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Hazards of titanium dioxide nanoparticles usage in concrete – Pulmonary toxicity literature review

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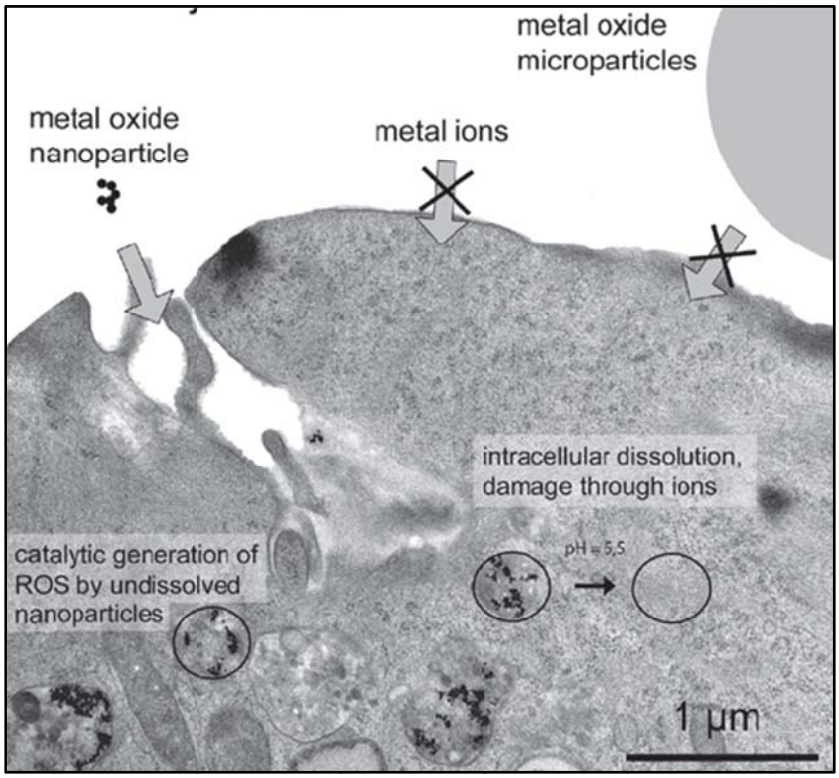

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Figure 1 (front page). The figure describes processes of nanoparticle interaction with a cell. The image shows a section of a human fibroblast after exposure to ceria nanoparticles. Particles (black dots, arrow) are found in vesicles and transported 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].

Hazards of titanium dioxide nanoparticles usage in concrete – Pulmonary toxicity literature review

Risker med användning av nanopartiklar av titandioxid i betong – en litteraturstudie i lungtoxicitet

Preface

The vocabulary used in this review requires experience of the life science literature and vocabulary. However, it is assumed that the readers of this report in general lack such knowledge. In fact, also the author had limited knowledge within this field before conducting this literature review. Therefore, a list of common terms 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.

As the author of this report I am grateful to have had the privilege and 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 is greatly acknowledged for having supported this work into completion, and its president professor Johan Silfwerbrand is particularly acknowledged for support and proof reading. Likewise Cementa AB and SBUF, the Development Fund of the Swedish Construction Industry are acknowledged for their support.

Professor Lars Österlund at the Ångström Laboratory at Uppsala University is acknowledged for valuable advises, scientific input and encouragement. I am also greatful to Dr. Hanna Karlsson, at Karolinska Institutet in Solna, Stockholm, who also supported me with scientific clarifications and for help to find important literature. Also my former colleague, professor Håkan Engqvist is acknowledged for guiding me in the right direction at the start of the literature search.

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

Lars Kraft, Stockholm, Aug. 29, 2013

Abstract

Nano-crystalline titanium dioxide is mixed into concrete in order to reduce nitrogen-oxides in urban areas, due to its photocatalytic activation of ultraviolet light. Therefore, it is important to make sure that such additions of titania nanoparticles will not cause any negative side-effects, if any, such as an increase in epidemiological harm. Accordingly, the objective of this report is to answer whether generated nanoparticles (NPs) from concrete containing titania, i.e. photocatalytic concrete, is more toxic than corresponding NPs from conventional concrete, and beside this, deduce whether titania NPs in general are more toxic compared to corresponding fine particles (FP) of titania. A state-of-the-art review is conducted, basically based on papers dealing with how micro- and nanoparticles of TiO_2 affect biological systems and on papers with explicit information of nano-sized TiO_2 when exposed to living tissue, *in vivo* or *in vitro*[*]. The primary route for body exposure to airborne particles is by inhalation, although ingestion and dermal penetration are also possible routes for NPs to enter the body. Therefore primarily papers concerning lung cell interaction with 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 titanium dioxide toxicity. Initially, contradicting facts were found regarding the influence of particle size and surface area on the toxicity of TiO_2 NPs. However, on closer inspection it was found that such contradicting results most probably may be explained by differences in particle sizes, crystal shape, dosages, methods etc., in the experimental setups. For example, in many papers NPs seem to induce higher inflammatory response at equivalent mass dose exposure of fine and ultrafine particles but at the same surface area no differences are found between NP and FP toxicity. Likewise, regarding crystal form, in most papers the anatase crystal form seems to be more potent in causing inflammation in cells and animals (guinea-pigs) than the rutile crystal form, whereas a combination of these two phases occasionally seems to be even more potent than the anatase phase alone. Therefore, 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 characterizations are insufficient. This 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 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 over the next coming years. Specifically, from the papers reviewed, TiO_2 NPs do not seem to be much more toxic than their larger counterparts, based on equal surface area. Furthermore, some papers conclude that many studies demonstrate a low hazard potential in mammals or aquatic species even at acute exposure to the ultrafine TiO_2 particles. For example, in many rat studies the inflammatory response is transient with time. Compared with other metal oxides, TiO_2 is most often less toxic than other elements, such as ZnO, CuO and SiO_2 in the different toxicological screening tests. Also, the doses used in the toxicological *in vivo* studies are 10-1000 times higher compared to any dosages that humans might be exposed to. In summary, we have the following general conclusions:

- Nanosized particles are often more potent in inducing toxicological response compared to fine particles of the same composition when delivered at equal mass.

* See section 4.3.

- At equal surface area but different particle size, similar compounds exhibit similar toxicological characteristics.
- Nanosized particles (< 50 nm) have the ability to penetrate lung cell membranes whereas larger particles cannot but will instead accumulate outside the cell wall, which also may promote damage to lung tissue.
- Generally, titanium dioxide is less toxic than silica. Nanosized silica (microsilica) is frequently used in concrete in large quantities.
- Addition of titanium dioxide nanoparticles will increase the amount of nanosized particles in the fresh concrete, but will be bound in the cement matrix in hardened concrete.
- Antropogenically generated particles from photocatalytic concrete pavements will contain less silica-based NPs but more TiO_2 NPs.
- The amount of nanoparticles generated in concrete pavements will likely be in same order of magnitude regardless of a recipe with or without TiO_2 NPs.
- Naturally or anthropogenically occurring nanoparticles agglomerate as aerosols or agglomerates in medium

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 nanoparticles generated from pavement wear from vehicle tires.

Sammanfattning

Nano-kristallin titandioxid blandas idag in i betong för att åstadkomma en miljövänlig betong med luftrenande egenskaper genom en fotokatalytisk syntes där kväveoxider i luften bryts ner. Därför är det viktigt att säkerställa att sådan inblandning av titandioxid inte orsakar några nya okända negativa sidoeffekter. Syftet med denna rapport är alltså att klargöra ifall nano-partiklar (NP) från betong som innehåller titandioxid, s k fotokatalytisk betong, är farligare än NP som genereras från annan betong, men också försöka dra en slutsats ifall NP av titandioxid är mer toxiska än motsvarande större titandioxidpartiklar i mikrometerstorlek, , s k ”fina partiklar” (FP). En litteraturstudie av aktuella vetenskapliga artiklar som redogör för skillnader i toxicitet mellan mycket små (nano-) och små (mikro-) titandioxidpartiklar har genomförts. Artiklar med specifik information angående hur NP av titandioxid påverkar levande vävnad, *in vivo* (latin för ”i den levande kroppen”) och *in vitro* (latin för ”i glas[kärl]”), har gåtts igenom. Den huvudsakliga exponeringen från små partiklar på människokroppen sker genom inandning av luft varvid lungorna utsätts för dessa partiklar. Därför är det i huvudsak artiklar som behandlar växelverkan mellan lungceller och partiklar som har studerats. Genomgången av litteraturen visar att partikelstorlek, agglomerationstillstånd, kristallstruktur, ytreaktivitet, exponeringsdos, exponeringsmetod, exponeringstid, typ av försöksdjur eller cell, spelar alla en roll för resultatet av de olika studierna. Inledningsvis visade denna litteraturstudie på en del motsägelsefulla resultat avseende toxisk påverkan pga. partikelstorlek och/eller yt-area. Men vid noggrannare granskning av sådana studier ser man att det ofta skiljer i partikelstorlek, kristallstruktur, undersökningsmetod etc., vilket givetvis har stor betydelse för resultaten. Till exempel demonstrerar många artiklar en större inflammatorisk respons för NP än för FP vid exponering av samma massa, men vid exponering av samma specifika yta uppvisar inte NP eller FP någon skillnad i toxicitet. Vidare, när det gäller kristallform; anatas eller rutil, visar de flesta artiklarna på en större toxisk inverkan från anatas än från rutil, medan andra visar på än större toxicitet för pulver med en blandad sammansättning an anatas- och rutilpartiklar. Av stor betydelse för korrekta slutsatser i de olika studierna är att forskarna genomför en noggrann fysikalisk-kemisk karakterisering av de ingående materialen i studierna såsom t.ex. förekomst av ytbeläggningar, zeta-potential och agglomerationstillstånd. I många av de undersökta artiklarna är sådan karakterisering otillräcklig, huvudsakligen i artiklar publicerade före 2005. Dock är utvecklingen snabb globalt inom vetenskapsgrenen nanopartikeltoxicitet och kunskapen om hur undersökningar bör genomföras har de senaste åren ökat markant. Därför kommer också mer specifik kunskap om olika nanopartiklars toxicitet tillta avsevärt de närmaste åren. Sammanfattningsvis visar studien att toxiciteten hos NP av titandioxid inte verkar vara större än toxiciteten hos motsvarande FP vid exponering av samma specifika yta av partiklarna. Vissa artiklar framhåller att många studier demonstrerar en låg risk vid exponering av NP av TiO_2 på däggdjur och fiskar också vid en akut (hög initial) exponering. Till exempel, vid akut exponering av partiklar på råttor avtar och försvinner initialt uppkommen inflammation med tiden. Jämfört med många andra undersökta metalloxider såsom ZnO, CuO and SiO_2 framstår oftast TiO_2 som minst toxiskt. Dessutom är de undersökta doserna i de olika *in vivo* studierna 10 – 1000 gånger högre än de doser som människor normalt riskerar att utsättas för. Rapporten resulterar i följande slusatser:

- Partiklar av nanostorlek inducerar oftare toxisk respons än motsvarande finpartiklar.
- Vid exponering av samma specifika yta av NP och FP av samma ämne uppvisar partiklarna likartade toxiska egenskaper.

- Nanopartiklar (<50 nm) har förmågan att penetrera cellväggar i lungan emedan större partiklar istället ackumuleras utanför cellväggen. Båda fallen kan orsaka inflammation.
- Generellt är titandioxid mindre toxiskt än kiseldioxid. Nanopartiklar av kiseldioxid (mikrosilika) används ofta i betong.
- Inblandning av nanopartiklar av titandioxid ökar mängden nanopartiklar i färsk betong, men de binds fast i betongen när den hårdnar.
- Mekaniskt alstrade partiklar från fotokatalytisk betong (t.ex. slitage av vägbetong) kommer innehålla färre partiklar med kiseldioxid men fler partiklar av TiO_2 än motsvarande mängd alstrade partiklar från konventionell vägbetong.
- Antalet genererade nanopartiklar från slitage av vägbetong kommer sannolikt att vara av samma storleksordning oavsett recept med eller utan tillsats av NP av TiO_2.
- Naturligt eller mekaniskt alstrade nanopartiklar agglomererar normalt i luft och i lösning.

Sammanfattningsvis dras slutsatsen att det inte är sannolikt att tillsats av fotokatalytisk TiO_2 i vägbetong kommer att medverka till att öka toxiciteten av genererade nanopartiklar vid slitage.

Contents

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 [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 TiO_2 in combination with cement based materials has shown a favourable synergistic effect in the reduction of pollutants. In comparison with other photocatalytic building materials, photocatalytic mortar and concrete are able to absorb nitrogen oxides (NOx) 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® (NPs of TiO_2) 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 NOx 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 [15]. 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 raised issues concerning potential toxicity. Therefore proactive risk assessment and regulatory guidelines are essential for a continual and increased use of environmentally beneficial products such as photocatalytic concrete [2].

2 Objective, methodology and limitations

From the background as described in chapter 1 above, the objective for this review concerns questions regarding toxicity, if any, of concrete compositions that contain photocatalytic titanium dioxide (titania). The most important questions to be answered are whether nanoparticles (NPs) from concrete containing titania is more toxic than NPs from concrete without 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. The focus is given to papers studying how micro- and nanoparticles of TiO_2 affect biological systems, respectively and to papers with explicit information of nano-sized TiO_2 when exposed to living tissue, *in vivo* or *in vitro*.

The literature searches were conducted with the scientific search engines Science Direct and Scopus. Principally only papers dealing with titanium dioxide nanoparticles exposed to lung tissue, *in vivo* or *in vitro*, have been of interest. Also, during this work some important papers have been handed to me by collegial friends involved in toxicology.

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_2” 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 Jan 1st. 2002 – June 6th, 2012.

Year	nanoparticles	nanoparticles AND toxicity	nanoparticles AND toxicity AND TiO2
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. Also some papers relating to the olfactory tract have been reviewed.

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 manmade courses of action or be manufactured on purpose for different medical or technical reasons. Airborne particles, aerosols or particulate matter usually refer to particles present in air in urban areas. Such anthropogenic NPs, that is 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 nano-materials (ENM) [19, 20] . Therefore, particles generated from concrete containing photocatalytic TiO_2 are engineered particles that have been transformed from manmade courses of action.

Worn air-borne particles containing TiO_2 NPs will not have the same physicochemical characteristics as the TiO_2 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. But no such studies, focusing on TiO_2 NPs worn from concrete pavements have been conducted yet, to the author's knowledge. Therefore, information of titanium dioxide toxicity in general, as found in the literature, is valuable for any judgment of the toxicity regarding using concrete containing TiO_2 NPs.

3.3 Nanoparticle 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 fibres have contributed to the current science of nanotoxicology [19].

The term nanotoxicology started to be used more frequently after some studies in the 1990'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_2 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_2 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_2 are more toxic than other types of NPs. However, from reading paper [1] it seems as if 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 righteously 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] do not review the papers [1, 33-35] where this influence of aggregation on studies is explained [1] and studied [33-36], the author hopes that this review will contribute to a broader knowledge regarding this aspect, not the least among novices of toxicology like myself. A recently published paper [37] also discusses the importance of having control of the agglomeration of particles in aerosols in *in vitro* lung cell exposure studies of NP. Today, however, the aspect of particle agglomeration almost always is enlightened by the scientists within the field of nanotoxicology.

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 among the least understood interfacial systems. The behaviour 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, 38]. 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 behaviour of NPs without 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]. For in vitro studies of lung cell toxicity it has recently been shown that air-liquid interface (ALI) cell exposure methodology more closely resemble in-vivo conditions compared to conventional submerged cell culture assays [37].

Limbach et al. [1] in 2009 presented well motivated strategies for *in vitro* toxicological studies on materials. They briefly summarize the current understanding of nanoparticle cell interactions. They explain that NPs behave completely different compared to soluble chemicals in biological fluids, such as phosphate buffer solution (PBS) or culture media typically used in *in vitro* assays. For molecules, the physical driving force is diffusion. This rapid movement of molecules assists homogeneous distribution into its corresponding phase (cytosol, blood, body fat, *etc*.). For fine particles (FPs) above 1 micron the main driving force is sedimentation. However, for particles within the size range of nanomaterials a cross-over takes place from diffusion-controlled movement to sedimentation controlled movement.

4.2 Material characterization for assessment of NPs biological behaviour

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*. [39].

Therefore many scientific organizations 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.* [32] write, “too often this recommendation becomes an extensive laundry list of material characteristics that does not have adequate prioritization”.

Therefore *Warheit* from 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 surface modifications (e.g. coatings).
- Purity of sample.

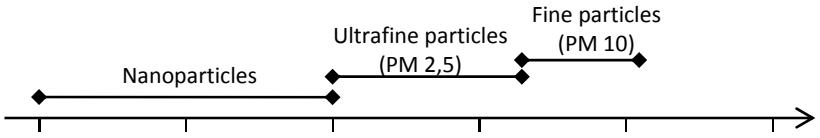
Furthermore, in [40] *Sayes and Warheit* classify particle characterization evaluations into three categories; primary, secondary and tertiary characterization as follows:
Primary characterization is performed on particles in its dry native state.
Secondary characterization is performed on particles 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.

4.3 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 micro spectroscopy* [36, 41], *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 and weaknesses as tools in determining different materials' different properties. However, *Fadeel et al.* [7] gives some information regarding usage of the different techniques.

4.3.1 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 [17]. 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 NPs are referred to particles having at least one dimension with a diameter of less than 0,1 µm, likewise ultrafines are particles with a diameter of 0,1 – 2,5 µm (PM 2.5) and fine particles with a diameter of 2,5 – 10 µm (PM10) [26, 43]. See Figure 2. These size definitions might differ somewhat in different scientific branches. All these particles are respirable and are capable of deposit in the gas-exchange region of the lungs in humans. Ultrafines and NPs frequently occur as agglomerates or aggregates, although surface coatings and other treatments are sometimes used to improve dispersion of the NPs [17].



1nm 10nm 0,1µm 1µm 10µm 100µm

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

4.3.2 Relation between particle size and surface area

Under the approximation that all particles are spherical 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 represents the particle size. Both axes are in logarithmic scale. As seen in the figure, having logarithmic scale on both axes, there is a tremendous increase in numbers of particles when the particle size is reduced [18]. This relation between particle size and surface area and particle numbers, respectively, is even greater if the smaller particles are irregular and not spherical.

For accurate toxicological studies of particle exposure also the particle size distribution (PSD) ought to be defined since all distributed particles seldom are of exactly the same size.

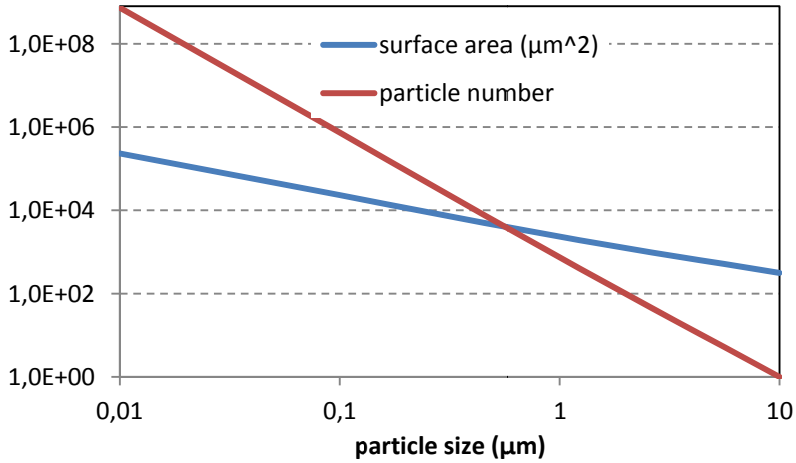


Figure 3. Relation of particle size, surface area and number for spherical particles at constant volume.

4.3.3 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 of aerosols 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. Unless particles are surface modified, NPs tend to form agglomerates when suspended in gases or liquids as already mentioned [1]. See Figure 4 [19].

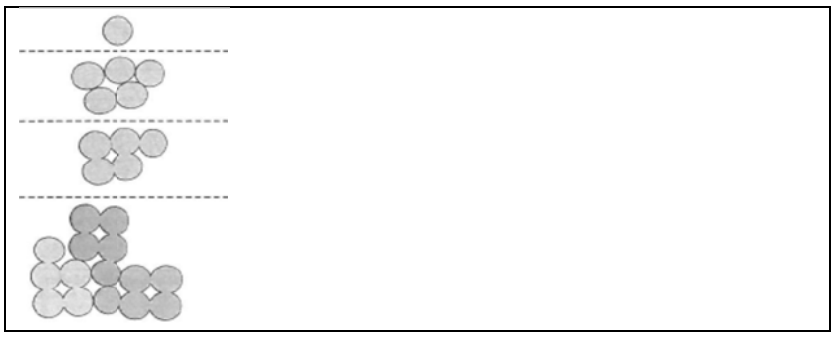


Figure 4. Primary NPs can agglomerate (coagulate) to form clusters, but may also aggregate (fuse, sinter) during generation and then agglomerate additionally. Some mass-produced engineered NPs (e.g. carbon black, TiO_2) are generated partly as aggregates that subsequently agglomerate [19].

Furthermore, as agglomerates in biological environment particles interact with biomolecules such as proteins and DNA [34, 41].

The rate of agglomeration linearly scales with the number concentration squared and temperature, while it is slowed down by viscosity. See [1, 44] for details. The rapid process of agglomeration is illustrated in Figure 5 from [1] in which the mechanisms controlling the agglomeration are described.

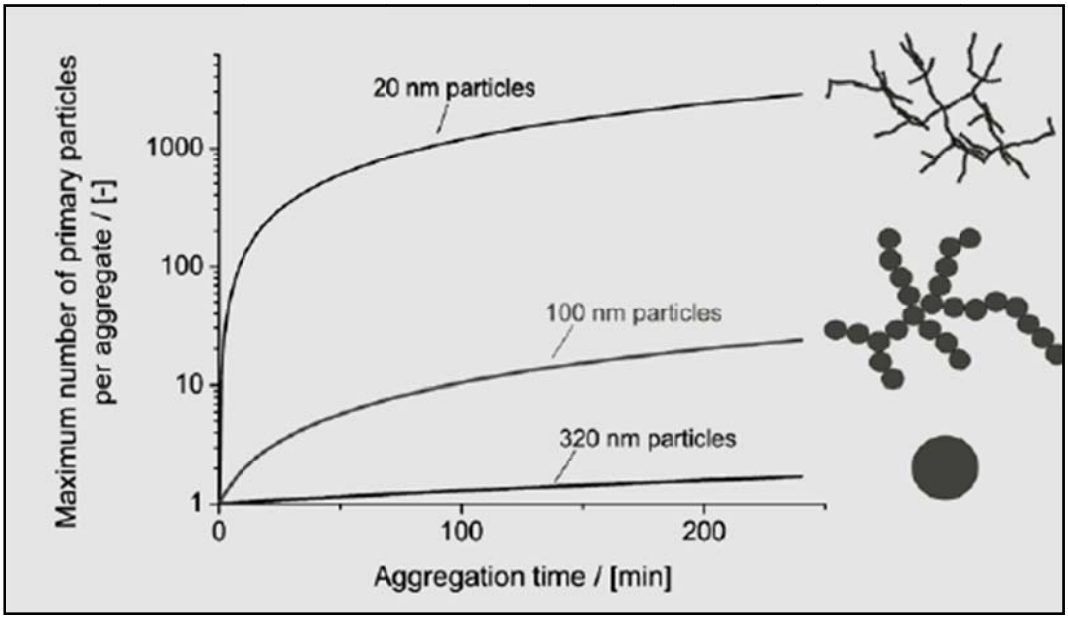


Figure 5. Agglomeration of ceria nanoparticle suspensions with different primary particle sizes. The mass concentration was kept constant at 1 ppm (1 mg/l) in water. At these conditions diffusion limits the agglomeration. At the beginning, the number concentration of 20 nm particles is much higher when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

4.3.4 Titanium dioxide characteristics

Among different metal-oxide nanomaterials, the greatest number of available toxicological studies have focused on TiO_2 [24] because of its abundant use in different applications. 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. One of the most interesting and perhaps potentially largest application, as mentioned earlier, is to use photo-activated TiO_2 NPs in concrete for air-cleaning in heavily trafficked urban areas.

Titanium dioxide (TiO_2) is a poorly soluble, low-toxicity (PSLT) particle [45]. It exists in a variety of crystal structures, but the most common ones are rutile, anatase and brookite. The most common structures are rutile and anatase. 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]. Some characteristics of the different crystal forms of TiO_2 are shown in Table 2. Furthermore, each of the rutile and anatase structures can form nanoparticles of different shapes. Figure 6 depicts five different shapes present in samples made by solution-based chemical preparation [46]. This certainly complicates comparison between toxicological studies of the “same” crystal structure even more.

Table 2: Some characteristics of the crystal forms of TiO_2 [47, 48].

	Rutile	**Anatase**	**Brookite**
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 [$Å^3$]	62	136	257
Molar volume [$Å^3$/mol]	18,7	20,2	19,4
Density [g/cm^3]	4,27	3,89	4,12
Band gap energy [eV]	3,00	3,23	3,13
Linear therm. exp [10^{-6} K^{-1}]	8,2	6,4	-
Refractive index	2,61	2,49	2,58

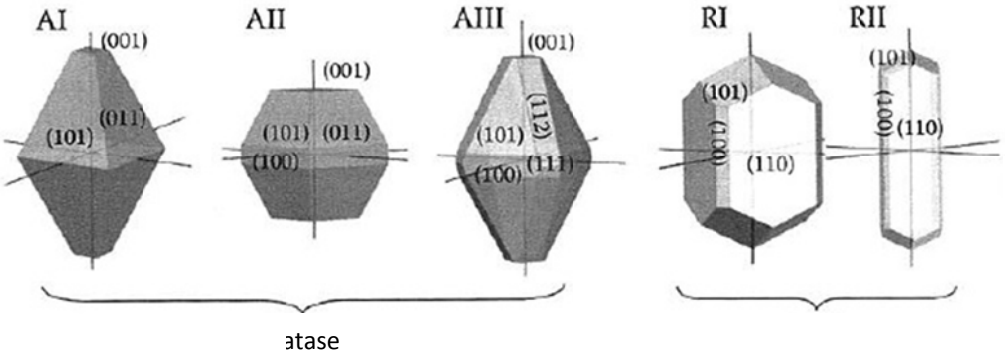


Figure 6. Compilation of the most probable shapes of TiO_2 nanoparticles with anatase (A) and rutile (R) structures. The crystallographic face indices are indicated. From [46].

Pigment grade (fine) TiO_2 is used in large quantities in paints due to its optical properties and small production cost, but also in food additives, cosmetics, sunscreens, concrete and pharmaceuticals (as carriers). In these applications TiO_2 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].

4.4 Toxicology and nano-bio interfaces

This section briefly explains how nano-bio interfaces interact and how toxicological studies are conducted and evaluated, since some knowledge is essential in order to be able to interpret the results presented in the literature. However, the subject of toxicology is too complex and advanced for a thorough description in this context. Figure 7 from *Shvedova et al.* [38] below demonstrates the complexity of studying toxicity of substances. It shows the different sizes of living tissue for comparison and for better understanding of interaction of particles with bio-interfaces.
Toxicology can be defined as the study of the adverse effects of substances on living organisms [37, 49]. 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 [50].

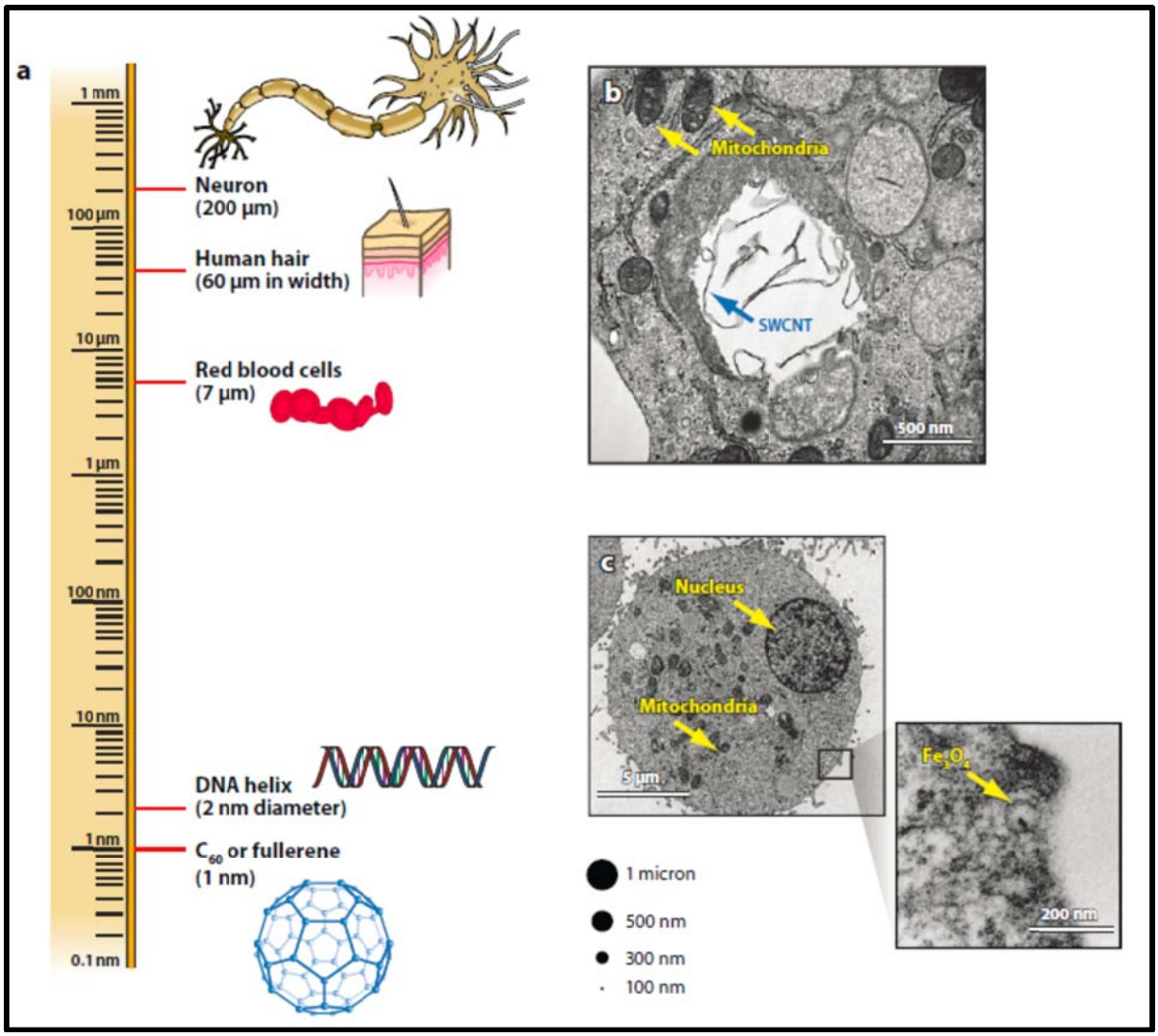


Figure 7, from [38]. In the figure three classes of nano-scale systems are considered; endogenous (cellular and extracellular) nanostructures or nanomachines, parasites (viruses, bacteria, etc.), and man-made or engineered nanomaterials. The schematic figure (a) depicts a logarithmic length scale showing the size of a classical nanomaterial (C60 fullerene) compared with various biological components. 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_3O_4) NPs of ~ 20 nm in diameter.

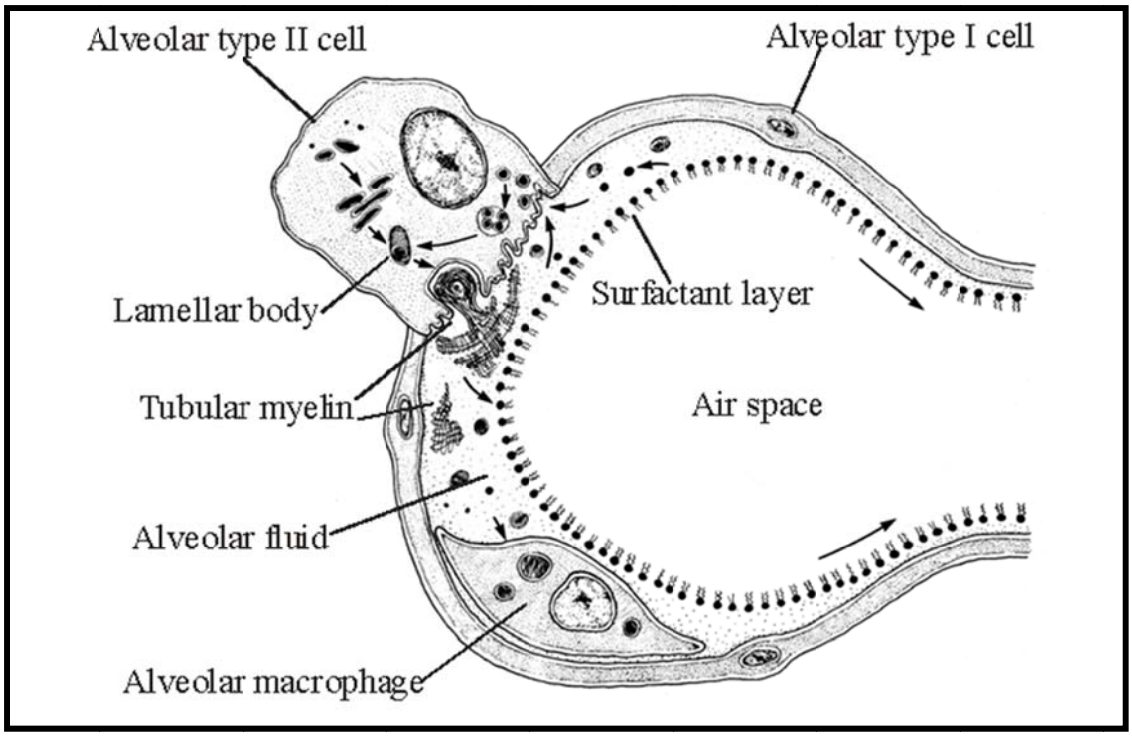


Figure 8 Structure of alveolus from [51]and modified in [52].There are three major alveolar cell types in the alveolar wall (pneumocytes) [52]:

- 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.

Reviewed papers on 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 rodents.

An example of a cell in the lung is presented in Figure 8. It demonstrates some of the complexity toxicologists have to consider when they conduct a study of toxicity in the lung, whether it is an *in vivo* or *in vitro* study.

4.4.1 Toxicological methods

As already stated, the subject of toxicology is a very broad and interdisciplinary science, which cannot be described more in detail in the scope of this report. However, the report aims to give short-hand information for what type of *in vitro* or *in vivo* method that was used in every reviewed study. Important parts of the vocabulary and concepts of toxicology are described in Appendix 1 and serve as help in the evaluation of all the presented results.

4.4.1.1 *In vivo* and *in vitro* methods

For the assessment and evaluation of the toxic characteristics of aerosols, the toxicity is usually determined by animal experiments. The determination of repeated dose inhalation toxicity requires long term studies ranging from several days up to two years employing large numbers of test animals, followed by extensive examination of tissue samples. For example, in 2005, 2 billion € were spent worldwide and 20 million animals were used for toxicological testing [37]. Despite the fact that

laboratory animals and humans show significant differences in lung physiology and immune system they do provide critical information for risk assessment.

During the subsequent half-century, tens of millions of dollars have been invested by corporations, governments, and other stakeholders with the goal of replacing animal studies (*in vivo* studies) with cheaper and more ethical *in vitro* studies. Efforts are continually made for advances in research and testing according to the so-called 3Rs where the 3R's stands for [50]:

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

Tests *in vivo* or *in vitro* can be made in a multitude of different ways. Many *in vivo* toxicity tests examine specific types of adverse effects, known as "endpoints", such as eye irritation, acute systemic toxicity, genotoxicity or cancer [50]. In typical toxicological assays, the test material (a chemical or a mixture) usually is exposed to biological fluids, consisting mainly of water, salts and biomolecules. Both *in vitro* and *in vivo* tests inherently follow this route [1]. In *in vivo* studies, 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 intra-tracheally into lungs or directly into the bloodstream (intravenous), the abdomen ("intra-peritoneal"), the bronchioles ("intra-bronchial") or the muscles ("intra-muscular"). Likewise *in vitro* studies are made in a multitude of different medical and veterinary laboratory tests upon different types of cells and tissues extracted from humans or animals, for example; cells derived from multicellular organisms (cell culture or tissue culture), subcellular components (e.g. mitochondria or ribosomes), cellular or subcellular extracts and purified molecules in test tubes (often proteins, DNA, or RNA, either individually or in combination). Traditionally test tubes containing nanomaterials and cells are used [53]. Furthermore, there are different methods for analysis of the results.

4.4.1.2 Acute, subacute, sub-chronic or chronic *in vivo* exposure

Usually experiments are set up to measure the toxicity of substances in terms of acute, sub-acute, sub-chronic or chronic exposure conditions. Studies with a maximum duration of two weeks are normally referred to acute toxicity studies. Sub-acute studies last for a maximum of 4 weeks (28 days), sub-chronic toxicity studies for a maximum of 13 days and chronic toxicity studies last longer than 4 months [54].

4.4.1.3 Exposure and dose-response measurements

In section 4.1 it was explained that NPs behave completely different compared to soluble chemicals in biological fluids, such as phosphate buffer solution (PBS) or culture media typically used in *in vitro* assays. *In vitro* lung models are traditionally performed under submerged culture conditions since it is a simple and well-proven approach for soluble molecular toxins. But submerged exposure represents an unrealistic way of exposure of airborne particles, since the *in vivo* exposure to airborne nanoparticles occurs at the air-liquid interface (ALI). Therefore, recently several *in vitro* exposure systems which allow for controlled nanoparticle exposure of cells cultured in the ALI have been developed. The ALI exposure represents a more realistic exposure scenario of inhaled NPs into the lung. Moreover, it also provides more control over the effective NP dose interacting with the cells which facilitates more reliable dose-response measurements. An account of a variety of such systems is presented by *Paur et al*. in [37].

4.4.2 Identifying toxicological interactions of NPs and cells

Two paradigms were proposed by *Shvedova et al*. [38] 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* and *in vivo* [55].

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 defence of the cell or induces the mitochondrial apoptotic [56] 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 analyse 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].

4.4.3 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, 41, 57]. 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, but also on cell type. For example macrophages, made for cleansing the body from foreign material, take up most kind of particles whereas blood cells may not. 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 modelled by diffusion, sedimentation and agglomeration as confirmed in [44] and as described earlier [1]. 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.

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 the particles have formed larger groups, their reduced mobility and larger mass will favour 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]. In most cases agglomeration is strongly favoured 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 behaviour of such oxide particles is similar at comparable primary particle concentration, if contacted with biological fluids [44]. This is in fact also confirmed by other research groups in papers [34-36, 41].
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*.[57] 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. In [41] TiO_2 NPs are also found within cells.

Furthermore, several studies show that surface coatings affect the uptake and interaction with cells [20, 35, 58, 59], which also differs from classical cell uptake of molecules.

For toxicological studies a very important aspect is the identification of the most relevant dose metric at exposure. For soluble and biodegradable NPs and chemicals mass dose metric works fine, but for (partially) inert or non-biodegradable nanoparticles surface area has been shown as a more relevant dose metric. However, most likely no single dose parameter can adequately describe the toxic effects of any type of nanoparticle because numerous pathways of nanoparticle toxicity exist [37].

4.4.4 Some examples of case studies on nanoparticle-cell interactions

Some straightforward examples of different tissue responses to different types of NPs are cited in [38], which give proof to interaction at the nanometric scale. Some examples of interaction due to different particle properties are given below:

1. *Importance of size:*
   According to Pan et al. [60] 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 μm micro silica particles located exclusively in the cytoplasm, that is on the cell wall [38]. 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].

2. *Dose-dependent toxicity*:
   Negative health effects of NPs do not appear to correlate with particle mass dose which normally is the case for fine (larger) particles. Indeed, a high concentration of NPs may promote particle aggregation and could therefore reduce toxic responses compared to lower concentrations of the same particle [38]. (Thus, this is rather a problem to solve by better experimental design, for example as in [34, 61].)
3. *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 TiO_2 particles, the half times for alveolar clearance of polystyrene test particles were proportional to the 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 [38].
4. *Crystalline structure and surface activity dependent toxicity:*
   In a study of 3-10 nm TiO_2 particles it was demonstrated that anatase titania was 100 times more toxic than an equivalent sample of rutile titania [55]. Pulmonary toxicities of fine and ultrafine (nano-sized) particles appeared to correlate better with surface activity than with particle size and surface area [62, 63].

This last conclusion, nr 4, made by *Warheit et al*. [63] regarding the importance of surface activity or particle size at equal mass dose or at equal surface area is discussed further in an paper by *Sager et al*. [64]. They found that on a mass dose basis ultrafine TiO_2 (21 nm, 80/20 anatase/rutile) was at least 41 fold more potent than fine TiO_2 (1 μm, 100% rutile), to obtain the same inflammogenic response. 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 they suggested that the surface area of particles administered in toxicological experiments may be the more appropriate dose metric for nanoparticle pulmonary studies. However, not only the size of the particles used differed, but also the crystal structure of the particles. Thus the paper illustrates how not only the different particle properties influence the toxicity, but also the interpretation of the results of the experiments. However, regarding the conclusion of the importance of surface activity by *Warheit et al.,* they argue in 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 μm which leads to an over estimation of the delivered surface areas. From the papers published later by *Warheit and Sayes* [32, 40] it seems as if they don't think this being an issue anymore. The toxic response may correlate either to particle size, surface area and/or surface activity. It is just a question of how the study and the particle characterization are performed. In one perspective it is all a function of the same coin, namely particle size. Because at the same mass; the smaller the particles are, the greater is the surface area. Likewise, the smaller the particle, the greater is its surface activity in general. Is it not? It's all a question for the ability of individual particles to interact with the tissue structure at the interface. But if smaller particles agglomerate into tightly bound clusters, then they will act as larger particles. Even more important is whether the particle cell interaction will trigger the immune system or not. And what is worst in the long term; accumulation of non-recognized particles within cells or recognition by the immune system with oxidative stress, inflammation and activation of phagocytosis?

The issue of particle size and surface area dependent toxicity will be discussed further later.

Another important note in [38] dealing with the recognition of nanomaterials by the immune system, as quoted above, concerns a paper by *Geiser et al*. [65] where it was found that 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_2 particles circulating in the body.

Nurkiewicz et al. [61] showed that pulmonary exposure of rats by inhalation of aerosols to fine and ultrafine TiO_2 causes systemic microvascular dysfunction. In rats exposed to ultrafine TiO_2 (25 nm, aerosol median diameter: 138 ± 3 nm) infusion elicited vasodilations (*dilations of blood vessels*) were blunted in proportion to pulmonary particle deposition. In rats exposed to fine TiO_2 (1 μm, aerosol median diameter: 0,7 μm) infusion produced arteriolar constrictions or significantly impaired vasodilator responses. However they exposed the rats to fine and ultrafine particles at 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. See section 5.1.

Shimizu et al. [66] report some examples from other studies where inhaled NPs of carbon were translocated to secondary organs such as the brain [67] 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 [68]. *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 *Pietropauli et al*. [69] 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.

4.5 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 fibres (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 fibres cannot be adequately phagocytised by the alveolar macrophages, preventing clearance and so the dose accumulates. Biopersistence is also important because long fibres 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 fibres [19]. (In this context long fibres are greater than 20 microns.)
However, the appearance of carbon nanotubes, single walled and multi-walled, 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 biopersistance of HARN in many ways exhibit similar toxic properties as asbestos [70].
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_2

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 by *H.J. Johnston et al*. was compiled in 2009 [24]. Since then at least two more specific reviews concerning TiO_2 NP toxicity have been published [29, 30] (that is until June 2012). In the following sections 5.1 – 5.3 facts from the some of the studies reviewed in those three reviews are compiled together with facts and conclusions from other studies, many of them published after 2009.

5.1 *In vivo* studies on pulmonary exposure to TiO_2 NPs

In *Ferin* et al. [71] rats were exposed via inhalation to ultrafine 21 nm (23,5 mg/m^3) and fine 250 nm (23 mg/m^3) TiO_2 particles for 12 weeks, and evaluated after 64 weeks; alternatively rats were administered to TiO_2 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 pe. 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, dosage and dosage rate impacted on the translocation process, and therefore TiO_2 toxicity.
- ⇨ Inflammation was affected by the process occurring during exposure and less by the lung burden or by particle redistribution after exposure.

Renwick et al. [72] investigated the size dependency of TiO_2 (29 nm NPs and 250 nm FPs) and carbon black (CB) 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 pe.

- At the highest dose, only TiO_2 NPs but not TiO_2 FPs stimulated the recruitment of neutrophils into the lungs, epithelial damage, increased permeability of the lung epithelium and cytotoxicity, measured with BALF.

– Both particles impaired the phagocytic ability of isolated rat alveolar macrophages (from exposed animals).
⇨ The NPs demonstrated to elicit a greater pulmonary inflammatory response than FPs.
⇨ Generally ultrafine carbon black particles induced greater inflammatory response than ultrafine TiO_2.
⇨ It was suggested that the greater inflammatory response seen with ultrafines is attributed to a greater surface area.

Bermudez et al. [73] determined if the choice of species influenced the pulmonary response to TiO_2 NPs. Rats., mice and hamsters were exposed to TiO_2 NPs (21 nm) via inhalation for 13 weeks, 6 h/day, 5 days/week, at conc. of 0.5, 2 or 10 mg/m^3. The pulmonary response was assessed up to 52 weeks pe. The aerosol diameters of the agglomerated particles at exposure were measured and found to be 1,29, 1,45 and 1,44 at hamster, mouse and rat exposure, respectively.
- Pulmonary inflammatory response was stimulated by TiO_2 NPs within rats and mice, but was absent in hamsters.
- In rats, a greater neutrophilic response was apparent, with progressive epithelial fibroproliterative 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 apperent 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 mimes 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 were compared with those from an earlier companion study performed two years earlier by *Bermudez* et al. [74] where the same species, rats, mice and hamsters were exposed to fine TiO_2 particles (1 micron) at concentrations of 10, 50 or 250 mg/m^3 via inhalation for 13 weeks, 6 h/day, 5 days a week as in Bermudez et al. 2004. The aerosol diameters of the agglomerated particles at exposure were measured and found to be 1,36, 1,39 and 1,44 at hamster, mouse and rat exposure, respectively.
⇨ Species differences were witnessed and also here the severity of the response were ranked as rat > mouse > hamster
⇨ The 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. [75] investigated the influence of exposure methods, inhalation and intratracheal instillation, and surface coating modification upon TiO_2 particles (290 – 440 nm) upon pulmonary toxicity within rats. For inhalation, rats were exposed to particles at concentrations up to 1300 mg/m^3 for 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_2 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_2 preparations stimulated a transient pulmonary response, typified by infiltration of neutrophils and lactate dehydrogen release (LDH) and this response was resolved within one week pe.

⇨ The response following particle exposure was very much dependent on the TiO_2 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.

Ahn et al. [76] investigated which processes were responsible for particulate mediated stimulation of excessive mucus secretion within humans. Excessive mucus secretion is one of the major clinical manifestations of chronic airway diseases such as asthma, chronic bronchitis, and cystic fibrosis. Rats were exposed to TiO_2 particles (290 nm, 4 mg/kg) via intratracheal instillation, and toxicological observations were made from 4 to 72 hours pe.

- The TiO_2 exposure stimulated an increase in goblet cell hyperplasia, which is a hallmark of airway remodeling in chronic airway diseases.

⇨ Therefore it was speculated that particle mediated increases in mucus secretion can contribute to the aggravation of chronic airway decease symptoms also in humans.

– Only one type of TiO_2 particles were included in the study and compared to sham-treated rats.

– No information was given regarding agglomeration state, surface area or crystal shape of the particles tested. But particles were from Du Pont, Wilmington, DE, and most likely rutile.

Chen et al. [77] exposed mice via intratracheal instillation (0.1 and 0.5 mg/mouse) to nano (19-21 nm; 50 m^2/g) and micro (180-250 nm; 6,5 m^2/g) TiO_2 particles. Also cell lines of human monocyte THP-1 and human A549 pulmonary epithelial cells were grown in conditioned culture medium and then treated with or without nano-TiO_2 for 24 h. The nano-TiO_2particles were purchased from Degussa and, according to the authors, of rutile crystal structure. The crystal structure of the larger particles was omitted by the authors.

- Histological assessment illustrated that morphological alterations were evident on exposure to nano-TiO_2, 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.

⇨ The results showed that a single intratracheal expose of 0,1 mg nano-TiO_2 can induce severe pulmonary inflammation in the mouse lung and pulmonary emphysema as a consequence.

⇨ No significant pathological changes were seen using the same dose of micro-TiO_2.

⇨ The in-vitro studies also showed higher toxicity for nano-TiO_2 compared to the micro-TiO_2 in all endpoints, except in levels of secreted P1GF protein in the A549 cells where micro-TiO_2 induced higher but not significantly higher levels than nano-TiO_2.

– The study lacks a proper characterization of the particles crystal shape and agglomeration state.

Grassian et al. [78] investigated size dependent effects of two TiO_2 NPs, 5 or 21 nm, of inflammation and toxic response through acute inhalation exposures and instillation. Inhalation exposure was done with particle concentrations of 0.8 to 12,7 mg/m^3 and nasal instillation with particle concentrations from 0,1 to 3,0 mg/ml. (These doses corresponds to ~1-13 mg/mouse and 5-30 mg/mouse for the inhalation exposure and the nasal instillation, respectively. Inhalation was administered for 4 hours with toxicological observations made immediately and 24 h post beginning of exposure. Toxicological observations after nasal instillation were also made 24 h pe. The 5 nm particles were pure anatase, whereas the 21 nm particles were a mix of rutile and anatase particles.

– The TiO_2 NPs formed agglomerated aerosol sizes of 120 and 145 nm for the 21 nm and 5 nm particles, and in saline solution in the inhalation study the hydrodynamic radius of agglomerates

increased from 35 – 600 nm for 21 nm particles and from 140 – 900 nm for 5 nm particles with mass concentrations of 0,005 g/L – 0,2 g/L, respectively.

- ⇨ An elevated macrophage population was associated with the inhalation of the particles. The macrophages internalized the particles, but an infiltration of neutrophils was associated with the nasal instillation of TiO_2 NPs. Thus experimental set-up influences the results of toxicity tests.
- ⇨ The nature of the response varied for the different exposure methods; inhalation -> macrophage driven response; instillation -> development of a neutrophil driven response.
- ⇨ In both exposure scenarios the inflammatory response induced was greater for the 21 nm particles compared to the 5 nm particles, at equal surface area doses - and for the inhalation studies also at equal mass dose. (Similar mass dose gave the same surface area for the two particle sizes.)
- ⇨ The moderately higher toxicity of the larger particles was suggested to depend on differences in the nature of the nanoparticle agglomeration, where the larger 21 nm particles formed slightly smaller and looser bound agglomerates than the smaller 5 nm particles which formed denser and slightly larger agglomerates.
- ⇨ The crystallinity of the samples was also suggested to influence the toxicity of TiO_2 NPs since it has been shown before [79] that a mix of crystals may generate higher toxic response than either pure anatase or pure rutile crystals.
- ⇨ A higher surface reactivity, due to higher surface free energy in smaller particles, was suggested as an explanation to more tightly bound agglomerates for the smaller 5 nm anatase TiO_2 NPs.

In another study by *Grassian et al.* [80] mice were exposed by inhalation to TiO_2 NPs 2 – 5 nm at an acute dosage of 0,77 or 7,22 mg/m^3 for 4 hours and at subacute exposure for 4 hours/day during ten days at an dosage of about 8,9 mg/m^3. The particles agglomerated as aerosols 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 subacute exposure showed evidence of inflammatory response after 0, 1 and 2 weeks pe, with recovery after 3 weeks.

- ⇨ Inhaled TiO_2 NPs < 10 nm in mice induce relatively modest but significant responses which disappeared after 3 weeks pe.

Warheit et al. [81] investigated the influence of crystalline form of TiO_2 NPs (1 or 5 mg/kg) to pulmonary toxicity within rats, subsequent to intratracheal instillation. Two rutile NP samples with an average particle size of ~140 nm and surface area 18,2 m^2/g and 35,7 m^2/g respectively (Du Pont company), a mixed rutile anatase sample (80 % anatase; 20 % rutile, P 25 Degussa) with an primary particle size 25 nm and surface area 53 m^2/g, a rutile microparticulate (382 nm; 5,8 m^2/g; negative control) and a microparticulate α-quartz sample (0,48 μm; 5,2 m^2/g; positive control) were considered. All TiO_2 NPs were highly agglomerated (> 2 micron) in PBS. Pulmonary response to particles was evaluated up to three months post exposure (pe).

- ⇨ Exposure of the higher dose 5 mg/kg of the three rutile particles produced transient and reversible inflammatory responses 24 h pe, which recovered one week after exposure. This was in contrast to the quartz particles which produced significant adverse inflammatory effects which continued through the 3-month pe study period. The anatase/rutile mixed sample produced intermediate pulmonary toxicity effects through 1 month pe as well as histopathologically adverse lung tissue effects compared to PBS vehicle controls.
- ⇨ It was suggested that differences in the particle surface area accounted, in part, for the responses, but since the rutile NP samples, with intermediate surface area, did not exhibit intermediate levels of toxicity, also other factors such as particle surface crystallinity, surface chemistry and photo-activity contribute to the toxicity of TiO_2. That is, rutile being less toxic than anatase.
- ⇨ Post-production removal of chloride from the particle surfaces may influence the toxicity which is not performed in the production of P25 mixed particles, but is practiced in the production of the ultrafine and fine rutile particles. of enhanced agglomeration of particles.
- ⇨ The particle concentrations were excessively high and unlikely to be encountered by humans.

In the study [62] *Warheit et al*. evaluated the acute lung toxicity in rats of intratracheally instilled pigment-grade TiO_2 particles (rutile, ~300 nm; 6 g/m^2) versus nanoscale TiO_2 rods (anatase, 200 x 35 nm; 26.5 g/m^2) or nanoscale TiO_2 dots (anatase, ~10 nm; 169 g/m^2) compared with a positive control particle type, quartz (1-3 microns; 4 g/m^2). Instillation dosages were 1 and 5 mg/kg rat. 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_2 rods or nanoscale TiO_2 dots produced transient inflammatory and cell injury effects at 24 h pe and was not different from the pulmonary effects of larger sized TiO_2 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.

Inoue et al. [82] studied the ability of TiO_2 NP exposure to influence the pulmonary toxicity of lipopolysaccharide (LPS). The impact of intratracheally instilled TiO_2 (8mg/kg; 15, 50, 100 nm) on LPS mediated pulmonary inflammation in mice was determined.

- Combined TiO_2 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.

Studies regarding the fate of TiO_2 NPs, subsequent to pulmonary exposure have been apparent, in particular on particle transfer to the brain [24]. In *Wang* et al.[83] and *Wang* et al. [84] the distribution of rutile (80 nm; 22,7 m^2/g) and anatase (155 nm; 10,5 m^2/g) 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_2 NPs accessed the brain and accumulation was highest in the hippocampus.
- The accumulation was time dependent and an inflammatory response within the brain was also stimulated by TiO_2 particle exposure.
- After 30 days of exposure no obvious changes were observed in heart, liver, spleen and lung except in the kidneys and brain.

⇨ The studies indicate that the TiO_2 NPs can enter the brain via the olfactory bulb.

⇨ It was concluded that the small differences in response to anatase particles versus the rutile ones could be related to crystal structure.

⇨ The results suggest that the techniques for occupational protection should be developed for workers in workplace.

Dankovic et al. [45] evaluated the rat dose-response data and conducted a quantitative risk assessment for TiO_2. Fine TiO_2 (<2.5 micron) has been shown to produce lung tumours in rats exposed to 250 mg/m^3, and ultrafine TiO_2 (< 0.1 micron diameter) has been shown to produce lung tumours in rats at 10 mg/m^3. Efforts to estimate the excess risk at exposure from seven different dose-response models were presented. Preliminary conclusions were:

⇨ Fine and ultrafine TiO_2 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_2 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_2 depend on both the dosimetric approach and the statistical model that is used.

Nurkiewicz et al. [85] determined the impact of TiO_2 FPs (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 arteriolar 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.

The translocation of TiO_2 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*. [61] it has been shown in other studies that, once in the lung, TiO_2 NPs initiate pulmonary responses such as airway inflammation in rats, alveolar macrophage recruitment, and the activation of various growth factors and chemokines in mice. It has also been shown, in other studies, [61] that ultrafine particles deposited in the lung may have the ability to translocate to systemic sites within 24 hours of deposition (animal studies) and, furthermore, within 24 hours of deposition in the lung a substantial portion of inhaled TiO_2 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_2 NPs. In [85] (see above) they showed that;

⇨ Pulmonary exposure to fine $PM_{2,5}$ 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_2 and residual oil fly ash (ROFA) caused similar, dose-dependent degrees of microvascular dysfunction [85]. While this indicates that larger TiO_2 particles elicit potent systemic microvascular effects, it cannot be used to conclude that ultrafine TiO_2 exposure will produce qualitatively or quantitatively similar biological effects. Therefore, *Nurkiewicz et al*. [61], studied the microvascular function after pulmonary exposure (via inhalation) to fine (<1 μm; Sigma-Aldrich; primary and secondary mode at 710 nm and 120 nm, respectively; 2,3 m^2/g) or NP (21 nm, Degussa Aeroxide P25; with primary and secondary mode at 100 nm and 400 nm, respectively; 48,1 m^2/g) TiO_2 aerosols over a range of concentrations, and determined if NP TiO_2 particles are inherently more toxic than larger, fine TiO_2 particles. It was found that;

– Particle size distributions of the aerosols generated by the inhalation exposure system showed that fine particles did not form agglomerates but that the NP did.

⇨ The study proved impaired vasodilator capacity in the systemic microcirculation after NP inhalation.

⇨ Inhalation of NP TiO_2 produced greater remote microvascular dysfunction than fine TiO_2 particles, despite the fact that the NPs agglomerated and the fine did not.

⇨ However, if the microvascular response were normalized to equivalent surface area, as measured by BET, the fine TiO_2 were more potent than the NPs.

Similar results were obtained in another study by *Nurkiewicz et al*. [86] 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*. [87] confirmed:

⇨ Evidence for translocation of TiO_2 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_2 NPs observed within the capillaries is still very low.

Elina M. Rossi et al. [59] investigated how airway exposure to silica-coated TiO_2 NPs induces pulmonary neutrophilia in mice. Four commercially availableTiO_2 particles, one in-situ produced TiO_2 and one type of amorphous silica (SiO_2) were used. The commercial TiO_2 materials were rutile (initial partide size < 5 µm; 2 m^2/g), nanosized rutile/anatase (~30 nm; 23 m^2/g), nanosized anatase (< 25 nm; 222 m^2/g) and silica-coated nano-sized needle-like rutile, "cnTiO_2" (10 x 40 nm; 132 m^2/g). In addition, inhalation exposure was carried out with in-situ produced anatase/brookite T iO_2 (21 nm; 61 m^2/g) and nanosized SiO_2 particles (10 nm; 515 m^2/g). 7-week-old female mice, eight mice per group, were exposed for inhalation in a chamber. The first two groups were exposed once for 2 h, and the second two groups were exposed for 2 h on 4 consecutive days, one of the replica groups was always sacrificed 4 h and the other 24 h after the last exposure. The fifth group was exposed for 2 h a day on 4 consecutive days for 4 weeks and sacrificed 24 h after the last exposure. All particles agglomerated as aerosols and the agglomerate sizes were 100 nm or even close to a micrometer. The chamber aerosol concentration was 10 ± 2 mg/m^3, chosen to mimic occupational conditions for workers exposed to a max concentration of 5 mg/m^3. Furthermore, effects from particles on human macrophages and fibroblasts were examined *in vitro*. Therefore also the z-potential for the different powders was measured at different pH.

– At pH = 6 all particles had a z-potential close to - 40 mV but at lower pH the z-potential differed between the different particles.
– Differences were found in cellular location for different NP agglomerates. Four weeks of inhalation exposure to uncoated TiO_2 NPs resulted in entrapment of NPs into membrane-bound phagosomes within mouse alveolar macrophages. In contrast a great majority of "cnTiO_2" NP agglomerates were present freely in the macrophage cytosol.

⇨ SiO_2-coated rutile TiO_2 nanoparticles, was the only sample tested that elicited clear-cut pulmonary neutrophilia. Uncoated rutile and anatase as well as nanosized SiO_2 did not induce significant inflammation.
⇨ The observed lung inflammation could not be explained by the surface area of the particles, their primary particle or agglomerate size, or radical formation capacity but rather is explained by the surface coating upon the rutile TiO_2 NPs, and not by the SiO_2 coating solely since the amorphous nano SiO_2 itself did not induce any signs of inflammation.
⇨ Regarding the issue of crystal shape Rossi et al also compared the "cnTiO_2" with another needle like rutile TiO_2 NP with about the same surface area but with an alumina coating. In those (unpublished results 2010) complementary *in vitro* studies no inflammatory effects were detected for the alumina coated needle like TiO_2 NPs, which again emphasizes the importance of the surface activity.
⇨ 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. [88] evaluated if TiO_2 NPs can induce acute pulmonary toxicity in rats. Therefore rats were intratracheally instilled with 0.5, 5 or 50 mg/kg of 5, 21 and 50 nm primary TiO_2 NPs. The 5 nm particles were anatase with surface area 210 m^2/g. Corresponding data for the 21 nm and 50 nm TiO_2 NPs were: 80% anatase/20% rutile; 50 m^2/g, and >98% rutile; 30 m^2/g, respectively. According to authors particle agglomerattion were limited due to treatment of 0,15 % NaCl solution and 15 min of ultrasonic vibration. Seven different endpoints of toxicity were evaluated. The authors found that:

⇨ The toxic effect of TiO_2 NPs in lung tissue exhibited a dose-response relationship.
⇨ At exposure of 5.0 mg/kg both 5 nm and 21 nm TiO_2 NPs induced standing pulmonary lesions
⇨ At the highest exposure of 50 mg/kg, 5 nm TiO_2 NPs suppress the phagocytic ability of alveolar macrophages.
⇨ Pulmonary toxicity caused by 5 nm TiO_2 NPs was more severe than that caused by 21 and 50 nm TiO_2 NPs.

5.2 Other *in vivo* administered exposures to TiO_2 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_2 NPs is negligible on healthy skin. Therefore the TiO_2 NPs propensity for toxicity responses on healthy skin is anticipated to be minimal. Oral exposure has shown that TiO_2 NPs (25, 80, 155 nm) are able to pass through the gut wall. Both oral and intravenous exposure of TiO_2 NPs has shown an accumulation of particles in the liver. See the reviews [24, 29, 54] which give lots of information regarding such studies.

5.3 TiO_2 NP toxicity studies from *in vitro* lung models

Cytotoxicities of TiO_2 NPs (21 nm) of different concentrations (5, 10, 20 and 40 µ/ml) were evaluated in a study by *Park et al.* [89] 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_2 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_2 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. [79] 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_2 particles may cause an inflammatory response.

Simon-Deckers et al. [90] investigated the toxicity of a panel of NPs, including TiO_2 (in a variety of sizes in both anantase and rutile forms) and Al_2O_3, 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_2 NPs were more toxic than their larger counterparts.

⇨ The chemical composition makes a difference since TiO_2 NPs of 12 nm were more toxic than 11 nm Al_2O_3, 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. [91] assessed the TiO_2 and SiO_2 mediated cytotoxicity to alveolar macrophages from rats, when exposed to concentrations 0.5 – 5 mg/ml.

⇨ TiO_2 elicited a dose dependent decrease in cell viability.

⇨ SiO_2 had a greater cytotoxic potential than TiO_2.

Karlsson et al. [92] compared NPs of the metaloxides Fe_2O_3, Fe_3O_4, TiO_2 and CuO with corresponding micrometer particles. The fine TiO_2 and nano TiO_2 particles had a mean size of 1 micron and 63 nm, respectively. The fine TiO_2 consisted of 99.9 % rutile and the ultrafine TiO_2 consisted of a mix of anatase and rutile. The influence of different dosages was also studied for TiO_2 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 epithelian 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.
- ⇨ Undeniably NPs of certain matter can be more toxic than micrometer particles of the same matter.
- ⇨ CuO particles are much more toxic and genotoxic compared to other metal oxides. (See also [93].)
- ⇨ TiO_2 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_2 NPs. But the study by Park et al., already mentioned, showed toxicity effects of 21 nm TiO_2 particles in BEAS-2B cells. [89])
- ⇨ However, both size fractions of TiO_2 showed high ability to damage DNA.
- ⇨ Interestingly the fine TiO_2 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 fine particles of some metal-oxides. The Table shows whether NPs are more or less toxic than the corresponding FPs. [92]

Nanoparticles vs fine (micro) particles	Cytotoxicty 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_2O_3	Slightly more toxic	Equally toxic	Less toxic	More toxic
Fe_3O_4	Not toxic	Slightly less toxic	Equally toxic	Much more toxic
TiO_2	Not toxic	Equally toxic	Less toxic	More toxic

Falck GC et al. [58] studied genotoxic effects of nanosized and fine TiO_2 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^2) of titanium (IV) oxide nanosized rutile (>95%, <5% amorphous SiO_2 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^2 for fine rutile and 10 μg/cm^{2}for nanosized anatase.
- ⇨ Nanosized rutile showed a significant induction in DNA damage only at 80 μg/cm^2 in the 24-h treatment and at 80 and 100 μg/cm^2 in the 72-h treatment (with a dose-dependent effect).
- ⇨ Uncoated nanosized anatase TiO_2 and fine rutile TiO_2 are more efficient than SiO_2-coated nanosized rutile TiO_2 in inducing DNA damage, whereas only nanosized anatase is able to slightly induce micronuclei.

The aim of a study conducted by *Park et al.* [94] was to evaluate *in vitro* biological effects of various (TiO_2, 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_2.

⇨ Uptake of no other NPs but fine and ultrafine TiO_2 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_2, 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[a]	apoptotic damage[b]	other remarks
TiO_2 , nano	++++	+	++	apop.dam. in dose
TiO_2 , micro	+++++	0	++	dependent manner
Silica, nano				
Silica, micro	+++	+++	+++++	
Ag, nano	+	< 0	0	
Al, nano	+	<< 0	+	
Zn, nano	+	+++++	+++++	intracellular generation of ROS
Ni, nano	+	++++	++++	

a) measured with two-coluor flow cytometry
b) measured with DNA fragmentation

In a study by *Bhattacharya et al.* [95] human lung fibroblasts (IMR-90) were exposed to TiO_2 (anatase) or Fe_2O_3 (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_2-NP demonstrated low amount of cyto- and genotoxicity as compared to the Fe_2O_3-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_2O_3-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_2-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_2-NP.

The results are summarized in Table 5.

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

	TiO_2-NP	Fe_2O_3-NP
Particle diameter (hydrodynamic)	91 nm	50 nm
Surface area	49,7 m^2/g	34,4 m^2/g
Zeta potential	+ 48,8	-26,7 mV
Genotoxicity (Comet assay: DNA breakage)	no	yes
Cytotoxicity (Trypan blue assay)	no (yes)*	yes
Acellular radical formation (ESR)	increasing	decreasing (increasing)**
Intracellular radical formation (H2DCFDA)	increasing	delayed
Oxidative DNA-damage (8-OHdG)	yes	no

*) Cytotoxicity negative in BEAS-"B 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_2 (anatase, 5-10 nm) and SiO_2 (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_2 and SiO_2 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_2 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_2	SiO_2	MWCNT (nm)			TiO_2	SiO_2	MWCNT (nm)			TiO_2	SiO_2	MWCNT (nm)		
			<8	20-30	>50			<8	20-30	>50			<8	20-30	>50
Cytotoxicity	+	+	+	+	+	+	++	++	+	-	3+	3+	++	++	3+
ROS	+	+	+	+	+	-	+	+	-	-	+	+	+	-	+
LMD[i]	-	-	3+	+	+	-	-	-	-	+	-	-	-	-	+
MMP[ii]	-	-	++	-	-	-	+	++	-	-	-	+	+	-	+
Caspase 3/7	++	++	-	+	-	-	++	3+	-	-	-	-	-	-	-
Apoptosis[iii]	-	+	+	+	+	-	3+	++	-	-	3+	3+	+	+	++
Necrosis	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-

i) Lysosomal Membrane Destabilisation
ii) Mithocondrial 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 [56]

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_2. 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 $\mu g/cm^2$, since higher doses potentially could have led to false positive or negative results.

⇨ From other work *Kroll et al*. [35] found that, for TiO_2 NPs, overload effects in rats occur at 10 mg/m^2. From estimation of the volume and the rat lung surface they calculated that such an overload corresponds to ~3 $\mu g/cm^2$.

Thus the three doses 0.1, 1 and 10 $\mu g/cm^2$ 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 $BaSO_4$.

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	CB	CB	CB	CB	CB	CB	CB	CB	CB
	TiO_2 3	TiO_2 3	TiO_2 3	TiO_2 3	TiO_2 3		TiO_2 3	TiO_2 3	TiO_2 3	TiO_2 3
	Ti-Zr 3	CeO_2-A		Ti-Zr 3	Ti-Zr 3		Ti-Zr 3	Ti-Zr 3	Ti-Zr 3	Ti-Zr 3
	CeO_2-A	CeO_2-C		CeO_2-A	CeO_2-A		CeO_2-A	CeO_2-A	CeO_2-A	CeO_2-A
	CeO_2-C			CeO_2-B	CeO_2-B		CeO_2-B	CeO_2-B	CeO_2-B	CeO_2-C
				CeO_2-C	CeO_2-C		CeO_2-C	CeO_2-C	CeO_2-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_2 no. 3 displays more ROS in the lung cells than the other materials in the study.
- ⇨ However, the two other TiO_2 NPs induced no ROS at all.

TiO_2 no. 1 and TiO_2 no. 2 were almost identical consisting of pure anatase being surface modified with a polyoxa acid, but TiO_2 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_2 no. 1 and TiO_2 no. 2, and with -26,3 mV for TiO_2 no. 3.
- ⇨ These findings and data suggest a fundamental role of coatings in modifying cytotoxic effects of TiO_2 NMs.

Chen et al. [96] obtained results showing that

- ⇨ Exposure of TiO_2 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_2 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 TiO2 nanoparticles used in [36] and [41].

Sample	Crystal structure	SSA [$m^2 g^{-1}$]	$d_{(XRD)}$ [nm]	$d_{(TEM)}$ [nm]	D_{SLS} [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.

$d_{(XRD)}$ – mean diameter calculated from Sherrer analysis.

$d_{(TEM)}$ – 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 reagglomerate 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.* [41], investigated the uptake and responses of the very same five TiO_2 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_2 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_2 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_2 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_2.

5.4 The carcinogenic potential of TiO_2 NPs

Becker et al. reviewed the carcinogenic potential of nanomaterials, where TiO_2 NPs were one of the elements in focus [97]. They found that only a handful of long-term epidemiological studies have investigated carcinogenic studies on TiO_2. 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 $TiO_2$was found. However, in an *in vivo* study by Heinrich et al. [98] it was concluded that after inhalation (10 mg/m^3) of nano-TiO_2 (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_2 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_2 to reach particle lung loads similar to those found in the diesel soot-exposed rats. In another study (see [97]) where rats were exposed by inhalation at 5mg/m^3 for 24-months of fine TiO_2 (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_2, used intratracheal instillation as the “19-dusts

study" by *Pott and Roller* [99] where rats were exposed to 19 different types of dusts. Among these elements there were ultrafine (uf) carbon black, small-fine (sf) diesel soot, uf TiO_2 - hydrophilic and hydrophobic, and sf Anatase. In Table 9 the percentage of rats that developed tumors from the exposure of some of the particles are presented. From Table 9 it can be concluded that uf carbon black is more and sf diesel soot is less carcinogenic compared with uf TiO_2. Again it seems as if particle sizes matters. Furthermore, hydrophobic TiO_2 is strongly toxic and carcinogenic.

Table 9: Results of the study by Pott and Roller as presented in [100].

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_2, uf, hydrophilic	5 x 3, 15	52
TiO_2, uf, hydrophilic	5 x 6, 30	67
TiO_2, uf, hydrophobic	15 x 0.5, 7.5	All died. Toxic.
TiO_2, sf, anatase	10 x 6, 60	30
TiO_2, sf, anatase	20 x 6, 120	64

However, *Becker et al.* conclude 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_2 is suspected of being potentially carcinogenic to humans [97].

There are several more papers available presenting data on overload studies on exposure of TiO_2 NPs resulting in tumours in animals [45], but there is not room in this report to make more comments on this subject.

6 Discussion of the reviewed presented results

The following discussion of results is sorted under subjects related to the different studies reviewed, respectively. The given 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. Basically that is due to the lack of toxicological competence of this reports author. Therefore, 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 conducted. For example, regarding the genotoxicity of NPs, even though it has been examined in several papers [54], it is hard to compare the results due to the different types of TiO_2 particles used, the various cell systems and the variable assay conditions in the different studies [20]. Still, the information from all the studies gathered here represents another step in the direction to a better understanding of TiO_2 NP toxicity.

6.1 Influence of particle size and surface area

The following papers support that nanoparticles of TiO_2 are more toxic than corresponding fine particles: [45, 61, 71-73, 77, 79, 82, 88, 90]. The papers [22, 81, 92] support it partly.

The following papers conclude that there is no absolute influence of particle size or in part have found higher toxicity for larger particles: [33, 34, 58, 62, 63, 92, 94, 101]. For example, fine particles of TiO_2 were more toxic than nanoparticles in DNA damage tests [58, 92].

According to *Gurr et al.* [79] ultrafine anatase (10-20 nm) particles induced toxicity, whereas fine (200 nm) anatase particles did not. Similarly, conclusions by *Simon-Deckers et al.* [90] points out higher toxicity for TiO_2 NPs than for their larger counterparts. But in a study by *H Karlsson et al.* [92] it was concluded from the results obtained that it cannot be generalized that NPs always are more toxic than fine particles. The same conclusion can be drawn from the results presented in the study by *Bhattacharya et al.* [95]. However, in both studies [92, 95], some testing models showed a higher toxicity for smaller TiO_2 particles. Furthermore, *Junko Okuda-Shimazaki et al.* [102] investigated the effects of TiO_2 nanoparticle size on gene expression. They analyzed the gene expression focusing on stress, inflammation, and cytotoxicity of two sizes, small (166 nm) and large (596 nm) of titania aggregate particles. 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_2 NPs is only correct if particles are administered to lungs in a mass dose manner [64]. However, as stated earlier in this review, according to *Limbach et al*. [1], *Kroll et al.* and *Sager et al*. [35, 64], 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 nano-particles of different size at *equal surface area*. In [64], where animals were exposed to TiO_2 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 [62], 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 [81] they do suggest that surface area influence toxicity.

Furthermore, conclusions in [78] by *Grassian et al.* contradict 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 characeristics of the primary NPs in determining the toxicity. *Grassian et al.* [80] in their study where mice were subacutely exposed by inhalation to the smallest available, 2 – 5 nm, TiO_2 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_2 NPs [72, 103]. But they also found four papers, one of them dealing with TiO_2 NPs [62], where this was not the case. In the review by *Johnston et al.* they point out six papers where size dependency of TiO_2 toxicity has been frequently demonstrated and, as they write, “appears to be applicable to a variety of TiO_2 forms, and occurs regardless of the model used”. This is a misconception according to *Warheit* [104], and is contradicted by results obtain by *Andersson et al.* [36].

Hussain et al. [22] conclude in their review concerning safe use of NMs that size does matter and can induce toxicity, but also emphasizes the crucial importance of complex particle characterisation.

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 [105]. Still it is unlikely that exposure to

cells will occur in a nano form since administration of TiO_2 NPs is associated with agglomeration [1, 36, 106]. Still, as *Johnston et al*. [24] write "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 degree, although they emphasize that they found no systematic relation between particle uptake and the specific area or primary particle morphology, since in their *in vitro* studies showed that uptake of particles depend on 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 should be considered [41].

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 [45] that fine and ultrafine TiO_2 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* [64] 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 [107]. However, according to *Warheit* this might be another misconception [104], since he argues that TiO_2 NPs of the same size not necessarily have the same surface characteristics. Something which is clearly shown in papers [36, 41]. 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.

6.2 Influence of crystal structure

The following papers support that anatase is more toxic than rutile: [55, 58, 81, 84]. For example anatase was 100 times more toxic than an equivalent sample of rutile in the study by *Sayes et al.* [55]. But the paper [79] by *Gurr et al*. shows a higher toxicity for fine rutile particles compared to fine anatase.

Several papers [41, 78, 79, 81] conclude higher toxicity for a mix of anatase and rutile, than for both/or either one of the pure crystal structures. Papers [36, 62] found no difference in toxicity due to different morphology of particles. Paper [108] shows significant differences in toxicity due to different shapes of particles. But these papers study TiO_2 particles of different sizes and are therefore hard to compare to each other. However, in summary most papers reviewed suggest that a mix of anatase and rutile crystal forms of TiO_2 NPs is more toxic than both/or either one of the pure crystal structures.

In a recently published paper by *Jin et al.* [109] the cellular toxicity of TiO_2 NPs of anatase and rutile was compared. The results indicated that only the anatase TiO_2 NPs allow spontaneous reactive oxygen species (ROS) generation, but rutile TiO_2 NPs do not after dispersion. The interaction between TiO_2 NPs and cellular components might also generate ROS for both anatase TiO_2 NPs and rutile TiO_2 NPs. No consideration regarding agglomeration influence was found.

In [62, 81] *Warheit et al*. concluded that the toxicity of TiO_2particles was not dependent on size and surface area, but rather on surface properties. Here, the ultrafine TiO_2 particles consisted partly of anatase, whereas the fine TiO_2 only consisted of rutile. In an earlier study the anatase crystal structure showed higher toxicity [55]. Furthermore another study showed that a mix of these crystal forms was more potent to induce oxidative DNA lesions than treatment with either anatase or rutile forms alone [79].

In [101] *Warheit et al.* first studied quartz particles of varying size and then they studied three different types of NP TiO_2 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_2 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.* [108], the toxicological response of three different types of TiO_2 particles were compared. TiO_2 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_2 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_2 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.

6.3 Surface coating and surface chemistry influence of particles

Rossi [59], *Warheit* [75, 110] , *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 make NPs inherently more toxic whereas other coatings make particles, or agglomerates of particles, less toxic [35, 58, 59].
In the study [75] it was concluded that response following particle exposure of fine TiO_2 particles (~350 nm) with different surface modifications of alumina and amorphous silica was very much dependent on the TiO_2 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_2 powders. Thus, from this study, TiO_2 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_2 powders tested – since it was the powder not having the smallest particle size (440 nm) but the greatest surface area (27.8 m^2/g) among all of the TiO_2 formulations studied that caused the largest response. Furthermore, the coatings consist of smaller units compared to the TiO_2 particles they are deposited upon, which also might contribute to the findings.

In the study [110] by *Warheit et al.* the influence of hydrophobic surface-coated (triethoxyoctylsilane – OTES) and hydrophilic base pigment-grade (~300 nm) TiO_2 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.

6.4 Surface properties, zeta-potential

Thus it is clear that intrinsic surface properties and morphology are more important than crystal form, as the studies comparing anatase with rutile have indicated [41]. In particular nano-wires, -fibres, -belts or –tubes likely will affect living tissue in a detrimental manner [108].

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, 75].

6.5 The toxicity of TiO_2 compared to silica

One of the most common elements in concrete, besides water and calcium, is quartz because cement consists of calcium-silicates. Furthermore, the aggregates in the concrete come from rocks which basically consist of quartz and/or other silica-based or silica-containing minerals, since silica is the most abundant element in the earth crust. Therefore it is of primarily interest to compare TiO_2 NPs with SiO_2 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*. [111] dusts of silica and TiO_2 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_2 particles. The inflammation was transient for TiO_2 and amorphous silica but not for crystalline silica.

In [112] *Warheit et al*. compared the toxicity of TiO_2 particles encapsulated with pyrogenetically deposited, amorphous silica with other TiO_2 and silica particles. It was found that this type of silica coated TiO_2 exhibited similar transient rat lung inflammatory response as the reference rutile TiO_2 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 [62, 75, 81, 91, 113] show higher toxicity for reference silica than for TiO_2 particles.

But in the paper by Rossi et al. [59] only the silica coated TiO_2 NPs elicited toxicity whereas the pure amorphous silica did not induce pulmonary inflammation.

6.6 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] has shown 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.* [73, 74].

6.7 Agreement between *in vitro* and *in vivo* studies

Adverse effects on cells *in vitro* 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 everybody to copy, something which might have been overlooked by others [114]. 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, 114, 115]. Likewise, general standard methods particularly developed for *in vivo* toxicity studies on NPs have to be recognized and applied, since established methods for exposure for micro-particulates are not always suitable.

Sayes et al. [115] compared the toxicity of some metal-oxides (not TiO_2) 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_2 particles, in general *in vitro* studies have proven toxicity in different endpoints, either of fine or ultrafine TiO_2, whereas *in vivo* studies, conducted at reasonable dosages, show less severe toxicity, since in many papers inflammation seems to decrease with time and disappear.

Chen et al. [77] and Rossi et al. [59] had good agreement in their respective studies of toxicity in both the *in vivo* and *in vitro* experiments showing toxicity for only one of the different TiO_2 NPs examined.

7 General discussion of the results

Obviously there is a consensus that TiO_2 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, 55, 61]. That is from an inflammatory response or oxidative stress caused by the TiO_2 NPs. Important are the findings by Nurkiewicz et al. which prove associations between particle matter such as TiO_2 NPs and cardiovascular diseases. However, in some studies (*in vivo*) no evidence of toxicity is observed [116, 117] and in some studies the inflammatory response seem to decline with time and disappear [80, 118]. Often also TiO_2 NPs show less or little toxic or inflammatory response in certain endpoints, while there is toxic response in other endpoints [92, 95]. Furthermore it is hard to discern if the doses used in the different tests are appropriate in regard to real exposure quantities. See section 7.1.

Johnston et al. [24] in their review regarding *in vivo* pulmonary exposure of TiO_2 NPs conclude that the toxic potential of TiO_2 particles is primarily dictated by size and crystallinity whereby

- ⇨ Decreasing particle size enhanced particle pulmonary toxicity.
- ⇨ Anatase forms of TiO_2 enhanced particle pulmonary toxicity.

Furthermore, they emphasize that the experimental model, including species used, exposure method and dose administered, all have impact on the toxicity of TiO_2, which complicates comparisons between different investigations. They also underline that chronic exposure to TiO_2 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_2 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_2.
- ⇨ It is of interest that the mechanisms that drive to toxicity of TiO_2 *in vitro* (inflammation and oxidative stress), are also witnessed *in vivo*.

Card et al. [23] emphasize the importance of form (phase) of TiO_2 NP in the *in vitro* toxicity studies, such as in the study by *Gurr et al*. [79]. 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_2 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*. [81] to show that TiO_2 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 is 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 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 the author agrees with this last conclusion by *Card et al.* and also question the bold conclusions stated by *Johnston et al.* above regarding the *in vivo* studies reviewed . In the paper [104] *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 has 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 [64].

8 Risk assessment of airborne TiO_2 NPs

The 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 mg/m^3 for respirable pigment grade (fine) TiO_2 and 0.3 mg/m^3 for NP TiO_2.

In the article by *Kuempel et al.* [119] key steps in quantitative risk assessment were illustrated, using dose-response data in rats chronically exposed to either fine or ultrafine titanium dioxide (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 mg/m^3 for ultrafine TiO_2, CB, or DEP, and 0.7 to 1.3 mg/m^3 for fine TiO_2.

In a Russian paper [120] *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^3, nano-TiO_2 0.19 mg/m^3, and C 60 0.08 mg/m^3.

Liao et al. [121] 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.* [122] investigated the exposure of airborne particles to industrial workers during the packing of pigment and nanoscale TiO_2. 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^3. That is lower than the estimated "one in a thousand risklevel" of 0,84 mg/m^3 mass concentration of ultrafine TiO_2 in humans associated with a 1/1000 risk of lung tumors after 45-year working lifetime, as presented by *Dankovic et al.* in [45]. Over 90% of the particles (numbers) were smaller than 100 nm. There were mainly soot and particles formed from process chemicals. Mass concentration originated primarly from the packing of the fine and nano TiO_2 agglomerates. The nano TiO_2 exposure resulted in a calculated dose rate of 3.6 x 10^6 particles/min and 32 μg/min where 70% of the particles and 85% of the mass were deposited in head airways (not in the lung). The peak mass concentrations were at around 3 mg/m^3 which originated mainly from the packing, air cleaning and material pouring of fine TiO_2 and nano TiO_2 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_2 NPs in the lung, which corresponds to a maximum daily dose of 60 x 8 x 4,8 µg = 2,3 mg of TiO_2 NPs in the lungs. The lung alveolar surface area in humans is calculated to about 70 m^2 [123].

Under the assumption that humans are as sensitive as rats we can do the following comparison; from *Kroll et al.* [35] the rat overload is calculated to about 3 µg per cm^2 rat lung, which corresponds to 0,3 mg/dm^2 and 30 mg/m^2 rat lung. The daily particle load of fine and nano TiO_2 agglomerates in workers of 2.3 mg/70 m^2 lung equals 33 $\mu g/m^2$ lung. Thus comparing the daily particle load of pigment packing workers with the calculated daily overload of particle exposed to rat lungs, it is found that the fine and nano TiO_2 agglomerate daily overload dosage of rat lungs is 30 mg / 33 µg ≈ 900 times greater than the daily agglomerate exposure to the workers as presented by *Koivisto et al.* [122].

Most *in vivo* studies using inhalation of particles into rats use a dose close to, or one or two magnitudes below the rat overload dose, which is reasonable from the viewpoint that larger species are more vulnerable than smaller ones, i.e. presuming that humans are more sensitive than rats.

More information is certainly available, but that is for another reviewer to study more in detail.

9 Conclusions

The scientific branch of nanoparticle toxicology is a fast developing area of research and results, and it is hard to find any shortcuts regarding assessment of different NPs toxicity in general because it seems necessary to examine every new type of ENM carefully in several types of cell lines to determine its toxicological properties. This also includes all the different types of TiO_2 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 is likely to be available increasingly in a continual manner the next coming years.

Specifically regarding the toxicity of TiO_2 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_2 particles by *Warheit et al.* [117] it was concluded that most studies demonstrated low hazard potential in mammals or aquatic species following acute exposure to the ultrafine TiO_2 particles.

Compared with other metaloxides, TiO_2 is most often less toxic than other elements, such as ZnO [124] , CuO [92] and SiO_2 [62, 101] in the different toxicological screening tests. Regarding using small amounts of TiO_2 in concrete it will result in presence of more TiO_2 particles but less silica and Ca-particles in the air. In respect to silica and Ca-silicate (cement), titania is (most often) less toxic. For the comparison of the toxicity of TiO_2 NPs with NPs of calciumhydroxide or calciumcarbonate this review lacks information. In many toxicological studies on TiO_2 NPs, silica particles are used as a positive control for toxicity [62, 81]. But amorphous silica NPs are not necessarily more toxic than crystals of TiO_2 NPs [59].

Furthermore, the doses used in the toxicological studies are about 10 – 1000 times higher compared to normal dosages that humans might be exposed to [122], considering the difference in lung capacity of rats and humans. (See section 8.)

In summary, we have the following general facts:

- Nanosized particles are often more potent in inducing toxicological response compared to fine particles of the same composition when delivered at equal mass.
- At equal surface area but different particle size similar compounds exhibit similar toxicological characteristics.
- Nanosized particles (< 50 nm) have the ability to penetrate lung cell membranes whereas larger particles cannot but will instead accumulate outside the cell wall, which also may promote damage to lung tissue [36, 44, 88].
- Generally titanium dioxide is less toxic than silica. Nanosized silica (microsilica) is frequently used in concrete in large quantities.
- Addition of titanium dioxide nanoparticles to will increase the amount of nanosized particles in the fresh concrete, but will be bound in the cement matrix in hardened concrete.
- Antropogenically generated particles from photocatalytic concrete pavements will hold less silica-based NPs but more TiO_2 NPs.
- The amount of nanoparticles generated in concrete pavements will likely be in same order of magnitude regardless of a recipe with or without TiO_2 NPs.
- Naturally or anthropogenically occurring nanoparticles agglomerate as aerosols or agglomerates in medium.

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 nanoparticles generated from wear of vehicles.

Afterword

Due to unforeseen circumstances, such as three weeks of sickness, work of higher priority, lack of time, money and perhaps even interest, this review was delayed at least 8 months. During this time several new scientific papers and some reviews concerning pulmonary toxicity of TiO_2 NPs have been published, of which the most interesting may be the review “Titanium dioxide particles: a review of current toxicological data” by Shi et al. [54]. A very limited literature search in Scopus on the keywords “pulmonary toxicity” AND “titanium dioxide nanoparticles” in Title, Abstract and Keywords resulted in 14 papers published in 2012 -2013.

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Appendix 1. Small list of vocabulary and concepts in nanotoxicology

Most of the explanations and definitions are cited from Wikipedia, 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.
An adduct (in c*hemistry*, from the Latin adductus, "drawn toward") is a product of a direct addition of two or more distinct molecules, resulting in a single reaction product containing all atoms of all components. The resultant is considered a distinct molecular species. Examples include the adduct between hydrogen peroxide and sodium carbonate to give sodium percarbonate, and the addition of sodium bisulfite to an aldehyde to give a sulfonate. Adducts often form between Lewis acids and Lewis bases. Compounds or mixtures that cannot form an adduct because of steric hindrance are called frustrated Lewis pairs.
Adenocarcinoma is a cancer of an epithelium that originates in glandular tissue.
An aerosol is a suspension of fine solid particles or liquid droplets in a gas. Examples are clouds, and air pollution such as smog and smoke.
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).
Anatase see section 4.2.5.
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.
An assay is an investigative (analytic) procedure in laboratory medicine, pharmacology, environmental biology, and molecular biology for qualitatively assessing or quantitatively measuring the presence or amount or the functional activity of a target entity (the analyte), which can be a drug or biochemical substance or a cell in an organism or organic sample.
BAL/BALF - bronchoalveolar lavage 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 (1013) cells. Most plant and animal cells are between 1 and 100 µm 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.
Chemotactic signal – a chemical stimulant that affect and/or causes movement of a cell.
The cytosol or intracellular fluid (or cytoplasmic matrix) is the liquid found inside cells.
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.
The cytoplasm comprises cytosol – the gel-like substance enclosed within the cell membrane – and the organelles – the cell's internal sub-structures. Synthesized proteins have many different final locations, even though the majority of protein synthesis in eukaryotic cells occurs in the cytoplasm.
Dermal – related to the skin.
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.
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.
EDS/EDAX – Energy Dispersive x-ray spectroscopy. Usually conducted in a SEM.
Enzymes are large biological molecules responsible for the thousands of chemical interconversions that sustain life. They are highly selective catalysts, greatly accelerating both the rate and specificity of metabolic reactions, from the digestion of food to the synthesis of DNA. Most enzymes are proteins, although some catalytic RNA molecules have been identified. Enzymes adopt a specific three-dimensional structure, and may employ organic (e.g. biotin) and inorganic (e.g. magnesium ion) cofactors to assist in catalysis.
Endocytosis is an energy-using process by which cells absorb molecules (such as proteins) by engulfing them. It is used by all cells of the body because most substances important to them are large polar molecules that cannot pass through the hydrophobic plasma or cell membrane.
The endothelium is the thin layer of cells that lines the interior surface of blood vessels and lymphatic vessels,[1] forming an interface between circulating blood or lymph in the lumen and the rest of the vessel wall. The cells that form the endothelium are called endothelial cells. Endothelial cells in direct contact with blood are called vascular endothelial cells, whereas those in direct contact with lymph are known as lymphatic endothelial cells.
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 [38].
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. Epithelia can also be organized into clusters of cells that function as exocrine and endocrine glands. Exocrine and endocrine epithelial cells are highly vascular. The Epithelial cells represent the boundary between ambient air and inner tissue of the organism [37].

A eukaryote is an organism whose cells contain a nucleus and other organelles enclosed within membranes. The defining membrane-bound structure that sets eukaryotic cells apart from prokaryotic cells is the nucleus, or nuclear envelope, within which the genetic material is carried.
Exotoxin is a toxin secreted by bacteria.
Fibril is a fine fiber, such as a myofibril or neurofibril.
Fibroproliterative – growth of fibrils.
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 ηπαρ (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.
Histopathology refers to the microscopic examination of tissue in order to study any type of abnormity such as manifestations of disease.
IMR 90 (human bronchial fibroblasts)
In vitro (Latin: in glass) studies in experimental biology are those that are conducted using components of an organism that have been isolated from their usual biological surroundings in order to permit a more detailed or more convenient analysis than can be done with whole organisms. See section 4.3.
In vivo work is that which is conducted with living organisms in their normal, intact state, while ex vivo studies are conducted on functional organs that have been removed from the intact organism. See section 4.3.
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.
Ingestion – the process of swallowing.
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 sorrounded by visceral peritoneum. Intraperitoneal organs are: 1.Esophagus; 2.Stomach; 3.Jejunum; 4.Ileum; 5.Caecum; 6.Appendix; 7.Transverse colon; 8.Sigmoid colon
Intratracheal – into the trachea.

LD50, median lethal dose, the dose required to kill half the members of a tested population after a specified test duration.
LDH – lactate dehydrogenase release, is an *enzyme* present in a wide variety of organisms, including plants and animals. Tissue breakdown releases LDH, and therefore LDH can be measured as a surrogate for tissue breakdown, e.g. hemolysis. Thus it is used as an indicator of cell injury.
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.
Lysosomes are the cells' garbage disposal system. They contain a number of enzymes capable of breaking down proteins. They vary in size and shape and several hundred may be present in a typical cell.
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 (μm) 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*.
Mucus – secretion ([swe.] *slem*, *sekret*)
Myofibril (also known as a muscle fibril) is a basic rod-like unit of a muscle.
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.
Necrosis, is a form of traumatic cell death that results from acute cellular injury.
Neurofibril (NF) are the 10 nanometer filaments found in neurons.
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).
The olfactory bulb transmits smell information from the nose to the brain, and is thus necessary for a proper sense of smell. As a neural circuit, the glomerular layer receives direct input from olfactory nerves, made up of the axons from approximately ten million olfactory receptor neurons in the olfactory mucosa, a region of the nasal cavity.
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.
An organelle is a specialized subunit within a cell that has a specific function, and it is usually separately enclosed within its own lipid bilayer. There are many types of organelles, particularly in eukaryotic cells. While prokaryotes do not possess organelles per se, some do contain protein-based microcompartments, which are thought to act as primitive organelles.
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 **[92]**.
Parenchyma are the functional parts of an organ in the body. This is in contrast to the stroma, which refers to the structural tissue of organs, namely, the connective tissues.
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.
A phagosome is a membrane-bound vesicle that holds foreign matter. It is formed phagocytosis, where the cell membrane folds inward and envelops foreign material such as bacteria. A phagosome is a cellular compartment in which pathogenic microorganisms can be killed and digested. Phagosomes fuse with lysosomes in their maturation process, forming phagolysosomes.
PBS – phosphate buffer solution, an artificially blended solution aimed to mimic saliva.
PMNs - polymorphonuclear neutrophils, see Neutrophil.
The prokaryotes are a group of organisms *(or cells)* whose cells lack a membrane-bound nucleus (karyon). The organisms whose cells do have a nucleus are called eukaryotes. Most prokaryotes are unicellular organisms, although a few such as myxobacteria have multicellular stages in their life cycles[1] or create large colonies like cyanobacteria. Prokaryotes include two major classification domains: the bacteria and the archaea.
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.
Proliferation – propagation, production, increase, creation. For example, cell proliferation = cell growth.
Pulmonary - pertaining to, having, or affecting the lungs.
Reticuloendothelial system – is an essential component of the immune system comprised of phagocytic cells located in different organs in the human body. Also see the *mononuclear phagocyte system*.
ROS – Reactive Oxidative Species.
ROFA – residual oil fly ash.

THP1 - Human acute monocytic leukemia cell line.
Titania is short term for titanium dioxide. (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 ±5,	Rapid coagulation or flocculation
from ±10 to ±30	Incipient instability
from ±30 to ±40	Moderate stability
from ±40 to ±60	Good stability
more than ±61	Excellent stability