European Commission

ENPRA Report Summary

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Final Report Summary - ENPRA (Risk assessment of engineered nanoparticles)

Executive Summary:

Aim and Objectives
Engineered Nanoparticles (ENP) are increasingly used in a wide range of industrial and consumer products. It is known that exposure to some types of particles can cause health effects. Therefore it is essential to ascertain whether exposure to ENP can lead to possible health risks for workers and consumers. The aim of the project was to develop an approach for the Risk Assessment of ENP (ENPRA).

Project Context and Objectives:
1. Description of project context and objectives
Nanotechnology is one of the most important new technologies of the 21st Century, with the global market for these products expected to grow to over 3 trillion by 2015. These technologies promise new very small (nanometer-sized) powder materials for industrial applications that have different or enhanced physico-chemical properties comparison to powders with larger (micrometer-sized) particles. As with all technological applications, there is some potential for exposure of humans and the environment to these nano-materials. Through their life-cycle, from development of new engineered nanoparticles (ENP), to manufacture, to consumer use, to final disposal, different human groups (i.e. workers, bystanders, consumers), environmental compartments (i.e. air, soil, sediment, water) and species (e.g. worm, fish or human through secondary exposure) may be exposed. Emerging data have shown a range of toxic (hazard) effects from ENP, suggesting that exposure may result in a risk to human health or the environment. However, the risk (chance of harm for an individual or group of individuals) resulting from the combination of exposure and the hazard. While standard methods exist for hazard and risk analysis for chemicals, these tools need to be modified and verified before they are applied to ENP. Previously used standard approaches to risk assessment need to be relevant for nanomaterials. Thus, the development of nanotechnology-based products must be complemented with appropriate validated methods to assess, monitor and reduce the potential risks to human health from ENP. Furthermore, new methods for ENP risk assessment must be integrated into an overarching, coherent strategy for regulators and industry to use. Public mistrust of any new technology is often high, and the scientific demonstration of safe nanotechnology products should promote an informed debate within society about these technologies and will help enhance the confidence of consumers, workers and other stakeholders. A safe nanotechnology will safeguard current and future global investments and will be the key to the sustainability of this industry. There is a need to develop a specific Risk Assessment method to manage the potential hazards associated with ENP. The aim of the ENPRA project was to develop and validate such methods.

1.1. The principal aim of ENPRA

The project aimed to develop and implement a novel integrated approach for ENP Risk Assessment (ENPRA). This approach was based on the Exposure-Dose-Response approach for ENP (Figure 1). This states that human exposure to ENP of different physico-chemical characteristics by inhalation, ingestion or dermal exposure is likely to lead to their distribution to other body systems. The cumulative dose in a target organ may eventually lead to an adverse response in a dose-response manner. Our approach has adapted the traditional chemical risk assessment approach to ENP and covers: hazard Identification; dose-response assessment; exposure assessment along with risk assessment and risk management.
1.2. The specific objectives of ENPRA
The objectives of ENPRA were (i) for Hazard Identification: To measure the physico-chemical characteristics of a panel of commercially available ENP carefully chosen to address the relevant hazards, properties and potential mechanisms of toxicity; (ii) for Dose-response Assessment: To assess the hazards of these ENP by means of in vitro toxicology tests with cells based on five body systems: pulmonary; hepatic; renal; cardio-vascular developmental, and five potential adverse health effects: oxidative stress; inflammation and immune-responses; genotoxicity; fibrogenicity and developmental toxicity; (iii) To verify the in vitro findings with in vivo (animal) models; (iv) for Exposure and Risk Assessment: To use data from this project and other sources (including US data) to model exposure and the exposure-dose-response relationships by means of mathematical modelling and to conduct example risk assessments; (v) for Risk Management: To develop and implement a strategy for dissemination of our findings in relation to appropriate risk

management actions to maximize the anticipated high impact on stakeholder actions.

Project Results:
2. Description of work performed and main results
2.1 Hazard Identification
High-quality data on physico-chemical characteristics and properties of the tested nanomaterials are essential for understanding the qualities of the test materials and interpretation of toxicological test results. Elaborate high-quality physico-chemical data are also one of the potential keys for establishment of predictive QSAR-like hazard assessment models. Establishment of such models, however, requires toxicological tests of several diverse and carefully characterized test materials and with detailed information on the exposure characteristics in the given test systems. Final risk assessment also requires data on the characteristics of the possible exposure for the specific test material and the potential exposure levels.

In response to these requirements, the primary objectives of WP3 were to:
1) Establish a suite of ten different industrially applied and well-characterized nanomaterials, which should address major nanomaterial types and enable answers on specific toxicological questions.
2) Establish a validated generic dispersion protocol applicable for both in vitro and in vivo toxicological studies
3) Characterise the ENP for a number of specifically selected physico-chemical characteristics and properties that describe the material characteristics and qualities and/or may drive development of adverse health effects.

Selection of the ten nanomaterials
The nanomaterials selected for the ENPRA studies are listed in Table 1. Beyond industrial relevance, the selection criteria were that test materials should represent sets of particulate and fibrous nanomaterials that have:

- Different chemical composition representing major commercial nanomaterials
- Different crystal structures with the same composition
- Different primary particle sizes and morphology with the same composition
- Different surface charges and same core material
- Different engineered surface coatings with the same core material
- No or Low solubility versus partial solubility
- Dispersibility in a liquid batch medium to enable both in vitro and in vivo testing

In addition, the aim of ENPRA was also to support the test program under the OECD Working Party on Manufactured Nanomaterials. Therefore, most of the nanomaterials (six) were selected from the so-called OECD nanomaterials, and included nanocrystalline anatase (NM-101), uncoated (NM-110) and coated (NM-111) nanocrystalline zincite, Ag nanoparticles in a dispersion (NM-300K), and two Multi-Walled Carbon Nanotubes, MWCNT (NM-400 and NM-402). Additionally, a small (ca. 10 nm) and a large (80 -100 nm) nanoparticle size TiO2 with the same crystal structure were selected. As indicated from equilibrium thermodynamics such a material set was indeed very rare, but we were able to establish a set of rutile nanomaterials (NRCWE-001 and NRCWE-004) satisfying our requirements based crystallite size analysis by XRD and a high degree of dispersibility at optimized pH conditions. The small-size rutile was subsequently coated as part of the project to establish test materials with an engineered positive (NRCWE-002) and negative (NRCWE-004) surface charge. Consequently, the TiO2 samples comprise a test matrix of small and large nanosize insoluble nanomaterials of the same chemical composition, small nm-size nanomaterials of anatase and rutile and expected differences catalytic properties, small nano-size rutile co-valently functionalized to have both negative and positive surface charge. The role of surface coating may also be investigated using the partially water-soluble ZnO samples (NM110 and NM111) and Ag NP (NM300K), where water-solubility also is an issue to be addressed. The two MWCNT nanomaterials selected found to have relatively similar dimensions and adsorption capacity although the manufacturer had identified them as having different size.

Incorporation of the OECD nanomaterials naturally ensured the research project was relevant to societal needs, but all the selected nanomaterial groups are also already widely used. Titanium dioxide, zinc-oxide and silver are currently among the most used ENPs; they are incorporated into many different types of products including paints and lacquers, sunscreens, photocatalytic cleaning surface coatings, pesticide and antibacterial coatings and other products. MWCNTs have numerous potential applications and is on the way to become a so-called high-volume chemical. Products such as sports goods, electronics, nanocomposites, epoxy paints, textiles, antistatic coatings with carbon nanotubes (CNT) are already available on the market.
Two different types of MWCNT have been tested in ENPRA, namely NM-400 which is supposed to have a shorter tube length according to the manufacturer than NM-402 (four times longer that NM-400). However, the particle characterization carried out in this project revealed a similar length of both carbon nanotubes making them much more similar.To summarise, the panel of test materials consists of several OECD nanomaterials, such as two types of nano zinc oxide (uncoated ZnO NM-110 and coated ZnO NM-111), Nanosilver (Nano Ag NM-300), multiwall carbon nanotubes (MWCNT NM-400 and NM-402) and several titanium dioxide (TiO2) nanoparticles that were synthesized with specific surface characteristics such as a neutral, positive or negative charge.

A generic batch dispersion protocol for toxicity testing
The second aim of the hazard identification work in the project was the establishment of a validated generic dispersion protocol to be used for preparation of the particles to expose cells and animals in the toxicological testing. It has been found that toxicological testing of partially or fully insoluble materials requires more effort in sample preparation and analyses across different studies than soluble chemicals. One cannot by default assume a simple mass per volume

exposure concentration from the batch dispersion or in a cell-assay. Particles may initially be well-dispersed and then dissolve or agglomerate at the bottom of the batch dispersion or test system. Therefore sufficient stability must be ensured to enable reliable dosing and interpretation of results from both animal and cell testing using exposure via pre-dispersion in a liquid medium. Therefore considerable effort was taken to establish and document a nanomaterial dispersion protocol where we could assure that the materials were well suspended.

At the starting time of ENPRA, a few methods had been established for specific nanomaterials and test systems. For example pre-dispersion in a cell-media, purified water, saline or phosphate-buffered saline had often been used. However, it is not possible to disperse and stabilize all types of nanomaterials in any of these dispersion media. Clearly hydrophobic materials are not readily dispersed and extensive agglomeration is often observed in saline media. Moreover, some media are not suitable for all toxicological test methods. Therefore, the project set out to establish a dispersion protocol that was able to satisfactorily disperse the nanomaterials in medium biologically acceptable for most bioassays. The work resulted in the ENPRA dispersion protocol. The protocol uses a 2% (w/v) serum-water (fetal calf serum) solution as dispersion medium involving pre-wetting with ethanol for hydrophobic materials. After mixing test material and medium at a concentration of 2.56 mg/ml, the particles are dispersed in the medium using a 300 W probe-sonicator set at low amplitude (10%) for 16 minutes. To prevent extensive heating, the dispersion is cooled in an ice-water bath during the whole sonication procedure.

The ENPRA-protocol can ensure meta-stable dispersion for at least 1 hour and re-dispersion can easily be re-established by simple vortex shaking of the batch solution. For some materials stability was observed for much longer durations. However, as a general recommendation, nanomaterials should be tested as soon as possible after dispersion to prevent extensive un-wanted reactions in the dispersion, such as dissolution or radical formation prior to toxicological testing. For generic use, however, the serum used must be compatible with the test system. Therefore further developments and documentation is necessary for additional sera to suit all relevant exposures.

Primary physico-chemical characterization of the test materials
The third objective was generation of the physicochemical characterization and properties of the test materials and the exposure media. Naturally numerous characterizations may be completed on nanomaterials and this alone can become a very extensive and costly activity. We decided to focus on the primary end-points thought to be most important for interpretation of the toxicological test results. The characterized properties and methods applied for these are summarized in Figure 2 and some of the key results already published are listed in Table 1, above. Clearly several techniques can be used and support the observations made for each of the different properties.

An important general outcome of the analytical results has been the recognition that the materials often have a wide size-distribution, different levels of impurities and homogeneities. Regarding size analysis, challenges still remains in proper analysis of MWCNT lengths. The CNT are often entangled and sample preparation methods using sonication may shorten the tubes. Other issues are the elemental compositions and impurity levels. The impurity levels of the MWCNT (mainly due to presence of inorganic catalysts) were high and on the level of 17 and 10 wt% in NM400 and NM402, respectively. The MWCNT were also very inhomogeneous requiring sample size larger than 10 mg to obtain repeatable results by TGA. For comparison, the metal impurity levels observed in the other nano materials were below 0.2 wt%.

Exposure characterization
In addition to knowledge on the inherent material characteristics, information about the exposure characteristics is also important for data interpretation and risk assessment. We addressed the emission potential and the characteristics of the airborne exposure in a powder handling scenario and the reactivity and sedimentation rate in cell test media.

The emission potential and characteristics of the airborne exposure was assessed by using dustiness testing with a miniaturized EN15051 rotating dustiness drum. This system was specifically developed for testing small amounts of nanomaterial powders. In our set-up the test includes traditional filter-based measurements of the inhalable and respirable dustiness indices in mg dust per kg powder combined with real-time measurements of particle size-distributions and number concentrations from 5.6 nm to 20 µm. Particles from 5.6 to 560 nm were measured using a Fast Mobility Particle Sizer and the coarser particles from 0.523 to 20 µm were measured using and Aerodynamic Particle Sizer. Only granular powders were tested due to risk of exposure to MWCNT during the test. The results showed that powder aerosol is dominated by agglomerates and in only 5 - 10 percent of the particles, by number, were smaller than 100 nm electrical mobility size. The dominant dust particle peak-size was normally between 100 and 200 nm and particle size-modes were always present in the µm-range. This clearly suggests that complete exposure assessment to airborne emissions from nanomaterial should include measurement of the aggregates and agglomerates in the entire respirable range.
The exposure characterization in liquid media included analyses of agglomeration, sedimentation and particle reactivity in simple to synthetic biological media.

The electron donor/acceptor capacity was assessed by two alternative methods: First measurement of the ability of the particles to produce hydroxyl radicals using benzoic acid to capture the radical. Second direct measurement of the pH and O2 concentration evolutions in a synthetic lung lining fluid and cell media doped with test materials followed by elemental analyses of the media to determine the amount of test material dissolved. Incubation was done in a cell incubator so the exposure copied the actual conditions in biological tests. The results showed the nanomaterials were very weak OH-radical producers and it could only be detected for one of the MWCNT. However, analysis of the reactivity using pH and O2 showed that all nanomaterials to a certain extent were able to destabilize the conditions in well-

established test media. As expected TiO2 and Ag only showed weak reactivity, whereas ZnO and maybe surprisingly MWCNT were able to cause very clear disturbances in the pH and O2 concentrations over time.

The dissolution studies were performed in a cell-incubator (37??°C, 5% CO2) using different cell media and synthetic lung lining fluids simultaneously as part of the reactivity studies above. Solubility could not be observed for MWCNT from elemental analysis. As expected from bulk materials ZnO and Ag nanomaterials were partially soluble. The analyses indicate that the ZnO and Ag nanomaterials would be partially of fully dissolved at the lower µg/ml doses tested in in vitro studies. Consequently, the solubility in the exposure media appears to be below the concentrations that would be expected to cause detrimental effects in biological tests.

2.2 in vitro Hazard Assessment

ENPRA aimed to use and develop non-animal (in vitro) models to assess the toxicity of ENP. The in vitro systems were in general cultured cells and were chosen to represent five different body systems: lung, liver, kidney, blood circulation/heart and development/growth. In all of the in vitro models we measured the ability of the different ENP to generate stress responses such as oxidation (oxidative stress), activation of the immune system (inflammation), genetic damage and scar formation (fibrosis).

The work was broken into 3 phases. Phase I assessed a range of concentrations for all 10 ENP in each model to measure cell death. This information allowed a ranking of the cell toxicity of the ENPs and this information was used to prioritise the ENP used in the in vivo studies (WP5). Phase II required mechanisms of toxicity to be investigated in more detail, and for this work a sub-set of the ENP were selected. Phase III involved testing the reproducibility of the protocol to assess cell death induced by ENP across different toxicology laboratories.

Phase I tested all 10 ENP for their ability to induce cell death in a variety of cell types from many parts of the body. The data generated clearly demonstrated that for all the cell types tested the particles could be divided into two groups; a relatively lower toxicity group (TiO2 and MWCNT) and a relatively higher toxicity group (Ag and ZnO). An example data set is provided in Figure 4. This is interesting because each research group used different culture conditions and cell types. This demonstrates clearly that the conclusion that Ag and ZnO are relatively toxic in different cells compared to TiO2 and MWCNT is relatively robust (see the two graphs in column 1 in contrast to the graphs in the two other columns of Figure 4). However, one exception was noted macrophages. Macrophages are cells from the immune system which identify foreign particles, including ENP, bacteria and viruses, ingest them and then either clear them from the body or active an immune response. This cell type was sensitive to MWCNT. We have hypothesised that this might be due to the long fibre like dimensions of the MWCNT leading to ineffective uptake and therefore toxicity to this particular cell type.

In the literature there are examples of studies in which Ag and ZnO dissolve under the experimental conditions. The studies conducted using hepatocyte medium indicated that the ZnO ENPs were about 50-60% soluble therefore leading to substantial release of Zn ions which could account for the observed toxicity. In contrast the Ag was less than 1% soluble, suggesting that solubility plays less of a role in the toxicity of these particles in vitro. For some studies it seems that the biological effects cannot be accounted for by soluble ions alone, but this is something that could be investigated further in other studies following from our project.

Looking at the data in more detail, there were some differences in the absolute ranking of the particles in terms of their cytotoxicity between cell types/laboratories when comparing either the different TiO2 particles with each other, or when comparing the MWCNT with the TiO2. This possibly reflects the difficulty in distinguishing between the potential risks of relatively low toxicity materials.

Oxidative stress: The major large molecules that make up cells and organisms (e.g. proteins, lipids (fats) and DNA) are susceptible to damage from reactive oxygen species (ROS; e.g. hydrogen peroxide or bleach). ROS occur naturally in cells but cells are protected against their damaging effects by antioxidants. ROS can be generated by external stresses such as ENP. this can lead to an overwhelming of the antioxidant defences and therefore oxidative stress. Low level oxidative stress activates cells to launch defence mechanisms, but higher levels of oxidative stress can lead to activation of disease processes and cell death. The cytotoxicity data related well to the ability of the particles to induce oxidative stress in the lung cell models. The relatively toxic particles, Ag and ZnO ENP were the most effective at inducing the production of ROS in lung and kidney cells. Measurement of antioxidant levels also identified ZnO to be effective at inducing oxidative stress in a complex 3-cell culture model of the lung (Figure 5), while both Ag and ZnO also induced antioxidant depletion in liver cells.

ROS levels inside cells were measured in some studies using a fluorescent dye (DCFH), but these data contrasted those generated using a different assay (DHE). The partners questioned whether cell death interfered with effective uptake and activity of the DCFH dye, and so they concluded that this assay was more suitable for lower toxicity particles and sub-lethal treatment concentrations when there would be less cell death.

In liver cells, the role of oxidative stress in stimulating cells to produce proteins that promote inflammation was investigated. Inflammation involves activation of the immune system. If this response is short lived then it is a useful way to clear particles, but if it is large in amplitude or prolonged it can lead to disease such as fibrosis (scar formation), cancer or worsening of a wide array of diseases including cardiovascular disease and asthma. The role of oxidative stress was investigated by pre-treating the cells with the antioxidant Trolox. Trolox in part prevented liver cell death, and decreased the ENP-induced production of the pro-inflammatory proteins after exposure to all of the ENP with the exception of Ag. In contrast studies with endothelial cells suggested that up-regulation of cell surface adhesion molecules, key in inflammation did not depend on oxidative stress. This suggests that while oxidative stress plays a role in the regulation of inflammatory responses, this is not the mechanism involved in all cellular responses.

For all cell culture studies, ENP were prepared in a specialised medium supplemented with serum. Serum is the liquid part of blood that contains lots of different types of molecules that help to keep cells healthy. There were some questions about whether dispersion of the particles in the presence of serum might lead to coating of the particle surface and therefore either a masking of the surface reactivity or whether the serum components might possess

antioxidant activity. Surface coating with the serum used in the dispersion protocol did tend to reduce the ROS production by most of the ENPs. For some of the insoluble TiO2 ENPs, the presence of FCS (foetal calf serum is commonly used as a surfactant) also prevented their toxicity to lung cells, possibly by preventing ROS production and oxidative stress. However, for the relatively soluble ZnO ENPs, the presence of serum increased their toxicity. Therefore the dispersion protocols used in ENPRA may have contributed to the relative toxicity of the soluble ZnO and Ag particles compared to the insoluble MWCNT and TiO2 particles. With respect to the pro-inflammatory effects of the ENP, Ag and ZnO were again relatively potent at stimulating a range of cell types to make proteins that activate inflammation.
In contrast, the lung cell responses to TiO2 in terms of pro-inflammatory production were lower and/or less consistent. Protein coating and shielding of the ENP surface might be responsible for the lack of effects here, but this would also have been true for the liver and kidney cells that were responsive to TiO2.
The MWCNT did not triggered pro-inflammatory production in some cell types but they did in others suggesting a cell type specific response. Also the two MWCNT differed in terms of this response, it will therefore be important to analyse the material, shape and surface properties of the MWCNTs to get further insights into the specific toxicity developed in further studies.

In conclusion, remarkable comparability was achieved across a range of cell types from the lung, blood circulation/heart, liver, kidney and immune system in terms of their susceptibility to the toxic effects of a panel of 10 ENP. All studies used the same protocol to generate ENP suspensions. Modifications of this protocol led to significant changes in the results and conclusions achieved, indicating the importance of comparing and validating such protocols in animal models in future. Some of the in vitro results have been compared directly with in vivo responses (see later), such as antioxidant content of animal livers and cultured liver cells. The in vitro models predicted correctly the liver cell oxidative stress response to the Ag, ZnO and most of the TiO2 particles (7 out of 10 were correctly predicted using the in vitro assay). In contrast one TiO2 (positive charged) induced a greater oxidative stress in the rodent liver while the MWCNT induced a lower oxidative stress. The partners have hypothesised that these differences could be accounted for by charge and shape respectively. However, overall, the protocol chosen seems to be quite appropriate for systemic response, although perhaps less appropriate for lung cells which would not normally be associated with serum.
In terms of sub-lethal effects such as oxidative stress and inflammation, the lower toxicity materials were also able to illicit responses suggesting that they may not be without toxicological effect. The consequences of such changes require further investigation to assess whether they are significant in inducing short term or long term disease.

2.3 in vivo Hazard Assessment
The inhalation route is foreseen as the most important entry portal for ENP to exert adverse health effects in humans. Here, we set out to determine:
1. The movement of ENP from the lung to the bloodstream and subsequently possibly to all other organs in the body,
2. The acute damage (24 hours after a single exposure)
3. Sub-acute damage (two months after a single exposure) of a panel of nine different ENP following exposure via the inhalation route to the lung and other organs and
4. Damage to the cardiovascular system from MWCNT, the response was tested in compromised animals that are a model for a sensitive human subpopulation, e.g. in people that already suffer from cardiovascular disease or in the elderly.
Due to their very small size ENPs may cross the lung membranes after inhalation and thereby enter the blood stream to a much higher extent than micron-sized. Since the blood is able to reach every distant organ and tissues of the body, ENP may also be able to reach organs and tissues, too. This is an important factor for determining the harmful effects of ENPs in organs and tissues, which are not initially considered to be exposed to ENP. The more ENP reach these secondary organs and tissues, the higher may be the adverse response and subsequently the harmful effect. In addition, there is evidence that the much larger specific surface area (surface area per mass) of ENP than of micron-sized particles initiates or mediates adverse biological responses such as oxidative stress and inflammation.
In order to address this, we have investigated the fraction of nanomaterial that can cross the air-blood-barrier (ABB) in the lungs as well as the fractions of ENP (relative to the administered ENP dose), which specifically accumulate in organs and tissues. We administered gold (as control) and titanium dioxide (TiO2) ENP into the lungs as well as directly into the blood stream. For application into the lungs, we chose inhalation, which is the most realistic physiological scenario. Since dosing animals by inhalation is technically very complicated, we also used ENP suspended in a small volume of water, and administered this suspension directly via the trachea into the lungs while the rodents were anesthetized, a technique called intratracheal instillation. In addition, we injected the ENP directly into the blood stream of the animals. Neither of the ENP application methods directly harmed the animals (rats or mice).
We found that the ENP fraction that crossed the lung barrier was strongly dependent on the size of the ENP. The smaller the ENP were the higher was the fraction, which reached the blood stream. Therefore, the highest fraction of approximately 10 % of the administered ENP dose could be detected for the 1.4 nm gold ENP. After blood injection of the gold ENP directly into the blood stream, most of the ENP were found in liver and spleen. Again, there was a size dependency, the less ENP were trapped in liver and spleen the more accumulated in other organs, soft tissue and the skeleton resulting in increasing fractions in these organs with decreasing ENP size.
After administering TiO2 suspensions into the lungs, most of these ENP stayed there. Likewise, a small but significant amount of the TiO2 ENP moved to the blood stream and accumulated subsequently in organs and tissues. We investigated the fractions of TiO2 ENP up to four weeks and detected rather little clearance of these accumulated ENP indicating biopersistence of the TiO2 ENP not only in the lungs but also in all other organs and tissues.
After blood injection of the suspended TiO2 ENP most of them accumulated in the liver. This is in agreement with the results from the gold ENP. After inhalation of 20 nm TiO2 ENP again most of the ENP stayed in the lungs. Interestingly, in

liver, kidneys, heart, uterus, and in the remains, a relevant increase of accumulated TiO2 ENP was detected between 0 hours and 4 hours. Most importantly, the results of the prolonged study indicated that no significant removal exists up to 28 days for liver, kidneys, spleen, brain, and heart. This indicates an accumulation of 20 nm TiO2 ENP, which had been inhaled initially. Our data suggest that the ENP are biopersistent and may stay in the body and do not leave these organs.
The most harmful nanoparticles were ZnO (NM-110 and NM-111) based on acute inflammation and lung damage. Attempts to visualize the inflammation in the lung in a living animal using fluorescent probes failed as the technique is currently not sufficiently sensitive. Ultimately, this method would make it possible to follow an inflammatory response over time. Then it could be determined whether the inflammation is resolved or becomes chronic, which could lead to tissue damage without the need of extra animals. ZnO also causes a reduction in spleen weight and an increase in red blood cells and haemoglobin. This could be a sign of the body trying to compensate for the onset of anaemia, an effect that has been reported in literature after zinc exposure. The two ZnO materials resulted in overlapping dose-response curves and the same benchmark dose (BMD) values, suggesting that the triethoxycaprylylsilane coating has no effect on the observed harm (but we cannot exclude an effect on biodistribution and biopersistence). A ZnO dose of 25µg per mouse can be lethal when waiting longer than 1 day after administration. At lower doses, inflammatory effects seem to diminish over time and two months after a single instillation the effects were no longer detectable.
MWCNTs have a lower acute inflammatory potential (compared to ZnO) which is based on effects seen 24 hours after a single administration. However, two months after a single exposure, both of the MWCNT tested induced a dose-dependent lung inflammation and fibrosis similar to the responses observed after exposure with crystalline silica particles.
Positively charged TiO2 nanoparticles (NRCWE-2) can cause a mild inflammatory response and some lung cell damage, yet to a lesser extent than ZnO. Interestingly these effects are somewhat more pronounced in the absence of 2% serum in the solution, the standard test developed for ENPRA. The serum is needed to stabilize the solution, but as we have hypothesised this might reduce the damage seen in the lung by forming a layer around the particles.
Before the experiments with TiO2, the particles were checked for stability in different suspension (Figure 9). Uncharged TiO2 nanoparticles of 10 nm can induce cell damage, but no inflammation is observed. Neither the negatively charged 10 nm TiO2 nor the 94 nm material gave a harmful response. The initial differences in charge of the TiO2 particles that was determined by the chemical group that is attached, is no longer apparent when the material is prepared in 2% serum. Then all the TiO2 particles are negatively charged, and although the exact charge level is unknown this might explain the different results.
Ag ENP causes cell damage in vitro, and cell death at relatively low doses, comparable to the effects of ZnO. These effects are expected to be driven by the dissolution of ions. In vitro, no cell damage or inflammation was observed after 24 hours, and this is possibly because the Ag particles take longer to dissolve in the lung (inside macrophages) of an animal and therefore effects might be observed after a longer period.
As predicted by the previous studies, the lung is not the only target where damage can occur. For ZnO, the spleen seems to be a target organ. There are responses observed in the liver, such as an increase in Glutathione (GSH) depletion, a marker indicative of a response of the body to compensate for the oxidative stress that some particle administrations may cause. Changes found gene expression also point to compensating mechanisms or adaptive responses of the body. For longer exposure periods, it could be that the compensating mechanisms of the body become overwhelmed. Performing studies with multiple administrations or preferably inhalation for multiple days with a longer recovery period should be the next step in the hazard screening ENP with simultaneous collection of information on where the particle go in the body.
Inhalation of ENP in the rat is the model of first choice to determine adverse health effects and to be able to extrapolate the results to humans. Due to budget constraints and limitations in the technical feasibility, it was not possible to perform inhalation exposures for the entire panel of nanomaterials. Therefore, we choose to perform intratracheal instillations (IT). An advantage of this method is that the dose is delivered to the lung in a more controlled manner compared to inhalation. The method of instillation is suitable for comparative toxicology and ranking the ENP from the most (acutely) harmful material to the least harmful material. These results can also be compared to in vitro studies conducted in is part of this research study. IT exposure provides additional information about the mechanisms of toxicity following sub-acute exposures.
Following IT exposures of ENP of the lung, the acute responses (within 24 hours) of different organs were followed at every dose to generate a so-called dose-response curve. The statistical method called benchmark dose modelling (BMD) was used to derive a dose that gives a well-defined change compared to the control treatment e.g. 20% effect above baseline values. This method differs from the No Observed Adverse Effect Level or Lowest Observed Adverse Effect Level (LOAEL) method that is more often applied in risk assessment. A NOAEL or LOAEL is usually derived from studies using three dose groups or sometimes less, leaving considerable uncertainty about the true dose at which a certain effect takes place. By using the BMD method with multiple dose groups and integrating the data into a dose-response curve, a confidence interval around the dose that gives an effect can be given. This gives an idea about the quality of the data and the applicability for extrapolation from in vitro to in vivo effects or for risk assessment purposes.
Based on studies with particulate air pollution, it is known that the cardiovascular system can be affected for the smallest sized particles. It is hypothesized that nanoparticles are also able to exert these effects as they translocate from the lung into the blood stream, although particles may induce an inflammatory response and oxidative stress in the lung and affect the cardiovascular system indirectly. The focus here is on nano ZnO and nano Ag, that were identified as most harmful in vitro and multiwall carbon nanotubes (MWCNT) which can cause harm in secondary organs as reported in other studies.

2.4 Risk Assessment

2.4.1. Modelling

Risk assessment of ENP must rely on tools to quantify the information on their Exposure and Hazard. This is done, to a large extent, on computational methods to integrate the various potential adverse health effects of ENP to assess risk in a given Exposure scenario. Within ENPRA we have employed two mathematical models to attempt to describe the behaviour of airborne ENP in the workplace and after they are taken up within the body, with the aim that these methods will aid in the risk assessment of ENP. These models are essential tools for describing the nature of ENP Exposure and the dynamics process from Exposure to the internal Dose and its distribution across different body organs.

1. Exposure Model

Increasingly, estimates of exposure of workers and consumers are being modelled mathematically rather than the assessor having to rely solely on small numbers of actual environmental measurements. There are many advantages associated with modelling exposures: models allow the exposures related to specific scenarios to be predicted and also to examine the effect on those exposures by various factors, such as local control measures, general room ventilation and room size. In most cases these models include factors to account for loss of airborne ENP through ventilation and from deposition to surfaces, which for larger particles is through sedimentation. The coagulation of small particles over time is also an important loss mechanism and is also included in the Exposure model. The schematic representation of the model is given in Figure 10.

The exposure model developed within ENPRA is based on a model outlined by Maynard and Zimmer (2003). This model represents the dynamics process underlying the generation of an aerosol concentration of ENP in a indoor environment, treated as a single compartment in which the air is completely mixed, i.e. there is a uniform concentration of ENP throughout. The model includes terms for the air ventilation rate and describes the coagulation of particles as well as loss by gravitational deposition and diffusion. Figure 11 illustrates the coagulation of particles as estimated by the model, where the highest line shows the initial distribution of particles introduced into the model and the other lines show the distribution at successive time points. The ENP size distribution shifts to the right over time and the total number of particles decreases as the particles coagulate.

The model was used to simulate the observations given in Figure 12. Figure 13 illustrates the concordance between model predictions and observations. The development of the Exposure model in ENPRA is of considerable interest to the Exposure Assessment community. During the course of ENPRA, the model was developed in user-friendly codes and is made available to researchers who request it.

2. Physiologically-Based Pharmaco-Kinetic (PBPK) model

After an airborne exposure to ENP occurs it is of interest to know where they go within the body. In-vivo kinetics experiments are designed to examine the particle burden in organs over time after a given exposure. While this work is of great importance these experiments can be expensive and as such it is useful to find a cheaper, and easier, alternative. Mathematical models that can predict the translocation of particles within the body over time is one such alternative approach, and such approaches epitomise the ethos of "Replace, Refine and Reduce", the use of animals in research.

Within ENPRA a Physiologically-Based Pharmaco-Kinetic (PBPK) model developed in earlier research has been further developed and refined with the aim of making such predictions. PBPK models use a series of differential equations to describe the movement of particles and chemicals throughout the body, using known information such as typical organ weights and blood flows as well as details of the exposure. Figure 14 illustrates the PBPK model used in ENPRA to model the movement of particles throughout the body. This model was originally developed in the project FP7 NANOMMUNE. In ENPRA we use it to simulate the bio-distribution of the ENP used in this project.

Using kinetics data generated within ENPRA for a TiO2 nanoparticle the model was calibrated for three methods of exposure; intratracheal instillation, IV injection and inhalation. The need for three separate calibrations became apparent due to the different entry routes into the body and, as a result, the differing organ systems involved. Figure 16 illustrates the calibration of the model predictions with the data measured after exposure via inhalation. It is clear that the model can be calibrated well to the data.

Using the calibrated model we then made some further assumptions, based on other data and literature, to adjust the parameters of the model in order to make predictions for each of the ENP used within ENPRA.

2.4.2. Introduction to the structure-activity concept

An understanding of the relationships between the structural characteristics and physicochemical properties of ENP on the one hand, and their fate and (potentially adverse) effects in the environment and biological systems on the other, provides the basis for structure-activity modelling. Such models may include both qualitative and quantitative structure-activity relationships, i.e. SARs and QSARs, that make qualitative (e.g. the potential for oxidative stress) or quantitative predictions (e.g. cytotoxic potency), depending on the data and modelling approach. These models provide an efficient and cost-effective means of prioritising toxicological testing, and also for filling data gaps in hazard and risk assessment. They may also help in giving an insight into the underlying mechanisms of action of ENP. For these reasons, ENPRA has included exploratory analyses on the development of structure-activity relationships for ENP.

The development of structure-activity models for ENP requires three components: 1) a data set providing a measure of the toxicity of a group of ENP; 2) molecular structure and/or property data of ENP that are used as descriptors; and 3) a (mathematical) model that can relate the descriptors to the toxicological effect of interest.

Although the structure-activity paradigm is well established in the areas of drug discovery and risk assessment of small molecules, its application in modelling the behaviour of ENP is still in its infancy, due to a number of scientific and technical challenges, such as the lack of theoretical descriptors for ENPs and coherent toxicological datasets (Winkler et al, 2012).

2.4.3 In Vitro In Vivo (IVIV) Comparison

In this study, we set out to develop a method for comparing the in vitro results of WP4 with their in vivo counterparts in WP5. Two sets of assays were used. One set of assays was carried out in vitro and a second set of assays was carried out in vivo. We were interested in a comparison of the in vitro and in vivo results. To do this, we identified which measured endpoints in the in vitro test systems were comparable to those for similar endpoints at the target site in vivo, and then carried out a comparison of the two sets of results. To do this we used a software package called PROAST to calculate the level of dose for the in vitro data for which the response for the ENP was 10% higher (or lower depending on the response being studied) than the response in the control group. This level of dose is called the benchmark dose (BMD). In the same way we calculated the benchmark concentration for in in vivo date. We then compared the BMD to the BMC for a selection of ENPs and endpoints.

2.4.4 Risk Assessment

The main goal of ENPRA has been to develop a framework for quantitative Risk Assessment (RA) of ENP in occupational settings. It is not meant to be a preliminary risk screening or research prioritization tool, but a methodology supporting regulatory decision-making. The specific objectives of the work were:
- Weigh and aggregate all available occupational exposure and effects data provided by project partners that refer to the panel of ENP considered as case studies in ENPRA in order to assess their risk.
- Allow inclusion of expert judgment into the RA process.
- Estimate uncertainty related to input data and use of models, and propagate this through the aggregation procedure up to the final results.
- Ensure compliance with the basic requirements of REACH (Registration, Evaluation, Authorisation and Restriction of Chemicals).

The first tiered framework for RA of ENMs was based on quantitative Weight of Evidence (WoE) methods (e.g. Multi Criteria Decision Analysis) and includes a strategy for probabilistic uncertainty evaluation. Based on the conventional RA paradigm, the approach uses acute effects data for five organ systems (i.e. pulmonary; cardio-vascular; hepatic; renal and developmental) and five endpoints (i.e. oxidative stress; inflammation; genotoxicity; fibrogenecity; and developmental toxicity) that refer to the ENP selected as case studies in the project. These data have been compared to exposure estimates derived with the NanoSafer model (Jensen et al., in preparation) for a number of hypothetical exposure scenarios based on data from the EU NANEX and NANOSH projects, and from the U.S. NIOSH to calculate endpoint-specific Margin of Exposure (MoE) values, which are directly used in the WoE approach. The MoE is a well-established method used for risk ranking in regulatory contexts. It is currently used by the U.S. Environmental Protection Agency (EPA) for non-carcinogens and carcinogens with non-linear dose-response characteristics and was applied for risk analysis of Silver nanoparticles (US EPA, 2011). Risky materials/scenarios were further analyzed and their actual health risks were estimated using the Derived No Effect Level (DNEL) approach, suggested in the REACH Guidelines for regulatory Chemical Safety Assessment (European Chemicals Agency, 2007). All uncertainties related to the input data, use of models and the application of the WoE-based aggregation procedures were thoroughly characterized using a Monte Carlo simulation approach.

The application of the proposed approach for risk analysis of nanomaterials provided some of the first quantitative risk assessment results for ENPs. They showed that the most risky situations occur with handling of dry powders in large quantities (e.g. bag/bin filling, manual un/loading, dumping), which is confirmed by the state-of-the-art literature (Brouwer, 2010; Hanai et al., 2009). For such scenarios risks are often not controlled and suitable risk mitigation measures need to be implemented. Scenarios, where risks are controlled, but are still relatively high involve handling of dry powders in smaller amounts (e.g. transferring of materials for solution preparation, weighing). In contrast, synthesis wet chemistry and laser ablation scenarios were shown to pose negligible risks due to very low emissions. The analysis clearly shows that the differences in exposure potential significantly influenced the risk assessment results.
The WP5 in vivo results described above showed that nano-ZnO is by far the most acutely harmful ENP in a the panel of the nine different ENP tested, which is most likely attributable to its solubility near or inside cells. However, the available exposure data indicated that for the investigated scenarios (i.e. preparation of nano-ZnO ink solution and deposit of the "nano-ZnO ink" on a silicon substrate) exposures to ZnO are not significant and therefore risks are controlled.
The hazard of MWCNTs has been ranked second due to observed severe fibrosis in the lungs after a single exposure. Nevertheless, for the investigated hypothetical scenarios (i.e. weighing raw MWCNTs and their sonication the risk analysis showed that although they are relatively high, the risks are actually controlled. These results take into account the higher cardiovascular hazard of the NM-402, which is more potent in keeping an increased size of plagues in the

aorta of mice post exposure. Again, the relatively low estimated workplace exposure is the main reason for concluding that the risks from the NM-400 and NM-402 materials are controlled, which is valid only for the investigated exposure scenarios and cannot be generalized.
Although the different types of nano TiO2 were much less harmful compared to nano ZnO and MWCNT, our analysis showed that for several scenarios (manual un/loading trays inside booth, dumping into mixing tank using focused LEV, dumping large amount of powder into vessel, and bag/bin filling) these materials pose highest and uncontrolled occupational risks with chronic risks being higher than the acute.
Nano-Ag was not harmful within a time period of 24 hours and therefore ranks as least hazardous. Moreover, the studied nano-Ag exposure scenarios (laser ablation and wet deposition synthesis activities) resulted in negligible exposure. Therefore the estimated risks from this material are also negligible.
For the first time probabilistic Monte Carlo uncertainty analysis was applied in RA of ENP. It showed that the results of the above risk analysis do not change significantly due to variations in the input parameters, confirming that the procedure is stable and reliable. However, the considerable uncertainties in the input parameters imply that the above results should be interpreted with caution and the above analysis should be repeated in an iterative process as better data become available. Such data may include high-resolution exposure estimates based on aerosol dynamic modelling (considering agglomeration/aggregation and deposition mechanisms as have been developed in this project) in combination with chronic inhalation toxicity studies. The production of these data will significantly reduce the present uncertainties and will ensure more informed regulatory decision-making.

In this context of the enormous diversity of ENP and the potentially costly safety evaluations associate with their use implies that the application of a tiered RA framework appears relevant. It can both target future research and testing and provide near-term regulatory RA results.
The proposed approach clearly demonstrates the ease and the analytical rigor of the WoE process and the many benefits derived from applying quantitative Monte Carlo Data Association (MCDA) tools. The main benefit arising from using WoE for early stage nanosafety evaluations is that its framework allows incorporation of different types of evidence in order to make informed decisions in the face of uncertainty. The quantitative nature of the proposed WoE/MCDA method formalizes the decision making process and allows incorporation of precaution in the assessment.

The call for quantitative, robust decision making tools in the nanosafety area is unlikely to wane, and the importance placed on them will increase with time. The production of new information will naturally move modeling activities towards the quantitative region of the WoE spectrum. MCDA can potentially overcome many limitations of the existing qualitative WoE approaches, while at the same time provide a flexible framework applicable to various needs. Therefore, practitioners should consider MCDA methods as a valuable data integration technique, which can potentially significantly contribute to the early stage RA of ENP#.

2.5 Dissemination

2.5.1 Scientific Dissemination

Any research project would be ineffective unless its results are timely and widely communicated to the appropriate audiences. Quite often research results are reported solely through publications in specialized scientific press in a language only accessible to the selected group of initiates on the matter. However, the importance of nanotechnology and nanomaterials as a rapidly developing industry requires a wider communication approach.

Our project included a number of activities aimed disseminating the projects results. But, as communication is a two way activity some of our activities also set out to receive feed-back both on the scientific developments and their relevance to stakeholders.

The impact on the scientific world has been relevant, ENPRA has produced a number of publications in specialised scientific journals and presented communications to many scientific workshops, conferences and symposia (about 29 papers, 45 presentations, 30 posters). Additionally, the ENPRA partners contributed to disseminate the scientific principles embodied in the project in training courses as, for example, in the annual Nanosafety Autumn School organised by University of Venice and the Doctoral School of the Catholic University of Leuven.

2.5.2 Dissemination to other Stakeholders

However, our project intended to have a relevant impact not only among the wide range of international scientists involved in nanomaterials safety research, but we intended to communicate as well to a larger audience. This would include (as far as possible) all stakeholders interested on how the safety of nanomaterials can be assessed and managed. That means e.g. the regulatory authorities, industry (large or small and medium) producing or using nanomaterials, nongovernmental organisations that represent e.g. environmental or workers protection organisations, the international organisations that deal with harmonisation of nanosafety (e.g. the OECD or the WHO), venture capitalists, insurance companies or consumer organisations.

As a first step of our effort, we identified a wide range of individuals and organisations potentially interested in the results of the project and provide feedback on the work. They all inserted received our periodic newsletters and reports and were then involved in other activities of the project.

We organized three annual workshops, as well as the final ENPRA event, where we invited all stakeholders to participate. In some cases we were able to provide financial support for the attendance of individuals. The findings of the ENPRA project were presented in these open workshops along with other relevant scientific developments in the field. While discussing several aspects of the hazard, exposure and risk assessment of nanomaterials, there was always an emphasis on linking the scientific results to the regulatory needs and the practical experiences from industrial producers. The presentations and discussions in these workshops were not limited to the ENPRA results, but also included invited presentations from other related projects and presentations from the stakeholders themselves. In this way the workshops served to share information with the stakeholders but also stimulated active dialogue and allowed us to receive feed-back. The discussions at each workshop were summarised in reports. Some of these have already been published and are available both in printed form and on-line. The last reports are in the pipeline for publication and will be widely distributed.

In order to help the ENPRA members to keep pace with the most relevant scientific developments, we organised six-monthly meetings (ENPRA Experts Panel Meetings) of selected experts where the most up-to-date information available in the public domain was discussed. We then captured this information in summary reports (two per year) called EONS (European Observatory on Nanotechnologies) reports that were widely circulated.

2.5.3 EU-US Relation

We established the sharing of the dispersion protocols in the early part of the project. We also conducted some essential joint experiments for calibration of measurement methods. Most importantly, our US partners have supplied us with their experimental data for QSAR modelling. This arrangement has been very important for ENPRA and to further the ENPRA approach the US EPA has incorporated the ENPRA dataset and uses it within their TOXCAST programme.
Of importance to the EU-US relation is also the modelling to support Risk Assessment. The collaboration has yielded fruitful results in area such as Exposure Modelling, PBPK and Uncertainty Analysis. These are important tools to be used in a common Risk Assessment process in the EU and US.
Finally, for the duration of ENPRA, the results have also been presented at important events in America both at international events but also at special events arranged by our US partners. In the final stage of the ENPRA project, all the results from the experimental Workpackages have been brought together for the ENP Risk Assessment.
ENPRA will provide a comprehensive database on the characteristics and toxicity of the tested ENP based on in vitro and in vivo models. The protocols for ENP characterisation, dispersion and dose-response will also be made available to other researchers. The key in vitro experiments have been chosen for a round-robin validation test, which will add extra reliability to the use of these models for hazard assessment.
The data generated by the experiments in ENPRA were also used to construct mathematical models of exposure-dose-response, which will be used for extrapolation to other exposure levels and ultimately extrapolation to humans. A QSAR model was also developed and used for assessing the relationship between the ENP physico-chemical properties and the toxic responses. All the ENPRA results were collected to form a Risk Assessment Framework for ENP.
The potential impacts of ENPRA on research into NANOSAFETY are of importance to worker and consumers health. In ENPRA, we have implemented a rigorous dissemination strategy to raise awareness of the results of ENPRA and their implication to major stakeholders such as government regulators, researchers and worker/consumer protection organisation.

ENPRA has also a direct impact in the areas of the EC action plan:

1. Research, Development and Innovation play a decisive role in ENPRA, which deals with the potential impact of nanomaterials and ENP on human health and the environment. It performs targeted studies and develops corresponding methodology as well as an information hub for collection and use in a targeted manner for safety and risk assessment and as a basis for knowledge application and innovation.
2. ENPRA addresses the integration of societal dimensions, expectations and concerns. By definition, concerns are among the most important drivers for the scientific analysis and developments performed practically in this project. Expectations and concerns are examined with regard to possible underlying mode of actions and possibilities to address related effects at the earliest possible stage.
3. ENPRA contributes to address health and safety issues or at the earliest possible point in order to improve assessment methodology and subsequently safe and responsible use of ENP. It thereby covers the items brought forward by the SCENIHR in its reports 2007 and 2009, the issues reported by the European Parliament in its motion dated April 2009, and implements an integrated programme to prepare for appropriate methodology for risk and safety assessment including instruments for risk governance. It provides best practice information on terminology, guidelines, measurement systems, model systems, validation of assessment methodology, integrated testing approaches and risk assessment taking into account the full chain of actors and lifecycle. It recommends adaptations of methodology according to identified science-based nano-specific requirements taking into account the potential of emerging nanotechnology towards sustainable use.
4. ENPRA operates in transparent communication with developed countries in direct exchange to guarantee relevance of the developments made, a critical mass and coherence of progress together with international activities, such as the OECD WPMN sponsorship programme and the activities in the US with direct involvement of participants from these countries. It takes into consideration all needs to avoid a nano-divide and grants access to knowledge, such as to hub. The hub will be hosted by the European Commission at the Joint Research Centre-Institute for Health and Consumer Protection (IHCP). Access is freely available for use by all stakeholders and bears full ability to exchange data.

List of Websites:
Project public website address: http://www.enpra.eu

Related information

Result In Brief	Risk analysis toolbox for nanoparticles

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