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## Minimal Analytical Characterisation of Engineered Nanomaterials Needed for Hazard Assessment in Biological Matrices

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## **Minimal analytical characterisation of engineered nanomaterials needed for hazard assessment in biological matrices**

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## **Abstract**

**Background:** This paper presents the outcomes from a workshop of the European Network on the Health and Environmental Impact of Nanomaterials (NanoImpactNet) held in June 2008. During this workshop 45 experts in the field of safety assessment of engineered nanomaterials from academia, non-profit organizations and industry addressed a list of essential metrics of engineered nanomaterials that need to be characterized as a minimum.

**Results:** The group discussed the need to systematically study sets of engineered nanomaterials to generate a dataset that allows for the establishment of dose-response data related to specific metrics of engineered nanomaterials. Concomitantly the availability of analytical methods to determine the physicochemical characteristics was discussed. Given the measurement challenges specific for engineered nanomaterials the issue of harmonizing protocols was raised.

**Conclusion:** The group concluded that international cooperation and worldwide standardization of terminology, reference materials and protocols are needed to make progress in establishing lists of essential metrics. The need for high quality data necessitates the development of harmonized study approaches and adequate reporting of data. Priority metrics can only be based on well-characterized dose-response relations (as regards biological interactions and physicochemical characteristics) of engineered nanomaterials. This requires the systematic study of the biokinetics and biointeractions of nanomaterials at both organism and (sub)cellular levels. Additionally, much effort needs to be put into the standardization and validation of analytical methods to determine these metrics. Especially for the characterization of engineered nanomaterials in a complex matrix much work needs to be done.

## Background

The field of nanotechnology is rapidly developing and engineered nanomaterials (ENMs) are already applied within industrial applications and consumer products. This will lead to many new products possessing new and exciting features that are not realisable using conventional forms of chemicals. Consumer exposure to ENMs is very likely to occur, while it needs to be acknowledged that different applications of ENMs in different products have a differential likelihood of exposure. Although the obvious beneficial effects of nanotechnologies are well recognised, the potential human and environmental toxicological effects and impacts of ENMs have so far received little attention [1, 2]. There is a growing body of evidence to suggest that new interaction processes may occur between biological systems and engineered ENMs [3].

As a consequence of their small size, ENMs can exhibit different physicochemical properties and biological effects compared to their respective bulk materials, even at the same mass dose [4]. At present, researchers have not been able to establish a single parameter that best describes the dose and the observed dose-response relationship for toxicological testing. Instead a variety of physicochemical parameters have been suggested to contribute to the biological behaviour of ENMs see [5, 6]. Given this uncertainty, a full characterisation of ENMs is essential in order to relate the possible benefits as well as the potential toxicity of ENMs in both human and ecological systems to the specific features of ENMs [7-9], not only at production and in buffer suspensions, but also in the complex biological medium relevant to the test system [10].

The ability to fully characterize the ENMS is severely limited by the limitations of the presently available analytical methods. Generally, with the available methods, only a single characteristic of an ENM can be determined in one run. The workshop of the European Network on the Health and Environmental Impact of Nanomaterials (**NanoImpactNet** [ <http://nanoimpactnet.eu>]) focused on making progress in this area by trying to reach consensus on the minimal analytical characterisation of engineered nanomaterials needed for hazard assessment in biological matrices. During the workshop 45 experts in the field of safety assessment of engineered nanomaterials from academia, non-profit organization and industry addressed this issue.

**Standardisation.** The need for standardisation and prioritization of protocols and materials in the field of nanotechnologies is well acknowledged by a number of major organisations (e.g. The Organisation for Economic Co-operation and Development [OECD] and the International Standards Organisation [ISO]). This was highlighted by Peter Hatto, who is the chair of the ISO technical committee on nanotechnologies (TC 229). This technical committee contributes to the development of standards in the areas of terminology, nomenclature, metrology and characterisation, and environmental health and human safety of ENMs to support regulatory regimes, research, commercialisation and trade in nanomaterials. Realisation of the widely recognised need for standardisation is currently difficult because there is no agreed terminology, no reference standard materials for toxicity testing and there are no protocols available for (reproducible batch-to-batch) ENM production, characterisation and hazard assessment, and indeed no consensus exists yet regarding the minimum set of information needed to characterise ENMs.

The ISO and OECD activities have full industry support. For producers standardisation and characterisation are very important for the quality of products containing ENMs. Development of clear terminology is to their advantage given the diversity of applications as indicated by representatives from industry: the possibility to produce materials of light weight but suitable for extreme conditions, more efficient solar cells, materials to protect against environmental degradation and targeted drug delivery. Numerous ENMs are currently used in these applications, such as metal oxide ENMs (e.g. ZnO, TiO<sub>2</sub>, SiO<sub>2</sub>), quantum dots and carbon ENMs, like (C<sub>60</sub>) fullerenes and carbon nanotubes (CNTs).

Christoph Klein from the European Commission Joint Research Centre added views from a regulatory, risk assessment perspective. For this the lists of physicochemical parameters as prepared by both OECD and ISO are very important. These lists are derived from currently existing requirements for conventional chemicals with the inclusion of other specific characteristics of ENMs, such as surface area. There is currently no consensus within the scientific community on which characteristics should be determined with higher priority.

**Metrics and dose response relations.** Consensus on the minimal characterisation of ENMs can only be reached on the basis of a sound understanding of the mechanisms of interaction of ENMs with biological systems. According to Vicki Stone (Professor of Toxicology and (co)director of the Biomedicine & Sports Science Research Group, Napier University, Edinburgh, UK) biological interactions of ENMs cannot be attributed to one single parameter/characteristic. Several physicochemical factors have been related to biological responses; e.g. size, surface area, high aspect ratio, charge, solubility, surface chemistry and reactivity. Within the literature attempts have been made to establish dose response relations for various physicochemical properties and observed effects of ENMs. Some examples of dose response relations are mentioned below. As a generalised pattern, it is observed that ENMs of chemical elements with low toxicity and low solubility induce reactive oxygen species (ROS) and pro-inflammatory cytokine release (see for example [11]). [12] showed a size dependent  $\text{TiO}_2$  pulmonary clearance ( $T_{1/2}$ ) in rats exposed to aerosolized materials. The relation between physicochemical properties (size, surface area, crystal phase) of  $\text{TiO}_2$  and ROS production was further substantiated in the systematic assessment by Jiang and colleagues [13]. Both Wilson, Stone and colleagues [14, 15] showed size dependent induction of oxidative stress by carbon black (although the study by [15] used equivalent masses e.g. the number of particles increases as the particle sizes decreases). Size dependent induction of ROS was also observed by Brown and colleagues [16] using polystyrene particles (also using equivalent masses). Lung inflammation following exposure to particulate air pollution (PM10) also shown to be size-dependent [17, 18]. In all of these studies, toxicity was shown to increase with decreasing ENM particle size.

A specific feature of ENMs is the large surface area and thus high proportion of surface atoms. Surface area has been suggested to be an important metric for the induction of inflammation by low solubility particles such as carbon,  $\text{TiO}_2$  and polystyrene beads [16, 19-21]. Good correlations with dose were also shown when expressing different sized materials (carbon black and  $\text{TiO}_2$ ) as surface area in a study on oxidative stress induction [22].

Expression of dose as surface area is an interesting alternative to mass dose, especially when considering exposure. For example, the uptake of differently sized ENMs by *Daphnia magna*, suggests that mass uptake of 20nm polystyrene nanoparticles is much lower than for



1000nm particles, but when expressed as surface area the two are almost identical (Rosenkranz *et al.*, manuscript in preparation). In the studies as performed by Duffin [19, 21] using low toxicity particles such as carbon black, titanium dioxide and polystyrene particles, there was a linear relationship between surface area dose instilled into the rat lung and the resultant neutrophil influx which was not related to the mass of the particles. In these studies the intrinsic toxicity of the particles was also shown to play a role, as expected, in that relatively toxic alpha-quartz silica particles induced a relatively more severe inflammation than low toxicity particles with the same surface area, which suggests that quartz has a more reactive surface, and that therefore the inflammogenic potential is a function of both particle surface area and surface reactivity.

In the case of carbon nanofibre/nanotube (CNT) induced effects, the nanomaterial morphology including length of fibres and state of aggregation is important for determining uptake processes by macrophages. Long (50 $\mu$ m) straight nanotubes were not effectively taken up by the macrophages, resulting in frustrated phagocytosis as indicated by an increase in the production of superoxide anion radicals, and inflammatory signalling as indicated by TNF $\alpha$  production [23]. Entangled nanotubes were easily and extensively phagocytosed and therefore did not affect ROS or TNF $\alpha$  production by the macrophages. Subsequent studies using CNTs have also shown that longer nanotubes induce intraperitoneal inflammation and granuloma formation associated with the mesothelial lining of the body cavity in mice, whereas no effects were observed following exposure to shorter entangled tubes [24]. This data suggests that nanotubes might behave according to the fibre toxicity paradigm, which highlights that respirable fibres which are biopersistent and possess an aspect ratio (ratio between length and thickness) of greater than 3:1 and a length of greater than 15 $\mu$ m are more likely to induce fibrosis and cancer than shorter or soluble fibres.

**Measurement challenges.** Nanomaterials have specific, unique, measurement challenges. This was exemplified by Hugh J. Byrne, Director of the Focas Institute of the Dublin Institute of Technology with data on the assessment of single walled carbon nanotubes (SWCNTs) toxicity. In their studies a variety of analytical techniques were used. For example, transmission electron microscopy (TEM) analysis shows that SWCNTs were not internalized

by A549 human epithelial lung cells, but that bundles of tubes adhered to the cells. These different interactions might explain the large variability in toxicological effects and TEM analysis proved to be the right technique to elucidate this phenomenon [25]. TEM with electron energy loss spectroscopy yield valuable information regarding the nanomaterial and its environment. Confocal microscopy of ENMs in species like *Daphnia magna* can be used to track and identify nanomaterials [26], but is often not very sensitive. In general, spectroscopic techniques can be used and could in the future offer high throughput techniques with relatively lower capital and personal cost. This however is dependent on identifying indicators or markers of ENMs toxicity that can be picked up using spectroscopic techniques.

To assess directly cytotoxicity, colorimetric (e.g. spectroscopic) techniques are frequently used as read out systems. In the case of SWCNTs this might be problematic because CNTs interact with the dyes used for colorimetric assays [27]. This interference might severely limit the reliability of the outcome for assays in which dyes are used. Hugh Byrne stated “everything” sticks to carbon nanotubes, even components from cell culture media, thereby likely interfering with the outcome of assays (e.g. cytotoxicity) (e.g. [28]). Given the limitations of these toxicology tests, alternative screening approaches need to be developed for CNTs, such as the clonogenics assay that has been demonstrated as a potential alternative [29]. Other possibilities include Fourier transform infrared spectroscopy and Raman spectroscopy which are both increasingly used for diagnostic and biochemical analyses in conjunction with multivariate statistical tools [30]. Raman spectroscopy requires minimal sample preparation and, in most cases, the samples are recoverable allowing it to be used and compared with other important techniques. There are, however, also drawbacks for the Raman technique. Firstly, it requires a clear marker, or indicator in order to complete a quantitative analysis and this marker or the ENM needs to be Raman active. Secondly, the Raman effect is quite weak and the limit of detection is highly dependent on the material under investigation (CNTs are highly Raman active but TiO<sub>2</sub> is not). Therefore, it may not be useful as a generic technique.

## **Objectives**

Following the introduction of the subjects,, participants were asked to address the following specific issues (see Annex 1 for a list of speakers and titles of their presentations):

- 1) Standardisation: What are priority engineered nanomaterials?
- 2) Future perspectives on metrics, dose response relations. Is there a common, effect driven metric within reach (e.g. surface area, biomolecular corona)?
- 3) Measurement challenges specific to engineered nanomaterials.
- 4) Priority directions for research with respect to alternative approaches. What other 'detection' approaches can be used, if instrumental detection is not feasible?

For the discussions the workshop, employed multidisciplinary breakout sessions with two randomly generated groups to consider these questions in detail. Outcomes of discussions were summarized and briefly discussed at the plenary at the end of the workshop. The discussions were guided by a number of pre-defined questions and managed by an appointed chair and rapporteur. Consensus of viewpoints was noted but not required. Different viewpoints have been captured in the discussions.

## Results

**Standardisation: Priority engineered nanomaterials.** Worldwide standardization of terminology, reference materials and protocols is needed to develop a minimum set of characteristics of ENMs to be determined. It was pointed out that all international and national regulatory and standards bodies should work together to address these issues (see also [31]). Commercially available ENMs are very variable in ENMs characteristics, with different chemical composition, size, shape and contamination/impurities including batch to batch variability, as well as the differences arising from different synthesis routes (e.g. use of other surfactants and therefore other protein adsorption behaviour). Perhaps with the only exception of C<sub>60</sub> fullerene (which is clearly defined), the variation in types of ENMs is huge. Therefore, it is rather difficult to make general statements regarding reference materials and the toxicity associated with ENMs.

**Lists of engineered nanomaterials.** Over the past few years, several lists of priority ENMs have been published. The most prominent is the OECD list (Table 1). When constructing this

list the OECD took into account those materials which are in, or close to, commercial use, as well as other criteria including, production volume, the likely availability of such materials for testing and the existing information that is likely to be available in dossiers on such materials. Thus the OECD list could be perceived as a list driven by industry needs. The International Council on Nanotechnology (ICON) used this list as a starting point, but they went on to prioritise further the most produced nanomaterials, which were then considered the most important group.

**Table 1 - OECD List of representative manufactured nanomaterials for testing**

(OECD; ENV/JM/MONO(2008)13)

- Fullerenes (C<sub>60</sub>)
- Single-walled carbon nanotubes (SWCNTs)
- Multi-walled carbon nanotubes (MWCNTs)
- Silver nanoparticles
- Iron nanoparticles
- Carbon black
- Titanium dioxide
- Aluminium oxide
- Cerium oxide
- Zinc oxide
- Silicon dioxide
- Polystyrene
- Dendrimers
- Nanoclays

*Note: The order in which the nanomaterials are listed above does not indicate a priority.*

The OECD list forms a starting point in various discussions related to nanosafety and nanorisk assessment and is used in setting many research agendas. During the discussion, several issues were raised. Inevitably with any list, important nanomaterials can be overlooked, and newly emerging ENMs are not included due to the constant rapid development of nanotechnologies. Moreover, the relevance of these lists for risk assessment purposes was questioned during the discussion as the list represents primary nanomaterials (as produced nanomaterials) and does not assume a priori that toxicity will be observed at realistic dose levels. This raises questions about the validity of test methods developed, as no observed effects may be due to the ENM not being toxic or a result of the test not being adequate for ENM. Separating these issues will be challenging. It was concluded that

concomitant with using the OECD priority list of ENMs, other aspects should be taken into consideration when preparing a list of priority ENMs. These aspects are listed in Table 2.

**Table 2 – Proposal for lists of ENMs that require prioritisation**

<b>List of ENMs</b>	<b>Description</b>
<b><u>Scientific</u></b>	Which nanomaterials should be used to study mechanisms of action or scientific paradigms? It was argued that ‘ad hoc’ constructed materials which have specific characteristics, such as being fluorescently labelled, will be extremely helpful for mechanistic research questions. Alternatively, for risk assessment purposes, research should focus on ENMs in the form they have in the environment or in the matrix where they occur.
<b><u>Availability for consumers</u></b>	Taking nano silver as an example, it was remarked that some ENMs should be given high priority because of the high expected consumer exposure, their widespread commercial availability, and thus their expected high volumes of use. This triggered a discussion on the need of a market driven priority list like the ICON list.
<b><u>Consumer concern</u></b>	It was recognised that the different types of application for the variety of nanomaterials currently available may trigger different degrees of consumer concerns (for example, the comparison of the use of nanomaterials in medical settings against their use within food applications and the resulting different risk-perception by consumers). Different applications may give rise to different degree of consumer concern,. Therefore the inclusion of anticipated consumer concern as a criterion for the selection of priority nanomaterials could be very illustrative.

***Perspectives on metrics: which characteristics of ENMs should be determined?*** The

great challenge now is, firstly to prioritise some metrics based on biological dose response relations and, secondly, to develop less labour intensive analytical methods to characterise ENMs in biological matrices. During the group discussion it was identified that, in principle, for

most, if not all, characteristics of ENMs, analytical methods are available [32-34]. The issue, however, is that these methods are normally only able to determine one single characteristic, making the process of full characterisation very labour intensive. This leads to the conclusion that currently not all ENMs characteristics can be readily determined. Furthermore, different techniques that are available to measure the same nanomaterial characteristic can produce contrasting results (e.g. reported sizes of ENMs) – the variations typically emerge as a result of intrinsic biases and modelling assumptions of the techniques. Agreement on standard testing methods is lacking and the comparability between different methods to assess a specific metric is still being evaluated. Even using reference materials, such as size standards, significant differences in the size are reported using different techniques (NIST, 2007). Therefore, it is recommended to clearly describe in all study reports which method was used and under which circumstances.

Although it was generally agreed by the participants of the workshop that one metric is not sufficient, it is vital that those metrics which have been previously used (e.g. size, morphology, mass, surface area, aspect ratio, charge, solubility and surface chemistry) are evaluated. It is important that a focused and validated list of metrics is initially proposed and progress is made from that point onwards. In addition, it is important to know which parameters can be altered without changing ENM-induced biological effects (e.g. which metrics are of lesser significance). The metrics must be practical, and easy to characterise, reproducible and applicable to the ENM as it will be used in subsequent toxicity tests. A pragmatic way forward might be to characterise fully the ENMs at production. At subsequent stages the group deemed it sufficient to assess a very limited set of parameters before use (exposure) in the exposure medium, in order to take into account the effects of different environments/conditions including effects of storage and sterilisation and adsorption of biomolecules on the physicochemical characteristics of ENMs [35, 36]. It is, however, important to note that subtle effects such as changing surface charge might need additional investigation as recent findings show that such small changes can have a significant impact on the biological response [37]. Specific problems encountered in characterising ENM dispersions are the agglomeration / aggregation state in suspensions and the interactions of

ENMs with the surrounding matrix before and during exposure, which need to be studied and reported.

**Box 1** – Definition of Agglomeration or Aggregation [38].

<p><b>Definitions:</b></p> <p>Agglomerate: A group of materials held together by weak forces such as van der Waals forces, some electrostatic forces and or surface tension.</p> <p>Aggregate: A group of materials held together by strong forces such as those associated with covalent or metallic bonds.</p>
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It was accepted that not all characteristics that can be measured are necessary for all purposes. For example the use of data for predictive toxicology metrics requires as much data as possible while for industry (production) requirements are likely to be less demanding, and even for the assessment of risks this might be the case. When reporting data on the characterisation of ENMs it is important that a rationale is presented regarding the choice to focus on a selected metric. Furthermore, any use of surface modification using ligand molecules, surfactants, stabilizing counter ions, surface coatings etc. must be reported. The group concluded to propose a list of metrics which (minimally) should be described in every study related to the health impact of nanomaterials. Only this ensures that the findings can be used by other related domains, and to enable comparisons between studies and ENM and cell / tissue / animal combinations. The list is presented in Table 3.

**Table 3** - List of minimal characteristics and metrics recommended for every field of research investigating the health impact of nanomaterials and metrics that may be useful for specific fields. This may be reduced in the future as more knowledge becomes available.

Characteristic	Metric	Challenges and comments
<i>Essential metrics</i>		
Size distribution (of primary particles)	Diameter – not appropriate for high or	- Different measurement methods investigate different sub-metrics, e.g. mobility diameter

	even medium aspect ration ENMs.	<p>vs. visual diameter.</p> <ul style="list-style-type: none"> <li>- Distribution of sizes needs to be reported.</li> <li>- Nanomaterials can agglomerate or aggregate (see box 1 for definition).</li> <li>- Nanomaterials coated (e.g. corona) with biomolecules, depending on matrix – which diameter to assess?</li> <li>- Depend on medium</li> </ul>
Chemical composition	Chemistry  Purity/impurities	<ul style="list-style-type: none"> <li>- Chemical composition can be determined, but structural information is difficult to obtain due to complex measurements, ideally this would be provided by manufactures,</li> <li>- Impurities may be as important for health impact as the basic material</li> </ul>
Nanomaterial surface	Surface area  Chemistry  Surface Charge	<ul style="list-style-type: none"> <li>- Different measurement methods investigate different submetrics, e.g. BET-surface, Fuchs's surface, visual surface, mobility diameter surface.</li> <li>- There are no simple methods to assess the chemistry of nanomaterials surface. Thus, provide at least information on the synthesis method used, and if / what surface treatment or stabilisation method had been used.</li> <li>- Zeta potential and pH measurements should be reported for all particles in appropriate test media.</li> </ul>
Structure	Agglomerate size	<ul style="list-style-type: none"> <li>- Agglomeration status is in equilibrium with</li> </ul>



	(distribution)	the matrix. No commonly agreed-on metric exists to define the agglomeration status. Also, information about the stability of agglomerates in different media would often be very useful. Aggregation is a more fixed status, and should not be mixed up with agglomeration.
<i>Often important metrics</i>		
Shape	Aspect ratio	- Aspect ratio determines if an object falls within the WHO-definition of a fibre, and is very important for health impact assessment purposes.
Persistence	Solubility UV-stability thermal-stability	- These different types of metrics give information about the persistence of materials in biological media, and environmental compartments. These factors (UV, heat) may also affect ENM surface properties and agglomeration.

***Dose response relations.*** It is essential that further research is devoted to determining dose response relations (e.g. biokinetics and biodynamics), in order to be able to identify biologically relevant metrics. It was agreed that it may be possible to derive general effect patterns of ENMs from a wide range of studies, especially if these studies use exactly the same ENM (same source, same batch & lot number) in combination with studies using a range of different forms/shapes (i.e. carbon nano tubes with different shapes), and analysed coherently. These systematic studies might subsequently be used for read-across and modelling purposes. For this, a robust physicochemical characterisation is a prerequisite. However, it was also noted that once in contact with living systems, ENM are immediately

coated with proteins and other biomolecules implying that direct physiochemical characterisation alone may not be sufficient in terms of a realistic characterisation for mechanistic studies[39].

To successfully perform such systematic studies it was recognized by the participants of the workshop, that it is important to fill in the gaps in the current OECD guidelines, including any decision regarding which endpoints to investigate, the procedure to measure dose, and the best approach to the characterisation of ENMs, including at which point in the studies characterisation should be conducted, i.e. at production, at exposure internally, or before and after the test. It was acknowledged that the amount of information that can be requested from producers is not unlimited from a practical point of view.

Moreover, for the interpretation in terms of relevance for hazard assessment, the use of high doses in the majority of the tests was challenged, similar to the current situation in the testing paradigms of conventional chemicals. For *in vivo* studies, biokinetics is a challenge, e.g. some ENMs might simply dissolve during transport in bodily fluids such as the contents of the gastro intestinal tract.

***Practical and measurement challenges specific to nanomaterials.*** For reliable studies with ENMs the availability of stable suspensions and harmonized dispersion protocols was considered very important by the group. The following methods are currently widely used for dispersing ENMs:

- Ultrasonication (by use of a ultrasonic bath or tip);
- Shaking and vortexing;
- Various solvents;
- Use of dispersants.

Concern was raised about the use of standardised dispersion protocols. Some interlaboratory variance in the protocols used is valuable/necessary for scientific progress. Obviously, researchers need to be mindful of the experiments being carried out. In some cases the harmonised protocols should be adapted to suit the ENMs to be studied and the cells/organisms/animals being used. The precise methods used to disperse ENMs should be specifically, yet succinctly described within the experimentation section of a publication.

Dispersing agents such as surfactant are sometimes utilised to facilitate the dispersion of ENMs [40]. This, however, might alter the resulting toxicological effects, and test of the dispersing agent at appropriate concentrations should be conducted in parallel as controls. Furthermore it was noted that the details of these dispersion aids and their effects on end measurements need to be incorporated within any materials sections published. A further note was made that the effect of sonication, or any other preparation technique should also be considered and appropriate controls included in the experimental design as there may be issues related to “fresh” surfaces having higher reactivity.

Some suitable methods to characterise the degree of dispersion of the ENMs were discussed. Clearly, one should not fully rely on one technique only, but rather multiple measurements from at least two analytical approaches (e.g. techniques) should be employed to characterise ENMs dispersions. Dynamic Light Scattering, Transmission and Scanning Electron Microscopy, Atomic Force Microscopy were all identified as suitable techniques (see also [8, 33, 34, 41, 42], although the microscopy techniques typically measure the dry materials rather than the dispersion. To assess the agglomeration state the only method is cryo-TEM, in which the sample is frozen in liquid helium and observed by TEM in the frozen state. Analytical centrifugation is also being developed as an alternative approach.

More challenging is the characterisation of ENMs in biological matrices (e.g. tissues, food, soil). It was recognized that there is currently no standard approach/protocol for sample preparation to control agglomeration/aggregation and (re)dispersion upon transfer from the “as-provided” state to the test medium. This was highlighted as an issue which needs to be addressed, but also that a pragmatic solution is not readily achievable in the short term, as there is no single solution for all ENM due to their enormous diversity. Much progress on harmonisation and exchange could be gained by addressing day to day issues/problems. Given the current difficulties in detection and characterisation of ENMs, adding a label could be a way of facilitating the study of the (internal) fate of ENMs in biological tissues. Several labelling and/or imaging techniques are possible, such as magnetic labelling, neutron activation, electromagnetic resonance spectroscopy?? and fluorescent-labelling. In this context it is essential that the ultimate fate and the effect of the label are known, and that introduction of the label does not fundamentally change the physicochemical characteristics

of the ENM, as otherwise there is no correlation to the unlabelled version. A recommendation was made not to rely on technologies which employ bio-persistent materials like for instance insoluble iron oxide ENM. Moreover, the example of nano-silver was discussed, given its ability to form silver ions readily in a variety of different environments with the result that the test material may change from nano-silver to ionic silver [43]. The conclusion of the discussion was that all experimental scientists need to ensure that the ENM that is characterised is similar to the ENM that is presenting itself to the entity under test (*in vitro/in vivo*), by monitoring the form and state of the test nanomaterial using correct analytical techniques at the various stages of sample preparation, pre- and post-exposure.

**Priority directions for research into alternative measurement approaches.** Currently no standard analytical equipment-based approaches have been established for the characterisation of ENMs. The evaluation of possible alternatives at this stage is a difficult task and it is unclear if there are at present any alternatives to instrumental detection. Biomarkers may provide information on response and the degree of a response but not on the dose (i.e. it will not be possible to distinguish between a low dose of a very reactive species and a high dose of a low reactive species). Yet, no generalized biological response specific to ENM exposure (or even a series of responses or markers) which can act as indicators has been identified. The group concluded that at the current stage of development it is seen as unrealistic that non-instrumental based systems such as biomarkers can offer anything other than a supporting role to detailed multi-technique based studies on establishing the indicators of ENM toxicity. Highly specific (next generation) ENMs may support the notion of establishing response specific phenomena in biological systems. Techniques in biotechnology, such as gene expression and engineered micro-organism (e.g. bacteria) might offer an insight into how such systems could work, offering a battery of responses from biological systems (e.g. a finger print of the ENM in a biological matrix). Cross reading approaches (like quantitative structure activity relationships [QSAR]) allow the prediction of a substance toxicity using a computer model. The system is still under development for conventional chemicals and is driven by the REACH initiative. Potentially such an approach could work for ENMs using the physiochemical properties integrated with doses response

information from biokinetic and biodynamic studies. Clearly, these approaches are important and could be a long term goal for simple ENMs. However, ENMs can be complex products whose properties evolve over time and thus the implementation of QSAR-like approaches in nanotechnology may be a difficult task. To make progress in the cross reading approaches toxicological data and data on ENM characteristics need to be collected in freely available databases that allow for a systematic analysis of all scientific data. Such databases need careful consideration and require strict control over the data quality and reporting of all appropriate experimental details and full characterisation. Networks like NanoImpactNet, with its associated members, will be important facilitators for this process.

## **Conclusions/ recommendations**

The interdisciplinary discussions in this workshop led to the following conclusions and recommendations:

### ***Standardisation: Priority engineered nanomaterials***

- Due to high batch variability of commercially available and to a lesser degree laboratory made ENMs it is not possible to make general statements regarding the toxicity resulting from exposure to ENMs.
  - It was recommended that the OECD priority list of ENMs should be complemented with ENM selected by other criteria such as suitability for mechanistic (scientific) studies or risk assessment-based studies, widespread availability (and thus high expected volumes of use) or provoking consumer concern (route of consumer exposure depending on application).

### ***Priority metrics***

- It was concluded that the first big challenge is to prioritise metrics based on biological dose response relations and secondly, to develop analytical methods for characterising ENMs in biological matrices.
- It was generally agreed that one metric is not sufficient to fully describe the ENMs.
  - It is recommended that initially similar well characterised batches of ENMs with varying forms/ shapes are used in a wide range of effect studies, before deciding

what metrics are most important. Subsequently, this process needs to be systematically repeated with other sets of priority ENMs.

- It is expected that a systematic analysis of the data will allow an assessment of any relationships between observed effects and physicochemical characteristics of ENMs.

#### **Standardisation: Characterisation techniques**

- For most, if not all, characteristics of ENMs, analytical methods are available, though not necessarily validated and standardized. Practically, it is currently not feasible to characterise ENMs fully, because generally individual methods are only able to determine one single characteristic and some of them can be rather expensive. In addition an agreement in respect to what constitutes a complete ENM characterisation has yet to be reached.
- It was concluded that the type of matrix that surrounds the ENM might critically influence the appearance of the nanomaterial and its interaction with the surrounding matrix. This further complicates the characterisation problem.
  - It is recommended that, where possible, at least two analytical approaches (e.g. techniques) are used to determine a given metric of ENMs (e.g. measuring the same parameter), because available techniques to measure the same nanomaterial characteristic often produce contrasting results.
  - It is recommended that the techniques used to determine physicochemical characteristics and methods used for (re) dispersion are clearly stated in the methods sections of published studies.

#### **Standardisation: Characterisation approaches**

- It was concluded that ENMs need to be characterised in the matrix as it is presented to the test system (*in vitro/ in vivo*).
  - A pragmatic recommendation is to characterise fully the ENMs at production, and subsequently further explore a very limited set of parameters before use (or exposure), in order to take into account the different environments/conditions including effects of storage and sterilization on the physicochemical characteristics of ENMs.

- It was concluded that there is currently no standard approach/protocol for sample preparation to control agglomeration/aggregation and (re)dispersion.
  - It is recommended that harmonization is initiated and exchange of protocols takes place. The precise methods used to disperse ENMs should be specifically, yet succinctly described within the experimental section of a publication.
- The use of dispersing agents as surfactants to facilitate the dispersion of ENMs might alter the toxicological effects.
  - Therefore it is recommended that details of these extra dispersion aids should be incorporated within any materials sections published, and their toxicity tested in parallel with the ENM.
- It was concluded that labelling of ENMs could be a way of facilitating the study of (internal) fate of ENMs in biological tissues. The ultimate fate and effect of the label needs to be known and it is recommended that no reliance is put on technologies which employ bio-persistent materials.

***Alternative measurement approaches.***

- It was concluded that alternative approaches (e.g. biological or in silico systems) for the characterisation of ENMs are simply speculation at the present time, given the lack of experimental data against which to test and validate the outcome of alternative approaches.
- It was recognized that gene expression studies (omics) could lead to the identification of biomarkers, but it was noted that at the current state of development that these approaches only have a supporting role to detailed multi-technique-based toxicological studies.
  - To make progress in the cross reading approaches it is recommended to collect toxicological data and data on ENM characteristics in freely available databases (open science) that allow for a systematic analysis of all scientific data. Such databases need careful considerations and require strict control over the data quality and reporting of all appropriate experimental details and full characterisation. It was recognized that these developments are a long term goal even for simple ENMs.

**Competing interests**

The author(s) declare that they have no competing interests.

### **Authors contributions**

HB co-organized the workshop, was rapporteur and wrote major parts of the manuscript, IS co-organized the workshop, was rapporteur and critically reviewed the manuscript, HM co-organized the workshop, was discussion leader, and critically reviewed the manuscript, KD hosted the workshop, introduced a session and was discussion leader, and critically reviewed the manuscript. PH, VS, CK, HB and LJ introduced a session of the workshop and critically reviewed the manuscript. MB, DB, AC, GC, MC, GE, TF, LF, WG, CN and MR either were discussion leaders and/or rapporteurs of a session, contributed to the writing and critically reviewed the manuscript. All authors read and approved the manuscript.

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**Annex 1:** list of speakers and titles of their presentations.

**NanoImpactNet WP1 Workshop**

Hosted by University College Dublin, Ireland; 19th June 2008

**Minimal analytical characterisation of NP needed for (future) hazard assessment of NP in biological matrices.**

Day-chair: RIKILT Institute of food Safety Wageningen

<b>8.30 - 9.00 am</b>	Registration and coffee	
<b>9.00 – 9.15 am</b>	Welcome Introduction to NanoImpactNet	Kenneth Dawson Michael Riediker
<b>9.15 – 9.30 am</b>	Aims and goals What information on NP characteristics is needed to describe dose-response relations, to model structure- effect relations? Is it possible to set a minimally required data-set?	Hans Bouwmeester
<b>9.30 – 11.00 am</b>	<b>Session 1 – Priority nanoparticles?</b>	
9.30-9.50	International Standardization activities relevant to hazard assessment of manufactured nanomaterials	Peter Hatto
9.50-10.10	Industry perspectives on nanomaterials	Mike Garner
10.10-11.00	Discussion: Which particles should EU / NanoImpactNet focus on?	All participants
<b>11.00 – 11.20 am</b>	Coffee	
<b>11.20 – 13.00 pm</b>	<b>Session 2 - Dose response relations</b>	
11.20-11.40	Are there common mechanisms of action for NPs?	Vicki Stone
11.40-12.00	OECD / SCENIHR views on priority characteristics	Christoph Klein
12.00-13.00	Discussion: Future perspectives on metrics, dose response relations.	All participants
<b>13.00 - 14.00 pm</b>	Lunch	
<b>14.00 – 15.40 pm</b>	<b>Session 3 – Measurement approaches</b>	
14.00-14.20	“Considerations in the assessment of nanoparticle toxicity – the case of SWCNTs”	Hugh J. Byrne
14.20-14.40	Medical Approaches	Lucienne Juillerat
14.40-15.40	Discussion: Measurement challenges specific to nanoparticles	All participants
<b>15.50 – 16.10 pm</b>	Coffee	
<b>16.10 – 17.00 pm</b>	<b>Session 4 - Alternative approaches</b>	
16:50-18.00	Discussion: Priority directions for research into alternative approaches.	All participants

<b>17.00</b>	Concluding remarks	Hans Bouwmeester Kenneth Dawson
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