HEINRICH HEINE
UNIVERSITÄT DÜSSELDORF

Investigation on the toxicity of Engineered Nanoparticles

Exploring the role of the glutathione antioxidant system

Inaugural dissertation

for the attainment of the title of doctor
in the Faculty of Mathematics and Natural Sciences
at the Heinrich Heine University Düsseldorf

presented by

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from Bangkok

Düsseldorf, August 2017

From the IUF – Leibniz Research Institute for Environmental Medicine

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Faculty of Mathematics and Natural Sciences at
Heinrich Heine University Düsseldorf

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Date of the oral examination: 20/10/2017

“Our greatest weakness lies in giving up. The most certain way to succeed is always to try just one more time.”

Thomas A. Edison

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General Introduction

1 Engineered nanoparticles

Recent decades have witnessed tremendous increases in the production and use of nanomaterials. Nanoparticles can be subdivided into naturally formed nanoparticles, unintentionally formed nanoparticles and engineered nanoparticles. Engineered nanoparticles (NPs) refer to intentionally created particles, with one or more dimensions in the range between 1 nm and 100 nm. Naturally occurring nanoparticles are for instance found in fine dusts and volcanic ashes. Combustion processes are a major source of unintentionally generated nanoparticles. In urban environments, traffic-derived nanoparticles (e.g. from diesel engine exhaust) are a major example of unintentionally generated nanoparticles. Engineered (or manufactured) nanoparticles, are those that are made deliberately at the nano-scale because they exhibit properties that provide technological advantages compared to the bulk form of the same material (Stone et al. 2016; The Royal Society and The Royal Academy of Engineering 2004). Engineered NPs are increasingly introduced in a lot of commercial products (Salata 2004). The unique properties of NPs have resulted in numerous interesting applications in various research fields and industrial sectors such as in "nanomedicine" applications, in food industry, in cosmetics and textiles (Boisselier and Astruc 2009; Frohlich 2012). There are numerous reports and studies showing the advantage of NPs over larger particles because of new properties and improved functions, such as in optics, conductivity, drug carrier function or bactericidal properties (Assolini et al. 2017; Besinis et al. 2014; Besinis et al. 2017; Cavassin et al. 2015; Jana et al. 2017; Wang et al. 2017). However, there are also ongoing discussions about the potential adverse health risks of NPs. The benefits and the risks of NPs are the two sides of the same coin.

1.2 Beneficial aspects of engineered nanoparticles

1.2.1 Carbon nanotubes

Carbon nanotubes (CNT) belong to the so-called high aspect nanoparticles. The most important types of CNT are the single walled carbon nanotubes (SWCNT) and multi-walled carbon nanotubes (MWCNT). Double walled carbon nanotubes (DWCNT) are a special class of CNT with promising applications. The global market for CNT was $158.6 million in 2014 and reached $167.9 million in 2015 and is expected to reach $670.6 million in 2019 with a growth

rate of 33.4% (Oliver 2015). CNT are used in numerous applications according to their specific properties such as thermal conductivity, mechanical strength and great electrical conductivity (Donaldson et al. 2006). Many sport equipment nowadays contains CNT for the strength and lightweight properties. There is also a steadily increasing number of studies that discuss the possible applications of CNT in the nanomedicine field, for example in cancer diagnosis and treatment (Sheikhpour et al. 2017) and in filtration applications (Rashid and Ralph 2017), as sensors and in electronic devices (Abdulrauf and Tan 2016).

1.2.2 Metal based engineered nanoparticles

Silver (Ag), titanium oxide (TiO_2) and zinc oxide (ZnO) represent three major types of metal based engineered NPs, besides silicon dioxide (SiO_2) and gold (Au). Silver NPs exhibit strong antibacterial properties. They are used for example in textiles coatings, food packaging, water disinfectants and room sprays, and for the treatment of wounds and burns. The Ag NPs are thought to become of further importance in medical approaches in relation to the growing concern about antibiotic resistance (Chen and Schluesener 2008; Dizaj et al. 2014). TiO_2 NPs are highly insoluble and are uses in ceramics applications, in fire retardants products, insulation materials and self-cleaning products, in the food industry as food additive and in food packaging, in cosmetics and sunscreen products and in pharmaceutical applications (Bumbudsanpharoke et al. 2015; Robertson et al. 2010). For, ZnO NPs also a wide range of applications exists in various branches of industry, for example rubber, ceramics, paints, pharmaceuticals, diet supplements, cosmetics, textile, electronic and photocatalytic applications (Contado 2015; Hahm 2014; McSweeney 2016).

1.3 Risk aspects of engineered nanoparticles

The field of nanotechnology has developed at an enormous pace over the past decades with the introduction of many novel products. Currently, more than 1,600 nanotechnology-based consumer products have been introduced to the market. A workshop report by the USA National Science Foundation (NSF) estimated that the global nanotechnology market value would reach $ 1 trillion annually by 2015 (Roco 2011). With the increasing production and use there is also an increasing potential exposure of humans to engineered NPs. Such exposure can occur incidentally or accidently, for example during the production of NPs at the working place (nano-

manufacture) or in research laboratories (nano-research) (Kuhlbusch et al. 2011). Moreover, exposure can occur following the natural release of particles from products during its use or afterwards following its release from waste products into the environment (Borm et al. 2006). Important sources of intentional exposure to engineered nanoparticles include cosmetics, personal care, food, medical and implantation applications (Contado 2015). The concern about the potential adverse health effects of engineered nanoparticles originated from the knowledge about the adverse health effects of particles of large size like asbestos and crystalline silica and from ambient particulate matter (PM)(Donaldson et al. 2003; Unfried et al. 2007; Xia et al. 2006). The inhalation of these "classic" particulate toxicants can lead to various deliberating lung diseases including lung fibrosis, chronic obstructive pulmonary disease (COPD) and cancer (Donaldson and Borm, 2006). Toxicological research of engineered NPs has therefore also focused on the identification of the hazards and risks related to the development of these lung diseases. However, because of their small size, exposure to engineered NPs has also been discussed in relation to diseases of other organ systems and tissues than the lung. The various routes of exposure and uptake, routes of translocation, redistribution and excretion are shown in the scheme of Figure 1-1. Experimental studies in rodents have demonstrated that engineered nanoparticles following their inhalation may translocate into the blood circulation and reach other organs and tissues (reviewed in Borm et al. 2006; Oberdorster et al. 2005a). For instance, with TiO_2 after intratracheal instillation exposure, a small fraction of the NPs was detected in the blood circulation and to reach other organs such as liver and kidneys (Li et al. 2010). Inhaled uranium nanoparticles have been shown to translocate to blood, brain, skeleton and kidneys (Lestaevel et al. 2005). In specific nanomedical applications, NPs may also reach various organ sites after intravenous application (reviewed in Murugan et al. 2015). In recent years there is also an increasing discussion about the effects in the gastrointestinal tract caused by nanoparticles used in food industry applications, such as the approved food additives SiO_2 (European Union food additive "E-Number" E551) and TiO_2 (E171) and the "antibacterial" Ag nanoparticles in food packaging (Bouwmeester et al. 2009; Chaudhry et al. 2008). Increasing evidence also indicates that specific types of nanoparticles may translocate from the intestine into the blood and to secondary organs (Kreyling et al. 2017). In contrast, the skin is considered to act as a firm barrier to most types of NPs e.g. in cosmetics and sunscreens (Borm et al. 2006; McSweeney 2016). There is also evidence that inhaled NPs can migrate to the brain via the olfactory bulb as demonstrated in animal experiments (Oberdorster et al. 2004; Elder et al. 2006) and the mechanism may correlate with the development of neurodegenerative diseases (Heusinkveld et al. 2016). The relevance of this translocation route has been shown for various

nanoparticles such as silver-coated gold, carbon, MnO_2 and TiO_2 NPs (De Lorenzo 1970; Elder et al. 2006; Oberdorster et al. 2004; Wang et al. 2008). The most important clearance routes of NPs are the gastrointestinal tract following inhalation (see also below), and the kidney and liver for particles that have entered the bloodstream following inhalation, oral exposure or intravenous application (Longmire et al. 2008; Oberdorster et al. 2002)

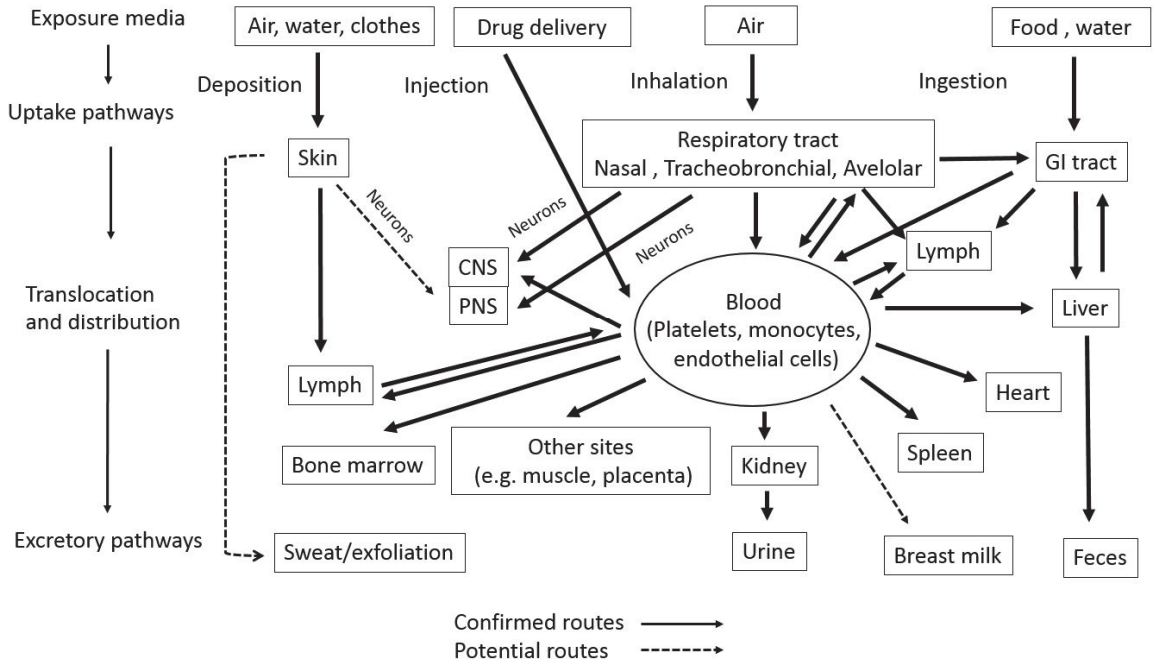

Figure 1-1: Major routes of uptake, translocation, distribution and excretion of nanoparticles. Taken from Oberdorster 2012

1.4 Nanoparticles and the respiratory tract

Inhalation is considered as the main exposure route of NPs and the lung is therefore a critical target organ for investigation of hazard and risks of NPs. While we can survive without food and water for several weeks and days, respectively, we will not survive for few minutes without breathing air. The lung is one of the most important organs in the human body, its function is to provide gas exchange, specifically oxygen and carbon dioxide between the air and our blood. Oxygen is a vital element for human metabolism. As we breathe the air (on average at least 10,000 liters per day), there is an enormous chance for incidentally and intentionally generated nanoparticles to deposit in within the respiratory tract.

1.4.1 Deposition and clearance of nanoparticles

The location of the deposition of particles within the respiratory tract depends on their aerodynamic properties (reviewed by Carvalho et al. 2011 and Londahl et al. 2014). Deposition is influenced by the size, density, shape, charge and the surface properties of the particles as well as the breathing pattern of the individual (Carvalho et al. 2011). The aerodynamic size depends on the shape and density of the particles. The mechanisms that play a role in the deposition of particles are sedimentation, impaction, Brownian diffusion, interception, suspension and electrostatic precipitation. Larger particles have the tendency to deposit in the upper airways, including the nasal compartment, while smaller particles like fine and ultrafine size particles (nanoparticles) can deposit deeper in the respiratory system, i.e. the bronchial and alveolar regions (Chow et al. 2007). Figure 1-2 shows the fractional deposition of inhaled particles based on a predictive data modelling (ICRP 1994).

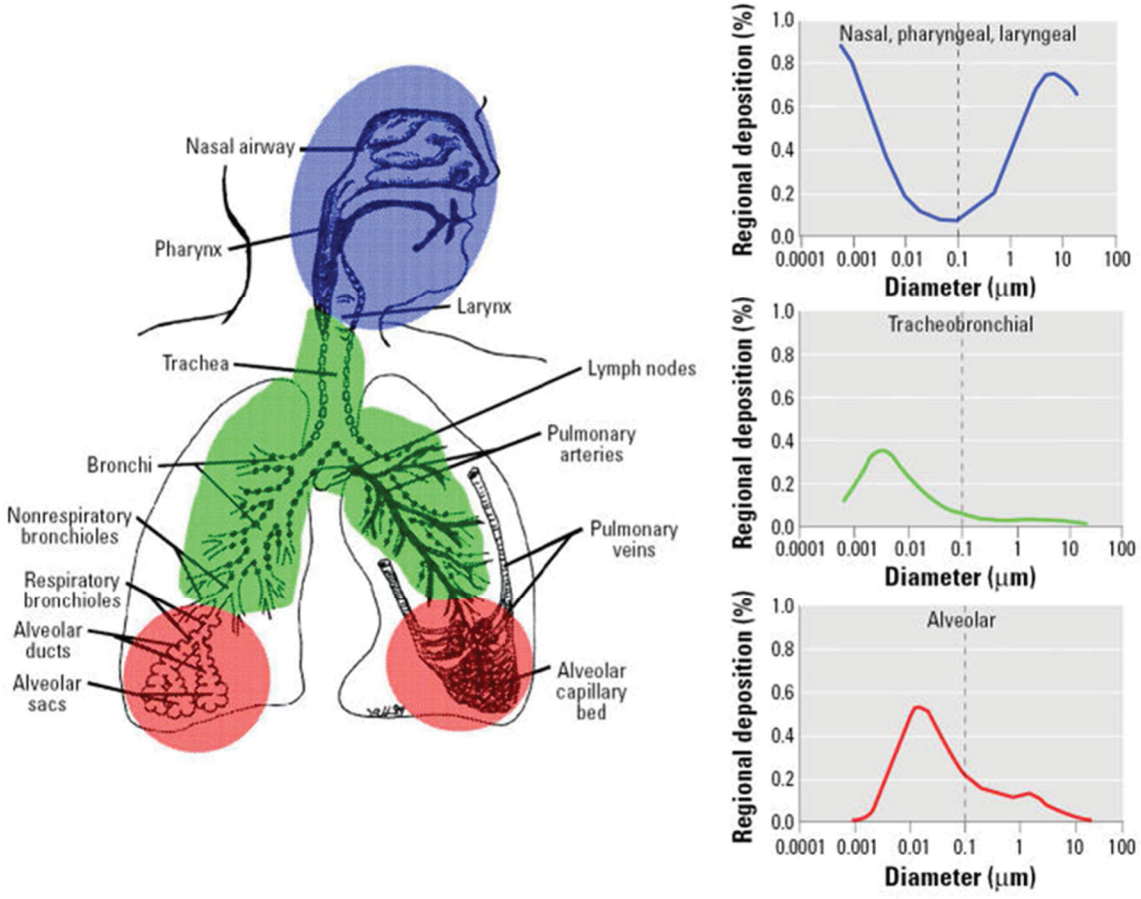

Figure 1- 2: Deposition of inhaled particles in the nasopharyngeal, tracheobronchial, and alveolar regions of the human lung during nose breathing. The data are based on a predictive data modelling (ICRP 1994). Taken from Oberdorster et al. 2005b.

As can be seen in this figure large particles (e.g. 10 µm) deposit almost exclusive and at high efficiency in the nasopharyngeal compartment. The data also indicate that NPs (< 0.1 µm) can deposit in all three regions but that efficiencies of deposition depend strongly on the size of the NPs. For instance, NPs with a diameter of 20 nm have the highest deposition in the alveolar region (about 50%). In the tracheobronchial and nasopharyngeal regions particle of this size deposit with approximately 15% efficiency. In contrast, when NPs with a diameter of 1 nm are inhaled, about 90% deposits in the nasopharyngeal compartment and only 10% in the tracheobronchial region. The deposition of such extremely small NPs in the alveolar region is essentially absent.

Upon deposition of particles in the respiratory system, the body tries to eliminate their presence by clearing them. Clearance describes the translocation, transformation and removal of the deposited particles from the various regions of the respiratory tract. Particle clearance depends on their size, solubility, durability and the region of deposition, i.e. nasopharynx, the tracheobronchial conductive airways, and the pulmonary gas exchange region. Soluble particles (e.g. lipid droplets) will dissolve rapidly after inhalation in the liquid lining layer consisting of the surfactant film and the solubilised components may also reach the blood circulation via the air-liquid interface or the lymphatic circulation. Poorly soluble and insoluble particles (e.g. carbon-based particles, TiO_2) that deposit on the inner airway surface of the nasopharynx or tracheobronchial regions will be cleared by mucocilliary transport. The upper airway are covered with goblet cell along the conducting airways. These cells produce the mucus to protect from dehydration of epithelial cells and to trap deposited particles. The trapped particles are moved by mucocilliary escalator to the pharyngeo-laryngeal region, from which the get expelled by coughing or swallowing (Satir and Sleigh 1990). Thus a considerable proportion of inhaled particles, including nanoparticles, will reach the gastrointestinal tract (see also Figure 1-1). The flow rate of the mucus in the human trachea is around 5 to 20 mm/min (Wood et al. 1975). Poorly soluble and insoluble particles that deposit lower than the ciliated airways are mainly taken up by macrophages and are then cleared either via the lymph nodes or the mucocilliary escalator. In contrast to large sized particles, NPs can translocate to significant extent from the lung to other organs and tissues (Figure 1-1). NPs may translocate across the respiratory tract epithelium by the process of transcytosis and thereby reach the interstitium, the blood circulation and the lymphatics. Finally, as already mentioned, NPs deposited in the nose can also be cleared to the central nervous system (Figure 1-1).

1.4.2 The alveolar region of the lung

The alveoli are the most tiny and thin part of the lungs. Alveoli are the most crucial structure of the respiratory tract, permitting the gas exchange between the air in the lung and the blood from the capillaries of the lung. The total number of alveoli is estimated to be around 200-600 million. The total surface area is estimated to be about 40-80 m^2. Alveoli are located into small clusters, called alveolar sacs at the end of the bronchioles. The alveolus typically has a cup-shaped cavity, its inside is hollow and its outside is covered by capillaries. The structure of alveolar wall is represented by a thin layer of epithelial cells. The single layer that supports the alveoli are the connective tissue that connect the capillaries around the alveolus.

The alveolus consists of type I and Type II cells. The respiratory function takes place at the respiratory membrane (air-blood barrier). The alveolar-capillary membrane is composed of a layer of squamous epithelia cells (Type I cells) and the cobweb of pulmonary capillaries. The type I epithelial cells line the alveolar- capillary barrier and cover more than 95 % of the surface area. These cells are large, thin and flat in structure to optimise gaseous exchange. Type I epithelial cells are incapable of division due to their specialized nature. If damaged by toxins or particles, the type I cells are replaced by division of type II cells, that subsequently differentiate in to type I cells. Type II cell hyperplasia can be seen as an indicator of damage to the epithelium.

The cell type II epithelial cells are functionally different to type I cells. A major function of the type II cells is the synthesis of surfactant. The surfactant is required to reduce the surface tension of the alveolar surface. It is also the layer that comes into contact first with (nano) particles that are deposited in the alveolar region. The type II cells are also very different from type I cells in structure. They possess a cuboidal structure with microvilli that extends into the alveolar lumen. Type II cells are also capable of synthesizing a range of inflammatory mediators, and are therefore considered to play a key role in particle-induced inflammation in the lung (Gilmour et al. 2003). Inhalation studies with crystalline silica dust and other toxic particles in rats indicate that particle-induced lung tumours originate from type II cells hyperplasia (Driscoll et al. 1996; Lee et al. 1986). In vitro studies to investigate the toxicity of inhaled NPs are often performed with primary type II epithelial cells or cell lines derived from immortalised type II cells of rodents (Driscoll et al. 1996). For the evaluation of toxicity of particles in human cells, the cell line A549 has been most commonly used as an epithelial II cell model. This cell line was first developed in 1972 from an adenocarcinoma tumour of a Caucasian male (Foster et al. 1998). Despite its potential limitations (i.e. transformation status), A549 cells have been used

in many nanosafety research projects. Isolation and culturing of human primary type II cells has been achieved only recently with limited success (Mao et al. 2015).

1.5 Nanoparticles, reactive oxygen species and oxidative stress

Oxygen is the vital element for life. All aerobic organisms including humans, require oxygen (O_2) for energy production. Daniel Gilbert, Rebeca Gerschman and colleagues first proposed that the poisonous effects of oxygen was due to free radical formation (Gerschman et al. 1954; Gutteridge and Halliwell 2000). Nowadays it is well accepted that reactive oxygen species (ROS) play a role in various deliberating diseases including particle-induced diseases like lung fibrosis, COPD and lung cancer (Fubini and Hubbard 2003; Knaapen et al. 2004; Liu et al. 2013). Numerous studies have demonstrated that various types of particles, including NPs, are able to cause ROS generation in cells (Manke et al. 2013; Unfried et al. 2007). Thus it is likely that ROS generation also plays an important role in the adverse effects of toxic NPs.

1.5.1 Nanoparticles and ROS

Reactive oxygen species (ROS) are oxygen containing molecules with a high chemical reactivity. They include free radicals, that containing unpaired electrons and non-free radical species (Gutteridge and Halliwell 2000). Oxygen free radicals include superoxide anion radicals ($O_2^{\cdot-}$), singlet oxygen ($^1O_2$) and hydroxyl radicals ($\cdot OH$). In biological systems, hydrogen peroxide (H_2O_2) represents a major non-free radical oxidant. $O_2^{\cdot-}$, $\cdot OH$ and H_2O_2 can be generated during the reduction of oxygen to water (see Figure 1-3).

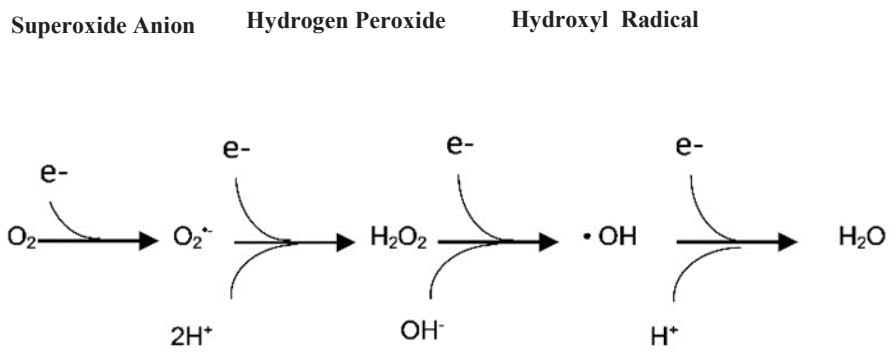

Figure 1-3: The generation of reactive oxygen species via the reduction of molecular oxygen. Adapted from Bartz and Piantadosi 2010.

There are various endogenous sources of ROS. Important sources of endogenous ROS are the mitochondria, where superoxide is generated during mitochondrial respiration, and activated macrophages and neutrophils at sites of inflammation (Gutteridge and Halliwell 2000). Exogenous sources of ROS include ultraviolet radiation, cigarette smoking and various types of particles including crystalline silica dust, asbestos fibres and ambient particulate matter (Fubini and Hubbard 2003; Knaapen et al. 2004; Liu et al. 2013; Shi et al. 2003). Particles may cause ROS in cells in two principal ways. They can generate oxidants due to their reactive physicochemical properties, or as a result of their interaction in cells with endogenous ROS sources (Knaapen et al. 2004). The formation of ROS by particles can be measured using various methods. Among these, electron spin resonance spectroscopy (ESR) is one of the techniques that can be used to measure ROS from particles. It can be used to determine ROS that are directly generated from the particles in the absence of cells, but also the levels of ROS that are present in particle-exposed cells (Shi et al. 2003; Singh et al. 2007). In this case, the radicals that are generated are being detected using spin trapping agents such as DMPO. As such, the measurement from ESR can be used to evaluate and rank the oxidative potential of ambient particulate matter or engineered NPs (Liu et al. 2013; Shi et al. 2003).

1.5.2 Nanoparticles and the oxidative stress paradigm

ROS can cause oxidative damage to cellular macromolecules including proteins, lipid, carbohydrates, membranes and nucleic acids. To protect the cells from these harmful effects, they can be scavenged by the cellular antioxidant defence system. This system includes enzymatic antioxidants which are the superoxide dismutases, catalase and the peroxidases and a variety of non-enzymatic oxidants which include glutathione, the vitamins C and E and uric acid (Rahman et al. 2006). Oxidative stress is defined as the condition where the production of ROS exceeds the capacity of the cellular antioxidant defence (Limon-Pacheco and Gonsebatt 2009; Rahman et al. 2006). This can be due to an excess of ROS generation as well as due to the depletion of antioxidants in the cells that results in an insufficient level of antioxidant protection, or a combination of both. This is schematically shown in Figure 1-4.

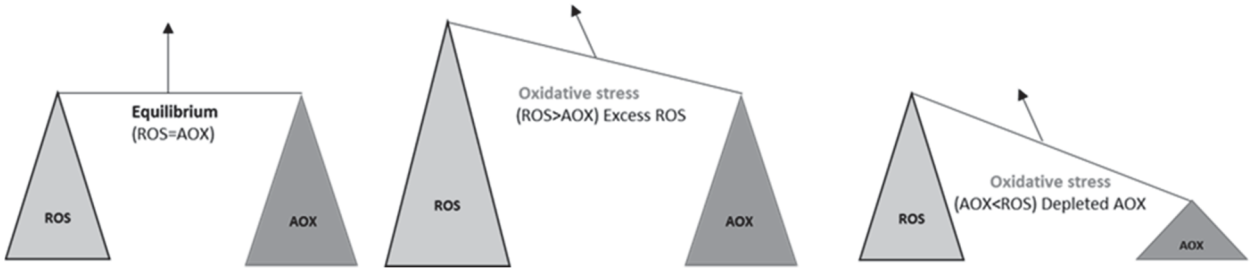

Figure 1-4: Oxidative stress as a result of the imbalance between the levels of reactive oxygen species (ROS) and antioxidants (AOX). Oxidative stress occurs when the cells are subjected to excess levels of ROS or as a result of the depletion of antioxidant defences. Adapted from Scandalios 2002.

Oxidative stress is considered as a key mechanism in the toxic effect of particles in general (Donaldson et al. 2003; Knaapen et al. 2004) as well as specific for NPs (Unfried et al. 2007; Xia et al. 2006). A hierarchical model of oxidative stress has been proposed by Andre Nel and colleagues as a mechanism to explain for the effect of NPs on the level of the cell. This is shown in Figure 1-5. The concept of this model is that NPs at low levels of oxidative stress (Tier 1) activate a protective response in the cell. This occurs via the activation of the nuclear factor erythroid 2-related factor 2 (Nrf2) pathway, which results in the increased expression of antioxidant defence enzymes. Moderate levels of the oxidative stress (Tier 2) activate the transcription factors Nuclear factor κB (NF-κB) and activator protein 1 (AP-1) leading to the increased expression of inflammatory genes. High levels of oxidative stress triggered by NPs cause cell death resulting from severe oxidative damages and induction of apoptosis. This oxidative stress paradigm has been proposed to explain for the mechanisms whereby NPs cause various effects in cells. In addition, the measurement of specific markers at the different tiers may also be used to rank and group NPs into safe (non-hazardous) and harmful (hazardous) NPs. This latter aspect has for instance been the focus of research in the European nanosafety project “NanOxiMet” (http://www.nanoximet.eu/).

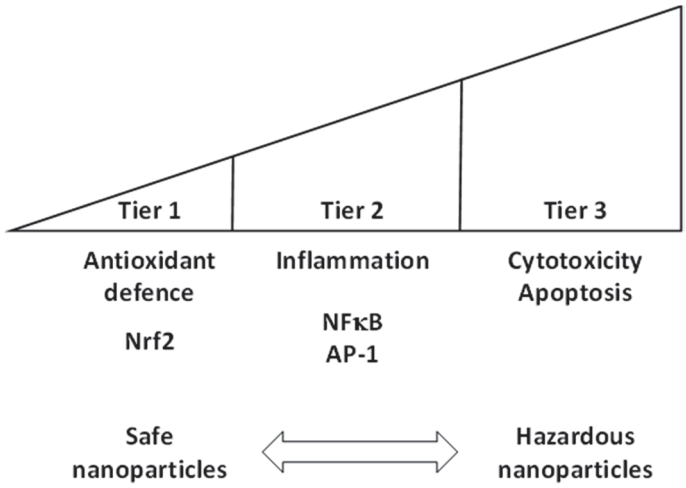

Figure 1-5: Hierarchical model of oxidative stress for engineered nanoparticles. Effects occurring at the different Tiers for nanoparticles may be used to determine whether they are safe or potentially harmful. Adapted from Nel et al. 2006 and the NanOxiMet project website (http://www.nanoximet.eu/).

1.6 The Nrf-2-Keap1 pathway

The transcription factor Nrf2 plays a central role in the protection of cells from oxidative stress induced damages and cell death (Figure 1-6). Upon its activation Nrf2 binds to the antioxidant response elements (AREs) in the promoters of genes of antioxidant and detoxification genes (Itoh et al. 1997). In unstressed conditions, Nrf2 is bound to Kelch-like ECH-association protein 1 (binding with Keap 1). Oxidative stress results in the dissociation of Nrf2 from the Nrf2-Keap1 complex and its translocation into the nucleus were it binds to the ARE (Jaiswal 2004). (See Figure 1-6). As such, the Nrf2-Keap1 activates the transcription of various antioxidant and detoxification genes including γ-glutamylcysteine synthetase (γ-GCS), glutathione S-transferase (GST), heme oxygenase-1, superoxide dismutase (SOD) and NAD(P)H:quinone oxidoreductase1 (NQO1) (Ma 2010).

The relevance of the Nrf2 pathway in particle induced lung disease is indicated from studies with diesel engine exhaust particles. In Nrf2 deficient mice, the diesel engine exhaust particles caused increased pulmonary toxicity (Aoki et al. 2001). Similar effects were seen with cigarette smoke in the lungs of Nrf2 deficient mice (Iizuka et al. 2005; Rangasamy et al. 2004). Nrf2 knockdown cells show a significant increase of intracellular ROS levels and also higher levels of DNA damage compared to wildtype cells following exposure to nickel (Kim and Seo 2012). In humans, Nrf2 incompetence has been associated with the development of chronic obstructive pulmonary disease (COPD) in relation to cigarette (Masuko et al. 2011). Interestingly, an

increasing number of studies also show that the Nrf2 signalling pathway also plays a role in the adaptation responses (Higgins and Hayes 2011; Lawal and Ellis 2011; Rigalli et al. 2016; Woods et al. 2009; Zhang et al. 2017). However, other studies also suggest the upregulation of Nrf2 is related to cancer development and contributes to chemo-and radiotherapy resistance (Cho and Kleeberger 2015; Ganan-Gomez et al. 2013; Taguchi et al. 2011).

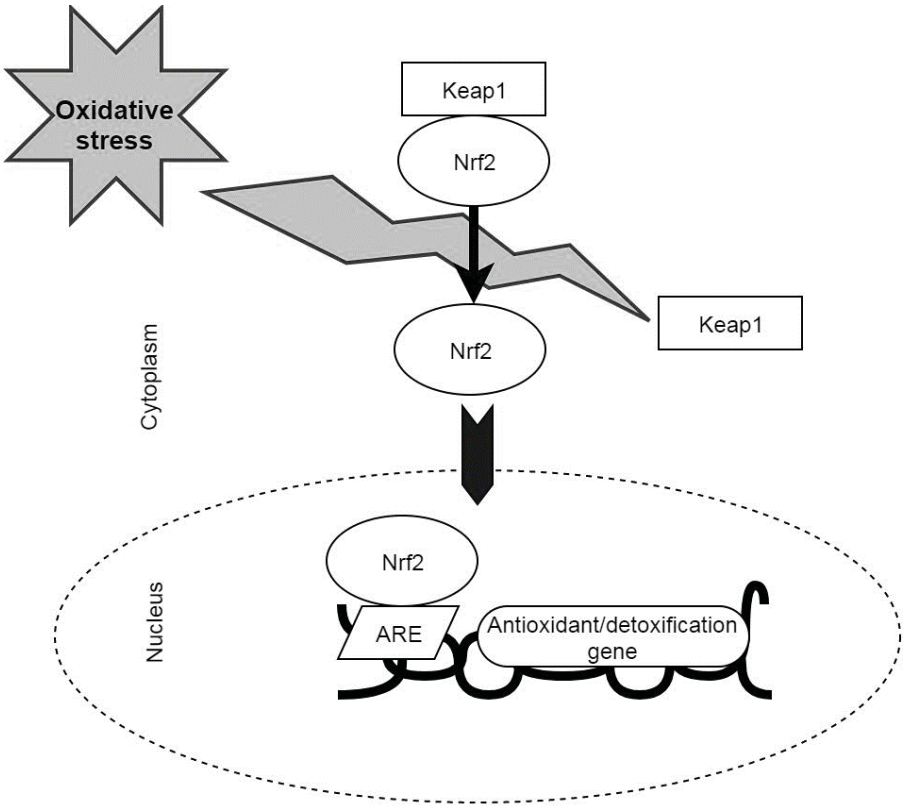

Figure 1-6: The Nrf2-Keap1 pathway. Adapted from Kim and Vaziri 2010.

1.7 Glutathione

The glutathione (GSH) antioxidant system has a key function in maintaining intracellular redox homeostasis. It is one of the major protective defence mechanisms against oxidative stress. GSH is a tripeptide composed of the amino acids glutamic acid, cysteine, and glycine. It is synthesised in subsequent steps via the enzyme γ-glutamylcysteine synthetase (γ-GCS) also known as glutamate cysteine ligase (GCL) and the enzyme glutathione synthetase (see Figure 1-7). In the detoxification of peroxides for example H_2O_2 and lipid peroxides it is oxidised to GSSG with the enzyme glutathione peroxidase (GPx). The GSSG is subsequently reduced by the glutathione reductase (GR) enzyme, which requires the hexose monophosphate from

NADPH (Figure 1- 7). In normal conditions the GR enzyme maintains the GSH/GSSH ration above 90%. Excessive ROS formation and other intracellular stress results in the depletion of GSH and causes as shift in the GSH/GSSH ratio.

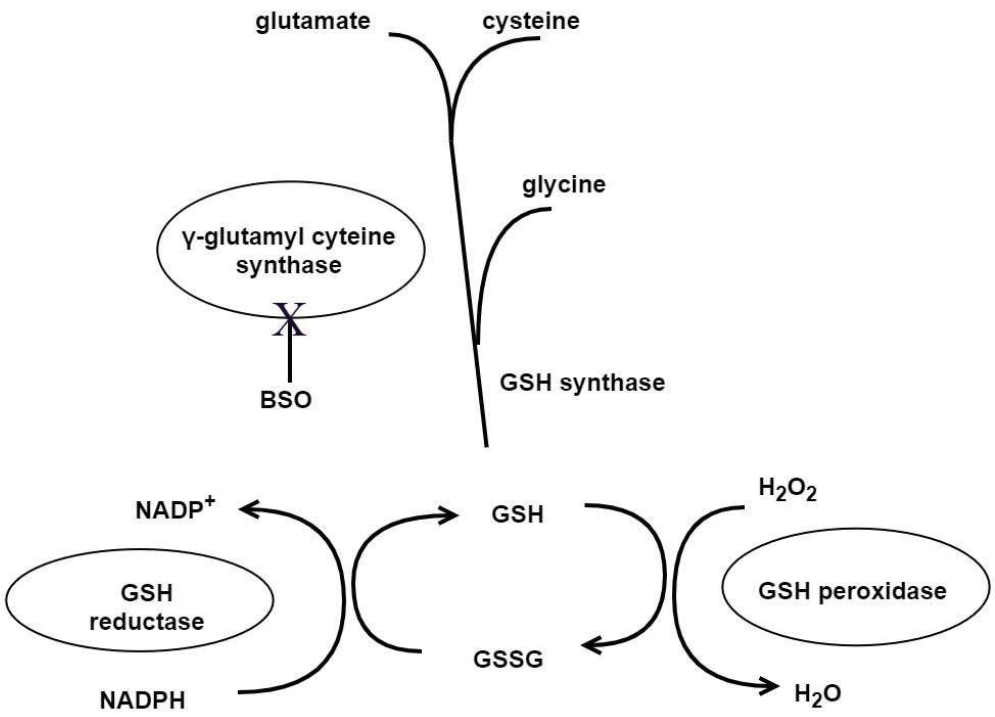

Figure 1-7: The Glutathione redox cycle. Adapted from Kurz et al. 2004.

The lung is one of the organs that is particularly rich in the antioxidant GSH, due the high concentrations of oxygen (Cook et al. 1991). The level of GSH in the epithelial lining fluid of the lung is approximately 100 times higher than in plasma (Cross et al. 1994). Changes in the intracellular GSH levels and in the GSH/GSSH ratio are used as markers of oxidative stress (Dubey et al. 2015; Pastore et al. 2001). The rate-limiting enzyme in the de novo synthesis of GSH is the γ-GCS enzyme. It is one of the enzymes that is regulated by the Nrf2 transcription factor. The importance of the intracellular GSH status in toxicity protection can be evaluated with the use of L-buthionine-SR-sulfoximine (BSO). This is a specific inhibitor of γ-GCS and can cause the depletion of GSH in cells (Figure 1-7).

1.8 DNA damage and cell death by nanoparticles

When damage occurs to the genomic DNA, it can lead to mutations and genetic disorders including cancer. DNA damage can be the result of endogenous stress, for example during

replication, and exogenous stress, for example exposures to ultraviolet radiation, chemicals or reactive oxygen species (Tuteja et al. 2001). Evaluation of genotoxicity is an important aspect in the toxicity testing of particles including engineered NPs (Donaldson et al. 2010; Schins and Hei 2006). DNA damage caused by NPs can results in mutations which may ultimately lead to cancer (see Figure 1-8). DNA repair and induction of apoptosis represent the two principal pathways that prevent mutagenesis. Importantly, NPs may also cause major cytotoxic effects in the absence of DNA damage induction. In this case, DNA damage may be detected in genotoxicity assays as a secondary outcome to the impaired cellular homeostasis caused by the primary cytotoxic effect. To avoid such false positive results, genotoxicity testing should always be analysed in relation to concurrent cytotoxicity.

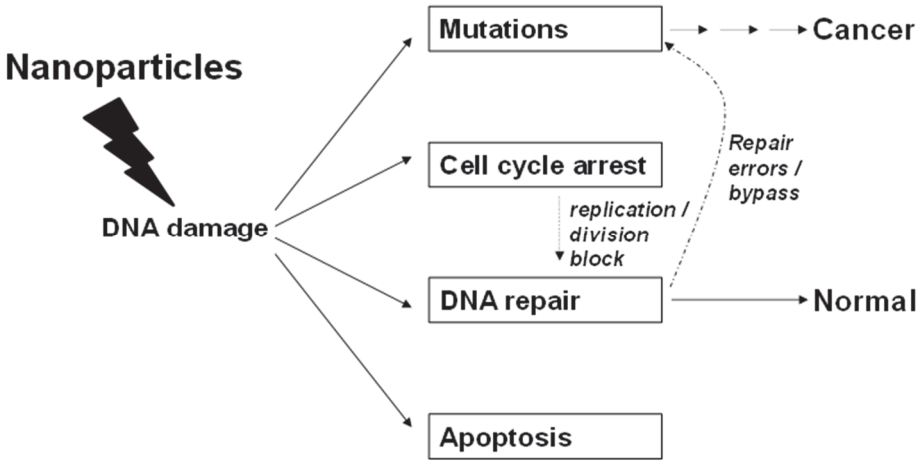

Figure 1-8: Possible cellular responses and consequences of DNA damage by nanoparticles. DNA damage caused by particles can lead to mutations if DNA repair processes are inadequate or bypassed. If DNA repair is successful mutagenesis is prevented. Major DNA damage in cells can also activate signal pathways resulting in apoptotic cell death and thus protect against fixation of mutations. Adapted from Schins and Hei 2006.

The generation of ROS and induction of oxidative stress are considered as major mechanisms of toxicity of NPs (see also 1.5.2). Thus, these properties can also play a role in the induction of oxidative DNA damage. ROS can cause DNA damage by oxidation, nitration, methylation, depurination and deamination processes (Knaapen et al. 2004; Knaapen et al. 2006). One of the most common DNA lesions induced by ROS is the oxidative DNA adduct 8-hydroxydeoxyguanosine (8-OHdG). This lesion was first reported by Kasai and Nishimura (Kasai and Nishimura 1984). Nowadays, 8-OHdG is widely used as a biomarker for oxidative

DNA damage and the risk of carcinogenesis (Valavanidis et al. 2009). Oxidative DNA damage is repaired in cells by base excision repair (BER). The base excision repair pathway is initiated by a series of lesion-specific DNA glycosylases. The specific lesion as 8-OHdG is recognised by the 8-oxoguanine glycosylase (Ogg1), which catalyse the hydrolysis of the N-glycosyl bonds and remove the damaged base. This process leads to the production of an apurinic/apyrimidinic (AP) site. The removal of AP sites is initiated by the enzyme AP endonuclease 1 (APE/Ref-1), which lead to the incision or nicks of the single nucleotide gap that have the 5' terminal deoxyribose-phosphate residue. The DNA-polymerase enzyme creates the complementary nucleotide and the gap is finally sealed by the DNA ligase III and XRCC1 enzymes (Friedberg et al. 2006).

DNA damage can be measured by various assays. The comet assay (single cell gel electrophoresis) is one of the most sensitive methods to detect the DNA damage and has been widely applied in genotoxicity testing of NPs (Donaldson et al. 2010). The alkaline variant of the comet assay is used to measure DNA single strand brakes (SSB), but also detects alkali-labile sites (Collins 2004; Liao et al. 2009). The pH-neutral version of the comet assay can be used to analyse double strand breaks (DSB) and oxidative DNA damage can detected in a specific manner using the Formamidopyrimidine glycosylase (FPG) modified comet assay. FPG is the enzyme from bacteria that recognises 8-OHdG lesions (Collins 2004).

Cell death assessment represents another important parameter in toxicology (Orrenius et al. 2011), whereby two major types can be distinguished, i.e. necrosis and apoptosis (McConkey 1998). As already mentioned, genotoxicity should always assessed together with cytotoxicity. Healthy and death cell can be observed in term of cell viability. There are more than ten types of methods to investigate cellular viability (Stoddart 2011). Among the assays the water-soluble tetrazolium salt (WST-1) assay is a widely used, sensitive method to screen for cell viability. It is a colorimetric method that determines the respiration and metabolic activity of cells based on the principle that water soluble tetrazolium salts are cleaved into a formazan within the mitochondria of metabolically active cells (Francoeur and Assalian 1996). Many studies have used the WST-1 assay to investigate the cytotoxicity of various engineered NPs including nanosilver (Haase et al. 2012), gold NPs, carbon nanotubes (Kandasamy et al. 2010), Fullerene-C60, titanium dioxide (Kato et al. 2014), SiO_2 and ZnO (Gerloff et al. 2013).

1.9 Hormesis and adaptation

Hormesis is referred to as the process were: “exposure to a low dose of a chemical that is damaging at higher doses, induces an adaptive upgrade of cellular protection” (Calabrese and Baldwin 2002). In toxicology, from the risk assessment point of view, hormesis describes a biphasic dose-response association, where a low dose activates or stimulates a beneficial effect and at a high dose an inhibitory or toxic effects (Calabrese 2008; Mattson 2008). The dose response presents graphically by a U-shape or J-shaped dose response or inverted forms of such curves (Calabrese 2013). The term hormesis has been introduced by Southem and Ehrlich as an adaptive response inducing either repair mechanisms or enhanced protection after exposure to low doses of a compound (Southem and Ehrlich 1943). The hormesis concept was already introduced as early as in 1888 by Hugo Schulz (Calabrese 2004), who reported the experiment of the biphasic dose responses in yeast exposure to the disinfectant agents. In the field of medicine hormesis has been defined as an adaptive response of cells to a moderate stress (Mattson 2008).

It has been suggested that the phenomenon of hormesis also may occur with various types of NPs (Iavicoli et al. 2014; Jiao et al. 2014; Neibert and Maysinger 2012; Theophel et al. 2014). The possible induction of adaptive responses has been mainly discussed for silver NPs (Aude-Garcia et al. 2015; Brzoska et al. 2015; Jiao et al. 2014). One of the molecular mechanisms whereby adaptation to the effects of ROS can occur is the activation of the Nrf2 pathway and subsequent induction of the GSH antioxidant defence system (Mattson 2008; Neibert and Maysinger 2012; Sthijns et al. 2016). The role of GSH in adaptation could be recently demonstrated in the human bronchial epithelial cell line BEAS-2B for acrolein (Sthijns et al. 2016). Activation of the Nrf2 pathway by silver NPs has been demonstrated in recent years by several investigators (Bohmert et al. 2015; Kang et al. 2012a; Kang et al. 2012b; Prasad et al. 2013; Sahu et al. 2015). Thus, the concept of hormesis could fit very well in the tiered oxidative stress model for NPs (see paragraph 1.5.2 and Figure 1-5). Verification of an adaptive hormetic response for specific NPs, such as nanosilver will have implications with regard to their use of medical applications as well as for risk assessment.

1.10 Aims of thesis

The production and use of nanomaterials has tremendously increased over the past decades. However, there is also an increasing concern about adverse health effects resulting from the exposure to engineered NPs. Toxicological research is needed to address the safety of these NPs. This is important for workers that may be exposed during the manufacturing of NPs, but also as for consumers that may be exposed to NPs and nanoparticle-containing products that are planned to be introduced on the market. The major goal of the toxicological investigations with NPs is the identification and possible classification of their hazard [H], i.e. the potential harm that they may cause. In combination with knowledge on actual levels of exposure [E], these data can then be used to estimate the actual adverse human health risks [R], which is a function of hazard and exposure [R = H x E] (Kuhlbusch et al. 2011; O'Shaughnessy 2013).

Beyond this, toxicological studies can also contribute to the identification of the underlying mechanisms of toxicity of NPs. The identification of such "mode of action (MoA)" for a specific nanomaterial is an important further aspect in nanotoxicology research. It can be used to compare effects of different types of NPs or effects of NPs with known toxic particles, like silica dust and asbestos. This research contributes to improved risk assessment and can also provide ideas for possible prevention or treatment of diseases. For both purposes of testing, it is crucial that the experimental models and assays are reliable and accurate. Several previous studies have demonstrated that toxicity studies with NPs can lead to artefacts because of the specific physical and chemical properties of these materials e.g. (Alkilany et al. 2016; Azhdarzadeh et al. 2015; Doak et al. 2009; Stone et al. 2009; Tournebize et al. 2013).

The ***aim of this thesis*** has been to ***investigate the cytotoxic and DNA damaging effects of various types of engineered nanoparticles in relation to their composition***. Formation of ROS and induction of oxidative stress has been proposed as a common mechanism to explain for the adverse effects of toxic NPs (Donaldson and Poland 2013), and therefore formed the central topic of investigation. The research focused on the role of the ***ROS generation*** by the NPs and the importance of ***glutathione***, as major antioxidant in the lung (Biswas and Rahman 2009). In addition, the effects were evaluated in relation to specific ***testing protocols***.

Three studies have been performed within the framework of this thesis:

In the first study (described in **Chapter II**) the toxicity of a panel of 10 engineered NPs was evaluated. The materials were two types of MWCNT, five types of TiO_2 NPs, two types of ZnO NPs, and one type of Ag NPs. The main goal of this study ***was to investigate and compare the DNA damaging properties*** of these materials ***in three different human epithelial cell lines in relation to the cytotoxicity*** of the materials. The three cell lines were derived from lung (A549), liver (HepG2) and kidney (HK-2) as representative target organs. The ROS generation of all NPs was analysed in order to determine their relationship with cytotoxicity and DNA damage. In specific subsets of experiments also the role of the dispersing surfactants was investigated.

In the second study (**Chapter III**), it was investigated ***whether exposure to low concentrations of Ag nanoparticles could induce an adaptive response***. The study was based on earlier observations made with the ubiquitous toxicant acrolein in lung epithelial cells and some suggestion in the literature that prolonged exposure can make cells more resistant to Ag NPs. The study was performed in A549 lung epithelial cells with two types of Ag NPs and focused on the involvement of the Nrf2 transcription pathway and associated induction of the GSH. A previous study with acrolein demonstrated that adaptation to this compound was related to induction of mRNA expression of the enzyme γ-GCS and increased GSH levels.

The third study (**Chapter IV)** was performed to ***further evaluate the role of GSH in the protection against DNA damage caused by TiO_2 nanoparticles***. This study was performed in the three different epithelial cell lines (A549, HepG2 an HK-2) to provide further mechanistic insight in the results obtained in the first study. Therefore, experiments were performed with the GSH synthesis inhibitor compound BSO. In addition, the uptake of the NPs into the three cell lines was evaluated by flow cytometry.

1.11 References

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Chapter II

Oxidant generation, DNA damage and cytotoxicity by a panel of engineered nanomaterials in three different human epithelial cell lines

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Mutagenesis 32 (2017): 105-115

Study 1: Oxidant generation, DNA damage and cytotoxicity by a panel of engineered nanomaterials in three different human epithelial cell lines

Declaration

The manuscript is published in Mutagenesis.

DNA damage analyses were performed by Waluree Thongkam

The impact on authoring this paper can be estimated in total with 40 %

2 Abstract

Due to the steeply increased use of nanomaterials for commercial and industrial applications, toxicological assessment of their potential harmful effects is urgently needed. In this study, we compared the DNA damaging properties and concurrent cytotoxicity of a panel of ten engineered nanomaterials (NMs) in three different cell lines in relation to their oxidant generating properties. The human epithelial cell lines A549, HepG2 and HK-2 were chosen to represent relevant target organs for NMs in the lung, liver and kidney. Cytotoxicity, evaluated by WST-1 assay in the treatment concentration range of 0.3 to 80 μg/cm^2, was shown for Ag and ZnO NM in all three cell lines. Cytotoxicity was absent for all other NMs, i.e., five types of TiO_2 and two types of multiwalled carbon nanotubes. DNA damage, evaluated by the alkaline comet assay, was observed with Ag and ZnO, albeit only at cytotoxic concentrations. DNA damage varied considerably with the cell line. The oxidant generating properties of the NMs, evaluated by electron spin resonance spectroscopy in cell free conditions, did not correlate with their cytotoxic or DNA damaging properties. DNA damage by the nanosilver could be partly attributed to its surfactant-containing dispersant. The coating of a TiO_2 sample with the commercial surfactant Curosurf augmented its DNA damaging properties in A549 cells, while surface modification with serum tended to reduce damage. Our findings indicate that measurement of the intrinsic oxidant generating capacity of NMs is a poor predictor of DNA damage and that the cytotoxic and DNA damaging properties of NMs can vary substantially with experimental conditions. Our study also underlines the critical importance of selecting appropriate cell systems and aligned testing protocols. Selection of a cell line on the mere basis of its origin may provide only poor insight on organ-specific hazards of NMs.

2.1 Introduction

Recent decades have witnessed tremendous increases in the production and use of nanomaterials, but also ongoing discussions about their potential adverse health risks to humans. Within the nanotoxicology field, an increasing body of research has devoted to the identification of potential genetic and carcinogenic risks of engineered nanomaterials (NMs). Through various *in vivo* and *in vitro* studies, genotoxicity has been identified for specific types of NMs and relevant underlying mechanisms have emerged (e.g. see reviews by Donaldson et al. 2010; Magdolenova et al. 2014; Singh et al. 2009)

Among a variety of appealing toxicological mechanisms, the formation of reactive oxygen species (ROS) and associated induction of oxidative stress in NM-exposed cells, has been nowadays implicated as a pivotal mode of action. ROS generation and oxidative stress induction has been shown to mediate pro-inflammatory and proliferative effects of specific NMs and also likely play a role in DNA damage induction and mutagenesis. This oxidative stress paradigm in nanotoxicology has emerged from research with well-known toxic particles of larger dimensions like crystalline silica, ambient particulate matter (PM) and, more recently, with engineered nanoparticles (Donaldson et al. 2003; Unfried et al. 2007; Xia et al. 2006). Thus, various methods to measure the formation of ROS by particles have been developed and proposed as a new metric in particles toxicology and as a grouping tool in nanosafety research (Borm et al. 2007; Xia et al. 2008). Previously, we could demonstrate that the oxidant generating capacities of crystalline silica and PM, measured by electron spin resonance (ESR) spectroscopy, is a good predictor of their DNA damaging properties in lung epithelial cells (Schins et al. 2002; Wessels et al. 2010). However, whether this method can also be applied to predict the genotoxic properties of NMs, has not yet been thoroughly investigated.

Current literature yields remarkable contrasting *in vitro* findings regarding the DNA damaging properties and effects of NMs, even within individual groups and types of materials. This variability has been contributed to critical-though sometimes subtle-differences in physicochemical properties of the particles, to heterogeneity in nanoparticle storage, handling and testing protocols, as well as to potentially confounding interactions with testing assay components (Doak et al. 2009; Stone et al. 2009). Contrasts in genotoxicity have for instance been contributed to the presence or absence of serum in the NMs dispersion protocols that are required for reproducible testing (Magdolenova et al. 2012). Moreover, in recent years, also the impact of surfactants on NM toxicity is gaining interest. On the one hand, chemicals that mimic key components of lung surfactant fluid have been introduced in testing protocols to better reflect the initial mode of interaction of inhaled particles towards lung epithelial cells. Pioneering research by Wallace and colleagues already revealed that the genotoxic effects of crystalline silica particles can be inhibited upon its pre-treatment with a simulated lung surfactant (Gao et al. 2000; Liu et al. 1996). On the other hand, surfactants are also often included in various NM preparations in order to improve their stability and dispersion status. Testing of the specific impact of such compounds within NMs dispersions alongside pristine nanomaterial should be a prerequisite, as this can improve our understanding on mechanisms of action as well as aid in the identification of potential assay artefacts. With such approach,

genotoxic properties of stabilizing agents of Ag NMs could be recently identified, for instance (Huk et al. 2015).

The aim of our study was to investigate the DNA damaging properties and concurrent cytotoxicity of a panel of nanomaterials in three different cell lines, in relation to their oxidant generating properties. The studies formed a component of the *in vitro* testing approach of the EU seventh framework project ENPRA. The overall aim of this project was to evaluate human health risks associated with exposure to engineered nanoparticles, focusing on the pulmonary, cardiovascular, hepatic, renal, and developmental system (Kermanizadeh et al. 2016). Accordingly, the consortium selected a variety of cell lines originating from these body compartments for *in vitro* investigations. In view of the relevance for carcinogenic risk assessment, genotoxicity studies within the project focused on human cell lines of epithelial origin and the lung, kidney and liver as relevant target organs. Accordingly, we evaluated DNA damage by the alkaline comet assay and cytotoxicity by WST-1 assay in the human A549, HK-2 and HepG2 epithelial cell lines. The ROS generating properties of the materials were investigated by ESR. We also evaluated the effects of serum, included in the standardised dispersion protocol as well as surfactants for specific NMs in order to address their possible impact on DNA damage.

2.2 Materials and Methods

Chemicals. Trypsin, Dulbecco's Modified Eagle Medium with glucose, RPMI-1640 medium, Penicillin/streptomycin, Dulbecco's Ca^{2+}/mg^{2+} -free phosphate buffered saline (PBS), agarose, low melting point (LMP) agarose, NaOH, Tris, NaOH, HCl, NaCl, Triton X-100, DMSO, ethidium bromide, ethylenediaminetetraacetic acid disodium salt dehydrate (EDTA), 5,5-Dimethyl-1-pyrroline N-oxide (DMPO), foetal bovine serum (FBS) and adult bovine serum (ABS) were obtained from Sigma (Germany). DMEM/F12H medium was obtained from Gibco Life Technologies (Germany), cell Proliferation Reagent WST-1 was obtained from Roche (Germany).

Nanomaterials. The ten NMs that were investigated in this study are listed in Table 2-1. The materials are based on TiO_2, ZnO, Ag and carbon (nanotubes) and represent the panel of NMs of the EC-FP7 funded project ENPRA. The main reasons to select these specific NMs for the project were their widespread commercial use and associated likelihood of exposure as well as

their relevance regarding the evaluation of the role of specific physicochemical properties on their toxicity. A detailed description of selection criteria for the NMs is provided elsewhere (Kermanizadeh et al. 2016). The ENPRA NM panel includes six materials that also form part of the European Commission Joint Research Centre (JRC) repository list and five different types of TiO_2 NMs procured by the Danish National Research Centre for the Working Environment (NRCWE, Copenhagen). The original sources of the materials are also specified in Table 2-1.

Table 2-1. Characteristics of the nanomaterials

Material basis	**Sample code**	**Physicochemical properties**	**Source / (Company) name**
TiO_2	NM-101	7 nm; Anatase	JRC repository / Hombikat UV100
	NRCWE 001	10 nm; Rutile	NanoAmor (Houston, USA)
	NRCWE 002	10 nm; Rutile, positively charged	
	NRCWE 003	10 nm; Rutile; negatively charged	
	NRCWE 004	94 nm; Rutile	NaBond (Hong Kong, China)
ZnO	NM 110	100 nm, uncoated	JRC repository / BASF Z-Cote
	NM 111	130 nm, coated with triethoxycaprylylsilane	
Carbon *(Multiwalled Nanotubes)*	NM 400	Diameter: 5-35, Length: 700-3000; entangled	JRC Repository / Nanocyl
	NM 402	Diameter: 6-20, Length: 700-4000; entangled	JRC repository / Arkema Graphistrength C100
Ag	NM 300	<20 nm	JRC repository / RAS GmbH
	NM 300 dis	Dispersant used for NM 300	

Characteristics were obtained within the framework of the ENPRA project as described in (Kermanizadeh et al. 2012; Kermanizadeh et al. 2016; Kermanizadeh et al. 2013).

Preparation of NMs. Suspensions of the NMs were prepared according to the protocol that was developed within the ENPRA project, and included a detailed characterization of the NMs in the generated dispersions (Kermanizadeh et al. 2016). The established protocol allowed for the testing for the entire panel of NMs with a single sonication-base dispersion method that results in their appropriate dispersion state and stability. Hydrodynamic diameters and zeta-potentials achieved in the NMs suspensions by the protocol have been published elsewhere (Kermanizadeh et al. 2013). All NMs except NM-300 are present as a dry powder. The Ag nanoparticles (NM-300) reside in a suspension of de-ionised water with stabilizing agents (7% ammonium nitrate, 4% polyoxyethylene glycerol trioleate and 4% Tween 20). The dispersant of this sample (NM-300dis) was purchased together with the NM-300 for inclusion as appropriate control in this study (see Table 2-1). The powder type NMs were dispersed in Milli-Q de-ionised water with 2% adult bovine serum added, with the exception of the ZnO samples NM-110 and NM-111. These samples were first pre-wetted with 0.5% ethanol prior to addition of the dispersant. Stock suspensions of 2.56 mg particles per ml dispersion media were sonicated for 16 min without stop, on ice, using a Branson 450 Sonifier equipped with a 13 mm disruptor horn. After sonication, all NMs were kept on ice until dilution in complete medium. Suspensions were applied to the tests within 1 h after sonication.

Oxidant generation by NMs. The oxidant generating properties of the nanoparticles were evaluated by ESR, using the spin trapping agent DMPO, according to a protocol that was previously applied for crystalline silica particles (Schins et al. 2002), with minor modifications. Briefly, 100 µl of freshly prepared suspensions of the NMs were mixed with 200 µl DMPO (0.05 M in PBS) and 100 µl of 0.5 M H_2O_2 (in PBS). The suspensions were incubated for 15 min at 37 °C and subsequently analysed using a MiniScope MS200 Spectrometer (Magnettech, Berlin, Germany) at room temperature and quantified as described previously (9). Quantification was carried out on first derivation of ESR signal of the characteristic DMPO–OH quartet, as the mean of amplitudes, and outcomes are expressed in arbitrary units (a.u.). As positive control a coal fly ash (EVA91) was used as characterized previously (Kermanizadeh et al. 2013). As a negative control, a mixture of distilled water containing 2% serum, H_2O_2, and DMPO was used, except for the ZnO and AgNP samples. For the ZnO samples, the negative control also included the 0.5% v/v. As the negative control for the AgNP (NM 300), the mixture of the dispersant control (NM 300dis, see Table 2-1), H_2O_2, and DMPO was used. Because of the potential effects of the added serum (2%) in the ENPRA protocol suspension preparations, all analysis were also performed in the absence of serum, with inclusion of the appropriate (non-

serum containing) negative and positive controls. ESR measurements of all NMs were also performed without addition of hydrogen peroxide in the reaction mixture with DMPO, both in the presence and absence of serum.

Cell culture. Three human lung epithelial cell lines were selected for the study, to represent the three target systems under investigation within the ENPRA project regarding the potential genotoxic effects of the NMs, i.e. the pulmonary, hepatic and renal system. The human alveolar adenocarcinoma cell line A549 (Kermanizadeh et al. 2012) was cultured in DMEM supplemented with 4.5 g/L glucose and 10 % FBS. The human hepatocellular carcinoma cell line HepG2 (Shi et al. 2003) was maintained in RPMI-1640 supplemented with 10 % FBS, and the human proximal tubule epithelial cell line HK-2 (Giard et al. 1973) was grown in DMEM/F12H supplemented with 10% FBS. 100 U/ml penicillin-streptomycin was added to all media. Cells were cultured at 37 °C and 5% CO_2. During experimentation, the aforementioned culture media were replaced with phenol red free media.

C**ytotoxicity.** Cytotoxicity was determined using the water-soluble tetrazolium salt (WST-1) assay which measures metabolic activity of cells. Cell lines were seeded in 96 well plates 10^4 cells/well in 100 µl of the cell culture medium and incubated for 24 h in complete medium at 37 °C and 5% CO_2. The cells were exposed to the NMs or controls for 24 h at 37 °C and 5% CO_2. Cytotoxicity was evaluated in all three cell lines at 9 different treatment concentrations using two-fold serial dilutions of the materials, with the highest dose of 80 µg/cm^2 (equivalent to 256 µg/ml). After the 24 h incubation, the plates were washed twice with PBS, followed by the addition of 10 µl of the WST-1 cell proliferation reagent and 90 µl of fresh medium. Plates were incubated for 1 h at 37 °C and 5% CO_2. The absorbance of the supernatants was measured by dual wavelength spectrophotometry at 450 and 630 nm using a micro-plated reader (Labsystems Multiskan Ascent).

DNA damage analysis by alkaline comet assay. The cells were seeded in 6-well plates with complete culture media (3.8 x 10^5 cells per well, equivalent to 40,000 cells per cm^2) and incubated at 37 °C, 5% CO_2 for 24 h. The cells were then exposed for 4 h to the NMs or controls. DNA damage was evaluated in all three cell lines at four different treatment concentrations in four-fold serial dilution starting at the dose of 40 µg/cm^2 (i.e. equivalent to 128 µg/ml). Two independent experiments were performed for each cell line. Following treatment, cells were rinsed twice with PBS, detached by using trypsin and immediately resuspended in normal culture medium at 1x10^6 cells/ml. An aliquot of 10 µl cell suspension was mixed with 100 µl

low melting point agarose at 37 °C, and 100 µl of this mixture was immediately applied to a glass slide. Then the slides were held horizontal for 5-10 min on an ice-cold metal plate to allow for solidification. The slides were lysed overnight at 4 °C in lysis buffer (2.5 M NaCl, 100 mM EDTA, 10 mM Tris-base, pH 10, containing 10 % DMSO and 1 % Triton X-100). The slides were washed three times for 5 min in water and then transferred into a pre-cooled electrophoresis tank and subjected to DNA denaturation for 20 min using alkaline (pH 13) denaturation buffer, followed by 10 min electrophoresis at 1.2 V/cm (270 mA, 26 V). Slides were neutralized for 3 x 5 min using neutralisation buffer (0.4 M Tris, pH 7.5), immersed in 90 % ethanol for 5 min and then air dried. Prior to analysis the slides were stained with ethidium bromide (10 µg/ml, 40 µl per slide). All washing and incubation steps were performed in the dark or red light, to avoid artificial DNA damage induction as previously could be identified e.g. for TiO_2 nanoparticles (Gerloff 2009; Karlsson et al. 2015). Comet appearances were analysed using an Olympus Bx60 fluorescence microscope at 400 x magnification. A comet image analysis software program (Comet Assay II, Perceptive Instruments, UK) was used for quantification of DNA damage by analysis of % DNA in tail (tail intensity). A total of 50 cells were randomly selected and analysed per slide per experiment.

Effect of serum and surfactant on DNA damage induction. In the framework of ENPRA project effects of serum coating of NMs on cytotoxicity and inflammatory responses could be demonstrated (Gerloff 2009). Other studies also investigated effects of the lung surfactant Curosurf on nanoparticle uptake and toxicity (Karlsson et al. 2015). Therefore, in the present study we analysed the effects of serum and Curosurf on DNA damage induction in the A549 cells. Two materials were therefore selected, i.e. the positively charged rutile sample (NRCWE002) and the nanotubes sample NM402. The experiments were performed as described above, with the sole exception that additional dispersion were prepared in the absence of 2%, or alternatively, upon addition of Curosurf.

Statistical analyses. Treatment concentrations causing 20% and 50% loss in cell viability (TC_{20} and TC_{50} values) were calculated from fitted dose-response curves obtained from the WST-1 experiments. Dose-dependency of DNA damage was evaluated by ANOVA with Dunnett post-hoc comparison versus untreated controls. Correlations between ESR measurement results, DNA damage and cytotoxicity (i.e. TC_{20},) were analysed by Spearman regression analyses. Effects of serum and Curosurf, versus the respective effects of the pristine NMs were evaluated by ANOVA with Tukey post-hoc comparison. Value of $p<0.05$ were considered as significant.

2.3 Results

Oxidant generation by NMs in cell free conditions. The oxidant generating properties of all NMs were evaluated in acellular conditions by ESR using DMPO as spin trapping agent, both in the presence and absence of serum. As can be seen in Figure 2-1, the different materials showed rather contrasting oxidant generating properties. The strongest effect per unit mass was found for the TiO_2 sample RWCE002. Oxidant generation was also observed with all other TiO_2 NMs except RCWE002, with both ZnO samples (NM110, NM111), and with one of the MWCNT samples (NM400). The presence of the 2% serum was found to reduce the oxidant generation of the samples, suggesting that the biological reactivity of these particles may be lowered due the interaction of specific serum constituents with the nanomaterial surfaces. The ESR-based oxidant generating measurements were also performed without addition of H_2O_2. However, with this approach none of the NMs showed increased oxidant generation (data not shown).

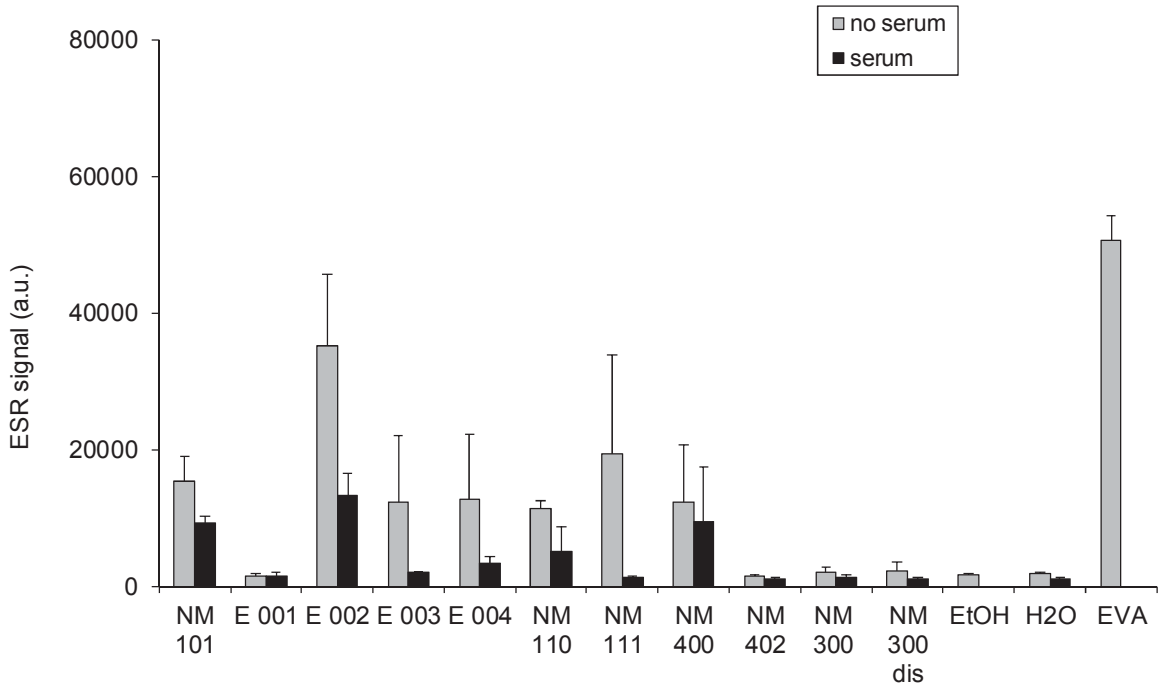

Figure 2-1: Reactive oxygen generating of the nanomaterials. ROS generating was determined by ESR spectroscopy using DMPO in the presence of H2O2. Samples were measured in NM suspensions containing 2% serum (grey bars), or without serum (black bars). EtOH: Respective control suspensions for NM110 an NM110, each containing 0.5% ethanol. H2O: Respective control suspensions for all other NMs, except for NM300, for which NM300dis serves as control. Pos ctrl: Suspensions of coal fly ash EVA91.

Cytotoxicity effect of NMs on three different cell lines. The WST-1 assay was used to determine the cytotoxic effects of the panel of NMs in the three different human epithelial cell lines. Results of these investigations revealed that most of the NMs did not show considerable toxicity, with the exception of the Ag nanoparticles and both ZnO samples. Results for the NMs are shown in Figure 2-2. From the dose-response curves TC_{50} values were calculated, representing 50 % loss of viability in the WST-1 assay. The Ag sample (NM300) was found to be the most toxic material for all three cell lines investigated. The toxicity of the NM300 was rather similar for all three cell lines, with calculated TC_{50} values of 23 $\mu g/cm^2$ (A549 cells), 29 $\mu g/cm^2$ (HepG2 cells), and 23 $\mu g/cm^2$ (HK-2 cells). Interestingly, the effects of the nanosilver (NM300) were observed in absence of marked toxicity for its corresponding dispersant control (i.e. NM300dis), except for the HepG2 cells. For this cell line, the dispersant showed some moderate effects at the highest NM300-equivalent treatment concentrations, with a calculated TC_{20} value (i.e. 20% viability loss at 61 $\mu g/cm^2$). For the ZnO based samples notable differences in effects could be observed between the different cell lines (See Figure 2-2). The HK-2 cells appeared to be the most sensitive to the uncoated ZnO (i.e. NM110) while the HepG2 cells were the most resistant. The respective TC_{50} values calculated for this nanomaterial were 44 $\mu g/cm^2$ (A549), 75 $\mu g/cm^2$ (HepG2) and 19 $\mu g/cm^2$ (HK-2). Moreover, while the uncoated ZnO (NM110) was toxic towards all three cell lines, for the coated ZnO sample (i.e. NM111) toxicity was only noted for the HK-2 cells with a TC_{50} of 34 $\mu g/cm^2$ (see Figure 2-2).

The results of the other investigated NMs are shown in Figure 2-3. As can be seen in the figure, none of the TiO_2 based NMs (NM 101, NRCWE 001, 002, 003 and 004) were toxic in the three cell lines, up to the highest treatment concentration that was applied (80 $\mu g/cm^2$). Also the nanotubes (NM400, NM402) showed no marked toxicity. Some moderate toxic effect could be observed for the NM400 sample in the HepG2 and HK-2 cells at high dose treatment, as also reflected by their respectively calculated TC_{20} values of 47 $\mu g/cm^2$ (HepG2 cells) and 46 $\mu g/cm^2$ (HK-2 cells).

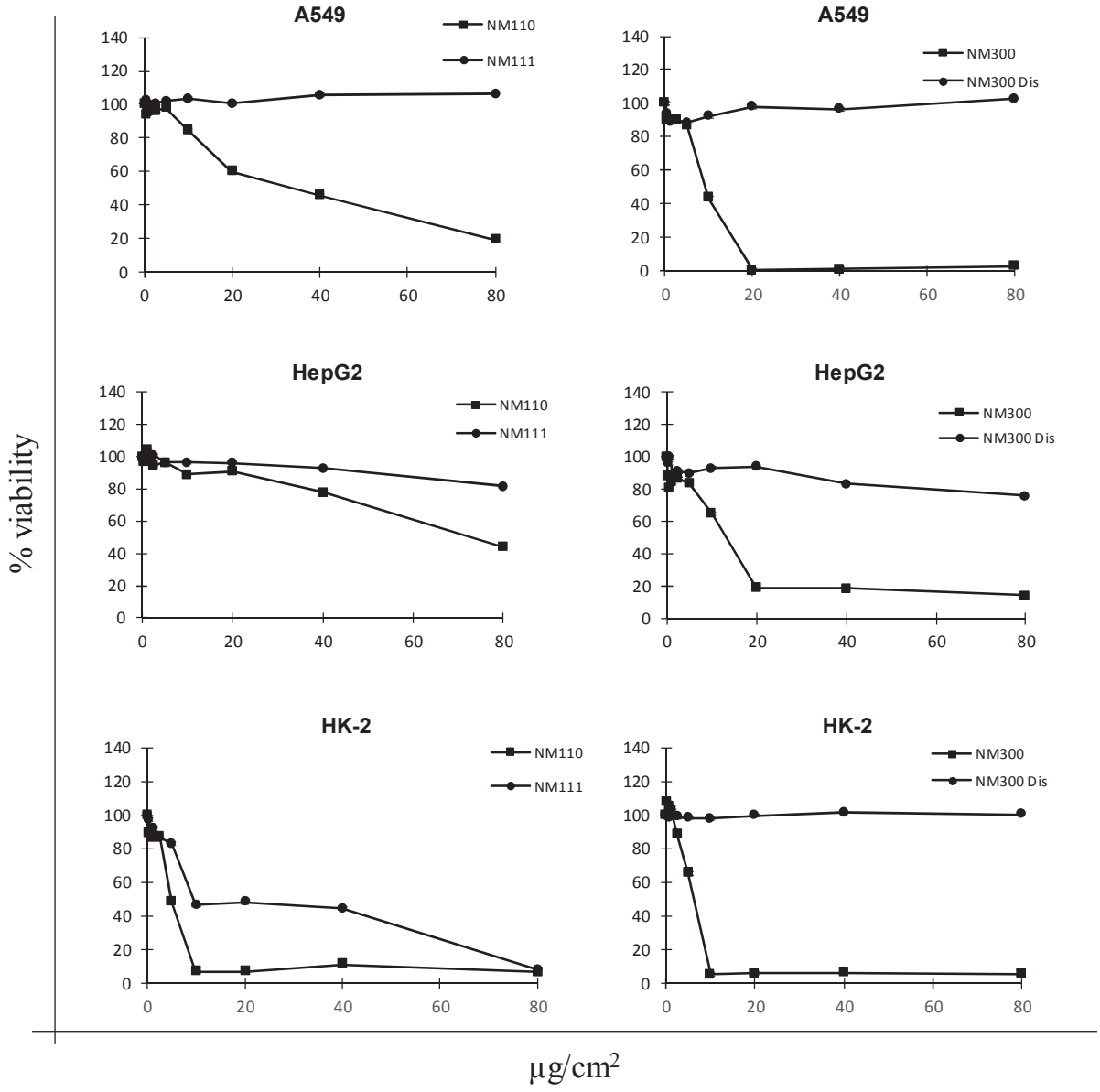

Figure 2-2: Cytotoxic effects of uncoated ZnO (NM110), coated ZnO (NM111), Ag (NM300) and the Ag NM dispersant (NM300dis) in A549, HepG2 and HK-2 cells. Cytotoxicity was evaluated by WST-1 assay following 24 h treatment at the indicated concentrations.

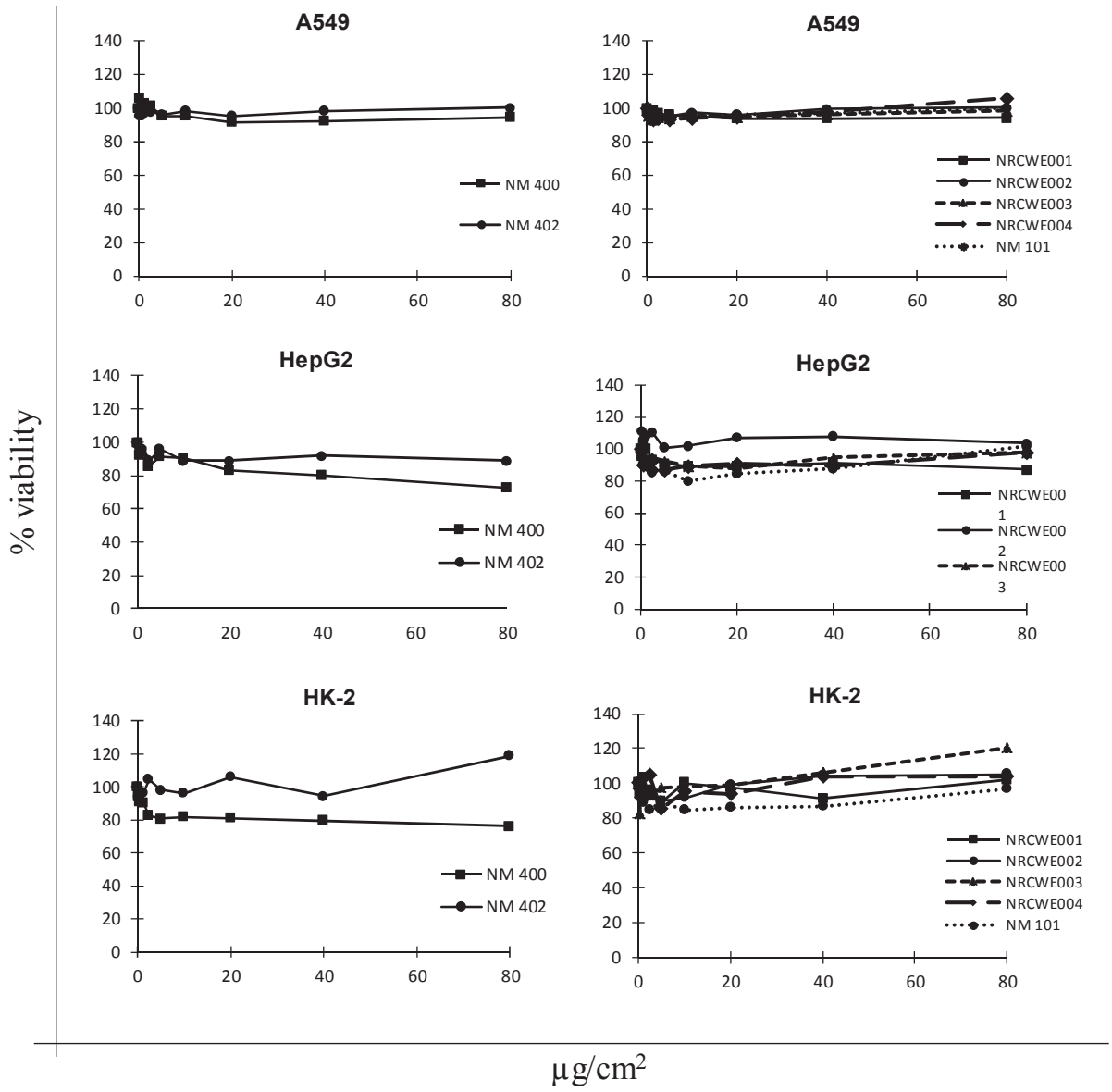

Figure 2-3: Cytotoxicity data for multiwalled carbon nanotubes (NM400, NM402) and for TiO_2 NMs (NM101, NRCWE001, NRCWE002, NRCWE003, NRCWE004) towards A549, HepG2 and HK-2 cells. Cytotoxicity was evaluated by WST-1 assay following 24 h treatment at the indicated concentrations.

DNA damage by NMs in A549, HepG2 and HK-2 cells. The DNA damaging potential of the ENPRA set of engineered nanoparticles was evaluated by the comet assay in the three cell lines following 4 h treatment at 0.625, 2.5, 10 and 40 µg/cm^2, with the exception of NM300. For this nanomaterial, in concordance with the steep cytotoxicity curves that were obtained (see Figure 2-2), 10 µg/cm^2 was chosen at highest relevant dose for DNA damage evaluation. The DNA damage observed in the untreated cells for these experiments were 2.37± 1.78, 6.8 ± 2.19 and 4.97 ± 0.98 for A549, HepG2 and HK-2 cells, respectively. This underlines the robustness of

the A549 cells, compare to both other cell lines. Various remarkable differences in effects could be observed among the different NMs was well as the investigated cell lines. Figure 2-4 shows the DNA damaging effects for the nanosilver sample, its dispersant control, the two ZnO samples and the two multi-walled carbon nanotubes. DNA damaging effects tended to be most pronounced with the nanosilver (NM300) and the uncoated ZnO (NM110) in the three cell lines. Importantly, however, these effects were observed at concentrations that were also associated with cytotoxicity. Moreover, a considerable variation was observed among experimental repeats, in particular at the highest concentration tested (e.g. for the NM300, in the HK-2 cells and for NM100 in the HepF2 cells). For the NM 300, curve fitting analysis from WST-1 data revealed TC_{20} values that were lower than the tested concentration of 2.5 $\mu g/cm^2$ for all three cell lines. In addition, notable DNA damage was also observed with the NM300 dispersant in the absence of cytotoxicity. The uncoated ZnO (NM110) also caused DNA damage in all three cell lines, but this effect was fare more pronounced in the HK-2 cells. However, the TC_{20} value in the HK-2 cells for NM110 was less than 1 $\mu g/cm^2$. For A549 and HepG2 cells, values of 15 $\mu g/cm^2$ and 30 $\mu g/cm^2$ were obtained, respectively. In contrast to the uncoated ZnO, the coated sample (NM111) did not reveal any notable DNA damage induction among the three cell lines.

The DNA damaging properties of the five, non-cytotoxic, TiO_2 nanomaterials are shown in Figure 2-5. Interestingly, the levels of DNA damage induction were found to vary with the material as well as with the cell line. The most pronounced effects were observed with the NM101 sample. For the TiO_2 nanoparticles, in contrast with the ZnO samples, the strongest effects in the comet assay were not always observed in the HK-2 cells. Notably, the various DNA damaging effects as observed among the TiO_2 samples were not associated with cytotoxicity; TC_{20} values could not be established for any of the TiO_2 samples (see also Figure 2-2). To evaluate whether the oxidant generating properties of the NMs measured in cell free conditions by ESR predicted toxicity in the three epithelia cell lines we performed Spearman's ρ rank correlation analyses (see Table 2-2). As show in the table, neither DNA damage, nor cytotoxicity were found to be significantly related to the ESR measurements, indication that these toxicological effects can be poorly predicted on the basis of this assay. One the other hand, there was good agreement concerning the ranking of toxicity among the three cell lines. With regard to DNA damage induction, there was also a significant correlation between A549 and HepG2 cells, but interestingly, not with the HK-2 cell.

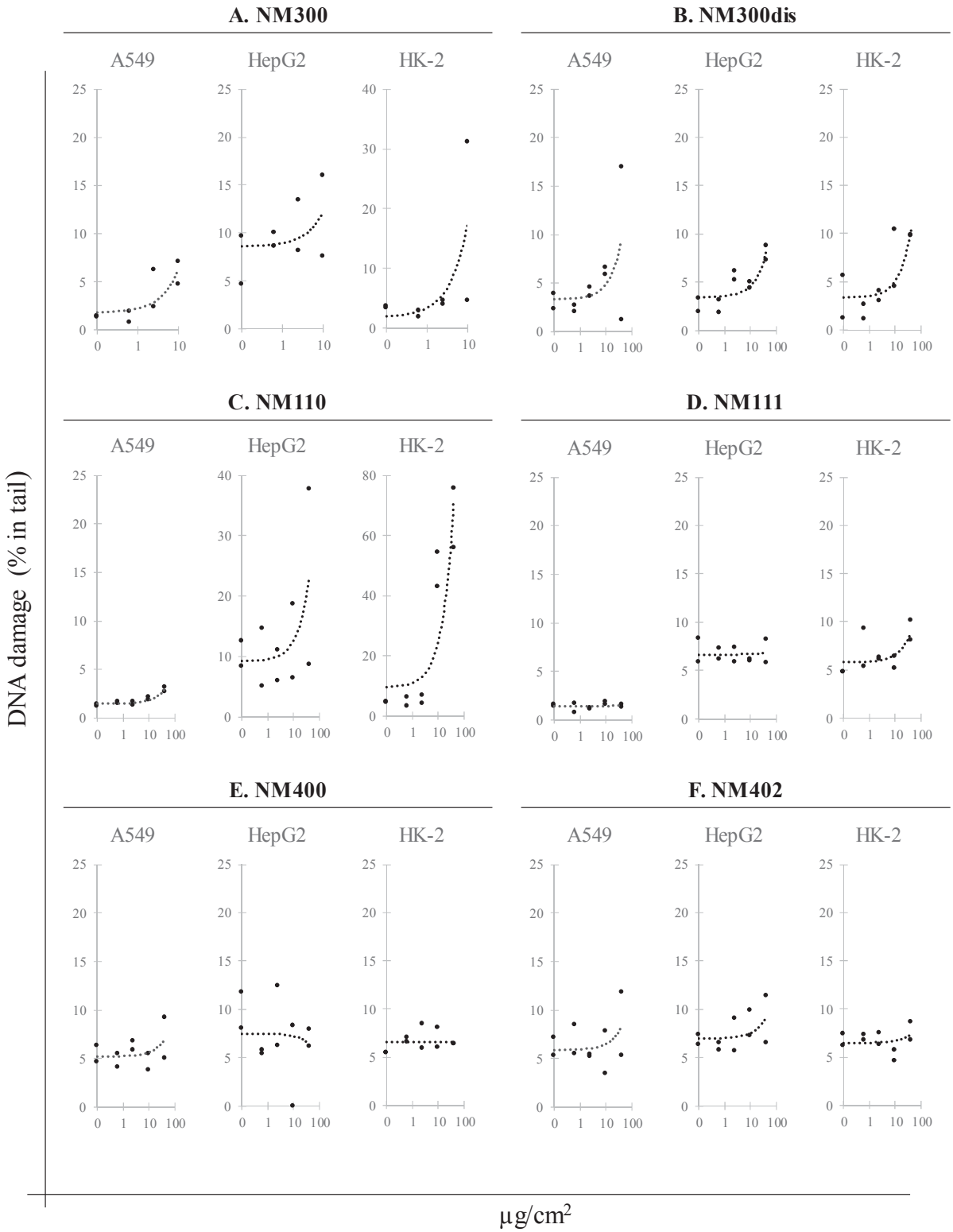

Figure 2-4: DNA damage by NM300, NM300dis, NM110, NM111, NM400 and NM402 in A549, HepG2 and HK-2 cells. DNA damage was evaluated by alkaline comet assay following 4 h treatment with NMs. Data are shown as % DNA in tail for NM300 (A), NM300dis (B), NM110 (C), NM111 (D), NM400 (E), and NM402 (F). Note the different scales of the y-axes for the different cell lines for NM300 and NM110.

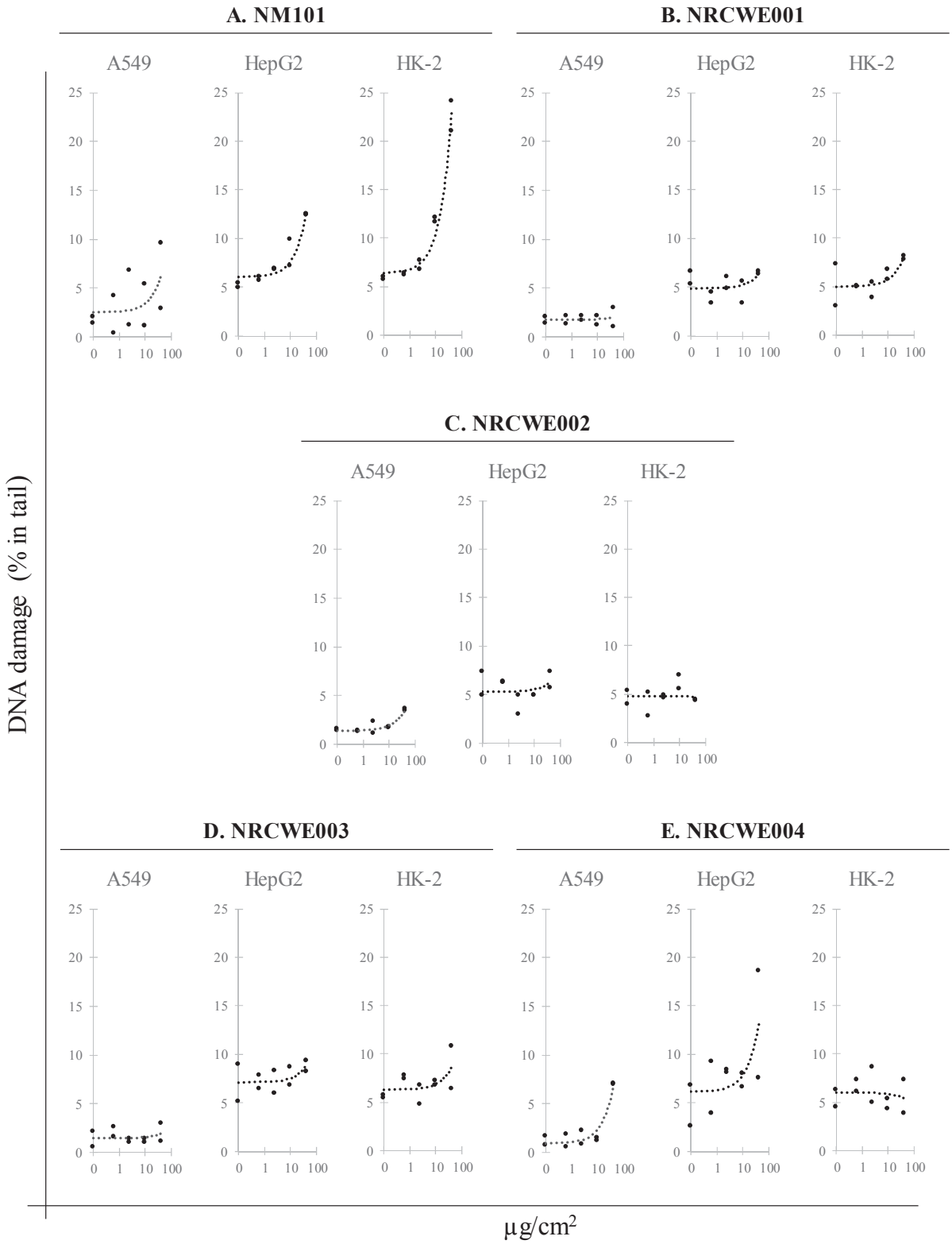

Figure 2-5: DNA damage by TiO2 NMs in A549, HepG2 and HK-2 cells. DNA damage was evaluated by alkaline comet assay following 4 h treatment with NMs. Data are shown as % DNA in tail, for NM101 (A), NRCWE001 (B), NRCWE002 (C), NRCWE003 (D), and NRCWE004 (E).

Table 2-2. Spearman's rank correlation matrix of intrinsic oxidant generation, DNA damage and cytotoxicity of NMs

		(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)
(1)	ESR NMs pristine	1.000							
(2)	ESR NMs serum dispersed	.613 *.059*	1.000						
(3)	DNA damage A549 cells	.159 *.662*	.345 *.329*	1.000					
(4)	DNA damage HK-2 cells	-.238 *.508*	-.178 *.622*	-.030 *.934*	1.000				
(5)	DNA damage HepG2 cells	-.098 *.789*	-.018 *.960*	**.818**** ***.004***	.261 *.467*	1.000			
(6)	LC_{20} values A549 cells	.357 *.311*	.123 *.735*	-.372 *.290*			1.000		
(7)	LC_{20} values HK-2 cells	.138 *.704*	.094 *.796*		**-.679*** ***.031***		**.783**** ***.007***	1.000	
(8)	LC_{20} values HepG2 cells	.368 *.296*	-.068 *.852*			-.067 *.854*	**.862**** ***.001***	**.843**** ***.002***	1.000

The intrinsic oxidant generating properties of the NMs were measured by ESR in pristine form (1) as well as following dispersion in the presence of serum (2). DNA damage, measured by comet assay, was calculated as % of control (3, 4 and 5). Treatment concentrations causing 20% loss in cell viability (TC20 values) were calculated from fitted dose-response curves obtained from the WST-1 experiments (6, 7 and 8). In each cell of the table the Spearman ρ-correlation coefficients are shown and, in italics, the level of significance (*p<0.05 and **p<0.01).

The comet assay was also used to evaluate the potential impact of the 2% serum as used in the ENPRA project dispersion protocol in the A549 lung epithelial cells. The effect of the surfactant Curosurf on DNA damage induction was evaluated in parallel, as this compound has been shown to modify nanoparticle uptake and associated toxicity (Vranic et al. 2013). The experiments were performed with NRCWE-002 and NM402, and results are shown in

Figure 2-6. Absence of serum or replacement of serum with the Curosurf did not affect baseline DNA damage. However, both compounds tended to have contrasting effects with regard to DNA damage induction in the A549 cells by the TiO_2 sample. The presence of the Curosurf resulted in a significant enhancement of the DNA damage by TiO_2. In contrast, the serum tended to reduce the DNA damaging properties of the TiO_2, however, this effect did not reach statistical significance. The MWCNT sample failed to induce DNA damage in the A549 cells, irrespective of the presence or absence of the serum or surfactant.

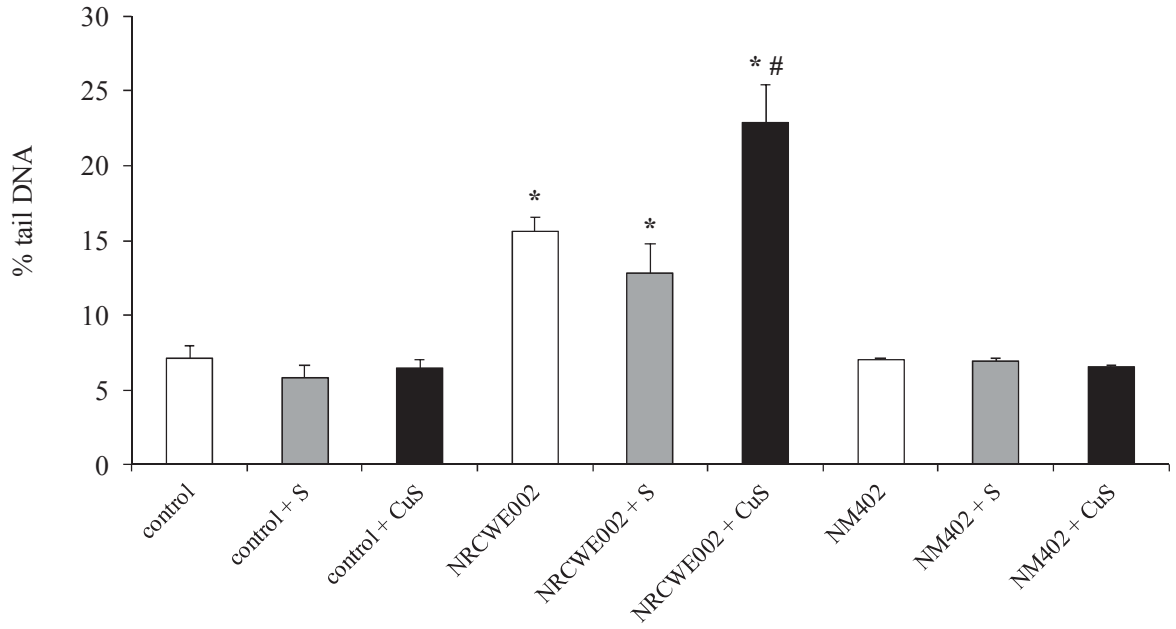

Figure 2-6: Effects of serum and surfactant on DNA damage by TiO_2 and MWCNT NMs in A549 cells. DNA damage was evaluated by alkaline comet assay following 4 h treatment with the ENPRA samples NRCWE002 (TiO_2) and NM402 (MWCNT) in the presence or absence of 2% serum (S, grey bars) or the surfactant Curosurf (CuS, black bars). *p<0.05 versus corresponding control and #p<0.05 versus NRCWE002 (ANOVA with Tukey post-hoc test).

2.4 Discussion

In this study, we have investigated DNA damage and cytotoxicity of ten NMs towards A549 lung, HK-2 kidney and HepG2 liver epithelial cells, in relation to the intrinsic oxidant generating properties of these materials. DNA damage was evaluated by the comet assay, cytotoxicity was analysed by the WST-1 assay, and ESR spectroscopy was used to determine

the intrinsic oxidant generating properties of the tested NMs. The comet assay represents a sensitive and robust genotoxicity indicator assay and has been judged appropriate for the specific testing of NMs (Cowie et al. 2015; Karlsson et al. 2015). Special care was taken to avoid assay artefacts for this *in vitro* test, e.g. as we described earlier e.g. for TiO_2 during sampling processing (Gerloff 2009; Stone et al. 2009). Likewise, the WST-1 assay protocol was modified within the ENPRA project in order to minimize potential assay artefacts for the entire panel of NMs (Kermanizadeh et al. 2016). In this study, we only used the WST-1 assay, while incorporation of additional tests has been recommended in view of potential assay artefact induction by specific NMs in specific test (Stone et al. 2009; Wilhelmi et al. 2012; Guadagnini et al. 2015). We and others could previously show that ambient ultrafine particles and specific types of NMs can act on NAD(P)H activities (Mo et al. 2009; Wilhelmi et al. 2013; Rinna et al. 2015). Such effect could possibly also influence WST-1 assay readouts by affecting cell membrane-associated NAD(P)H-dependent oxidoreductase (Berridge et al. 2005). Although not investigated in this study, we consider such potential effects as low. Moreover, concordance in toxicity ranking has been demonstrated for the full panel of NMs within the ENPRA project, as well as for various related types of NMs in side-by-side comparisons with alternative assays (Kermanizadeh et al. 2016; Gerloff et al. 2009; Wilhelmi et al. 2012; van Berlo et al. 2014; Zou et al. 2014).

In recent years, there has been increasing interest in methods that evaluate the oxidant generating properties of NMs. They are considered to be highly promising in nanosafety testing, towards improved NMs grouping and lower-cost, animal- alternative research prioritization strategies (Riebeling et al. 2016; Rushton et al. 2010; Nymark et al. 2014). These analyses revealed marked contrasts in intrinsic oxidant generating properties among the NMs. We hypothesized that this method would predict their DNA-damaging properties. However, unlike earlier finding with silica (Schins et al. 2002) and PM (Wessels et al. 2010) we found no significant correlations between the ESR data and DNA damage induction or cytotoxicity in any of the cell lines. For most NMs, the serum modification resulted in diminished ROS generation. This may be due to inhibition of surface-catalysed ROS generation following coating of the NMs as well as the antioxidant and ROS scavenging properties of specific serum constituents. Along these lines, the absence of an increased ESR signal for the nanosilver could be related to its dispersant. Subsequent investigations showed that the dispersant control (NM300dis) can strongly inhibit the H_2O_2-mediated DMPO-OH adduct formation by $CuSO_4$ (data not shown). We therefore also evaluated oxidant generation in NM Treated A549 cells using a previously established method (Singh et al. 2007). However, for none of the NMs

treatments any significant increase in ESR signal was detected (data not shown). It remains to be investigated whether approaches to measure oxidant generation capacity of NMs (Riebeling et al. 2016; Rushton et al. 2010; Nymark et al. 2014) allow for a better prediction of their DNA-damaging potencies. It also should be taken in to account, that the enhancement of ROS generation by NMs can occur as a results of their intrinsic chemical reactivity as well as upon physical interaction with cellular structures involved in the catalysis of redox processes (Unfried et al. 2007). Hence, poor intrinsic oxidant generation properties of specific NMs do not necessary need to correlate with absence of oxidative stress dependent cellular responses.

In the present study, we observed contrasting cytotoxic and DNA damaging effects for the various NMs among all three cell lines. For the specific panel of NMs our results also aligned well with findings from other ENPRA project partners (Kermanizadeh et al. 2012; Kermanizadeh et al. 2016; Kermanizadeh et al. 2013). In concordance with our findings, Karlsson *et al*. (Karlsson et al. 2008) found DNA damage in A549 cells with ZnO and TiO_2 NMs, respectively with and without concurrent cytotoxicity. In our hands, ZnO previously also turned out to be the most DNA damaging NMs in human Caco-2 intestinal epithelial cells, among four other NMs, i.e. TiO_2, MgO, SiO_2 and carbon black (Gerloff et al. 2009). The effects observed with the nanosilver are in agreement with findings reported for a variety of cell lines including A549 cells, HepG2 cells, HEK293 human kidney cells, HT29 human intestinal cells and TK6 human B-lymphoblastoid cells (Brzoska et al. 2015; Hudecova et al. 2012; Huk et al. 2015; Kruszewski et al. 2013). The two MWCNT samples did not reveal any notable DNA-damaging properties in all three cell lines in our study, whereas some DNA damage by comet assay was shown by others for this type of NMs, for instance in A549 cells (Karlsson et al. 2008; Ursini et al. 2012). However, in those studies also significant cytotoxicity was observed, unlike our investigations. The genotoxic properties of MWCNTs may vary considerably depending, e.g., on aspect ratio (i.e. length vs. thickness), rigidity, entanglement and associated agglomeration, the presence of metal impurities and specifically engineered chemical modifications, i.e. nanotube functionalization (van Berlo et al. 2012). However, also specific experimental conditions, such as treatment time, cell culture conditions and the specific experimental protocol for NM handling and dispersion should be taken into account. Each of these may explain for differences seen with MWCNTs, as well as with other types of NMs (Doak et al. 2009; Stone et al. 2009). The differences in NM properties and variations of experimental conditions among different laboratories have contributed to the existence of a substantial heterogeneity of published data. Accordingly, there is an ongoing debate on the genotoxicity of specific groups and event specific types of engineered NMs. The variations in

effects that we observed with the five TiO_2 NMs should be attributed to differences in e.g. size, surface charge, crystal phase or aggregation/agglomeration behaviour. The importance of the physical properties in the genotoxicity of this type of NM TiO_2 was shown earlier by Falck et al. (Falck et al. 2009).

Our study also revealed that the effects of NMs can vary substantially with the cell line. All experiments presented in this paper were performed in a single laboratory. With exception of the differences in cell culture media composition (see methods section), all cell culture and NMs treatment conditions and equipment were identical. With this uniform set up, the silver nanoparticles were found to cause marked DNA damage in all three cell lines whereas the uncoated ZnO caused DNA damage in the HK-2 cells and HepG2 cells, but not in the A549 cells. Cell line specific differences in extent and profile of DNA damage effects were previously shown for silver nanoparticles in HepG2, A549 and HT29 cells (Brzoska et al. 2015; Kruszewski et al. 2013;). Recently, a large panel of NMs with widely differing physicochemical properties, including TiO_2, was also subjected to a thorough genotoxicity evaluation within the EU project NanoTEST (Cowie et al. 2015). Interestingly, however, they observed a considerable overlap regarding the detection of positive versus negative (i.e. non-genotoxic) responses over a broad range of cell lines, even though experiments were performed in multiple laboratories. When considering the full panel of NMs in our study, a good concordance was found in DNA damage ranking between the A549 cells and HepG2 cells (Spearman rank correlation: 0.818 P = 0.004), but not with the HK-2 cells (Spearman Table 2-2). However, it needs to be emphasised that our study was not designed, per se, for genotoxicity classification purposes and did not fully comply with current comet assay testing recommendations.

Our study should also be viewed in the light of cell line selection criteria for nano-genotoxicity studies in relation to organ specificity of effects. The differences in NM effects observed for the three human epithelial cell lines are not specifically meaningful regarding hazard assessment towards the organs from which those respective cell lines originate. For the mutagenesis community this concept will be obvious, but it is also important to address this once more to the nanotoxicology research field. All the more since studies on systemic effects of NM are steadily expanding. Rather, on the contrary, findings in our present study highlight the importance of the choice of cell treatment protocols when organ-specific effects are addressed. The DNA damaging effect by a TiO_2 NM depended strongly on the presence of the serum in the dispersion protocol and was significantly enhanced if replaced by a commercial lung surfactant. For inhaled NMs, alveolar epithelial cells are the predominant initial target and

upon deposition in the alveolar region the particles will immediately interact with the covering lung surfactant. In contrast, NMs that enter the body via other compartments and/or NMs that translocate into the bloodstream towards secondary organs (including liver and kidney) are prone to be coated by a corona of proteins and other biological components (Donaldson et al. 2010; Kreyling et al. 2014; Vranic et al. 2016). Our results demonstrate that the choice of NM dispersion method can have major impact on genotoxicity. Earlier, Magdolenova *et al*. also found a significant effect of the dispersion protocol in the comet assay for TiO_2 NMs (Magdolenova et al. 2012). Our dispersion protocol (i.e. 2% serum) mimics, at least to some extent, the effects of NMs that enter the bloodstream and thus attain a corona. The protocol was appropriate to disperse all NMs used in this study, and allowed for a direct comparison of their toxic potencies among the three cell lines. However, for the hazard screening of inhaled NMs, the alternative, lung surfactant-based protocol appears more justified. Thus, it can be argued that the genotoxic effects towards the A549 lung cells may be underestimated for TiO_2 and possibly other NMs. The importance of cellular uptake in nanomaterial toxicity is well recognised (Unfried et al. 2007) , but was not investigated in our current study. Interestingly, however, using the exact same dispersion protocol as ours, in NCI-H292 bronchial epithelial cells the uptake of the TiO_2 NM was found to be enhanced in the presence of the serum, whereas the Curosurf tended to decrease its cellular uptake (Vranic et al. 2013; Vranic et al. 2016).

A final remarkable observation in our study concerns the effects of the nanosilver dispersant. It caused notable DNA damage without concurrent cytotoxicity, and further highlights the importance of including appropriate controls in nano(geno)toxicity testing. Unlike our findings, Huk *et al.* found no DNA damage by comet assay with a number of Ag NM dispersants (Huk et al. 2015). However, they did observe mutagenicity with sodium citrate and Tween 80. Recently, Zhao *et al.* showed dose-dependent histone H_2AX phosphorylation with various surfactants in MCF-7 breast carcinoma cells, including Tween 20, Triton X-100 and Nonidet P-40 (Zhao et al. 2015). They proposed that these agents cause DNA double-strand breaks via a mechanism involving DNase I release and actin disruption. Accordingly, it would have been interesting to evaluate DNA double strand breakage induction by the NM300, but this was beyond the scope of our current study. No increased formation of γH2AX foci was found in a recent study with two types of Ag NMs (Kruszewski et al. 2013). Nevertheless, we agree with *Huk et al.* who concluded that stabilizers with mutagenic potency should not be included in NMs preparations (Huk et al. 2015).

Conclusions. The results of our study indicate that the DNA damaging properties of NMs can be markedly influenced by experimental conditions. This includes the choice of the cell line as well as the dispersion protocol. Measurement of the intrinsic oxidant generating capacity of NMs by ESR turned out to be a poor predictor of their DNA damaging potency. Our study also revealed that one should be cautious to select a cell line on the mere basis of its origin in an attempt to assess organ/tissue-specific hazards of nanomaterials and further underline the critical importance of selecting appropriate cell models and testing protocols.

Funding

This work was supported by the European Commission Framework Programme (FP7) funded Risk Assessment of Engineered Nanoparticles project (ENPRA - NMP4-SL-2009-228789).

Acknowledgements

The authors thank Christel Weishaupt and Gabriele Wick for their analytical support to this work. The Curosurf was kindly provided by Dr. S. Vranic (Laboratory of Molecular and Cellular Responses to Xenobiotics, University Paris Diderot, Sorbonne Paris Cité).

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Chapter III

Silver nanoparticles induce hormesis in A549 human epithelial cells

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Toxicology in vitro 40 (2017): 223-233

* With equal contributions.

Study 2: Silver nanoparticles induce hormesis in A549 human epithelial cells

Declaration

The manuscript is published in Toxicology in vitro

Analyses were performed by Waluree Thongkam with the exception of mRNA and GSH measurement

The impact on authoring this paper can be estimated in total with 45 %

3 Abstract

Despite the gaps in our knowledge on the toxicity of silver nanoparticles (AgNPs), the application of these materials is fast expanding, from medicine, to food as well as the use in consumer products. It has been reported that prolonged exposure might make cells more resistant to AgNPs. This prompted us to investigate if AgNPs may give rise to a hormetic response. Two types of AgNPs were used, i.e. colloidal AgNPs and an AgNP powder. For both types of nanosilver it was found that a low dose pretreatment of A549 human epithelial cells with AgNPs induced protection against a toxic dose of AgNPs and acrolein. This protection was more pronounced after pretreatment with the colloidal AgNPs. Interestingly, the mechanism of the hormetic response appeared to differ from that of acrolein. Adaptation to acrolein is related to Nrf2 translocation, increased mRNA expression of γGCS, HO-1 and increased GSH levels and the increased GSH levels can explain the hormetic effect. The adaptive response to AgNPs was not related to an increase in mRNA expression of γGCS and GSH levels. Yet, HO-1 mRNA expression and Nrf2 immunoreactivity were enhanced, indicating that these processes might be involved. So, AgNPs induce adaptation, but in contrast to acrolein GSH plays no role.

3.1 Introduction

Silver nanoparticles (AgNPs) have found increasing applications in industry, medicine and consumer products and their use is fast expanding (Austin et al. 2014; Johnston et al. 2010). The main reason why silver is used in medical settings is because of its antibiotic properties (Batchelor-McAuley 2014). Nevertheless, there are fundamental gaps in the knowledge on their toxicity and mode of action (Beer et al. 2012; Braakhuis et al. 2014; Cronholm et al. 2013; De Matteis et al. 2015; Govender et al. 2013; Levard et al. 2013; McShan et al. 2014; Recordati et al. 2016). It is long known, that the adverse health effects of toxic fine particles like crystalline silica dust and asbestos are linked to reactive oxygen species (ROS) formation and induction of oxidative stress. Subsequently, redox dependent cellular processes and effects became the focus of investigations on the toxicity of ambient and engineered nanoparticles (Donaldson et al. 2003; Unfried et al. 2007). Indeed, also studies with AgNPs relate toxicity to their ability to generate ROS and to induce oxidative stress (Bohmert et al. 2015; Foldbjerg et al. 2012; Johnston et al. 2010; Liu et al. 2010; Xia et al. 2006). Paradoxically, AgNPs also activate the transcription factor nuclear factor erythroid-derived 2 related factor 2 (Nrf2) (Bohmert et al. 2015; Kang et al. 2012a; Kang et al. 2012b; Prasad et al. 2013; Sahu et al. 2015). This leads to upregulation of cellular antioxidant and detoxification enzymes and upgrades the cellular protection against oxidative stress (Xia et al. 2006; Zhang et al. 2012).

This prompted us to examine the potential relevance of both processes induced by AgNPs i.e. increased formation of ROS versus upregulation of the antioxidant defense. In recent years, peculiar findings on the toxicity of AgNPs have been reported. Aude-Garcia et al. compared the toxicity of AgNPs administered as a single high dose versus the same amount of AgNPs administered as repeated low doses on primary mouse macrophages (Aude-Garcia et al. 2015). Intracellular accumulation of Ag was similar for both treatment regimes, while only the single high dose treatment caused a pro-inflammatory activation of these cells. Brzoska and co-workers reported a short term (2 h) increase in DNA damage returning to baseline after prolonged exposure (24 h) to AgNPs in HepG2 liver epithelial cells (Brzoska et al. 2015). This may point to a hormetic response to AgNPs. Hormesis is that “exposure to a low dose of a chemical that is damaging at higher doses, induces an adaptive upgrade of cellular protection” (Calabrese and Baldwin 2002). In recent years, this adaptation concept has also gained more attention (Bell et al. 2014; Iavicoli et al. 2010).

The process of hormesis can be illustrated with the ubiquitous environmental pollutant acrolein. Recently, we showed that in BEAS-2B lung epithelial cells a low dose of acrolein

induces protection against a high toxic dose of acrolein (Haenen et al. 1988; Sthijns et al. 2014). Acrolein can react with thiols of important cellular proteins, causing toxicity. The protagonist in the protective mechanism induced by this reactive unsaturated aldehyde is the thiol containing compound glutathione (GSH). GSH levels are upregulated by a low dose of acrolein via increasing the Nrf2-mediated gene expression of the rate limiting enzyme of GSH synthesis *gamma*-glutamylcysteine synthetase (γGCS) (Stevens and Maier 2008). This protects against a second exposure to acrolein and thus gives the hormetic response.

The aim of our study was to investigate the hormetic adaptive response to AgNPs by investigating whether pretreatment with a low dose of AgNPs can protect against exposure to a high dose. Two contrasting types of AgNPs, i.e. colloidal versus powder, were selected and characterized for primary particle size, solubility and agglomeration behaviour in this study. Additionally, the mechanism of adaptation was compared to acrolein. Experiments were performed in human A549 lung epithelial cells, which have been widely used to investigate the toxicity of various types of engineered nanoparticles including AgNPs (Beer et al. 2012; Brzoska et al. 2015; Foldbjerg et al. 2012; Han et al. 2014; Huk et al. 2014; Liu et al. 2010; Maurer et al. 2014; Singh et al. 2007). The use of this cell line also has practical relevance due to inhalation exposure to nanosilver caused by a growing number of consumer products such as desinfectants, deodorants, antimicrobial sprays and other applications where these particles also become airborne (Braakhuis et al. 2014; Christensen et al. 2010). Despite its potential limitations (i.e. transformation status), A549 cells were used in several recent and ongoing large-scale nanosafety projects to allow for the necessary bridging of data from different research labs. Their robustness for toxicity evaluation of nanomaterials was evaluated in a round robin approach within the EU-7th framework projects ENPRA (Kermanizadeh et al. 2016).

3.2 Materials and Methods

Nanomaterials. Two types of silver nanoparticles were used, referred to in this study as AgNP1 and AgNP2. The first material, AgNP1, was purchased from Skyspring Nanomaterials, Inc. (US). This material is available as a powder. The AgNP2 represents a sample of the NM-300 reference nanomaterial and was received from the European Commission Joint Research Centre (Ispra, Italy) in the context of the EU FP7 project ENPRA. The nanomaterial NM-300 is a dispersion of silver nanoparticles in deionised water (85%) with 7% stabilising agent (ammonium nitrate) and 8% emulsifiers (4% each of polyoxyethylene glycerol Trioleate and

Tween 20). This colloidal nanosilver samples were purchased and studied along with its dispersant control (i.e. NM-300dis) within the framework of the ENPRA project (Kermanizadeh et al. 2016). Because of absence of cytotoxicity for NM-300dis towards the A549 cells up to the highest test concentration equivalent to 80 μg/cm^2 for NM-300 (data not shown), it was decided not to include this dispersant control further in the present study. Representative scanning electron microscopy (SEM) images of both AgNPs are shown in Figure 3-1. As can be seen in the figure, the AgNP1 consists of compact, near-spherical to elongated primary particles which tend to form compact agglomerates/aggregates. Detailed SEM analysis of the material revealed a primary particle size of 37.0 nm ± 13.0 nm. In contrast, the AgNP2 consists of homogenously distributed, merely spherical particles. For this sample a primary particle size of 16.6 nm ± 4.4 nm was determined. Both types of nanoparticles also revealed contrasting dissolution properties. We determined the solubility of the samples in deionised water (at room temperature) after continuous shaking, followed by syringe filtration and analysis by ICP-OES. Upon 72 h incubation, for the AgNP1 about 0.2% was found to be dissolved, whereas for the AgNP2 sample this was much larger, i.e. up to 5%.

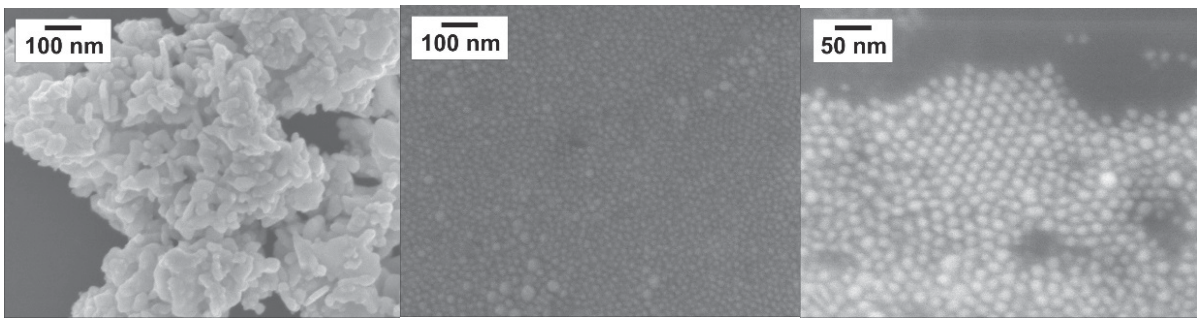

Figure 3-1: Representative scanning electron micrographs of AgNP1 (A) and AgNP2 (B and C).

Culture and treatment of cells. Human lung adenocarcinoma cells (A549) were cultured in Dulbecco's Modified Eagle Medium (DMEM)/F12 (Invitrogen, Bleiswijk, The Netherlands) supplemented with 10 % Fetal Calf Serum (FCS, Sigma-Aldrich, St. Louis, MO, USA) and 1 % Penicillin/Streptomycin (Life Technologies, Bleiswijk, The Netherlands) in an environment containing 5% CO_2 and 95% air at 37 °C. Experiments were performed with cells from passage 15-20.

All particle suspensions for the *in vitro* experiments were prepared on the basis of the nanoparticle dispersion protocols as developed within the EU-7th framework project ENPRA (http://www.enpra.eu) (Kermanizadeh et al. 2016; Kermanizadeh et al. 2013) and the SIINN-ERANET project NanOxiMet (http://www.nanoximet.eu) with following specific modifications: Stock solutions of 1 mg/ml were prepared in sterile RNase free water with 2% Adult Bovine Serum (ABS, Sigma-Aldrich, Darmstadt, Germany). The suspensions were then sonicated for 10 min with a Branson 450 Sonifier with Cuphorn at a power of 5.71 (200 W) and 20% duty cycle. The stock suspensions were quickly further diluted in exposure medium to a concentration of 256 µg/ml, equivalent to the treatment concentration of 80 µg nanoparticles per cm^2 of cell culture monolayer. The exposure medium for the AgNP samples consisted of phenol red free DMEM/F12 with 1% 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid, N-(2-Hydroxyethyl)piperazine-N′-(2-ethanesulfonic acid) (HEPES, Sigma-Aldrich, Darmstadt, Germany) and 1 % Penicillin/Streptomycin. The acrolein (Sigma-Aldrich, St. Louis, MO, USA) exposures were performed in Hank's Balanced Salt Solution (HBSS, Gibco, Bleiswijk, The Netherlands). For dose response relationship evaluations, the A549 cells were exposed to 0, 1, 3, 10, 30, 100 and 200 µM acrolein for 30 min and to 0, 1.25, 2.5, 5, 10, 20, 40, 80 µg/cm^2 AgNPs for 24 h, respectively. Toxicity and intracellular GSH levels were then determined as described in the subsequent sections.

To investigate adaptation processes, repeated exposures were performed at concentrations and treatment times based on the outcome of initially performed dose response analysis, and, for acrolein, in part also on the basis of earlier observations in BEAS-2B cells (Sthijns et al. 2014). Details of these treatment protocols are shown in Figure S1. Briefly, the A549 cells were pretreated with 10 µM acrolein for 30 min after which the HBSS-containing acrolein was replaced with exposure medium. After 3.5 h incubation, a second dose of 100 µM acrolein was then added and cytotoxicity and intracellular GSH levels were determined. For AgNP2, a pretreatment of 5 µg/cm^2 was used and after 24 h of exposure, either a second AgNP2 dose of 80 µg/cm^2 or 100 µM acrolein was added. Acrolein was incubated for 30 minutes, while particles were incubated for 24 h after which again cytotoxicity and GSH levels were measured (Figure S1). For AgNP1, the same experimental set-up as for AgNP2 was performed, however in this case the pretreatment concentration was 2.5 µg/cm^2 and the second treatment was 60 µg/cm^2. For the mRNA expression analyses, A549 cells were exposed for 8 h to suspensions of AgNP1 and AgNP2 at the respective concentrations of 2.5 µg/cm^2 and 5 µg/cm^2. For acrolein,

cells were treated at 10 μM in HBSS for 30 min and subsequent further incubation for 7.5 in fresh exposure medium. For the immunohistochemistry evaluation, cells were exposed for 4 h to 2.5 μg/cm^2 (AgNP1) or 5 μg/cm^2 (AgNP2), or for 30 min to acrolein (10 μM) in HBSS followed by incubation of the cells in fresh exposure medium for a further 3.5 h.

Characterization of the AgNP dispersions by dynamic light scattering analysis. We evaluated the dispersion states of the AgNP1 and AgNP2 by dynamic light scattering (DLS) using a Delsa-Nano C (Beckman Coulter Inc., Krefeld, Germany). The measurements were performed on three different types of suspensions. To verify the effectiveness of the applied sonication protocol, freshly prepared, sonicated stock suspensions of the nanoparticles (i.e. 1 mg/ml water containing 2% ABS) were analysed. To evaluate the dispersion states of the AgNPs under the applied cell culture testing conditions, we also measured the diluted AgNP suspensions containing the phenol red free DMEM/F12 with 10% FCS and 1% penicillin/streptomycin. Finally, to evaluate the impact of serum on the agglomeration status of the nanoparticles, we analysed the diluted culture medium dispersions without 10% FCS. The results of these complementary analyses are shown in Table 3-1. The two samples differed in their aggregate/agglomerate size in the various tested fluids. Under cell treatment conditions (i.e. 10% FCS containing medium), the Z-averages (hydrodynamic diameters) were 642 nm for AgNP1 and 408 nm for AgNP2, respectively. The relative high polydispersity index (PDI) for both materials under these conditions, suggests that the A549 cells were exposed to agglomerates with a rather wide range of diameters.

Table 3-1. Characterisation of the silver nanoparticle dispersions by dynamic light scattering (DLS)

	Sonicated suspension[a]	Medium-FCS[b]	Medium + FCS[c]
AgNP1	690.6 ± 74.5 (PDI:0.298 ± 0.02)	548.1 ± 100.3 (PDI: 0.396 ± 0.01)	642.1 ± 230.2 (PDI: 0.317 ± 0.04)
AgNP2	242.5 ± 93.5 (PDI: 0.151 ± 0.00)	138.6 ± 48.3 (PDI: 0.174 ± 0.05)	408.5 ± 108.6 (PDI: 0.243 ±0.01)

The diameter is expressed as the Z-average hydrodynamic diameter (nm) ± standard deviation (n=3); PDI = Polydispersity index. [a] Sterile RNAse free water supplemented with 2% ABS, measured after sonication; [b] Phenol red free DMEM/F12 medium supplemented with 1 % Penicillin/Streptomycin (without FCS); [c] Phenol red free DMEM/F12 medium supplemented with 10 % FCS and 1 % Penicillin/Streptomycin, as used in cell culture experiments.

Cytotoxicity. Cytotoxicity was evaluated using the WST-1 assay (Roche, Mannheim, Germany). The assay is based on the cleavage of the tetrazolium salt WST-1 to formazan by cellular mitochondrial dehydrogenases. The amount of formazan directly correlates to the number of living cells in the culture. A549 cells were seeded in 96 well plates at a density of at 10 000 cells/well and incubated for 24 h. After treatment of the cells 10 µl of the WST-1 reagent was added to each well (1:10 dilution), and the plates were then incubated for a further 30 min at 37°C. Absorbance was measured at 450 nm by microplate reader (Infinite 200 pro NanoQuant, Tecan). The treatment protocol for the WST-1 assay included evaluation of assay artefacts by analysis of additional control wells as developed in the ENPRA project (Kermanizadeh et al. 2016). Artefacts were not observed with the WST-1 assay, unlike the lactate dehydrogenase (LDH) cytotoxicity test, where assay interference could not be ruled out (data not shown).

Intracellular GSH levels. Cells were seeded at a density of 300 000 cells/well in 6-well plates. Thereafter, cells were treated as described above. Then, cells were washed using 2 ml Phosphate Buffered Saline (PBS, Sigma-Aldrich, Darmstadt, Germany). In addition, 500 µl lysis buffer (0.1 M Potassium Phosphate buffer containing 10 mM EDTA disodium salt, pH 7.5 and 1% Triton-X-100 (Sigma-Aldrich, St. Louis, MO, US)) was added to the cells and they were incubated for 30 min on ice. After scraping the cells, lysates were transferred to Eppendorf tubes that were centrifuged for 10 min at 14 000 rpm and 4 °C to get rid of cellular debris. Part of the supernatant was used to determine protein content using the bicinchoninic acid assay (BCA; Pierce, Thermo Fisher Scientific, Etten-Leur, The Netherlands). Finally, 6% sulfosalicylic acid (Sigma-Aldrich, St. Louis, MO, USA) was added in a ratio of 1:1 to 300 µl of the remaining supernatant and the mixture was diluted 1:5 in 0.1 M lysis buffer without Triton-X-100. The previously described enzymatic recycling method was used to determine GSH levels (Rahman et al. 2006).

Expression of Heme Oxygenase-1 and *gamma*-glutamylcysteine synthetase. Cells were plated at a density of 300 000 cells/well 24 h before the start of the exposure. After treatment, cells were lysed using 500 µl Qiazol (Qiagen, Venlo, The Netherlands). Phase separation was induced by addition of 100 µl chloroform (Sigma-Aldrich, St. Louis, MO, US) for 2-3 min at 20 °C and samples were then centrifuged (15 min, 12 000 g) at 4 °C. The upper phase was then

collected and supplemented with 250 µl of isopropanol (Sigma-Aldrich, St. Louis, MO, US). After overnight incubation at 4 °C followed by centrifugation, supernatants were discarded and pellets were washed in 0.5 ml 75% ethanol (Sigma-Aldrich, St. Louis, MO, US). Samples were then centrifuged for 5 min at 7 500 g and 4 °C upon which supernatants were removed. Pellets were allowed to dry at room temperature air for 1 h and then resuspended in 50 µl RNase/DNase free water. Resuspended samples were incubated for 10 min at 60 °C and the amount of RNA was measured using the NanoDrop (Thermo scientific Nanodrop 1000 spectrophotometer, isogen life science, De Meern, The Netherlands). Hereafter, complementary DNA (cDNA) was made of 500 ng of RNA using the iScript cDNA synthesis kit (Biorad, Veenendaal, The Netherlands). Quantitative RT-PCR was performed in Bio Rad MyiQ Real-Time PCR Detection System (American Laboratory Trading, Boston/Cambridge) using the iQ™ SYBR® Green Supermix (Biorad) with the following primers: Heme oxygenase-1 (HO-1; sense: 5'-CTTCTTCACCTTCCCCAACA-3' and antisense: 5'-GCTCTGGTCCTTGGTGTCAT-3'); *Gamma*-glutamylcysteine synthetase (γGCS; sense: 5'-GCACATCTACCACGCCGTC-3' and antisense: 5'-CCACCTCATCGCCCCAC-3'). Beta-actin was used as a housekeeping gene (β-actin; sense: 5'-CCTGGCACCCAGCACAAT-3' and antisense: 5'-GCCGATCCACACGGAGTACT-3'). The relative mRNA expression of HO-1 and γGCS was calculated according to the method of Livak and Schmittgen (Livak and Schmittgen 2001).

Immunohistochemistry. A549 cells were seeded in 4-chamber-slides (BD Falcon) at a density of 1 600 000 cells/well. The following day, cells were exposed to acrolein or the silver nanoparticles for 30 min or 4 h, respectively. The control cells were treated in parallel for 4 h with FCS-containing phenol red free control medium. After gently washing with PBS buffer by means of three consecutive incubations for 5 min at 20 °C cells were fixed with 4 % formaldehyde in PBS (pH 7.4) for 20 min. The washing procedure was repeated and cells were then permeabilised with 0.1% Triton X-100 in PBS for 5 min. The cells were washed again and incubated with normal goat serum (1:65 dilution) for 30 min. Cells were then washed and incubated overnight at 4 °C with a 1:50 diluted primary Nrf2 antibody (C-20, Santa Cruz Biotechnology, Inc.). Cells were washed again three times with PBS for 10 min, followed by addition of AlexaFluor 594 secondary goat anti-rabbit IgG antibody (Life Technologies, Darmstadt, Germany) at 1:200 dilution and incubated for 1 h at room temperature. After performing the washing procedure again, cells were stained at room temperature with 1 µg/ml Hoechst 33342 nuclear dye dissolved in PBS for 15 min. The washing procedure was repeated

and cells were mounted with Prolong Gold Anti-fading (Life Technologies). Next day, images were acquired by Zeiss AxioVert Microscope equipped with an AxioCam MRm camera and analysed using Zeiss (ZEN) software.

Statistics. All experiments were at least performed in duplicates at $n \geq 3$ and all data are represented as mean ± SEM. To test differences between the AgNP1 and AgNP2 groups a Two-Way Anova was used. Additionally, independent samples with equal variances were assessed for statistical significance with a t-test. P values < 0.05 were considered to be statistically significant.

3.3 Results

The cytotoxicity of both types of silver nanoparticles and acrolein towards the A549 lung epithelial cells was evaluated by the WST-1 assay. Results are shown in Figure 3-2. A significant loss of metabolic activity could be observed with AgNP1 at the cell surface area treatment doses of 40 and 80 μg/cm^2. In contrast, AgNP2 did not cause statistically significant toxicity up to the highest achievable treatment dose of 80 μg/cm^2. The loss in viability was approximately 20% at this concentration. To enable a direct comparison of the toxic potency of the silver nanoparticles, in Figure 3-2B the acrolein dose is also expressed in μg/cm^2, despite its non-(nano) particulate i.e. chemical nature. On this mass per cell surface area dose, the markedly higher toxicity of acrolein becomes apparent. The toxicity of acrolein towards the A549 cells reached statistical significance at concentrations of and above 10 μM, equivalent to 0.175 μg/cm^2. Herein, it should be taken into account that the toxicity of acrolein was determined upon 30 min treatment, while for the two types of AgNP toxicity was evaluated after 24 h. The calculated TC_{50} values for acrolein, AgNP1 and AgNP2 are 0.15 ± 1.62 μg/cm^2, 55 ± 12 μg/cm^2 and > 80 μg/cm^2 respectively. Based on the observed thresholds for cytotoxicity and the shape of the dose response curves, 10 μM acrolein, 2.5 μg/cm^2 AgNP1 and 5 μg/cm^2 AgNP2 were selected as pretreatment doses to evaluate potential adaptation mechanisms. As the toxic challenge concentrations, 100 μM acrolein, 60 μg/cm^2 AgNP1 and 80 μg/cm^2 AgNP2 were chosen.

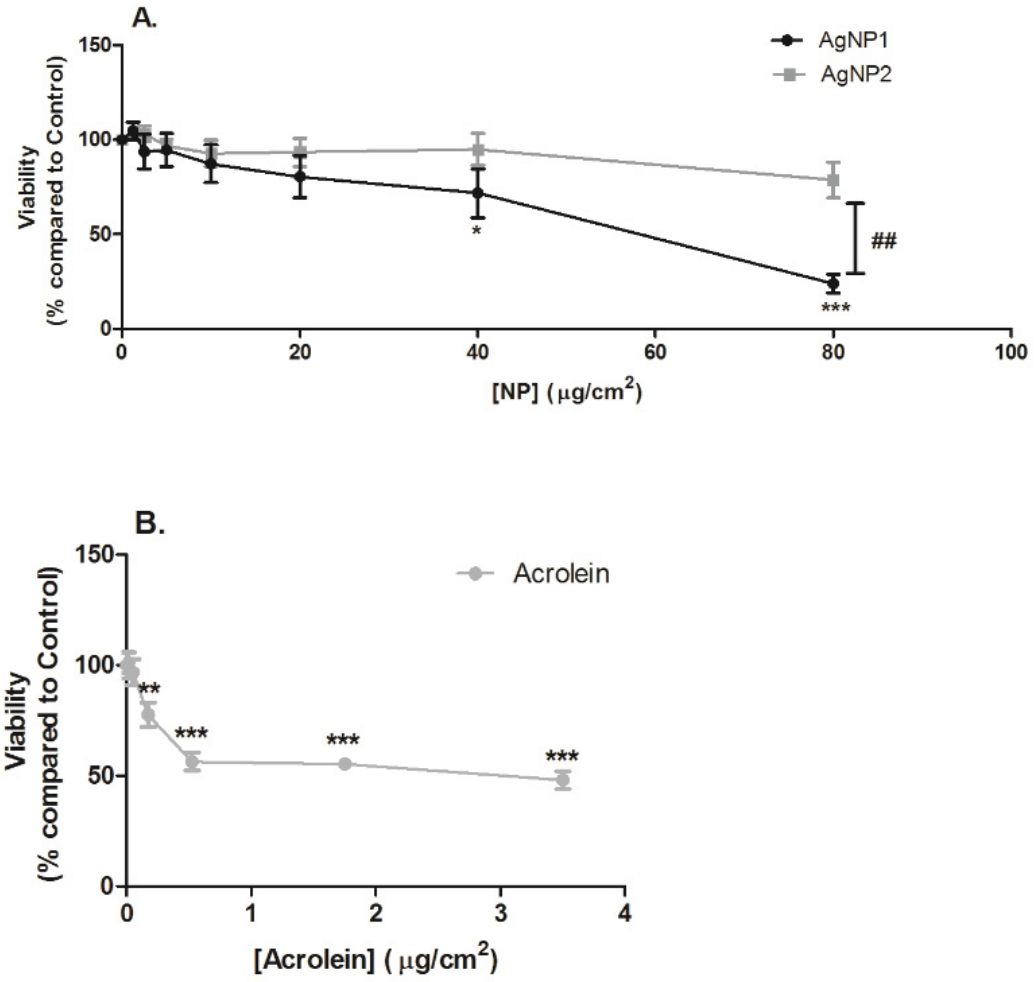

Figure 3-2: Effects of acrolein and AgNP1 and AgNP2 silver nanoparticles on cytotoxicity in A549 cells. The graphs shown in panel A (AgNP1 and AgNP2) and panel B (Acrolein) indicate mean ± SEM from 4 independent experiments. *P < 0.05; **P < 0.01; ***P < 0.001 compared to control and #P < 0.05 compared to indicated condition.

To address hormesis of AgNPs, repeated exposure experiments were performed according to the time schemes shown in Figure S1. A549 cells that were pretreated with 5 μg/cm^2 AgNP2 for 24 h showed less toxicity than non-pretreated cells induced directly by a toxic challenge of 80 μg/cm^2 of AgNP2 (Figure 3-3 D) or 100 μM acrolein (Figure 3-3 E). Similarly, the cells that were pretreated with 2.5 μg/cm^2 AgNP1 followed by a toxic challenge of 60 μg/cm^2 AgNP1 or 100 μM acrolein showed an increasing trend in viability compared to the cells that were exposed to 60 μg/cm^2 AgNP1 or 100 μM acrolein without pretreatment (Figure 3-3 B and C). Finally, a significant diminishment of toxicity of 100 μM acrolein was observed in response to pretreatment with 10 μM acrolein (Figure 3-3 A).

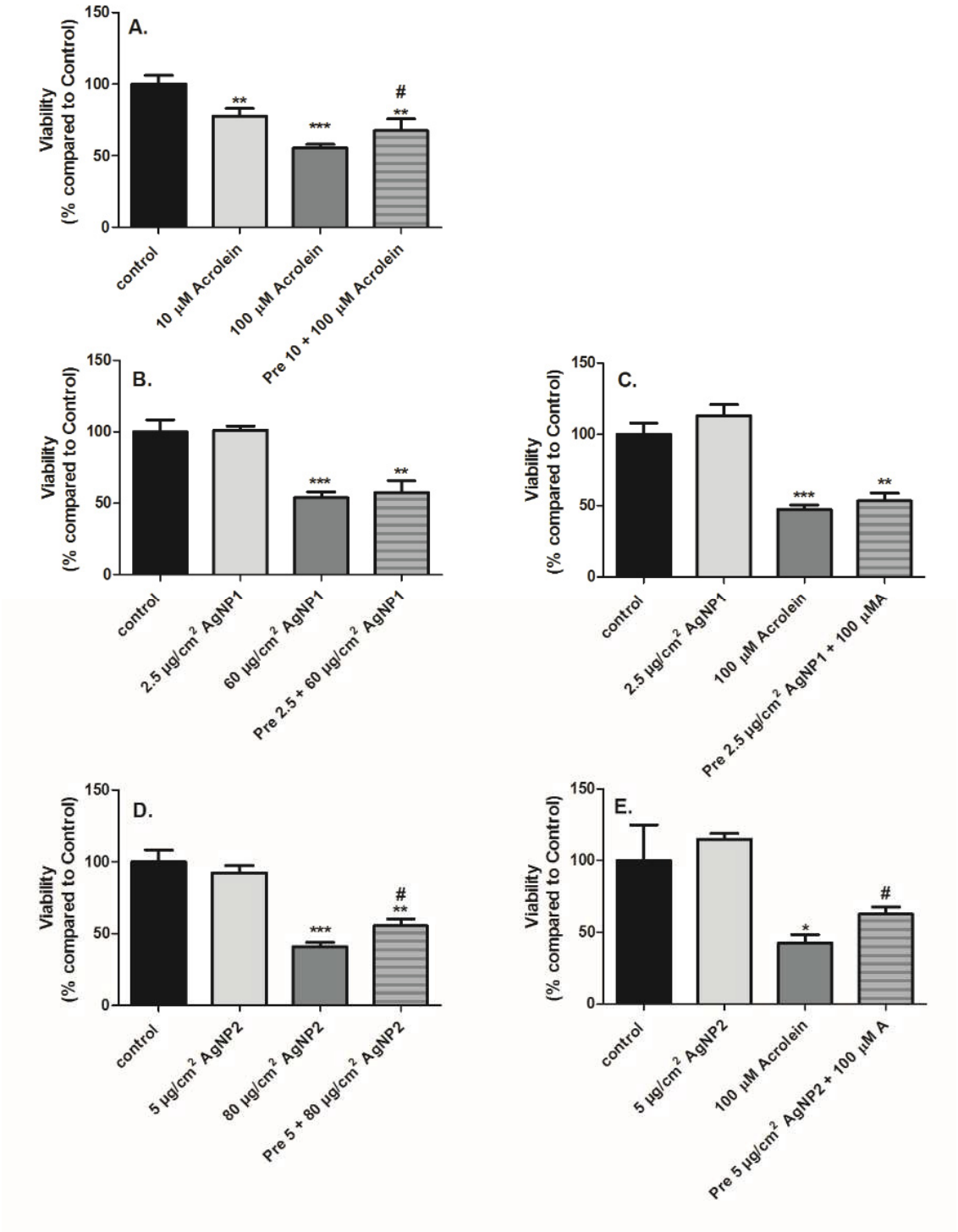

Figure 3-3: The effect of a pretreatment (=Pre) with 10 µM Acrolein (A), 2.5 µg/cm² AgNP1 or 5 µg/cm² AgNP2 on cell viability in A549 cells. N=4 and data are presented as mean±SEM. The graphs indicate the effects of acrolein pretreatment on viability loss by acrolein (A), AgNP1 pretreatment on viability loss by AgNP1 (B), AgNP1 pretreatment on viability loss by acrolein (C), AgNP2 pretreatment on viability loss by AgNP2 (D), and the effect of AgNP2 pretreatment on viability loss by acrolein (E). *P < 0.05; **P < 0.01; ***P < 0.001 compared to control #P < 0.05 compared to the respective non-pretreated cells.

The role of GSH in the toxicity and hormetic response was evaluated. The effects of the AgNPs and acrolein on GSH depletion in the A549 cells are shown in Figure 3-4. Both types of AgNPs caused a dose dependent decrease in intracellular GSH levels. A significant loss in GSH for AgNP1 was observed at 2.5 μg/cm^2 and for AgNP2 at 20 μg/cm^2. At the highest treatment concentrations (80 μg/cm^2) the effect of AgNP1 was significantly stronger than the effect of the AgNP2 material. Acrolein treatment led to a significant decrease in intracellular GSH at 100 μM, equivalent to 1.18 μg/cm^2 (Figure 3-4).

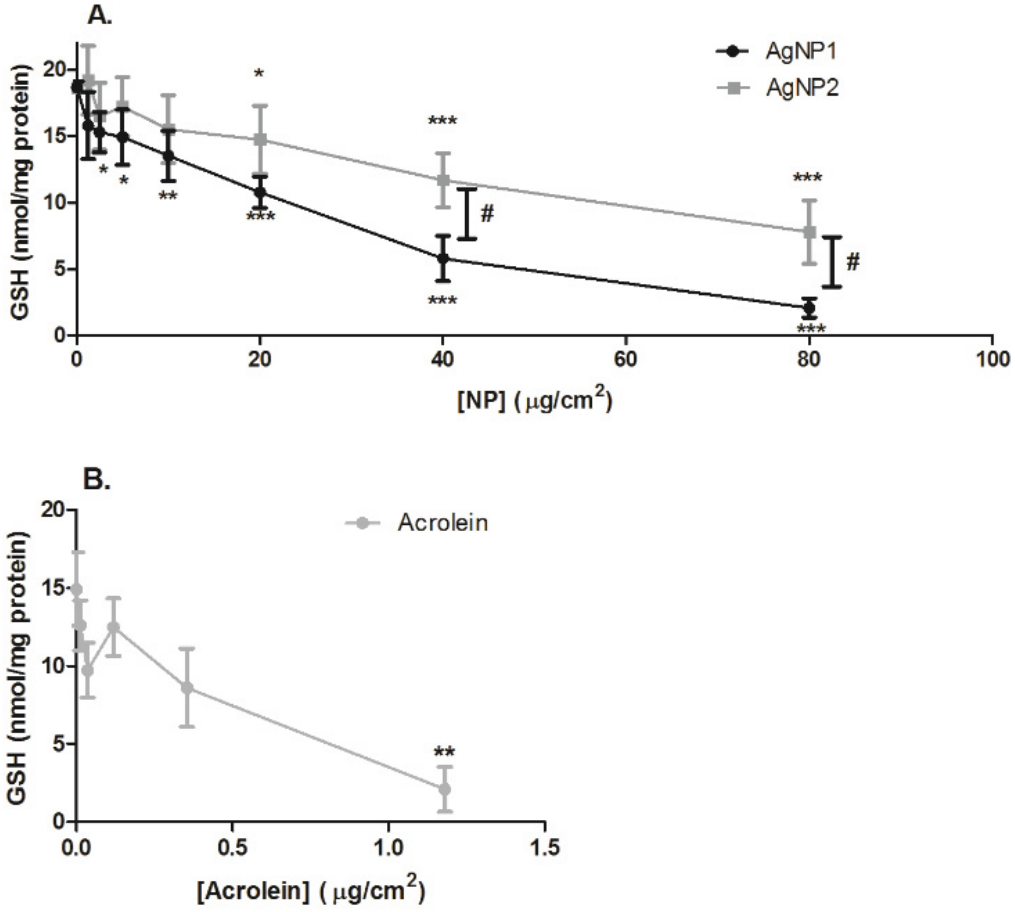

Figure 3-4: Effects of acrolein and AgNP1 and AgNP2 silver nanoparticles on intracellular GSH levels in A549 cells. The graphs shown in panel A (AgNP1 and AgNP2) and panel B (Acrolein) indicate mean ± SEM from three independent experiments. *P < 0.05; **P < 0.01; ***P < 0.001 compared to control and #P < 0.05 compared to indicated condition.

Again, it should be noted that the GSH depletion of acrolein was measured upon 30 min treatment whereas the effects of the silver nanoparticles were evaluated following 24 h incubation. For acrolein as well as both types of AgNPs decreases in GSH levels were observed in association with viability loss. However, the extent of reduction in GSH in the A549 cells does not result in the same level of cytotoxicity for the three compounds studied. The concentrations at which a significant reduction in viability was seen did not correlate with the concentrations at which a significant decrease in GSH is observed (Figure S2, Figure 3-2 and Figure 3-3).

Treatment of the A549 cells with acrolein at 100 μM significantly diminished intracellular GSH levels. The pretreatment with 10 μM acrolein prevented the significant loss of GSH after the subsequent toxic challenge at 100 μM acrolein (Figure 3-5 A). In contrast, pretreatment of the cells with AgNP1 (2.5 μg/cm^2) or AgNP2 (5 μg/cm^2) for 24 h, followed by a challenge with 60 μg/cm^2 AgNP1 or 80 μg/cm^2 AgNP2 consecutively, did not prevent intracellular loss in GSH, when compared to the sole treatments with 60 μg/cm^2 AgNP1 or 80 μg/cm^2 AgNP2 respectively (Figure 3-5 B and D). Pretreatment with AgNP1 or AgNP2 for 24 h also failed to protect the cells against the GSH depletion induced by acrolein (Figure 3-5 C and E). The GSH levels in A549 cells were not changed following 24 h exposure to either 2.5 μg/cm^2 AgNP1 or 5 μg/cm^2 AgNP2 (Figure 3-5 F and G).

To further evaluate the potential underlying mechanisms of the observed effects, mRNA expression analyses and immunohistochemistry were performed. Treatment of the cells for 8 h with 10 μM acrolein, 2.5 μg/cm^2 AgNP1 and 5 μg/cm^2 AgNP2 resulted in a significant increase in HO-1 expression (Figure 3-6). Moreover, acrolein also caused a significant increase in γGCS mRNA level. However, γGCS levels were not significantly changed in response to treatment with AgNP1 or AgNP2. Treatment of the A549 cells for 30 min with acrolein also increased in the immunoreactivity for Nrf2, indicative of the activation of this transcription factor. Representative staining results are shown in Figure 3-7. Digital analyses of revealed 19 % increased red fluorescent signal compared to control cells (>400 cells analysed/treatment). Enhanced immunoreactivity was also observed after 4 h treatment with AgNP1 (2.5 μg/cm^2) and AgNP1 (5 μg/cm^2) albeit to different extent, i.e. 30% and 17%, respectively.

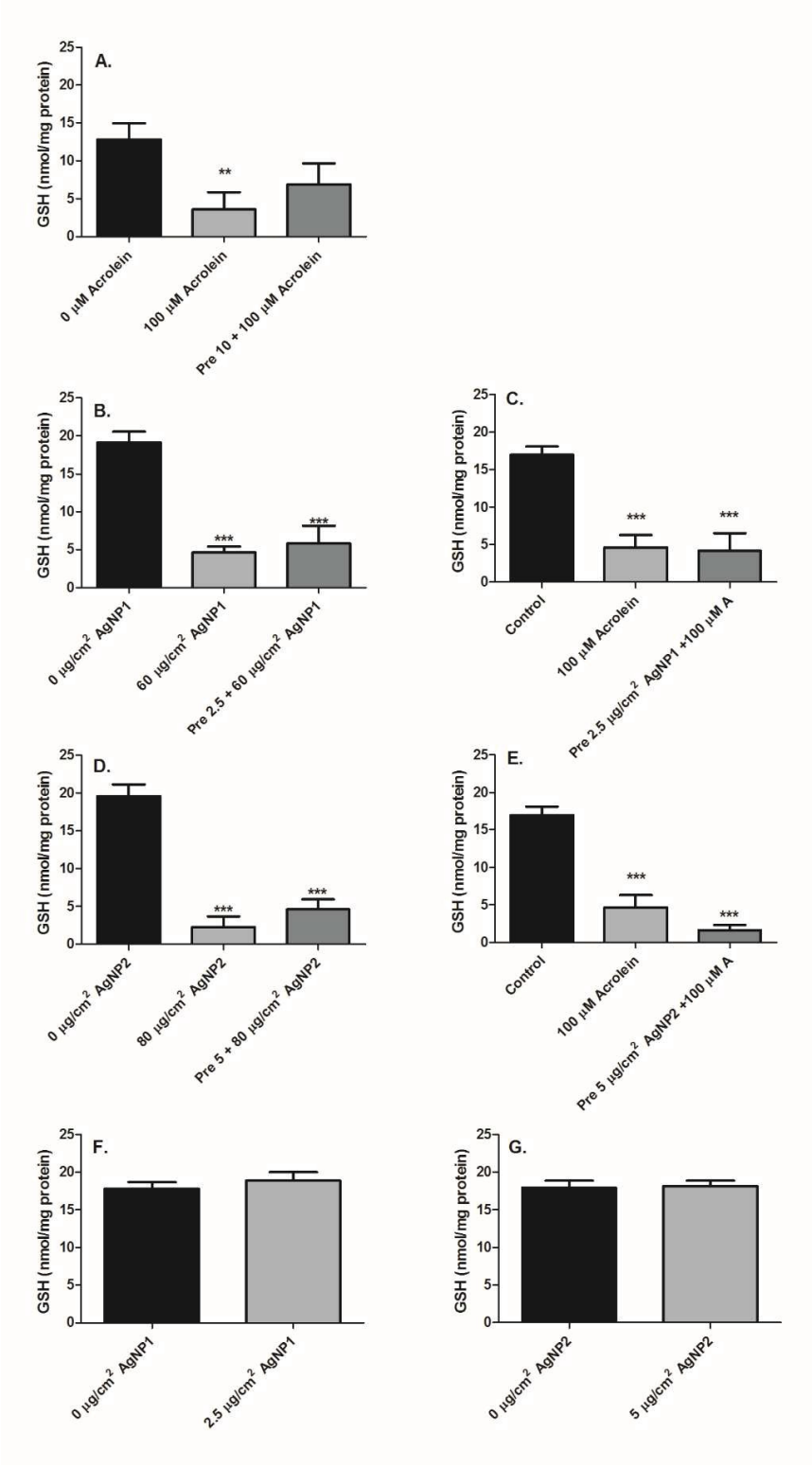

Figure 3-5: The effect of a pretreatment with 10 µM Acrolein (A), 2.5 µg/cm² AgNP1 or 5 µg/cm² AgNP2 on GSH levels in A549 cells. The graphs indicate the effects of acrolein pretreatment on intracellular GSH change by acrolein (A), AgNP1 pretreatment on GSH change by AgNP1 (B), AgNP1 pretreatment on GSH change by acrolein (C), AgNP2 pretreatment on GSH change by AgNP2 (D), AgNP2 pretreatment on GSH change by acrolein (E) and the effects of 2.5 µg/cm² AgNP1 (F) or 5 µg/cm² AgNP2 (G) on GSH levels in A549 cells. N ≥ 3 and data are presented as mean ±SEM. *P < 0.05; **P < 0.01; ***P < 0.001 compared to control.

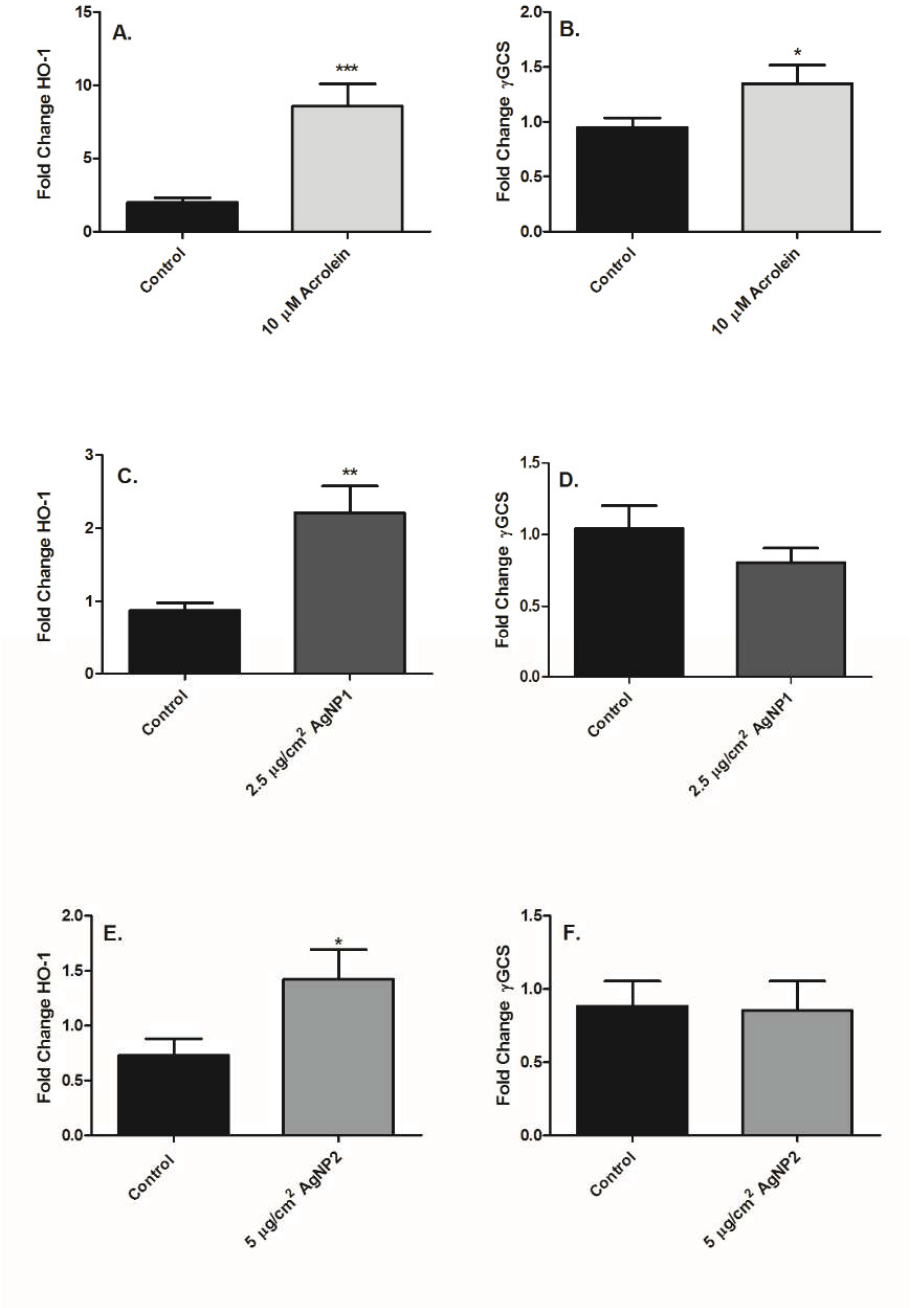

Figure 3-6: mRNA levels of Heme Oxygenase-1 (HO-1) (A, C and E) and gamma-glutamylcysteine synthetase (γGCS) (B, D and F) in A549 cells exposed to either 10 µM Acrolein (A and B), 2.5 µg/cm^2 (A and B), AgNP1 (C and D) or 5 µg/cm^2 AgNP2 (E and F) for 8 h. N=3 and data are presented as mean±SEM. *P < 0.05; **P < 0.01; ***P < 0.001 compared to control.

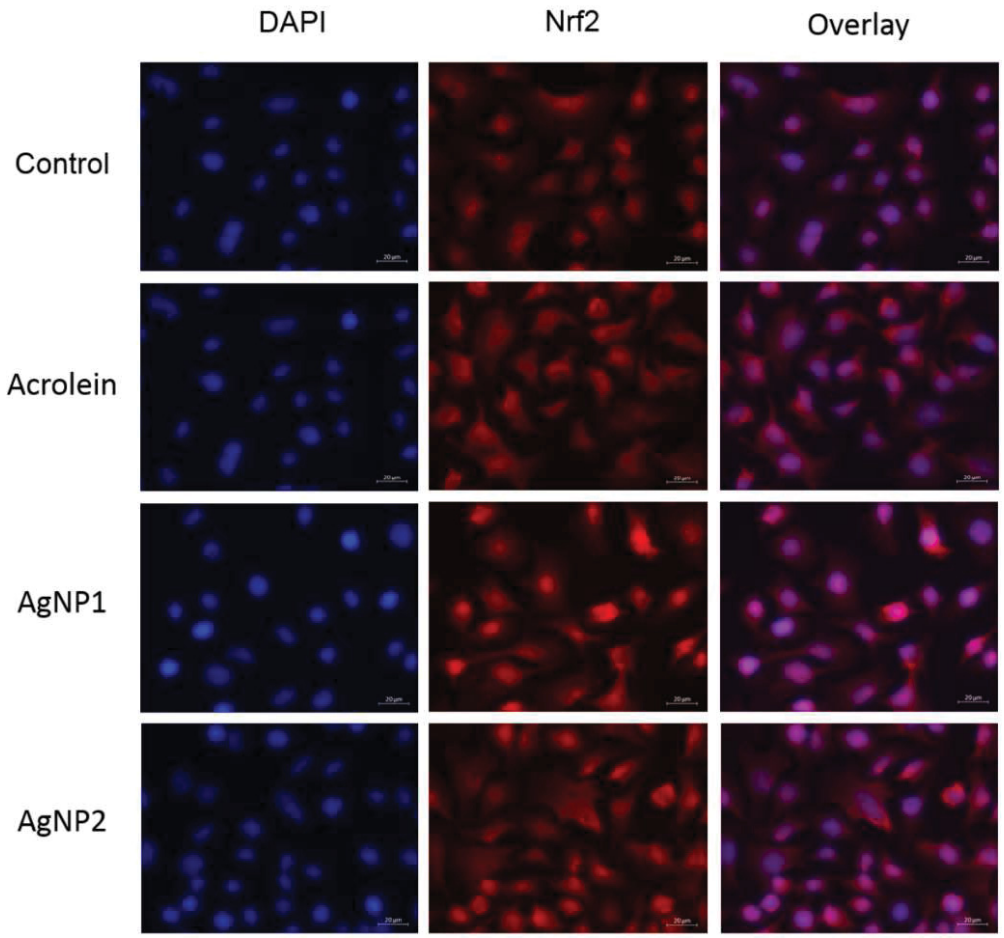

Figure 3-7: Immunohistochemical staining for Nrf2 in A549 cells after 4 h-treatment with 10 µM acrolein, 2.5 µg/cm² AgNP1 or 5 µg/cm² AgNP2 at 400X magnification, in comparison to controls (4 h-incubation in fresh test medium in the absence of AgNPs). The cells were stained for Nrf2 (red) while counterstaining with Hoechst 33342 is shown in blue.

3.4 Discussion

Increased application in medicine, consumer products including cosmetics, and even food underlines the need to examine thoroughly the toxicity of AgNPs (Bohmert et al. 2015; Johnston et al. 2010; McShan et al. 2014). The present study provides evidence for hormesis of this type of nanomaterial, meaning that exposure to a low non-toxic dose of AgNPs might protect against a second toxic dose. Various physicochemical properties including particle size, surface area and dissolution rate have been linked to the toxicity of AgNPs e.g. an inverse correlation between their primary particle size and toxicity has been demonstrated in several studies (Liu et al. 2010; Maurer et al. 2014; Mishra et al. 2016; Wildt et al. 2015). In our hands,

the toxicity of the AgNP2 sample (16.6 nm) was found to be markedly lower than that of the AgNP1 sample (37.0 nm). In line with this, the toxicities of the two samples were also inversely related to their solubility, as the AgNP2 sample showed a markedly higher dissolution in deionised water. Several studies have also shown that the toxicity of engineered nanoparticles, including AgNPs, relates to their ability to generate ROS (Johnston et al. 2010; Xia et al. 2006). However, using electron spin resonance spectroscopy (Papageorgiou et al. 2007), results on radical formation for both AgNPs were less conclusive (data not shown).

Apart from the differences in primary particle size, solubility and reactivity, also contrasts in agglomeration/aggregation state and particle shape should be taken into account as well as the original state of the material, i.e. a powder versus a stabilized colloidal dispersion. DLS analyses revealed a better dispersion state of the AgNP2 sample in water. However, in the FCS-containing culture medium, which reflects cell treatment conditions, the Z-average and PDI increased for this sample. Comparative analysis in FCS-free culture medium indicated the impact of the 10% FCS on agglomeration behaviour for this colloidal sample. In contrast, the AgNP1 had a larger hydrodynamic size and polydispersity, irrespective of the suspension conditions. To unravel the mechanism of the hormetic response, acrolein was also investigated in this study. Both AgNPs appeared to be less toxic than acrolein at equal dose ($\mu g/cm^2$), and may relate to different cellular uptake processes and kinetics (Gliga et al. 2014). AgNPs are taken up and compartmentalized in the cells, primarily by endocytosis and through several metal transporters or ATPases, whereas acrolein uptake probably mainly involves rapid, passive diffusion through the lipid membrane (Behra et al. 2013; Stevens and Maier 2008).

Previous research in BEAS-2B bronchial epithelial cells showed that GSH is the first line of defense against acrolein (Prasad et al. 2013; Sthijns et al. 2014). In the present study with A549 alveolar epithelial cells, acrolein toxicity is also accompanied by a decrease in GSH. The BEAS-2B cells seem to be less vulnerable than the A549 cells, because more acrolein is needed to induce the same level of toxicity. This difference in toxicity has also been seen for other substances including multi-walled carbon nanotubes (MWCNTs) that are more toxic in BEAS-2B cells than in A549 cells (Ursini et al. 2014). This difference in toxicity can be explained by differences in antioxidant defense systems between the different cell types (Zhang et al. 2015). One of these antioxidant defense mechanisms is the GSH system. Remarkably, which further underlines the role of GSH as protector against toxicity is that in this study in A549 cells an increase in viability is correlated with a decrease in GSH. In BEAS-2B cells the GSH level dropped at lower levels of acrolein compared to A549 cells. This corresponds to relating the vulnerability to toxic compounds of A549 cells to BEAS-2B cells (Schlinkert et al.

2015). For AgNP2 and AgNP1, an increase in viability is also associated with a decrease in GSH. Such an effect for AgNPs has for instance also been reported in Caco-2 intestinal epithelial cells (Auevirivavit et al. 2014; McCracken et al. 2015), HepG2 liver epithelial cells (Vrcek et al. 2014), and HK-2 kidney epithelial cells (Kang et al. 2012a). The importance of GSH in the protection against AgNP toxicity is supported by the protective effect of the glutathione precursor N-acetylcysteine (Foldbjerg et al. 2011; George et al. 2012). Apart from the protection provided by GSH, cells also have alternative pathways to protect against toxicity of AgNPs (Chairuangkitti et al. 2013). For example, in A549 cells more Nrf2-mediated upregulation of the endogenous antioxidant response has been observed (Singh et al. 2006).
In the present study, it was found that acrolein, such as in BEAS-2B cells (Sthijns et al. 2014), also induces hormesis in A549 alveolar epithelial cells. GSH functions as the first line of defense against acrolein. Then, Keap1 is adducted by acrolein (McMahon et al. 2010) and Nrf2-mediated gene expression of the antioxidant enzyme HO-1 and the rate-limiting enzyme of GSH synthesis γGCS are induced. The last one probably contributes to the higher GSH level in pretreated A549 cells and explains why pretreated A549 cells show less cytotoxicity in case of pretreatment with acrolein. In case of the AgNPs, pretreatment with a low dose of AgNP1 or AgNP2 before a treatment with a toxic dose of these respective samples did show less cytotoxicity than without pretreatment, also indicating hormesis. Notably, however, the effect was stronger and only statistically significant for AgNP2, i.e. the colloidal sample with the smaller primary particle size and associated higher solubility, and the relatively lower agglomeration behaviour in the testing medium. This indicates that adaptation depends on the specific physicochemical properties of this type of nanomaterial.

A major finding in our study was that the pretreatment of the A549 cells with a low dose of the silver nanoparticles also offered protection against the toxic effects of a treatment with a toxic concentration of 100 μM acrolein. Thus, in this case, a low dose of a substance that is damaging at high doses also protects against the damaging effects of another toxic substance at its toxic concentration. Therefore, we would like to introduce the term transhormesis, hormesis induced by a different chemical than the chemical of exposure. This finding would implicate that both acrolein and nanosilver, depending on its dose and (physico-)chemical properties can induce adaptation by inducing a similar protective mechanism. Only then, exposure to the low dose of one substance could induce protection for a toxic dose of another chemical. Since GSH has been shown to be the first line of defense against acrolein in BEAS-2B cells, we expected this to be the mechanism by which silver nanoparticles upregulate cellular protection. However, only for acrolein it could be confirmed that GSH plays a role as a protective mechanism in

response to low dose pretreatment of this compound: The GSH level was significantly higher in A549 cells exposed to 100 µM of acrolein preceded by a pretreatment with 10 µM of acrolein compared to A549 cells that were exposed to 100 µM of acrolein without pretreatment. However, for neither of the AgNPs, such significant difference could be observed. From this it can be concluded that GSH is not the key in the adaptation induced by the silver nanoparticles. Moreover, our findings also indicate that there has to exist another protective mechanism that is induced by nanosilver to protect the cells from the toxic concentration of acrolein.

Immunohistochemical analyses indicated activation of the cellular redox sensor Nrf2 in the A549 cells by acrolein as well as the AgNPs, which is in concordance with other investigations (Bohmert et al. 2015; Kang et al. 2012a; Prasad et al. 2013; Sahu et al. 2015; Zhang and Forman 2008). Remarkably, both γGCS and HO-1 were found to been upregulated by acrolein and AgNPs only increase HO-1, whereas both genes are regulated by Nrf2 (Bohmert et al. 2015; Kang et al. 2012a; Kang et al. 2012b). Timing could play an important role, because for adaptation induced by acrolein only 4 h are required, while for AgNPs adaptation is induced over a time period of 24 h (Bell et al. 2014; Brzoska et al. 2015). Besides timing also other factors including the specific physicochemical properties, cellular uptake and dissolution of the applied nanoparticles or the cell lines used could be involved, which can explain why acrolein showed enhanced mRNA expression for γGCS as well as for HO-1, while AgNPs only increase HO-1. Kang and co-workers (Kang et al. 2012b) could demonstrate that blocking of Nrf2 resulted in increased DNA damage and apoptosis by AgNPs in ovarian carcinoma cells. Moreover, they showed that HO-1 induction by cobalt protoporphyrin offered protection against AgNP-induced toxicity, whereas specific inhibition of HO-1 exacerbated the effects of the nanoparticles. This would be in support of the role of the HO-1 protein in the observed adaptation by nanosilver in the A549 cells. Interestingly, in this regard, these effects shown by Kang and co-workers, occurred in association with AgNP-mediated activation of the PI3K and p38MAPK signalling pathways. In HBE1 human bronchial epithelial cells a role for protein kinase C delta (PKC-delta) in Nrf2-mediated induction of HO-1 by acrolein was previously shown by Zhang and Forman (Zhang and Forman 2008). Thus, HO-1 could also be a main player in the observed transhormesis effect by AgNPs and acrolein, although a direct protective effect of HO-1 on acrolein in the A549 cells remains to be investigated.

Potential adaptation mechanisms to the toxicity of AgNPs have been discussed in a number of recent studies (Aude-Garcia et al. 2015; Brzoska et al. 2015; Jiao et al. 2014). Comparing the effects of a single high versus repeated low doses of AgNPs, Aude-Garcia and colleagues revealed the importance of addressing the dose rate in the toxicity of nanoparticles.

Although adaptation was not in the focus of their work, it would be interesting to investigate to what extent changes in dose rate would affect the observed effects in our study. In line with this, it would also be of great relevance to further investigate the role of specific physicochemical properties, target cell-specific interactions on adaptation and further examine the time dependency of the adaptation. It should be underlined that GSH plays a role in short term adaptation, whereas Nrf2-mediated mechanisms provides protection on a longer term, which hypothetically confirms our findings (Sthijns et al. 2016), but should still be verified experimentally. Acrolein is taken up rapidly by passive diffusion, whereas silver nanoparticles are taken up by endocytosis and then subject to intracellular dissolution (Behra et al. 2013; Stevens and Maier 2008) which agrees more with a long term exposure.

In conclusion, AgNPs can induce hormetic adaptation. GSH is related to toxicity but not to hormesis, which is in contrast to findings with acrolein in which GSH is linked to both toxicity and hormesis. These findings have implication with regard to the use of AgNPs in medicine as well as to risk assessment, because consumers are repeatedly exposed to a relatively high concentration of AgNPs. The precise molecular mechanism of hormesis by AgNPs is still enigmatic, but Nrf2 mediated signaling seems to be involved. In view of the established importance of oxidative stress and redox-signaling in the toxicity of many nanomaterials it will be interesting to investigate whether other types of nanoparticles also can induce hormetic adaptation via a similar mechanism as AgNPs.

Supplement

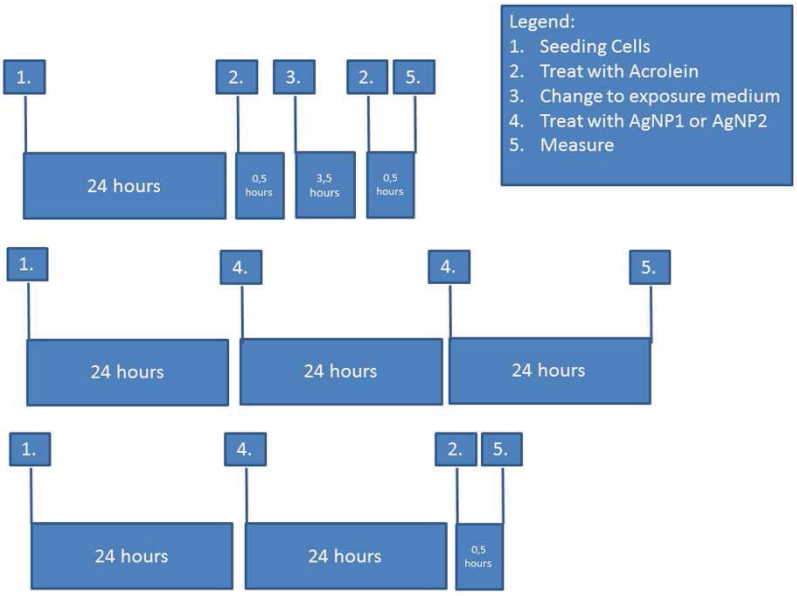

Figure S1. Timeline for treatments to investigate the adaptation principle in A549 cells.

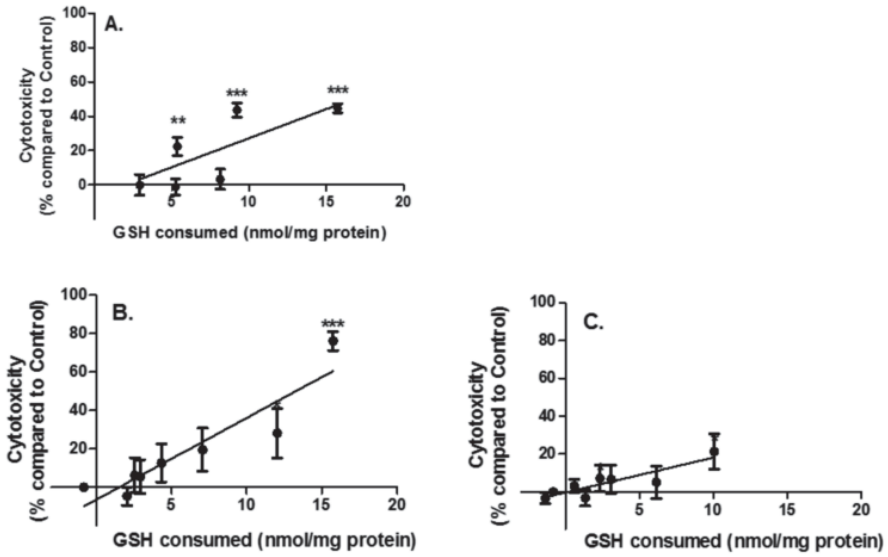

Figure S2. Association of cytotoxicity and the amount of GSH consumed in A549 cells exposed to Acrolein (A), AgNP1 (B) and AgNP2 (C). N=3 and data are presented as mean±SEM. *P < 0.05; **P < 0.01; ***P < 0.001 compared to control.

Acknowledgements

Part of this study was financially supported by the Federal Ministry of Education and Research (BMBF) under the framework of the ERA-NET SIINN call “Safe Implementation of Innovative Nanoscience and Nanotechnology.” The authors thank Dr. Burkhard Stahlmecke (IUTA) for the electron microscopical characterization of the silver nanoparticles.

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Chapter IV

The role of uptake and intracellular glutathione level in the DNA damaging effects of titanium dioxide nanoparticles

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Study 3: The role of uptake and intracellular glutathione levels in the DNA damaging effects of titanium dioxide nanoparticles.

Declaration

The manuscript is submitted to a peer reviewed journal.

All experimental work was performed by Waluree Thongkam, except the analysis of the particle uptake by flow-cytometry

The impact on authoring this paper can be estimated in total with 90 %

4.1 Introduction

There has been some growing concern about the carcinogenicity of specific types of nanoparticles (NPs). At the same time, results from genotoxicity tests with NPs have been increasingly scrutinized (reviewed e.g. in Landsiedel et al. 2009; Magdolenova et al. 2014; Singh et al. 2009). Genotoxicity studies form an important component in cancer risk assessment of chemicals, and especially studies that have been performed with nanomaterials are under ongoing strong debate. In relation to the complex physico-chemical properties of nanomaterials it has been discussed that false negative or false positive findings may be found, for instance, due to assay interferences or specific handling protocols of the NP (Doak et al. 2009; Donaldson et al. 2010; Kumar and Dhawan 2013; Landsiedel et al. 2009; Stone et al. 2009). One of the nanomaterials that has gained such major attention in relation to genotoxicity testing is TiO_2. Indeed, the in vitro genotoxicity studies with TiO_2 NPs have revealed both negative and positive findings regarding DNA damage and other genotoxicity tests such as the micronucleus assay e.g. (Biola-Clier et al. 2017; Demir et al. 2013; Falck et al. 2009; Gerloff et al. 2009; Kansara et al. 2015; Landsiedel et al. 2009; Magdolenova et al. 2012; Warheit et al. 2007).

We recently investigated the cytotoxic and DNA damaging effects of a panel of 10 nanomaterials, including five different types of TiO_2 NPs, in three different human lung epithelial cell lines (Thongkam et al. 2017). These studies were carried in the setting of the EU nanosafety project ENPRA (Kermanizadeh et al. 2016) to evaluate human health risks of NPs in various organ systems including lung, liver and kidney. In this context, DNA damage by the NPs was evaluated in the A549 lung, HK-2 kidney and HepG2 liver human epithelial cell lines. Among other findings, this study revealed that different cell lines responded differently to the same type of nanomaterial, including the TiO_2 NPs (Thongkam et al. 2017). These findings let us to conclude, however, that the observed differences in strengths of effects cannot be directly extrapolated to organ-specific hazards. Instead, we hypothesised that the effects are due to cell line specific differences in cellular pathways that impact on DNA damage by the NPs.

The DNA damaging properties of many types of NPs have been linked to their ability to induce oxidative stress upon their interaction with cells (Donaldson et al. 2010; Knaapen et al. 2004). In a more general manner, a hierarchical model of oxidative stress has been proposed by Nel and co-workers as a mechanism to explain for the effect of NPs on the level of the cell (Xia et al. 2006). This also includes the concept that low levels of oxidative stress activate a protective response in cells, while higher levels of oxidative stress respectively trigger responses leading to inflammation and cell death.

The protective cellular response involves the transcription factor nuclear factor erythroid 2 -related factor 2 (Nrf2). Oxidative stress causes dissociation of Nrf2 from its inhibitor Kelch-like ECH-association protein 1 (Keap 1) and its subsequent translocation into the nucleus were it binds to antioxidant response elements (AREs) within the promoters of antioxidant genes including γ–glutamylcysteine synthetase (γ-GCS) and heme oxygenase-1 (HO-1) (Itoh et al. 1997; Jaiswal 2004; Ma 2010). The γ-GCS enzyme, also known as glutamate cysteine ligase, is the rate limiting enzyme in the de novo synthesis of glutathione (GSH) (Rahman and MacNee 1999). The thiol-containing tripeptide GSH is a key antioxidant factor in many organs including the lung, the liver and the kidney. In alignment with the oxidative stress paradigm for NPs we therefore hypothesise that the cell line specific differences in DNA damage by NPs as we recently observed (Thongkam et al. 2017) can be explained by cell line specific differences in the constitutive intracellular levels of GSH and/or activation of the Nrf2-γGCS-GSH axis.

The aim of the present study was to investigate the role of the GSH antioxidant defence system in the heterogenic DNA damage responsiveness to TiO_2 NPs as previously observed in the A549, HK-2 and HepG2 cell lines. We hypothesise that the DNA damaging properties of the TiO_2 NPs are related to differences in constitutive levels GSH between the three cell lines. From the five TiO_2 NP samples that were investigated in our recent study (Thongkam et al. 2017), we selected the one that tended to show the strongest DNA damaging effects (in the absence of cytotoxicity). For comparison we also included the uncoated ZnO NP sample, which in our previous study revealed clear DNA damaging properties in all three cell lines (Thongkam et al., 2017). In addition, we evaluated the role of TiO_2 uptake in the three cell lines to address whether this would provide further insight in the cell line specific differences in DNA damage induction in relation to dosimetry differences.

4.2 Materials and Methods

Chemicals. Trypsin, Dulbecco's Ca^{2+}/Mg^{2+} -free phosphate buffered saline (PBS), agarose, low melting point (LMP) agarose, Triton X-100, DMSO, ethidium bromide, ethylenediamineteraaceteic acid (EDTA) disodium salt dehydrate, 5,5-Dimethyl-1-pyrroline N-oxide (DMPO), Buthionine sulphoximine (BSO), Glutathione-reductase, β-nicotinamide adenine dinucleotide 2´-phosphate reduced tetrasodium salt hydrate (NADPH), 5,5'-Dithiobis-(2-Nitrobenzoic Acid (DTNB), 5-Sulfosalicylic acid dehydrate (SSA), KH2PO4, K2HPO; bicinchoninic acid (BCA) assay kit, Dulbecco's Modified Eagle Medium (DMEM), RPMI-

1640 medium, foetal calf serum (FCS), bovine serum albumin, adult bovine serum (ABS), isopropanol and ethanol were obtained from Sigma (Germany). TRIzol® and DMEM/F12H were purchased from Gibco Life Technologies (Germany), iScript cDNA was purchased from Invitrogen (Germany), QuantiFast SYBR Green PCR Kit from Qiagen (Germany), goat serum from Vector Laboratories,Nrf2 antibody from Santa Cruz Biotechnology,AlexaFluor 594 from Life Technologies and water-soluble tetrazolium salt WST-1 reagent was obtained from Roche (Germany).

Nanomaterials. The TiO_2 and ZnO NPs that were investigated form part of the European Commission Joint Research Centre (JRC) repository list of nanomaterials, i.e. TiO_2 (anatase, 7 nm; NM101) and ZnO (uncoated 100 nm; NM110). Suspensions of the NPs were prepared according to the protocol that was developed within the ENPRA project, which included a detailed characterization of their dispersion within the generated dispersions (Jacobsen et al. 2010; Kermanizadeh et al. 2016). Briefly, stock solutions of 2.56 mg/ml were prepared in sterile RNase free water with 2% Adult Bovine Serum (ABS) and sonicated for 16 min without pause, on ice, using a Branson 450 Sonifier equipped with a 13 mm disruptor horn. After sonication, the NPs were kept on ice until dilution in complete medium. Suspensions were applied to the tests within 1 h after sonication. Hydrodynamic diameters and zeta-potentials achieved in the NM suspensions by the protocol have been published elsewhere (Jacobsen et al. 2010; Kermanizadeh et al. 2013).

Cell lines. Three human epithelial cell lines were used, i.e. A549 alveolar adenocarcinoma cells, HepG2 hepatocellular carcinoma cells and HK-2 proximal tubule epithelial cells. The A549 cells were cultured in DMEM supplemented with 4.5 g/L glucose and 10 % FBS, the HepG2 cells in RPMI-1640 medium supplemented with 10 % FBS and the HK-2cells in DMEM/F12H with 10% FCS; 100 U/ml penicillin-streptomycin was added to all media. Cells lines were cultured at 37 °C and 5% CO_2. During experimentation, the aforementioned culture media were replaced with phenol red free media.

Treatment of cells. Treatment concentrations of the NPs were selected from cytotoxicity results by WST-1 assay following 24 h treatment at 80, 40, 20, 10, 5, 2.5, 1.25, 0.63 and 0 $\mu g/cm^2$ as previously reported (Thongkam et al., 2017). Concentrations causing loss in cell viability of 20 % (TC_{20}) and 50% (TC_{50}) were calculated from the fitted dose-response curves

and are shown in table 1 for the three cell lines. Based on these results, for the present study all three cell lines were treated with the TiO_2 at the nominal mass per surface area dose of 40 µg/cm^2, equivalent to 128 µg/ml. For the ZnO, the concentration of 10 µg/cm^2 (i.e. 32 µg/ml) was chosen for the three cell lines. The cells were treated for 4 h with the freshly prepared suspensions of the NPs to evaluate intracellular GSH depletion, mRNA expression analysis of γ-GCS and HO-1, activation of Nrf2, uptake of the NPs and DNA damage. To evaluate the role of the intracellular GSH status cells were pre-treated for 18 h with the GSH synthesis inhibitor buthionine sulfoximine (BSO) at a concentration of 10 µmol/ml.

Table 4-1. TC_{20} and TC_{50} values in µg/cm^2 of NM101 and NM110 in A549, HK-2 and HepG2 cells. The treatment concentrations causing loss in cell viability of 20 % (TC_{20}) and 50% (TC_{50}) were calculated from fitted dose-response curves obtained from the WST-1 tests. The nominal mass per surface dose of 80 µg/cm^2 is equivalent to 256 µg/ml.

	NM101 (TiO_2)		**NM110 (ZnO)**	
	TC_{20}	**TC_{50}**	**TC_{20}**	**TC_{50}**
A549 cells	> 80	> 80	15	44
HK-2 cells	> 80	> 80	< 1	19
HepG2 cells	> 80	> 80	30	75

Intracellular GSH levels. For the determination of GSH, the cells were seeded in 6 well plates with complete culture media (1.5 x 10^5 cells per well, equivalent to 15,625cells per cm^2). Following treatment, cells were rinsed twice with ice cold PBS and then scraped in 1 ml KPE-buffer (1:6.5 phosphate buffer A [0.1 M KH_2PO_4] and phosphate buffer B [0.1 M K_2HPO_4*$3H_2O$] 10 mM EDTA, pH 7.5), plus 0.1% Triton-X-100 and 0.6% SSA. The suspensions were then sonicated in Bandelin Sonorex water bath and vortexed three times (30 s each), and then centrifuged at 6200 rpm for 5 min at 4°C. Supernatants were collected and 50 µl aliquots were used for GSH analysis, while 10 µl aliquots of the supernatants were used for protein content analysis. A mixture of 0.8 mM NADPH and 0.6 mM DTNB (1:1) was add to each sample or GSH standard in a 96-well plate, followed by the immediate addition of 50 µl GSH reductase (4 units/ml). The absorbance was measured at 412 nm for five times during a 2

min interval to determine GSH levels. Calculated values were adjusted to the total protein content as determined by the BCA-assay according to the manufacturer's protocol.

RNA isolation and Semi- quantitative Real-Time Reverse Transcription polymerase Chain Reaction (qRT-PCR). For the mRNA expression analysis, cells were seeded in 6 well plates with complete culture media (1.5 x 10^5 cells per well, equivalent to 15,625 cells per cm^2).Following treatment of the cells total RNA was isolated by Trizol© method as described previously (van Berlo et al. 2014). RNA purity and quantity were measures by ultraviolet spectrophotometry at optical density 260 and 280 nm. The quartz cuvette for analysis contain 5 µl of the isolated RNA sample diluted in 95 µl RNase free water. The synthesis of complementary DNA (cDNA) were process by iScript cDNA synthesis kit from BioRad. Amount of 0.5 µg per sample was transcribed into cDNA using iScript cDNA synthesis Kit according to the kit protocol. The optimal transcription process program for temperature steps was adjusted in the thermal cycler as follow 5 min at 25 °C, 30 min at 42°C, 5 min at 85°C and hold at 4 °C for cooling step. Total amount of mRNA was analysed in the Rotor-Gene Q (Qiagen) in 47 cycle using 7.5 ml of QuantiFast SYBR Green PCR Kit, 25 ng of cDNA and, 0.5 µM Primer. Human β-Actin used as the internal reference gene. Primer sequences for HO-1 were 5′-AAC TTT CAG AAG GGC CAG GT-3′ (forward) and 5′-CCT CCA GGG CCA CAT AGA T-3` (reverse), for γ-GCS (light chain) were 5′-GAC AAA ACA CAG TTG GAA CAG C-3′ (forward) and 5′-CAG TCA AAT CTG GTG GCA TC-3′ (reverse) and for β-actin 5′- CCC CAG GCA CCA GGG CGT GAT-3′ (forward) and 5′-GGT CAT CTT CTC GCG GTT GGC CTT GGG GT-3′ (reverse).

DNA damage analysis by alkaline comet assay. For the comet assay, cells were seeded in 6-well plates with complete culture media (3.8 x 10^5 cells per well, equivalent to 40,000 cells per cm^2), incubated at 37 °C, 5% CO_2 for 24 h and then treated for 4 h with the NPs. DNA damage by the NPs was evaluated by the comet assay according to the method as described previously (Thongkam et al., 2017). All washing and incubation steps were performed in the dark or red light, to avoid potential artificial DNA damage induction. The comet appearances were analysed using an Olympus Bx60 fluorescence microscope at 400 x magnification using the comet Assay II comet image analysis software program (Perceptive Instruments, UK). DNA damage was quantified by analysis of % DNA in tail (tail intensity) following counting of 50 randomly selected cells per slide per experiment.

Immunohistochemistry for Nrf2. Detection of Nrf2 protein was performed as already described previously (Sthijns et al. 2017). Briefly, cells were seeded in 4-chamber-slides (BD Falcon) at a density of 1 600 000 cells/well. After 4 h exposure to the NPs on the next day, cells were fixed with 4 % paraformaldehyde/PBS (pH 7.4) for 20 min. Following permeabilisation with 0.1% Triton X-100/ PBS for 5 min, unspecific blocking was performed using normal goat serum (Vector Laboratories, Burlingame, CA, USA) /PBS (1:65 dilution) for 30 min. Next, cells were incubated overnight at 4 °C with a 1:50 diluted primary Nrf2 antibody (C-20, Santa Cruz Biotechnology, Inc.), which was detected by a AlexaFluor 594 secondary goat anti-rabbit IgG antibody /PBS (Life Technologies) at 1:200 dilution. Counterstaining was performed using Hoechst 33342 (Cell Signaling)/PBS nuclear dye at a concentration of 1 µg/ml. After mounting with Prolong Gold Anti-fading (Life Technologies) images were analysed using fluorescence microscope (Zeiss AxioVertMicroscope) equipped with an AxioCam MRm camera controlled by an image analysis system (Zeiss (ZEN) software) at 400 X magnification.

Particle uptake measurement. Particle uptake measurement. For the evaluation of uptake of the TiO_2 NPs, a flow-cytometry measurement was used based on the approach developed by Stringer and co-workers for macrophages(Stringer et al. 1995) and modified for epithelial cells (Li et al. 2007; Neumeyer et al. 2011). The side scatter of the cells relates to their granularity, which is used as a measurement of particle uptake. The cells were seeded in 6-wells plates (3.8 x 10^5 cells/cm^2) for 24 h with or without pre-treatment with BSO and then treated with TiO_2 for 4 h. An additional treatment on ice was used to identify the proportions of attached or internalized particles. Following treatment, the cells were rinsed 2 times with PBS buffer. Cells were scraped in 1 ml ice-cold PBS buffer and transferred to FACs tubes followed by two washes with PBS buffer, and then centrifuged at 5000 rpm for 5 min. Cells were then resuspended in 1 ml PBS buffer and analysed using Becton Dickinson FACSCalibur II and FACS Diva software. Dotplot histograms of side (SSC) and forward (FSC) scatter of cell free TiO_2 suspensions and control cells were used to discriminate free particulate events from cellular events. For all subsequent measurements of untreated and treated cells, the "free particle events" fraction was excluded to measure the cellular particle uptake. Univariant histograms of SSC provided the median of cell granularity used as indicator of the particle uptake.

Statistical analysis. Data were obtained from at least three independent experiments for each cell line, and were analysed using IBM-SPSS (version 22). Data are expressed as mean ± SEM

unless stated otherwise. Effects of TiO_2 and ZnO NPs on intracellular glutathione levels and mRNA expression were evaluated using one-way analysis of variance (ANOVA) with post-hoc analysis according to Tukey's method. DNA damage and uptake effects of TiO2 including the role of BSO and cold temperature were evaluated by Student's t-test. Differences were considered statistically significant at $p < 0.05$.

4.3 Results

The thiol-containing tripeptide GSH represents a major cellular antioxidant compound and analysis of its depletion has been widely applied as a marker of oxidative stress in the toxicity screening of NPs. The effects of TiO_2 (40 $\mu g/cm^2$) and ZnO (10 $\mu g/cm^2$) on the intracellular GSH levels in the three human epithelial cell lines are shown in Figure 4-1. TiO_2 did not cause any significant depletion of GSH in the A549, HK-2 and HepG2 cells. As can be seen in the figure, there were substantial differences in the GSH levels among the three cell lines in the absence of NM treatment (i.e. A549 > HepG2 > HK-2). In contrast to the TiO_2, the ZnO caused significant depletion of GSH in both the A549 cells and the HepG2 cells. It should be noted that these effects occurred at a 4-fold lower treatment dose than that of the TiO_2 NPs. Interestingly, GSH depletion by ZnO was not significant in the HK-2 cells while the applied dose causes marked cytotoxicity in this cell line at 24 h treatment (see Table 4-1).

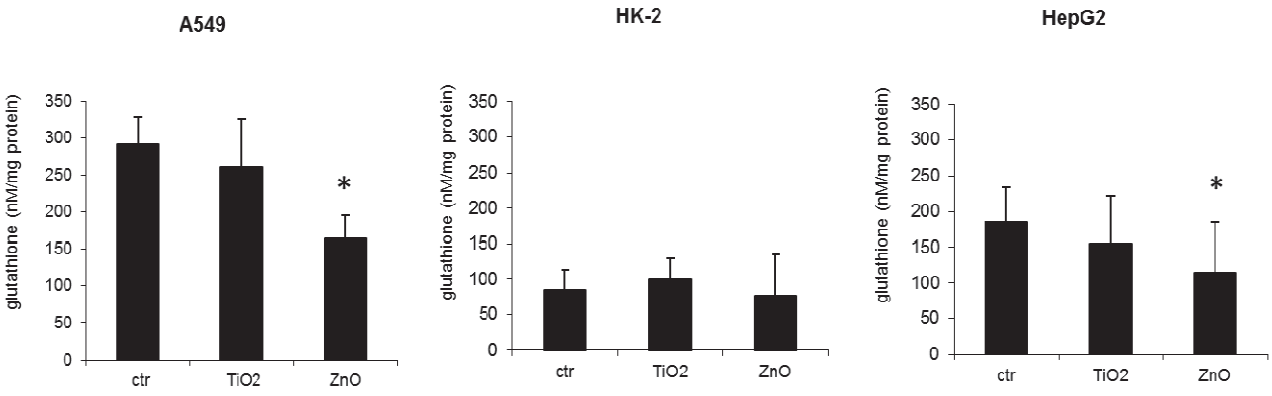

Figure 4-1: Intracellular GSH levels in A549, HK-2 and HepG2 cells following 4 h treatment with TiO_2 (40 µg/cm^2) and ZnO (10 µg/cm^2). * p<0.05 versus controls (ctr).

To further evaluate oxidative stress related effects of both NPs in the three cell lines, we evaluated the mRNA expression of the genes γ-GCS and HO-1 (see Figure 4-2). In concordance with the GSH depletion findings, the TiO_2 treatment did not lead to a significant induction of the mRNA levels in any of the cell lines, although some upregulation was noted particularly in the HK-2 cells. The ZnO triggered in a pronounced upregulation of both mRNAs in all three cell lines. Interestingly, for the stress-response marker gene HO-1 the strongest effect was observed in the HK-2 cells, whereas γ-GCS, involved in GSH synthesis, was most strongly enhanced in the HepG2 cells. Taken together, the results indicated that TiO_2 did not cause a marked oxidative stress in any of the three cell lines in contrast to the ZnO at 4-fold lower treatment concentration.

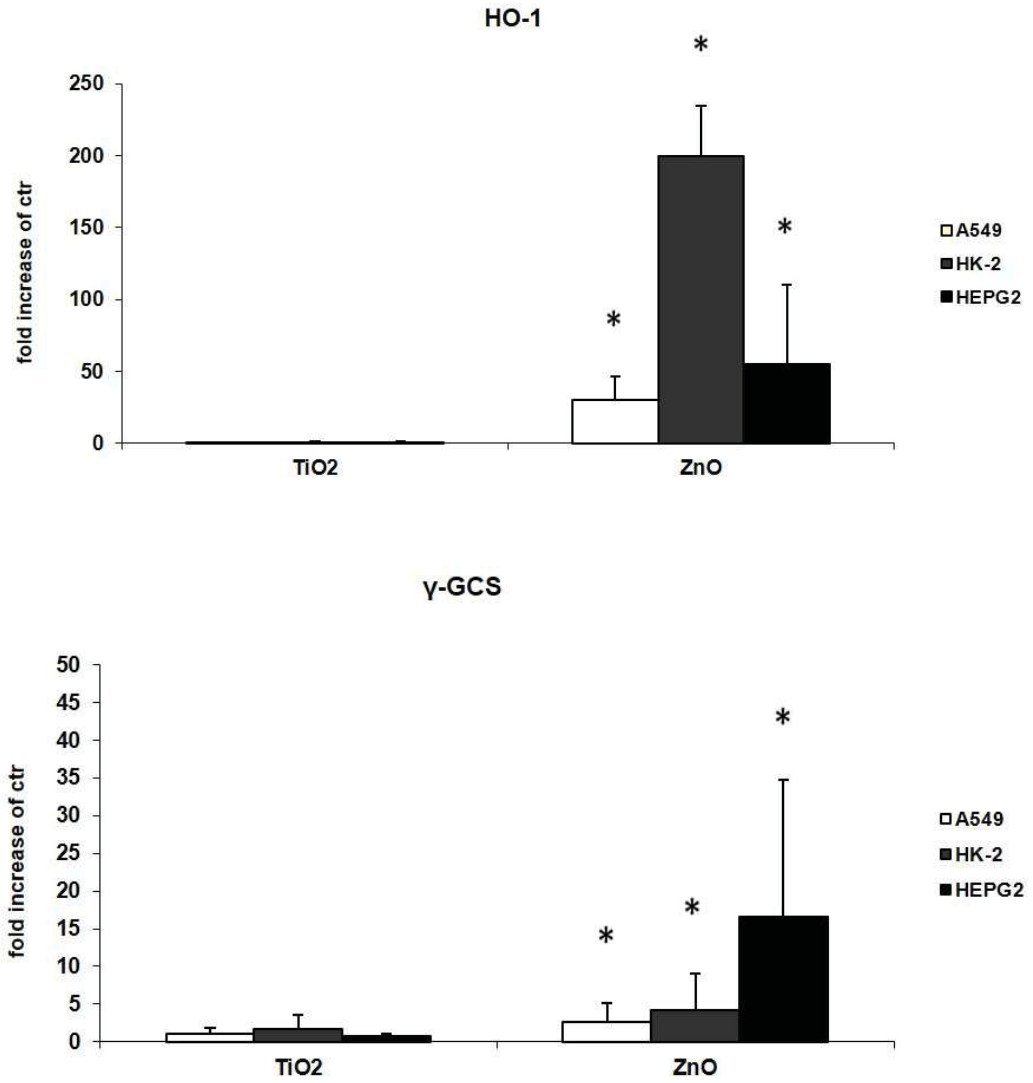

Figure 4-2: mRNA levels of Heme Oxygenase-1 (HO-1) and gamma-glutamylcysteine synthetase (γ-GCS) in A549, HK-2 and HepG2 cells following 4 h treatment with TiO_2 (40 µg/cm^2) and ZnO (10 µg/cm^2).
*** p<0.05 versus controls (ctr).**

The expression of both HO-1 and γ-GCS is known to be regulated by the transcription factor Nrf2. Activation of this pathway involves its translocation into the nucleus, which was evaluated by immunohistochemistry in the A549 cells for further evaluation of the effects of TiO_2 versus ZnO (See Figure 4-3). In cells exposed for 4 h to TiO_2, activation seemed negligible while for the ZnO a clearly enhanced nuclear staining could be observed, indicative of activation of this transcription factor

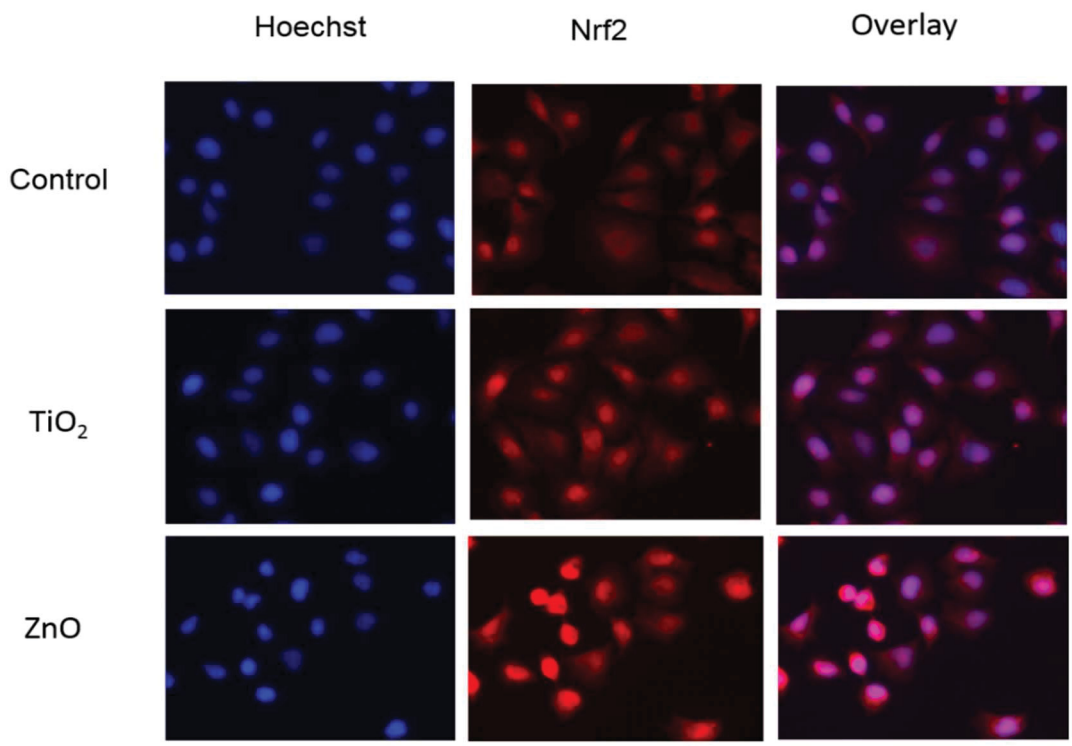

Figure 4-3: Immunohistochemical staining for Nrf2 in A549 cells after 4 h treatment with TiO_2 (40 µg/cm²) or ZnO (10 µg/cm²) at 400×magnification, in comparison to controls. Cells were stained for Nrf2 (red) and counterstained with Hoechst 33342 (blue).

To further evaluate the role of intracellular glutathione in the DNA damaging properties of the TiO_2 NPs, we compared DNA damage induction by comet assay in all three cell lines after 4 h treatment with or without 18 h pre-treatment with the GSH-synthesis inhibitor BSO. Results are shown in Figure 4-4. In line with our previous findings, the TiO_2 damaging effects tended to be most pronounced in the HK-2 cells. A statistically significant increase in DNA damage was observed only in these cells without BSO pre-treatment. In all three cell lines, the BSO pre-treatment tended to results in some increasing DNA damage, but these effects were not statistically significant. In the cells that were pre-treated with BSO, TiO_2 caused significant DNA damage in the HK-2 and HepG2 cells, but not in the A549 cells. In both cases, the TiO_2 effects in the BSO pre-treated cells were also significantly stronger than in the respective cell lines following pre-treatment with BSO alone.

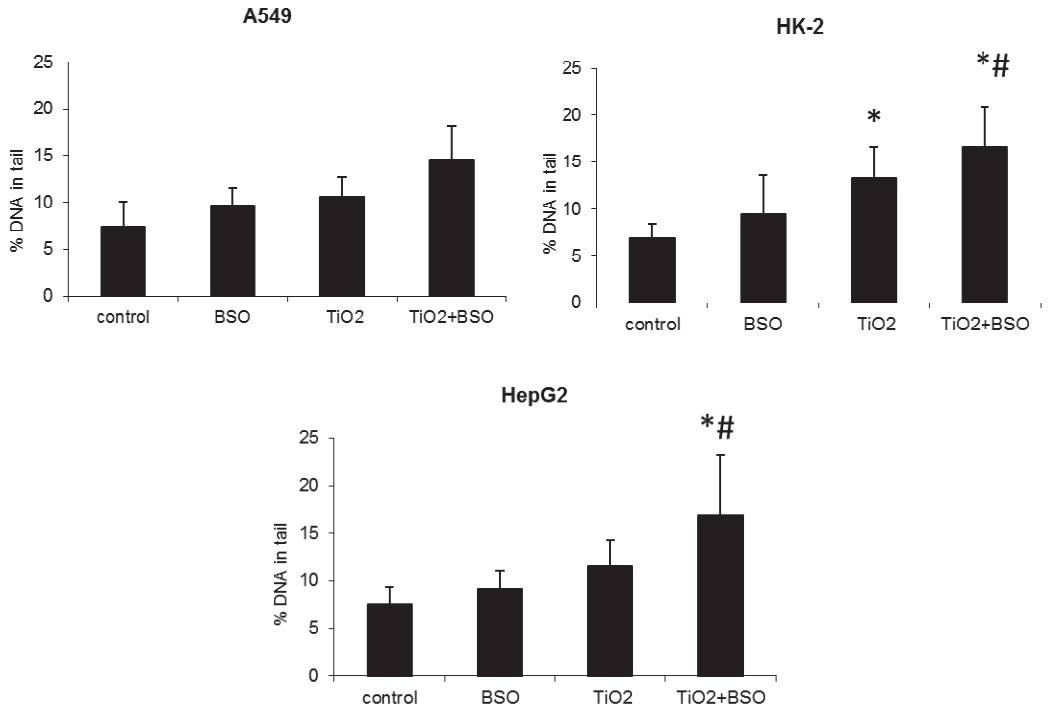

Figure 4-4: DNA damage in A549, HK-2 and HepG2 cells after BSO pre-treatment and/or treatment with TiO_2. DNA damage was evaluated by comet assay in the three cell lines, either with or without pretreatment with BSO (10 µmol/ml) for 18 h with BSO and subsequent treatment for TiO_2 (40 µg/cm^2) for 4 h.
*** p<0.05 versus controls and # p<0.05 versus BSO pre-treated controls.**

In order to address the role of cell uptake in the DNA damaging effects of TiO_2 in the three epithelial cell lines we used flow cytometry. Uptake was evaluated after 4 h treatment of the cells with TiO_2. To discern between internalisation and adherence on the cell surfaces, we also evaluated the cells upon treatment with TiO_2 on ice, and to address the potential influence of GSH status also BSO pre-treatment groups were included. The results of the uptake analyses are shown in Figure 4-5, while representative flow-diagrams are shown in Figure 4-6.

The flow cytometry analysis indicated uptake of the TiO_2 in all three cell lines (see Figure 4-5). Comparison of the SSC for the three cell lines (see Figures 4-5 and 4-6), indicated that that uptake after the 4 h treatment was more pronounced for the HK-2 cells than the A549 and HepG2 cell lines. The BSO pre-treatment alone did not affect the SSC signals and also did not cause a significant effect on TiO_2 uptake for any of the cell lines. Granularity was also not significantly affected in the control cells that were incubated on ice, whereas this resulted in a significantly reduced TiO_2 uptake in all three cell lines. Interestingly, the inhibitory effect seemed markedly stronger for the A549 cells and the HK-2 cells, than for the HepG2 cells. This suggests that for these latter cell type the proportion of internalised cells was relatively lower than for the other two cell lines.

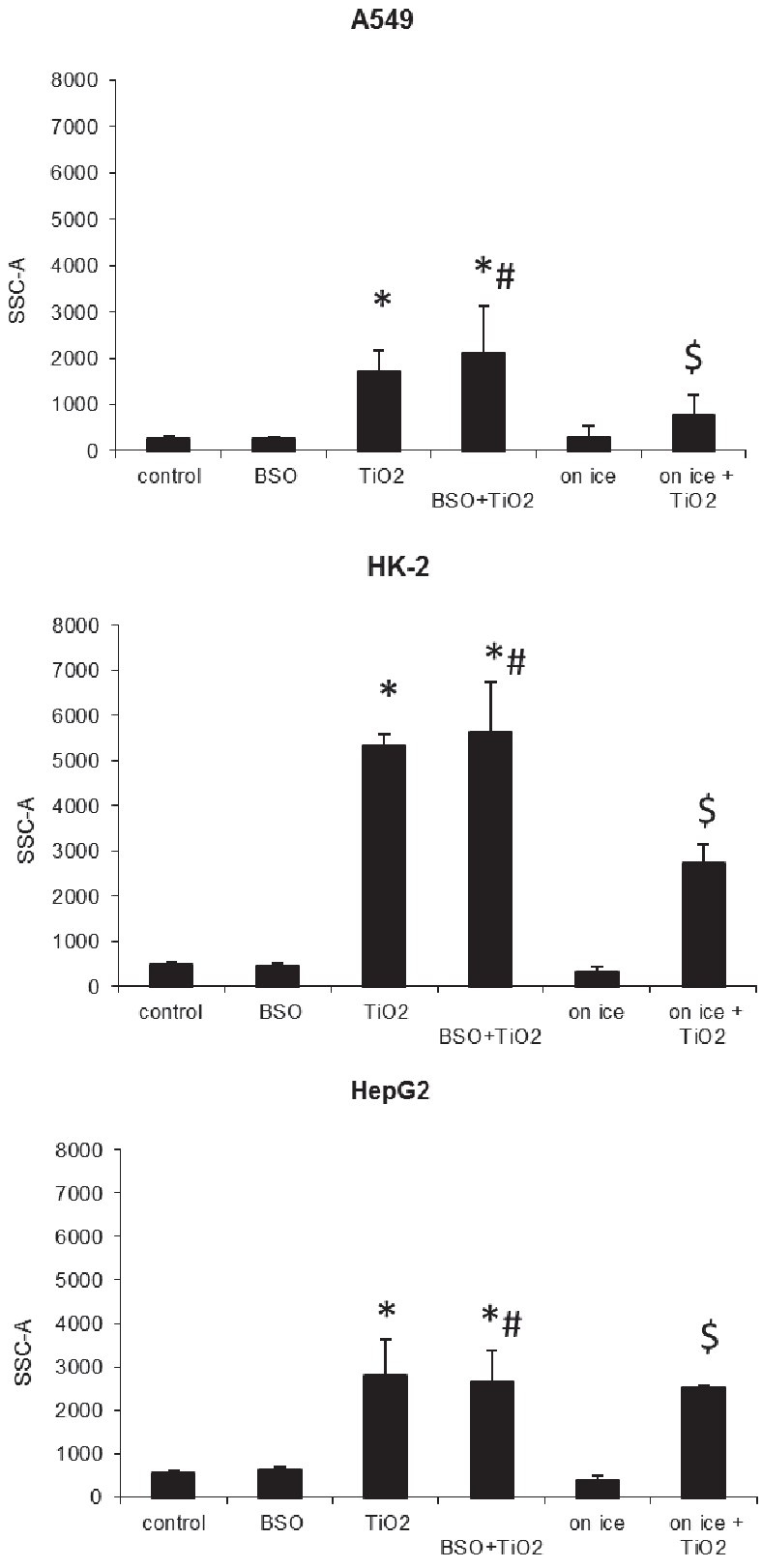

Figure 4-5: Evaluation of uptake of TiO_2 in A549, HK-2 and HepG2 cells by flow cytometry. Uptake was evaluated by flow cytometry analysis. Cells were exposed for 4 h to TiO_2 (40 µg/cm^2) or control medium at 37°C with or without 18 h pre-treatment with BSO (10 µmol/ml), or alternatively on ice for 4 h with either TiO_2 (40 µg/cm^2) or control medium. * $p<0.05$ versus controls, # $p<0.05$ versus BSO pre-treated controls and $ versus TiO_2 treated cells at 37 °C (TiO_2).

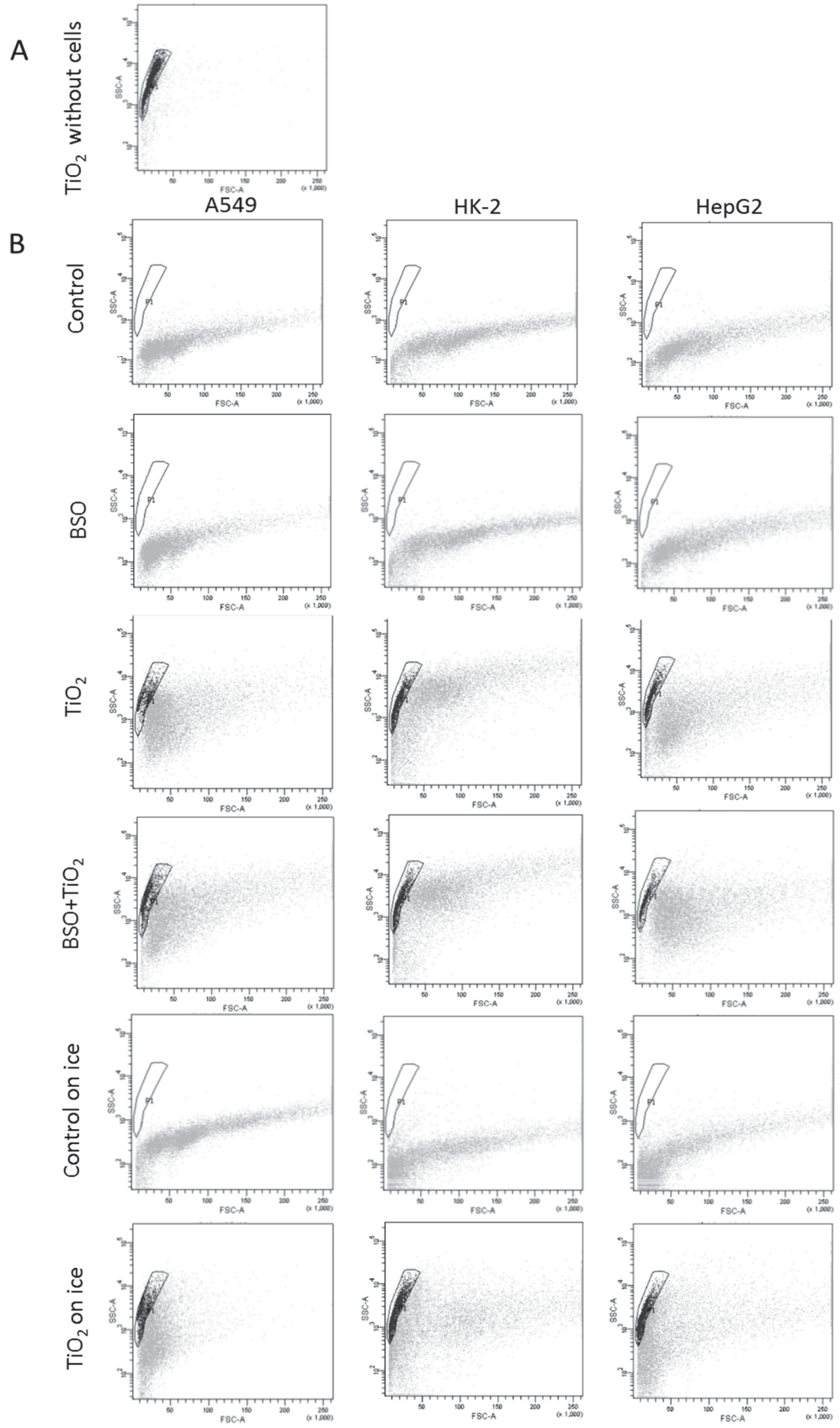

Figure 4-6: Representative flow-diagrams for the analyses of uptake of TiO_2 NPs. The dotplot histograms of side (SSC) and forward (FSC) scatter of cell free TiO_2 suspensions (panel A) and control cells (panel B, top row histograms) were used to discriminate free particulate events from cellular events and subsequent quantification of uptake.

4.4 Discussion

The present study was undertaken to investigate the mechanisms of DNA damage induction by TiO_2 NPs with specific focus on the involvement of the GSH antioxidant defence system. Our investigations were predominantly triggered by our recent observation on the contrasting DNA damaging properties of TiO_2 NPs in the three investigated epithelial cell lines (Thongkam et al., 2017), and independent published literature that show both negative and positive findings regarding DNA damage and other genotoxicity assays with this type of NP (Biola-Clier et al. 2017; Demir et al. 2013; Falck et al. 2009; Gerloff et al. 2009; Kansara et al. 2015; Magdolenova et al. 2012).

The results from our study indicate that GSH plays a major role in the defence against the DNA damaging properties of TiO_2 NPs, as revealed in two complementary ways. First, it was observed that the DNA damaging properties of the TiO_2 differed among three cell lines in association with their constitutive intracellular levels of GSH. The TiO_2 NPs induced DNA damage only in the HK-2 cells, the cell line with the lowest constitutive GSH level. In addition, the DNA damaging properties of TiO_2 were found to be enhanced upon blocking of the *de novo* GSH synthesis of BSO. Under these conditions, the TiO_2 also caused DNA damage in the HepG2 cells, the cell line with intermediate constitutive intracellular GSH levels, while the DNA damage in the HK-2 cells became significantly more pronounced. In the A549 cells, the cell line with the highest GSH levels, DNA damage did not reach statistical significance, irrespective of BSO pre-treatment.

Our findings on the association between GSH levels and DNA damage by TiO_2, suggests that these NPs cause DNA damage in an oxidative stress dependent manner. Surprisingly, however, the same treatment concentrations of TiO_2 did not cause a significant reduction in the levels of GSH, and also did not trigger an increased mRNA expression of HO-1 and γ-GCS in any of the three cell lines. Intracellular GSH depletion and increased expression of HO-1 and γ-GCS have been long recognised as markers of oxidative stress (reviewed in Rahman and MacNee 1999; Tyrrell and Basu-Modak 1994). These markers have also been used specifically to evaluate oxidative stress responses by NPs (Gerloff et al., 2009; Stone et al., 2009; Gerloff et al., 2013; Dubey et al. 2015). Our present study findings indicate that TiO_2 causes DNA damage in a glutathione dependent manner, while triggering only minimal oxidative stress. Importantly, this phenomenon is observed under non-cytotoxic conditions (see also Table 4-1). It is likely that, in line with findings by several other investigators (Monteiller

et al., 2007; Jin et al., 2008; Dubey et al., 2015), that higher (sub)toxic concentrations of TiO_2 exert significant effects on intracellular GSH levels and the Nrf2-γGCS-GSH axis.

In contrast, the ZnO NPs caused significant increases in HO-1 and γ-GCS mRNA levels in all three cell lines, and a significant depletion of GSH in the A549 and HepG2 cells. This indicates that the strong DNA damaging effects of the ZnO NPs that we observed previously (Thongkam et al. 2017) take place in a condition of marked oxidative stress. The lack of significance in GSH depletion in the ZnO treated HK-2 cells can be likely explained by the markedly higher cytotoxicity of this nanomaterial in this renal cell line (see also Table 4-1). The contrasting oxidative stress inducing properties of the TiO_2 and ZnO nanomaterials was also revealed from their contrasting abilities to activate the transcription factor Nrf2. As such, the findings from our study with the TiO_2 NPs do not seem to align with the hierarchical oxidative stress model proposed for NPs (Xia et al. 2006), in relation to DNA damage induction.

Previous investigations in our laboratory with Caco-2 human intestinal epithelial cells already indicated that TiO_2 NPs do not trigger marked oxidative stress responses in non-cytotoxic concentrations. Unlike TiO_2 NPs, ZnO caused a marked induction of oxidative DNA damage, measured by the formamidopyrimidine glycosylase (FPG) modified comet assay (Gerloff et al. 2009). In a subsequent study with 5 different TiO_2 samples, none of the NPs were found to affect markers of oxidative stress including HO-1 mRNA expression in the Caco-2 cells (Gerloff et al. 2012). In addition, all samples also failed to cause significant oxidative DNA damage induction measured by fpg-comet assay, whereas one of the samples caused DNA strand breakage (fpg-independent comet). Although the fpg-modified comet assay was not applied in present study, altogether, these findings suggest that the DNA lesions observed by the comet assay in our present study, are merely non-oxidative. Further research is needed to explore the mechanisms and types of DNA damage induced by TiO_2 at these non-cytotoxic concentrations. Interestingly, findings from a recent study in A549 and BEAS-2B lung epithelial cells revealed a downregulation of DNA repair proteins following TiO_2 NPs treatment (Biola-Clier et al., 2017). Early investigations with radiation or cisplatin treatment already revealed that glutathione levels can also influence the repair of DNA lesions, including DNA strand breaks (Evans et al. 1984; Lai et al. 1989). Thus, the observed differences in sensitivity of cell lines to DNA damage by TiO_2 NPs may be related to cell-specific, GSH-mediated, effects on DNA repair.

It may be argued that the observed differences in effects of the TiO_2 NPs between the three cell lines could also be due to dosimetry differences. This aspect has been increasingly discussed in recent years in relation to comparison of different types of nanomaterials. Due to differences in

particle size, density and agglomeration behaviour, of NP suspensions of different composition will settle at different velocities onto the cell monolayer surfaces. As such, this may lead to substantial differences in the nominal dose (concentration of NPs in the suspension) versus the effective does (particle number that interacts with cell surface) (Lison et al. 2008; Teeguarden et al. 2007). However, in the present study, one single type and batch of TiO_2 was used with one identical NP-suspension protocol for all three cell lines. From this it can be concluded that that the different cell lines received the same TiO_2 doses. However, flow-cytometry was used to determine uptake of the TiO_2 NPs in the three cell lines. The comparison of uptake at 37°C versus ice indicated that TiO_2 internalisation was the most pronounced for HK-2 cells, and the least pronounced in the HepG2 cells. In line with our current findings, Lankoff and co-workers reported differences in kinetics of uptake of TiO_2 NMs using flow cytometry in HepG2, A549 and THP-1 cells (Lankoff et al. 2012). Our findings indicate that, despite equal dosing, the "effective intracellular concentrations" could have been substantially different between the three different cell lines. Thus, the stronger DNA damaging effect in the HK-2 cell line may be related to some extent to the larger internal TiO_2 dose. However, this cannot explain why the A549 cells were the least sensitive. Moreover, TiO_2 uptake was not significantly increased upon BSO pre-treatment for any of the cell lines cells. Together, this indicates that the DNA damaging potency of TiO_2 is independently related to its level of uptake (internal dose) as well as the intracellular level of GSH. To further unravel the contribution and mechanisms of uptake in the observed DNA damaging effect of the TiO_2 NPs, endocytosis mechanism-specific inhibitors can be applied as done previously in macrophage cells (Scherbart et al. 2011).

In summary, our study shows that depending on the cell line and its constitutive level of intracellular GSH, TiO_2 nanoparticles can cause DNA damage in the absence of cytotoxicity. In concordance with our recent study findings (Thongkam et al., 2017) it also further highlights that test outcomes with NPs can strongly depend on the choice of the cell. Glutathione seems to offer major protection against the DNA damaging properties of TiO_2 NPs. Cell lines with low consecutive levels of this cellular thiol-tripeptide have, depending on the amount of internalised particles, increased vulnerability for this ubiquitous nanomaterial.

Acknowledgement

This study was financially supported by the Federal Ministry of Education and Research (BMBF) under the framework of the ERA-NET SIINN call "Safe Implementation of Innovative Nanoscience and Nanotechnology."

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Chapter V

General Summary

5 General discussion and conclusions

Nanotechnology is an important research field for the development of innovative industrial and consumer products. With the increasing production of nanomaterials and their introduction on the market, exposure of the population to engineered NPs will likely increase. There is a concern about the safety of specific engineered NPs. Workers can be exposed to engineered NPs during manufacturing. There is also concern about the release and exposure to NPs from various consumer products during their use, or afterwards, from nanomaterial containing waste products. Therefore, toxicological research with NPs is of importance:

(1) To determine hazards and risks of new types of engineered NPs, and

(2) To identify (new) mechanisms of action.

The lung is considered as the most important organ with regard to the inhalation exposure and potential adverse health implications of engineered NPs. Decades of toxicological, epidemiological and clinical research have led to the recognition that inhaled particles like crystalline silica, asbestos and coal dust, can cause various severe lung diseases, such as COPD, emphysema, fibrosis or lung cancer (Donaldson et al. 2003; Rushton 2007). Experimental studies with silica and asbestos have demonstrated the importance of particle size and shape and ROS generation in the development of these diseases. Therefore, many toxicity studies with engineered NPs also focus on the evaluation of oxidative stress related effects in lung cells. In accordance with the increasing surface area of smaller particles, especially NPs, oxidative stress is considered as important factor in particle toxicity (Borm et al. 2007; Brauner et al. 2007; Weissenberg et al. 2010; Wessels et al. 2010) In vitro studies using lung cells as well as in vivo studies in rats and mice have demonstrated that NPs can cause stronger toxicity and oxidative stress than the same material in the bulk form. This is for instance shown for TiO_2 NPs in comparison to pigmentary grade (fine) TiO_2 particles (Oberdörster et al. 2005; Singh et al. 2007). Another important difference between NPs and larger particles is the location where toxic effects can occur. For inhaled particles, the respiratory tract system is the primary organ of exposure and associated pathologies (Knaapen et al. 2004). However, tiny particles like ultrafine particles in ambient air and engineered NPs can translocate via the air-blood-barrier to other organs in the body (Elder and Oberdorster 2006). In addition, it has been shown that oral exposure to NPs may also lead to their translocation into the bloodstream (Kreyling et al. 2017). This route of exposure is becoming of increasing interest in recent years because of the increasing use of engineered NPs in food industry and consumer applications (Bouwmeester et

al. 2009; Chaudhry et al. 2008). Various organs may also be targets for engineered NPs that are directly introduced into the body, for instance in intravenous applications and in implants (Polyzois et al. 2012; Simovic et al. 2011). It is therefore also relevant to study the toxicity of engineered NPs for other organ systems.

Nanomaterial safety and risk assessment research is an important factor for current and future successful developments in the nanotechnology research and industry sectors. Toxicological research should therefore concentrate on the identification of the mechanisms of action. The linking of specific physical and chemical properties of engineered NPs to the induction of specific adverse effects can be used for the design of safer nanomaterials. This is also known as the "safer-by-design" approach in nanotoxicology (Bastus and Puntes 2017). This concept also requires that toxicity screening tests are reliable and reproducible regarding the correct identification of toxic versus nontoxic NPs. Specific methods and assays that aim to increase our understanding about the underlying mechanisms of action should, of course, also be reliable. Assay artefacts caused by NPs in toxicity tests have been described and reviewed by several research groups (Alkilany et al. 2016; Doak et al. 2009; Stone et al. 2009; Tournebize et al. 2013).

The **aim of this thesis** was to investigate the cytotoxic and DNA damaging effects of various types of engineered NPs in relation to their composition. To unravel the underlying mechanisms, the research in this thesis focused on the evaluation of ROS generation and oxidative stress. Specifically, the role of GSH was investigated since this is the major antioxidant in the lung (reviewed by Biswas and Rahman, 2009). For the evaluation of the levels and mechanisms of toxicity of NPs it is also of crucial importance that the test methods are sensitive, specific and reliable (Azhdarzadeh et al. 2015; Stone et al. 2009). Therefore, in this thesis special attention was also drawn to the importance of the specific testing protocols.

In the first experimental study (**Chapter II**), the cytotoxicity and DNA damage induction was investigated for a panel of engineered NPs which included two types of MWCNT, two types of ZnO, 5 types of TiO_2 and one type of Ag NPs. The experiments were performed in three different cell lines. To study the effects of the various engineered NPs in relation to inhalation exposure, A549 human lung epithelium cells were used. HK-2 human proximal tubule epithelial cell and HepG2 human hepatocellular carcinoma cells were used, respectively, to address effects in liver and kidney as relevant secondary target organs. It was found, in line with literature findings, that there are strong differences in the toxicity of different types of engineered NPs. A main finding of this study also was, that different cell lines responded

different to the same NPs. The three epithelial cell lines were from different organs, namely lung, liver and kidney. It is thus tempting to conclude that the observed differences in strengths of effects relate to different hazards of specific NPs to these respective organs. However, the results from this study indicate that this may not be correct, because it is also important to consider the source and differentiation status of the cell lines. The A549 and HepG2 cell lines are both derived from tumours, whereas the HK-2 cell line is derived from normal kidney tissue and immortalised via transfection. Several investigators that oxidative stress, cytotoxicity and DNA damage effects of NPs can strongly vary with the source of the cell line. For instance, a study with cobalt-oxide NPs showed high sensitivity regarding cytotoxic and pro-inflammatory effects in the human bronchial epithelial cell line BEAS-2B, while the A549 cells responded on genotoxicity and oxidative DNA damage (Cavallo et al. 2015). Ursini and co-workers also showed that TiO_2 NPs cause strong cytotoxic effects in BEAS-2B cells while A549 cells are more sensitive regarding oxidative DNA damage induction (Ursini et al. 2014). Others investigators could show strong differences in the DNA damaging effects of Ag NPs among different epithelial cell lines, namely A549, HepG2 and HT-29 colon epithelial cells (Kruszewski et al. 2013). Not only for NPs, but also for chemical toxicants it has been shown that cytotoxic an DNA damage effects can differ to considerable extent in different cell lines, as shown for instance of cadmium (Fischer et al. 2016).

To evaluate the underlying mechanisms that could explain differences in toxicity of NPs, two of the tested nanomaterials, i.e. a ZnO and a TiO_2 sample, were further investigated (**Chapter IV**). Oxidative stress is considered as a major mechanism in DNA damage induction by NPs and GSH is known as a major antioxidant in cells and specifically in the lung. Large differences in the levels of GSH have been found in different cell types in the body, and also among different cell lines in cell culture. Therefore, the study focused on the role of this antioxidant system and the comparison between the three cell lines. Large differences in GSH levels were found between the A549, HepG2 and HK-2 cell lines. Also the effects of the GSH synthesis inhibitor BSO were found. The HepG2 cells were more resistant to BSO than the other two cell lines, which is also observed in several other studies (Ishida et al. 2012; Jin et al. 2011). The study also demonstrate that different types of NPs have different effects on the GSH antioxidant defence system and that this is cell specific. The ZnO caused a significant diminishment of GSH levels in the A549 and HK-2 cells, and in all three cell lines it caused upregulation of γGCS, the rate-limiting enzyme in the de novo synthesis of the GSH. The ZnO also activated the transcription factor Nrf2 and the oxidative stress marker gene HO-1. None of these effects were observed with the TiO_2 NPs. This also indicates that this nanomaterial does not cause

strong oxidative stress in cells in contrast to the ZnO. However, in the BSO pre-treated cells, TiO_2 treatment caused increasing DNA damage. This was significant in the HepG2 cells and the HK-2 cells, but not in the A549 cells. Together, the findings from the study indicate that, while TiO_2 does not induce strong oxidative stress, the DNA damaging properties of these NPs depend on the intracellular level of glutathione. Importantly, these contrasting effects of the TiO_2 NPs occurred at non-cytotoxic concentrations. Further research is needs to identify the mechanisms whereby GSH can protect cells from DNA damage by TiO_2. Investigations with radiation or cisplatin treatment showed that glutathione levels can also influence DNA repair, especially DNA strand breakage (Evans et al. 1984; Lai et al. 1989). Thus, it could be relevant to investigate the effects of TiO_2 on DNA repair in a future study.

The glutathione antioxidant defence system was also the main component of investigation in the study with silver NPs (**chapter III**). This study was performed on a study with acrolein, a ubiquitous environmental pollutant and for instance a main component of cigarette smoke (Stevens and Maier 2008). In BEAS-2B lung epithelial cells, hormetic effects were observed for acrolein that depended on the regulation of intracellular GSH levels (Haenen et al. 1988; Sthijns et al. 2014). The cytotoxic properties of two types of silver NPs were investigated in A549 cells and compared with the effects of acrolein. The first nanosilver was already investigated in A549, HepG2 and HK-2 cells with regard to DNA damage induction (**Chapter II**). An independent nanosilver sample was included for comparison. The two Ag NPs used in the study were of similar size but one was in powder form and the other as a colloidal suspension. The results showed that, in addition to acrolein, also silver NPs can trigger adaptation. However, the effects differed between the two Ag NPs. This demonstrates that the specific composition (physical and chemical properties) of Ag NPs determine cellular responses. A surprising finding was also that low, nontoxic concentrations of one type of Ag NPs, also protected the cells against higher doses of acrolein. This phenomenon may be defined as transhormesis. A final important finding was that the mechanisms of protection by the nanosilver and the acrolein was not the same. For the silver nanoparticle the role of GSH synthesis could not be confirmed. Both acrolein and the Ag NPs could activate the transcription factor Nrf2 in the A549 cells. However, acrolein also caused a significant induction of γGCS mRNA expression in the A549 cells, whereas Ag NPs only caused increased HO-1 mRNA expression. Other investigators have previously demonstrated that Nrf2 is an important factor in cell adaptation mechanisms (Calabrese et al. 2010; Schmidt et al. 2015). Theories suggest that adaptation not only depends on the chemical but also on the cell responses, which may involve more than one mechanism. Hormesis effects have been shown in various cells types

with specific chemicals, but also with other conditions such as radiation and temperature (Feinendegen 2005; Iwasaka et al. 2011; Liang et al. 2016; Nielsen et al. 2006; Schmidt et al. 2015).The study in this thesis is the first that demonstrates that NPs can also trigger hormesis, and possibly also can induce transhormesis effects. Since these findings are also important for risk assessment of NPs, it is 6of relevance to further investigate this aspect for other types of NPs. Also studies with longer exposure intervals should be considered, to further evaluate dose and exposure time (Rozman and Doull 2000).

The main findings of this thesis can be summarised in Figure 5-1, in relation to the tiered oxidative stress response model from Nel and colleagues (Nel et al. 2006; Xia et al. 2006). In concordance with the literature (reviewed in Stone et al. 2009; Stone et al. 2016; Unfried et al. 2007) the results obtained within the framework of this thesis showed the importance of oxidative stress induction in the toxicity of NPs. It was revealed that GSH is an important factor and can be related to the tiered oxidative stress response model by Nel et al (2006). Depending on the level of oxidative stress, the NPs do not trigger adverse effects, trigger activation of inflammatory processes (e.g. via NFκB activation) or cause more severe damages including DNA damage and cell death. DNA damage can lead to cell death or mutations depending the extent of damage and the success of DNA repair processes. The results from present thesis show that the ability of NPs to cause DNA damage depends on the level of GSH in various cell lines. However, the findings with the TiO_2 NPs suggest that this may not be directly related to oxidative stress induction by this nanomaterial (**chapter IV**). Further research is needed to investigate the mechanism of DNA damage by TiO_2 NPs. For nanosilver, it was shown that the Nrf2-pathway may lead to an adaptive response and offer further protection to the effects of NPs (**chapter III**). However, GSH seemed to play no role here, and further research is needed to identify factors that explain for the adaptation. The role of inflammation was not investigated in this thesis but is an interesting topic of future research as well. Inflammatory effects of NPs are considered to play a key role in the development of COPD and fibrosis by particles, and is also contributes to carcinogenesis. Adaptation to inflammatory mechanisms would also prevent against the adverse effects of NPs.

The studies performed in the framework of this thesis have also generated important findings with regard to nanoparticle testing strategies. This concerns the endpoints that were evaluated, the selection of the cell line and the way that the nanoparticle suspensions are prepared for the testing.

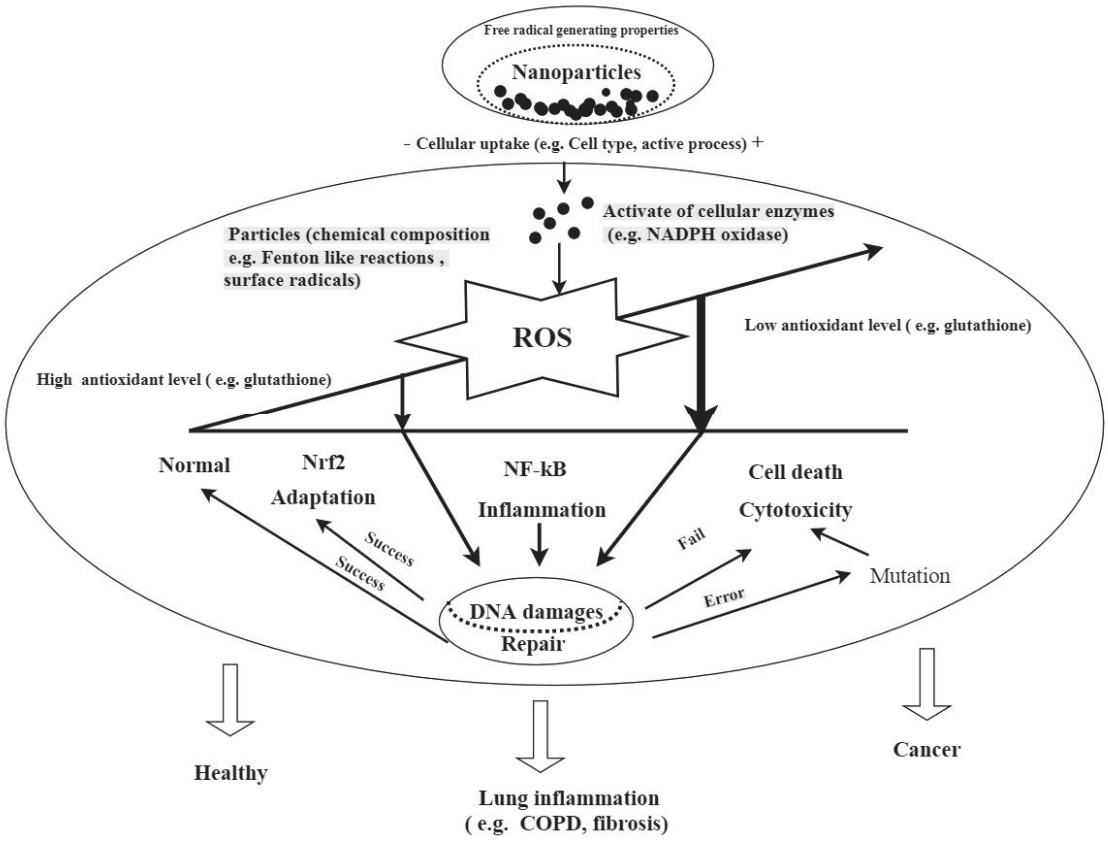

Figure 5-1: The tiered oxidative stress model with inclusion of DNA damage and the role of glutathione

For the panel of NPs that was selected for cytotoxicity and DNA damage analysis, electron spin resonance spectroscopy (ESR) was used to measure their ROS generation properties (**chapter II**). It has been proposed that the measurement of ROS generation in cell free conditions can be used to predict toxicity of NPs. However, the investigations showed that the oxidant generation data from the ESR analysis were not correlated to cytotoxicity or DNA damage. Our findings indicate that the cell free ESR method to detect ROS is poor predictor of toxicity of NPs. For the risk assessment and nanosafety testing cell based assays are important since cell free assays may not predict all types of effects.

The investigations in this thesis also revealed that the effects of various NPs were very different between the different cell lines. This has also important implications for nanosafety testing strategies. The results of the investigations with the A549, HepG2 and HK-2 cell lines (Chapter II and IV) showed that the test outcome, and therefore the classification of the hazard of a nanomaterial, may depend on the choice of the cell line. This also indicates that it is important to use more than one cell line in nanosafety testing. The results with the cell lines from the three different cell lines, were designed to address possible effects in organs. However, the results of

our study show that they cannot be directly translated to organ specific hazards of the NPs. For instance, the DNA damaging effects of TiO_2 NPs depended strongly on the intracellular levels of GSH of the different cell lines (**chapter IV**).

It was also found that the effects of TiO_2 depended on the dispersion agent (**chapter II**). The serum used in the dispersion tended to reduce the DNA damaging properties of TiO_2 in A549 cells compared to TiO_2 dispersions without serum. The coating of the TiO_2 with the commercial lung surfactant Curosurf in the same cells enhanced the DNA damage. This indicates that it is important to consider the choice of the dispersant. The human lung is covered with lung lining fluid and inhaled NPs will be covered by components of the lung surfactant. Therefore, inclusion of surfactants like Curosurf is relevant for lung toxicity testing of NPs and can be recommended in future toxicity studies. For the testing in cell lines or tissues from organs like liver and kidney, serum is a more relevant dispersing component.

Finally, the investigation of the toxicity of colloidal silver NPs showed that the dispersion mixture had DNA damaging properties (**chapter II**). This indicated, that the part of the observed DNA damaging effects of this nanosilver sample could be due to components of the dispersant. This example demonstrates that it is important to include testing of the dispersants in the absence of NPs. The effect was only observed in the comet assay (DNA damage) but not in the cytotoxicity assay (WST-1), and it was therefore not needed to include this in the hormesis study (**chapter III**).

In **conclusion**, the studies that were performed in the framework of this thesis have generated important information regarding the choice of experiments for hazard assessment as well as novel findings about the mechanisms of effects of NPs in cell culture. In relation to nanoparticle toxicity, the importance of the selection of the cell line was demonstrated and the importance of the dispersant protocol. In relation to mechanisms of toxicity of NPs, the intracellular GSH status emerged as an important factor. The levels of GSH could explain for differences in sensitivity of different cell lines toward induction of DNA damage by TiO_2 NPs. Effects of NPs on GSH decline and activation of the transcription factor Nrf2 are indicators of oxidative stress induction. However, glutathione appeared to play no role in possible adaptation to Ag NPs. Future research is needed to better understand the role of the glutathione antioxidant system and also on other pathways that play a role in the cytotoxic and DNA damaging effects of NPs and possible adaptive protective mechanisms.

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5.2 Abstract

There is an ongoing concern about the safety of engineered nanoparticles (NPs). Humans can be exposed to NPs during their manufacturing or following their release from consumer products. While the lung is considered as most important entrance and target organ for inhaled NPs, other organs may also be affected upon NP translocation from the lung or, for instance, following oral exposure and intravenous administrations. The aim of this thesis was to investigate the cytotoxic and DNA damaging effects of various types of engineered NPs in relation to their composition and to study the underlying role of oxidative stress and the glutathione (GSH) antioxidant defense system. In the first study, a panel of 11 NPs was investigated in human epithelial cell lines from lung (A549), liver (HepG2) and kidney (HK-2) in relation to their oxidant generating properties. Ag NPs and uncoated ZnO NPs caused DNA damage with concurrent cytotoxicity, whereas specific TiO_2 caused DNA damage in the absence of cytotoxicity. The effects of specific NPs also varied considerably with the cell line, and on the presence of the NP dispersant. The oxidant generating properties of the NPs, measured under cell free conditions, did not predict their cytotoxic and DNA damaging capacity. The second study evaluated whether Ag NPs may give rise to an adaptive response in a similar fashion to previous findings with the ubiquitous toxicant acrolein in lung cells. It was found that a low dose pretreatment of A549 human epithelial cells with Ag NPs induced protection against a toxic dose of the Ag NPs as well as acrolein. In contrast to acrolein, adaptation by Ag NPs was not associated with an increase in γ-glutamylcysteine synthetase (γGCS) mRNA and GSH level, although heme oxygenase 1 (HO-1) mRNA expression and Nuclear factor (erythroid-derived 2)-like 2 (Nrf2) immunoreactivity were enhanced. The third study explored whether differences in intracellular GSH status could explain for differences in toxicity of nanoparticles in the A549, HepG2 and HK-2 cells. Data showed an inverse relation between DNA damage induction by TiO_2 NPs and the constitutive level of GSH in the respective cell lines. DNA damage by TiO_2 was further enhanced upon pre-treatment with the GSH synthesis inhibitor buthionine-sulfoximine (BSO). However, in contrast to ZnO NPs, TiO_2 did not cause a substantial decline of GSH levels, nuclear translocation of Nrf2, and increased mRNA expression of γGCS and HO-1. Together, these findings indicate that TiO_2 does not induce strong oxidative stress in cells, but that the DNA damaging properties of these nanoparticles depend on the intracellular GSH level. The studies performed in the present thesis provided novel insight on the importance of the selection of the cell line and dispersion method in nanoparticle toxicity screening approaches. Moreover, it could be demonstrated that GSH is involved in the protection from DNA damage by TiO_2 NPs, but not in an adaptive protective mechanisms to Ag NPs.

5.3 Zusammenfassung

Es gibt anhaltende Bedenken bzgl. der Sicherheit industriell hergestellter Nanopartikel (NP). Zu einer humanen Exposition gegenüber NP kann es bei deren Herstellung oder Freisetzung aus erbraucherprodukten kommen. Während die Lunge als bedeutendstes Eintritts- und Zielorgan für inhalierte NP gilt, können auch andere Organe infolge Translokation von der Lunge, nach oraler Aufnahme oder intravenöser Applikation von NP beeinflusst werden. Ziel dieser Doktorarbeit war die Untersuchung von Zytotoxizität und DNA-Schädigung unterschiedlicher Typen industriell-hergestellter NP in Bezug auf ihre Zusammensetzung und der zugrundeliegenden Rolle von oxidativem Stress und intrazellulärem Glutathion (GSH)-Status. In der ersten Studie wurden 11 NP in humanen Zelllinien von Lunge (A549), Leber (HepG2) und Niere (HK-2) bezüglich ihrer Oxidantien-generierenden Eigenschaften untersucht. Ag-NP und unbeschichtete ZnO-NP induzierten DNA-Schäden und Zytotoxizität, während TiO_2 DNA-Schäden ohne zytotoxische Effekte verursachte. Die Effekte der einzelnen NP variierten auch deutlich zwischen den Zelllinien und in Abhängigkeit des verwendeten Dispergens. Die Oxidantien-generierenden Eigenschaften der NP, gemessen unter zellfreien Bedingungen, ergaben keine Vorhersage bzgl. des zytotoxischen oder DNA-schädigenden Potentials der NP. In der 2. Studie wurde untersucht, ob Ag-NP eine adaptive Antwort zur Folge haben können, ähnlich wie zuvor mit dem ubiquitären Toxin Acrolein in Lungenzellen demonstriert wurde. Die Ergebnisse zeigten, dass eine Vorbehandlung von A549-Zellen mit einer geringen Dosis von Ag-NP sowohl gegen toxische Dosen von Ag-NP als auch von Acrolein schützte. Im Gegensatz zu Acrolein war die Adaptation durch Ag-NP nicht durch eine erhöhte mRNA-Expression von γ-Glutamylcystein-Synthetase (γGCS) und steigende GSH-Level begleitet, jedoch durch eine verstärkte Expression von Hämoxigenase 1 (HO-1)-mRNA und eine erhöhte Nuclear factor (erythroid-derived 2)-like 2 (Nrf2)-Immunoreaktivität. In der 3. Studie wurde untersucht, ob der intrazelluläre GSH-Status die Unterschiede bzgl. der Toxizität von NP in A549-, HepG2- und HK-2-Zellen erklären könnte. Die Ergebnisse zeigten einen inversen Zusammenhang zwischen DNA-Schäden durch TiO_2-NP und dem konstitutiven GSH-Level in den jeweiligen Zelllinien. Dieser Effekt wurde durch Vorbehandlung mit dem GSH-Synthese-Inhibitor Buthionin-Sulfoximin (BSO) weiter verstärkt. Im Gegensatz dazu induzierten TiO_2-NP keine substantielle Senkung des GSH-Spiegels, nukleare Translokation von Nrf2 und steigende mRNA-Expression von γGCS und HO-1. Zusammenfassend zeigen die Daten, dass TiO_2-NP keinen starken oxidativen Stress in Zellen induziert, aber die DNA-schädigenden Eigenschaften dieser NP vom intrazellulären GSH-Status abhängen. Die im Rahmen der Doktorarbeit durchgeführten Studien erbringen neue Erkenntnisse bzgl. der Bedeutung der Auswahl der Zelllinie und Dispersions-Methodik in NP-Toxizitäts-Screening-Ansätzen. Darüber hinaus konnte gezeigt werden, dass GSH am Schutz vor DNA-Schädigungen durch TiO_2-NP beteiligt ist, jedoch nicht an adaptiven Schutzmechanismen gegenüber Ag-NP.

5.4 Abbreviations

8-OHdG	8-hydroxydeoxyguanosine
ARE	Antioxidant response element
BCA	Bicinchoninic acid
BSO	Buthionine sulfoximine
cDNA	Complementary deoxyribonucleic acid
CNT	Carbon nanotube
COPD	Chronic Obstructive Pulmonary Disease
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
EDTA	Ethylendiaminetetraacetic acid
FCS	Foetal calf serum
γ-GCS	γ-glutamylcysteine synthetase
GSH	Glutathione
GSSG	Glutathione disulfide
HBSS	Hank's Buffered Salt Solution
HO-1	Heme Oxygenase-1
Keap 1	Kelch-like ECH-associated protein 1
nm	Nanometers
NADPH	Nicotinamide adenine dinucleotide phosphate
NPs	Nanoparticles
NMs	Nanomaterials
Nrf2	Nuclear factor erythroid-derived 2 related factor 2
PBS	phosphate buffered saline
PCR	Polymerase chain reaction
ROS	Reactive Oxygen Species
WST-1	Water-soluble tetrazolium salt

Publications

Thongkam, W, Gerloff, K, van Berlo, D, Albrecht, C, Schins, RPF (2017). Oxidant generation, DNA damage and cytotoxicity by a panel of engineered nanomaterials in three different human epithelial cell lines. Mutagenesis 32(1):105-115.

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Thongkam, W, Kolling, J, Neumeyer-Sickinger, A, Chirino, YI, Albrecht, C, Schins, RPF. The role of uptake and intracellular glutathione level in the DNA damaging effects of titanium dioxide nanoparticles. Submitted for publication

Acknowledgements

First of all I deeply thank and express my gratitude to Prof. Dr. Jean Krutmann for giving me the opportunity to work at the IUF under his support and supervision.

I also would like to express my appreciation and gratitude to Prof. Dr. Peter Proksch for supporting me as co-supervisor.

I would like to express my deepest appreciation and gratitude to Dr. Roel Schins for giving me this valuable opportunity to pursue my doctoral research in his research group at IUF. I would like to express my sincere thanks and gratitude to him for his remarkable support, his excellent supervision and outstanding knowledge in both work and life. I could not have wished for a better and nicer mentor and supervisor.

I would like to express my sincere cordial thanks to Dr. Catrin Albrecht for her insightful comments, fruitful discussions and constructive advices with regards to scientific and publication issues as well as the great bureaucratic support, delicious cake and food.

My sincere thanks also go to Frau Christel Weishaupt and Frau Gabriele Wick for their help and support in the laboratory and the friendly teamwork atmosphere.

I wish to extend my thanks to my past and present colleagues, the “party(cle)” people Dr. Damien van Berlo, Dr. Kirsten Gerloff, Dr. Bryan Hellack, Dr. Julia Kolling, Dr. Andrea Neumeyer-Sickinger and Dr. Yolanda Irasema Chirino for their support in laboratory work and contributions to the publications. I also thank Dr. Agnes Scherbart, Dr. Agnes Boots, Dr. Verena Wilhelmi, Dr. Maja Hullmann, Dr. Harm Heusinkveld, Dr. Tina Wahle, and Adriana Sofranko for the nice and great hospitality during my PhD.

Thank you very much for the great atmosphere at working place and to your personal support!

Mireille Sthijns, thank you for the great collaboration, it is my great pleasure to working with you in research and knowing you as person. I have really enjoyed working with you and to produce our interesting results together. We are co-workers and friends.

My special thanks to Dr. Julia Kolling and her family who gave me opportunity to join the family event and to learn more about the German culture during my stay in Germany.

Mr. Michael Bauer, thank you very much for your spiritual and mental support as well as great cheering up during my PhD study.

I also want to acknowledge the Selma-Meyer-Mentoring program at HHU Düsseldorf and staff for this great program and beautiful time that we spent together.

Finally my heartily big thanks to my parents to whom I dedicate this dissertation, for the unconditional love, unlimited support, continuous encouragement and for always being with me in every decision.

ข้าพเจ้าขออุทิศวิทยานิพนท์และผลงานที่เกิดขึ้นตลอดระยะเวลาการศึกษาปริญญาเอก แก่บิดา-มารดา นายมงคล ทองคำ และ นางสำรวย ทองคำ รวมถึงครอบครัวของข้าพเจ้าผู้ให้การสนับสนุนทั้งกำลังกาย กำลังใจ และเชื่อมั่นในตัวข้าพเจ้าโดยปราศจากเงื่อนไขใด ๆ

Thank you very much to all of you ☺

Eidesstattliche Erklärung

Hiermit versichere ich, dass ich die vorliegende Arbeit eigenständig verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel verwendet habe.

Ferner versichere ich, dass ich weder an der Heinrich-Heine-Universität noch an einer anderen Universität versucht habe, diese Doktorarbeit einzureichen.

Ebenso habe ich bisher keine erfolglosen Promotionsversuche unternommen.

Düsseldorf, den

Waluree Thongkam