A common European approach to the regulatory testing of nanomaterials

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Time wise, the NANoREG project is now halfway. After setting the basic conditions for its R&D work, the project now focuses on the generation of reliable and comparable experimental data on the EHS aspects of the selected NANoREG nanomaterials. These data will form the basis for the main “end products” of the NANoREG project: the Regulatory Framework and the NANoREG Toolbox. Highlights of this experimental work and results will be shared with you in this 3rd NANoREG Newsletter.

The Regulatory Framework and the NANoREG Toolbox just mentioned will be developed in close cooperation with organisations involved in standardisation and in the regulatory aspects of nanomaterials like ECHA, OECD, CEN and ISO. The results of other EU FP7 and H2020 projects will also be taken into account when developing these products. One of these projects is the H2020 project NANoREG II that focuses on Safe by design and that will start in the 2nd or 3rd quarter of 2015.

The coordinated and integrated approach in developing the Framework and the NANoREG Toolbox is one of the main elements of the H2020 funded Coordination and Support Action (CSA) “ProSafe” that recently had its Kick-Off meeting in Aix-en-Provence, France. Just like NANoREG this CSA is coordinated by the Dutch Ministry of Infrastructure and the Environment and as such executed by me. Other elements of this CSA are - among others - the expansions of the involvement of EU and non-EU countries in the NANoREG project in order to broaden the platform of support for the NANoREG results world-wide (“NANoREG+”), the exploitation of synergies between the NANoREG project and other “nanosafety” projects and data management.

The outcome of the CSA will be a White Paper that can be used by policy makers, regulators and industry to establish methods for measuring and assessing the EHS aspects of nanomaterials and that will give guidance to industry how to implement “safe by design”. A forerunner of the White Paper will be subject of a three days scientific conference to be held at the end of 2016. It will include the results of the NANoREG project, the results of the evaluation of EHS data available at the OECD and results from other sources. After consulting Risk assessors and policymakers, the White Paper will be published in the first quarter of 2017.

It is quite a challenge we face. Given the expertise and scientific authority of our partners, including the Czech-, Brazilian- and South Korean parties that recently joined the NANoREG project, I am confident however that we will succeed in reaching our goal: creating a solid basis for a balanced combination of nanosafety and innovation that will be beneficial to society.

Tom van Teunenbroek
Coordinator NANoREG and Prosafe
On the 17th to 19th September 2014, the OECD (Organisation for Economic Co-Operation and Development) in collaboration with the US Environmental Protection Agency (US EPA) held an Expert Meeting on Categorization of Manufactured Nanomaterials.

The Meeting aimed to analyse various categorization strategies in a risk assessment framework and discuss these in close collaboration with the OECD Working Party on Manufactured Nanomaterials (WPMN) Steering Groups and invited international experts in physical-chemical characterization, fate, exposure, ecotoxicity, human health toxicity, exposure assessment, risk assessment and risk management.

The goal of the meeting was to provide recommendations on how manufactured nanomaterials should be categorized for purposes of testing, read across/ Structure-Activity Relationships (SAR), risk assessment and risk management.

Focus areas for this Meeting addressed the full range of risk assessment-relevant endpoints: the Meeting initially explored the ‘context for need for the use of categories, and perspectives on their application to nanomaterials’ from the point of view of different jurisdictions (i.e. USA, Japan, Canada, Europe), and then discussed detailed scientific aspects of categorisation and assessment approaches, such as physical-chemical characterisation, fate assessment, ecotoxicity assessment, in-vitro testing, human health assessment, release assessment and exposure assessment. Each aspect was subsequently analysed and concluded upon by expert breakout groups with regard to the relevance of categorization.

The Meeting concluded and agreed on the following recommendations:

**Recommendation:** Discussion and conclusions from the workshop to be used to develop fit-for-purpose decision frameworks for categorization that can be utilized under different regulatory systems for manufactured nanomaterials.

To support this, the workshop recommends:

- That we identify and develop, where needed, methods for characterization of relevant phys-chem properties for toxicokinetics, fate, hazard and exposure assessments.
- Use of methods which enable comparability, are reliable, and use the OECD Guidance on Sample Preparation and Dosimetry.
- Agreeing on or developing experimental models (e.g., in-vitro and in-vivo assays) which are predictive of human health and environment effects and support categorization.

**Acknowledge** tools and methodologies for categorization might be different for the different parts needed for the assessment of nanomaterials.

**Acknowledge** that definitions and terminologies need to be clarified and consistently applied.

**Support** adapting existing approaches for conventional substances to fit specificities of categorization frameworks for manufactured nanomaterials.

**Support** case-studies which inform categorization schemes as they are developed and refined.
Summary of the evaluation results of the 10 OECD protocols

Physico-chemical characterization of manufactured nanomaterials requires the development of cost-effective standard methods, detailed protocols and reference materials for calibration and analysis of both pristine materials and materials in relevant media or complex matrices. In these months, the following ten OECD test guidelines (TGs) are under revision by WP2 in Task “NM characterization SOPs for regulatory purposes”: TG109; TG110; TG106; TG105; TG115; TG107/117/123; TG112; TG108. This work is related to key questions from regulative authorities with the aim to identify an intelligent characterization strategy for risk assessors within the context of regulatory toxicology.

Size is a key factor in determining the potential toxicity of nanomaterials, therefore particle size determination is crucial for understanding their biological effects. TG 110 reports two methods for determining particle size distribution and fibre length and diameter distributions. This protocol needs to be modified in order to satisfy nanomaterial requirements. In particular, two set of experiments on determination of primary particle size (electron microscopy) and aggregate size (e.g. dynamic light scattering or centrifugal liquid sedimentation) are required.

Accurate in vitro dosimetry is essential for the development of cost-effective toxicological screening methods for nanomaterials. An important key point to accurate in vitro dosimetry is the characterization of sedimentation and diffusion rates of nanoparticles suspended in culture media, which largely depend upon the effective density and diameter of formed agglomerates in suspension. Among methodological approaches reported in TG109, the only appropriate technique for measuring nanomaterial density is helium pycnometer.

Solubility is an important parameter of nanomaterials, strictly related to the other physico-chemical properties such as chemical composition and size. In this task, solubility has been defined as both dispersion stability and nanomaterial dissolution. TG 105 is only useful for determining ion leaching from nanomaterial particles, while a definition for dispersion stability and a protocol to measure it need to be developed. Linked to solubility is the determination of dissociation constant in water as described in TG 112. This protocol is thought for solutions and not for colloidal suspensions, therefore a new protocol needs to be proposed and tested. Same considerations have been done for TG 108 on the ability of a substance to form complexes, taking into account that nanomaterials have multisite complexing entities.

After evaluation, TGs 107, 117 and 123 regarding the classification of chemical substances as hydrophobic or hydrophilic have been considered useless for nanomaterials. Adsorption/desorption test guideline (TG 106) is used for generating essential information on the mobility of chemicals and their distribution in soil, water and air compartments of our biosphere. This protocol is crucial for understanding environmental fate, and determining the most suitable approach for hazard assessment of the sediment and soil compartments. Several scientific models, such as USETox and EUSES, have been developed to characterize human and ecotoxicological impacts of chemicals in life cycle impact assessment. Unfortunately, these models are based on octanol-water partitioning coefficient (TG 107, 117 or 123) and then not applicable to nanomaterials in general. Measurements of surface tension using TG 115 have been considered applicable only to some nanomaterials, as described in RIPO-N-2.

In conclusion, most of TGs need revisions to be suitable for nanomaterials. In the next months, revised and new SOPs will be elaborated and presented.
Outstanding results from NANoREG’s scientific work packages

Report on the most relevant results obtained within WP 2 - 5 activities

NANoREG delivers adequate answers to demand driven questions. In particular, the NANoREG project aims to give an answer to regulators and legislators on Environmental, Health and Safety aspects of nanomaterials by linking regulatory questions and needs to a scientific evaluation of data and test methods.

The most important regulatory questions identified by consultation with regulators and gap analysis within WP1 belong to three general knowledge gaps:

- characteristics that influence the risk of NMs in the environment and humans
- standardized methods to determine these characteristics
- nano-specific risk assessment strategies and approaches.

From these three main gaps, sixteen regulatory questions and needs were generated. They are related to measurements, characterization, transformation, dose metrics, grouping, persistence and long-term effects, kinetics, mode of action, hazard, exposure, risk assessment and management and health surveillance.

The scientific work packages 2 to 5 are carrying out research to fill in the gaps, so providing scientific answers to regulatory questions and needs. The most relevant results obtained within these work packages are reported herein.

The first regulatory question concerns the identification of nanomaterials according to the European Commission’s recommended definition requires a new and well-defined methodological approach. The core of the definition relies on the measurement of particle number size distribution. Instrumental methods have to be developed and adapted within Task 2.2 “Identification of MNM according to the EC regulatory definition”.

A round-robin test is currently ongoing to validate this method and determine its reproducibility. Once validated, the standard operating procedure can directly address the key regulatory question No.1 on Measurement and characterization – Identification. In particular, a standard operating procedure will be used for identifying nanomaterials according to the EC recommendation.

Within work package 2 “Synthesis, supplying and characterisation” – Task 2.3 “NM characterisation SOPs for regulatory purposes” – OECD test guidelines have been theoretically evaluated. This activity has been performed for answering key regulatory question No.2 Measurement and characterization concerning the definition of an intelligent characterization strategy. Among the OECD test guidelines that have been taken into considerations (around 10), only OECD test guideline 109 concerning relative density has been considered suitable for measurements on nanomaterials using helium pycnometry, while the other test guidelines need modifications for use with nanomaterials or are not relevant for nanomaterials.

Three key regulatory questions – No. 11, 12 and 13 – are related to exposure throughout the different life cycle stages of nanomaterials. To answer these regulatory questions, Task 3.1 “Identification and elaboration of exposure scenarios” focused its activities on the identification and elaboration of exposure scenarios, generating input for Deliverable 3.1 “Gap analysis report, identifying the critical exposure scenarios within the key value chains”. This Deliverable contains the inventories of European production volume, hazardous properties, applications and exposure routes of (selected) nanomaterials; the identification of relevant data to support the development of the exposure scenarios; the identification of critical life cycle exposure scenarios; and identification of data gaps. This activity highlighted the lack of information on effective quantities of nanomaterials in circulation, representing
one of the major problems in assessing possible risks to human health and the environment. The deliverable will contribute to the prioritization of the exposure potential by considering the processes that are conducted across the life cycle stage of the target nanoparticles, identifying common uses and generic exposure scenarios.

WP1: Scientific answers to regulatory issues

As can be seen from its full name and mentioned in the editorial note, NANOReg aims to link regulators, stakeholders and the scientific community to analyse the applicability of current testing and assessment methodologies to manufactured nanomaterials (NMs). This should allow producing a Framework and a Toolbox that are useful both for the regulators and for the producers and contain the instruments for a meaningful assessment of NMs. Such Framework and Toolbox are the main objects of the work of WP1.

To this end, the first step taken (Task 1.1 “Refinement of problem identification and formulation of questions and requirements, including interaction with stakeholders”) was generating a collection of regulatory relevant questions (on the basis of a gap analysis on the data and tools which are currently available, Task 1.2 “Gap analysis”) that need to be answered by the scientific work packages to accomplish a reliable safety assessment of NMs in the regulatory context.

Using as a basis the results of previous projects (e.g. the RIPoN3.2 and 3.3), the recommendations of the EU Scientific Committees and opinions of several national and international regulatory agencies and competent authorities, a consultation via the project’s National Coordinators allowed to eventually identify a set of sixteen questions or areas where scientific input was needed. Questions originate from issues that the regulatory authorities are facing when assessing the safety of nanomaterials and deciding on risk management strategies. Those questions also define the project’s desired outputs and ensure that NANOReg stays focused, since they constitute the backbone for the organisation and tailoring of the scientific work in the project. Indeed, the project tasks and sub-tasks have been mapped against the topics of the regulatory questions in Task 1.3. A second iteration process for the continuous update of the regulatory relevant questions has just started under the supervision of WP 7.

The outputs from the scientific work packages, together with considerations on the existing applicable regulatory framework for safety assessment of NMs, are going to be used to generate the NANOReg framework (task 1.4 “Framework development”) and the related toolbox (task 1.7 “NANOReg Instruments Toolbox for regulators and legislators”) and will be integrated in an information platform. A first backbone for the framework has already been compiled by the task leader and is being discussed with the task partners and other project partners whose inputs are considered to be relevant.

WP2: Synthesis, supplying and characterisation

WP2 is currently in the process of including the new partners from South Korea and Brazil who will both contribute significantly to WP2. In the past period AIDICO left the project who was one of our key major partners in the project. Besides these structural changes, all support facilities have been established and WP2 scientific work is now in midterm process.

In Task 2.1 “MNM synthesis and procurement” a newly-established nanomaterial subsampling facility has become fully operational at the Joint Research Centre in Ispra (Italy) and the production of new sub-samples (NM vials) from both existing and new nanomaterials is on-going. New high-quality tailored fluorescent silica nanoparticles with negative and positive charge have been produced and were shown to be stable after test-item preparation using the NANOReg dispersion protocols. These silica nanoparticles will be used in task 5.3 „The relevance for barriers“ for the tracking of nanoparticles through the epithelial barrier in vitro. Procurement of graphe-
ne is still in process under lead by TCD. In task 2.2 “Identification of MNM according to the EC regulatory definition” the major activities have been establishment of the working protocols for size-distribution analysis by TEM and determination of the volume-specific surface area in response to the recommended EC definition of nanomaterial. Implementation of the protocols is currently in process in several laboratories. In addition collaboration agreements have been established between NANoREG and the NANODEFINE project. For regulatory substance identification and categorization of nanomaterials, a major review on the existing nomenclature as well as criteria has also been completed. A revised system is under development to enable differentiation between different nanomaterials and in particular nanomaterial of the same composition.

In task 2.3 “NM characterisation SOPs for regulatory purposes”, the variations in quality and characterization methods and requirements for CNTs is under review using the NANoREG and OECD WPMNM nanomaterials as a starting point. The entire suite of CNT types are not available as part of the project. It is, however, evident that significant variations in chemical and structural compositions occur in the different products and it appears difficult to consider event structural material sub-classes, such as SWCNT and MWCNT, as a single substance from scientific point of view. CNT and inorganic materials has also been used to establish procedures for identification and quantification of chemical functionalization.

In task 2.4 “Test item preparation, exposure, dose and fate for regulatory purposes and toxicology”, WP2 has reached a major milestone in testing and documentation of the in NANOGENOTOX (in vitro and in vivo), ENPRA (in vivo) and NANoREG Ecotox (ecotox) nanomaterial dispersion protocols. The results will soon be offered as benchmark values for dynamic light scattering analysis (DLS) of batch dispersions within the project. Finally, work is in progress to demonstrate different methods and procedures to test and document the ROS-formation capacity and redox activity of nanomaterials, their protein-interaction as well as dissolution/biodegradability in cell mediums and biologically relevant liquids. Regarding dissolution testing a WP2 and WP5 (“Advancement of Regulatory Risk Assessment and Testing”) review collaboration has showed that no universal method exists. Although some techniques are more commonly used than others, a huge research gap remains, related with the need to ensure data reliability.

All in all, WP2 work is in good progress, but significant effort is needed to get the WP back on track after initial delays in materials availability and lately some restructuring of the partnerships.

Representative TEM image of monodispersed fluorescent nanoparticles.

Example of the very bright and stable signal generated by the IIT fluorescent silica nanoparticles. The nanoparticles are suitable for tracking uptake of nanoparticles across cellular membranes in vivo.
WP3: Exposure through life cycle analysis

It is very important to adequately characterize the release of MNMs in the workplace and during later stages of the life cycle of a MNM-containing product. To this end, CEA (partner 23) took the lead in reviewing available standard operating procedures for dustiness and material aging testing.

These procedures include approaches:

- to simulate the use of products (i.e. textiles washing; polymer aging in water; paints, coating and nanocomposites sanding; paints and coating environmental aging) => simulation approaches for consumer exposure and to evaluate the nanoparticle release to the various relevant environmental compartments (air, water and soil)
- to evaluate nanoparticle release rates for processes placed under fume cupboards and hoods => simulation approaches for occupational exposure
- to generate controlled aerosol from nanomaterials using the shaker method (fibrous nanomaterials such as CNT and CNF; granular bio-persistent particles) => generic method to evaluate dustiness and to simulate emission to indoor air
- to evaluate dustiness indexes of nanomaterial powders

The instrumentation to implement these protocols is available and operational at the different partner institutions and will be used to generate data on occupational exposure, consumer exposure and environmental exposure on actual products.

Assessment of the efficacy of risk management measures toward nanomaterials

In occupational settings, the control of exposure is a relevant part to establish a safe environment for workers. Risk management measures (RMM) are also an important element of exposure scenarios in REACH, demonstrating the safe use of a product in well-defined conditions. If conditions do not allow the safe use, RMM can be implemented identifying additional protections require for reducing the exposure. While for traditional chemicals the RMM efficacy is well known, the same is not so for nanomaterials. There is the need to measure the ability of available RMM to reduce the exposure to nanomaterials, by using experimental data and agreed protocols.

Within Task 3.5 “Effectiveness of risk management measures” of WP3, ITENE carried out a systematic review of the literature and protocols addressing the efficacy of the RMM on nanomaterials. This work allowed identifying criteria to evaluate the performance of different type of RMM (performance factors) such as local ventilation, organizational measurements, and personal protection equipment.

Diagram of a typical sanding experiment on nanocomposites
Examples of evaluation criteria are: capture efficiency (ventilation), barrier efficiency (e.g. gloves), total inward leakage (respirators).

Taking into account the available literature and existing standards used to assess RMM effectiveness, 10 standard operative procedures were developed for key RMM. The procedures indicate operatively and in detail how to carry out the measurement, from the required equipment, to the nanoparticle exposure conditions (e.g. nanoparticle generation conditions), to the method for measuring the performance factors. Moreover, the work clearly describes conditions for applying the protocol. These protocols can be used by manufacturer and users to evaluate the effectiveness of existing and new RMM, allowing a standardized approach and comparable results.

On a similar line, INRS evaluated experimentally the efficacy of ventilated enclosures with respect to airborne nanomaterials. On the basis of their extensive experience, and using an INRS verified method, the release of nanoparticles from a pollution source placed inside a fume hood was measured and modelled by developing a time-related mathematical relation. This work will be verified in real conditions, leading to the development of a specific operative procedure, to be added to the already developed SOPs.

**WP4: Biokinetics and toxicity testing in vivo**

**Task 4.5.5 90-day oral toxicity study in rat with SAS nanoparticle (NM-203)**

Dpt. Veterinary Public Health and Food Safety Istituto Superiore di Sanità, Rome Italy

To be able to perform a chemical risk assessment, information of the profile of toxicity after longer-term exposure is essential. A 90-day oral toxicity study has been considered the minimum requirement to obtain a reliable risk assessment of nanomaterials relevant for food safety. It provides a resilient basis to derive threshold values for consumer uses. Nanomaterials can enter the food and feed chain as contaminants through several processes (e.g., waste disposal, anthropogenic or natural sources) generating concern for human health. Furthermore, some nanomaterials are used as additives in food products. For instance, amorphous silicon dioxide is used as flow agent in powdered foods.

The aim of the task 4.5.5 “Repeated-dose 90-day oral toxicity study” in NANoREG WP4 is the identification of putative hazards of amorphous silicon dioxide after oral application according to the specific requests of the European Food Safety Authority. In this respect, the data of a study performed according to the 408 OECD guideline can provide support for regulatory purposes.

General toxicity showed no meaningful differences among groups in body weight gain, feed consumption and absolute/relative organ weight in both sexes.

Concerning clinical biochemistry, no effect was recorded in liver; in kidney – target tissue for SAS excretion - creatinine was significantly altered in males in higher dose groups and in females in all treatment groups. Tissue histopathological and biodistribution analyses will help to clarify magnitude and mechanism of the effect.
No alterations in T3 serum levels were detected in both sexes. TSH serum level analysis is in progress and together with evaluation of thyroid tissue, will contribute to put into evidence potential thyreotoxic effects.

Moreover, to obtain a comprehensive picture of reproductive endpoints, estrogen and testosterone serum levels, analyses of epididymis and sperm, histopathological analysis of reproductive tissues are in progress. However, treated males did not show impaired mating ability at all doses.

Preliminary immunotoxicity endpoints - proliferative response of spleen and lymph node cells to PHA mitogen, inflammatory response by peritoneal macrophages (NO production after LPS stimulation), blood count - showed an overall picture of gender differences and the difficulty in establishing both a critical dose (effective in different parameters) and a dose-response relationship. Interestingly, a significant reduction in the numbers of circulating white blood cells, in particular lymphocytes and granulocytes, was observed in both sexes.

Genotoxicity data showed spotted increases of DNA damage in bone marrow of male rats and ovary cells. Moreover, a slight but significant dose related increase of DNA damage in spleen cells of male rats was observed. Data of DNA damage from several organs (intestine, bone marrow, spleen, kidney and liver) of female rats have also been collected but statistical analyses are in progress.

Overall the preliminary data show no marked general toxicity in both sexes at the selected dose levels, although gender-specific differences both in immune and biochemical endpoints are observed. As commonly reported with nanomaterials, this study highlights again the difficulty in identifying a clear dose-response relationship for key endpoints, which would help for risk assessment of SAS.

The genotoxicity endpoints will be evaluated in collaboration with: Agenzia Nazionale per le Nuove Tecnologie, L’energia E Lo Sviluppo Economico Sostenibile (ENEA – partner 18), Italy, Agence Nationale De Securite Sanitaire De L’alimentation, De L’environnement Et Du Travail, France (ANSES – partner 35); Dept. Environment and Primary Prevention, Istituto Superiore di Sanità Rome Italy (ISS - partner 17).

The reproductive toxicity endpoints will be evaluated in collaboration with: University of Tor Vergata, Rome Italy

The immunological and inflammatory parameters will be evaluated in collaboration with the Dept. of Infectious, Parasitic and Immune-mediated Diseases Immune-mediated Diseases Unit - Istituto Superiore di Sanità Rome, Italy.

**WP5: Advancement of Regulatory Risk Assessment and Testing**

The work package 5 achieved significant results in the following areas:

The combination of ultrafiltration (UF) followed by analytical measurement of the concentration of elements (e.g. ICPMS, AAS etc.), which had hitherto been regarded as one of the easiest accessible and likely one of the most robust methods to study solubility, was shown to highly depend on the material that is being tested and on the matrix it is tested in. Complex formation of the soluble fraction with the matrix will give a misrepresentation (i.e. underestimation) of the soluble fraction. Thus, when measuring in complex matrices a complementary approach to measurement appears to be highly recommendable.

Preliminary results on potential penetration in oral mucosa model were obtained with spherical TiO2 NPs and NanoREG NM200, NM300k, fluorescent SiO2@IIT 50nm (+), fluorescent SiO2@IIT 50nm (-) and NM203 NPs. The suitability of the airway epithelium model was investigated by evaluating the penetration of a commercial CeO2 NM. Further studies will include 4 core NanoREG NM (NM100, NM101, NM212 and NM220).
Regarding the Blood Brain Barrier model (BBB), the suitability of hCMEC/D3, bEnd.3 and a primary microvascular bovine model was assessed for NM permeation and toxicology assessment. Following preliminary results, the hCMEC/D3 endothelial cell line and bEnd.3 cells were selected as both have been extensively characterized. Further studies are currently under way with these cells both grown as monocultures or as co-cultures supported by primary astrocytes with NM100, NM101, NM212 and NM220.

The stress-testing of in vitro inhalation test methods resulted in a preliminary indication of a greater sensitivity of co-culture compared to the monoculture and higher cytotoxicity and inflammatory effects in cells exposed to aerosol (max. dose deposited: 2.65 µg/cm² in 3h), compared to cells exposed to suspension.

When dealing with the use of traditional in vitro assays to assess NM toxicity initial experimental studies on CNT and CeO2 showed that biodegradation of CNT by LPO (lacto peroxidase) was not affected by the presence of a bio corona due to lung surfactants; these results were observed both in vitro and ex-vivo in murine cell-free BALF. However, the antibacterial activity of LPO may be compromised as a result of the pre-occupancy of LPO with CNT.

When toxicity to Ag was assessed by exposing cells to different sizes and coating of Ag, results showed cytotoxicity only for the 10 nm particles independent of surface coating (citrate vs PVP). In contrast, all Ag NM tested caused an increase in overall DNA damage after 24 h assessed by the comet assay, suggesting independent mechanisms for cytotoxicity and DNA damage.

Pre-screening toxicity experiments to assess viability and genotoxic potential of several core NMs have been achieved for alveolar and bronchial cell lines.

Immunotoxicity studies have provided further insights into the way immune cells handle NMs. Regarding cell transformation assays, the HBEC cell line has been distributed between all partners. Toxicity of positive controls (MNU and B(a) P) and NM401 are being carried out before starting the 4 week treatment.

The stress-testing of different High-Throughput and High Content Screening (HTPs/HCSs) methodologies regarding their applicability to the testing of nanomaterial toxicity, it became clear that the bottleneck for HTP screening is dispersion. Nevertheless, experiments started and first data have been obtained.

The intracellular CeO2 NP concentration and their localization in lung tissue have been measured with Ion Beam Microscopy (IBM) in rat lungs obtained from the 28 day study on inhalative toxicity and carcinogenicity by BASF. CeO2 NPs were almost exclusively found in alveolar walls, but were absent in the mucosal linings. CeO2 NP showed a rather inhomogeneous distribution observing “hot spots” with Ce concentrations being

![Fluorescence labeled NP internalization into colon cancer cells](image)

Further preliminary experiments included a sub chronic toxicity study covering 90 days of exposure using a 3D airway epithelial model with a commercial CeO2 NM. Significant changes in cell culture viability were recorded at 0.1 mg/cm² after day 80 using the Resazurin and LDH tests. Regarding inflammation, a significant increase in TNFα and IL-1β was also observed at 0.1 mg/cm² CeO2 after day 75.
ten times larger than on average. The average CeO2-concentration in single alveoli was found to be about 1270 ppm, which is comparable with the P or S cellular element concentrations. “Hot spots” concentrations in sub-acute study should be related to inflammation and carcinogenicity as a predictive tool. In vitro culture cell experiments in A549 cells revealed that an applied dose of 10 µg/ml CeO2 NPs lead to a toxicologically relevant intracellular dose comparable to the hot spot concentrations in in vivo experiments. This quantitative comparative in vitro – in vivo study by means of IBM thus provides a bridge between cell culture and animal experiments on the basis of quantification of intracellular doses. IBM was proved to be a successful tool for visualization of two-dimensional distributions of a broad spectrum of nanoparticulate elements as well as for quantification of uptake and internalization of NPs in tissues and culture cells.

NM100, NM101, NM200, NM203, NM300K, and NM302 were used for impedance based measurements with the AMPHASYS 30 and xCEL-Ligence instruments - for A549, SAOS2 and primary gingival fibroblasts. The afferent DLS measurements are completed. A series of experiments with the NANoREG nanomaterials was performed and for the HTS additional negative and positive controls were included such as nanodiamonds and NH2 polystyrene nanobeads.

WP6: Keeping pace with innovation

Safe-by-design, an update

The safe-by-design concept is now gathering momentum amongst scientists, regulators, and industry for use in the development of manufactured nanomaterials. The turn towards applying the concept of safe by design to the development of nanomaterials has been encouraged by some of the significant challenges facing regulation as a means of guaranteeing safety in this field. In addition to its development and use in construction and engineering sectors, this concept has also had a long history of successful deployment in the domain of drug development. In early phase drug development, new chemical entities are screened in parallel for both their efficacy and their potential for toxicity. High throughput testing in this type of research serves the question of whether benefit-risk ratios will likely to be positive. Such an approach might therefore include relevant building blocks for the uptake and development of the safe by design concept for MNMs. Turning this concept into effect will, however, be a significant challenge.

It should be kept in mind that in drug development these types of tests have a kind of guiding function, leading to the most optimal functionality-efficacy-toxicity combination. However, full verification for drugs has to be proved by extensive in vivo testing in experimental animals and in humans. How such methods can be used for chemicals or MNMs used in consumer products is one of the topics addressed in NANoREG in the Work package 6 “Keeping pace with innovation”.

While regulation plays an ongoing important role for ensuring product safety, within the approach of responsible innovation, considerations of safety questions are also moved further up in the innovation chain. Here they become integrated into the research and development process itself, e.g. through the operationalization of concepts such as safe by design.

Our approach of the Safe by Design concept is development of new products where functionality and safety are tested in an integrated way through the development process phase. This integrated approach demands multi-disciplinary collaboration, knowledge and resources. Moreover, during the development of this approach several aspects have to be addressed, for example i) the motivation for different parties to participate, ii) the role of trust, and iii) whether this can be a self-regulating system or whether leadership or responsibility is expected from a stakeholder. These process-oriented sides deserve more attention in Safe by Design projects, because it is a prerequisite for Safe by Design structurally being viewed as a concept rather than a project.

Within WP 6 of NANoREG, our approach to the concept of safe by design is:

- for all stakeholders to contribute to a process...
in which public health and environmental safety should be considered at all stages of the innovation process

- for there to be a good interaction between risk-based research and product development (innovation)

- for innovation processes to be able to specifically adapt design factors to take safety aspects into account

The toxicity screening processes for drug development and for development of chemicals (with a specific eye for MNM) have been compared for common goals and common roles in a product development process. Next steps planned will focus more in-depth to parameters and methods specifically required to address safety of MNM at various stages of innovation.

Contact: Cornelle Noorlander, Institute for Public Health & the Environment (RIVM), The Netherlands, cornelle.noorlander@rivm.nl

**WP 7: Liaisons, Dissemination, Exploitation and Communication**

*Report from the ECHA October 2014 Workshop on the Regulatory Challenges in Risk Assessment of Nanomaterials*


The workshop, held in Helsinki Finland was attended by about 130 participants, mainly from EU, but also from Canada and US. Several NANoREG partners attended the workshop, including e.g. BASF, JRC, KI, NIA, NRCWE, ITENE and MIN (NL) to mention a few. Several NANoREG partners were also members of the Scientific Committee supporting ECHA in the preparation of the programme.

The workshop was chaired by Wim de Coen, head of unit, Executive Office. There was also a poster presentation that was well attended during the workshop breaks. The two days were divided into five topics:

**Topic 1: Introduction to international views on scientific challenges in regulatory risk assessment of nanomaterials**

The presentations in this session set the scene for the workshop by providing an overview from an EU and a joined US/Canadian perspective based on the US/Canada Regulatory Cooperation Council (RCC).

**Topic 2: Measurement and characterization of nanomaterials**

The presentations in this session discussed some of the key challenges faced by regulators, industry, and other stakeholders in characterising nanomaterials. The focus was on characterising nanomaterials for the purpose of identification, in situ and during release over the lifecycle.

**Topic 3: Metrology and dose metrics for hazard and exposure assessment throughout the life cycle**

This session discussed the scientific challenges related to the metrology and dose metrics when dealing with the safety assessment of nanomaterials. The concept of “dose metrics” specifically addresses the particle based characterisation of nanomaterials. Topics included key parameters that may influence hazard properties and identification of the most suitable metrics describing exposure and the possible bridging principles between different metrics.

**Topic 4: Environmental fate, persistence and bioaccumulation throughout the life cycle**

This session presented an overview of the state of the art on environmental fate assessment of NM and discussed testing strategies to assess environmental fate of NM in regulatory context. Advances and challenges in measurements and modelling of environmental fate and testing strategies for regulatory purposes were presented.
Topic 5: Read across and categories of nanomaterials

Read-across and grouping of substances are valuable approaches used to predict specific properties of substances for which there is insufficient experimental data. The possibilities, limitations and prerequisites of read-across and grouping approaches for nanomaterials in a regulatory context were discussed. A comparative short term inhalation study of nanomaterials was presented and physicochemical properties as predictors for hazard were discussed.

The Scientific Workshop closed with a wrap-up and conclusions where this all fits in the bigger picture for ECHA’s future activities in relation with ECHA’s working groups on nanomaterial issues.

- The presented characterisation approach of combining the volume specific surface area with electron microscopy is seen as an elegant tiered approach. The ability to define when a material is not a nanomaterial is important, but it is recognized there will be grey areas.

- In relation to release, it is important to consider both the material and the release processes.

- Metrology and dose metrics are both critical issues. ECHA recognises the importance and use of different metrics.

- Benchmarking controls to enable comparative hazard analysis is useful, but the prioritisation for ECHA is very much towards characterisation as this is the basis of everything.

- ECHA believes in the current environmental fate descriptors. In case there is evidence that these are not relevant or appropriate for nanomaterials, then ECHA can amend this (for specific materials, specific environmental factors, etc.).

- New models bring new challenges and these need to be validated and challenged, e.g. the issue of background measurements need to be met.

- Knowledge on the mechanisms of toxicity will help to build groups and justifications for read-across, etc. In vitro models will play a role here, but it is important to know their limitations.

ECHA recognises the issue of the relevance of current test concentrations and what this can mean for effects, both on environmental and human toxicity. There is a possibility that we are missing something at lower doses.

In general ECHA still sees a lack of useful data, which are crucial to gain and improve insight in behaviour and toxicological mechanism and risks. Within REACH only 13 registrations have been seen on nanomaterials up till now. In the context of read-across for REACH there is high urgency to get all useful data. A breakthrough is needed with regard to this point. Therefore, ECHA invites the research community to come up with new or improved methods as well as standardised protocols and reference materials. ECHA emphasises that the standard risk assessment tools are mostly appropriate for nanomaterials, but careful use of them is needed, in particular where media effects, ageing, etc. become important.

All the presentations from the workshop, the agenda as well as a background document prepared for the meeting are available via ECHA workshop website. Proceedings from the symposium are under preparation and are foreseen to be published in the first quarter of 2015.
**EuroNanoForum - participation of NANoREG, SIINN, ProSafe, OECD, NanoDefine and NanoValid**

**12 June 2015, Riga Latvija**

**10:30 a.m. - 12:00 p.m.**

Joint seminar with:

- NanoSafety Cluster (introduction and results)
- SIINN (Final Conference)
- NANoREG (Mid Term Conference)
- ProSafe (Joint call)

**Introduction by the NanoSafety Cluster and Review Results**
(Speaker: Lynch Iseult, Uni Dublin)

**ERA-NET SIINN - Safe Implementation of Innovative Nanoscience and Nanotechnology, final results** (Speaker: Katharina Schumacher, Projektträger Jülich)

**NANoREG - Current status and trends in precautionary measures, Safe-by-Design and regulation - project mid-term information** (Speakers: Adrienne Sips, Karl Hoehener)

**Outlook ProSafe with first Call of common projects** (Tom van Teunenbroek)

**12:30 p.m. - 4:40 p.m.**

Joint seminar with:

- SIINN and NANoREG (New Frameworks and Guidelines)
- OECD (GD towards RA LCA of nano enabled applications)
- SIINN (Results of funded projects)
- ProSafe Call (Brokerage Event)
- NanoDefine and NanoValid (Complementary information and demonstrators)

**New Frameworks and Guidelines** (Juergen Hoeck, SIINN framework; Keld Jensen, NANoREG Guidance Document; Ingeborg Kooter, NANoREG Data base for safe-by-design)

**OECD Guidance Document on RA into LCA** (Achim Boenke (EC, DG GROWTH, Chemical Industry Unit I.2)).

**Overview and Networking of funded SIINN Projects** (Speakers: Katharina Schumacher (JUELICH) and Dina Carrilho (FCT)) - Part 1

**Overview and Networking of funded SIINN Projects** (Speakers: Katharina Schumacher (JUELICH) and Dina Carrilho (FCT)) - Part 2

**Brokerage for the first ProSafe call** (Dina Carrilho, FCT Portugal)

**Efficient characterisation and classification of materials according to the EC nano-definition:** The EU FP7 NanoDefine project (Speaker: Stefan Weigel (RIKILT))

**EU FP7 NanoValid project (263147) (2011-2015)** (Speaker: Rudolf Reuther (NordMiljö AB))

**Other events**

- Industrial oriented workshop May 18, 2015, Porto Salvo, Portugal

- 5th NANoREG Consortium Meeting, May 19 - 21, 2015, Lisbon, Portugal
  The first NICC meeting will take place as part of the Consortium Meeting on May 20th, 2015
NANOREG AT A GLANCE

Grant agreement number: 310584

Project start: March 1st, 2013

Project duration: 42 months

The NANoREG project is funded by the EU Framework 7 Programme with € 10.000.000

14 European states (EER) in collaboration with industry matched with 40 million resulting in € 50 million project

CONTACT

Project Coordination

Tom van Teunenbroek
Ministry of Infrastructure and the Environment
tom.van.teunenbroek@nanoreg.eu

Project Management

Aart H. J. Dijkzeul
Ministry of Infrastructure and the Environment
aart.dijkzeul@minienm.nl

IPR Management

Karl Hoehener
TEMAS AG
karl.hoehener@temas.ch