

# Review of hazards concerning titaniumdioxide nanoparticle inhalation – addressing questions of using photocatalytic concrete in construction

Lars Kraft, Ph D, Swedish Cement and Concrete Research Institute

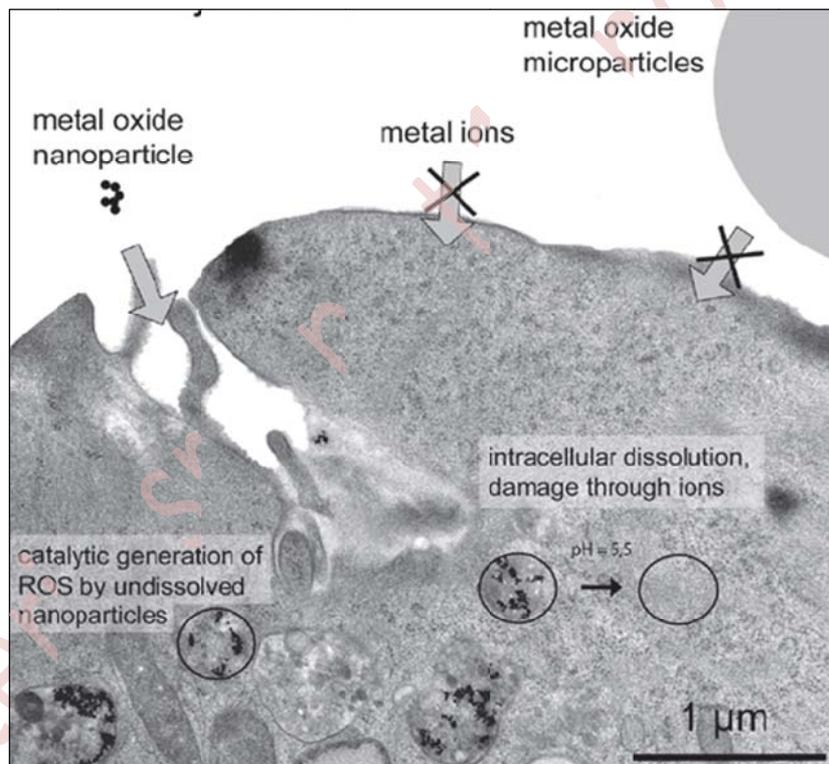


Figure 1. The figure describes processes of nanoparticle interaction with a cell.

The image shows a section of a human fibroblast after exposure to ceria nanoparticles. Particles (black dots, arrow) are found in vesicles and transported through large sections of the cell. Specific heavy metal oxide particles can be dissolved within lysosomes (lower pH). This results in an otherwise rare transport of heavy metal ions into the cytosol [1].

**Note**

The vocabulary used in this review requires experience of the life science literature and vocabulary. However, it is assumed that the readership of this report in general lack such knowledge. In fact, also the author has very limited knowledge within this field. Therefore, a list of some common expressions used in toxicology, describing toxicological methods and biological systems, is put together in Appendix 1.

Furthermore, most of the results cited from the different papers are often simplified, leaving out central experimental facts which certainly are significant for the toxicologist, but due to the limited knowledge of the materials or civil engineer, usually of minor importance for their interpretation of the results.

Interimsrapport 19 nov 2012

## Abstract

Nano-crystalline titaniumdioxide is mixed into concrete in order to reduce nitrogen-oxides in urban areas, due to photocatalytic activation of ultraviolet light . Therefore it is important to make sure that such addition of titania nanoparticles won't cause any negative side-effects, if any, such as an increase in epidemiological harm. Accordingly, the objective for this report is to answer whether nanoparticles (NPs) from concrete containing titania is more toxic than NPs from concrete not containing titania, and besides this, deduce whether titania NPs in general are more toxic compared to fine titania particles? A state-of-the-art review is conducted, basically based on papers dealing with how micro- and nanoparticles of  $\text{TiO}_2$  affect biological systems and to papers with explicit information of nano-sized  $\text{TiO}_2$  when exposed to living tissue, *in vivo* or *in vitro*. The primary route for body exposure from airborne particles is by inhalation, although ingestion and dermal penetration also are possible routes for NPs to enter the body. Therefore primarily papers concerning lung cell interaction with  $\text{TiO}_2$  NPs are reviewed. From the literature it is highlighted that the particle size, agglomeration state, crystallinity, surface reactivity, dose, exposure route, experiment duration, species or cells used all play a role in titaniumdioxide toxicity. In summary contradicting facts were found regarding the influence of particle size and surface area on toxicity of  $\text{TiO}_2$  dusts, although at equivalent mass dose exposure of fine and ultrafine particles, smaller particles seem to induce higher inflammatory response. Likewise, regarding crystal form in many papers anatase seems to be more potent to cause inflammation in cells and animals (guinea-pigs) than rutile, whereas a combination of these two phases occasionally seems to be even more potent than the anatase phase alone. Of great importance for correct interpretation of the results is the physicochemical characterization of the particles such as coatings applied at the surface, the z-potential and the agglomeration grade. In many of the papers reviewed such characterization are insufficient. That is particularly more common in papers published before 2005. But the scientific branch of nanoparticle toxicology is a fast developing area and the good news is that the knowledge of how to assess the toxicity accurately has increased much during the last 4-5 years. Therefore more concise knowledge regarding different NPs inherent toxicity to living tissues and the environment will be available increasingly in a continual manner the next coming years. Specifically, from the papers reviewed, the toxicity of  $\text{TiO}_2$  NPs seems not much more toxic than their larger counterparts, if compared at equal surface area. Furthermore, toxicological researchers conclude that many studies demonstrate a low hazard potential in mammals or aquatic species even at acute exposure to the ultrafine  $\text{TiO}_2$  particles. That is because in many rat studies the inflammatory response is transient with time. Compared with other metaloxides,  $\text{TiO}_2$  is most often less toxic than other elements, such as  $\text{ZnO}$ ,  $\text{CuO}$  and  $\text{SiO}_2$  in the different toxicological screening tests. Also, the doses used in the toxicological studies are 1000 – 100 000 times higher compared to any dosages that humans are exposed to. Therefore, regarding using  $\text{TiO}_2$  in concrete, which results in little less silica and Ca-particles but somewhat more small amounts of  $\text{TiO}_2$ , it is concluded that it is not likely that using additives of photocatalytic titania in pavements made of concrete will increase the inherent toxicity of the particles generated from the wear of vehicles.

## Contents

Review of hazards concerning titaniumdioxide nanoparticle inhalation – addressing questions of using photocatalytic concrete in construction .....	1
Note .....	2
Abstract .....	3
1 Background for this report .....	6
2 Objective .....	6
3 Introduction.....	7
3.1 Exposure routes.....	7
3.2 Origin of nanoparticles .....	8
3.3 Conventional particle toxicology.....	8
3.4 Epidemiological studies on airborne particles .....	8
3.5 Earlier reviews specifically regarding TiO <sub>2</sub> NP toxicity .....	9
4 Toxicity of nanomaterials, a complex study.....	9
4.1 Toxicity of nanomaterials, a science in progress.....	10
4.2 Material characterization for assessment of NPs biological behavior .....	11
4.2.1 Methods for materials characterization.....	11
4.2.2 Definition of particle sizes .....	11
4.2.3 Relation between particle size and surface area .....	12
4.2.4 Particles, aggregates and agglomerates.....	13
4.2.5 Titanium dioxide characteristics.....	14
4.2.6 Nano-bio interfaces.....	15
4.3 Toxicological methods.....	15
4.4 Identifying toxicological interactions of NPs and cells .....	17
4.4.1 Mechanisms controlling the nanoparticle-cell interactions.....	18
4.4.2 Some examples of case studies on nanoparticle-cell interactions.....	18
4.4.3 High aspect ratio nanoparticles (HARN).....	20
5 Studies on the toxicity of TiO <sub>2</sub> .....	21
5.1 In-vivo studies on pulmonary exposure to TiO <sub>2</sub> NPs .....	21
5.2 Other <i>in vivo</i> administered exposures to TiO <sub>2</sub> NPs .....	26
5.3 <i>In vitro</i> investigations of TiO <sub>2</sub> NP toxicity .....	26
5.3.1 TiO <sub>2</sub> NP toxicity studies from <i>in vitro</i> lung models.....	27
5.4 The carcinogenic potential of TiO <sub>2</sub> NPs .....	33
5.5 Summary of conclusions from both <i>in vivo</i> and <i>in vitro</i> studies reviewed .....	34

5.5.1	Influence of particle size and surface area.....	34
5.5.2	Influence of crystal structure .....	36
5.5.3	Surface coating and surface chemistry influence of particles.....	37
5.5.3.1	Surface properties, zeta-potential .....	37
5.5.3.2	The toxicity of TiO <sub>2</sub> compared to silica .....	37
5.5.4	Influence of experimental model .....	38
5.6	Agreement between <i>in vitro</i> and <i>in vivo</i> studies.....	38
6	Discussion.....	39
6.1	Risk assessment of airborne NPs, TiO <sub>2</sub> NPs in particular .....	40
7	Conclusions.....	41
8	Acknowledgements .....	42
	References.....	43
	Appendix 1. Small list of vocabulary and concepts in nanotoxicology.....	51

Interimsrapport 19 nov 2012

## 1 Background for this report

Today nanotechnology is making a great impact on diverse science, engineering and commercial sectors, including the construction industry where the current knowledge is applied in many different areas [2]. For example, nanotechnology has made the nanoscale analysis of Portland cement hydration possible. Nanoparticles are used to increase both the strength and the durability of cement based materials. Functional paints and coatings on windows are other examples of construction materials where nanoparticles contribute to many improved properties such as self-cleaning as one example [3]. From the discovery of the photocatalytic process in the beginning of the 1970's by Fujishima and coworkers [4] many different applications of photocatalysis have been found [5]. For example, in medicine the antibacterial effect is used in photocatalytic sterilization, cancer treatment, and as antibacterial coatings on implants [6, 7]. Furthermore, in environmental applications photocatalytic filters, coatings, films, paints, windows, mortar or concrete are used in self-cleaning surfaces, as odor removers, and in air and water cleaning devices or structures [5, 8].

Perhaps the most interesting application for construction materials is to use concrete to obtain self-cleaning and air-cleaning surfaces in urban environments. This application of photocatalytic concrete represents a potentially expansive area in the construction industry and many studies have already been presented from laboratory studies [9, 10], full-scale experiments [11, 12] and from real construction projects [13-15]. According to Gian-Luca Guerrini, a senior scientist at Italcementi, the use of nanoparticles of  $\text{TiO}_2$  in combination with cement based materials has shown a favorable synergistic effect in the reduction of pollutants. In comparison with other photocatalytic building materials, photocatalytic mortar and concrete are able to absorb nitrogen oxides ( $\text{NO}_x$ ) on the surface to transform them into harmless non-noxious ions and to block them in form of salts (nitrates), which can easily be removed from the surface by rain or of washing actions [16]. Indeed, Italcementi, at its homepage, states that in a large city such as Milan, researchers have calculated – on the basis of test results – that covering 15% of visible urban surfaces with products containing TX Active® would enable a reduction in pollution of approximately 50%. In fact, the studies on real construction projects cited above have reported good results with a reduction of  $\text{NO}_x$  in street canyons of 30, 20 and 5-10 %, respectively. In the latter case, in Malmö Sweden, photocatalytic pavers (Econox®) were used only in one pavement on one side of the street which explains the rather low values obtained. Moreover, the irradiance from the sun is lower in northern countries compared to in Italy, which also influences the efficiency of the photocatalytic activity. Still, all these studies confirm that photocatalytic concrete has a strong potential to improve the urban environment. Besides the air-cleaning and self-cleaning effect, concrete pavements are stronger and brighter compared to asphalt, contributing to higher durability and a higher albedo to reduce the heat generation in great cities.

However, the use of nanoparticles has also raised issues concerning potential toxicity. Therefore proactive risk assessment and regulatory guidelines are essential for a continual and increased use of environmentally beneficial concrete products such as photocatalytic concrete [2].

## 2 Objective

From this background the objective for this review concerns questions regarding toxicity, if any, of concrete compositions that contain photocatalytic titaniumdioxide (titania). The most important questions to be answered is whether nanoparticles (NPs) from concrete containing titania is more

toxic than NPs from concrete not containing titania, and if NPs of titania are more toxic compared to fine particles. Although this review might not be able to give explicit answers, the aim is also to give a short guidance into how toxicity studies are conducted and evaluated, and which kind of material properties that play the most important role in being harmful.

Another question of interest concerns if concrete containing titania produce more NPs compared to concrete not containing titania, when subjected to wear under equal conditions. But that is for other studies to find out.

The report reviews some of the recently published papers concerning potential toxicity of nanomaterials (NMs) and nanotechnology in general. But the focus is given regarding papers dealing with how micro- and nanoparticles of TiO<sub>2</sub> affect biological systems, respectively and to papers with explicit information of nano-sized TiO<sub>2</sub> when exposed to living tissue, *in vivo* or *in vitro*.

### 3 Introduction

During the last decade there has been a tremendous increase in papers published on the subject of NPs and toxicity. TABLE 1 presents data from an article search at the Scopus website for different keywords. It reveals that during the last decade at least 160 papers have been published having the words “nanoparticles”, “toxicity” and “TiO<sub>2</sub>” in its “Title, Abstract or Keywords”. Furthermore, when the search was limited to the keywords “lung”, “lung toxicity”, “lung lavage”, “pneumonia” and “bronchoalveolar lavage fluid” only 22 published papers in between 2006 and 2011 were found.

TABLE 1: Numbers of papers published from 2002-01-01 - 2012-06-05.

Year	nanoparticles	nanoparticles AND toxicity	nanoparticles AND toxicity AND TiO <sub>2</sub>
2002	3069	24	1
2003	4023	31	
2004	5893	81	
2005	8814	142	3
2006	10503	223	4
2007	13898	326	18
2008	16432	441	24
2009	18642	690	44
2010	22665	883	44
2011	27314	1164	19
2012	12552	529	3

#### 3.1 Exposure routes

The primary route for body exposure for airborne particles or fibers is by inhalation, although ingestion and dermal penetration are also possible routes for NPs to enter the body. As NPs enter the body they have the potential to translocate to the blood circulation and non-pulmonary organs. Still, the respiratory tract remains the primary target organ for potential adverse effects of airborne particles, such as nanoparticles [17]. Therefore, papers concerning lung cell interaction with NPs have been of prior interest for this review.

### 3.2 Origin of nanoparticles

The sources of nanoparticles that will interfere with biological systems are manifold. They can stem from both natural sources, be created from man-made courses of action or be manufactured on purpose for different medical or technical reasons. Airborne particles or particulate matter usually refers to particles present in urban areas. Such NPs, which basically stem from manmade courses of action, i.e. from wear of tires, brakes and pavements and combustion sources, typically are called ultrafine particles [18]. Manufactured particles are referred to as engineered nanomaterials (ENM) [19, 20]. Therefore, particles generated from concrete containing photocatalytic TiO<sub>2</sub> are engineered particles that have been transformed from man-made courses of action.

Worn air-borne particles containing TiO<sub>2</sub> NPs will not have the same physicochemical characteristics as the TiO<sub>2</sub> NPs that were originally blended into the concrete. Since an assessment of the toxicity of NPs is not meaningful without a careful characterization of their physico-chemical properties [7, 17, 21-24], the authentic particles should be studied, for a correct judgment. However, regarding this report, information of titaniumdioxide toxicity in general, as found in the literature, is valuable for any judgment of the toxicity regarding concrete containing TiO<sub>2</sub> NPs.

### 3.3 Conventional particle toxicology

The roots of the current science of nanotoxicology can be traced to a variety of origins. Conventional particle toxicology provide useful knowledge [19] and has given increased awareness regarding the association between health effects (increased hospital admissions and acute mortality as a result of respiratory and cardiovascular conditions) and traffic-related particulate air pollution. Also, virology and other sciences, the toxicology of metal fumes, radionuclides, nuisance dusts, rat lung overload, the toxicology of silica, asbestos and synthetic vitreous fibers have contributed to the current science of nanotoxicology [19].

The term nanotoxicology started to be used more frequently after some studies in the 90's where it was shown that inhaled ultrafine particles (20 nm) caused greater pulmonary inflammation and more easily translocated interstitially compared to fine particles (250 nm) in rats [25]. Based on such results it was suggested that occupational standards for 'nuisance' dusts should be reconsidered to take into account possible damaging effects of ultrafine particles.

### 3.4 Epidemiological studies on airborne particles

Epidemic studies have shown convincing correlation between the amount of particles present in the air and adverse effects in patients with cardiovascular and respiratory diseases [19]. In a report from the Swedish Road Administration from 2005 [26], concerning inhalable particles from pavement wear, it is explained that particle polluted air consists of a heterogeneous mix of different types of substances such as coal, metals, nitrates, sulphates and both volatile and particulate organic matter. According to the same report, research has identified inhalable particles smaller than 10 microns (PM 10) as the most severe matter in polluted air from a medical point of view. In Sweden, research concluded that particulate matter, generated from wear of roads and exhaust gas in Stockholm and Gothenburg, annually causes 230 and 100 premature deaths, respectively [26]. Also, at least until 2005, when the above cited report was written, it was concluded that toxicological studies have, so far, not been able to indicate similar effects as in the epidemical studies at corresponding dosages of particles. Higher particle dosages have been required in the toxicological studies to show similar toxicological effects.

Furthermore, there are no epidemiological studies yet available giving explicit information regarding the specific influence of ultrafine particles in particulate matter; that is particles smaller than 100 nm. More information on epidemiological studies and particulate matter can be found in [27] and [28].

### 3.5 Earlier reviews specifically regarding TiO<sub>2</sub> NP toxicity

Among the papers selected and reviewed here, there are a couple of other reviews [29], [30] and [24] which, from their titles, presumably would give explicit answers to the question if NPs of TiO<sub>2</sub> are more toxic than NPs of other inorganic particles. In general, however, the conclusions in those reviews seldom give any explicit answers due to the complexity of this type of research. Still these reviews give guidance in judging whether certain NPs of TiO<sub>2</sub> are more toxic than other types of NPs. However, after reading paper [1] I believe that the aspect regarding the agglomeration state of the particles at the exposure in all the different types of studies is not as emphasized as it rightfully ought to be, although mentioned in [29] and interestingly discussed in [24]. In papers [31] and [32] the importance of proper material characterization prior to the toxicity testing is emphasized, including determination of the particle size and size distribution *in the wet state* as well as *the aggregation status in the relevant media* for the experiments. Since, the reviews [29], [30] and [24] doesn't review the papers [1, 33-35] where this influence of aggregation on studies is explained [1] and studied [33-36], I hope this review will contribute to a broader knowledge especially among novices of toxicology like myself.

## 4 Toxicity of nanomaterials, a complex study

There is a common assumption that the small size of NMs allows them to easily enter and traverse tissues, cells, and organelles since the actual size of engineered NPs is similar to that of many biological molecules (e.g. proteins) and structures (e.g. viruses) [7]. Therefore there is an increasing awareness that human exposure to some types of engineered particles, inadvertently or intentionally, may lead to significant health effects [19]. However, NPs may not freely or indiscriminately cross all biological barriers. These processes are governed by the specific physico-chemical properties of the NPs themselves as well as the corresponding biological system. Since there is a multitude of different types of NPs and biological systems, general conclusions are very difficult to work out. Nano-bio interfaces are the most complex and the least understood interfacial systems. The behavior of such interfaces depend on the properties of the NPs, the biophase (protein, cell membranes, endocytic vesicles or organelles), the medium and most importantly on changes in them due to their interaction [7, 22, 35, 37]. One major limitation to assess the toxicity is the characterization of the NM prior to and after exposure to living cells or animals, in particular regarding agglomeration and surface characteristics. The most relevant physicochemical characteristics of NMs are size, surface chemistry, crystallinity, morphology, solubility, purity, aggregation tendency and homogeneity of dispersions. All of these properties need to be assessed in order to determine the materials' contribution to toxicity and to enable comparison of the results from the different studies [7, 23] by use of well characterized standard reference materials [22]. Furthermore it is necessary to establish optimal experimental conditions in order to identify if NMs pose a threat to human health. It is an urgent need for novel strategic methods for NM-living tissue interaction experiments for correct assessment of the toxicity for all the different types of NM. Therefore, it seems as if studies need to be rather complex and comprehensive in order to give valuable results. In this context the papers [33-35] are recommended. Certainly also the other papers

reviewed in this report are of value, although they may no longer represent state-of-the art. That is due to the very rapid progress within this research area, with many new papers published continually. See TABLE 1.

#### 4.1 Toxicity of nanomaterials, a science in progress

Again; it is not meaningful to assess the biological behavior of NPs without a careful characterization of the physico-chemical properties of the NPs in question. For example it is essential to consider whether living tissue in fact do encounter individual NPs as opposed to aggregates or agglomerates [1, 7]. The toxic response should be evaluated by normalizing it with the uptake of nanomaterials and their state of agglomeration [1]. Therefore, due to inadequate characterization of the nano-particles some results presented in the literature are of minor importance [7, 23]. Likewise, some data presented from toxicological studies are inconsistent and can hardly be compared. Again, other studies use excessively high concentrations which always give toxic responses [24]. This underlines the urgent need for a standardized NM cytotoxicity assessment, which requires carefully evaluated *in vitro* methodologies. For example, all classical cytotoxicity assays for testing micro-particulates are not suitable for testing ENMs without prior evaluation and adaption, as shown by Kroll *et al.* [35].

Limbach *et al.* [1] present well motivated strategies for toxicological studies on materials. They briefly summarize the current understanding of nanoparticle cell interactions. In stationary biological fluids, such as phosphate buffer solution (PBS) or culture media typically used in *in vitro* assays, chemicals and NPs behave completely different. For molecules, the physical driving force is diffusion. This rapid movement of molecules counteracts any concentration gradient and assists homogeneous distribution into its corresponding phase (cytosol, blood, body fat, *etc.*). For microparticles in stagnant media, the driving forces involve sedimentation. However, for particles within the size range of nanomaterials a cross-over takes place from diffusion-controlled movement to sedimentation controlled movement. Particles in medium are always in a metastable form and they can only be temporarily stabilized, typically by charge or steric repulsion, before they agglomerate. Thus they cannot be characterized in the same manner as molecules or chemicals by solubility or reactivity. Therefore, in most applications of nanomaterials, great efforts are made to make sure that particles are stable to agglomeration. However, during uptake of particles in an organism or at prolonged environmental exposure, such stabilization typically breaks down and particles agglomerate [1]. Once particles have formed larger groups their reduced mobility and larger mass favors sedimentation. For charge stabilized nanomaterials the so called *zeta potential* can be used to assist understanding of agglomeration processes. Therefore, zeta-potential measurements can enable a qualitative comparison between different nanomaterials, as long as the steric hindrance is similar [34]. Biological fluids consist of salts and numerous biomolecules that drastically change the dynamics of NPs. In most cases agglomeration is strongly favored in biological media because of the increased ionic strength. This effect can sometimes be compensated by the adsorption of biomolecules on the surface of NPs. Therefore, different oxides that have very varying zeta potential in water, will obtain almost similar zeta potential in culture media after agglomeration and adsorption of proteins on the surface of the agglomerates. Therefore, it can be assumed that the agglomeration behavior of such oxide particles is similar at comparable primary particle concentration, if contacted with biological fluids [38]. This is in fact confirmed by other research groups in papers [34-36, 39].

## 4.2 Material characterization for assessment of NPs biological behavior

In almost every paper reviewed it is stated that a proper characterization of the physico-chemical properties of all ingoing experimental materials is essential. Despite that, the material characterization many times is insufficient. In the review by Card et al. [23] a careful distinction between papers with and without sufficient characterization is given. For example, a thorough characterization of particles in the wet state was conducted in the paper by Sayes et al. [40].

Therefore many scientific organizations or task forces strongly recommend that investigators conduct thorough characterizations of physicochemical properties of the nanoparticle-types that are being assessed for toxicity testing. But, as *Warheit et al.* writes [32], “too often this recommendation becomes an extensive laundry list of material characteristics that does not have adequate prioritization”. Therefore Warheit, from his own experience, strongly recommends the following prioritized characterization of the physicochemical properties prior to toxicological investigations of NPs [31]:

- Particle size and size distribution (wet state) and surface area (dry state) in the relevant media.
- Crystal structures and crystallinity.
- Aggregation status in the relevant media used.
- Surface reactivity.
- Method of NM synthesis and/or preparation including postsynthetic modifications (e.g. surface coatings).
- Purity of sample.

However, in [41] *Sayes and Warheit* classify particle characterization evaluations into three categories; primary, secondary and tertiary characterization as follows:

Primary characterization is performed on particles in its dry native state.

Secondary characterization is performed on particles in the wet phase as a solution or suspension in aqueous media, e.g. pure water, phosphate buffered saline (PBS) solution or cell culture media.

Tertiary characterization is performed on particles following interactions with cells under *in vivo* or *in vitro* conditions. This could include characterization in blood, lung fluids, urine etc.

The last category is complex and nontrivial but still the most relevant characterization depending upon the questions addressed.

### 4.2.1 Methods for materials characterization

The physic chemical properties of NPs are characterized using all kinds of different analysis techniques, for example; *electron microscopy* (TEM, SEM, STM), *energy dispersive X-ray spectroscopy* (EDS) *nitrogen adsorption-desorption isotherm* (BET), *infrared spectroscopy* (IR), *UV-visible spectroscopy*, *Raman microspectroscopy* [36, 39], *Dynamic light scattering* (DLS), *inductive coupled plasma mass spectroscopy* (ICP-MS) and *x-ray photoelectron spectroscopy* (XPS) [7, 42]. This demonstrates the broad intersectional scientific base needed for toxicity studies and proper characterization of nanomaterials. It is beyond the scope of this review to explain and describe all these particular techniques in more detail or to explain their respective strengths as tools in determining different materials' different properties. *Fadeel et al.* [7] gives some information regarding usage of the different techniques.

### 4.2.2 Definition of particle sizes

The terms ultrafine particle and NP are sometimes used interchangeably, although NP is a newer term that generally refers to engineered particles of homogenous size and composition. Ultrafines,

however, are usually heterogeneous in size and sometimes referred to particles with a solid core of either inorganic material or soot surrounded by a layer of adsorbed or condensed semi-volatile organic constituents [18]. Most often however, NPs are referred to particles with a diameter of less than  $0,1 \mu\text{m}$ , ultrafines are particles with a diameter of  $0,1 - 2,5 \mu\text{m}$  (PM 2.5) and fine particles have a diameter of  $2,5 - 10 \mu\text{m}$  (PM10) [26, 43]. All these particles are respirable and are capable of deposit in the gas-exchange region of the lungs in humans. See Figure 2. Ultrafines and NPs frequently occur as agglomerates or aggregates, although surface coatings and other treatments are sometimes used to improve dispersion of the NPs.

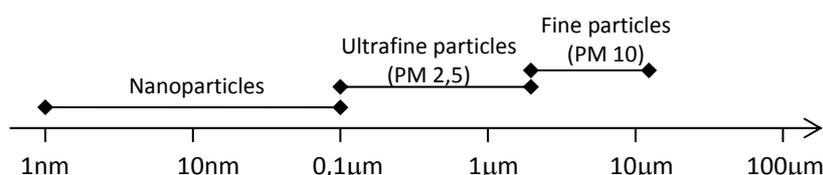


Figure 2. Dimensional definitions of particle sizes [17].

#### 4.2.3 Relation between particle size and surface area

Under the approximation that all particles are spherical and perfectly packed in hexagonal or cubic close packing, the relation between particles size, total particle surface area and the total number of particles at constant volume is illustrated in Figure 3, where the x-axis represent the inverse of the particle size. Both axes are in logarithmic scale. For the same type of particles, a constant volume equals constant mass dose at a theoretically perfect packing. In reality, however, the small difference between constant mass and volume are negligible.

As seen in Figure 3, having logarithmic scale on both axes, there is a tremendous increase in numbers of particles when particle size is reduced [18]. The relation between particle size and surface area differs if particles are irregular and not spherical.

It is also important to define the particle size distribution (PSD) in toxicological studies of particle exposure of, since all particles seldom are of exactly the same size.

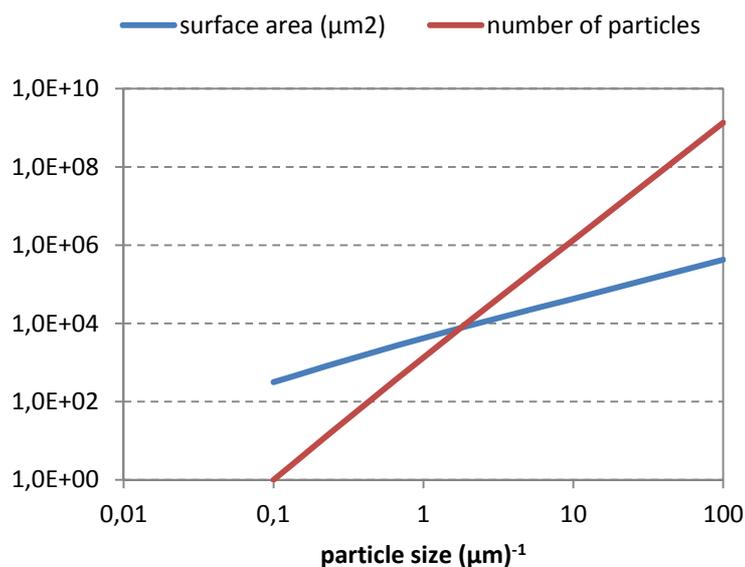


Figure 3. Relation between particle size, surface area and number of particles at constant volume.

#### 4.2.4 Particles, aggregates and agglomerates

Determination of the state of agglomeration and aggregation and the particle size distribution is an essential part of NP characterization. For example, for inhalation studies, the particle size distribution is important for determination of where in the pulmonary region the particles are anticipated to be deposited [23, 26].

It is a great difference in physical and chemical performance whether the NPs are mono-dispersed as individual particles, generated as heterogeneous aggregates or as agglomerates. Usually, unless particles are surface modified, NPs tend to form agglomerates when suspended in gases or liquids. See Figure 4 [19]. Furthermore, as agglomerates in biological environment they interact with biomolecules such as proteins and DNA [34].

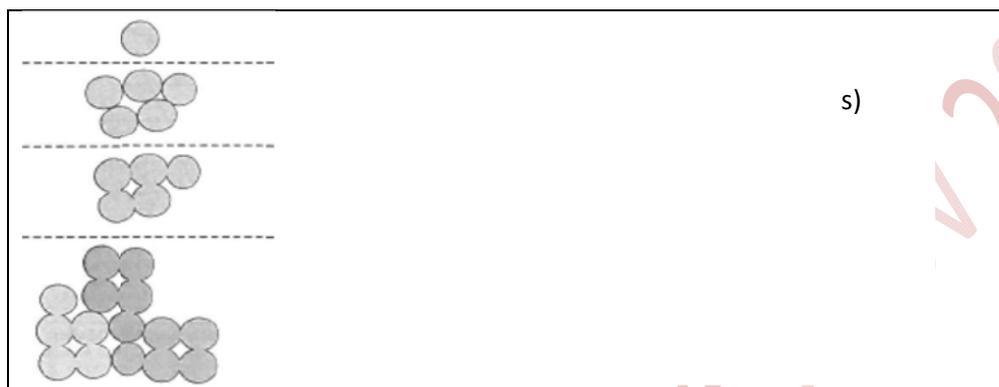


Figure 4. Primary NPs can agglomerate (coagulate) to form clusters, but may also aggregate (fuse, sinter) during generation and then agglomerate additionally. Some mass-produced engineered NPs (e.g. carbon black,  $\text{TiO}_2$ ) are generated partly as aggregates and subsequently agglomerate [19].

The rate of agglomeration linearly scales with the number concentration squared and temperature, while it is slowed down by viscosity. See [1, 38] for details. The rapid process of agglomeration is illustrated in Figure 5 from [1] in which the mechanisms controlling the agglomeration are described.

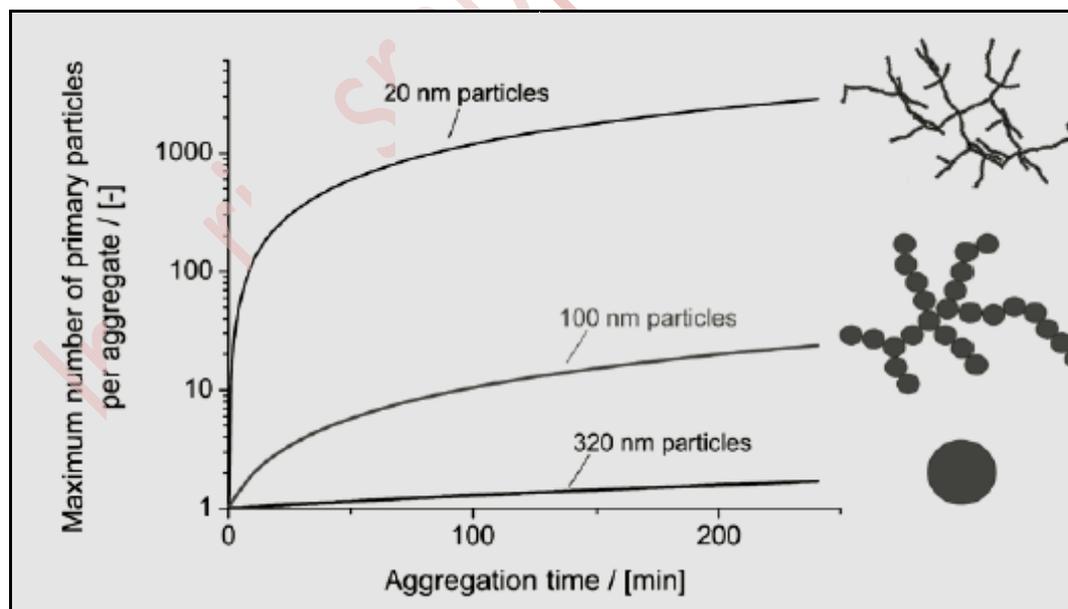


Figure 5. Agglomeration of ceria nanoparticle suspensions with different primary particle size. The mass concentration was kept constant at 1 ppm (1 mg/l) in water. At these conditions diffusion limits agglomeration. At the beginning, the number concentration of 20 nm particles assembles them in bigger clusters. Therefore, agglomeration alters the mobility of the otherwise rapidly diffusing 20 nm particles.

#### 4.2.5 Titanium dioxide characteristics

Among different metal-oxide nanomaterials, the greatest number of available studies have focused on revealing the toxicity of  $\text{TiO}_2$  [24]. Titanium dioxide ( $\text{TiO}_2$ ) is a poorly soluble, low-toxicity (PSLT) particle [44]. It exists in a variety of crystal structures, but the most common are rutile, anatase and brookite. Only the first two play a significant role as ENMs. The crystal forms are shown in Figure 6 [45]. In the bulk form, rutile is the most stable while in the nanoparticle form anatase appears to be the most stable structure. The surface energy for anatase is lower than for rutile [7].

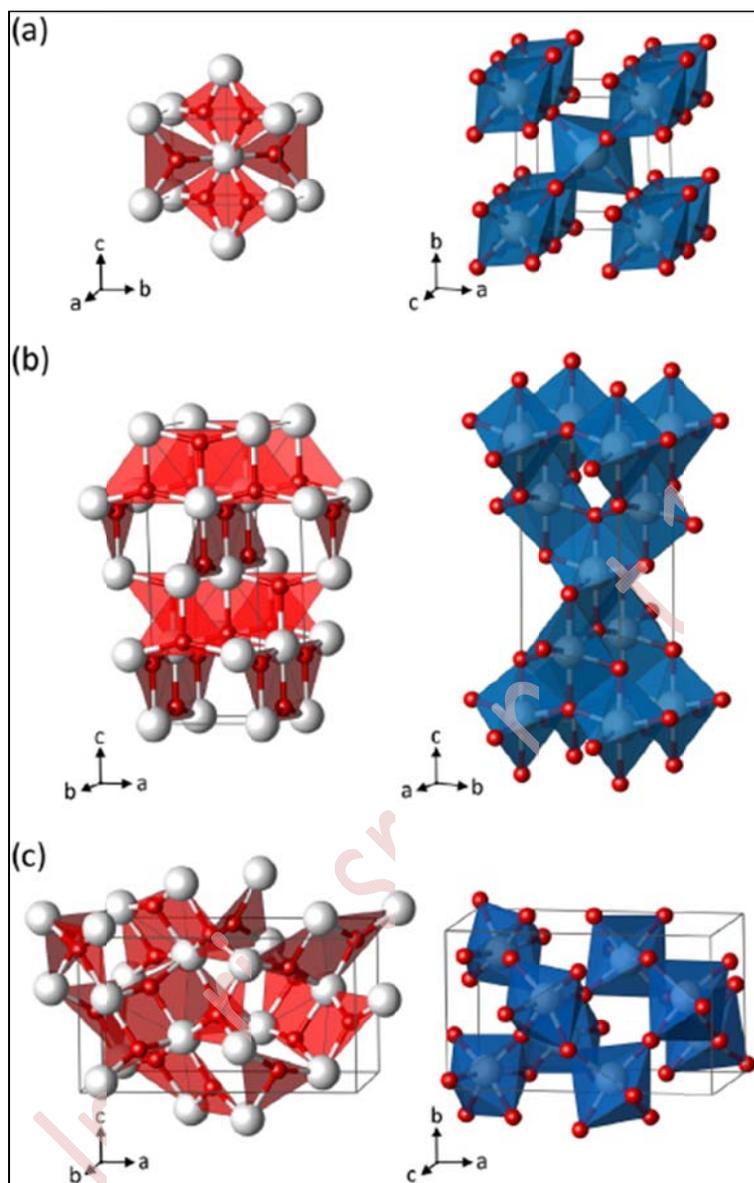


Figure 6. Planar  $\text{Ti}_3\text{O}$  building-block representation (left) and  $\text{TiO}_6$  polyhedra (right) for the  $\text{TiO}_2$  phases rutile (a), anatase (b) and brookite (c), Ti –white; O – red. From [45].

Titanium dioxide NPs are the focus of much research due to their photocatalytic, dielectric and optical properties. They are a component of sunscreens, pigments and paints, solar panels, catalyst supports and water purification devices. Perhaps the most interesting and potentially largest application, as mentioned earlier, is to use photo-activated  $\text{TiO}_2$  NPs in concrete for air-cleaning in heavily trafficked urban areas. Some characteristics of the different crystal forms of  $\text{TiO}_2$  is shown in TABLE 2. Pigment grade (fine)  $\text{TiO}_2$  are used in large quantities in paints due to its optical properties and small production cost, but also in food additives, cosmetics, sunscreens and pharmaceuticals (as

TABLE 2: Some characteristics of the crystal forms of TiO [45, 46].

	<b>Rutile</b>	<b>Anatase</b>	<b>Brookite</b>
Molar mass [g/mol]	79,9	79,9	79,9
Z [mol/unitcell]	2	4	8
Crystal system	Tetragonal	Tetragonal	Orthorhombic
Unitcell: a: b: c [Å]	4,59: 4,59: 2,96	3,78: 3,78: 9,51	9,16: 5,43: 5,13
Volume [Å <sup>3</sup> ]	62	136	257
Molar volume [Å <sup>3</sup> /mol]	18,7	20,2	19,4
Density [g/cm <sup>3</sup> ]	4,27	3,89	4,12
Band gap energy [eV]	3,00	3,23	3,13
Linear therm. exp [10 <sup>-6</sup> K <sup>-1</sup> ]	8,2	6,4	-
Refractive index	2,61	2,49	2,58

carriers). In these applications TiO<sub>2</sub> is considered chemically inert [36]. Therefore it is often used as a negative control in assessing pulmonary toxicity of pathogenic particulates such as α-quartz [24].

#### 4.2.6 Nano-bio interfaces

Reviewed papers of nano-bio interfaces are dealing with many different types of cells, in both *in vivo* and *in vitro* studies. *In vitro* studies often deals with human cells, but most *in vivo* studies deals with animals, rats or other bovines.

Figure 6 from *Shvedova et al.* [37] on next page describes the different sizes of living tissue for comparison and for better understanding of interaction of particles with bio-interfaces.

An example of cells exposed in the lung is presented in Figure 7. It demonstrates some of the complexity toxicologists have to consider when they have to prepare a study of toxicity in the lung, whether it is an *in vivo* or *in vitro* study.

This review only briefly explains how nano-bio interfaces interact and how toxicological studies are conducted and evaluated in the next sections 3.3 and 3.4., since some knowledge is essential in order to be able to interpret the results presented in the literature. But the subject of toxicology is too complex and advanced for a thorough description in this context.

### 4.3 Toxicological methods

Toxicology can be defined as “the study of the adverse effects of chemical, physical or biological agents on living organisms and the ecosystem, including the prevention and amelioration of such adverse effects” [47]. It is based on the principle that any substance can be toxic if consumed in sufficient quantity. Regulations are based upon toxicological tests which aims at identifying the lowest exposure level at which no adverse effects are observed (known as “no observed effects level” or “NOAEL”) and on determination whether the benefits of any particular substance outweigh its risks to human health and/or the environment [48].

Tests *in vivo* or *in vitro* can be made in a multitude of different ways. Many toxicity tests examine specific types of adverse effects, known as “endpoints”, such as eye irritation, acute systemic toxicity, genotoxicity or cancer [48]. In a typical toxicological assay the tested material has to be exposed to biological fluids, consisting mainly of water, salts and biomolecules. Both *in vitro* and *in vivo* tests inherently follow this route [1]. Substances are administered to testing animals in many different ways, as single-exposure or repeat dose and via many different exposure routes: orally, by inhalation,

on skin; or by injection into the bloodstream (intravenous), the abdomen (“intra-peritoneal”), the bronchioles (“intra-bronchial”) or the muscles (“intra-muscular”). Likewise *in vitro* studies can be

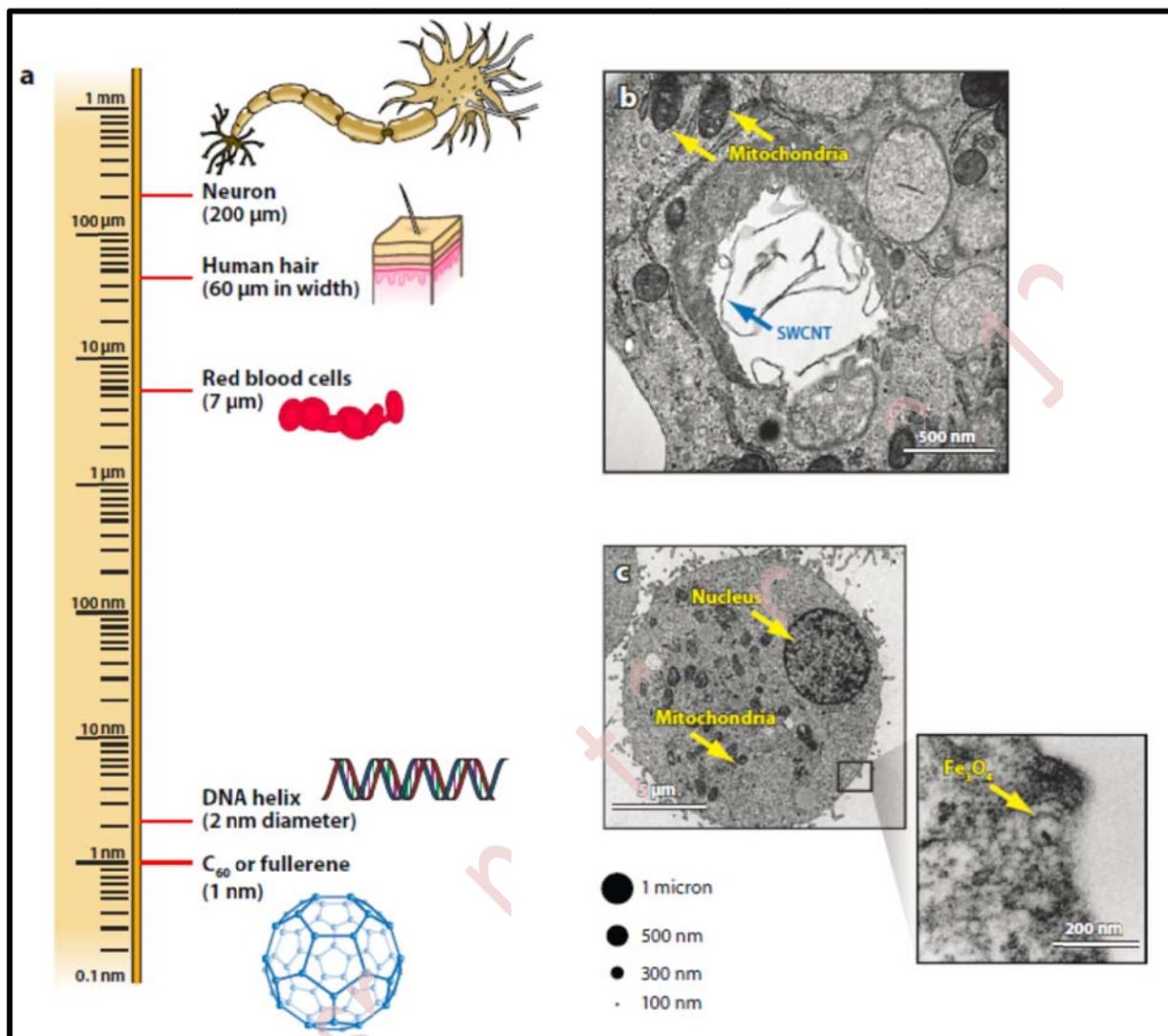


Figure 6, from [37]. In the figure three classes of nano-scale systems are considered; endogenous (cellular and extracellular) nanostructures or nanomachines, parasites (viruses, bacteria, etc.), and man-made or engineered nanomaterials. The schematic figure (a) depicts a logarithmic length scale showing the size of a classical nanomaterial (C<sub>60</sub> fullerene) compared with various biological components (adapted from Buzea et al., *BioInter-Phases*, 2007). Particles of various sizes are drawn to scale. (b) Rat macrophage cells with internalize drop-like bundles of single-walled carbon nanotubes (SWCNT). For comparison, mitochondria are marked with arrows. Human macrophages are up to two times larger than the rat counterparts. (c) Human lung carcinoma cells with evidence of internalization of ironoxide (Fe<sub>3</sub>O<sub>4</sub>) NPs of ~ 20 nm in diameter. Transmission electron microscopy images were recorded at the University of Pittsburgh/National Institute for Occupational Safety and Health (NIOSH) and Swiss Federal Laboratories for Materials Testing and Research (EMPA), respectively.

made in a multitude of different manners upon different types of cells and tissues extracted from humans or animals. Furthermore, there are many different methods for analysis of the results. In summary, the subject of toxicology is a very broad and interdisciplinary science, which cannot be described more in detail in the scope of this review. However, some important parts of the vocabulary of toxicology are described in Appendix 1.

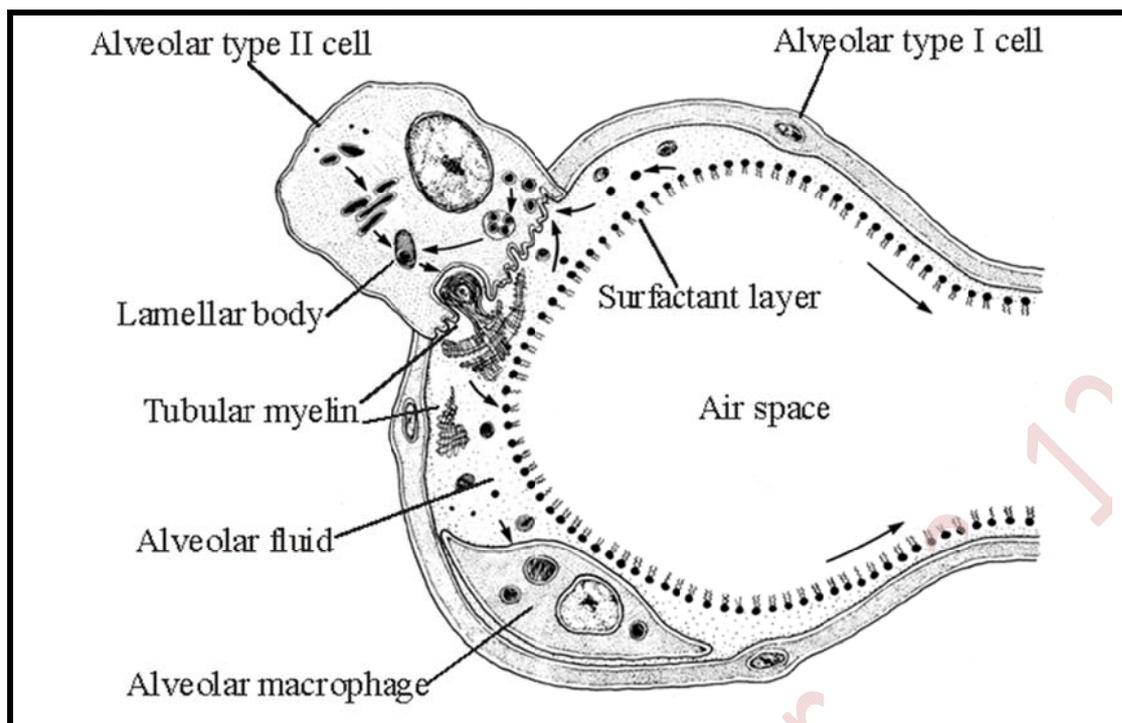


Figure 7. Structure of alveolus from [49] and modified in [50]. There are three major alveolar cell types in the alveolar wall (pneumocytes) [50]:

- Type I (Squamous Alveolar) cells that form the structure of an alveolar wall
- Type II (Great Alveolar) cells that secrete pulmonary surfactant to lower the surface tension of water and allows the membrane to separate, thereby increasing the capability to exchange gases. Surfactant is continuously released by exocytosis. It forms an underlying aqueous protein-containing hypophase and an overlying phospholipid film composed primarily of dipalmitoyl phosphatidylcholine.
- Macrophages that destroy foreign material, such as bacteria.

#### 4.4 Identifying toxicological interactions of NPs and cells

Two paradigms were proposed by *Shvedova et al.* [37] as common principles to understand the interactions of nanomaterials with cells and tissues better.

1. *Recognition versus non-recognition* of nanomaterials by the immune system, which might dictate their toxic potential.
2. *Oxidative stress* (production of reactive species) the mechanism that drives the toxicities of nanomaterials *in vitro* as *in vivo* [51].

Examples of recognition or oxidative stress *in vivo* from NPs are given in a complex biological context, since all toxicological papers published deal with interaction of nanomaterials with cells and tissues in one way or another. Generation of reactive oxidative species (ROS) may lead to cellular toxicity if the level of ROS production overwhelms the antioxidant defense of the cell or induces the mitochondrial apoptotic [52] mechanisms.

Toxicological assays, initially developed for chemicals, have long since been used for *in vitro* measurements on acute toxic effects of NPs. In order to analyze the effects of ingested nanomaterials, studies have followed the influence of single parameters, such as size, shape, chemical composition or crystallinity [22, 24]. But the toxic response is difficult to rank since varying physical parameters also influence the uptake. An increased toxic response can be attributed either to a higher toxicity or an increased uptake of the material. (See next section.) Therefore, again, the toxic response should be evaluated by normalizing it with the uptake of nanomaterials and their

state of agglomeration [1]. Then chemical parameters such as crystallinity and composition can more easily be compared and evaluated. Another question is how to determine long-term toxicological effects of biopersistent nanomaterials [1].

#### 4.4.1 Mechanisms controlling the nanoparticle-cell interactions

NPs strongly interact with interfaces or, as in the case of cell membranes, they typically also enter cells [1, 53]. Classical molecules are taken up in cells either by *active* processes, e.g. *endocytosis*, or by *passive* processes where hydrophobicity and molecular weight are the major parameters. Are NPs taken up by the same, generally applicable mechanisms?

Investigations through several analytical techniques indicate that uptake of NPs depends on particle size, surface charge, shape and, obviously, on exposition time and concentration of the NM. None of these properties are particularly biological. Do physical material properties determining the uptake of NMs in cells?

However, the uptake is a sequential problem: Transport (a) of the nanomaterials to the cells followed by their uptake (b). This is similar to the kinetics of heterogeneous chemical reactions, which are limited by transport and the reaction rate, and have found detailed mathematical treatment in chemical engineering. Hence, what is controlling the uptake of nanomaterials into cells, the biological uptake or the physical transport? The transport of NMs can be modeled by diffusion, sedimentation and agglomeration as confirmed in [38]. That study demonstrated that the transport of NPs to cells is actually the limiting step of NP uptake in classical *in vitro* testing. The study explains how the measured raw data are limited by the slower of the two sequential steps, the transport. This transport is strongly influenced by the physical properties size, surface charge, exposure concentration and the shape (structure) of the NMs.

The cellular uptake mechanisms in not well understood at the present time. However, the cellular membrane works as a protective barrier against foreign particles and most molecules. The transport mechanism for entry of nutrition into the cell is called *endocytosis*. Foreign materials, like NPs, are taken care of by phagocytes which neutralize or consume them through *phagocytosis*, a special type of endocytosis. In the study by Geiser *et al.*[53] it was found that NPs entered cells without a normal cell interaction. The authors proposed an unspecific mechanism for the NP uptake (not biological, but physical) based on interfacial effects, such as electrostatic and steric interactions. NPs were found within cells, either free in the cytoplasm or enclosed by a membrane. See Figure 1.

Several studies show that surface coatings affect the uptake and interaction with cells [20, 35, 54, 55], which also differs from classical cell uptake of molecules.

#### 4.4.2 Some examples of case studies on nanoparticle-cell interactions

Some straightforward examples of different tissue responses to different types of NPs are cited in [37], which give proof to interaction at the nanometric scale. Some examples of interaction due to different particle properties are given below:

- *Importance of size:*

According to Pan *et al.* [56] a gold nanocluster compound with a distinct particle size of 1.4 nm intercalates with the major groove of DNA and is a potent inducer of cell death in cancer cells. Interestingly, gold particles marginally smaller or larger showed significantly reduced toxicity, whereas considerably larger gold NPs were completely non-toxic in various cell lines tested.

Silica NPs of 40-80 nm in diameter can enter the cell nucleus and localize to distinct sub nuclear domains in the nucleoplasm of a cell. In contrast, fine 0.5 – 2  $\mu\text{m}$  micro silica particles located exclusively in the cytoplasm, that is on the cell wall [37].

Increased lung inflammation resulting from exposure to nano-sized particles compared with that resulting from an equivalent mass of micron-sized particles has been demonstrated in some studies, whereas others have found this not be the case [23].

- *Dose-dependent toxicity:*

Negative health effects of NPs do not appear to correlate with particle mass dose. Indeed, a high concentration of NPs may promote particle aggregation and could therefore reduce toxic responses compared to lower concentrations of the same particle [37].

(Thus, this is rather a problem to solve by better experimental design, as in [34, 57].)

- *Surface-area dependent toxicity.*

There are changes in surface chemical and physical properties as materials are reduced in size. For instance, following inhalation exposure of rats to 20 nm or 250 nm  $\text{TiO}_2$  particles, the half times for alveolar clearance of polystyrene test particles were proportional to the  $\text{TiO}_2$  particle surface area per million macrophages [25]. Surface area is suggested as a more appropriate dose metric than mass for pro-inflammatory effects of low-solubility, low-toxicity particles [37].

- *Crystalline structure-dependent toxicity:*

In a study of 3-10 nm  $\text{TiO}_2$  particles it was demonstrated that anatase titania was 100 times more toxic than an equivalent sample of rutile titania [51]. In the same study, the extent to which nanoscale titania affected cellular behavior was not dependent on sample surface area in this study.

Pulmonary toxicities of fine and ultrafine (nano-sized) particles appeared to correlate better with surface activity than with particle size and surface area [58, 59].

The last conclusion from 2006 by *Warheit et al.* regarding surface chemistry versus particle size is questioned in a paper by *Sager et al.* [60]. From their study they conclude that on a mass dose basis ultrafine  $\text{TiO}_2$  (21 nm, 80/20 anatase/rutile) was at least 41 fold more potent than fine  $\text{TiO}_2$  (1  $\mu\text{m}$ , 100% rutile), i.e. fine  $\text{TiO}_2$  demanded at least 41 times the mass of ultrafine  $\text{TiO}_2$  to obtain the same inflammogenic response. They concluded that the surface area of particles administered in toxicological experiments may be the more appropriate dose metric for nanoparticle pulmonary studies, since their findings support the fact that on a mass dose basis nano-sized particles are more toxic than fine-sized particles of the same material. Regarding the conclusions made by *Warheit et al.* they argue that once the particles, both ultrafine and fine, were suspended in phosphate buffer solution (PBS), their dynamic light scattering data indicated that both types of particles had agglomerated particle sizes  $> 2 \mu\text{m}$ . This leads to an over estimation of the delivered surface areas. From the papers published later by *Warheit and Sayes* [32, 41] I interpret this as not being an issue any more. The toxic response may correlate either to particle size, surface area and/or surface activity. It's just a question of how the study and the particle characterization are performed. In one way it's all a function of the same coin, namely particle size. That is for the same mass of particles, the smaller the particles are, the greater is the surface area. Likewise, the smaller the particle, the greater is the surface activity for the same mass. Isn't it? It's all a question for particles to interact. The issue of particle size and surface area dependent toxicity will be discussed further later.

Another important note in [37] dealing with the recognition of nanomaterials by the immune system, as quoted above, concerns a paper by *Geiser et al.* [61] where it was found that  $\text{TiO}_2$  NPs of 20 nm

were shown to escape from clearance by alveolar macrophages in peripheral lung of exposed mice, and this phenomenon could potentially explain the translocation of such particles into circulation. This raises questions regarding potential damage from TiO<sub>2</sub> particles circulating in the body.

*Nurkiewicz et al.* [57] showed that pulmonary exposure to fine and ultrafine TiO<sub>2</sub> causes systemic microvascular dysfunction. In rats exposed to fine TiO<sub>2</sub> (25 nm, agglomerate: 138 ± 3 nm) infusion elicited vasodilations (*dilations of blood vessels*) were blunted in proportion to pulmonary particle deposition. In rats exposed to ultrafine TiO<sub>2</sub> (1 µm, agglomerate: 402 ± 3 nm) infusion produced arteriolar constrictions or significantly impaired vasodilator responses. However they also exposed the rats to fine and ultrafine particles for same pulmonary mass deposition. If the particles had been administered based on surface area perhaps no significant difference had been found. They also argued that deposited particle mass is not an appropriate dose metric for pulmonary exposure.

*Shimizu et al.* [62] report some examples from other studies where inhaled NPs of carbon were translocated to secondary organs such as the brain [63] and the central nervous system via the olfactory nerve .

The issue regarding whether NPs can cross biological barriers and accumulate in secondary target organs following, for instance, inhalation was debated in a symposium on Nanomedicine held in Stockholm 9-11 September 2009 [64]. Dr. Wolfgang Kreyling (German Research Centre for Environmental Health) presented interesting quantitative data on the accumulation of small fractions of NPs (iridium, carbon, gold, titanium dioxide) in all secondary organs studied including the brain and the heart, also accumulation was even found in fetuses. The fractions were generally low but this depended strongly on particle size and surface modifications of the particles and on the route of administration. He concluded that it is possible that chronic exposure to NPs may lead to accumulation in secondary organs at levels that are sufficient to induce adverse health effects. However, long-term exposure studies have yet to be conducted.

The review from 2008 about NPs and the lung by Card et al. [23] presents some facts regarding studies in humans, which in fact are rather limited. Studies both confirm and contradict a computational model which predicts increased deposition of inhaled NPs in deceased or constricted airways. From a study by *Pietropaoli et al.* [65] it was shown that NPs can affect respiratory functions without a concomitant induction of inflammation. Regarding the issue whether NPs may translocate from the lungs to the systemic circulation in humans Card et al. only found one study out of seven which detected inhaled carbon NPs outside of the lungs. Still, that was just for carbon NPs (CB) and only after a single inhalation exposure.

#### **4.4.3 High aspect ratio nanoparticles (HARN)**

There has been considerable interest in the fibrous, high aspect ratio NPs given the previous experience with asbestos. Asbestos is a term used to describe a range of crystalline fibrous silicate minerals classified as serpentines, containing the single type chrysotile asbestos, and the amphiboles that contains crocidolite, amosite, tremolite and anthophyllite. Of these chrysotile (white asbestos) has been the most-used commercially. It has been suggested that asbestos is a nano material and hence the first commercially-used HARN since the chrysotile asbestos fibrils are < 100 nm in diameter. A full understanding of the asbestos hazards became apparent from studies on synthetic vitreous fibers (SVF) and bringing the results together with asbestos within a single 'fibre paradigm'. This paradigm highlighted the importance of length and biopersistence. Length was important, because above a key length, the fibers cannot be adequately phagocytosed by the alveolar

macrophages, preventing clearance and so the dose accumulates. Biopersistence is also important because long fibers that are biosoluble weaken, break and become short enough to be cleared and so do not persist in the milieu of the lungs. The overall conclusions from many studies were that the two factors length and biopersistence dominated the pathogenicity of respirable mineral and vitreous fibers [19]. (In this context long fibers are greater than 20 microns.)

However, the appearance of carbon-nano-tubes has questioned this fibre paradigm. It is by no means certain that fibres other than the asbestos and SVF adhere to this model, although some studies already point in the direction that biopersistence of HARN in many ways exhibit similar toxic properties as asbestos [66].

This also emphasizes that studies on all specific interactions of every novel nanomaterial and the corresponding relevant biological system are necessary.

## 5 Studies on the toxicity of TiO<sub>2</sub>

From a series of reviews conducted as part of the “*Engineered Nanoparticles: Review of Health and Environmental Safety*” (ENRHES) project, funded by the European Commission FP7 funding program, the review “Identification of the mechanisms that drive the toxicity of TiO<sub>2</sub> particulates: the contribution of physicochemical characteristics” by H.J. Johnston et al. was compiled in 2009 [24]. Since then at least two more specific reviews concerning TiO<sub>2</sub> NP toxicity have been published [29, 30]. In the following sections 4.1 - 4.3, facts from these reviews are compiled. Whenever results from any particular paper presented in these reviews is presented, the authors are written in *italics*. is referred to directly in this text and accordingly not included in the reference list at the end of this report, except if the particular paper has been read. Certainly also facts and conclusions from some other studies reviewed in this report are presented.

All conclusions from the findings are numbered in consecutive order in the following sections in order to facilitate the discussion.

### 5.1 In-vivo studies on pulmonary exposure to TiO<sub>2</sub> NPs

In *Ferin et al.* [67] rats were exposed via inhalation to ultrafine 21 nm (23,5 mg/m<sup>3</sup>) and fine 250 nm (23 mg/m<sup>3</sup>) TiO<sub>2</sub> particles for 12 weeks, and evaluated after 64 weeks; alternatively rats were administered to TiO<sub>2</sub> particles of various sizes (12, 21, 230, 250 nm) via single intratracheal instillation (up to 1 mg/rat) and the toxic response was studied 24 h post exposure. It was found that:

- ⇒ Nanoparticles (ultrafine) induced greater pulmonary inflammatory response than the micro-particulates which did not elicit any changes in the rat lungs.
- ⇒ The smaller particles also remained within the lung for longer periods compared to the larger particles (501 versus 174 days).
- ⇒ It was suggested that the smaller particles were able to translocate to the pulmonary interstitium more effectively than the larger particles.
- ⇒ Particle size and the number of particles administered impacted on the translocation process, and therefore TiO<sub>2</sub> toxicity.

*Renwick et al.* [68] investigated the size dependency of TiO<sub>2</sub> (29 nm NPs and 250 nm microparticles = MPs) and carbon black particles. Rats were exposed to particles via intratracheal instillation at 0,125 mg/rat or at relatively high dose of 0,5 mg/rat. Toxicological investigations were conducted 24 h post exposure.

- At the highest dose, only TiO<sub>2</sub> NPs but not TiO<sub>2</sub> MPs stimulated the recruitment of neutrophils into the lungs, epithelial damage, increased permeability of the lung epithelium and cytotoxicity, measured with BALF.

- The NPs were also able to diminish the phagocytic ability of isolated rat alveolar macrophages (from exposed animals).
- ⇒ The NPs demonstrated to elicit a greater pulmonary inflammatory response than MPs.

*Bermudez et al.* [69] determined if the choice of species influenced the pulmonary response to TiO<sub>2</sub> NPs. Rats, mice and hamsters were exposed to TiO<sub>2</sub> NPs (21 nm) via inhalation for 13 weeks, 6 h/day, 5 days/week, at conc. of 0.5, 2 or 10 mg/m<sup>3</sup>. The pulmonary response was assessed up to 52 weeks post exposure.

- Pulmonary inflammatory response was stimulated by TiO<sub>2</sub> NPs within rats and mice, but was absent in hamsters.
- In rats, a greater neutrophilic response was apparent, with progressive epithelial fibroproliferative changes also apparent.
- In mice there was neutrophil and macrophage components of the inflammatory response, and these cell populations remained elevated throughout the observation time.
- ⇒ The severity of the response was ranked as rat > mouse > hamster.
- ⇒ The limited toxicity apparent in hamsters was thought to derive from the low lung burden of particles, as hamsters had the greatest propensity to efficiently clear particles from the lung.
- ⇒ The difference in response of the different species is highlighted; therefore the sensitivity of different species within investigations must be considered and the model that mimics the human situation most accurately requires assessment.
- ⇒ The effects observed were dose dependent. The toxicity of NPs was enhanced by increasing the concentration.

These results are comparable with those of a companion study by *Bermudez et al.* [70] where the same species, rats, mice and hamsters were exposed to fine TiO<sub>2</sub> particles (1 micron) at concentrations of 10, 50 or 250 mg/m<sup>3</sup> via inhalation for 13 weeks, 6 h/day, 5 days a week as in *Bermudez et al.* 2004.

- ⇒ Species differences were witnessed and again ranked rat > mouse > hamster, and also that toxicity was concentration dependent.

From both these studies by *Bermudez et al.* it was concluded that

- ⇒ No size dependency could be distinguished, due to different mass dose in the different studies
- ⇒ It is reasonable to assume that the NP sample is more toxic when administered at the same mass dose.
- ⇒ The mass doses used were exceptionally high in both studies and thus their physiological relevance is questionable.

However, the studies aimed at investigating the efficiency of clearance processes in the different species, which justifies the high doses. It was thus concluded that hamsters had efficient clearing processes.

*Warheit et al.* [71] investigated the influence of exposure methods, inhalation and intratracheal instillation, and surface coating modification upon TiO<sub>2</sub> particles (290 – 440 nm) upon pulmonary toxicity within rats. For inhalation, rats were exposed to particles at concentrations up to 1300 mg/m<sup>3</sup> for a duration of 4 weeks. The high aerosol concentrations were used in order to provide a hazard screen to determine whether surface treatments drastically modify the toxicity of inhaled TiO<sub>2</sub> particles.

- After inhalation there was an accumulation within the lungs of macrophages containing particles.
- Following intratracheal instillation up to 10 mg/kg (~3 mg/rat), some TiO<sub>2</sub> preparations stimulated a transient pulmonary response, typified by infiltration of neutrophils and lactate dehydrogenase release (LDH) and this response was resolved within one week post exposure.
- ⇒ The response following particle exposure was very much dependent on the TiO<sub>2</sub> formulation. Specifically, samples containing the highest alumina or amorphous silica elicited the greatest adverse pulmonary response.

- ⇒ Overall, both methods (inhalation and intratracheal instillation) were associated with minimal adverse effects. That is despite the excessively high particle concentrations. But it is probably explained by the rather large non-nano size of the particles, being greater than 0.1 micron.

*Grassain et al.* [72] investigated which particle attributes that drive the toxicity of TiO<sub>2</sub> NPs, 5 and 21 nm, within mice subsequent to inhalation (0.7 or 7 mg/m<sup>3</sup>) or nasal instillation (12,7 mg/mouse). Inhalation was administered for 4 hours with toxicological observations made immediately and 24 h post exposure.

- An elevated macrophage population was associated with the inhalation of the particles. The macrophages internalized the particles.
- An infiltration of neutrophils was associated with the nasal instillation of TiO<sub>2</sub> NPs.
- ⇒ In both exposure scenarios the response induced was greater for the 21 nm particles. Thus the surface area was not the sole determinant of TiO<sub>2</sub> NP toxicity, which was unexpected.
- ⇒ The crystallinity of the samples was suggested to influence the toxicity of TiO<sub>2</sub> NPs, as the 5 nm particles were pure anatase, whereas the 21 nm particles were a mix of rutile and anatase particles.
- ⇒ The nature of the response varied for the different exposure methods; inhalation -> macrophage driven response; instillation -> development of a neutrophil driven response.
- ⇒ Experimental set-up influences the results of toxicity tests.

In another study by *Grassain et al.* [73] mice were exposed by inhalation to TiO<sub>2</sub> NPs 2 – 5 nm at a dosage of 0,77 or 7,22 mg/m<sup>3</sup>. Aerosol particles agglomerated to a mean diameter of 120 – 130 nm. Acute exposures demonstrated no adverse effects 4 h after the exposures commence. Analysis of mice 0, 1 and 2 weeks post exposure showed evidence of inflammatory response, with recovery after 3 weeks.

- ⇒ Inhaled TiO<sub>2</sub> NPs < 10 nm in mice induce relatively modest responses which disappeared after 3 weeks post exposure.

*Warheit et al.* [74] investigated the influence of crystalline form of TiO<sub>2</sub> NPs (1 or 5 mg/kg) to pulmonary toxicity within rats, subsequent to intratracheal administration. Two rutile NP samples, a mixed (80 % anatase; 20 % rutile) sample and a microparticulate rutile (negative control), in addition to a  $\alpha$ -quartz sample (positive control) were considered. All TiO<sub>2</sub> NPs were highly agglomerated (> 2 micron) in PBS and their primary particle size of less than 100 nm. Pulmonary response to particles was evaluated up to three months post exposure.

- ⇒ Although quartz had the greatest inflammogenicity, it was evident that several TiO<sub>2</sub> particle samples elicited a short term, pulmonary inflammatory response that was characterized by the infiltration of neutrophils, and was greatest and more sustained for the mixed NP sample.
- ⇒ The properties responsible for driving the TiO<sub>2</sub> toxicity were then considered and it was suggested that differences in the particle surface area accounted, in part, for the responses. Accordingly, the mixed sample had the greatest surface area (53 m<sup>2</sup>/g compared to 18,2 and 35,7 m<sup>2</sup>/g respectively for the rutile NPs).
- ⇒ Since the rutile NP samples, with intermediate surface area, did not exhibit intermediate levels of toxicity, it was speculated that additional factors, besides surface area, such as crystallinity contribute to the toxicity of TiO<sub>2</sub>. That is, rutile being less toxic than anatase. However, that could also be a result of post-production removal of chloride from the particle surfaces, and of enhanced agglomeration of particles.
- ⇒ The particle concentrations were excessively high and unlikely to be encountered by humans.

In the study [58] *Warheit et al.* evaluated the acute lung toxicity in rats of intratracheally instilled pigment-grade TiO<sub>2</sub> particles (rutile, ~300 nm) versus nanoscale TiO<sub>2</sub> rods (anatase, 200 x 35 nm) or nanoscale TiO<sub>2</sub> dots (anatase, ~10 nm) compared with a positive control particle type, quartz (1-3 microns). Evaluation of lung tissue of the rats was conducted as 24 h, 1 week, 1 month and 3 months post instillation exposure.

- Exposure to nanoscale TiO<sub>2</sub> rods or nanoscale TiO<sub>2</sub> dots produced transient inflammatory and cell injury effects at 24 h post exposure (pe) and were not different from the pulmonary effects of larger sized TiO<sub>2</sub> particle exposures.
- In contrast, pulmonary exposures to quartz particles in rats produced a dose-dependent lung inflammatory response characterized by neutrophils and foamy lipid-containing alveolar macrophage accumulation as well as evidence of early lung tissue thickening consistent with the development of pulmonary fibrosis.
- ⇒ The results provide the first example of nanoscale particle types which are not more cytotoxic or inflammogenic to the lung compared to larger sized particles of same composition.

*Chen et al.* [75] exposed mice via intratracheal instillation to nano (19-21 nm) and micro (180-250 nm) TiO<sub>2</sub> particles (0.1 and 0.5 mg/mouse).

- Histological assessment illustrated that morphological alterations were evident on exposure to nano- TiO<sub>2</sub>, which were emphysema like in nature, including, for example, alveolar enlargement.
- Lesions were more pronounced in areas where particles preferentially accumulated, and increased in severity with increasing time and dose.
- An inflammatory response was also observed.
- ⇒ Despite the large doses used, no pathological lesions were found in response to micro-particulate TiO<sub>2</sub>.
- ⇒ This study again illustrates the size dependent toxicity. (*According to Johnston et al.*).

*Ahn et al.* [76] investigated which processes were responsible for particulate mediated stimulation of excessive mucus secretion within humans. Rats were exposed to TiO<sub>2</sub> particles (290 nm, 4 mg/kg) via intratracheal instillation, and toxicological observations were made from 4 to 72 hours post exposure.

- The TiO<sub>2</sub> exposure stimulated an increase in goblet cell hyperplasia.
- ⇒ Therefore it could be speculated that particle mediated increases in mucus secretion can contribute to the aggravation of chronic airway disease symptoms also in humans.

*Inoue et al.* [77] studied the ability of TiO<sub>2</sub> NP exposure to influence the pulmonary toxicity of lipopolysaccharide (LPS). The impact of intratracheally instilled (8mg/kg) TiO<sub>2</sub> (15, 50, 100 nm) on LPS mediated pulmonary inflammation in mice was determined.

- Combined TiO<sub>2</sub> and LPS treatment was able to exacerbate LPS mediated inflammation.
- Cytokines were also increased, as well as coagulatory factors such as fibrinogen.
- ⇒ There was a size dependency whereby smaller particles were more toxic.

*Nurkiewicz et al.* [78] determined the impact of TiO<sub>2</sub> MPs (1 micron) on the systemic microvasculature, following intratracheal instillation of rats (0,1 or 0,25 mg/rat, for 24 h). They encompassed the possibility that systemic effects were a component of the pulmonary response to particles.

- No cytotoxic response in the lungs was evident, but particles were able to induce an impairment of endothelium dependent arterior dilation.
- ⇒ The responses were suggested to be related to increased neutrophil adhesion to the vessels, myeloperoxidase (MPO) deposition and oxidative stress within the vessel wall.
- ⇒ These findings are of concern as they indicate that an inflammatory response may be stimulated within the vessel. However, the response was independent of the level of pulmonary inflammation and was not thought to be reliant on the migration of particles from the lung.

Studies regarding the fate of TiO<sub>2</sub> NPs, subsequent to pulmonary exposure have been apparent, in particular on particle transfer to the brain [24]. In *Wang et al.*[79] and *Wang et al.* [80] the distribution of rutile (80 nm) and anatase (155 nm) particles within the mouse brain, after nasal instillation (0.5 mg/mouse every other day for 30 days), was investigated to determine if any neurotoxicity was associated with the exposure.

- Both forms of TiO<sub>2</sub> NPs accessed the brain and accumulation was highest in the hippocampus.

- The uptake was likely mediated via neuronal transport following the nasal instillation.
- The accumulation was time dependent and an inflammatory response within the brain was also stimulated by TiO<sub>2</sub> particle exposure.
- ⇒ It was concluded that the small differences in response to anatase particles versus the rutile ones could be related to crystal structure.

*Dankovic et al.* [44] evaluated the rat dose-response data and conducted a quantitative risk assessment for TiO<sub>2</sub>. Fine TiO<sub>2</sub> (<2.5 micron) has been shown to produce lung tumors in rats exposed to 250 mg/m<sup>3</sup>, and ultrafine TiO<sub>2</sub> (< 0.1 micron diameter) has been shown to produce lung tumors in rats at 10 mg/m<sup>3</sup>. Efforts to estimate the excess risk at exposure from seven different dose-response models were presented. Preliminary conclusions were:

- ⇒ Fine and ultrafine TiO<sub>2</sub> and other poorly soluble, low-toxicity (PSLT) particles show a consistent dose-response relationship when dose is expressed as particle surface area;
- ⇒ The mechanism of TiO<sub>2</sub> tumor induction in rats appears to be a secondary genotoxic mechanism associated with persistent inflammation;
- ⇒ The inflammatory response shows evidence of a nonzero threshold.
- ⇒ Risk estimates for TiO<sub>2</sub> depend on both the dosimetric approach and the statistical model that is used.

The translocation of TiO<sub>2</sub> NPs, subsequent to pulmonary exposure must encompass the possibility that distal sites, like the CNS, liver and the cardiovascular are affected. According to *Nurkiewicz et al.* [57] it has been shown that once in the lung TiO<sub>2</sub> NPs initiate pulmonary responses such as airway inflammation in rats, alveolar macrophage recruitment, and the activation of various growth factors and chemokines in mice. According to *Nurkiewicz et al.*:

- ⇒ It has also been shown in other studies that ultrafine particles deposited in the lung may have the ability to translocate to systemic sites within 24 hours of deposition (animal studies).
- ⇒ Similarly, within 24 hours of deposition a substantial portion of inhaled TiO<sub>2</sub> NPs escape phagocytosis and enter the alveolar interstitium and pulmonary capillaries.

Therefore, while the pulmonary responses to nano-sized titania particles starting to appear fairly well understood, *Nurkiewicz et al.* focused on systemic microvascular effects of exposure to TiO<sub>2</sub> NPs.

They had previously shown that [78];

- ⇒ Pulmonary exposure to fine PM<sub>2.5</sub> causes systemic microvascular dysfunction, most evident in the form of impaired or abolished endothelium-dependent arteriolar dilation.

Those studies also provided evidence that this systemic effect is not necessarily the result of the inherent pulmonary toxicity of the particles (*chemistry*), in that equivalent doses of TiO<sub>2</sub> and residual oil fly ash (ROFA) caused similar, dose-dependent degrees of microvascular dysfunction [78]. While this indicates that larger TiO<sub>2</sub> particles elicit potent systemic microvascular effects, it cannot be used to conclude that ultrafine TiO<sub>2</sub> exposure will produce qualitatively or quantitatively similar biological effects. Therefore, *Nurkiewicz et al.* [57], studied the microvascular function after pulmonary exposure (via inhalation) to fine or ultrafine TiO<sub>2</sub> aerosols over a range of concentrations, and determined if ultrafine TiO<sub>2</sub> particles are inherently more toxic than larger, fine TiO<sub>2</sub> particles. It was found that

- ⇒ Inhalation of ultrafine TiO<sub>2</sub> produced greater remote microvascular dysfunction than fine TiO<sub>2</sub> particles.

Similar results were obtained in another study by *Nurkiewicz et al.* [81] where the purpose was to identify alterations in the production of reactive species and endogenous nitric oxide (NO) after nanoparticle exposure. The results indicated that in conjunction with microvascular dysfunction, as shown before, nanoparticle exposure also decreases NO bioavailability through at least two functionally mechanisms, that may mutually increase local reactive species.

A study by *Mühlfeld et al.* [82] confirmed:

- ⇒ Evidence for translocation of TiO<sub>2</sub> NPs from the lungs into the vascular system.

- ⇒ However, in the relation to the total number of NPs within the lung, the number of TiO<sub>2</sub> NPs observed within the capillaries is still very low.

Elina M. Rossi et al. [55] investigated how airway exposure to silica-coated TiO<sub>2</sub> NPs induces pulmonary neutrophilia in mice.

- ⇒ SiO<sub>2</sub>-coated rutile TiO<sub>2</sub> nanoparticles (cnTiO<sub>2</sub>) was the only sample tested that elicited clear-cut pulmonary neutrophilia. Uncoated rutile and anatase as well as nanosized SiO<sub>2</sub> did not induce significant inflammation.
- ⇒ Their findings emphasize that it is vitally important to take into account in the risk assessment that alterations of NPs, e.g., by surface coating, may drastically change their toxicological potential.

Liu et al. [83] evaluated if TiO<sub>2</sub> NPs can induce acute pulmonary toxicity in rats. Therefore rats were intra-tracheally instilled with 0.5, 5 or 50 mg/kg of 5, 21 and 50 nm primary TiO<sub>2</sub> NPs. The 5 nm particles were anatase with surface area 210 m<sup>2</sup>/g. Corresponding data for the 21 nm and 50 nm TiO<sub>2</sub> NPs were: 80% anatase/20% rutile, 50 m<sup>2</sup>/g; >98% rutile, 30 m<sup>2</sup>/g. No data on particle agglomeration were discussed in the paper. The authors found that

- ⇒ The toxic effect of TiO<sub>2</sub> NPs in lung tissue exhibited a dose-response relationship.
- ⇒ At exposure of 5.0 mg/kg both 5 nm and 21 nm TiO<sub>2</sub> NPs induced standing pulmonary lesions
- ⇒ At the highest exposure of 50 mg/kg, 5 nm TiO<sub>2</sub> NPs suppress the phagocytic ability of alveolar macrophages.
- ⇒ Pulmonary toxicity caused by 5 nm TiO<sub>2</sub> NPs was more severe than that caused by 21 and 50 nm TiO<sub>2</sub> NPs.

## 5.2 Other *in vivo* administered exposures to TiO<sub>2</sub> NPs

Many other types of *in vivo* exposure may be performed in toxicological studies, for example intraperitoneal, oral and dermal exposure. Intraperitoneal exposure is the injection of a substance into the peritoneum, i.e. the body cavity. Dermal exposure is testing substances on skin and intravenous exposure is injection of substance into the veins. Regarding dermal exposure most studies have shown that the penetration of TiO<sub>2</sub> NPs is negligible on healthy skin. Therefore the TiO<sub>2</sub> NPs propensity for toxicity responses on healthy skin is anticipated to be minimal. Oral exposure has shown that TiO<sub>2</sub> NPs (25, 80, 155 nm) are able to pass through the gut wall. Both oral and intravenous exposure of TiO<sub>2</sub> NPs has shown an accumulation of particles in the liver. See the reviews [24, 29] which gives lots of information regarding such studies.

## 5.3 *In vitro* investigations of TiO<sub>2</sub> NP toxicity

During the subsequent half-century, tens of millions of dollars have been invested by corporations, governments, and other stakeholders with the goal of advancing the 3Rs in research and testing, where the 3R's stands for:

- Replacement of animals,
- Reduction in the numbers used, or
- Refinement of techniques to alleviate or minimize potential pain, distress, and/or suffering.

*In vitro* cell and tissue cultures are:

- Freshly harvested "primary" cells, tissues, or organ (e.g., liver slices for metabolism studies; corneas from slaughtered cow or chicken eyes for eye irritation studies);
- Cell lines (e.g., mouse 3T3 cell line for evaluating the potential for sunlight-induced phototoxicity); stem cells (e.g., stem cells for embryotoxicity testing);
- Complex reconstructed tissue models (e.g., EpiDerm<sup>TM</sup> human skin corrosion test).

These and other cell and tissue-based methods have already achieved international acceptance as full or partial replacement methods for their animal-based counterparts.

Regarding the studies reviewed and presented in the following, again, there is no aim for this review to explain the different methods, except to give an account for the toxicologist what type of *in vitro* method that was used. But many concepts of *in vitro* syllabus are explained briefly in Appendix 1.

### 5.3.1 TiO<sub>2</sub> NP toxicity studies from *in vitro* lung models

Cytotoxicities of TiO<sub>2</sub> NPs (21 nm) of different concentrations (5, 10, 20 and 40 µ/ml) were evaluated in a study by *Park et al.* [84] using a cultured human bronchial epithelial cell line, BEAS-2B. Exposure of the cultured cells to NPs led to

- Cell death, reactive oxygen species (ROS) increase, reduced glutathione (GSH) decrease, and induction of oxidative stress-related genes.
- It was found that TiO<sub>2</sub> NPs exert cytotoxicity by an apoptotic process.
- Expressions of inflammation-related genes were also elevated.
- ⇒ Uptake of the NPs into the cultured cells was observed and TiO<sub>2</sub> NPs seemed to penetrate into the cytoplasm and locate in the peri-region of the nucleus as aggregated particles.
- ⇒ This last result may induce direct interactions between the particles and cellular molecules, to cause adverse biological responses.

*Jia-Ran Gurr et al.* [85] found that

- ⇒ Ultrafine anatase of sizes 10 and 20 nm, in the absence of photo-activation, induced oxidative DNA damage, lipid peroxidation (oxidative degradation of lipids), and micronuclei formation and increased hydrogen peroxide and nitric oxide production in BEAS-2B cells. But large anatase particles, > 200 nm, did not.
- ⇒ It seems that smaller particles more easily induce oxidative damage than larger particles.
- ⇒ In contrast rutile particles of 200 nm induced hydrogen peroxide and oxidative damage in the absence of light.
- ⇒ The results suggest that intratracheal instillation of ultrafine TiO<sub>2</sub> particles may cause an inflammatory response.

*Simon-Deckers et al.* [86] investigated the toxicity of a panel of NPs, including TiO<sub>2</sub> (in a variety of sizes in both anatase and rutile forms) and Al<sub>2</sub>O<sub>3</sub>, to A549 cells. In general the cytotoxicity was low. The study showed that:

- ⇒ It is possible for particles to be internalized by cells without having a detrimental impact on normal cell function.
- ⇒ The crystal phase was observed to influence the cytotoxicity exhibited by the particles. In particular, the greater the anatase content, the greater was the ability to induce cell death.
- ⇒ Also, the NP size had influence, since TiO<sub>2</sub> NPs were more toxic than their larger counterparts.
- ⇒ The chemical composition makes a difference since TiO<sub>2</sub> NPs of 12 nm were more toxic than 11 nm Al<sub>2</sub>O<sub>3</sub>, despite their similarity in size.

In summary it was found that the crystal phase (a), the size (b) and the chemical composition (c) influenced the toxicity, which in fact was low generally for all the NPs used.

*Kim et al.* [87] assessed the TiO<sub>2</sub> and SiO<sub>2</sub> mediated cytotoxicity to alveolar macrophages from rats, when exposed to concentrations 0.5 – 5 mg/ml.

- ⇒ TiO<sub>2</sub> elicited a dose dependent decrease in cell viability.
- ⇒ SiO<sub>2</sub> had a greater cytotoxic potential than TiO<sub>2</sub>.

*Karlsson et al.* [88] compared NPs of the metaloxides Fe<sub>2</sub>O<sub>3</sub>, Fe<sub>3</sub>O<sub>4</sub>, TiO<sub>2</sub> and CuO with corresponding micrometer particles. The fine TiO<sub>2</sub> and nano TiO<sub>2</sub> particles had a mean size of 1 micron and 63 nm, respectively. The fine TiO<sub>2</sub> consisted of 99.9 % rutile and the ultrafine TiO<sub>2</sub> consisted of a mix of anatase and rutile.

The influence of different dosages was also studied for TiO<sub>2</sub> and CuO. The ability of the particles to cause cell death, mitochondrial damage, DNA damage and oxidative DNA lesions were evaluated after exposure of the human alveolar epithelial type-II cell line A549.

From the results (summarized in TABLE 3) the following conclusions were suggested.

- ⇒ It cannot be generalized that NPs always are more toxic than fine/micrometer particles.
- ⇒ Indeed NPs with certain compositions can be more toxic than micrometer particles of the same composition.
- ⇒ CuO particles are much more toxic and genotoxic compared to other metal oxides. (See also [89].)
- ⇒ TiO<sub>2</sub> particles were not cytotoxic and did not cause mitochondrial damage. (Other studies have also shown low cytotoxicity and little ability to mitochondrial damage of TiO<sub>2</sub> NPs. But the study by Park et al., already mentioned, showed toxicity effects of 21 nm TiO<sub>2</sub> particles in BEAS-2B cells. [84])
- ⇒ However, both size fractions of TiO<sub>2</sub> showed high ability to damage DNA.
- ⇒ Interestingly the fine TiO<sub>2</sub> particles caused significantly higher damage than the NPs, which is in contrast to most *in vitro* and *in vivo* studies regarding inflammatory potential.

TABLE 3. Comparison of toxicity of nano- and microparticles of some metal-oxides. The table shows whether NPs are more or less toxic than the corresponding microparticles. [88]

Nanoparticles vs Microparticles	Cytotoxicity test	Mitochondrial Damage test	DNA Damage test	Oxidative DNA damage test
CuO	Much more toxic	Much more toxic	Much more toxic	Much more toxic
Fe <sub>2</sub> O <sub>3</sub>	Slightly more toxic	Equally toxic	Less toxic	More toxic
Fe <sub>3</sub> O <sub>4</sub>	Not toxic	Slightly less toxic	More toxic	Much more toxic
TiO <sub>2</sub>	Not toxic	Slightly more toxic	Less toxic	More toxic

Falck GC et al. [54] studied genotoxic effects of nanosized and fine TiO<sub>2</sub> in human bronchial epithelial BEAS 2B cells using the single-cell gel electrophoresis (comet) assay and the cytokinesis-block micronucleus test. BEAS 2B cells were exposed to eight doses (1-100 µg/cm<sup>2</sup>) of titanium (IV) oxide nanosized rutile (>95%, <5% amorphous SiO<sub>2</sub> coating, 10 x 40 nm), nanosized anatase (99.7%; <25 nm), or fine rutile (99.9%; < 5 µm) for 24, 48, and 72 h. In conclusion, their studies showed that:

- ⇒ Fine rutile reduced cell viability at lower doses than nanosized anatase, which was more cytotoxic than nanosized rutile.
- ⇒ In the comet assay, nanosized anatase and fine rutile induced DNA damage at several doses with all treatment times.
- ⇒ Dose-dependent effects were seen after the 48- and 72-h treatments with nanosized anatase and after the 24-, 48- (in one out of two experiments), and 72-h treatments (one experiment) with fine rutile.
- ⇒ The lowest doses inducing DNA damage were 1 µg/cm<sup>2</sup> for fine rutile and 10 µg/cm<sup>2</sup> for nanosized anatase.
- ⇒ Nanosized rutile showed a significant induction in DNA damage only at 80 µg/cm<sup>2</sup> in the 24-h treatment and at 80 and 100 µg/cm<sup>2</sup> in the 72-h treatment (with a dose-dependent effect).
- ⇒ Uncoated nanosized anatase TiO<sub>2</sub> and fine rutile TiO<sub>2</sub> are more efficient than SiO<sub>2</sub>-coated nanosized rutile TiO<sub>2</sub> in inducing DNA damage, whereas only nanosized anatase is able to slightly induce micronuclei.

The aim of a study conducted by Park et al. [90] was to evaluate *in vitro* biological effects of various (TiO<sub>2</sub>, Ag, Al, Zn, Ni) inhalable metallic NPs. Human alveolar epithelial cells (A549) were exposed to various concentrations of NPs for 24 h. Toxicity was estimated by the extent of morphological damage, apoptotic damage measured with two-colour flow cytometry or with DNA fragmentation.

- ⇒ The extent of toxicity was affected in a dose-dependent manner in all three tests.
- ⇒ The extent of apoptotic damage measured with DNA fragmentation indicated no significant difference in the damages from fine and ultrafine TiO<sub>2</sub>.

- ⇒ Uptake of no other NPs but fine and ultrafine TiO<sub>2</sub> into the cells was observed after 27 h exposure.
- ⇒ The intracellular generation of ROS was significant with n-Zn but not with the other particles. The results are given in an approximate manner in TABLE 4 where the extent of toxicity is weighed by + signs where one + is weak and 5+ is strong toxicity. The results demonstrated that various inhalable NPs (TiO<sub>2</sub>, Ag, Al, Zn, Ni, silica) could cause cell damages directly or indirectly.

TABLE 4. Approximate indications of toxicity of the different metallic NPs in relation to each other.

Substance	morphological damage	apoptotic damage <sup>a</sup>	apoptotic damage <sup>b</sup>	other remarks
TiO <sub>2</sub> , nano and micro	m-Ti: 5+ ; n-Ti: 4+	n-Ti: + ; m-Ti: <+	n-Ti: 2+ ; m-Ti: 2+	apop.dam. in dose dependent manner
Silica, nano and micro	m-Si: 3+	m-Si: 3+	m-Si: 5+	
Ag, nano	+	<<<+	<+	
Al, nano	+	<<+	+	
Zn, nano	+	5+	5+	intracellular generation of ROS
Ni, nano	+	4+	4+	

a) measured with two-colour flow cytometry

b) measured with DNA fragmentation

In a study by *Bhattacharya et al.* [91] human lung fibroblasts (IMR-90) were exposed to TiO<sub>2</sub> (anatase) or Fe<sub>2</sub>O<sub>3</sub> (hematite) NP to compare and analyze their cyto- and genotoxic potential, their ability to generate ROS and to form DNA-adducts. Furthermore additional studies regarding the physicochemical properties of the particles were undertaken, such as surface area, particle size distribution, zeta potential, EDX for surface chemistry, trace elemental determination (Fe[II] / Fe[III]) and electron microscopy studies. Cytotoxicity and genotoxicity studies were carried out in both types of human cells: IMR 90 (human bronchial fibroblasts) and BEAS-2B cells.

- IMR-90 cells were more sensitive and showed stronger effects after particle exposure than the virus-transformed BEAS-2B cells.
- ⇒ It was concluded that even though the TiO<sub>2</sub>-NP demonstrated low amount of cyto- and genotoxicity as compared to the Fe<sub>2</sub>O<sub>3</sub>-NP, they were more capable of generating stable 8-OHdG adducts (*see App. 1*). This may be related to the surface charge of the NP (*zeta-potential*).
- ⇒ The study also demonstrated that the Fe<sub>2</sub>O<sub>3</sub>-NP required a special reduced condition to undergo conversion from Fe[III] to Fe[II] according to the Fenton reaction to generate OH radicals.
- ⇒ Generation of intracellular ROS by TiO<sub>2</sub>-NP may induce the observed oxidative DNA adduct formation.
- ⇒ Even low adducts may lead to persistent DNA lesions.
- ⇒ Other factors that need to be considered are potential DNA repair inhibitions caused by TiO<sub>2</sub>-NP. The results are summarized in TABLE 5.

TABLE 5: Element properties and summary of the observed effects from anatase and hematite NPs.

	TiO <sub>2</sub> -NP	Fe <sub>2</sub> O <sub>3</sub> -NP
Particle diameter (hydrodynamic)	91 nm	50 nm
Surface area	49,7 m <sup>2</sup> /g	34,4 m <sup>2</sup> /g
Zeta potential	+ 48,8	-26,7 mV
Genotoxicity (Comet assay: DNA breakage)	-	+
Cytotoxicity (Trypan blue assay)	-(+)*	+
Acellular radical formation (ESR)	↑	↓(↑)**
Intracellular radical formation (H2DCFDA)	↑	delayed
Oxidative DNA-damage (8-OHdG)	+	-

\*) Cytotoxicity negative in BEAS-2B and positive in IMR-90 cells.

\*\*\*) Elevated radical formation under reducing conditions.

Lanone et al. [33] evaluated the toxic effect of 24 NPs of similar equivalent spherical diameter and various elemental compositions on two human pulmonary cell lines: A549 and THP-1. They also evaluated the experimental design. According to their results they found that:

- ⇒ Copper and zinc based NPs were most toxic.
- ⇒ Titania, alumina, ceria and zirconia based NPs show moderate toxicity.
- ⇒ No toxicity was observed for tungstencarbide.
- ⇒ No correlation between cytotoxicity and equivalent spherical diameter was found.
- ⇒ Their study highlighted the difference of sensitivity between cell types and cytotoxicity assays, which demand careful valuation when assessing NPs toxicity.

Sohaebuddin et al. [34], aimed to systematically investigate the influence of nanomaterial properties on the degrees and pathways of cytotoxicity, in order to clear some of the inconsistent and sometimes contradictory results obtained from earlier research efforts. They assessed the characteristics of the NPs in aqueous solution and evaluated their ability to absorb serum proteins from culture media, through monitoring their cellular uptake, cell viability, intracellular responses, and potential mechanisms of toxicity. They used three model cell lines representing different physiological compartments, 3T3 fibroblasts, RAW 264,7 macrophages and telomerase-immortalized (hT) bronchiolar epithelial cells. They selected TiO<sub>2</sub> (anatase, 5-10 nm) and SiO<sub>2</sub> (30 nm). They also chose MWCNT (Multi-Walled-Carbon-Nano-Tubes) 0,5 – 2 microns in length with three different ranges of diameters, < 8 nm, 20-30 nm, > 50 nm to examine the material size on nanoparticle toxicity. Since all cell types were exposed to the NPs suspended in solutions, all nanomaterials were characterized in solution, both in PBS (phosphate buffer solution) and in cell culture media containing serum. In PBS both TiO<sub>2</sub> and SiO<sub>2</sub> agglomerated to a size of 200-400 nm, whereas the agglomerated MWCNT had sizes in between 100-240 nm. The thickest MWCNT (>50 nm) had less tendency to agglomerate than the thinner MWCNTs. Thus, the metal-oxide NPs had the highest tendency to form agglomerates. Although the metal oxides differed in particle size, their average size in PBS was identical. The size of aggregates seemed to be concentration dependent. Conclusions were

- ⇒ Toxicity increases dependent on concentration. RAW macrophages appear particularly susceptible to higher concentrations, regardless of particle size or composition.
- ⇒ RAW macrophages were more sensitive to non-spherical geometries.
- ⇒ 3T3 fibroblasts and hT epithelial cells only showed susceptibility to TiO<sub>2</sub> at elevated concentrations where viability fell to near 50 %.
- ⇒ Regarding the influence of size and shape on toxicity, their results demonstrate the importance of both cell type and composition regarding size considerations.

A summary of the results is put together in TABLE 6, as presented in [34].

TABLE 6: Cell responses from the three different model cell lines.

Cell responses	3T3 fibroblasts					hT bronch. epithel. cells					RAW macrophages				
	TiO <sub>2</sub>	SiO <sub>2</sub>	MWCNT (nm)			TiO <sub>2</sub>	SiO <sub>2</sub>	MWCNT (nm)			TiO <sub>2</sub>	SiO <sub>2</sub>	MWCNT (nm)		
			<8	20-30	>50			<8	20-30	>50			<8	20-30	>50
Cytotoxicity	+	+	+	+	+	+	++	++	+	-	3+	3+	++	++	3+
ROS	+	+	+	+	+	-	+	+	-	-	+	+	+	-	+
LMD <sup>i</sup>	-	-	3+	+	+	-	-	-	-	+	-	-	-	-	+
MMP <sup>ii</sup>	-	-	++	-	-	-	+	++	-	-	-	+	+	-	+
Caspase 3/7	++	++	-	+	-	-	++	3+	-	-	-	-	-	-	-
Apoptosis <sup>iii</sup>	-	+	+	+	+	-	3+	++	-	-	3+	3+	+	+	++
Necrosis	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-

i) Lysosomal Membrane Destabilisation

ii) Mitochondrial Membrane Potential

iii) Apoptosis is the process of programmed cell death (PCD) that may occur in multicellular organisms. In contrast to necrosis, which is a form of traumatic cell death that results from acute cellular injury, apoptosis, in general, confers advantages during an organism's life cycle [52]

Due to the lack of standards for NM in-vitro testing, current data on NM toxicity are often inconsistent and can hardly be compared. Recent findings suggest that multiple *in vitro* assays should be employed in nanomaterial toxicity assessments to be able to detect the potentially multiple responses of cells exposed to NMs. Therefore, in the study by *Kroll et al.* [35], they evaluated and standardized common *in vitro* assays measuring three different cytotoxic endpoints (oxidative stress, metabolic activity, cell death) and adapted them for the toxicity assessment of 23 engineered NMs using a test matrix of ten cell lines in an attempt to identify the specific NM properties responsible for NM toxicity. The NMs represented metal oxides, sulfates, and carbonates. Carbon black was used as reference (positive). The primary particle size, morphology, surface chemistry, crystallinity, impurities, zeta-potential, pH and solubility in water and/or in cell culture medium were measured and determined, before the toxicity studies started. The influence of physicochemical properties on particle toxicity was investigated using different particles of the same composition. For example there were three different pure titania powders, five different CeO powders and six mixed oxides containing TiO<sub>2</sub>. Before testing the engineered NMs, the different cytotoxic assays were tested and evaluated in a manner to administrate proper doses of materials, so that no particles interference would occur (due to their intrinsic optical activity) in the analysis of the results. The particle concentration was limited to 10 µg/cm<sup>2</sup>, since higher doses potentially could have led to false positive or negative results.

⇒ From other work *Kroll et al.* [35] found that, for TiO<sub>2</sub> NPs, overload effects in rats occur at 10 mg/m<sup>2</sup>. From estimation of the volume and the rat lung surface they calculated that such an overload corresponds to ~3 µg/cm<sup>2</sup>.

Thus the three doses 0.1, 1 and 10 µg/cm<sup>2</sup> were used, which covered the range up to overload. Over all, the screening of the 23 engineered NMs biological effects on the ten different cell lines, resulted in 7 NMs inducing cytotoxic responses. Of the different cell lines four represent humans (Lung: A549, CaLu3 | Skin: HaCaT | Colon: CaCo2s), and the six others were of animal origin including lung epithelial cells (RLE-6TN), fibroblasts (NIH-3T3), macrophages, (RAW264.7) as well as three different epithelial cell lines (MDCK, MDCK II, NRK52E) representing the kidney as an important secondary target organ.

⇒ Significant increases of cell death were not detected with any of the particle types and reduced metabolic activity was only displayed by a single cell line (NIH-3T3) and only when exposed to BaSO<sub>4</sub>.

The results from the ten different cell lines on reactive oxidative stress (ROS) are summarized in TABLE 7, as presented by the authors.

TABLE 7. Summary of the cell-line specific oxidative responses after exposure to different NMs. Mean DCF fluorescence values significantly different from control values are displayed as follows: black: >200 % of control; dark grey: 150-200 % of control; light grey: 120-150 % of control; white: >100-120 % of control.

	A549	CaCo2	CaLu3	HaCaT	MDCK	MDCKII	NIH3T3	NRK52E	RAW264.7	RLE-6TN
Oxidative Stress (DCF)	CB									
	TiO <sub>2</sub> -3		TiO <sub>2</sub> -3	TiO <sub>2</sub> -3	TiO <sub>2</sub> -3	TiO <sub>2</sub> -3				
	Ti-Zr-3	CeO <sub>2</sub> -A		Ti-Zr-3	Ti-Zr-3		Ti-Zr-3	Ti-Zr-3	Ti-Zr-3	Ti-Zr-3
	CeO <sub>2</sub> -A	CeO <sub>2</sub> -C		CeO <sub>2</sub> -A	CeO <sub>2</sub> -A		CeO <sub>2</sub> -A	CeO <sub>2</sub> -A	CeO <sub>2</sub> -A	CeO <sub>2</sub> -A
	CeO <sub>2</sub> -C			CeO <sub>2</sub> -B	CeO <sub>2</sub> -B		CeO <sub>2</sub> -B	CeO <sub>2</sub> -B	CeO <sub>2</sub> -B	CeO <sub>2</sub> -B
			CeO <sub>2</sub> -C	CeO <sub>2</sub> -C		CeO <sub>2</sub> -C				

Regarding the three lung derived epithelial cell lines (A549, CaLu3, RLE-6TN) a different sensitivity towards the different NMs was found. Overall the cell line RLE-6TN exhibited the strongest increase in ROS. In the most common used lung cell line, A549 (features type I and type II alveolar epithelial cells, see Figure 7), the ROS formation was slightly lower compared with the RLE-6TN cells. A closer look in TABLE 7 reveals that,

- ⇒ Besides carbon black (CB) the reference, TiO<sub>2</sub> no. 3 displays more ROS in the lung cells than the other materials in the study.
- ⇒ However, the two other TiO<sub>2</sub> NPs induced no ROS at all.

TiO<sub>2</sub> no. 1 and TiO<sub>2</sub> no. 2 were almost identical consisting of pure anatase being surface modified with a polyoxa acid, but TiO<sub>2</sub> nr 3 consisted only of 80 % anatase and 20 % rutile, was slightly larger, had a different morphology and had no surface modification.

- ⇒ The surface modification had a big impact on their zeta-potential with 17,5 mV for TiO<sub>2</sub> no. 1 and TiO<sub>2</sub> no. 2, and with -26,3 mV for TiO<sub>2</sub> no. 3.
- ⇒ These findings and data suggest a fundamental role of coatings in modifying cytotoxic effects of TiO<sub>2</sub> NMs.

*Chen et al.* [92] obtained results showing that

- ⇒ Exposure of TiO<sub>2</sub> NPs to mast cells increased the degranulation of histamine in the cells.

This may explain how chronic inflammatory diseases (e.g. asthmatic responses) are elicited by particulate matter, as earlier found in epidemiological studies and in animal models.

*Andersson et al.* [36] investigated the uptake of five different types of TiO<sub>2</sub> NPs in A549 cells. Toxicity assessment, using in-vitro assays, analysis of inflammatory mediators and activation of intracellular signal transduction pathways, were correlated with size-dependent uptake of anatase and rutile NPs. The NPs used are presented in TABLE 8 showing some of the particle data presented in the article. The concentration of NPs in PBS was 0,1mg/mL at the exposure.

TABLE 8. Physicochemical properties of TiO<sub>2</sub> nanoparticles used in [36] and [39].

Sample	Crystal structure	SSA [m <sup>2</sup> g <sup>-1</sup> ]	d <sub>(XRD)</sub> [nm]	d <sub>(TEM)</sub> [nm]	D <sub>SLS</sub> [μm]	A	ζ-potential [mV]
A14	Anatase	270	14	20	5,8	412	-21
A60	Anatase	11	60	66	0,6	12	-24
R5	Rutile	300	5	3 x 5	1,9	380	-22
R9	Rutile	40	9	6 x 80	6,1	678	-20
P25	A/R*	50	21	20-80	0,2; 3,0	19	-24

SSA – Specific Surface Area.

d<sub>(XRD)</sub> – mean diameter calculated from Sherrer analysis.

d<sub>(TEM)</sub> – the two numbers a x b for rutile express their asymmetric rectangular morphology.

SLS – static light scattering measuring the agglomerate mean diameter.

A – the agglomeration parameter (A = D/d)

- ⇒ All particles agglomerate in both PBS and in cell culture media
- ⇒ Sonication break up agglomerates temporarily, but particles reaggregate in less than a minute.
- ⇒ Small agglomerates only form from softer agglomerates which are not densely compacted.
- ⇒ In general, NPs that exhibit small and soft agglomerates are more readily taken up by the cells, which suggest that small loosely bonded agglomerates are generally available for cell uptake under physiological conditions (in vivo).
- ⇒ This implies that basic physicochemical properties, such as agglomeration parameter, surface roughness and surface charge may be used to predict NP uptake in cells.
- ⇒ No systematic relation between particle uptake and specific area or primary particle morphology was found.

In another paper performed by the same research group, Ekstrand.Hammarström et al. [39], investigated the uptake and responses of the very same five TiO<sub>2</sub> NPs, as in [36], in two more cell types; BEAS-2B cells and human bronchial epithelial cells (NHBE). Both A549 and BEAS-2B are immortalized cells, whereas the NHBE are normal human cells. They found that the cell uptake of particles in NHBE was the same as in A549 cells. However, the responsiveness, i.e.release of

cytokines after exposure, was significantly larger in NHBE cells. The concentration of NPs in PBS was in the range 5 – 500 µg/mL. Dosage dependency was investigated.

- ⇒ For all cell lines none of the TiO<sub>2</sub> particles (agglomerates) had any significant effect on the cell viability (cytotoxicity response).
- ⇒ All particles induced ROS in all three cell types and most often increasingly at increased particle concentrations.
- ⇒ The mixed P25 was more potent in inducing secretion of pro-inflammatory cytokines compared to the pure anatase and rutile TiO<sub>2</sub> agglomerates.
- ⇒ The responsiveness of lung epithelial cells is strongly dependent on both the physicochemical properties and the type of responder cells.
- ⇒ Agglomerate size and softness are important factor for uptake of TiO<sub>2</sub> NP uptake in cell. But once taken up in cells, other factors such as primary particle size and should be considered.
- ⇒ The primary bronchial epithelial cells (NHBE) respond differently than the immortalized lung epithelial cell lines.
- ⇒ Immortalized cell lines may not be representative for the responses of the human lung epithelium to inhales TiO<sub>2</sub>.

#### 5.4 The carcinogenic potential of TiO<sub>2</sub> NPs

Becker et al. reviewed the carcinogenic potential of nanomaterials, where TiO<sub>2</sub> NPs were one of the elements in focus [93]. They found that only a handful of long-term epidemiological studies have investigated carcinogenic studies on TiO<sub>2</sub>. Most of these refer to “dust” which workers have been exposed to, without any closer information on particle size. In none of the four epidemiological studies any correlation or association between lung cancer deaths and TiO<sub>2</sub> was found. However, in an *in vivo* study by Heinrich et al. [94] it was concluded that after inhalation (10 mg/m<sup>3</sup>) of nano-TiO<sub>2</sub> (15 - 40 nm, 80% anatase/20% rutile) by rats, after 18 and 24 months, resulted in a high incidence of lung tumors. The incidence of tumors induced by nano-TiO<sub>2</sub> was similar to that induced by diesel soot, which is classified as “probably carcinogenic to humans” according to IARC. Particle exposure concentration was increased during the course of the experiment for carbon black and TiO<sub>2</sub> to reach particle lung loads similar to those found in the diesel soot-exposed rats. In another study (see [93]) where rats were exposed by inhalation at 5mg/m<sup>3</sup> for 24-months of fine TiO<sub>2</sub> (1,1 micron) inflammatory reactions were observed, but no higher rate of tumors as compared with the control group. Other *in vivo* studies on lung exposure of TiO<sub>2</sub>, used intratracheal instillation as the “19-dusts study” by Pott and Roller [95] where rats were exposed to 19 different types of dusts. Among these elements were ultrafine (uf) carbon black, small-fine (sf) diesel soot, uf TiO<sub>2</sub> - hydrophilic and hydrophobic, and sf Anatase. In TABLE 8 the percentage of rats that developed tumors from the exposure of some of

TABLE 8: Results of the study by Pott and Roller as presented in [96].

Dust, sizeclass (uf, sf, lf)	Dose (nr of inst x mg), total [mg]	% of rats with tumors
Carbon black, uf	5 x 3, 15	78
Carbon black, uf	5 x 6, 30	83
Diesel soot, sf	5 x 3, 15	26
Diesel soot, sf	5 x 6, 30	40
TiO <sub>2</sub> , uf, hydrophilic	5 x 3, 15	52
TiO <sub>2</sub> , uf, hydrophilic	5 x 6, 30	67
TiO <sub>2</sub> , uf, hydrophobic	15 x 0.5, 7.5	All died. Toxic.
TiO <sub>2</sub> , sf, anatase	10 x 6, 60	30
TiO <sub>2</sub> , sf, anatase	20 x 6, 120	64

particles are presented. From TABLE 8 it can be concluded that uf carbon black is more and sf diesel soot is less carcinogenic compared with uf TiO<sub>2</sub>. Again it seems as if particle sizes matters. Furthermore, hydrophobic TiO<sub>2</sub> is strongly toxic and carcinogenic.

However, Becker et al. concludes their review by saying that at present, from the epidemiological studies no conclusions can so far be drawn regarding the carcinogenic effect on humans. On the basis on animal data, it can merely be said that nano-TiO<sub>2</sub> is suspected of being potentially carcinogenic to humans [93].

There are several more papers available presenting data on overload studies of exposure of TiO<sub>2</sub> NPs resulting in tumors in animals [44], but there is not room in this report to make more comments on this subject.

## 5.5 Summary of conclusions from both *in vivo* and *in vitro* studies reviewed

The following summary of conclusions is sorted under subjects related to the different studies reviewed respectively. The summarized conclusions are not precise since no stringent judgment between the results from the different studies reviewed, regarding what type of tests and endpoints used in the different studies, are conducted. A deeper analysis of the results quoted where attention is taken to compare the dosages administered, the physicochemical characterizations, the experiment preparations, the exposures and the evaluation of the different endpoints still remains to be performed. For example, regarding the genotoxicity of NPs, even though it has been examined in several papers, it is hard to compare the results due to the different types of TiO<sub>2</sub> particles used, the various cell systems and the variable assay conditions in the different studies [20]. Still the information from all the studies must contain truths in one way or another.

### 5.5.1 Influence of particle size and surface area

The following papers supports that nanoparticles of TiO<sub>2</sub> are more toxic than corresponding fine particles : [44, 57, 67-69, 75, 77, 83, 85, 86]. The papers [74, 88] supports it partly.

The following papers conclude that there is no influence of particle size or in part have found higher toxicity for larger particles: [33, 34, 54, 59, 88, 90, 97]. In particular fine particles of TiO<sub>2</sub> were more toxic than nanoparticles in DNA damage tests [54, 88].

According to Gurr et al. [85] ultrafine anatase (10-20 nm) particles induced toxicity, whereas fine (200 nm) anatase particles did not. Similarly, conclusions by Simon-Deckers et al. [86] points out higher toxicity for TiO<sub>2</sub> NPs than for their larger counterparts. But in a study by H Karlsson et al. [88] it was concluded from the results obtained that it cannot be generalized that NPs always are more toxic than fine/micrometer particles. The same conclusion can be drawn from the results presented in the study by Bhattacharya et al. [91]. However, in both studies [88, 91], some testing models showed a higher toxicity for smaller TiO<sub>2</sub> particles. Furthermore, Junko Okuda-Shimazaki et al. [98] investigated the effects of TiO<sub>2</sub> nanoparticle size on gene expression. They analyzed the gene expression focusing on stress, inflammation, and cytotoxicity of two sizes of titania aggregate particles, small (166 nm) and large (596 nm). They found that the large titania aggregates showed a larger effect on cell viability and gene expression than the small particles. Still, more papers point out that NPs are more toxic due to their higher reactivity compared to their larger counterparts of same chemistry.

The size dependency of the toxicity of TiO<sub>2</sub> NPs is only correct if particles are administered to lungs in a mass dose manner [60]. However, as stated earlier in this review, according to *Limbach et al.* [1],

Kroll et al. and Sager et al. [35, 60], a more appropriate method for studying nanoparticle cell-interactions in the lungs for better analysis of the influence of particle size is to administer the nanoparticles of different size at *equal surface area*. In [60], where animals were exposed to TiO<sub>2</sub> particles of different sizes, it was shown that on a mass basis the ultrafine particles caused significantly more inflammation and were significantly more cytotoxic than the fine sized particles. But when the particles were normalized based on surface area, the ultrafine particles were only slightly more inflammogenic and cytotoxic, but the difference was not significant. Thus, as Warheit et al. suggests [58], toxicity might not depend on particle size or surface area in the first place, but rather upon surface characteristics. However, in their study from 2007 [74] they do suggest that surface area influence toxicity.

Furthermore, conclusions in [72] by Grassain et al. contradicts the assumption that smaller particles are more toxic than the larger ones, since the larger 21 nm particles were found to induce a larger response in the mice compared to smaller 5 nm particles. However, it was also mentioned that the NPs had different agglomeration states which may be a factor as important as the surface and physical characteristics of the primary NPs in determining the toxicity. Grassain et al. [73] in their study where mice were subacutely exposed by inhalation to the smallest available, 2 – 5 nm, TiO<sub>2</sub> NPs, no particularly toxic effects were found three weeks after exposure.

In the review by Card et al. [23] they found seven papers with results confirming increased lung inflammation from exposure of NPs compared from equivalent mass of micron-sized particles. Two out of these seven papers investigated TiO<sub>2</sub> NPs [68, 99]. But they also found four papers, one of them dealing with TiO<sub>2</sub> NPs [58], where this was not the case. In the review by Johnston et al. they points out six papers where size dependency of TiO<sub>2</sub> toxicity has been frequently demonstrated and, as they write, “appears to be applicable to a variety of TiO<sub>2</sub> forms, and occurs regardless of the model used”. This is a misconception according to Warheit [100], and is contradicted by results obtain by Andersson et al. [36].

Important for any determination of particle size influence on toxicity is the deposition into the lungs, whether particles are inhaled as singlets or as agglomerates [101]. Still it is unlikely that exposure to cells will occur in a nano form since administration of TiO<sub>2</sub> NPs is associated with agglomeration [1, 36, 102]. Still, as Johnston et al. [24] writes “what seems to be critical to the toxic potential of particle samples is the size of particles that make up the agglomerates” in their discussion about size dependency. Andersson, Österlund and co-researchers [36] agree to a certain agree, although they emphasizes that they found no systematic relation between particle uptake and the specific area or primary particle morphology, since their in-vitro studies showed that uptake of cells depend of agglomerate size and the bonding between the primary particles in the agglomerates. But once the agglomerates are taken up in cells, other factors such as primary particle size and should be considered [39].

Regarding the issue of administering the testing material according to mass dose or surface area dose, Dankovic et al. made an preliminary conclusion from their study [44] that fine and ultrafine TiO<sub>2</sub> and other poorly soluble, low-toxicity (PSLT) particles show a consistent dose-response relationship when the dose is expressed as particle surface area. Therefore they suggested that test materials should be administered at equal surface area for determination of the influence from other parameters such as crystal shape, chemical composition, zeta-potential or surface coating. The same opinion is seen in papers by Limbach et al. [1], Kroll [35], Sager [60] and Card et al. [23].

There is also some arguments about that particles are more toxic at a certain size interval, for example particles larger than 30 nm are most often found to be less toxic compared to smaller particles since they have more or less the same properties as bulk material of the same chemical composition [103]. However, according to *Warheit* this might be another misconception [100], since he argues that TiO<sub>2</sub> NPs of the same size not necessarily have the same surface characteristics. Something which is clearly shown in papers [36, 39]. Whether there are certain particle or agglomerate size intervals which are more prone to affect living tissue - for better or worse - needs to be examined further.

### 5.5.2 Influence of crystal structure

The following papers' supports that anatase is more toxic than rutile: [51, 54, 74, 80]. For example anatase was 100 times more toxic than an equivalent sample of rutile in the study by Sayes et al. [51]. But the paper [85] by *Gurr et al.* shows a higher toxicity for fine rutile particles compared to fine anatase.

The papers [39, 72, 74, 85] concludes higher toxicity for a mix of anatase and rutile, than for both/or either one of the pure crystal structures. Papers [36, 58] found no difference in toxicity due to different morphology of particles. Paper [104] that shows significant differences in toxicity due to different shapes of particles. But these papers study TiO<sub>2</sub> particles of different sizes and are hard to compare to each other.

In words, most papers reviewed suggests that a mix of anatase and rutile crystal forms of TiO<sub>2</sub> NPs is more toxic than both/or either one of the pure crystal structures.

In a recently published paper by *Jin et al.* [105] the cellular toxicity of TiO<sub>2</sub> NPs of anatase and rutile was compared. The results indicated that only the anatase TiO<sub>2</sub> NPs allow spontaneous reactive oxygen species (ROS) generation, but rutile TiO<sub>2</sub> NPs do not after dispersion. The interaction between TiO<sub>2</sub> NPs and cellular components might also generate ROS for both anatase TiO<sub>2</sub> NPs and rutile TiO<sub>2</sub> NPs. No consideration regarding agglomeration influence was found.

In [59, 74] *Warheit et al.* concluded that the toxicity of TiO<sub>2</sub> particles were not dependent on size and surface area, but rather on surface properties. Here, the ultrafine TiO<sub>2</sub> particles consisted partly of anatase, whereas the fine TiO<sub>2</sub> only consisted of rutile. In an earlier study the anatase crystal structure showed higher toxicity [51]. Furthermore another study showed that a mix of these crystal forms were more potent to induce oxidative DNA lesions than treatment with either anatase or rutile forms alone [85].

In [97] *Warheit et al.* first studied quartz particles of varying size and then they studied three different types of NP TiO<sub>2</sub> with the similar particle size distribution but with different crystal structures. Particles were intratracheally instilled in rats. The largest quartz particles (Min-U-Sil; 1,5 µm) produced enhanced pulmonary toxicity compared to the 50 nm quartz particles, but less toxicity compared to the smaller 12 nm particles. For the different TiO<sub>2</sub> NPs, the surface activity characteristics correlated with the hazard biomarkers in the dose-response, time-course studies. They concluded that particle surface reactivity rather than size/surface area correlated best with lung inflammatory potency following exposures to particles.

In a study by *Hamilton et al.* [104], the toxicological response of three different types of TiO<sub>2</sub> particles were compared. TiO<sub>2</sub> nanospheres (60-200 nm), short (< 5 micron) and long (> 15 microns) nanobelts with a diameter of ~ 60 – 300 nm were synthesized, characterized and testing for biological activity in

vitro using primary alveolar macrophages and *in vivo* in mice. The study demonstrates that alteration of anatase TiO<sub>2</sub> nanomaterial into a fibre structure of greater than 15 microns creates a highly toxic particle and initiates an inflammatory response by alveolar macrophages. The long TiO<sub>2</sub> nanobelts interact with lung macrophages in a manner very similar to asbestos or silica. It was concluded that the toxicity and pathogenic potential change dramatically as the shape of the material is altered into one which is difficult to process for a phagocytic cell.

### 5.5.3 Surface coating and surface chemistry influence of particles

Rossi [55], Warheit [71, 106], Savolainen [20], and Kroll [35] and their co-researchers, respectively, have all demonstrated the importance of how a surface coating affects the NP-cell interface interactions. Certain coatings sometimes makes NPs inherently more toxic (Rossi) whereas other coatings make particles, or agglomerates of particles, less toxic [35, 54].

In the study [71] it was concluded that response following particle exposure of fine TiO<sub>2</sub> particles (~350 nm) with different surface modifications of alumina and amorphous silica was very much dependent on the TiO<sub>2</sub> formulation. Specifically, samples containing the highest alumina or amorphous silica elicited the largest adverse pulmonary response, although only mildly larger response compared to the uncoated TiO<sub>2</sub> powders. Thus, from this study, TiO<sub>2</sub> seems less toxic than corresponding particles with a coating of alumina or silica or a combination of both. However, it could perhaps also depend on the surface area – that is the shape of the different TiO<sub>2</sub> powders tested – since it was the powder not having the smallest particle size (440 nm) but the greatest surface area (27.8 m<sup>2</sup>/g) among all of the TiO<sub>2</sub> formulations studied that caused the largest response. Furthermore, the coatings consist of smaller units compared to the TiO<sub>2</sub> particles they are deposited upon, which also might contribute to the findings.

In the study [106] by Warheit *et al.* the influence of hydrophobic surface-coated (triethoxyoctylsilane – OTES) and hydrophilic base pigment-grade (~300 nm) TiO<sub>2</sub> particles, intratracheally installed on rats, was studied. The results demonstrated that only at high dose (10 mg/kg) the base pigment grade and both types of particles containing a surfactant (Tween 80) produced a transient pulmonary response, which was revertible within a week. It was concluded that the hydrophobic coating did not cause significant pulmonary toxicity.

#### 5.5.3.1 Surface properties, zeta-potential

Thus it is clear that intrinsic surface properties and morphology are more important rather than crystal form, as the studies comparing anatase with rutile have indicated [39]. In particular nano-wires, -fibrés, -belts or –tubes likely will affect living tissue in a detrimental manner [104].

Also it seems as if a particle's surface chemistry and/or the environment, rather than size might be the determining factor in its propensity to form aggregates in liquids [34, 36]. Surface coatings and zeta potential seem to have a crucial influence on NPs reactivity [1, 35, 71].

#### 5.5.3.2 The toxicity of TiO<sub>2</sub> compared to silica

One of the most common elements in concrete, besides water and calcium, is quartz because cement consists of Calcium-silicates and most aggregates in the concrete consists of quartz or other silica-containing minerals. Therefore it is of primarily interest to compare TiO<sub>2</sub> NPs with SiO<sub>2</sub> particles in the respect of judging whether replacing parts of the cement in concrete with titanium dioxide particles may increase the particle generation and, due to its inherent properties, contribute to an increased environmental threat.

In *Yuen et al.* [107] dusts of silica and TiO<sub>2</sub> were exposed to lungs of rats. The results showed that both crystalline and amorphous silica elicited higher degrees of pulmonary inflammation when compared to TiO<sub>2</sub> particles. The inflammation was transient for TiO<sub>2</sub> and amorphous silica but not for crystalline silica. Furthermore, in [108] *Warheit et al.* compared the toxicity of TiO<sub>2</sub> particles encapsulated with pyrogenetically deposited, amorphous silica with other TiO<sub>2</sub> and silica particles. It was found that this type of silica coated TiO<sub>2</sub> exhibited similar transient rat lung inflammatory response as the reference rutile TiO<sub>2</sub> particles of the same size and surface area, but substantially less tissue reactions than crystalline quartz (Min-U-Sil; 1,5 µm), the positive control. Also papers [58, 74, 87] shows higher toxicity for reference silica than for TiO<sub>2</sub> particles.

#### 5.5.4 Influence of experimental model

Certainly the experimental model, analysis method, species used, dose administered and exposure method have a great impact on the results. As aforementioned, a proper physicochemical characterization including agglomeration data is crucial to determine the different effects from particle size, chemical composition and crystal shape and other surface characteristics in all the different types of studies. Many papers published discuss ways of improving the experimental models and implementing standard reference tests with standardized positive and negative reference materials. Recently published papers [33-36] shows promising improvements of *in vitro* studies.

Regarding animal studies, tests on rats ought to be considered as more interesting compared to tests on mice or hamsters, since rats are more sensitive to particles, both fine and ultrafine particles, as shown by Bermudez et al. [69, 70].

#### 5.6 Agreement between *in vitro* and *in vivo* studies

Adverse effects on cells have been observed mostly with NM concentrations much higher than in tissue concentrations in animal models. Since also the experimental setup has been shown to influence experimental outcome [34] standardized protocol for the preparation and characterization of NM dispersions, exposure conditions and reference material have to be developed to improve the comparability between *in vitro* and *in vivo* studies [22].

Considering the huge number of potential NM variables that may determine the biological impact, each new type of NM has to be tested individually. This requires integrated testing strategies minimizing the need for animal studies. Recent studies demonstrate that classic cytotoxic assays may not be suitable to assess NM toxicity since NMs can interfere with assay reagents or detection systems thereby generating false positive/negative results. Due to the lack of standards for NM testing, current data on NM toxicity are often inconsistent and can hardly be compared [35]. However *Kroll et al* [35] did conduct a thorough study where valuable information at realistic doses was obtained. Also *Lanone et al.* [33] presented well planned and performed *in vitro* studies. Both these *in vitro* studies may statue good experimental examples for others to copy, something which might have been overlooked by others [109]. Still it seems clear that *in vitro* cellular systems will need to be further developed, standardized, and validated relative to *in vivo* effects in order to provide useful screening data on the hazards of inhaled particles [30, 109, 110]. Likewise, general standard methods particularly developed for *in vivo* toxicity studies of NPs have to be recognized and applied, since established methods for exposure for micro-particulates are not always suitable.

Sayes *et al.* [110] compared the toxicity of some metal-oxides (not TiO<sub>2</sub>) in *in vitro* endpoints and in *in vivo* experiments. They emphasized that none of the *in vitro* endpoints could mimic a transient inflammatory/cytotoxic response as often seen in *in vivo* studies. They concluded that current (2009) *in vitro* cell culture systems do not accurately forecast the pulmonary hazard responses in instilled particle-types.

Regarding the findings from all the toxicological studies of TiO<sub>2</sub> particles, in general *in vitro* studies have proven toxicity in different endpoints, either of fine or ultrafine TiO<sub>2</sub>, whereas *in vivo* studies, conducted at reasonable dosages, shows less severe toxicity, since in many papers inflammation seems to decrease with time and disappear.

## 6 Discussion

Obviously there is a consensus that TiO<sub>2</sub> NPs are able to affect different types of cells and interact with biological tissue in a negative manner to mediate inflammatory, genotoxic and/or cytotoxic responses [7, 24, 25, 51, 57]. That is from an inflammatory response or oxidative stress caused by the TiO<sub>2</sub> NPs. However, in some studies (*in vivo*) no evidence of toxicity is observed [111, 112] and in some studies the inflammatory response seem to decline with time and disappear [73, 113]. Often also TiO<sub>2</sub> NPs show less or little toxic or inflammatory response in certain endpoints, while there is toxic response in other endpoints, such as in the study by Karlsson *et al.* [88, 91].

Furthermore it is hard to discern if the doses used in the different tests are appropriate in regard to real exposure quantities. See section 6.1.

Johnston *et al.* [24] in their review regarding *in vivo* pulmonary exposure of TiO<sub>2</sub> NPs conclude that the toxic potential of TiO<sub>2</sub> particles is primarily dictated by size and crystallinity whereby

- ⇒ Decreasing particle size enhanced particle pulmonary toxicity.
- ⇒ Anatase forms of TiO<sub>2</sub> enhanced particle pulmonary toxicity.

Furthermore, they emphasize that the experimental model, including species used, exposure method and dose administered, impact on the toxicity of TiO<sub>2</sub>, which complicates comparisons between different investigations. They also underline that chronic exposure to TiO<sub>2</sub> also has the ability to promote tumor development when particles are administered in overload conditions.

Regarding the reviewed *in vitro* investigations by Johnston *et al.* [24] they conclude that ;

- ⇒ it is evident that TiO<sub>2</sub> particles are able to detrimentally affect both lung derived macrophage and epithelial cells.
- ⇒ in general particles mediated oxidative, inflammatory and genotoxic effects that eventually culminated in cytotoxicity.
- ⇒ In some studies there was no evidence of toxicity mediated by TiO<sub>2</sub>.
- ⇒ It is of interest that the mechanisms that drive to toxicity of TiO<sub>2</sub> *in vitro* (inflammation and oxidative stress), are also witnessed *in vivo*.

Card *et al.* [23] emphasizes the importance of form (phase) of TiO<sub>2</sub> NP in the *in vitro* toxicity studies, such as in the study by Gurr *et al.* [85]. Also Fadeel and Garcia-Bennet [7] suggests that it appears as if the size is less important than phase composition in the case of TiO<sub>2</sub> NPs.

In the review by Card *et al.* [23], they first point out that out of 16 papers reviewed by them, 10 papers had sufficient nanoparticle characterization. They also cite the paper by Warheit *et al.* [74] to show that TiO<sub>2</sub> is a good example of a metal-oxide where the size and form of nano-particles

influence its pulmonary toxicity despite the fact that all particles had agglomerated before instillation, as *Johnston et al.* [24] mention. *Johnston et al.* also remark that there is a dispute regarding the importance of surface area and size not the least from *Warheit* and his co-workers at DuPont Haskell – manufacturer of particles and pigments. Regarding the influence of particle size and surface area *Card et al.* explain that the increased ratio of surface area to mass for NPs means that a greater percentage of the atoms or molecules of a given particle are present on the surface of the particle, thereby providing an increased number of potential reactive groups at the particle surface that may influence toxicity. Although surface area appears to be a useful metric for assessing the toxic potential of some metal-oxide NPs like  $\text{TiO}_2$ , there seem to exist a consensus among experts in the field that no single dose metric (i.e. particle number, size, surface area, surface reactivity or other) has emerged to the most important factor for assessment of the reactivity and potential toxicity for nanomaterials in general [23]. From this review I agree with this last conclusion by *Card et al.* and also question the bold conclusions stated by *Johnston et al.* above. In the paper [100] *Warheit* writes that “particle size does play a contributing role in producing toxicity impacts, because reduced size corresponds to greater particle number and enhanced surface area metrics. However, the reactivity of the particle surface has a greater likelihood to directly influence particle – cellular interactions, generate ROS and consequent cytotoxic and inflammatory responses”. Furthermore, the role of particle agglomeration have a crucial impact of how particles are taken up by cells, and agglomerates of smaller particles do not exhibit higher uptake compared to agglomerates composed of larger agglomerates as shown in paper [36] although they only studied particles of different particle sizes in the nano scale, from 5 nm to 60 nm. It would have been interesting to see how larger ultrafine and fine particles behave in the same in vitro experiments.

Still, if nano and fine sized particles are administered in mass dose in lung toxicological tests, of same shape, morphology, chemistry, and surface characteristic, smaller particles will cause a larger impact due to their larger surface area, and thus larger contact area with the biomaterial. But at equal surface area exposure, the surface characteristics do play a more important role in NP toxicology [60].

### 6.1 Risk assessment of airborne NPs, $\text{TiO}_2$ NPs in particular

The  $\text{TiO}_2$  material has been classified as “possibly carcinogenic to humans” (IARC – the International Agency for Research on Cancer, 2010). The National Institute for Occupational Safety and Health (NIOSH) recommends an airborne exposure limit of  $2.4 \text{ mg/m}^3$  for respirable pigment grade (fine)  $\text{TiO}_2$  and  $0.3 \text{ mg/m}^3$  for NP  $\text{TiO}_2$ .

In the article by *Kuempel et al.* [114] key steps in quantitative risk assessment were illustrated, using dose-response data in rats chronically exposed to either fine or ultrafine titanium dioxide ( $\text{TiO}_2$ ), carbon black (CB), or diesel exhaust particulate (DEP). The assessment is judged from mass dose exposure studies which have shown higher toxicity for smaller sized particles. The rat-based estimates of the working lifetime airborne concentrations associated with 0.1% excess risk of lung cancer were approximately  $0.07$  to  $0.3 \text{ mg/m}^3$  for ultrafine  $\text{TiO}_2$ , CB, or DEP, and  $0.7$  to  $1.3 \text{ mg/m}^3$  for fine  $\text{TiO}_2$ .

In a Russian paper [115] *Radilov et al.* suggested threshold values for airborne concentrations of nano aerosols were suggested. The authors developed an approach to comparative toxicity assessment and expressed calculation of occupational exposure standards for nano-aerosols based on criteria for the maximum allowable concentrations (MACs) of aerosols in the workplace air. The

approach was used to obtain the following prognostic MACs of aerosols: nano-Ag 0.08 mg/m<sup>3</sup>, nano-TiO<sub>2</sub> 0.19 mg/m<sup>3</sup>, and C 60 0.08 mg/m<sup>3</sup>.

Liao et al. [116] presented a model-based assessment for human inhalation exposure risk to airborne nano/fine particles. The findings pointed out that dry/wet treatment and ore handlers in US and maintenance mechanics in EU factories were unlikely to pose substantial lung cancer risks.

In a recently published paper Koivisto et al. [117] investigated the exposure of airborne particles to industrial workers during the packing of pigment and nanoscale TiO<sub>2</sub>. The particles had sizes between 5 nm and 10 μm. The quantitative particle exposure from inhalation in the industry workers was defined from the concentrations measured at the work area where the packing of the pigments and nanoparticles takes place. It was found that workers average exposure varied from 0,22 to 0,70 mg/m<sup>3</sup>. Over 90 % of the particles (numbers) were smaller than 100 nm. There were mainly soot and particles formed from process chemicals. Mass concentration originated primarily from the packing of the fine and nano TiO<sub>2</sub> agglomerates. The nano TiO<sub>2</sub> exposure resulted in a calculated dose rate of 3.6 x 10<sup>6</sup> particles/min and 32 μg/min where 70 % of the particles and 85 % of the mass was deposited in head airways (not in the lung). The peak mass concentrations were at around 3 mg/m<sup>3</sup> which originated mainly from the packing, air cleaning and material pouring of fine TiO<sub>2</sub> and nano TiO<sub>2</sub> being over 500 and 200 nm in diameter, respectively. Therefore respirators are recommended at those work events. However, the exposure limits recommended by NIOSH (as cited above) were not exceeded. The authors declared no conflicts of interest.

The calculated dose rate of 32 μg/min results in 0,15 x 32 μg/min = 4,8 μg/min deposit of TiO<sub>2</sub> NPs in the lung, correspond to a maximum daily dose of 60 x 8 x 4,8 μg = 2,3 mg of TiO<sub>2</sub> NPs in the lungs. The lung alveolar surface area in humans is calculated to about 70 m<sup>2</sup> [118].

If we assume that humans are as sensitive as rats; from Kroll et al [35] the rat overload is calculated to about 3 μg/cm<sup>2</sup>, which corresponds to 0,3 mg/dm<sup>2</sup> and 30 g/m<sup>2</sup>. The daily particle load of fine and nano TiO<sub>2</sub> agglomerates in workers of 2,3 mg/70 m<sup>2</sup> lung, equals 33 μg/m<sup>2</sup> lung. Thus the fine and nano TiO<sub>2</sub> agglomerate exposure to the workers are 30 g / 33 μg = 9,1 x 10<sup>5</sup> times smaller than the assumed overload.

Most in vivo studies using inhalation of particles into rats uses a dose close to, or one or two magnitudes below the overload dose.

## 7 Conclusions

The scientific branch of nanoparticle toxicology is a fast developing area of research and results. Still it seems hard to find any shortcuts regarding assessment of different NPs toxicity in general. Nonetheless it seems necessary to examine every new type of ENM carefully in several types of cell lines to determine its toxicological properties, which also includes all the different types of TiO<sub>2</sub> particles manufactured by many different producers. The good news is that the knowledge of how to assess the toxicity accurately has increased much during the last 4-5 years, and more concise knowledge regarding different NPs inherent toxicity to living tissues and the environment will be available increasingly in a continual manner the next coming years.

Specifically regarding the toxicity of TiO<sub>2</sub> NPs it seems as if they are not much more toxic than their larger counterparts, if compared at equal surface area. Furthermore, in a base set of hazard tests on a set of newly developed well characterized ultrafine TiO<sub>2</sub> particles by *Warheit et al.* [112] it was

concluded that most studies demonstrated low hazard potential in mammals or aquatic species following acute exposure to the ultrafine TiO<sub>2</sub> particles.

Compared with other metaloxides, TiO<sub>2</sub> is most often less toxic than other elements, such as ZnO [119], CuO [88] and SiO<sub>2</sub> in the different toxicological screening tests. Regarding using TiO<sub>2</sub> in concrete it will result in just a little less silica and Ca-particles in the air replaced by just very small amounts of TiO<sub>2</sub>. In respect to silica, titania is (most often) less toxic, but (most often) more toxic than calcium-oxide particles.

Furthermore, the doses used in the toxicological studies are 1000 – 100 000 times higher compared to any dosages that humans are exposed to.

From all of this it is concluded that it is not likely that using additives of photocatalytic titania in pavements made of concrete will increase the inherent toxicity of the particles generated from wear of vehicles.

## 8 Acknowledgements

I have been a privileged to have had the opportunity to complete this review. It has been most interesting and stimulating for me to attain new knowledge within a different scientific field, from the one I am supposed to be an “expert” in. Therefore the Swedish Cement and Concrete Research Institute are greatly acknowledged to have supported this work into completion. Likewise Cementa AB and SBUF, the Swedish construction industry's organisation for research and development, are acknowledged for their support.

Professor Lars Österlund at the Ångström Laboratory at Uppsala University is acknowledged for valuable advises and encouragement and I am also grateful to Hanna Karlsson, Ph D at Karolinska Institutet in Huddinge, Stockholm, for helping me to find important literature.

Wikipedia is also acknowledged as a very useful tool to obtain new knowledge, besides all the scientific literature.

## References

- [1] Limbach Ludwig K., Grass Robert N., and Stark Wendelin J., "Physico-Chemical Differences Between Particle- and Molecular-Derived Toxicity: Can we make Inherently Safe Nanoparticles?," *CHIMIA*, vol. 63, pp. 38-43, 2009.
- [2] Lee Jaesang, Mahendra Shaily, and Alvarez Pedro J.J., "Nanomaterials in the Construction Industry: A Review of Their Applications and Environmental Health and Safety Considerations," *ACS Nano*, vol. 4, pp. 3580–3590, 2010.
- [3] Pacheco-Torgal F. and Jalali Said, "Nanotechnology: Advantages and drawbacks in the field of construction and building materials," *Construction and Building Materials*, pp. doi: 10.1016/j.conbuildmat.2010.07.009, 9 pages, 2010.
- [4] Fujishima A., Rao Tata N., and Tryk Donald A., "Titanium dioxide photocatalysis," *J. of Photochemistry and Photobiology C: Photochemistry Reviews*, pp. 1-21, 2000.
- [5] Fujishima A., Hashimoto K., and Watanabe T., *TiO<sub>2</sub> Photocatalysis: Fundamentals and Applications*. Tokyo: BKC 1999.
- [6] A. Markowska-Szczupak, K. Ulfig, and A. W. Morawski, "The application of titanium dioxide for deactivation of bioparticulates: An overview," *Catalysis Today*, vol. 169, pp. 249-257, 2011.
- [7] Fadeel Bengt and Garcia-Bennett Alfonso E., "Better safe than sorry: Understanding the toxicological properties of inorganic nanoparticles manufactured for biomedical applications " *Advanced Drug Delivery Reviews*, vol. 62, pp. 362-374, 2010.
- [8] Fujishima A. and Zhang Xintong, "Titanium dioxide photocatalysis: present situation and future approaches," *Comptes Rendus Chimie*, pp. 750-760, 2006.
- [9] Hüsken G., Hunger M., and Brouwers H.J.H., "Experimental study of photocatalytic concrete products for air purification," *Building and environment*, pp. 2463-2474, 2009.
- [10] Hunger M., Hüsken G., and Brouwers H.J.H., "Photocatalytic degradation of air pollutants - From modelling to large scale application," *Cement and Concrete Research*, pp. 313-320, 2010.
- [11] Bygott Claire E., Maltby Julie E., Stratton John L., and McIntyre Robert, "Photocatalytic coatings for the construction industry," in *International RILEM Symposium on Photocatalysis*, Florence, 2007, pp. 251-258.
- [12] Maggos Th., Plassis A., Bartzis J.G., Vasilakos Ch., Moussiopoulos N., and Bonafous L., "Photocatalytic degradation of NO<sub>x</sub> in a pilot street canyon configuration using TiO<sub>2</sub>-mortar panels," *Environ Monit Assess*, pp. 35-44, 2008.
- [13] Guerrini G.L. and Pecceci E., "Photocatalytic cementitious roads for depollution," in *International RILEM Symposium on Photocatalysis*, Florence, 2007.
- [14] Beeldens Anne, "An environmental friendly solution for air purification and self cleaning effect: the application of TiO<sub>2</sub> as photocatalyst in concrete," in *TRA 2006*, Göteborg 12-15 June 2006.
- [15] Nilsson H. and Häger A., "Utvärdering av försöket med plattor med fotokatalytisk titandioxid på Amiralsgatan i Malmö (Evaluation of a trial with photocatalytic concrete tiles at Amiralsgatan in Malmö)," *Miljöförvaltningen*, Malmö stad 2010.
- [16] Guerrini G., "Development of photocatalytic cement-based materials: situation and perspectives," in *XIII international congress on the chemistry of cement*, Madrid, June 13th 2011, p. p. 175.
- [17] Kuempel E.D., Geraci C.L., and Schulte P.A., "Risk assessment approaches and research needs for nanomaterials: An examination of data and information from current studies," in *Nanotechnology - Toxicological Issues and Environmental Safety*, 2007.
- [18] Nel A., Xia T., Mädler L., and Li N., "Toxic Potential of Materials at the Nanolevel," *Science*, vol. 311, pp. 622-627, 2006.
- [19] Oberdörster Günter, Stone Vicki, and Donaldson Ken, "Toxicology of nanoparticles: A historical perspective," *Nanotoxicology*, vol. 1, pp. 2-25, 2007.

- [20] Savolainen Kai, Alenius Harri, Norppa Hannu, Pylkkänen Lea, Toumi Timo, and Kasper Gerhard, "Risk assessment of engineered nanomaterials and nanotechnologies - A review," *Toxicology*, vol. 269, pp. 92-104, 2010.
- [21] Somasundaran P., Fang X., Ponnurangam S., and Li B., "Nanoparticles: Characteristics, Mechanisms and Modulation of Biototoxicity," *KONA Powder and Particle Journal*, pp. 38-49, 2010.
- [22] Hussain Saber M., Braydich-Stolle Laura K., Schrand Amanda M., Murdock Richard C., Yu Kyung O., Mattie David M., Schlager John J., and Terrones Mauricio, "Toxicity Evaluation for Safe Use of Nanomaterials: Recent Achievements and Technical Challenges," *Advanced Materials*, vol. 21, pp. 1549-1559, 2009.
- [23] Card Jeffrey W., Zeldin Darryl C., Bonner James C., and Nestmann Earle R., "Pulmonary applications and toxicity of engineered nanoparticles," *Am J Physiology - Lung Cellular and Molecular Physiology*, pp. 400-411, 2008.
- [24] Johnston Helinor J, Hutchinson Gary R, Christensen Frans M, Peters Sheona, Hankin Steve, and Stone Vicki, "Review. Identification of the mechanisms that drive the toxicity of TiO<sub>2</sub> particulates: the contribution of physicochemical characteristics," *Particle and Fibre Toxicology*, vol. 6, pp. 1-27, 17 dec 2009.
- [25] Oberdörster G., Ferin J., and Lehnert BE., "Correlation between particle size, in vivo particle persistence and lung injury.," *Environ. Health Perspect.*, pp. 173-79, 1994.
- [26] Gustafsson Mats, Blomqvist Göran, Dahl Andreas, Gudmundsson Anders, Ljungman Anders, Lindbom John, Rudell Bertil, and Sweitlicki Erik, "Inhalable particles from the interaction between tyres, road pavement and friction materials," VTI, Linköping2005.
- [27] Negri E, Gallus S, Boffetta P, McLaughlin J.K., and L. V. C., "A comprehensive review of European epidemiological studies on particulate matter exposure and health," Brussels2011.
- [28] Sehlstedt Maria, Forsberg Bertil, Westerholm Roger, Boman Christoffer, and Sandström Thomas, "The Role of Particle Size and Chemical Composition for Health Risks of Exposure to Traffic Related Aerosols - A Review of the current Literature," Dep of Respiratory Medicine and Allergy, Umeå, Sweden2007-12-12 2007.
- [29] Iavicoli I., Leso V., and Bergamaschi A., "toxicological Effects of Titanium Dioxide Nanoparticles: A Review of In-Vivo Studies," *Journal of Nanomaterials*, vol. Article ID 964381, p. 36, 2012.
- [30] Iavicoli I., Leso V., Fontana L., and Bergamaschi A., "Toxicological effects of titanium dioxide nanoparticles: A review of In-Vitro mammalian studies," *European Review for Medical and Pharmacological Sciences*, vol. 15, pp. 481-508, 2011.
- [31] Warheit David B, "How meaningful are the Results of Nanotoxicity Studies in the Absence of Adequate Material Characterization?," *Toxicol. Sci.*, vol. 101, pp. 183-185, 2008.
- [32] Warheit David B., Sayes C. M., Reed Kenneth L., and Swain Keith A., "Health effects related to nanoparticle exposures: Environmental, health and safety considerations for assessing hazards and risks," *Pharmacology & Therapeutics*, vol. 120, pp. 35-42, 2008.
- [33] Lanone Sophie, Rogerieux Françoise, Geys Jorina, Dupont Aurélie, Maillot-Marechal Emmanuelle, Boczkowski Jorge, Lacroix Ghislaine, and Hoet Peter, "Comparative toxicity of 24 manufactured nanoparticles in human alveolar epithelial cell lines," *Particle and Fibre Toxicology*, vol. 6, p. 12, 30 april 2009.
- [34] Sohaebuddin Syed, Thevenot Paul T, Baker David, Eaton John W, and Tang Liping, "Nanomaterial cytotoxicity is composition, size, and cell type dependent," *Particle and Fibre Toxicology*, vol. 7, p. 17, 2010.
- [35] Kroll Alexandra, Dierker Christian, Rommel Christina, Hahn Daniela, Wohlleben Wendel, Schulze-Isfort Christian, Göbbert Christian, Voetz Matthias, Hardinghaus Ferdinand, and Schnekenburger Jürgen, "Cytotoxicity screening of 23 engineered nanomaterials using a test matrix of ten cell lines and three different assays," *Particle and Fibre Toxicology*, vol. 8, p. 19, 2011.

- [36] Andersson Per Ola, Lejon Christian, Ekstrand-Hammarström Barbro, Akfur Christine M., AhlinderLinnéa, Bucht Anders, and Österlund Lars, "Polymorph- and Size-Dependent Uptake and Toxicity of TiO<sub>2</sub> Nanoparticles in Living Lung Epithelial Cells," *Small*, vol. 7, pp. 514-523, 2011.
- [37] Shvedova Anna A., Kagan Valerian E., and Fadeel Bengt, "Close encounters of the Small Kind: Adverse Effects of Man-Made Materials Interfacing with the Nano Cosmos of Biological Systems," *Annual Reviews of Pharmacology and Toxicology*, pp. 63-88, 2010.
- [38] Limbach L.K., Li Y., Grass R.N., Brunner T.J., Hintermann M.A., Muller M., Gunter D., and Stark W.J., "Nanoparticle Uptake in Human Lung Fibroblasts: Effect of particle Siza, agglomeration and Diffusion at Low Concenttration," *Environ. Sci. Technol.*, vol. 39, pp. 9370 - 9376, 2005.
- [39] Ekstrand-Hammarström Barbro, Akfur Christine M., Andersson Per Ola, Lejon Christian, Österlund Lars, and Bucht Anders, "Human primary bronchial epithelial cells respond differently to titanium dioxide nanoparticles than the lung epithelial cell lines A549 and BEAS-2B," *Nanotoxicology*, vol. 6, pp. 623-634, 2012.
- [40] Sayes C. M., Reed K.L., and W. D.B., "Assessing Toxicity of Fine and Nanoparticles: Comparing In Vitro Measurements to In Vivo Pulmonary Toxicity Profiles," *Toxicol. Sci.*, vol. 97, pp. 163-180, 2007.
- [41] Sayes C. M. and Warheit D.B., "Characterization of nanomaterials for toxicity assessment," *Nanomedicine and Nanobiotechnology*, vol. 1, pp. 660-670, 2009.
- [42] Sibilia J.P., *A Guide to Materials Characterization and Chemical Analysis*. New York: Wiley-VCH, Inc., 1996.
- [43] Ahlinder Linnéa and Österlund Lars, "Hyperspectral analysis of cells and tissue that are exposed to airborne particles," *Report no FOI-R2995-SE*, p. 31, February 2011.
- [44] Dankovic D, Kuempel E, and Wheeler M, "An approach to risk assessment for TiO<sub>2</sub> ," *Inhalation toxicology*, vol. 19, pp. 205-212, 2007.
- [45] Landmann M, Rauls E, and Schmidt W.G., "The electronic structure and optical response of rutile, anatase and brookite TiO<sub>2</sub>," *Journal of Physics: Condensed Matter*, vol. 24, p. 6, 2012.
- [46] Humer D.R. and Heaney P.J., "Thermal expansion of anatase and rutile between 300 and 575 K using synchrotron powder X-ray diffraction," *Powder Diffraction*, vol. 22, pp. 352 - 357, 2007.
- [47] "Society of Toxicology " [http://www.toxicology.org/AI/PUB/si05/Si05\\_Define.asp](http://www.toxicology.org/AI/PUB/si05/Si05_Define.asp).
- [48] <http://alttox.org/ttrc/tox-test-overview/>.
- [49] Hawgood Samuel and Clements John A., "Pulmonary Surfactant and Its Apoproteins," *J Clin. Invest.*, vol. 86, pp. 1-6, 1990.
- [50] [http://en.wikipedia.org/wiki/Pulmonary\\_alveolus](http://en.wikipedia.org/wiki/Pulmonary_alveolus).
- [51] Sayes C. M., Wahi R., Kurian P.A., Liu Y., West J. L., Ausman K. D., Warheit D.B., and Colvin Vicki L., "Correlating nanoscale titania structure with toxicity: a cytotoxicity and inflammatory response study with human dermal fibroblasts and human lung epithelial cells," *Toxicol. Sci.*, vol. 92, pp. 174-185, 2006.
- [52] Thyrell Lena, "To catch a killer: On the mechanisms of interferon alpha induced apoptosis," Ph D, Department of Oncology-Pathology, , Karolinska Institute, Stockholm, 2005.
- [53] Geiser Marianne, Rothen-Rutishauser Barbara, Kapp Nadine, Schürch Samuel, Kreyling Wolfgang, Semmler Manuela, Im Hof Vinzenz, Heyder Joachim, and Gehr Peter, "Ultrafine Particles Cross Cellular Membrandes by Nonphagocytic Mechanisms in Lungs and in Cultured cells," *Environ. Health Perspect.*, vol. 113, pp. 1555-1560, 2005.
- [54] Falck GC, Lindberg HK, Suhonen S, Vippola M, Vanhala E, Catalán J, Savolainen K, and Norppa H, "Genotoxic effects of nanosized and fine TiO<sub>2</sub>," *Human and Experimental Toxicology*, vol. 28, pp. 339-352, 2009.
- [55] Rossi Elina M., Pyökkänen Lea, Koivisto Antti J., Vippola Minnamari, Jensen Keld A., iettinen Mirella, Sirola Kristiina, Nykäsenoja Heli, Karisola Piia, Stjernvall Tuula, Vanhala Esa, Pertti Pasanen Mirja iilunen, Mäkinen Maija, Hämeri Kaarle, Joutsensaari Jorma, Tuomi Timo, Jokiniemi Jorma, Wolff Henrik, Savolainen Kai, Matikainen Sampsa, and Alenius Harri,

- "Airway Exposure to Silica-Coated TiO<sub>2</sub> Nanoparticles Induces Pulmonary Neutrophilia in Mice " *Toxicological Sciences*, vol. 113, pp. 422-433, 2009.
- [56] Pan Yu, Neuss Sabine, Fischler Monika, Wen Fei, Simon Ulrich, Schmid Günter, Brandau Wolfgang, and Jahnen-Dechent Willi, "Size-dependent Cytotoxicity of gold particles," *Small*, vol. 3, pp. 1941 -1949, 2007.
- [57] Nurkiewicz Timothy R, Porter Dale W, Hubbs Ann F, Cumpston Jared L, Chen Bean T, Frazer David G, and Castranova Vincent, "Nanoparticle inhalation augments particle-dependent systemic microvascular dysfunction," *Particle and Fibre Toxicology*, vol. 5, p. 12, 12 February 2008.
- [58] Warheit David B., Webb Thomas R., Sayes Christie M., Colvin Vicki L., and Reed Kenneth L., "Pulmonary Instillation Studies with Nanoscale TiO<sub>2</sub> Rods and Dots in Rats: Toxicity is not dependent upon Particle Size and Surface Area," *Toxicological Sciences*, vol. 91, pp. 227-236, 2006.
- [59] Warheit David B., Webb Thomas R., Colvin Vicki L., Reed Kenneth L., and Sayes Christie M., "Pulmonary bioassay studies with nanoscale and fine-quartz particles in Rats: Toxicity is not dependent upon particle size but on surface characteristics," *Toxicological Sciences*, vol. 95, pp. 270-280, 2006.
- [60] Sager Tina M, Kommineni C, and Castranova Vincent, "Pulmonary response to intratracheal instillation of ultrafine versus fine titanium dioxide: role of particle surface area," *Particle and Fibre Toxicology*, vol. 5, p. 15, 1 December 2008.
- [61] Geiser Marianne, Casualta Marco, Kupferschid Barbara, Schulz Holger, Semmler-Behnke Manuela, and Kreyling Wolfgang, "The Role of Macrophages in the Clearance of Inhaled Ultrafine Titanium Dioxide Particles," *Am J Respir Cell Mol Biology*, vol. 38, pp. 371-376, 2008.
- [62] Shimizu Midori, Tainaka Hitoshi, Oba Taro, Mizou Keisuke, Umezawa Masakazu, and Takeda Ken, "Maternal exposure to nanoparticulate titanium dioxide during the prenatal period alters gene expression related to brain development in the mouse," *Particle and Fibre Toxicology*, vol. 6, pp. 1-8, 29 July 2009.
- [63] Oberdörster G., Sharp Z., Atudorei V., Elder A., Gelein R., Kreyling Wolfgang, and Cox C., "Translocation of Inhaled Ultrafine Particles to the Brain," *Inhalation Toxicology*, vol. 16, pp. 437-445, 2004.
- [64] Fadeel Bengt, Kasemo B., Malmsten B., and Strømme M., "Nanomedicine: reshaping clinical practice," *J. of Internal Medicine*, pp. 2-8, 2009.
- [65] A. P. Pietropaoli, M. W. Frampton, R. W. Hyde, P. E. Morrow, G. Oberdörster, C. Cox, D. M. Speers, L. M. Frasier, D. C. Chalupa, L. S. Huang, and M. J. Utell, "Pulmonary function, diffusing capacity, and inflammation in healthy and asthmatic subjects exposed to ultrafine particles," *Inhalation toxicology*, vol. 16, pp. 59-72, 2004.
- [66] Poland Craig A., Duffin Rodger, Kinloch Ian, Maynard Andrew, Wallace William A.H., Seaton Anthony, Stone Vicki, Brown Simon, MacNee William, and Donaldson Ken, "Carbon nanotubes introduced into the abdominal cavity of mice show asbestos-like pathogenicity in a pilot study," *Nature Nanotechnology*, vol. 3, pp. 432-428, 2008.
- [67] Ferin J., Oberdörster G., and Penney D.P., "Pulmonary retention of ultrafine and fine particles in rats," *Am J Respir Cell Mol Biology*, vol. 6, pp. 535-42, 1992.
- [68] Renwick LC, Brown D, Clouter A, and Donaldson K, "Increased inflammation and altered macrophage chemotactic responses caused by two ultrafine particle types," *Occu Environ Med*, vol. 61, pp. 442-447, 2004.
- [69] Bermudez Edilberto, Mangum James B., Wong Brian A., Asgharian Bahman, Janszen Derek B., Hext Paul M., Warheit D.B., and Everitt Jeffrey I., "Pulmonary Responses of TMice, Rats and Hamsters to Subchronic Inhalation of Ultrafine Titanium Dioxide Particles," *Toxicol. Sci.*, vol. 77, pp. 347-357, 2004.
- [70] Bermudez Edilberto, Mangum James B., Asgharian Bahman, Wong Brian A., Reverdy Edward E., Janszen Derek B., Hext Paul M., Warheit D.B., and Everitt Jeffrey I., "Long-Term Pulmonary

- Responses of Three Laboratory Rodent Species to Subchronic Inhalation of Pigmentary Titanium Dioxide Particles," *Toxicol. Sci.*, vol. 70, pp. 86-97, 2002.
- [71] Warheit David B., Brock W.J., Lee K.P., Webb T.R., and Reed K.L., "Comparative Pulmonary Toxicity Inhalation and Instillation Studies with different TiO<sub>2</sub> Particle Formulations: Impact of Surface Treatments on Particle Toxicity," *Toxicol. Sci.*, vol. 88, pp. 514-524, 2005.
- [72] Grassian V.H., Adamcakova-Dodd A., Pettibone J.B., O'Shaughnessy P.T., and Thorne P.S., "Inflammatory response of mice to manufactured titanium dioxide nanoparticles: Comparison of size effects through different exposure routes," *Nanotoxicology*, vol. 1, pp. 211-226, 2007.
- [73] Grassian V.H., O'Shaughnessy P., Pettibone J., Dodd A., and Thorne P., "Inhalation Exposure Studies of Nanoparticulate Titanium Dioxide with a Primary Particle Size of 2 to 5 nm," *Environmental Health Perspectives*, vol. 115, pp. 379-402, 2007.
- [74] Warheit David B., Webb T.R., Reed K.L., Frerichs S., and Sayes C.M., "Pulmonary toxicity studies in rats with three forms of ultrafine-TiO<sub>2</sub> particles," *Toxicology*, vol. 230, pp. 90-140, 2007.
- [75] Chen Huei-Wen, Su S.F., Chien C.T., Lin W.H., Yu Sung-Liang, Chou C.C., Chen Jeremy J.W., and Yang Pan-Chyr, "Titanium dioxide nanoparticles induce emphysema-like lung injury in mice," *The FASEB Journal*, vol. 20, pp. E1732 - E1741, 2006.
- [76] Ahn Mi-Hyun, Kang Chun-Mi, Park Chon-Sik, Park Sang-Jun, Rhim Taiyou, Yoon Pyeong-Oh, Chang Hun Soo, Kim Soo-Ho, Kyono Hiroko, and Kim Kwang Chul, "Titanium dioxide particle - induced goblet cell hyperplasia: association with mast cells and IL-13," *Respiratory Research*, vol. 6, pp. 1-9, 2005.
- [77] Inoue K., Takano H., Ohnuki M., Yanagisawa R., Sakurai M., Shimada A., Mizushima K., and Yoshikawa T., "Size effects of nanomaterials on lung inflammation and coagulatory disturbance," *Int J Immunopath Pharmacol*, vol. 21, pp. 197-206, 2008.
- [78] Nurkiewicz Timothy R., Porter Dale W., Barger Mark, Millecchia Lyndell, Rao K. Murali K., Marvar Paul J., Hubbs Ann F., Castranova V., and Boegehold Matthew A., "Systemic Microvascular Dysfunction and Inflammation after Pulmonary Particulate Matter Exposure," *Environ. Health Perspect.*, vol. 114, pp. 412-419, 2006.
- [79] Wang Jiangxue, Chen Chunying, Liu Ying, Jiao Fang, Li Wei, Lao Fang, Li Yufeng, Li Bai, Ge Cuicui, Zhou Guoqiang, Gao Yuxi, Zhao Yuliang, and Chai Zhifang, "Potential neurological lesion after nasal instillation of TiO<sub>2</sub> nanoparticles in the anatase and rutile crystal phases," *Toxicology Letters*, vol. 183, pp. 72-80, 2008.
- [80] Wang Jiangxue, Liu Ying, Jiao Fang, Lao Fang, Li Wei, Gu Yiqun, Li Yufeng, Ge Cuicui, Zhou Guoqiang, Li Bai, Zhao Yuliang, Chai Zhifang, and Chen Chunying, "Time-dependent translocation and potential impairment on central nervous system by intranasally instilled TiO<sub>2</sub> nanoparticles," *Toxicology*, vol. 254, pp. 82-90, 2008.
- [81] Nurkiewicz Timothy R., Porter Dale W., Huibbs Ann F., Stone Samuel, Chen Bean T., Frazer David G., and Boegehold Matthew A., "Pumonary Nanoparticle Exposure Disrupts Systemic Microvascular Nitric Oxide Signaling," *Toxicological Sciences*, vol. 110, pp. 191-203, 2009.
- [82] Mühlfeld Christian, Geiser Marianne, Kapp Nadine, Gehr Peter, and Rothen-Rutishauser Barbara, "Re-evaluation of pulmonary titanium dioxide nanoparticle distribution using the "relative deposition index": Evidence for clearance through microvasculature," *Particle and Fibre Toxicology*, vol. 4, p. 8, 2007.
- [83] R. Liu, L. Yin, Y. Pu, G. Liang, J. Zhang, Y. Su, Z. Xiao, and B. Ye, "Pulmonary toxicity induced by three forms of titanium dioxide nanoparticles via intra-tracheal instillation in rats," *Progress in Natural Science*, vol. 19, pp. 573-579, 2009.
- [84] Park EJ, Yi J, Chung KH, Ryu DY, Choi J, and P. K., "Oxidative stress and apoptosis induced by titanium dioxide nanoparticles in cultured BEAS-2B cells.," *Toxicological letters*, vol. 180, pp. 222-229, 2008.

- [85] Gurr Jia-Ran, Wang Alexander S.S., Chen Chien-Hung, and Jan Kun-Yan, "Ultrafine titanium dioxide particles in the absence of photoactivation can induce oxidative damage to human bronchial epithelial cells " *Toxicology*, vol. 213, pp. 66-73, 2005.
- [86] Simon-Deckers A., Gouget B., Mayne-L'Hermite M., Herlin-Boime N., Reynaud C., and Carrière M., "In vitro investigation of oxide nanoparticle and carbon nanotube toxicity and intracellular accumulation in A549 human pneumocytes," *Toxicology*, vol. 253, pp. 137-146, 2008.
- [87] Kim Jong Kee, Lee Woon-Kyu, Lee Eun-Jung, Cho Young-Jin, Lee Kweon-Haeng, Kim Ho-Shik, Chung Young, Kim Kyoung-Ah, and Lim Young, "Mechanism of silica- and titanium dioxide-induced cytotoxicity in alveolar macrophages," *Journal of Toxicology and Environmental Health*, vol. 58, pp. 437-450, 1999.
- [88] Karlsson H. L., Gustafsson J., Cronholm P., and Möller Lennart, "Size-dependent toxicity of metal oxide particles - A comparison between nano- and micrometer size," *Toxicology Letters*, vol. 188, pp. 112-118, 2009.
- [89] Karlsson H. L., Cronholm P., Gustafsson J., and Möller L., "Copper oxide particles are highly toxic: a comparison between metal oxide nanoparticles and nanotubes," *Chem. Res. Toxicol.*, vol. 21, pp. 1726-1732, 2008.
- [90] Park S, Lee YK, Jung M, Kim KH, Chung N, Ahn EK, Lim Y, and L. KH., "Cellular toxicity of various inhalable metal nanoparticles on human alveolar epithelial cells.," *Inhalation toxicology*, vol. 19, pp. 59-65, 2007.
- [91] Bhattacharya Kunal, Davoren Maria, Boertz Jens, Schins Roel PF, Hoffman Eik, and Dopp Elke, "Titanium dioxide nanoparticles induce oxidative stress and DNA-adduct formation but not DNA-breakage in human lung cells," *Particle and Fibre Toxicology*, vol. 6, pp. 1-11, 2009.
- [92] Chen Eric Y., Garnica M., Wang Yung-Chen, Mintz Alexander J., Chen Chi-Shou, and Chin Wei-Chun, " A mixture of anatase and rutile TiO<sub>2</sub> nanoparticles induces histamine secretion in mast cells," *Particle and Fibre Toxicology*, vol. 9, p. 10, 2012.
- [93] Becker Heidi, Herzberg Frank, Schulte Agnes, and Kolossa-Gehring Marike, "The carcinogenic potential of nanomaterials, their release from products and options for regulating them," *International Journal of Hygiene and Environmental Health*, vol. 214, pp. 231-238, 2011.
- [94] Heinrich U., Fuhst R., Rittinghausen S., Creutzenberg O., Bellman B., Koch W., and Levsen K., "Chronic Inhalation Exposure of Wistar Rats and two Different Strains of Mice to Diesel Engine Exhaust, Carbon Black, and Titaniumdioxide," *Inhalation toxicology*, vol. 7, pp. 533-556, 1995.
- [95] F. Pott and M. Roller, "Carcinogenicity study with nineteen granular dusts in rats," *European Journal of Oncology*, vol. 10, pp. 249-281, 2005.
- [96] Mohr Ulrich, Ernst Heinrich, Roller Markus, and Pott Friedrich, "Pulmonary tumor types induced in Wistar rats of the so-called "19 dust study", " *Experimental and toxicological Pathology*, vol. 58, pp. 13-20, 2006.
- [97] Warheit David B., Reed Kenneth L., and Sayes Christie M., "A role for surface reactivity in TiO<sub>2</sub> and quartz-related nanoparticle pulmonary toxicity," *Nanotoxicology*, vol. 3, pp. 181-187, 2009.
- [98] Okudo-Shimazaki J., Takaku S., Kanehira K., Sonezaki S., and Taniguchi A., "Effects of Titanium Dioxide Nanoparticle Aggregate size on Gene expression," *Int J of Molecular Sciences*, vol. 11, pp. 2383-2392, 2010.
- [99] Ferin J., Oberdörster Günter, and Penney DP, "Pulmonary retention of ultrafine and fine particles in rats," *J Control Release*, vol. 83, pp. 276-286, 1992.
- [100] Warheit D.B., "Debunking Some Misconceptions about Nanotoxicology," *Nano Lett.*, vol. 10, pp. 4777-4782, 2010.
- [101] Warheit David B., "Nanoparticles: Health impacts?," *Materials Today*, vol. 7, pp. 32-35, 2004.
- [102] Grassian Vicki H., O'Shaughnessy P.T., Adamcakova-Dodd A., Pettibone J.M., and Thorne P.S., "Titanium dioxide nanoparticles: Grassian et al. respond," *Environmental Health Perspectives*, vol. 116, pp. A152-A153, 2008.

- [103] Auffan Mélanie, Rose Jérôme, Bottero Jean-Yves, Lowry Gregory V., Jolivet Jean-Pierre, and Wiesner Mark R., "Towards a definition of inorganic nanoparticles from an environment, health and safety perspective," *Nature Nanotechnology*, vol. 4, pp. 634-641, 2009.
- [104] Hamilton Raymond F, Wu Nianqiang, Porter Dale W, Buford Mary, Wolfarth Michael, and Holian Andrij, "Particle length-dependent titanium dioxide nanomaterials toxicity and bioactivity," *Particle and Fibre Toxicology*, vol. 6, p. 11, 31 December 2009.
- [105] C. Jin, Y. Tang, F. G. Yang, X. L. Li, S. Xu, X. Y. Fan, Y. Y. Huang, and Y. J. Yang, "Cellular toxicity of TiO<sub>2</sub> nanoparticles in anatase and rutile crystal phase," *Biological Trace Element Research*, vol. 141, pp. 3-15, 2011.
- [106] Warheit David B., Reed Kenneth L., and Webb T.R., "Pulmonary toxicity studies in rats with triethoxyoctylsilane (OTES)-coated, pigment-grade titanium dioxide particles: Bridging studies to predict inhalation hazard," *Experimental Lung Research*, vol. 29, pp. 593-606, 2003.
- [107] Yuen I.S., Hartsky M.A., Snajdr S.I., and Warheit David B., "Time Course of Chemotactic Factor Generation and Neutrophil Recruitment in the Lungs of Dust-exposed Rats," *American Journal of Respiratory Cell and Molecular Biology*, vol. 15, pp. 268-274, 1996.
- [108] Warheit David B., Webb T.R., and Reed Kenneth L., "Pulmonary toxicity screening studies in male rats with TiO<sub>2</sub> particulates substantially encapsulated with pyrogenically deposited, amorphous silica," *Particle and Fibre Toxicology*, vol. 3, 2006.
- [109] Valant J., Iavicoli I., and Drobne D., "The importance of a validated standard methodology to define in vitro toxicity of nano-TiO<sub>2</sub>," *Protoplasma*, vol. 249, pp. 493-502, 2012.
- [110] Sayes C.M., Reed K.L., Subramoney S., Abrams L., and Warheit D.B., "Can in vitro assays substitute for in vivo studies in assessing pulmonary hazards of fine and nanoscale materials?," *Journal of Nanoparticle Research*, vol. 11, pp. 421-431, 2009.
- [111] Fabian E., Landsiedel R., Ma-Hock L., Wiench K., Wohlleben W., and van Ravenzwaay B., "Tissue distribution and toxicity of intravenously administered titanium dioxide nanoparticles in rats," *Archives of Toxicology*, vol. 82, pp. 151-157, 2008.
- [112] Warheit David B., H. R.A., Finlay C., Donner E.M., Reed K.L., and Sayes C.M., "Development of a base set of toxicity tests using ultrafine TiO<sub>2</sub> particles as a component of nanoparticle risk management," *Toxicology Letters*, vol. 171, pp. 99-110, 2007.
- [113] Kobayashi N., Naya M., Endoh S., Maru J., Yamamoto K., and Nakinishi J., "Comparative pulmonary toxicity study of nano-TiO<sub>2</sub> particles of different sizes and agglomerations in rats: Different short and long-term post-instillation results," *Toxicology*, vol. 264, pp. 110-118, 2009.
- [114] Kuempel E.D., Tran C.L., Castranova V., and Bailer A.J., "Lung dosimetry and risk assessment of nanoparticles: evaluating and extending current models in rats and humans," *Inhalation toxicology*, vol. 18, pp. 717-724, 2006.
- [115] A. S. Radilov, A. V. Glushkova, S. A. Dulov, and N. S. Khlebnikova, "Development of prognostic occupational air standards for nanoparticles," *Journal of Physics: Conference Series*, vol. 304, 2011.
- [116] Liao CM, Chiang YH, and Chio CP, "Model-based assessment for human inhalation exposure risk to airborne nano/fine titanium dioxide particles," *Science of the Total Environment*, vol. 407, pp. 165-167, 2008.
- [117] Koivisto Antti J., Lyyräinen J., Auvinen A., Vanhala E., Hämeri Kaarle, Toumi Timo, and Jokiniemi Jorma, "Industrial worker exposure to airborne particles during the packing of pigment and nanoscale titanium dioxide," *Inhalation toxicology*, vol. 24, pp. 839-849, 2012.
- [118] Notter Robert H., *Lung surfactants: basic science and clinical applications*: New York, N.Y: Marcel Dekker. pp. 120. ISBN 0-8247-0401-0., 2000.
- [119] I. L. Hsiao and Y. J. Huang, "Effects of various physicochemical characteristics on the toxicities of ZnO and TiO<sub>2</sub> nanoparticles toward human lung epithelial cells," *Science of the Total Environment*, vol. 409, pp. 1219-1228, 2011.

Interimsrapport 19 nov 2012

## Appendix 1. Small list of vocabulary and concepts in nanotoxicology

Most of the explanations and definitions are cited from Wikipedia, [www.wikipedia.org](http://www.wikipedia.org), where original references are found.

**3T3 cell line** - 3T3 cells come from a cell line established in 1962 by two scientists then at the Department of Pathology in the New York University School of Medicine, George Todaro and Howard Green. The 3T3 cell line has become the standard fibroblast cell line. Todaro and Green originally obtained their 3T3 cells from Swiss mouse embryo tissue.

**8-OHdG adducts** - 8-Oxo-2'-deoxyguanosine (8-oxo-dG) is an oxidized derivative of deoxyguanosine. 8-oxo-dG is one of the major products of DNA oxidation. Concentrations of 8-oxo-dG within a cell are a measurement of oxidative stress.

**A549 cells** are *adenocarcinomic* human alveolar basal *epithelial* cells. A549 cell line are widely used as an in vitro model for a type II pulmonary epithelial cell model for drug metabolism and as a *transfection* host.

**Adenocarcinoma** is a cancer of an epithelium that originates in glandular tissue.

An **agglomerate** is a group of particles held together by relatively weak forces (e.g. electrostatic, surface tension). An **aggregate** is a heterogeneous particle with components held together by relatively strong forces, and thus is not easily broken apart. (ISO 2006).

**Antibody (Ab)**, also known as an immunoglobulin (Ig), is a large Y-shaped protein produced by B-cells that is used by the immune system to identify and neutralize foreign objects such as bacteria and viruses.

**Apoptosis** is the process of programmed cell death (PCD) that may occur in multicellular organisms. In contrast to **necrosis**, which is a form of traumatic cell death that results from acute cellular injury, apoptosis, in general, confers advantages during an organism's life cycle.

**BAL/BALF** Bronchoalveolar lavage (BAL) is a medical procedure in which a bronchoscope is passed through the mouth or nose into the lungs and fluid is squirted into a small part of the lung and then recollected for examination. BAL is typically performed to diagnose lung disease. In particular, BAL is commonly used to diagnose infections in people with immune system problems, pneumonia in people on ventilators, some types of lung cancer, and scarring of the lung (interstitial lung disease). BAL is the most common manner to sample the components of the epithelial lining fluid (ELF) and to determine the protein composition of the pulmonary airways, and it is often used in immunological research as a means of sampling cells or pathogen levels in the lung. Examples of these include T-cell populations and influenza viral levels.

**Basophils** - Basophil granulocytes, mostly referred to as basophils, are the least common of the granulocytes, representing about 0.01% to 0.3% of circulating white blood cells.

**B cells** belong to a group of white blood cells known as lymphocytes

**BEAS-2B cells** – a human bronchial epithelial cell line, transformed by an adenovirus 12-SV40 hybrid.

The **cell** is the basic structural and functional unit of all known living organisms. It is the smallest unit of life that is classified as a living thing (except virus, which consists only from DNA/RNA covered by protein and lipids), and is often called the building block of life. Organisms can be classified as unicellular (consisting of a single cell; including most bacteria) or multicellular (including plants and animals). Humans contain about 10 trillion (10<sup>13</sup>) cells. Most plant and animal cells are between 1 and 100  $\mu\text{m}$  and therefore are visible only under the microscope.

**Cell viability** is a determination of living or dead cells, based on a total cell sample. When a sample is stained with various dyes or treated with chemicals, it is then subject to microscopic examination to evaluate cell viability. These measurements can be used to evaluate the effectiveness or lack thereof of certain treatments to cells.

**Chemokines** (Greek -kinos, movement) are a family of small *cytokines*, or proteins secreted by cells.

The **cytosol** or intracellular fluid (or cytoplasmic matrix) is the liquid found inside cells.

**LD50**, median lethal dose, the dose required to kill half the members of a tested population after a specified test duration.

In molecular genetics, a **DNA adduct** is a piece of DNA covalently bonded to a (cancer-causing) chemical. This process could be the start of a cancerous cell, or carcinogenesis.

**Cytokines** - are small cell-signaling protein molecules that are secreted by numerous cells and are a category of signaling molecules used extensively in intercellular communication. Cytokines can be classified as proteins, peptides, or glycoproteins; the term "cytokine" encompasses a large and diverse family of regulators produced throughout the body by cells of diverse embryological origin.

DNA adducts in scientific experiments are used as biomarkers of exposure and as such are themselves measured to reflect quantitatively, for comparison, the amount of carcinogen exposure to the subject organism, i.e. rats or other living animals. Under experimental conditions for study, such DNA adducts are induced by known carcinogens, of which commonly used is DMBA (7,12-dimethylbenz(a)anthracene). For example, the term "DMBA-DNA adduct" in a scientific journal refers to a piece of DNA that has DMBA attached to it. The presence of such adducts indicates prior exposure to a potential carcinogen, but does not by itself indicate the presence of cancer in the subject animal.

EDS/EDAX – Energy Dispersive x-ray spectroscopy. Usually conducted in a SEM.

Endotoxin. An "endotoxin" is a toxin that is a structural molecule of the bacteria that is recognized by the immune system. The term endotoxin was coined by Nats Umangay, who distinguished between *exotoxin*, which he classified as a toxin that is released by bacteria into the environment, and endotoxin, which he considered to be a toxin kept "within" the bacterial cell and to be released only after destruction of the bacterial cell wall. Today, the term 'endotoxin' is used synonymously with the term *lipopolysaccharide*, which is a major constituent of the outer cell membrane of *Gram-negative bacteria*. Larger amounts of endotoxins can be mobilized if *Gram-negative bacteria* are killed or destroyed by detergents. The term "endotoxin" came from the discovery that portions of Gram-negative bacteria themselves can cause toxicity, hence the name endotoxin. Studies of endotoxin over the next 50 years revealed that the effects of "endotoxin" are, in fact, due to *lipopolysaccharide*.

Engineered nanomaterials are manufactured materials in the same size range (1 – 100 nm in at least one dimension) as cellular nanomachines (e.g. mitochondria) and biomolecules [37].

EpiDermTM – se:

[http://ecvamdbalm.jrc.ec.europa.eu/public\\_view\\_doc2.cfm?id=671E9D0F0B37F87BEAC1E9382D1B03597180B B0BC12CB10496CDA74B54630A05A3291B895581F634](http://ecvamdbalm.jrc.ec.europa.eu/public_view_doc2.cfm?id=671E9D0F0B37F87BEAC1E9382D1B03597180B B0BC12CB10496CDA74B54630A05A3291B895581F634)

Epithelium is one of the four basic types of animal tissue, along with connective tissue, muscle tissue and nervous tissue. Epithelial tissues line the cavities and surfaces of structures throughout the body, and also form many glands. Functions of epithelial cells include secretion, selective absorption, protection, transcellular transport and detection of sensation. In Greek "epi" means, "on, upon," and "thele" meaning "nipple". Epithelial layers are avascular, so they must receive nourishment via diffusion of substances from the underlying connective tissue, through the basement membrane.[2][unreliable source?] Epithelia can also be organized into clusters of cells that function as exocrine and endocrine glands. Exocrine and endocrine epithelial cells are highly vascular.

Exotoxin is a toxin secreted by bacteria.

Gene expression is the process by which information from a gene is used in the synthesis of a functional gene product. These products are often proteins, but in non-protein coding genes the product is a functional RNA.

GIT – Gastro intestinal tract, refers to the stomach and intestine and sometimes to all the structures from the mouth to the anus.

Glutathione (GSH) - is a tripeptide with a gamma peptide linkage between the amine group of cysteine (which is attached by normal peptide linkage to a glycine) and the carboxyl group of the glutamate side-chain. It is an antioxidant, preventing damage to important cellular components caused by reactive oxygen species such as free radicals and peroxides.

Gram-negative bacteria are bacteria that do not retain crystal violet dye in the *Gram staining* protocol. Gram-positive bacteria will retain the crystal violet dye when washed in a decolorizing solution. Compared with gram-positive bacteria, **gram-negative are more resistant against antibodies**, because of their impenetrable wall. The pathogenic capability of Gram-negative bacteria is often associated with certain components of Gram-negative cell envelope, in particular, the *lipopolysaccharide* layer (also known as *LPS* or *endotoxin layer*). In humans, LPS triggers an innate immune response characterized by cytokine production and immune system activation. Inflammation is a common result of cytokine (from the Greek *cyto* = cell and *kinesis* = movement) production, which can also produce host toxicity.

Gram staining (or Gram's method) is a method of differentiating bacterial species into two large groups (Gram-positive and Gram-negative). It is based on the chemical and physical properties of their cell walls. Primarily, it detects peptidoglycan, which is present in a thick layer in Gram positive bacteria. A Gram positive results in a purple/blue color while a Gram negative results in a pink/red color.

Granulocytes are a category of white blood cells characterized by the presence of granules in their cytoplasm. They are also called polymorphonuclear leukocytes (PMN or PML) because of the varying shapes of the nucleus, which is usually lobed into three segments. In common parlance, the term polymorphonuclear leukocyte often refers specifically to neutrophil granulocytes,[2] the most abundant of the granulocytes. Granulocytes or PMN are released from the bone marrow by the regulatory complement proteins.

Heparin (from Ancient Greek *ἥπαρ* (hepar), liver), also known as unfractionated heparin, it is widely used as an injectable anticoagulant, and has the highest negative charge density of any known biological molecule.[2] It can also be used to form an inner anticoagulant surface on various experimental and medical devices such as test tubes and renal dialysis machines.

Histamine is an organic nitrogen compound involved in local immune responses as well as regulating physiological function in the gut and acting as a neurotransmitter. Histamine triggers the inflammatory response. As part of an immune response to foreign pathogens, histamine is produced by basophils and by mast cells found in nearby connective tissues. Histamine increases the permeability of the capillaries to white blood cells and some proteins, to allow them to engage pathogens in the infected tissues.

IMR 90 (human bronchial fibroblasts)

In vitro assay is an experimental set-up for cell viability (cytotoxicity) examinations in *in vitro* toxicology. Examples are MTT-assay, MTS-assay and ATP-assay.

Interstitium is the space between cells in a tissue.

Intraperitoneal injection or IP injection is the injection of a substance into the peritoneum (body cavity).

Intraperitoneal organ is an organ, within the abdomen, that is completely surrounded by visceral peritoneum. Intraperitoneal organs are: 1.Esophagus; 2.Stomach; 3.Jejunum; 4.Ileum; 5.Caecum; 6.Appendix; 7.Transverse colon; 8.Sigmoid colon

LDH – lactate dehydrogenase release, ...

A lesion is any abnormal tissue found on or in an organism, usually damaged by disease or trauma.

Lipids are a broad group of naturally occurring molecules which includes fats, waxes, sterols, fat-soluble vitamins (such as vitamins A, D, E and K), monoglycerides, diglycerides, phospholipids, and others. The main biological functions of lipids include energy storage, as structural components of cell membranes, and as important signaling molecules.

LPS – Lipopolysaccharide. LPS is the major component of the outer membrane of Gram-negative bacteria. They are large molecules consisting of a lipid and a polysaccharide and act as *endotoxins* and elicit strong immune responses in animals.

Macrophages is a phagocyte. Macrophages are versatile cells that play many roles. As scavengers, they rid the body of worn-out cells and other debris. Along with dendritic cells, they are foremost among the cells that "present" antigen, a crucial role in initiating an immune response. As secretory cells, monocytes and macrophages are vital to the regulation of immune responses and the development of inflammation; they produce a wide array of powerful chemical substances (monokines) including enzymes, complement proteins, and regulatory factors such as interleukin-1. At the same time, they carry receptors for lymphokines that allow them to be "activated" into single-minded pursuit of microbes and tumour cells.

Mast cell is a resident cell of several types of tissues and contains many granules rich in histamine and heparin. Although best known for their role in allergy and anaphylaxis, mast cells play an important protective role as well, being intimately involved in wound healing and defense against pathogens.[2]

The mast cell is very similar in both appearance and function to the basophil, a type of white blood cell. However, they are not the same, as they both arise from different cell lines.

Mitochondria - In cell biology, a mitochondrion (plural mitochondria) is a membrane-enclosed organelle found in most eukaryotic cells.[1] These organelles range from 0.5 to 1.0 micrometer ( $\mu\text{m}$ ) in diameter. Mitochondria are sometimes described as "cellular power plants" because they generate most of the cell's supply of adenosine triphosphate (ATP), used as a source of chemical energy.[2] In addition to supplying cellular energy, mitochondria are involved in a range of other processes, such as signaling, cellular differentiation, cell death, as well as the control of the cell cycle and cell growth.[3] Mitochondria have been implicated in several human diseases, including mitochondrial disorders[4] and cardiac dysfunction,[5] and may play a role in the aging process. The word mitochondrion comes from the Greek *μίτος* mitos, thread, + *χονδρίον* chondrion, granule.

Monocytes and macrophages are phagocytes. Monocytes are a type of white blood cell and are part of the innate immune system of vertebrates including all mammals (including humans), birds, reptiles, and fish. Monocytes play multiple roles in immune function. Such roles include: replenish resident macrophages and dendritic cells under normal states, and in response to inflammation signals, monocytes can move quickly (approx. 8-12 hours) to sites of infection in the tissues and divide/differentiate into macrophages and dendritic cells to elicit an immune response. Half of them are stored in the spleen. Monocytes are usually identified in stained smears by their large kidney shaped or notched nucleus.

Mononuclear phagocyte system (MPS) is a part of the immune system that consists of the phagocytic cells located in reticular connective tissue. The cells are primarily monocytes and macrophages, and they accumulate in lymph nodes and the spleen. The Kupffer cells of the liver and tissue histiocytes are also part of the MPS.

MTT assay, see *in vitro* assay.

Nanotoxicology is an interdisciplinary study of toxicity induced by nanomaterials, particles and fibers. A discipline studying the interference of engineered nanomaterials with the functions of cellular and extracellular nanomachineries [37].

Necrosis, is a form of traumatic cell death that results from acute cellular injury.

Neutrophil granulocytes are the most abundant type of white blood cells in mammals and form an essential part of the innate immune system. They are generally referred to as either **neutrophils** or polymorphonuclear neutrophils (or PMNs), and are subdivided into segmented neutrophils (or segs) and banded neutrophils (or bands). They form part of the polymorphonuclear cell family (PMNs) together with basophils and eosinophils.

Nucleic acids (nukleinsyra [swe.]) are large biological molecules essential for all known forms of life. They include DNA (deoxyribonucleic acid) and RNA (ribonucleic acid).

Olfactory nerve is the first of twelve cranial nerves. It is instrumental in the sense of smell. The specialized olfactory receptor neurons of the olfactory nerve are located in the olfactory mucosa of the upper parts of the nasal cavity.

Oxidative stress is a shift in the redox balance of cells. It occurs when reactive oxygen species (ROS) disturb the balance between oxidative pressure and antioxidant defense [88].

Phagocytes are the white blood cells that protect the body by ingesting (phagocytosing) harmful foreign particles, bacteria, and dead or dying cells. Their name comes from the Greek phagein, "to eat" or "devour", and "-cyte", the suffix in biology denoting "cell", from the Greek kutos, "hollow vessel". They are essential for fighting infections and for subsequent immunity. Phagocytes are important throughout the animal kingdom and are highly developed within vertebrates. One litre of human blood contains about six billion phagocytes. Phagocytes were first discovered in 1882 by Ilya Ilyich Mechnikov while he was studying starfish larvae. Mechnikov was awarded the 1908 Nobel Prize in Physiology or Medicine for his discovery. Phagocytes occur in many species; some amoebae behave like macrophage phagocytes, which suggests that phagocytes appeared early in the evolution of life.

Phagocytosis (from Ancient Greek φαγεῖν (phagein), meaning "to devour", κύτος, (kytos), meaning "cell", and -osis, meaning "process") is the cellular process of engulfing solid particles by the cell membrane to form an internal phagosome by phagocytes and protists. Phagocytosis is a specific form of endocytosis involving the vesicular internalization of solids such as bacteria, and is, therefore, distinct from other forms of endocytosis such as the vesicular internalization of various liquids. Phagocytosis is involved in the acquisition of nutrients for some cells, and, in the immune system, it is a major mechanism used to remove pathogens and cell debris. Bacteria, dead tissue cells, and small mineral particles are all examples of objects that may be phagocytosed. The process is homologous to eating only at the level of single-celled organisms; in multicellular animals, the process has been adapted to eliminate debris and pathogens, as opposed to taking in fuel for cellular processes, except in the case of the Trichoplax.

PBS – phosphate buffer solution, an artificially blended solution aimed to mimic saliva.

PMNs - polymorphonuclear neutrophils, see Neutrophil

Professional phagocytes, Phagocytes of humans and other jawed vertebrates are divided into "professional" and "non-professional" groups based on the efficiency with which they participate in phagocytosis. The professional phagocytes are the monocytes, macrophages, neutrophils, tissue dendritic cells and mast cells.

Reticuloendothelial system - see the *mononuclear phagocyte system*,

ROS – Reactive Oxidative Species.

THP1 - Human acute monocytic leukemia cell line.

Titania is short term for titaniumdioxide. (May also refer to the moon of Saturn.)

Toxin is poisonous substance that is produced within living cells or organisms; man-made substances created by artificial processes are thus excluded. Toxins can be small molecules, peptides, or proteins that are capable of causing disease or even death.

Transfection is the process of deliberately introducing *nucleic acids* into cells.

Zeta potential is electric potential in the interfacial double layer (DL) at the location of the slipping plane versus a point in the bulk fluid away from the interface. In other words, zeta potential is the potential difference between the dispersion medium and the stationary layer of fluid attached to the dispersed particle. The zeta potential indicates the degree of repulsion between adjacent, similarly charged particles (the vitamins) in a dispersion. For molecules and particles that are small enough, a high zeta potential will confer stability, i.e., the solution or dispersion will resist aggregation. When the potential is low, attraction exceeds repulsion and the dispersion will break and flocculate. So, colloids with high zeta potential (negative or positive) are electrically stabilized while colloids with low zeta potentials tend to coagulate or flocculate as outlined in the table.

Zeta potential [mV]	Stability behavior of the colloid
from 0 to $\pm 5$ ,	Rapid coagulation or flocculation
from $\pm 10$ to $\pm 30$	Incipient instability
from $\pm 30$ to $\pm 40$	Moderate stability
from $\pm 40$ to $\pm 60$	Good stability
more than $\pm 61$	Excellent stability

Interimsrapport 19 nov 2012