ERA-NET SIINN
Safe **I**mplementation of **I**nnovative
Nanoscience and **N**anotechnology

Updated Deliverable D1.4 (M 36)
Knowledge Gaps 1
Identification of knowledge gaps of in-vitro assays, on toxicity and translocation of nanomaterials, such as size, shape, surface reactivity, surface area, adsorption and their transportation through the human body (deliverable updates in month 36)

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1. Introduction

What Nanomaterials (NMs) are: A recently adopted definition by European commission about nanomaterial is "*A natural, incidental or manufactured material containing particles, in an unbound state or as an aggregate or as an agglomerate and where, for 50% or more of the particles in the number size distribution, one or more external dimensions is in the size range 1 nm – 100 nm. In specific cases and where warranted by concerns for the environment, health, safety or competitiveness the number size distribution threshold of 50% may be replaced by a threshold between 1 and 50%.*" [1].

The impact of using NMs in applications in global economy is huge, for example they are used in: cosmetics and personal care products, paints and coatings, catalysts and lubricants, security printing, textiles and sports, medical and healthcare, food and nutritional supplements, food packaging, agrochemicals, veterinary medicines, water decontamination, construction materials, electrical and electronics, fuel cells and batteries, paper manufacturing, weapons and explosives. According to the research report Nanotechnology Market Forecast to 2013 [RNCOS E-Services, 2010], the global nanotechnology market is projected to grow at a compound annual growth rate (CAGR) of over 18% during 2010–2013. The report expects that the global market for nanotechnology incorporated in manufactured goods will be worth US $1.6 trillion, representing a CAGR of around 50% in the forecast period (2010–2013) [2]. NMs can be either the result of a physical process, or man-made by using the principles of the supramolecular chemistry [3-5]. The latter are called artificial nanostructures, engineering or manufactured nanomaterials (MNMs). It is important to expand the knowledge on how the usage of NMs or MNMs in commercial applications influences the lifecycle (human health, environment etc.). It is worth to notice that the physical and chemical properties of NMs do not follow the corresponding physical and chemical properties of their bulk counterpart from which have been come. NMs creation is based on the bottom up approach, and in contradiction to products created by using the top-down approach and used in daily life, where necessary international regulatory codes have been adopted based on the principle that similar compounds will behave similarly [6]. No similarity rules about their properties can be adopted for NMs.

The synthesized MNMs are usually classified in the following categories: a) nanotubes, b) nanoclays, c) metal oxides, d) composites, e) polymers/dendrimers, and f) quantum dots. Each MNM of them corresponds to a unique structure with different physicochemical properties (e.g. conductivity, reactivity, melting point) (see for details [7]). Furthermore, MNMs properties are closely related to the surroundings where a MNM functions, while other parameters, e.g. enhancing due to quantum effects and rearranging of atoms, alter radically the properties of a MNM. The key

parameter for these novel properties of MNMs is their high reactivity due to their large surface to volume ratio. In practice, most of the atoms of MNMs are on the surface making the reaction with the surroundings easy. Furthermore, the latter is a high-risk potential factor for bioaccumulation. It is clear that the potential risk of using MNMs is high enough and special actions are needed towards the safe use of them. Up to now, it is widely accepted that surface characteristics of MNMs clearly affect their toxicity. Although the last 15 years the synthesis and production of MNMs have been rapidly increased, while the chemical composition and the capabilities of most of them are still unknown and reports on their side effects are rare and controversial.

Independently of the area of interest (human health, biological systems, environmental issues e.t.c.) toxicity is maybe the common factor present in all fields of MNMs' applications. It is worth to notice that very few are known about MNMs toxicity although a growing concern about their potential toxicological effects on humans has been raised in recent years [8-14]. The human body may be intentionally or unconsciously exposed to diverse types of nanoparticles by several routes such as inhalation, ingestion, dermal penetration, and injection due to the expanded production and use of artificial NMs in a variety of fields [15]. And it is certain that the toxicity of MNMs, with large surface area and high reactivity may not be predictable from the known properties of bulk-sized materials. It is, therefore, highly necessary to do research on the dose-response relationships between nanoparticles and biological systems to provide information about their potential adverse effects and to develop their human-related applications with safe level.

Toxicological effects of inorganic nanoparticles have been reported in cultured cell lines as well as in animal models. The most frequently evaluated harmful effects of inorganic nanoparticles are the induction of reactive oxygen species (ROS), oxidative stress, inflammation response, membrane or DNA damage, and cell death [16-18]. However, the toxicity of nanoparticles could also be strongly influenced by their physicochemical properties such as chemical composition, chemical stability, particle size, surface area, surface charge, shape, crystal structure, aggregation, etc [19,20]. Controlling the physico-chemical characteristics of MNMs as well as the understanding of their cellular response would be of importance to minimize their toxicity potential for biomedical applications.

The chemical and structural stability of nanoparticles is crucial not only for delivery efficiency, but also toxicological effects under systemic conditions. High stability of nanoparticles as delivery systems may be indispensable to protect encapsulated molecules from harsh physiological conditions such as enzyme attack, low pH, and vascular endothelial barriers. Meanwhile, nanoparticle-based carriers should easily be decomposed and degraded in biological and

environmental systems after delivering bioactive molecules to the target specific organs, in order to avoid potential bioaccumulation in the body, which may induce harmful effects later.

A critical issue regarding the properties of MNMs is their size-dependency, a trend that in some cases seem to have great impact on their toxicity. For example Renwick et al. [21] comparatively have discussed the effect of transitional metals oxides / ultrafine carbon black as a source of oxidative stress. For micron sized particles the effect of carbon black has been shown to be more severe than that of titanium dioxide, while for both compounds the nanoparticles induced lung inflammation and epithelial damage in rats at greater extent than their larger counterparts. Other studies have shown very similar toxicities between very different materials when presented as ultra-fine particles (UFPs), for example latex and TiO_2. For several different nanoscale particles (polyvinyl chloride, TiO_2, SiO_2, Co, Ni), differences in cytotoxicity are obtained due to size difference at the nanoscale, as the particle size ranged from a mean diameter of 14 nm to 120 nm [22]. Furthermore, it has been demonstrated that the size plays an important role in cellular uptake behaviours of nanoparticles, because nanosized particles with high surface reactivity could easily penetrate the cell membrane compared to micron-sized particles, resulting in high uptake [16,23,24]. As the particle size decreases, the surface area to volume ratio increases rapidly, and therefore, the total number of small particles per given mass is considerably higher than that of larger particles. Thus, a hypothesis that nanoparticles would be generally more toxic than micron-sized particles is widely accepted. However, at the same time, recent studies have provided evidence that intentionally produced nanomaterials can display unique toxicity that cannot be explained by differences in particle size alone. Although a correlation between increasing surface area and biological effects is shown in many cases, there are also research reports in which this relationship between size, surface area and toxicity is not straightforward or even reverse. Therefore, it is not always possible to predict effects on the basis of size or surface area alone [25].

The surface properties of nanoparticles such as surface charge, hydrophobicity or hydrophilicity, roughness, etc. is another principal factor in determining cellular interaction between nanoparticles and the plasma membrane *in vitro* and *in vivo*. As efficient delivery vehicles, nanoparticles have to possess generally enough hydrophilicity to prolong half-life time in circulation system, whereas, hydrophobicity of nanoparticles could facilitate the plasma membrane penetration by fusion-like mechanism [26]. Toxicity studies also showed that nanoparticles with hydrophobic surface characteristics tended to be accumulated in specific organs such as the liver and spleen [27]. Thus, the uptake, distribution, and toxicity of nanoparticles could be highly affected by their surface. Moreover, it is well known that a high surface area can be attained either by fabricating small particles where surface to volume ratio of each particle is high, or by creating materials of high

porosity. So not only the particle diameter but also the porous surface adds to the total surface area of the particles. It can be therefore speculated that particles with complex porous surfaces will give different results than the particle of same size but of negligible porosity. Since pores or crevices on the particle surface adds more to the surface area, we can predict that the surface area is much more important factor than the size. For particles of very small size and complex pore structure, the size of the gas molecule may affect the penetration of the gas molecule into the pores and therefore not being able to predict the actual surface area. In this case the surface area would depend on the precision and sensitivity of method used.

2. *In vitro* studies of nanomaterials

a) Carbon MNMs

I) Carbon nanotubes (CNTs)

Due to their unique physical and chemical properties, CNTs have sparked much interest recently with a large amount of research dedicated to their novel applications. CNTs are among newly developed products and are currently of interest for a variety of applications in electronics, reinforced rods, micro-fabricating conjugated polymer activators, biosensors, enhanced electron/scanning microscopy imaging techniques, etc. A very broad range of physical properties of CNT—affecting their electronic, thermal, and structural characteristics—is defined by their diameter, length, and chirality or twist. In addition to single-walled carbon nanotubes (SWCNT) with a single cylindrical carbon wall, multi-walled carbon nanotubes (MWCNT) have multiple walls—cylinders nested within other cylinders [28]. It has been pointed out that CNTs may cause inflammation and granulosomas [29-32], similar causes with the exposure to asbestos, which precede the development of cancers e.g. mesothelioma, as well as skin-cell toxicity via dermal exposure [33] and genotoxicity [34]. In addition, the effects of SWCNT exposure on interleukin-8 (IL-8) expression in human alveolar epithelial cells (A549) showed that the levels of inflammatory response could be altered under different exposure conditions [35]. Furthermore, their effects may be altered by different amount of residuals coming from catalysts used for their synthesis and remaining on their body. Since, these factors alter CNTs toxicity, different types of CNTs pose various levels of risks. The risks posed by them are related not only to the limited information (synthesis, structure, physicochemical and electronic properties) but also to a debate about which methods and approaches are better for testing. For example, if CNTs are tested according to fiber-toxicology methods then long nanotubes are toxic, instead, if they are tested according to methods relevant to particles then short nanotubes appear to be the most toxic. *It is clear that the toxicity of CNTs, as well as its impact on human health should be clarified.*

b) Metal oxides

I) Fe_2O_3

Hematite, being a mineral form of Fe(III) oxide, exists in several polymorphous subtypes (α-, γ-Fe_2O_3), has about 70% iron content, and, due to its utilization as a pigment, is one of the most industrially used forms of iron oxide besides magnetite. The use of Fe_2O_3 nanoscale particles also includes drug targeting of cancer cells, tracking target cells using labeling, and imaging techniques like magnetic resonance tomography. Studies performed with microscale and nanoparticulate Fe_2O_3 described them both to be nontoxic under *in vitro* test conditions in human small airway epithelial

and mouse fibroblast cells (exposure concentration up to 400 μg/cm^2) [36]. Bhattacharya et al. [37] characterized and compared the biophysical reactivity and toxicological effects of α-Fe_2O_3 nano- (d < 100 nm) and microscale (d < 5 mm) particles in human lung cells and they demonstrated that the surface reactivity of nanoscale a-Fe_2O_3 differs from that of microscale particles with respect to the state of agglomeration, radical formation potential and cellular toxicity. The presence of proteins in culture medium and agglomeration were found to affect the catalytic properties of the hematite nano- and microscale particles. Both the nano- and microscale α-Fe_2O_3 particles were actively taken up by human lung cells in vitro, although they were not found in the nuclei and mitochondria. Significant genotoxic effects were only found at very high particle concentrations (> 50 mg/ml). The nanoscale particles were slightly more potent in causing cyto- and genotoxicity as compared with their microscale counterparts. Both types of particles induced intracellular generation of reactive oxygen species. However, the immediate environment of the particles (biomolecules, physiological properties of medium) modulates their toxicity on the basis of agglomeration rather than their actual size.

II) TiO_2, ZnO, CuO and Co_3O_4

They are some of the most common industrial MNMs additives for various applications. With greater surface area per unit weight than their bulk counterparts, these nanometal oxides have superior performance. TiO_2 is an opacifier used in paints, paper, plastic, and cosmetic products. ZnO nanoparticles are included in personal care products such as toothpaste, beauty care products, sunscreens and textiles. Nano-CuO has industrial applications in gas sensors and catalytic processes. Wang et al. [38] investigated the toxicity of nano-ZnO, TiO_2, CuO and Co_3O_4 using a primary culture of catfish hepatocytes and human HepG2 cells as the *in vitro* model systems. In that study, the overall ranking of the toxicity of metal oxide NPs to the test cells was as follows: TiO_2<Co_3O_4<ZnO<CuO which does not show a relationship consistent with their relative sizes. Nano-CuO and ZnO showed measurable toxicity to both HepG2 cells and catfish primary hepatocytes but with some marked difference. The aforementioned nanoparticles showed significant toxicity to HepG2 cells at low concentrations, but required much higher concentrations to show significant toxicity to the fish primary hepatocytes. This clearly demonstrated that HepG2 cells are more sensitive than catfish primary hepatocytes to the toxicity of these metal oxide NMs and suggested that the size-dependent toxicity of NMs is litigable by other factors. Moreover, the main mechanism of NMs cytotoxicity may differ, depending on the types of NMs and cells involved. The results revealed the difference in toxicity of NMs to different cells and may aid in developing rapid and efficient test strategies to assess the impact of these emerging materials on human health and the natural environment. Cell cultures can serve as experimental systems both to assess the potential for toxicity of MNMs and to

study their mechanisms of toxicity at the molecular and cellular levels by allowing the cells to be studied in a controlled environment in isolation from multiple physiological systems that help determine their fate *in vivo*.

Zheng et al. [39] found that nano-TiO_2 had a similar adsorption capacity in different media, although a higher aggregation level was observed in cell culture medium. In that study nano-TiO_2 alone did not induce significant DNA and chromosome damage, but the mixture of nano-TiO_2 and BPA increased toxicity via increasing oxidative stress, DNA double strand breaks and micronuclei formation. The BPA-bound nano-TiO_2 were proven to be uptaken into nuclei of exposed cells, which may increase intracellular BPA and nano-TiO_2 levels and thus lead to synergistic toxicity.

c) Metal nanoparticles

I) Nickel NPs

In vivo and *in vitro* studies have demonstrated that Ni containing nanoparticles are more toxic than particles greater than 3 μm. However, since now there is limited information with respect to nano-Ni containing particles both in regard to their cytotoxicity and cellular uptake. Recently there were several studies that assessed their effect on cell survival with respect to drug delivery and cancer treatment. It is found that the nanoparticle forms were more toxic than the soluble forms, and of the nanoparticle varieties the dendritic clusters demonstrated the highest level of toxicity indicating that shape is an important factor for toxicity of Ni nanoparticles. Surface charge, physical structure, size thresholds, calcium levels, and metabolic activity are all factors which influence the level of particulate uptake. The adjustment of any one parameter can alter uptake and thereby alter *in vitro* toxicity of a Ni compound. This sensitivity in the parameters governing uptake mandate that a clear understanding of uptake is integral in toxicity assessments [40].

Ni hydroxide nanoparticles (nano-NH), which are increasingly used in the power and energy industries, induced significant inflammatory responses in mouse lungs in a dose-dependent manner [41]. Later research assessed the role of particle solubilization from the nanoparticles to determine if toxicity resulted from the generation of oxidative stress and inflammation. Nano-NH was compared to nickel sulfate nanoparticles (nano-NS). Results indicated that the greater toxic potential of nano-NH was not attributed to increased solubilization or to generic properties of nanoparticles, but rather was more likely related to increased levels of deposition and a stronger inflammogenic potential than nano-NS that was independent of lung Ni burden. This indicates that the toxicity of nano-NH is chemical-specific and important factors to consider are the deposited dose and solubility.

II) Silver NPs

Farkas et al. [42] reported that silver nanoparticles were highly cytotoxic to rainbow trout hepatocytes due to the reduction of metabolic activity and membrane integrity at low concentrations. Hayashi et al. [43] used earthworm primary cells, cross-referencing to human cell cultures with a particular emphasis on the conserved biological processes, and provide the first *in vitro* analysis of molecular and cellular toxicity mechanisms in the earthworm *Eisenia fetida* exposed to AgNPs (83 ± 22 nm). While they observed a clear difference in cytotoxicity of dissolved silver salt on earthworm coelomocytes and human cells (THP-1 cells, differentiated THP-1 cells and peripheral blood mononuclear cells), the coelomocytes and differentiated (macrophage-like) THP-1 cells showed a similar response to AgNPs. Intracellular accumulation of AgNPs in the coelomocytes, predominantly in a phagocytic population, was evident by several methods including transmission electron microscopy (TEM). Molecular signatures of oxidative stress and selected biomarker genes probed in a time-resolved manner suggest early regulation of oxidative stress genes and subsequent alteration of immune signaling processes following the onset of AgNP exposure in the coelomocytes and THP-1 cells.

Finally, the results of Park et al. [44] suggest that the most pronounced effect of silver nanoparticles is inflicting damage towards a range of different cell types, potentially resulting in a myriad of secondary effects, such as generation of ROS, DNA damage and inhibiting stem cell differentiation. Despite the likelihood that macrophages will be amongst the highest exposed cell types, their studies showed that macrophages may not be the most sensitive to the effects of silver nanoparticles, indicating that other cell types may need to be included in the evaluation of the biocompatibility of nanomaterials. They also observed large differences in toxic concentrations between nanoparticles of different sizes, indicating that deriving exposure limits for silver nanoparticles based on mass concentrations does not appear to be appropriate. Whether the risk of being exposed to silver nanoparticles is higher compared to silver ions is a complex question that depends on a number of factors, including bioavailability, distribution pattern, exposed cell type, silver nanoparticle size and likely surface chemistry. Deriving safe exposure limits for silver nanoparticles should therefore be handled on a case-by-case approach.

III) Gold NPs

Biodistribution study up on intravenous administration in mice revealed gold NPs of all sizes were mainly accumulated in organs like liver, lung and spleen [45]. The accumulation of gold NP in various tissues was found to be depending on particle size. 15nm gold NP revealed higher amount of gold and number of particles in all the tissues including blood, liver, lung, spleen, kidney, brain, heart, stomach. Interestingly, 15 and 50nm gold NP were able to pass blood–brain barrier as evident from gold concentration in brain. Two-hundred nanometers gold NP showed very minute presence in

organs including blood, brain, stomach and pancreas. The results revealed that tissue distribution of gold nanoparticles is size-dependent with the smallest 15nm nanoparticles showing the most widespread organ distribution. Chithrani et al. [46] also observed a large difference in the cellular uptake of the different size and shaped gold nanoparticles. For example, the uptake concentrations for 74 x 14 nm rod-shaped nanoparticles were different than those for 74 or 14 nm spherical nanoparticles.

d) Silica (SiO_2) NPs

Silica (SiO_2) nanoparticles, one of the most widely used nanomaterials, can enter biological systems through different exposure routes via their use in industrial manufacturing, disease labeling, drug delivery, cancer therapy, and biosensors, which also have been receiving increased attention for their potential toxicity [47]. SiO_2 nanoparticles can reportedly cause inflammatory responses, hepatotoxicity, as well as fibrosis, and their tissue distribution and excretion differ depending on particle size. However, conflicting information has been provided by a handful of early studies, in which disagreements regarding size-dependent toxicity are observed. For instance, Nishimori *et al.* [48] found that only 70 nm silica particles caused acute liver failure at 24 h when compared with submicro- and micro-sized silica particles (300 and 1000 nm, respectively). By contrast, Cho *et al* [49] demonstrated that submicro-sized silica particles with a diameter of 100 or 200 nm significantly increased the incidence and severity of liver inflammation within 12 h, whereas the effects of nano-sized particles with a diameter of 50 nm were non-significant. The silica particles in these two studies were both intravenously administered at similar doses. The discordant observations are likely due in part to the low sensitivity of classical toxicological assessments, such as histopathological examinations and clinical chemistry analysis. Histopathological examinations and clinical chemistry analysis allow only for the observation of severe changes in the body. Finally, in the work of Lu et al. [47] no major differences were found in the response of biological systems caused by the different Silica particles sizes among the metabolite profiles. Their results suggest that not only nano-sized but also submicro-sized silica particles can cause similar extents of liver injury, which is dependent on the exposure dose, and the mechanism of toxicity may be almost the same. A full summary of *in vitro* and *in vivo* studies of the toxicity of silica NPs can be found in [50].

3. Transport of MNMs in human body.

The human body has several semi-open interfaces for direct substance exchange with the environment, i.e. the skin, respiratory tract and gastrointestinal tract (GIT).

I) Skin. Skin is the largest primary defense organ in our body and directly comes into contact with many toxic agents. Skin exposure to nanomaterials can occur during the intentional application of

topical creams and other drug treatments [8,51,52]. According to a study by van der Merwe et al. [53], nanocrystalline magnesium oxide and titanium dioxide applied to dermatomed human skin (as dry powder, water suspension, and water/surfactant suspension) for 8 h did not show dermal absorption through human skin with intact functional stratumcorneum. In another study, Gontier et al. [54] tested penetration of topically applied titanium dioxide (TiO2) nanoparticles (size range 20–100 nm) in porcine-, healthy human-, and human grafted-skin samples. It was seen that penetration of TiO2 nanoparticles was restricted to the topmost 3–5corneocyte layers of the stratum comeum. In contradiction to this finding, there are many reports that show deeper penetration of nanoparticles. Oberdorster et al. [8] demonstrated penetration of a variety of nanoparticles in the dermis and translocation to the systemic vasculature via lymphatic system and regional lymph. Further, Ryman-Rasmussen et al. [55] demonstrated that quantum dots with diverse physicochemical properties could penetrate the intact stratum corneum barrier and get localized within the epidermal and dermal layers. In a clinical study, treatment of burns using nanosilver coated dressings [56] led to abnormal elevation of blood silver levels and argyria (blue or gray discoloration of the skin due to silver accumulation in the body over time which is a 'cosmetic problem'). Though nanosilver-based dressings and surgical sutures have received approval for clinical application and good control of wound infection is achieved, their dermal toxicity is still a topic of scientific debate and concern. Despite laboratory and clinical studies confirming the dermal biocompatibility of nanosilver-based dressings [57-59] several other researchers have demonstrated the cytotoxicity of these materials. Paddle-Ledinek et al. [60] exposed cultured keratinocytes to extracts of several types of silver containing dressings. Of these, extracts of nanocrystalline silver coated dressings were most cytotoxic. Similar observations were also reported by Lam et al. [61] in another study. Fullerene-based peptides were also shown to be capable of penetrating intact skin and mechanical stressors could facilitate their traversal into the dermis [62]. Intradermally administered quantum dots could enter subcutaneous lymphatics [63] and regional lymph nodes [64]. Topically applied fine and ultrafine beryllium particles can be phagocytosed by macrophages and Langerhans cells possibly leading to perturbations of the immune system [65]. Epidermal keratinocytes have also been shown to be capable of phagocytosing a variety of engineered nanoparticles and setting off inflammatory responses [66]. It is worth noting that some other types of nanoparticles, i.e. single-/multi-wall carbon nanotubes, quantum dots with surface coating and nanoscale titania, have been shown to have toxic effects on epidermal keratinocytes and fibroblasts and are capable of altering their gene/protein expression [67].

II) Respiratory tract. The respiratory system serves as a major portal for ambient particulate materials. Recently, the pathogenic effects and pathology of inhaled manufactured nanoparticles have received attention [68-71]. Being different than micron sized particles that are largely trapped

and cleared by upper airway mucociliary escalator system, particles less than 2.5 µm can get down to the alveoli. The deposition of inhaled ultrafine particles (aerodynamic-diameter b100 nm) mainly takes place in the alveolar region [51-52]. After absorption across the lung epithelium, nanomaterials can enter the blood and lymph to reach cells in the bone marrow, lymph nodes, spleen and heart [52,70]. The latter could be of significance since the association between inhaled ambient ultrafine particles and cardiovascular events such as coagulation and cardiac rhythm disturbances has been proven [67]. Other targets after translocation include the sensory nerve endings embedded in the airway epithelia, followed by ganglia and the central nervous system via axons [8]. Takenaka et al. [72] have demonstrated that in both inhalation and instillation experiments, ultrafine silver particles were taken up by alveolar macrophages and aggregated silver particles persisted there for up to 7 days. Aggregated silver nanoparticles and some other nanomaterials have been shown to be cytotoxic to alveolar macrophage cells as well as epithelial lung cells [73].

III) Gastrointestinal tract (GIT). Nanomaterials can reach the GIT after mucociliary clearance from the respiratory tract through the nasal region, or can be ingested directly in food, water, cosmetics, drugs, and drug delivery devices [8,52]. The utility of biodegradable nanoparticles in the delivery of oral vaccines has been proposed for antigens known to be susceptible to proteolysis [74]. Apparently studies on toxicity of nanomaterials post oral ingestion are limited. Chen et al. [57,58] determined the acute toxicity of copper particles (bulk) and nanocopper in mice and found that nanocopper was several folds toxic than bulk copper (LD50 for nanocopper 413 mg/kg; bulk copper >5000 mg/kg). Nanocopper was also reported to cause pathological damage to liver, kidney and spleen. Finally, Chung et al. [75] recently reported occurrence of systemic argyria after ingestion of colloidal nanosilver proves its translocation from the intestinal tract.

4. Knowledge gaps

MNM's functionality and structure: All the properties of a MNM are closely related to its structure. Thus, it will be very useful if, by understanding theoretically the connection between structure and properties, we set the rules that minimize the appearance of problematic structures and incorporate environmentally benign functionalities. For chemical species this framework exists, but it is not clear if it can be applied to MNMs. *Thus, it is necessary first to check which part of the existing framework about chemical species can predict functional stability of MNMs. Of special interest should be structures that increase toxicity, bioavailability, and bioaccumulation.*

Toxicity and MNMs' surroundings: The physical and chemical properties of MNMs may change when incorporated into products [76,77]. The medium used in a cell line study can influence how MNMs are dispersed and how this may impact their subsequent toxicity. The importance of considering the solutions in which MNMs are suspended was demonstrated by Foucaud et al. [78] who indicated that dipalmitoyl phosphatidylcholine (DPPC) and bovine serum albumin (BSA) were able to better disperse carbon black MNMs. When compared to particles contained within saline only, this arrangement increased the ability of carbon black MNMs to induce production of ROS within monocytic MonoMac 6 cells. In addition, Sager et al. [79] reported similar results in their study. Moreover, chemical groups added to the surface of CNTs changed their properties and decreased their toxicity [80] while the addition of water-soluble functional groups can decrease the toxicity of pristine C-60. These findings indicate that the surroundings can highly affect MNMs' toxicity, *thus it is important to ensure that their composition is relevant to the expected exposure scenario [81]. The same MNMs should be checked in different surroundings in order to create a database, where the changes of their properties will be recorded.*

Toxicity and form of MNMs: The same MNMs could be found in diversity of forms and locations, and thus it can influence the toxicity of a material by different ways during life cycle. For toxicological purposes, M*NMs should be categorized according to where the MNM is located or what form it takes in a product e.g. being airborne, surface bound, suspended in liquids or being bound in a nanostructured surface [82].*

Dose metric and toxicity evaluation: *There is as yet no consensus on what is the most appropriate dose metric when testing nanomaterials.* Currently three metrics are frequently used: mass, surface area (SA) or particle number. So far, SA has been pointed out as the most relevant [8,83]. For instance, for a given mass, MNMs were reported to be more toxic than fine-sized particles of the same composition; however, once normalized by SA (assessed in majority of the studies with BET method), the toxicity responses were similar for all the sizes studied [84,85] also showed a good correlation between inflammatory response and SA for MNMs tested. On the other hand, the universal correla-

tion between SA and MNMs effects has been questioned [25,86]. Nevertheless, toxicity evaluation based on a mass basis can sometimes be misleading to interpret whether the toxicological effects are induced by small particle size or large surface area. Hence, the surface area or particle number of nanoparticles may be a more appropriate dose metric for toxicity evaluation rather than mass burden of nanoparticles and an in-depth particle characterization prior to toxicity study is important. Oberdörster et al. [8] suggested that that particle SA is a more appropriate dose metric than particle mass or particle number when evaluating dose–response relationships of nanoparticle-induced pulmonary inflammation. Wittmaack et al. [87] however, disagreed with this statement and claimed that he found particle number to work best as a dose metric. In toxicology, SA as a dose metric is not yet accepted to be the most relevant dose metric, leaving only few studies with surface area as the dose metric. Toxicological studies using this type of dose metric often do not use a SA per volume metric, but only total or calculated surface area, or surface area, normalized for lung weight. In addition, the toxic responses of some MNMs may be a result of obstruction of cellular processes by physical presence of poorly soluble particles in sufficient numbers to cover the cellular surface of alveolar macrophages. In that case, nanoparticle number or mass can be relevant dose metrics. A recent study exploring the applicability of different physical exposure metrics to a range of nanoparticle classes and relating them to health impact-relevant attributes shows that no single method for monitoring nanoaerosol exposure will suit all nanomaterials [88].

Quantification, tracing of MNMs and the current available techniques and methodologies: Towards understanding the potential impact of MNMs is important to measure and characterize them in different environmental and biological matrices. State of the art techniques are capable for such characterization: a) AFM (Atomic Force Microscopy) can give information about the size distribution of NMs. b) DLS (Dynamic Light Scattering) and TEM (Transmission Electron Microscopy) can be used to determine the aggregation state of NMs. Particles can be identified in tissues using the electron microscope, but the sample preparation takes time, and there is always the risk of introducing aggregation artifacts during processing of the material. c) XPS (X-ray photoelectron Spectroscopy) is an appropriate technique to unravel the predominant functional groups on MNMs. can be used, d) Inductively Coupled Plasma Mass Spectrometry (ICP-MS) or similar techniques are based on the analysis of an acid digest of the tissue. However those approaches may work well for materials that are not usually exist at high concentration in organisms (e.g., silver), but it may be more difficult to distinguish the metal MNMs result from the background levels of metal already present in the organism. *Therefore the development, standardization and validation of a toolbox of robust analytical methodologies and probably new in situ techniques and instruments are of very high*

priority. Furthermore, analysis in real time is needed towards a dynamical characterization of the behavior of MNMs in various matrices.

Prediction models and the influence of MNMs' concentration: The precise knowledge of the concentration of NMs at the exposure points and its impact on the environment is one of the most crucial parameters in the complex MNM-environment interaction problem. For this reason, computational models capable of predicting accurately the MNMs' concentration values and their influence on MNMs properties as also the reaction with the surroundings are of high priority. Furthermore, such models could give answers about receptors responsible for the accumulation of different MNMs. Unfortunately, most of the existing models are not able to fill these gaps, due to the composition of MNMs by a small number of components that usually live and function out of equilibrium. So, classical principles of Thermodynamics and Statistical Physics fail to describe them. *Stochastic methods are seen to be the appropriate approach towards predicting and connecting NMs properties with their concentration.*

Environmental disposal and Release Dynamics: For different types of products containing MNMs the most likely entry point into environment is: a) air, surface water, and sewage for cosmetics (titanium dioxide, zinc oxide, fullerenes, gold), b) surface water, and sewage for catalysts and lubricants (cerium oxide, platinum, molybdenum trioxide), c) air, surface water, and sewage for paint and coatings (titanium dioxide, gold, quantum dots), d) surface water, sewage, and soil for environmental and water remediation (iron, polyurethane, carbon nanotubes), e) air, surface water, and soil for agrochemicals (silica as carrier), f) surface water and soil for food packaging (gold, copper nanoclays, silver, titanium dioxide), and g) sewage and soil for pharmaceuticals and medicine (hybrid NMs and carriers). Treatment technologies for protection from MNMs exposure must be developed. These should include studies with different routes of exposure (e.g., diet versus water) and chronic exposure experiments which are generally lacking at present. First, we must measure the repercussions coming from both point like and sized sources of MNMs in different environments. Development of methodologies to measure MNMs in complex environmental media (soil, sediments, natural waters) is a vital and essential requirement to environmentally realistic studies of MNMs ecotoxicity. *So, of high priority in this field is the developing of appropriate analytical tools or identifying sentinel species that will allow us to identify different species and to monitor pollution by NMs.*

Bioavailability and the uptake mechanisms of MNMs: Currently, there is no evidence about testing procedures on the assessment of MNMs bioavailability and their impacts on organisms (different labs get different results for the same type of MNMs). For example knowledge of the ecotoxicology of

NMs to bacteria and other microbes is limited, even though some manufactured MNMs have been designed as cleaning agents with antibacterial properties. However, toxicity in laboratory solutions may be somewhat different from that in real environmental samples. *For this reason, standardization protocols are necessary, which will enable comparison of results. As far as bioavailability is concerned fields related to food chain, nutrient cycling, and waste degradation, must also be taken into account. Interesting is also the study of uptake mechanisms by different organisms, this topic today is poorly understood, as MNMs affect their surroundings and vice versa.*

Lack of data regarding the other possible pathways that MNMs can insert into the human body except inhalation: Taking into consideration the routes of exposure to nanoparticles, to better understand dermal absorption of nanomaterials more research on regular skin, dry skin and damaged skin is necessary as pointed out by Zwart et al. [89] Hagens et al. [52]. More studies on gastrointestinal lymphatic uptake and transport and direct toxicological effects on the GIT are required [90]. *Similarly questions such as penetration of placental barrier by nanomaterials would require attention. For such studies suitable in vitro models need to be developed with subsequent in vivo studies.* Cellular interactions with certain nanomaterials may not introduce any new pathological conditions, but one cannot ignore novel mechanisms of injury that require special tools, assays and approaches to assess their toxicity.

Distribution of MNMs to human body: There is little research to date about the potential for distribution of MNMs to other tissues. When MNMs have passed the barriers (i.e., lung epithelia, intestinal lining, dermis) at the site of entry, the systemic circulation may be reached. Further distribution throughout the entire body may take place via the systemic circulation. The literature on distribution following exposure to MNMs does not reveal whether MNMs reach tissues and organs as nanoparticles. Only element concentrations were measured in blood and in organs/tissues. When it has become clear that MNMs can pass the ports of entry, then it will be relevant to perform additional research on their distribution in the body. Such research should include interaction with plasma proteins and distribution to critical tissues like reproductive organs, brain and transplacental passage. Although several routes are confirmed in animal studies, to date, the current knowledge of the kinetics of NMs is too limited to allow a proper foundation of human risk assessment of MNMs. *To close the knowledge gaps, an important research question is the elucidation whether and to what extent MNMs enter the body. In case MNMs really are absorbed into the body, then research of the whole kinetic spectrum of MNMs in the body will be required, including absorption, distribution and metabolism and excretion processes over time [91].*

Lack of in-situ measurements. Currently, neither the fate of nanosize materials nor their impact on animals, plants and soil communities have been investigated in situ although it would be necessary for the validation of models proposed for the environmental risk assessment of nanoparticles [92]. *Physico-chemical characteristics of particles after they react with cultured cells in vitro needs to be evaluated, and there is also a need for more research on effects of long term exposure to nanomaterials.*

Monitoring of biological markers: To identify the mechanisms of nanoparticle-induced inflammatory response under different exposure conditions, other inflammatory markers and various candidate biological markers (cellular component, macromolecules, other metabolic parameters, and cellular function) need to be monitored. *Comparative studies with normal cell lines and novel experimental set-ups for a multiplexed screening of biological markers [93] will be required to unravel the uncertainty of nanoparticle-induced inflammatory responses.* In their review for the *in vitro* toxicity of nano-TiO_2, Valant et al. [94] indicate that even when the *in vitro* tests measure the same biomarkers with the same exposure duration and known primary particle sizes, it is insufficient merely to use such data for risk assessment. Papers published to date provide an understanding of the mode on nano-TiO_2 action but are not suitable for assessment and management of risk. *In the future, validated standard methods should include a limited number of cell lines and an obligatory selection of biomarkers.*

In vivo, *in vitro* correlations: When evaluating MNM toxicity using *in vitro* assays, *in vivo* relevance is an essential criterion for accepting their utility. The *in vivo* relevance can be questioned because of the differences between *in vitro* and *in vivo* conditions. *These differences warrant developing novel methods to define equivalent doses between in vitro and in vivo exposures in order to improve correlations between the two testing systems.* One difference is the high concentrations/dose used in most traditional *in vitro* studies. An extremely high dose rate (dose administered per unit of time) is another issue of *in vitro* studies. Because the full dose is delivered as a bolus in traditional *in vitro* assays. The dose rate in such studies is much higher than in *in vivo* inhalation studies in which animals are exposed to a low concentration of chemical or particle via inhalation for an extended period of time (hours, days, weeks, or longer). Another important difference is the wide *use of dispersants in vitro but not necessarily in vivo* (e.g., inhalation of pristine NPs generated from dry powders) [95]. MNMs dispersed in cell culture medium would adsorb some components in the medium while MNMs inhaled into the lung would adsorb components of pulmonary surfactant. However, the use of high doses and high dose rates *in vitro* by itself does not invalidate *in vitro* assays. The *in vitro* data could still be useful as long as the data are confirmed to correlate well with *in vivo* results. In particular, *in vivo dosing* of the respiratory tract by intratracheal instillation or oropharyngeal aspiration are also bolus-type delivery methods and results should correlate better with

in vitro dosing. There have been some studies that addressed the issue of the *in vivo* relevance of some *in vitro* assays for evaluating the toxicity of MNMs [96] or ambient particulate matter [97]. Both found poor correlations. However, these findings do not necessarily indicate intrinsic flaws of the *in vitro* assays for predicting *in vivo* toxicity. *Instead, there can be multiple reasons for the differences between in vitro and in vivo results* [97]. Rushton et al. [98] have proposed an alternative approach of slope analysis and found a good in vitro–*in vivo* correlation when applied to the data in the paper of [96]. Finally Han et al. [99] introduced a new method to derive the steepest slope – a mathematical method and using this approach they showed a good correlation of acute toxicity between *in vitro* and *in vivo* results. *Future work needs to also consider extension to long-term effects.*

Toxicity testing: The early onset and persistence of pulmonary lesions in rodents suggest that sub-chronic (90 days) or short-term *in vivo* data may be predictive of the long-term effects of some nanomaterials. Daily inhalation exposures for 2 and 4 weeks were recommended by an expert panel in Tier 1 and Tier 2 studies of nanomaterials, respectively [70]. Depending on the type of nanomaterials, even a 5-, 7-, 14- or 28-day rodent studies may be able to reveal some of their toxic activities. Technically, because of the high propensity of hydrophobic nanomaterials to agglomerate, preparation of adequate aerosol dispersions suitable for inhalation studies is immensely difficult. As a result, alternative exposure methods (e.g., intratracheal instillation, intratracheal inhalation, pharyngeal/laryngeal aspiration) have been used by various investigators for delivery of particles to the lungs [32, 100]. Although inhalation is the preferred toxicity method of testing nanoparticles, it appears that, with appropriate dosing schemes and match controls, some of these alternative methods are reasonable alternatives to more costly and time consuming inhalation studies and may be useful for hazard identification or establishing the relative toxicity ranking among different nanomaterials for further testing [88]. To date, carefully monitored tests are used to establish the inhalation toxicity of airborne nanoparticles. However, only very recently a new standard, ISO 10808:2010, *Nanotechnologies – Characterization of nanoparticles in inhalation exposure chambers for inhalation toxicity testing*, was adapted to ensure that the results of such tests are reliable and harmonized worldwide. ISO 10808 establishes a battery of inhalation toxicity testing chamber monitoring, including a differential mobility analyzing system (DMAS), for determining particle number, size, size-distribution, surface area and estimated mass dose, as well as morphological examination using transmission electron microscopy (TEM) or scanning electron microscopy (SEM) equipped with an energy dispersive X-ray analyzer (EDXA) for chemical composition. *More such standards for toxicity testing are needed involving different exposure routes and specialized in different classes of MNMs.*

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5. July 2014 update

Because the work in this report is an additive process and will continue to expand in the following years, as the amount of papers in the literature for this highly interested and significantly important subject is increasing enormously every year, the author chose to add the updates as supplements to the original report. This is beneficial for the author as well as the reader. The former can add, every time the report is updated, the more recent information and state-of-the-art experiments and techniques without the need to track every change in the previous document and concerning about the coherence of the overall report. Meanwhile, the latter can read, every time, only the latest studies on the subject, without reading the whole text again. In addition, as the properties and the effects of the MNMs are strongly material-dependent, the reported works are categorized accordingly, except from some works that examine and compare different MNMs. Finally, some new strategies and approaches are described.

In this update, due to the rapidly increasing interest of the scientific community for the biomedical applications of nanomaterials, such as cell labeling and drug delivery, and the evaluation of their impact on human health, the author focused on the latest works related with the *toxicological studies in vitro and in vivo* of carbon "biocompatible" nanomaterials, i.e. carbon nanotubes, fullerenes, the recently highly promising graphene and their derivatives. Moreover, an extensive report of the most recent toxicological studies (January 2013-July 2014) in various metal and metal oxide MNMs as well as Silica MNMs was carried out.

6.Toxicological studies of nanomaterials

a) Carbon MNMs

I) Carbon nanotubes (CNTs)

Mohammadian et al. [1a] showed in their study that CNTs might have cytotoxic effects on lung cells that are comparable to the cytotoxic effects of asbestos fibers. The result of their experiments revealed that SWCNTs can induce an indirect cytotoxicity by alteration of cell culture medium, which potentially results in a false positive toxic effect. The mode of dispersion of nanomaterials for test exposures is decisive for the outcome of *in vitro* studies as the complete dispersion of CNTs in the test solutions and avoiding the agglomeration of particles is a vital factor and affects the toxicity data. In addition, the dimensions of particles might be an influencing parameter on the dispersion and, hence, on the degree of cellular toxicity. They also suggest that other properties such as specific surface area and metal impurities might yield different dose–response data. Interesting results of their study are that MWCNTs were more cytotoxic after a 24-h exposure period compared to a 6-h exposure period and that the cytotoxicity of SWCNTs is more dependent on exposure time compared to MWCNTs.

In their recent review, Lanone et al. [2a] address the issue of the formation of a bio corona which is considered crucial in the determination of CNT toxicity. Generally, MNMs, including CNTs, when they are in a physiological environment, they can selectively adsorb biomolecules, leading to the formation of a bio-corona. Such dynamic exchanges between CNT surfaces and bio-fluids (i.e. proteins or lipids) may lead to a modified biological “identity” of CNTs (new biological effects as well as potential modifications of the material by the biomolecules present in the corona — in terms of structure and/or functions. Several characteristics of the corona are extremely important and are routinely measured such as the thickness, density, identity, quantity, conformation and affinity. A large proportion of studies have focused on isolated blood proteins, isolated plasma or serum as models for blood protein adsorption (fibrinogen, gamma globulin-transferrin, serum albumin, complement proteins, etc.) [3a]. From these studies, it is clear that CNTs can adsorb proteins to their surface, although this binding depends on protein-specific as well as CNT-specific characteristics. Cai et al. [4a] demonstrated that cytoskeletal proteins were dramatically overrepresented on the MWCNT bio-corona [71]. By contrast, nuclear proteins (chromatin, ribosomal and sliceosomal proteins) were heavily represented in the fraction of proteins that did not bind effectively to CNTs. The authors suggest that the preferential binding of CNTs to cytoskeletal proteins might represent a biochemical basis for CNT toxicity. Interestingly, CNT diameter was also an important factor of efficiency for proteins: CNTs with a diameter of 20–40 nm or above bound a greater amount of proteins than materials with a diameter less than 10 nm.

Moreover, Hamilton et al. [5a] demonstrated that increasing the diameter or length of MWCNT significantly increases NLRP3 inflammasome driven IL-1β production *in vitro*using THP-1 cells and primary alveolar macrophages from C57BL/6 mice. These results imply that structural differences due to changes in diameter or length are important in defining interactions of the MWCNT with phagolysosomal membranes. In addition, they showed that carboxylation of the MWCNT was sufficient to significantly decrease the bioactivity of MWCNT in vitro, demonstrating that, regardless of size, -COOH functionalization decreases both toxicity and bioactivity.

In their work, Dong et al. [6a] determined the toxic effects of SWCNTs and acid-functionalised SWCNTs (AF-SWCNTs) on the immune system, especially on macrophage functions using cultured murine peritoneal macrophages and purified splenic T cells. In macrophages exposed to 0–50 mg/ml of CNTs for 24 h and no significant cytotoxicity was found, while AF-SWCNTs were engulfed mostly through phagocytosis and located in lysosomes of macrophages damaging the mitochondrial function and the proteasome formation in a concentration-dependent manner. Wan et al. [7a] also evaluated the toxicity of AF-SWCNTs in murine peritoneal macrophages. They found that AF-SWCNTs induce autophagosome accumulation and the conversion of LC3-I to LC3-II. Further analysis on lysosomes revealed that AF-SWCNTs accumulate in macrophage lysosomes, leading to lysosome membrane destabilization, which indicates reduced autophagic degradation.

In their concise review on the hemotoxicity and the effects on hemodynamics of carbon nanotubes, Bussy et al. [8a] conclude that among the critical parameters which determine CNT hemocompatibility, the surface characteristics are the major parameter influencing the interactions with blood components and therefore the overall biological impact, as the addition of functional groups or coating on the surface has been proven to dramatically change the CNT reactivity. On the same aspect, Vakhrusheva et al. [9a] demonstrated that pre-treatment of SWCNTs with albumin results in the reduction in platelet aggregation and thus the formation of non thrombogenic CNTs.

Søs Poulsen et al. [10a] used high-content genomics tools to compare *in vivo* pulmonary responses of MWCNT to those *in vitro* in cultured lung epithelial cells (FE1) at the global transcriptomic level. Primary size, surface area and other properties of MWCNT- XNRI -7 (Mitsui7) were characterized using DLS, SEM and TEM. Mice were exposed via a single intratracheal instillation to 18, 54, or 162 µg of Mitsui7/mouse. FE1 cells were incubated with 12.5, 25 and 100 µg/ml of Mitsui7. Tissue and cell samples were collected at 24 hours post-exposure. Three major concepts emerged from their analysis: (1) the primary toxic response *in vivo* was an early acute phase and inflammation dose-response which was not activated *in vitro*; (2) the *in vitro* model is less responsive than the *in vivo* at lower doses and does not show a dose-response pattern in the induction of toxicity pathways at increasingly higher concentrations; and (3) both models respond by activating similar core cellular functions (oxidative stress and fibrosis) at the pathway level, however, regulation of most individual

genes, the underlying mechanisms and consequent nature of the biological response are different showed that although there were similarities observed between the two models at the pathway-level, the specific genes altered under these pathways were different, suggesting that the underlying mechanisms of responses are different in cells in culture and the lung tissue. In another study, Ponti et al. [11a] investigated the toxicological effects of nude and chemically functionalized (-NH2, -OH and -COOH groups) MWCNTs using immortalized mouse fibroblasts cell line (Balb/3T3) as *in vitro* model, alternative to the use of animals, to assess basal cytotoxicity, carcinogenic potential, genotoxicity and cell interaction of MNMs. Combining *in vitro* tests such as cell transformation assay and micronucleus with physicochemical and topological analysis, they obtained results showing no cytotoxicity and genotoxicity. In contrast Carcinogenic potential and MWCNTs interaction with cells were evident. The two previous studies point out that careful consideration should be given in selecting relevant endpoints when substituting animal with *in vitro* testing to detect to detect long-term effects, such as carcinogenicity and more immediate effects like basal cytotoxicity or genotoxicity.

Cancino et al. [12a] focused on the effects of SWCNT– polyamidoamine dendrimers (PAMAM) MNMs and their individual components on the C2C12 murine cell line, which is a mixed population of stem and progenitor cells. The results indicate that PAMAM and SWCNT–PAMAM had increased toxicity to the cell cultures compared to SWCNTs possibly due to the positive charge from the dendrimer molecules surface. In another work related with functionalized SWCNTs, Song et al. [13a] investigated the cytotoxicity of four types of SWCNTs functionalized with hydroxyl, amino, carboxyl and polyethyleneglycol on MCF7 cells. These f-SWCNTs have insignificant effects on mitochondrial activity and ROS production in MCF7 cells at all test concentrations. However, explicit results revealed that all the tested f-SWCNTs could cause changes of cell morphology, induce cell membrane damage, decrease cell adhesion, and increase cell apoptosis.

Snyder-Talkington et al. [14a] determined the cellular and gene expression changes in small airway epithelial cells (SAEC) after *in vitro* exposure to MWCNT. A direct interaction between SAEC and MWCNT was confirmed by both internalization of MWCNT and interaction at the cell periphery. Following exposure, SAEC showed time-dependent increases in reactive oxygen species production, total protein phosphotyrosine and phosphothreonine levels, and migratory behavior. Analysis of gene and protein expression suggested altered regulation of multiple biomarkers of lung damage, carcinogenesis, and tumor progression, as well as genes involved in related signaling pathways.

Concerning the interactions between CNTs and the immune system Dumortier [15a] in her review article describes the “ideal” immunologically neutral CNT, as being short, functionalized, highly dispersible in the physiological milieu and carrying chemical groups that allow its oxidative biodegradation.

Finally, steps towards the investigation and prediction of CNT lung fibrogenesis have been made by 3D modeling. S. Luanpitpong et al. [16a] developed a 3D model of CNT lung fibrogenesis that is fast, robust, and resembles the clinical fibrotic foci of lung fibrosis. The model employs primary human lung fibroblasts that form a collagen-rich 3D structure upon stimulation with CNTs or TGF-β. Using this model, they unveiled the presence of fibroblast stemlike cells in the fibroblastic nodules and demonstrated its role in CNT-induced fibrogenesis. The developed model could potentially be used as an alternative assay to predict the fibrogenicity of CNTs and other nanomaterials for their safer design and risk assessment. In addition, the model could be used to aid mechanistic investigations of the cellular and molecular events leading to fibrogenesis.

II) Fullerenes and derivatives

Fullerenes, such as Bucky balls, C_{60}, are a group of carbon nanomaterials formed as spherical nanoensembles which range from C_{20} to C_{720} (and larger), typically forming empty closed cage structures, which are made up of multiple 5-member and 6-member rings [17a]. The chemical reactivity of fullerene C_{60} has been known for several years, and it is a common agreement that there are several different chemical classes of C_{60} derivatives. Like benzene derivatives, for example, toluene, nitrotoluene or phenylalanine, each C_{60} derivative has its own physicochemical characteristics and toxicological behavior. Even extremely similar fullerene derivatives can have completely opposite results, which stimulates efforts to understand how changing the chemical composition and structural arrangement of fullerenes affects molecular interactions at the cellular, tissue and organ system levels. The ability of fullerenes to be derivatized with side chains provides opportunities to diversify, manipulate and harness the electronic properties of the cage for selected applications. Of course, each derivation results in changes of the compound's physical and chemical properties, including particle size/length, z-potential, molecular weight, surface characteristics and solubility, contributing to how they affect biological systems [18a].

C_{60} inability to dissolve in polar substances, most notably water, limits its practical applications in materials science, biotechnology, and medicine [19a]. Therefore, water-soluble fullerene-based derivatives, especially fullerene-containing biomolecules, have been widely investigated. Nevertheless, with appropriate modifications C_{60} derivatives have a wide field of applications, including transport of drugs through dense tissues, environmental remediation, and in energy systems as in organic solar cells [17a]. Due to their small size (<5 nm), fullerenes decorated with hydrophillic entities around their cage, form water-soluble fullerene derivatives which can easily penetrate, without damaging, the cell membrane, and thus they become very attractive for drug delivery usage. Moreover, fullerenes are among the strongest antioxidants, characterized as "radical sponges". By reacting

with the ROS specific fullerene derivatives can prevent ROS attacks and thus protecting lipids, proteins, DNA, or other macromolecules.

However, studies for health-adverse aspects in the last years have shown that fullerenes interfere with several mechanisms in the body of animals and humans. These effects vary according to the format of the fullerenes, and their chemical modifications. However, modified, the principal toxicological property of fullerenes is due to their predominantly apolar character which is compatible with merging with biologic membranes in organism [20a]. In the study by Nakagawa et al. [20a], hydroxylated fullerenes, also named fullerenoles, were tested on rat hepatocytes. The fullerenes showed strong detrimental effects and induced cell death within the 3 h in the rat liver cells. Furthermore, several crucial cellular factors, such as ATP and thiol levels, were depleted by the fullerenes. The fullerenes were also found to cause substantial damage on the mitochondrial membrane, particularly the highly hydroxylated fullerenes types which induced chemical modification of the lipid molecules. It was found that out of the several membranes in hepatocytes, the mitochondrial membrane was affected most negatively. This led directly to dysfunction of the Adenosine-50-triphosphate (ATP) synthesis and cell death. This study therefore introduces the important notion that the modification from pristine C_{60} to hydroxylated C_{60} makes the hydrophobic particle more hydrophilic while simultaneously introducing other side effects. This mechanism presents a unique and perhaps novel form of toxicity, where innocuous molecules such as water are transported by the toxic agent into domains within the cell where they become detrimental and act as co-contaminant [17a]. This mechanism could in principle work with other co-contaminants. Due to the various modifications, fullerenes may resemble cellular components, particularly proteins, because of the spherical structure, amphiphilic nature and stable conformation. This may therefore make it is easier for fullerenes to pass ‘‘unhindered’’ by the body’s defense system, making modified fullerenes harder to be identify and degrade, compared to non-hydroxylated fullerenes [17a]. Such a mechanism of increased toxicity through increasing bio-availability via ‘‘bio-resemblance’’ was observed by Drug et al. [21a], where toxins were more efficiently transported throughout the lungs due to their compatibility with the tissue.

C_{60}, although highly hydrophobic, due to its electronic configuration it can form strong C_{60}-H_2O bonds when in colloidal water suspensions resulting in stable nano-aggregates that can promote deleterious effects in biological systems [22a]. Despite being poorly studied, the uptake rate and toxicity of other environmental contaminants seem to be somehow affected when co-exposed to fullerene. Baun et al. [23a] indicated that co-exposure with fullerene C_{60} enhanced the toxicity of phenanthrene to the *microcrustacean Daphnia magna* and to the algae *Pseudokirchneriella subcapitata*. This was due, at least in part, to the high adsorption of phenanthrene molecules onto C_{60} nano-aggregates, which facilitated phenanthrene uptake. Similarly, Costa et al. 24[a] observed that arsenic

(As^{III}) uptake was higher in zebrafish hepatocytes co-exposed to fullerene (1 mg/l). Furthermore, Ribas Ferreira et al. [22a] investigated the effects of fullerene C_{60} in a *Danio rerio* (zebrafish) hepatocyte cell lineage exposed to benzo[a]pyrene (BaP) in terms of cell viability, oxidative stress parameters and BaP intracellular accumulation. Fullerene C_{60} provoked a significant ($p < 0.05$) loss in cellular viability when co-exposed with BaP at 0.01, 0.1 and 1.0 µg/l, and induced an increase ($p < 0.05$) in BaP accumulation in the cells after 3 and 4 h of exposure. The levels of ROS in the cells exposed to BaP were diminished ($p < 0.05$) by the fullerene addition, and the increase of the GST activity observed in the BaP-only treated cells was reduced to the basal levels by co-exposure to fullerene.

III) Graphene, graphene oxide and derivatives

Graphene, one of the carbon nanomaterial allotropes, is a single-atom-thick, two-dimensional sheet having sp^2-hybridized carbon atoms arranged hexagonally. It is the thinnest possible configuration of carbon molecules, and is a basic building block for other graphitic materials [25a]. Graphene has unique physicochemical properties including a high surface area, extraordinary electrical and thermal conductivity, and strong mechanical strength [25a]. The excellent electronic transport properties and high surface-to-volume ratios endow it with unique mechanical and rheological properties, and resistance to degradation [26a]. The two active parts, surfaces and edges, facilitate graphene attaching to biological molecules and adhering to cells [27a]. Graphenes vary in shape, size, surface area, layer number, lateral dimensions, surface chemistry, stiffness, defect density or quality of the individual graphene sheets, and purity; and all these properties significantly influence the interaction of graphenes with biological systems [28a]. Generally, graphenes with small size, sharp edges, and rough surfaces easily internalize into the cell as compared to larger, smooth graphenes. graphenes, particularly monolayer graphene, have the theoretical maximum surface area because every atom lies on the surface, providing an extremely high capacity for drug delivery [29a]. The surface chemistry varies greatly among the members of graphenes even before any surface modification; it determines their hydrophilicity or hydrophobicity, stability, and dispersibility in physiological conditions [28a]. The planar structure and ultra-high surface area (2600 m^2/g) of graphene facilitate molecular loading and bioconjugation [30a]. graphenes, specifically graphene oxide (GO) and graphene, have been evaluated as novel nanocarriers for a variety of theranostic applications, including the delivery of conventional drugs, because their use may alleviate problems due to multidrug resistance and nonspecific targeting [31a]. Below, some of the more recent and interesting works on the topic of graphene and graphene sub-products are presented. For further reading the reviews of Guo and N. Mei [32a] and Seabra et al. [33a] are highly recommended.

Horváth et al. [34a] investigate the in vitro short-term cellular toxicity associated with GO and reduced graphene oxide RGO. This study focuses on the toxicity of GO and RGO on two cell types

(i.e., epithelial cells and macrophages) found in the luminal aspect of the respiratory system, where the initial exposure to these materials is most prominent. GO exhibits a mild cytotoxic action in comparison to carbon nanotubes on epithelial cells and macrophages. An important parameter determining the biological effects of GO is its two-dimensional shape. GO nanosheets are only present in phago(endo)somes not causing any apparent adverse changes in the cellular morphology and ultrastructure. The interaction of the nanomaterial with the cell surface generated reactive oxygen species during the initial phase of the exposure and transmission electron microscopy studies shows that GO flakes of different sizes are taken up by cells via an endocytic pathway, both in epithelial cells and macrophages.

Li et al. [35a] investigate the interactions of graphene and few-layer graphene (FLG) microsheets with three cell types and with model lipid bilayers by combining coarse-grained molecular dynamics (MD), all-atom MD, analytical modeling, confocal fluorescence imaging, and electron microscopic imaging. The simulations revealed direct bilayer penetration that begins with localized piercing at sharp corners or at protrusions along graphene edges followed by propagation along the edge to achieve full penetration. For a small graphene flake, Brownian motion and entropic driving forces in the near-membrane region first position the flake orthogonal to the bilayer plane, which then leads to spontaneous corner piercing. All-atom steered molecular dynamics simulations track the free energy evolution during corner piercing and reveal only a small energy barrier, comparable to k_BT. Interestingly, in the absence of sharp corners or edge protrusions, the cell membrane has a high intrinsic energy barrier against penetration by long graphene edge segments even though they are atomically thin. Such uniform, atomically smooth, horizontally aligned, long-length graphene edges are rare, however, so in practice cell penetration is spontaneous due to the presence of atomic- or nanoscale edge roughness that essentially eliminates the energy barrier. Experimental imaging studies confirm graphene penetration of cell membranes in a dominant edge-first or corner-first mode for each of three cell types studied: lung epithelial cells, keratinocytes, and macrophages. The experiments also show penetration and successful uptake of graphene flakes as large as 5–10 µm in lateral dimension, which supports the model prediction that penetration activation barriers are not intrinsically length dependent, because of initiation at local sharp features. Once the initial energy barrier for spontaneous membrane penetration has been overcome, they suggest that interaction between the hydrophobic basal surfaces of graphene microsheets with the inner hydrophobic region of the plasma membrane promotes cellular uptake. Hydrophobic surfaces are considered to represent damage-associated molecular patterns (DAMPs) that nonspecifically activate the innate immune response. Hydrophobic cellular surfaces and surface functionalized nanoparticles are more readily internalized and initiate more potent innate immune responses than weakly charged, hydrophilic surfaces. By this mechanism, they hypothesize that graphene microsheets that penetrate into hydro-

phobic lipid domains may be recognized as DAMPs by target cells that are the first line of defense against particles and microbes deposited on the skin or on the epithelial lining of the lungs following inhalation. They conclude that the ability of graphene microsheets with large lateral dimension to penetrate and enter cells, may lead to cytoskeletal disruption, impaired cell motility, compromised epithelial barrier function, or other geometric and steric effects that deserve further study.

Lammel and Navas [36a] study the toxicity of GO and carboxyl graphene (CXYG) nanoplatelets to non-mammalian species using the fish cell line PLHC-1 as in vitro model. The cytotoxicity of GO and CXYG was assessed using different assays measuring alterations in plasma membrane integrity, metabolic activity, and lysosomal and mitochondrial function. The induction of oxidative stress was assessed by measuring intracellular ROS levels. Interaction with the plasma membrane and internalization of nanoplatelets were investigated by electron microscopy. Graphene nanoplatelets spontaneously penetrated through the plasma membrane and accumulated in the cytosol, where they further interacted with mitochondrial and nuclear membranes. PLHC-1 cells demonstrated significantly reduced mitochondrial membrane potential (MMP) and increased ROS levels at 16 µg/ml GO and CXYG (72 h), but barely any decrease in cell viability. The observation of intracellular graphene accumulations not enclosed by membranes suggests that GO and CXYG internalization in fish hepatoma cells occurs through an endocytosis-independent mechanism.

Mao et al. [37a] investigate whether the effect of the interactions between graphene-sheets with various human plasma concentrations (i.e. both in vitro (cells/tissues) and in vivo simulating states) is toxic. The results showed that by increasing the human plasma concentration, the affinity of proteins with low molecular weights to graphene-sheets surface is significantly increased. Fluorescence microscopy of HeLa and Panc-1 cell lines showed a reduction of nuclei number and an increase of ROS production respectively after a longer incubation of graphene-sheets with plasma proteins. ROS production was higher in Panc-1 cell line, when used as protein source for graphene-sheets than HeLa cell line. In overall, they conclude that the hard corona on the surface of graphene-substrates can evolve quite significantly as one passes from protein concentrations appropriate to in vitro cell studies to those present in in vivo studies, which has deep implications for in vitro–in vivo extrapolations and will require significant consideration in the future. The cellular responses to the graphene-sheets are strongly dependent to either cell type or hard corona composition.

Das et al. [38a] investigated in vitro the toxicological assessment of GO and RGO human umbilical vein endothelial cells (HUVEC). GO is found to be more toxic than RGO of same size. GO and RGO induce significant increases in both intercellular ROS levels and messenger RNA (mRNA) levels of heme oxygenase 1 (HO1) and thioredoxin reductase (TrxR). Moreover, a significant amount of DNA damage is observed in GO treated cells, but not in RGO treated cells. In addition, it is found that, oxidative stress induced cytotoxicity reduces with a decreasing extent of oxygen functional group

density on the RGO surface. They suggested that oxidative stress mediates the cellular toxicity of GO and that although size of the GO sheet plays a role, the functional group density on the GO sheet is one of the key components in mediating cellular cytotoxicity.

Mao et al. [39a] combined large-scale computer simulations, theoretical analysis, and experimental discussions in a systematic study on the interactions of graphene nanosheets having various oxidization degrees with a model lipid bilayer membrane. In the mesoscopic simulations, they investigate the detailed translocation pathways of these materials across a 56 x 56 nm^2 membrane patch considering the role of membrane perturbation during this process. A phase diagram regarding the transmembrane translocation mechanisms of graphene nanosheets is thereby obtained in the space of oxidization degree and particle size. They find that a graphene nanosheet with a higher oxidization degree can induce a larger scale of irregular membrane perturbation destroying the integrity of the membrane, and that the perturbation degree of membrane increases with increasing the edge length of nanosheet. Theoretical analysis reveals that the endocytosis process is more likely to occur for the graphene nanosheet taking hemisphere vesicle structure through phagocytosis while the cellular endocytosis seems to be more difficult to occur for the graphene nanosheet sandwiched within the membrane.

Chng and Pumera [40a] investigated the cytotoxicity of the GOs prepared by four different top-down route oxidative treatments, which include the usage of concentrated H_2SO_4, by measuring the mitochondrial activity in adherent lung epithelial cells (A549) by using commercially available viability assays. The dose–response data was generated by using two assays, the methylthiazolyldiphenyl-tetrazolium bromide (MTT) assay and the watersoluble tetrazolium salt (WST-8). From the viability data, it is evident that there is a strong dose-dependent cytotoxic response resulting from the four GO nanomaterials tested after a 24 h exposure, and it is suggested that there is a correlation between the amounts of oxygen content/functional groups of GOs with their toxicological behavior towards the A549 cells.

Yoon et al. [41a] evaluate the cytotoxicity of graphene nanoflakes by cell-based electrochemical sensing of extracellular H_2O_2 with a nanocomposite-modified indium tin oxide (ITO) electrode. The ITO working electrode was modified with a nanocomposite film of Nafion and chemically-driven graphene (G) using the electrospray method. Quantitative sensing of extracellular H_2O_2 released from HeLa cells treated with different sizes and concentrations of G nanoflakes was performed by cyclic voltammetry, and optical bioassays were also used to complement the electrochemical measurements. The H_2O_2 concentrations after treatment by nanoflakes with different concentrations continuously increased up to 1 M, 20-fold higher than the H_2O_2 concentration of about 50 mM for untreated cells after 24 h. The results also showed that the increased tendency in measured H_2O_2 concentra-

tion as the concentration of the G nanoflakes increased was consistent with the results of the toxicity analysis data obtained by optical bioassays and increased further for smaller G size.

Khim Chng et al. [42a] compared the cytotoxicity of the highly hydrogenated graphene (HHG), with that of GO *in vitro*. In their results, HHG was discovered to be cytotoxic towards A549 cells across a wide range of concentrations tested; and at the same time it is much more cytotoxic than GO. They hypothesize that the hydrophobic nature as a result of the saturated carbon bonds in highly hydrogenated graphene plays a key role in its cytotoxicity by means of the adsorption of micronutrients from cell media through hydrophobic forces. In contrast, the presence of hydrophilic oxygen-containing groups on graphene oxide served as mediators for its cytotoxicity by reducing the effective hydrophobic domains for the adsorption of micronutrients.

iv) Other carbon MNMs

Dworak et al. [43a] evaluated genotoxic and mutagenic potential of nanodiamond (particle size <10 nm) in human lymphocytes in vitro. Starting from concentration of 50 µg/ml, diamond nanopowder was cytotoxic, inhibited cell proliferation and induced apoptotic cell death. Nanodiamond associated oxidative stress was revealed, the effect was dose-dependent and statistically significant. Nanodiamond-mediated DNA oxidative damage (8-oxoG level) and changes in chromatin stability was observed even at as low as 1 µg/ml concentration, which was especially seen for DNA single strand breaks estimated with alkaline comet assay. In contrast, no induction of DNA double strand breaks (neutral comet assay) was documented after nanodiamond treatment. Nanodiamond at concentration of 10 µg/ml was able to stimulate micronuclei production.

b) Metal oxides

I) ZnO

Fernández-Cruz et al. [44a] study aimed at evaluating the cytotoxic effect of micro- and nanosized ZnO in a fish and a mammalian hepatoma cell line. A detailed characterization of the particles in exposure media showed that ZnO NPs formed large aggregates. ZnO cytotoxicity was evaluated with a battery of in vitro assays including LUCS, a new approach based on DNA alteration measurements, which provides extra information regarding cellular events. In fish cells, ZnO NP aggregates contributed substantially to the cytotoxic effects whereas toxicity in the human cells appeared to be mainly produced by the dissolved fraction. The results from this study also indicated that ROS production is not the main factor causing cytotoxicity of ZnO particles, but cellular damage at the mitochondrial and DNA levels. Finally they suggest that in order to have realistic conditions which consider all possible mechanisms of action, experiments involving exposure to ZnO NPs in

aqueous suspensions should be performed without homogeneous dispersion of the NPs and measuring concentrations of NPs is essential to understand the mechanisms underlying their toxicity.

Zhang et al. [45a] investigated the cytotoxicity of two ZnO-NPs products (Z-COTE and Z-COTE HP1). As all samples were highly agglomerated they demonstrated that agglomerated ZnO-NPs had toxic effects on mammalian cells, and this effect was dependent on the ZnO concentration and the cell line used dose-dependent cytotoxicity was observed 24 h after exposure to ZnO NPs, and monocytes were more sensitive than lung epithelial cells or lymphoblasts in both human and mouse cells. There was a significant difference in cytotoxicity between nano- and fine-forms, but only at the threshold cytotoxic dose with cellular metabolism assays. Compared to uncoated ZnO-NPs, the surface coating with triethoxycaprylylsilane marginally attenuated cellular oxidative stress and protected cellular metabolic activity.

Hsiao and Huang [46a] evaluate the influence of serum—in this case, fetal bovine serum—in a cell culture medium on the toxicity of nano-sized (50–70 nm) ZnO on human lung epithelial cells (A549). The nano-sized ZnO exhibited their highest toxicity when exposed to serum-free media, in contrast to exposure in media containing 5 or 10 % serum. This mainly comes not only from the fact that ZnO particles in the serum-free media have a higher dosage-per-cell ratio, which results from large aggregates of particles, rapid sedimentation, absence of protein protection, and lower cell growth rate, but also that extracellular Zn^{2+} release contributes to cytotoxicity. Although more extracellular Zn^{2+} release was observed in serum-containing media, it did not contribute to nano-ZnO cytotoxicity. Furthermore, non-dissolved particles underwent size-dependent particle agglomeration, resulting in size-dependent toxicity in both serum-containing and serum-free media. A low correlation between cytotoxicity and inflammation endpoints in the serum-free medium suggested that some signaling pathways were changed or induced. Since cell growth, transcription behavior for protein production, and physicochemical properties of ZnO particles all were altered in serum-free media, they recommend the use of a serum-containing medium when evaluating the cytotoxicity of NPs.

Gümüş et al. [47a] investigate possible genotoxic effects of ZnO nanoparticles in cultured human peripheral lymphocytes by using chromosome aberrations and micronucleus assays (MN). ZnO induced significant increase of the ratio of chromosomal aberrations as well as percentage of abnormal cells at concentrations of 1, 5, 10 and 20 µg/ml in 24 h treatments. In 48 h treatments, while ZnO nanomaterials induced significant increase of the percentage of abnormal cells only at a concentration of 10 µg/ml, and of chromosome aberration per cell in comparison to the control at concentrations of 5 and 10 µg/ml. On the other hand, this material significantly increased the micronuclei frequency (MN) at concentrations of 10 and 15 µg/ml in comparison to the control. Cytokinesis- block proliferation index was not affected by ZnO treatments. It also decreased the

mitotic index in all concentrations at 24 h but not at 48 h. The present results indicate that ZnO nanoparticles are clastogenic, mutagenic and cytotoxic to human lymphocytes *in vitro* at specific concentrations and time periods.

Kwon et al. [48a] evaluated the genotoxicity of four kinds of ZnO nanoparticles: 20 nm and 70 nm size, positively or negatively charged. Four different genotoxicity tests (bacterial mutagenicity assay, *in vitro* chromosomal aberration test, *in vivo* comet assay, and *in vivo* micronucleus test, were conducted, following Organization for Economic Cooperation and Development (OECD) test guidelines with good laboratory practice (GLP) procedures. No statistically significant differences from the solvent controls were observed. These results suggest that surface-modified ZnO nanoparticles do not induce genotoxicity in *in vitro* or *in vivo* test systems. However they indicate the possibility of genotoxicity at lower doses than those used in their study. Since nanoparticles can be agglomerated at high dose. Furthermore, there may be a possibility that different exposure route or long-term exposure duration can induce genotoxicity in different kinds of organs.

Valdiglesias et al. [49a] studied the cytotoxic and genotoxic effects of ZnO NPs on human SHSY5Y neuronal cells under different exposure conditions. Results obtained by flow cytometry showed that ZnO NPs do not enter the neuronal cells, but their presence in the medium induced cytotoxicity, including viability decrease, apoptosis and cell cycle alterations, and genotoxicity, including micronuclei production, H2AX phosphorylation and DNA damage, both primary and oxidative, on human neuronal cells in a dose- and time-dependent manner. Free Zn^{2+} ions released from the ZnO NPs were not responsible for the viability decrease, but their role on other types of cell damage cannot be ruled out.

Sahu et al. [50a] in their study investigate the effect of particles size (nano and micro) of ZnO on viability, phagocytosis, and cytokine induction in human monocytes, THP-1 cells, a model of the innate immune system. Cells were incubated with nano (approximately 100 nm) and micro (approximately 5 µm) sized ZnO particles in a concentration range of 10–100 µg/ml. The parameters measured included the MTT assay, phagocytosis assay, enzyme-linked immunosorbent assay (ELISA), gene expression, and DNA analysis. ZnO particles of both sizes induced cytotoxicity and altered the phagocytic capacity with no marked effect on DNA in human monocytes. Exposure of THP-1 cells to both sizes of ZnO stimulated and increased release of proinflammatory cytokines interleukin (IL)-1β, tumor necrosis factor (TNF)-α, and IL-6, as well as chemokine IL-8, and upregulated the expression of monocyte chemoattractant protein-1 and cyclooxygenase-2 genes, thus indicating the potential of this material in induce inflammation. The nano ZnO particles demonstrated more potential to induce toxicity and inflammation than micro-size ZnO in THP-1 cells and increased with duration of exposure.

Talebi et al. [51a] evaluate the effects of ZnO nanoparticles on mouse spermatogenesis. Thirty two adult male NMRI mice were used. Thirty two adult male NMRI mice divided into four groups were used. Experimental Groups (ZNP-1-ZNP-3) received one of the following treatments daily for 35 days: 5, 50 and 300 mg/kg zinc oxide nanoparticles respectively while control group received only distilled water. Epididymal sperm parameters, testicular histopathology, morphometric analysis and spermatogenesis assessments were performed for evaluation of the zinc oxide nanoparticles effects on testis. The results established that ZnO nanoparticles have cytotoxic actions on testicular germ cells in a dose dependent manner. The multinucleated giant cell formation and sloughing of immature germ cells from the seminiferous tubules indicates that these NPs might also affect Sertoli cell functions. Alterations in the Johnsen's scoring and morphometric studies may relate to induction of apoptosis or autophagy in testicular germ cells.

Boutard et al. [52a] carried out *in vitro* cytotoxicity of various ZnO commercial sunscreens using keratinocytes NCTC2544 cell line, which is the first portal of entry for such nanoparticles through sunscreens products. Assessment of cell viability demonstrated that all tested ZnO commercial sunscreens reduce cell viability in a dose-and time-dependent manner.

II) CuO

Semisch et al. [53a] explored the extra- and intracellular bioavailability of CuO NP and CuO MP. In addition, different endpoints related to cytotoxicity as well as direct and indirect genotoxicity of the copper oxides and copper chloride ($CuCl_2$) were compared. The samples of CuO NP and CuO MP were analyzed regarding their copper ion release in model fluids. In all media investigated, CuO NP released far more copper ions than CuO MP, with most pronounced dissolution in artificial lysosomal fluid. CuO NP and $CuCl_2$ caused a pronounced and dose dependent decrease of colony forming ability (CFA) in A549 and HeLa S3 cells, whereas CuO MP exerted no cytotoxicity at concentrations up to 50 µg/mL. Cell death induced by CuO NP was at least in part due to apoptosis, as determined by subdiploid DNA as well as via translocation of the apoptosis inducing factor (AIF) into the cell nucleus. Similarly, only CuO NP induced significant amounts of DNA strand breaks in HeLa S3 cells, whereas all three compounds elevated the level of H2O2-induced DNA strand breaks. Finally, all copper compounds diminished the H2O2-induced poly(ADP-ribosyl)ation, catalyzed predominantly by poly(ADP-ribose)polymerase-1 (PARP-1); here, again, CuO NP exerted the strongest effect. Copper derived from CuO NP, CuO MP and $CuCl_2$ accumulated in the soluble cytoplasmic and nuclear fractions of A549 cells, yielding similar concentrations in the cytoplasm but highest concentrations in the nucleus in case of CuO NP. For these differences in cytotoxicity, extracellular copper ion levels due to dissolution of particles as well as differences in physicochemical properties of the particles like surface area may be of major relevance. Regarding direct and indirect

genotoxicity, especially the high copper content in the cell nucleus derived after cell treatment with CuO NP appears to be decisive.

Di Bucchianico et al. [54a] carried out a series of cytotoxic and genotoxic responses following in vitro exposure to differently shaped CuO nanoparticles (mass concentrations from 0.1 to 100 µg/ml) in murine macrophages RAW 264.7 and in peripheral whole blood from healthy volunteers. Cytotoxicity, cytostasis and genotoxicity were evaluated by the colorimetric assay of formazan reduction [3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT)] and by the cytokinesis-block micronucleus cytome (CBMN Cyt) assay. The comet assay was applied for detecting DNA strand breaks and information on oxidative damage to DNA (oxidised purines and pyrimidines). The MTT assay revealed a decrease in cell viability in RAW 264.7 cells and peripheral blood lymphocytes (PBL) with significant dose–effect relationships for the different CuO NP shapes. The comet assay revealed a dose-dependent increase in primary DNA damage, and a significant increase in oxidative damage to DNA was also detectable, as well as increased frequency of micronuclei in binucleated cells, often in a dose-related manner. Proliferative activity, cytotoxicity and apoptotic markers showed a significant trend in the two cell types. Finally, they have differentiated clastogenic events from aneugenic events by fluorescence in situ hybridisation with human and murine pancentromeric probes, revealing for the first time characteristic aneugenic responses related to the shape of CuO NPs and cell type.

III) MnO_2

S.P. Singh et al. [55a] investigated the acute oral toxicity of MnO_2 NMs and MnO_2-bulk particles in female albino Wistar rats. The genotoxic effects were examined using comet, micronucleus and chromosomal aberration assays. Nanosized MnO_2 (45 nm) significantly ($p < 0.01$) increased DNA damage in peripheral blood leukocytes and micronuclei and enhanced chromosomal aberrations in the bone marrow cells at 1000 mg/kg bw. These findings showed that the neurotoxicity of MnO2-45 nm in the brain and red blood cells, as determined through acetylcholinesterase activity, was significantly ($p < 0.01$) inhibited at 1000 and 500 mg/kg bw doses. MnO2-45 nm disrupted the physicochemical state and neurological system of the animals through alterations in ATPases via the total Na^+–K^+, Mg^{2+} and Ca^{2+} levels in the brain P_2 fraction. In addition, 500 and 1000 mg/kg bw doses of MnO_2-45 nm caused significant changes in AST, ALT and LDH levels in the liver, kidney and serum of treated rats. Significant tissue distribution was found in all tissues in a dose- and time-dependent manner. MnO_2-45 nm exhibited much higher absorptivity and tissue distribution compared with MnO_2-bulk. A large fraction of MnO_2-45 nm was cleared in the urine and feces. The histopathological analysis revealed that MnO_2-45 nm caused alterations in the liver, spleen and brain.

IV) CeO_2

Demokritou et al. [56a] present an integrative study evaluating the toxicity of nanoscale CeO_2 both *in vitro*, using the A549 lung epithelial cell line, and *in vivo* using an intact rat model. Realistic nano-ceria exposure atmospheres were generated using the Harvard Versatile Engineered Nanomaterial Generation System (VENGES), and rats were exposed via inhalation. The *in vitro* results of this study suggest that CeO_2 nanoparticles are relatively non-toxic. However, results from the inhalation experiments show lung injury and inflammation with increased PMN and LDH levels in the bronchoalveolar lavage fluid of the CeO_2-exposed rats. Discrepancies between *in vitro* and *in vivo* toxicity evaluations may be attributed to particle transformations in the micro-environments of cell culture media enriched with serum proteins versus the alveolar lining fluid of the animal lung. MNMs suspended in physiological media may experience agglomeration and the formation of a protein corona on the particle, and these transformations may mediate subsequent bio-nano interactions. Future research will be necessary to determine just how much the protein corona and particle kinetics observed in an *in vitro* system can influence toxicity. One hypothesis to be investigated in future studies is whether distinct protein-particle interactions in cell culture media differ from those of alveolar lining fluid found in the animal lungs. Such a difference may explain some of the different biological responses between *in vitro* and *in vivo* reported in this study. Another possible explanation for the observed discrepancies between *in vitro* and *in vivo* results might be that single cell line *in vitro* assays lack interactions among the many cell types forming the lung epithelial barrier. Such interactions among different cell types may induce inflammation, and elicit further damage to the epithelial cells which cannot be captured by a single cell line assay. Finally, the use of a nanothin amorphous SiO_2 encapsulation coating as a means of mitigating CeO_2 toxicity was assessed. Exposure to SiO_2-coated CeO_2 did not induce any pulmonary toxicity to the animals, representing clear evidence for the safe by design SiO_2-encapsualtion concept.

Ji et al. [57a] demonstrated that there is a relationship between the aspect ratio (AR) of CeO_2 nanoparticles and in vitro hazard potential. CeO_2 nanorods with AR ≥ 22 induced lysosomal damage and progressive effects on IL-1β production and cytotoxicity in the human myeloid cell line, THP-1. In order to determine whether this toxicological paradigm for long aspect ratio (LAR) CeO_2 is also relevant in vivo, they performed [58a] comparative studies in the mouse lung and gastrointestinal tract (GIT) of zebrafish larvae. Although oropharyngeal aspiration could induce acute lung inflammation for CeO_2 nanospheres and nanorods, only the nanorods with the highest AR (C5) induced significant IL-1β and TGF-β1 production in the bronchoalveolar lavage fluid at 21 days but did not induce pulmonary fibrosis. However, after a longer duration (44 days) exposure to 4 mg/kg of the C5 nanorods, more collagen production was seen with CeO_2 nanorods vs nanospheres after correcting for Ce lung burden. In addition, using an oral-exposure model in zebrafish larvae, they

demonstrated [58a] that C5 nanorods also induced significant growth inhibition, a decrease in body weight, and delayed vertebral calcification. In contrast, CeO_2 nanospheres and shorter nanorods had no effect. Histological and transmission electron microscopy analyses showed that the key injury mechanism of C5 was in the epithelial lining of the GIT, which demonstrated blunted microvilli and compromised digestive function.

Courbiere et al. [59a] performed in vitro assays on mature mouse oocytes incubated with CeO_2 NPs to study, the physicochemical biotransformation of NPs in culture medium, the ultrastructural interactions with follicular cells and oocytes using TEM and the genotoxicity of CeO_2 NPs on follicular cells and oocytes using a comet assay. DNA damage was quantified as Olive Tail Moment. They show that CeO_2 NPs aggregated, but their crystal structure remained stable in culture medium. TEM showed endocytosis of CeO_2 NP aggregates in follicular cells. In oocytes, CeO2 NP aggregates were only observed around the zona pellucida (ZP). The comet assay revealed significant DNA damage in follicular cells. In oocytes, the comet assay showed a dose-related increase in DNA damage and a significant increase only at the highest concentrations. DNA damage decreased significantly both in follicular cells and in oocytes when an anti-oxidant agent was added in the culture medium. They hypothesize that at low concentrations of CeO_2 NPs oocytes could be protected against indirect oxidative stress due to a double defense system composed of follicular cells and ZP.

Ould-Moussa et al. [60a] examined the interactions between 7 nm surface-modified anionically charged cerium oxide particles and living mammalian cells. The modification of the particle coating included low-molecular weight ligands and polymers. Two generic behaviors are compared: particles coated with citrate ions that precipitate in biofluids and particles coated with poly(acrylic acid) that are stable and remain nanometric. The authors find that CeO_2 NPs covered with both coating agents are taken up by mouse fibroblasts and localized into membrane-bound compartments. However, flow cytometry and electron microscopy reveal that as a result of their precipitation, citrate-coated particles interact more strongly with cells. At cerium concentration above 1 mM, only citrate-coated nanoceria (and not particles coated with poly(acrylic acid)) display toxicity and moderate genotoxicity.

Boutard et al. [52a] carried out in vitro cytotoxicity of CeO_2 nanopowder using keratinocytes NCTC2544 cell line. They find that CeO_2 particles did not produce any effects on NCTC2544 growth at the highest concentration tested (80 mg/ml). In addition, no visual differences in the cell morphology of the exposed cells compared to the unexposed control were observed.

V) TiO_2

Roszak et al. [61a] assessed the cytotoxic and genotoxic potential of commercially available TiO_2 and TiO_2/Ag MNMs in pristine form as well as polypropylene fibers modified with the MNMs.

Both TiO_2 MNMs showed a cytotoxic effect on BALB/3T3 clone A31 and V79 fibroblasts after 72-h exposure. Both MNMs induced a weak genotoxic effect in comet assay, with TiO_2/Ag being more active. In vitro micronucleus test on human lymphocytes revealed a weak mutagenic effect of both materials after 24 h of exposure. In contrast, no significant increase in micronuclei frequency was observed in the in vitro micronucleus test on V79 fibroblasts. The 24-h extracts prepared from polypropylene fibers modified with TiO_2/Ag induced a cytotoxic effect on BALB/3T3 cells which strongly depended on the mode of the fibers manufacturing.

Park et al. [62a] compared *in vivo* and *in vitro* based on the physicochemical properties the toxicity of three different types of TiO_2 nanowires, $H_2Ti_3O_7$ nanowires (1HTO), hydrothermal treatment (2HTO), and calcination (3HTO) of 1HTO. On day 28 after a single instillation (1 mg/kg), the nanowires induced a Th2-type inflammatory response together with the relative increase in $CD4^+$ T cells, especially by 2HTO. *In vitro*, three TiO_2 nanowires (10 μg/ml) commonly induced the generation of cell debris in eight cell lines which may be the potential target organ of nanoparticles, especially by 2HTO. It seemed that the generation of cell debris coincides with the increase in autophagosome-like vacuoles in the cytosol. In further study using BEAS-2B cells originated from the lung, the protein amount from cells exposed to 2HTO decreased more clearly although the generation of ROS was less compared to 1HTO and 3HTO. Based on these results, they suggest that surface area may act as an important factor depends on the biological response by TiO_2 nanowires. Furthermore, the increase in autophagosome-like vacuoles may be an important cause of cell death by nanoparticles with ROS.

Ghosh et al. [63a] evaluated the toxic effect of commercially available TiO_2 nanoparticles (~100 nm) using a battery of cytotoxic, genotoxic, hemolytic and morphological parameters. The cytotoxic effects of TiO_2 nanoparticles in human lymphocyte cells were studied with respect to membrane damage, mitochondrial function, metabolic activity and lysosomal membrane stability. Genotoxicity in lymphocyte cells was quantitated using a comet assay. The mode of cell death (apoptosis/necrosis) was evaluated using PI/Annexin V staining. TiO_2 nanoparticles were also evaluated for their hemolytic properties, osmotic fragility and interaction with hemoglobin. Human erythrocyte cells were studied for morphological alterations using AFM. Results suggest that the particles could induce a significant reduction in mitochondrial dehydrogenase activity in human lymphocyte cells. Membrane integrity remained unaffected by nanoparticle treatment. DNA damage and apoptosis were induced by TiO_2 nanoparticles in a dose-dependent manner. A study on human erythrocyte cells revealed a hemolytic property of TiO_2 nanoparticles characterized by spherocytosis and echinocytosis. Spectral analysis revealed a hemoglobin TiO_2 nanoparticle interaction. Finally, the authors state that commercially available blood contacting TiO_2 nanoparticles should be carefully evaluated for their toxic potential.

George et al. [64a] investigated TiO_2 NPs potential toxicity to a fish cell line (BF-2) and zebrafish embryos under dark and Simulated Solar Light (SSL) exposure conditions. Using high throughput screening (HTS) platforms, they showed that the oxidative stress-dependent cytotoxicity and embryonic toxicity of NPs were significantly increased upon exposure to SSL. TiO_2 NPs were not toxic to BF2 cells and zebrafish embryos under dark. However, exposure to SSL induced dose-dependent increase in ROS generation, oxidative stress in cells, and mortality in zebrafish embryos. The induction of oxidative stress in BF2 cells were evidenced by an increased number of cells positive for mitochondrial superoxide generation and by the reduced cytotoxicity in NAC pretreated cells. Furthermore, they also found that SSL could alter the aggregation behavior and ζ potential of TiO_2 NPs. The role of SSL in elevating the toxic potential of TiO_2 NPs could be explained based on ROS generation.

Faria et al. [65a] examined sublethal oxidative stress effects of aqueous exposure to three different types of MNM-TiO_2 differing in their coating or crystal structure but of similar primary size (20 nm) plus a micron-sized bulk material to zebra fish embryos without and with SSL. The results evidenced a low bio-availability of MNM-TiO_2 to embryos with detrimental effects on growth at 1 mg/ml. Phototoxicity increased moderately, by 3 and 1.5 fold, under co-exposures to fluoranthene (100 µg/l) and to the NM-TiO_2 P25 (1 mg/ml), respectively, being unchanged in the other TiO_2 aggregates. *In vitro* exposures under SSL confirmed that the MNM-TiO_2 P25 had the highest potential to generate ROS. Antioxidant enzyme activities of superoxide dismutase increased shortly after exposure to the studied materials, whereas the levels of glutathione tend to be altered after longer exposures. All compounds were able to produce oxidative stress enhancing the senescence-associated β galactosidase pigment (SA-β-gal). Under SSL radiation the MNM-TiO_2 P25 affected antioxidant and oxidative stress responses as the phototoxic compound fluoranthene. These results indicated that despite the low bio-availability of MNM-TiO_2 to zebrafish embryos, P25 was phototoxic due to the production of reactive oxygen species.

Kim et al. [66a] investigate the effect of particle size on developing zebrafish exposed to 6, 12 and 15 nm citrate-functionalized anatase TiO_2 NPs under either SSL or in the dark. All three sizes of TiO_2 NPs caused photo-dependent toxicity. Under SSL, the acute toxicity of the 6 nm citrate-TiO_2 NPs (120 h LC_{50} of 23.4 mg/l) exceeded that of the 12 and 15 nm citrate-TiO_2 NPs. Exposure to 6 nm particles under illumination also caused a higher incidence of developmental defects than the larger particles. These abnormalities included pericardial edema, yolksac edema, craniofacial malformation, and opaque yolk. To gain insight into the mechanisms of toxicity, they measured hydroxyl radicals (cOH) generated by NPs in vitro and ROS produced in vivo. They found that on a mass basis, smaller particles generated higher levels of ROS both in vitro and in vivo, and the 6 nm citrate-TiO_2 NPs induced more oxidative stress than larger particles in the zebrafish embryo. They also examined

oxidative DNA damage by measuring 8-hydroxydeoxyguanosine in zebrafish exposed to different-sized citrate-TiO_2 NPs and found that 6 nm particles caused more DNA damage than did larger particles (12 and 15 nm) under illumination. These results indicate a photodependent toxicity of citrate-TiO_2 NPs to zebrafish embryos, with an inverse relationship between particle size and toxicity. Production of more ROS, resulting in more oxidative stress and more DNA damage, represents one possible mechanism of the higher toxicity of smaller citrate-TiO_2 NPs.

Sekar et al. [67a] compared the toxicity of two different families of TiO_2 nanoparticles on erythrocytes from *Oncorhynchus mykiss* trout. The toxicity results indicate that both TiO_2 nanoparticles increase the hemolysis rate in a dose dependent way (1.6, 3.2, 4.8 μg/ml) but they do not influence superoxide anion production due to NADH addition measured by chemiluminescence. Moreover, TiO_2 nanoparticles (4.8 μg/ml) induce DNA damage and the entity of the damage is independent from the type of TiO_2 nanoparticles used. Modified comet assay (Endo III and Fpg) shows that TiO_2 oxidizes not only purine but also pyrimidine bases. However, in these experimental conditions, the exposure to TiO_2 nanoparticles does not affect the DNA repair system functionality.

Wei et al. [68a] investigated the possible adverse effects of TiO_2 NPs on human lung epithelium cells (A549) and to investigate NP size-dependent effects on these cells, considering both the primary and hydrodynamic particle size. NPs were found to inhibit cell viability and proliferation at the highest concentration level (10 mg/ml) included in this study, as measured by a clonogenic assay. Moreover, cell viability, proliferation and metabolism were impaired to a greater extent by the smaller NPs (5nm TiO_2) relative to the larger particles (200nm TiO_2) included in this study, as measured by cell proliferation and metabolism. Notably, the observed cytotoxic effects correlated to the primary size, rather than the hydrodynamic size. Similarly, NP cytotoxicity was found to be correlated with the NP surface area.

Botelho et al. [69a] explored the effects of different doses of TiO_2 nanoparticles on human gastric epithelial cells *in vitro*. They analyzed proliferation by MTT assay, apoptosis by Tunel, migration by injury assay, oxidative stress by determining GSH/GSSG ratio and DNA damage by Comet assay on nanoparticle-treated AGS human gastric epithelial cell line in comparison to controls. They show tumor-like phenotypes of nanoparticles-exposed AGS cells *in vitro*, as increased proliferation and decreased apoptosis.

Chen et al. [70a] evaluated the genotoxicity of TiO_2 NPs using *in vivo* and *in vitro* test systems. *In vivo* study, the adult male Sprague-Dawley rats were exposed to anatase TiO_2 NPs (75 ± 15 nm) through intragastric administration at 0, 10, 50 and 200 mg/kg body weight every day for 30 days. The γ-H2AX assay showed TiO_2 NPs could induce DNA double strand breaks in bone marrow cells after oral administration. However, the micronucleus test revealed that the oral-exposed TiO_2 NPs did not cause damage to chromosomes ormitotic apparatus observably in rat bone marrow cells. In

vitro study, Chinese hamster lung fibroblasts (V79 cells) were exposed to TiO_2 NPs at the dose of 0, 5, 10, 20, 50 and 100 µg/ml. Significant decreases in cell viability were detected in all the treated groups after 24 h and 48 h exposure. Significant DNA damage was only observed at the concentration of 100 µg/ml after 24 h treatment using the comet assay. The obvious gene mutation was observed at the concentration of 20 and 100 µg/mL after 2 h treatment using hypoxanthine-guanine phosphoribosyl transferase (HPRT) gene mutation assay.

Hamzeh and Sunahara [71a] investigated the cytotoxicity and genotoxicity of commercially available TiO_2 nanoparticles with respect to their selected physicochemical properties, as well as the role of surface coating of these nanoparticles. While all types of tested TiO_2 samples decrease cell viability in a mass-based concentration- and size-dependent manner, the polyacrylate-coated nano-TiO_2 product was only cytotoxic at higher concentrations. A similar pattern of response was observed for induction of apoptosis/necrosis, and no DNA damage was detected in the polyacrylate-coated nano-TiO_2 model.

Jin et al. [72a] studied the cytotoxicity of homogeneous and weakly aggregated (< 100 nm) TiO_2 nanoparticles by analyzing the changes in metabolite profiles both in mouse fibroblast (L929) cells and their corresponding culture media using gas chromatograph with a time-of-flight mass spectrometry (GC/TOFMS)-based metabolomic strategy. Based on the variable importance in the OPLS-DA models, a series of differential metabolites were identified by comparison between TiO_2 nanoparticle-treated L929 cells or their corresponding culture media and the control groups. It was found that the major biochemical metabolism (carbohydrate metabolism) was suppressed in TiO_2 nanoparticle-treated L929 cells and their corresponding culture media.

Shukla et al. [73a] showed that TiO_2 NPs induce DNA damage ($p < 0.05$) and cause apoptosis in HepG2 cells even at very low concentrations (Fpg-Comet assay at 1 mg/ml). A corresponding increase in the micronucleus frequency was also observed. This could be attributed to the reduced glutathione levels with concomitant increase in lipid peroxidation and reactive oxygen species generation. Furthermore, immunoblot analysis revealed an increased expression of p53, BAX, Cyto-c, Apaf-1, caspase-9 and caspase-3 and decreased the level of Bcl-2 thereby indicating that apoptosis induced by TiO_2 NPs occurs via the caspase-dependent pathway.

Al-Jubory and Handy [74a] demonstrated Ti uptake across the intestine from TiO_2 NP exposures, involving CO_2-dependent and nystatin sensitive mechanisms. In vitro gut sac preparations and isolated perfused intestines were used to investigate the absorption mechanism(s). Exposure of whole gut sacs to 1 mg/l TiO_2 NPs for 4 h caused total Ti metal concentrations to increase in the intestine, with 80% or more of the Ti in the mucosa. Perfused intestines showed a saturable time-dependent accumulation of total Ti, which increased when the CO_2 in the gas mixture was lowered to 0.5%. Adding cyanide did not stop Ti uptake, and 100 µmol/l vanadate (ATPase inhibitor) caused a

2.8-fold reduction in the net uptake rate of Ti for TiO_2 NP exposure. Luminal additions of nystatin (endocytosis inhibitor), blocked the uptake of Ti from both bulk and TiO_2 NP treatments. Notably, the gas mixture effect observed in the present study, adds a new dimension to hazard assessment not previously considered for MNMs.

Chang et al. [75a], using meta-analysis of in vitro and short-time animal studies, investigated the toxicity of nano-TiO_2 and its potential harmful impact on human health. Data were retrieved according to included and excluded criteria from 1994 to 2011. The combined toxic effects of nano-TiO_2 were calculated by the different endpoints by cell and animal models. From analysis of the experimental studies, more than 50% showed positive statistical significance except the apoptosis group, and the cytotoxicity was in a dose-dependent but was not clear in size-dependent manner. Nano-TiO_2 was detained in several important organs including the liver, spleen, kidney, and brain after entering the blood through different exposure routes, but the coefficient of the target organs was altered slightly from animal models. It is possible that nano-TiO_2 can induce cell damage related to exposure size and dose.

c) Metal nanoparticles

I) Gold NPs

Chueh et al. [76a] described the impacts of AuNPs in various mammalian cell models using an automatic and dye-free method for continuous monitoring of cell growth based on the measurement of cell impedance. AuNPs induced a concentration-dependent decrease in cell growth. This inhibitory effect was associated with apoptosis induction in Vero cells but not in MRC-5 or NIH3T3 cells. Interestingly, cDNA microarray analyses in MRC-5 cells supported the involvement of DNA damage and repair responses, cell-cycle regulation, and oxidative stress in AuNP-induced cytotoxicity and genotoxicity. Moreover, autophagy appeared to play a role in AuNPs-induced attenuation of cell growth in NIH3T3 cells.

Chuang et al. [77a] evaluated the biological effects of gold nanoparticles in six different mammalian cell lines (AGS (human gastric adenocarcinoma cells), A549 (human lung adenocarcinoma epithelial), NIH3T3 (mouse embryonic fibroblast), PK-15 (porcine kidney), Vero (African green monkey kidney) and MRC5 (human normal lung tissue) cells). Among the six cell lines, gold nanoparticles induced a dose-dependent suppression of cell growth with different levels of severity and the suppressive effect of gold nanoparticles was indirectly associated with their sizes and cellular uptake. Mechanistic studies revealed that the action of gold nanoparticles is mediated by apoptosis induction or cell cycle delay, depending on cell type and cellular context. Although redox signaling is often linked to the toxicity of nanoparticles, in this study, they found that gold nanoparticle-mediated reac-

tive oxygen species generation was not sustained to notably modulate proteins involved in antioxidative defense system.

Buzulukov et al. [78a] studied the bioaccumulation of gold (Au) NPs with mean sizes of 6 nm, after their intragastric administration to rats at a dose of 100 µg/kg of body weight for 28 or 14 days. The organs and tissues (liver, kidney, spleen, heart, gonads, brain, and blood) were subjected to thermal neutron activation, and, then, the activity of the ^{198}Au isotope generated was measured. The NPs of were detected in all biological samples studied, the highest specific weight and content of Au being found in the kidneys of the animals. The content Au NPs found in organs and tissues of rats could be regarded as nonhazardous (nontoxic) in accordance with the known literature data.

II) Silver NPs

Maurer et al. [79a] applied enhanced separation techniques with the aim to study levels of ions originating from the Ag NPs using separation by a recirculating tangential flow filtration (TFF) system. They thoroughly characterized NPs in biologically-relevant solutions to understand the dissolution of Ag NPs (10nm and 50 nm) over time. The results suggest that the ion dissolution from Ag NPs is dependent on parameters such as exposure time, chemical composition, and temperature of the exposure solution. Further, the well characterized separated ionic and NP solutions were exposed to a lung epithelial cell line (A549) to evaluate the toxicity of each fraction. The results suggest that although Ag NPs show concentration-dependent toxicity, dissolution of ions appears to exacerbate the toxicological effect. This finding adds data to the set of probable toxic exposure mechanisms elicited by metallic nanomaterials and provides important consideration when assessing findings of key cell function modulation.

Buzulukov et al. [78a] studied the bioaccumulation of Ag NPs with mean sizes of 35 nm, after their intragastric administration to rats at a dose of 100 µg/kg of body weight for 28 or 14 days. The organs and tissues (liver, kidney, spleen, heart, gonads, brain, and blood) were subjected to thermal neutron activation, and, then, the activity of the ^{110m}Ag isotope generated was measured. The NPs of were detected in all biological samples studied, the highest specific weight and content of Ag being found in the liver of the animals. The content of Ag NPs detected in the brain was 66.4 ± 5.6 ng (36 ng/g tissue), no more than 7% of these NPs being localized in the lumen of brain blood vessels. The content Ag NPs found in organs and tissues of rats could be regarded as nonhazardous (nontoxic) in accordance with the known literature data.

Lee et al. [80a] investigated the effects of Ag NPs on NIH 3T3. The results demonstrated that Ag NPs were taken up by NIH 3T3 cells and localized within the intracellular endosomal compartments. Ag NPs induced morphological and biochemical markers of autophagy in NIH 3T3 cells and induced autophagosome formation, as evidenced by TEM analysis, the formation of microtubule-associated

protein-1 light chain-3 (LC3) puncta and the expression of LC3-II protein. Thus, autophagy activation may be a key player in the cellular response against nano-toxicity.

George et al. [64a] investigated Ag NPs potential toxicity to a fish cell line (BF-2) and zebrafish embryos under dark and SSL exposure conditions. They showed that the oxidative stress-dependent cytotoxicity and embryonic toxicity of NPs were significantly increased upon exposure to SSL. The enhanced toxicity of Ag NPs under SSL exposure was due to surface oxidation and physicochemical modification of Ag NPs and shedding of Ag^+, leading to an increased bioavailability of silver.

Ávalos et al. [81a] studied how Ag NPs interact with four different human cell lines (hepatoma, leukemia, dermal and pulmonary fibroblast) in order to understand their impact on cellular biological functions. For toxicity evaluations, mitochondrial function (MTT assay) and membrane leakage of lactate dehydrogenase (LDH assay) were assessed under control and exposed conditions (24, 48 and 72 h of exposure). Results showed that mitochondrial function decreased in all cell lines exposed to Ag NPs at 6.72-13.45 µg/ml. LDH leakage also increased in all cell lines exposed to Ag NPs (6.72-13.45 µg/ml). These findings indicate that Ag NPs are cytotoxic on human tumor and normal cells, being the tumor cells more sensitive to the cytotoxic effect of Ag NPs.

Comfort et al. [82a] developed and implemented a chronic *in vitro* model coupled with lower, regulatory dosages in order to provide a more realistic assessment of NM-dependent consequences and illuminate the implications of long-term NM exposure. When keratinocytes were exposed to 50 nm Ag NPs, they determined that chronically dosed cells operated under augmented stress and modified functionality in comparison to their acute counterparts. Specifically, Ag NP exposure through a chronic mechanism increased p38 activation, actin disorganization, heightened ki67 expression, and extensive gene modification. Additionally, chronic Ag NP exposure altered the way in which cells perceived and responded to epidermal growth factor stimulation, indicating a transformation of cell functionality. Most importantly, this study demonstrated that chronic exposure in the pg/mL range to Ag-NPs did not induce a cytotoxic response, but instead activated sustained stress and signaling responses, suggesting that cells are able to cope with prolonged, low levels of Ag-NP exposure.

Massarsky et al. [83a] investigated the relationship between Ag NP cytotoxicity and oxidative stress and damage in rainbow trout (*Oncorhynchus mykiss*) hepatocytes and erythrocytes in comparison to silver ions (Ag^+). Generally the cytotoxicity of Ag NPs and Ag^+ was similar, such that both silver types generated reactive oxygen species, decreased glutathione levels, and decreased activities of glutathione reductase and glutathione-S-transferase. Nonetheless, the two silver types had different cellular targets; AgNPs increased lipid peroxidation without apparent uptake into the cells whereas Ag^+ increased DNA damage. Furthermore, the toxicity of both silver typeswas generally decreased in cells treatedwith cysteine while treatment with buthionine sulfoximine increased the toxicity of both silver types.

Monteiro-Riviere et al. [84a] quantitated human epidermal keratinocyte (HEK) uptake of Ag NP complexed to different human serum proteins. Prior to HEK dosing, Ag NP (20 nm and 110 nm citrate BioPureTM; 40 nm and 120 nm silica-coated) were preincubated for 2 h at 37 ^{o}C without (control) or with physiological levels of albumin (44 mg/ml), IgG (14.5 mg/ml) or transferrin (3 mg/ml) to form protein-complexed NP. Uptake of Ag in HEK was <4.1% of applied dose with proteins suppressing citrate, but not silica coated Ag uptake. IgG exposure dramatically reduced 110 nm citrate Ag NP uptake. In contrast, greatest uptake of 20 nm silica-Ag NP was seen with IgG, while 110 nm silica-Ag NP showed minimal protein effects. Electron microscopy confirmed cellular uptake of all NP but showed differences in the appearance and agglomeration state of the NP within HEK vacuoles. This work suggests that NP association with different serum proteins, purportedly forming different protein coronas, significantly modulates Ag uptake into HEK compared to native NP uptake, suggesting caution in extrapolating *in vitro* uptake data to predict behavior *in vivo* where the nature of the protein corona may determine patterns of cellular uptake, and thus biodistribution, biological activity and toxicity.

Yin et al. [85a] demonstrated that Ag NPs induce significant cellular toxicity to rat cerebellum granule cells (CGCs) in a dose-dependent manner without damaging the cell membrane. Flow cytometry analysis with the Annexin V/propidium iodide (PI) staining indicates that the apoptotic proportion of CGCs upon treatment with AgNPs is greatly increased compared to the negative control. Moreover, the activity of caspase-3 is largely elevated in Ag NP-treated cells compared to the negative control. Ag NPs are demonstrated to induce oxidative stress, reflected by the massive generation of reactive oxygen species (ROS), the depletion of antioxidant glutathione (GSH), and the increase of intracellular calcium. Histological examination suggests that Ag NPs provoke destruction of the cerebellum granular layer in rats with concomitant activation of caspase-3, in parallel to the neurotoxicity of Ag NPs observed *in vitro*.

S. Grosse et al. [86a] examined the cytotoxic effects of spherical, citrate-coated Ag NPs (10, 50 and 100 nm) in rat brain endothelial (RBE4) cells and investigated whether the observed effects can be explained by the intrinsic toxicity of the particles or the silver ions released from the particles. The results indicated that exposure of RBE4 cells to Ag NPs lead to significant reduction in dye uptake as measured with the Neutral red (NR) assay. The effect was found to be related to particle size, surface area, dose and exposure time. In contrast, silver ions increased NR uptake (ca. 10%) in RBE4 cells after 1 h, while a reduction in NR uptake was observed after 24 h exposure at high concentrations (20–30 µM). Finally, colony formation, as an indicator of proliferation ability, was completely inhibited by Ag NPs at concentrations higher than 1 µg/ml, while silver ions had less effect on the colony formation of RBE4 cells than Ag NPs.

Scanland et al. [87a] carried out a comprehensive evaluation of the physico-chemical stability of four silver nanowires (AgNWs) of two sizes and coatings and their toxicity to *Daphnia magna*. Inorganic aluminum-doped silica coatings were less effective than organic poly(vinyl pyrrolidone) coatings at preventing silver oxidation or Ag^+ release and underwent a significant morphological transformation within one-hour following addition to low ionic strength *Daphnia* growth media. All AgNWs were highly toxic to *D. magna* but less toxic than ionic silver. Toxicity varied as a function of AgNW dimension, coating and solution chemistry. Ag^+ release in the media could not account for observed AgNW toxicity. Single-particle inductively coupled plasma mass spectrometry (spICPMS) distinguished and quantified dissolved and nanoparticulate silver in microliter-scale volumes of *Daphnia magna* hemolymph with a limit of detection of approximately 10 ppb. The silver levels within the hemolymph of *Daphnia* exposed to both Ag+ and AgNW met or exceeded the initial concentrationin the growth medium, indicating effective accumulation during filter feeding. Silver-rich particles were the predominant form of silver in hemolymph following exposure to both AgNWs and Ag^+. SEM imaging of dried hemolymph found both AgNWs and silver precipitates that were not present in the AgNW stock or the growth medium. Both organic and inorganic coatings on the AgNW were transformed during ingestion or absorption. Pathway, gene ontology and clustering analyses of gene expression response indicated effects of AgNWs distinct from ionic silver on *Daphnia magna*.

Gliga et al. [88a] investigated size- and coating-dependent toxicity of thoroughly characterized AgNPs following exposure of human lung cells and to explore the mechanisms of toxicity. BEAS-2B cells were exposed to citrate coated Ag NPs of different primary particle sizes (10, 40 and 75 nm) as well as to 10 nm PVP coated and 50 nm uncoated Ag NPs. The particle agglomeration in cell medium was investigated by photon cross correlation spectroscopy (PCCS); cell viability by LDH and Alamar Blue assay; ROS induction by DCFH-DA assay; genotoxicity by alkaline comet assay and γH2AX foci formation; uptake and intracellular localization by TEM; and cellular dose as well as Ag release by atomic absorption spectroscopy (AAS). The results showed cytotoxicity only of the 10 nm particles independent of surface coating. In contrast, all Ag NPs tested caused an increase in overall DNA damage after 24 h assessed by the comet assay, suggesting independent mechanisms for cytotoxicity and DNA damage. However, there was no γH2AX foci formation and no increased production of intracellular ROS. The reasons for the higher toxicity of the 10 nm particles were explored by investigating particle agglomeration in cell medium, cellular uptake, intracellular localization and Ag release. Despite different agglomeration patterns, there was no evident difference in the uptake or intracellular localization of the citrate and PVP coated AgNPs. However, the 10 nm particles released significantly more Ag compared with all other Ag NPs (approx. 24 wt% vs. 4–7

wt%) following 24 h in cell medium. The released fraction in cell medium did not induce any cytotoxicity, thus implying that intracellular Ag release was responsible for the toxicity.

Hamilton et al. [89a] examined the effect of size (20 and 110 nm) and surface stabilization (citrate and PVP coatings) on toxicity, particle uptake and NLRP3 inflammasome activation in a variety of macrophage and epithelial cell lines. The results indicated that smaller Ag (20 nm), regardless of coating, were more toxic in both cell types and most active in the THP-1 macrophages. TEM imaging demonstrated that 20 nm Ag nanospheres dissolved more rapidly than 110 nm Ag nanospheres in acidic phagolysosomes consistent with Ag ion mediated toxicity. In addition, there were some significant differences in epithelial cell line *in vitro* exposure models. The order of the epithelial cell lines' sensitivity to Ag was LA4 > MLE12 > C10. The macrophage sensitivity to Ag toxicity was C57BL/6 AM > MARCO null AM, which indicated that the MARCO receptor was involved in uptake of the negatively charged Ag particles. These results support the idea that Ag nanosphere toxicity and NLRP3 inflammasome activation are determined by the rate of surface dissolution, which is based on relative surface area.

Leo et al. [90a] studied the stability of Ag NPs in dipalmitoylphosphatidylcholine (DPPC), the major component of pulmonary surfactant, was investigated as a function of pH. Spherical, citrate-capped Ag NPs with average diameters of 14 ± 1.6 nm (n=200) were prepared by a chemical bath reduction. The kinetics of Ag^+ ion release was strongly pH-dependent. After 14 days of incubation in sodium perchlorate ($NaClO_4$) or perchloric acid ($HClO_4$) solutions, the total fraction of Ag NPs dissolved varied from ~10 % at pH 3, to ~2 % at pH 5, with negligible dissolution at pH 7. A decrease in pH from 7 to 3 also promoted particle aggregation and coarsening. DPPC (100 mg/l) delayed the release of Ag^+ ions, but did not significantly alter the total amount of Ag+ released after two weeks. In addition, DPPC improved the dispersion of the Ag NPs and inhibited aggregation and coarsening. TEM images revealed that the Ag NPs were coated with a DPPC layer serving as a semi-permeable layer. Hence, lung lining fluid, particularly DPPC, can modify the aggregation state and kinetics of Ag+ ion release of inhaled Ag NPs in the lung. These observations have important implications for predicting the potential reactivity of AgNPs in the lung and the environment.

d) Silica

Athinarayanan et al. [91a] investigated the morphology and dimensions of silica (E551) particles in food. The silica content of commercial food products was determined using inductively coupled plasma optical emission spectrometry. The result indicates that 2.74–14. 45 µg/g silica was found in commercial food products; however, the daily dietary intake in increase causes adverse effects on human health. E551 was isolated from food products and the morphology, particle size, crystalline nature, and purity of the silica particles were analyzed using XRD, FTIR, TEM, EDX and DLS.

The results of these analyses confirmed the presence of spherical silica nanoparticles (of amorphous nature) in food, approximately 10–50 nm in size. The effects of E551 on human lung fibroblast cell viability, intracellular ROS levels, cell cycle phase, and the expression levels of metabolic stressresponsive genes (CAT, GSTA4, TNF, CYP1A, POR, SOD1, GSTM3, GPX1, and GSR1) were studied. The results suggest that E551 induces a dose-dependent cytotoxicity and changes in ROS levels and alters the gene expression and cell cycle. Treatment with a high concentration of E551 caused significant cytotoxic effects on WI-38 cells.

Ha et al. [92a] performed extensive *in vitro* safety profiling of ~50 nm spherical silica nanoparticles with OH-terminated or Polyethylene Glycol decorated surface, with and without a magnetic core, and synthesized by the Stöber method. Nineteen different cell lines representing all major organ types were used to investigate an in vitro lethal concentration (LC) and results revealed little toxicity in any cell type analyzed. To calculate an *in vitro* therapeutic index they quantified the effective concentration at 50% response (EC50) for nanoparticle-stimulated mineral deposition activity using primary bone marrow stromal cells (BMSCs). The EC50 for BMSCs was not substantially altered by surface or magnetic core. The calculated Inhibitory concentration 50% (IC50) for pre-osteoclasts was similar to the osteoblastic cells.

Duan et al. [93a] evaluated the cardiovascular effects of silica nanoparticles *in vitro* and *in vivo*. *In vivo* study, mortality, malformation, heart rate and whole-embryo cellular death were measured in zebrafish embryos. Results showed that silica nanoparticles induced pericardia toxicity and caused bradycardia. They also examined the expression of cardiovascular-related proteins in embryos by western blot analysis. Silica nanoparticles inhibited the expression of p-VEGFR2 and p-ERK1/2 as well as the downregulation of MEF2C and NKX2.5, revealed that silica nanoparticles could inhibit the angiogenesis and disturb the heart formation and development.

Lankoff et al. [94a] studied the kinetics of cellular binding/uptake of the vinyl- and the aminopropyl/vinyl-modified silica nanoparticles into peripheral blood lymphocytes in vitro, to explore their genotoxic and cytotoxic properties and to compare the biological properties of modified silica nanoparticles with those of the unmodified ones. The size of nanoparticles varied from 10 to 50 nm. Surface-modified silica particles were internalized by lymphocytes with varying efficiency and expressed no cytotoxic nor genotoxic effects, as determined by various methods (cell viability, apoptosis/necrosis, oxidative DNA damage, chromosome aberrations). However, they affected the proliferation of the lymphocytes as indicated by a decrease inmitotic index value and cell cycle progression. In contrast, unmodified silica nanoparticles exhibited cytotoxic and genotoxic properties at high doses as well as interfered with cell cycle.

Okoturo-Evans et al. [95a] investigated the effects of amorphous nano-SiO_2 particles on A549 lung epithelial cells using proteomics to understand the interactions that occur and the biological

consequences of exposure of lung to nanoparticles. Suitable conditions for treatment, where A549 cells remained viable for the exposure period, were established by following changes in cell morphology, flow cytometry, and MTT reduction. Label-free proteomics was used to estimate the relative level of proteins from their component tryptic peptides detected by mass spectrometry. It was found that A549 cells tolerated treatment with 100 µg/ml nano-SiO2 in the presence of 1.25% serum for at least 4 h. After this time detrimental changes in cell morphology, flow cytometry, and MTT reduction were evident. Proteomics performed after 4 h indicated changes in the expression of 47 proteins. Most of the proteins affected fell into four functional groups, indicating that the most prominent cellular changes were those that affected apoptosis regulation (e.g. UCP2 and calpain-12), structural reorganisation and regulation of actin cytoskeleton (e.g. PHACTR1), the unfolded protein response (e.g. HSP 90), and proteins involved in protein synthesis (e.g. ribosomal proteins). Treatment with just 10 µg/ml nano-SiO_2 particles in serum-free medium resulted in a rapid deterioration of the cells and in medium containing 10% serum the cells were resistant to up to 1000 µg/ml nano-SiO_2 particles, suggesting interaction of serum components with the nanoparticles. A variety of serum proteins were found which bound to nano-SiO2 particles, the most prominent of which were albumin, apolipoprotein A-I, hemoglobin, vitronectin and fibronectin.

Guidi et al. [96a] investigated the cytotoxic and genotoxic potential of silica particles of different sizes (250 and 500 nm) and structures (dense (DS), and mesoporous (MCM-41)). Murine macrophages (RAW264.7) and human epithelial lung (A549) cell lines were selected for investigation. Genotoxicity was evaluated by Comet assay and micronucleus test. Cytotoxicity was tested by the trypan blue method. Cells were treated with 0, 5, 10, 20, 40 and 80 µg/cm2 of different silica powders for 4 and 24 h. Amorphous particles penetrated into the cells, being compartmentalised within endocytic vacuoles. DS and MCM-41 particles induced cytotoxic and genotoxic effects in A549 and RAW264.7 although to different extent in the two cell lines. A549 were resistant in terms of cell viability, but showed a generalized induction of DNA strand breaks. RAW264.7 were susceptible to amorphous silica exposure, exhibiting both cytotoxic and genotoxic responses as DNA strand breaks and chromosomal alterations. The cytotoxic response of RAW264.7 was particularly relevant after MCM-41 exposure.

Andrade et al. [97a] studied the functionalized (using two different silanizing agents: 3-amino-propyl-triethoxysilane (APTES) and n-propyltriethoxysilane (PTES)) mesoporous silica SBA-16 nanoparticles in terms of cytocompatibility. *In vitro* tests were conducted in an MRC-5 cell line, human fetal lung fibroblast cells, at different concentrations. The results indicated no significant change in cell morphology, chromosomal changes, or increase of ROS in the MRC-5 cells related to the control group.

Tay et al. [98a], using TR146 cells as an in vitro model of the human oral buccal mucosa, examined the uptake, spatial intracellular distribution, ROS production, inflammatory response, and cytotoxic effects of commercial SiO_2 NPs. SiO_2 NPs are shown to dock and cross the cellular membrane barrier in a dose–time-dependent manner. Confocal sectioning reveals translocation of SiO_2 NPs into the cell nucleus after 12 h of exposure. A concentration threshold of more than 500 × 10 −6 M is observed, above which SiO_2 NPs are shown to exert significant oxidative stress with concomitant upregulation of inflammatory genes IL6 and TNFA. Further analysis of the p53 pathway and a series of apoptotic and cell cycle biomarkers reveals intracellular accumulation of SiO_2 NPs exert marginal nanotoxicity.

Skuland et al. [99a] compared non-crystalline silica particles of nano (50 nm)- and submicro (500 nm)-size (Si50 and Si500) for the potential to induce cytokine responses in bronchial epithelial lung cells (BEAS-2B). The cell cultures were exposed to equal mass and surface area concentrations of the two particles in different exposure media; LHC-9 and DMEM:F12. The state of agglomeration was different in the two media; with marked agglomeration in LHC-9 and nearly no agglomeration in DMEM:F12. On a mass basis, Si50 was more potent than Si500 in inducing cytokine responses in both exposure media. In contrast, upon exposure to similar surface area concentrations, Si500 was more potent than Si50 in DMEM:F12. This might be due to different agglomeration/sedimentation properties of Si50 versus Si500 in the two media. However, influence of differences in particle reactivity or particle uptake cannot be excluded. The data indicated no qualitative changes in the cytokine gene-expression patterns induced by the two particles, suggesting effects through similar mechanisms.

e) Studies Comparing different MNMs

Panas et al. [100a] screened different oxide MNMs, namely Fe_2O_3, SiO_2 and TiO_2 NPs, for toxic effects in A549 lung epithelial cells and RAW264.7 macrophages in the presence and absence of FCS. In summary, silica-NPs were acutely cytotoxic especially in the absence of serum. Furthermore, silica-NPs initiated an inflammatory response as evidenced by increased expression of COX-2, TNF-a, IL-1b, IL-6 and IL-8. However, iron oxide- or titania-NPs were not cytotoxic and did not induce a broad range of inflammatory mediators but surprisingly only specifically induced TNF-a mRNA in A549 cells and iNOS in macrophages. Noteworthy, the level of ROS in cells did not correlate with the observed cytotoxicity and strength of inflammatory response as for example silica-NPs did not enhance ROS levels but where the most toxic NPs. On the other hand, titania-NPs clearly increased the amount of ROS without concomitant cytotoxicity. Finally, the pre-coating of silica-NPs with serum proteins completely abrogated their toxicity and inflammatory potential suggesting that the NP surface and its interaction with sensitive biomolecules is key for the observed biological effects.

Hou et al. [101a] investigated the influences of three different types of nanoparticles (mesoporous SiO_2, Fe_3O_4, and TiO_2) with diameters of around 100 nm on the biological functions of endothelial cells, in particular of the organelle of cells. The results indicated that different types of nanoparticles had cytotoxic effects in a dose- and time-dependent manner, and there was no significant difference in cytotoxicity between SiO_2 and Fe_3O_4 at concentrations <0.20 mg/ml. The shape and surface charges of nanoparticles greatly affected cellular internalization. They found that cytoskeleton and integrity of cells were destroyed by different nanoparticles. Additionally, the production of reactive oxygen species damaged the mitochondria of cells, in turn leading to cells apoptosis and death.

Shrivastava et al. [102a] investigated the toxic effects of three metal oxide nanoparticles, TiO_2, ZnO and Al_2O_3 on mouse erythrocytes, brain and liver. Male mice were administered a single oral dose of 500 mg/kg of each nanoparticles for 21 consecutive days. The results suggest that exposure to these nano metallic particles produced a significant oxidative stress in erythrocyte, liver and brain as evident from enhanced levels of ROS and altered antioxidant enzymes activities. A significant increase in dopamine and norepinephrine levels in brain cerebral cortex and increased brain oxidative stress suggest neurotoxic potential of these nanoparticles. TEM analysis indicated the presence of these nanoparticles inside the cytoplasm and nucleus. These changes were also supported by the inhibition of CuZnSOD and MnSOD, considered as important biomarkers of oxidative stress. The toxic effects produced by these nanoparticles were more pronounced in the case of zinc oxide, followed by aluminum oxide and titanium dioxide, respectively.

Kermanizadeh et al. [103a] evaluated the effects on the human renal proximal tubule epithelial cells (HK-2) treated with a panel of engineered nanomaterials (NMs) consisting of two zinc oxide particles (ZnO - coated - NM 110 and uncoated - NM 111), two multi walled carbon nanotubes (MWCNT) (NM 400 and NM 402), one silver (NM 300) and five TiO_2 NMs (NM 101, NRCWE 001, 002, 003 and 004). They found the two ZnO NMs (24 hr LC50 – 2.5 $\mu g/cm^2$) and silver NM (24 hr LC50 – 10 $\mu g/cm^2$) were highly cytotoxic to the cells. The LC50 was not attained in the presence of any of the other engineered nanomaterials (up to 80 $\mu g/cm^2$). All nanomaterials significantly increased IL8 and IL6 production. Meanwhile no significant change in TNF-α or MCP-1 was detectable. The most notable increase in ROS was noted following treatment with the Ag and the two ZnO NMs. Finally, genotoxicity was measured at sub-lethal concentrations. They found a small but significant increase in DNA damage following exposure to seven of the ten NMs investigated (NM 111, NRCWE 001 and NRCWE 003 being the exception) with this increase being most visible following exposure to Ag and the positively charged TiO_2.

Chusuei et al. [104a] used a systematic approach to delineate physicochemical properties of nanoparticles that govern cytotoxicity. The cytotoxicity of fourth period metal oxide nanoparticles

(NPs): TiO_2, Cr_2O_3, Mn_2O_3, Fe_2O_3, NiO, CuO, and ZnO increases with the atomic number of the transition metal oxide. This trend was not cell-type specific, as observed in non-transformed human lung cells (BEAS-2B) and human bronchoalveolar carcinoma-derived cells (A549). Addition of NPs to the cell culture medium did not significantly alter pH. Physiochemical properties were assessed to discover the determinants of cytotoxicity: (1) point-of zero charge (PZC) (i.e., isoelectric point) described the surface charge of NPs in cytosolic and lysosomal compartments; (2) relative number of available binding sites on the NP surface quantified by X-ray photoelectron spectroscopy was used to estimate the probability of biomolecular interactions on the particle surface; (3) band-gap energy measurements to predict electron abstraction from NPs which might lead to oxidative stress and subsequent cell death; and (4) ion dissolution. Our results indicate that cytotoxicity is a function of particle surface charge, the relative number of available surface binding sites, and metal ion dissolution from NPs.

Tavares et al. [105a] evaluated the genotoxicity of nanosized TiO_2, synthetic amorphous silica (SAS) and MWCNTs, in human lymphocytes. They observed significant increases in the frequencies of micronucleated binucleated cells (MNBCs) for some TiO_2 NMs and for two MWCNTs, although no clear dose–response relationships could be disclosed. In contrast, all forms of SAS analyzed in this study were unable to induce micronuclei.

Ucciferri et al. [106a] studied the toxicity and sub-lethal effects of six types of NPs (15 nm and 80 nm Au, NM 101 TiO_2 (7 nm), NM 300 Ag (20 nm) and 50 nm and 200 nm fluorescent labelled (with fluorescein isothiocyanate or FITC) polystyrene (PS) NPs), on human umbilical vein endothelial cells (HUVECs) were investigated using different *in vitro* assays. The results showed that all the particles investigated induce some level of damage to the cells and that silver particles were most toxic, followed by titanium dioxide. Furthermore, endothelial cells were shown to be more susceptible when exposed to silver nanoparticles under flow conditions in a bioreactor. The study underlines that although simple *in vitro* tests are useful to screen compounds and to identify the type of effect induced on cells, they may not be sufficient to define safe exposure limits.

Sauer at al. [107a] tested the usefulness of in vitro systems to predict acute inhalation toxicity by using nineteen substances in three-dimensional human airway epithelial models, EpiAirway™ and MucilAir™, and in A549 and 3T3 monolayer cell cultures. IC50 values were compared to rat four-hour LC50 values classified according to EPA and GHS hazard categories. Best results were achieved with a prediction model distinguishing toxic from non-toxic substances, with satisfactory specificities and sensitivities. Using a self-made four-level prediction model to classify substances into four in vitro hazard categories, in vivo–in vitro concordance was mediocre, but could be improved by excluding substances causing pulmonary edema and emphysema in vivo. However, none of the test systems

was outstanding, and there was no evidence that tissue or monolayer systems using respiratory tract cells provide an added value.

Wang et al. [108a] conducted a toxicity relationship analysis using principal component analysis (PCA) for a panel of nanoparticles that included dry powders of oxides of titanium, zinc, cerium and silicon, dry powders of silvers, suspensions of polystyrene latex beads, and dry particles of carbon black, nanotubes and fullerene, as well as diesel exhaust particles. Acute *in vitro* toxicity was assessed by different measures of cell viability, apoptosis and necrosis, haemolytic effects and the impact on cell morphology, while structural properties were characterised by particle size and size distribution, surface area, morphology, metal content, reactivity, free radical generation and zeta potential. Different acute toxicity measures were processed using PCA that classified the particles and identified four materials with an acute toxicity profile: zinc oxide, polystyrene latex amine, nanotubes and nickel oxide. It was found that metal content was an explanatory variable for acute toxicity associated with zinc oxide and nickel oxide, whilst high aspect ratio appeared the most important feature in nanotubes. Particle charge was considered as a determinant for high toxicity of polystyrene latex amine.

f) New strategies and approaches for assessing MNM toxicology and correlation with in vivo systems

Zimmer et al. [109a] introduced a new approach, single cell mechanics, derived from atomic force microscopy based single cell compression. The single cell based approach is intrinsically advantageous in terms of being able to directly correlate to *in vivo* investigations. Its reliability and potential to measure cytotoxicity is evaluated using known systems: ZnO and SiO_2 NPs on human aortic endothelial cells (HAECs). This investigation clearly indicates the reliability of single cell compression. For example, ZnO NPs cause significant changes in force vs. relative deformation profiles, while SiO_2 NPs do not. New insights into NPs-cell interactions pertaining to cytotoxicity are also revealed from this single cell mechanics approach. This method is very sensitive, and able to capture the response of individual cells, which is highly complementary to cytotoxicity assays which usually only present a single output for a large population (> 104) of cells. Further, single cell mechanics is label-free. There is neither need for lysis to extract cellular contents for assay, nor for adding test reagents, eliminating false positives due to the biochemical reagents used in cellular assays. Finally, this method enables time-dependent study, as well as direct comparison between *in vitro* and *in vivo* exposures, as getting single cells after exposure is much easier than alternative approaches.

In the same direction, Qiao et al. [110a] described the use of a single cell array based assay for genotoxicity study of nanomaterials using normal human fetal fibroblast cells obtained from two dimensional (2D) monolayer cultures and three-dimensional (3D) microtissue. After being exposed to

a suspension of nanomaterials, cells are attached onto microfabricated patches with proper modification through electrostatic attraction and embedded in hydrogel. The damaged DNAs diffuse in gel matrix and form observable halo structures, where the level of DNA damage is quantified from the dimensions of core and halo. A concentration dependent genotoxicity has been found in nanomaterials. Compared to the traditional cytotoxicity (live/dead) assay, the genotoxicity results from the single cell array based assay are more robust and sensitive at the same exposure concentration, indicating that nanomaterials cause significant DNA damage without detectable cytotoxicity. In addition, cells from 3D microtissues are less damaged than 2D culture due to different cell microenvironments.

Agreeing with the latter authors, Shah et al. [111a], in their review, emphasize that using a chip based approach to sort and analyze single cells out of a population seems to be more cost effective, simpler and can yield a higher throughput performance. Microelectrodes on a chip integrated with microfluidic control and automation not only would make the initial nanotoxicity screening easy, but also would increase the participation from various authorities, and encourage the nanotoxicity assessment efforts throughout the world. CoC-based assays will provide more dynamic information on cell and particle interaction.

Cancino et al. [112a] introduced a methodology to evaluate the interactions between CNTs/dendrimers nanoconjugates and phospholipid biomembrane models, using the Langmuir film balance technique. Their main goal was to elucidate the action of engineered nanomaterials in cell membranes, at the molecular level, using a membrane model system. The penetration of single-walled carbon nanotubes (SWCNTs)/polyamidoamine dendrimer nanocomplexes into dipalmitoylphosphatidylcholine monolayers was pronounced, as revealed by adsorption kinetics and surface pressure measurements. These findings suggest that SWCNTs were able to interact even at high surface pressure values, ~30 mN/m. Therefore, the results confirm that the presence of the nanomaterial affects the packing of the synthetic membranes.

Cohen et al. [113a] presented a methodology that enables accurate determination of delivered to cell dose metrics. This methodology includes (1) standardization of MNMs suspension preparation; (2) measurement of MNM characteristics controlling delivery to cells in culture; and (3) calculation of delivered dose as a function of exposure time using the ISDD model. The approach is validated against experimentally measured doses, and simplified analytical expressions for the delivered dose (Relevant *In Vitro* Dose (RID)f function) are derived for 20 MNMs. These functions can be used by nanotoxicologists to accurately calculate the total mass (RIDM), surface area (RIDSA), or particle number (RIDN) delivered to cells as a function of exposure time. The proposed methodology was used to derive the effective density, agglomerate diameter and RID functions for 17 industrially-relevant metal and metal oxide MNMs, two carbonaceous nanoparticles, and non-agglomerating

gold nanospheres, for two well plate configurations (96 and 384 well plates). For agglomerating MNMs, the measured effective density was on average 60% below the material density. The authors report great variability in delivered dose metrics, with some materials depositing within 24 hours while others require over 100 hours for delivery to cells. A neutron-activated tracer particle system was employed to validate the proposed *in vitro* dosimetry methodology for a number of MNMs (measured delivered to cell dose within 9% of estimated. The findings confirm and extend experimental and computational evidence that agglomerate characteristics affect the dose delivered to cells.

Zhu et al. [114a] developed a novel lateral flow immunoassay (LFIA) to measure the concentration of 8-OHdG in cells and thus reveal the nanotoxicity on the genomic level. The feasibility of this new method is validated by comparison with two other established methods: Alamar Blue assay and a recently developed electrical impedance sensing (EIS) system on the level of cell proliferation/viability. Furthermore, the toxicological effects of three metallic nanoparticles (CuO, CdO, and TiO_2) are investigated and compared using these three methods with completely different mechanisms. The results show that there is a high variation among different nanoparticles concerning their ability to cause toxic effects. CuO nanoparticles are the most potent regarding cytotoxicity and DNA damage. CdO shows a fallen cell viability as well as DNA damage, however, to a lesser extent than CuO nanoparticles. TiO_2 particles only cause very limited cytotoxicity, and there is no obvious increase in 8-OHdG levels.

Mukherjee et al. [115a] developed a computational multiscale toxicodynamic modeling of silver and carbon nanoparticle effects on mouse lung function to quantify and predict pulmonary effects due to uptake of MNMs in mice. The model consists of a collection of coupled toxicodynamic modules that were independently developed and tested using information obtained from the literature. The modules were developed to describe the dynamics of tissue with explicit focus on the cells and the surfactant chemicals that regulate the process of breathing, as well as the response of the pulmonary system to xenobiotics. Alveolar type I and type II cells, and alveolar macrophages were included in the model, along with surfactant phospholipids and surfactant proteins, to account for processes occurring at multiple biological scales, coupling cellular and surfactant dynamics affected by nanoparticle exposure, and linking the effects to tissue-level lung function changes. Nanoparticle properties such as size, surface chemistry, and zeta potential were explicitly considered in modeling the interactions of these particles with biological media. The model predictions were compared with *in vivo* lung function response measurements in mice and analysis of mice lung lavage fluid following exposures to silver and carbon nanoparticles. The predictions were found to follow the trends of observed changes in mouse surfactant composition over 7 days post dosing, and are in good agreement with the observed changes in mouse lung function over the same period of time.

Pokhrel et al. [116a] described a strategy for creating nanoparticle libraries (pure or Fedoped ZnO or TiO2) utilizing flame spray pyrolysis (FSP) for the generatrion of MNMs and using these libraries to test hypotheses related to the particles' toxicity. They, further, describe how the dissolved metal ion from these materials (Zn^{2+}) can effectively bind with different cell constituents, causing toxicity. They use Fe-S protein clusters as an example of the complex chemical reactions taking place after free metal ions migrate into the cells. As a second example, they show that decreasing the bandgap energy of TiO_2 increases the phototoxicity in the presence of near-visible light. Another suggestion for nanoparticle libraries, this time gold NPs, is made by Alkilany et al. [117a]. In their work highlight that gold nanoparticles are excellent nanoparticle models to probe the interaction of nanomaterials with cells, biological media, whole organisms, and ecosystems. The selection of gold nanoparticles is based on the availability of various simple methods to prepare them in libraries, the ease of surface modification, the ability to track and detect them with various analytical tools, and finally their biocompatible core nanoparticles.

DeLoid et al. [118a] presented a rapid and inexpensive method for accurately measuring the effective density of nano-agglomerates in suspension. This novel method, the volumetric centrifugation method (VCM), is based based on the volume of the pellet obtained by low speed, benchtop centrifugation of an MNM suspension in a packed cell volume (PCV) tube. The findings demonstrate close agreement between the VCM approach and gold-standard analytical ultracentrifugation (AUC) data for various classes of materials including flame-generated fractal MNM agglomerates as well as non-agglomerating nanospheres. The authors also demonstrate that the effective density estimated by this method can be used to accurately determine the rate of MNM agglomerate deposition and thus delivered dose of MNM in an *in vitro* system. Numerical estimates of delivered dose are validated for various materials using a novel neutron-activated tracer particle system, highlighting the utility and accuracy of employing direct measurement of effective density via VCM to calculate the dose delivered to cells over time. This simple and cost-effective method allows nanotoxicologists to correctly model nanoparticle transport, and thus attain accurate dosimetry in cell culture systems, which will greatly advance the development of reliable and efficient methods for toxicological testing and investigation of nano–bio interactions *in vitro*.

Khatri et al. [119a] studied *in-vitro* photocopier-emitted nanoparticles and their ability to induce cytotoxicity, pro-inflammatory cytokine release, DNA damage, and apoptosis. They showed that nanoparticles in photocopy centers induced production of several inflammatory cytokines and chemokines in three human cell lines (THP-1, primary human nasal- and small airway epithelial cells), as well as moderate apoptosis, but no DNA damage under *in vitro* conditions. Up-regulation of inflammatory (TNF-α), apoptotic (CASP8 and p53), and oxidative stress genes (HO1) in THP-1 cells further substantiates these findings.

Rogers et al. [120a], instead of using ROS generation as an indicator of oxidative stress, used human blood serum as reaction media to determine the depletion of the antioxidant capacity of MNM exposed human blood serum. The decrease in antioxidant capacity was defined as biological oxidative damage (BOD). BOD was found to be a highly informative, biologically relevant metric reflecting the combined effects of multiple physicochemical characteristics. Going one step further, Hsieh et al. [121a] utilized BOD as an indicator and established the first large database by mapping out the oxidative potential of a range of different MNMs, to develop for the first time a surface activity index for a large library of commercially relevant MNMs, and to explore the relationship between oxidative potential and physicochemical parameters.

Hu et al. [122a] using combined in vitro and in silico methods, showed how hydrophobicity and surface charge of nanoparticles differentially regulate the translocation and interaction with the pulmonary surfactant film. While hydrophilic nanoparticles generally translocate quickly across the pulmonary surfactant film, a significant portion of hydrophobic nanoparticles are trapped by the surfactant film and encapsulated in lipid protrusions upon film compression. Their results support a novel model of pulmonary surfactant lipoprotein corona associated with inhaled nanoparticles of different physicochemical properties. Their data suggest that the study of pulmonary nanotoxicology and nanoparticle-based pulmonary drug delivery should consider this lipoprotein corona.

7. Concluding remarks

Overall, the study of nanoparticle toxicity both *in vitro* and *in vivo* is a field that has grown substantially in recent years, and this research is constantly producing new results regarding the interaction of nanoparticles with complex biological systems, such as human body. However, more studies analyzing various types of nanomaterials in *in vitro* and corresponding in *vivo tests* will further help to establish the correlation and to improve understanding of *in vitro* predictability. Up to the present, experimental findings can provide us with very useful information but are still limited to help prediction of certain physicochemical properties on the cellular behavior of MNMs, especially on the processes of biotransformation and elimination of MNMs [123a]. A major challenge of identifying the causative relationships between physicochemical properties of MNMs and their toxicity responses from the viewpoint of cellular trafficking is the lack of better probing techniques or methodology, in particular, the real time, in situ, rapid, and quantitative analysis methodology for characterizing the cellular behavior of MNMs. Because of the large number of variables in nanomaterials, experimental exploration requires a long time and great cost to clarify the cellular uptake, transport, and fate of each MNM [124a]. Thus, modeling from *in vitro* data to *in vivo* metabolism using computer simulation becomes a great challenge.

Furthermore, even if a link is established between in vitro and in vivo toxicological parameters, adverse outcomes in humans and the environment are dependent on real-life exposures at toxicologically relevant doses. Fate and transport as well as exposure assessment are key ingredients not incorporated in the predictive toxicological paradigm. There are also toxicological scenarios under chronic exposure conditions that could involve multiple steps that are not properly simulated in a single-step toxicological exposure. To encounter with these problems, continuous improvement and introduction of proteomic and genomics discovery platforms to widen the scope and build a systems biology approach is required [125a]. It is also possible that machine learning will help to identify systems biology approaches to improve nano safety screening.

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