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First Approaches to Standard protocols and Reference Materials for the Assessment of Potential Hazards Associated with Nanomaterialss

Gordon Chambers
Technological University Dublin, Gordon.chambers@tudublin.ie

Hugh Byrne
Technological University Dublin, hugh.byrne@tudublin.ie

Iseult lynch
University College Dublin

Micheal Riediker
Institute for occupational safety and health, Augustin, Germany.

Hans Bouwmeester
1) RIKILT – Institute of Food Safety, Wageningen UR, P.O. Box 230, 6700 AE Wageningen, The Netherlands, hans.bouwmeester@wur.nl

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See next page for additional authors

Authors

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First approaches to standard protocols and reference materials for the assessment of potential hazards associated with nanomaterials

Iseult Lynch[1*], Hans Bouwmeester[2], Hans Marvin[2], Alan Casey[3], Gordon Chambers[3], Markus Berges[4], Martin J.D. Clift[5], Teresa F. Fernandes[6], Lise Fjellsbø[7], Lucienne Juillerat[8], Gert Roebben[9#], Christoph Klein[10#], Qinglan Wu[11], Vince Hackley[12], Jean-Pierre Kaiser[13], Wolfgang Kreyling[14], C. Michael Garner,[15] Peter Hatto[16], Kenneth Dawson[1], and Michael Riediker[17]

1 Centre for BioNano Interactions; School of Chemistry & Chemical Biology; University College Dublin, Ireland
2 RIKILT – Institute of Food Safety, Wageningen UR, P.O. Box 230, 6700 AE Wageningen, The Netherlands
3 Focas Research Institute, Dublin Institute of Technology, Kevin Street, Dublin 8, Ireland
4 Institute for Occupational Safety and Health, Alte Heerstraße 111; 53754 Sankt Augustin Germany
5 Institute of Anatomy, Division of Histology, University of Bern; Baltzerstrasse 2; CH-3000 Bern 9; Switzerland
6 Edinburgh Napier University, School of Life Sciences, Edinburgh EH10 5DT; United Kingdom
7 NILU - Norwegian Institute of air research; Instituttveien 18, NO-2027 Kjeller, Norway
8 Institut Universitaire de Pathologie / CHUV / UNIL, Bugnon 25, CH-1011 Lausanne, Switzerland
9 European Commission JRC- Institute for Reference Materials and Measurements (IRMM), Retieseweg 111, B-2440 Geel , Belgium.
10 European Commission JRC- Institute for Health and Consumer Protection (IHCP) Nanotechnology and Molecular Imaging (NMI), Via E. Fermi 2749, I-21027 Ispra, Italy
11 DNV Research & Innovation, Det Norske Veritas AS, Veritasveien 1, N-1322 Høvik, Norway
12 National Institute of Standards and Technology, 100 Bureau Drive, Stop 8520, Gaithersburg, MD 20899-8520, USA.
13 Materials Science and Technology (Empa), Lerchenfeldstrasse 5, St. Gallen, CH-9014, Switzerland
14 Institute of Lung Biology and Disease, Focus Network Nanoparticles and Health, Helmholtz Center Munich, Ingolstädter Landstr.1, D-85764 Neuherberg / Munich, Germany
15 External Materials Research, Intel Corp., Santa Clara, California
16 IonBond Ltd., Consett, UK United Kingdom
17 Institute for Work and Health, Rue du Bugnon 21, CH-1011 Lausanne, Switzerland

*) Corresponding author
#) The views expressed by this author are purely those of the author and may not in any circumstances be regarded as stating an official position of the European Commission.

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Hugh J. Byrne, Focas Research Institute, Dublin Institute of Technology, Dublin.

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Contents

1 Summary

All new technologies have an inherent risk, which is typically assessed alongside the development of applications of the technology. This is also the case for nanotechnology: a key concern in the case of engineered nanomaterials (ENMs) is that due to their very small size, NMs can reach areas such as the cell that are inaccessible to other materials, such as implants and drugs. As a result of their large surface area, NMs may be more reactive than other larger materials. The large physical and chemical variability of NMs, and the fact that small changes can have large consequences, mean that there is insufficient data on which to make safety or risk assessments at present. Thus, a widely supported scientific basis and sufficient high quality data upon which to base regulatory decisions are required urgently. NanoImpactNet (NIN) can support the development and dissemination of such data.

This report presents the outcome of the discussions of 60 experts in the field of safety assessment of manufactured NMs from academia, industry, government and non-profit organizations on some of the critical issues pertaining to the development of standard protocols and reference materials for assessment of the potential hazards associated with ENMs. It should be noted here that there was a separate NIN workshop on determining the best metrics for assessing NP safety, and that this workshop was directed specifically to how best to standardise testing protocols and develop reference materials for human health assessment.

Some of the major conclusions drawn are listed below:

- There is an urgent need for nanoparticle (NP) reference materials, not just for calibration of physical or chemical measurement methods, but also with carefully controlled surface properties for use as positive and negative controls for various biological impacts. However, the validity of positive and negative control reference NMs was questioned, given the very significant batch-to-batch variability being reported for nanoparticles from all commercial and laboratory sources, not just in terms of their size but also in terms of their surface chemistries.
- There is an urgent need to share details of protocols and best practice in the assessment of NM impacts on living systems. NIN offers an excellent platform to achieve this, and the project website should enable access to consortium members to help develop a structured approach to method development. This has since been implemented within the NIN consortium.
- It was recommended by the workshop participants that Organisation for Economic Co-operation and development (OECD) could / should provide templates for the type of data and supporting documentation that they require for the validation of protocols, and could also provide some training workshops in respect of the process necessary for development and validation of test methods and reference materials (e.g. biological positive and negative NP controls), such that these can be built into the protocols that are emerging within the scientific community. NIN could facilitate this, via one of its training schools.
- The need for another workshop on this topic closer to the end of the NIN project was highlighted during this workshop, as clearer consensus of the best practice, and recommendations regarding which

protocols for which end-point should be significantly advanced by then. Additionally, a second workshop would give the consortium a chance to reflect on the role of NIN in facilitating the onward development and framing of the field in relation to human health impacts assessment.

2 Background

A nanomaterial is defined as a material that has one or more external dimensions in the nanoscale or which is nanostructured.[1] A nano-object is defined as a discrete piece of material with one or more external dimensions in the nano-scale,[2] and includes nanoparticles (NPs), which for the purpose of NIN reports are defined as "a sub-classification of ultrafine particles with lengths in two or three dimensions greater than 0.001 micrometre (1nm) and smaller than about 0.1micrometre (100nm) and which may or may not exhibit a size-related intensive property." The physical properties of materials at the nanoscale can differ from their bulk counterparts with large reactive surface area to volume ratios in NMs which may lead to size-dependent effects.

While there is extensive literature on the toxicity of ambient aerosol particles[3] and a growing literature on the interactions of ENMs in biological systems,[4] there remains considerable uncertainty regarding the potential toxicity of ENMs,[5] and the approaches by which this is evaluated.[6] Much of the current literature is clouded by issues such as chemical elution from NMs and aggregation of NMs into micron scale aggregates under the test conditions. As nanotechnology is developing very rapidly, with increasing levels of commercial production of EMNs, a series of standardized tests and protocols to assess the potential toxicity of these unique materials in biological systems is needed urgently.

The lack of standard approaches and protocols in the assessment of potential NM hazards for human health is rapidly becoming a critical road block to the success of the entire field, and needs to be addressed urgently in order to ensure that the trust of the public, regulators and policy makers internationally is not lost irrevocably. Issues such as the significant variability of surface properties between nominally "identical" particles from different batches (batch-to-batch variability); the tendency of NMs to aggregate under physiological conditions (ionic strength, pH and interaction with biomolecules all affect the dispersion characteristics and stability); differences in the cell passage number and degree of confluence of cells and a host of other factors all contribute to the enormous variability of biological impacts reported in the literature. This situation is untenable, as generating increasing amounts of data that are irreproducible and incomparable to other data sets will do little to address the multiple knowledge-gaps that have been identified, and may in fact result in the downfall of the nanotechnology revolution unless significant and concerted action is taken immediately. Thus, the need for standardized methodologies and experimental protocols that remove potential sources of irreproducibility in testing the potential toxicity of NMs, and for in-depth characterization of NMs under the relevant experimental / test conditions, in order to ensure that comparisons can be made amongst *in vitro* investigations is evident.

Other fields that have recently emerged have successfully applied the principles of round-robin approaches to validate themselves and their methodologies, such as the genomics and proteomics fields which operated via the Human Genome Organisation (HUGO) and Human Proteome Organisation (HUPO) respectively. The pharmaceuticals industry has also successfully applied the round-robin approach to validate new drugs, driven via the European Medicines Agency (EMEA). NIN, which is the largest European grouping of research, academic and other organisations in the field of nanosafety, is ideally placed to play a similar role for the issue of nanosafety, and the goals of this and the subsequent workshops and training schools are to identify approaches and areas where NIN can play a leading role in terms of clarifying many of the most pressing and outstanding issues that are acting as roadblocks to the successful and safe implementation of nanotechnology.

3 Procedure

The workshop was advertised on the NIN website, and the announcement was distributed widely among the NIN partners and collaborators, and beyond. Registration was open to all who could demonstrate that they are active in the field of nanosafety, nanotoxicology, nanobiology or related. In order to ensure that the discussions were manageable, participation was on a first-come-first-served basis and was limited to 60 participants.

In each session the topic was introduced by two plenary lectures followed by a general discussion in two randomly generated groups. Discussions during the workshop were structured into four specific sessions, as described in Table 1. The discussions were guided by a number of pre-defined questions and managed by an appointed chair. The chairs were assisted by rapporteurs who were appointed to prepare a report (including the major discussion points). The rapporteurs' reports, as well as the summaries of presentations from the plenary lectures, were used as the foundation for this report (see annex 1 for list of speakers and the titles of their presentations). The authors of the present report include the chairs, rapporteurs and presenters from the workshop.

Table 1 – Topic areas covered during the Workshop.

	Discussion topic
Session 1	Are there data we are certain of, that could be the basis of a comparative testing approach? Is there an emerging direction that offers real promise that we could focus on? Are there pitfalls that should definitely be avoided?
Session 2	Can we make nanosafety/nanotoxicology a quantitative science? Can we identify new end-points to investigate? How can these be incorporated into OECD testing? Is high-throughput screening a viable way forward?
Session 3	Within the research arena of human health effects, are there certain aspects where we (EU, NIN team members) are world-leading (niche areas – e.g. the protein corona)? Are there known pitfalls in protocols/ experiments that should definitely be avoided? Should we focus on validation of new methodologies or the refinement of existing metrologies for nanomaterials? How should we progress with the topic of biological system characterization?
Session 4	How can we best gather the existing protocols and standard operating procedures (SOPs) from all sources and cross-validate them? Are reference standard materials for specific biological end-points (positive and negative control NMs) a real possibility when batch-to-batch variability of NMs is so high? How can NIN optimally contribute to existing efforts (OECD, ISO, IAHN etc.)?

4 Results

4.1 Session 1: Key points to build upon from existing state-of-the-art

An important element of NIN is to coordinate research findings from the ongoing EU projects in which its partner organisations are engaged, in order to ensure that the knowledge gained is transferred into other projects in an appropriate manner. Thus, the aim of the first session was to highlight some key findings from ongoing / ending EU projects, as the basis for future progress. Presentations from the EU projects NanoTransport and NanoInteract were given, focussing on the experiences of these projects ("lessons learned") as well as the current state of the art in nanosafety evaluation.

The initial discussions following these presentations centred on occupational exposure, and it was highlighted that the existing occupational exposure threshold limit values are not specific to ENMs and that many of the existing studies have significant limitations, as it is not possible at present to measure all of the relevant parameters for determining occupational exposure to ENMs. It was highlighted that measurement methods employed in determining occupational exposure need to be routinely calibrated with recognised and appropriately sized reference materials.

It was widely agreed that more than one measurement technique should be employed to determine the size distribution of ENMs; however these techniques need to be chosen and optimised for each specific NM sample. The (U.S.) National Institute of Standards and Technology (NIST) has a list of sizes determined with specific techniques (including dynamic light scattering (DLS), atomic force microscopy (AFM), and electron microscopy (EM)) for one particular reference material, where some significant variation in the reported size emerged depending on the technique used.[7] Correct sample shipping, storing, handling and preparation were all highlighted as being key elements to ensure successful size measurements: the issues of shipment and storage conditions of ENMs, as well as the time required for shipping have to be accounted forby researchers to ensure that NMs are stored in the correct environment and only used for the manufacturer's specified lifetime, which will be dependant on the specific reference material. Additionally, the measurement technique and its inherent assumptions need to be are reported together with the size data in publications. This was deemed necessary for the reporting of all stages of sample testing from both a metrological and biological viewpoint; detailed descriptions should be provided to aid inter-laboratory reproducibility.

Next the discussion focused on biological endpoints, and an attempt was made to identify the most suitable technology/protocol(s) available to determine different endpoints such as cell viability, Reactive Oxygen Species production and others. The discussion focused on trying to define a test method which could give inter-laboratory reproducibility. It was widely agreed that to do this a set of complementary tests needed to be established. Genotoxicity studies were identified as a possible candidate for comparison with cell viability results, as a first round-robin study using the Comet assay has already been performed successfully.[8] Again it was noted that the endpoint used for viability measurements needed to be free from

absorptive interferences (the case of Single Walled Carbon Nanotubes (SWCNTs) interfering with colorimetric assays was highlighted[9]) and perhaps alternative endpoints such as colony forming assays should be employed.[10] It was suggested that toxicity should also be assessed in a more mechanistic approach, using for example confocal and electron microscopies as these approaches would provide information regarding the morphological changes that may occur following exposure of ENMs to cells, helping further to elucidate the possible mechanism(s) of toxicity.

Selection of the types of cell lines/cultures employed in assessing impacts of NMs also needs careful consideration: to enable *in vitro* experiments to potentially be extrapolated to human exposure the recommendation was made that a variety of different cell types be studied, (epithelial, endothelial, macrophages etc.) and that co-culture models be established and validated. The mechanisms of NMs uptake may also be affected by the cell type employed in the study. Furthermore the stage of the cell lifecycle was noted to be of importance (confluence levels, proliferation rates etc.) as this will play a role in the interactions of ENMs with cells, and on the observed effects.

In a more general approach, the type, source and content of protein supplements employed in cellular experiments was noted to play a significant role in the generation of inter-laboratory differences. A suggestion was made that researchers explore different types of protein supplements (e.g. Foetal Bovine Serum (FBS), Bovine Serum Albumin (BSA)) to try and reduce/overcome this problem of variation, and to define a standard protein supplement for comparative cell culture experiments (taking into account that different cells need different environments). It was further highlighted that the attachment time of a protein to NMs is extremely rapid (coverage by abundanat proteins within milliseconds, with rearrangements occurring over several hours[11]), indicating that the ENMs will be coated in a biological material during the assay andthat this needs to be considered as a contributing factor to the resultant measurement outcome.

In terms of identifying priority biological testing protocols, a number of endpoints were agreed upon by the discussion group (see below). However, the lack of standardised methods (protocols, SOPs) was identified as a limiting factor. It was widely agreed that the degree of calibration required for successful physical and chemical experiments would also need to be implemented in biological assays for successful impact assessment of NMs. The following biological endpoints were agreed as being optimal markers for cellular nanotoxicology:

Reactive Oxygen Species (ROS) Screening

Cytotoxicity (Viability, Proliferation, Morphological Studies)

Genotoxicity

Cellular Signalling

The discussion identified a variety of problems associated with ENMs that are not applicable to their bulk counterparts. Again it was highlighted that in order to assess the nano-scale effect, adequate controls must be incorporated into the experiment. Such controls could include parallel testing of the NMs bulk counterpart and nano form to assess the nano (size / curvature) effect, testing of the molecular oir ionic form of the material, where available, and consideration of the possibility that the ENMs adsorb to their surface enzymes

released by the cell following ENMs-cell interactions. A further point was made that the experimental endpoints employed in all studies need to be validated and assessed for correct operation, and to ensure that the presence of the ENMs does not interfere with the readout. Again, the case of SWCNT was noted as an example in which significant interference has been observed for a variety of cytotoxic endpoints. A final note was made on the testing of ENMs: it is vital that researchers ensure that the ENMs they seek to test are actually in the nanoparticulate form in the test system, by continually monitoring the dispersion state of the particles at all stages of the experiment

Software and hardware differences were also identified as a source of inter-laboratory variability and the establishment of standardised protocols may not necessarily resolve all issues associated with the testing of NMs, but it was felt that such an approach would significantly aid in the development of the area.

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4.2 Session 2: Reference materials – Are other approaches needed?

In this session, presentations were given on the topic of the current progress in terms of the development of standard and reference NMs. Most of the materials that are used as standard and/or reference materials in toxicity testing are industrial materials (e.g. carbon black, P25 titanium dioxide), and are mostly agglomerates rather than individually dispersed (monodisperse) ENMs. While these reflect the majority of the materials as currently available, they might not be suitable for all purposes that reference materials are required for.

During the presentations on reference materials, several different types and uses of reference materials were identified, as described in Table 2. The distinction between calibrants and matrix reference materials was made, whereby a calibrant is used for the quantitative step in a measurement process, while a matrix reference material is used for quality control of the full analytical process, including sample preparation, as it is considered to be more representative of the “real” experimental situation or end-use application.

Table 2: The different types of reference materials and their uses.

Reference Material (RM)	A material that is sufficiently homogeneous and stable with respect to one or more specific properties which has been established to be fit for its intended use in measurement. RMs can be used as size calibrants for example, to calibrate instruments for size determination. Note: RMs are typically produced by chartered bodies such as Institute for Reference Materials and Measurements (IRMM) or National Institute of Standards and Technology, but may also be provided by commercial entities often with traceability to formally certified RMs.
Certified Reference Material (CRM)	An RM accompanied by a certificate providing the value of the specified property, its associated uncertainly and stating the metrological traceability. Note: CRMs are typically developed by chartered bodies such as IRMM or NIST.
Quality Control Materials	Reference materials without a certificate and without a property value having an uncertainty small enough for use in calibration. This is typically the class of reference materials used during in-house method validation exercises, for interlaboratory comparisons for method assessment, etc.
Study or Test Material	A well-characterized material that is provided from a single source for use in studies (tests) designed to test a hypothesis or explore a specific material attribute across one or more testing labs. RMs could serve this purpose, but more likely this material would be procured or manufactured for a limited use and for a limited time frame that is not generally compatible with RM or CRM development.
Benchmarks (controls)	Materials that express a desired property and are sufficiently homogeneous and stable to be used as a marker for that property when incorporated into experimental studies. Benchmarks typically would have stability measured in years or decades and be widely accessible; it may not be necessary for a benchmark to have an associated or certified property value. RMs could serve as benchmarks and controls. An important criterion for this type of standard material is broad acceptance by the research community. Any useful benchmark or control material must be a reference material (at least sufficiently homogeneous and stable for its intended use) to be useful.

Uses for reference materials were identified as follows:

- calibration or qualification of analytical instruments (certified value of the reference material should be traceable) (Note: (metrological) traceability is the property of a value, not of a material, and is to be distinguished from the material traceability throughout its production – also called trackability.)
- validation of methods and procedures
- manufacturing quality control
- inter-laboratory comparisons and laboratory accreditation
- biological studies (e.g., NIST distributes gold NP RMs that can be used to conjugate chains to, in order to manipulate the physico-chemical properties in a highly controllable manner)
- benchmarks (e.g. used as positive (biological) controls; need to be fully characterized, with agreed dispersion protocol and a reproducible biological end-point, e.g. genotoxicity).

It is important to note that the intended use determines the choice of which type of reference material is needed. From the presentations, the list of available (and forthcoming) reference NMs that emerged is shown in Table 3.

Table 3: The list of reference NMs currently available or in preparation at IRMM or NIST (as of June 2008).

Nanomaterial type	**Available sizes**	**Supplier**
Silica	40 nm	IRMM
Gold	10, 30, 60 nm	NIST
In preparation:		
Bimodal Silica		IRMM
Single-walled carbon nanotubes		NIST
Titanium dioxide (TiO_2) nanopowder (industrial)	25 nm	NIST
Future considerations:		
Pure phase dispersible TiO_2	< 25 nm, companion to industrial grade RM	NIST
Conjugated or functionalised Gold	Prototype development stage	NIST
Silver	Currently undergoing feasibility study.	NIST
Cerium oxide	Still assessing need and applications	NIST

It was recognized by all that developing reference materials is a laborious (and costly) task, and that currently limited budgets are available for this task. A suggestion emerged that it could be useful to have some workshops on defining what reference materials would be needed and with what priority – e.g. for upcoming FP7 projects. To some extent this has already been attempted in Europe through the Department of the Environment, Food and Rural Affairs (DEFRA) funded REFNANO project,[12] and in the U.S. as part of an National Nanotechnology Initiative (NNI)-sponsored workshop held at NIST in September 2007.[13] It is essential that further workshops are performed in this area, taking into consideration of the ongoing efforts, and building on these efforts.. Additionally, as many industrial materials are agglomerated / aggregated, the need for well characterized agglomerates / aggregates was highlighted, and the question raised as to the possibility to produce, store and ship agglomerates / aggregates as reference materials. In addition, the need for polymeric reference ENMs (e.g. capsules, dendrimers) was highlighted.

The discussion also covered to the topic of whether there is a need for standardized dispersion media and standardised dispersion protocols to be supplied with reference materials. As different tests require different dispersion media, it was suggested that it would be most useful if a consensus on three or four representative dispersion media could be reached. The National Iinstitute for Occupational Safety and Health (NIOSH) have developed a simulated lung fluid which showed excellent promise as a dispersion medium,[14] although other studies have shown that lung surfactants cannot break up TiO_2 agglomerates.[15] In one study of NM exposure to mice, a very encouraging outcome was demonstrated using bronchoalveolar lavage (BAL) fluid instead of blood serum to disperse the ENMs.[16] However, it was pointed out that simulated lung fluids are very dilute, whereas when the "stabilized" ENMs are introduced into the lungs *in vivo* they come in contact with a very concentrated system which can induce immediate agglomeration / aggregation. Additionally, the small number of components in lung fluids may not be able to reproduce the complexity of the situation in blood. It was suggested that for *in vivo* studies, the best practice was to first disperse the NMs in BAL-fluid, then in medium (including supplements such as serum, antibiotics etc.)) prior to exposure studies. However, the broader application of BAL-fluid as a generic dispersing medium for studies beyond the original intended use requires further investigation.

A discussion on standard endpoints is currently underway, co-ordinated by the OECD. The need for transparent and open discussion with OECD was emphasized. The possibility of identifying new toxicological end-points, that should be incorporated in the OECD test list was also discussed. Also risk assessment, behavioral effects and chronic endpoints from a health perspective could be very relevant, however, it was considered that for the moment, disease-based endpoints might be too complicated. Interaction of NMs with proteins and other biological molecules is not on the OECD NM characterisation list. This can be raised at the relevant committees, and perhaps could be a unique focal point for NIN. It was noted that molecular toxicology is rarely directly attributable to the level of proteins, and as such identification of protein binding alone is unlikely to be sufficient to characterise / predict potentail toxicological impacts of ENMs.

Included in the potential "new" endpoints was the suggestion of protein fibrillation.[17, 18] Fibrillation as an end-point has recently been highlighted as an issue to watch by the Scientific Committee on Emerging and Newly Identified Health Risks (SCENIHR) Opinion on Risk Assessment of Products of

Nanotechnology.[19] However, the opinion also mentions that neurodegeneration research is still at an early stage, and it is unclear as yet whether it is relevant for humans or should be a cause for concern.

OECD is actively assessing the appropriateness of the current standard protocols for the risk assessment of ENMs, and are looking to the scientific community for support in this process. A challenge for NIN could be to assess the validity and relevance of the OECD list of endpoints and protocols, and to identify ones that are of particular interest for NIN participants. The OECD list is included as Annex II in the report, and the tests of particular relevance to the issues discussed at the workshop are highlighted.

4.3 Session 3: Protocols for in vitro and in vivo experiments

In this session, presentations were given on the topics of the key requirements / issues for (i) *in vitro* studies, (ii) quantitative *in vivo* studies, and (iii) biological system characterization. Important issues highlighted included the need to provide precise information relating to the exact concentrations applied in experiments, as well as the optimal dose metric to use (μg/cm^2 or μg/mL or both). The importance of studying biokinetics was also discussed, as such investigations can provide essential information regarding dose-estimates in specific organs of interest, and a rational basis for toxicological studies (at relevant doses). It was concluded that a combination of both macro and microscopic dosimetric analysis will significantly advance the current 'state-of-the-art' analytical technique(s).

Subsequent discussions centred on identification of key areas where NIN can advance the state-of-the-art in the assessment of the effects of ENMs on human health and safety. From this it emerged that NIN could address issues related to sharing details of experimental procedures, and development of methods to close current knowledge gaps. While it was agreed that the NIN network, indeed the area as a whole, is not at the stage of developing SOPs there are protocols established in research groups, laboratories and even micro-networks within NIN. These protocols, while *ad hoc* and used primarily to cross-validate results from different laboratories, could be shared with the wider network via the NIN website. This would allow others in the network to learn from the wider community, as would also serve to confirm and validate (and improve where necessary) the protocols. Since the time of the workshop, NIN has indeed established such a portal for internal use initially (www.nanoimpactnet.eu). Once the protocols have been peer-reviewed by NIN members, and thus become more robust and generally accepted by the NIN community they will be made publicly available. An important aspect of this knowledge and protocol sharing network will include reporting on the success, or failure, of a protocol, due to the open access nature of the protocol sharing aspect in NIN which allows protocols to be edited and improved, ensuring that other researchers can be aware of any limitations and/or problems associated with the protocol(s).

Comparison with other fields of research were made, such as protein expression and cell biology, in which if a member clones a protein or engineers a cellular transcript, they are obliged to make it available to others in the area. It was suggested that NIN should operate in a similar fashion such that results and

protocols are available throughout the consortium. In addition, the members may also have cell lines or cultures they wish to share and/or may wish others to confirm a particular set of results - such details could also be included on the NIN website. WP1 (Human Hazards and Exposure) and WP5 (Integration, Nomenclature and ELSI) will collaborate to ensure that this is implemented

Considerable time was devoted to the discussion of the pitfalls of (nano)toxicology research. With respect to improving reproducibility of cell culture, the network needs to share procedural knowledge in particular regarding the choice of cells. Many cells used in toxicology studies are tumour cells because of ease of handling, low time requirements for the cells to reach confluence and low costs. Nevertheless, these cells can undergo significant phenotypical and genotypical changes which are not being taken into consideration in the interpretation of nanotoxicity studies. However, moving away from such cells could cause difficulties in procedure development, as primary cells are significantly harder to culture, are more sensitive to additives in the media, and could increase the overall number of variables, thereby decreasing comparability across studies. To develop robust procedures with a high degree of credibility it was recommended that cell lines which are readily available and traceable, i.e. same source and commercially available, should be used rather than specifically isolated / modified cell lines. It was also highlighted that commercially purchased cells / cell lines come with strict protocols for their culture. Depending on the cell line, data sheets/ test sheets are available from the supplier. However the cell line characterization is crucial and should be reported and included in any data for publication.

Generally, impurities in the NMs also need to be characterised/ studied as their toxic effects can be severe with the NMs potentially acting as carriers for the impurities, leading to possible contamination of the cell culture and heightened toxicity.

4.4 Session 4: Worldwide and round-robin approaches

A very interesting presentation on stakeholder needs in terms of Nano Environmental Health and Safety (EHS) introduced this session. It was clear from the presentation that there is currently limited toxicology data that corresponds with exposure to ENMs and their potential exposure routes, and that current testing protocols do not correlate with acute and/or chronic effects. It is imperative therefore, that industry acknowledges this pitfall and defines the constituents of the ENMs and how these (may) related to the toxicity of the ENMs. An additional point that was raised was the need for validated procedures for ENM handling, such as their shipping, sampling, exposure and disposal. Furthermore, the possibility of learning from studies on non-human models, such as invertebrates, via cross-reading or correlation of data was also discussed as an important key step, once validated tests that correlate *in vitro* effects with *in vivo* effects have been developed and implemented. Currently, dosmetric data research has reported extrapolation from *in* vitro to *in* vivo, as well as extrapolation of data gained from various laboratory animals and humans. At this time however, there is no such data in regards to (nano)toxicology research.

The discussion resulting from this session centred on whether NIN can share protocols and data with the OECD and if so what are the mechanisms other then peer-reviewed publication of results in the open scientific literature. The point was made that OECD is a broad body with considerable tasks before them beyond nanotechnology. They are therefore looking to others to provide relevant data and they will deliver the strong administrative process to reinforce the results and conclusions found from the data.

It was suggested that NIN could be considered as one of the very early stages in the process used by the ISO and OECD, as could other similar networks where these issues are debated and eventually published. However, it was noted that realistically OECD, ISO, ASTM and other regulatory bodies need to eventually take control of the process. This is also the approach recommended by a recent workshop on Enabling Standards for Nanomaterial Characterization held at NIST in October 2008, and co-sponsored by several U.S. federal agencies (report pending).

The conclusion from the discussion was that the various bodies such as the OECD could / should provide templates for the type of data and documents that are required to progress this field, and could also provide some training workshops with respect to the process necessary for standard and method development, such that these can be built into the protocols that are emerging within the scientific community. NIN could certainly be a vehicle to facilitate this, via one of its training schools.

An additional (unexpected) benefit of the meeting was that it presented a nice opportunity for members of the International Alliance on NanoEHS Harmonisation (IANH, www.nanoehsalliance.org) to meet and to obtain an update on the most relevant issues, which helped them to finalize their workplan before going public during the Nanotoxicology conference in September 2009.

5 Conclusions and Recommendations

A number of conclusions and recommendations emerged from the workshop. The main conclusions are listed below (in order of discussion). No priority or ranking has been applied to this list.

- The importance of sharing experiences was highlighted, along with the need for closer cooperation between ongoing and future projects. The lack of validation of existing test methods and the added complication that ENMs can affect the test performance was highlighted as the baseline for further discussions.
- Cell culture work can have a number of pitfalls which need to be reported and procedures or protocols need to be adhered to strictly. All reports and publications should include a section on the cell line characterisation (passage (division) number, phenotype confirmation, etc.).
- ENMs can act as carriers of contaminants or impurities which can impact on the cellular responses. Thus, reports and publications should also include sufficient repeats and controls, and checks for endotoxin impurities or other contaminants such as catalysts, surfactants etc. should be conducted and reported as standard.

- There is an urgent need for reference NMs not just for calibration of physical and chemical measurements, but also as positive and negative controls for various measurement methods assessing biological impacts.
- Details of protocols and open knowledge should appear on the website with access to all NIN members to help develop a structured approach to method development – this has since been implemented within the NIN project in line with the deliverable set-out by the NIN consortium.
- OECD and other international organisations are working on a list of end-points for assessing ENMs. Within the discussions specific end-points were highlighted as being advantageous for 'round-robin' studies, to validate the tests and to generate data regarding a range of endpoints for an expansive panel of ENMs. Examples of endpoint specific areas discussed are genotoxicity, oxidative stress and cell viability.
- Additional end-points, not yet in the OECD list were also highlighted, including ENM interaction with biomolecules, and ENM-induced protein fibrillation.
- Generally it was agreed that the OECD and ISO are approachable and are interested in close collaboration in order to ascertain a 'knowledge-base' for all ENMs. NIN should therefore, act as a vehicle to tie all EU projects together and contributing credible and valid data to the OECD process.
- Thus, it was recommended that NIN track all communications and develop closer links with other ongoing and future projects who are willing to share details on ENMs testing. This would allow NIN to be in a position within the scientific community to make a valid representation and contribution to existing efforts by the OECD and other organisational bodies, such as ISO.
- It was recommended that OECD could / should provide templates for the type of data and documents that they require and also provide some training workshops in respect of the process necessary for standard and method development, culminating in a 'knowledge-base' of standard and methodological protocols for use within the scientific community. NIN could facilitate this, via its training schools.
- The need for another workshop on this topic closer to the end of the NIN project was highlighted, as clearer consensus of the best practices, and recommendations regarding which protocols for which end-point should be significantly further advanced by then. Additionally, a second workshop would enable us to reflect on the role of NIN in facilitating the onward development and framing of the field of assessment of the human health impacts of ENMs.

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Annex 1: List of speakers and titles of their presentations

NanoImpactNet WP1 Workshop

Hosted by University College Dublin, Ireland; 20th June 2008

Workshop on Standardization of materials and protocols		
Day-chair: University College Dublin		
08:30- 09:00	Registration and coffee	
09:00- 09:05	Welcome	Michael Riediker
09:05- 09:20	Aims and goals o Why do we need standards and protocols? o Are round-robin approaches a viable approach	Kenneth Dawson
09:20- 11:10	**Session 1 – Existing EU activities on standardisation**	
09:20-09:35	NanoTransport outcomes and future perspectives	Qinglan Wu
09:35-09:50	NanoInteract – dispersion, cell culture standards, protocols	Iseult Lynch
09:50-11.00	Discussion: Key points to build upon from existing state-of-the-art	All participants
11:00- 11:20	Coffee	
11:20- 13:20	**Session 2 – Standard materials**	Gert Roebben
11:20- 11:35	EU efforts towards reference materials	Vince Hackley
11:35- 11:50	NIST activities on materials standardisation	All participants
11:50- 13.00	Discussion: Other approaches needed?	
13:00- 14:00	Lunch	
14:00- 16:00	**Session 3 – Protocols for in vitro and in vivo experiments**	Jean-Pierre Kaiser
14:00- 14:15	Key requirements / issues for in vitro studies	Wolfgang Kreyling
14:15- 14:30	Key requirements for quantitative in vivo studies	All participants
14:30- 15:30	Discussion: Key areas NIN can advance state-of-the-art	
15:30- 16:30	Coffee	
16:30- 18:00	**Session 4 – Worldwide and round-robin approaches**	Mike Garner
16:30- 16:50	Needs for NanoEHS research from an industrial perspective including the need for standard test methodologies, etc.	All participants
16:50- 17:50	Discussion: Next steps in standardization for nanosafety	
17:50- 18:00	Concluding remarks and end of meeting	Kenneth Dawson Michael Riediker

Annex 2 – OECD activities in determining minimum physicochemical characteristics

The OECD report on the list of manufactured materials and list of endpoint for phase one of the OECD testing programme[20] describes the initial set of parameters that should be characterised. According to the report, addressing this set should help ensure consistency between the various tests to be carried out on specific NMs and should lead to the development of dossiers for each ENM describing basic characterization, fate, ecotoxicity and mammalian toxicity information.

It is expected that the list of endpoints be refined based on the practical results obtained through the testing programme. As such, phase one testing is expected to be of an exploratory nature, science-based and without any consequences for existing regulatory datasets.

The list of OECD endpoints is given below, exactly as laid out in the OECD report. In this list, the ones that are considered of particular importance, based on the discussions from the present workshop are highlighted.

Endpoints

Nanomaterial Information/Identification

• Nanomaterial name (from list)

• CAS Number

• Structural formula/molecular structure

• Composition of nanomaterial being tested (including degree of purity, known impurities or additives)

• Basic morphology

• Description of surface chemistry (e.g., coating or modification)

• Major commercial uses

• Known catalytic activity

• Method of production (e.g. precipitation, gas phase)

Physical-Chemical Properties and Material Characterization

• Agglomeration/aggregation

• Water solubility

• Crystalline phase

• Dustiness

• Crystallite size

• Representative TEM picture(s)

• Particle size distribution

• Specific surface area

• Zeta potential (surface charge)

• Surface chemistry (where appropriate)

• Photocatalytic activity

• Pour density

• Porosity

• Octanol-water partition coefficient, where relevant

• Redox potential

• Radical formation potential

• Other relevant information (where available)

Environmental Fate

• Dispersion stability in water

• Biotic degradability

• Ready biodegradability

• Simulation testing on ultimate degradation in surface water

• Soil simulation testing

• Sediment simulation testing

• Sewage treatment simulation testing

• Identification of degradation product(s)

• Further testing of degradation product(s) as required

• Abiotic degradability and fate

• Hydrolysis, for surface modified nanomaterials

• Adsorption- desorption

• Adsorption to soil or sediment

• Bioaccumulation potential

• Other relevant information (when available)

Environmental Toxicology

• Effects on pelagic species (short term/long term)

• Effects on sediment species (short term/long term)

• Effects on soil species (short term/long term)

• Effects on terrestrial species

• Effects on microorganisms

• Other relevant information (when available)

Mammalian Toxicology

• Pharmacokinetics (ADME)

• Acute toxicity

• Repeated dose toxicity

If available:

• Chronic toxicity

• Reproductive toxicity

• Developmental toxicity

• Genetic toxicity

• Experience with human exposure

• Other relevant test data

Material Safety

Where available:

• Flammability

• Explosivity

• Incompatibility