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Working Report on the Status Quo of Nanomaterials Impact on Health and Environment

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Working Report on the Status Quo of Nanomaterials Impact on Health and Environment

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1 Introduction

Author: Nicoleta Lupu and Horia Chiriac

1.1 General considerations on nanotechnology and nanomaterials

Nanotechnology is regarded as one of the key technologies of the future and associated with high expectations by politics, science and economy. Artificially produced nanosized particles and nanoscale system components have new properties which are of importance for the development of new products and applications. Such new properties of materials and substances result from the special properties of surfaces and interfaces and in part, from the geometric shape of the material.

In theory nanoparticles (NPs) can be produced from nearly any chemical; however, most NPs that are currently in use today have been made from transition metals, silicon, carbon (single-walled carbon nanotubes; fullerenes), and metal oxides (zinc dioxide and titanium dioxide).

Potentially harmful effects of nanotechnology might arise as a result of the nature of the NPs themselves, the characteristics of the products made from them, or aspects of the manufacturing process involved (Borm and Kreyling, 2004). The large surface area, crystalline structure, and reactivity of some NPs may facilitate transport in the environment or lead to harm because of their interactions with cellular material. In the case of nanomaterials, size matters, and could facilitate and exacerbate any harmful effects caused by the composition of the material.

The highest risks for humans and the environment are associated with nanomaterials contained in products in the form of free particles. As long as NPs remain firmly embedded in materials, hardly any risk should be expected (Brouwer, 2004). However, it has to be clarified in these cases whether and in which form nanomaterials can be released into the environment during the production process, the use of a product, due to ageing and degradation as well as during disposal and recycling processes. Of course, also in the case of nanomaterials, environmental risk assessment should take into account their entire life cycle.

The assessment of the risk involved in NPs will decisively depend on the form in which these materials come into contact with humans and the environment. In this respect, important open questions still to be answered include the following: How stable and persistent are these forms? Do they decompose or agglomerate? Are they soluble in water? Will they interact with other NPs, chemicals, or surfaces? Are they degradable, and how will their properties change during degradation? Because of their size, NPs may cross barriers by the airborne route, also by adherence to aerosols. Unlike gases, liquids and many solid materials, the desirable properties of engineered nanomaterials closely depend on size, shape and structure (both physically and chemically) at the nanoscale. Similarly, there is a strong likelihood that biological activity will depend on physicochemical parameters not usually considered in toxicity screening studies. Nanoparticles can penetrate into live cells. Therefore, they have a potential to accumulate in organisms and thus, also in the food chain.

Typically, the biological activity of particles increases as the particle size decreases. Smaller particles occupy less volume, resulting in a larger number of particles with a greater surface area per unit mass and increased potential for biological interaction.

The unusual properties of nanomaterials are predominantly associated with their nanometer-scale structure, size and structure-dependent electronic configurations and an extremely large surface-to-volume ratio relative to bulk materials. Particles in the nanosize range can deposit in all regions of the respiratory tract including the distal lungs (Brown *et al.*, 2002). Due to their small size, NPs may pass into cells directly through the cell membrane or penetrate between or through cells and translocate to other parts of the body. Limited data have suggested possible translocation of inhaled NPs to the nervous system and other organs/tissues.

The size of NPs alone may not be the critical factor determining their toxicity; the overall number and thus the total surface area may also be important. As a particle decreases in size, the surface area increases (per unit mass only; if you normalize to number of particles, the surface area decreases) and a greater proportion of atoms/molecules are found at the surface compared to those inside. Thus, NPs have a much larger surface area per unit mass compared with larger particles. The increase in the surface-to-volume ratio results in the increase of the particle surface energy which may render them more biologically reactive.

Chemical composition is another important parameter for the characterization of nanomaterials, which comprise nearly all substance classes, e.g., metal/metal oxides, compounds, polymers as well as biomolecules. Some nanomaterials can also be a combination of the above components in core-shell or other complex structures. Dependent on the particle surface chemistry, reactive groups on a particle surface will certainly modify the biological effects (Donaldson *et al.*, 2001a). Under ambient conditions, some NPs can form aggregates or agglomerates. These agglomerates have various forms, from dendritic structure to chain or spherical structures. To maintain the characteristics of NPs, they are often stabilized with coatings or derivative surface to prevent agglomeration. The properties of NPs can be significantly altered by surface modification and the distribution of NPs in the body strongly depends upon the surface characteristics. Changes of surface properties by coating of NPs to prevent aggregation or agglomeration with different types and concentrations of surfactants have been shown to change their body distribution and the effects on the biological systems significantly.

Therefore, it is recommended that the following physico-chemical properties of the test materials should be characterized:

- Size distribution
- Agglomeration state
- Shape
- Crystal structure
- Chemical composition – including spatially averaged (bulk) and spatially resolved heterogeneous composition
- Surface area
- Surface chemistry
- Surface charge
- Porosity

The agglomeration state of a nanomaterial during and following administration may have a significant impact on its biological activity. Agglomeration state at different structure scales should be characterized, including primary (primary particles), secondary (primary particle agglomerates and self-assembled structures) and tertiary (assemblies of secondary structures) scales. Ideally, agglomeration state in the biological environment following administration should be evaluated. If possible, some insight into the binding forces within agglomerates (e.g. relatively weak van der Waals forces or relatively strong sintered bonds) should be obtained. Material agglomeration or de-agglomeration in different liquid media should also be investigated where possible.

1.2 Nanomaterials

The focus within this report is on specific materials which have several prominent properties. The first criterion is the persistency within the environment or within an organism. Thus, we concentrate on stable materials that may have the chance to reach the environment in higher concentrations and stay there for months or years. Moreover, medical applications have been excluded because there will be used specific materials with often biological substances as backbone. This will need a completely different risk governance. Taking this into consideration the following materials have been decided to be discussed in this report.

1.2.1 Nanocrystalline Materials (Ceramics, Metals and Metal Oxides)

Authors: Speranta Tanasescu

Key parameters to explain the chemical reactivity and toxicity of nanocrystalline materials

Included here are ceramics, metals, and metal oxide NPs. These materials are assembled from nanometer-sized building blocks, mostly crystallites. The building blocks may differ in their atomic structure, crystallographic orientation, or chemical composition. In cases where the building blocks are crystallites, incoherent or coherent interfaces may be formed between them, depending on the atomic structure, the crystallographic orientation, and the chemical composition of adjacent crystallites. In other words, materials assembled of nanometer-sized building blocks are microstructurally heterogeneous, consisting of the building blocks (e.g. crystallites) and the regions between adjacent building blocks (e.g. grain boundaries). It is this inherently heterogeneous structure on a nanometer scale that is crucial for many of their properties and distinguishes them from glasses, gels, etc. that are microstructurally homogeneous.

Studies have demonstrated that nanoparticle toxicity is extremely complex and multifactorial, potentially being regulated by a variety of physicochemical properties such as size and shape, as well as surface properties such as charge, area, and reactivity (Cai *et al.*, 1992; Derfus *et al.*, 2004; Nemmar *et al.*, 2003; Sayes *et al.*, 2004; Sclafani and Herrmann, 1996). Nevertheless, these and other studies do lend substantial weight to the hypothesis that the health hazard of some engineered nanomaterials will be dependent on chemistry and structure (Maynard, 2007), as well as on the energetics of the crystalline phases (Navrotsky, 2004; Tanasescu and Marinescu, 2006; Wang *et al.*, 2007).

Understanding the properties of nanoparticles presents a host of challenging questions and problems. In the following some key parameters involved in the control of the toxicity of NP will be analyzed. A focus will be given to nanosized metal oxides emphasizing on some critical aspects and new topics related to these compounds.

Particle size versus particle composition

Studies assessing the role of particle size on toxicity have generally found that ultrafine or nanoparticles (Ø < 100 nm) are more toxic on a mass-based exposure metric when compared to larger particles of identical chemical composition (Oberdörster *et al.*, 1994; Li *et al.*, 1999; Höhr *et al.*, 2002). In their inhalation studies, Oberdörster *et al.* (1994) and Ferin *et al.* (1992) observed a significant increase in inflammation signs or parameters during administration of 20 nm TiO_2 particles in comparison with the same mass of 250 nm particles. Until these studies performed by the same team, titanium oxide was considered to be nontoxic dust and served as an inert control in several toxicological studies. Damage to the pulmonary epithelium, obstruction of Kohn's pores, development of sources of interstitial fibrosis and alteration of macrophage functions (inflammation mediators) were significantly greater. These results show that inert particles can become biologically active when nanoscaled. **Reduction in size** to the nanoscale level results in **an enormous increase of surface to volume ratio**, so relatively more molecules of the chemical are present on the surface, thus enhancing the intrinsic toxicity (Donaldson *et al.*, 2004b). The extraordinarily high number concentrations of NPs will likely be of toxicological significance when these particles interact with cells and subcellular components. Many studies have shown that particle surface area dose is a better predictor of the toxic and pathologic responses to inhaled particles than is particle mass dose (Brown *et al.*, 2001; Donaldson *et al.*, 1998; Driscoll, 1996; Duffin *et al.*, 2002; Lison *et al.*, 1997; Oberdörster *et al.*, 1996; Tran *et al.*, 2000). Likewise, their increased surface area per unit mass can be toxicologically important if other characteristics such as surface chemistry and bulk chemistry are the same. Donaldson *et al.* (2001a) and his team had proved that nanoparticulate forms (< 50 nm) of titanium oxide, aluminium oxide and carbon black increased the pulmonary inflammation parameters 10 times more than administration of fine particles of the same products. Borm *et al.* (2004), in a lung cancer journal, point out that low solubility particles, such as carbon black and titanium oxide, are recognized to cause fibroses, neoplasic lesions and pulmonary tumours in rats. The quantity of these products required to generate the same effects is much smaller with NPs.

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 (Singh, 2005). 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 (Singh, 2005).

The chemical composition and the intrinsic toxicological properties of the chemical are also of importance for the toxicity of particles. Early indications were that transitional metals and their oxides might be more toxic as ultrafine particles (UFPs) than other materials (Donaldson *et al.*, 1999). However the evaluation of toxicity for both micron and nanosized materials gave interesting results. For micron sized biomaterial particles, the *in vivo* distribution was dependent on the composition of the material. Research is now showing that when normally harmless bulk materials are made into ultrafine particles they tend to become toxic. The effect of carbon black has been shown to be more severe than that of titanium dioxide (Kim *et al.*, 1999), while for both compounds the NPs 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 UFPs, for example latex and TiO_2 (Oberdörster, 2003). 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 (Peters, 2004). What seems clear from all these papers is that exposure of living systems to UFPs tends to increase oxidative stress and from this point of view **the size effect is considerably more important to UFP toxicity than the actual composition of the material** (Howard, 2003).

However, recent studies have provided evidence that intentionally produced nanomaterials can display **unique toxicity that cannot be explained by differences in particle size alone** (Lam *et al.*, 2004; Warheit *et al.*, 2004)[1]. 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 (Warheit *et al.*, 2006; Yin *et al.*, 2005).

Particle size versus crystalline structure

Shvedova *et al.* (2005) reported unusual inflammatory and fibrogenic pulmonary responses to specific nanomaterials, suggesting that they may injure the lung by new mechanisms. The combination of small particle size, large surface area, and ability to generate reactive oxygen species have been suggested as key factors in induction of lung injury following exposure to some incidentally produced nanomaterials (Nel *et al.*, 2006). Uchino *et al.* (2002), Warheit *et al.* (2006) and Sayes *et al.* (2006b) have reported that specific crystal structure and the ability to generate reactive oxygen species are important factors to consider in evaluating nanomaterial toxicity. Inhalation studies using rodents have demonstrated that 20 nm diameter TiO_2 particles had a greater impact on the animals' lungs than pigment-grade particles with the same composition, even though both particle sizes were administered as micrometer–diameter agglomerates (Bermudez *et al.*, 2004). So, the published toxicity studies clearly show that **particle size alone is not a good criteria for differentiating between more or less hazardous materials**. Oberdörster *et al.* (2005a) address the potential health impact of nanostructured particles—those having sub-100 nm scale

[1] The toxicity of carbon nanotubes and especially of their contaminants will be addressed in chapters 1.2.3.1 and 2.1.3

structures—rather than solely focusing on nanometre–diameter particles. Maynard and Kuempel (2005) further suggest that the structure-dependent behaviour of nanomaterials indicates an emphasis on nanostructured rather than nanosized particles. The association between structure and functionality provides a useful handle for beginning to explore occupational health risk (Maynard, 2007). There is a dependency between the physical and chemical structure of engineered nanomaterials and the health hazard they present (for instance, see Maynard *et al.*, 2005, 2006; Oberdörster *et al.*, 2005a, b; Lam *et al.*, 2006).

With these observations in mind, an important conclusion is being raised. The biological behaviour of NPs is determined not only by the chemical composition, but also by the corresponding shifts in chemical and physical properties, associated to the increase in surface to volume ratio (Navrotsky, 2004; Tanasescu and Marinescu, 2006). Or, in other words, for every compound, every property has a critical length scale where the fundamental physics of that property starts to change. It is noteworthy that the surface cannot be considered only as a two-dimensional boundary limiting the solid, but rather as a zone several atomic layers deep with specific properties different from the bulk. Then the question is to know how the influence of surface properties can be modified when the crystallite size decreases to the limit of stability of the nanocrystal. Therefore, it may be expected that the energetics of nanostructured materials is a key factor of the life of oxide materials and of their reactivity. In fact it is an enormous field of research which is starting to be explored.

Particle size versus energetics of nanomaterials

Energetics of the polymorph phases

As a first example of the size effects on the energetics of nanosized oxide materials one can quote works on the competition between polymorphism and surface energy (McHale, 1997; Navrotsky, 2004; Zhang, 1998). Many oxides are polymorphic, often showing different structures for small particles. Examples are α- and γ-Al_2O_3 and Fe_2O_3, the TiO_2 polymorphs (rutile, anatase and brookite), the ZrO_2 polymorphs (cubic, tetragonal and monoclinic), manganese oxides, as well as Al and Fe oxyhydroxides. The reason for such polymorphism can be related to the competition between small free energies of phase transitions and differences in surface free energies of different polymorphs. Some examples are illustrated in Table 1 in which the transformation and surface enthalpies as a function of surface area for titania, alumina, zirconia and iron oxide (Gribb *et al.,* 1997; Navrotsky, 2003; Ranade *et al.* 2002) are presented. The closely balanced energetics directly confirms the crossover in stability of nanophase polymorphs. For instance, TiO_2 is known to exist in three polymorphs, namely, rutile (tetragonal), anatase (tetragonal), and brookite (orthorhombic). The energetic driving force represented in the Table 1 and Fig. 1 by the surface enthalpies and the enthalpies of the sequence transformation rutile/brookite/anatase evidences the crossover of the phase stability at level nano. In Fig. 1, the dark solid lines represent the phases of lowest enthalpy (meaning the highest stability) as a function of surface area. Rutile is energetically stable for surface area <592 m^2/mol (7 m^2/g), brookite is energetically stable from 592 to 3,174 m^2/mol (7-40 m^2/g), and anatase is energetically stable for greater surface areas (Table 4 and Fig. 1). The anatase and rutile energetics are crossing at 1, 452 m^2/mol (18 m^2/g) (Fig. 1). The energetic stability crossovers are confirmed. Assuming spherical particles, the calculated average diameters of rutile and

brookite for 7 m^2/g surface area are 201 nm and 206 nm, and of brookite and anatase for 40 m^2/g surface area are 36 nm and 39 nm. When particles become smaller, the surface free energy becomes dominant, and if the surface energies of the polymorphs are sufficiently different, phase stability can be reversed for nanocrystalline particles. Rutile is the stable high-temperature phase, but anatase and brookite are common in fine-grained natural and synthetic samples.

Tab. 1: Surface enthalpies, transformation enthalpies and surface area for several oxides.

Sample	ΔH_s (J/m^2) (surface enthalpy)	ΔH_{transf} (kJ/mol) (enthalpy of phase transformation)	Surface area (m^2/mol)
TiO_2 (rutile)	2.2 ± 0.2	0	< 592
TiO_2 (anatase)	0.4 ± 0.1	2.61 ± 0.41 (bulk rutile - anatase)	>3,174
TiO_2 (brookite)	1.0 ± 0.2	0.71 ±0.38 (bulk brookite – rutile)	592-3174
α-Al_2O_3	2.6 ± 0.2	0	<10000
AlOOH (boehmite)	0.5 ± 0.1	4.9±2.4	5140
ZrO_2 (monoclinic)	6.5 ± 0.2	0	Coarse
ZrO_2 (tetragonal)	2.1 ± 0.05	9.5 ± 0.4	4313-5545
ZrO_2 (amorphous)	0.5 ± 0.05	34 ± 4	37700
α-Fe_2O_3 (tetragonal)	1.0	14.9±1.5	4000-6400
γ-Fe_2O_3 (cubic)	0.6	18.7±3.6	8000-39200
ZrO_2 (monoclinic)	6.5 ± 0.2	0	Coarse

Zhang *et al.* (1998, 1999) and Gribb *et al.* (1997) observed that the synthesis of ultrafine titania resulted in anatase and/or brookite, which transformed to rutile on coarsening. Once rutile was formed, it grew much faster than anatase. From the thermodynamic analysis, they conclude that anatase becomes more stable than rutile for particle size smaller than 14 nm. Ranade *et al.* (2002) directly confirmed the energetic crossover in nanophase polymorph stability of TiO_2 by high-temperature oxide melt drop solution calorimetry. The energetics of the TiO_2 polymorphs (rutile, anatase, and brookite) explains the differences between the bulk and surface properties (Table **2**). The closely balanced energetics directly confirms the crossover in stability of nanophase polymorphs inferred by Zhang *et al.* (1998, 1999), explaining the differences between the bulk and surface properties (Fig. **1**). An amorphous sample with surface area of 34 600 m^2/mol is 24.25 ± 0.88 kJ/mol higher in enthalpy than bulk rutile.

Tab. 2: Comparison between the bulk and surface properties of the TiO_2 polymorphs.
Data from Navrotsky (2003).

TiO_2 polymorphs	Bulk enthalpies	Surface enthalpies
rutile	**0.71 ± 0.38 kJ/mol**	**2.2 ± 0.2 J/m^2**
brookite	**0.71 ± 0.38 kJ/mol**	**1.0 ± 0.2 J/m^2**
anatase	**2.61 ± 0.41 kJ/mol**	**0.4 ± 0.1 J/m^2**

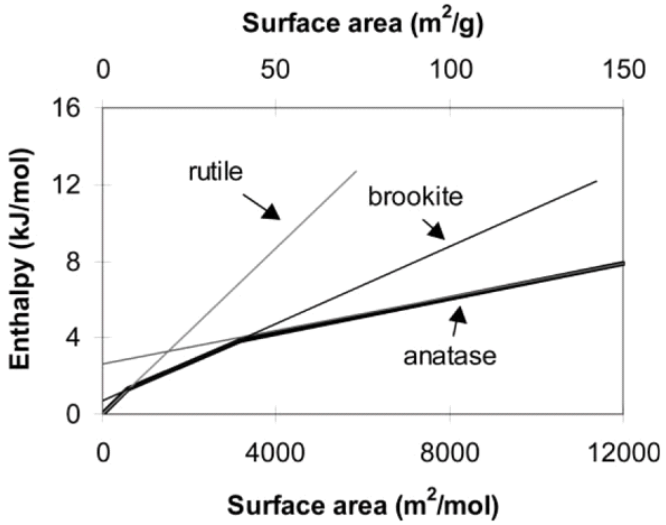

Fig. 1: Enthalpy relative to bulk rutile for rutile, anatase, and brookite of various surface area. Figure modified from Ranade *et al.* (2002).

Because many oxides (silica, iron and aluminium oxides and oxyhydroxides, manganese oxides, titania, zirconia, zeolites) show polymorphism with relative small free energy differences between polymorphs, such crossovers in stability at the nanoscale may be a rather general phenomenon. The metastable phases are somehow structurally more similar to their precursors in solution, melt, or glass and are able to nucleate more readily. Thus, a strategy for making a given polymorph in the laboratory, and one that may be used by organisms as well, is to control the size of the initial crystal (typically by controlling concentration of reactants, ionic strength, and organic and inorganic additives) to precipitate the desired polymorph with a relatively uniform size distribution within the size range in which that polymorph is stable and then to aggregate and coarsen the particles without phase transformation. In ceramic processing, coarsening is often accomplished by controlled heating; nature finds other aggregation and growth mechanisms at biological temperatures.

If there is a general correlation between increasing metastability and decreasing surface energy, as discussed above, then crossovers in thermodynamic stability at the nanoscale may be a key parameter controlling the global reactivity and toxicity.

Correlation between the stability, energetic parameters and the toxicity of nano-TiO_2 nanoparticles

Different authors further evaluated the toxicity of the different TiO_2 polymorphs. Uchino *et al.* (2002) studied the relationship between the amount of radicals produced in UV irradiated TiO_2 particles and cytotoxicity. The viability of Chinese hamster ovary (CHO) cells with internalized TiO_2 particles decreased significantly after UV irradiation. Although the intensity of UV light did not influence cytotoxicity, the anatase fraction in TiO_2 particles had a significant effect on cytotoxicity. In addition, cell viability was proportional to the formation of DMPO-OH radical adducts. The Electron Spin Resonance (ESR) results confirmed the presence of DMPO-OH radical adducts, consistent with the formation of OH· radicals. The optimum crystal size for OH· radical formation was 30 nm for anatase. Most anatase samples produced more hydroxyl radicals than rutile or amorphous TiO_2 (Fig. **2**). These findings indicate that when anatase forms TiO_2 with UV exposure, hydroxyl radicals have cytotoxic effects.

Recent studies (Singh, 2005; Dreher, 2005; Warheit *et al.* 2006; Sayes *et al.* 2006b) assessed the photocatalytic properties, reactive oxygen species generation, and *in vitro* cytotoxic potentials of nano-TiO_2 particles of the three different crystal phases, namely, anatase, anatase/rutile, and rutile phases. These investigators reported that, when compared to the other crystal phases, the nano-TiO_2 particles in the anatase phase, produced the most reactive oxygen species generation and the largest cytotoxic responses following *in vitro* exposures to human dermal fibroblasts or to A549 human lung epithelial cells. Sayes *et al.* (2006b) concluded that the nano-TiO_2 particles in the anatase crystal phase are a superior photocatalyst to the rutile particle types because of differences inherent in the crystal structures. It remains to be determined whether similar results will be measured under *in vivo* conditions.

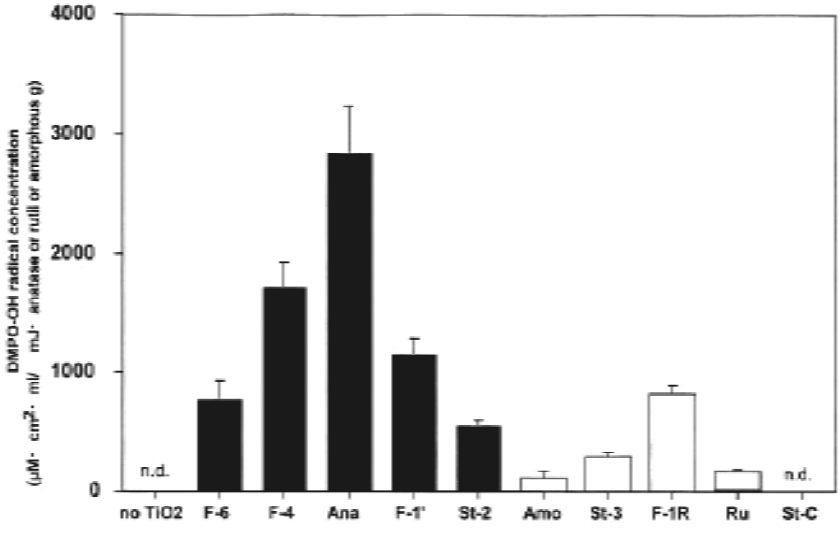

Fig. 2: Oxidative stress hydroxyl radical production (ESR) in different TiO_2 polymorph phases Uchino et al. (2002).

Barnard and Zapol (2004) performed various studies in order to elucidate the physical and chemical parameters affecting the stability of TiO_2 at the nanoscale. Using a thermodynamic model, they have presented predictions of the transition enthalpy of nanocrystalline anatase and rutile as a function of shape, size, and degree of surface

hydrogenation, showing that transition enthalpies may differ as a result of morphological changes and surface structure.

Nanoparticles in the aqueous environment have hydrated surfaces. The enthalpy of hydration and the enthalpy of adsorption of other inorganic ions and organic molecules on NPs can be also studied by calorimetry. Such adsorption is expected to be sensitive to polymorphism and, for particles below 10 nm or so in size, new phenomena may come into play. Unfortunately, the enthalpy of hydration has been explored only for a small number of oxides (McHalle, 1997; Navrotsky, 2003). Further work by adsorption and immersion calorimetry is needed.

The influence of different compositional variables on the reactivity and thermodynamic data

In the description of the multicomponent oxides reactivity by referring to ideal surfaces, it is necessary to consider several interconnected structural and compositional features:

- intrinsic defects
- foreign atoms
- nonstoichiometry

The reactivity of iono-covalent oxide surfaces at moderate temperatures is made possible by the existence of defects that play a basic role in the adsorption of molecules, which often in the step required before the nucleation of new solid phases. Therefore, an efficient control of the oxide reactivity characterizes both nature and number of **structural defects** pre-existing at surfaces, or those forming or vanishing as a consequence of the solid state transformation.

In addition to these **intrinsic defects**, **foreign atoms** are often present as a consequence of their existence as additives or impurities, or combined or not with segregational effects. Foreign atoms can also be intentionally deposited onto surfaces for specific purposes. Their influence on the thermodynamic properties and toxic effect is generally poorly known and underexploited. However, when they are controlled they can be used not only for obtaining or improving the suitable properties in specific applications (such as catalysis, elaboration of sensors, sintering of ceramic oxides or electronics), but also to predict the possible bio/non-bio interaction.

TiO_2 displays its high photoactivity only when it is irradiated by ultraviolet light due to its wide band gap (3.2 eV for anatase). Doping with impurities has been widely used to modify the properties of TiO_2 by introducing new states in its electronic structure (Diebold, 2003; Xie *et al.,* 2003). Because of the unique 4f electron configuration, lanthanide metal ions are ideal dopants to modify the electronic structure of TiO_2. For example, cerium element doping (Hou *et al*., 2006; Wang *et al*., 2007) could introduce new energy level into band gap of nano TiO_2, making it possible for light with a wavelength over 400 nm (generally named visible light) to excite an electron jump to conduction band from valence band. At the same time, Ce(IV) can separate e^-/h^+ pair by grasped electron, then liberate h^+ to react with H_2O to generate OH and H_2O_2, leading to accumulation of ROS in cell membranes and in cytoplasm.

When we discuss the influence of different compositional variables on the reactivity and thermodynamic data under the conventional risk assessment paradigms, understanding the risk presented by these materials will be a function of both hazard (incorporating

toxicity and health outcomes) and exposure (including exposure routes and dose). There is also a third component that deserves specific attention when addressing engineered nanomaterials: Characterization (EPA, 2007; Maynard, 2007; Oberdörster *et al.*, 2005a; SCCP, 2007; SCENIHR, 2007; Tanasescu *et al.* 2008).

In constructing a framework for nanomaterials toxicity testing, Oberdörster *et al.* (2005a) recommend sixteen physicochemical parameters that should be evaluated in toxicity tests. These range from surface area and surface chemistry to particle size distribution and particle charge. Engineered nanomaterials are notoriously difficult to characterize—even two materials that are notionally the same may have subtle but significant differences that determine their behaviour. For instance, introducing a small percentage of impurities to the surface of nano-TiO_2 particles may fundamentally alter their propensity to generate free radicals under UV radiation (Wakefield *et al.*, 2004). Without rigorous nanomaterials characterization, it will be near-impossible to interpret toxicity studies, compare similar studies and develop predictive models of nanomaterials hazard. Besides the other physicochemical parameters, the control of the energetic parameters could be important step in understanding the stability and reactivity of the micro and nanostructured materials (Navrotsky, 2003; Tanasescu *et al.* 2008).

One of the major problems results from the difficulty in characterizing the surface in perfectly controlled conditions and in using the classical techniques to analyze its properties at equilibrium *in situ.* Another difficulty is that, in contrast to macro-thermodynamics, the thermodynamics of a small system will usually be different in different environments (Hill, 2001a, 2001b). The thermodynamic functions of a small system differ from those of the corresponding macroscopic system, and also are depending on the "environmental" variables of the small system (e.g., for a single component: N, p, T; μ, V, T; μ; p, T in conventional thermodynamic notation). If we take the environmental variables N, p, T as an example, applied to, say, small crystallites of various fixed sizes N, the thermodynamic properties of interest, such as free energies, enthalpies, entropies, heat capacity are in fact size effects. Or, in other words, for every compound, every property has a critical length scale where the fundamental physics of that property starts to change.

In the Chemical Thermodynamics Laboratory from Institute of Physical Chemistry of the Romanian Academy a detailed investigation of the thermodynamic properties of micro and nanostructured multicomponent oxides was initiated in order to evidence new features related to the effect of different compositional variables on the thermodynamic behaviour. The focus of the research was to emphasize modifications of the thermodynamic properties connected with the nanocrystalline state in some oxide systems with perovskite structure. In previous papers (Tanasescu *et al.*, 2008, 2008), examples from nonbiological systems illustrate features potentially important for the interaction of small-scaled particles within large-scaled settings. The size (1 nm - 100 nm) and surface area alter some behaviours such as work function, energetic parameters, chemical potential, and oxygen capacity storage. The results obtained for the nanocrystalline ceramics can be discussed only being related with the significant changes in the overall defect concentration, suggesting reduced formation energy of oxygen vacancies and an increase of order in the oxygen sublattice. Searching for the potential harm associated with UFPs, there are indications that size

matters as much as or more than the material of which the particle is composed. Interesting or unusual properties appear because the size of the system approaches some critical length.

Recommendations

- There is need for more and advanced tools for the surface characterization under controlled conditions, thus, the properties and environmental effects of/on nanoparticles can be related to each other
- To enhance the endeavours in development and application of theory and modelling, expanding conceptual framework, and calibrating the state of our understanding to the nanoscale
- Integration of theory, modelling, and simulation into experimental design

Knowledge gaps

- regarding the dependence of particle energy on size; in contrast to macrothermodynamics, the thermodynamics of a small system will usually be different in different environments.
- Information about the correlation between surface energy, reactivity and biological activity is needed
- No or little information regarding the fundamental understanding of the reaction specificity of nanoparticles in solution is available

1.2.2 Fullerenes

Authors: Maja Remskar

Fullerenes and fullerene-like particles are spherical NPs with self-terminated shells. Due to their geometry they undergo to a strong Brownian motion, they easily become airborne, especially the smallest C_{60} fullerenes. Agglomeration is the usual process to minimize the large surface energy, but it changes the desired physical and chemical properties typical for nanoobjects. Therefore they are usually functionalized with polar molecules-surfactants.

1.2.2.1 Carbon fullerenes

C_{60} is a molecule that consists of 60 carbon atoms, arranged as 12 pentagons and 20 hexagons. The spherical molecules, 3.52 Å in diameter, do not exhibit "superaromaticity", the effect that the electrons in the hexagonal rings would be delocalized over the whole molecule. Therefore they have a high electron affinity at pentagon rings, which can lead to reaction with oxygen dissolved in water and creation of oxygen free radicals. These are responsible for oxidative cell damage. They are hydrophobic and very sparingly soluble in water. Common solvents for the fullerenes include toluene and carbon disulfide. Solutions of pure C_{60} have a deep purple color. Under a variety of conditions, upon contact with water, C_{60} spontaneously form stable aggregates with nanoscale dimensions (d=25-500 nm), termed as “nano-C_{60}”, with a partially oxidized shell. The colour, hydrophobicity, and reactivity of individual C_{60} are substantially altered in this aggregation form. Smallest aggregates are typically circular in cross section, intermediate and large particles are mostly rectangular, and the very

largest particles often appear to be triangular. The properties of C_{60} are summarised in Tab. **3**. As the pH of water is varied, a change in the average particle size is observed (Fortner *et al.*, 2005). The removal of electrons from the surface via a mild oxidizing agent is necessary to destabilize nano-C_{60} and cause disassembly to single C_{60} molecules. These aggregates allow for concentrations up to 100 mg/L, which is 11 orders of magnitude more than the estimated molecular solubility ($<10^{-9}$ mg/L). These photoactive nanocrystals can then produce oxygen radicals that damage lipid bilayer of cells, although it does not appear to oxidize the cellular proteins and other organelles. This crystallization can be prevented by partially oxidizing the C_{60} molecule in advance. For example, $C_{60}(OH)_{24}$ is not toxic at all, but the question appears if it shows still the same promising chemical and physical properties for applications? It was discovered (Bogdanovic *et al.*, 2004) that water soluble derivatives of $C_{60}(OH)_{22}$ inhibited the growth of human breast cancer cell lines at a range of nanomolar concentrations, so such molecules are still biologically active. Only two (OH) groups more completely prevent toxicity in water bio-media. How to control the stage of complete oxidation in water or $(OH)^-$ rich media inside human body?

Nevertheless, the ability of oxidising the C_{60} molecule is a perspective for health and cosmetic industry for neutralisation of free radicals caused in human body due to internal and external processes, like UV irradiation, aging, food additives, etc. From the available data it is not clear how the oxidising of C_{60} takes place without to go through partial toxic $C_{60}(OH)_x$ phases.

The world's first Fullerene-based cosmetic ingredient is already on market[2]. Part of the advertising: “The effects of Fullerene are maximized in Radical Sponge®, which can easily be applied to and absorbed by the skin. The product is designed to remain in the epidermis and protect the keratinocyte that forms the corneum and the melanocyte, the key players in skin protection, from harmful radicals. "Radical" is a generic name given to any reactive molecular species, including active oxygen, that is harmful to living organisms. Examples include $\bullet O_2^-$ (superoxide anion radicals), •OH (hydroxide radicals), •NO (nitrogen oxide radicals), and lipoperoxide. These radicals are produced in abundance by ultraviolet radiation, irritant chemical substances (including make up ingredients), and stress. Due to their high reactivity, radicals begin to attack and destroy nearby living tissue immediately after production, causing various skin problems and illnesses. Radical Sponge can take you to realms unreachable by vitamin C, a still superb radical-eliminating agent.”

[2] http://www.vc60.com/english/radicalcontrol/

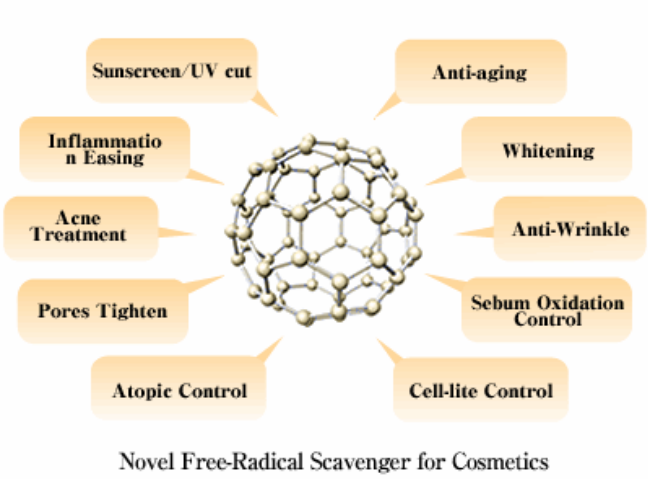

Fig. 3: Fullerene as possible radical scavenger?

Drug delivery was the second important use of C_{60}, but USA scientists producing DNA buckyballs[3] instead of C_{60} are concerned on the C_{60} potential toxicity to humans and animals. They report: "Even miniscule alterations to the surface of the buckyballs can dramatically affect how toxic they are to individual cells. Toxicity is desirable for example, for particles that kill cancer cells or harmful bacteria. In other cases, like applications where particles may make their way into the environment - toxicity is undesirable."

Tab. 3: Characteristic properties of Fullerene C_{60}.

No.crt.	Properties of C_{60}	Details
1	Morphology	0.704 nm in diameter Aspect ratio: L/l/h(D): 1 Specific surface (m^2/g): $5,2.10^5$ Agglomeration: nC_{60}
2	Crystallinity	Rectangular crystals
3	Wettability	Hydrophobic
4	Reactivity/Degradation	Radical scavenger, persistent
5	Solubility	10^{-9} mg/L as C_{60} and 100 mg/L as nC_{60} colloidal aggregates
6	Toxicity	Moderate or no toxicity (Sayes *et al.*, 2007a; Zhu *et al.*, 2007)

1.2.2.2 Inorganic fullerenes: WS_2, MoS_2

WS_2 and MoS_2 inorganic fullerenes (Tenne *et al.*, 1992) are onion-like NPs with several molecular layers (S-W-S or S-Mo-S) in wall thickness. They can be hollow or partially filled with MoO_x or WO_x compounds, which serve as precursor material during the synthesis. Inorganic fullerenes were firstly produced by the sulphidization of thin films of trioxides and later-on from NPs of transition metal trioxides or suboxides in gas flow of

[3]http://www.in-pharmatechnologist.com/news-by-product/news.asp?id=62142&k=researchers-create-dna

mixture of H_2 and H_2S. The particle diameters range from 20 nm up to 150 nm for WS_2 (Fig. 4) and up to 80 nm for MoS_2. The particles are frequently agglomerated and sintered into clusters composed of few up to several tens of NPs of different diameter. Surface of the particles is inert due to saturated sulphur bonds composing the top most atomic layer. The only reactive sites are surface defects in form of screw dislocations, edges of broken molecular layers or exfoliated layers. These sites are saturated by oxygen forming transition metal oxides. Pure material is hydrophobic. It is possible to des-aggregate the assemblies in solvents containing surfactants using ultrasound. The inorganic fullerenes are relatively heavy and therefore too heavy to become airborne easily, if the wall thickness exceeds 10% of radius in MoS_2 and 6% in WS_2, which is always the case. The values were estimated for the empty spheres, so real values are even smaller. Charging of the particles or use a flowing gas including convection can take up them into air for a time dependent on their geometry. The sedimentation rate of the particles in air or in water is not known. The properties of inorganic fullerenes are summarised in Tab. **4**.

One of the most promising applications is as advanced solid-state lubricant in automobile and aerospace industries, as well as in military use as lubricants. The spherical geometry of the particles enables their use as the best known shock absorbers (Zhu *et al.*, 2005). Under uniaxial shockwave they survive pressure up to 30 GPa, what evidences that they are superior to the all-carbon cage structures, which collapse and convert into diamonds under similar or much lower pressures.

Currently ApNano can manufacture only a few kilograms of the new material a day at their lab in Nes Ziona. In an interview by IsraCast[4], Dr. Menachem Genut, ApNano CEO, explained that the company is moving into semi-industrial manufacturing within the next six months producing between 100-200 kilograms of the material per day, gradually moving to full-scale industrial production by 2007, creating several tons each day.

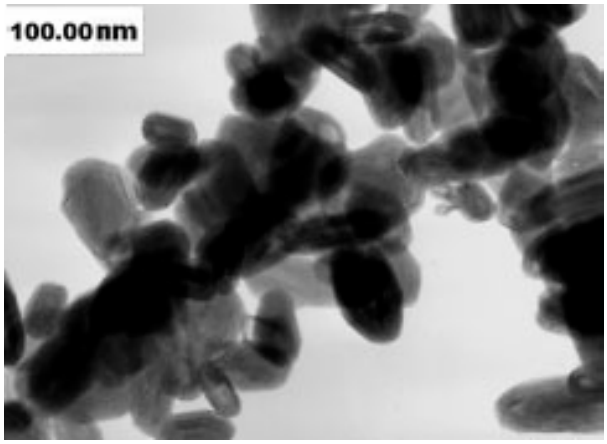

Fig. 4: HRTEM of typical WS_2 nanoparticles.

Due to inertness of the structure, toxicity is not expected. The first analysis performed on rats have shown that the WS_2 NPs are not toxic for oral digestion, breathing, neither they produce any irritation of the skin. Open question is accumulation of the particles in human lung if the particles become airborne, and possibility of self-cleaning of sulphur

[4] http://www.isracast.com/tech_news/091205_tech.htm

rich material from human body. The possible reactions with body liquids are not known. If the accumulated particles would undergo the decomposition, the appearance of hyper-sensitivity on metals is possible. Sensitivity of human body to metallic tungsten or molybdenum oxides is not known.

No reports were located in which death in humans could be specifically associated with exposure to airborne tungsten or tungsten compounds[5]. Increased mortality has been attributed to occupational exposure to dusts containing tungsten carbide and cobalt among hard metal workers. It is generally believed that the health effects observed in hard metal workers are the result of exposure to cobalt or other metals (e.g., nickel), not tungsten. Mechanisms concerning absorption, distribution, and toxic action of tungsten have not been studied to date; studies should be designed to identify such mechanisms.

No established methods or treatments for reducing the body burden of tungsten were identified in literature searches. No information was located regarding treatments to repair damage or improve compromised function resulting from exposure to tungsten.

There is not enough information to determine whether inhalation, oral, or dermal exposure to tungsten or tungsten compounds can cause cancer in humans. Tungsten has not been classified for carcinogenic effects by the Department of Health and Human Services (DHHS), the International Agency for Research on Cancer (IARC), or the US Environmental protection Agency (EPA).

Data documenting molybdenum toxicity in humans are limited[6]. The physical and chemical state of the molybdenum, route of exposure, and compounding factors such as dietary copper and sulphur levels may all affect toxicity. Mild cases of molybdenosis may be clinically identifiable only by biochemical changes (e.g. increases in uric acid levels due to the role of molybdenum in the enzyme xanthine oxidase). Excessive intake of molybdenum causes a physiological copper deficiency, and conversely, in cases of inadequate dietary intake of copper, molybdenum toxicity may occur at lower exposure levels.

Sulphate and molybdate follow similar metabolic pathways[7]. Sulphate will alleviate molybdenum toxicity. Molybdate and sulphate act together in creating copper deficiency in cattle and sheep giving rise to molybdenosis or "teart" condition. Molybdenum inhibits the activity of the enzyme liver sulphide oxidase and the toxicity of molybdenum compounds is enhanced by sulphide. In assessing possible biological effects of molybdenum it is important to take into account its metabolic interrelationships with other trace elements (phosphorus, sulphur, potassium, iron, copper, zinc, and iodine). Acute molybdenum poisoning in human beings is extremely unlikely because of the massive dose required. The effect of repeated exposure to small concentrations of molybdenum compounds is more difficult to assess. In animals and human beings molybdenum is adsorbed and excreted rapidly and so is not likely to be a cumulative poison. In checking for possible molybdenum toxicity it is important to know where and in what form toxic effects may occur. In experimental animals molybdenum toxicity

[5] http://www.atsdr.cdc.gov/tfacts186.html
[6] http://risk.lsd.ornl.gov/tox/profiles/molybdenum_c_V1.shtml
[7] International Molybdenum Association, U.K., http://www.imoa.info/Default.asp?Page=110

causes loss of weight, harmful changes in the liver, kidneys, and bones and diminution of the strength of conditioned reflexes.

Tab. 4: Characteristic properties of inorganic Fullerenes.

No.crt.	Properties of WS_2 and MoS_2 fullerenes	Details
1	Morphology	20nm-150 nm in diameter Aspect ratio: L/l/h(D): 1-2 Specific surface (m^2/g): 35 – 9 for MoS_2 and 23-9 for WS_2 Agglomeration: yes
2	Crystallinity	Yes, faceting, exfoliation
3	Wettability	Hydrophobic
4	Reactivity/Degradation	Inert/no information, probably oxidation of metal
5	Solubility	In water with ionic or cationic surfactants
6	Toxicity	No, according to available information

1.2.3 Nanotubes

Nanotubes are quasi one-dimensional objects with extremely large aspect ratio: diameters in a range from 1 nm up to several microns, and length from a few microns up to several millimetres. They are hollow; wall thickness varies from 0.3 nm up to 100 nm, density of defects depends strongly on the way of synthesis and on the selected materials. The tubes prepared from layered materials (graphite, MoS_2, WS_2, TiS_2, etc.) show a tendency of spontaneous formation of self-terminated cylinders, while metal or metal oxide nanotubes need a template growth process to force the structure to form the cylindrical shape.

1.2.3.1 Carbon nanotubes

Authors: Gordon Chambers, Eva Herzog, Declan McCormack.

Carbon nanotubes (CNT) were first observed by Sumio Iijima in 1991 (Iijima, 1991). They are unique, one dimensional macromolecules, comprised entirely of carbon. They consist of extended tubes of rolled graphene sheets with an axial symmetry and a diameter in the nanometer range and can grow up to several centimetres long (Saito, 1998). There are two main types of CNT, differentiated by their structure, single wall carbon nanotubes (SWCNT) and multiwall carbon nanotubes (MWCNT). SWCNT consist of a singular graphene cylindrical wall (diameter 0.7 - 2 nm), whereas MWCNT have walls made up of several coaxial graphene cylinders, so that the diameter of MWCNT is about ten nanometres or more, depending on the number of layers (Ebbesen et al, 1993). CNT exhibit a number of unique properties including strength, toughness, chemical robustness, thermal conductivity and electrical conductivity

(Dresselhaus, 1996). These properties, coupled with their nanoscale geometry make them ideal candidates for a number of potential applications in the nano area although production costs and processibility are restricting factors.

Synthesis and purity of carbon nanotubes

Several methods have been developed for the synthesis of CNT, including electric arc discharge, laser ablation and catalytic vapour decomposition of hydrocarbons. No synthetic processes for the production of CNT however can guarantee specific dimensions or physical properties. Furthermore, the separation of tubes from tangles (or bundles) in which they emerge from the production process is still problematic and it is often necessary to remove catalyst or amorphous carbon residues present after synthesis. The most commonly used catalysts for CNT production are metal particles such as cobalt (Co), molybden (Mo), iron (Fe), nickel (Ni) and yttrium (Y). These metals can be encapsulated and therefore coated with carbon, or adhere to the surface of the tubes so that an apparent metal oxide layer forms on the outside of the tube. The type and content of residual metal particles is thus important in terms of the relative toxicity of 'as produced' CNT since the health risk associated with CNT prior to removal of catalyst material will likely be due to both the carbonaceous and metallic components. (Shvedova et al., 2003; Kagan et al., 2005) (compare Chapter 2.1.3).

Ultrasonication or acid washing is frequently employed to purify SWCNT samples, but these methods increase the risk of damaging or chemically altering the tubes (Donaldson *et al.*, 2006). Furthermore these purification processes are of unequal efficiency, so that the final chemical composition of nanotube samples often varies (Muller et al., 2005). Hence even purified SWCNT may contain some amount of catalyst residues and amorphous carbon.

Aggregation

As-produced CNTs pack into crystalline ropes via electrostatic and Van der Waals forces, these ropes can further aggregate into tangled networks and reduce the unique properties of the individual tubes acting as an obstacle to the realisation of many applications. The degree of aggregation however also determines how CNT are presented to exposed cells or tissue (Casey et al., 2007; Davoren et al., 2007). For example their lung deposition characteristics can change, as aggregates have a greater aerodynamic diameter than single particles beyond the range of respirabiltiy (> 5 µm), and making it more difficult for them to be inhaled deep into the lung (Muller *et al.*, 2005). However much of the CNT research over the last decade has focused upon the separation and purification of 'as produced' samples with a range of methods now available to disperse CNT for application purposes. This could inadvertently increase the risk of inhalation exposure (Muller *et al.*, 2005). Furthermore evidence suggests that lung surfactant itself might also separate single fibres (Jia *et al.*, 2005).

Exposure to carbon nanotubes

Due to the growing interest in development and production of CNT, there is increasing potential for human exposure (see Chapter 3). Currently the greatest potential for CNT exposure is undoubtedly occupational. Owing to their extremely light weight, CNT can become airborne and be inhaled as either single particles or aggregates, if they are incorrectly stored and/or handled in an industrial setting (Fiorito *et al.*, 2006). The

propensity of SWCNT to form aerosols was shown in a study on HiPco[8] single walled carbon nanotubes which indicated that CNT aerosols formed under laboratory conditions gave rise to between 0.7 and 53 µg/m^3 of nanotubes in air samples (Maynard *et al.*, 2004). The same study showed that potential dermal loading during material handling in the field was 217 to 6020 µg on individual gloves for the same HiPco SWCNT.

Toxicology of carbon nanotubes

Pulmonary toxicity

In vivo studies

To date there is only a handful of published *in vivo* studies assessing the impact of CNT on the lung and results of these investigations are conflicting (Huczko *et al.,* 2001; Lam *et al.*, 2004; Muller *et al.*, 200; Shvedova *et al.*, 2005; Warheit *et al.,* 2004). Nevertheless nearly all *in vivo* studies have found histological evidence of inflammation and granuloma formation in rodent lungs via intratracheal administration. However, an important limitation of all tests using intratracheal administration is that a single dose of CNT suspended in a vehicle such as PBS (Phosphate buffered saline), directly introduced into the trachea or pharynx of anaesthetized animals, results in an unrealistic situation compared to natural inhalation exposure. Artefacts are possible due to the non-physiological rapid delivery of particles. In addition, many of the reports have the intrinsic problem that CNT form aggregates in aqueous solutions, despite the use of surfactants and sonication (Smart et al., 2006; Royal Society, 2004). By injecting a bolus dose of aggregated material, foreign body granulomas can be induced that are non-specific and may not reflect the intrinsic effects of the test material. Even the investigators themselves questioned the toxicological relevance of their findings because of this fact. The absence of dose response relationships noted in some of the literature might also be the consequence of the CNTs clumping into large masses instead of being evenly distributed throughout the lung (Muller et al., 2005). Although pharyngeal aspiration results in some exposure to single CNT fibres, it still cannot mimic the real situation, because the nose is bypassed and CNT are delivered as a bolus dose.

Another drawback in experiments by Huczko *et al.* (2001), Warheit *et al.* (2004), Lam *et al.* (2004), and Shvedova *et al.* (2004) is that the degree of purity of CNT preparations used was not reported. The toxicological findings, particularly inflammatory effects observed, could be influenced by the presence of graphite and metal particle contaminants present in the samples (Fiorito *et al.*, 2006). Finally rat and mouse instillation studies also have their limitations due to difficulties occurring when extrapolating data to the human situation. Nikula et al. (2001) have shown that rat lungs process inhaled particles very differently from larger mammals, particularly humans.

In vitro studies

Muller et al. (2005), as in their *in vivo* studies, tested the inflammatory potential of MWCNT on peritoneal and alveolar macrophages, derived from Sprague-Dawley rats.

[8] HiPco® a form of commercial SWCNT produced via the high pressure decomposition of carbon monoxide available from Carbon Nanotechnologies, Inc. (Houston, TX)

In comparison with asbestos and carbon black, ground MWCNT was seen to be as effective in inducing dose-dependent cytotoxicity and up-regulation of TNF-α expression, an indicator of inflammatory potential. In contrast as-produced MWCNT showed significantly lower activity. The authors suggested that increased agglomeration in the 'as produced' samples resulted in a decrease in CNT availability to the cells, reducing their cytotoxic and inflammatory potential.

Jia et al. (2005) also conducted cytotoxicity studies of both SWCNT and MWCNT on an alveolar macrophage cell line. Due to the tendency of CNT to aggregate, the effect of particle size was attempted to be removed by the researchers by adopting a modified dosing regime of 1.41 to 226 μg/cm^2 for SWCNT. At an exposure concentration of 22.6 μg/cm^2, cytotoxicity using the MTT assay revealed that SWCNT were more toxic than MWCNT on a mass basis. However the SWCNT sample used was synthesised via the electric arc discharge method and had trace amounts of iron, yttrium and nickel catalysts and so the influence of trace catalysts may have played a role in this outcome. Indeed other reports indicate that purified SWCNT (with catalyst removed) did not stimulate inflammatory responses in murine and human macrophages and are actually low in cytotoxicity (Fiorito *et al.* (2006)). Furthermore the presence of iron in the samples could affect redox dependent macrophage responses (Kagan *et al.*, 2006).

Jia et al. (2005) however also explored the phagocytic ability of macrophage cells with the results indicating that SWCNT were more effective in impairing phagocytosis and caused a larger number of cells to lose their phagocytic abilities. Furthermore, TEM analysis showed that with increasing SWCNT concentrations, swelling of the endoplasmic reticulum (ER), vacuolar changes, and phagosomes were produced. At higher doses of 3.06 μg/cm^2, surface protrusions, as seen following apoptosis, could also be observed (Jia et al., 2005). A drawback of this study however was that authors did not provide enough information to gauge whether the dose dependent increase in toxicity corresponded to an increase in CNT particle size or an increase in total mass to which cells were exposed.

In a study by Wörle-Knirsch et al. (2006) A549 lung carcinoma cells were exposed to SWCNT, containing mainly Co catalyst residues. Cell viability was determined using different cytotoxicity assays, namely MTT, WST-1 and LDH. After 24 hour exposure to SWCNT, no decrease in viability could be observed, indicating no reduction in mitochondrial viability and membrane integrity, respectively. In addition, propidium iodide (PI) and annexin-V staining was employed to confirm the results obtained and neither necrosis nor apoptosis could be detected. TEM studies performed by these authors revealed that SWCNT bundles are taken up into A549 cells and are surrounded by a membrane. Immunohistochemistry showed that focal adhesion kinase (FAK) and cytoskeletal actin filaments strongly accumulated near SWCNT which adhered to the cells. As described by Tian *et al.* (2006), cells seemed to detach from the culture dish and grow out of plain, so that authors supposed this might be the explanation for granuloma formation seen *in vivo*.

The inconsistencies reported for the *in vitro* studies can potentially stem from a number of interference effects such as aspects of sample purity and aggregation. However, for cytotoxicity tests which utilise fluorescent end points the interactions between the CNT and the cell culture medium's components may also represent a considerable degree of interference. The *in vitro* cytotoxicity testing of SWCNT (Cui *et al.*, 2005; Bottini *et al.*,

2006) typically involves their dispersion within a cell culture medium, followed by their subsequent addition to a cell line of interest in the medium in which they have been dispersed. The interaction between the SWCNT (bundles) and the cell culture medium and the influence of such interactions on cell viability has been explored by Casey *et al.* (2007). Both the intrinsic components of the medium and the added growth supplement are seen to interact with the SWCNT most likely through physisorption. The interaction of these SWNT bundles with the growth medium and supplement is postulated to result in a reduction in the availability of the constituents to the cells, causing a secondary rather than primary toxicity of the SWCNT. In summary due to medium depletion by the absorption of constituents onto the nanotube surface a false positive toxic effect could be observed for a range of cytotoxic dyes and assays. Indeed most authors now agree that a more detailed physico-chemical characterisation of CNT samples is crucial, before testing their biocompatibility and drawing any conclusion concerning their toxicity.

Dermal toxicity

In vivo studies

There is extremely limited information available on the skin irritating potential of CNT. A preliminary study was published by Huczko and Lange (2001) who conducted two routine dermatological tests. A 96 hour patch test and a modified Draize rabbit eye test. In both tests, no irritation due the CNTs was observed. Koyama *et al.* (2006) evaluated the biological responses to four different types of CNT by measuring $CD4^+$ and $CD8^+$ T-cells in peripheral blood obtained form BALB/c mice. In addition, they carried out histopathological studies on tissues surrounding subcutaneously implanted CNT.

After one week only the SWCNT activated the major histocompatibility complex class one (MHC I) pathway of the antigen-antibody response system resulting in the appearance of oedemas. After two weeks, $CD4^+$ and $CD4^+/CD8^+$ values were significantly high with no change in $CD8^+$ suggesting an activated MHC class II could be measured for all samples. The time dependent changes in peripheral T-lymphocytes correlated with a processing phase of granuloma formation reported by Warheit *et al.* (2004) and Sayes *et al.* (2006a). Physico-chemical analysis of the SWCNT sample revealed Iron residues were present however no significant correlation between the iron amount and T-cell responses could be found, suggesting that the metal catalysts were a minor factor in the T-cell responses to CNT *in vivo*.

In vitro studies

The first dermal cytotoxicity warnings were reported by Shvedova *et al.* in 2003 after investigating the effects of unrefined HiPco SWCNT on immortalized human epidermal keratinocytes (HaCaT). SWCNT exposure resulted in accelerated oxidative stress, including increased free radical and peroxide generation, depletion of glutathione levels, oxidation of protein SH groups, and depletion of total antioxidant reserve in vitamin E. Furthermore, loss in cell viability and morphological alterations to cellular structures was observed. It was concluded that oxidative stress might be associated with the concentration of the iron catalyst which for this sample was up to 30 percent iron residues per mass. Similar dermal toxicity results were reported by Monteiro-Riviere *et al.* (2005) for MWCNT after a study on human neonatal epidermal keratinocytes (HEK). In addition vacuoles could be seen within the cytoplasm of cells

containing MWCNT as detected by TEM. As the exposure time and dose was increased it was noted that the cell viability decreased although localization and initiation of an irritation response by MWCNT could not be shown. However the MWCNT samples used in this study were free of catalyst particles, suggesting that CNT themselves are potentially dermatotoxic (Monteiro-Riviere *et al.*, 2005).

Another study on the effects of SWCNT exposure on human keratinocytes (HaCaT) was carried out by Manna *et al.* (2005), were significant increases in Reactive oxygen species (ROS) could be seen at SWCNT concentrations ranging between 1 and 10 μg/ml after 72 hour exposure. In addition significant dose-dependent decreases in cell viability at a SWCNT concentration of 0.5 μg/ml was also observed. Other cell lines used in this study including HeLa (epithelial cells), A549 and H1299 lung carcinoma cells showed a similar behaviour. Finally Tian *et al.* (2006) evaluated the *in vitro* cytotoxicity of a number of carbon nanomaterials, including SWCNT and MWCNT on human dermal fibroblasts. SWCNT exposure resulted in the highest drop in cell survival with 58% viability after five days. In addition cell adhesion assays showed that only SWCNT exposure caused a significant decrease in the ability of cells to adhere and form colonies.

Environmental Aspects of CNT

A limited number of ecotoxicological studies of carbon nanomaterials have been published. Oberdörster *et al.* (2006) showed that fullerenes as well as SWCNT can be taken up into aquatic organisms following exposure in the water column. It appeared that SWCNT elicit fewer biochemical or gene expression level changes than fullerenes. The main problem however occurring during these studies was the non-solubilisation of the test material. Not solubilised particles were floating on top of the water and mistaken as food and ingested. An interesting observation made by Oberdörster and colleagues (2006) regarding C_{60} however was the increase in lipid peroxidation in fish brains after 48 hour exposure to 0.5 mg/l.

A study on the life-cycle effects of SWCNT on an estuarine meiobenthic copepod was reported by Templeton *et al.* (2006). The test SWCNT's were functionalized making them dispersable in water and were processed to remove metal and carbon residues. Toxicity assays were carried out using meiobenthic copepods Amphiasus tenuiremis, a critical food source for shrimps, fish and crabs which passes through three distinct life stages. A 96-well microplate life-cycle bioassay was employed where larvae were exposed to 200 µl of SWCNT concentrations of 0.58 to 10 mg/l prepared in seawater. In addition, a simulated matriarchal stage structured population growth model, derived from population life-cycle characteristics was used to define the growth rate of exposed copepods. Finally, adult Amphiascus tenuiremis and their foecal material were investigated by confocal laser scanning microscopy (CLSM). The authors concluded that purified SWCNT were exhibiting very low toxicity to deposit-feeding estuarine copepods, only producing adverse effects at environmentally unrealistic concentrations of 10 mg/l. As seen by CLSM, copepods were ingesting SWCNT, which formed clusters in the gut and were eventually incorporated in a morphologically altered state into faecal pellets.

Other toxicity studies

In 2001, Mattson *et al.* reported that CNT can inhibit the growth of embryonic rat-brain neuron cells, but no further studies were performed on this issue. Tamura *et al.* (2004) briefly investigated the cytotoxic effects of purified CNT on neutrophils isolated from human blood. Significant increase in super-oxide anion and TNF-α production could be observed after cell contact for one hour, while cell viability decreased. The lack of details on the type of CNT, synthesis or handling methods was identified as a major drawback of this publication (Smart *et al.*, 2006).

Confounding problems occurring during CNT toxicology studies

A common problem in all studies on carbon nanotubes is their hydrophobic surface which makes them hardly soluble in aqueous solutions. Manipulation and characterization of large numbers of individual CNT is difficult because high molecular weights and strong intertubular forces, both van der Waals and electrostatic, promote the formation of bundles and ropes. This occurs particularly in saline, media or serum solutions as commonly used in toxicity testing. In addition, it is not known how CNT in general behave when dispersed in exposure media -and if absorptive interferences render some assays inconclusive or inappropriate for assessing CNT toxicity (Belyanskaya *et al.*, 2007; Casey *et al.,* 2007; Wörle-Knirsch *et al.*, 2006). Researchers have used various different methods to disperse their CNT samples, which ultimately determines how CNT are presented to exposed cells, leading to discrepancies in toxicity data due to differences in sample preparation and handling, making inter-study comparisons problematic (Smart *et al.*, 2006) and raising the question of the materials natural behaviour if it enters an eco-system.

Another important issue in comparing and interpreting CNT toxicity data is the presence of residual metal catalyst particles. The type and content of residual metals may be important in terms of relative toxicity. The health risk associated with CNT prior to removal of catalyst material may be due to both the carbonaceous and metallic components. However it also has to be kept in mind, that purified CNT also appear to generate oxidative stress on cultured cells, by as yet unknown mechanisms. Nevertheless it is widely accepted that suitable nanotoxicology assays for carbon based nanostructures must incorporate adequate physical and chemical characterization studies on the pristine material to allow traceability and standardization between studies.

Added after revision of the report by H.F. Krug on request of the EC:

Recently, Ken Donaldson and his coworkers (Poland *et al.,* 2008) have presented new data on CNT toxicity and draw the conclusion that very long carbon nanotube fibres may act as asbestos fibres in provoking granuloma-like tissue alterations directing to mesothelioma formation. As most of these results have been presented and discussed at different events during the past months the published data have been known already within the scientific community before this publications appeared in Nature Nanotechnology Online in May 2008. This work makes several points pretty clear: In the field of “Nanotechnology” we are confronted with totally different materials even within a „consistent“ family of materials like the CNTs. There is a big difference in the activity of single-walled versus multi-walled CNTs, and several papers claimed that it is the relatively high proportion of contaminants (such as iron, nickel or amorphous

carbon) that is affecting biological systems, as recent studies have shown (Belyanskaya *et al.,* 2007; Kagan *et al.*, 2006; Pulskamp *et al.*, 2007a; Pulskamp *et al.*, 2007b; Wick *et al.*, 2007; Wörle-Knirsch *et al.*, 2006).

Members of the consortium have already published last year that there is a remarkable difference in biological activityof CNTs if they are agglomerated or highly dispersed; hence, the effect shown in Donaldson's paper is not totally new (Wick *et al.,* 2007).

Moreover, Donaldson's "very long" CNTs were synthesised in a very special way, and the fraction containing the longest CNTs also contain the highest amount of iron residues (used as catalysts for synthesis of the CNTs). Donaldson himself stated that it is totally unclear and not shown if these long fibres will reach tissues beyond the lung in a real life-scenario, hence there is so far no final indication for the induction of mesothelioma under "normal" conditions.

To my believe, Andrew Maynard, one of the co-authors of this publication, is absolutely right, stating that it would be totally wrong not to investigate the advantages of these new materials and we can not abstain from the use of these materials. But it goes without saying that we also have to be aware of their possible biological effects that may end in a catastrophe as it did in the case of asbestos.

The study does clearly indicate that not all different CNT variations have the same severe effect in this mouse model (Poland *et al.*, 2008). Especially the shorter tubes have no effect at all, thus confirming the earlier results obtained in the laboratories of the members of the consortium with several cell cultures. The study also demonstrates undoubtedly that we have to keep in mind that specific forms or special shapes of some of the (nano)materials may induce important biological effects and give rise for severe concern. Thus, we should not cease to work in the field of nanotoxicology and the international funding has to be continued to guarantee a sustainable nanotechnology.

1.2.3.2 Inorganic nanotubes

Authors: Maja Remskar

Since the first report on WS_2 and MoS_2 nanotubes in 1992, several compounds have been found stable in cylindrical geometry. Six families of inorganic nanotubes (NTs) have been synthesized up to now (Remskar, 2004).

1. transition metal chalcogenide NTs: MoS_2, $MoSe_2$, WS_2, WSe_2, NbS_2, $NbSe_2$, TaS_2, ZrS_2, HfS_2, TiS_2, ZnS, NiS, CdSe, CdS;
2. oxide NTs: transition metal oxides: TiO_2, ZnO, GaO/ZnO, VO_x, $W_{18}O_{49}$, V_2O_5, Al_2O_3, In_2O_3, Ga_2O_3, $BaTiO_3$, $PbTiO_3$; silicon oxide: SiO_2; MoO_3; RuO_2; rare earth oxides: (Er, Tm, Yb, Lu) oxide;
3. transition metal halogenous NTs: $NiCl_2$;
4. mixed phase and metal doped NTs: $PbNb_nS_{2n+1}$, $Mo_{1-x}WS_2$, $W_xMo_yC_zS_z$; Nb-WS_2, WS_2-carbon NTs, NbS_2-carbon NTs;Au-MoS_2, Ag-WS_2, Ag-MoS_2; $Cu_{5.5}FeS_{6.5}$;
5. boron and silicon based NTs: BN, BCN, Si;
6. metal nanotubes: Au, Co, Fe, Cu, Ni, Te, Bi.

The aspect ratio in different NTs depends on the synthetic route and ranges from several tens up to several tens of thousands. The longest tubes show several millimetres in length. Regarding diameters, NTs with diameters below 20 nm have been synthesized quite rarely. BN NTs with inner diameters from 1 to 3 nm and outer

diameters from 6 to 8 nm were made by the arc-discharge process; 15 nm diameter WS_2 NTs were grown by sulphurization of WO_3; NbS_2 NTs with inner diameters in the range ~4-15 nm were made by decomposition of NbS_3; CdSe NTs with outer diameters in the range 15-20 nm have been made from cadmium oxide using surfactant assisted synthesis. Titania nanotubes with diameters of about 8 nm have been produced by hydrothermal treatment of nanosize TiO_2 powder in NaOH solution. A long term discussion of their composition, balancing between the anatase and rutile phases of TiO_2, is coming to the conclusion that the titania nanotubes are actually $H_2Ti_3O_7$ single sheets rolled up into cylindrical geometry.

The nanotubes differ from the corresponding compounds in high aspect ratio, small dimensions and due to defect structure, in enhanced chemical reactivity. The lighter nanotubes are those composed of BN, BCN and silicon based ones due to low molecular mass and titania ones due to low dimensions. The least stable ones in the ambient atmosphere are $NiCl_2$ NTs, which facilitate water intercalation resulting in slow self-dissolution. Until now, no g-quantities of inorganic nanotubes have been reported and their physical and chemical characterisation is still in research stage.

Inorganic NTs exist in different states of crystallinity. Semi-single crystal structures are typical for non-helical or mono-helical NTs (WS_2, Fig. 5, left image), while polycrystallinity appears either in the structure of the nanotube wall, composed of small thin crystal flakes or in the radial direction as multi-helicity. Many of the inorganic NTs prepared by the decomposition process appear as an assembly of nanocrystallites forming the nanotube wall (e.g. HfS_2, NbS_2, Fig. 5, right image).

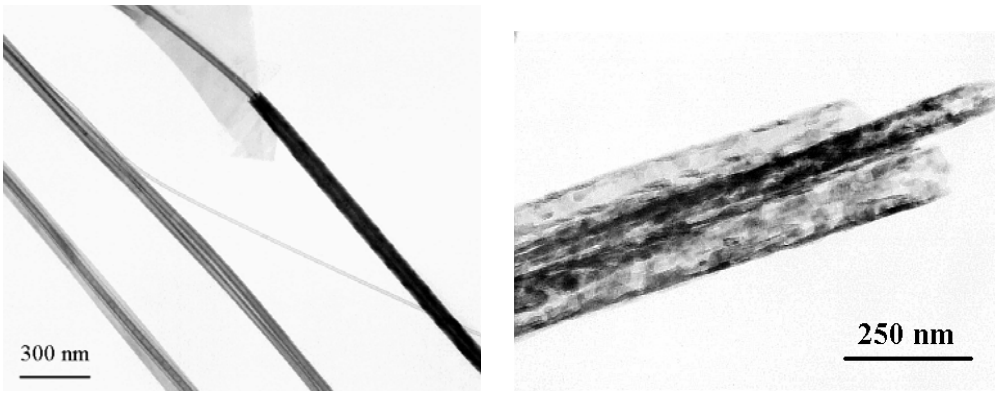

Fig. 5: Inorganic NTs.

No data are available about possible toxicity of inorganic nanotubes, which would differ from bulk material of the same compound. Their needle like geometry enables expectations about similar effect on lung as in case of asbestos, but only in a case that the NTs are light enough to become airborne, which is not likely in most inorganic nanotubes due to large molecular mass.

Inorganic materials have a wide range of useful properties that might be exploited in nanotube and nanowire form, including high temperature superconductivity for low-loss electrical power delivery, enormous magnetoresistivity for information storage, and ferroelectric and ferromagnetic properties for quantum computing and spintronics

applications. Currently, the field of synthesis of inorganic nanotubes is one the most quickly developed area of chemistry and material science.

Tab. 5: Characteristic properties of inorganic nanotubes.

No.crt.	Properties of Inorganic nanotubes	Details
1	Morphology	8 nm-few μm in diameter Aspect ratio: L/l/h(D): 10 - 10^4 Specific surface (m^2/g): Depends on the compound Agglomeration: yes, into ropes
2	Crystallinity	Yes, single crystallinity or polycrystallinity
3	Wettability	Depends on the compound
4	Reactivity/Degradation	Depends on the compound
5	Solubility	Depends on the compound
6	Toxicity	Depends on the compound and aspect ratio (not enough data available)

1.2.4 Engineered Quantum Dots

Author: Declan McCormack

Whilst on the nanoscale there has been a true convergence of medicine, engineering and the sciences leading to a blending of these disciplines, from another perspective there has been a divergence and proliferation in the potential applications of new technologies enabled by nanomaterials. Quantum dots have been one of the most interesting classifications of such nanomaterials. A quantum dot (QD) may be defined as a semiconductor nanostructure which confines the motion of valence band electrons, conduction band holes or excitons (bound pairs of electrons and holes) in all 3 spatial directions. Quantum dots, per se, cannot be considered as an entirely novel classification of compounds. Biogenic and anthropogenic nanosized inorganic particles occur in water streams, silts, clays and other natural sources. It should be noted that there is little evidence to indicate that such nanosized materials have had a more detrimental ecotoxicological or environmental impact than their corresponding bulk analogues.

The situation for engineered quantum dots (EQDs) currently lacks much corresponding data. As their prevalence increases it is important to investigate the potential risks associated with such nanomaterials. It should be noted that studies on their cytotoxicity, bioaccumulation, biopersistence and ecotoxicity are at quite preliminary stages. The recent comprehensive review by Hardman (2006) suggests strongly that one must take account of many specific factors when assessing the toxicity of EQDs.

Commercially manufactured quantum dots generally are based upon CdSe spherical nanoparticle cores coated or capped by a thin layer of ZnS as a stabilizing agent (Alivisatos *et al.*, 2005) although recently a number of non-heavy metal based InGaP/ZnS materials have also become commercially available (Evident-www.evidenttech.com). Their global production is relatively small in comparison to other nanomaterials and the concentrations at which such materials are deployed are quite low (of the order of μM).

Surface Modification

In most situations quantum dots such as CdSe/ZnS are surface-modified for a number of reasons. Firstly it is important to stabilize such EQDs and this may be achieved by a variety or combination of routes including capping agents, affinity ligands, polymer coatings and shells. Such stabilizing agents can also play other important functions in that they improve dispersion properties, assist binding and biocompatibility, whilst also minimizing dissolution and reactivity of the metals present in both the shell and core. The addition of robust surface chemistries e.g. dithiols, cross-linked ligands, oligomeric phosphines, dendrimer and peptide coatings have been quite successful in improving the stability of such EQDs and furthermore have also resulted in increased quantum yields for QDs such as CdSe (Tsay and Michalet, 2005). Polymeric coatings such as PEG not only enhance the aqueous solubility of QDs but also reduce non-specific adhesion to biological cells. Much evidence is available (Guo *et al.,* 2007; Hoshino *et al.,* 2004) to suggest that the properties (including toxicity) of EQDs are controlled by the surface modifiers rather than the QD cores.

Applications

Currently the main applications of EQDs are as biological markers where they may replace traditional fluorescent dyes (O'Brien, 2003). In comparison with organic fluorophores, these EQDs display unique properties such as size- and composition-tunable fluorescence emission from visible to infrared wavelengths, large absorption coefficients across a wide spectral range and high levels of brightness and photostability (Gao *et al.,* 2004). Indeed the use of such EQD-based probes in biological applications has escalated from 4 in 2000 to approximately 100 in 2005 (Pinaud *et al.*, 2006). Their narrow emission range and tunability allows multi-colour imaging of biological samples and fluorescence labelling of biomedical samples using, for example, confocal fluorescence microscopy (Bruchez *et al.*, 1998). Other potential applications include advanced displays (QDLEDs), site specific gene and drug delivery, ultrafast optical switches and logic gates (> 15 TB/sec), encryption and anti-counterfeiting technologies (O'Brien, 2003)), molecular diagnostics (Smith *et al*, 2006) and drug discovery (Ozkan, 2004).

Toxicity

Uncoated CdSe dots have been reported to be highly toxic to cells under certain conditions. One of the earlier reports by Derfuss *et al.* (2004) noted that such acute toxicity to primary hepatocytes occurs upon exposure to ultraviolet light. This process facilitates the release of Cd^{2+} ions due to degradation of the nanostructure. Such UV degradation of QDs may have some benefit as a therapeutic agent. For example potential exists for photodynamic therapy in that the release of Cd^{2+} allied to the

formation of free radicals and reactive oxygen species which may assist destruction of targeted cancer cells (Pinaud *et al.*, 2006; Rhyner *et al.*, 2006). Cadmium has a half life of 20 years in the human body and is a suspected carcinogen which accumulates in the liver and kidney. It biodistributes in all tissue since there are no known active mechanisms to excrete Cd from the body (Nath, 1984). Both cadmium and selenium are known to cause acute and chronic toxicities in vertebrates and are of considerable concern for both human health and environmental impact (Hardman, 2006). Lovrick *et al.* (2005) have attempted to quantify the differences in sub-cellular distribution and toxicity of CdSe based QDs of varying size. In particular they found that coated EQDs were significantly less (by a factor of 10) cytotoxic to rat phenochromocytoma cell cultures than their uncoated equivalents. Another important finding was that smaller EQDs (2.2 nm) show more pronounced cytotoxicity than larger (5.2 nm) counterparts. This relationship between cytotoxicity and EQD size was also observed by Shiohara *et al.* (2004) in their cell viability investigations using mercapto-undecanoic acid coated EQDs. Clarke *et al.* (2006) note that little toxicity is seen when EQDs are retained in lyosomes whereas severe damage can occur when EQDs associate with mitochondria.

In his summary of EQD toxicity Hardman (2006) states that “the studies reviewed here suggest that QD toxicity depends on multiple factors derived from both the inherent physicochemical properties of QDs and environmental conditions. QD size, charge, concentration, outer coating bioactivity (capping material and functional groups), and oxidative, photolytic, and mechanical stability are each factors that, collectively and individually, can determine QD toxicity. Of these physicochemical characteristics, functional coating and QD core stability figure prominently and likely will be significant factors in assessing the risk of QD toxicity in real-world exposure scenarios”.

Exposure Routes

The main exposure routes of EQDs are gastrointestinal and to a lesser extent, dermal. Inhalation can be discounted as EQDs are formed and exist in solution-based media. Gao *et al.* (2004) indicate that EQD probes accumulate at tumours both by the enhanced permeability and retention of tumour sites and by antibody binding to cancer-specific cell surface biomarkers. Such bioconjugated probes can combine in-vivo targeting along with direct imaging of human cancer cells. In situations where a substantial decrease in fluorescence has been observed on administration of EQDs in-vivo such a diminution has been attributed to degradation of surface ligands and coatings by body fluids. However in the study by Gao *et al.* (2004) hydrophobic protective layer ensures stability of these EQDs, which have been observed in the blood for as long as 72 hours. Such polymer-based coatings show no effect on cell division or no ATP reduction. Indeed a dose of up to 3×10^6 EQDs in a single cell did not reduce its viability or growth.

Ryman-Rasmussen *et al.* (2006, 2007) report that both spherical (4.6 nm) and ellipsoidal ($12\text{nm} \times 6\text{nm}$) Qds when flowed across porcine skin cells resulted in penetration across the stratum corneum and localization within the dermal and epidermal layers. Whilst there was some difference in rates of translocation depending on particle size and coating the main finding reported was that penetration of intact skin, at an occupationally relevant dose, can occur within the span of an average working day.

Biopersistence

A quantum dot is approximately the same size as a small protein (< 10 nm) hence QDs can easily enter most cells and indeed reach vital organs via the blood stream as it circulates through the body. Some authors (Sharma *et al.*, 2006) have suggested a strong correlation between surface nano-architecture and biocompatibility i.e. increasing aspect ratio indicating increased toxicity. However EQDs are, by and large, spherical in structure, which diminishes the importance of shape as an influencing factor.

Little is known about the mechanism of metabolism or clearance of EQDs from living animals although EQDs have been detected in mouse bone marrow, spleen and liver at least 4 months after administration whilst other reports suggest that particles larger than 50 nm could not be easily cleared by the kidney (Yu *et al.,* 2006).

Risk

A detailed analysis of the risk factors associated with the industrial scale production and use of one specific type of (ZnSe based-) EQD was undertaken by Ogilvie Robichaud *et al.* (2005). The QD material chosen was produced by Karanikolos *et al.* (2004). Relative risk factors such as volatility, carcinogenicity, flammability, toxicity and persistence were considered based on a listing of input materials, output materials and waste associated with the manufacturing process of such materials. The overall risk score was determined by an insurance database risk evaluation and by a separate qualitative method. Both yielded similar results and the overall risk associated with QDs was found to be on a par with the production of aspirin and significantly lower than the production of refined petroleum.

Future

Future work needs to address the potential long-term toxicity, degradation and metabolism of nanoparticle agents, to identify and develop new biomarker-probe systems and to develop multifunctional nanoscale platforms for integrated imaging, detection and therapy.

Recommendations / Knowledge Gaps

Future work needs to

- address the potential long-term toxicity, degradation and metabolism of nanoparticle agents,
- identify and develop new biomarker-probe systems
- develop multifunctional nanoscale platforms for integrated imaging, detection and therapy.

1.3 Nanoparticle Properties

Author: Nicoleta Lupu and Horia Chiriac

Particle Size

As extensively covered in Chapter 1.2.1 two main parameters from particles are of importance for their possible toxicity: one being the size and the other the chemical composition, which is responsible for the intrinsic toxicity of the compound (Donaldson *et al.*, 2002; Donaldson *et al.*, 2004a; Donaldson and Stone, 2003). Reduction in particle size to the nano-level results in an enormous increase of surface area, so relatively more molecules of the chemical are present on the surface, increasing the risk of an enhanced intrinsic toxicity. This may be one of the explanations why NPs are more toxic per unit mass than larger particles of the same material when used on a mass base (compare Chapter 1.2.1, page 9. Similar dose response relationships between particles of different sizes have been observed when the dose was expressed in surface area (Oberdörster, 1996; Oberdörster *et al.*, 2000, 2005a).

Chemical Composition

The chemical composition of the surface is important for the adverse effects of NPs. Fractions isolated from particulate pollutants (diesel exhaust particles) were demonstrated to exert toxic effects on cells *in vitro*. So, besides the particulates also the chemical composition, or for air pollutants the chemical absorbents can be responsible for the toxic effects.

Ultrafine particles can interact with metals. Iron was able to potentiate the effect of carbon black NPs, resulting in enhanced induction of reactive oxygen species (ROS) in a cell free system (Wilson *et al.*, 2002). In addition, surface modification of NPs can result in a diminution of cytotoxicity. The *in vitro* cytotoxicity of superparamagnetic iron oxide NPs could be abrogated by coating the NPs with pullulan (Gupta and Gupta, 2005). Also for dextran and albumin derivatised iron oxide NPs a reduction in their *in vitro* cytotoxicity was noted (Brown *et al.*, 2002).

Geometric Form

A special category to consider for adverse effects is nanotubes or nanofibres of a few nanometers in diameter but a length which could be several micrometers. It is speculated that even at this very small level differences may occur regarding the aspects ratio of NPs, especially when not roundish but fibrous.

Nanoparticle Properties and Biological Effects

Little is known about the pathways of non-degradable nanoparticles, their distribution throughout the body and the dependency of the biological behaviour from their physicochemical properties. It has to be assumed that accumulation will take place predominantly in the organs of detoxification. Whether or not a risk may arise from such accumulation of particles in the body has not yet been sufficiently examined. The biological activity of a tissue in contact with NPs depends both on the tissue as well as on the NPs chemistry, surface, shape and size. It is, therefore, rather difficult to define a material “biocompatible”, since, in the vast majority of cases, such definition takes into account its chemistry and, to a lesser extent, its surface, but very seldom the shape that particular material takes when it is transformed into an implantable device

or, in any case, into something that comes in contact with a living tissue, and even more rarely its size or surface topography (Kaiser *et al.*, 2006; Maniura *et al.*, 2006). In order to be biocompatible, a synthetic material that is put in touch with a biological tissue must induce a specific protein adsorption from the extracellular matrix. There are many cellular processes, which are triggered by the type of protein adsorbed, by its conformation and by its biological activity. If the presence of a certain protein is requested to guarantee a proper interaction of such a synthetic material, generally, but not exclusively, an implantable medical device, with the biological environment, it may be possible to manipulate the implant surface in order to induce in advance that situation (Maniura *et al.*, 2006). The so-called “biomimetic” surfaces base their activity in the human body upon this concept. The ability to design such a system is greatly supported by biotechnologies and nanotechnologies.

Nanospheres are already employed in humans, though only experimentally, in the diagnostic field. In fact, it was observed that highly lymphotrophic super-paramagnetic NPs (monocrystalline iron oxide) can easily gain access to lymphnodes by means of interstitial lymphatic transport in patients suffering from prostate cancer. Their presence in lymph nodes can be detected by MRI (Magnetic Resonance Imaging) and their concentration can indicate a metastasis. But the possibility to interact with the smallest components of the human body is not much known and makes NPs potentially dangerous, while verifying the results of this interaction is an awkward matter. It has already been demonstrated that those materials interact in different ways with the endothelium, macrophages and gut and liver epithelial cells. It was also discovered that metals are more hazardous than plastics for the survival activity of the cells. The production of the inflammatory and defence mediators depends on the particle chemistry. *In vitro* tests suggest that some new phenomena can occur when the nanoscale range of interaction is investigated. From the clinical point of view, it has been known for a long time that inhaled particles can induce diseases like asbestosis and silicosis. Another well-known clinical phenomenon is that a number of implantable medical devices wear *in vivo*, thus creating debris of micro- and nanosized particulate matter: an example of that is the wear of hip-joint prostheses and of dental restorations. The possibility that inorganic, chemically inert, microscopic debris can induce granulomas even in regions beyond the implant site is familiar to orthopaedic surgeons who must remove worn hip-joint prostheses because the debris their erosion produce brings about the local formation of granulomatous tissue and a bone degeneration with the ensuing loosening of the device. The data induced us to think that a pathology can be started by the presence of inorganic particles that cannot be metabolised or, in any case, disposed of, and these findings have us strongly suspect that the size of the particles, their local concentration and their velocity to reach the critical concentration can have an influence on the type of pathology. As a consequence, the concept of biocompatibility should be revised, keeping into account the fact that a material, which is certainly accepted in bulk form, may be no longer biocompatible when its size is reduced below a certain “critical” threshold. Probably, also the different chemistry of the particles (either ceramic or metal or plastic materials) can influence the relationship between the cells and the material's surface, which can lead to a different cellular reaction and, as the next step, to a clinical expression, although no literature has ever considered this so far. Those foreign “intruders”, not only bacteria, viruses and parasites, can be the cause of various pathologies.

As particles become smaller, the surface to volume ratio increases. Since catalytic reactions, particularly evident with transition metals, occur at surfaces, a given mass in nanoparticulate form will be far more reactive than the same mass of material made up of larger particles (for review: Nel *et al.*, 2006).

A further, well-known property of nanoparticles is their quantum effect. As their size approaches the smaller end of the nanoscale, the effect on their electric, optical and magnetic behaviour becomes more and more visible. Finally, NPs, being on the same scale as cellular components, have the ability to cross cell membranes and evade natural defences and this is a peculiarity that deserves the greatest attention. The properties briefly summarized above, probably among other that are not as well-known or are still ignored at all, are of the utmost importance to understand why NPs behave in such a distinctive way and can interact so oddly with cells and organisms, from humans down to bacteria. To assess the noxiousness of those particles, a number of factors must be taken into consideration. Probably, the most important of all is their being a foreign body, which the organism regards as an outsider or even an enemy to be somehow eliminated or, failing that, as far as possible, safely isolated; all reactions that may be unsuccessful and trigger the onset of a pathological condition.

Prevented agglomeration and Environmental Exposure

Many of the nanoparticles that occur in nature are soluble in water, but scientists claim that manufactured NPs could adversely affect the environment (for details see Chapter 4).

NPs tend to agglomerate and change into larger microparticles, which are less reactive, less mobile and less well-distributed. To prevent agglomeration, manufacturers will often coat commercially available NPs. This makes them reactive and highly mobile in the environment. If these NPs are released into the water or air, they could contaminate soil and groundwater. Pollutants could spread globally if these NPs enter into the water cycle. If plant roots were to absorb NPs, the human and animal food chain could become contaminated through crop consumption. Artificially manufactured NPs used in disposal items could also contaminate soil and groundwater if they are not properly recycled or removed as waste.

1.4 Risks of Nanomaterials

Author: Nicoleta Lupu and Horia Chiriac

Health and environmental threats can occur from the production, use and disposal of NPs. Workers in nanotech industries are especially at risk, as they can be exposed to high concentrations of NPs that may be taken up. NPs used in consumer products may threaten public health, yet there are no labelling requirements for products using nanomaterials. Already it is known that ultrafine (nano) particles in air pollution can be up to 50 times more damaging to lung tissue than fine particles of the same chemicals but no such rule exists for synthetic engineered materials. Scientists believe that ambient air ultrafine particles are more toxic due to both their small size and their ability to carry large loads of toxic metals and hydrocarbons into the lungs, exacerbating breathing problems and asthma. NPs may also damage the body's natural defences or can lead to the formation of free radicals, highly reactive elements that can damage or destroy cells and cause inflammation, heart and lung disease but none is still

evidenced in living organisms. Scientists are developing NPs used as drug delivery devices, in some cases hoping to cross the blood-brain barrier, yet some warn that NPs could carry toxins into the brain that promote Alzheimer's or other diseases. There has been little study of the health affects of manufactured NPs, but there are already reasons to be concerned. Animal studies suggest that NPs can trigger unpredictable inflammatory and immune responses (Borm and Kreyling, 2004; Resnik and Tinkle, 2007). Studies have found NPs in the livers of lab animals and show that they can seep into living cells. In a 2004 study, fifteen percent of rats exposed to nanotubes in the lungs unexpectedly died immediately (Warheit *et al.*, 2004), and another study showed damage to the brain in fish exposed to NPs (Oberdörster, 2004). But both these studies indicate the real problems with the investigation of nanoparticle toxicity: whereas the rats in David Warheits study ceased from suffocation by thick bundles of carbon nanotubes within their airways (Warheit *et al.*, 2004), Eva Oberdörster tested the toxicity of the peroxides in the solvent used (THF, tetrahydrofuran) to suspend the fullerenes in water (Oberdörster, 2004). These two examples make very clear that there is a great need for standardised methods and the experimental protocols have to be proven if they were usable or adjustable for the testing of NPs.

Little is also known about the environmental persistence or impact of engineered nanoparticles. It is difficult to predict which of these new materials may persist and bioaccumulate, as there have been no long term studies observing the unique physicochemical characteristics of these new materials. There have also been no life-cycle analyses that look at the possibilities for environmental release from production through disposal of NPs. Their large, "sticky" surface area and mobility through air, water or food leads to fears that NPs would be particularly adept vehicles for transporting toxic pollutants globally. Remediation problems from such nanopollution would be difficult if not impossible.

For medical applications immobilized nanostructures on surfaces may pose a minimal risk as long as they remain fixed on the surface. The potential risk would be related to the possibility of release from such structures depending on the strength of fixation of the NPs within or on the carrier material. Such release may occur in the form of wear debris due to continuous chemical processes and/or mechanical stresses at the interface of implant and surrounding tissue. In addition, for implants wear particles may be generated with a size in the submicron range, including particles at nanolevel below 100 nm. Whether such wear particles have similar increased reactivity/toxicity as ultrafine ambient air particles or model polymer particles is unknown and needs further investigation.

From a regulatory point of view, the implementation of a risk management strategy that includes a risk assessment by the manufacturer is already a requirement for all medical technology applications. Health care providers and European and National Authorities also have a significant role in the management of risks associated with healthcare technology. With regard to applications utilizing nanotechnology, this focus on risk management is considered sufficient, as long as all the stakeholders are made aware of the possible specific toxicological properties of nanostructures and nanoparticles. From our current knowledge of the effects of size reduction on material properties, we can conclude that an evaluation of the possible specific behaviour of nanostructures in the products needs to be incorporated into any risk assessment performed. Risk

assessment needs to be carried out for each separate formulation of a nanosized product. One should not rely on existing knowledge of the toxicity of the constituent chemicals or materials but include particle size among the parameters to be considered during the risk assessment. This may result in the recognition of a new or additional risk to those who are exposed. It is strongly recommended that specific guidance at European level is developed, pointing out the above conclusions to the relevant stakeholders.

1.5 What is typical for “nanotoxicology” and different from bulk or other “toxicology”?

Author: Juergen Hoeck

Given the overall definition of toxicology as the *science involving the study of the actual or potential danger presented by harmful effects of substances on living organisms and ecosystems, the relation of the effects to exposure, the mechanisms of action diagnosis, prevention and treatment*, nanotoxicology is a 100% subdivision of "normal" toxicology. As such, the terminology nanotoxicology is not an artefact but a distinct name for one of the areas of toxicology.

The term "Nanotoxicology" has been proposed by Donaldson *et al.* (2004b) as a new subcategory of toxicology to address gaps in knowledge and the special problems likely to be caused by NPs, because these have a greater potential to travel through the organism than other materials or larger particles and might be “nanonoxes (Kern *et al.* 2004).

For a distinction from (or a more precise description of) bulk toxicology, nanotoxicology is already an established expression in use worldwide for the area of the toxicological effects of materials going beyond the effects of the bulk material when coming to the nanoscale. The following examples give clear evidence for this statement:

- The journal Nanotoxicology invites contributions addressing research relating to the potential for human and environmental exposure, hazard and risk associated with the use and development of nanostructured materials (including materials with at least one dimension in the nanometer size range = nano-objects)
- a book entitled "Nanotoxicology - Interactions of Nanomaterials with Biological Systems", edited by Zhao and Nalwa, has been released in June 2006
- an international conference named "Nanotoxicology" has been established in January 2006 with the aim to address all aspects of occupational, environmental and consumer risks in relation to nanoparticle and nanotube exposure. The second conference in this series will be held in Zurich Switzerland, in 2008 and is organized by a member of the IMPART-consortium.
- a definition of nanotoxicology can already be found on the free on-line Encyclopaedia "Wikipedia": *nanotoxicology is the study of the toxicity of nanomaterials. Because of the small size and large surface area of nanomaterials, these materials have unique properties compared with their larger counterparts. The nanomaterials, even when they are made of inert elements like gold, become very active at the nanometer range. Nanotoxicological studies are intended to determine whether and to what extent these may pose a threat to the environment and to human beings*

- the recent review of Oberdörster *et al.* (2005b) on “Nanotoxicology as an emerging new discipline” outlines the importance of this topic.

Being specifically nano-oriented, nanotoxicology as a term as well as a scientific discipline is the appropriate means and a prerequisite for the discussion (scientific or public) of typical nanotechnological risks.

To stress the delimitation of particularities, a more stringent definition of nanotoxicology, including ecotoxicological aspects, is necessary. As one possible definition the following can be suggested: nanotoxicology is the *science involving the study of the specific toxicological effects of nanomaterials with a special focus on nanoparticles in living organisms and ecosystems, which go beyond the effects of the corresponding bulk materials*, taking into account the different effects which different forms and modifications of the same material can have on the nanoscale (e.g. nanotubes, nanofibres, fullerenes, nanographite,...).

So the actual scope of nanotoxicology should comprise the influence of quantum effects, surface factors, and special interactions with all types of biomolecules and -structures on the toxicity of nanoscale particles, as opposed to micro or macro materials as well as dissolved substances, which belong to the field of standard (bio-) chemistry. It has to be stressed that the restriction given here clearly excludes toxic effects which arise from the solution of NPs, and therefore belong to "normal" toxicity issues originating from the purely chemical properties of the substances, disregarding their size. However, as these "normal" toxic effects are produced by the application of nanoscale particles, a strict distinction between nanotoxicology and other toxicologies in this case is not advisable when talking about risks of nanotechnologies.

The above mentioned book "Nanotoxicology - Interactions of Nanomaterials with Biological Systems" gives a concise overview of the topics which need to be covered when discussing "nanotoxicology":

- biological activities of nanoparticles
- interactions between nanoparticles and living organisms
- interactions of nanoparticles with cells and their cellular nanotoxicology
- uptake and cytotoxicity
- toxicology of carbon nanomaterials
- interaction with biomolecules and molecular nanotoxicology
- effects on the immune system
- the role of oxidative stress

The results evolving from current studies of ultrafine particles will give an important input for the elucidation of the effects of NPs. The incorporation and extension of evolving knowledge about differences between classical particle (inhalation) toxicology and specific nanoparticle-cell-interactions should be a focal point.

Risk assessment of the potential toxicities of NPs must include the identification of toxic effects, the establishing of dose-effect-relationships, assessment of the exposition, calculation of the risk, and development of new test strategies (Oberdörster *et al.* 2005b).

As a conclusion it can be stated that nanotoxicology is a distinct part of the whole of toxicology, showing specific and typical effects that are based on nanoscale properties of the particles. Nanotoxicology does not *differ* from normal toxicology, it only displays *additional features* which are not yet entirely understood and have to be further investigated.

Missing information and knowledge gaps:

- a deeper understanding of the differences between nano and bulk, based on experimental and theoretical work, including quantum, surface and size effects, also taking into account the consequences of agglomeration and deagglomeration of the particles is missing
- other primary effects apart from oxidative stress are underrepresented in research on nanotoxicology, and should be addressed in more detail (such as adverse effects on the immune system, lung disease, inflammation)
- an evaluation of current test methods for "normal" toxicology with respect to their applicability for nanotoxicological testing is required, if necessary new dedicated test methods must be developed

2 Health

2.1 Nanoparticles and Human Health

Author: Nicoleta Lupu and Horia Chiriac

The economic growth in the field of nanotechnologies will lead to an increased variety and increased volumes of engineered NPs that are produced. Even if exposure assessments and data are still lacking it is foreseeable that some degree of exposure to engineered NPs -- for various segments of the population and for the environment – will occur to an increasingly extent over the coming years.

Keeping in mind that these “free nanoparticles” can enter the human body over various pathways (inhalation, ingestion or via the skin) or disperse into the environment, it is important to understand the implications for human health and the ecosystems.

To assess the risks of NPs, established methods of chemical safety assessments have to be modified to address the special characteristics of NPs. The main difference to the assessment of bulk materials is the fact that additional parameters like size, shape or surface properties will come into play (see Chapter 1.2). The same reason that makes NPs technologically interesting leads to the fact that they represent a new category of (potentially) toxic substances. The interaction with the human body and their health effects are perhaps expected to be different from molecules as well as from bulk materials of the same composition.

It is necessary to understand both, the hazards associated with nanomaterials and the levels of exposure, which are likely to occur. In both areas, the existing knowledge is quite limited and it will be necessary to generate and establish new data in the future.

2.1.1 Hazards from Engineered Nanoparticles

When coming from bulk materials down to the nanoscale, nanomaterials tend to become chemically more reactive – this is why they are very interesting as catalysts. Even chemically inert materials like gold or platinum are able to catalyse chemical reactions in nano-powder form. Many studies indicate that NPs generally are more toxic when incorporated into the human body than larger particles of the same materials. Experts are overwhelmingly of the opinion that the adverse effects of NPs cannot be reliably predicted or derived from the known toxicity of the bulk material.

The biggest concern is that free NPs or nanotubes could be inhaled, absorbed through the skin or ingested.

2.1.2 Toxicity of Nanomaterials

Iron oxide NPs have been used extensively for biological applications and as pigments. NPs with a wide degree of morphologies and crystal structures exist. According to Cornell and Schwertmann (2003), there are fifteen known polymorphs of ferric oxide. Ferric oxide NPs are in fact one of the few classes of nanomaterials approved by the FDA for parenteral (IV) administration to humans.

The magnetic properties of mixed valent Fe(II), Fe(III) oxides are finding increased applications for imaging, drug delivery, and separations. The toxicity of these mixed valent materials is far less clear. The ability of many microorganisms (i.e. magnetosomes), fish and mammals to produce and/or utilize magnetite, Fe_3O_4, demonstrate that they are not toxic under all conditions. Magnetic NPs are also thought to be exploited by more advance organisms such as trout, migrating birds, and whales. Conversely, the well known Fenton reaction of Fe(II) yields hydroxyl radicals that damage DNA and can oxidize a wide variety of organic and biological reagents. Below we review recent cellular studies of magnetic iron oxide NPs. The vast majority of these studies are focused on superparamagnetic Fe_3O_4 particles that respond rapidly to magnetic fields but retain no residual magnetism when the field is removed. Such materials have long been commercially available as micron-sized magnetic beads, in which the superparamagnetic particles are encapsulated within an organic sphere. The use of nanometer-sized materials presents new opportunities for separations and imaging technologies, where possible toxicity is a critical concern.

Gupta and Gupta (2005) reported a cytotoxicity decrease and internalization increase for pullulan-coated superparamagnetic NPs with human fibroblasts. Uncoated, 20 nm iron oxide particles were toxic to human dermal fibroblasts. Internalization of these particles resulted in disruption of the cell cytoskeleton. Pullulan coated particles were non-toxic and had a different effect on the cytoskeleton. TEM data indicated that the internalization mechanisms were different for the two particles – behaviour that was attributed to the hydrophilic nature of the pullulan coating.

The effect of surface-coated superparamagnetic iron oxide NPs on the human cancer cells was investigated recently (Fond and Meyer, 2006). Nine-nm iron oxide NPs were coated with poly (vinyl alcohol) (PVA) or PVA with carboxylate, amine or thiol functional groups. The PVA and the carboxyl and thiol functionalized PVA NPs were non-toxic to the melanoma cells. Some cytotoxicity was observed for the amine functionalized PVA NPs, particularly when the polymer concentrations were high. The amine groups increased cellular uptake of the NPs.

Stroh *et al.* (2005) reported on studies of rat macrophages incubated with citrate coated iron oxide NPs (9 nm). Atomic absorption and NMR studies showed a large uptake of the NPs that could be easily visualized by confocal microscopy.

Cytotoxicity of metal ions and other chemicals differs among cell lines. Larger particles (only if phagocytised) tended to have higher cytotoxicity than smaller particles.

Hanawa (2006) and Yamamoto (2004) studied the toxicity of metal oxide particles ranging from 500 to 3000 nm in diameter. The particles were incubated in human fibroblasts for 24 h and stained with haematotoxylin and eosin to determine the magnitude of toxicity. With this assay, cells that adhered to the coverglass would stain, while dead cells would detach from the glass during staining. A digitizer was used to assess the area that was stained. The area stained was considered to be proportional to the magnitude of cytotoxicity of the metal oxide particles. Cells incubated with Al_2O_3, TiO_2, Fe_2O_3, Fe_3O_4, Co_2O_3, NiO, Ga_2O_3, SnO, SnO_2, HgO showed no cytotoxic effects. A difference in formal oxidation state of some of these metals yielded different effects, e.g., CoO, Co_3O_4, and Ni_2O_3 appeared to be toxic. In addition, Cr_2O_3, Cu_2O, CuO, ZnO, and Ag_2O proved to be cytotoxic. A potential problem would be that the study was based on particles dissolving into elements/ions, which have a cytotoxic response. Therefore, larger areas of affected cells indicated a more cytotoxic effect, which does not necessarily correlate with the components of the particles being more effective at killing cells. Larger cytotoxic effects could have been a result of a higher particle concentration in the medium.

Research into the toxicity of nanoparticles has, in the past, been largely uncoordinated. Recent articles highlight the need for a systematic approach to studying this important aspect of nanotechnology and additionally, the need for a regulatory framework for the protection of workers exposed to NPs during their production. Nanotoxicology is now an established discipline having evolved from studies on NPs, where it is well known that such particles cause morbidity and mortality in susceptible populations. In comparison, there is a paucity of data for human or environmental exposure levels of NPs. There are significant differences between NPs and larger particles in terms of their deposition and clearance from the respiratory system. NPs, in contrast to larger particles, move throughout the body and can reach other organs. During the life-cycle of a product it is likely that nanomaterials will enter the environment. The stability of coatings on NPs therefore needs to be investigated both *in vivo* and in the environment. A significant factor in risk assessment is the likelihood of exposure - which has been shown to be negligible with due care in handling procedures.

Benign NPs in cosmetic products (10 and 20 nm particles) induce oxidative stress in human bronchial epithelial cells without photoactivation, noting that oxidative stress is implicated in aging, atherosclerosis, carcinogenesis and inflammatory disorders. The larger particles (200 nm) do not have the same effect, and it therefore seems that the smaller the particle the easier it is to induce oxidative damage. This suggests that ultrafine particles could cause an inflammatory response intratracheally, whereas particles larger than 100 nm are classified as benign in humans and animals.

We observed NPs penetrating into the corneocyte layers of stratum corneum with no particles in the cytoplasm of the granular cells (Kreuter, 2001). Schulz *et al.* (2002) found that surface characteristics, particle size or shape of micronised pigments result in the dermal absorption of the substance. Nanoscale particles of PVC, TiO_2, SiO_2 and

cobalt metal (Co) are taken up into human endothelial cells. Only exposure of HDMECs (human dermal microvascular endothelial cells) to Co particles leads to enlargement of vacuoles, and HDMECs exposed to Co, SiO_2 and TiO_2 NPs induce pro-inflammatory effects. Co and SiO_2 particles produce a larger effect than TiO_2 particles. The inflammatory lung disease caused by SiO_2 particles might share a common mechanism with that shown by the endothelial cells.

Most nano-products can be designed in such a way as to either increase or decrease toxicity depending on the desired outcome. For treatment in drug delivery, minimizing toxicity of the carrier is necessary and in most cases this can be done. On the other hand for chemotherapeutic agents, toxicity is designed to be magnified and targeted to specific tissues or areas. Altering the coating of many of these therapeutic agents can increase selectivity and toxicity.

However, as a general principle, reduction in size results in an increased reactivity, which may be expressed as increased toxicity after exposure. Observations with several chemicals show small (nano)particles to be more toxic than larger particles of the same chemical composition after inhalation exposure. Even materials of established low toxicity can become highly toxic when are administered as NPs, because they are similar in size to proteins in the body. NPs are considerably smaller than many cells in the body (for example, human alveolar macrophages are 24 μm in diameter and red blood cells are 7-8 μm in diameter), and consequently cells growing in tissue culture will pick up most NPs (Borm and Kreyling, 2004; Donaldson and Stone, 2003; Goodman *et al.*, 2004; Kirchner *et al.*, 2005; Tinkle *et al.*, 2003).

It is evident that micro- and nano-sized inorganic particles can enter biological tissues. According to what we observed, the most common way of entry is inhalation. (a human breathes approximately 15 m^3 of air per day). Because of their size, micro- and nanoparticles, no matter how they are produced, stay suspended in the air for a very long time. From the air, they are breathed in and, once they are inside the bronchial alveoli, depending on their size, they can be phagocytized by the macrophages the way any foreign body in that anatomical district is, or, in the smaller sizes, pass directly, with a mechanism that is still to be made clear. As it was proved, micro- and nanometric particles can be found in the blood (and cause thrombosis in predisposed subjects) and nanometric debris can even enter the red cells, an excellent Trojan horse to negotiate virtually any barrier.

Whatever the modality through which they enter the blood, sooner or later those particles are sequestered by a tissue and, being not biodegradable, are impossible, or, in any case, very hard, to remove through the physiological ways of elimination. A further and certainly not negligible problem is that those non biodegradable foreign bodies are also non biocompatible. That means that, just because of their non-biocompatibility and by definition, they can induce adverse reactions, and this is what, under certain conditions, they do. As it happens with any foreign body, inflammation is how tissues generally react against that unwanted presence, and that reaction grows visible when the concentration the debris has reached is high enough. But when the particle is of nanometric size, it passes unnoticed and can enter cells, even being able to go as deep as their nucleus. It may be interesting to note that NPs can pass into macrophages directly and interfere with functions as motility and ability to remove bacteria.

2.1.3 Do impurities in commercial nanomaterials contribute to toxicological properties?

Author: Aris Tsatsakis

The investigation of structure and toxicology of impurities in drug formulation takes an important place in pharmaceutical chemistry and technology (Basak *et al.*, 2007).

The solution of these problems should play the decisive role for development of harmless drug systems and delivery systems in genetic engineering of those on the basis of nano-sized carriers including, first of all, liposomes, solid liposome nanoparticles, polymer nanospheres, aggregates of amphiphilic polymers, and dendrimers.

The nano-sized objects are commonly defined as those up to 100 nm in size. But in practice the size of particles applied in bio-medical area is larger and may reach some hundred nanometers. This is reasonable due to simple technology, which does not affect the activity since the production of smaller particles requires a more complicated process.

With respect to toxicological properties of impurities in nano-sized carriers used in pharmaceutical technologies it can be noted that despite its importance this field now is only under consideration.

Despite their promising features NPs have so far displayed several toxicological drawbacks. For instance, there is a number of complications related to administration of carbon nanotubes (CNT) (Donaldson *et al.*, 2006; Kagan *et al.*, 2006; Monteiro-Riviere *et al.*, 2005; Wörle-Knirsch *et al.*, 2006). Also the ultra small particles can generate harmful oxyradicals (ROS), which can cause cell injury by attacking DNA, proteins, and membranes (Brown *et al.*, 2001). On the contrary there is little information on toxicological properties of impurities in nanoparticles (Zhao and Nalwa, 2006).

There are two main causes for impurities in bio-medical nanosystems, i.e.:

- Contamination in process of preparation
- Formation of impurities throughout the system's life cycle.

2.1.3.1 Contamination of nanomaterials during preparation process

During formation a nanoparticle captures various by-products, which may in general have a negative effect on the surrounding tissue and the organism itself (compare Chapter 1.2.3.1 for carbon nanotubes). Such foreign materials may be first of all the initial chemicals used for production of nanoparticles such as residual monomers, catalysts, solvents, surfactants, etc. Usually toxicity of these substances, if they are admitted to application, is known and can be taken into consideration.

Largely diffusion retention of impurities in bulk nanoparticles is common for branched macromolecular systems, e.g. dendrimers or particles produced on the basis of crosslinked polymers or water insoluble polymers, purification of which can be complicated.

There is a number of possibilities for inclusion of initial substances into the resulting NPs. For example among the water insoluble polymers used for production of NPs are the polymers of hydroxycarboxylic acids, e.g. glycolic acid, lactic acid and the like, as

well as the esters of poly-α-cyanacrylic acid. These polymers can undergo biodegradation in the body tissues, which may be accompanied by release of impurities formed in bulk of NPs during synthesis into the surrounding tissues. For example the particles produced on the basis of poly-α-cyanacrylates by polymerization of appropriate monomer esters containing medium-sized alkyl radicals, e.g. butyl- or ethoxyethyl-α-cyanacrylates may in general occlude the monomers (Díaz-Torres *et al.*, 2005). This also applies to all polymer particles produced in dispersion systems (suspension or emulsion polymerizations) produced on the basis of water-insoluble polymers. Formation of NPs from polymers insoluble in water but soluble in organic solvents like polyesters of hydroxycarboxylic acids, may lead to inclusion of solvents (e.g. methylene chloride) and surfactants used for formation of two-phase systems into the bulk of particles.

If NPs are obtained in the form of associates of amphiphilic polymers, e.g. low-molecular weight amphiphilic derivatives of polyethylene oxide or poly-N-vinyl pyrrolidone, a certain amount of the polymer may remain unassociated (Torchilin *et al.*, 2001). Moreover formation of polymer NPs just as any other chemical process may be accompanied by side processes resulting in formation of such substances, toxicological properties of which may not be even identified. All these substances are often highly toxic and may have a severe negative impact.

2.1.3.2 Formation of impurities throughout the system's life cycle

Degradation products of nanoparticle systems forming throughout the system's life cycle within the body can also be toxic. This first of all refers to biodegradable polymers (Panyam and Labhasetwar, 2003). For example degradation products of polymers of hydroxycarboxylic acids, such as glycolic acid (polyglycolides) or D- and L-lactic acid (polylactides) show little or no toxicity since these acids are natural metabolites. On the contrary degradation products of polycyanacrylates are not so harmless. It is well known that polymers of esters of α-cyanacrylic acid are biodegradable. It is assumed that this process results in formation of formaldehyde. Degradation of poly-α-cyanacrylates starts at pH 7.0 and considerably accelerates at pH 8.0. A smaller alcohol radical also increases the rate of hydrolysis. Polymers of methyl-α-cyanacrylate show the fastest decomposition. This process can result in damage of surrounding tissue (Leonard *et al.*, 1966; Wide and Leonard, 1980). Some natural polymers applied for production of biodegradable NPs such as chitosan also biodegradable.

Nanoparticles based on aggregates of amphiphilic polymers are rather unstable and can dissociate to yield the initial polymers that in turn are separately carried by biological fluids. Finally the toxicity of polymers used for surface modification of NPs, which can be released into the surrounding body tissues, must also be considered. Thus 9 nm iron oxide NPs were coated with polyvinyl alcohol (PVA) or PVA with carboxylate, amine or thiol functional groups. The PVA and the carboxyl and thiol functionalized PVA NPs were non-toxic to the melanoma cells. Some cytotoxicity was observed for the amine functionalized PVA NPs, particularly when the polymer concentrations were high. The amine groups increased cellular uptake of the NPs (Gupta and Curtis, 2004; Gupta and Gupta, 2005).

An example of such modifying coating is polyethyleneglycol (PEG) that was seen in a prolonged presence in the circulation by avoiding recognition and phagocytosis by the

mononuclear phagocytic system (Bazile *et al.*, 1995). Special attention should be paid to substances used for chemical modification of nanoarticle surface (if any) as well as to by-products of these reactions.

2.1.3.3 Removal of impurities from nanomaterials

If a nanoparticle carries a bioactive (drug) substance, which is to be gradually released into the surrounding tissue (controlled release action), then removal of contaminants by extraction with organic solvents is inapplicable since the extracting agent may be itself caught by the particles. Beside that the extracting solvent may remove the immobilized substance.

Furthermore this method can not be used for immobilization of bioactive protein-like substances in NPs since the organic solvent may lead to denaturation of proteins. Therefore, the most applicable way for elimination of impurities from NPs is to apply membrane methods and sometimes gel-filtration and ultracentrifugation. However, such methods as gel-filtration and ultracentrifugation applied in laboratory practice become useless in case of scaling.

Dialysis in bags and tubes with various pore sizes is the most common practice. However ultrafiltration is more efficient on a larger production scale (Dalwadi *et al.* 2005; Hammady *et al.* 2006; Heydenreich *et al.* 2003; Limayem and Charcosset 2004; Miglietta *et al*. 2000; Sweeney *et al.* 2006). It is noteworthy that membrane methods that usually require a lot of time may result in partial loss of the immobilized substance due to its diffusion detachment from the nanoparticle.

2.1.3.4 Analysis of impurities

Analysis and identification of impurities in NPs is quite complicated. A foreign substance can be separated during purification, concentrated and then analyzed by a standard method. In case of impurities formed throughout the life cycle of a nanoparticle it is often impossible.

The processes that occur during interaction of NPs and body tissues can be simulated to a certain extent *in vitro*. However a comprehensive recreation of the conditions of interactions between the NPs and intracellular and extracellular enzymes as well as with the reticuloendothelial, scavenger and giant cells of the foreign body is more than difficult.

2.2 Is there a difference between “local” and “systemic” toxic effects?

Author: Peter Hoet

Exposure sites of engineered nanomaterials are skin (and eye), lung and intestinal tract. From these exposure sites nanomaterials can penetrate the body (systemic circulation). This differs significantly from medical applications because often direct injection into the systemic circulation is performed (Hoet *et al.*, 2004a).

2.2.1 Local toxicity at the site of exposure

Pulmonary exposure

Multiple reports exist on toxic effect of pulmonary exposure, mostly reporting on environmental exposure to pollutants; less frequent (but still a significant number) reports on exposure on engineered nanomaterials. In studying the local effects of nanomaterials in the lung a few important notes must be made: particulate matter vs tubular matter, exposure level and the phenomena of overload (Hoet *et al.*, 2004a; Oberdörster *et al.*, 2005a, b).

Nanomaterials in the lung can affect other tissues indirectly by two pathways. Inflammation in the lungs caused by NP causes atheromatous plaque development and destabilization and the inflammation in the lungs causes alteration in the clotting status or fibrinolytic balance favouring thrombogenesis (Duffin *et al.* 2007a; Nemmar *et al.,* 2004)

Skin exposure

Skin exposed to solid materials such as fibre glass or ceramic fibres can be irritated (see ref below), but no such an effect has been studied for nanomaterials (Jolanki *et al.,* 2002). Nevertheless, there is a multitude of publications on skin and NPs (Cross *et al.*, 2007; Díaz-Torres *et al.*, 2005; Gamer *et al.*, 2006; Lademann *et al.*, 1999; Mavon *et al.*, 2007; Pflücker, 2001; Pflücker *et al.*, 2001; Ryman-Rasmussen *et al.*, 2007; Tinkle *et al.*, 2003) as well as a big European project calles “NanoDerm” (Nanoderm, 2007).

The cytotoxicity of CNT on skin cells has been evaluated and reviewed by Lam *et al.* (2006).

Thus, indications exist that the skin itself can be a target of nanomaterials but it is general accepted that these effects are relatively uncommon which is maybe a good reason to stimulate studies on local skin effects of both particle as well as fibrous materials.

Oral exposure

The local effects of nanomaterials in the intestinal track are not studied in detail. Recently, Duncan and Izzo (2005) reviewed the toxic effects of dendrimers. The anionic PAMAMs had no effect on viability and TEER of Caco-2 cells while cationic PAMAM dendrimers reduced significantly the viability and TEER. As for the skin it is general accepted that ingested nanomaterials will not be harmful, since we daily swallow particulate material, but clear evidence has never been generated.

2.2.2 Local sites as portals of entry to systemic exposure

Pulmonary exposure

It has been shown several time that nanomaterials can enter the circulation via the lung (Geys *et al.*, 2006; Nemmar *et al.*, 2001; Nemmar *et al.*, 2002a), although also at least equal amount of papers show the opposite (Mills *et al.,* 2006). The NP themselves or metals/organics released by the particles enter the circulation and have direct effects on the endothelium, plaques, the clotting system or the autonomic nervous system/

heart (Duffin *et al.,* 2007a; Nemmar *et al.*, 2002b; Silva *et al.*, 2005; Vermylen *et al.*, 2005).

Skin exposure

Until recent no reports could be found on the translocation of nanomaterials through intact skin, quantum dots (size 5 – 12 nm) were proven to penetration through the skin. It has been suggested that the skin may serve as portal for systemic exposure of quantum dots for humans (Ryman-Rasmussen *et al.*, 2006). The local effects of nanomaterials on the skin can be very diverse, mainly depending on the material, e.g. oxidative stress (TiO_2 and UV) or sensitisation (Be particles) (Lademann *et al.*, 1999, 2001; Tinkle *et al.*, 2003).

Silver nanomaterials (in e.g. cotton gauze) are examined as possible anti-bacterial treatment on damaged skin (Lee *et al.* 2007). Although there is no *in vivo* evidence to suggest nanocrystalline silver is toxic to human keratinocytes and/or fibroblasts, there is some *in vitro* evidence; moreover no study on uptake of the silver particles via the wounded skin can be found. Thus these type of dressings should be studied better and be used with caution (Fong and Wood 2006).

Oral exposure

Oral uptake of nanomaterials has never been questioned, and has been studied for many years in pharmacology (not scope of this overview).

Other routes of entering the body

It has been show that in the nose nanosized materials can be taken up by the olfactory bulb. The observation has been reported by different researchers, but the toxicological importance has not been cleared (Oberdörster *et al.*, 2004).

Probably nanosized materials can enter the eye (Pignatello *et al.*, 2006), although this is not considered as an important portal (SCCP 2007).

2.2.3 Systemic effects of nanomaterials

The systemic effects of nanomaterials, after entering the circulation via one of the portals have not been studied in large detail. We certainly do not have a good view on the toxicokinetics of nanomaterials.

The following knowledge gaps are obvious:

1. Recently the distribution of nanomaterials in the lung tissue has been studied in some detail. Unfortunately no clear mechanisms could be appointed (Mühlfeld *et al.,* 2007)

2. Entering the system: air – blood; nose – brain; skin – blood; intestine – blood (Hoet *et al.*, 2004a; Oberdörster *et al.*, 2004, 2005b)

3. Moving from one compartment to another: blood to organs-tissue: brain, liver, spleen, kidney; from organ-tissue to blood?

4. The excretion of nanomaterials, the half-life time in the body has not been studied for most of the known materials.

Theoretically, we can we speculate that any organ can be a potential target organs for circulating nanomaterials, but we can also speculate that most probably the lung and liver will be affected. The lung has an important vascular bed and can be therefore an important target for nanomaterials. It is also very well documented that the liver can function as a trap for particulate matter in the circulation. Both organs can play an important role via e.g. stimulation of macrophages, release of prothrombic mediators, and further mechanisms.

Organ, tissue, cellular accumulation has been described in a number of reports, unfortunately, most of these reports are rather separate studies? The uptake of nanomaterials in the brain of rodents (olfactory bulb or via the BBB) (Oberdörster *et al.*, 2004), or in the brain fish has been described (Oberdörster, 2004). Until now only a small number of long (rather sub acute and sub chronic) term studies (Borm and Tran, 2002; Ji *et al.,* 2007; Li *et al.,* 2007; Schins *et al.*, 2002) are available to discuss in detail this issue. Cellular accumulation in phagocytotic cells has been described in some detail; nanosized materials seem to inhibit the phagocytosis. In a number of studies the uptake of nanomaterials has been described in non-phagocytotic cells (epithelium, endothelium) (Baeza-Squiban *et al.*, 1999; Jia *et al.*, 2005).

Recently more studies describe the biocompatibility of nanomaterials (use of specific coatings of e.g. implants, nano-dots, drug delivery devices) (Ballou *et al.*, 2005; Jain, 2003, 2005). This has certainly also a consequence on the half life time of the material (some quantum dots circulate a long time in the blood of animals) and can maybe result in significant serum levels in chronic and repeated exposure (Dubertret *et al.*, 2002; Michalet *et al.*, 2005). Another point of concern is the decay of quantum dots since the core constituents such as cadmium and selenium are toxic. Some evidence exists that the toxicity of QD is often related to the capping material rather than to the core metalloid complex (Hoshino *et al.* 2007).

2.3 How to express dosing

Authors: Harald F. Krug; Peter Wick

2.3.1 Is mass relevant versus surface?

The small size and corresponding large specific surface area of solid NPs confer specific properties to them, for example, making them desirable as catalysts for chemical reactions. The importance of surface area becomes evident when considering that surface atoms or molecules play a dominant role in determining bulk properties (Amato, 1989); the ratio of surface to total atoms or molecules increases exponentially with decreasing particle size. Thus, the increased surface area per unit mass of NPs can be toxicologically important if other characteristics such as surface chemistry and bulk chemistry are the same. Moreover, an increased surface reactivity predicts that NPs exhibit greater biologic activity per given mass compared with larger particles, should they be taken up into living organisms and provided they are solid rather than solute particles. This increased biologic activity can be either positive and intended (e.g., antioxidant activity, carrier capacity for therapeutics, penetration of cellular barriers for drug delivery) or negative and unintended (e.g., toxicity, induction of oxidative stress or of cellular dysfunction) (Limbach *et al.*, 2007), or a mix of both. Not only may adverse effects be induced, but interactions of NPs with cells and subcellular

structures and their biokinetics are likely to be very different from those of larger-sized particles (Oberdörster *et al.,* 2005a).

Tab. 6: Particle size, number and particle surface area per 10 µg/m³ airborne particles.

Particle diameter (nm)	Particle no. (cm^{-3})	Surface Area ($µm^2/cm^3$)
5	153 000 000	12 000
20	2 400 000	3 016
250	1 200	240
5,000	0.15	12

Compared with larger particles on a mass basis, NPs have a higher predicted pulmonary deposition, greater potential to induce pulmonary inflammation, larger surface area, and enhanced oxidant capacity. NPs also have the potential to cross the epithelium and enter the systemic circulation. The lower effect per unit mass dose seen for larger particles is consistent with earlier studies showing that particle surface area of low toxicity particles is a more appropriate dosimetric for induction of inflammation in the lungs than particle mass (Frampton *et al.* 2004; Gallagher *et al.,* 2003; Hohr *et al.,* 2002; Li *et al.,* 1999; Oberdörster, 1996; Oberdörster *et al.,* 2000; Oberdörster, 2000; Renwick *et al.,* 2001).

2.3.2 Surface expressed as outer or / and inner surface

Agglomerates of NPs may cause adverse health effects because of their large surface area. Despite the fact that there is a large number of studies dealing with NPs and their biological effects especially on lung tissue (Beck-Speier *et al.,* 2001; Brown *et al.,* 2001; Dick *et al.,* 2003; Donaldson *et al.,* 2002; Donaldson and Stone, 2003; Frampton *et al.,* 2004; Hamoir *et al.,* 2003; Hext *et al.,* 2005; Hohr *et al.,* 2002; Kreyling *et al.,* 2004; Li *et al.,* 1999; Oberdörster *et al.,* 1992; Oberdörster *et al.,* 1994; Oberdörster, 1996; Oberdörster, 2000; Oberdörster *et al.,* 2005a; Yin *et al.,* 2005), no single publication compares these effects in dependence on the outer or the inner surface of NPs or agglomerates or aggregates with the activity of primary particles. To my knowledge there is no study comparing the biological effects of NPs in dependency on their active surface on a systematic level (Sayes *et al.,* 2007b; Warheit *et al.,* 2006, 2007) and surely no study on that compares the outer and the inner surface.

2.3.3 Does porosity play a role

As for inner surface there is no real information on the porosity of NPs and the effect of porosity on the intensity of their biological effects as stated recently (Oberdörster *et al.,* 2005a). Nevertheless, the porosity of some materials is important for their use in cell culturing of nerve cells (Yang *et al.,* 2004).

2.3.4 Exposure limits restricted to surface doses?

Taking together all above mentioned aspects, the first approach is surely the surface dose as limit value, as the original publications of Oberdörster recommended (Oberdörster *et al.,* 2000; Oberdörster, 2001). Some more recently published results confirm this model (Yin *et al.,* 2005; Zhang *et al.,* 2003), but more data are needed to establish such a threshold or limit value solely on the basis of surface area (see 2.3.5).

2.3.5 Correlation between reactivity and toxicity (dose dependency on reactivity)?

Besides the surface area several other properties may determine the toxicity of NPs. The number of atoms on the surface compared to the interior of the particles, the reactivity of the elements or atoms on the surface and the binding capacity of biological molecules, such as proteins or DNA, may also be important characteristics in toxicity (Chen *et al.,* 2005; Cheng, 2004; Dick *et al.,* 2003; Donaldson and Borm, 1998; Duffin *et al.,* 2001; Knaapen *et al.,* 2002).

Moreover, it is known that surface reactivity plays an important role in catalytic activity of NPs technically used in the destruction of pollutants (Rajagopalan *et al.,* 2002). Thus, such particles with high surface reactivity will exhibit additionally a higher biological activity (Cheng, 2004).

2.3.6 Do we need information on size distribution; hydro-phobicity; zeta potential; wettability

Some first results indicate a dependency of toxicity of NPs on their surface area and not on their hydrophobic status or coatings (Höhr *et al.,* 2002). Nevertheless, there is too little data for a realistic estimation of these properties and their contribution to health effects. Actually, Warheit has recommended the knowledge of a set of properties of NPs to understand publications and compare data (Warheit, 2008; see our "knowledge gaps" at page 90). We claimed for such a "minimum" catalogue of properties at the beginning of the report (page 7).

2.3.7 Discussion on measurement/determination of the surface area

Reliable, standardised methods are not available to measure the surface area of NPs, except the Brunauer-Emmett-Teller-method (BET; Robert *et al.,* 1971). For this method larger amounts of the nanomaterials are needed (mg to g range) that normally are not available. A sensitive technique for small amounts is therefore urgently required.

Recommendations/ knowledge Gaps:

- Which information is enough to clearly define a given dose or concentration within an experiment?
- Should we refer to mass, surface area or particle number?
- Should sedimentation or deposition play a role for considerations regarding the actual dose which reaches the cells in an experiment?
- Is information needed at the end of an *in vitro* experiment about the amount of material still measurable within the supernatant for better calculation of the reactivity of the investigated nanomaterial?
- What is the minimum knowledge about dose or concentration for a reader of a study to interpret the data properly?
- Should we have guidelines for *in vitro* experiments for dosing nanomaterials?
- What about *in vivo* experiments and dosing?
- We (toxicologists) need a sensitive and cheap method to determine particle number and surface to include this information into the discussion of the results.

2.4 How good are the present protocols? Have we to apply new Tox-test? Do we need a new strategy?

Authors: Petya Krasteva and Margarita Apostolova

Included here are ceramics, metals, and metal oxide NPs. These materials are assembled from nanometer-sized building blocks, mostly crystallites. The building blocks may differ in their atomic structure, crystallographic orientation, or chemical composition. In cases where the building blocks are crystallites, incoherent or coherent interfaces may be formed between them, depending on the atomic structure, the crystallographic orientation, and the chemical composition of adjacent crystallites. In other words, materials assembled of nanometer-sized building blocks are microstructurally heterogeneous, consisting of the building blocks (e.g. crystallites) and the regions between adjacent building blocks (e.g. grain boundaries). It is this inherently heterogeneous structure on a nanometer scale that is crucial for many of their properties and distinguishes them from glasses, gels, etc. that are microstructurally homogeneous.

The manufacture of novel nanomaterials has gained increasing and considerable attention by the scientific community over the past decade. The applications of these various nano-scale materials continue to grow, and their potential to beneficially impact the medical and technological world is seemingly boundless. However, with increased manufacturing of these diverse NPs worldwide, comes an urgent need for investigations regarding their toxicity. The realization that these materials may pose a threat, and that exploration of the subject is sorely lacking, has only just come into popular attention (Gorman, 2002; Service, 2003). Different studies have been undertaken that investigate the toxicity of nanomaterials. The impact of these nanomaterials should be established, in order both to avoid problems in the future. Biological activity and biokinetics are modified by many parameters; hence detailed physicochemical characterization is necessary. The parameters of concern include the following: Size, Shape, Chemistry, Mono/Polydispersity, Crystallinity, Surface properties (area, porosity, charge, coating), Agglomeration state, Biopersistence, Dose, etc. (compare criteria list on page 7). Most generalized data for the analytical methods used come from the review by Oberdőrster *et al.* (2005). They summarized the currently used analytical techniques to provide specific physicochemical information on engineered nanomaterials, in the context of toxicity screening studies (Table 7):

Tab. 7: Analytical Techniques

	TEM	SEM	XRD	XPS	Auger Spectro-scopy	SIMS	Scanning Probe Microscopy	DLS	Zeta Potential	Size Exclusion Chromatography	Analytical Ultra-centrifugation	DMA	Isothermal adsorp.	Spectro-scopic techniques
Size Distribution	▲	•	•				•	•		•	•	•		
Shape	▲	•					•	√				√		
Surface area	•	◊					◊	◊			◊	√	•	
Composition	•	•	•			▲								•
Surface chemistry	•	√		•	•	√	√		◊					√
Surface contamination	√			•	•		√							√
Surface charge									▲					
Crystal structure	•	◊	▲											
Particle physico-chemical structure	▲	•				√								
Agglomeration state	▲	•	√				•	√		√	√	•		
Porosity	◊											•	•	
Heterogeneity	▲	•					◊							√

▲ Highly applicable
• Capable of providing information in some cases
◊ Capable of providing information in some cases, with validation from more accurate/ applicable techniques
√ Capable of providing qualitative or semi-quantitative information

Even though so many techniques exist, it is clear that there are several research gaps, namely the need of development of

techniques for the *in vivo* detection of nanomaterials:

1. Inexpensive real-time monitoring instruments and methods for aerosol mass concentration (low concentrations, nanoscale particles), surface area concentration and size distribution.
2. Standardized, well characterized nanomaterial samples.
3. Radio-labelled nanomaterial samples or samples that can be tracked and detected through neutron-activation.
4. More advanced surface chemistry characterization techniques, in particular techniques capable of detecting and speciating biological molecules on the surface of nanoparticles and nanomaterials.
5. Electron microscopy techniques for biologically-relevant nanoscale analysis.

Standards and protocols that exist assume that *particulate mass* is most closely associated with health risk. This is not the case with nanomaterials: early toxicity studies show that particulate *surface area* and *number* are more relevant to health effects than mass is. The ratio of surface to total atoms or molecules increases exponentially with decreasing particle size. The increased surface area counts for increased chemical and biologic activity of NPs. The increased biologic activity can be positive and desirable (e.g. antioxidant activity, carrier capacity for therapeutics, penetration of cellular barriers), negative and undesirable (e.g., toxicity, induction of oxidative stress or of cellular dysfunction), or a mix of both.

It has to be noted that both *in vitro* and *in vivo* studies should make attempts to differentiate the primary particle size ranges, aggregate sizes, or morphologies. Fibres,

fibre bundles or aggregates, fibre/uni-sized particulate aggregates, or other complex nanoparticle composites pose a variety of airstream responses and deposition behaviours (Tab. 8).

Tab. 8: Deposition mechanisms of inhaled particles to be studied.

Impaction	particle velocity, directional changes, inertia
Sedimentation	gravity, settling velocity, residence time
Diffusion	collision with air molecules, residence time
Interception	fibrous particles
Electrostatic precipitation	charged particles, image forces

Another issue that should be considered is the *in vitro*/*in vivo* alterations of nanomaterials: deagglomeration, solubility, weathering of protective coatings, adsorption of proteins, lipids, etc. (for example, adsorption of pulmonary surfactant constituents that could facilitate the translocation of NPs via the pulmonary epithelium)

A key issue in both *in vitro*/*in vivo* assays is the exposure-dose-response relationship, but most important the relevance of dose levels. It has been a continual practice to perform both in vitro and *in vivo* experiments with high doses of NPs without any consideration of realistic organismic, organ, or cellular exposures. Hence knowledge about exposure levels and about the correlation of exposure with doses (an exposure is not a dose) is absolutely necessary. Lack of data in the low- oftentimes most relevant-dose range can result in severer misinterpretation if a threshold or even a hormetic response is present.

In vitro assays:

1. **Non-cellular assays are to consider:**
    - Free radical production: (EPSR- electron paramagnetic spin resonance, DCFD- di-chloro-fluorescein-di-acetate, plasmid DNA scission, 8-OH-dG production in ‘naked’ DNA, etc.)
    - Durability: appropriate deagglomeration / solubility assessment methods should be developed
    - Adsorptive properties
    - Complement activation
2. **Portals of entry and target organs cellular assays are to consider:**
    - dose-response relationships and appropriate controls (negative and positive)
    - target organs and cells (epi-/endothelial, neuronal, keratinocytes, other)
    - endpoints: oxidative stress, viability, cytokines, cell signalling pathways, other
    - cytoskeletal effects (microtubule, mitochondria, endoplasmic reticulum)

***In vivo* assays- issues of consideration:**

- exposure-dose-response relationships, realistic dose/ exposure values
- exposure route (inhalation, intratracheal instillation, or pharyngeal and laryngeal aspiration ingestion, dermal exposure, biomedical applications (intravenous, subcutaneous, intramuscular))
- deposition mechanism (Tab. 8)
- portal of entry vs. remote organ effects, with special attention to possible theratogenic activity
- acute vs. chronic exposure/ effects
- kinetics (Tab. 9): translocation, accumulation, biopersistence
- *in vivo* interaction of nanomaterials with proteins and biologically active substances
- endpoints: inflammation, function impairment (BAL damage markers, oxidative stress markers, histopathology, cell proliferation)
- proteomic, genomic, and metabolomic alterations
- design of the experiments: animal model, gender of the test animals, appropriate positive and negative controls
- long-term studies with attention to the effect on genomic stability

Tab. 9: Clearance mechanisms to be taken into consideration for inhaled solid particles in the respiratory tract:

Physical clearance processes	**Chemical clearance processes**
Mucociliary movement (translocation) Macrophage phagocytosis (tracheobronchal, alveolar) Epithelial endocytosis (tracheobronchal, alveolar) Interstitial translocation (nasal, tracheobronchal, alveolar) Lymphatic drainage (tracheobronchal) Blood circulation (tracheobronchal, alveolar) Sensory neurons (nasal, tracheobronchal)	Dissolution Leaching Protein binding (nasal, tracheobronchal, alveolar)

2.5 Dissolution / precipitation of material within the biological system (organspecific)?

Author: Peter Hoet

Dissolution can be defined as a dynamic process by which a molecule (of a particle) goes into the solution phase to form a homogeneous mixture. The molecules of the solid migrate from surface of the material to solution.

We have to note a few points:

1. Particles have a surface energy which partly depends on their morphology (the surface curvature). Particles with a smaller convex radius (positive curvature) have a higher interfacial surface free energy and therefore are subject to preferential dissolution (Gibbs-Thompson effect - relationship between curvature, interfacial free energy and dissolution), surface features with a small concave radius (negative curvature) are less prone to dissolution due to a lower localized surface free energy. (Ruckenstein and Djikaev, 2005; Tang *et al.*, 2004)
2. It is noted that amorphous materials dissolve more easily compared to the crystalline forms. E.g. amorphous silica has a solubility ranging from 2 mmol/l (pH 7, 25°C) to 4.5 mmol/l (pH 7; 45°C), differs from Cristobalite at 0.45 mmol/l and Quartz at 0.09 mmol/l. But it is important to state the following: most natural amorphous particles are unstable and tend to transform with time towards more crystalline forms, either by aging or possibly, by dissolution and re-crystallization (Mavrocordatos *et al.*, 2004).
3. The solubility of particles can depend on the pH of the dissolution milieu. E.g. for TiO_2 an increased solubility is found in the acidic region pH < 3. For most metallic particles a low pH will increase the potential of the material to solubilise. The presence of different ionic species in the solution can play a role. Also temperature plays a role in dissolution, for most materials the dissolution increases with increasing temperature (remark: maybe this is not relevant in humans).
4. For many nanomaterials the chemical purity will play a role in their toxicological profile. Impurities have to be taken into account also when measuring the dissolution of the nanomaterial (compare Chapter 2.1.3). This can be e.g. important for metallic compounds present in the sample (Hostynek, 2003; Lam *et al.*, 2006; Pulskamp *et al.*, 2007a). In addition to impurities (point 4), Limbach *et al.* (2007) have shown that particles can act as a Trojan-horse to efficiently deliver metallic ions into cells.
5. The discussions in points 2 to 4 brings us to a problem which has not been studied yet: “Do dissolved nanomaterials precipitate at (other) target sides in the body (not defined which ones)”. This hypothetic consideration has to be taken into account because the saturation concentration of a compound can change (lower) in view of differences in pH, osmolarity, etc. in the body (Ruckenstein and Djikaev, 2005).

Importance for toxicology (and health effects)

From points 1 and 2 we learn that the external appearance of the particle (and thus also the surface energy) can be very important towards the availability of the dissolved material and/or the (bio)persistence of the material. This is closely linked to the physical

organisation of the material, amorphous vs crystalline (and even different types of crystals). Therefore a first suggestion could be to try to include this information (shape and surface energy) in building a database linking material properties and biological effects.

The conditions of the milieu play an important role in dissolution of materials (point 3). No or little information has found on organic materials, or the organic coatings used to make materials biocompatible. The lack on data on organic materials in somewhat different environmental conditions (as found in cellular vesicles) is probably due to fact that not many organic nanomaterials are produced compared to metal based materials. But since the growing interest gap needs to be filled as soon as possible.

Another important finding has been published recently (Lison *et al.* 2008). In this paper it is describe that mono-disperse nanomaterials behave (at least in the conditions used in the paper) as solubilised molecules. This is important for all dispersed materials but the impact of the small nanomaterials (smaller than 20 nm) seems to be bigger because the toxic effects seen could be related to the total surface area of the materials. This finding needs to be verified using different types of materials.

It has certainly also be stressed that dissolution can be a desired property. The formulation of drugs as a nanomaterial can help to dissolve the active substance (Perrut *et al.*, 2005). Micronisation alone cannot guarantee a significant enhancement of dissolution rate or bio-availability of hydrophobic drugs. Many other factors play a major role in the bioavailability (this is not necessarily dissolution) among which probably the most important one is wettability; as shown e.g. by Yang *et al.* (2007); they used ionic-complementary peptides (EAK16-II) to modify surfaces. Due to the amphiphilic nature of these molecules they can interact with both hydrophobic and hydrophilic surfaces allowing the use in several applications. The main question that can be put forward is: how stable are these interactions in a biological system and what is the fate of the amphiphilic material. This is a valid question since a well described effect of cationic amphiphilic molecules is the induction of phospholipidosis (Reasor *et al.* 2006).

Knowledge gaps:

- How does surface energy correspond with toxicity, if it does at all?
- Does conversion from amorphous to crystalline status in all cases enhance toxicity of the NPs?
- The stability of most of the nanomaterials in biological fluids is unknown

2.6 Cellular uptake and accumulation in “organelles” and “organs”?

Author: Peter Wick, Harald F. Krug, Jörg Wörle-Knirsch

For most, if not all nanomaterials an uptake into cells and tissues has been observed and is well documented in the literature (for overview see: Kumar, 2006). So far there are no real material specific mechanisms described. Nanomaterials that are poorly water soluble and can therefore not be administered to cells are usually chemically modified to fulfil the criteria of being available to the cells. Then an uptake can take place. In a recent work, the biodistribution of carbon nanotubes was described (Wang *et al.,* 2004). It was found that carbon nanotubes (CNT) distribute significantly stronger

in the stomach, kidneys and bones than in any other tissue or organ. The modality of distribution is more or less independent on the site of intrusion of CNT into the mouse body but Wang and his colleagues used hydroxylated and water-soluble forms of the SWCNT. Another study used various functionalized CNTs and investigated their uptake by different cell types (Kostarelos *et al.,* 2007). They found all CNT variations in all cells despite the fact that active mechanisms were inhibited. The underlying mechanism of nanoparticle uptake is still controversially discussed. Some neglect an active uptake (Geiser *et al.,* 2005) and postulate a passive not specified nonphagocytic mechanism (Rothen-Rutishauser *et al.,* 2006) as others find indications for active phagocytic ways like clathrin coated vesicles and endosome formation (Shi Kam *et al.,* 2004). Apparently some nanomaterials do not remain in endosomes as they are widely distributed within organisms. If this is true for non carbonaceous materials it has not been shown yet. But from our work, we see metallic or metal oxide particles in most cases within endosomes (Wörle-Knirsch, personal data). This has been confirmed by Limbach *et al.* (2005) with their experiments on cerium oxide NPs of different sizes and their uptake by fibroblasts. They found the larger particles more rapidly within the cells and most particles were enclosed in endosome-like structures.

A clear correlation between nanoparticle size and surface area has been described with regard to toxicity by Oberdörster and his coworkers (Oberdörster, 2000, 2001; Oberdörster *et al.,* 2000). They described the intrusion of leukocytes into the lung upon treatment with TiO_2 NPs of different size regimes. No correlation between the applied dosages could be observed, but with regard to the applied nanoparticle surface data of both materials matched. It has been postulated that carbon nanotubes interfere in vitro with the DNA-polymerase, inhibiting as well as boosting performance of this important enzyme for DNA replication (Cui and Tian, 2004). Unfortunately *in vivo* no such data are present or available today. Lu as well as Gao described an interaction of carbon nanotubes and DNA (Gao *et al.,* 2003; Lu *et al.,* 2005). These materials can be easily linked with DNA and therefore can be used as gene delivery tools. If this is true for in vitro experiments it is also likely to happen *in vivo*. For other nanomaterials so far no interaction with DNA has been postulated.

So there is still a clear need to investigate nanomaterial interaction with cells and cell organelles and perform work on nanomaterial persistence within living organisms and the environment.

Key-issues as postulated from the 2nd IMPART expert meeting in Karlsruhe, December 2005:

- Material specific mechanisms
- Size and surface dependency
- Active and passive processes (phagocytosis, caveoli, endocytosis)
- Interaction with metabolic pathways
- Remaining or using endosomes
- Accumulation in membrane-enclosed compartments
- Effects on genetic material (e.g. chromosomes)
- Interaction with DNA

2.7 How will we administer the materials for *in vivo* and *in vitro* tests?

Author: George Robillard

2.7.1 Introduction

The entire REACH regulation does not make a single reference to NPs but since NPs are being examined as potentially hazardous chemicals the REACH protocol standards could at least serve as the minimum standards which could be applied to NPs as well. Nevertheless, there can be extenuating circumstances which prevent such a simple approach.

2.7.2 Inhalation versus instillation versus aspiration

For instance the requirements of large amounts of material for testing might make it difficult if not impossible to apply the same testing methods for standard potentially hazardous chemicals and NPs. An example is the *in vivo* inhalation test in which the REACH regulation, Volume IV, requires a dynamic air flow with 12 volumes of air changing per hour for the entire experimental set-up and the set-up should have a size large enough that the volume of the test animal is less than 5% of the total volume of the test chamber. For well-characterized NPs, such a procedure can be prohibitively expensive. In such a case REACH offers intratracheal **instillation** as an acceptable alternative.

If inhalation is used, the **particle size distribution** has to be determined in the same atmosphere as the test chamber involving liquid or solid aerosols. Conditions and standards are specified in section B.30 of REACH volume IV which can be directly applied to nanoparticle aerosol inhalation studies concerning the size distribution measurements etc.

In inhalation studies involving aerosols **formulations** or the use of an emulsifier of uncharacterized biological activity, REACH requires that an additional control group which is not exposed to the vehicle be utilized.

2.7.3 Dosing (see additional information in Chapter 2.3)

The IOM (Institute of Occupational Medicine) report (Tran *et al.,* 2005) summarizes all studies dealing with inhalation of NPs. On the issue of **dosing** they conclude that “it is difficult to make firm recommendations about the most appropriate metric by which to assess exposure by inhalation to NPs. Current toxicological evidence suggests that, for most NPs, the most appropriate metric is surface area. (This is probably not universally true e.g. for particles which could be considered to be fibres, such as some forms of carbon nanotubes, particle number may be more appropriate).” Other suggestions are to use mass, volume or particle number. “Depending on the circumstances, surface area, mass, volume, particle number, or some other property could be the appropriate basis for expressing the dose of a nanoscale material. The nature of the material itself could be the most important determinant of this decision” (Gainsville, Florida, 2004). It is stated that for some NPs, health effects correlate best with the surface area measured by the BET nitrogen absorption methodology. This statement appears to be in relation to the findings of epidemiology studies of air pollution effects on humans and may not be universally applicable to other types of NPs (NIA Comments on SCENIHR Opinion May 2007.doc, further information: http://www.nanotechia.co.uk).

Additionally, dosimetry of NPs in in vitro studies includes also understanding of ability of NPs to settle, diffuse and aggregate to different extents. Recently, Teeguarden *et al.* (2007) introduced the concept of cellular dose in vitro in which they outlined an approach for simulation of nanoparticle particokinetics in cell culture systems as a function of media density and viscosity and particle size, shape, charge and density.

In the context of dosing the nanomaterials:

- there is a lack of international consensus on measurement techniques or standards for monitoring nanoparticles in the workplace
- there is a strong need for a measurement device that can differentiate between engineered nanoparticles and the background level of natural nanoparticles
- there is a need for the evaluation of nanoparticle formulations on a case-by-case basis in order to choose the most appropriate metrics

2.7.4 Surface modification to prevent aggregation within a test

Aggregation/clumping of NPs is a common occurrence with dry, aerosolized or aqueous suspensions of NPs. In general, NPs, even when aggregated have been seen to be more toxic than larger complexes. Many **surfactants** can be found in the environment that might promote particle deagglomeration. Deagglomeration is likely to aid dispersion of NPs in the environment and hence increased interaction with different organisms. Deagglomeration may also promote uptake of NPs into an organism via specific routes if particle size determines access to the site of absorption (e.g. lung). The role of aggregation in influencing the ability of NPs to cross biological membranes has not been studied in any system.

The recommendation is to prevent agglomeration by using tissue-specific components to cover the surface of the particle; for instance, a lung surfactant should be used if working with lung cells, etc.

2.8 What about long term studies at low (more realistic) concentrations?

Author: Jamila Smisterova

2.8.1 Long-term toxicity testing for chemical substances

According to Guidelines for the testing of toxicity of chemical substances (REACH) **the long-term toxicological information** includes sub-chronic (repeat dose toxicity study for 90 days) and chronic study, performed with one species, male and female, testing the most appropriate route of administration. The long-term toxicity studies are required for the quantities above 10 tons. Concerning the ecotoxicological information, the long-term aquatic ecotoxicity study on Daphnia should be conducted already for the quantities already above 1 ton if the short-term aquatic toxicity data indicates the need to further investigate and if the substance is poorly water soluble (< 1mg/l). The sub-chronic study is proposed if

- the frequency and duration of human exposure indicates that a longer term study is appropriate;
- other available data indicate that the substance may have a dangerous property that can not be detected in a short-term toxicity study;

- toxicokinetic studies revealed accumulation of the substance or its metabolites in certain tissues or organs which would remain undetected in a short-term toxicity study but which are liable to result in adverse effects after prolonged exposure.

2.8.2 What are low (more realistic) doses of NPs?

Studies on toxicity of nanomaterials should use the appropriate doses of NPs, i.e. not overload organs, to gain realistic hazard information and hence the potential risks posed. However, there is a complete lack of data for human or environmental exposure levels of NPs. Current human exposure to NPs is likely to be from manufacturing, processes at the workplace or research environment. First measurements of carbon nanotubes at a model workplace resulted in very low concentrations, < 50 µg/m³, and these were most likely in the form of aggregates (Maynard, 2004). Airborne concentrations of nanotube material generated during handling have been estimated to be lower than 53 µg/m³. Glove deposits of SWCNT during handling were between 0.2 mg and 6 mg per hand (Tran *et al.*, 2005). It is, however, estimated that exposure concentrations of NPs at workplace conditions might reach up to several hundreds micrograms per cubic meter (Muller, 2005).

Probably the most direct exposure studies could be done on NPs derived from the products applied to the skin (cosmetics), swallowed, injected, inhaled or implanted medicines.

Data regarding exposure of NPs to soil and water is extremely limited and little is known about their behaviour. In terms of exposure to humans, the route is most likely to be through drinking water. Currently there is no apparent direct data on measurements of NPs in water systems.

Thus, what is needed are:

- Methods, combinations and strategies to provide knowledge about real-world exposure to different classes of nanoparticles and nanoparticle aerosols
- Methods to evaluate dermal and ingestion exposure
- Knowledge about the biokinetics of NP in order to estimate appropriate doses at the target site.

2.8.3 Long-term toxicity testing for nanoparticles

Most studies concerning the nanotoxicity follow short-term *in vivo* effects. Long-term **animal toxicity** studies are sparse, although some of the harmful effects on nanomaterials may only be confirmed after many years of exposure. To compensate for this, animals are exposed to high dose over a shorter period of time. Shvedova *et al.* (2005) tested the effect of low concentrations of NPs in mice by the administration of SWCNT either by laryngeal aspiration or intrapharyngeal instillation in mice (10-40 µg/mouse). Maximum dose in these studies extrapolates to 20 days exposure at the current occupational safety and health administration standards. The aspiration of SWCNT resulted in the substantial pulmonary toxicity, including granulomas surrounding SWCNT, diffuse interstitial fibrosis, dose –dependent increase in airway resistance persisting for 60 days, aortic mt-DNA damage and reduced clearance of bacteria. These studies gave indication of human toxicity at realistic doses of inhaled

NPs and demonstrated that workers exposed to the current permissible exposure levels may be at risk of developing pulmonary fibrosis.

Prolonged exposure of rats (Muller *et al.,* 2005, Warheit *et al.,* 2004) and mice (Lam *et al.,* 2004) to intratracheal instillation of higher doses of CNTs, 0.5-5 mg/rat and 0.1-0.5 mg/mouse, respectively revealed the persistence of inflammation and nanotubes in the lung after 60 days (Muller *et al.,* 2005). Similarly in mice, dose-dependent epithelioid granulomas has been observed which was pronounced in 90 days. On other hand, long-term exposure of rats to water soluble fullerenes appeared to cause no adverse lung tissue effects up to 3 months postinstillation at the highest dose applied, 3 mg/kg (Sayes *et al.*, 2007a).

Although lower inhalation exposure has been applied in studies with ultrafine titanium dioxide, applied doses were still far from the realistic ones. Muhle *et al.* (1995) exposed rats to ultrafine silica and titanium dioxide at the doses of 1 mg/m³ and 5 mg/m³, respectively for 24 months and observed increased lung tumour incidence. Warheit *et al.* (1997) exposed rats to air containing 5-250 mg/m³ titanium dioxide NPs for 4 weeks and observed sustained pulmonary inflammatory response through a period of 3-6 months. Similarly Baggs *et al.* (1997) and Warheit *et al.* (1996) observed persistent adverse pulmonary effects in rats, 1 year after the 3 months exposure to about 23 mg/m³ of ultrafine titanium oxide. Bermudez *et al.* (2002, 2004) observed the differences in the persistence of the adverse effect of the exposure to ultrafine titanium dioxide in different rodents. The inflammation, retardation of particle clearance and progressive epithelial damage as a result of the inhalation of 0.5 to 10 mg/m³ NPs for 90 days were still present by 52nd week in mice, while rats recovered by 26th week.

Similarly, pulmonary toxicity has been observed for rats intratracheally instilled with nano TiO_2 rods and dots for prolonged period of 90 days (Warheit *et al.,* 2006). After this period the inflammation was still evident for the highest dose of 5 mg/kg.

As nanomaterials are unlikely to leave **the environment** after exposure, a long-term study may result in better understanding of the bioaccumulation of NPs and their chronic effects on organisms. The toxicity tests for bioaccumulation of nano-ZnO and C60 from the alga to the Daphnia, for example, revealed that the effects of these NPs were greatest in the long-term (20 days) when compared to to the initial period of 48 hours (Luo, 2007). In addition to lengthening the experimental period, *new methods to evaluate the responses to nanoparticles, such as DNA microarray, and to keep the particles suspended in the medium will be required to test the effects of bioaccumulation.*

To date, **human studies** were limited to acute exposures, measuring both pulmonary and systemic inflammatory endpoints. Only a few of them used NPs in concentrations which are close to the realistic exposure. For example, effect of two hours inhalation exposure of normals and asthmatics to ultrafine carbon NPs in the concentration range between 10 and 50 µg/m³ was studied by Pietropaoli *et al.* (2004) and Frampton *et al.* (2004). No significant effects on respiratory symptoms, blood pressure, oxygen saturation, pulmonary function, markers of airway inflammation (NO), soluble markers of systemic inflammation or coagulation have been observed.

The issue of **skin absorption** of NPs is of obvious importance when these materials are introduced into cosmetics and sunscreens. A number of studies have investigated skin penetration by nanoscale TiO_2. For example, Pflücker *et al.* (2001) found that

micronised titanium dioxide is solely deposited on the outermost surface of the stratum corneum and could not be detected by EM or light microscopy in deeper stratum corneum layers. Lademann *et al.* (1999) found less than 1% of the applied coated titanium dioxide NPs from sunscreen in skin follicles. Cross *et al.* (2007) found that less than 0.03% of the applied zinc content of nanoparticulate zinc oxide sun screen penetrated the epidermis and no particles could be detected in the lower stratum corneum or viable epidermis. Gamer *et al.* (2006) report similar findings on the penetration of zinc oxide and titanium dioxide microfine particles into porcine skin. Mavon *et al.* (2007) reported the absence of titanium dioxide penetration from a broad spectrum sunscreen formulation into the viable skin layers through either transcorneal or transfollicular pathways. Tan *et al.* (1996) have found that the penetration into the epidermis of TiO_2 from sunscreen containing 8% micronised 10–50 nm TiO_2 upon exposure for a prolonged period of 6 weeks was greater when applied as an oil-in-water emulsion than an aqueous suspension.

It is highly recommended to perform:

- *In vivo* long-term toxicity assays for inhalation, oral, skin and injection exposure with the evaluation of markers for inflammation, oxidative stress, cell proliferation in portal-of-entry and selected remote organs, like liver, spleen, bone marrow, heart, kidney, CNS
- Reproductive and immunotoxicity studies
- Cardiovascular and coagulation tests mainly for those NPs for which translocation to the blood circulation, after e.g. inhalation has been shown.
- Studies on deposition, translocation and biopersistence of NPs.
- Validation of results of high dose studies using order of magnitude lower concentrations resembling those realistic *in vivo* exposures, for quantum dots, fullerenes, metal oxides NPs. For inhalation, a 2 week exposure is recommended in multiple doses (1-10 µg/kg body weight), e.g. dosing daily for 4 weeks with 3 months follow up.

The National Toxicology Program is planning short and long-term studies, including oral, dermal, and inhalation exposures for some NPs (http://ntp-server.niehs.nih.gov/files/nanoscale05.pdf).

2.8.4 In vitro assays for long-term toxicity studies

The necessity of long-term toxicity studies is supported by the ability of NPs to persist and to accumulate. Since the metabolism in the cell determines the **biopersistence**, long –term in vitro studies might give indication about the long-term effect *in vivo*. However, current in vitro test systems are mainly focused on **acute cytotoxicity** testing rather than short-term or long-term repeated dose toxicity investigations. In vitro methods including the use of cells of human origin relevant for respiratory (Brown *et al.,* 2004; Duffin *et al.,* 2007b; Geys *et al.*, 2007), dermal (Manna *et al.,* 2005; Monteiro-Riviere *et al.,* 2005; Papageorgiou *et al.,* 2007; Rouse *et al.,* 2006; Shvedova *et al.,* 2003), cardiovascular (Gojova *et al.,* 2007; Radomski *et al.*, 2005; Yamawaki and Iwai , 2006) and neuronal (Hussain *et al.*, 2006; Pisanic *et al.,* 2007) systems confirmed the

cytotoxic potential of carbon nanotubes, fullerenes and metal oxides, especially at higher concentrations (above 10 µg/ml).

An example of promising **long-term** in vitro cytotoxicity studies is the use of human HepG2 cells for the comparison of the in vitro long-term cytotoxicity of chemicals with acute human toxicity data by following the 6 weeks exposure of the cells and measuring the concentration of compound which was needed to reduce the total cell protein content by 50% (Scheers *et al.,* 2001).

It is highly recommended to:

- Perform chronic nanotoxicity assays, including the development and validation of nonanimal test methods
- Gain more knowledge about the reproductive toxicity, mutagenicity, immunotoxicity and irritancy of nanomaterials

3 Exposure

3.1 Occupational exposure

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As the nanotechnology workforce is growing rapidly, the nanotech industry is projected to employ two million workers and have a $1 trillion impact on the global economy by 2015. Nanotechnology is the manipulation of matter on a near-atomic scale to make new structures, new materials and devices in the nanometre scale. Today, about 80 consumer products and over 600 raw materials, intermediate components and industrial equipment items, are used by manufacturers and produced or handled by workers. There are only some data about real amount of exposed workers in US and UK but information is not available about other countries. The US national nanotechnology initiative has estimated that around 20 000 researchers are working in the field of nanotechnology. For UK Institute of Occupational Medicine has estimated that approximately 2 000 researchers are employed in new nanotechnologies companies and universities where they may be exposed to NPs but this number may double over the next five years. A maximum of 500 workers are considered to potentially be exposed through existing manufacturing of ultrafine carbon black; around 100 000 individuals may potentially exposed to fine powders through various powder handling processes, including pharmaceutical industry. More than 1 000 000 workers in the UK may be exposed to NPs via incidental production in processes such as welding and refining (Aitken *et al.*, 2004; HMGovernment, 2007; NIOSH CDC, 2006).

As with all technologies new risks might appear that we have not thought about yet, underlining the need for continuous dynamic risk reviews (Allianz, 2005; ICON/RICE, 2005; IRSST, 2006) and books (Maynard *et al.*, 2007). Concerns about the lack of knowledge and possible risks arising from exposure to NPs led to the formation of working groups in different organisations of occupational health and safety to assess the health risk from exposure to these materials and collect the information on websites for example:

http://www.iom-world.org/research/nanoparticles/;
http://www.nanotech.org.uk/;
http://hse.gov.uk/research/;

http://icon.rice.edu/research.cfm;
http://www.cdc.gov/niosh/topics/nanotech/;
http://www.vcu.edu/oehs/chemical/nanotech.pdf;
http://www.irsst.qc.ca/;
http://www.defra.gov.uk/environment/chemicals/achs/index.htm;
http://www.temas.ch/nano/nano_homepage.nsf/

There is organized special Online Libraries collecting information on nanotechnology and occupational risks: IOM Library http://www.iom-org/ , Centre of Diseases Control and Prevention and National Institute of Occupational Health and Safety http://www.cdc.gov/niosh/topics/nanotech/safenano/; Nanoscale Science and Engineering Center at the University of Wisconsin - Madison to explore the self-assembly of complex materials and building blocks at the nanoscale and develop the means of communicating advances in nanotechnology to the public http://www.nsec.wisc.edu/NS-Home.php; International Council on Nanotechnology, RICE University and CBEN database on Environmental Health and Safety (EHS) with research records on nanotechnology and nanomedicine based on TOXNET, Medline and NTIS EHS – http://icon.rice.edu/research.cfm. Health and Safety Executive (2006, 2007) designed bulletin service to provide overview of publications of studies that have examined the exposure and potential health effects of nanomaterials, which have been published in two areas of interest: measurements, characterisation and control of exposure to NPs; potential for toxic effects of NPs in humans.

3.1.1 Is occupational exposure an existing problem?

Epidemiological data is limited for occupational exposure to NPs. Primary routes of occupational exposure to NPs include inhalation, transdermal desorption and ingestion (Borm *et al.*, 2006). There are four main groups of nanoparticle production processes (gas-phase, vapour deposition, colloidal and attrition) all which may potentially result in exposure by inhalation, dermal or ingestion routes. From an occupational hygiene perspective, the processes are not dissimilar to existing chemical production processes. Only gas-phase processes have the potential to cause exposure to primary NPs by inhalation during the synthesis stage. All processes may give rise to exposure (by inhalation, dermal and ingestion) to agglomerated NPs during recovery, powder handling and product processing (Aitken *et al.*, 2004, IRSST, 2006). Flame synthesis is one of the most versatile and promising technologies for large-scale production of nanoscale materials. Pyrolysis has recently been shown to be a useful route for the production of single-walled nanotubes, quantum dots and a wide variety of nanostructured ceramic oxides for catalysis and electrochemical applications (Beaucage *et al.*, 2004).

Non-engineered NPs (release of diesel, gasoline and gas combustion, frying, cooking, welding) are widespread occupational risk factors (Dennekamp *et al.*, 2001; Ono-Ogasawara and Smith, 2004). In modern society, printers are widely used in the office environment and the newest researches showed the problem of carbon NPs in indoor air generated by different types of printers. The mean size detected in different studies varied from 35 nm to 120 nm and the range of number concentration is 350 – 3.8 10^4 particles/cm^3 (He *et al*., 2007; Koponen *et al*., 2001; Uhde *et al*., 2006).

Specific characteristics of NPs (size, shape, surface area charge, chemical properties, solubility and degree of agglomeration) can influence their effects in biological systems.

Nanosized particles may behave differently to larger sized materials. Information about production volume, occupational exposure potential of nano-sized material as well as safety and potential hazards is urgently needed. New problems of occupational health arise with nanotechnology (Maynard and Pui, 2007; Oberdörster *et al.*, 2005b). Separate question is release of NPs throughout the application life cycle. Köhler *et al.* (2008) showed that release of carbon nanotubes (lithium-ion secondary batteries, synthetic textiles) can occur not only in the production phase, but also in the usage, recycling and disposal phase of nanotube applications.

Development of nanotechnologies and growing number of employed workers is cause for organization special conferences. The first European Conference focusing on occupational safety and health from the viewpoint of nanotechnologies and engineered NPs in workplaces was NanOSH Conference – Nanotechnologies "A Critical Area on Occupational Safety and Health" on 3-5 December 2007 in Helsinki (Finland). Selected papers will be published as proceedings after the Conference in a thematic issue of Human & Experimental Toxicology Journal. The estimated publishing date is in the end of 2008 (http://het.sagepub.com/).The 4th International Conference on Nanotechnology – Occupational and Environmental Health (NanOEH 2009) will be held on 26–29 August 2009 in Helsinki, Finland (http://www.ttl.fi/Internet/English/Information/International+meetings+and+symposia/Nanoeh2009/).
Special question for today is managing occupational and safety risks and how effective are filters at removing nanometer-diameter particles from the air (Maynard and Pui, 2007).

Recommendations / knowledge gaps:

- Information about production volume, occupational exposure potential of nano-sized material is urgently needed to determine potential risk of employees.
- The workplace represents a critical interface between people and nanotechnology, and an area where potential impact needs to be understood and managed. In the future, it is also likely that even wider distribution of these particles may have significant effects on organisms.
- The potential effects of engineered nanoparticles on the ecosystem must also be considered in order to assure the safety of humans and the environment from production emissions.
- Development of innovative technologies for effective health and safety management in workplaces.
- Studying the potential release of nanoparticles throughout the application life cycle including recycling process workplaces and emissions in environment.

3.1.2 How to assess / measure? Is there a need for new / other sampling devices?

There is not one sampling method that can be used to characterize exposure to NPs. Inhalation is the traditional route of occupational exposure to NPs. Occupational nanoparticle aerosols should be monitored by three exposure metrics – mass, surface and numbers. Current best practice to measure the exposure by inhalation is to use a personal sampling device to collect a sample of aerosols as the most appropriate,

biologically relevant fraction. Aerosol samples can be collected using inhalable, thoracic, or respirable samplers. Respirable fraction samplers will also collect a nominal amount of nanometre-diameter particles that can deposit in the upper airways. The commercially available filters could not give quantitative sampling of smallest nanoparticle because the smallest size of pores is 25 nm. Respirable fraction samplers allow mass-based exposure measurements to be made using gravimetric and/or chemical analysis; they do not provide information on aerosol number, size, or surface area concentration. **Generally accepted, realistic methods for exposure assessment are still lacking for workplaces; no commercially available personal samplers are designed to measure the particle number, surface area, or mass concentration of nanometre aerosols,** no standardized, well-characterized reference standards of NPs are developed (Aitken *et al.*, 2004; Allianz, 2005; NIOSH CDC, 2005)**.** However, several methods are available that can be used to estimate number, surface area and mass for particles smaller than 100nm (Aitken *et al.*, 2004; IRSST, 2006; Wake, 2006):

Mass could be analysed by weighing or chemical analysis; limitation is the mass which should be collected in accordance with limit of detection (0.01 mg on filter, in case of usual sampling rate 5 l/min the lowest measurable concentration based on a full shift collection would be 0.02 mg/m^3

Number of particles could be measured with different types of methods:

- optical particle counter (OPC) – is governed by the wavelength of their source, usually detection size limit 300 nm (particle counter the Grimm 1.104 Work-Check (http://www.dustmonitor.com/monitors/occupational/1104.htm);
- condensation particle counter (CPC) – device operate by condensing vapour onto sampled ultrafine particles to grow them to size range that can be detected optically by a standard optical counter (TSI Model 3007 – www.tsi.com), claimed size range 10 - 1 000 nm, concentration range ~ 0 - 100 000 particles per cc, limitation of this device is lack of size information;
- scanning mobility particle sizer (SMPS) – operates by charging particles and separating them based on their mobility passing between electrodes, separated particles are than counted to give size range of mobilities; devices are capable of measuring aerosol size distribution from 3 to 800 nm (www.tsi.com, TSI model 3934), although not simultaneously over the complete range, limitation of use in occupational hygiene due to lack of mobility, expense and complications in use;
- electronic low pressure impactor (ELPI) – sampled particles are charged and then passed into low pressure impactor with electrically isolated collection stages; the electrical current by the charged particles onto each impactor stage is measured in real time by sensitive multi channel electrometer, the particle collection into each impactor stage is dependent on the aerodynamic size of the particles, particles can be removed from the impactor stages for further analysis; device can measure particle size distribution and concentration in the size range 7 nm to 10 µm (addresses of devices production www.dekati.com; http://appliedphysisusa.com/moudi.asp).

A major limitation of all these measurement methods is that they cannot discriminate agglomerates of NPs from single larger particles.

Surface area:

- Epiphaniometer is the only instrument which has been successfully used to measure particle surface area directly with lead isotope charging chamber, the amount of radioactivity measured is proportional to the particle surface area – this is a complex and difficult instrument**;**
- Brauner-Emmet-Teller (BET) bulk method of surface area measurements in which used process depends on gas adsorption using nitrogen, krypton, argon or carbon dioxide gases; the sample sizes used are typically greater than expected in occupational hygiene sampling.

Image analysis – includes range of imaging processes which may be used along with scanning (SEM) or transmission electro-microscopes (TEM) to obtain size, shape, structure and in some cases compositional information from single or collections of aerosol particles. Resolution for SEMs – 5-10 nm whereas TEMs can resolve down to about 1 nm; samples can be collected directly onto filters, filter substrates or impactor substrates; methods need complex arrangements for sample collection (especially TEM) and calibration of particles onto sample filter.

All of the methods which may be used clearly fall short of what would be an ideal sampling and measurement system for NPs. There is a pressing need for more research into the development of new and improved measurement methods to provide reliable assessments of exposure of NPs (HMGovernment, 2007). Only few exposure measurements are from real engineered NPs workplaces (Brouwer *et al.*, 2004; Maynard *et al.*, 2004) but more research is dedicated to measurement of diesel and gasoline exhaust carbon NPs. The above mentioned methods or part of them are used in research of exposure of different type of NPs:

- the combined use of all methods applied to metals of very low concentrations (gold nanocrystals in pyrite and uranium nanocrystals, U and Fe nanocrystals embedded in an aluminosilicate, As-bearing nanophase, westerveldite -FeAs) was identified (Utsunomiya and Ewing, 2003).
- emissions from a silicon smelter in Southern Tasmania, Australia were characterized using optical microscopy for the initial assessment of particle density and transmission electron microscopy was used for primary particle and aggregate sizing. The authors conclude that the size **distributions could be important in the consideration of health effects** from silica fume exposure (Cunningham *et al.*, 1996).
- researchers of Work Environment Institute of Norway (STAMI) detected nanoparticles by counting in the aluminum processing workplace area by anode processes (because during electrolysis vapour containing fluorine quickly changes to ultrafine particles which exist freely in the work environment). Size of NPs - 10-250 nm, concentration was 20 000 particles per cm^3 in pot rooms work area and rises up to 10 times higher top concentrations during anode changing on open bath (Thomassen *et al.*, 2006; www.ams-aluminium.no/html/happa__survey_of_occ__exposure.html).
- diesel engine exposure are investigated by use of different combinations of above mentioned methods to identify the nanocrystalline or nanoparticulate

components, especially their degree of crystallinity, size, structural/morphologic features, and chemistries. Reference aggregates of TiO_2 rutile and anatase as well as Si_3N_4 nanoparticles were used to establish these characterization protocols, which were applied to several hundred individual particulates: homogeneous aggregates of carbonaceous/diesel particulate matter, complex mixtures of carbonaceous matter, including carbon nanocrystals, and inorganic nanocrystals; and heterogeneous, nanocrystal/nanoparticulate aggregates (Murr *et al.*, 2004a). A nano-differential mobility analyzer was used to size-select NPs (mass median diameter similar to 25-60 nm) from diesel engine exhaust for subsequent chemical analysis by thermal desorption particle beam mass spectrometry. Mass spectra were used to identify and quantify nano particle components (Tobias *et al.*, 2001). The particle number concentrations were not significantly different for the compressed natural gas (CNG) and diesel engines (Hasegawa *et al.*, 2004). Originally constructed an electrical low-pressure impactor designed to monitor the particle size distribution of heavy-duty diesel truck exhaust (Brown *et al.*, 2000b).

Research on sampling strategy showed possible influences of both distance to source and time course on particle number concentration and particle size distribution. For the studies CPC devices are well for the identification of particle emission sources. The range of ultrafine particle number concentration can be detected by both SMPS and ELPI. An important advantage of the ELPI is that aerosols with ultrafine sizes can be collected for further analysis. Specific surface area of the aerosols can be estimated using gas adsorption analysis; however, with this technique ultrafine particles cannot be distinguished from particles with non-ultrafine sizes. **Consequently, estimates based on samples collected from the breathing zone and scanning electron microscopic analysis may give a more reliable estimate of the specific surface area of the ultrafine particles responsible for personal exposure.** The results suggest both spatial and temporal variation in total number concentration and aerosol size distribution. Therefore, the results obtained from static measurements and grab sampling should be interpreted with care as estimates of personal exposure. For evaluation of workplace exposure to ultrafine particles it is recommended that all relevant characteristics of such exposure are measured as part of a well-designed sampling strategy (Brouwer *et al.*, 2004).

Famous nanoparticle researchers (Maynard and Aitken, 2007) explore the idea of a universal aerosol monitor, which would enable personal exposure measurements to be collected for all three metrics simultaneously, while being inexpensive enough to encourage widespread use. Such a device would provide an economical and adaptable solution to monitoring exposure to nanostructured aerosols, as both the materials and information on the potential risks they present are developed.

Recommendations / knowledge gaps:

- There is not one sampling method that can be used to characterize the traditional inhalation route of occupational exposure to NPs therefore research should be in progress to determine most appropriate of three exposure metrics (mass, surface and numbers) to be monitored as well as realistic and suitable sampling equipment.

- Generally accepted, realistic methods for exposure assessment are still lacking for workplaces; no commercially available personal samplers are designed to measure the particle number, surface area, or mass concentration of nanometre aerosols.
- There are lacks of the occupational exposure limits for nanoparticles (different type) and no standardized, well-characterized reference standards of nanoparticles are developed.
- Skin absorption of nanoparticles has to be evaluated as acceptable or unnecessary method for detection of summarized exposure.

3.1.3 What to measure (size, mass, surface…) and what is feasible?

Engineered NPs are not uniform group of substances. Differences in size, shape, surface area, chemical composition and biopersistence require that the possible environmental and health impact be assessed for each type of nanomaterial separately.

There is a lack of reliable, affordable and standardized methods for measuring of NPs size and shape and characterization of NPs (their composition and surface behaviour). These properties are essential for valid risk assessment and receiving comparable and repeatable results. It is important to define the most appropriate metrics in exposure as well as in hazard studies (surface area of NPs or fibre number for nanotubes). Research activity defined in **UK** (characterisation and metrology) is to identify the most suitable metrics and associated methods for the measurement and characterisation of NPs (Aitken *et al.*, 2004, HMGovernment, 2007, Wake, 2006). International Standards Organization (ISO, 2006) has developed document "Workplace Atmospheres – Ultrafine, nanoparticle and nano-structured aerosols – Exposure characterization and assessment" that will be starting point for more comparable measuring of NPs.

For evaluation of **workplace exposure** to ultrafine particles it is recommended that all relevant characteristics of such exposure (total number concentration and aerosol size distribution) are measured as part of a well-designed sampling strategy (Aitken *et al.*, 2004, Allianz, 2005, Brouwer *et al.*, 2004). After investigation of emissions from a silicon smelter in Southern Tasmania the authors conclude that the **size distributions could be important in the consideration of health effects** from silica fume exposure (Cunningham *et al.*, 1996). **The number deposition fraction increased as particle size decreased**. The deposition at rest was greater in these subjects with asthma than in previously studied healthy subjects. The efficient respiratory deposition of ultrafine particles increases further in subjects with asthma (Chalupa *et al.*, 2004). Currently, ultrafine particles (UF, PM0.1) should be characterised by particle number instead of particle mass. However, data on UF exposure and health effects are still limited. **The mechanisms by which particles influence human health are only poorly understood. Under discussion is the role of particle size and particle composition** (Allianz, 2005, Englert, 2004).

Carbon nanotubes are leading to the development of mass production and handling facilities but little is known of the risk associated with exposure. Occupational exposure and the potential exposure routes of single-walled carbon nanotube material (SWCNT) were investigated while handling unrefined material. Estimates of the airborne concentration of nanotube material generated during handling suggest that concentrations (mass concentration) were lower than 53 $\mu g/m^3$ in all cases. Glove

deposits of SWCNT during handling were estimated at between 0.2 mg and 6 mg per hand (Maynard *et al.*, 2004). Exposure to graphite and carbon materials has been associated with increased incidence of skin diseases and these findings indicate the necessity for **skin exposure detection** and use of protective measures. The investigations showed dermal toxicity of SWCNT due to accelerated oxidative stress in the **skin** of exposed workers (Shvedova *et al.*, 2003).

Beryllium is a lightweight metal which causes a chronic granulomatous lung disease among workers who become sensitized to it. Research confirm a previous finding in certain plants that **particle number concentrations are higher in areas where historical estimate of risk showed a high risk of disease despite relatively lower mass concentrations**. By providing side-by-side measurements of both particle number and mass, this research adds support to the proposal that **particle number rather than particle mass may be more reflective** of target organ dose and subsequently a more appropriate measure of exposure for chronic beryllium disease. The investigation also shows that particle mass exposure measurements and particle number exposure measurements were not correlated (McCawley *et al.*, 2001).

Health effects of ultrafine particles were larger than those of the mass of the fine particles. In addition, the effects of the number of the ultrafine particles on peak expiratory flow PEF were stronger than those of particulate matter smaller than 10 µm (Peters *et al.*, 1997).

Research on human pulmonary responses to controlled experimental high-dose exposure to fine (< 2,5 µm) and ultrafine (< 0,1 µm) magnesium oxide particles showed no evidence of any pulmonary inflammatory response (bronchoalveolar lavage (BAL) cell and cytokine concentrations, pulmonary function, and peripheral blood neutrophil concentrations) in six healthy volunteers. These findings are in contrast to those previously seen in a similar study using zinc-oxide particles. The results of study **support the concept that particle chemical composition, in addition to particle size, is an important determinant of respiratory effects** (Kuschner *et al.*, 1997). There is still insufficient evidence to preferentially select one exposure metric over another—particularly for airborne exposures—and that where there is uncertainty, all three should be measured (Maynard and Kuempel, 2005; Maynard *et al.*, 2007; Oberdörster *et al.*, 2005a). Wake (2006) recommended that none of the parameters (mass, number and active surface area) taken in isolation can give insufficient information to predict toxicity of NPs after laboratory comparison the results of mass, number and surface area measurements of five types of NPs.

Recommendations / knowledge gaps:

- Unlike research results on different chemical composition do not provide sufficient evidence to preferentially select one exposure metric and research have to be continued.
- Research has to be continued on skin absorption of different compositions of nanoparticles as way of occupational exposure and for development of safety measures.

3.1.4 Indirect measures of exposure: Lung function or other applicable endpoints

Epidemiological studies have shown adverse health effects associated with exposure to the ultrafine particulate fraction of air pollution. Most nano-sized spherical solid materials can easily enter the lungs and reach the alveoli (Allianz, 2005, Hoet *et al.*, 2004b). Inhaled particles can have two major effects on the human body. The primary toxic effect is to induce inflammation in the respiratory tract causing tissue damage and subsequent systemic effect: the secondary effect is transport through the blood stream to other organs or tissues of the body. The property that determine the inflammogenicity of NPs is unknown but is expected relate to particle surface area and number of particles. NPs can impair the ability of macrophages to phagocytose and clear particles, and this may contribute to inflammatory reactions (Allianz, 2005, Nemmar *et al.*, 2002b). Further investigations will confirm different impact of NPs on health.

The association between fine and ultrafine particles and respiratory health was studied in adults with a history of asthma in Erfurt, Eastern Germany. Both fractions were associated with a decrease of peak expiratory flow (PEF) and an increase in cough and feeling ill during the day. Health effects of the 5-d mean of the number of ultrafine particles were larger than those of the mass of the fine particles. In addition, the effects of the number of the ultrafine particles on PEF were stronger than those of particulate matter smaller than 10 µm (PM10). (Peters *et al.*, 1997). The negative association between the number of ultrafine particles and ventilatory function in other study demonstrates a need for further investigation into the pulmonary health effects of ultrafine particles (Hauser *et al.*, 2001). The results suggest that the total deposition fraction of ultrafine particles increases with a decrease of particle size and with breathing patterns of longer respiratory time, a pattern that is consistent with diffusion deposition of ultrafine particles. The results also suggest that there is a differential lung dose of ultrafine particles and thus there may be a differential health risk for men versus women (Jaques and Kim, 2000.). Six panel studies with patients suffering from chronic pulmonary diseases have been performed in Germany, Finland and the United Kingdom. Overall, a decrease of peak expiratory flow (PEF) and an increase of daily symptoms and medication use were found for elevated daily particle concentrations. Effects were seen with both fine and ultrafine particles (UFP). One large study on daily mortality from Germany showed comparable effects of fine and ultrafine particles in all size classes considered. However, fine particles showed more immediate effects while **ultrafine particles showed more delayed effects** on mortality. The limited number of epidemiological studies suggests that there are health effects of fine and ultrafine particles which might be independent of each other. If these effects are confirmed by ongoing research, monitoring and regulation of particulate air pollution (air quality standards) may need to be revised (Ibald-Mulli *et al.*, 2002). Araujo et al. (2008) demonstrate that UFP exposures have a higher proatherogenic potential than fine particles and these effects could be linked to a greater propensity of UFP to generate systemic oxidative stress and to interfere with the anti-inflammatory capacity of plasma HDL. UFP promote early atherosclerosis and systemic oxidative stress. Authors noted that further epidemiological and experimental data collections are required to determine the critical physicochemical and toxicological properties of UFPs in humans.

The exact mechanism by which ultrafine particles have adverse effects is unknown, but these particles have recently been shown to **enhance calcium influx** on contact with

macrophages. **Oxidative stress** is also to be anticipated at the huge particle surface; this can be augmented by oxidants generated by recruited inflammatory leukocytes. Athermanous plaques form in the coronary arteries and are major causes of morbidity and death associated epidemiologically with particulate air pollution. In populations exposed to air pollution episodes, blood viscosity, fibrinogen, and C-reactive protein (CRP) were higher. More recently, increases in heart rate in response to rising air pollution have been described and are most marked in individuals who have high blood viscosity (Li *et al.*, 2003). In the study of elderly individuals, there were **significant rises in CRP, an index of inflammation**. In this present review, authors consider the likely interactions between the ultrafine particles the acute phase response and cardiovascular disease (Donaldson *et al.*, 2001b). Inconsistency with previous finding **an absence** of particle-associated symptoms or changes in lung function were indicated despite a relatively high overall deposition fraction in initiated **human clinical studies** of the health effects of UFPs (Frampton, 2001).

Several nanomaterials characteristics can culminate in reactive oxygen species (ROS) generation and direct relationship may exist between surface area, ROS generation capability, and pro-infammatory effect of NPs in the lung, several types of NPs target mitochondria directly, and other forms of injury include protein denaturation, membrane damage, immune reactivity, and formation of foreign body granulomas (Brown *et al*., 2001; Nel *et al*., 2006).

Impressive progress has been made in recent years when **objectives changed from classical tests like lung function**, etc. to endpoints comprising of particle induced **oxidative stress, cell signalling and activation, release of mediators initiating inflammatory processes** not only in the respiratory tract but also in the cardio-vascular system. Particularly, the large surface area of ultrafine particles provides a unique interface for catalytic reactions of surface-located agents with biological targets like proteins, cells, etc. (Donaldson *et al*., 2006; Kreyling *et al.*, 2004). The available data are consistent with the occurrence of a systemic inflammatory response and an alteration of autonomic cardiac control, but evidence on endothelial dysfunction, pro-coagulatory states, and particulate-related myocardial malfunction is as yet scarce. Further studies are therefore needed to substantiate our current understanding of the pathophysiological links between PM exposure and adverse cardiovascular outcomes (Mossman *et al*., 2007; Schulz *et al.*, 2005).

Exposure to graphite and carbon materials has been associated with increased incidence of skin diseases, such as carbon fibre dermatitis, hyperkeratosis, and naevi. These data indicate that dermal exposure to unrefined SWCNT may lead to dermal toxicity due to accelerated oxidative stress in the **skin** of exposed workers (Shvedova *et al.*, 2003)**.** The chances of penetration again depend on size and surface properties of the particles and strongly on the point of contact as well as of nature of particles (Allianz, 2005; Pflücker, 2001; Ryman-Rasmussen *et al*., 2007). NANODERM project is dedicated to clarify the problem of skin exposure (NanoDerm, 2007).

Recommendations / knowledge gaps:

- Investigation of lung function could be indirect measure for evaluation of health risk of nanoparticles but there still need wider research in this area to confirm air quality standards of nanoparticles and monitoring methods.

- Further studies are therefore needed to substantiate our current understanding of the pathophysiological links between PM exposure and adverse cardiovascular outcomes.
- Further epidemiological and experimental data collections are required to determine the critical physicochemical and toxicological properties of NP in humans.

3.1.5 Synergism or precipitation by other particles/compounds

Most airborne particulates were aggregates and agglomerates ranging in aerodynamic diameters from a few nanometres (nm) to a few microns (μm); containing as few as two nanocrystals to several thousand nanocrystals or nanoparticulates such as carbonaceous spherules arranged in complex branched homogeneous aggregates composing diesel exhaust, with spherule diameters ranging from 10 to 30 nm. The potential for ultrafine airborne aggregates to fragment into hundreds or thousands of nanoparticulate components in human airways and act as toxic agents in deep lung tissue is demonstrated by Murr *et al.* (2004a).

Carbon nanotubes and other aggregated fullerene-related multi-layer shell structures have been collected in propane and natural gas flame emissions from cooking stoves and observed by transmission electron microscopy: some aggregated NPs were mostly multi-walled nanotubes; many tangled and distorted and aggregated with other closed-concentric, multi-shell forms. Such clean-burning regimes may be major contributors to complex particulate matter in indoor and outdoor air (Bang *et al.*, 2004).

Engineered NPs have the strong tendency to aggregation or to agglomerate. The degree to which NPs aggregate or agglomerate in the occupational or ambient aerosol and subsequently do or do not deagglomerate following inhalation and particle deposition in the lung will strongly influence particle deposition rates and patterns as well as interactions with lung cells. If the NPs deagglomerate upon interaction with alveolar lung fluids at sites of particle deposition, than they could behave as discrete individual NPs. Alternatively, aggregated nanoparticle-types could behave as fine-sized particles. Inhalation studies with aggregated NPs cause more inflammation, but no general conclusions regarding nanoparticle toxicity in connection with agglomeration and deagglomeration especially for different kinds of NPs (Borm *et al*., 2006).

Recommendations / knowledge gaps:

Development of a model describing the dispersion and transformation of nanoparticles and their agglomerates in the working environment to assess location of maximum exposure and to develop safe workplaces.

3.1.6 Can we define (possible) biomarkers?

Impressive progress has been made in recent years when objectives changed from classical tests like lung function, etc to endpoints comprising of particle induced oxidative stress, cell signalling and activation, release of mediators initiating inflammatory processes not only in the respiratory tract but also in the cardio-vascular system in epidemiological studies indicated an association between adverse health effects and ambient ultrafine particle concentrations in susceptible individuals. Particularly, the large surface area of ultrafine particles provides a unique interface for catalytic reactions of surface-located agents with biological targets like proteins, cells,

etc (Kreyling *et al.*, 2004). The possible biomarkers of above mentioned health impact of NPs are included below with described use of them in few of studies.

The pulmonary health effects of ultrafine particles can be investigated by measurement of peak expiratory flow (PEF) and an increase of daily symptoms and medication use, the predicted forced expiratory volume in 1 second (FEV1.0) allow evaluate the association between the number of ultrafine particles and ventilatory function (Donaldson *et al.*, 2001b; Hauser *et al.*, 2001; Ibald-Mulli *et al.* 2002).

Researchers quantified pulmonary inflammatory response by bronchoalveolar lavage (BAL) cell and cytokine concentrations, pulmonary function, and peripheral blood neutrophil concentrations for evaluation of the impact after inhalation of fine and ultrafine metal oxides to exposed volunteers (Kuschner *et al.*, 1997). The cellular responses to particle exposure could be measured by the levels of IL-8 chemokines produced as a function of exposure time (Cheng *et al.*, 2003). Ultrafine particles (UFPs) are related to their uptake in macrophages and epithelial cells and their **ability** to induce oxidative stress. UFPs were most potent toward inducing cellular heme oxygenase-1 (HO-1) expression and depleting intracellular glutathione. HO-1 expression, a sensitive marker for oxidative stress, is directly correlated with the high organic carbon and polycyclic aromatic hydrocarbon (PAH) content of UFPs in case of diesel exhaust studies. The dithiothreitol (DTT) assay, a quantitative measure of in vitro reactive oxygen species (ROS) formation, was correlated with PAH content and HO-1 expression. UFPs also had the highest ROS activity in the DTT assay. Because the small size of UFPs allows better tissue penetration, authors used electron microscopy to study subcellular localization. UFPs localize in mitochondria, where they induce major structural damage. This may contribute to oxidative stress. The study demonstrates that the increased biological potency of UFPs is related to the content of redox cycling organic chemicals and their ability to damage mitochondria (Cheng *et al.*, 2003).

Nanotechnology encompasses an increasingly sophisticated ability to manipulate matter at the nanoscale, resulting in new materials, products and devices that demonstrate new and unusual behaviour. While emerging nanotechnologies have great potential for good, there are increasing concerns that the selfsame attributes that make them attractive will also lead to new risks to human health. People involved in making and using these materials need to know what the risks are and how to manage them, if safe nanotechnology-based businesses are to emerge. Maynard (2007) concluded in the last review that we currently know enough to suggest that some engineered nanomaterials will present new and unusual risks, but there is very little information on how these risks can be identified, assessed and controlled.

Dr. Frank Chen of Berkeley Lab has established biomarkers and specific gene expression patterns in response to various nanoparticles/nanomaterials in the biological pathways of inflammation, apoptosis, immune response, ubiquitination, cell proliferation, cell cycle regulation, cell differentiation, golgi vesicle transport, membrane fusion, secretory pathway, intracellular transport, nucleocytoplasmic transport, response to DNA damage, and response to stress and stimuli. His quantitative matrix includes the key factors that have been determined to contribute to nanomaterial-related cytotoxicology such as concentration, size/mass, shape, surface charge,

surface functionalization, and surface coating (http://www.lbl.gov/tt/techs/lbnl2419.html).

Recommendations / knowledge gaps:

- research have to be continued on the biomarkers for detection the impact of nanoparticles on health.

3.2 Exposure and detection in biological samples/fluids

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3.2.1 Detection limits (depending on technique used and on the material investigated)

Many nanomaterials are made of non-essential metals or transition metals. Thus, traces of these elements can be easily detected by modern analytical methods *ex vivo*. A live or real time observation of these materials is more difficult but still manageable. This changes totally when materials are made of biologically more relevant materials like carbon or iron. Despite the fact that some carbon modifications have distinct physicochemical properties (CNT – Raman shift), others are hard to detect but sampling is possible when air borne with filter collectors (Kuhlbusch *et al.* 2004). An exposure event in cells is therefore still not proven.

Tab. 10: Primary isotopes produced by nanopowder irradiation and detection limit estimate.

Nanoparticle	Tracer Isotope	Isotope Production Reaction	Isotope Half-life	Minimum Mass of Nanoparticles Detected in Experiment [µg]	Calculated Nanoparticle Detection Limit [µg]
Al_2O_3	^{24}Na	$^{27}Al(\gamma,^{3}He)^{24}Na$	14.95h	42	9.3
ZnO	^{67}Cu ^{65}Zn	$^{68}Zn(\gamma,p)^{67}Cu$ $^{66}Zn(\gamma,n)^{65}Zn$	2.58d 244d	46	4.1
TiO_2	^{46}Sc ^{47}Sc ^{48}Sc	$^{47}Ti(\gamma,p)^{46}Sc$ $^{48}Ti(\gamma,p)^{47}Sc$ $^{49}Ti(\gamma,p)^{48}Sc$	83.8d 3.35d 43.7h	23	0.3

3.2.2 Local exposure concentration lung, GIT, skin

Local exposure levels and intracorporal distribution change dramatically with alteration of the surface characteristics of nanomaterials. Nanomaterials present in sun screens for example have certainly the highest dermal concentration, decreasing steeply to GIT and lung. Whereas no penetration through the skin could be measured (NanoDerm, 2007) there might be some translocation from GIT or the lung into the body where they can later be found in other organs. Exposure at workplace will clearly not take place via the skin or GIT to a considerable amount. But inhalation and deposition in the lung does and is most likely to happen. Such exposure conditions must therefore be prevented and kept to a minimum.

It was found that for some nanomaterials independent on the site of exposure a biodistribution can occur and high organ specific levels arise.

3.2.3 Identification and quantification

As long as nanomaterials have a distinct appearance within an analytical system they can be identified (Kuhlbusch *et al.,* 2004). But therefore a reference material is needed. Currently there is no model available to determine deposition levels and therefore to quantify the real dose that is or can be administered to cells or living animals. As nanomaterials tend to agglomerate it is always very difficult to distinguish between administered and achieved dosage. Hence models and experiments overcoming this obstacle are needed.

3.2.4 Possible biomarkers

Several inflammation markers, e.g. members of the interleukin family, have been described to appear upon treatment with nanomaterials. Among these IL-6, IL-8 and IL-10 are the most prominent ones; but secondary inflammation markers like TNF-α and Iκb have also been reported (Brown *et al.,* 2000a; Ernst *et al.,* 2002; Nemmar *et al.,* 2003; Peters *et al.,* 2004; Renwick *et al.,* 2004; Tran *et al.,* 2000).

Coherent with the high catalytic surface, often described for nanomaterials it is reasonable that oxidative stress (Beck-Speier *et al.,* 2005; Brown *et al.,* 2001; Diabaté *et al.,* 2004; Manna *et al.,* 2005; Oberdörster *et al.*, 2006; Stone *et al.,* 2000) has also been reported. These free oxygen radicals produced by either nanomaterials themselves or induced in the biological samples are often related to DNA damage and the onset of cancer and tumours. On the other hand, nanomaterials can also have a more anti-oxidative nature (Lin *et al.,* 1999). DNA damage (Gallagher *et al.,* 2003; Greim *et al.,* 2001; Schins, 2002; Schwerdtle *et al.,* 2002) as mentioned before has not been directly linked to an interaction of DNA with nanomaterials but is rather a secondary effect from the earlier mentioned oxidative stress caused by many metallic or metal oxide materials. Investigations on direct interaction between nanoscale materials and DNA are still missing.

Lack of knowledge:

- There are no (standardised) methods to determine NPs within body fluids or tissue
- The limited number of measurements at workplaces gave no reason for concern as no NP could be found in the air, but more measurements are needed
- No thresholds are so far defined

4 Environment

Authors: Victoria Hand, David Vaughan

Earth materials with at least one dimension in the nanoscale are essentially ubiquitous. They have been studied for several decades and more are being discovered all the time (Hochella, 2002a). Our ability to synthesize and manipulate engineered NPs, and the worldwide increase in investment in nanotechnology research and development, has brought NPs to our attention (Nowack and Bucheli, 2007). There is already a large body of information concerning the physical and biological properties of naturally occurring NPs, and analogies for potential exposure to pollutant “engineered” nanoparticles may be drawn from results of studies on naturally occurring NPs, for

example exposure to mineral dusts such as quartz or asbestos. In reality, we are already exposed to the ultrafine particles that are present in the environment on a daily basis; for example, particulates from diesel exhausts. Indeed, it has been suggested that we may have more to fear from the NPs we encounter on a daily basis than from new ones arising from the potentially cleaner nanotechnologies of the future (Oberdörster, 2004).

4.1 Natural Nanoparticles in Geological Systems

Recent work in the geosciences has shown that many geochemical processes are governed by phenomena at the nanometer scale, often involving nanoscale particles. Nearly all processes of weathering, soil formation and water/rock interaction are linked to nanoscience; at the Earth's near-surface, materials are often in the nanoscale regime (Hochella, 2002b). Because processes are intrinsically molecular at the nanoscale, there is a diffuse boundary between geosciences and the fields of chemistry, physics, and materials science, and also life sciences as microbial processes often proceed by manipulating surface forces at the nanoscale. Consequently, geoscientists have unique skills in aqueous and solid state chemistry, particularly in multicomponent systems.

Much of the chemistry in the shallow Earth occurs at disequilibrium and minerals form and dissolve in large solubility gradients. These gradients are caused by bacterial metabolism, by the transition from oxygen-rich to anoxic environments in sediments, by the large temperature and pressure changes found in hydrothermal environments and in settings where pollution causes sharp changes in pH and metal concentrations. Metal transport in the environment is often of great environmental concern especially when related to radioactive waste disposal, acid mine drainage and industrial pollution. A few scientists have realised the importance of the transport of species complexed in or on organic (biopolymers, humic substances, etc.) and inorganic (mineral) NPs and not as dissolved entities (e.g. Boult *et al.*, 2006; Buffle and van Leeuwen 1992; Grolimund *et al.,* 1996).

Good examples of nanoparticle metal transport in the environment occur in acid mine drainage (AMD) systems. The weathering of metal sulfide minerals in hydrated, oxygenated surface environments results in a highly acidic effluent. At low pH metals can be transported in the aqueous phase, but AMD waters increase in pH the further they travel from the source. This is due to dilution with uncontaminated water and buffering reactions in the streambed or aquifer. Precipitating phases, such as iron-dominated oxides, oxyhydroxides, and/or hydroxysulfates result in iron-rich sediments and mineral/rock coatings that may contain high concentrations of toxic metals trapped in their structures or attached to their surfaces. These toxic metals can be further transported over long distances in a relatively short space of time. Hochella *et al.* (1999) observed the process by which this occurs due to the formation of nanocrystalline Fe-oxyhydroxides with toxic metals sorbed to their surfaces, and also the formation of toxic metal-oxides directly in the nanometer size range (Hochella *et al.*, 1999). Suzuki *et al.* (2002) showed that direct microbial reduction of U^{6+} to U^{4+} can result in the formation of nanoparticulate uraninite (UO_2). Such particles are potentially extremely mobile and are likely to have a much higher solubility than bulk uraninite. This has obvious implications for the mobility of uranium since its precipitation as

insoluble uraninite does not necessarily make it immobile. Furthermore, nanocrystals of uraninite (5-10 nm in diameter) encapsulated in carbonaceous matter with a structure similar to fullerene (about 50 nm in diameter) were identified in aerosols collected in Detroit (Utsunomiya *et al.* 2002). A further investigation by Utsunomiya *et al.*(2004) detected several heavy metals (including some that are toxic e.g. As, Cr, Pb, Se) in particles ranging down to a few nanometres in size. The inflammatory potential of these NPs in lung tissue is expected to be high. Their chemical toxicity may be underestimated if, due to their size, they show enhanced dissolution or exchange reactivity (Hochella and Madden, 2005).

These fine-grained materials are very small and represent only a small fraction of the mass of the material on Earth. However, they do represent a large fraction of the particles in atmospheric and aqueous environments and account for most of the exposed surface area of Earth materials. It has become apparent that the kinetics and mechanisms of sorption of aqueous species onto nanocrystalline particles can be significantly different compared to the same sorbate attached to larger particles (Zhang *et al.,* 1999). Because chemical reactions generally occur at surfaces and interfaces rather than in the bulk, chemical reactions in both natural and laboratory systems disproportionately involve NPs.

The large surface-to-volume ratio of nanoscale particles ensures that surface forces exert considerable influence over the chemistry and structure of NPs and nanomaterials in general, to the point that they exhibit properties that are distinct from those of the macroscopic solid (NSF, 2002). The distortion of metal surfaces sites as particles get smaller has important implications for metal binding and electron transfer in associated redox reactions (Hochella and Madden, 2005). Valden *et al.* (1998) investigated the use of gold catalysts on titania substrates to oxidize CO to CO_2. They demonstrated that the gold catalysts were most active at driving the oxidation reaction at 2-3 nm in diameter. At sizes larger than this, their reactivity drops off considerably.

Hochella (2002a) provides an overview of how diversely nanoscience has impacted the geological sciences in the last few years. These examples range from minerals in the troposphere, including nanominerals, with implications for radiative forcing effects and consequently global heating/cooling (Buseck *et al.*, 2000), to carbonaceous nanofilms in many crystalline rocks of deep crustal and mantle origin, with implications for the relatively high electrical conductivity of these rocks (Anastasio and Martin, 2001). An extensive discussion of NPs in the environment can be found in Banfield and Navrotsky (2001). The book centres primarily on the effects of particle size on particle properties and reactivity with detailed coverage of recent nanoparticle systems of interest such as TiO_2, ZnS and also the mineralogical species in AMD.

The study of nanosized particles in geological systems continues to yield important results and contributing to developing fundamental principles and the experimental techniques used are becoming more sophisticated all the time. The use of techniques such as X-ray diffraction and X-ray absorption spectroscopy and electron and scanning probe microscopies (SEM, TEM, STM, AFM) provide a powerful combination of methods to observe and characterise nanoscale materials and processes. There is still a great need for additional tools with greater versatility and instrumental physicists are continually refining established techniques and inventing new ones that will become important in nanomaterial characterization (Hochella, 2002a).

It is only relatively recently that more detailed observations of natural atmospheric nanoparticulates and nanocrystals have been made. Carbon nanotubes and other fullerene-related nanocrystals have recently been reported as being ubiquitous in the Earth's atmosphere, and have even been observed in a 10,000 year-old ice-core sample, indicating their occurrence in antiquity, probably as natural gas/methane combustion products (Murr *et al.,* 2004b). The results from this study begin to establish an environmental context for considering the potential impact of engineered nanostructured particles on human health and the environment.

4.2 Nanotechnology and the Environment

The manufacture of engineered NPs is a rapidly expanding industry as their properties are exploited with the continuing development of nanotechnology. The novel properties of nanomaterials means they are likely to have distinctive transport and accumulation behaviour. As well as concerns about the negative impact these materials might have on health that have already been outlined, there are also concerns about the potential negative impact of engineered NPs on the natural environment.

Many health and environmental concerns are related to exposure to NPs that are free, rather than fixed to or within a material. Manufactured NPs are mostly fixed in materials (e.g. composites) although these fixed particles may become free in the environment due to damage, recycling and degradation following disposal. Exposure to free manufactured NPs is mostly limited to workplaces of manufacture and research, and to a small number of cosmetic uses, such as in sunscreens. However, there has been no research into the life cycles of products containing engineered NPs and the possible exposure risk from their potential to release free NPs. Assessing the risks of ENP's in the environment requires an understanding of their mobility, reactivity, ecotoxicity and persistency (Nowack and Bucheli, 2007). There is a requirement for life cycle analysis of products containing engineered NPs in order to determine the likelihood of this happening. This should consider the processes and materials used in manufacture, the likely interactions between the product and individuals or the environment during its manufacture and useful life, and the methods used in its eventual disposal (Royal Society Report, 2004).

4.2.1 Binding of toxic elements and compounds to nanomaterials

The high surface area of NPs maximizes any chemical interactions with the environment, and could make such particles less mobile in groundwater systems due to increased interaction with porous media, therefore slowing transport. On the other hand, like naturally occurring colloids, high surface area may lead to significant adsorption of molecular contaminants leading to concentration of contaminant molecules and an avenue for long-range contaminant migration (Colvin, 2004). Like naturally occurring colloids, engineered NPs may provide an avenue for rapid and long-range transport of waste/contaminants in groundwater. Conversely, binding to engineered NPs might neutralise contaminants, reducing the harm they cause (Kleiner and Hogan, 2003).

Probably the greatest potential source is from the proposed introduction of NPs into soils or waters for remediation, for soil stabilisation, or to deliver fertilisers. There are many sites that are contaminated with chemicals and heavy metals, and the potential for nanotechnologies to contribute to remediation is large. However, very little is known

about the behaviour of engineered NPs in air, water and soil and what impact the high surface reactivity of NPs might have on plants, animals, micro-organisms and ecosystem processes. Another potential source is the waste streams from factories and research laboratories.

There are a wide variety of routes by which NPs may reach humans and other organisms; for example, organisms may ingest materials that have entered a water system or that have been deposited on vegetation. Once materials have been inhaled or ingested, they may enter the food chain, leading to the possibility of bioaccumulation and then ingestion by organisms further up the food chain. The bioaccumulation of NPs will depend on their surface properties, which will determine whether they are likely to be taken up by the fatty tissue, bone or proteins in the body. Low aqueous solubility generally favours the persistence of a chemical in the environment and its uptake by biological systems, where it can persist for long periods of time and even bioaccumulate, as has been shown for DDT or dioxins (Hoet *et al.*, 2004b).

4.2.2 Mobility of nanoparticles within the environment

It is probable that environmental processes such as bioaccumulation, biodegradation, fate and transport will affect the concentration and form of engineered NPs that are exposed to the environment. It is not yet known whether engineered NPs can be converted to aerosol by routine handling of powders and liquids; however, rapid and irreversible aggregation of engineered NPs in air may significantly increase size and thus limit the possibilities of inhalation of isolated NPs (Colvin 2004). Research from the field of air pollution provides compelling evidence of the severe health effects of atmospheric NPs and it is well established that exposure to ultrafine particles (UFPs) should be avoided (Oberdörster *et al.* 1995). With cosmetic uses, dermal exposure is already occurring, and the range of NPs used in such applications is likely to increase.

Fortner *et al.* (2005) are currently conducting studies on how C_{60} (fullerene) affects bacteria and simple organisms like worms. They are also exploring whether these fullerenes tend to move up the food chain. Initial results show that NPs accumulate in living cells over time, with ever-increasing concentrations in microbes, in the worms that eat those microbes, and in animals higher up the food chain. It is possible that these NPs reach humans (Fortner *et al.,* 2005). NPs have been shown to inhibit the motility and phagocytosis of macrophages in the lungs, which suggests that similar effects might be expected in simple soil organisms (Lam *et al.*, 2004). Lovern and Klaper (2006) recently found that exposure of *Daphnia magna* to filtered C_{60} and filtered TiO_2 caused an increase in mortality with increasing concentration.

Surface modification of engineered NPs has succeeded in making them soluble in water, and thus of use in drug delivery and other biomedical applications. The absence or presence of surface coatings on nanomaterials complicates their toxicity. It is unknown how long surface coatings are retained on particles. The likelihood of coating breakdown has been studied in cell culture systems, where quantum dots were initially rendered non-toxic with coatings but, if the quantum dots were exposed either to air or UV radiation for as little as 30 minutes, they became very destructive to living cells (Derfus *et al.,* 2004). The same effect has been seen with fullerenes, and generally, it is not known how long such coatings are retained on particles (Oberdörster, 2004). It is important to note that most NPs in technical applications are functionalised and

therefore studies using pristine nanoparticles may not be relevant for assessing the behaviour of the NPs actually used (Nowack and Bucheli, 2007). Considering that surface modification is the fastest growing area of nanoparticle technology, the effects of these modifications on the toxicology of NPs should be investigated (Donaldson *et al.,* 2004b).

There are several ongoing studies evaluating fullerene toxicity in aqueous systems. Due to the possibility of C_{60} solubilisation through colloid formation under environmental conditions, many studies have focused on the effects of n-C_{60} (highly stable colloidal aggregates of C_{60}). The possibility of n-C_{60} formation following extended contact with water suggests that n-C_{60} could be a significant form of C_{60} if these fullerenes were introduced to aquatic systems. The first interest attracting published work on n-C_{60} toxicity to organisms concluded that n-C_{60} produced oxidative damage in the brains of exposed largemouth bass (Oberdörster, 2004). Nevertheless, the same group has to correct these results as the tetrahydrofuran (THF) solubilised fullerenes are toxic through the peroxides coming from the solvent THF (Oberdörster *et al.*, 2006; see comment page 38).

The tendency of n-C_{60} to aggregate and deposit will play a key role in determining its longevity in aquatic systems and, therefore, provide key information on the exposure risk presented by these colloids. In one case, it was shown that hydrophobic contaminants can irreversibly interact with fullerene aggregates in water, and these species showed a high capacity for concentrating a model aromatic hydrocarbon (Cheng *et al.,* 2004).

The mobility of eight particulate products of nanochemistry in a well-defined porous medium were evaluated by Lecoanet *et al.* (2004) to assess their potential for migration in porous media, such as groundwater aquifers and water treatment plant filters. They found that the particles exhibited widely differing transport behaviour. Their results showed that nanomaterials exhibit widely differing transport behaviours. They suggest that the potential for exposure to n-C_{60} through groundwater transport may be less than that of other fullerenes. Observations made by Brant *et al.* (2005) suggest that, under some conditions present in natural aquatic systems, these materials have limited mobility as they form large aggregates that may settle out of suspension or deposit on surfaces. These phenomena may, at least partially, offset any risk presented by n-C_{60} toxicity due to a reduced potential for exposure (Brant *et al.,* 2005). Such investigations will increase understanding of the potential uses of such NPs to clean-up groundwater pollution, as well as aiding in the assessment of any environmental risks the materials may present.

More recent data have shown for the first time how multiwalled carbon nanotubes might behave in natural aquatic environments (Hyung *et al.,* 2007). This research suggests that natural organic matter (NOM) present in river water could aid the dispersion of carbon nanotubes by stabilizing the nanotubes and increasing their potential for dispersal dramatically. In fact, their experiments showed that natural organic matter stabilizes the model carbon nanotube in the aqueous phase more efficiently than a surfactant. They also found that the nanotubes remain as discrete units. However, the toxicity of the new materials in natural environments remains relatively unknown. The paper on the occurrence, behaviour and effects of NPs in the environment by Nowack and Bucheli (2007) is a comprehensive and useful review of this area.

4.3 Conclusions and Future Research

NPs have different levels of interaction with biological systems and have different mobilities based on their size, shape and chemical composition. Therefore, it is not possible to address the hazards and risks of NPs in a general way as each type of nanoparticle needs to be evaluated as regards its toxicity. There is not enough research on engineered NPs to know whether they present a serious problem to human health and the environment. There is a strong need to prevent a backlash of negative public opinion, and a political and regulatory backlash, which may have an effect on the development of the field. The nanotechnology industry is keen to accumulate risk data so as to answer questions and address problems early, and so that nanotechnology can flourish responsibly and with public support. It is also important to remember the positive impacts of the improved environmental technologies that are developing from new nanotechnologies. There is considerable promise not only for removing persistent pollutants from soil and water supplies, but also for improving the efficiency of energy production using nanostructured catalysts and energy storage capabilities reducing waste production, which will benefit the environment and increase sustainability (Dror *et al.,* 2005; Masciangioli and Zhang, 2003).

More research into the hazards and exposure pathways of NPs and nanotubes is required to reduce the many uncertainties and knowledge gaps related to their potential impact on health, safety and the environment. Current funding and hence research is inadequate. Zhang (2003) suggests that more attention should be directed to the fundamentals of nanochemistry in the environment, such as the process of contaminant transformation at the nanoparticle-water interface. An interdisciplinary approach is necessary for an appropriate risk assessment. There are many opportunities for collaboration between the different centres of expertise in nanotechnology, environmental science, pharmaceutical science and toxicology within the European Community. It is important to appreciate that environmental scientists and engineers already investigate nanostructures and nanoscale systems, as in studies of the natural weathering of minerals or the production of nanoscale colloids by microorganisms that are important in the fate, transport and transformation of potentially toxic substances (Masciangioli and Zhang, 2003).

The report produced by the Royal Society and Royal Academy of Engineering in 2004 recommends that, until more is known about the environmental impact of NPs, their release into the environment should be avoided as far as possible. They also recommend that NPs should be treated as hazardous and be reduced in waste streams, and that the use of free NPs in environmental applications such as remediation of groundwater be prohibited (Royal Society Report, 2004).

Knowledge gaps/recommendations

- We need to understand what happens to nanomaterials during their journey from manufacture to waste disposal. This can help focus studies that can tell us about transport pathways, biogeochemical cycling and environmental fate. Ultimately, such work will help us to identify which, if any, environmental compartments are at risk of contamination by nanomaterials.
- There is a paucity of information in a number of areas that are fundamental to the development of detailed guidelines on the risk assessment of nanoparticles.

These include nanoparticle characterisation, the detection and measurement of nanoparticles, the dose-response, fate, and persistence of nanoparticles in humans and in the environment, and all aspects of toxicology and environmental toxicology related to nanoparticles.

- Significant efforts are required in order to collect the huge amount of data required in order to confirm the risk assessment of nanomaterials.
- A standardised framework for the risk assessment of nanomaterials such as standard reference samples and toxicology protocols should be settled quickly.

5 Law and Regulation

Author: Jan Stetkiewicz

Rules for marketing of dangerous substances and preparations are established within the framework of total harmonisation of national legislations.

Up to now the system distinguished between «existing» substances i.e. substances declared to be on the European market prior to September 1981, and “new” substances i.e. those introduced since this date. Testing “new” substances and assessing their risks to human health and the environment, according to Council Directive 67/548/EEC (1967), Some general frameworks are the Existing Substances Regulation (Council Regulation (EEC) No. 793/93, 1993) on the evaluation and control of the risks of existing substances and the Dangerous Substances Directive (Council Directive 67/548/EEC, 1967), covering classification and labelling of substances in general and, through its amendments, providing for a pre-marketing notification system for New Chemicals. Risk management, including request for information and risk reduction measures, may be triggered by e.g. classification of substances and risk assessments.

The Council Directive 67/548/EEC (1967) requires all new substances introduced on the market in a volume of 10 kg/year or more to be notified. The purpose is to provide information on chemicals, to allow for classification and labelling and safe handling and use. The Directive contains information requirements for different tonnage intervals (tiered testing) in the Annexes. Full notification dossiers are required for substances produced in volumes from 1 ton per year, and allow for risk assessment to be performed, followed by risk reduction strategies where necessary.

Council Regulation (EEC) No 793/93 (1993) on the evaluation and control of the risks of existing substances involves the data reporting, priority setting, risk evaluation and, where necessary, development of strategies for limiting the risks of existing substances. The regulation obliged industry to report data (for production volumes above 10 tons/year) and update information on significantly new uses. Priority lists have been published for substances to go through a Community risk assessment and risk reduction strategy are developed where appropriate. The regulation can also require industry to provide additional testing or information if data is lacking. A prerequisite is that the substance is on a priority list or for any other EINECS substance, that there is a valid reason for believing that the substance may present a serious risk to man or the environment.

Classification and labelling criteria have been laid down in Annex VI of Council Directive 67/548/EEC (1967). Guidance for risk assessment of new and existing substances as well as biocides has been provided in the Technical Guidance Documents (TGD), and a computerised risk assessment tool, the EU System for Evaluation of Substances (EUSES) is used. Furthermore guidance for development of Risk Reduction Strategies is available. It needs to be investigated if and how the current legislation and implementation tools for new and existing chemicals may be applied to ensure that possible risks of NPs are adequately addressed at an early stage.

The current legislative framework has been now replaced by REACH, the Regulation concerning the Registration, Evaluation, Authorisation and Restrictions of Chemicals (Regulation No 1907, 2006). The provisions of REACH shall apply to the manufacture, import, placing on the market or use of substances on their own, in preparations or in articles, if so stated. REACH abolishes the distinction between «existing» and «new» substances and establishes a single legislative system for the marketing of chemical substances in Europe. It replaces Council Regulation 793/93 (1993) as well as Council Directive 76/769 (1976). The existing restrictions will remain in force and will be listed in an annex to the REACH Regulation. Council Directives 67/548/EEC (1967) and 1999/45/EC (1999) (dangerous preparations) will be amended. The provisions related to the safety data sheets (Directive 91/155/EEC amended by Directive 93/112/EEC and Commission Directive 2001/58/EC, 2001) were incorporated into the REACH Regulation.

Any substance that is produced or imported in annual volumes of at least 1 ton/year and manufacturer has to be registered. If the annual volume is > 10 tonnes, mandatory preparation of a chemical safety report (CSR) which contains chemical safety assessments (CSA) for each identified use is required. A CSR shall include the following steps in accordance with the respective sections of this Annex: human health hazard assessment, human health hazard assessment of physicochemical properties, environmental hazard assessment, PBT (Persistent, Bioaccumulative, Toxic) and vPvB (very Persistent and very Bioaccumulative) assessment. If as a result of these steps the manufacturer or importer concludes that the substance or the preparation meets the criteria for classification as dangerous according to Directive 67/548/EEC (1967) or Directive 1999/45/EC (1999) or is assessed to be a PBT or vPvB, the CSR shall also consider the following steps: exposure assessment and risk characterisation. The main element of the exposure part of the CSR is the description of the manufacturer's or importer's exposure scenario(s) and the exposure scenario(s) recommended by the manufacturer or importer to be implemented for the identified use(s). The exposure scenarios contain a description of the risk management measures which the manufacturer or importer has implemented and recommends to be implemented by downstream users. If the substance is placed on the market, these exposure scenarios including the risk management measures shall be summarised in an annex to the safety data sheet in accordance with Annex IA. NPs having different hazard properties than the "bulk" substance would require individual treatment under REACH.

The European Commission has adopted a so-called “incremental approach”, which focuses on adapting existing laws to regulate nanotechnologies, and therefore this paper aims to test the effectiveness of the “incremental approach” (Franco *et al.,* 2007)

[7]. The authors on the example of three commercially available products containing fullerenes (C_{60} and carbon nanotubes) were analysed in a life cycle perspective in order to (1) map current applicable regulations, (2) analyse their applicability to nanomaterials, (3) identify their gaps, and (4) suggest proper solutions.

After mapping the life cycle of the three products, Franco *et al.* (2007) analysed applicable regulations in the order in which they became relevant in their life cycle, i.e.:

- The Safety at Workplace Directives
- Council Directive 96/61 (1996) on the Integrated Pollution Prevention and Control
- The European Union's Directive on the Registration, Evaluation, Authorization and Restriction of Chemicals (Regulation No 1907, 2006)
- The Waste Management Directives: Council Directive 2006/12/EC (2006), Council Directive 91/689/EEC (1991), Council Directive 75/439/EEC (1975), Council Directive 2000/53/EC (2000).

It was found that the applicability of environmental laws is limited due to difficulties in generating sufficient data on the nanomaterials residing in the products according to their life cycles. Authors pointed out that metrology tools are unavailable; thresholds are not tailored to the nanoscale; and toxicological data and occupational exposure limits cannot be established with existing methodologies. Conclusion of this paper is that the "incremental approach" can only be applicable with the implementation of due amendments.

5.1 Protection of workers' health and safety against risks due to chemicals

European rules concerning the protection of workers' health and safety aim at minimum harmonisation of the different Member States' legislations; the Member States are therefore entitled to impose national rules more stringent than the European ones if they see fit to do so.

The most important piece of legislation in the area of health and safety at work is the Framework Council Directive 89/391/EEC (1989) "on the introduction of measures to encourage improvements in the safety and health of workers" to ensure a higher degree of protection of workers at work.

The model for health and safety management in the Framework Directive places prevention in a central position. Equally important are the provisions regulating the obligations of the employers for planning, organising and regulating the protection of workers at work. The employer is obliged to make an a priori overall risk assessment and to undertake measures to prevent occupational risks; in the first place to combat risks at source either by eliminating/avoiding or, if not possible, by taking the appropriate control measures in order to reduce them (e.g. selecting personal protective equipment (Council Directive 89/656/EEC,1989).

In the case of nanotechnologies the risks are neither known nor predictable; further research should be carried out in order to evaluate the risks they could entail to the health and safety of the workers. Methods for risk assessment in relation to worker protection need to be developed and made available to the employers. In addition to

the Framework Council Directive 89/391/EEC (1989), there are two other Directives that could be applied:

1) Council Directive 98/24/EC (1998) on the protection of the health and safety of workers from the risks related to chemical agents at work and

2) in case any nanoparticle would be shown carcinogenic or mutagenic, Council Directive 2004/37/EC (2004) on the protection of workers from the risks related to exposure to carcinogens or mutagens at work, could be applicable.

5.2 ISO Technical Committee 229

Standard terminology and a standard nomenclature system are essential for the development of an effective regulatory framework. Precise identification of nanomaterials is needed in order to determine the appropriate regulatory track and ensure any appropriate risk management.

Technical Committee 229 (TC 229) of ISO was formed in 2005 to address standardization in the field of nanotechnologies. Within TC 229, three working groups are convened by different countries to address specific subject matters.

Terminology and Nomenclature

Measurement and Characterization

Health, Safety and Environment

Under the TG 229 following standards are projected:

- Nanotechnologies -- Outline of nanomaterials classification (Nano tree)
- Terminology and definitions for carbon nanomaterials
- Nanotechnologies - Terminology and definitions for nanoparticles
- Nanotechnologies -- Endotoxin test on nanomaterial samples for in vitro systems
- (EDXA) in the charaterization of single walled carbon nanotubes (SWCNTs)
- Nanotechnologies -- Generation of silver nanoparticles for inhalation toxicity testing
- Nanotechnologies -- Monitoring silver nanoparticles in inhalation exposure chambers for inhalation toxicity testing
- Nanotechnologies -- Use of Raman spectroscopy in the characterization of single-walled carbon nanotubes (SWCNTs)
- Nanotechnologies -- Use of evolved gas analysis-gas chromatograph mass spectrometry (EGA-GCMS) in the characterization of single-walled carbon nanotubes (SWCNTs)
- Nanotechnologies -- Use of thermo gravimetric analysis (TGA) in the purity evaluation of
- single-walled carbon nanotubes (SWCNT)
- Nanotubes -- Use of transmission electron microscopy (TEM) in walled carbon nanotubes (SWCNTs)

- Measurement methods for the characterization of multi-walled carbon nanotubes (MWCNTs)
- Nanotubes -- Scanning electron microscopy (SEM) and energy dispersive X-ray analysis
- Nanotubes -- Use of NIR-Photoluminescence (NIR-PL) Spectroscopy in the characterization of single-walled carbon nanotubes (SWCNTs)
- Nanotubes - Use of UV-Vis-NIR absorption spectroscopy in the characterization of single-walled carbon nanotubes (SWCNTs)

6 Knowledge gaps and recommendations

We recommend the following physicochemical properties to be published together with toxicological data in series of importance:

- Chemical composition – including spatially averaged (bulk) and spatially resolved heterogeneous composition (incl. contaminations)
- Size and size distribution
- Specific surface area
- Shape / aspect ratio
- Surface chemistry
- Agglomeration state

Additionally of importance in specific cases:

- Crystal structure
- Surface charge
- Porosity

The following knowledge gaps have been identified:

1. Regarding nanocrystalline materials:
   - Particle size alone is not a good criteria for differentiating between more or less hazardous materials
   - Some specific characteristics of nanomaterials will necessitate new test strategies to determine the mechanisms of potential injury that they may cause
   - Integration of theory, modelling, and simulation into experimental design
   - Information about surface energy, reactivity and biological activity is needed
   - No or little information regarding the fundamental understanding of the reaction specificity of nanoparticles in solution is available
   - A deeper understanding of the differences between nano and bulk, based on experimental and theoretical work, including quantum, surface and size effects, is needed
   - The consequences of agglomeration and deagglomeration of the particles are unclear
   - An evaluation of current test methods for "normal" toxicity with respect to their applicability for nanotoxicological testing is required, if necessary new dedicated test methods must be developed
2. Regarding dosing of nanomaterials or nanoparticles in biological experiments:
   - Which information is enough to clearly define a given dose or concentration within an experiment:
     Should we refer to mass, surface area or particle number?
   - Should sedimentation or deposition play a role for considerations regarding the actual dose which reaches the cells in an *in vitro* experiment?
   - Is information needed at the end of an *in vitro* experiment about the amount of material still measurable within the supernatant for better calculation of the reactivity of the investigated nanomaterial?
   - What is the minimum knowledge about dose or concentration for a reader of a study to interpret the data properly?

- Should we have guidelines for *in vitro* experiments for dosing nanomaterials (e.g. SOPs)?
- What about *in vivo* experiments and dosing?
- We (toxicologists) need a sensitive and cheap method to determine particle number and surface to include this information into the discussion of the results

3. Need for techniques for the *in vivo* detection of nanomaterials:

   - Inexpensive real-time monitoring instruments and methods for aerosol mass concentration (low concentrations, nanoscale particles), surface area concentration and size distribution
   - Standardized, well characterized nanomaterial samples.
   - Radio-labelled nanomaterial samples or samples that can be tracked and detected through neutron-activation
   - More advanced surface chemistry characterization techniques, in particular techniques capable of detecting and speciating biological molecules on the surface of nanoparticles and nanomaterials
   - Electron microscopy techniques for biologically-relevant nanoscale analysis

4. Regarding administration of nanomaterials to test systems:

   - There is a lack of international consensus on measurement techniques or standards for monitoring nanoparticles in the workplace (aerosol measurements)
   - There is a strong need for a measurement device that can differentiate between engineered nanoparticles and the background level of natural nanoparticles (species discrimination)
   - There is a need for the evaluation of nanoparticle formulations on a case-by-case basis in order to choose the most appropriate metrics

5. Regarding low-dose/low-concentration experiments:

   - Methods, combinations and strategies to provide knowledge about real-world exposure to different classes of nanoparticles and/or nanoparticle aerosols
   - Methods to evaluate dermal and/or ingestion exposure
   - No or little information available on the biokinetics of NP in order to estimate appropriate doses at their target site (that even have to be identified)

6. It is highly recommended to establish long-term strategies:

   - *In vivo* long-term toxicity assays for inhalation, oral, skin and injection exposure with the evaluation of markers for inflammation, oxidative stress, cell proliferation in portal-of-entry and selected remote organs, like liver, spleen, bone marrow, heart, kidney, CNS
   - Chronic nanotoxicity assays, especially the development and validation of non-animal test methods
   - Reproductive and immunotoxicity studies
   - Cardiovascular and coagulation tests mainly for those NPs for which translocation to the blood circulation, after e.g. inhalation, has been shown
   - Studies on deposition, translocation and biopersistence (degradation, metabolism) of NPs
   - Validation of results of high dose studies using order of magnitude lower concentrations resembling those realistic *in vivo* exposures, for quantum

dots, fullerenes, metal oxides and other NPs. For inhalation a 2 week exposure is recommended in multiple doses (1-10 µg/kg body weight), e.g. dosing daily for 4 weeks with 3 months follow up

7. Knowledge gaps regarding the workplace scenario:
    - Information about production volume, occupational exposure potential of nano-sized material is urgently needed to determine potential risk of employees
    - The workplace represents a critical interface between people and nanotechnology, and an area where potential impact needs to be understood and managed. In the future, it is also likely that even wider distribution of these particles may have significant effects on organisms
    - The potential effects of engineered nanoparticles on the ecosystem must also be considered in order to assure the safety of humans and the environment from production emissions
    - Development of innovative technologies for effective health and safety management in workplaces
    - Studying the potential release of nanoparticles throughout the application life cycle including recycling process workplaces and emissions in environment
8. Knowledge gaps regarding sampling and “real-life” measurements:
    - There is not one sampling method that can be used to characterize the traditional inhalation route of occupational exposure to NPs therefore research should be in progress to determine most appropriate of three exposure metrics (mass, surface and numbers) to be monitored as well as realistic and suitable sampling equipment
    - Generally accepted, realistic methods for exposure assessment are still lacking for workplaces; no commercially available personal samplers are designed to measure the particle number, surface area, or mass concentration of nanometre aerosols
    - There are lacks of the occupational exposure limits for nanoparticles (different type) and no standardized, well-characterized reference standards of nanoparticles are developed
    - Skin absorption of nanoparticles has to be evaluated as acceptable or unnecessary method for detection of summarized exposure
    - Research has to be continued on skin absorption of different compositions of nanoparticles (especially lipophilic ones) as way of occupational exposure and for development of safety measures
9. It is recommended to improve and adapt measurement systems:
    - Investigation of lung function could be indirect measure for evaluation of health risk of nanoparticles but there still need wider research in this area to confirm air quality standards of nanoparticles and monitoring methods
    - Further studies are therefore needed to substantiate our current understanding of the pathophysiological links between NP exposure and adverse cardiovascular outcomes
    - Further epidemiological and experimental data collections are required to determine the critical physicochemical and toxicological properties of NP in humans

- Development of a model describing the dispersion and transformation of nanoparticles and their agglomerates in the working environment to assess location of maximum exposure and to develop safe workplaces
- Research is needed to possibly establish biomarkers for detection of the impact of nanoparticles on health

10. There is still a lack of knowledge on exposure limits and behaviour in biological systems:
    - There are no (standardised) methods to determine NPs within body fluids or tissue
    - The limited number of measurements at workplaces gave no reason for concern as no NP could be found in the air, but more measurements are needed
    - No thresholds are so far defined
11. There are knowledge gaps regarding environmental aspects of NPs
    - We need to understand the behaviour of nanomaterials during their journey from manufacture to waste disposal. This can help to increase knowledge on transport pathways, biogeochemical cycling and environmental fate. Ultimately, such work will help us to identify which, if any, environmental compartments are at risk of contamination by nanomaterials.
    - There is a paucity of information in a number of areas that are fundamental to the development of detailed guidelines on the risk assessment of nanoparticles. These include nanoparticle characterisation, the detection and measurement of nanoparticles, the dose-response, fate, and persistence of nanoparticles in humans and in the environment, and all aspects of toxicology and ecotoxicology related to nanoparticles.
    - Significant efforts are required in order to collect the huge amount of data required in order to confirm the risk assessment of nanomaterials.
    - A standardised framework for the risk assessment of nanomaterials such as standard reference samples and (eco)toxicology protocols should be settled quickly.

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