Conflict of Interest

The authors declare no conflict of interest.

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Commentary

Genotoxicity of Nanomaterials: Refining Strategies and Tests for Hazard Identification

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A workshop addressing strategies for the genotoxicity assessment of nanomaterials (NMs) was held on October 23, 2010 in Fort Worth Texas, USA. The workshop was organized by the Environmental Mutagen Society and the International Life Sciences Institute (ILSI) Health and Environmental Sciences Institute. The workshop was attended by more than 80 participants from academia, regulatory agencies, and industry from North America, Europe and Japan. A plenary session featured summaries of the current status and issues related to the testing of NMs for genotoxic properties, as well as an update on international activities and regulatory approaches. This was followed by breakout sessions and a plenary session devoted to independent discussions of in vitro assays, in vivo assays, and the need for new assays or new approaches to develop a testing strategy for NMs. Each of the standard assays was critiqued as a resource for evaluation of NMs, and it became apparent that none was appropriate without special considerations or modifications. The need for nanospecific positive controls was questioned, as was the utility of bacterial assays. The latter was thought to increase the importance of including mammalian cell gene mutation assays into the test battery. For in-vivo testing, to inform the selection of appropriate tests or protocols, it was suggested to run repeated dose studies first to learn about disposition, potential accumulation, and possible tissue damage. It was acknowledged that mechanisms may be at play that a standard genotoxicity battery may not be able to capture. Environ. Mol. Mutagen. 54:229–239, 2013. © 2013 Wiley Periodicals, Inc.

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INTRODUCTION

The term "nanomaterials" (NMs) is used as an umbrella term for a diverse group of nanosized particulate materials. NMs are designed to have unique properties different from the bulk material. NMs are found in an increasing number of products, including clothing, drug-delivery systems, wound dressings, cosmetics, and paint, as well as many other products. The novel properties of NMs have resulted in questions about their potential effects on human health and the environment. The ability of the standard genotoxicity assays to predict potential hazards of NMs has not been established. This workshop did aim to evaluate the usefulness of the standard regulatory genotoxicity tests, identify gaps for potential research, and if feasible, develop recommendations for the genotoxicity testing of NMs.

There are many published studies on the genotoxicity of NMs indicating they can be genotoxic in vitro as well as in vivo [Gonzalez et al., 2008; Landsiedel et al., 2009; Singh et al., 2009]. Yet for the purposes of designing an appropriate testing strategy, it is clear there are key data gaps and confounding factors that need to be addressed, e.g., as discussed in Doak et al. [2009]. For instance, as is typical in a new focus area, many publications are directed at reporting positive results, even when the results include a minimal enhancement over the controls. There are insufficient data on NMs that are negative in genotoxicity tests. Also, positive results for NMs tested in nonstandard assays at high, non-physiologically relevant concentrations/doses may not contribute meaningfully to the risk assessment of these materials. There is a great deal of attention given to the unique aspects of NMs, which often fuels a sense of concern that these materials may be associated with adverse toxic effects not observed with non-NMs of the same composition. Although there are a number of initiatives underway that are designed to help address issues related to NM safety assessment, the growing interest in developing new products that utilize NMs creates an immediate need for a testing approach that can be used today by industry, regulatory agencies, and academic scientists.

To promote progress in this direction, a workshop was held on October 23, 2010 in Fort Worth Texas, USA, in connection with the annual meeting of the Environmental Mutagen Society (EMS). The workshop was organized by the Relevance and Follow-up of Positive Results in In Vitro Genetic Toxicity Testing Project Committee (IVGT) at the International Life Sciences Institute (ILSI) Health and Environmental Sciences Institute (HESI) with the intention of developing recommendations for a genotoxicity testing scheme for NMs, as well as the identification of potential research needs. The workshop was attended by over 80 participants representing stakeholders from academia, regulatory agencies and industry from a wide range of geographies (list of attendees: Appendix A). The workshop was designed to give all participants the opportunity to actively contribute to the discussion during the breakout sessions. These sessions followed a plenary session featuring summaries of the current status of testing of NMs for genotoxic properties in vitro and in vivo, as well as an update on international activities.

Workshop Focus

The workshop, limited by time constraints, made the deliberate decision to focus on the following topics:

- Evaluation of the usefulness of standard in vitro and in vivo assays to capture genotoxic effects caused by NMs.
- Discussion on whether standard protocols are suitable to detect the anticipated modes of action.
- Discussion of the need for integration of "new technologies" (e.g. "omics") into the genotoxicity assessment of NMs.
- Definition of gaps left by the standard assays.
- Identification of additional techniques that might fill gaps.
- Identification of research needs in this area.

What Was Not Discussed

- It was recognized that there are many definitions of what is to be considered as "nanosized" and we did not seek to define the term at this workshop. The term "NMs" was considered as an umbrella term for a diverse group of nanosized particulate materials. We want to emphasize, though, that the discussion was focusing on particulate materials that do not solubilize under physiological conditions or leach ions, as the genotoxic hazard of those may be adequately captured by standard testing approaches.
- The importance of appropriate characterization of NMs was acknowledged, and there was agreement that the characterization should generally cover morphology and metric related characteristics (form, chemical composition, shape, specific surface area, primary particle size, and size distribution), as well as physico-chemical properties such as purity, state (amorphous vs. crystalline), charge, and solubility. This has been discussed before by many authors [e.g., Powell et al., 2006]. Thus we decided not to address this issue directly, although it was raised in several breakout groups.

Workshop Structure

The breakout sessions were organized into three groups charged with discussion of core questions that were defined by the steering team ahead of the meeting.

- Group 1 evaluated the usefulness of standard in vitro assays (both bacterial and mammalian) and whether standard protocols are suitable to detect the anticipated modes of action.

- Group 2 assessed the utility of standard in vivo approaches and which assays could be recommended.
- Group 3 discussed the need for integration of new technologies into the genotoxicity assessment of NMs, as well as the question which tests or additional research would be useful.

After summarizing and discussing the outcome of the breakout group activities, the final plenary brought everyone back together to seek consensus towards the goal of defining a strategy for genotoxicity testing of NMs. The proceedings of this workshop provide some initial recommendations and considerations on a testing approach for NMs that reflect the available information and experience of the global expert participants. It was beyond the scope of a 1-day workshop to develop definitive recommendations, but it is hoped that these proceedings help lay the foundation for future research and more formal discussions on testing guidelines.

WORKSHOP SUMMARY CONCLUSIONS

- All of the standard genotoxicity assays require modification in methods or special considerations for the assessment of NMs.
- Bacterial assays may be limited in utility due to the apparent lack of uptake of NMs but additional studies were deemed necessary before final conclusions can be drawn.
- If bacterial assays are not performed, then the inclusion of mammalian cell gene mutation assays into the test battery would become more important.
- None of the protocols of in vitro standard assays was considered appropriate without special considerations or modifications like: possible interference of cytochalasin B treatment with the uptake of NMs, potential lack of uptake of agglomerated NMs by cells, and possible interference of NMs with endpoint measurement, e.g., for assays measuring fluorescence.
- Issues with exposure and removal of the NM test articles may result in complications in both monolayer and suspension cell cultures.
- The in vitro micronucleus assay was seen to be advantageous as it is capable of detecting chromosomal damage in the form of clastogenicity (e.g., triggered by ROS), aneugenic effects such as physical disturbance of spindles/mitotic apparatus).
- Because NMs may exhibit unusual ADME characteristics, such as organ-specific accumulation, it was recommended that other testing, such as repeat dose toxicity (RDT) studies be performed prior to in vivo genotoxicity testing to aid in design of an informative test.
- Thus, it was suggested that the testing paradigm should be changed so that other in vivo mammalian studies would be conducted prior to conducting an in vivo genotoxicity test. Alternatively, genotoxicity endpoints could be integrated into RDT testing.
- NM-specific positive controls were not considered to be necessary for genotoxicity assays, because positive controls relate to assay performance.
- Among the research needs identified were methods to measure DNA interaction and distribution of NMs, and methods to differentiate effects of substances in different physical states.
- Unconventional in vivo systems, such as Drosophila or Zebra fish, might be useful in the study of NM effects on both somatic and germ cells.
- It was acknowledged by the workshop participants that mechanisms may be at play for NMs that a standard genotoxicity battery may not be able to capture; therefore it was recommended that additional tools be sought which should help unravel potential modes and mechanisms of action.

SUMMARIES OF THE PRESENTATIONS FROM THE PLENARY SESSION

Approaches and Caveats to Investigating the Genotoxicity of NMs: Shareen H. Doak, Swansea University, UK

This presentation introduced a number of the confounding factors that are likely to account for some of the inconsistencies in the current literature, which contribute to the difficulty in reaching firm conclusions on the safety of NMs. The importance of complete physico-chemical characterization of NMs under investigation was highlighted, in addition to considering their secondary structure and form under experimental conditions, as this is what biological test systems are actually exposed to. In addition, sample preparation techniques used can influence this secondary structure and the degree of NM agglomeration. Thus, to facilitate comparison between studies and full interpretation of the biological responses observed, full detail with regards to physico-chemical features and sample preparation technique is vital. The presentation also highlighted a number of sources for variability in genotoxicity test systems including the selection of cell types (for in vitro studies); NM interaction with assay components including serum, colorimetric and fluorometric dyes, the use of cytochalasin B in the cytokinesis blocked micronucleus assay [Doak et al., 2009, 2012]; and the applicability of the Ames test for NM genotoxicity testing [Doak et al., 2012].

Accounting for such confounding factors within the experimental system when initiating nano(geno)toxicology investigations should improve the quality of in vitro assays, providing more reliable and reproducible data sets.

Testing for Genotoxic Effects of NMs In Vitro and In Vivo: Robert Landsiedel, BASF, Germany

This presentation focused on the use of standard genotoxicity assays to identify NM hazards. Generally, NMs are not expected to cause a new quality of genotoxic effects other than the well-established gene mutations, clastogenic and aneugenic effects by direct or indirect

mechanisms. Therefore, the battery of standard genotoxicity assays should be generally applicable. The questions are, what modifications of these assays are needed to meet special requirements of NMs, which assay (or combination of assays) is predictive for NM mutagenic potential in humans, and is there a need for additional data (genetox or other assays) to allow a valid hazard identification for humans? This was explicated by results of selected NMs in various test systems in vitro and in vivo. These included several Ames bacterial tests, HPRT gene mutation tests, micronucleus tests (MNT), and comet assays in vitro, as well as MNT and comet assays in vivo, published elsewhere [Landsiedel et al., 2010a,b; Schulz et al., 2011].

In vitro methods for genotoxicity testing require the dispersion of the NM in the culture media. The use of efficient, reproducible and well-documented dispersion protocols is crucial. Various protocols to prepare dispersions from 10 different NMs were tested; methods and pitfalls in preparing and characterizing the dispersions were presented. In summary, the agglomeration state and the surface coating of particles in dispersion may vary with the surrounding fluid (water, dimethylsulfoxide [DMSO], fetal calf serum, lung surfactant). The particle size distribution in the dispersions was analyzed by light scattering and analytical ultracentrifugation. The methods and their limitations were evaluated. In general, the highest amounts of ultrafine particles, i.e., the least agglomerated particles, were observed when using fetal calf serum [Schulze et al., 2008]. Several instances of interference of NMs with in vitro test systems have been described, e.g., in the in vitro chromosome aberration test, precipitation of (agglomerated) particles on the slides interfered with scoring for chromosome breakage.

The selection of appropriate in vitro genotoxicity tests for a NM requires information on its material properties and potential fate in the body. The particle size distribution and surface coating of the NMs in vitro may be very different from the ones found in the body. We have assessed the genotoxic potential of two differently sized amorphous silica NMs with potential inhalation exposure, by use of the in vitro single cell gel electrophoresis (Comet Assay) in a permanent cell line (V79 cells), and in Precision Cut Lung Slices (PCLS) from Wistar rats [Schulz et al., 2011].

In addition to in vitro methods, in vivo methods include uptake, distribution, excretion, and modification processes and allow the observation of concurrent general toxicity. Both of the amorphous silica NMs were instilled into the lungs of Wistar rats and genotoxic effects in the lung (Comet Assay) and bone marrow (Micronucleus Test) were examined along with general toxicity (e.g., inflammation of the lung). The comparison of the in vitro and in vivo methods revealed that while some genotoxic effect were observed in vitro those could not be reproduced in vivo [Schulz et al., 2011].

Developing a Coordinated International Research Effort in Nano-Genotoxicology: Tim Singer, Health Canada

This presentation highlighted several international initiatives currently underway, which are expected to produce data that will be informative in the context of: (a) better understanding the genotoxicity of a selection of NMs, and (b) better understanding the relevance and reliability of genotoxicity tests for NMs. The Organization for Economic Cooperation and Development (OECD)'s Working Party on Manufactured Nanomaterials has initiated nine projects, of which three were noted to be particularly relevant for the two objectives above, including the Safety Testing of a Representative Set of Manufactured Nanomaterials (Project 3), Manufactured Nanomaterials and Test Guidelines (Project 4), and the Role of Alternative Methods in Nano Toxicology (Project 7). Project 3 intends to test thirteen representative NMs across 59 endpoints for which there are OECD Test Guidelines; nine of these NMs were to be tested for genotoxicity using one or more of the tests in the standard test battery. Project 4 intends to review existing OECD test guidelines for their applicability to NMs and to identify the need for new or revised guidelines. This project has produced *Preliminary Guidance Notes on Sample Preparation and Dosimetry* as guidance to researchers [OECD, 2010]. Project 7 intends to support and advice on further developments of alternative test methods and integrated testing strategies. Furthermore, in Europe, a multilateral project called *Nanogenotox* was launched in 2010 with the aim of obtaining sound and reliable methods for detecting the potential genotoxicity of manufactured NMs within 3 years. This project is currently undertaking round-robin genotoxicity tests of titanium dioxide, silicon dioxide, and carbon nanotubes.

Scientific Issues Related to Genotoxicity Safety Assessment of NMs: Rosalie K. Elespuru, USFDA

This talk presented (1) some general concerns for safety assessment of NMs, (2) a description of the varying regulatory contexts for NM assessment at FDA, and (3) discussion points on the adequacy of current genotoxicity assays for NM assessment. Besides NM characterization, other concerns for safety assessment include product variability as related to manufacturing, stability, shelf life, and sample preparation. The regulatory basis for product assessment at FDA varies both between and within centers, reflecting the varying legal contexts. For example, for some products the assessment is risk/benefit based, whereas for other product categories the assessment is related to risk only. Concerning genotoxicity testing, we are aware that NM modes of action may be unknown and thus not always predictable. Thus, we seek tests that will detect a broad set of genotoxic mechanisms. We need to address whether the standard tests are appropriate and sufficient. Can we detect reactive oxygen species efficiently? If not, what other tests might be considered? Generally we need to determine how

to deal with the broad scope of NM effects, as well as variations based on differences in particle size, composition, and properties.

The Principal Approaches to Genotoxic Hazard Assessment of NMs in Japan: Masamitsu Honma, NIHS, Japan

This presentation introduced "Research on Hazard Characterization and Toxico-Kinetic Analysis of the Manufactured NMs for the Establishment of Health Risk Assessment Methodology," a project supported by the Ministry of Health, Labor, and Welfare in Japan. In accordance with the OECD sponsorship program on the testing of manufactured NMs, this project examined mainly fullerene and carbon nanotubes. The presentation highlighted principal genotoxicity test strategies. The in vitro tests used were (1) a chromosome aberration test to detect structural aberrations as well as polyploidy, and (2) a cell transformation or micronucleus assay to detect non-DNA interacting genotoxic mechanisms, including aneugenicity. OECD test guidelines and good laboratory practices (GLPs) are needed to ensure reproducible and reliable data. In the in vivo tests, target organs (e.g., lung and mesothelium), as demonstrated by histopathological observation and toxicokinetics analysis are the ones generally examined. A gene mutation assay using transgenic rodents is useful for the target organs. As a follow-up, oxidative DNA damage is measured. Finally, weight of evidence (WOE) and mode of action (MOA) approaches are considered for both in vitro and in vivo results. Negative results are not dismissed and should be published.

BREAKOUT GROUP DISCUSSIONS

Characterization of NMs

All groups emphasized the importance of characterization of NMs. Characterization generally covers morphology and metric related characteristics (form, chemical composition, shape, specific surface area, primary particle size, and size distribution) but also physico-chemical properties such as purity, state (amorphous vs. crystalline), charge, and solubility.

Breakout Group 1: Standard In Vitro Assays: Assessment/Strategy Development

Question: If you had to conduct genotoxicity testing on a nanoparticle today using only in vitro tests, or a battery of in vitro and in vivo tests, what in vitro tests would you choose and why?

Outcome of discussion

The Salmonella typhimurium Bacterial Reverse Mutation Assay (Ames Assay)

On the basis of existing data as well as practical aspects of the Ames assay, it is not clear whether bacterial assays add significantly to a testing approach. Since bacteria do not have active systems facilitating particle uptake, there is the question of whether nanoparticles are taken up into the bacteria at all. Thus, bacterial assays might not be suitable for genotoxicity assessment of nanoparticles, particularly considering agglomeration, and thus could lead to false negative results due to exclusion of nanoparticles from the bacterium. The vast majority of studies utilizing the standard Ames assay have demonstrated negative results [Gonzalez et al., 2008; Landsiedel et al., 2009; Singh et al., 2009]. One study examining iron–platinum nanoparticles capped with tetramethylamonium hydroxide did report a weak positive using the TA100 strain [Maenosono et al., 2007]. This nanoparticle has not been tested in any other cytogenetic system and thus the value of the single positive result cannot be determined. It must be noted that a number of recent reports utilizing the Ames assay along with other in vitro and in vivo genotoxicity assays indicated that all tests were negative for genotoxicity when examining fullerenes [Shinohara et al., 2010] and both TiO_2 and ZnO [Landsiedel et al., 2010a,b]. However, Al_2O_3 provides an example of a NM that is negative in the Ames test [Balasubramanyam et al., 2010] while being positive in the in vitro micronucleus assay [Di Virgilio et al., 2010], and in vivo micronucleus and comet assays [Balasubramanyam et al., 2009]. Of course, with the limited data currently available, additional studies are necessary to arrive at a definitive conclusion on the utility of the bacterial assays in a test battery. It was recommended that a coordinated study comparing a set of NMs prepared in the same manner and tested concurrently in the other in vitro genotoxicity assays be conducted to address this issue.

In vitro Micronucleus Assay

The existing data as well as mechanistic considerations indicating that the in vitro micronucleus assay responds to a variety of relevant mechanisms involved in NM genotoxicity indicate that this is a useful test for the evaluation and investigation of NMs. For example, the in vitro micronucleus assay is able to detect chromosomal damage in the form of clastogenicity induced by ROS, one of the main mechanisms of NM genotoxicity. In addition, the test system is capable of detecting potential aneugenic effects such as physical disturbance of spindles/mitotic apparatus [Sargent et al., 2009]. These different mechanisms can be discriminated by performing kinetochore or centromere staining on the resultant cell preparations. Importantly, the in vitro micronucleus assay has been shown to be useful for addressing linear versus nonlinear dose–response relationships [Elhajouji et al., 1995; Doak et al., 2007] which appear to be relevant to understanding the biological relevance of NMs. The group felt that if a

single assay had to be selected, the in vitro micronucleus assay would be the most appropriate choice.

There was discussion of a number of important technical aspects to the in vitro micronucleus assay that need to be taken into consideration. For instance, avoiding the use of chamber slides and washing the cells using centrifugation steps prior to slide preparation will minimize the interference with micronucleus scoring caused by agglomerates that sediment out of solution, as noted by some investigators [e.g., Lindberg et al., 2009]. Cytochalasin B is often used in the in vitro micronucleus assay (to block cytokinesis and create binucleated cells), which may affect the uptake of the nanoparticles by the cells due to inhibition of filaments involved in endocytosis [Doak et al., 2009]. To address this, it is essential to allow a suitable exposure period to the NM in the absence of cytochalasin B. The preferred duration of the exposure period without cytochalasin B is presently not clear. If the exposure period without cytochalasin B were at least one cell cycle it would ensure that the NMs are present during mitosis. This is the most likely stage at which those NMs could come into direct contact with the DNA, as the nuclear membrane breaks down and the genetic material intermingles with all the cytoplasmic components [Scientific Committee on Consumer Products, 2007; Singh et al., 2009; Doak et al., 2009, 2012; Gonzalez et al., 2011]. On the other hand, if the particles exert an indirect genotoxic effect shortly after cellular uptake or are small enough to enter the interphase nucleus, prolongation of the period without cytochalasin B could reduce the detection of a genotoxic effect, as the cells would have time to divide before cytochalasin B is added.

Finally, a variety of cell types can be used in the in vitro micronucleus assay and there does not appear to be a significant advantage of one cell type over others, provided that the cell type is able to take up small particles. Monolayer cells can perhaps be washed more easily, but the actual exposure to NMs is more uncertain unless agglomeration occurs, in which case the particles may then settle on top of the cells, perhaps in a nonuniform manner. Exposure of NMs to suspension cells may be more uniform, but it is harder to centrifuge out the particles. At this point, for hazard identification and regulatory purposes it was recommended that standard cell types as recommended in the recent OECD In Vitro micronucleus guideline (OECD 487) be used. Investigation of the intracellular content for various NMs across a variety of cell types and exposure scenarios (long/short/with and without cytochalasin B) would be useful to address this issue. It is recognized however that NM genotoxicity does not always require internalization of the particles [Bhabra et al., 2009].

Mammalian Mutation Assays

Not all forms of DNA damage can be detected by any one test. Cytogenetic assays will quantify gross chromosomal damage which is a relatively large event. However, more subtle genetic changes can also arise in response to a genotoxin such as point mutations including base substitutions, frameshifts or small amplifications and deletions. Thus, it was suggested that mammalian mutation assays should also be considered in a test battery, particularly as there appear to be some NMs that are missed by the in vitro micronucleus assay [Doak et al., 2012], and especially if the bacterial mutagenicity assays are not informative or not performed.

Positive Controls

One question that has been raised in a number of publications is the appropriate positive control for NM assays [Singh et al., 2009; Doak et al., 2009; Gonzalez et al., 2011]. In fact, there appears to be some thinking that testing of NMs is not appropriate until NM positive controls can be identified. Since the purpose of a positive control is to ensure that the assay system can respond and the analysts are able to appropriately score the assay, it was concluded that standard positive control chemicals are appropriate for use at this point. In the future, other positive controls, or NM-specific controls may be identified.

In Vitro Only Test Strategy

Taking into consideration the emphasis in the scientific field on reducing animal use, including the March 2009 ban on in vivo genotoxicity testing for cosmetic ingredients (EU Cosmetics Directive to the EU 7th Amendment), REACH legislation, etc., we discussed the testing approach if only in vitro assays could be used. One might consider that a battery of in vitro tests alone would not appropriately reflect the in vivo situation, for instance when genotoxicity is induced as a secondary mechanism by inflammation [Driscoll et al., 1997; Greim and Norppa, 2010]. For this situation, it was felt that there were other tools to evaluate potential for inflammation in in vitro assays (e.g., cytokine release) that could be used to address this. However, there is presently not enough information to judge how well in vitro co-culture models or assays, e.g., release of inflammatory cytokines, can predict in vivo effects. In general, the details of the interplay between inflammation, genotoxicity, and carcinogenicity are not well understood. It is not adequately known which stages and mechanisms in the inflammatory process are critical for genotoxicity, how strong the inflammatory effect would need to be, and how long the inflammation should continue to result in genotoxic effects. Materials provoking strong inflammation at low doses can be expected to be more hazardous than those inducing milder effects at high doses, but prolonged inflammation even at a low level may also be important.

On the basis of the known difficulties in preparing test solutions of NMs that reflect human exposure for use in

in vitro and in vivo assays, we discussed whether there was value in considering an approach that if a physiologically relevant dosing solution of a nanoparticle could not be prepared for in vitro assays, in vivo tests alone be conducted. However, it was concluded that in vitro testing was still appropriate even in situations where the level of agglomeration of the nanoparticle may not be exactly representative of human exposure (often times the nature of the NMs in human exposures and in the body is not fully known). It was felt that there would still be enough relevance for in vitro assays to provide useful information for hazard identification. Many of the various mechanisms by which NMs may be genotoxic [Gonzalez et al., 2008, Sargent et al., 2009] are detectable in vitro. The genotoxicity of readily or partly soluble NMs may depend on the soluble form [Greim and Norppa, 2010]. Thus, it was concluded that in vitro tests are an important part of a test battery.

Important Gaps to Consider for Future Work

There are many gaps in our knowledge about NMs and covering them all was beyond the scope of discussion in the limited time frame for this meeting. A few key gaps were highlighted. A continuing problem is the lack of complete physico-chemical characterization of the NM being tested and how to express concentration metrics. Additionally, the lack of established NM carcinogens/NM noncarcinogens was seen as a key gap in the development and validation of genotoxicity testing approaches. It was concluded, however, that hazard identification of NMs using current tests was still important. Another area lacking is a coordinated effort on testing a range of NMs within a battery of standard in vitro and in vivo assays for direct comparative purposes, as opposed to studies that are typically conducted on specific NMs using individual assays, many of which are not standardized. It was felt that NMs programs like that organized by OECD may help in this regard. Most importantly, it was felt there was a need for studies to be published even when showing negative results, and for attention to physiological relevance of the concentrations tested.

Breakout Group 2: Standard In Vivo Assays: Assessment/Strategy Development

Question: How would in vivo assays fit into a testing scheme for genotoxicity assessment of NMs?

Outcome of discussion:

The conventional testing strategy today is to assess in vitro and in vivo genotoxicity in a battery of standard assays, which will usually include the in vivo micronucleus assay (OECD TG 474) in bone marrow or peripheral blood. For the time being, this is required for regulatory submissions. However, current data support the view that the standard in vivo micronucleus assay is not capable of capturing all relevant NM modes of action (MOA), as, for example, may be the case when nanoparticles accumulate in organs other than the hematopoietic system.

Genotoxicity in vivo might be associated with NM initiated site of contact effects, e.g., through indirect mechanisms like bursts of reactive oxygen species (ROS) caused by inflammation [Downs et al., 2012], or with a NM leaching product. It is therefore likely that the conventional in vivo testing schemes used today do not capture all potential effects of NMs. Despite these uncertainties with in vivo testing, some potential in vivo approaches are discussed below.

It was suggested that the testing paradigm be modified so that other in vivo mammalian tests would be conducted prior to conducting an in vivo genotoxicity test. The purpose of these tests would be to characterize the pharmacokinetics using different routes of administration, and to address internal exposure, target tissue concentration and toxicity, as well as the persistence of NMs in the body/organ/cell. On the basis of this information, which is expected to help determine an MOA, a decision would be made concerning the need for a genotoxicity test and how it would be designed or selected.

Another topic that was debated was the route of exposure. For particulate materials it appears that this is of even higher importance than for other chemicals and it was suggested that the exposure route relevant for the human exposure scenario should be used, if possible.

The relevant length of exposure was also discussed. Most tests are done with short-term exposures although the most concerning exposure for humans is chronic exposure. Although this is also true for the testing of chemicals, the notion that nanomaterial uptake and distribution will usually be slower than for chemicals points towards the need for longer exposure schemes for NMs. Thus, for testing NMs, an alternative approach might be more appropriate, i.e., a short-term repeat-dose rodent study (7- or 28-days) conducted first to determine fate and distribution of the NM. Targeted tissue-specific in vivo genotoxicity studies would be conducted only when the target tissue, NM accumulation, and histological or microscopic changes had been determined. It also would be important to know if ROS formation or inflammation is the primary MOA, with genotoxicity as a secondary effect.

Depending on the outcome of the initial rodent study, the standard in vivo micronucleus assay (OECD TG 474) might be insufficient to capture accumulated genetic damage of the NMs. Consideration should therefore be given to variants of the MNT that allow the determination of effects after repeat-dose exposures (like the 28d integrated MNT). In addition, assays should be considered that can detect DNA damage that has accumulated over time in different organs, like transgenic assays using rodent models such as MutaMouse, BigBlue®, or *Gpt-*

delta mice which can measure long-term mutational effects in neutral transgenes. An alternative option is to use, for example, the recently developed Pig-a assay (if the hematopoietic cells are targets for the NMs).

Another assay that seems well suited for detecting the genotoxicity of NMs is the modified Comet assay. This assay can provide mechanistic information when coupled with DNA repair enzymes (e.g., formamidopyrimidine-DNA glycosylase (FPG), endonuclease III (ENDOIII), and 8-oxoguanine DNA glycosylase (hOGG1)) that generate DNA strand breaks in the presence of specific oxidative DNA adducts. The Comet assay has been reported to detect DNA damage resulting from ROS formation [Collins, 2009], one of the main MOAs postulated for NMs.

It was also proposed during the discussion that a NMs-specific positive control would not be required. There is no known positive NMs control that would be suitable for genotoxicity testing. In general, positive controls for in vivo genotoxicity tests are not considered to be of great importance for the validity of the data, but used as evidence of assay performance.

Important Gaps for Future Work

It could be worthwhile to explore the suitability of more unconventional model organisms such as *Drosophila* or Zebra fish for assessment of the cytotoxic, genotoxic, or germ cell effects of NMs. However, there are no data that show the suitability of these systems, and it was argued that especially for inhaled particles these models do not mimic human systems.

Breakout Group 3: Integration of New Technologies: Which Ones and When Are They Needed?

Question: If we could start over and create a "strategy" for assessment of the genotoxicity of NMs, *what tests might be chosen and why?*

Outcome of discussion:

Several issues were discussed for which the standard assays may not be optimal, and thus there is a need for consideration of new or adapted technologies. This includes assays for measuring NMs uptake into cells/systems, distribution into target organs, and macromolecule (DNA or protein) binding. Uptake studies require either NMs which can be monitored with existing methods (ICP-MS) or auto-fluorescent particles without leakage for imaging. The same requirement for labelled particles or tracking mechanisms applies to understanding the distribution and disposition of NMs within a surrogate animal or human. It is not clear if older DNA-binding mechanisms (e.g., ^{32}P postlabeling) would suffice for NMs interactions. It is likely that NMs DNA-binding capacity would require new approaches (e.g., fluorescence for some NMs), the results of which might be critical to define primary genotoxicity of the NMs. The screening of protein binding capacity may be of great help to select adequate genotoxicity assays and to interpret the results; protein arrays are in development to establish specific NMs profiles.

Concerning in vitro tests, the group considered that bacterial cells are not adequate, and that the eukaryotic systems need adaptations to avoid undesired interference of NMs with some vital components of the assay (interaction with formamidopyrimidine-DNA glycosylase (fpg) for the Comet assay; Cytochalasin B timing for the in vitro micronucleus assay). Cell suspensions (e.g., as used in the mouse lymphoma assay, MLA) could be a problem because of poor contact with NMs. The identification of DNA interactions and DNA strand breaks was considered important. The micronucleus and Comet assays were discussed as useful in this context. New in vitro assays that might be considered include genomics and proteomics as related to detection of immune system effects, oxidative stress, inflammation and other pathways. These technologies also can be used to assess pathway interactions. The identification of biomarkers was also discussed.

Concerning in-vivo genotoxicity assays, they are considered critical for assessment of NMs since they allow the detection of inflammation and, therefore, secondary genotoxicity. The strategy for in vivo testing requires consideration of the route of exposure, NMs uptake kinetics and the involvement of target tissues. Many studies are based on inhalation, but exposure occurs *via* different routes of which oral exposure is of particular interest.

As far as positive controls are concerned, at the present time their choice should be guided by the necessity to prove the quality and reliability of the assay.

Other Question Discussed by Group 3: How Do We Address the Concern for Reactive Oxygen Species (ROS)?

NMs can produce ROS either directly or through interaction with cell membranes or mitochondria. Photoreactive NMs could generate ROS triggered by ultraviolet (UV) or other radiation. Current tests designed for the detection of ROS are probably not sensitive enough. Moreover, they can be problematic due to the interference of the NMs with some of the fluorophores used in those assays or because of autofluorescence of some NMs. As alternatives, Electron Spin Resonance and spin trapping might be better suited to assess the cellular production of free radicals. A key question concerns the differentiation of exogenous and endogenous ROS. Research in this field using reporter cell lines or chemical assays such as aldehyde production might be helpful.

How Can We Cover Potential Novel Mechanisms of Action of NMs?

Besides ROS production, novel mechanisms of action of the NMs are being identified, in particular remodelling

of cytoskeletal proteins (tubulins and actins), which might lead to changes in cellular trafficking, transmembrane transport, cytokinesis (binucleation and/or polyploidy), and chromosome non-dysjunction (aneuploidy) [Gonzales, 2010]. The in vitro/in vivo micronucleus tests detect both chromosome breakage and aneuploidy and, if combined with molecular probing (centromere fluorescence in-situ hybridization, FISH, or kinetochore staining), the two mechanisms can be distinguished. Additional molecular and/immunofluorescent detection of cellular organelles might be helpful for elucidating the mechanisms. "Omics" technologies might be used to elucidate novel mechanisms of action of NMs.

Are There "Gaps" That New Assays or New Technologies Could Fill With Regard to Assessment of NMs?

The major gaps relate to the characterization of NMs, their interaction with macromolecules and the interaction among response pathways induced by the NMs. Differentiating effects of substances in different physical states, e.g., amorphous versus crystalline, and with different sizes and surface areas, remains a considerable challenge. A lot of work remains to address these challenges and to validate new assays and approaches.

OUTCOME OF THE PLENARY SESSION—CONCLUSIONS

The chairs of the individual breakout groups presented an overview of the results of the group work to all participants of the workshop. During an open discussion among all participants the following points were made:

i. Need for Analytical Assessment of NMs Recognized

The premise under which the discussions have taken place was that any study will need to be supported with adequate physical and analytical data in order to understand how the NMs in question behave in the vehicle/system used.

ii. Current Genotoxicity Test Battery Needs Modification for NMs

- *Bacterial assays*: There was agreement among the workshop participants that, while it seems that the bacterial assays may provide limited value for testing NMs, it is too early to eliminate them from the test battery. The generation of Ames study data for a wider range of materials, ideally in a coordinated manner in parallel with other in vitro genotoxicity tests, was encouraged.
- *In vitro micronucleus assay*: The utility of the in vitro micronucleus assay was underlined as it is able to capture damage from ROS induced stress as well as potential direct interaction with the spindle apparatus that may lead to aneuploidy. It was also emphasized that the standard OECD protocol for the in vitro micronucleus assay may need to be modified to capture effects triggered by NMs.
- *In vitro mammalian cell mutation assays*: These assays should be considered in the test battery as well, especially if it can be demonstrated that the bacterial assays are not informative for NMs-induced damage. Also the micronucleus assay will likely not cover the full spectrum of potential genotoxic effects.
- *In vivo assays*: It was suggested that repeated dose toxicity studies could provide very valuable information about potential mode of action and target organs. This information then could be used to guide the performance of in vivo genotoxicity assays which would only be performed if tissue toxicity were observed. Alternatively, genotoxicity endpoints can be built into the RDT studies, which would also take care of a concern that came up during the workshop, i.e., whether the classical acute protocols of in vivo genotoxicity assays are suitable at all to capture genotoxic events triggered by NMs which may accumulate in certain tissues over time. The workshop participants also wanted to emphasize the utility of assays that allow easy access to a wide range of potential target organs, like e.g., the Comet assay and transgenic rodent mutation assays.
- *Test battery*: The workshop participants agreed that results from a battery of standard in vitro and in vivo genotoxicity assays may not be sufficient to adequately assess the genotoxic potential of engineered NMs. Modifications to test methods, tests chosen, and additional information may be needed beyond that obtained from the standard genotoxicity studies.

iii. Mechanistic Context for NMs Should be Addressed

It was acknowledged by the workshop participants that mechanisms may be at play for NMs that a standard genotoxicity battery may not be able to capture, or that may generate wrong conclusions (i.e., direct versus indirect genotoxic effects). Therefore it was recommended that additional tools be used which should help unravel potential modes and mechanisms of action. Tools that were suggested during the workshop include markers for generation of ROS, e.g., electron spin resonance techniques and spin trapping, and "omics" technologies that help elucidate the affected pathways which could be targeted to prove or discard a hypothesis for a specific NM.

iv. Research Needs Acknowledged

It is recognized that, while there is current knowledge supporting the above recommendations, more research will need to be done before a genotoxicity testing approach for NMs can be finalized, and that there may not be a "one size fits all" strategy that will work for this very diverse class of materials. The discussion from this workshop helps lay the foundation for future workgroups and international bodies expected to address the safety assessment of NMs.

APPENDIX A: LIST OF PARTICIPANTS BY BREAKOUT GROUP

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Structural characterization of the C_{60} nanowhiskers heat-treated at high temperatures for potential superconductor application

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Structure of the C_{60} nanowhiskers (C_{60}NWs) heat-treated at high temperatures was investigated by high-resolution transmission electron microscopy (HRTEM), electron energy loss spectroscopy (EELS), Raman spectrometry, thermogravimetric analysis, Brunauer-Emmett-Teller (BET) method, and so forth. Although the C_{60}NWs heated at 800 °C in vacuum showed a remaining of C_{60} molecules, the C_{60}NWs became amorphous by heating at 900 °C in vacuum, exhibiting the highest specific surface area of 195.2 m^2g^{-1}. The porous nanofibers synthesized by heating C_{60}NWs at high temperatures are new candidates of superconductive graphitic carbon.

Key words: C_{60} nanowhiskers, liquid-liquid interfacial precipitation method, LLIP method, fullerene, superconductor, carbon nanofiber

1. INTRODUCTION

In 2011, we discovered that C_{60} nanowhiskers (C_{60}NWs) exhibit good superconducting properties by doping potassium (K) [1,2]. The C_{60}NWs doped with K at 200 °C showed a superconducting transition temperature (T_c) of 17 K, and the highest shielding fraction of a full shielding volume was found in the C_{60}NWs with a nominal composition of $K_{3.3}C_{60}$, although that of a K-doped fullerene crystal was less than 1% [1]. It was considered that such a high superconducting volume fraction of C_{60}NWs was owing to the homogeneously distributed nanosized pores that could assist the rapid diffusion of K through the whole body of C_{60}NWs. Hence, the fabrication of porous C_{60}NWs is an important theme in their superconducting application. The C_{60}NWs have a low density less than 2.0 gcm^{-3}. The fibrous C_{60}NWs are quite suited to fabricate the lightweight and flexible superconducting wires for power transfer, power generators, electric mortars and so forth.

Carbon superconductors other than C_{60} have been investigated for many years. Graphite specimens doped with alkali metals such as C_8K (T_c < 0.55 K [3], 0.128-0.198 K [4]), C_8Cs (T_c=0.020-0.135 K [3]) and C_8Rb (T_c=0.023-0.151 K [3]) were reported. Recently, graphite superconductors such as C_6Ca (T_c = 11.5 K) and C_6Yb (T_c = 6.5 K) were synthesized [5].

C_{60}NWs can be turned into glassy carbon nanofibers by heat-treatment at high temperatures [6, 7]. The C_{60}NWs heated at 3000 °C turned to the carbon nanofibers with a few to about 18 graphene layers [6]. The number of stacked graphene layers was observed to increase with increasing the heat treatment temperature between 2000 °C and 3000 °C [6]. Those C_{60}NWs heated at high temperatures with developed graphitic ribbons are also a promising nanocarbon material that may exhibit superconductivity by doping alkali metals and alkaline-earth metals.

On the other hand, the C_{60}NWs heat-treated at 900 °C in vacuum showed a specific surface area for water adsorption as high as 155 m^2g^{-1}, although the water adsorption ability of C_{60}NWs rapidly decreased to 2 m^2g^{-1} with increasing the heat treatment temperature up to 2500 °C [8]. Hence, since the heat-treated C_{60}NWs show such unique surface properties, it is necessary to investigate their detailed microstructure. This knowledge should be useful for their future superconductive application as well.

2. EXPERIMENTAL

The C_{60}NWs were synthesized by the liquid-liquid interfacial precipitation (LLIP) method, using C_{60} (99.5% pure, MTR Co.,USA)-saturated toluene solutions and isopropyl alcohol as previously described [9, 10]. The structural characterization of C_{60}NWs was performed by Raman spectroscopy (JASCO NRS-3100, Japan) and transmission electron microscopy (TEM, JEOL JEM-2800, Japan).

The as-synthesized C_{60}NWs were dried in air and heated in vacuum for 120 min at temperatures between 800 and 900 °C at a heating rate of 10 °$Cmin^{-1}$ in evacuated fused silica tubes by use of a muffle furnace.

The specific surface area measurement of the heat-treated C_{60}NWs was conducted by the Brunauer-Emmett-Teller (BET) method (Autosorb iQ AG Gas Sorption System, Quanta Chrome, USA).

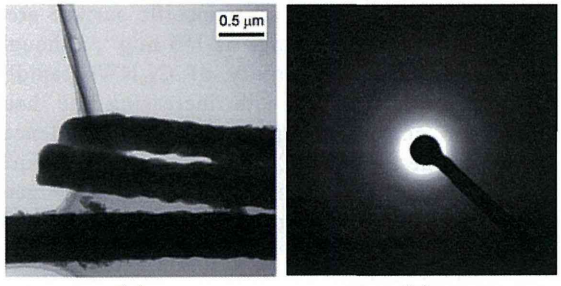
The pore size distribution of heat-treated C_{60}NWs was analyzed by the Density Functional Theory (DFT) method and the Barrett-Joyner-Halenda (BJH) method, using the software (AsiQwin) of the Quanta Chrome gas sorption system and the N_2 adsorption-desorption isotherms recorded at 77 K, where a slit-shaped pore geometry for the micropores and a cylindrical pore geometry for the mesopores were assumed in the pore size calculation in the DFT method [13].

The thermal properties of C_{60}NWs and the heat-treated C_{60}NWs were investigated by the thermogravimetric analysis (TGA, SII TG/DTA 6200) in air at a heating rate of 10 °C min^{-1}.

3. RESULTS AND DISCUSSION

The C_{60}NWs heat-treated at 900 °C were observed to retain their linear morphology as shown in a TEM image of Fig. 1 (a). As shown in the selected-area electron diffraction pattern (SAEDP) of Fig. 1 (b), however, the C_{60}NWs lost their original crystalline structure and exhibited an amorphous structure by the heat treatment at 900 °C.

As shown in the Raman profile of Fig. 2, the C_{60}NWs heat-treated at 800 °C show the $A_g(2)$ peak characteristic to C_{60}. However, the Raman profile of the C_{60}NWs heated at 900 °C shows no clear $A_g(2)$ peak (Fig. 3), indicating that all the C_{60} molecules turned to amorphous carbon.



(a) (b)

Fig. 1 (a) Bright-field TEM image and (b) SAEDP of the C_{60}NWs heat-treated at 900 °C in vacuum.

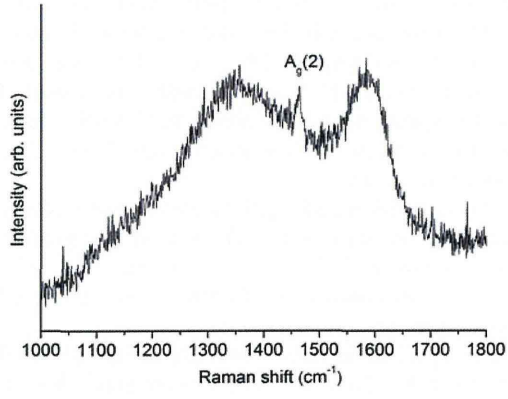


Fig. 2 Raman profile of the C_{60}NWs heat-treated at 800 °C in vacuum.

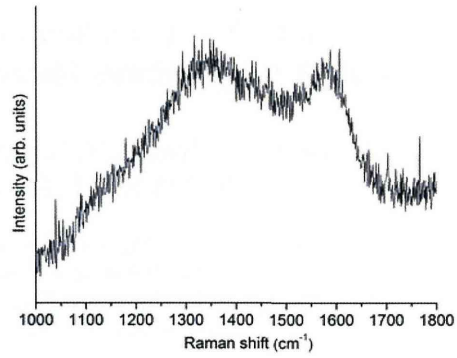


Fig. 3 Raman profile of the C_{60}NWs heat-treated at 900 °C in vacuum.

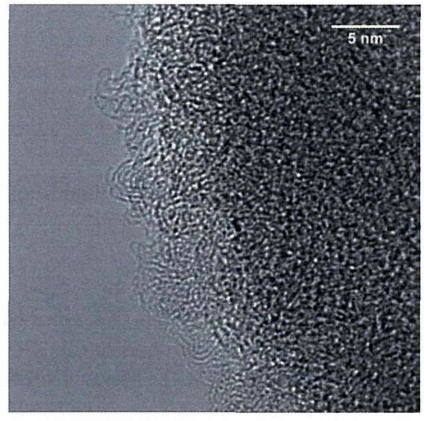


Fig. 4 HRTEM image of a C_{60}NW heated-treated at 900 °C in vacuum.

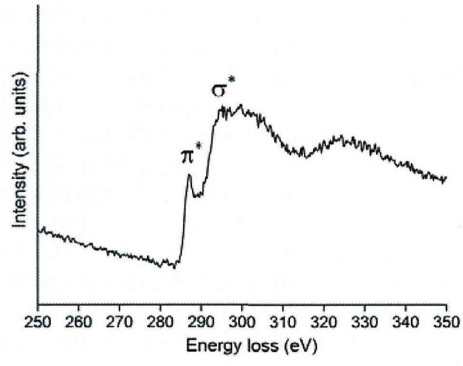


Fig. 5 EELS spectrum of a C_{60}NW heated-treated at 900 °C in vacuum.

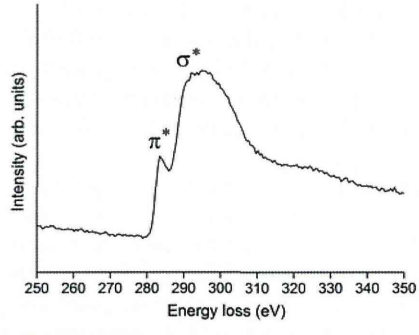


Fig. 6 EELS spectrum of a TEM amorphous carbon microgrid.

The high-resolution TEM (HRTEM) image of a C_{60}NW heat-treated at 900 °C of Fig. 4 shows the formation of randomly oriented graphene layers. The number of stacked graphene layers in the image is less than several layers, and the graphitic structure is not well developed in contrast to the C_{60}NWs heated-treated at 3000 °C [6].

The EELS spectrum of a C_{60}NW heat-treated at 900 °C of Fig. 5 strongly resembles the profile of amorphous carbon as shown in Fig. 6.

As shown in the TEM image of Fig. 7, the C_{60}NWs heat-treated at 900 °C show a highly porous structure with the distributed nano-sized pores.

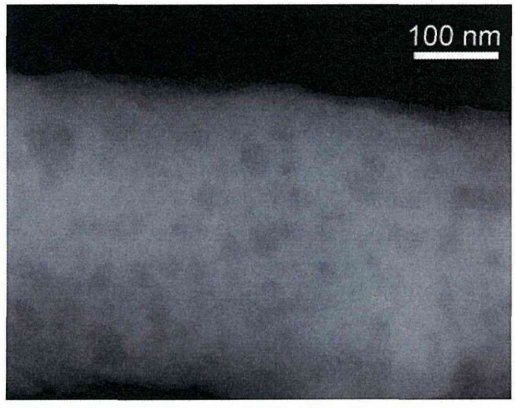


Fig. 7 TEM image of a part of C_{60}NW heat-treated at 900 °C in vacuum. The bright-field contrast was reversed to show the porous structure more clearly.

The pore size distribution for the heat-treated C_{60}NWs was measured as shown in Figs. 8 and 9.

The pore size distribution was markedly changed by the heat treatment at 900 °C from 800 °C, i.e., the broad peak indicated by arrow in Fig. 8 became much sharper in the C_{60}NWs heat-treated at 900 °C, which means the formation of more uniform-sized mesopores with similar radii of about 19 Å.

The specific surface area of the C_{60}NWs heat-treated at 800 °C was measured to be 171.0 m^2g^{-1} and that of the C_{60}NWs heat-treated at 900 °C was measured to be 195.2 m^2g^{-1} by N_2 adsorption at 77 K. The value of 195.2 m^2g^{-1} is very close to that of 195 m^2g^{-1} of the C_{60}NWs heat-treated at 900 °C in our previous paper [8]. This very good coincidence in the specific surface area between the different experiments shows that the LLIP method can synthesize the C_{60}NWs with similar structural properties with a good reproducibility.

Fig. 10 shows the change of specific surface as a function of heat treatment temperature. The specific surface area is the highest at 900 °C and decreases with increasing the heat treatment temperature. The decrease of specific surface area shows the matrix densification accompanying the development of multilayered graphitic structure [6].

Fig. 11 shows the TGA curves that were measured for the C_{60}NWs heat-treated at 800 °C and 900 °C. It is noted that the small hump shown by arrow in Fig.11 (a) is not observed in the curve of Fig. 11 (b). This disappearance of hump coincides with the disappearance of Raman $A_g(2)$ peak in the C_{60}NWs heat-treated at 900 °C (Fig. 3).

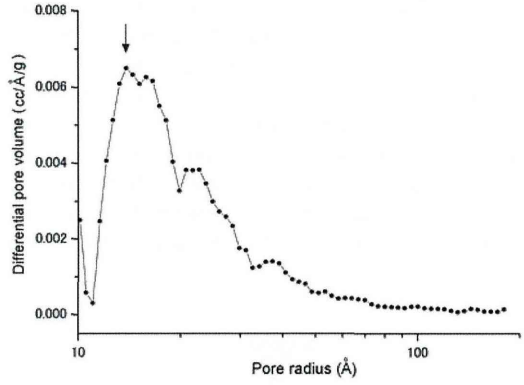


Fig. 8 Pore size distribution measured by the DFT method for the C_{60}NWs heat-treated at 800 °C.

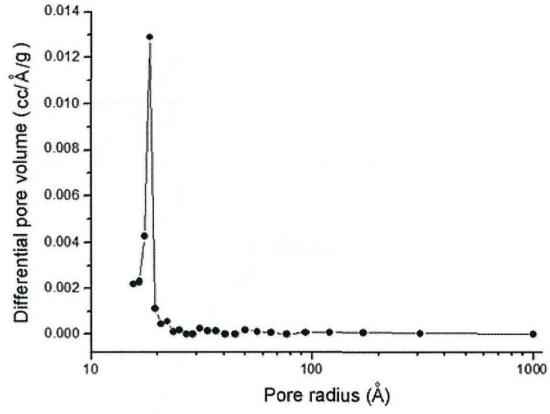


Fig. 9 Pore size distribution measured by the BJH method for the C_{60}NWs heat-treated at 900 °C in vacuum.

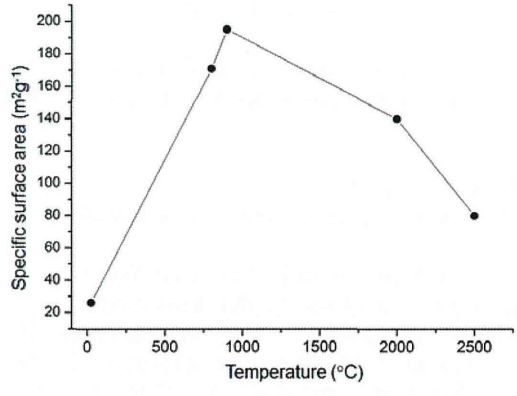


Fig. 10 Change of the specific surface area of C_{60}NWs as a function of heat-treatment temperature. The points for the heat treatment temperatures of 2000 °C and 2500 °C were obtained from ref.[8], while the point for the room temperature was obtained from ref.[11].

Hence, it is considered that the hump observed in Fig. 11 (a) is partly due to the chemisorption of oxygen to the C_{60} molecules remaining in the C_{60}NWs heat-treated at 800 °C [12].

The onset temperature (T_e) of decomposition in the heat-treated C_{60}NWs is 386 °C in Fig. 11 (a) and 387 °C in Fig.11 (b), while the offset temperature (T_o) of decomposition is 611 °C in Fig.11 (a) and 624 °C in Fig.11 (b). The increase in T_e and T_o shows that the C_{60}NWs become a little more stable against the oxidation by increasing the heat treatment temperature. It is conjectured that the slight increase in T_e and T_o is due to the structural change of the residual C_{60} molecules into the amorphous carbon phase by the heat treatment at 900 °C.

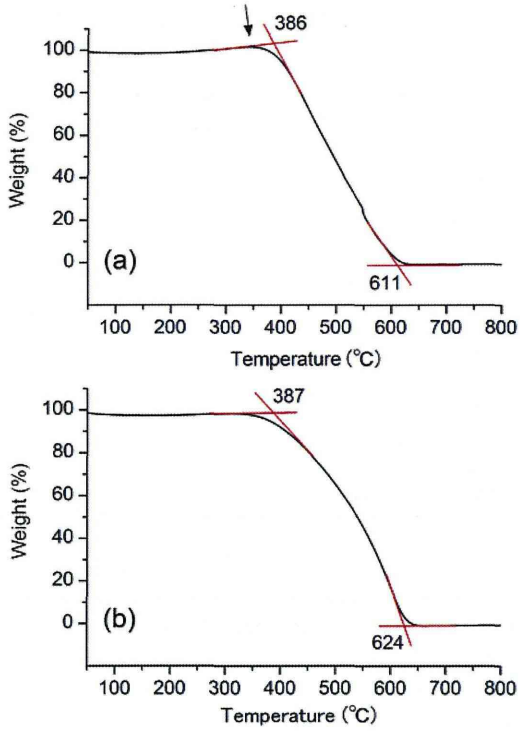


Fig. 11 TGA curves measured in air for the C_{60}NWs heat-treated in vacuum at (a) 800 °C and (b) 900 °C.

4. CONCLUSIONS

The above research can be summarized as follows.

(1) It has been found that C_{60}NWs turn to amorphous carbon by the heat treatment at 900 °C in vacuum. The remaining of C_{60} molecules, however, was observed in the C_{60}NWs heat-treated at 800 °C in vacuum.

(2) The C_{60}NWs heat-treated at 900 °C in vacuum showed the highest specific surface area of 195.2 m^2g^{-1}, having the mesopores with similar radii of about 19 Å.

(3) The C_{60}NWs heat-treated at 900 °C showed the slightly higher stability for oxidation than the C_{60}NWs heat-treated at 800 °C.

ACKNOWLEDGEMENT

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—Review—

ナノマテリアルの健康影響評価指針の国際動向

広瀬明彦

International Trend of Guidance for Nanomaterial Risk Assessment

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In the past few years, several kinds of opinions or recommendations on the nanomaterial safety assessment have been published from international or national bodies. Among the reports, the first practical guidance of risk assessment from the regulatory body was published from the European Food Safety Authorities in May 2011, which included the determination of exposure scenario and toxicity testing strategy. In October 2011, European Commission (EC) adopted the definition of "nanomaterial" for regulation. And more recently, Scientific Committee on Consumer Safety of EC released guidance for assessment of nanomaterials in cosmetics in June 2012. A series of activities in EU marks an important step towards realistic safety assessment of nanomaterials. On the other hand, the US FDA announced a draft guidance for industry in June 2011, and then published draft guidance documents for both "Cosmetic Products" and "Food Ingredients and Food Contact Substances" in April 2012. These draft documents do not restrictedly define the physical properties of nanomaterials, but when manufacturing changes alter the dimensions, properties, or effects of an FDA-regulated product, the products are treated as new products. Such international movements indicate that most of nanomaterials with any new properties would be assessed or regulated as new products by most of national authorities in near future, although the approaches are still case by case basis. We will introduce such current international activities and consideration points for regulatory risk assessment.

Key words——nanomaterial; risk assessment; cosmetic product; food ingredient; food contact substance

1. はじめに

ナノマテリアルの安全性に関する国際的な関心が高まって以降，各国及び国際的な評価機関から様々なオピニオンや提言が公表されてきているが，一般的な概説やデータの収集が必要であるといったような一般論に終始しているものがほとんどであった．しかしながら最近になって，EFSA の科学委員会より 2011 年の 5 月に，より実際的なリスク評価ガイドラインが公開された．その後，化粧品や化学物質も含めて，欧米の規制当局では様々なガイダンスが公表されるようになってきている．このような国際的な動向は，現状ではケースバイケースのアプローチではあるものの，近い将来にはほとんどの新規物性を持つナノマテリアルが，ほとんどの国の規制当局によって新規物質又は新製品としてリスク評価が行われるようになることを示唆していると思われる．本稿では有害性評価の観点からの健康影響評価指針の作成に関する最近の国際動向について，特に欧州や米国の動きを中心に紹介する．

2. 欧州の動向

欧州ではナノマテリアルの安全性に関する論議は比較的活発で，欧州委員会では，2008 年に「ナノマテリアルは REACH で規制する」との基本姿勢を示しており，2009 年には，ナノマテリアルに関して Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH) を実施する際の要点について具体的かつ包括的な科学的並びに技術的アドバイスを提供するための「ナノマテリアルに対する REACH 実施プロジェクト (REACH Implementation Project on Nanomaterials; RIPoN)」を開始した．2011 年 7 月には，ナノマテリアルの場合に

The author declares no conflict of interest.

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本総説は，日本薬学会第 132 年会シンポジウム S25 で発表したものを中心に記述したものである．

REACHに記載すべき情報の要件（試験方法の適切さを含む）や化学的安全評価について詳細に明示したガイダンスとなる報告書の最終版を公開している.[1] 規制動向としても2009年12月に他地域より早く，欧州議会においてナノマテリアルの安全性データの届出と表示を含めた新化粧品指令（Regulation (EC) No 1223/2009）が採択されている（施行は2013年7月より）．一方，食品関係では予防原則の下に2008年12月には既存の食品添加物であっても，ナノテクノロジー等を用いて製法や粒子サイズを変更した場合は再評価が必要であることが指令（Regulation (EC) No 1333/2008）された．その後，2009年に欧州食品安全機関（European Food Safety Authority; EFSA）からナノ食品のリスクに関するオピニオンが公表された.[2]

このような状況の中2011年5月に，「食品/飼料へのナノ科学とナノテク応用から生ずる可能性のあるリスクに関するリスク評価ガイダンス」がEFSAより発表された.[3] このガイダンスではそれまでの概論的な提言とは異なり，より具体的な評価ガイダンスが示されている．その物質の物性や曝露シナリオ，化学組成としての新規性に従った以下の6つのケースを想定し，適切な毒性評価試験を組み合わせることによって具体的な評価方針を示している．

Case 1：ナノテクノロジーを利用しているが，製品中にはナノマテリアルは残存していない．

Case 2：容器等からのナノマテリアルの溶出はない．

Case 3：製品中では，完全に（適切な分析法により）非ナノサイズ（分解，溶解等）になっている．

Case 4：消化管の中で，分解することが（適切な分析法により）証明されている．

Case 5：同じ化学組成を持つ非ナノサイズの物質についての毒性情報が既に知られている場合．

Case 6：同じ化学組成を持つ非ナノサイズの物質についての毒性情報が不明な場合（全くの新規物質）．

基本的な考えとしては，Case 1–Case 4の場合においては，体内に吸収する前に非ナノサイズ物質となることで，通常の化学物質の評価手法が適用できることになると考えている．ただし，Case 4の場合は，消化管内まではナノサイズである可能性があり，刺激性などのナノマテリアルとしての局所影響を評価する必要があるとしている．そしてこれらのCaseの検討の結果，消化管内でもナノサイズである可能性が否定されない場合は，最小限の毒性試験項目として*in vitro*遺伝毒性試験と*in vivo*試験としてADME試験，90日間反復経口投与毒性試験が要求される（*in vitro*試験が陽性，又は*in vitro*試験ができない場合は*in vivo*遺伝毒性試験が必要）．これらの試験結果は，同じ化学組成を持つ非ナノサイズの物質についての毒性情報と比較され，必要に応じて更なる毒性試験を行うことが推奨されている．一方，化学組成からして全くの新規物質である場合は，ナノサイズであるなしにかかわらず，元来，慢性毒性や生殖発生毒性も含めたすべての毒性試験項目が必要となるので，上記のような曝露シナリオに依存した毒性試験の評価スキームは適用されない．

しかし現状では，食品中や消化管内等の複雑なマトリックス中のナノマテリアルのサイズを的確に測定する手法はまだ確立されておらず，安全側に立てば，ナノマテリアルが分解されないことを前提に毒性試験を行う可能性が高いこと．さらにこれまでのガイドライン化された毒性試験を適用した結果が有効であるかどうかについて，特に食品を対象とした経口投与試験について十分な知見も得られていないこと．これらの現状を考慮すると，当面はケースバイケースの対応を積み重ねていく必要があることが，今後の課題とされている．

さらに，2012年の6月には消費者安全科学委員会(Scientific Committee on Consumer Safety; SCCS)が化粧品中のナノマテリアルの評価ガイダンスを公表し，そこにも上記のような曝露シナリオに基づいた安全性評価スキームが取り入れられた.[4] こちらは主要な曝露経路が経皮曝露であるので，皮膚透過性の有無に重点が置かれているほか，局所影響としては皮膚刺激／腐蝕性，感作性，光毒性などが重要なエンドポイントとなっている．さらに，2013年7月より施行される動物実験の全面禁止を背景に，*in vitro*試験など実験動物代替試験法への取り込みが，緊急的な課題としてとりあげられている．

3. 米国の動向

米国でもナノテクノロジーやナノマテリアルの安全性に対する議論の必要性は早くから認識されているが，欧州のような包括的な法的規制にまでには至っていない．化学物質管理の観点からは，2007年にU. S. Enviromental Protection Agency（EPA）が有害物質管理法（Toxic Substances Control Act; TSCA）の下に，ナノマテリアルを取り扱う企業等に対し自主的なデータの提出を促すスチュワードシッププログラムを開始したが，期待したほどの情報を得られないようであった．しかしEPAは2010年から，単層多層のカーボンナノチューブについて，製品毎の製造前届出（PMN）を必要とする重要新規利用ルール（significant new use rule; SNUR）を公布した.[5] 届けデータとしては，懸念されている吸入曝露影響に対して90日間吸入試験等が要求されており，実質的に必要な試験項目として認識されつつある．しかし，この製造前届出データの範囲は各製品に対応したケースバイケースの措置の結果であり，さらに化学的特性等は企業秘密として開示されていないことも含め一般化される評価指針として利用することはできない．

一方U. S. Food and Drug Administration（FDA）は，2007年に公表されたナノテクノロジータスクフォースの報告書を受けて，2011年6月にようやく業界向けに「FDA規制権限製品にナノテクノロジーが利用されているかどうかの検討」に関するガイダンス案を公表した.[6] このガイダンスでは，ナノマテリアルの定義としてそれまでに通常使用されてきた「三次元のうち少なくとも一次元のサイズが1から100 nmの範囲に入る」という定義に加えて，たとえ100 nmを超えるサイズであったとしても，そのサイズに起因した物理学的あるいは生物学的特性を示すかどうかという点にも着目している．その際の検討するサイズの上限を1 μmとしている．その後，具体的なFDA規制関連製品に関するガイダンスとして，2012年4月に食品材料及び食品接触物質（食器など）[7]と，化粧品[8]の安全性評価に関する業界向けのガイダンスをそれぞれ公表した．化粧品は，着色料を除いては事前承認が必要ではないが，安全性確保のための製造業者等の責任において，考慮すべき安全性評価上の論点が示されており，以下に示す因子が重要であるとされている．

- 物理化学的特性
- 最終製品に対応した毒性試験条件での凝集状態と粒径分布
- 不純物
- 製品からの曝露可能レベルと最終製品中での凝集性
- *in vitro* 及び *in vivo* 試験に供する用量の測定法
- 化粧品成分とそれらの不純物に関する *in vitro* と *in vivo* の毒性データ，皮膚透過性，眼及び皮膚刺激性，感作性，変異原性／遺伝毒性
- 化粧品成分や最終製品に対するヒトボランティア臨床試験

また，化粧品の安全性試験としてナノマテリアルの評価に適した *in vitro* 試験開発の重要性にも触れているが，試験系における凝集性について対応することが必要であるとされている．特に不溶性のナノ粒子については *in vivo* 試験の方が適切であるかもしれないとコメントしている．

食品材料及び食品接触物質のガイダンスにおいては，既認可食品及び食品接触成分に対してナノテクノロジーを利用して製造工程を変更した場合に対して，安全性評価上検討すべき論点について記されている．重要な製造工程の変更としては，出発物質の種類や濃度変更，及び触媒の変更と並んで食品成分の粒子サイズに影響を与えるような新技術が例示として挙げられている．勧告案は，21CFR（連邦規則集）に記載された食品添加物及び色素，21CFRのgenerally recognized as safe（GRAS）として同定された物質，food contact notification（FCN）申請された食品接触物質，既にGRASとしての使用が，認証された物質の4分類に分けて記載されている．しかし，各々の物質に対して検証すべき項目はほぼ同様で，既に認証あるいは申請が認められた成分との同一性に影響を及ぼした物理化学的特性と，その変化に対応した安全性評価を行うこと，製造過程の変更の度合いやその結果製造された製品の規格や不純物が法的基準の範囲に入るかどうかを検証することといったような，抽象的な記載となっている．さらに最終的には，必要に応じてFDAと相談することが推奨されている．

4. 国際機関の動向

化学物質管理関係では，欧米におけるナノマテリ