

From THE DEPARTMENT OF BIOSCIENCES AND NUTRITION  
Karolinska Institutet, Stockholm, Sweden

# **TOXICITY OF METAL CONTAINING MICRO- AND NANOPARTICLES**

**- Studies from an inhalation perspective**

Johanna Kain



**Karolinska  
Institutet**

Stockholm 2013

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Cover photo: SEM picture of stainless steel particles 316L (investigated in study IV), taken by the colleagues at the Division for Surface and Corrosion Science at the Royal Institute of Technology (KTH) in Stockholm, Sweden.

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*To those who are closest to my heart;  
To my lovely family and dearest friends*

*Nothing in life is to be feared, it is only to be understood.  
Now is the time to understand more, so that we may fear less.  
/Marie Curie\**

*\* **Marie Curie** (1867 – 1934), Scientist and the first woman to be awarded a Nobel Prize (1903, in Physics). She was also the first person ever to get the award a second time (1911, in Chemistry).*





## ABSTRACT

Particles in urban environments have for a long time been associated with different health problems and diseases, such as worsening of asthma and chronic obstructive pulmonary disease as well as cancer and cardiovascular diseases. In addition, metal containing particles present in occupational settings and industries, particularly particles produced during welding, have been linked to several adverse health effects. In recent years it has also become obvious that humans get exposed to metal containing particles in subway systems although possible health effects of such exposure is unknown. Furthermore, engineered nanoparticles (often containing metals) are increasingly being used within various applications including consumer products, thus constituting a risk for human exposure. In all, this means that the exposure to metal containing particles is present in society and may increase in the future due to the increased use of nanoparticles in various products.

There is thus today a great need for more knowledge concerning toxicity of these particles. The main aim of this thesis was to investigate the toxicity and underlying biological mechanisms following exposure to different metal containing micro- and nanoparticles from an inhalation perspective.

In studies I-IV (paper I-IV), the toxicity and underlying mechanisms were investigated for a wide range of metal and metal oxide containing micro- and nanoparticles following exposure of cultured human lung cells (A549). In particular, the importance of material composition, size and ion release for induction of toxicity in terms of cytotoxicity, DNA damage, oxidative DNA damage, mitochondrial depolarisation, generation of intracellular ROS and haemolysis of red blood cells was explored. In general, there was a high variation in the toxic potential of the investigated particles and the high and size-dependent toxicity of CuO was especially highlighted in study I-II. One suggested reason for this is the release of toxic ions inside cells, facilitated by a Trojan horse like mechanism where the particles transport the ions in to the cell. The investigations of industrially relevant iron and chromium based particles revealed that ion release from these particles, in an acidic and complexing fluid, were dependent on particle size and most likely the surface oxide of the particles. Further, investigations of stainless steel (316L) in several other artificial biological fluids, with neutral and weakly alkaline pH, revealed that the ion release was only increased in the acidic and complexing fluid. The toxicity of the iron and chromium based microparticles was in general low but increased DNA damage potential of 316L and NiO nanoparticles was observed, and the latter was also confirmed in study III.

In study III (paper III), several metal containing particles were observed to have an oxidative capacity. Despite this, no considerable oxidative DNA damage was detected using the FPG comet assay. The results suggest that this may be due to interference of some of the nanoparticles with the FPG enzyme that is supposed to find the oxidative damage within the assay. Ag nanoparticles particularly inhibited the enzyme, mainly due to Ag ions.

In study V (paper V), immunological effects in healthy humans were studied during 24 hours after a 2 hour exposure to the PM rich environment of a subway station. The results indicated a small, but transient, direct inflammatory response seen as a decrease in the level of lymphocytes in blood as well as small changes in the lymphocyte subpopulation in the end of the follow-up time. In addition, an increase in DNA damage in mononuclear cells of the blood was detected in some of the subjects.

In conclusion, the results from these studies indicate that the chemical composition of metal containing particles is crucial for the toxicity of the tested particles. This thesis especially highlights toxic effects of CuO and NiO nanoparticles. Release of ions from particles may be one important factor for toxicity and this thesis shows that the release of metals ions from stainless steel particles increased in acidic pH. Additionally, interference of nanoparticles and toxicity assays may be important to consider and this thesis highlights possible interactions with FPG in the comet assay especially with Ag nanoparticles. Exposure to the air at a subway station, rich in metal containing particles, did not cause any large acute toxic effects in humans. Still, some parameters indicated that the immune system reacted to the exposure.

## LIST OF PUBLICATIONS

Surname changed from Gustafsson to Kain in 2010.

\* These authors contributed equally to this work.

- I. Hanna L. Karlsson, Pontus Cronholm, **Johanna Gustafsson** and Lennart Möller. *Copper oxide nanoparticles are highly toxic: A comparison between metal oxide nanoparticles and carbon nanotubes*. Chemical Research in Toxicology, 2008, 21(9), 1726-1732.
- II. Hanna L. Karlsson\*, **Johanna Gustafsson\***, Pontus Cronholm and Lennart Möller. *Size-dependent toxicity of metal oxide particles – A comparison between nano- and micrometer size*. Toxicology Letters, 2009, 188(2), 112-118.
- III. **Johanna Kain**, Hanna L. Karlsson and Lennart Möller. *DNA damage induced by micro- and nanoparticles – interaction with FPG influences the detection of DNA oxidation in the comet assay*. Mutagenesis, 2012, 27(4), 491-500.
- IV. Yolanda Hedberg, **Johanna Gustafsson**, Hanna L. Karlsson, Lennart Möller and Inger Odnevall Wallinder. *Bioaccessibility, bioavailability and toxicity of commercially relevant iron- and chromium-based particles: in vitro studies with an inhalation perspective*. Particle and Fibre Toxicology, 2010, 7:23.
- V. **Johanna Kain**, Anna Klepczynska-Nyström, Hanna L. Karlsson, Britt-Marie Larsson, Benita Engvall, Anders Lundin, Anders Eklund, Johan Grunewald, Magnus Svartengren and Lennart Möller. *Inflammation and DNA damage in healthy humans after subway air exposure – a time series study*. Submitted in 2013.

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1. Roger W. L. Godschalk, Clara Ersson, Maciej Stepnik, Magdalena Ferlińska, Jadwiga Palus, João Paulo Teixeira, Solange Costa, George D. D. Jones, Jennifer A. Higgins, **Johanna Kain**, Lennart Möller, Lykke Forchhammer, Steffen Loft, Yolanda Lorenzo, Andrew R. Collins, Frederik-Jan van Schooten, Blanca Laffon, Vanessa Valdiglesias, Marcus Cooke, Vilas Mistry, Mahsa Karbaschi, David H. Phillips, Osman Sozeri, Michael N. Routledge, Kirsty Nelson-Smith, Patrizia Riso, Marisa Porrini, Adela López de Cerain, Amaya Azqueta, Giuseppe Matullo, Alessandra Allione and Peter Möller. *Comparison of DNA damage levels in mononuclear cells from humans in five European countries*. Submitted in 2013
2. Johan Olsson, **Johanna Kain**, Clara Ersson, Birgitta Sundberg, Arvo Hänni, Samar Basu, Bengt Vessby, Mark Stavro, Ina Schoppe and Lennart Möller. *Dark chocolate consumption does not influence blood pressure, oxidative damage, inflammation, body weight or blood lipids*. Manuscript under preparation



## PREFACE

When I was introduced to the research of particles, it was to the field of nanotoxicology. The year was 2007 and the research field was brand new, the term nanotoxicology had actually been introduced only a few years earlier, in 2004. Particles in the size of nanometres (comparable to the size of viruses) were increasingly being used in different consumer products, and therefore questions were raised about their potential impact on our health. It was in that context that I started my research. In the first studies I was involved in, the toxicity of different nanoparticles were investigated and compared, and some fundamental questions were answered. Retrospectively, I have realised the extent to which these studies have contribution to the research field, a field that to a large degree was undiscovered at the time. The two articles presenting our initial discoveries (paper I and II) have, at the time of this writing been cited more than 400 times in other scientific articles, which I find amazing and it makes me proud!

Particle toxicology research has become more and more advanced during the past years, and collaborations between researchers with different backgrounds are a necessity today. This has proven to be especially important when it comes to characterisation of particles and it is a demand today that we know exactly what particles we are investigating! A lot of research has been performed since 2004, research that has given us new knowledge, but also new questions. One example is which methods that are most suitable for testing toxicity of nanoparticles, an issue addressed within paper III in the present thesis.

The increased amount of knowledge and questions has caused a demand for action in the society. Alongside my PhD-studies I have had the great opportunity to work (outside the lab) with an investigation for the Swedish Government regarding this issue; The Swedish Government Official Report SOU2012:89, with the purpose to present a National action plan for the use and handling of nanomaterials in Sweden. This work has put my research in a new context and has provided me with a lot of experience and insight.



The focus of my thesis is metal containing particles; nanoparticles and particles in micrometre size, including intentionally produced nanoparticles as well as unintentionally produced particles from the steel industry and the subway system of Stockholm. This thesis is a journey through particle toxicology, in which the approach of the studies become more and more complex going from studies of effects and mechanisms in cultivated cells to health effects in humans.

With that said, it is time to turn page and to start this exciting journey. I wish you a pleasant experience!

*/Johanna Kain, Stockholm August 2013*

PS. If your time is limited, I recommend you to take a shortcut and read the abstract of this thesis, or the Swedish summary at the end.

# TABLE OF CONTENTS

<b>1</b>	<b>Introduction .....</b>	<b>1</b>
1.1	Air pollution and health effects – a historical overview up until today.....	1
1.2	Particles.....	3
1.2.1	Definitions and origin .....	3
1.2.2	Limit values.....	4
1.3	Metal containing particles and health effects .....	4
1.3.1	Occupational exposure, industrial work and welding.....	5
1.3.2	Nanoparticles.....	7
1.3.3	The subway.....	11
1.4	Lung exposure and mechanisms .....	12
1.4.1	Lung exposure – deposition, dose, clearance and translocation ...	13
1.4.2	Mechanisms of toxicity – oxidative stress, inflammation and DNA damage.....	17
1.5	Burning questions.....	23
<b>2</b>	<b>Research aim.....</b>	<b>25</b>
2.1	General aim.....	25
2.2	Specific aims.....	25
2.2.1	The thesis will answer the following questions .....	25
<b>3</b>	<b>General study approach and analytical methods.....</b>	<b>26</b>
3.1	General study approach .....	26
3.1.1	Exposure of cells, <i>in vitro</i> .....	26
3.1.2	Chemical studies, <i>in vitro</i> .....	27
3.1.3	Studies of human exposure, <i>in vivo</i> .....	28
3.2	Particle characterisation and air monitoring .....	29
3.2.1	Particle size and morphology.....	29
3.2.2	Surface area and charge .....	31
3.2.3	Air monitoring.....	32
3.3	Toxicity testing .....	33
3.3.1	Particle exposure <i>in vitro</i> - dose, time and sample preparation ...	33
3.3.2	Cell viability - cytotoxicity .....	34
3.3.3	Intracellular ROS .....	35
3.3.4	Damage of DNA .....	35
3.3.5	DNA repair – hOGG1 activity.....	37
3.3.6	Mitochondrial depolarisation.....	38
3.3.7	Haemolysis .....	39
3.3.8	Inflammation .....	39
3.3.9	Other human health markers – lung permeability, lung function, self graded symptoms.....	40
3.3.10	Interference of particles with toxicity assays .....	41

<b>4</b>	<b>Results and discussion – particle toxicity.....</b>	<b>43</b>
4.1	The role of chemical composition.....	43
4.2	The role of size, surface area, charge and metal ion release .....	48
4.3	Interaction with toxicity testing and other methodological considerations for the comet assay .....	54
4.3.1	Interference of particles with the comet assay.....	54
4.3.2	Comparison of FPG and hOOG1 for detection of oxidative DNA damage... ..	56
4.4	Human exposure to subway particles and health effects .....	58
<b>5</b>	<b>Summary and conclusions .....</b>	<b>62</b>
5.1	Concluding remarks.....	62
5.2	Future remarks .....	64
<b>6</b>	<b>Svensk populärvetenskaplig sammanfattning .....</b>	<b>65</b>
<b>7</b>	<b>Acknowledgements – Tack!.....</b>	<b>67</b>
<b>8</b>	<b>References.....</b>	<b>69</b>

## LIST OF ABBREVIATIONS

A	Adenine
ALF	Artificial lysosomal fluid
ALS	Alkali labile sites
ASW	Artificial sweat
ATF	Artificial tear fluid
AP	Apurinic or apyrimidinic
BAL	Bronchoalveolar lavage
BER	Base excision repair
BET	Brunauer Emmet Teller
BSA	Bovine serum albumin
C	Cytosine
CC16	Clara cell protein 16
COPD	Chronic obstructive pulmonary disease
CRP	C-reactive protein
DCF	Dichlorofluorescein
DCFH-DA	2',7' – dichlorofluorescein diacetate
DLS	Dynamic light scattering
DMEM	Dulbeccos minimal essential medium
DNA	Deoxyribonucleic acid
DSB	Double strand break
EC	Electrochemical
ECHA	European Chemical Agency
ECNIS	Environmental Cancer risk, Nutrition and Individual Susceptibility
ECVAG	European Comet assay Validation Group
ELISA	Enzyme-linked immunosorbent assay
ESCODD	European Standards Committee on Oxidative DNA Damage
EtBr	Ethidium bromide
EU	European Union
FACS	Fluorescence-activated cell sorting
FapyA	Fapy-adenine
FapyG	Fapy-guanine
FBS	Foetal bovine serum
FEV1	Forced expiratory volume during the first second
FPG	Formamidopyrimidine DNA glycosylase
FVC	Forced vital capacity
G	Guanine
GC	Guanine-Cytosine base pair



GMB	Gamble's solution
HPLC	High performance liquid chromatography
IL	Interleukin
ISO	International Organization for Standardization
LALLS	Low angle laser light scattering
LDH	Lactate dehydrogenase
µm	Micrometre, $10^{-6}$ x metre, 0.000 001 metre
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NER	Nuclear excision repair
nm	Nanometre, $10^{-9}$ x metre, 0.000 000 001 metre
hOGG1	Human oxoguanine glycosylase
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PEF	Peak expiratory flow
PM	Particulate matter
PM10	Particulate matter, diameter <10 µm
PM2.5	Particulate matter, diameter <5 µm
PMN	Polymorphonuclear neutrophils
Ro	A photosensitiser
ROS	Reactive oxygen species
SCENIHR	Scientific Committee on Emerging & Newly Identified Health Risks
SEM	Scanning electron microscopy
SSB	Single strand breaks
Smog	Smoke and fog
T	Thymine
TA	Thymine-Adenine base pair
TEM	Transmission electron microscopy
TMRE	Tetramethylrhodamine ethyl ester
UV	Ultraviolet
VAS	Visual analogue scale
VC	Vital capacity
WHO	World Health Organization
8-OxodG	8-oxo-7,8-dihydro-2'-deoxyguanosine



# 1 INTRODUCTION

Exposure to air pollution, containing particles, chemicals and biological material, is for many of us known to be a health hazard. This has, however, not always been the case and today's knowledge of the link between exposure to air pollution and ill health has evolved during centuries. To be able to understand the research field of particle toxicology, which is the topic of the thesis you hold in your hands, there is a need to start from the beginning and to understand the research of air pollution and what has been achieved within it, particularly during the last decades. Much of what is known about particle toxicology today derives from knowledge of air pollution.

## 1.1 AIR POLLUTION AND HEALTH EFFECTS – A HISTORICAL OVERVIEW UP UNTIL TODAY

Exactly when the first connection was made between air pollution and ill health is not possible to know. However, work in dusty mines and health effects seen in workers, were described many years ago by Hippocrates who lived between 470-360 BC. When the printing press was invented in 1450, Georgius Agricola, later referred to as the father of mineralogy and occupational hygiene, printed his famous book *De re metallica* (the nature of minerals)<sup>1</sup> and for the first time lung disease and work in dusty mines were linked to each other. A direct connection between the exposure of dust and health effects was, however, not made at this point. His second book *De Animantibus Subterraneis*, about the demons of mines, demonstrated the various ideas circulating during this time period concerning “exposures” in this work environment. After the book by Agricola, the knowledge of diseases in workers exposed to air pollution, particularly in mines, increased. Nonetheless, it was not until the industrial revolution (1760-1820) that legislators started to get interested in the matter and it took until the early 20<sup>th</sup> century until something was done to protect workers from danger in their work environments. Knowledge of health effects from exposure of air pollution from this time period is mainly based on exposure to quartz (crystalline silica), coal and asbestos, together responsible for the greatest amount of lung disease in history so far<sup>2</sup>.

Up until this point, health effects from air pollutants had been associated with work environments. In the mid 20<sup>th</sup> century, however, intense industrial air pollution led to some incidents causing exposure to the population. One of the most well documented incidences is often referred to as the “London smog”, which occurred in 1952. Cold, stagnant weather conditions and high levels of air pollution caused by coal heating induced high levels of air pollutants (as high as 1620 µg/m<sup>3</sup>) for several days. Analysis of the death statistics have shown that the death rate was increased not only during the episode, but for several month after the episode and that the total numbers of deaths were around 12 000<sup>3</sup>. The London smog and additional incidents at that time lead to the first public action to reduce air pollution: The British Clean Air Act of 1956. It was the first effort in the industrialised world to reduce emissions from industries and domestic heating. This and further legislations made in the Western society has to a

great extent eliminated these dense winter smogs that caused increased mortality in numbers of thousands.

The source of pollution gradually changed with the introduction of the automobile in the beginning of the 20<sup>th</sup> century, from the coal burning to pollution from combustion in engines of cars. The increased awareness of the influence of air pollution on health led to large epidemiological studies in the 1960s and 70s<sup>2</sup>. These were large population studies that established a correlation between death rates and concentrations of particles and sulphur dioxide in the air. These studies showed short-term relationships between these variables, as well as long-term effects. One of the most famous studies is the so called *Six cities study*<sup>4</sup> in which six cities in US were compared, and a correlation of death rates with increased PM levels was shown. This study and other recently conducted studies have revealed negative health effects at quite modest exposure levels.

Until the beginning of the 1990s, studies had primarily focused on respiratory effects of exposure to air pollution. A clear link between exposure and worsening of allergic and inflammatory conditions of the lung, such as asthma and chronic obstructive pulmonary disease (COPD) has additionally been established since then<sup>5</sup>. Several studies have also indicated an association between exposure to traffic related particles and cardiovascular diseases in recent years<sup>6</sup>. Since the prevalence of cardiovascular diseases is increasing in the world the role of air pollution in regard to this matter is highly important to investigate. A recent study looking at cancer risk among 300 000 European citizen and the link to ambient particles, has revealed that particulate matter (PM) contributes to the cancer incidence and even at levels below the PM limit values set by EU<sup>7</sup>. The World Health Organization (WHO) estimates that over 1.3 million deaths of today can be endorsed to health effects caused by ambient air pollution<sup>8</sup>.

In the 1990s, engineered materials in nanometre size started to become increasingly important for technical applications. This is an industry, which yet today is highly important and the new technology has opened up for a large amount of new beneficial applications. The materials used are often in the form of particles (nanoparticles), raising concern of their impact on human health and nature. The term *nanotoxicology*, established in 2004<sup>9</sup>, has become a field of science relevant to both workplaces, general environments and the consumer safety<sup>10</sup>.

An interesting observation made by K. Donaldson and A. Seaton<sup>2</sup> is that the introduction of engineered nanoparticles has changed the way that research deals with particle toxicology. Previously, adverse health effects were reported prior to investigating the toxicity and potential mechanisms of particle exposure. Today, a large amount of research of particles aims to understand a potential hazard of particle exposure before health effects have been observed. This approach has for some decades led to investigations of work environments, including industries and production sites, as well as other potential places where exposure to air pollution is suspected, and where there might be a risk for human health. One such area is the exposure of unintentionally produced metal containing particles, which will be discussed later on in this thesis (in the studies of metal industries and in the subway system).

## 1.2 PARTICLES

### 1.2.1 Definitions and origin

Air normally consists of a range of different atmospheric gases, water vapour and a background level of particles. The composition of air is essential for life and when the composition is disrupted by e.g. human activities or natural causes, the amount of any of these components may be altered. If the levels of gases or particles are increased the air becomes polluted. The word *air pollution* includes components originating from many different sources, some related to human activities (e.g. car engines and fires) and some to natural sources (e.g. sandstorms and volcanoes).

The term *aerosol* is an expression used to describe the subset of air pollution containing particles. The definition includes both liquid and solid particles that are suspended in a gas, which most often is air <sup>11</sup>. *Particles or particulate matter (PM)* less than 100  $\mu\text{m}$  in size can often become airborne and a wide range of objects may be considered as particles; *naturally occurring particles* such as snow, sand and sea-salt, *biological particles* including pollen, bacteria and viruses as well as *man-made particles* of e.g. smoke and dust in work and home environment produced either intentionally or unintentionally <sup>12</sup>.

Particles are often defined by their size and the terms coarse ( $>2.5\ \mu\text{m}$ ), fine ( $<2.5\ \mu\text{m}$ ) and ultrafine particles ( $<100\ \text{nm}$ ) are often used for ambient PM. *Coarse particles* are often mechanically produced e.g. by wear of a material. They are relatively heavy and have a short lifetime, travelling only a small distance in air before they fall to the ground. *Ultrafine particles* are often formed from gas phase components e.g. during fossil fuel combustion, from evaporation of metals during for example welding or from intentional production in factories for use in e.g. technical products. Their lifetime is also short; they are in air for only minutes to hours before they deposit onto surfaces or combine into larger particles. *Fine particles* can be formed both by mechanical wear and by growth of ultrafine particles via coagulation (combination of small particles into a larger one) or condensation (a gas molecule condense on a solid particle). Fine particles seldom get larger than a couple of micrometres, which make their lifetime and travel distance in air much greater than coarse and ultrafine particles. They can stay in the atmosphere for days up to weeks and travel more than 100 km with wind before removed from the atmosphere, often by rainy weather <sup>12</sup>.

Nowadays, there is a great intentional production of particles below 100 nm for use in e.g. technical and medical applications. This is due to the unique properties particles may have in this small size range. The term *engineered nanoparticle* is most often used for these particles. However, the term nanoparticle is sometimes used in the literature for ambient ultrafine particles as well. Throughout this thesis, the term nanoparticles only refer to the engineered ones.

Particle concentration in air is often measured by two fractions referred to as PM<sub>10</sub> (mass concentration of particles  $<10\ \mu\text{m}$ ) and PM<sub>2.5</sub> (mass concentration of particles  $<2.5\ \mu\text{m}$ ). Limit values exist for both PM<sub>10</sub> and PM<sub>2.5</sub> in ambient air.

### 1.2.2 Limit values

The European Commission has decided on *air quality standards*, limit values, for PM<sub>10</sub> and PM<sub>2.5</sub> in ambient air <sup>13</sup>. For PM<sub>10</sub> the limit value of 50 µg/m<sup>3</sup>, as a mean value over 24 hours, is not allowed to be exceeded more than 35 days/year. The mean value over one year should not be over 40 µg/m<sup>3</sup>. For PM<sub>2.5</sub> a so called target value of 25 µg/m<sup>3</sup> has been set, which member states should try not to exceed as a mean value over one year. This target value will become a set limit value that enters into force in 2015. Since PM<sub>10</sub> and PM<sub>2.5</sub> are based on mass concentration of particles, the measured values are mostly affected by the larger particles in the sample. Ultrafine particles often dominate the number concentration of particles in ambient air, but represent only a small fraction of the total mass concentration. Hence PM<sub>10</sub> and PM<sub>2.5</sub> mainly give information about the amount of particles in micrometre size.

Occupational environments do also have limit values <sup>14</sup>. Some examples of exposure limit values that should not be exceeded during a workday (8 h) are 3.5 mg/m<sup>3</sup> for iron oxide, 0.2 mg/m<sup>3</sup> for copper and 0.1 mg/m<sup>3</sup> for manganese (respirable dust). Silver should not be above 0.1 mg/m<sup>3</sup> and chromium should not be over 0.5 mg/m<sup>3</sup> (total dust). For chromium VI (the ionic form) the limit value is 5 µg/m<sup>3</sup>.

No specific limit values exist for nanoparticles. If nanoparticles are present in ambient air, the standards for PM should be followed. If present in a workplace, occupational limit values are valid and the particles chemical composition (e.g. which metal it consists of) then decides which limit value that should be followed.

## 1.3 METAL CONTAINING PARTICLES AND HEALTH EFFECTS

The focus of this thesis is metal containing particles, both unintentionally and intentionally produced. Metal containing particles can be found in many different environments, such as occupational settings where particles can originate from welding or manufacturing work of metals. During recent years a new potential exposure of metal containing particles has arisen from the intentionally produced particles, present as nanoparticles, e.g. in consumer products. These particles may not only be a risk during production but may also pose a risk for consumers, as well as during disposal of these products. Another milieu studied in this thesis is the subway system, and the exposure to metal containing particles that may take place there. Particles are produced unintentionally in this environment and both people working in the subway system as well as the public, often travelling on a daily basis with the subway may be exposed.

The following text will shed light on metal containing particles relevant for the present thesis.

### 1.3.1 Occupational exposure, industrial work and welding

#### *Steel industries*

During work in industrial environments, release of metal containing particles is a likely event during cutting, milling and melting of metals. Since workers often work close to the source generating particles, the occupational exposure may be high. In a study by Elihn and Berg <sup>15</sup>, the characteristics of airborne particles were measured in seven Swedish industrial plants where mainly iron based material was processed. These measurements show that the highest concentrations of PM10 and PM1 (particles < 1 µm) were present during laser cutting and welding of steel, which are processes generating heat, while colder processes generated lower levels. Further studies investigated effects of inflammation in the workers at these industries. The results showed increased levels of interleukin-6 (IL-6), by 52% after the first day of a work shift, where after an adaptation seemed to occur and the levels decreased <sup>16</sup>. C-reactive protein (CRP) was also increased, by 17% after the second day into a work shift, which may indicate an increased risk of coronary heart disease for these workers. In other studies, biological samples such as hair, blood and urine, from steel mill workers with long-term exposure to steel, have shown elevated levels of arsenic (As), cobalt (Co), manganese (Mn) and copper (Cu) <sup>17</sup>. Additionally, studies of health effects in workers of a Swedish copper smelter have shown increased respiratory problems linked to the corrosive gases and dust at the workplace, as well as excess mortality when compared to the general Swedish population <sup>18</sup>.

The knowledge of potential respiratory effects from exposure to iron and chromium based particles (ferrochromium alloys) such as stainless steel, investigated in this thesis, is limited. Studies of workers, exposed to low levels of particles during stainless steel production, have however shown such exposure to cause small clinical effects in terms of nasal symptoms and disease <sup>19</sup>. Increased lung accumulation of particles in workers at specific positions of the stainless steel plants have also been shown <sup>20</sup>. Assumptions from toxicological findings relevant for individual constituents of stainless steel, such as chromium (Cr) are often made trying to understand the toxicity of stainless steel. Hexavalent chromium (Cr(VI)) is a known carcinogen when inhaled <sup>21</sup>, but there is inadequate evidence for carcinogenicity of trivalent chromium (Cr(III)) <sup>22</sup>. In a cohort study of 4800 workers producing stainless steel, no indication was found regarding increased mortality due to lung cancer <sup>23</sup>. However, long term daily exposures to low levels of dust and fumes containing Cr(III) have been shown to generate enhanced production of phlegm in the respiratory tract, shortness of breath and breathlessness on exertion <sup>24</sup>.

Many studies regarding the impact on health from occupational exposure to metals, are however, studies conducted on welders, which is discussed in the following section.

#### *Welding*

Welding is a common manufacturing tool used for joining pieces of metal together by heating. In a report conducted by the Swedish Work Environment Authority in 2013 <sup>25</sup>, it was estimated that 20 000-25 000 persons work as full-time welders in Sweden. In addition, approximately 250 000 perform welding as one part of their job. Welding is,

for example, a common procedure used in the work of auto mechanics. In industrialised countries, one percent of the working population are estimated to work as welders<sup>25</sup>.

During welding, fumes and particles are released and the heat that is produced causes the fume to rise in a column from the welding source. The materials found in the welding fume may originate from the electrode of the welding device, the so called shielding gases, from the base metals that are joined or from paint and surface coatings of the metals. Vaporised metals that are produced react with air and metal oxides are formed. When the vapour condenses, particles are produced. The chemical properties of the particles can be rather complex since welding material often consists of metal alloys (often steel), containing different amounts of for example iron, manganese, silica, chromium, nickel, zinc or copper. The particle concentration in the fume column formed during welding can be high, as much as 1000 times higher than in the air just a small distance away<sup>25</sup>. The particles produced are often in a size range of 5 - 40 nm. They do, however, tend to aggregate and form chains of particles with a size of 0.1-1 µm. This is mainly due to the heat formed during the welding procedure, which increases the movement of the particles and hence the probability of collision, causing formation of larger particles<sup>26</sup>. Welding fume not only consists of particles, but also toxic gases such as carbon monoxide (CO), ozone (O<sub>3</sub>) and nitrogen oxides (NO<sub>x</sub>) in evaluated levels.

The current limit value for total dust concentration in the breathing zone during a 8 h workday is 5 mg/m<sup>3</sup><sup>25</sup>. Studies conducted in the 1970's of 500 welding workplaces showed that the levels often exceeded the limit value. When a similar evaluation was performed in 1994, the levels were lower and the limit value was not exceeded in the majority of the sites<sup>25</sup>. Nonetheless, during some types of welding the *number* of particles in nanometre size can be very high even though the limit value is not exceeded. Threshold values for certain components of stain less steel are, however, much lower than that for total fume. For example, Cr(VI) has a limit value of 5 µg/m<sup>3</sup> (= 0.005 mg/m<sup>3</sup>). In a study by Gavelin et al.<sup>27</sup> Cr(VI) levels were measured during welding and the limit value was reported to be exceeded regularly, even when proper safety equipment was being used, such as specialised ventilation.

#### *Health effects and toxicity associated with welding*

Welding is associated with numerous health effects and there are several factors during welding that may cause disease: gases produced, ultraviolet (UV) light exposure, the heat of the fumes, particle exposure etc. Health problems for welders are often also linked to the heavy physical work they perform, as well as to other factors, such as noise and other more general exposures in the working environment. Nevertheless, particles and gases are considered to be the most harmful components of exposures during welding<sup>26</sup>. It is also possible that workers smoke, a fact that cannot be neglected when investigating health effects. It may contribute to the effects seen in welders, but it may also be that smoking and welding act synergistically when introducing health effects, as suggested by Mur et al. in the case of bronchitis<sup>28</sup>.

Several health effects have been linked to the different metals present in the particles that are released during welding. Welding of stainless steel containing *chromium* is associated with asthma, lung cancer and impaired sperm quality<sup>25</sup>. Alloys of steel may



release chromium, both as Cr(III) and Cr(VI). Cr(VI) is toxic and classified as a human carcinogen (as previously discussed) and studies of welding of metals rich in Cr(IV) indicate a possible increase in mortality from lung cancer among exposed workers <sup>29</sup>. The report by the Swedish Work Environment Authority <sup>25</sup> concludes that research indicate that welding of stainless steel can cause lung cancer as well as cancer of the nose. In epidemiological studies of exposure to *nickel*, the same cancer forms have also been shown to be affected <sup>26</sup>. Nickel is classified as a human carcinogen <sup>30</sup> and may also be released from alloys during welding. *Manganese* is present in most welding fumes and effects on the nervous system have been seen from it, contributing to symptoms resembling Parkinson's disease <sup>31</sup>. In addition, chronic inflammation of the nose has been observed following welding of steel alloys containing manganese (Werner 1977). The main component of fumes generated from most welding processes is, however, *iron oxides* and exposure to these during welding has been linked to airway and heart disease <sup>25</sup>. In a case-control study by Palmer et al. <sup>32</sup> lung inflammation of hospitalised welders was linked to their exposure to welding fumes containing iron during the past year. *Lead* may also be released upon welding, and is well known to cause damage to the nervous system, reproduction and foetus <sup>25</sup>. *Zinc*, in the form of zinc oxide, is the main factor causing the condition *metal fume fever*, which is the most commonly described health effect from welding. Symptoms of metal fume fever include transient flu symptoms such as fever and muscle pain. The importance of other metals for this condition is, however, unclear and suggestions that vaporised copper might play an important role in its development remain uncertain and needs to be studied more thoroughly <sup>33,34</sup>. Metal fume fever has been suggested to be associated with later onset of respiratory symptoms similar to asthma <sup>35</sup>. In epidemiological studies associations of respiratory disease and welding have been confirmed <sup>26</sup>. Respiratory effects seen in full-time welders include, for example asthma, airway irritation and an increased incidence of lung cancer <sup>25</sup>. Pulmonary infections are also seen to be more severe, have a longer duration time and hit welders more often.

Since several welding methods exist and the metals used can differ, each welding method may present its own potential health hazards. In addition, other factors, including the conditions under which the welding takes place, affect the exposure. An example of such a condition is whether the welding takes place outdoors or indoors. Which components of the welding fume that are responsible for health effects after exposure is difficult to elucidate since welders are exposed to a mixture of particles and gases. In addition, a possible impact from smoking habits and exposure to other factors in the work environment may also contribute.

### 1.3.2 Nanoparticles

The prefix “nano” originates from the Greek word for dwarf and refers to something small. A nanometre (nm) is a billionth of a metre ( $10^{-9}$ ). The small size of a nm might be hard to imagine. However, the thickness of this page is actually 100 000 nm (0.1 mm).

### Definitions

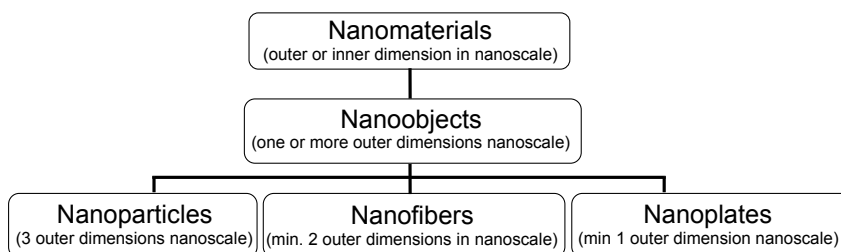
Several international institutions and organisations have suggested different definitions to be used for describing nanomaterials. However, in October 2011 the European Commission adopted a recommendation defining the concept of a nanomaterial as <sup>36</sup>:

*"A natural, incidental or manufactured material containing particles, in an unbound state or as an aggregate or as an agglomerate and where, for 50 % or more of the particles in the number size distribution, one or more external dimensions is in the size range 1 nm - 100 nm. In specific cases and where warranted by concerns for the environment, health, safety or competitiveness the number size distribution threshold of 50 % may be replaced by a threshold between 1 and 50 %.*

*By derogation from the above, fullerenes, graphene flakes and single wall carbon nanotubes with one or more external dimensions below 1 nm should be considered as nanomaterials"*

This definition is the one used for European Union (EU) legislation. Within EU, nanomaterials fall under the chemical legislation of REACH, which is applicable to the manufacture of, placing on the market of and use of substances, on their own, in preparations or in products. The general obligations in REACH include that substances manufactured at one tonne or more need registration. The European Chemical Agency (ECHA) receives these registrations and the purpose is to generate more information useful for risk assessment. Nanomaterials that fulfil the criteria for classification as hazardous, must in addition be classified and labelled independently of the amount in which the substances are manufactured or imported <sup>36</sup>. There have been discussions on how REACH should be adapted for nanomaterials since the production in many cases does not exceed the tonnage level <sup>37,38</sup>. Modifications of REACH regarding nanomaterials are currently under discussion within the European Commission.

The recommendation by EU mentioned above is partly based on the definition set by The International Organization for Standardization (ISO) where three distinctions of nanoobjects exist: 1) Nanoparticles (with a size between 1-100 nm in three dimensions), 2) Nanofibres (with two dimensions in the nanoscale) and 3) Nanoplates (with one dimension in the nanoscale) <sup>39</sup> (see Figure 1). This definition of nanoparticles is often used and is the one used within the studies of this thesis.



**Figure 1.** The ISO definitions of nanomaterials, nanoobjects, nanoparticles, nanofibres and nanoplates. Nanoscale refers to a size between 1 and 100 nm. Image reproduced from Krug and Wick <sup>39</sup> with minor modifications, with permission from John Wiley and Sons.

### *Agglomerates/aggregates*

The terms *agglomerates* and *aggregates* are often used to describe assemblies of particles. Historically there has been some confusion of what these terms stand for since the Latin meaning of these words are approximately the same <sup>40</sup>. However, the *Scientific Committee on Emerging and Newly Identified Health Risks* (SCENIHR) working for the European Commission, states <sup>41</sup> that if particles are held together by weak forces such as van der Waals forces, electrostatic forces and/or surface tension this should be referred to as agglomerates. If stronger forces such as covalent or metallic bonding hold them together aggregates should be the term to use. These are the definitions used in the studies of the present thesis.

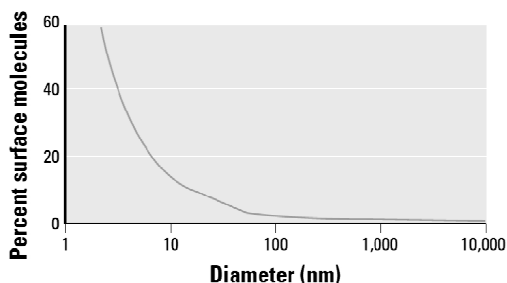
Often nanoparticles agglomerate to its neighbours, get glued to a surface or to other small molecules, due to the adhesive forces that is present close to the particle surface. Agglomerates often have the same level of toxicity as the separate nanoparticles <sup>42</sup>. However, formation of agglomerates may affect the exposure levels since deposition during inhalation as well as uptake by cells may change due to the size of the agglomerate.

### *What is special about nano?*

One specific property of nanoparticles is of course their small size. For particles in the size of 1 – 100 nm the physical behaviour changes, from classical physics to quantum physics, giving nanoparticles unique properties that larger particles do not possess despite of the same material origin <sup>42</sup>. The unique properties related to size mean that nanoparticles, to some extent, might behave like totally new particles.

Another feature of particles is that the ratio of surface atoms or molecules of the particle (i.e the number of atoms/molecules on the surface in relation to all atoms/molecules present in the particle) increases exponentially with decreasing particle size (see Figure 2). This means that a greater proportion of its atoms and molecules are present at the surface and available for chemical reactions with the surroundings.

The size related properties of nanomaterials are the reason for its wide use in applications of various kinds. Nanomaterials, including nanoparticles are today increasingly used both in technical and medical applications as well as in consumer products.



**Figure 2.** Surface molecules as a function of particle size. Surface molecules increase exponentially when particle size decreases < 100 nm. Reproduced from <sup>10</sup>, with permission from Environmental Health Perspective.

### *The use of nanomaterials and nanoparticles*

The global market for nanomaterials is currently estimated to be 11 million tonnes/year<sup>43</sup>. Some nanomaterials have been produced and in use in large volumes for almost a century, such as carbon blacks used for the manufacturing of rubber and pigments. Silica and oxides of titanium are examples that have been in extensive use for almost half a century, as agents in paint and cosmetics<sup>42</sup>. According to the European Commission, also aluminium oxide, barium titanate, cerium oxide, zinc oxide, carbon nanotubes and nanofibres, as well as silver are produced in thousands of tonnes yearly<sup>43</sup>. Lately, many new applications containing nanomaterial have been developed, e.g. consumer products such as cosmetics, sunscreens, anti-odour textiles, coating of self-cleaning windows and roofs, and food packaging. The amount of nanomaterial-containing consumer products on the market is today estimated to be over 1000, with the most commonly used nanomaterials being silver, carbon, titanium and silica<sup>44</sup>. In addition, medical and technical applications including cancer therapies, lithium-ion batteries for electrical cars and solar panels utilising nanomaterials also exist. Environmental benefits from using nanotechnology are suggested to include reduction of energy consumption as well as emission of green house gas emission<sup>45,46</sup>. All the applications mentioned have the potential to create major technological breakthroughs for the society<sup>47</sup> and be beneficial in a lot of different sectors, such as in the electronic industry, the construction industry, engineering and environmental technology. Within EU 300 000 - 400 000 jobs is currently linked to nanotechnology<sup>48</sup>.

### *Human exposure - toxicity and health effects*

The same properties that make nanoparticles so attractive to use in different contexts are properties that potentially can cause toxicity and damage to cells. If nanoparticles are compared to other biological materials they are in the same size range as viruses, smaller than bacteria and the cellular nucleus. Hence, there is no surprise that cell interaction may be possible. Nanotechnology has been around for quite some time, but studies of the toxic potential of nanomaterial lag behind. For most manufactured nanoparticles there is a lack of toxicity data available, or the data is inadequate. Toxicity studies of particles in nanometre size have indicated that particles cause toxicity in lower doses than larger particles of the same material<sup>42</sup>. However, the toxicity seems to differ depending on which characteristics the nanoparticles possess. Analysis of published data of *in vitro* toxicity of metal containing nanoparticles suggest that nanoparticles that are oxidised, reduced or dissolved are cytotoxic and genotoxic while stable metal containing nanoparticles indicate no significant cellular toxicity<sup>49</sup>. Moreover, other properties of metal containing particles, such as the possible presence of transition metals that may cause the formation of reactive oxygen radicals (which will be discussed later on) may be particularly important for these particles. Aust et al.<sup>50</sup> showed, for example, that transition metals of particles were linked to radical formation and subsequent release of cellular mediators important for the induction of respiratory symptoms. Studies of prolonged exposure of rats (of e.g. carbon black, titanium dioxide, iron oxides and amorphous silica) have, in addition, shown airway inflammation and lung tumours as a result<sup>42</sup>. Yet, the knowledge of the effects of human exposure to nanoparticles is limited. However, epidemiological studies on ambient particles show that when the concentration of all kind of particle sizes are high in the atmosphere, morbidity and mortality increase<sup>51</sup>.

The wide use of nanomaterials speaks for a range of different situations where exposure to these particles might occur. Inhalation is considered the major route of exposure, but ingestion and dermal exposure are also potential exposure routes. In the case of nanomedicine, exposure via administration directly to blood, subcutaneously or intramuscularly must also be considered. Nanoparticles that are incorporated into technical equipment might not be a risk to the consumer whereas products containing free particles, such as cosmetics, certainly will cause exposure. Production and waste handling of products or of the nanomaterial itself might pose a risk to workers. Data on exposure of nanomaterials at some workplaces have recently been reported <sup>52,53</sup>. Nonetheless, the available data of exposure in the work environment is small relative to the number of jobs linked to nanomaterials.

### 1.3.3 The subway

In recent years high levels of particles have been observed in the subway (underground) systems of several major cities around the world, including Berlin <sup>54</sup>, Helsinki <sup>55</sup>, London <sup>56</sup>, Los Angeles <sup>57</sup>, Mexico city <sup>58</sup>, New York <sup>59</sup>, Paris <sup>60</sup>, Rome <sup>61</sup>, Seoul <sup>62</sup>, Stockholm <sup>63</sup> and Tokyo <sup>64</sup>. The monitored particle levels in these subway systems, in terms of PM<sub>2.5</sub> and PM<sub>10</sub>, are reported to range from average values of 47 to 480  $\mu\text{g}/\text{m}^3$ . In the subway of Stockholm, the levels of PM<sub>2.5</sub> and PM<sub>10</sub> was reported to be 260 and 470  $\mu\text{g}/\text{m}^3$ , respectively, which was 10 and 5 times higher when compared to levels at a busy street close by <sup>63</sup>. That the subway system presents particle levels comparable with, or above, PM levels of urban environments with heavy traffic is a phenomenon often observed.

#### *Subway particles*

Analyses of the origin and composition of particles from the subway system have concluded that the particles mainly originate from wearing of rails, wheels and brakes as well as from release from current collectors and conductor rails <sup>64</sup>. Predominantly, the particles consist of iron and iron oxide. In a study of the subway of Stockholm, approximately 60% of the total mass of collected particles was iron <sup>65</sup>. Manganese, chromium, zinc, copper, barium and silica are also often present in smaller amounts, as well as elements of aluminium, antimony, lead, nickel, tin and titanium, which are found in very small quantities <sup>56,61,66</sup>. The presence and amounts of the different elements differ between subway systems and depend on the type of rails, trains etc. In a study of the subway station Odenplan in Stockholm, particles in nanometre size were shown by Midander et al. <sup>67</sup> to dominate in total particle number compared to coarse particles. The mean of nanometre particles were 12 000 particles/ $\text{cm}^3$  and particles larger than 250 nm in size were 190 particles/ $\text{cm}^3$  <sup>67</sup>. From the size modes measured of the nanometre particles it was suggested that both metal containing particles and diesel particles were present in the air of the station.

#### *Toxicity and health effects*

In contrast to the large number of studies performed on traffic related PM, less is known about toxicity and health effects from exposure of subway PM. However, subway particles collected in London have shown toxicity *in vitro* in terms of release of the pro-inflammatory marker interleukin-8 (IL-8) from human lung cells at the same

levels as welding fume particles when compared <sup>56</sup>. In this study the subway particles also induced cell death and DNA damage. In another study of cultured human lung cells, subway particles were shown to be more potent to cause oxidative stress and DNA damage than traffic related particles, particles from wood combustion and tire-road wear <sup>68,69</sup>. Macrophages exposed to subway particles from the subway in Paris have been shown to increase the production of the inflammatory markers TNF- $\alpha$  and MIP-2 protein <sup>70</sup>. Inflammatory responses such as increased amounts of neutrophils in bronchoalveolar lavage (BAL), altered epithelial permeability and increased expression of oxidative stress markers after particle exposure have also been observed, in exposed mice <sup>70</sup>. Interestingly, the effects of subway particle exposure were more pronounced than those observed after diesel exposure. Studies regarding human health effects from subway particle exposure are scarce, but some studies have been conducted on the personnel of the subway. Bigert et.al <sup>71</sup> observed slightly increased levels of blood markers for cardiovascular disease and inflammation in exposed subway platform workers when compared to less or not exposed workers.

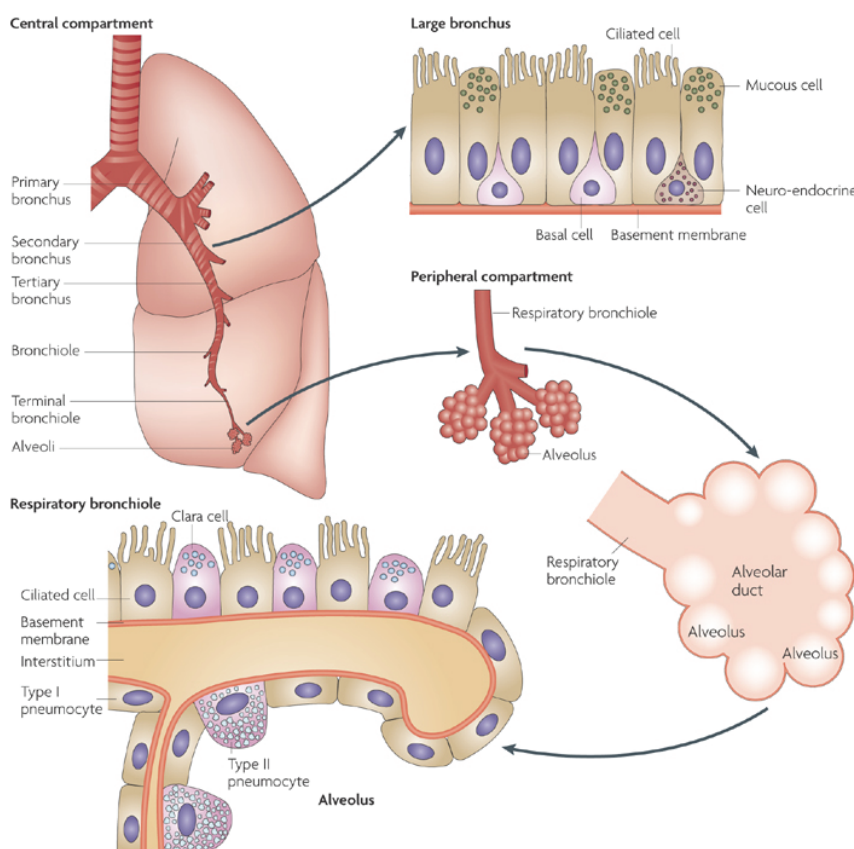
The subway system is in many cities a highly important transport system with a large number of daily commuters. In Stockholm the subway accounts for approximately 45% of all the public transportation in the area with around 1 137 000 boardings each weekday <sup>72</sup>. This means that human exposure to subway particles may occur daily, during long periods of the citizen's life. In a study conducted in New York measuring the exposure levels of metal containing particles <sup>73</sup>, the subway system appeared to be the dominant exposure environment for personal airborne exposures to iron, manganese and chromium when levels were compared to ambient and indoor air. The data suggest that the time spent in the subway system could be a primary determinant factor of the personal exposure to these metals for people who are not occupationally exposed. In another study <sup>74</sup> performed on taxi drivers and office workers in London, the exposure to manganese was studied before and after it was introduced as an additive in diesel. This study showed that the manganese exposure did not increase as a result of introduction of the additive. However, the mean exposure to manganese was higher among the office workers in both years compared to the taxi drivers. This was due to that approximately half of the office workers commuted via the underground railway system where airborne dust and manganese concentrations were measured to be 10-fold higher than in the general environment. Despite all this, studies addressing the question of whether exposure to particles in the subway environment could possess a risk to daily commuters, healthy persons or sensitive groups, are few and represent a knowledge gap.

## **1.4 LUNG EXPOSURE AND MECHANISMS**

Fundamental for the understanding of health effects from particle inhalation is to comprehend the lung exposure. If there is no exposure there is no risk. To what extent, and where in the lungs, particles are deposited, in addition to how well the immune defence system of the body can clear the particles is essential. Translocation of particles from the site of entry to distant organs is currently also a highly discussed issue.

### 1.4.1 Lung exposure – deposition, dose, clearance and translocation

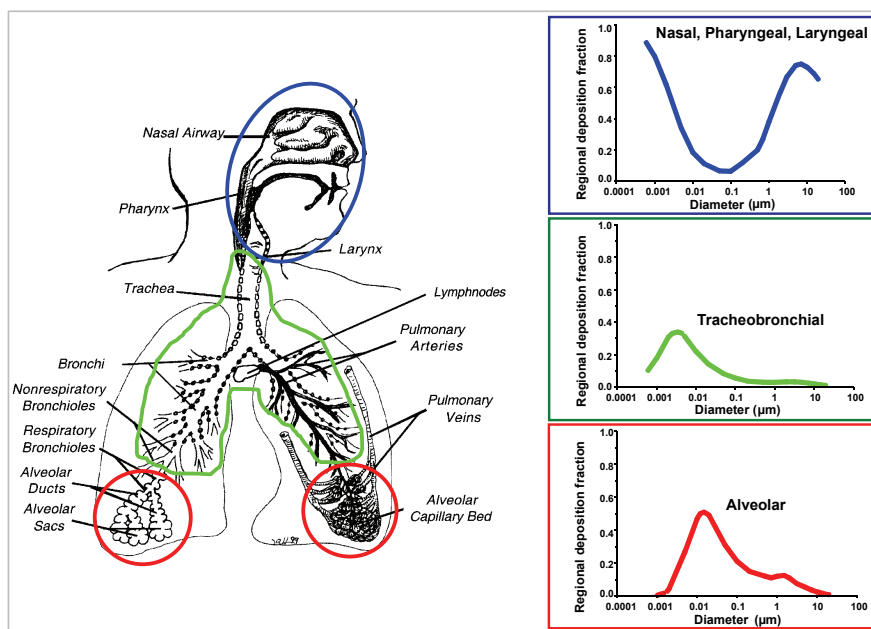
Breathing is essential for life and thousands of litres of air pass the lungs every day. The human respiratory system can be divided into two general zones, the *conducting zone* (consisting of nose, pharynx, larynx, trachea, bronchi and bronchioles) with the functions to filter, warm, moisten and conduct the air to the lungs and the *respiratory zone* where the gas exchange of oxygen and carbon dioxide takes place in the alveoli. The diameter of the alveoli is approximately 0.2 mm and the total surface area of the lungs is 140 m<sup>2</sup> <sup>75</sup>. The lung barrier, which consists of a layer that is only two cells thick, is a unique construction that together with the large surface area enables effective uptake of oxygen to the body. The anatomy and present cells of the different regions of the respiratory tract differ to some extent and approximately 40 cell types are represented in the respiratory tract all together. A schematic illustration of the anatomy and the most common cells within the respiratory tract can be seen in Figure 3.



**Figure 3.** The human respiratory tract. Image from Sun et al. <sup>76</sup>. Published with permission from Nature Publishing Group.

## Deposition

When air contaminated with particles is inhaled, a certain amount of the particles will deposit in the respiratory tract. The size of the particle is an important determinant for where the deposit takes place, as illustrated by the graphs in Figure 4. If the respiratory tract is separated into nasopharyngeal, tracheobronchial and alveolar regions, nanoparticles can target all three of these regions. From these data it can also be concluded that even small changes in the size of nanoparticles alter the percent of the deposit in the different regions. Larger particles, over 1  $\mu\text{m}$ , often deposit in the nasopharyngeal region and just a small fraction will reach deeper into the system reaching the tracheobronchial and alveolar regions. The particle deposition shown in Figure 4 is valid for nose breathing during rest and it is based on predictive mathematical models for single particles, not aggregates<sup>10,77</sup>.



**Figure 4.** Deposition of particles in the human respiratory tract. The image is a reproduced version from Oberdörster et al.<sup>10</sup>, with permission from Environmental Health Perspective.

Nanoparticles mainly deposit by diffusion, which is due to collision with air molecules. When nanoparticles carry a significant electric charge, electrostatic precipitation may also occur. Deposition of larger particles is mainly accomplished through other mechanisms, that do not occur for nanoparticles, such as impaction, gravitational settling and interception<sup>10</sup>.



### *Exposure dose*

There is a natural dose limit of  $10^6$  particles/cm<sup>3</sup> (particle number concentration) for which dispersed particles in a stable aerosol starts to merge into larger particles. This limit is not often reached for micrometre sized particles, but for nanoparticles this is achievable. For 20 and 100 nm sized nanoparticles, this particle number concentration corresponds to 4.2 µg/m<sup>3</sup> and 525 µg/m<sup>3</sup>, respectively <sup>78</sup>. This phenomenon is important to consider since the location of particle deposition may change if the original particles are increased in size.

The airflow follows certain patterns in the respiratory tract, which means that particles of micrometre size in an aerosol often will not deposit evenly, but rather in higher concentration at certain locations <sup>79</sup>. This is the concept often referred to as “hot spots” in the context of particle deposition. In a study of chromate manufacturing workers, it was shown that hot spots with higher content of chromium was found in bifurcations of the airways <sup>80</sup>. A small fraction of epithelial cells in a hot spot can receive doses that are a few hundred times higher than the average dose of the total airways <sup>81</sup>. In a review by Borm et al. <sup>82</sup>, a calculation from *in vitro* data of cellular exposure to quartz in the dose 40 µg/cm<sup>2</sup> (the cm<sup>2</sup> refers to the cell-layer in a petri dish) was transformed to corresponding doses in exposure of rats. Their calculation showed that the total burden of particle deposition in the rat lung would be 155 mg if the particles were spread evenly in the whole lung with homogeneous concentration. This amount of particles exceeds threshold doses for inflammation and does not represent a likely exposure scenario. When this was recalculated with the idea of hot spots in mind, it was concluded that a much lower amount of particles (approx. 12 mg) needs to be inhaled to get the same concentration in the hot spot areas. If a rat inhales a dose of 6.2 mg/m<sup>3</sup> of quarts, that lung burden would be reached after 15 months (if exposed 5 days/week, 7 h /day). This example is for quartz particles and whether the local dose will be the same for other particles is hard to predict since the characteristics of the particle influence this.

The calculation experiment above illustrates a concept and indicates that doses around 40 µg/cm<sup>2</sup> used *in vitro* (a dose used in the studies within this thesis) may occur *in vivo* after a certain time of particle exposure at levels comparable to some occupational exposures. It is, however, highly important to point out that cells *in vitro* are exposed to this dose all at once and hence this dose can be considered to be high. The effects are also evaluated during a quite short time frame after exposure *in vitro*, compared to a rat that inhales it over a longer period of time, enabling the immune system to take care of the particles. In a review by Gangwal et al. <sup>83</sup> this issue, of what *in vitro* doses to use in order to reflect *in vivo* doses, was addressed. Gangwal et al. here suggest that concentrations between 30 and 400 µg/ml, often used in *in vitro* toxicity testing (40 and 80 µg/ml have mostly been used in the studies of this thesis), are in fact relevant since these doses are comparable with that of daily exposures for 45 years of certain particles in occupational settings. As a response to this review, Oberdörster <sup>84</sup> highlights what was emphasised above, namely that results from years of exposure should not be extrapolated to dosage for *in vitro* testing of short-term exposure, and that extrapolations like these can be highly misleading. For nanoparticles it is also important to consider whether the concept of hot spots, relevant for particles in micrometre size, is an occurring event or not. Studies have shown that deposition

patterns become more and more homogenous (the particles are more spread out) when particles decrease in size, especially below 100 nm<sup>85</sup>. However, this highlights the importance to consider agglomerated nanoparticles, forming aggregates that may be more likely to deposit in certain hot spots.

#### *Clearance and translocation*

The respiratory tract has several defence mechanisms for catching and clearing foreign components, such as particles, from the inhaled air<sup>10</sup>. Particles trapped in the nose and mouth are often sneezed out, coughed out or swallowed. If particles pass this first gate of clearance and deposit in the tracheobronchial region, the major pathway of clearance is by mucociliary transportation. Particles that deposit in this region will initially interact with the lung surfactant, which is a liquid creating a thin layer at the surface of the tissue. Particles will then move into the mucus layer underneath. The mucus is an aqueous gel containing a network of glycoproteins as well as electrolytes, serum proteins, immunoglobulins and lipids. The mucus moves towards the upper end of the tracheal tube due to the movement of the ciliated cells in the epithelium. Hence, particles will rather quickly be removed from the site of its deposit (around 24 hours). When the mucus reaches the pharyngeal region the particles and mucus will either be swallowed or coughed up.

The ciliated cells are, however, rare in the alveoli and clearance here mainly depends on a slower clearance mechanism through phagocytosis by macrophages<sup>86</sup>. Macrophages with internalised particles move towards the regions with ciliated cells for further transport to the larynx. This mechanism is initiated within a few hours after particle deposition, but it can take days or even years before the particles are cleared out<sup>87</sup>. Nanoparticles may bypass phagocytic uptake by macrophages, which has been shown in several studies<sup>77,88</sup>. In a study by Ferin et al.<sup>89</sup>, lungs of rats were investigated after exposure of TiO<sub>2</sub> and Al<sub>2</sub>O<sub>3</sub> particles, and the result showed that particles of nanometre size were found in the lavage fluid (collected one day post exposure) to a much lesser extent than particles of micrometre size. The authors suggest that the reason for this is that the nanoparticles are taken up into the pulmonary tissue to a greater extent than particles of micrometre size. This was in fact also shown to be the case, as was seen when the content of these particles in lung tissue and lymphatic nodes were investigated in the same study. In a recently published review by Kreyling et al.<sup>78</sup>, pathways for particle clearance in the peripheral lungs include transportation to lymph nodes located close to the lung as well as possible further translocation from epithelial cells to the interstitium or beyond. For particles with a certain degree of solubility, dissolution of the particle may also be an important mechanism for clearance<sup>10,77</sup>.

Inhalation studies of rats have shown that different kinds of nanoparticles to a high degree penetrate into the alveolar epithelium and underlying interstitium<sup>78</sup>. Translocation across the air-blood barrier in the lung, have been shown to occur for gold, silver, TiO<sub>2</sub>, polystyrene and carbon nanoparticles in the size range of 5 - 100 nm, in animal studies<sup>77</sup>. Translocation mediated by alveolar macrophages from the lung to regional tracheobronchial lymph nodes has also been shown for micrometre particles<sup>90</sup>. In a study by Choi et al.<sup>91</sup>, the importance of chemical composition, shape, size and surface charge was evaluated in terms of particle translocation from lung to

lymph nodes in rats. The study showed that the majority of the tested inorganic as well as organic nanoparticles remained in the lung within 30 minutes after administration, but for particles < 34 nm there was a fast translocation from the alveolar luminal surface to regional lymph nodes. It was also seen that the smallest nanoparticles (<6 nm) with a zwitterionic surface charge (a neutral particle with a positive and a negative electrical charge at different positions) very rapidly (in minutes) translocated to the lymph nodes, and 30 minutes later particles could be traced in the kidneys and in urine. Liver, bile and intestine showed no accumulation of the particles<sup>91</sup>. Studies have also indicated that nanoparticles that penetrate into the interstitial spaces of the lungs of rats are phagocytised by macrophages and then transported back to the inner lining of the lung epithelium for further clearance<sup>78</sup>. Translocation of nanoparticles to the brain, via the olfactory nerve, has been observed in rats<sup>92,93</sup>. However, since the nose of rodents differ to a great extent to humans, with a higher developed nervous system in this region in rodents, it is difficult to extrapolate these findings to human.

Studies like these have, however, raised concern of possible translocation of nanoparticles also in the human body. Several studies have tried to study translocation of nanoparticles from the lungs and there has been some indication of possible translocation, but there is no strong evidence for any major translocation beyond the lung. In a study of carbon nanoparticles it was estimated that approximately 1% of the exposure dose had translocated in the exposed human subjects<sup>94</sup>. Labelling of nanoparticles (for tracing of the particles within the body) with e.g. radio-isotopes, is often difficult and leaching of the isotope may be a problem<sup>77</sup>. In translocation studies this is an issue that may contribute to false positive results if the probe is translocated instead of the nanoparticle. This may be one reason for the ambiguous results seen regarding e.g. translocation to blood and accumulation in the liver after inhalation of labelled carbon nanoparticles in humans<sup>95,96</sup>. Due to ethical issues, studies on human exposure have also been difficult to conduct.

Epidemiological studies have established that inhalation of ambient PM increases the risk for cardiovascular mortality and morbidity<sup>97</sup> and particles in the nanometre size are thought to contribute to cardiovascular effects more than larger particles<sup>98</sup>. Several mechanisms have been suggested to be responsible for the systemic cardiovascular effects including activation of neurons in the lung that may change cardiac function and rhythm, direct systemic effects by translocated particles, and the release of inflammatory mediators from the lung into the circulation<sup>99</sup>. If the amount of translocated particles is enough to give these effects is unsure and alveolar inflammation induced by particles initiating a systemic response with e.g. changed coagulation of the blood may be a more likely explanation.

#### 1.4.2 Mechanisms of toxicity – oxidative stress, inflammation and DNA damage

If the air is contaminated with toxic components it can have a significant impact on human health through inhalation. The following section will explain some of the most common and studied mechanisms of toxicity from particle inhalation and in which way they are linked to common human diseases. The mechanisms described below are

selected in terms of their importance for particle toxicity, but also in terms of relevance for the studies performed in this thesis. It may also be stressed that the mechanisms described below often are linked to and affect each other.

#### 1.4.2.1 Oxidative stress

Oxygen is one of the key components needed for human life and oxidation is therefore a constantly present event in our cells. Oxidation is *a loss of electrons or an increase in oxidation state of a molecule, atom or ion*. It is an important reaction for cellular function and oxidising agents are formed constantly during normal processes e.g. from energy production in the mitochondria of the cell<sup>51</sup>. Many of the oxidising agents are free radicals, which are atoms or molecules having one or more unpaired electrons<sup>100</sup>. Radicals are usually electrically neutral; no electrostatic interactions will be formed between radicals, but since a radical contains an atom with an incomplete outer electron shell, most radicals are unstable and reactive.

*Reactive oxygen species* (ROS) is a collective term for oxygen radicals and non-radical derivatives. The term reactive is relative, where some of the species are highly reactive attacking everything they come close to and others are more selective<sup>100</sup>. There are many different forms of ROS, for example the superoxide radical ( $O_2^{\bullet-}$ ), hydrogen peroxide ( $H_2O_2$ ) and hydroxyl radicals ( $OH^{\bullet}$ )<sup>51</sup>. The definition of ROS is today often described in the context of the expanded term *reactive species* including not only oxygen species, but also halogen, sulphur and nitrogen species<sup>100</sup>. This term includes several species, and it is important to remember their own chemical properties.

Cells can cope with a certain amount of ROS, owing to the many defence mechanisms that have evolved during evolution. Non-enzymatic scavenger and quenchers (also known as antioxidants), as well as enzymatic systems such as e.g. glutathione peroxidase and catalase are important in this defence against harmful ROS<sup>101</sup>. These convert ROS to less toxic compounds<sup>51</sup>, and an equilibrium state of ROS and defence are normally reached in the cell. If the balance is disturbed and an overload of reactive species is created, a situation called *oxidative stress* occurs. This term has been defined as:

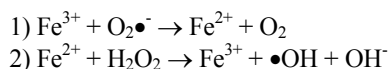
*“a disturbance in the prooxidant-antioxidant balance in favour of the former, leading to potential damage”*<sup>102</sup>

In the studies conducted in this thesis this commonly used definition of oxidative stress has been used. This definition is, however, not crystal clear and it has recently been suggested that the term *redox imbalance* maybe should be used instead<sup>103</sup>.

ROS can cause great damage if reacting with different components of the cells such as proteins, lipids and DNA<sup>104,105</sup>. Necrosis (unprogrammed cell death) and apoptosis (programmed cell death) can also be induced. An often discussed concept in the context of oxidative stress and particles is the hierarchical oxidative stress model, describing a tiered process with particle generated ROS giving rise to oxidative stress, which promotes inflammation reactions and subsequently development of pulmonary diseases. This process is divided into three steps where low amounts of oxidative stress induces antioxidant enzymes, intermediate amounts activates redox-sensitive

signalling pathways (including e.g. MAP kinases and NF-κB activation) with subsequent changes in pro-inflammatory gene expression, and high amounts causes toxicity in terms of apoptosis and necrosis <sup>106,107</sup>. ROS generated damages, at the cellular level, may lead to a range of different diseases, including atherosclerosis, autoimmune diseases, inflammation and malignant diseases. Actually, more than 100 different diseases in humans have been suggested to have a link to oxidative stress <sup>108</sup>. Especially cancer development and neurodegenerative diseases, such as Parkinson's and Alzheimer's disease, seem to have a strong connection <sup>109</sup>. Oxidative stress is considered the most important mechanism explaining health effects from exposure to ambient PM <sup>110</sup> and several mechanisms have been defined by which particles can generate ROS. The mechanisms can roughly be divided into *direct* or *indirect* pathways <sup>111</sup>. Examples of direct reactions includes ROS production mediated by molecules at the surface of the particle (e.g. free radicals, transition metals or other chemicals) <sup>107</sup>. Examples of indirect mechanisms include release of metals and organic compounds generating ROS, as well as activation of the immune system, releasing ROS as a defence mechanism. Particles in nanometre size have also been shown to target or disturb mitochondria <sup>112</sup>, which, if damaged, may cause a release of ROS. A combination of these mechanisms is most likely to occur from particle exposure <sup>111</sup>.

When it comes to metal containing particles, the concept of soluble transition metals, e.g. iron, copper and chromium, is of high relevance. Transition metals can generate ROS by the so-called *Fenton reaction* or *Haber-Weiss reaction* <sup>105</sup>:



Net reaction:  $\text{O}_2\bullet^- + \text{H}_2\text{O}_2 \rightarrow \text{O}_2 + \bullet\text{OH} + \text{OH}^-$

The significance of the Fenton reaction *in vivo* has been debated for many years <sup>113</sup>, since the prevalence of free catalytic metals within the body is small under normal conditions due to metal-binding proteins. Nevertheless, the Fenton reaction might be of importance in conditions where an overload of metals occurs.

Oxidative stress is, together with inflammation the main mechanisms linked to health effects caused by particle exposure <sup>111</sup>. Oxidative stress is, however, not an isolated event, but rather an important player in other mechanisms of cellular damage, such as inflammation and DNA damage, both which will be discussed in the following sections.

#### 1.4.2.2 Inflammation

Inflammation is a natural biological response in humans, with the purpose to protect against foreign pathogenic invaders including microorganisms (viruses and bacteria), mechanical injury, particles, pollen and chemicals. The immune defence consists of several types of protection. The first type of defence that the invaders often get in contact with is from the *innate immune defence*. It can be divided into four groups of barriers including *anatomic or physical* (e.g. membranes), *physiological* (e.g. increased

blood flow, degradation of materials, activation of the immune system by temperature, pH, lysozymes and interferon), *endocytic and/or phagocytic* (uptake by specialised cells) and *inflammatory* barriers (recruitment of macrophages and neutrophils, mainly)<sup>114</sup>. The innate immune system is nonspecific and has no memory; it will react similar every time it is exposed to foreign components, and it responds immediately upon invasion. In the respiratory tract, alveolar macrophages and polymorphonuclear neutrophil cells (PMNs) play important roles in the defence system. The PMNs are relatively short-lived phagocytic cells, which increase in number during inflammation. During an immune response they leave the blood and transfer to the lung. Macrophages, on the other hand, reside in the lung tissue. They also have the capacity of phagocytosis of particles and can, together with PMNs, initiate inflammatory responses in the lungs by initiating release of pro-inflammatory mediators such as cytokines (e.g. TNF- $\alpha$ ) or chemokines (e.g. interleukins such as IL-8, IL-6 and IL-2)<sup>115</sup>. The mediators enable recruitment of additional cells, e.g. monocytes that will mature to macrophages in the lungs. ROS can also be released by macrophages, to defend the body against the invading pathogens<sup>115</sup>. Many phagocytic cells, e.g. dendritic cells, can present antigens for other immune cells which, together with secreted chemotactic peptides, stimulate proliferation and recruitment of T-lymphocytes (helper T-cells and cytotoxic T-cells) from the lymphoid organs to the site of invasion and connect the innate response to the next line of defence; the adaptive immune defence. The *adaptive immune defence* responds slowly to pathogens at the first time of invasion, but effectively to re-exposure of pathogens. In the adaptive response (in addition to T-cells mediated destruction of pathogens), B-lymphocytes produce antibodies that bind specifically to antigens, using its specific memory from the last invasion. They mark the foreign components for further destruction of phagocytic cells.

Most often, under normal circumstances, inflammation resolves itself and is a transient condition. If the inflammation gets out of control it might, however, cause severe diseases such as asthma and various pulmonary lung diseases<sup>116</sup>, as well as cardiac infarction<sup>104</sup>. At the cellular level, the production of ROS can cause damage to surrounding cells that may alter their cellular function and finally cause disease.

Particles of various kind, both ambient PM and metal containing particles have shown inflammatory potential, as previously described. However, the inflammatory potential seems to differ between particles with different characteristics. In a study by Cho et al.<sup>117</sup> where 15 metal/metal oxide nanoparticles were compared in their inflammatory effects after lung exposure of rats, this was demonstrated as a rather diverse inflammatory potential between the particles. In the context of this thesis, markers for inflammation have been evaluated in a human exposure study in the subway system (study V).

#### 1.4.2.3 DNA damage

The human genome, consisting of the DNA, is the genetic construction code essential for cellular function and life. DNA consists of a double stranded helix, built up of sequences of nucleotides. A nucleotide is composed of a nucleobase, a five-carbon sugar (either ribose or 2-deoxyribose) and one or more phosphate groups. The nucleobases are: adenine (A), cytosine (C), guanine (G) and thymine (T). The

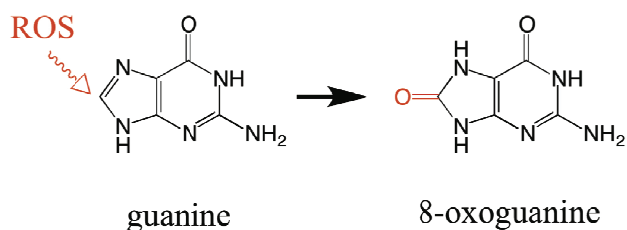
nucleobases of one of the backbone strands connect to the nucleobases of the other by base pairing of G-C and T-A. The genetic code is created by the sequence of the nucleobases. It is translated by the cells to specific sequences of aminoacids during the transcription, which creates proteins. This process is crucial for life and damage to the genome may cause lesions that are fatal, e.g. contributing to the development of cancer. Cancer development is, however, complex including several steps and taking a long time, often many years. There are several cellular changes that have been considered to be needed for a cell to become a cancer cell. The cell needs to be changed so it 1) sustains proliferative signalling, 2) does not respond to anti-growth signals, 3) is resistant to cell death (apoptosis), 4) has the capability to invade tissue and metastasise, 5) can induce angiogenesis and 6) has a limitless replication<sup>118</sup>. In recent years, two more characteristics have been discovered to be important as well, 7) a possibility to reprogram energy metabolism and 8) the characteristics to evade immune destruction<sup>119</sup>. Damage to DNA, which is not repaired or taken care of in any other way (e.g. by apoptosis) may lead to genetic instability and alteration, i.e. mutation, which can contribute to changes in the characteristics of a cell. If the cell is unlucky, the mutation occurs in a gene important for development of any of the eight characteristics mentioned above and then the cell is one step closer to becoming a cancer cell.

Exposure to ambient particles is associated with cancer<sup>120</sup> and two main mechanisms have been described by which particles might cause damage to DNA: primary and secondary genotoxicity<sup>121</sup>. *Primary genotoxicity* refers to genetic damage caused by particles without the involvement of inflammation. Primary genotoxicity can be either direct or indirect. *Direct, primary genotoxicity* is caused by direct interaction of the particle with genomic DNA. This includes reactions via the particle surface, e.g. by ROS bound to the particle surface causing an oxidative attack, or by physical interaction of the particle and DNA. Direct interaction between the surface and DNA is only possible if the particle is close to DNA, which means that particles need to be taken up into the nucleus. Nanoparticles may gain direct access to DNA via transport into the nucleus through nuclear pores. However, it is likely that nanoparticles need to be no more than a few nanometres in size to be able to pass the nuclear pore complex, which itself is less than 8 nm in diameter<sup>122</sup>. Particles may also, possibly, come in contact with DNA during mitosis when the nuclear membrane breaks down. To what extent nanoparticles can get into the nucleus is under debate, but studies of e.g. CuO, silica and polystyrene nanoparticles have shown particle lodging in the nucleus<sup>123-125</sup>. For silica, Borm et al.<sup>82</sup> have shown that a very high dose is needed for genotoxicity to occur by the direct, primary mechanism. Evaluation of the cells in this study, by electron microscopy, demonstrated that there were no particles present in the nucleus. Therefore, the DNA-damaging effects of these particles was not due to penetration of the particles into the nucleus, thus indicating that DNA damage of the particles in fact was caused by an indirect mechanism<sup>82</sup>. *Indirect, primary genotoxicity* refers to DNA damage that occurs due to enhanced production of ROS caused either directly at the surface of the particles or by contact of particles with e.g. mitochondria. Depletion of antioxidants caused by particle interaction may also increase the amount of ROS in the cell. Release of metals or organic species from the surface of the particle may be an additional source to ROS production. The ROS may reach the nucleus and damage DNA. *Secondary genotoxicity* is a consequence of inflammation, where the inflammatory cells (macrophages and polymorphonuclear neutrophils) produce

oxidants that cause genotoxicity. In the review by Borm et al.<sup>82</sup> it was concluded that animal and *in vitro* studies support their hypothesis that genotoxicity is foremost caused by inflammation-driven secondary genotoxicity when it comes to silica exposure. This work also highlight that a much lower exposure dose is needed to cause genotoxicity by this mechanism than by direct genotoxicity.

#### *Different types of DNA lesions*

A range of possible DNA lesions may be caused by particle exposure, including oxidation damage, alkylation damage, base loss, DNA cross-links etc. If the backbone of DNA is damaged, strand breaks, either as single strand breaks (SSB) or double strand breaks (DSB) may be the consequence. Oxidation of DNA by ROS generated by particle exposure is a likely event<sup>111</sup>, and due to the low redox potential of guanine (meaning it has a tendency to easily donate electrons)<sup>126</sup>, guanine is the base that most easily gets oxidised. The radical attack often occurs at the 8-position of the base (see Figure 5) and 8-oxoguanine is created. This damage of the nucleobase is sometimes also referred to as 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodG), but then the sugar is also linked to the base. 8-oxoguanine can also be introduced into DNA during replication or repair, in which the already oxidised nucleotide is incorporated from the DNA precursor pool. It should also be mentioned that oxidation may occur at other positions of the guanine base, causing lesions such as e.g. fapy-guanine (FapyG). The other bases may also be oxidised causing formation of e.g. 8-oxoadenine and fapy-adenine (FapyA)<sup>100</sup>. 8-oxoguanine is one of the most studied oxidation products of DNA and has been shown to be able to base pair with A in addition to the usual C. This transversion of GC to TA occurs upon replication and results in a mutation<sup>127</sup>. This type of point mutation is often found in the tumour suppressor gene p53, leading to its inactivation<sup>128</sup>. Mutated p53 causes G<sub>1</sub> arrest failure of the cell, which decreases the cell's capacity to repair DNA damage during the cell cycle. This can in turn give more DNA damage and contribute to the cascade by which cancer is developed. Hence, 8-oxoguanine is considered to have promutagenic potential<sup>129</sup>. 8-oxoguanine is the most frequently measured marker for oxidatively damaged DNA<sup>130</sup>, probably due to the simplicity of measuring it in biological samples in combination with its association to several cancers<sup>131</sup>. The focus in this thesis when it comes to oxidative damage has therefore been on 8-oxoguanine. Whether 8-oxoguanine can be used as a biomarker predicting cancer risk, in the same way as chromosome aberrations and micronucleus formation<sup>132,133</sup>, remains to be evaluated and its clinical importance needs further studies.



**Figure 5.** ROS attack of guanine at the 8-position causing the formation of 8-oxoguanine.  
Illustration made by Dr. Clara Ersson



### *Repair of DNA lesions*

Damage to DNA is most often effectively repaired by different repair systems of the cell. Oxidative lesions as well as strand breaks are mainly repaired by *base excision repair* (BER), bulky adducts or bulky single strand break by *nuclear excision repair* (NER) and double strand breaks by *homologous recombination* or *non-homologous end-joining*. In the following text the focus will be on repair of 8-oxoguanine.

BER is the major pathway for removal of damaged bases and it is performed by several enzymes that collaborate<sup>100</sup>. The principal steps of the BER starts with the damaged base being found and cut off by a lesion specific DNA glycosylase, creating an apurinic or apyrimidinic (AP) site. The AP site will cause a single strand break, either created by the DNA glycosylase itself or by an AP endonuclease enzyme. A DNA polymerase and a ligase then glue the parts together and insert a correct nucleotide. The human 8-oxoguanine DNA glycosylase (hOGG1) is the BER enzyme that removes 8-oxoguanine<sup>134</sup>. The bacterial version of this enzyme is called formamidopyrimidine DNA glycosylase (FPG) or MutM.

The NER is performed by several sets of repair enzymes and it works in a similar way to BER; it finds the damage, cuts DNA, a DNA polymerase fills this gap and a ligase glues it together. However, for BER only one base is often removed while NER makes a cut a certain distance from the lesion and a so called oligonucleotide (a short piece of the DNA) is removed. NER is important for removal of UV-induced damage, but can also remove oxidative lesions such as 8-oxoguanine<sup>100</sup>.

The BER and NER takes place before DNA replication<sup>100</sup>. However, *mismatch repair* is also used as a repair mechanisms for 8-oxoguanine<sup>135</sup>, and it acts on damage post replication<sup>100</sup>. In the creation of a GC to TA transversion caused by 8-oxoguanine (previously described), 8-oxoguanine is base-paired to adenine in an intermediate step. The Mut protein hMUTYH can recognise this incorrect base-pairing, making mismatch repair an additional repair mechanism for 8-oxoguanine lesions<sup>100</sup>.

## **1.5 BURNING QUESTIONS**

Ambient particles are today known to cause health effects when inhaled, which raises concern about the impact of other types of particle exposures. The most burning questions of particle toxicology of today are to do with whether nanoparticles pose a new kind of threat to human health. There is a great need for more knowledge concerning the nanoparticles and nanomaterials that already are produced, already found on the market and already used by consumers. Is there a potential risk to human health and the environment from nanoparticle exposure? To be able to solve these questions there is a need for reliable test methods, that can be used and work for the testing of nanoparticles. In addition, suitable legislation and guidelines for risk assessment is urgently needed in order to be able to use these materials in a safe and responsible way. The potential risks versus the great benefits that nanotechnology may bring is a current dilemma. In this era of problems regarding greenhouse gases and global warming, can we afford to stop techniques based on nanomaterials on vague and ambiguous grounds if they may help in these matters? Which risks are we willing to

take to serve other purposes? Other applications of nanomaterial may also be important for the development of more “environmental friendly” products, and nanomaterials in medical treatment may save lives in the future. However, distinctions may have to be made between products and applications that aid in achieving a sustainable development and those that are unnecessary in society. Do we need nano-containing products that do not improve existing products to a great deal and where the risk of adverse effects for humans and the environment are greater than the positive effects of use?

In the field of particle toxicology questions regarding what underlying mechanisms that cause health effects are also important. In the context of understanding the possible link between exposure and public health diseases such as cardiovascular diseases, this is crucial to investigate. It is, however, also important in order to increase the knowledge of new exposure scenarios from particles of materials that have not yet been studied to a great extent. These may include certain particles in work environments such as the metal industry and the subway system, both evaluated in this thesis.

## 2 RESEARCH AIM

### 2.1 GENERAL AIM

The overall aim of this thesis is to increase the knowledge concerning toxicity and underlying biological mechanisms following inhalation of metal containing particles. In the studies conducted, this has been investigated *in vitro* in lung cells after exposure to engineered particles (in micro- and nanometre size) as well as after exposure to particles from steel industries and the subway system of Stockholm. In addition, health effects of human exposure to subway particles have been investigated in an *in vivo* (human) study. Main indicators for toxicity and health effects have been markers for oxidative stress and DNA damage. Inflammation has also been investigated in the human study.

### 2.2 SPECIFIC AIMS

More specifically, the aims are to:

- Identify the role of chemical composition and size for the toxicity of metal oxides.
- Investigate metal ion release and toxicity of commercially relevant iron and chromium based particles.
- Explore the ability of various metal containing particles to cause DNA damage and oxidative stress, and to investigate whether the particles interact with the method (the comet assay) used for these analyses.
- Study health effects in humans after exposure to air pollution at a subway station in Stockholm.

#### 2.2.1 The thesis will answer the following questions

- 1) How important is the chemical composition, solubility and surface reactivity for toxicity of metal containing particles? (Paper I, II, III)
- 2) If particles have the same metal origin, is the particle size then an important factor for toxicity? (Paper II, III) Are nanoparticle more toxic than particles of a larger size? (Paper II)
- 3) Can methods used for toxicity testing of chemicals, be used for particles or do they interact with the test method, causing false results? Is this a problem especially for nanoparticles and the comet assay? (Paper III)
- 4) Are iron and chromium based particles from the stainless steel industry toxic? Can particle surface characteristics or released ions in synthetic biological media be predictors of toxicity towards lung cells? (Paper IV)
- 5) Does a short time exposure to metal containing subway particles cause acute inflammatory effects, oxidative stress or DNA damage in exposed humans? (Paper V)

### 3 GENERAL STUDY APPROACH AND ANALYTICAL METHODS

The detailed study designs as well as methods and materials used are described in each separate paper (see paper I-IV).

#### 3.1 GENERAL STUDY APPROACH

Responses following exposure to particles are complex, when seen to the human as a whole since several cell types and mechanisms are involved when particles interact with the human body. The use of different exposure models is therefore common practice when testing toxicity of components that humans are exposed to. The approach of this thesis has been both to use isolated cells in culture (*in vitro*) as well as to study humans (*in vivo*) after exposure to particles (study I-IV and study V, respectively). Chemical studies (non-cellular) where cellular components, such as bases of DNA, or enzymes, have been exposed to metal containing particles (study III) are also part of this thesis. Particle characterisation, important for understanding the toxicity (studies I-IV), as well as the air monitoring during the study of human exposure (study V) are self-evident parts of the studies included in this thesis.

##### 3.1.1 Exposure of cells, *in vitro*

When investigating the underlying mechanisms of particle exposure on the respiratory system, studies of exposed cells in culture are commonly used. Cell cultures serve as a simplified model system of exposure and can give answers to fundamental questions concerning e.g. particle-cell interaction, toxic potential of particles and they can put light on underlying mechanisms. Advantages of cell studies include that cellular functions and molecular pathways can be studied in a controlled and simplified way. The fact that cell lines often are stable and homogenous makes it easier to reproduce experiments compared to studies of primary cells, organ exposure or *in vivo* studies. On the other hand, a homogenous cell culture is also a disadvantage since the cells lack phenotypic differentiation, and since the effects seen are effects of that particular strain of cells only. An *in vitro* system can not replace or reproduce exactly what happens in the lung or in humans, but if the limitations of the *in vitro* procedure are taken into consideration and the cell lines are used in a proper way, they can be an excellent tool for answering basic questions and for understanding underlying mechanism of toxicity. Cell lines may help to understand what and why effects are seen *in vivo*. Cell culture can also be a good tool for screening of particle toxicity before animal studies are conducted and hence it may prevent some animal sacrifices. According to the three Rs (Replacement, Reduction and Refinement) animal tests should be avoided when possible<sup>136</sup>, and *in vitro* studies may be useful for this matter.

*In vitro* studies can be very valuable in hazard assessment, when evaluating if a certain particle may cause toxicity. However, to be able to make a complete risk

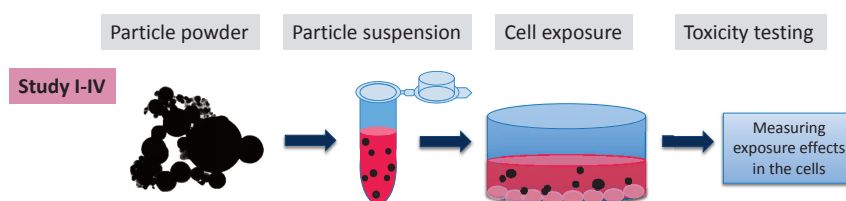
assessment of exposure, *in vitro* studies need to be complemented with information about possible exposure scenarios (if there are any risk of getting exposed), and in some cases the *in vitro* data needs to be verified by studies *in vivo*.

In this thesis, the focus is mainly at hazards (toxicity) and in the *in vitro* studies (studies I-IV) two human lung cell lines have been used: A549 and BEAS-2B.

A549 originates from a human lung carcinoma and has been used since 1972 when it was established<sup>137</sup>. The cell line has been thoroughly characterised<sup>138</sup> and is one of the most commonly used *in vitro* models when studying toxic effects of PM<sup>139</sup>. It is a lung epithelial cell line with structural and biochemical characteristics of type II alveolar epithelial cells<sup>138</sup> that are found in the alveoli of the lung. The type II cells are plump and secrete surfactant that creates a thin film on the inner surface of the lung, reducing surface tension. Type II cells are interspersed among the more frequent type I cells, which are the thin cells allowing gas exchange (see Figure 3).

BEAS-2B was originally derived in 1988 from normal human bronchial epithelial cells immortalised using the adenovirus *12-simian virus 40 hybrid virus* (Ad12-SV40 hybrid virus)<sup>140</sup>. These cells have characteristics of the epithelial cells of the bronchi and have often been used to study airway epithelial structure and function.

In the particle exposure studies of these cells (studies I-IV) the cells were grown in well-plates, in cell medium (supplemented with FBS, penicillin-streptomycin and sodium pyruvate) and in a humidified environment imitating the physiological atmosphere in the lung with 37°C and 5% CO<sub>2</sub>. The general exposure procedure and toxicity testing for studies I-IV is illustrated in Figure 6.



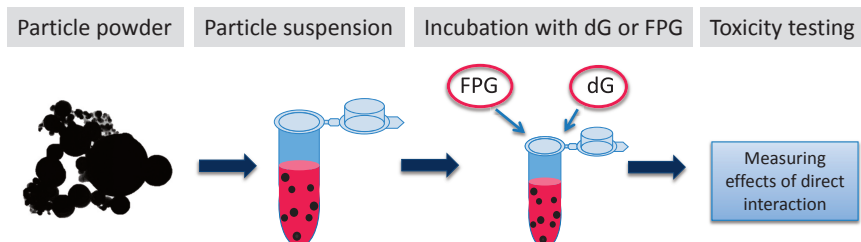
**Figure 6.** Illustration of the general approach in studies I-IV, which includes preparation of a particle suspension from dry particle powder and subsequent exposure of cells in culture. After a certain time of exposure the toxicity is evaluated.

### 3.1.2 Chemical studies, *in vitro*

To get a deeper understanding of how cellular components may interact with particles, studies where no cells are present can be conducted. Such studies can include bases of DNA or enzymes of the DNA repair system, and these components can be incubated with particles in order to understand what happens when they are in direct contact with each other. The advantage of this procedure is that no other components of the cell may

interfere. This approach was used in study III, where particles were incubated with the DNA base guanine (dG) and the DNA repair enzyme FPG. The general approach for the chemical studies is illustrated in Figure 7.

### Study III



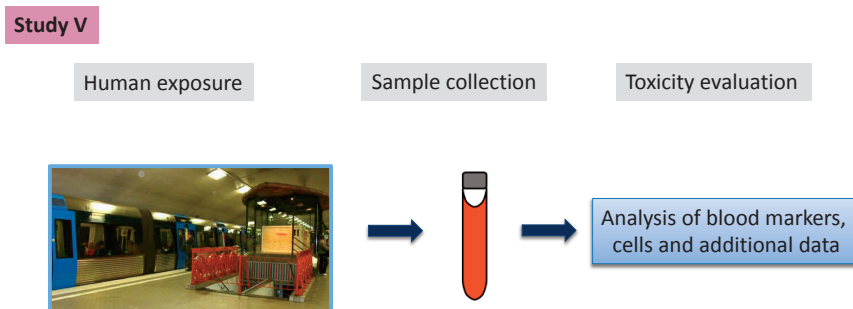
**Figure 7.** Illustration of the general approach used in study III for the chemical studies *in vitro*. A particle suspension is prepared from dry powder followed by incubation with cellular components such as DNA bases (dG) or enzymes (FPG). After a certain time the effects of particle interaction is measured.

### 3.1.3 Studies of human exposure, *in vivo*

The ultimate goal for studies of particle toxicity is to understand what happens in the human body due to the exposure. To evaluate possible health effects in humans that have been exposed to particles under controlled conditions, is as close as we can get in order to understand the real exposure. However, these kind of studies complicates the picture since the body is complex in itself, and since humans are different to each other. It is also difficult to know if there are any confounding factors present, if the study population evaluated is large enough for effects to be caught and if the markers of effect that have been chosen really are the ones that reflect what happens in the body. Despite this, a human study with a well thought-out design can give a lot of valuable information for the prediction of what would actually happen in a real life exposure of humans.

In study V of this thesis, a human study with a randomised crossover design was conducted. Eighteen healthy, non-smoking volunteers fulfilled the inclusion criteria and were included in the study. All participants gave their informed written consent to participate in the study (prior to start), which was approved by the Regional Ethical Review Board in Stockholm, Sweden. In this study the study subjects were exposed to both a subway environment with particle-contaminated air on one occasion and to clean air in an office on another occasion, with some weeks in between. The study was randomised, which means that it was decided by chance which exposure each subject should start with. The design allowed for the subjects to be their own control, opposed to the study design of so called case-control studies where subjects are allocated to be either a “control” or a “case”, in which the case group could be e.g. exposed to particles. The advantage of subjects being their own control is that the same individual is compared throughout the study. This rules out many confounding factors that may

arise due to mismatch of the case and control groups, including age and weight differences etc, in case-control studies. This approach also requires fewer subjects. The disadvantage of letting the study subjects be their own control is, however, that there might be so called drop-over effects from the exposure, meaning that effects from the first exposure is still present at the second exposure. The general study approach for the human study (study V) is illustrated in Figure 8. For more details of the study design, see paper V.



**Figure 8.** Illustration of the general study approach in study V. The human exposure of particle-contaminated air took place at a subway station, and blood samples as well as other data were collected at different time points for analysis of toxic effects.

## 3.2 PARTICLE CHARACTERISATION AND AIR MONITORING

In particle toxicology studies, it is necessary to characterise the particles thoroughly. In the studies summarised in this thesis the particle characterisation as well as evaluation of the air exposure in the human study, have been conducted using inter-disciplinary collaborations. Researchers from the Division for Surface and Corrosion Science at the Royal Institute of Technology (KTH) in Stockholm, assistance from the electron microscopy unit EMil at Karolinska Institutet and the expertise from co-authors have been used to gain an overall understanding of physicochemical properties of the particles investigated, as well as the air inhaled at the subway station.

### 3.2.1 Particle size and morphology

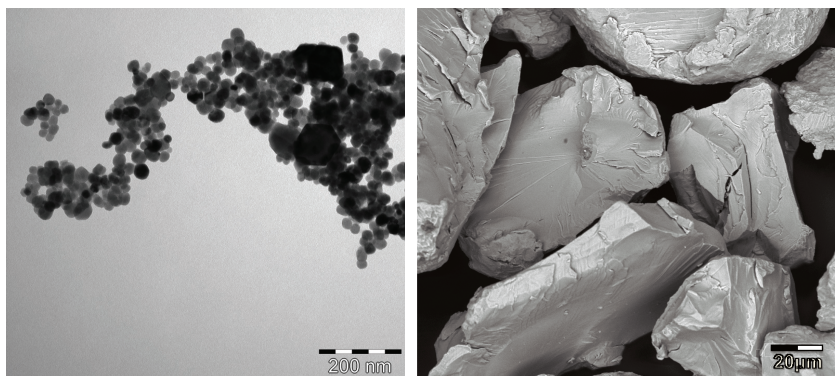
#### 3.2.1.1 Electron microscopes

The use of microscopes and microscopic images can give information on both size and morphology of particles. Ordinary light microscopes use wavelengths within the visible light range to image a sample, and they can often magnify a sample up to 1500 times. Due to the wavelengths used and hence the resolution limitation, objects smaller than 200 nanometres are not possible to detect with these devices. To be able to see objects in this size or smaller a shorter wavelength is needed. In comparison to the photons often used in light microscopes, electrons have a wavelength, which is 100 000 times shorter. Electron microscopes consequently have a greater resolution than optical microscopes and enable the analysis of structures down to the lower nanometre size

scale. In the present thesis, two microscopic techniques have been used to analyse particle size and morphology:

*Transmission electron microscopy* (TEM) was used to study the particle size and morphology in studies I-III. TEM uses a beam of electrons that transmit through the object and the transmitted electrons are detected creating an image (see Figure 9, left). TEM requires a thin, electron-transmitting sample and the darker the image, the less electrons have been transmitted through the sample (thicker particles).

*Scanning electron microscopy* (SEM) was used in study IV for micrometre sized particles and it provides high resolution images of the surface of the object investigated (see Figure 9, right). It has a greater depth of focus and hence gives a more spatially resolved image than TEM. In a SEM a high-energy beam of electrons is focused on the surface of the sample which then scans the object using a regular pattern. The resolution can be between 1-20 nm. SEM can image a large area and the thickness of the sample is not as crucial.



**Figure 9.** TEM image of magnetite nanoparticles (left) and SEM image of chromium (right). Magnetite nanoparticles are studied in studies I-III and chromium (Cr fine) in study IV.

#### 3.2.1.2 Particle size in solution

Microscopic images of particles give information of particle size and morphology at dry conditions. However, particles are often in solution when evaluated in *in vitro* studies or in contact with liquid when deposited in the lining of the lungs. Particles in nanometre size often agglomerate in a liquid, creating agglomerates. The extent of agglomeration depends on several factors, such as the composition of the liquid, the concentration of particles, the surface charge and surface groups of the particles. How particles react in liquid is of importance when it comes to toxicity and cellular uptake.

*Dynamic light scattering* (DLS) was used to measure size of nanoparticles and agglomerates in supplemented cell medium in studies I-III, by the means of light scattering. The particle size was calculated from the Brownian motion of the particles (the random movements of particles in liquid or gas resulting from the bombardment of the fast moving atoms and molecules in the liquid or gas) using the Stoke-Einstein equation. The method gives a hydrodynamic diameter of the particles, which is a



calculated particle diameter of a sphere that has the same measured motion in solution as the actual particle. Particles in micrometre size are difficult to measure by DLS due to sedimentation.

*Low angle laser light scattering (LALLS)*, which is a laser diffraction technique, was used in study IV to measure particle size in phosphate buffered saline (PBS). LALLS has the ability to measure particles in  $\mu\text{m}$  size.

### 3.2.2 Surface area and charge

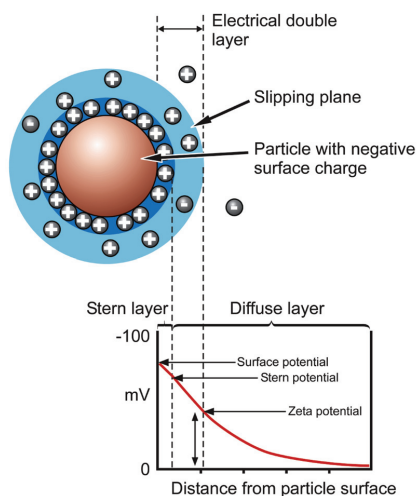
#### 3.2.2.1 Surface area

When it comes to nanotoxicology, surface area is a highly debated characteristic of particles. It is often discussed in the context of being an important factor for the toxicity due to the fact that the surface area becomes larger in relation to the mass of the particle when the particle decreases in size. If the same mass of nanoparticles and microparticles are compared, the total surface area per mass is much larger for the smaller particles. If the surface present reactive features and if surface reactions are important for toxic effects, there will most likely be a larger toxic effect from the nanoparticles than from the larger particles.

*Brunauer Emmet Teller (BET) analysis* was used in studies I-IV to measure the particle surface area. In most cases the research team of KTH was responsible for the measurements of the BET area and this information was only rarely taken from the manufacturer of the particles. BET analysis estimates the particle surface area by using the adsorbed amount of nitrogen on the particles at cryogenic conditions. Since the size of the nitrogen atom, the amount adsorbed and the mass of the particle is known, the surface area can be calculated. BET analysis is performed with dry powder and the weak forces within agglomerates do not interfere with the measurements of the individual particles in the agglomerate.

#### 3.2.2.2 Zeta potential

Most particles will acquire a surface charge in liquid, which is due to either ionisation of the surface or adsorption of charged species. The charge of the surface will result in an ion layer around the particle that is different to the rest of the surrounding liquid. The ion layer is stable and when the particle moves in the liquid (e.g. by Brownian motions) the layer will follow. The electrical potential created between the charged groups associated with the particle surface and the liquid is called the zeta potential (or  $\zeta$ -potential) (see Figure 10).



**Figure 10.** A schematic illustration of zeta potential. This figure is reproduced from <sup>141</sup> with the permission from Malvern instruments Ltd.

The zeta potential is sensitive to the components present in the liquid, and to the pH of the solution. The zeta potential influences which interactions that may occur between the particle and different components of the liquid, or with other particles. Particles with high zeta potential of the same charge (negative or positive) will cause a repulsive force, meaning that particles with a high zeta potential will remain dispersed in a solution. The zeta potential is hence a determinant for particle agglomeration, but also for the attachment of biological components to the particle creating a so-called corona around the particle.

Using the same instrument as for the DLS measurement, the zeta potential of particle suspensions were analysed in study I and III, by means of electrophoresis. The zeta potential is measured by applying an electric field through the solution containing particles, and allowing the particles to migrate towards an electrode. Dependent on the zeta potential, the particles will migrate at different speeds towards the electrode of the opposite charge, which is measured and converted to zeta potential using Smoluchowski's theory.

### 3.2.3 Air monitoring

Monitoring of the air in the human study (study V), at the subway station (Odenplan in Stockholm) and at the control environment (a clean office at the Karolinska University Hospital in Solna) was performed during each exposure session. Particle mass and number concentration in air were measured with the equipment listed in Table 1.

**Table 1.** For monitoring of the air in the human study (study V) stationary and portable instruments were used for measurements at the subway station. For the measurements at the control environment only portable instruments were used.

Equipment	Measurement	Particle size	Additional information
<b>Stationary instruments</b>			Used at the subway exposure
Harvard impactors	Mass concentration, $\mu\text{g}/\text{m}^3$	$\text{PM}_{2.5}$ and $\text{PM}_{10}$	Upper 50% cutoff aerodynamic diameter of 2.5 and 10 $\mu\text{m}$ , respectively  Teflon filters with a pore size of 2 $\mu\text{m}$ and a flow rate of 10 L/min was used
Scanning Mobility Particle Sizer (SMPS) system	Number concentration, particles/mL	Ultrafine particles: diameter 10-100 nm	Consisting of an Electrostatic Classifier model 3081 in combination with a Condensation Particle Counter model 3010 (TSI)
<b>Portable instruments</b>			Used at the control and subway exposure
DataRAM	Mass concentration, $\mu\text{g}/\text{m}^3$	$\text{PM}_{10}$	
P-Trak	Number concentration, particles/mL	Ultrafine particles: diameter 20-1000 nm	

Previous studies in the same subway environment <sup>65,142</sup> have used the sample filters from the Harvard impactor to analyse which metals that were present in the air.

NO and NO<sub>2</sub> measured with chemiluminescence instrument AC 31M and relative air humidity as well as temperature were also monitored at the subway station during each exposure session.

Personal exposure to NO<sub>2</sub> (indicator of air pollution exposure) was measured during both control and subway exposures and the following 24 hours, with a passive diffusion monitor carried by each subject. The time spent outdoors during this period was also stated.

### 3.3 TOXICITY TESTING

The essence of particle toxicology lies in the toxicity testing. Products of oxidative stress, inflammation and damage to DNA can be used as markers for indication of damage caused by particle exposure. This part of the thesis will therefore describe the different toxicity assays used to measure relevant markers of toxicity. In this context assays used for the cellular studies (studies I-IV) as well as the human study (study V) will be considered.

#### 3.3.1 Particle exposure *in vitro* - dose, time and sample preparation

To mimic a pulmonary exposure of particles, the cultured human lung cell lines A549 and BEAS-2B were used in the *in vitro* studies (studies I-IV).

##### *Exposure dose and time*

To set a dose for the particle exposure in *in vitro* studies can be very tricky, as previously discussed in the introduction section of this thesis. As discussed by Oberdorster et al. <sup>10</sup>, it is often tempting to use a rather high dose without any consideration of whether it is relevant for an *in vivo* exposure or not. In a study by Risom et al <sup>111</sup>, calculations were made in an attempt to conclude what the doses that have been seen to cause oxidative damage to DNA of cells in *in vitro* studies would correspond to in a human situation, as lung exposure. The dose of 25 µg/well in *in vitro* exposure was shown to correspond to 3.7 years of exposure to a typical ambient concentration of ultrafine particles <sup>111</sup>. The conclusion was that the doses used *in vitro* exceed what is believed to be realistic in a real exposure scenario. When exposing cells *in vitro* to particles in solution it might, however, be important to consider the possibility that not all nanoparticles in a sample will sediment due to their small size. Consequently, the cells might not be exposed to the full intended dose.

The use of high doses is often defended by the idea that the high dose does not matter as long as there is a mechanistic pathway being studied. However, Oberdorster state that that is likely to be untrue and that the mechanisms operating at low doses are likely to be different from when the cellular system is overwhelmed with a high particle dose. In a study by Kroll et al. <sup>143</sup> a dose range between 0.1 and 10 µg/cm<sup>2</sup> was calculated

from exposure studies *in vivo* and suggested to be relevant for use in *in vitro* studies. Nonetheless, what is a realistic exposure dose? How high can the dose be in hot spots of the lungs? There are so many unanswered questions regarding this issue at present. One way to cope with this issue is to evaluate cellular responses *in vitro* to different doses, low to high, hence evaluating if there is a dose response or not. In studies I-IV the doses range from 5 – 40  $\mu\text{g}/\text{cm}^2$ , but most often doses of 20 and 40  $\mu\text{g}/\text{cm}^2$  have been used. These are probably somewhat high doses, but they are, however, undoubtedly far from being the highest used in particle toxicity testing performed worldwide.

Furthermore, to estimate a relevant exposure time can also provide a challenge in *in vitro* studies. Dependent on the mechanism studied, it is important that the time is long enough for a certain cellular response to be initiated. Regarding exposure time in the studies of this thesis, the exposure times vary depending on the toxicity test performed and range from short exposure of 1 h to longer exposure times of up to 24 h.

#### *Sample preparation*

For the *in vitro* exposure of lung cell lines, the particles of interest were mixed with cell medium. Characteristics of the cell medium can vary in terms of ionic strength, pH etc., and it contains a mixture of nutritional components such as serum proteins, which is needed for growth and living of the cells. To mix particles in cell medium upon exposure is a common procedure used in *in vitro* toxicity testing of particles. However, the characteristics of cell medium have been shown to influence particle dispersion and agglomeration<sup>144</sup>. Some particles may tend to agglomerate in cell medium, causing the formation of larger complexes of particles. The forces that agglomerate the particles are often weak and *ultrasonication*, which is a method that sends ultrasound through the suspension, can be used to separate the particles just before the start of cellular exposure. During sonication, bubbles are formed that, when collapsing at the solid surface of the agglomerate, cause breakage of agglomerates<sup>145</sup>. This separation may, however, be temporarily since re-agglomeration may occur. Sonication using a probe was used in studies I-III and a bath was used for sonication in study IV.

### 3.3.2 Cell viability - cytotoxicity

Cytotoxicity is a word explaining the toxicity of a substance to the cell. Often cytotoxicity is measured as a decrease in cell viability i.e. the amount of cells that die. This death is often referred to as either being caused by controlled apoptosis or uncontrolled necrosis<sup>146</sup>. However, it may not be that simple and 13 different types of cell deaths have in fact been suggested<sup>147</sup>. There are several available methods to use to evaluate cell viability *in vitro* after particle exposure, some more precise than others in catching the different types of cell death.

Cell membrane integrity is one way to study and evaluate cytotoxic effects. One classical method in doing so is to study the exclusion of the dye trypan blue from cells<sup>148</sup>. Trypan blue does normally not penetrate the cell membrane. If the cell membrane is damaged, however, the dye penetrates the membrane and the cell will attain the colour blue. Damaged and undamaged cells can subsequently be visualised microscopically.

The result is frequently expressed as the % of damaged (non-viable) cells of the total cell count. Since this method measures the amount of cells with a disrupted membranes the cells do not necessarily need to be dead *per se*.<sup>149</sup> and discrimination between necrosis and apoptosis is not possible. Nonetheless, trypan blue staining is a well used method and is considered to measure necrosis and cells in late apoptosis. One way to specifically measure apoptosis is to look at caspase levels in cells, which is important in the event of apoptosis<sup>148</sup>. Other ways to study cell membrane integrity include to measure the leakage of cellular components such as e.g. lactate dehydrogenase (LDH).

Another indicator which is often used for cytotoxicity evaluation is the tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT). MTT is reduced by dehydrogenases to formazan in the mitochondria of living cells<sup>150</sup>. The end product is purple and is measured by light absorbance in a spectrophotometer. In the MTT-assay the metabolic activity (viability) of the exposed cells is compared to that of unexposed cells.

In this thesis, toxicity testing using the trypan blue method has mainly been used. Both in the context of evaluating the cytotoxicity of particle exposure (study I, II and IV) and prior to evaluation of DNA damage using the comet assay, assuring a high percentage of viable cells prior the comet assay.

### 3.3.3 Intracellular ROS

One challenge when studying ROS is their short lifetime<sup>101</sup>. Analysis of stabile end-products from oxidation can therefore be favourable, and can be used to give an estimate of the oxidative stress level in a cell. To detect the amount of ROS *in vitro* despite their short lifetime, fluorescent probes may be used. One of the most commonly used fluorescent probe is 2',7'-dichlorofluorescein diacetate (DCFH-DA). This probe easily penetrates the cell membrane, and upon entering the cell the DA groups of the molecule become enzymatically cleaved. DCFH is then oxidised to dichlorofluorescein (DCF) by ROS and it is not until this moment that the probe becomes fluorescent. The fluorescence can be measured by e.g. a computerised microplate fluorometer or a flow cytometer. This method measures general oxidative stress rather than any specific kind of ROS. DCFH-DA was used in studies I and III of the present thesis.

### 3.3.4 Damage of DNA

#### *HPLC-EC/UV system*

High performance liquid chromatography (HPLC) coupled to electrochemical (EC) and ultraviolet (UV) detection can be used to measure the formation of 8-oxodG<sup>151</sup>. In this approach, 8-oxodG and undamaged dG are separately detected by the EC detector and UV detector, respectively. From this method the amount of 8-oxodG in relation to undamaged dG is calculated and is reported as 8-oxodG/10<sup>6</sup> dG. The creation of artificial oxidation during the sample preparation is one disadvantage of this method. However, the method is considered specific and has been evaluated by the European Standards Committee on Oxidative DNA Damage (ESCODD)<sup>152</sup>. This

method was used in study III for evaluation of the formation of 8-oxodG by particle exposure of dG.

#### *Comet assay*

A frequently used method for measuring DNA damage is the comet assay<sup>153</sup>. It is considered to be a well-established method and has been evaluated in ESCODD<sup>154</sup> as well as in the European Comet assay Validation Group (ECVAG)<sup>155-157</sup> working within the frame of the European Network of Excellence: Environmental Cancer risk, Nutrition and Individual Susceptibility (ECNIS). The first version of the assay was developed in 1984 by Östling and colleagues for the detection of SSB and DSB<sup>158</sup> and some years later further developments were made by Singh et al.<sup>159</sup> for the measurement of additional alkali labile sites (ALS) under alkaline conditions. In recent years, the method has been increasingly used when evaluating DNA damage in *in vitro* studies of nanoparticle exposure<sup>160</sup>.

The version of the comet assay most often used is the alkaline comet assay that measures DNA damage, predominantly in the form of SSB and ALS. An ALS is a site on the DNA that, due to damage such as a missing base, will break if it is exposed to high alkaline pH. By adding glycosylases, such as FPG or hOGG1, that find specific types of lesions, the method is also a great tool to analyse oxidative DNA damage and oxidative stress.

In the comet assay (procedure illustrated in Figure 11), cells that have been exposed to e.g. particles are embedded in agarose on a microscope slide. Then the cells are lysed with detergents and high salt that liberates the DNA from the histones. Alkaline treatment unwinds the DNA and the high pH also contributes to SSB being formed at ALS. During the subsequent electrophoresis at high pH, the breaks in DNA contribute to the extension of so called DNA loops from the nucleoid, creating a comet like shape with a “head” and a “tail” (see Figure 11). This happens since the negatively charged DNA moves from the nucleoid (head) of the cell towards the anode of the electrophoresis. Following the electrophoresis, the gel is neutralised and fixed with methanol. To be able to visualise the DNA, the gel can be stained for example with the fluorescing dye ethidium bromide (EtBr) and analysed in a microscope using a computer software. The more DNA breaks present, the more DNA will be found in the tail. To estimate the amount of DNA damage, the tail of the comet is measured. Several units exist for this measurement, including *tail moment*, *tail length* and *%DNA in tail*. %DNA in tail has been recommended by Collins et al. as the preferred unit<sup>161</sup>. It reflects the percentage of DNA in the tail relative to the amount of DNA in the comet head, and is the unit used within this thesis.

To specifically analyse oxidatively damaged DNA, enzymes such as human OGG1 (hOGG1) and *E. coli* FPG can be used to find oxidised purines, such as 8-oxoguanine. hOGG1 has been suggested to recognise 8-oxoguanine with a higher specificity than FPG<sup>162</sup>. FPG primarily recognises 8-oxoguanine, fapy-guanine and fapy-adenine<sup>163</sup>. The enzyme is added and the cells are incubated in the comet assay before the alkaline treatment. The enzyme removes the lesion, creates an abasic site, which is converted to a strand break either by the enzyme’s associated lyase activity or by the alkaline

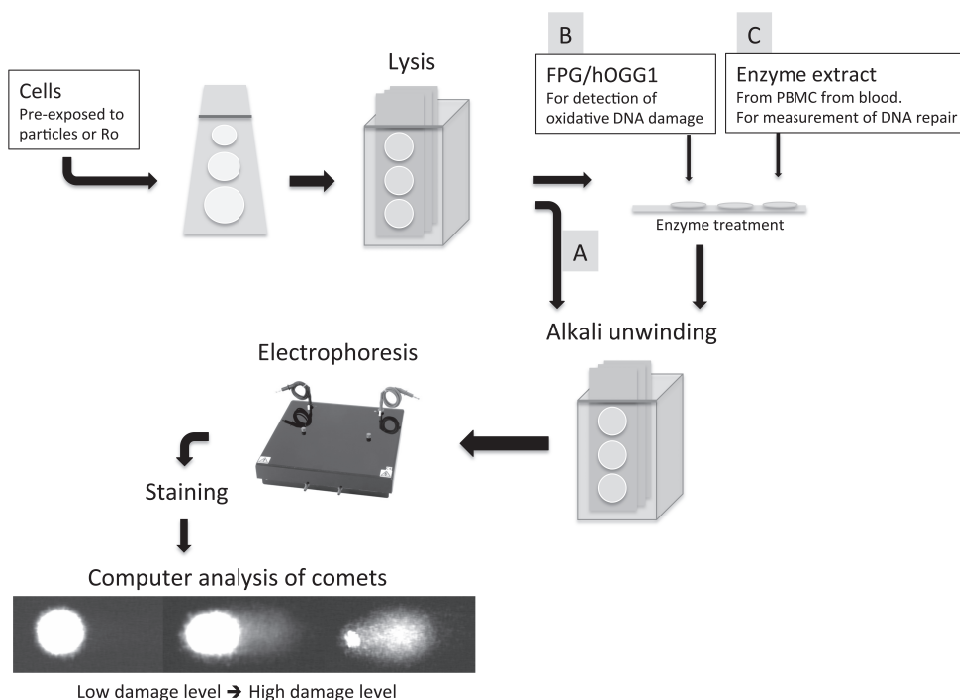
treatment. Since the other factors that are needed for repair are not present in the human cells at this stage of the assay, the FPG or hOGG1 enzyme will only perform this first step of the repair process, generating SSB and ALS. If the cells with and without the addition of the enzyme are compared, the amount of oxidative damage can be calculated by the following formula:

$$\% \text{ DNA in tail (enzyme treated)} - \% \text{ DNA in tail (without enzyme)} = \text{oxidatively damaged DNA}$$

The comet assay was used for the evaluation of DNA damage in all studies of this thesis, both for *in vitro* and *in vivo* samples. For evaluation of oxidative DNA damage, the FPG enzyme was used in study I, II, III and V. hOGG1 has been evaluated, but the results have not been published yet and will instead be discussed in the result/discussion section of this thesis.

### 3.3.5 DNA repair – hOGG1 activity

The effect of particle exposure on DNA repair (BER) or hOGG1 activity can be measured by a modified version of the comet assay <sup>164</sup>. In this version, DNA repair enzymes are extracted from cells, often peripheral blood mononuclear cells (PBMC) from blood of exposed humans. The sample of extracted enzymes is added after lysis in the comet assay, and its ability to find oxidatively damaged DNA in pre-damaged cells (damaged by a so called photosensitiser causing specific oxidative damage to DNA) can be evaluated. This makes it possible to detect whether the level of repair enzymes is elevated or not after a certain exposure. This repair assay is a new and promising technique. However, no standardised protocol exists. Nonetheless, a validation study has been performed by ECVAG, in which the reliability and reproducibility of the assay was evaluated <sup>165</sup>. The method has been used in some human studies of exposure to particles <sup>166,167</sup> and will likely be increasingly used in the future. DNA repair was evaluated in the human study (study V) of the present thesis (see Figure 11 for illustration of the procedure).



**Figure 11.** Procedures for the different versions of the comet assay. Cells exposed to particles or the photosensitiser Ro (creating oxidatively damaged DNA when exposed to light) are mixed with agarose and added to a microscope glass before lysis. For evaluation of SSB and ALS no enzyme is added after lysis (A), for evaluation of oxidative damage from particle exposure FPG or hOGG1 enzyme is added (B). For evaluation of the DNA repair in PBMC from human subjects, exposed to particles, an enzyme extract from these cells is added (C) to Ro exposed cells after lysis. Then alkali unwinding, electrophoresis and staining are performed. Finally, the cells are analysed microscopically using a computer software. The image of the comets at the bottom illustrates cells with increasing amounts of damage. For the repair assay (C), low levels of damage means that the repair has been disrupted since it can not find the damage that exists in the Ro exposed cells.

### 3.3.6 Mitochondrial depolarisation

Mitochondrial depolarisation is a cellular event where the negative potential (caused by the electron transport chain) of the mitochondria is disrupted. Mitochondrial depolarisation is important in apoptosis<sup>146</sup> and has also been shown to be involved in necrosis caused by nanoparticles<sup>168</sup>. Damage to mitochondria can also cause release of ROS, hence damage to mitochondria is highly relevant also for several other possible damages to the cell.

For analysis of mitochondrial depolarisation the fluorescent probe tetramethylrhodamine ethyl ester (TMRE) can be used. TMRE is a cationic lipophilic dye, which



accumulates in the negatively charged mitochondrial matrix. To analyse the accumulation of TMRE, a fluorescence-activated cell sorting (FACS) instrument can be used. If the potential is lost (i.e. the mitochondria are damaged) no fluorescence will be seen. Mitochondrial depolarisation was evaluated in study II.

### 3.3.7 Haemolysis

Surface reactivity of particles can be analysed by determining the ability of the particles to cause haemolysis of red blood cells. The haemolysis is considered to be caused by direct interactions of particle surface with the red blood cell, which can possibly be driven by oxidative stress on the surface <sup>169</sup>. In the assay, particles are incubated with red blood cells (often from human donors) and the amount of haemoglobin released is determined by a spectrophotometer. In the assay, the amount of haemoglobin is compared to that of cells that have been incubated with the detergent Triton X100 that causes 100% lysis. This method is often used to investigate the reactivity of particle surfaces, but can also be seen as a method to evaluate the toxicity of particles towards red blood cells. Additionally, a study by Lu *et al.* <sup>170</sup> evaluating the toxicity of different metal oxide nanoparticles has shown that the results from this haemolytic assay could, in almost all cases, predict the inflammation outcome from lung installation in rats of the same particles.

### 3.3.8 Inflammation

In the present thesis, markers for inflammation were measured in the human exposure study (study V).

#### *Cell differentiation*

Cell differentiation counts or concentration of neutrophils, eosinophils, basophils, lymphocytes and monocytes in blood can give information about inflammation status in the body. In healthy persons the concentration of neutrophils lies between 40-60%, lymphocytes 20-40%, monocytes 2-8%, eosinophils 1-4% and basophils 0.5-1%. In study V this was measured in blood samples after subway air exposure according to common procedure using an auto-analyser.

#### *Lymphocyte subsets*

A useful procedure to distinguish between types of lymphocytes, their stage of differentiation and activity in blood is by the presence (+) or absence (-) of certain cellular surface markers called cluster of differentiation (CD) markers. There are several hundreds of different CD markers <sup>115</sup>. Tests using monoclonal antibodies against specific CD molecules can be used for the detection of CD markers of interest. In the human study (study V), a so called TBNK 6-color Multitest, consisting of antibodies for T-cells, B-cells and Natural killer (NK) cells was used to determine lymphocyte subsets. In addition, separate monoclonal antibodies were used for further phenotypic characterisation (T-cells activity and regulatory functions). Since the antibodies were conjugated with a fluorochrome the results could be analysed by a FACS, evaluating approximately  $2.5 \times 10^5$  cells from each sample. For the TBNK test,

General markers	
CD45	White blood cells (leukocytes)
CD69	Early activation of lymphocytes
CD27	Co-stimulator for T and B-cells
T-cells markers	
CD3	T-cells
CD4	T-helper/inducer cells
CD8	T-cytotoxic/suppressor cells
CD25	Early activation of T-cells
Foxp3	T-regulatory cells
CD25/Foxp3	T-regulatory cells
B-cells markers	
CD19	B-cells
NK-cells markers	
CD56+/CD16+	NK or NKT-cells

a gate was set (a selection was made during the evaluation of the FACS-results) for CD45 (leukocytes). For the evaluation of T-cell activation and analysis of T-regulatory cells a gate was set for CD3 (T-cells). The CD markers measured in study V, are listed in table 2.

**Table 2.** The following CD markers and the intra-cellular transcription factor *Foxp3* were studied in the human exposure study (study V). Evaluation of the lymphocyte subsets in blood can give information about possible inflammation responses.

### Fibrinogen

The plasma glycoprotein fibrinogen is an important factor in normal blood clotting. Fibrinogen is also a so called acute-phase protein which initiates immune functions, and its levels have been seen to be elevated during inflammation<sup>171</sup>. The concentration in plasma is often analysed as a routine at hospital laboratories. This was also the case in study V where a kinetic fibrinogen assay was used.

### 3.3.9 Other human health markers – lung permeability, lung function, self graded symptoms

In the human exposure study (study V), some additional health markers were studied, as will be described below.

#### Lung permeability

Clara cells, located in the lining of the airways (see Figure 3), release *clara cells protein 16* (CC16) upon damages of the lung epithelium. Release of CC16 can be measured in serum and is used as a marker for lung epithelial damage, as well as for lung permeability. To measure CC16 in serum, *enzyme-linked immunosorbent assay* (ELISA) can be used. In this assay, CC16 in blood is allowed to bind to antibodies bound in wells of a plate. After washing, an antibody and a conjugated streptavidin-horseradish peroxidase is added and an enzyme-dependent colour-change reaction catalysed by peroxidases then makes it possible to measure the presence of CC16. The result is evaluated using a spectrophotometer.

#### Lung function test

Prior to the inclusion in the human study (study V) all subjects underwent a routine physical examination and a lung function test with a spirometer, measuring vital capacity (VC), forced vital capacity (FVC) and forced expiratory volume during the

first second (FEV1). This was performed to assure that the study subjects were without any symptoms of disease of the respiratory system.

During the study, lung function test was performed with a portable lung health monitor, measuring peak expiratory flow (PEF). PEF is the maximum speed of expiration measured in litre/min and it can be used to measure changes in the degree of obstruction of the airways.

#### *Self graded symptoms*

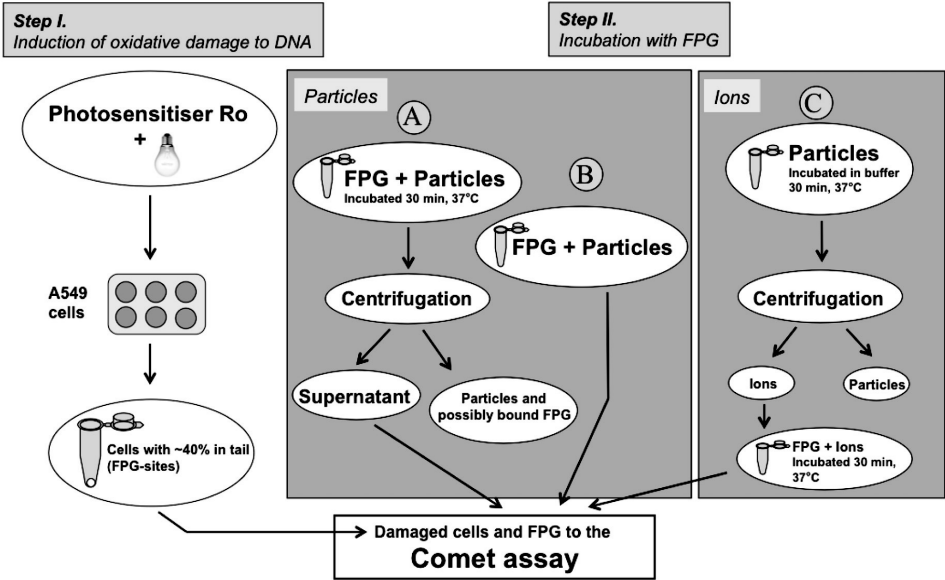
Using a Visual Analogue Scale (VAS), study subjects can evaluate their symptoms during a study<sup>172</sup>. In study V the following symptoms were graded at a scale from 0-10 (where 0 correspond to no symptoms and 10 to severe symptoms): irritation from eyes, nose, lower airways and experience of disturbing smell.

### 3.3.10 Interference of particles with toxicity assays

Many traditional *in vitro* toxicity assays were designed for testing of chemicals not particles. Since particles display unique physicochemical properties due to their solidity, i.e. size, shape, agglomeration properties, surface area etc. they are different to chemicals. The unique properties of particles may lead to interaction in assays, a fact that has been discussed especially within the field of nanotoxicology<sup>173</sup>. Interference of particles in test methods may give both false positive and false negative results. Nanoparticles can, for example, have optical absorbance at the same wavelength as a coloured product that is measured in an assay, or they may adsorb dyes that are to be measured. Reaction between the particle surface and a dye, as well as magnetic properties of the particle may also be issues<sup>174</sup>. In the concept of the studies of this thesis, particle interactions with the assays used have been evaluated when possible. One example is the discovery of autofluorescence of TiO<sub>2</sub> particles, interfering with the results of the assay evaluating intracellular ROS levels by DCFH-DA. Possible interference was also seen in study III when HPLC-EC/UV was used to study oxidation of the DNA base guanine. This was indicated by a lower level of oxidation from some particles when compared to the control without particles. Moreover, interference by particles in the comet assay using FPG is the focus of study III. In this study an interaction test of particles and ions with FPG was designed as described in Figure 12. The test contained two separate steps, as described below.

Step I) A549 cells were exposed to a photosensitiser (Ro) and light irradiation, inducing oxidatively damaged DNA (i.e FPG sites, at a level of 40% DNA in tail). The damaged cells from this step were then evaluated with FPG from step II (described at the next page and in Figure 12).

Step II) FPG was pre-incubated with all particles for 30 min at 37°C and then centrifuged. The supernatant without the particles was then used to detect FPG sites of the cells from Step I using the comet assay (A) in Figure 12. This procedure reveals if the particles adsorb/bind FPG, in which case the enzyme is discarded at the centrifugation, or if FPG gets dysfunctional after contact with the particles. The particles that caused a lower activity of FPG (in A) were further evaluated in procedure (B) and (C) (see Figure 12). In (B), FPG was mixed with the particles and directly used in the comet assay for detection of oxidatively damaged DNA. The results indicate if FPG still works despite being bound to the particle. In (C), the particles were incubated in buffer for 30 min for possible release of ions. The particles were centrifuged away and the released ions (if any) were incubated with FPG for 30 min. This procedure shows if the detected inhibition of FPG was due to released ions.



**Figure 12.** Procedure in study III, analysing particle and ion interaction with FPG in the comet assay.

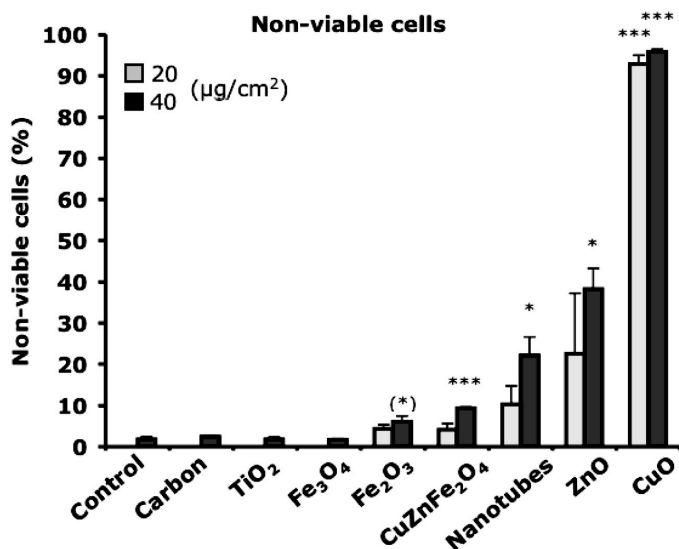
## 4 RESULTS AND DISCUSSION – PARTICLE TOXICITY

The following text will summarise and discuss the results from the studies I-V within this thesis (for details see paper I-V). The chapter begins with an evaluation of the importance of chemical composition, size, surface area, charge, metal ion release and protein corona for toxicity and interaction of metal containing particles with biological systems. Methodological interference of particles with the comet assay, when measuring oxidatively damaged DNA in cells, will also be presented and discussed, as well as some other considerations for the comet assay when measuring oxidatively damaged DNA. Finally, the results from the study of human exposure to metal containing particles in the subway system will be highlighted as an example of human exposure to metal containing particles. The conclusions that are made in this section are not necessarily the conclusions of the co-authors of the published papers, but rather reflections made by the author of this thesis.

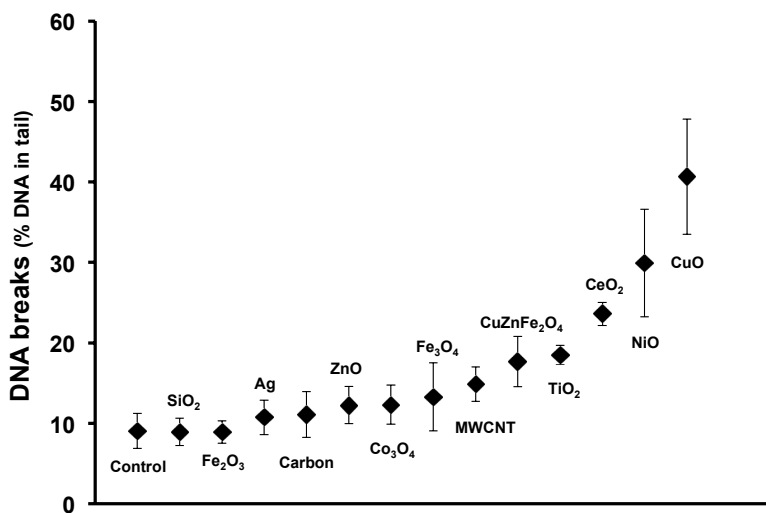
### 4.1 THE ROLE OF CHEMICAL COMPOSITION

In studies I, II and III of this thesis, toxicity and underlying mechanisms of toxicity of totally 12 nanoparticles and one nanofibre were evaluated. Human lung epithelial A549 cells and bronchial epithelium BEAS-2B cells (only in study III) were exposed in culture and the parameters tested were cytotoxicity, DNA damage, formation of oxidatively damaged DNA, intracellular ROS (studies I and III) and mitochondrial depolarisation (only study II). For more detailed information about the study design see the separate papers.

In study I, nanoparticles of carbon black, CuO, CuZnFe<sub>2</sub>O<sub>4</sub>, Fe<sub>2</sub>O<sub>3</sub>, Fe<sub>3</sub>O<sub>4</sub>, TiO<sub>2</sub> and ZnO, as well as multiwalled carbon nanotubes (MWCNT) were compared. The toxicity evaluation showed a high variability of toxicity from the exposure of the different metal oxides and MWCNT. The most important finding was the univocal results of the exposure of CuO nanoparticles, as shown in all the used toxicity tests, namely high cytotoxicity, DNA damage and oxidatively damaged DNA. The toxicity of CuO was always the highest when compared to the other metal oxides as well as MWCNT. However, toxicity was also seen after exposure to some of the other nanoparticles: *cytotoxicity* after exposure to Fe<sub>2</sub>O<sub>3</sub>, CuZnFe<sub>2</sub>O<sub>4</sub>, MWCNT and ZnO, *DNA damage* from ZnO, MWCNT, CuZnFe<sub>2</sub>O<sub>4</sub> and TiO<sub>2</sub>, and *oxidatively damaged DNA* by ZnO, Fe<sub>3</sub>O<sub>4</sub> and CuZnFe<sub>2</sub>O<sub>4</sub> exposure (the nanoparticles/fibre are mentioned in the order of lowest to highest levels of toxicity). Beyond the overall high toxicity of CuO nanoparticles, it was obvious that the nanoparticles affected the cells in different ways. For example, ZnO showed rather high cytotoxicity, but low DNA damaging effect, whereas TiO<sub>2</sub> caused pronounced DNA damage but no effect on cell viability (see Figure 13 and 14). A conclusion from these results is that the chemical composition of the particles (and the fibre) seems to affect the lung cells by different mechanisms, resulting in different toxicological effects. The cytotoxicity results for the nanoparticles and MWCNT in study I can be seen in Figure 13. Figure 14 shows DNA damage from all evaluated nanoparticles and MWCNT in study I-III.



**Figure 13.** Cytotoxicity in cultured A549 cells after exposure to  $20\mu\text{g}/\text{cm}^2$  ( $40\mu\text{g}/\text{mL}$ ) and  $40\mu\text{g}/\text{cm}^2$  ( $80\mu\text{g}/\text{mL}$ ) nanoparticles for 18 h, measured as percent nonviable cells by trypan blue staining. The asterisks (\*, \*\*, \*\*\*) indicate significantly higher levels compared to controls and correspond to  $p < 0.05$ , 0.01, 0.001, respectively. For more details see paper I.



**Figure 14.** DNA damage in cultured A549 cells after exposure to  $40\mu\text{g}/\text{cm}^2$  ( $80\mu\text{g}/\text{mL}$ ) nanoparticles for 4 h, measured as percent DNA in tail by the comet assay. For more details, see paper I, II and III.

Redox active metals (transition metals) of nanoparticles might be an attribute linked to possible toxicity. Studies suggest that transition metals present in particles, especially iron, generate ROS, subsequent oxidative stress and respiratory symptoms<sup>50</sup>. In the studies of the present thesis, iron oxides did, however, show small or no effects on cultured human lung cells and they were, for that reason often used as controls with low toxic potential. No or low toxic effect of iron oxide particles is in agreement with other studies<sup>175,176</sup>. One main explanation to this may be that only low amounts of redox active iron ions are dissolved from the particles intracellularly during the time course of the experiment. This is in contrast to CuO that rapidly dissolves in the cells (as will be discussed in more detail later on). A study by Limbach et al<sup>177</sup> reveals, similarly to study I of this thesis, that transition metal containing nanoparticles can cause quite different levels of ROS both in A549 cells and in cell free experiments. In that study, iron oxide also gave low levels of ROS in comparison to other transition metals.

The high toxicity of CuO particles leading to cell death after 18 h could be a response to the DNA damage these particles caused, as a way to prevent a mutagenic outcome. It is, however, likely that a pronounced oxidative stress generated by the CuO particles and dissolved Cu ions, lead to cell death independently of the DNA damage. In a study by Wang et al.<sup>123</sup> primary cytotoxic responses were shown to be due to oxidative stress rather than DNA damage. Which specific cellular process that is the driving force might, however, be hard to clarify and several effects may often take place simultaneously. Fahmy et al.<sup>178</sup> have found similar results from CuO nanoparticle exposure of epithelial cells as presented in the studies of this thesis in terms of generation of ROS. The particles were additionally shown to be able to overwhelm antioxidant defence to a higher degree when compared to particles of silicon oxide and iron oxide. This is likely an important event for the formation of oxidatively DNA damage seen in the present thesis.

The high toxicity of CuO nanoparticles seen in this thesis, is particularly alarming since nanoparticles of copper are used in several consumer products, as an ingredient in e.g. polymers, paints and bioactive coatings (with the purpose to inhibit the growth of microorganisms)<sup>179</sup>. Moreover, CuO is used in antimicrobial textiles, in the same way as the widely used silver<sup>180</sup>. Furthermore, some cosmetic products available for consumers contain Cu nanoparticles<sup>44</sup>. Copper is a metal that is also widely used in industries, particularly in the electrical sector. Emissions of copper and copper containing particles from smelters, iron foundries, power stations, and municipal incinerators<sup>181</sup> as well as in subway stations<sup>69</sup> may also cause human exposure. *In vivo* studies of Cu nanoparticles have shown acute toxicity, including severe injuries of the kidney, liver and spleen when mice were exposed orally<sup>182</sup>. Additionally, rats exposed to CuO nanoparticles by intratracheal installation have been seen to suffer from acute inflammation in the lungs<sup>183</sup>.

An important finding in studies I and II concerns TiO<sub>2</sub>. Namely that TiO<sub>2</sub> caused a significant increase in DNA damage both in nano- and microparticle form, which was not seen in the same way for the other particle types tested (see paper II). TiO<sub>2</sub> exists in both anatase and rutile forms, and studies have indicated mixes of these to induce DNA strand breaks<sup>184,185</sup>. In the studies of this thesis a mix of anatase and rutile TiO<sub>2</sub>

nanoparticles was used, and the positive results are thus in agreement with these studies. When mixes of anatase and rutile forms have been compared to either anatase or rutile forms alone, mixes have had the strongest effect on oxidatively damaged DNA<sup>186</sup> as well as on cytotoxicity measured as LDH release. It is likely that DNA damage from TiO<sub>2</sub> is linked to the photocatalytic action of these particles, i.e. TiO<sub>2</sub> can form radicals in the presence of light. It can therefore not be excluded that some additional damage was formed during the experimental procedure when these particles were evaluated within the studies of the present thesis. However, no signs of oxidative stress from TiO<sub>2</sub> particles were found in our studies (studies I and II). The *in vitro* toxicity of TiO<sub>2</sub> nanoparticles is of particular concern considering its wide use in sunscreens and cosmetics.

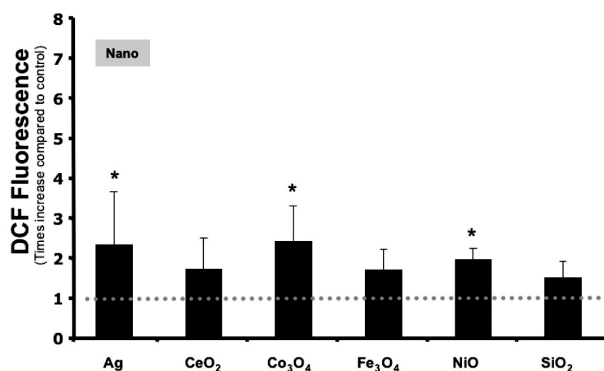
In study III, the toxicity of nanoparticles of Ag, CeO<sub>2</sub>, Co<sub>3</sub>O<sub>4</sub>, Fe<sub>3</sub>O<sub>4</sub>, NiO and SiO<sub>2</sub> in terms of DNA damage and oxidatively damaged DNA in both A549 and BEAS-2B cells was investigated. Intracellular ROS in A549 and oxidative capacity of the particles, measured as oxidation of the DNA base guanine in a non-cellular system, were also evaluated. Similar to the results reported in study I and II, the nanoparticles in this study also differed in toxic capacity depending on their chemical composition. For DNA damage, NiO showed the highest damaging effect in both cell lines, followed by CeO<sub>2</sub>. For NiO, the level was not too far from that seen from CuO in studies I and II (see Figure 14). Other studies have also reported toxic effects as a consequence of NiO exposure. Cultured human airway epithelial cells (HEp-2) and human breast cancer cells (MCF-7) have shown reduced cell viability by NiO nanoparticle exposure, as well as elevated oxidative stress as indicated by depletion of glutathione, induction of reactive oxygen species and an increase of lipid peroxidation, all in a dose-dependent manner<sup>187</sup>. In that study, biomarkers of apoptosis were also observed to be induced, including caspase-3 enzyme activity and DNA fragmentation. In a study by Horie et al.<sup>188</sup>, in which effects of NiO nanoparticle exposure were compared between cell cultures (A549) and after tracheal installation in rats, the particles were seen to induce oxidative stress in A549 and cause oxidative stress related injury in the rats. The conclusion was that the results of the *in vitro* and *in vivo* studies tended to correspond. Nanoparticles of NiO are increasingly being used in various applications, e.g. in alkaline batteries and as an electronic component. Despite this wide use and the indication of toxicity along with the fact that nickel compounds (including NiO) are considered carcinogenic to humans according to IARC<sup>189</sup>, there is limited information concerning the toxic mechanisms and detailed biological responses of NiO in the form of nanoparticles. However, the cellular uptake of the NiO particles and intracellular release of Ni is likely to be important for carcinogenic potential of Ni-compounds<sup>190</sup>.

One specific aim of study III was to investigate if toxicity of nanoparticles with different chemical compositions was dependent on cell line, and both A549 and BEAS-2B were therefore tested. Especially for nanoparticles of Ag, the cell line seemed important for the DNA damage since positive results were seen for BEAS-2B whereas no damage was observed in A549 (see paper III). Fe<sub>3</sub>O<sub>4</sub> also indicated DNA damage in BEAS-2B cells, but not in A549. Co<sub>3</sub>O<sub>4</sub>, CeO<sub>2</sub> and NiO showed DNA damage in both cell lines. These results indicate that the cell types are affected slightly differently depending on the chemical composition of the particles. The fact



that cell lines might respond differently to exposure of nanoparticles has been shown in other studies as well <sup>143,191</sup>. Furthermore, the resistance of A549 compared to other cell lines has been indicated for some nanoparticles <sup>191,192</sup>. A conclusion that can be made is that the choice of cell model will certainly influence the findings in toxicity studies.

In study III, significantly increased levels of intracellular ROS in A549 cells after one hour of particle exposure were seen for the nanoparticles of Ag, Co<sub>3</sub>O<sub>4</sub> and NiO (see Figure 15). However, Co<sub>3</sub>O<sub>4</sub> exposure in BEAS-2B was the only nanoparticle causing oxidatively damage to DNA measured by the comet assay. The absence of oxidatively DNA damage from all the other nanoparticles tested was suggested to, in part, be due to methodological interaction with the restriction enzyme FPG used in the comet assay (which will be discussed further on). The interference of Ag nanoparticles with FPG, which was detected, speaks for the possibility that Ag nanoparticles may interfere with the repair machinery for oxidatively damaged DNA, since FPG is a repair enzyme. ROS production and subsequent DNA damage, in the form of bulky DNA adducts, has been measured in A549 cells following Ag exposure by others <sup>193</sup> and oxidation of the DNA base guanine was induced by the Ag particles in study III (see paper III). The ability of Ag to cause oxidative DNA damage, in combination with possible interference with DNA repair, may give Ag nanoparticles a “double potential” to cause damage to DNA, direct and indirect by interaction with repair. It should, however, be noted that the genotoxicity of Ag nanoparticles reported in the literature differ, with some studies reporting pronounced toxicity whereas other report low or a lack of toxicity <sup>194,195</sup>. Another metal oxide with conflicting data reported is CeO<sub>2</sub>, described to possess antioxidative properties as well as toxic properties <sup>196</sup>. In study III, DNA damage was observed, but ROS was not significantly increased from CeO<sub>2</sub> exposure. This contradicts to the results from a study by Kroll and colleagues <sup>143</sup>. Three of the four types of CeO<sub>2</sub> nanoparticles that they investigated showed ROS production (using the same method as in study III) in A549 cells at a dose representing only 25% of the dose tested in the present thesis. Thus, the toxicity of CeO<sub>2</sub> seems to differ from study to study even when using the same cell line.



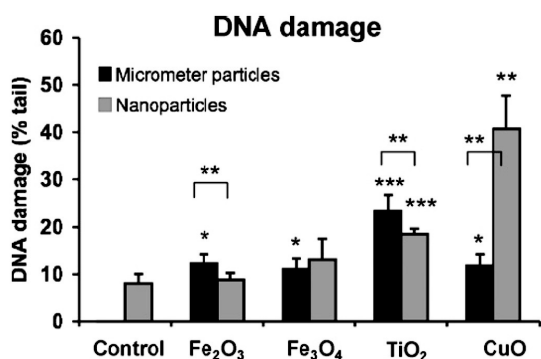
**Figure 15.** Generation of intracellular ROS in A549 cells from particle exposure of 40 µg/cm<sup>2</sup> (1 h), measured as DCF fluorescence. An asterisk (\*) indicates  $p < 0.05$ , a significantly higher level corresponding to the control. The y-axis shows time increase compared to the control, indicated by the dotted line. For more details see paper III.

Chemical composition has been suggested to be more important for larger particles than for particles in nanometre size <sup>111</sup>. However, the studies of this thesis and the discussion above highlight its importance also for toxicity of a wide range of metal containing nanoparticles. **A conclusion that can be drawn from the results of the presented studies so far, is that the chemical composition of the metal containing particles seems to be important for the toxicity, and this thesis especially highlights the toxicity of CuO and NiO nanoparticles.** Furthermore, the different chemical compositions will most likely lead to differences in charge and dissolution/metal ion release that may be crucial for the toxic potential. This will be discussed more in the next section as well as the impact of size and surface area of particles with the same chemical composition.

## 4.2 THE ROLE OF SIZE, SURFACE AREA, CHARGE AND METAL ION RELEASE

### *Particle size and surface area*

In study II, nanoparticles and particles in micrometre size of CuO, TiO<sub>2</sub>, Fe<sub>2</sub>O<sub>3</sub> and Fe<sub>3</sub>O<sub>4</sub> were compared in terms of their toxicity. The aim was to see if particle size was an important factor for toxicity when the material composition was the same. Cytotoxicity, mitochondrial depolarisation, DNA damage and oxidatively damaged DNA were the parameters evaluated after exposure to A549 cells. The results revealed a significantly higher toxicity for nanoparticles of CuO, when the parameters for cytotoxicity, mitochondrial depolarisation and DNA damage were compared to micrometre sized particles of the same composition. For oxidatively DNA damage there was also a difference, however, this difference was not of statistical significance when micro- and nanoparticles were compared. Norppa et al. stated in a recently published study <sup>197</sup> that it can not be generally assumed that nanoscale size would increase the genotoxicity of nanomaterials, or that nanoparticles are generally genotoxic. This is indeed in agreement with our results. Comparison of DNA damage from micro- and nanoparticles of Fe<sub>2</sub>O<sub>3</sub> and TiO<sub>2</sub> actually showed opposite results compared to that seen for CuO, namely a small, but significantly higher damage level from the micrometre sized particles (see Figure 16). For TiO<sub>2</sub> the reason for this effect might be that the particle samples consisted of different crystalline structure. The nanoparticles were a mix of rutile and anatase, and the microparticles were rutile with a small amount of anatase. In an inhalation study of mice by Grassian et al. <sup>198</sup>, it was shown that nanoparticles of TiO<sub>2</sub> with a diameter of 21 nm caused significantly more inflammation than the particles of 5 nm. In this study too, the crystalline structure is discussed to partly explain the results (5 nm = anatase, 21 nm = a mix of anatase and rutile). Thus, for TiO<sub>2</sub>, the crystalline structure is likely to be more important for the toxicity than size.



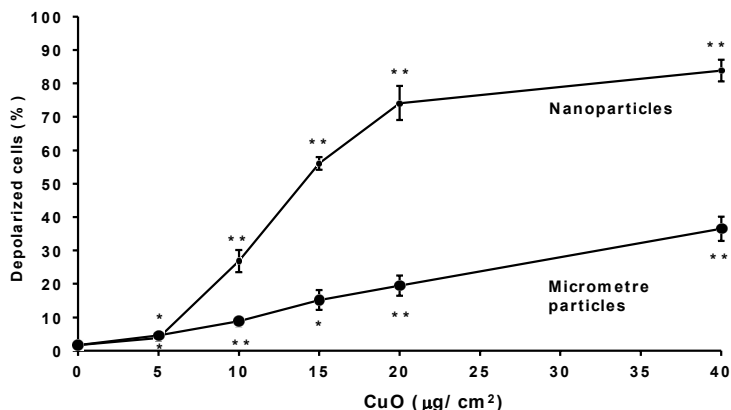
**Figure 16.** Comparison of DNA damage in A549 cells after 4 h exposure to 40  $\mu\text{g}/\text{cm}^2$  micro- and nanoparticles of different composition. Asterisks (\*, \*\*, \*\*\*) indicate significantly higher levels compared to control and corresponds to  $p < 0.05$ , 0.01 and 0.001. For more details see paper II.

In the study by Grassian et al.<sup>198</sup>, agglomeration was additionally suggested to be the reason to why the larger nanoparticles caused more toxicity than the smaller. Data from DLS showed that there were smaller agglomerates for the 21 nm particles than for the 5 nm nanoparticles. The interaction of the larger particles were weaker and hence allowed the 21 nm particle agglomerates to de-agglomerate and go into the deeper parts of the lung, as opposed to the more tightly packed larger agglomerates formed by the smaller 5 nm particles, which may be cleared more easily.

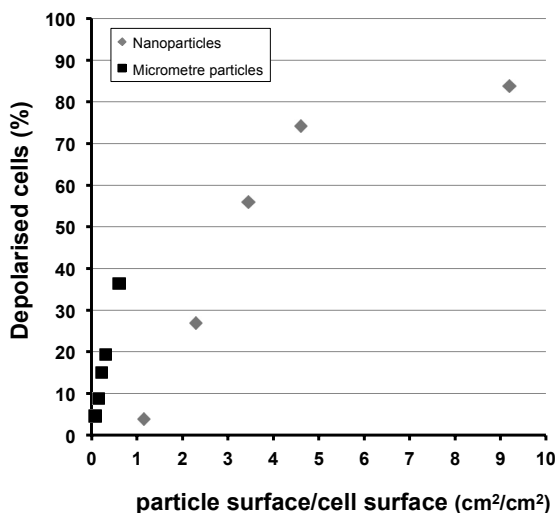
**A conclusion so far from the results in the present thesis is that particle size does not always seem to be a dominant factor driving toxicity.** However, particle size is highly important in the context of particle deposition in the lung as seen in Figure 4 illustrating the fact that particles often are in the nanometre size when deposited deep in the respiratory tract. Caution must, however, be taken since the size of the individual particle may, due to agglomeration, not be the actual size of the particle that is inhaled. As previously discussed, immunological response in the lung may also differ for nanoparticles and micrometre sized particles, enabling nanoparticles to escape the immune defence. In addition, their ability to translocate into cells and possibly further into the body might cause a hazard from nanoparticles but not from larger particles. The size seems to matter in terms of “availability” but does not seem to be important for toxicity *per se* for all materials.

Recent studies within nanotoxicology have drawn attention to the fact that the surface area of particles might be an important factor for toxicity. For example, studies of carbon black have suggested that the surface area is the most important determinant of effects for particles in nanometre size<sup>111</sup>. In study II where the importance of particle size was evaluated, only the dose metric mass/surface of cell layer (or mass/volume) was used and the results indicated higher toxicity, in particular for CuO particles in the nanometre size. In Figure 17, this difference in toxicity is illustrated by the mitochondrial depolarisation induced by CuO. However, if these results instead are based on the surface area of the particles (based on BET) the results indicate the opposite, i.e. that particles in micrometre size are more toxic than the nanoparticles (see Figure 18). The larger particles sediment on to the cells more rapidly and this may be part of the explanation for the higher effects observed when dose is expressed as surface area. Another explanation might be that the nanoparticles agglomerate in

biological media, and consequently the true surface area of the nanoparticles that the cells are exposed to might be lower than the measured BET area.



**Figure 17.** Mitochondrial depolarisation in A549 cells from exposure of CuO micro- and nanoparticle. Here the dose metric mass/surface of cell layer has been used, indicating a higher toxicity for nanoparticles. For more details see paper II.



**Figure 18.** Mitochondrial depolarisation in A549 cells from exposure of CuO micro- and nanoparticles. Here the results from Figure 17 have been recalculated for the dose metric particle surface/cell surface. The results shows that the nanoparticles have a much larger surface than the micrometre particles in the doses presented in Figure 17. If the toxicity of the micrometre particles would follow the trend of toxicity seen in this graph (Figure 18), the opposite to Figure 17, i.e. a higher toxicity for micrometre particles than for nanoparticles, would then be the result if expressed as toxicity/particle surface area instead of toxicity/mass dose.

It is often highlighted that surface area is a better dose metric than particle mass or particle number<sup>10,199</sup>. However cautions need to be taken to other aspects of the choice of dose metric, such as the importance of size for the deposition of the particles in the lungs (as previously discussed). Additionally, particle levels are still measured in metrics based on mass for ambient and occupational air. However, OECD recommend that exposure levels of materials in nanometre size should be expressed as surface area or particle number concentration<sup>200</sup>. So a combined use of several dose metrics (mass, particle number and surface area) would be preferable.

A large surface area can, however, not be dedicated the whole responsibility for toxicity of nanoparticles. If the surface is not reactive, the size of it should not matter. The surface reactivity changes when different functional groups or coatings are present on the particles. This has been shown by Warheit et al.<sup>201</sup>, who compared particles of TiO<sub>2</sub> in inhalation and instillation experiments and found that only nanoparticles with alumina and amorphous silica coatings caused adverse effects. The combination of a large surface area and high reactivity might possess a “double hazard”, which has e.g. been discussed in terms of inflammatory potential of particles<sup>202</sup>. However, the opposite was seen in study IV of this thesis. The microparticles of Cr<sub>2</sub>O<sub>3</sub> were shown to have the largest surface area of all the iron and chromium based particles tested in this study. They also caused increased levels of haemolysis of red blood cell, indicating a reactive surface, but in terms of toxicity, DNA damage and cell viability were not increased (see paper IV). The reason for this might be that Cr<sub>2</sub>O<sub>3</sub> induces other mechanisms of toxicity not tested in this study, or that proteins of the cell medium covered these particles, creating a corona, which will be discussed in the next section.

**In conclusion, the results in this thesis in combination with results presented by others suggest that surface area is one important factor for the determination of toxicity - if the surface is reactive.** However, for some particles additional factors might be of even greater importance. Particle charge, protein corona and ion release will be discussed in the next section.

#### *Particle charge, protein corona and ion release*

The nanoparticles of CuO, NiO and Co<sub>3</sub>O<sub>4</sub> investigated in studies I-III, were all measured to have high positive zeta potentials and show increased toxicity for many of the parameters tested in these studies. One explanation to this can be that cell membranes attract positively charged particles due to their negatively charged phospholipids located in the inner cell membrane<sup>203</sup>. There have been indications that positively charged nanoparticles cause cytotoxicity to a higher degree than negatively charged particles when evaluated *in vitro*<sup>204,205</sup>. In accordance with this the microparticle Cr<sub>2</sub>O<sub>3</sub> evaluated in study IV has a positive zeta potential (in NaCl)<sup>206</sup> and was the only particle causing haemolysis of red blood cells (see paper IV). However, some nanoparticles with negative zeta potential were also shown to increase toxicity in some of the tests in studies I-III. It should also be noted that the zeta potential in these studies were measured in water containing 10 mM NaCl. In serum rich cell media, metal oxides, with various zeta potential in water, tend to show a slightly negative zeta potential<sup>207</sup>. This is due to the proteins that are attracted to the surface, creating a layer around the particle, a layer often referred to as a *protein corona*<sup>208</sup>. The protein corona

might actually be the determinant of what will initially happen to a particle in a biological system. The corona may in some cases consist of proteins that are attractive for cell interaction, while in other circumstances that will not be the case<sup>209</sup>. In a study by Cho et al.<sup>117</sup> coating of nanoparticles with serum proteins were shown to reduce the particles' ability to cause haemolysis, showing that the surface charge and reactivity was changed by the protective corona. In the present thesis, cells were exposed to particles in media containing serum. Consequently the particles are likely to have a corona. This has been observed to be highly important, reducing the toxicity of some particles such as SiO<sub>2</sub><sup>210</sup>, while the toxicity of some particles, such as Cu nanoparticles, is not affected by the corona to the same extent<sup>211</sup>. The corona can have an impact on the uptake of particles and e.g. facilitate the uptake by macrophages<sup>212</sup>. Upon phagocytosis, particles become localised into so called phagosomes, which fuse with lysosomes<sup>213</sup> to form acidic phagolysosomes<sup>214</sup>. Enzymes in the lysosomal environment digest proteins of the corona revealing the reactive surface of the particle<sup>215</sup>. If the particle is positively charged it may interact with the negatively charged internal of the lysosomal membrane leading to its disruption. Conclusively, the zeta potential of particles might be important inside the lysosomes and for a subsequent toxicity. For nanoparticles that easily dissolve, the acidic environment of the lysosome may accelerate the dissolution rate of the particles, leading to release of soluble ions and, if the ions are toxic, they might disrupt the lysosomal membrane as well. Both these mechanisms may lead to release of the particles or ions into the cell, which subsequently can trigger inflammation. Cho et al.<sup>117</sup> suggested this mechanism for 15 metal/metal oxide nanoparticles, which were investigated regarding their potential to induce lung inflammation in rats. The study showed that positive zeta potential for particles with low solubility, as well as ion release for particles with high solubility in acidic environment, was correlated to the degree of acute inflammation in rats.

In study IV, toxicity of micrometre sized iron and chromium based particles were studied following exposure of A549 cells. The particles were the alloys FeCr, FeSiCr, stainless steel 316L, the metals Fe and Cr, and the oxide Cr<sub>2</sub>O<sub>3</sub>, all in a size below 4.1 µm with the exception of Cr, which was < 39 µm (measured in PBS). In this study, ion release was thoroughly investigated for these particles (see paper IV) as well as for particles of larger sizes in artificial lysosomal fluid (ALF), which mimics intracellular conditions in lysosomes. ALF has a pH of 4.5 and is strongly complexing, due to citric acid<sup>216</sup>. In ALF, it was observed that the overall release of chromium and iron ions was low for the alloys and chromium metals, which was due to a protective chromium-rich surface oxide. However, the amount of ions that were released per surface area (metal release rate) in ALF increased with decreased particle size for the alloys, but not for the pure metals for which it instead decreased. One reason for this might be that the surface oxide of the smaller particles more easily is complexed and subsequently dissolved than the larger particles in ALF. The importance of pH for release of ions from stainless steel 316L particles was further investigated in other more neutral or weakly alkaline fluids: Gamble's solution (GMB) simulating the fluid of the lung lining, artificial sweat (ASW) and artificial tear fluid (ATF). The ion release seen in ALF was significantly lower in the other fluids. From this follows that ion release from inhaled particles might change in an acidic and complexing environment. In study IV, stainless steel caused DNA damage in the exposed cells and the reason for this was suggested to be due to direct particle-cell interactions since the release of the DNA damaging ions chromium

and nickel was low. However, later studies by Hedberg et al.<sup>217</sup> have shown that manganese is released from the stainless steel particles. Manganese in the form of  $\text{Mn}_3\text{O}_4$  nanoparticles have been shown to induce relatively high levels of intracellular ROS levels in exposed cells *in vitro*<sup>177</sup>. In study III of this thesis, microparticles of  $\text{MnO}_2$  showed increased levels of DNA damage in both A549 and BEAS-2B cells (see paper III). Whether the DNA damaging effect of stainless steel has any significance in humans is hard to predict. However, studies of changes at the genomic level in terms of histone modifications have been studied in blood leukocytes of steel workers<sup>218</sup>. The exposure of nickel and arsenic, but not manganese, in the air of the work environment was in this study linked to increased levels of the modifications, which may in the long run affect gene expression and contribute to cancer.

In study I of this thesis, exposure of CuO nanoparticles was shown to induce much higher toxicity in terms of cell death in A549 than Cu ions (from  $\text{CuCl}_2$ ) (see paper I). This is probably due to the protective cell membrane, which is an excellent barrier against most ions. Cronholm et al.<sup>195</sup> showed that the same CuO nanoparticles as investigated in this thesis were localised inside A549 cells 4 h after exposure, while no CuO nanoparticles could be detected after 24 h, indicating that the CuO nanoparticles had dissolved by then. Hence, CuO nanoparticles seem to act like a “Trojan horse” bringing in the Cu ions into the cells. The fact that nanoparticles might transport toxic ions into the cell is a discussed concept also by others<sup>207</sup>. However, it is important to consider that the released ions need to have a certain toxic potential to cause damage, as was also stated in the study by Cho et al.<sup>117</sup> previously discussed. Even though it was not studied in the present thesis, an intracellular release of Ni ions is likely an important explanation to the high toxicity of NiO<sup>219</sup> observed in study III.

**In conclusion, the results within the present thesis suggest that the high toxicity of CuO is not due to Cu-ion released in the media. Instead, one likely explanation is that CuO nanoparticles might act like a Trojan horse, transporting toxic ions in to the cells where released Cu ions (and the reactive particle surface) cause oxidative stress. The results also indicate that the intracellular environment can change the ion release properties as noted from micro-sized stainless steel particles that released more ions at acidic and complexing conditions.**

### 4.3 INTERACTION WITH TOXICITY TESTING AND OTHER METHODOLOGICAL CONSIDERATIONS FOR THE COMET ASSAY

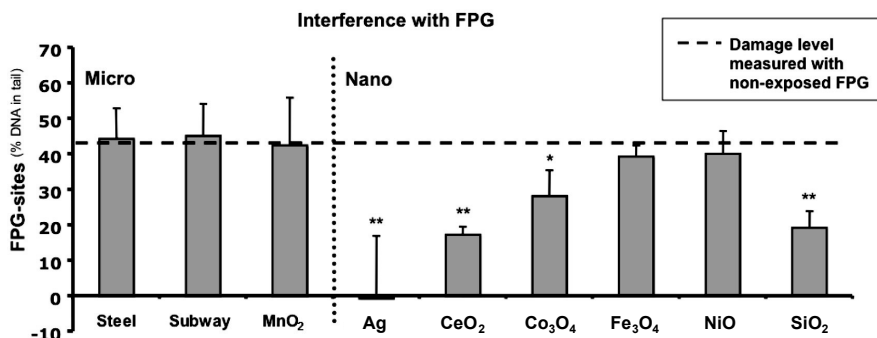
In study III of this thesis, the comet assay, used for analysis of DNA damage and oxidative damage of DNA, was evaluated regarding possible particle interaction. The comet assay is increasingly used within nanotoxicology. However possible interference of particles has only been mentioned briefly in a few published studies and interference with the restriction enzyme FPG has, as far as I am aware, not been considered at all prior to study III of this thesis.

#### 4.3.1 Interference of particles with the comet assay

In study III, the comet assay was initially evaluated with cells exposed to the chemical photosensitiser Ro, which, when in combination with light, induces oxidative damage to DNA (mostly in the form of 8-oxoguanine). When using FPG the results clearly indicated that the assay found the induced damage and it supported the general understanding that the comet assay is reliable to use for chemicals. Human lung cell lines (A549 and BEAS-2B) exposed to a wide range of metal containing particles both as micro- and nanoparticles showed that several of the particles induced DNA breaks but only low levels of oxidative damage to DNA (see paper III). This had also been a phenomenon seen in study I and II. These results can of course be due to low oxidative potential of the particles, but interaction within the method is also a possibility. In study III, the oxidative capacity of several of the particles was, however, shown by their ability to generate intracellular ROS. In addition Ag nanoparticles and subway particles also caused oxidation of the DNA base guanine when exposed non-cellularly and evaluated by HPLC.

The hypothesis that particles may interact with FPG used within the comet assay arises from the fact that particles have been observed in the comet nucleoid after particle exposure<sup>220,221</sup>. Most likely, the particles get in contact with the nucleoid during the comet assay, as discussed in a study by Karlsson<sup>160</sup>. The presence of particles close to DNA during the comet assay procedure consequently makes interaction with FPG a possible scenario. In study III, incubation of FPG with the particles, at an approximate intracellular dose, led to lower FPG activity, particularly for the nanoparticles of Ag, but also with CeO<sub>2</sub>, Co<sub>3</sub>O<sub>4</sub> and SiO<sub>2</sub> (see Figure 19). Further investigations of these particles revealed that the decreased activity of FPG from Ag incubation mainly was due to released Ag ions. For CeO<sub>2</sub> and Co<sub>3</sub>O<sub>4</sub>, the decreased activity was due to the particles, and for SiO<sub>2</sub> FPG seemed to be active although bound to the particles (see section 3.3.10 for procedure and paper III for more details).





**Figure 19.** Detection of oxidatively damaged DNA (FPG sites) in Ro exposed A549 cells by the comet assay, using FPG incubated with particles in an estimated intracellular dose. The dotted line represent the normal capacity of FPG to find the oxidatively damaged DNA.

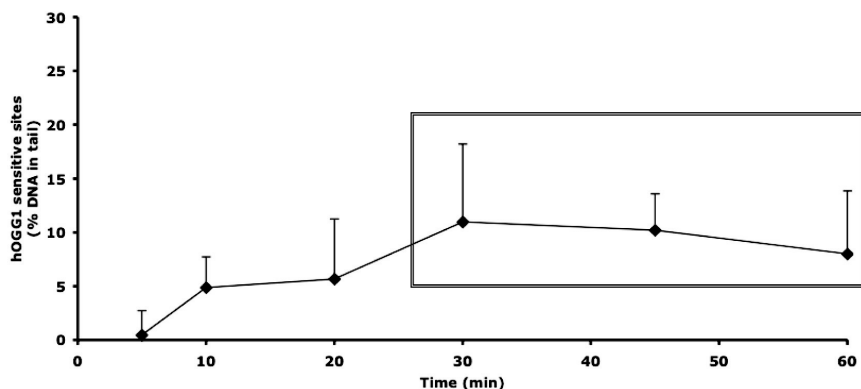
The mechanism by which particles or ions inactivate FPG may be through conformational changes of the enzyme or interference with the structure of the enzyme, which is important for the substrate specificity. FPG is a zinc finger protein and it is the “finger-like” structure that wraps around DNA and enables the binding of the enzyme to DNA during repair of oxidatively damaged DNA. It has been shown that the active site of the finger structure is positively charged<sup>222</sup> with one Zn atom<sup>223</sup> anchored between four SH groups<sup>224</sup>. This makes the active site a possible target of metal ions, which might take the place of the Zn atom. Low doses of metal ions of Cd(II), Cu(II) and Hg(II) have been observed to have a high affinity for the zinc finger of FPG, causing pronounced enzymatical inhibition<sup>225</sup>. In study III, Ag ions caused FPG to totally lose its ability to detect oxidatively damaged DNA in the comet assay. This disturbance is most likely due to the binding of Ag ions to the SH groups at the active site of the enzyme. Binding of Ag ions to SH groups in proteins is suggested to be the mechanism behind the well-known antibacterial effect of Ag<sup>226</sup>.

After the publication of this study another study also addressed this issue, with the focus on interaction of SiO<sub>2</sub> nanoparticles<sup>227</sup>. The procedure for testing this was quite different compared to the study in the present thesis. HeLa cells were exposed to SiO<sub>2</sub> nanoparticles before the induction of oxidative DNA damage by the photosensitiser Ro. The comet assay was then performed with FPG and the same amount of damage with and without the pre-exposure of SiO<sub>2</sub> was detected. This can be compared to study III where the cells are only exposed to the photosensitiser and not to the particles. Instead, the enzyme was incubated with particles and ions separately (see paper III). The authors, Magdolenova et al. conclude that their results indicate that the nanoparticles did not interfere with FPG. However, whether the highly reactive photosensitiser Ro react with SiO<sub>2</sub> nanoparticles before the comet assay was performed, was not stated to be tested. An interaction between the photosensitiser and SiO<sub>2</sub> might cause structural or chemical changes of the particle or ions. What form of the particle that is actually present in the nucleoid, if present at all, when using this study design is in fact difficult to know.

The results in the studies (I, II and III) within this thesis suggest that oxidative stress caused by nanoparticles may, for some nanoparticles, be underestimated if the comet assay with FPG is used for analysis of oxidatively damaged DNA. Thus, this method may not be the most suitable to use for measurements of oxidatively damaged DNA from particles after exposure to cell lines. This study highlights that established, reliable genotoxicity tests for chemicals may not always be suitable for studying genotoxicity of particles.

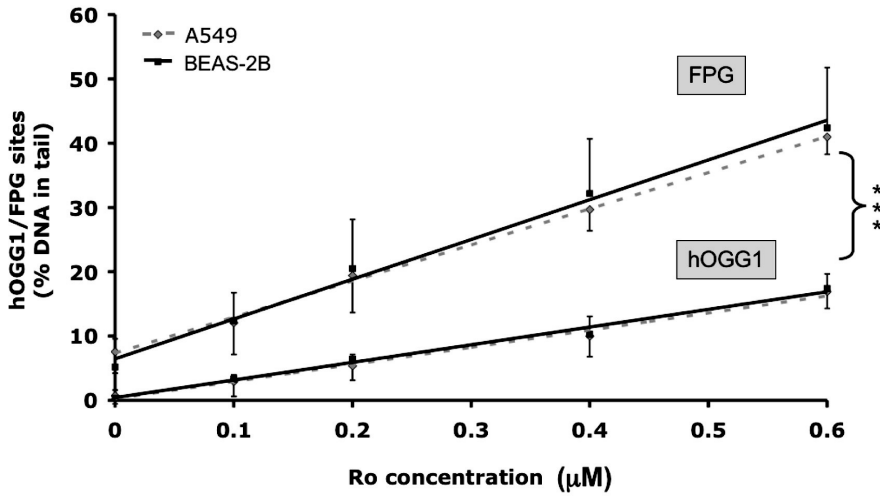
#### 4.3.2 Comparison of FPG and hOGG1 for detection of oxidative DNA damage

It has been suggested by Smith et al.<sup>162</sup> that hOGG1 finds 8-oxoguanine more specifically than FPG. The authors compared the ability of FPG and hOGG1 to find oxidatively damaged DNA in the form of 8-oxoguanine, but other damages such as alkylation were also evaluated. It was observed that hOGG1 found less damage and was therefore suggested to be more specific. However, the incubation time for the enzymes differed (10 min for hOGG1 and 30 min for FPG), and it can therefore not be excluded that experimental differences explain the level of damage detected. To investigate this further, within the concept of this thesis, A549 cells were exposed to 0.4  $\mu$ M Ro and light irradiation. Enzyme incubations for 5, 10, 20, 30, 45 and 60 min were applied and the enzyme dilution was set to 1:1000 (0.048 U/gel) in enzyme buffer (the same as in the study by Smith et al.). The results can be seen in Figure 20 and indicate that it is not until the incubation time of 30 min is reached that the damage levels seem to flatten out. These results indicate that an incubation of 10 min is not enough for hOGG1 to find all damage present in the cells.



**Figure 20.** Evaluation of different incubation times for hOGG1 in the comet assay. The A549 cells used for the evaluation were exposed to 0.4  $\mu$ M photosensitiser Ro and light. The incubated with hOGG1 ranged from 5-60 min and the enzyme had a concentration of 0.048 U/gel (diluted 1:1000). The box indicates a possible plateau. Each point represents three independent experiments  $\pm$  SD.

In further studies, the optimal concentration of hOGG1 was evaluated as well. The results showed that hOGG1 has not reached a stable plateau in the dilution 1:1000 and that a dilution of 1:2000-1:5000 probably would give a more correct result, since the higher concentration might cause unspecific “cutting” by the enzyme. When oxidative damage in A549 and BEAS-2B cells was evaluated using the comet assay after exposure to the photosensitiser Ro in a concentration range (0-0.6  $\mu$ M) and using a 30 min incubation of hOGG1 diluted 1:4000 times the same result as in the study by Smith et al. was obtained (see Figure 21); namely, a lower level of damage detected by hOGG1. The shorter exposure time in the study by Smith et al. was most likely compensated by the higher concentration of hOGG1. In conclusion, the result of the present study is in line with the study by Smith et al. However, the experimental conditions could have been optimised further, in the study by Smith et al.



**Figure 21.** Oxidative DNA lesions from photosensitiser Ro in cultured A549 and BEAS-2B cells evaluated with FPG and hOGG1 using the comet assay after exposure to 0 - 0.6  $\mu$ M Ro and light. Each point represent the average of four independent experiment  $\pm$  SD. All the lines for A549 and BEAS-2B have  $R^2$  values of 0.99 - 1.00. Stars (\*\*\*) indicate the statistical significant difference of  $p < 0.001$ , estimated by two-way-ANOVA, when the detection capacity of the two enzymes is compared. There is no significant difference between the cell lines when evaluated with the same enzyme.

#### 4.4 HUMAN EXPOSURE TO SUBWAY PARTICLES AND HEALTH EFFECTS

A project was conducted during the years of 2006-2010 attempting to fill the existing knowledge gap regarding health effects in humans after exposure to air at a subway station. This project consisted of three studies, by which the study present in this thesis (study V) is the last one. All studies were conducted at a subway station in Stockholm (Odenplan) and the air was measured to be rich of metal containing particles. The study design for the three studies was the same, but with somewhat different end points, study subjects and time frames of evaluation (see paper V for details of the third study).

The two first studies investigated the acute inflammatory responses in healthy and mildly asthmatic human subjects respectively, 14 h after a 2 h long exposure at the subway station. In healthy individuals, a small increase in fibrinogen and regulatory T-cells expression was detected in blood after the exposure, indicating a systemic biological response<sup>65</sup>. This was, however, not seen for the mild asthmatics, whom instead showed an increase in T-cells expressing the activation marker CD25 in BAL, indicating a local effect in the lungs<sup>142</sup>. A conclusion from these studies is that an immunological reaction occurs from the exposure, and that the mild acute responses differ for asthmatics and healthy individuals.

To further investigate the immunological effects in healthy individuals the study present in this thesis (study V) was conducted including repeated measurements during a longer follow up time (24 h). Since blood is a much more suitable method for repeated measurements than BAL, and since effects was previously observed in blood of the healthy human subjects, blood sampling was used for this study. The blood samples were used to investigate effects on lung permeability and inflammation responses, in terms of immune cell differentiation, characterisation of lymphocyte subsets as well as fibrinogen levels. In addition, the DNA damaging effect and oxidative stress seen in previous *in vitro* studies from the research group<sup>68,69</sup>, as well as DNA repair was investigated in human PBMC. The parameters were investigated after a 2 h exposure at the subway station and compared to the same exposure in a clean environment.

A total of 18 subjects were evaluated and in short the results indicated a direct inflammatory response, as a decrease in cell concentration of lymphocytes in blood after the subway exposure. In the end of the follow-up time discreet indications of inflammatory responses in blood were also detected, as changes in the lymphocyte subpopulation. More precisely, NK-T and T-regulatory cells were increased, and T-helper cells and the ratio of CD4/CD8 cells were decreased. DNA damage in PBMC of some of the study subjects was increased 23 h after exposure, indicating certain sensitive subjects within the study group.

The increased expression of regulatory T-cells, the tendency of increased NK-T cells and the small increase in T-helper cells in blood of healthy subjects 14 h after subway exposure, seen in the first study could not be detected in the present study 24 h post exposure when subway and control exposure was compared. However, the present study design allowed for a comparison within the same day i.e. *before exposure* versus

*24 h after the exposure*, ruling out the influence from possible changes in basic health status of the subject between the two different exposure occasions (there was a minimum of 3 weeks between the control and subway exposure). This comparison showed, in consistency with the first study, a significant increase of the regulatory T-cells and NK-T cells 24 h after subway exposure. No such difference was seen for the control exposure. The significant drop in the CD4/CD8 ratio in the present study was not detected after 14 h in the first study. The levels of this ratio are, however, within a normal range and the drop in CD4/CD8 ratio is a reflection of the drop in T-helper cells, and indicate that the immune system has reacted. In the second study where mildly asthmatics were exposed in precisely the same manner as the healthy subjects in the first study, no effect at all was detected in blood. In the first and second studies lymphocyte subsets were also measured in BAL fluid from lung. In the healthy subjects, no effects were detected, but the asthmatics showed an increase in T-helper cells expressing CD25 (CD4+/CD25 cells). The conclusions from the two first studies were that asthmatics seemed to get a local effect in the lungs whereas the healthy subjects had a systemic reaction in the blood. Changes in lymphocyte subsets, such as the decrease of T-helper cells and the increase of regulatory T-cells and NK-T cells in the present study of this thesis, may be seen before an inflammation has been established and other inflammation markers are present.

The decreased concentration and differential (%) of lymphocytes measured in peripheral blood in the present study (study V), directly after the subway exposure, indicates a direct effect of the subway exposure, most likely in the lungs. The level returned to normal within two hours, and the leucocytes, neutrophils, eosinophils, basophils and monocytes were not affected. All together, this points towards a non-extensive reaction at this time-point. This decrease of immunological cells in blood can be a result of cell migration out of blood into an inflammation site. Hence, in scope of this study the decrease of lymphocytes in blood after subway exposure might be an indication of an induced inflammation in the lung.

In a study by Bigert et al. <sup>71</sup>, the inflammatory markers PAI-1, IL-6 and fibrinogen showed a tendency to be increased in platform workers of the subway. Modest elevated levels of fibrinogen were also observed in the first study of healthy subjects, 14 h after the subway exposure. However, there was no increase in fibrinogen in the present study after 24 h. So the effect seen after 14 h seems to have declined 24 h after the exposure occasion.

The self reported symptoms that each subject stated during the study indicated a very small increase in perceived irritation in the eyes and nose during the subway exposure, compared to the control exposure. The experience of disturbing smell was low but showed the most prominent increase when ranked, as 2 (i.e. a small disturbance) in the 0-10 scale, compared to the control environment where all parameters were ranked as 0 (i.e. no disturbance). In the first study of healthy volunteers, the self reported experienced irritation from the lower airway was increased, which is in contrast to the lack of symptoms in the present study. Self reported measurements constitute a somewhat unspecific tool and since it is not possible to blind this part of the study, these results may be influenced by the fact that the participants are aware that they are in the subway. However, other measured health effects and the correlation of these to

self reported symptoms could be of interest, e.g. the correlation between PEF lung function test and perceived irritation from the lower airways. There was, however, no effects seen in PEF for the subway exposure, neither in the self reported symptoms of irritation of the lower airways.

The air monitoring of the subway station Odenplan, including particle number, PM<sub>2.5</sub>, PM<sub>10</sub>, NO and NO<sub>2</sub>, were in the same level for all studies. However, the particle levels, measured as PM<sub>2.5</sub> and PM<sub>10</sub>, were 3.5 and 2.0 times lower than at the Mariatorget station in Stockholm, as previously measured by Johansson and Johansson<sup>63</sup>. There may be several reasons for this, such as differences in the air flow and air conditioning at the stations, the architecture of the platform and number of passing trains. It may also be so that the new version of subway trains (C20 trains), which are used at the lane passing Odenplan cause less emission of particles compared to the older trains (CX trains) that passed Mariatorget station at the time of those measurements. Furthermore, it has been suggested that diesel particles from the street level may be present at subway stations<sup>67</sup>. However, the personal exposure to NO<sub>2</sub> in the present study, reflecting exposure to traffic pollution, did not indicate any extra exposure of traffic pollution for the exposure session in the subway when compared to the control exposure session.

Previous studies have shown DNA damage in A549 cells after exposure to subway particles *in vitro*. This was also seen in study III of this thesis, in both A549 and BEAS-2B cells. In study III, intracellular ROS in A549 cells and oxidation of the DNA base guanine was also shown after exposure of subway particles (see paper III). However, in the present human study a broader aspect of the toxicity was evaluated, since these damages were measured in cells of blood from exposed humans. An initiation of inflammation in the lungs includes release of cytokines and activation of cells of the immune system. It constitutes a local reaction in the lungs that may spread to the rest of the body and influence production and distribution of different immunological cells. Effects of PBMC, which were studied, highlight this systemic inflammatory response, and induction of damage to the DNA may be the result of an initiation of an oxidative stress response or other mechanisms of the immunological defence. Disturbance of the DNA repair system of these cells is also a possibility, and the DNA damage caused by direct effects or by the decreased ability of cells to repair damages, are important initial events in carcinogenesis. An increase of DNA damage in PBMC was detected 23 h post subway exposure for some of the subjects. In these subjects, the DNA damage increased by 24% in the PBMC. Since this is the first study of DNA damage in human subjects after exposure to air at a subway station, it is not possible to compare these results to studies from similar environments. However, studies of humans exposed to traffic related ultrafine particles from one of Copenhagen's busiest streets have shown increased levels of DNA damage in PBMC with 41-60% when measured with the comet assay, 6 and 24 h after the start of the particle exposure<sup>166</sup>. The level of DNA damage in PBMC has also been shown to be increased by 50% after high levels of benzene and ultrafine particle exposure in the city of Benin<sup>228</sup>. The statistical analyses in the present study showed that the increase in DNA damage after subway exposure was not statistically significant when compared to control exposure. This was due to overrepresentation of high levels of DNA damage in the study group that had been randomly chosen to start with the control exposure. Five out of seven subjects in this

group showed an increase in DNA damage 23 h after the subway exposure compared to only two out of eleven subjects in the group starting with the control exposure. The analysis did not point towards a drop-over effect from the subway exposure, and therefore the results may indicate a certain sensitive subpopulation among the subjects studied.

Oxidatively damaged DNA in PBMC is an indicator of systemic oxidative stress relevant for e.g. cardiovascular diseases. This parameter was not affected by the subway exposure at any of the time points. However, exposure to particles can increase the repair of oxidatively damaged DNA, masking the actual damage levels of exposure, a phenomenon which has previously been discussed in relation to human exposure of wood combustion PM<sup>228</sup>. To be able to draw conclusions about oxidative stress induced DNA damage and to really understand what the actual effect of the exposure is, it is important to investigate the repair capacity as well. The repair capacity of PBMC was, however, unchanged in the present study (study V).

The combination of small health effects in exposure studies and relatively small groups of subjects makes it hard to conclude if effects measured are of statistical significance or not. The number of study subjects in the present study was based on previous studies and power calculations performed in studies of health effects from exposure in a road-tunnel environment with high concentrations of particles. The size and composition of particles differ greatly between exposure in road and subway tunnels, therefore it is not surprising if different effects are observed. A larger number of study subjects would have been needed to establish a possible significance for some of the effects detected in the subway, particularly in the case of DNA damage.

*In conclusion, this human study of exposure to metal containing particles at a subway station indicates discrete signs of an induced inflammation. A decrease in cell concentration and differential (%) of lymphocytes in blood was seen immediately after the subway exposure. Some changes in the lymphocyte subpopulation were seen 24 h post exposure along with a slight increase in DNA strand breaks in PBMC for some of the exposed subjects. Whether the small effects observed impose any real health risk is difficult to predict. Nevertheless, the evidence does not indicate that a short subway exposure cause any major acute inflammatory health effects in healthy humans.*

## 5 SUMMARY AND CONCLUSIONS

The main aim of this thesis was to increase the knowledge concerning toxicity and underlying biological mechanisms from exposure of metal containing particles through inhalation. In the studies conducted, this was investigated *in vitro* in lung cells after exposure to engineered micro- and nanoparticles, as well as after exposure to particles from steel industries and the subway system. Additionally, as an example of exposure to metal containing particles, health effects from subway particle exposure was investigated in a human study where healthy humans were exposed to the air at a subway station in Stockholm, Sweden.

The main indicators for toxicity and health effects investigated were markers for oxidative stress and DNA damage. In the human study, inflammation was also investigated. The comet assay was used in all studies for the measurement of DNA damage, oxidatively damaged DNA and DNA repair. Consequently, methodological considerations regarding the comet assay were an important part of this thesis.

### 5.1 CONCLUDING REMARKS

Here follows some specific conclusions that can be draw from the presented studies (studies I-V):

- The *chemical composition* of the metal containing particles seems to be crucial for the toxicity tested. CuO and NiO nanoparticles particularly stood out, inducing high toxicity towards lung cells. One likely explanation for this is that these nanoparticles might act like Trojan horses, transporting toxic ions into the cells, causing oxidative stress.
- The *pH* and complexing agent of the intracellular environment can change the ion release properties of particles, as was seen for the stainless steel 316L microparticles that released more ions at acidic and complexing conditions.
- The combination of surface composition (surface oxide) and particle size of iron and chromium based microparticles seem to be linked to the amount of released ions in a acidic and complexing fluid.
- The *size of the particles* is important for the toxicity of CuO (when dose is expressed as mass), which was seen as higher toxicity for nanoparticles than for micrometre particles of CuO. However, particle size does not always seem to be a dominant factor driving toxicity *per se*, indicated by the investigation of other metal containing particles in the present thesis. The literature shows, however, that in the context of particle deposition, the immunological response towards the particles in the lung and the ability of particles to translocate, the size still might play an important role.



- The *surface area* is suggested to be an essential dose metric to consider for the toxicity of nanoparticles, particularly if the surface is reactive.
- Some *nanoparticles indicate interaction with FPG* used within the comet assay. Oxidative DNA damage may consequently be underestimated, and the comet assay with added FPG may not be suitable for detecting oxidatively damaged DNA in cell lines after particle exposure.
- The fact that Ag nanoparticles interfered with FPG, implies that *Ag nanoparticles might affect the repair of DNA damage* in cells. Consequently, this suggests a relatively new mechanism for the toxicity of nanoparticles.
- The human exposure to air rich in metal containing particles at a subway station caused discrete signs of inflammation: a decrease in lymphocytes in blood immediately after the exposure, some changes in the lymphocyte subpopulation 24 hours post exposure along with a slight increase in DNA strand breaks in PBMC for some of the exposed subjects. Whether these small effects observed impose any real health risk is difficult to predict. Nevertheless, the evidence indicates that a short subway exposure does not cause any major acute inflammatory health effects.

To be able to understand the toxicity of metal containing micro- and nanoparticles, this thesis attempted to arrive at some kind of conclusion concerning which particle characteristics that mainly drive the toxicity. However, the characteristics varied greatly for the investigated particles and the combination of the different physicochemical properties (chemical composition, size, solubility, surface reactivity etc.) made this kind of conclusion very complex to perform. In addition, there might be characteristics of particles that are yet to be revealed to be important, but that are not even considered or discussed today due to lack of knowledge.

Regarding nanoparticles, this thesis is in line with the growing consensus that nanoparticles are not a toxicologically uniform class of materials and therefore they should not be viewed upon as a homogenous group with simple toxic attributes. There is not one specific property of nanoparticles that can be held responsible for the toxicity. Knowing this, researchers have suggested again and again that nanomaterials should be tested individually (we suggested it in the first studies of this thesis as well). However, lately a healthy debate regarding this has arisen. Since it is not possible to evaluate all different nanoparticles one by one, that would be extremely time consuming and expensive, and possibly not even operable. The call for individual evaluation of nanoparticles previously made is most likely a call for bringing awareness to the differences that exist between different nanoparticles, in terms of their characteristics and toxicity. Today, there is a need for agreement and development of reliable standardised test methods and toxicity end points relevant for human health so we can start screening the nanoparticles that are out there, already on the market. It might feel impossible to agree upon this. However, there is no other options, we cannot test all nanoparticles individually. Some materials and characteristics show to be linked

to toxicity more often than others and these are probably particles and characteristics that we should be particularly cautious with.

## **5.2 FUTURE REMARKS**

Nanomaterials, including nanoparticles, may open tremendous opportunities for different applications in the fields of, for example, medicine, engineering, electronics, optics, consumer products, alternative energy and water cleaning. However, too little is known about their capability to cause adverse effects in humans and the ecosystem. The lack of toxicology data on engineered nanoparticles is a problem causing inadequate risk assessment, and is something that needs more attention and more studies, not in the distant future, but today!

I personally find it exciting to think about all the tremendous applications that nanotechnology might bring in the future. At the same time, when thinking of the enormous amount of nanomaterials used today in the society and the potential exposures this brings, it can scare me. We are using these materials in so many contexts in our society today without knowing if they will make us unwell in the future! What will happen if we discover that some of them are toxic to us and/or the environment? We are in urgent need for some action!

In relation to the industrially relevant particles and particles present in the subway, which were investigated in this thesis, I believe that more in-depth investigations of exposure are required. Studies on effects of long term exposure, occupational exposure of humans as well as exposure to sensitive groups are needed.

The future in the field of particle toxicology is exciting and slightly frightening at the same time, particularly regarding nanoparticles. I presume we should listen to Marie Currie, who is cited on the first page of this thesis: we can not let us be scared and give up in our endeavour to find the answers to our questions!

## 6 SVENSK POPULÄRVETENSKAPLIG SAMMANFATTNING

Partiklar i stadsmiljö, från bilavgaser och uppriven asfalt (från dubbdäcksanvändning), har länge förknippats med olika typer av hälsoproblem och sjukdomar, såsom försämring av astma och kronisk obstruktiv lungsjukdom (KOL) samt cancer och hjärt-kärlsjukdomar. Även partiklar i olika dammiga arbetsmiljöer och industrier, särskilt partiklar av metaller som bildas vid svetsning, är kopplade till flera negativa hälsoeffekter. Under senare år har det framkommit att människor även blir exponerade för partiklar som innehåller metaller när de vistas i tunnelbanesystem, men om det innebär någon risk för hälsan är inte känt.

Idag används allt oftare mycket små metallinnehållande partiklar, så kallade nanopartiklar, exempelvis i olika konsumentprodukter. Det finns nanopartiklar i solkräm, målarfärg, byggmaterial, elektriska apparater, tyger, kläder och kosmetika m.m. Nanopartiklar besitter en rad specifika kemiska egenskaper till följd av sin storlek, vilka kan komma till stor nytta i olika sammanhang. Den breda användningen betyder att exponering för metallhaltiga partiklar med stor sannolikhet sker i samhället idag och att det troligtvis kan komma att öka i framtiden till följd av den ökade användningen i olika produkter.

Det finns i dag ett stort behov av mer kunskap om huruvida metallinnehållande partiklar kan påverka vår hälsa negativt. Det främsta syftet med studierna som presenteras i den här avhandlingen var att undersöka giftigheten hos en rad partiklar och försöka förstå på vilket sätt eventuella skador uppstår i celler efter exponering för partiklarna. Detta undersöktes med hjälp av odlade, mänskliga lungceller i studierna I-IV (artiklarna I-IV) och i studie V undersöktes effekten hos försökspersoner som fått andas in luft innehållande partiklar på en tunnelbanestation.

I studierna I-IV undersöktes giftigheten och de bakomliggande skademekanismerna för ett brett spektrum av metallinnehållande partiklar, från mycket små nanopartiklar till lite större partiklar ("mikropartiklar"). I dessa fyra studier undersöktes framförallt hur materialsammansättningen, d.v.s. vilken metall/metaller partiklarna består av, påverkar deras nivå av giftighet. Även betydelsen av partiklars storlek och om de friger joner (d.v.s. löses upp) studerades. De effekter som mättes hos lungcellerna efter partikelexponering var: celdöd, skador på arvsmassan (DNA), förekomsten av skadliga radikaler i cellen och skador på DNA kopplade till detta. Dessutom mättes skador på "energifabrikerna" i cellen (mitokondrierna) samt huruvida röda blodkroppar sprack efter att de exponerats för partiklarna. Alla dessa skador kan vara viktiga för utvecklandet av olika sjukdomar och negativa hälsoeffekter hos människan, exempelvis för cancer och inflammation. Resultaten av undersökningarna visade att det fanns en hög variation i giftighet hos de undersökta partiklarna. Särskilt skadliga var partiklar av koppar (CuO) och de små partiklarna (nanopartiklarna) var betydligt mer skadliga än de större partiklarna. En trolig orsak till detta är att kopparpartiklarna löser upp sig och avger giftiga joner. En teori är att partikeln, likt en trojansk häst, tar sig in i cellen och sedan avger de giftiga jonerna. Dessa kan i sin tur skada cellen.

I studie IV undersöktes industriellt relevanta partiklar av järn och krom. Av dessa visade sig partiklar av rostfritt stål reagera olika i olika mänskliga vätskor. Resultaten avslöjade att mer joner frigavs från dessa partiklar i sura vätskor (surt pH). Giftigheten för de järn- och krombaserade partiklarna var relativt låg då odlade lungceller utsattes för dessa, men höga nivåer av skador på DNA kunde ses från både partiklar av rostfritt stål och från nickelpartiklar (NiO). Även partiklar av krom ( $\text{Cr}_2\text{O}_3$ ) tycktes vara skadliga på så sätt att de påverkade röda blodkroppar.

I studie III (artikel III) sågs en ökad förekomst av radikaler i odlade lungceller efter exponering för flera metallinnehållande partiklar, men några skador från dessa radikaler kunde inte upptäckas på DNA. Genom noggranna eftersökningar visade det sig att detta kunde bero på att partiklarna påverkade ett protein (enzym) som är viktigt för mätmetoden (den så kallade "kometmetoden"). Detta var särskilt tydligt för vissa partiklar och nanopartiklar av silver påverkade metoden allra mest, vilket visade sig bero på att silverjoner frigetts från partiklarna.

I studie V (artikel V) undersöktes effekter hos friska försökspersoner som vistats två timmar på en tunnelbanestation (Odenplan i Stockholm). Personerna fick under 24 timmar lämna blodprover vid särskilda klockslag, samt genomföra tester för att se om deras andningsförmåga påverkats av vistelsen på stationen. Resultaten visade att en liten inflammatorisk respons i form av en minskad nivå av immunceller i blodet uppstod direkt efter vistelsen, men att effekten var snabbt övergående. I slutet av uppföljningstiden kunde även små förändringar ses i vilken typ av immunceller som fanns närvarande i blodet. En ökning av skador på DNA kunde även ses i celler i blod hos några av försökspersonerna i slutet av uppföljningstiden.

Sammanfattningsvis visar resultaten från dessa studier att den kemiska sammansättningen av metallinnehållande partiklar är avgörande för giftigheten hos de testade partiklarna. Denna avhandling belyser särskilt giftiga effekter av nanopartiklar av koppar (CuO) och nickel (NiO). Frisättning av joner från partiklar inuti cellerna kan vara en viktig mekanism bakom de skador som ses i celler efter exponering. Frisläppandet av joner från partiklar observerades även öka för partiklar av rostfritt stål vid surt pH. Dessutom visar studierna i denna avhandling att vissa nanopartiklar kan påverka kometmetoden med vilken skador på DNA från radikaler kan mätas. Denna upptäckt tydliggöra därför vikten av att noga överväga vilka lämpliga testmetoder man väljer för att testa partiklars giftighet. Slutgiltigt visar denna avhandling att en kortvarig exponering för luft (rik på metaller) vid en tunnelbanestation ger en liten respons från immunförsvaret, men att några större akuta hälsoeffekter på människor inte tycks uppstå.

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