy (AAS) has been used to analyse the released amount copper and silver from Cu and CuO nano- and micro-sized particles, as well as Ag nanoparticle. Atomic absorption spectroscopy is a common and useful technique to analyse the total metal concentration in a fluid. During sample testing a known amount of energy is passed through the atomized sample and its concentration is determined by measuring the quantity of light remaining after absorption by the element. In order to estimate the amount metal that is released from the particles during cell exposure, metal release have been performed in PBS (paper III), as well as in cell medium (paper III, IV and V).
3.3 TOXICITY TESTING

In this thesis, in vitro methodological approaches were used to perform the toxicity studies in cultured human cells. To mimic pulmonary exposure the studies have been performed on two different cell-types originating from the lung: A549 type II human epithelial cell line (paper I-V) and BEAS-2B bronchial epithelial cell line (paper V). To facilitate exposure the particles have firstly been suspended in cell culture medium, and subsequently added to the cells that were cultured in 37 °C and humidified atmosphere.

In comparison to in vivo studies, in vitro systems are less complex and relatively easy to perform, control and interpret [60]. Advantages include avoidance of animals sacrifice and less cost and time consuming [72]. For practical, economical and ethical reasons in vitro systems are needed when assessing the hazard of an increasing number of different types of nanomaterials as well as other substances. For similar reasons, the European Chemical Agency (ECHA) also addressed in REACH documentation states that in vitro studies are preferable in toxicological assessments [73, 74]. The loss in complexity, however, has a range of drawbacks. The in vitro approach cannot fully imitate the complex interactions between multiple cells within and between organs. As an example it is not possible to measure inflammation per se as it is dependent on interactions between multiple cell types. Nonetheless, for mechanistic-toxicological as well as cell uptake studies the simplicity can serve to be beneficial [60].

3.3.1 DNA damage

The alkaline version of the comet assay has been performed to analyse DNA damage in form of (1) single strand brakes (SSB) and alkali labile sites (ALS) as well as (2) oxidative lesions (mainly oxidized purines). In paper I-V, SSB and ALS have been detected. ALS sites are e.g apurinic and apyrimidinic sites on the sugar backbone of the DNA that are converted to SSB in the alkaline method protocol. More specific measurements of oxidative DNA damages were performed with a modified version of the comet assay. This modification utilises formamidopyrimidine DNA glycosylase (FPG) that recognises and cuts 8-oxoGua and the purine backbone products FaPyAde and FaPyGua into SSB. FPG also detects the alkylation damage N7 methylGua [62]. In the FPG modified version of the comet assay (used in paper I, II and IV), both SSB and ALS, as well as the additional oxidative lesions, can be quantified. Quantification of the amount of damages has been done using computerised scoring (figure 8) and is presented as percentage of DNA in the tail. Using the Comet assay, approximately 100 to several thousand DNA breaks per human cell can be detected [57].

Figure 8. Comets as analysed in paper II, after exposure to CuO nanoparticles.
3.3.2 Cytotoxicity

Two different cytotoxicity assays have been used to measure cell death and cell viability. In the Trypan blue assay viable cells with intact cell membrane are impermeable to trypan blue dye, whereas dead cells with compromised membranes are efficiently stained. The quantity of stained cells is used to determine the percentage of non-viable/dead cell. As the trypan blue assay measures membrane integrity it is regarded as an assay that detects necrosis and late apoptosis and has been used in all studies included in this thesis. In addition, in paper V, the MTT assay was used to study cell viability by measuring the metabolic activity of a cell population relative to a control. MTT is a tetrazolium salt that is reduced by succinic dehydrogenases in active mitochondria\(^ {75}\). The end product is purple and is measured by light absorbance in a spectrophotometer. The assay gives measurement of the extent of active metabolism (viability) in an exposed cell population, including effects of cell death or reduced proliferation in comparison to un-exposed control cells.

3.3.3 Mitochondrial damage

Mitochondrial depolarisation have been analysed using the fluorescent probe tetramethylrhodamine ethyl ester (TMRE), (paper II). Damages to the mitochondria and inhibition of the respiratory chain can stimulate production of ROS causing oxidative stress. Depolarisation of the mitochondria membrane is one key event involved in apoptosis-induced cell death\(^ {52}\). Investigation of mitochondrial damages as a target for nanoparticle toxicity is therefore relevant. TMRE is a positively charged lipophilic dye and will accumulate in the negatively charged mitochondrial matrix. If the mitochondria is depolarised the inner membrane potential is lost and no fluorescence will be detected. A flow cytometer was used to analyse the percentage of cells with depolarised mitochondria after exposure.

3.3.4 Intracellular ROS

To measure intercellular levels of ROS, the oxidation-sensitive fluoroprobe 2’,7’-dichlorofluorescin diacetate (DCFH-DA) was used (paper I). The DCFH-DA assay is sensitive to a variety of ROS and was originally used by Wilson et al\(^ {76}\). ROS can induce DNA damages (as measured by the comet assay), lipid and protein oxidation, as well as cell death. Intracellular ROS can be produced directly by the particle or as a cellular response to the particle exposure. DCFH-DA is a nonfluorescent compound that is freely taken up by cells and hydrolyzed by esterases in the cell that remove the DA group. The level of cellular oxidants is proportional to the formation of the fluorescent oxidation-product dichlorofluorescin (DCF), which is measured with a computerized fluorescence reader.
3.4 CELLULAR DOSE AND INTRACELLULAR UPTAKE

Nanoparticle uptake in cells is a key issue in nanotoxicological research. If the particles are internalized by the cells, they can interact and damage intracellular organelles and molecules. Properties such as charge, agglomeration, diffusion and sedimentation are all capable of modulating uptake and cellular dose of nanoparticles.

3.4.1 Cellular dose

By measuring the elemental constituents of metal and metal oxide nanoparticles the actual cellular dose after exposure can be assessed. Similarly, the cellular dose can be determined after exposure to the corresponding metal salts. After exposure, the cells are collected and the elemental concentration is measured by atomic absorption spectroscopy (AAS). The cellular dose is then calculated by dividing the total metal mass with the number of cells in the same suspension. In paper IV, this technique was used in order to compare cellular dose of Cu nanoparticles after suspending the particles in serum-deficient or serum-containing medium. In addition, the effect of sonication of particles, to reduce agglomeration, on cellular dose was investigated. In paper V the cellular dose was compared between Ag and CuO nanoparticles, and corresponding soluble salts. A limitation of this method is that it cannot separate internalized particles from particles that are closely attached to the cell membrane and not removed by washing the cells.

3.4.2 Intracellular uptake

In order to detect intracellular uptake of particles, both TEM as well as Laser scanning confocal microscopy (LSCM) have been used. In TEM, thin sections of the cell can be investigated, detecting nanoparticles on the cell surface, or internalized and distributed to different cell compartments such as the cell nucleus. In TEM both primary particles, as well as particle agglomerates can be visualized and the basic morphological shapes can be distinguished (figure 9). In contrast to TEM, the LSCM enables simple investigation of cells in all three dimensions, (x, y, z). In paper IV and V, TEM imaging was used to detect intracellular particles. In paper V, intracellular detection of CuO and Ag nanoparticles, as well as uptake in the nucleus was assessed using LSCM. To visualize the cell and nucleus they were stained in different colours, using Cell tracker green and Hoechst. The Ag nanoparticles were visualized by taking benefit of its surface plasmon resonance as described for noble metals.\textsuperscript{77, 78} CuO nanoparticles were visualized in a similar manner as they also emitted light in a close proximity to the wavelength of the laser.

Figure 9. TEM image of intracellular Ag nanoparticles, as analysed in paper V.
4 PARTICLE PROPERTIES INFLUENCING TOXICITY – DISCUSSION AND RESULTS

In nanotoxicology, as specified by the name, special awareness is drawn to the size of the particles, although other important parameters to observe include: material composition, surface area, particle shape, metal release/solubility, surface reactivity and charge. These physicochemical properties are affecting the interactions between the particle and the biological system. Another aspect to take into consideration is possible modifications on the particle surface either by intentionally binding molecules, or through formation of a layer (“corona”) of proteins and lipids when introducing the particles into biological systems.

In the following sections, the influence of the most discussed physicochemical properties on toxicity will be presented. Also, the importance of the solid particles to mediate cellular uptake and enabling a toxic response will be discussed.

4.1 MATERIAL COMPOSITION

For a given material, properties may change when the particle size decrease. However, solely being nano-sized does not give the particles a set of properties that induce a special response. Multiple studies investigating nanoparticles of different material composition supports this statement, pointing to diverse toxic responses \([79-83]\). In a recent review on the toxicity assessment of metal-based nanoparticles, it was concluded that nanoparticles based on Zn, Cu and Ag, were in general more toxic compared to metal nanoparticles of other composition \([84]\). For example, human cardiac microvascular endothelial cells revealed a more extensive cellular response from ZnO, CuO and MgO nanoparticles as compared to Fe\(_3\)O\(_4\), Fe\(_2\)O\(_3\) and Al\(_2\)O\(_3\) \([85]\). The same study investigated cell viability, intercellular ROS production and permeability of the cell layer, and all particle-types ranged within similar sizes (39-47 nm).

In paper I, we compared six different types of metal oxide nanoparticles (CuO, ZnO, CuZnFe\(_2\)O\(_4\), TiO\(_2\), Fe\(_3\)O\(_4\), Fe\(_2\)O\(_3\)), as well as nanoparticles of carbon black, and MWCNT (nanotubes). With exception of the nanotubes, all particles had roughly the same size. We could see a high variation between the different particles with regards to induction of cytotoxicity, DNA damage and oxidative DNA lesions, with CuO nanoparticles being the most potent and producing an effect in all three measurements. Nanoparticles of CuO, TiO\(_2\), ZnO, CuZnFe\(_2\)O\(_4\) as well as MWCNT induced DNA damage, CuO, ZnO, CuZnFe\(_2\)O\(_4\) and Fe\(_3\)O\(_4\) induced oxidative DNA lesions and CuO, ZnO, CuZnFe\(_2\)O\(_4\) and MWCNT induced cytotoxicity. These particles induced some form of effect, although to a different extent depending on the assay. In figure 10 and 11, DNA damage and percentage of non-viable cells are presented, respectively.

In two in vitro studies, oxidative stress and cell viability of up to 24 different metal, metal oxide and carbon nanoparticles was investigated with the conclusion of vast diversity in the induced cellular response \([85, 86]\). In the study by Kroll et al, only seven of the 23 particle-types induced a cellular response after exposure to a concentration of
10 µg/cm²; six induced oxidative stress and one reduced the metabolic activity, measured by MTT. In the study by Horie at al, a vast diversity of the response could be observed. Interestingly in the highest dose tested (1 mg/mL), representing a huge overload, a number of particle-types, such as anatase TiO₂ and one of the two tested rutile TiO₂, as well as Fe₂O₃ and Al₂O₃ induced no reduction in cell viability in the HaCaT and A549 cells tested. Even though there might be possible interference of the particles with the MTT assay, the results highlight the fact that nanoparticles are heterogeneous in their toxicological response. Furthermore, as will be discussed in the following sections, the toxic response cannot solely be explained by material composition of the particles, but rather in conjunction with a range of physicochemical properties. As stated in [84] and considering the diversity of nanoparticles, whether engineered or formed, they should not be viewed as a homogeneous population with simple toxic attributes.

Figure 10. DNA damage in A549 cells after exposure to 1 µg/cm² (2 µg/mL), 20 µg/cm² (40 µg/mL) and 40 µg/cm² (80 µg/mL) nanoparticles for 4 h, measured by the comet assay. Stars (*, **, ***), indicate significantly higher levels compared to controls, and correspond to p<0.05, 0.01, and 0.001, respectively. The grey line represents the control value. For further details see paper 1.
4.2 SIZE AND SURFACE AREA

Nanoparticles are generally defined to have a diameter smaller than 100 nm. The small size allows for nanoparticles to have properties that are vastly different from larger particles of the same chemical composition. A decrease in size renders a larger relative surface area per unit mass, as more atoms are exposed on the surface compared to the interior of the particles. This increase in surface area, changes in crystal planes and structural defects (seen for particles smaller than 20-30 nm), will raise the number of positions and reactive surface groups that can function as reactive sites mediating and driving toxic reactions [17, 19]. A particular nanoparticle size can also influence the nanoparticle-cell interaction and its subsequent toxicity. As shown in studies by Tsoli et al. and Liu et al., Au nanoparticle clusters of 1.4 nm in size could coordinate into the major groove of the DNA, inducing cytotoxicity [87, 88]. Furthermore, comparing Au nanoparticles of sizes varying between 0.8 to 15 nm, 1.4 nm Au nanoparticles were observed to be the most cytotoxic by inducing elevated levels of necrosis. The 1.2 nm particles caused cell death mainly through apoptosis [53].

In paper II, our aim was to compare the in vitro toxicity of micro and nano-sized CuO, TiO₂, Fe₃O₄, Fe₂O₃ particles. For the CuO nanoparticles, we observed a significant increase in cytotoxicity, mitochondrial damage and DNA damage compared to micro-sized particles. The damages were assessed by looking at cell membrane integrity, mitochondrial depolarisation and DNA stand breaks. In figure 12 results regarding cytotoxicity and mitochondrial damage can be seen. As presented in paper II, the level of oxidative DNA lesions measured by the comet assay was also significantly increased for the CuO nanoparticles, whereas the difference towards the micro-sized particles was not statistically significant. The other particle types did not induce the same size specific effect and the level of damage induced were generally lower. Hence for the
Fe$_2$O$_3$ and TiO$_2$ particles, the micro-sized particles caused a low but still statistically significant increase in DNA damage compared to the nano-sized particles.

Concluding the results from paper II, it cannot be generalized that nanoparticles consistently are more toxic than micro-sized particles with the same chemical composition. The increased effect of the micro-sized TiO$_2$ particles can in part be explained by the fact that the crystal structure was different in the two TiO$_2$ particles types compared. The nanoparticles were a mix of rutile and anatase and the micro particles were rutile but with a small amount of anatase. As discussed in a later section (4.3), the crystalline structure can have a significant impact on the toxicity of particles, as well as on the mechanism of action.

In study III, we continued to compare the toxicity of nano- and micro-sized CuO particles, as well as metallic Cu nano- and micro-sized particles. For both CuO and Cu, the nanoparticles caused significantly more cytotoxicity and DNA damage as compared to the corresponding micro-sized particles. Performing a thorough characterisation of the particles’ physicochemical characteristics, it was concluded that higher toxicity of the nanoparticles was due to increased release of Cu compared to the micro-sized particles. The role of metal release and dissolution of particles on toxicity are more disused in a later section (4.4).

Depending on the type of particle, the importance of particle size on toxicity is different, and it is highly dependent on toxic mechanisms of action. As previously described, gold nanoparticles have been seen to interact differently with DNA depending on a specific size [$^{53,87,88}$]. However, as shown in an in vivo study on rats and mice by Oberdörster et al, an effect can also be size dependent even though it is not equally as size specific as in the case of the 1.4 nm Au nanoparticles. An increased lung inflammatory response of anatase 20 nm TiO$_2$ nanoparticles, as compared to 250 nm, could be explained by an increased surface area of the nanoparticles, as compared to
the micro-sized particles. If the dose metrics were changed from “mass” to “surface area” the observed difference in toxic response between the particles sizes diminished. This indicates that the neutrophil lung inflammatory response was linked to surface reactions related to the size of the surface area, and thus indirect to the size of the particle \(^{[89]}\). When investigating the importance of the actual surface area in a wide range of particles of different composition, it was clear that pro-inflammatory effects were closely related to the area. However, no relationship between the particles and the pro-inflammatory effect could be observed when relating the dose to mass. When expressing the dose as surface area, a clear linear relationship could be seen to toxic effects, indicating that the observed pro-inflammatory differences were solely dependent on particle surface area \(^{[90]}\). Preliminary, un-published results on nano- and micro-sized Cu particles point to a similar toxicity-to-surface area dependent effect. If exposing A549 cells to the same mass dose, Cu nanoparticles will induce cell death to a higher extent compared to the micro-sized counterpart (paper III). If the same particle type is exposed in a dose corresponding to the same surface area, then the difference diminishes (see figure 13).

In the case of Cu particles, which is probably true for other partly “soluble” particles as well, an increased surface area increases the release of Cu or other metal ionic species and causes toxicity. Likewise, for TiO\(_2\), the decrease in size increases the “dose” of reactive surface area, causing an inflammatory response. One should bear in mind that a surface area-dependent effect is highly related to the surface properties. If the surface area is inert, or covered by a protective layer of, say, proteins, it will most likely have no or little direct effect on toxicity. And as for a given particle size, changing the material composition relates to alterations in for instance solubility and surface reactivity. This indicates that size alone is not a determinant for a toxic response, but other physicochemical characteristics must be assessed to predict potential hazards of particle exposure.
4.3 CRYSTAL STRUCTURE AND SURFACE REACTIVITY

The crystal structure of particles and reactivity of their surface is of importance for the biological effect and potential toxicity of nanoparticles. This is particularly true for TiO$_2$ and SiO$_2$ (silica) particles. Particles composed of TiO$_2$ can differ in their crystal structure where the most common polymorphs are anatase and rutile, or a mixture of the two. In many studies on TiO$_2$, the difference in crystal structure has not been fully assessed, making it difficult to interpret the toxicity of TiO$_2$ particles although anatase has been proposed to be more toxic $^{[84,91,92]}$. As an example, in an in vivo lung study in rats, it was reported that TiO$_2$ nanoparticles of 80% anatase and 20% rutile, were consequently more potent in inducing inflammation and cytotoxicity, as compared to nanoparticles of predominantly rutile formation $^{[93]}$. In a comparison between 50 nm-sized particles of 100% anatase vs. 100% rutile, both decreased cell viability of keratinocyte (skin) cells. Interestingly, however, it was observed that TiO$_2$ anatase induced cell death through necrosis whereas rutile through apoptosis. Further treatment with the antioxidant N-Acetyl-L-Cystein counteracted the apoptotic effect, whereas the necrotic effect of anatase was not diminishing. This indicates that apoptosis caused by the rutile particles was mainly due to oxidative stress. An increased level of both cellular and acellular production of ROS was also seen for the rutile particles, compared to anatase, adding to the observed differences $^{[91]}$. The role of the crystal structure on pro-oxidative and inflammatory effects is, however, not fully known. It has been suggested that the oxidative activity is in part driven by the anatase crystal structure $^{[94]}$, although not always observed $^{[91]}$. In fact the reactivity and oxidative activity of TiO$_2$ nanoparticles can vary in different cell types and the observed effects may also depend on study design $^{[94]}$, which could explain some of the inconsistencies between results.

Similar to TiO$_2$, silica particles occur in different forms; alfa-quartz, ß-quartz, trydimite and cristobalite. In addition, silica particles can also be amorphous in structure $^{[95]}$. Studying micro-sized silica has shown that toxicity is highly linked to the crystal structure and the reactivity of the surface, and it is in micro-sized silica most research has been done. In a review on pulmonary response to silica particles, cristobalite was ranked first, followed by quartz and amorphous silica in their potency to cause lung injury $^{[96]}$. However, most nanoparticles of silica are in amorphous form and the same ranking of hazardous effects cannot be made on silica nanoparticles $^{[95]}$. Hydroxyl groups on the silica surface, named silanols (SiOH), are also linked to the biological response of the particles. The coverage of silanols can be vastly different depending on the type of silica particle and manufacturing process. Silica particles covered with silanol groups have been observed to induce more membrane damage and toxicity to cells. Moreover, modification of the silanol surface affects cytotoxicity as well as haemolytic capacity of a range of amorphous nano-sized silica materials. Covering the particle surface with a protein/lipid corona significantly reduced the haemolytic and cytotoxic capacity of the particles, most likely due to shielding the reactive surface $^{[97]}$.

In addition to the importance of (1) material composition and (2) size and surface area, the toxicity of nanoparticles is also dependent on the difference in crystal structure and surface reactivity. Modifications to alter these physicochemical properties of particles greatly influences biological and toxicological response. In a study focused on surface
area and reactivity it was suggested that the ability of (low-solubility) particles to induce inflammatory effects is the product of surface area and surface reactivity \[^{90}\]. When ranking the pro-inflammatory effect of a range of different nano- and micro-sized particles of different material composition, with low solubility as well as with low surface reactivity, and that did not induce any cytotoxic effect in the study, a clear increase and linear relationship was seen in the assembly of neutrophils in rat lungs when expressing the dose as surface area. On a similar note, production of IL-8 in A549 cells \textit{in vitro} was observed. Comparison between nano- and micro-sized quartz particles with a more reactive surface, showed similar surface area dependent pro-inflammatory effect. However, the slope of the curve, which refers to the magnitude of the response, was 63 times steeper. Interestingly, the pro-inflammatory effect of quartz was reduced 60 times per surface area unit if the particles were surface treated with aluminium lactate, demonstrating the role of the reactive surface.

### 4.4 Metal Release and Particle Solubility

Release of ionic species from the particles is an important aspect in the toxicity of metal and metal-containing particles. The release of metals can be the actual cause and the mechanism through which the toxicity occurs. However, often it seems to be an interplay between the two; particle \textit{vs.} ion effect, and the specific effects are often hard to separate. For ZnO nanoparticles, release of Zn ions is thought to be related to the particle-induced toxicologic, as well as eco-toxicologic response \[^{98-101}\]. Also, even though not as well assessed, the body of evidence on Cu and CuO nanoparticles suggests that release of Cu has a large part in the toxic potential of the nanoparticles \[^{102-105}\]. In three associated \textit{in vivo} studies on mice, Meng and Chen \textit{et al.} compared the oral toxicity of Cu nanoparticles \textit{vs.} micro-sized particles and corresponding soluble salt (CuCl$_2$). The nanoparticles were observed to be more toxic compared to the micro-sized particles, and the copper dose in kidney was consequently higher after exposure to the nanoparticles due to increased transportation to peripheral target organs. The LD$_{50}$ values for Cu nanoparticles was 413 mg/kg whereas for Cu micro-sized particles it was >5000 mg/kg. After exposure to Cu nanoparticles, grave pathological changes were seen in kidney, liver, and spleen, compared to no effect after Cu microparticle exposure. However, when compared to Cu ionic species from the soluble salt, the LD$_{50}$ value was 110 mg/kg and the copper dose in kidney was as high as after Cu nanoparticle exposure. Summarizing the results from the three studies, small particle size and high surface area mediate the reactivity of the nanoparticles. However, it is concluded that the released Cu is causing Cu ion poisoning and alkalosis.

In paper I and V, a comparison between CuO nanoparticles \textit{vs.} CuCl$_2$ (Cu soluble salt) was conducted, and in paper III the toxicity of Cu and CuO nano- and microparticles was compared to exposure to the released fraction of Cu from the particles. Consistent observations showed that exposure to the CuO and Cu particles (nano as well as micro) caused higher toxicity in terms of cell death and DNA damage when compared to copper ionic species (see figure 14). The difference between particles and soluble salt was also observed to be more pronounced in BEAS-2B compared to A549 cells. By measuring the concentration of Cu released in cell medium it was observed that a large fraction of the particles had been released/dissolved during the time of exposure.
Released, or added Cu ionic species in the growth medium give seemingly much lower toxicity compared to Cu and CuO nanoparticles. It cannot be excluded, however, that Cu released from the particles, once inside the cell, is mediating a toxic response. Cellular uptake and subsequent release of ionic species inside the cell is further discussed in next section (4.5); the Trojan horse type mechanism of metal and metal oxide nanoparticles.

Released Ag is also thought to have a role in the toxicity of Ag nanoparticles. In paper V, the toxicity of Ag nanoparticles as well as the corresponding soluble salt AgNO₃ was assessed. Despite previous findings of cell toxicity in similar or even lower particle concentrations as used in our study no cytotoxicity or DNA damage was seen in either BEAS-2B or A549 cells [30, 31, 106, 107]. It might be due to the measured low release of Ag (< 1%) of the Ag nanoparticles in the study. In contrast to the Ag nanoparticles, an extensive cytotoxicity was seen after AgNO₃ exposure. The low Ag release and subsequent low toxicity of the Ag nanoparticles is strengthening the idea that toxicity of Ag nanoparticles is dependent on release of Ag from the particles.

To design a study separating a neat particle-specific effect from that of metal release is methodologically difficult. This is because metal release from particles composed of, for example Ag, Cu and Zn, will naturally occur during exposure and separating the two effects are consequently difficult. A recent study trying to resolve this question used E. coli as a model, so that experiments could be performed in both aerobic and anaerobic condition. It was observed that the E. coli strain was equally sensitive to Ag ionic species under both conditions, but when exposed to Ag nanoparticles under anaerobic condition, the effect was eliminated. Observations also showed that there was no Ag release from the Ag nanoparticles under anaerobic condition [108]. The same study also compared the EC₅₀ value of six different Ag nanoparticles with increasing size. The EC₅₀ value decreased with decreasing particles size indicating a size

![Figure 14](image-url)
dependent effect. However, it was observed that the Ag concentration released was equal between the different particles at the EC$_{50}$ concentration, suggesting that the size dependent effect was related to increased Ag release with decreased Ag nanoparticle size. This study shows that released Ag from the solid Ag particles cause the toxic effect. However, it is to be noted that the effects observed in E. coli cannot be directly translated to a mammalian scenario, as toxic mechanisms of action can be different. Ag ionic species caused high cell death in both human cell lines investigated (A549 and BEAS-2B), through disruption of the cell membrane independent of intracellular uptake (paper V). Taken together, the release of Ag ionic species is likely to have a clear role in the toxic potential of Ag nanoparticles. Release of Ag from Ag nanoparticles has shown to generate peroxide intermediates such as H$_2$O$_2$. Hydrogen peroxide is very reactive and will rapidly react with biomolecules or metallic silver.$^{[109]}$ An oxidizing environment can facilitate the release process from the nanoparticle surface, further creating reactive ROS that interact with cellular components and accelerate the release process.$^{[110]}$ Even though there was no cytotoxicity or increase in DNA damage measured after Ag nanoparticle exposure (paper V), evidence of ROS, related to Ag-OH and Ag-O, was observed in the RAMAN spectrum following 24 h exposure.

As mentioned previously, metal release from both CuO and Cu nanoparticles have been observed to be high in cell medium. About 40% up to 100% of the particle mass was released during a 4 h exposure of CuO and Cu nanoparticles, respectively. In contrast, less then 1% of the Ag nanoparticle was released after 4 h. This difference in release/dissolution of the particles was confirmed by TEM imaging of A549 cells. After exposure for 4 h, no intracellular Cu nanoparticles were observed, whereas both CuO and Ag nanoparticles were frequently detected. After 24 h of exposure, only Ag nanoparticles were still observed. Even though no intracellular Cu nanoparticles were detected after 4 h, relatively high concentrations of Cu cellular dose remained, as measured using AAS. Thus, it indicates that Cu nanoparticles were taken up by the cells and subsequently degraded. Detection of CuO nanoparticles after 4 h, but not after 24 h, also confirms particle degradation.

Contamination and presence of metals on particles can also contribute to toxicological effects seen from different nano- as well as micro-sized particles and fibres. As an example, the most commonly used techniques to manufacture single-walled nanotubes (SWCNT) utilizes transition metals such as Fe, Co and Ni.$^{[66]}$ As a result, carbon nanotubes can contain large amounts of these metals modifying the toxicological response. As an example, the iron content in SWCNT was found to be responsible for the enhanced generation of oxidative stress, depletion of antioxidants and accumulation of lipid peroxidation products in macrophages. Comparable effects have been observed in various cell models$^{[49, 111]}$. As discussed earlier, in the introductory section to the thesis (1.1.1), metals that are leaching, or are present on ambient airborne particulate matter, are believed to be one key component on health effects related to particle exposure.

In contrast to what has been discussed previously, degradation of particles can be beneficial. Particles that are bio-persistent can cause accumulation and retention of the particles in the body. In the case of asbestos, macrophages in the lung cannot cope with
the long bio-persistent fibres, which are causing a chronic leakage of oxidative radicals and are likely to be responsible for the inflammation, fibrosis and eventually cancer development in the lung [40]. Persistent particles may also accumulate in distant organs, such as liver and kidney after having penetrated the lung-blood barrier, and provide a platform for surface reactions [40].

4.5 THE TROJAN HORSE TYPE MECHANISM - CELLULAR UPTAKE AND SUBSEQUENT INTRACELLULAR METAL RELEASE

For nanoparticles, as compared to regulated uptake of ions, the solid particle can mediate high cellular uptake. Subsequently, the release/dissolution of the particles can cause high local concentrations of ionic species in the cells that otherwise would not be possible [18]. In analogy with Greek mythology this mechanism is called the Trojan horse type mechanism. In most mammalian cell types, pinocytosis can mediate uptake of particles ranging from a few to several hundred nanometer, whereas in specialized immune cells like macrophages, monocytes and neutrophils, particles exceeding 750 nm can be internalized via phagocytosis [112, 113]. Uptake of nanoparticles has been studied in a vast number of studies. Comparing particles with different properties, it seems that uptake is dependent on properties such as size, shape, charge and different coating on the particle surface. As nanoparticles have a tendency to group together and create larger agglomerates, this is also likely to have an impact on the cellular uptake. This was observed in a recent study by Ekstrand-Hammarstöm et al., where uptake was seen to be dependent on the agglomerate size, stability and softness of the agglomerates, more than on the primary particle size [94].

In paper IV and V, uptake and cellular dose of Cu, CuO and Ag nanoparticles has been studied. Further, in paper V the cellular dose following CuO and Ag nanoparticle exposure was compared with uptake of Cu and Ag from the corresponding soluble salts (CuCl₂ and AgNO₃). For all three types of nanoparticles tested (Cu, CuO and Ag), a clear increase in cellular dose after exposure was measured with AAS (see figure 15 and 16). The cellular doses were also higher when compared to exposure to the soluble salts. As previously discussed (paper I, III and V), Cu and CuO nanoparticles cause more cell death and DNA damage than extracts of leached Cu or soluble Cu salts. The difference in toxic response is considered to depend on the difference in cellular uptake and a higher intra-cellular dose for the nanoparticles. For other nanoparticle types, similar data have been presented. Limbach et al reported an increased level of intracellular reactive oxygen species (ROS) induced from Co₃O₄ nanoparticles as compared to cobalt salt (CoCl₂). A Trojan horse type uptake of Co₃O₄ nanoparticles into the cells mediated a high uptake and release of cobalt in ionic form inside the cell [114]. The difference in toxicity was explained by the capability of these nanoparticles to be delivered into the cell by passing through the cell membrane, similar to what have been observed for Cu and CuO nanoparticles. These results are also in line with findings on cellular uptake of Co and Mn₃O₄ nanoparticles in comparison to soluble salts of CoCl₂ and MnSO₄, studied in human leukocytes, mouse fibroblast and in type II lung epithelial rat cells, respectively [115-117].
As seen in paper IV, despite elevated cellular dose measured with AAS, no or few cells were observed with intercellular Cu nanoparticles after 1 h and 4 h exposure, when using TEM. The lack of intracellular particles is likely due to a fast intracellular release/dissolution process. A comparable release process has been observed in a study by Van Winkele et al, where a faster release process of Cu from the Cu nanoparticles was detected when the particles were in contact with cells, as compared to when suspended in the cell medium alone \cite{118}. In the study, intracellular nanoparticles of Ag, TiO\textsubscript{2} and Mn were detected whereas no intracellular Cu nanoparticles were observed. The fast release/dissolution of the Cu nanoparticles is explained by the fact that the cell membranes contain approximately three times higher concentration of O\textsubscript{2} compared to cell medium alone and O\textsubscript{2} drives the oxidation of the particle-surface with subsequent
metal ion release \[^{118}\]. As seen in paper V, and in contrast to exposure to Cu nanoparticles, CuO and Ag nanoparticles were observed in the cells after 4 h, both detected with TEM and LSCM. Further, after prolonged exposure (24 h) Ag nanoparticles were still detected whereas no CuO nanoparticle were seen. It is evident that the kinetics of metal release is closely related to the kinetics of the toxicological response. As observed in paper III, the Cu nanoparticles exposed to A549 cells induced high cytotoxicity already after 4 h, whereas the CuO nanoparticles did not. However, after prolonged exposure, both particle types induced high cell death (see Figure 17). Although the Ag nanoparticles mediated high cellular uptake, no toxic response could be detected even after 24 h, likely depending on slow release of Ag from the particles. Ag ions seem, however, to be toxic to cells, and in contrast to Cu ions the toxicity acts via extracellular mechanisms causing cell membrane damage (paper V).

![Non-viable cells after 4 h and 18 h exposure](image)

**Figure 17.** Cytotoxicity in A549 cells after 4 h and 18 h exposure to Cu and CuO nanoparticles in concentrations of 80 ug/mL. Stars (***,***) indicate significantly higher levels compared to controls, and correspond to p < 0.05, 0.01, 0.001, respectively. For further details see paper III.

The *in vitro* data of Cu and CuO nanoparticles are in line with the so-called Trojan horse type mechanism, highlighting how the solid particle facilitates cellular uptake, leading to subsequent release of ionic species inside the cell and mediating toxicity. However, inside the cell, particles can be active in different ways: easily soluble particles can release ions mediating toxicity, while particles with a more persistent surface oxide or poor solubility can be stable for a longer time, accumulating and exerting toxicity through reactions on the particle surface \[^{40}\]. In a study investigating the acute pulmonary inflammation by 15 different types on nanoparticles, two parameters correlated with inflammatory response. The zeta potential was important for low-solubility particles, whereas high solubility and release of toxic species, was important for the soluble particles \[^{119}\]. Following cellular uptake, particles frequently end up in lysosomes with a lower pH (around pH 5.5) that can enhance the rate of dissolution of many particles \[^{120}\]. The microenvironment and pH in which the release occurs, can also influence particle reactivity. In an acidic environment a greater amount of released Cu will be present as free Cu\(^{2+}\), as compared to neutral pH where complexation of Cu in protein complexes will be more pronounced \[^{32, 121}\]. In that
sense, uptake of Cu and CuO nanoparticles within the cell and in acidic lysosomes, will not only enhance the concentration of Cu in the cell, but also increase the fraction of free Cu$^{2+}$ ions. In figure 18 and 19 intracellular CuO and Ag nanoparticles are seen, as visualized by means of LSCM and TEM respectively.

![LSCM images of A549 cells exposed to CuO and Ag nanoparticles for 4 h.](image)

**Figure 18.** LSCM images of A549 cells exposed to CuO and Ag nanoparticles for 4 h. The particles are visualized in red, cytosol in green and nucleus in blue. In (A) and (C), sections of the cells are visualized from three different angles and the lines cross at a points where intracellular particles/agglomerates are detected. (B) and (D) are images of the same cells as in (A) and (C), where all z-sections are merged into one single image to give an idea of the amount of particles/agglomerates that are attached on/in the cells. For further details see paper V.
SHAPE

Fibre shaped, or so-called high aspect ratio (HARN) nanomaterials represent a growing sector in nanotechnology. This has raised concern as HARN in resemblance with asbestos are considered to cause lung cancer and mesothelioma \[^7\]. Compared to other fibre shaped nanomaterials, carbon nanotubes (CNT) have raised concern since the global market for CNTs is predicted to be in the order of 2 billion US dollar in 2014 \[^122\]. The pathogenic fibre paradigm describes three characteristic features, making fibres more hazardous as compared to non-fibre materials. (1) If a fibre is long (>20 μm), it cannot be completely enclosed by macrophages, which creates a continuous, provoked leaching of ROS. (2) Thin fibres (<3 μm) have low aerodynamic diameter and can be deposited beyond the ciliated airways, where clearance is slow and mediated by macrophages. (3) If the fibre is bio-persistent it will not degrade or break into shorter fractions, but rather accumulate along with dose \[^7\].

It has been shown that long multi-walled carbon nanotubes (MWCNT) exposed to the mesothelial lining of the body cavity in mice induced a length dependent initiation of inflammation and granuloma formation \[^122\]. This length dependency resembles what has been observed for asbestos \[^123\]. A clear length-dependent effect has also been observed for nickel nanowires. Nickel nanowires of predominantly 20 μm length were compared to shorter nickel nanowires (5 μm) with identical chemical nature. In the study, the longer wires caused inflammation in the peritoneal cavity in mice. In
accordance with the fibre paradigm the difference in response was considered to depend on whether macrophages could enclose the fibres or not. It was detected that the shorter wires were completely enclosed by macrophages, whereas as for the longer fibres incomplete phagocytosis was observed \cite{124}. Similar length-dependent phagocytosis was also observed in vitro when macrophages were shown to internalize shorter wires but not longer fibres \cite{124}.

Fibre and needle-like effects have also been observed on cells that do not have any phagocytosis activity. In a study by Stoehr et al., on A549 cells, silver nanowires ranging in size between 1.5 to 25 \( \mu \)m were compared to spherical silver nanoparticles (30 nm), as well as a micro-sized powder (< 45 \( \mu \)m). No effect on cell viability or induction of cytotoxicity was observed in the cells after exposure to the spherical particles, but the wires reduced cell viability and increased cytotoxicity (measured by LDH release) \cite{125}. In line with the fibre paradigm, the effect was dependent on the fibre shape. However, the actual length of the silver wires did not seem to affect the response in the A549 cells. This can be due to the fact that the A549 cells mediate uptake via endocytosis and relatively short fibres can be prohibited complete entry in to the cells \cite{125}.

In paper I, MWCNT was investigated and compared to spherical carbon nanoparticles for the ability to induce cytotoxicity, DNA damage in form of single strand brakes, as well as oxidative DNA lesions and intracellular ROS. It was observed that MWCNT induced DNA damage in all tested concentrations and cytotoxicity in the highest dose (80 \( \mu \)g/ml). However, no effect could be seen on oxidative lesions or intracellular ROS. The carbon nanoparticles did not induce any response in any of the tested toxicity parameters, indicating that the fibre-shape of the MWCNT tested, might have a role in the toxicological response seen.

4.7 SURFACE CHARGE

The surface charge of particles has been seen to be decisive for the toxicity of nanoparticles. In a recent study investigating different types of nanoparticles, it was observed that for low-solubility particles, a significant correlation between acute pulmonary inflammation and positively charged nanoparticles was seen \cite{119}. During exposure the particles were initially covered by a corona composed of macromolecules from serum and lung surfactants, changing the charge of the particles. However, it was hypothesised that when internalized by cells and lysosomes, the acidic environment and enzyme digestion removes the protective corona from the positively charged particles. This will allow for interaction with the negatively charged interior of the lysosome, which leads to lysosomal destabilisation and triggering of inflammation. Moreover, nanoparticles with an acidic zeta potential greater that 10 was shown to induce significantly more inflammation than controls. A similar charge-dependent response was observed when investigating cytotoxicity and cell proliferation of amine-(positive), carboxyl acid- (negative) and azide- (neutral) functionalised silicon nanoparticles \cite{126}. The IC\textsubscript{50} value, measured using the MTT assay were for positively charged particles 20 \( \mu \)g/L, whereas for the neutral particles the value were 600 \( \mu \)g/L and the negatively charged particles did not induce any decrease in cell viability in
investigated concentrations. These results were achieved when the particles were suspended in medium with serum. Interestingly, when similar experiments were performed without serum, no decrease in cell viability was seen when exposing the cells to positively charged silicon particles. The neutral particles displayed only a small change, and the negatively charged particles did not lead to any decrease in cell viability\textsuperscript{[126]}. It was speculated that serum proteins bound to the particles could mediate a higher cellular uptake in to the Caco-2 cells investigated. Modifying the surface charge of particles has in several studies been observed to modulate both cellular uptake and the toxicological response\textsuperscript{[127, 128]}. A surface charge dependence of both uptake and induction of toxicity opens up for the possibility to modify particles to be safe by design or to manipulate their uptake and toxicity for use in for example cancer therapy.

### 4.8 METHODOLOGICAL ASPECTS AND TOXICITY – USE OF SERUM AND SONICATION

Different methodological aspects in the design of nanotoxicological studies are shown to have an impact on both nanoparticles properties as for the final toxicological results. As an example, proteins, ionic strength and different additives in the particle suspension can influence properties such as dispersion, agglomeration and sedimentation of the nanoparticles\textsuperscript{[129-132]}. This has been addressed in a recent study by Mogdolenova et al where a clear effect on TiO\textsubscript{2} agglomerate size and stability of the suspension could be observed when using two different protocols to disperse the nanoparticles. The main difference in the protocol was time of sonication and presence of serum in the stock particle suspension. It was observed that the protocol with shorter sonication and no serum resulted in larger agglomerate. For the same protocol a genotoxic, as well as a slight cytotoxic, effect of the TiO\textsubscript{2} nanoparticles could be observed. Using the other protocol, no such effects could however be detected\textsuperscript{[133]}.

Paper IV in this thesis also addresses how differently methodological settings can influence particle characteristics and toxicity. The aim was to study how sonication of the Cu nanoparticle suspension and presence of serum in the cell medium influenced the extent of metal release, particle agglomeration and stability, as well as cell viability and genotoxicity. It was shown that sonication of the particle suspension resulted in reduced cell viability as well as increased Cu release from the particles (see Figure 20 and 21). No evident difference in toxicity could be detected between Cu nanoparticles suspended in medium with or deprived of serum. Serum in the cell medium, however, affected the particle suspension. With serum the agglomerate size was smaller and stable for a longer period of time, and also increased the release of Cu (see figure 22). It could also be noted that serum free medium resulted in a higher cellular dose as measured with AAS, figure 16 in section (4.5).
Figure 20. Released Cu from Cu nanoparticles after 4 h per amount total Cu measured in particle suspensions (+/- sonication). A significant effect of sonication on Cu release was seen. For further details see paper IV.

Figure 21. Cell viability of A549 cells after 4 h, measured with trypan blue staining (A) and MTT assay (B) after exposure to sonicated and non-sonicated samples of Cu nanoparticles. A significant effect of sonication was observed measured both with MTT and trypan blue staining. For further details see paper IV.
Similar to what was observed in paper IV, no clear effect on the toxicity of either CuO nanoparticles or Ag nanoparticles was observed when exposing A549 and BEAS-2B cells in medium with or deficient in serum. However, in contrast to what was observed in paper IV no difference in cellular dose of CuO nanoparticles and Ag nanoparticles was observed after exposure with or without serum. Thus, in several other studies the absence or addition of serum in the medium are reported to affect both cellular uptake and the toxic response. In a recent study by Lesniak et al, it was nicely shown that both uptake and toxicity was reduced when silica nanoparticles were suspended in medium with serum. In condition with serum, a protein corona was formed covering the particle surface but under serum free conditions the bare particle surface had a stronger adhesion capacity toward the cell membrane and thereby affecting both cellular uptake and the toxicological outcome\cite{134}. A protective capacity of a protein corona covering the nanoparticle surface have also been detected in other studies investigating silica\cite{97, 135} as well as a range of carbon nanoparticles and nanotubes\cite{136}. Though it seems that depending on both particle and cell type, the effect of serum and formation of a surface-covering corona can be different. When investigating uptake of iron oxide nanoparticles in macrophages, the cellular uptake was enhanced when the proteins were attached to the particle surface\cite{137}. In a study by Shi et al, the presence of serum mitigated the cytotoxic effect of SiO₂ nanoparticles, whereas ZnO nanoparticles were more or less equally cytotoxic both with and without serum. The difference between the two particle types probably depends on the fact that the cytotoxic effect in silica is dependent on a reactive surface that is shielded when covered by the protein. The cytotoxic effect of the ZnO nanoparticles, on the other hand, are caused by released Zn ionic species that are not similarly affected by a corona\cite{135}. As the toxicity of the Cu and CuO nanoparticles are believed to depend on release of Cu causing the toxic response, it can partly explained why no protective effect have been seen of serum in paper IV and V.

A definitive statement on the effects of serum is, however, complicated since serum and the presence of amino acids and proteins in the particle suspension have been observed to enhance the release process of some metal/metal oxides, as observed by
Okazaki and Gotoh [138] and in paper III and IV. Increased release can potentially enhance a toxic response. However, the contrary has been observed for silver surfaces as reported by Liu et al, where adsorption of biomolecules partly hinder silver release [139]. Further, it is likely that strong metal complexes are formed between constituents in the serum and released ions, also affecting the toxicological outcome.

It seems evident that methodological aspects, such as sonication and serum in the particle suspension are potential to affect the toxicological readout of a study. This complexity can lead to obvious differences when reporting the toxicity of the same nanomaterial investigated in different studies. When assessing the toxicity of nanoparticles one should carefully consider how to handle and suspend the particles and establishing standardized testing conditions might be considered as necessary. On the other hand, the use of different protocols and settings can increase the understanding of mechanisms and toxicity of different nanoparticles.

4.9 CONCLUDING REMARKS

The potential of nanotechnology is great with a vast spectrum of technical applications, including medical, and environmental. The use of specifically designed nanoparticles can, for instance in medicine improve disease diagnosis and treatment, and nanomaterials in technical application are capable to decrease energy consumptions as well as to achieve cleaner an more effective industrial processes. However, the same properties that in a technical perspective can be positive may be unwanted and harmful for both humans and the environment. Nanotechnology is therefore associated with both human profits and risks. By neglecting or being ignorant of potential hazards, serious setbacks may arise with adverse financial and health effects that hinders future promises of nanotechnology. In order to avoid unnecessary risks and facilitate the use of safe nanotechnology there is a need for adequate toxicological research, as well as risk assessments of nanoparticles and nanotechnologies.

Based on the findings generated in the context of this thesis, there is high variation among different types of nanoparticles to induce a toxicological response. This is also in agreement with general findings in the nanotoxicology research field. One general conclusion is that nanoparticles cannot just be seen as one entity where solely the size generates a toxic response. The hazards are accordingly dependent on a range of specific physicochemical properties.

A key property of metal and metal oxide nanoparticles is the release of ions facilitating a toxicological response. Via a so-called Trojan horse type mechanism the solid particles can facilitate uptake into cells and subsequently release toxic ionic species. A key finding is that the toxicity of Cu and CuO nanoparticles, investigated in this thesis, are facilitated by such a mechanism. However, in contrast to what was observed for the Cu nanoparticles and CuO nanoparticles, no toxicity was seen for Ag nanoparticles, likely due to low intracellular release of Ag ions.
From the studies it can also be concluded that nanoparticles are not always more toxic as compared to micro-sized counterparts of the same chemical composition. Among the particles investigated, a size-dependent effect could be observed for the Cu nanoparticles, as well as CuO nanoparticles. In addition, an increased Cu release was observed for the Cu-derived nanoparticles, as compared to their micro-sized counterparts, which was likely to generate the enhanced toxicological response. The difference between the cytotoxic potential of the Cu particles was also observed to diminish when cells were exposed to the same specific surface area. This finding highlights the need to consider the increased surface area per mass unit of nanoparticles and its role to mediate an increased toxicological response compared to larger particles.

It can also be concluded that different methodological settings can alter both particle properties and the outcome of a study. This emphasises the importance to carefully consider the handling and suspension of the nanoparticles, as well as the choice of cell models, in order to achieve a testing protocol that mimics a real exposure scenario. Such improvements can increase the compliance between in vitro and in vivo models and most importantly, to improve the ability to anticipate adverse health outcomes.
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