Towards a method for detecting the potential genotoxicity of nanomaterials

NANOGENOTOX Stakeholders workshop
3 May 2012

Synthesis report on stakeholders workshop
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WP2: Dissemination of the Joint Action

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Workflow	
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Summary

The aims of this workshop were to inform the stakeholders of the preliminary results and latest scientific progress of the Joint Action, to provide a forum for discussion between scientific partners of the Action and stakeholders and to share experts' knowledge and experiences about the critical issues specific for the genotoxicity assessment of nanomaterials in a regulatory context.
The dissemination activities, as well as the knowledge transfer of the final results of the Joint Action and the preparation of the final conference were also discussed during this workshop.
The workshop involved about 70 invited participants, including partners of the Joint Action, industries as well as industrial associations, national regulatory bodies (governmental, European Commission DG etc.), NGOs, and researchers from universities and national institutes.
The workshop started with the presentations of scientific results of the different workpackages. Each presentation was followed by an open discussion with the speakers and the audience was involved in an open dialogue about the session topic (presentations are available on the Nanogenotox web site www).
This plenary session was followed by a panel discussion with five speakers : Steffi Friedrichs (NIA), Andrej Kobe (EC, DG Environment), Hartwig Muhle (BUND), Aida Ponce (ETUI), Michael Riediker (NanoImpactNet). These speakers represented the main categories of stakeholders (EU risk assessors and policy makers, scientific community, professional federations representing companies, NGOs for protection of workers, consumers, the environment).

The discussion focused on two topics: first, on the technical and scientific aspects of the results, and second, on knowledge transfer and dissemination of these results (both on technical aspects but also regarding relevance for policy making) ; some highlights:

- issues associated with the presentation of the results particularly for the physico-chemical characterisation data as well as the availability to the public of the protocols and SOPs developed for the project are important;
- Regarding the *in vitro* experiments, main concerns and comments were on cellular uptake of the particles but also on the positive controls chosen in Nanogenotox in *in vitro* and *in vivo* assays;
- It was also pointed out that the genotoxicity mechanisms are still not clear and the links between inflammation and genotoxicity as well as the role of inflammatory cells need to be explored as this was not studied in the Action;
- Toxicokinetics studies are important to identify the target organs but questions were raised on the inhalation route and the consequence of the bolus administration (instillation is used in Nanogenotox), was discussed.
- Finally, it was necessary to remind the stakeholders that the aim of Nanogenotox Joint Action is to provide a method to evaluate potential hazard (i. e. genotoxicity) but not to conduct risk assessment and risk management. The method can later be taken up by regulators or other stakeholders for risk assessment purposes and the final conference will provide a good opportunity in that perspective as well as for the dissemination of various communication and information tools.

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Plenary session

Welcomme – Juan Piñeros Garcet, senior expert in nanomaterials and REACH (Federal Public Service Health, Food Chain Safety and Environment, Belgium).

Juan Piñeros welcomed the participants and highlighted the fact that nanomaterials are a very important topic for the regulator and that there is a need for methods which can be used for regulatory purposes. He indicated that there are many discussions at the EU level about the applicability of REACH for the nanomaterials and there are a lot of expectations regarding the results of this Joint Action (JA). The cosmetic regulation may also benefit from the results of Nanogenotox. Methods used for dispersion, characterization, *in vitro* and *in vivo* genotoxicity can also contribute to OECD guidelines. The JA will also address the applicability of the new nanomaterial definition. The methods developed in Nanogenotox can be used at national level. This workshop will provide a better understanding of the stakeholders' expectations which will be useful for the preparation of the final project conference and for the dissemination activities. Many regulators hope that this new method can be used as a screening test that could avoid unnecessary tests and also reduce animal testing.

As a regulator in health end environment, J. Piñeros expects that the project will also deliver insights on the limits of the method and conditions of its applicability.

Introduction - Nathalie Thieriet, Scientific Project Manager, Coordinator of the Nanogenotox Joint Action (ANSES, France)

Nathalie Thieriet reminded participants of the main objective of the Action: to establish a robust (specific and sensitive) methodology to assess the potential genotoxicity (i.e. inducing DNA damage) of nanomaterials and to generate data on the genotoxic effect of certain reference manufactured nanomaterials (MNs).

A brief presentation of the Joint Action was made on: budget, partners, work packages (3 transversal and 4 scientific work packages), work schedule, manufactured nanomaterials (MNs) tested [carbon nanotubes (CNTs), titanium dioxide (TiO_2) and Synthetic Amorphous Silica (SAS)] which are commercially available with widespread exposure for consumers and workers, etc. She mentioned the interactions with others projects such as ENPRA and NanoReg (a submitted proposal) and highlighted the relations with the OECD sponsorship program for testing of MNs.

WP4 - Physicochemical characterization of manufactured nanomaterials and exposure media – Jan Mast, Senior Scientist, Electron microscopy unit (CODA-CERVA, Belgium)

The main objectives of the WP4 are to obtain detailed physico-chemical properties of each nanomaterial used in the JA, to determine the influence of exposure media on nanomaterials dispersability and to identify the optimum preparation protocols for the specific nanomaterials.

Indeed, acceptable dispersion of test materials without changing particle toxicology is one of the first main challenges in nanotoxicology.

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The main tasks of the WP4 are:

- to test and develop suitable methods and standard operation procedures (SOPs) for analysis and characterization of nanomaterials and dispersions thereof,
- to determine the intrinsic characteristics of nanomaterials selected for toxicological studies,
- to test the homogeneity of the batches distributed,
- to develop, test and verify highly suitable nanomaterial dispersion protocols to be used in toxicity testing.

The planned work within the WP4 has progressed well, including the characterization of nanomaterials with different techniques (TEM, AFM, Raman, BET, ICP-MS, XRD, etc.) and the development of the dispersion protocol. An overview of the characteristics of the tested MNs and a description of the new generic test preparation procedure were given.
Some difficulties were reported, for example to obtain primary particles characteristics of SAS or to determine the quantity of impurities present in the CNTs.
Concerning the dispersion protocol, the strategy was reiterated: to have one dispersion protocol for all test systems, with a high concentration in a "physiologically" acceptable medium, applicable for both hydrophobic and hydrophilic MNs. In this protocol, it was important to harmonize the sonication procedures (sonicator tip, sample volume, sample vial, probe position, time etc.). The sonication is used to obtain a stable dispersion of nanomaterials. Nevertheless, it has been indicated that the nanomaterial stability will vary in different exposure medium and should be verified case by case.

Discussion

Questions were raised about the presentation of the results: Standard Deviation, impurities present in certain MNs, LOI (Lost Of Ignition) of 90% in CNTs and 2% for TiO_2 and SAS.
J. Mast indicated that for the sake of the presentation, the results were over simplified: e.g. presenting means with SD is not really accurate because most of these distributions are not Normal. Regarding the validation of the methods, WP4 is working on the estimation of the different uncertainties on different steps of the method. LOI is 90% for CNTs because they can be burned so loss is important. For silica only 2% of substance can be burned, indicating a low amount of organic residue.
Other questions concerned the dispersion protocol and the stock solution particularly regarding the 0,05% of bovine serum albumin (BSA). For BSA, considering that *in vivo*, the serum concentration is at least 1000 times higher, why is the WP4 using such a low BSA concentration?
J. Mast indicated that 0.05% of BSA is needed to obtain a well dispersed stock solution. This solution is used as stock solution which will be used diluted in cell culture media (with serum) as well as for the preparation of test articles for *in vivo* administration.
Participants were interested to know when and where the protocols and SOPs will be published. N. Thieriet indicated that the dispersion protocol is already available on the Nanogenotox website and at the end of the JA, all results will be published on the website and in scientific journals.

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WP5 – *In vitro* methods for genotoxicity – Hannu Norppa, Head of the genetic toxicology laboratory (FIOH, Finland)

H. Norppa reminded participants of the specific aims of the WP5:

- to generate *in vitro* genotoxicity data on the selected MNs and more precisely to produce *in vitro* genotoxicity data on MNs using standard tests and modified assays utilizing specific cell models,
- based on *in vitro* genotoxicity data and physical/chemical characterization data previously obtained, to conduct a round robin test on selected MNs using the two *in vitro* assays (micronucleus assay and Comet assay).

He indicated that most of *in vitro* genotoxicity studies are completed. The round robin test for *in vitro* genotoxicity testing is ongoing.

Interesting new insights into dose response relationships, sensitivity differences among cell types, and possible mechanisms of action were revealed in the context of this WP. DNA or chromosome damage studied by the comet assay and micronucleus assay, and mutations by the thymidine kinase assay were also highlighted. These damages were studied in seven different cell systems. The different cell lines were chosen as a function of the different exposure routes: pulmonary, dermal and ingestion (human pulmonary cells, human dermal cells, lymphatic cells, etc.). Fifteen nanomaterials were tested. The first results indicated that for TiO_2, anatase and mix anatase-rutile induced DNA damage in almost all the various cell lines, and some DNA damage was induced by rutiles as well. Micronuclei were induced especially in keratinocytes. For SiO_2, DNA damage was induced in BEAS 2B and Caco-2 cells and micronuclei in Caco-2 and A549 cells. For CNTs, micronuclei were induced in BEAS 2B, Caco-2 and A549 cells. Results for comet assay are ongoing.

It's too early to say if the translation of the WP5 results into policy orientations will be possible. More complete conclusions will be available at the end of the project with the round robin test results and the comparison between *in vitro* and *in vivo* data (and the data from other projects and the scientific literature). H. Norppa concluded that the relevance of *in vitro* assay is that there should be some correlation with *in vivo* results.

Discussion

A lot of comments were made regarding the cellular uptake of the MNs by the cells. This is essential for the effect and it was asked if the differences in the cell uptake were tested. Some stakeholders confirmed that they have data that agrees with the data presented. For example, on human primary lymphocytes, as well as on keratinocytes (both non-tumor cell lines) very low toxicity was observed, these observations can be related with the low uptake of the MNs.

H. Norppa confirmed that some cell lines are known to be quite efficient in up taking materials (e.g. BEAS-2B, as quantified in earlier projects for TiO_2 and CNT). In Nanogenotox, the cellular uptake was not part of the project even if those data would have been very interesting. H. Norppa has the feeling that much of the response is due to the uptake of the material. Nevertheless he agreed with the assumption that lymphocytes are not taking up the MNs, but positive responses were obtained and it is difficult to explain how it works. The audience indicated that for CNTs the release of metals seems

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possible, so the MNs don't necessarily have to go into the cell. H. Norppa agreed and insisted on the importance of the deep physico-chemical characterization of the MNs tested in the JA.

Another set of questions concerned the technical details such as concentration ranges tested, exposure time etc. and the establishment of a standard protocol for the experiments.
H. Norppa indicated that for micronucleus assay, the exposure time should be about 1,5 times the cell doubling time (around 48 hrs in most cases). In comet assay, 2 different approaches were used: one short (3 hrs) and one longer (24 hrs) treatment. Both are justified, because for a soluble material (i.e. ZnO) effect is seen earlier. Less soluble MNs can induce damage on a longer period. The maximum concentration was determined by the dispersion protocol developed by WP4, in principle 250 μg/mL (in some cases it was possible to double the dose by increasing the quantity of water).
Regarding this dose that is quite high, cytotoxicity was questioned. H. Norppa agreed that cytotoxicity in these assays is fundamental. In these experiments cytotoxicity tests were conducted. Most of the MNs tested are not toxic, except the positive control ZnO.

WP7 - Toxicokinetics and tissue distribution – Wim de Jong, senior researcher at the Laboratory for Health Protection Research (RIVM, The Netherlands)
W. de Jong recalled the main objectives of the WP7:

- to determine the feasible dose for *in vivo* studies,
- to determine time points for tissue sampling for *in vivo* studies,
- to identify relevant target organs for possible genotoxic damage based on tissue distribution of nanomaterial.

The WP7 developed a method with ICP-MS to determine silica and titanium in tissues, whereas the carbone nanotubes were radio-labeled with ^{14}C. The dispersion protocol was slightly adapted using Rat Serum Albumin instead of BSA (in order to avoid immunological reaction). Both intravenous (IV) and oral routes were studied. The general approach for toxicity studies was based on OECD guideline 420 [1].
A summary of the results of toxicity studies was made. The main aim of these studies was to identify tolerable dose. TiO_2 were found to be non toxic after intra-venous administration (at the highest possible concentration of 2,3 mg/ml) and after administration by gavage in rats (for all 5 TiO_2 tested). CNTs were found to be non toxic after intra-venous administration at the highest possible concentration (2,3 mg/ml). SAS were found to be non toxic after a single intra-venous administration and after single and repeated oral administrations (at a dose of 20 mg/kg). One SAS (NM-203) showed some toxicity after a repeated intra-venous administration of 20 mg/kg (splenomegaly and discolored liver).

[1] OECD Guideline for testing of chemicals GUIDELINE FOR TESTING OF CHEMICALS (Acute Oral Toxicity – Fixed Dose Procedure) n°420, adopted 17 December 2001 .PDF.

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Tissue distribution and kinetics were studied after single dose administration. Tissue distribution was also studied after repeated dose. Both intra-venous and oral administrations were investigated. Some preliminary results were presented (experimental analysis is still ongoing):

- TiO_2 were still detected in various organs at day 90, with a gradual decrease in time and a variation in decrease (single and repeated intra-venous administrations). They showed a very low uptake even after repeated oral administration,
- SAS were more or less similar to TiO_2: SAS were still present at day 90 with a decrease in time after both single and repeated intra-venous administrations. They showed a low uptake after repeated oral administration,
- The 4 CNTs investigated showed different behavior, they seemed to be split into 2 groups, one with high uptake and the other with low uptake. Differences in washing out were also observed.

Discussion

One of the panelist indicated that the MultiWall CNTs used are not very long; and the concentrations used are very high if compared to 0.1 fibers (recommended values in air), for him this is interesting but not the most relevant for the scientists. W. de Jong answered that as shown in J. Mast presentation it's quite difficult to determine the length of the CNTs. Regarding the concentrations, for CNTs the concentration was much lower than for the other MNs studied, because the main purpose was to see the target organs and the CNTs were radio-labeled so there is no relation to the maximum inhalation exposure. Of course for the CNTs, there is concern about the similar behavior compared to asbestos, but CNTs that behave like asbestos are specifically designed to be long, rigid and bio-persistent. The majority of the CNTs that are used don't have these characteristics.

One participant highlighted that in the 90-days *in vivo* studies with SiO_2, dissolution of the material takes place and it would be difficult to say if the MNs was translocated as a particle or bound to protein. W. de Jong considered that ICP-MS has limitations. Directly after administration we can assume that particles are present so at the beginning the presence of Si or Ti indicates the presence of particles. However, after IV administration, they are rapidly coated by all kinds of proteins and that may reduce the dissolution of the particle. But W. de Jong agreed, at the end we are not sure to look at particles or at the element. Studies clearly show a high level of SiO_2 at Day1 when particles are normally not dissolved.

Regarding the toxicity observed during these experiments, another stakeholder indicated that the fact that the particles or the elements have disappeared does not mean that the toxic effect has disappeared. W. de Jong confirmed that during necropsy, for one SAS after administration an increase in the spleen weight was noticed; this is a sign of toxicity but no further histological examinations were conducted. Concerning the mechanisms of toxicity, he indicated that in an other project nano-silver induced also an increase in spleen weight and that may have an effect on immune functions.

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WP6 : *In vivo* testing of nanomaterials genotoxicity – Valerie Fessard, Head of the Toxicology of contaminants Unit (ANSES, France).

V. Fessard indicated that the work of this WP began in October 2011. It will combine two genotoxicity assays (comet and micronucleus) from various tissues of the same animal. Both gavage and instillation administrations will be investigated.

The general work program of this WP and the trainings and trials which were required prior to the nanomaterials experiments were presented. The aim of the trainings is to ensure that all partners are ready when the experiments have to be undertaken.

Comet assay and micronucleus assay will be performed on the same animal. Some complementary measurements will be conducted: histology, lipid peroxidation and analysis of mediators of inflammation. Histology will only be performed on the organs which will be found positive in the comet assay.

The choice of the positive controls was discussed. Carbon Black® was tested but was finally not selected as nanosized positive control. Methyl methane sulphonate (MMS) was selected as the chemical positive control. It will be given by gavage for both routes (oral and instillation).

The principles of nanomaterials experiments have also been detailed: same administration protocol as in WP7, animals (rats, around 200g), treatment (5 animals, 3 doses, 3 days in a row, etc.), dispersion protocol, organs to collect, etc.

Discussion

All the comments concentrated on inflammation. Participants commented that inflammation is really important for genotoxicity and they wanted to know if inflammation will be studied after gavage, or intra-tracheal installation? V. Fessard indicated that measuring inflammation was not initially planned in the project (at ANSES, some measurements will be done but only on blood and some histology will be available).

H. Norppa indicated that in some other EU-funded projects, the association of inflammation and genotoxicity is being investigated.

WP3 - Evaluation of the Joint Action – Mario Götz, Head of Molecular Toxicology Unit (BfR, Germany)

M. Götz explained that the ability of Nanogenotox to deliver a robust and reliable methodology to facilitate genotoxic hazard characterisation is evaluated by an internal evaluation team, together with an external academic reviewer panel.

The main aims of the WP3 are to evaluate the Joint Action results and added values as well as to give recommendations for future developments.

A view on potential evaluation criteria and the balance between expectations and feasibility was presented. Two types of indicators were identified:

- Quality indicators: applicability of OECD methods, SOPs, reproducibility;
- Quantity indicators: characteristics, organ burden, dose responses *in vitro* and *in vivo* studies.

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The evaluation methodology consists in:

- An internal evaluation team whose members represent each of the scientific WPs undertake actions to verify if the project is being implemented as planned and reaches the proposed objectives,
- To facilitate the work of the WP3 team, the Coordinator implemented templates for reporting the research results every 6 months,
- In addition, the WP3 team relies on the presentations by work package leaders at the General Assemblies outlining the progress of work and results,
- The WP3 team monitors and analyses the quantitative and qualitative specific indicators every 6 months,
- The results (data, reports, deliverables) will be communicated to the external evaluation team members who will participate to the evaluation. A 2-days final evaluation meeting will be held at the BfR in November 2012 in order to discuss the results of the Action.

Panel Discussion

Benoit Vergriette (WP2 Leader), Head of the Risks and Society unit (ANSES, France) introduced the panel discussion by asking the panelists to comment on the previous plenary session and the WP leaders to react on their comments. B. Vergriette asked also the panelists to comment on the dissemination strategy and the stakeholder consultation process.

Steffi Friedrichs, Director General Nanotechnology Industries Association (NIA)

S. Friedrichs applauded the scientists for the work done, as well as the organizers of the consultation and of the workshop. She recognized the links with other on-going activities (namely OECD sponsorship program) because it is impossible to answer the questions that are raised regarding MNs and the uncertainties except at a global level. It is important that all countries work together and it's positive that the Nanogenotox researchers communicate with other groups.

She would like to see more conclusions. During the presentation of the Evaluation (WP3), M. Götz made reference to risk assessment and risk management, but in fact what she saw was hazard profiling and results from hazard profiling. Nothing was shown on exposures, full risk assessment, and risk management options. She recommended addressing during the remaining 9 months issues that could help risk management.

S. Friedrichs also noted some conclusions from W. de Jong (WP7) about MWCNTs that seems displaying similar properties to asbestos only if they are long and rigid. This is interesting to go to safe design in order to avoid hazards during the production process as well as in the final product. For her, this is the kind of results that need to be communicated early into the right channels where they can be used for both risk management and safe design.

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Aida Ponce Del Castillo, senior researcher at the "Working conditions, health & safety" department of the European Trade Union Institute (ETUI).

A. Ponce gave her comments from the health and safety perspective. Introductory slides or comments on issues that are outside the scope of the project would have been useful; for example what was mentioned on inflammation, toxicity of MNs and histology. It would also be useful to know why these issues were not covered by the JA.

Concerning the dissemination, particularly communication to the general public, A. Ponce did not see too much effort on that point. She wonders how stakeholders are supposed to use the large amount of data produced by the JA. Is this point planned to be more comprehensive on the web site or in the newsletters, as well as information to the different stakeholders who can interpret these data. She would have appreciated to know how the scientists from Nanogenotox interpret the results for policy use. Is Nanogenotox built to give some guidelines to the regulators in charge for the member states or to the European commission on how to use these data?

Regarding the connection with OECD, A. Ponce would like to know how the researchers consider the OECD guidelines now that they have used them with MNs. Do these guidelines need adaptation, if so, in which sense, in which way?

Michael Riediker, coordinator of NanoImpactNet, leader of the networking activities of QNano and of the dissemination group of the European NanoSafetyCluster, researcher on particles and health at the Institute for Work and Health (IST) in Lausanne, Switzerland.

M. Riediker recognized that there is a real need to share protocols (in NanoImpactNet difficulties on that were encountered), but it's better to share protocols that people can apply and above all give guidance in order for scientists to check whether these protocols are applied correctly. He really hopes to see this aspect addressed in the method produced by Nanogenotox (with self-test for example, or ranges of expected results, results for controls etc.). M. Riediker is looking forward to seeing these protocols and would like them to be shared with the NanoSafetyCluster.

M. Riediker mentioned that many small companies ask him through the Institute for Work and Health, how they can find out if substances of potential interest can be tested regarding possible hazards before the end of the development of these products (meaning before having invested too much money). He wondered how various interested parties can be confident that the method proposed by Nanogenotox will give an indication about the safety of the tested *material*. Currently, he referred to standard published tests (by OECD for example) and tools such as precautionary matrix for synthetic nanomaterials developed in Switzerland. From the point of view of a researcher in occupational health, if in cells and animals it seems not toxic or genotoxic, are the workers really safe? To answer this question, the mode of action is needed.

Hartwig Muhle, working group "Environmental chemicals and toxicology" of Friends of the Earth Germany (BUND).

The first set of comments from H. Muhle was on the positive controls. Methyl methanesulfonate (MMS) is a soluble material and if there is a problem with the uptake of a nanomaterial into the cells this will not be detected with MMS. He wondered why Carbon black was not kept as particle control.

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Regarding toxicokinetic studies, it's known that intra-tracheal studies can induce artefacts (for example by bolus effect that can cause inflammation, surfactant dysfunction or differences in particle translocation). So it is important to link these intra-tracheal studies to inhalation exposure.
Regarding risk assessment, he wanted to know how the dose will be defined: is it the particle mass, the surface area (which is particularly important during inhalation as seen for asbestos), the particle size (which is important for translocation) or the particle shape? For him, toxicokinetic is influenced by the properties of the particles as well as the degree of inflammation in the lung.
Regarding genotoxicity: secondary genotoxicity is defined as resulting from reactive oxygen and nitrogen species generated by inflammatory reactions by activated phagocytes. There is a lot of indication that in the chronic experiment with TiO_2 or Carbon Black there may be chronic inflammation. In Nanogenotox, primary genotoxicity was explored and this is defined by genetic effect of particles in the absence of inflammation. Direct pathways would be a direct interaction between nanoparticles and DNA; but indirect pathways exist i.e. reactive oxygen species that are generated inside the target cells. Additionally, the internal repair processes may be reduced. H. Muhle concluded that Nanogenotox could miss a lot of information by looking only at the primary direct genotoxicity.
He said also that in *in vitro* studies, useful information on the mechanisms may be generated for hazard identification (but only on primary genotoxicity). In the cell lines used in Nanogenotox, which have limited capacity of phagocytosis, relatively high particle concentrations are used. H. Muhle insisted to discuss the cellular uptake in these conditions. Finally, he indicated his position that with regard to regulation, the evaluation of nanomaterials by *in vitro* tests is limited. For him Nanogenotox will provide some information but a small part of "the all game".

Andrej Kobe, Chemicals, Biocides and Nanomaterials Unit (DG Environment, European Commission)

A. Kobe indicated that he looked at the JA from a "systems engineer" perspective: does the system (i.e. functions)? For example, Commission's REACH implementation project on nanomaterials (RIP-oN) was addressing this question in a broad sense. Testing is a crucial element of the REACH regulatory scheme. This JA is respectively very important as it addresses the applicability of the method to cover genotoxicity end-points for nanomaterials.
A. Kobe noted that the project is developing well with an excellent EU cooperation, including involvement of new member states. The external evaluation is very important to ensure that there is a validation of the results generated already within the project. This step should facilitate the uptake of the work into the regulatory context (e.g. through the validation of the OECD methods).
On more technical points, regarding the presentation, for physico-chemical characterization, he would like the JA to come up with very explicit recommendations from this JA perspective about what should be characterized (and, if in position, the way to measure it) and also on how to present the characterization data and results (for example as discussed for Standard Deviation, metrics beyond mass concentration). These will help develop guidelines and define how information on safety will be presented in REACH dossier and how the regulation will be applied, e.g. respecting the test results' applicability domain.
About WP5, H. Norppa indicated that open issues will remain and A. Kobe wonders what kind of open issues remain.

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Feedback from WP leaders and general discussion

B. Vergriette asked the WP leaders to comment on the issues raised by the panelists.

J. Mast (WP4) agreed with the comments made and from the TEM specialist point of view he specified that detailed data will be available in the reports (number based distribution, and non parametric statistic was applied). Data on particle size will be available but also on shape, surface properties etc. 24 parameters in total. Regarding the size, different presentations of size can be used: diameter, perimeter etc. This is directly related with how the dose can be expressed. Three factors seem to be very important: size, shape and surface topology. These factors may change during the life time of the particle, the medium etc. J. Mast indicated that at the end of the JA all the data will be available in a mode friendly shape. These 3 parameters can be determined by TEM or by other techniques, however the original data set will be needed in order to do the correlation between the other techniques.

H. Muhle indicated that in air the process is very dynamic (agglomeration versus de-agglomeration) so this is not easy to define the size. J. Mast indicated that data on agglomeration and aggregates size are available.

M. Riediker suggested to look at the parameters defining MNs as vector, mathematically speaking. For chemicals concentration is used, but for nanomaterial a series of numbers may be better.

H. Mulhe indicated that from the workers point of view the concern is inhalation. The final goal is to find a dose response for the risk assessment so how the dose is defined and where the MNs go into the body is a very important issue. He agreed that toxicokinetics studies are important but with the issue of bolus it's questionable for risk assessment.

W. de Jong answered that toxicokinetics in Nanogenotox are not made for risk assessment; they are designed for the identification of the target organs, which is for hazard identification. He agreed that inhalation studies are very important but they are very expensive and to start it's better to do instillation studies. M. Götz confirmed that the project from a regulation point of view concerns hazard identification. As the coordinator, N. Thieriet reiterated that the aim of the Joint Action is not to do risk management but to provide a method to evaluate the potential hazard (genotoxicity). This method can be used by the stakeholders in different manners, regulation, prevention etc. It was agreed at the OECD Working Party on Manufactured Nanomaterials (WPMN) that guidelines could be the basis for risk assessment and Nanogenotox uses existing OECD guidelines that may need to be modified. H. Muhle thinks that OECD guidelines are not necessarily sufficient (i.e. data on chronic and sub-chronic inflammation, for him it is difficult to explain the effect because we do not have any data on the cellular uptake.

H. Norppa commented about the open issues that will still remain. We assume that the tests run are able to demonstrate primary genotoxicity as it is for chemicals. The problem is that not the same amount of data is available for MNs compared to chemicals (e.g. on human carcinogenicity). For chemical all these data can be correlated, Nanogenotox will provide comparative data but in general few *in vivo* data are available on MNs. The mechanisms of genotoxicity and the links between

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inflammation and genotoxicity and the role of the inflammatory cells need to be clarified particularly in order to do risk assessment.

Regarding the quality controls used in Nanogenotox: V. Fessard agreed that MMS is a soluble chemical positive control, but it's used as quality control and not to measure the uptake. Partners tried to find a nanosized particle control but Carbon Black did not have the characteristics of a good control because the responses were very different from one organ to another and from one participating laboratory to another. M. Riediker indicated that he characterized some Carbon Black and he did see extreme differences between the different types, for example in functionality, depending on the producer.
H. Norppa indicated that the ZnO was proposed as positive control in the *in vitro* study because most of the cells respond to ZnO. Two problems remained with ZnO, doses may be difficult to optimize and it's soluble. M. Götz suggested that a nano crystalline quartz could have been used to take carcinogenicity as an end point. H. Muhle indicated that in a published 90-day inhalation study on rats, a comparison was made on inhalation of amorphous silicon dioxide compared to crystalline silicon dioxide[2], both demonstrated the same degree in inflammation but only cristalline type induced mutations in epithelial cells.

Regarding the connections with the OECD, M. Götz indicated that once the data will be published they ought to be transferred into the OECD data base. For the robustness of the methodology in the OECD guidelines there is some flexibility regarding standard procedures or scoring method, WP5 tried to harmonize those items that can influence the outcome of the test but all the final steps cannot be harmonized. Nanogenotox could make recommendation for the methods. N. Thieriet indicated that Nanogenotox results will be shared with the WPMN and upon the collaborating partners, the ministries involved will contribute to the sustainability of the action.
The final conference of Nanogenotox will be held in Paris on February 22, 2013. N. Thieriet Proposed to the stakeholders present at the workshop to be involved in the preparation of a special session dedicated to regulatory purposes and policy making.

B. Vergriette indicated that for the final conference efforts will be made regarding communication to the various categories of stakeholders. There will be specific tools (leaflets, reports) in order to make data available to the different categories of stakeholders.

For his last comments, H. Muhle indicated his hope that industry, based on Nanogenotox results, will not draw a conclusion that nanomaterials are safe since it's much too early to do so, especially since a lot of information is still missing.
M. Riediker looks forward to seeing the published protocols as well as the associated guidelines on how to use them correctly. He asked also the partners to share information on the mistakes and problems encountered during the Action and how they were addressed, if that was possible.

[2] *Pulmonary chemokine and mutagenic responses in rats after subchronic inhalation of amorphous and crystalline silica,* Johnston et al., Toxicol. Sci. 2000 Aug; 56(2): 405-13

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Concluding remarks - Salma Elreedy – Assistant Director "European and International Affairs Department" (ANSES, France)

There are a lot of benefits for the different institutions participating in the Action, and it's a successful example of EU cooperation. The stakeholders input during the first consultation, and during this workshop, has been very valuable, in identifying the concerns and needs of various groups of stakeholders; for example, industry with regards to safe design or to the preliminary testing of a nanomaterial that is still under development before investing important efforts and money. The occupational health and safety issues are very important and communication tools will be produced for the various public.

In terms of communication, the messages to the general public, policy-makers, as well as other groups, should be defined in view of the final conference (22 February 2013, in Paris).

Some partners of the Action are purely research institutes but others are risk assessment institutes with strong links, as knowledge brokers, to ministries. As mentioned before, ministries of several member states are collaborating partners of the Action and some were present at this workshop so the participants to final conference will have to think together how to involve the policy makers even if the project will not answer all risk assessment and risk management issues. One of the issues will be on how the method produced can be taken up and followed upon within REACH or other regulatory mechanisms. The external evaluation team that supports Nanogenotox is very useful and constitutes a particularity of this project and we will try to make available the conclusions of the evaluation conducted.

Nathalie Thieriet closed the workshop and thanked all the participants

SAVE THE DATE! Nanogenotox final conference will be held in Paris on 22 February 2013.

Towards a method for detecting the potential genotoxicity of nanomaterials

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