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1 Description of task

1.1 Introduction

Nanotechnology is one of the key emerging technologies (KETs) identified in the European Union (EU) 2020 Strategy. As a set of technologies, it has enormous potential to help to address societal needs (water, energy, food etc.) and in development of new industrial applications and thus to contribute to innovation and economic growth. However, uncertainties or over-generalization around the potential environmental, health and safety (EHS) risks of manufactured nanomaterials (MNs) as a "uniform" class of materials have raised persistent concerns that can reduce the benefits associated with nanotechnology and may already be considered to have restricted the uptake and use of these technologies in the development of new products and processes in Europe and elsewhere. In order to redress this and avoid future liabilities, sound scientific analysis of the EHS implications of specific materials and uses of MNs is required, taking into consideration all stages of their life cycles, thus protecting the safety of workers, the general public and the environment. For an emerging technology such as nanotechnology, use of forward-thinking risk governance strategies such as OFF-LINESafe by Design (SbD) is both possible and encouraged. As measurement needs are being defined along with the discovery of emergent properties and uses of MNs, it is necessary to consider emerging equipment and methods to support SbD for MN uses.

1.2 Risk Analysis Context Informing Equipment Needs for SbD Applied to Manufactured Nanomaterials

Risk Assessment (RA) frameworks for chemicals are generally considered to be applicable to MNs provided that they are properly adapted to address their unique properties. Risk assessment systematically applies scientific principles to estimate the probability that adverse human health or environmental effects emerge from exposure to chemicals. Although different organisations give different names to the RA phases, there is a general agreement that the framework is composed of Hazard identification, Hazard Assessment, Exposure Assessment and Risk Characterisation. With MN risk management it is becoming increasingly apparent that transformations during fabrication and release from uses require Exposure Identification in parallel with Hazard Identification to identify the possibility of exposure to the MN forms. MNs that are put into materials used in commerce are frequently transformed by the use so that the hazard information for pristine MNs is not informative to risk management of MNs.[1]

Furthermore, the sometimes dynamic and continuous nature of MN transformations in environmental or biological systems can create risk analysis challenges where it is difficult to match the form of the MN studied in toxicity assays to the form of the MN that is measureable for multiple exposure times, places, media, and routes for populations at potential risk. For example, the particles emitted during abrasion of a car bumper are likely to have multiple forms that include free MN particles and MNs embedded in polymer. The free MN may also have variation in surface characteristics compared to the MN that was added to the polymer, due to interaction with the polymer or physical forces during the release process. The MN released from the same car bumper or bicycle frame because of UV weathering may differ from the MN released from abrasion. Each of these release forms may have different toxicity profiles (e.g., absorbed dose, distribution in the body, surface reactivity, most sensitive organ system) compared to the MN that was added to the polymer during the manufacture. Similarly, the toxicity profiles of the released polymer/MN particles may differ from the toxicity profiles of the MN that are released as the polymer/MN particles further transform in the environment. While these differences may be found to be irrelevant in many cases when full toxicity evaluations are done, the need to measure the material to which exposure occurs seems clear. It also seems clear that the MN could be transformed through release processes and in transformations in biological and environmental media and that these transformations may require new data to support risk assessment for a use of the MN.

We are discussing these MN transformations in review of equipment needs because they point to a need, in some cases, for quantitatively more complex analysis of potential MN risk than is needed for analysis of risk for standard chemicals. This complexity underscores a need for capabilities that comprise an emerging "equipment" category capable of generating high volumes of data and analysing patterns within and between parallel data generation arrays.

This is not necessarily an argument for equipment that can do full toxicity assays, but rather, it is an argument that data on multiple forms of a MN may be needed to fill in gaps of knowledge for risk

[1] http://www.nanorelease.org

management of an individual MN use. This kind of capability is not new to standard chemical analysis; however, in contrast to risk analysis for chemicals where high volume data acquisition is useful in screening multiple chemicals, the complexity of MN transformations may require its use for analysis of single MNs in use in commerce. High volume data acquisition/analysis capabilities may also have use in selecting "safer" MN candidates for use in products, and for grouping MN in consideration of regulatory needs.

Such high volume data acquisition/analysis capabilities are not limited to biological response. For example, one limitation noted by research into the form of MN released from uses in products is that collecting data on form and surface characteristics that may drive some nano-biological interaction is difficult to conduct using methods adapted for chemical analysis. The typical "average" mass based approaches of mass spectroscopy and other analytic techniques used in standard chemical analysis are not easily adapted to surface and form evaluation. Techniques such as field flow fractionation, single particle mass spectroscopy, and analytical ultracentrifugation can provide some information useful for decisions for some cases for MNs. However, evaluation of form and surface characteristics needed to support dose estimation for other cases may require a combination of resolution (e.g., electron microscopic scales) and sampling rates (e.g., to address heterogeneity of nanoscale forms across production batches for kilogram quantities) that exceed resources using current standard methods. Approaches using automated spectral and image analysis at high resolution are being developed in some laboratories and companies as possible solutions for this kind of sampling challenge.

These approaches for high volume data acquisition/analysis use novel combinations of existing equipment and software and thus represent a combination of standard equipment and data analytics that affect the meaning of the term "equipment" for the purpose of this report.

Furthermore, given the emergent properties of MNs,[2] the use of integrative technologies of Tox21[3] and NextGen[4] toxicity testing approaches may be essential for MN risk management and SbD approaches, and are therefore of primary interest consideration of future directions of equipment development.[5]

1.3 Programmatic context

Over the last 10 years there has been extensive research and regulatory activity in the nanoEHS area seeking to address the many questions across the risk assessment paradigm. This activity has been particularly intensive in Europe through a very large programme of risk-based research under FP6, FP7 and H2020[6], research to integrate risk into the innovation process across the value-chain through innovation led projects such as Nanofutures, Value4nano as well as regulatory focused activities such as the REACH Implementation Project on Nanomaterials (Rip-ONs) and the FP7 project NANOREG [7]. Internationally (also

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[2] S. Pokhrel A. E. Nel L. Mädler Custom-designed nanomaterial libraries for testing metal oxide toxicity Acc. Chem. Res. 2013 46(3) 632-641.
S. LinY. Zhao Z. Ji J. Ear C. H. Chang H. Zhang C. Low-Kam K. Yamada H. Meng X. Wang R. Liu S. Pokhrel L. Mädler R. Damoiseaux T. Xia H. A. Godwin S. Lin A. E. Nel Screening to reveal the mode-of-action of metal oxide nanoparticles toxicity in zebrafish embryos Small 2013 9(9-10) 1776-1785
H. Zhang Z. Ji T. Xia H. Meng C. Low-Kam R. Liu S. Pokhrel S. Lin X. Wang Y.-P. Liao M. Wang L. Li R. Rallo R. Damoiseaux D. Telesca L. Madler Y. Cohen J. I. Zink A. E. Nel Use of metal oxide nanoparticle semiconductor properties and band gap to develop a predictive paradigm for the oxidative stress injury and acute toxicological potential in cells and the murine lung ACS Nano 2012 6(5) 4349â€“4368.
S. George S. Pokhrel Z. Ji B. L. Henderson T. Xia L. J. Li J. I. Zink A. E. Nel L. Mädler Role of Fe doping in tuning the band gap energy of titanium dioxide for studying the light activated cytotoxicity J. Am. Chem. Soc. 2011 133(29) 11270â€“11278.
T. Xia Y. Zhao T. Sager S. George S. Pokhrel N. Li D. Schoenfeld H. Meng S. Lin X. Wang M. Wang Z. Ji J. I. Zink L. Mädler V. Castranova S. Lin A. E. Nel Decreased dissolution of ZnO by iron doping yields nanoparticles with reduced toxicity in the rodent lung and zebra fish embryos ACS Nano 2011 5 (2) 1223â€“1235.
S. George S. Pokhrel T. Xia B. Gilbert Z. Ji M. Schowalter A. Rosenauer R. Damoiseaux K. A. Bradley L. Mädler A. E. Nel Use of a rapid cytotoxicity screening approach to engineer a safer zinc oxide nanoparticle through iron doping ACS Nano 2010 4(1) 15-29.
[3] http://pubs.acs.org/doi/pdfplus/10.1021/acs.chemrestox.6b00135
[4] http://ehp.niehs.nih.gov/EHP233/
[5] http://onlinelibrary.wiley.com/doi/10.1002/wnan.1413/full
[6] http://www.nanosafetycluster.eu/
[7] http://nanoreg.eu/

involving European stakeholders) there has been extensive activity through the OECD Testing Programme of Manufactured Nanomaterials[8].

This extensive programme of activity has contributed greatly to the knowledge base and the output provides an improved foundation for the development of risk management tools and processes. Yet despite this progress significant uncertainties remain. There are still serious gaps in our basic understanding of key nano-bio interactions, mechanisms of biological uptake, fate, distribution and bioaccumulation and exposures for many MN in commerce now and for MN that are being considered for commercial use. These gaps are part of a current risk management context forced to rely on use of largely qualitative risk estimations based on expert judgments, which may fail to support a proper management of their risks.

The innovative and economic potential of MNs may be threatened by limited understanding of MN safety aspects along the value chains. Substantial efforts have given insights in toxicity of and exposure to MNs. However, there appear to be many cases where today's knowledge is not comprehensive enough for regulatory purposes, and answering open questions is urgently required. It is intended that NANOREG will provide the right answers to Society, Industry and the National Regulation and Legislation Authorities.

The PROSAFE project is linked to and follows from the NANOREG and is concerned with;

- Integrating and harmonising activities in the EU and other industrialised countries through specifically orientated action beyond the efforts foreseen in the context of individual projects.
- Coordination and support of the instrument by which the increasing efforts of emerging economies and emerging science countries will be integrated with the leading actors in the area of nanosafety.
- Integration of the SbD methods in industrial product innovation processes through twinning of ongoing and new projects as well as initiating new projects.

Ultimately, the project has the objective of preparing a white paper dealing with the issue of what is safe in SbD, in an effort to facilitate the acceptance of SbD as an incentive for industry to reduce costs and time for product validation and approval.

WP2 of the PROSAFE project consists of three tasks, addressing the following objectives:

- To establish links to and identify synergies between NANOREG and other key national and international projects;
- To review existing understanding of requirements for assessment for novel risks and regulation;
- To carry out a foresight exercise to identify requirements and synergies for novel MNs.

The results of these activities will ultimately feed into the White Paper. This report describes the results from Task 2.2, which consists of two subtasks. The first subtask aimed to review existing efforts on foresight exercises in the area of MNs and nanotechnologies with focus on risk assessment / governance. The second subtask aimed at determining the specific equipment, tools and methods need required for risk assessment and SbD for MNs.

2 Description of work & main achievements

2.1 Executive Summary

This report describes the results of two activities i) a review of previous foresight exercises on the governance and future applications and national strategies in relation to nanotechnology; and ii) an evaluation of the equipment needs for implementing appropriate risk assessment and management procedures. Both activities are linked to the Delphi poll that is carried out within Task 2.3 to determine priorities and existing knowledge gaps for health, safety and environment in relation to nanotechnology. All these activities will feed into the White Paper developed under WP1.

There is a general lack of foresight publications covering nanotechnology as a general use. Therefore, the review of foresight activity for nanotechnology in this report draws from conceptually relevant foresight topics ranging from methodological developments (e.g. how to apply a specific method to nanotechnology) to foresight studies related to specific sectors, such as military applications, as well as energy and building applications. However, the assessment is rarely linked to very specific MNs, and the level of assessment is

[8] http://www.oecd.org/chemicalsafety/nanosafety/testing-programme-manufactured-nanomaterials.htm

often limited to the domains and sub-domains. The nanotechnology domains mostly investigated or considered in the literature are energy and medicine. Specific technologies identified in different papers include nano-bio-sensors, nano-electro mechanic devices, super capacitors and energy storage devices in general, and nano drug-delivery systems. However, information on specific and promising MN, such as for example new generation MNs, is lacking in foresight literature.

Given the limitations of the available data, it is difficult to identify specific trends and timeline of appearance on the market of new products and applications. Some information can be inferred from some documents, such as national registries and national assessments. However, these data are based on registrations that are reflecting the current situation. In order to identify actual trends, and to achieve a better understanding of the new MN development, it is necessary to perform a focused search by using different sources (not only published papers), taking into account a limited set of technologies or a single sector. Such detailed analyses were not within the scope of the PROSAFE project as it requires specialised knowledge and expertise. However, in Task 2.3 a Delphi forum is carried out to determine the current understanding of the available knowledge on risk assessment and management for MNs as well as what the main gaps are.

The Task 2.3 Delphi forum was informed by early drafts of the Task 2.1 and 2.2 reports and specific questions in the forum were used to seek responses from the multi-national, multi-stakeholder expert group in the forum. The full results of the forum will be described in Task 2.3; however, information relevant to Task 2.2 includes expert opinion on the focus of instrumentation and methods needs. In general terms, the expert response indicates that exposure measurement instrumentation and methodologies specific to MN particle analysis are more urgently needed than are new methods for measuring MN toxicology. The need for exposure measurement capabilities is primarily driven by a perceived disconnect between the hazard information that has been derived using laboratory studies of MN generated in the laboratory and the transformations to those MN when placed in composites or after interactions with the environment. Experts in the forum noted that new toxicology assessment methods may be needed; however, it is first necessary to develop an understanding of the nature of exposures that occur from actual uses of MNs. Furthermore, risk management in the manufacturing life cycle stage, and to some degree also in the occupational use life cycle stage, were noted by most experts in the forum as being adequately served by existing instrumentation and analytic methods to measure exposure and toxicity of MNs. The uncertainty with regard to occupational risk management is more within the development of standard methods and their implementation consistently across manufacturers. This speaks to a need for development of standard methods and for consistent monitoring of application across manufacturing and occupational settings. In contrast, experts expressed greater uncertainty that instrumentation and methods are available to support risk management for the less controllable settings of consumer use and transformations and exposures in the environment. Furthermore, the instrumentation and methods to measure exposures from the environment for some MN types and uses may be as much as a decade away. This speaks to a pressing need for instrumentation and methods development to support risk management following environmental releases.

In parallel to these considerations through the Delphi forum, in the Task 2.2 report the equipment in place and under development which can and will provide support for improved regulatory oversight for nanotechnology, MN risk issues was reviewed. The assessment of regulatory relevance of the identified equipment was based largely on the needs framework identified in the 16 regulatory questions developed as part of the NANOREG project. The regulatory questions are broad in scope and cover the full spectrum of the risk assessment paradigm from characterisation, toxicology, exposure assessment and risk management. There has been no prioritisation between these questions, all are considered equally important. Consequently, the types of equipment necessary to answer these questions are also broad including characterisation techniques, methods for the detection and quantification of exposure, new and improved methods of toxicity and kinetics assessment.

At a first level of assessment, the regulatory questions were analysed to determine for each question which types of equipment were necessary to provide improved answers to that question. The analysis developed for each question, the data need the tools implied, and the measurement context. This enabled a matrix to be developed in which the type of measurement and therefore equipment needed was mapped to the questions. It was clear that each question required multiple measurement types in order to provide an answer to the question and that each measurement type would support the development of answers for multiple questions. It was also possible through this process to group the measurement types into 5 groups, namely, physico-chemical characteristics, detection and quantification of concentration and exposure, toxicity, kinetics and an catchall “others” category. Other types of grouping may have been possible. Again, this did not provide a basis of a prioritisation in that all of the identified methods had utility across a broad a large proportion of the questions.

In the final stages of the analysis we assessed the state of the art for the equipment options for each measurement type required. This did not produce a clear outcome in terms of the level of maturity of each of the methods.

Ultimately, we were not able to establish a convincing quantitative or semi-quantitative approach to develop a prioritisation between the highly diverse equipment needs implied to answer the set of regulatory questions. Without a coherent quantitative/semi-quantitative approach, this defaults to a question of opinion and we did not consider that the small group who prepared this analysis represented a broad enough base on which such an opinion should be reliably formed.

Therefore, we have engaged with the wider PROSAFE project, specifically in relation to the PROSAFE Delphi forum and developed a short set of questions to be included in the second round of the forum. These questions are intended to gather a wider opinion on the relative importance of the identified measurement types and equipment needs. The results will be discussed within the Task 2.3 report.

Nonetheless, the need to consider the complexity of forms and uncertainties for measurement expressed in the Delphi forum and in recent literature can inform prioritization of instrumentation based on broad classes of methods. First and foremost, more of the experts participating in the Delphi forum tended to say that the need is more pressing to develop methods to assess exposures from actual uses of MNs, rather than toxicity methods at present. However, comments in the forum also indicated that this attention to exposure methods does not rule out a need for development of new toxicity methods, rather, it simply speaks to the need to sequence methods development so that toxicity assessment methods can be focused what is measured in exposure assessments of actual uses of MNs in products.

Second, in addition to sequencing methods development starting with the general class of real-world exposures, an instrumentation “class” is emerging that combines data analytics with a capability to generate multiple measurements across forms, time scales, and sampling points which we will term “high throughput data/analytics” or HTD/A for the purpose of discussion in this report.

This class of methods appears to be necessary to capture transformations in environmental and biological systems as well as variation in forms of MNs at the necessary combinations of scale and sampling frequencies. One form of this new class of instrumentation is an extension of existing methods development for chemicals seen in Tox21 or NextGen risk assessment paradigms. When fully adapted to nanoparticulate testing, these methods could employ massively parallel (compared to traditional toxicity testing) *in vitro* testing systems to generate biological interaction, chemical reactivity, and physical data on multiple forms of a MN. Using these high throughput testing methods, variations in coating, weathering, aggregation, and degradation in the environment could be assessed to identify and connect variation in form in environmental or biological transformations for an individual product release to the forms tested in standardized *in vivo* toxicity studies. As the methods develop through standardization and application in regulatory frameworks, they can also be used to generate data that more directly informs exposure pathway doses and points of departure for toxicity assessment used in risk assessment.

Another form of HTD/A capabilities draws from particle sorting and spectral/image analysis combined to generate high sampling rates that are necessary to address size and form variation seen in high resolution spatial analysis. Sampling for nanoscale features across the resolution and scale needed to understand variation in production batches, dosing regimens, and sampling points in environmental media have been found in some cases to require sampling rates that quickly exhaust resources.

HTD/A methods will feed into and at times require modelling and visualization/decision tools for the data that differ from the standard statistical means testing and regression analyses used for traditional toxicology and risk assessment. The pharmaceutical industry has used such approaches to scan candidate chemicals for activity in particular endpoints while also developing screening level data for toxic effects. In fact, as they develop and become more trusted in the scientific community for MN their first applications may be more in the form of optimization or selection strategies for product development choices rather than in support of more traditional risk analysis of existing products. As such, their application in SbD may be both a bridge for development of safe and beneficial new products and for development of new methods for assuring safety of products on the market.

2.2 Review foresight exercises

2.2.1 *Introduction*

The current approach to MNs safety assessment is reactive, and regulators face the safety issues of MNs once the commercialization occurs, or the authorization process starts. For example, a REACH dossier about a substance in nanoform is submitted, or the use of a new filler for food packaging is requested. However, innovation pace is increasing, and novel materials are generated all the time in industry, with

different properties and applications. To support the faster commercialization of safer products, and to be prepared to address potential issues, also from regulatory side, pre-market information is necessary, and it needs to be organized and assessed, for the implications in terms of risks for different targets, and to the regulatory environment. This process can be generically called Foresight.

Foresight and similar tools are used by regulators to foresee the economic, technological, and social impacts of innovative applications, for example to decide public health policy (to invest in the use of a new medical instrument), or to identify potential future concerns of a product.

The pre-market information about innovation developments, new products, potential new applications, and promising materials, can be collected in different ways. There are two main ways to collect the data: expert-based, or web-based. These two methods can be applied alone, or in combination. Experts can be interviewed for example through a Delphi approach, and the feedback can be then elaborated producing a foresight report. In other cases, web-based sources (e.g. databases, blogs, web-sites, newsletters, interests groups) can be interrogated to collect what are called "weak signals", i.e. pieces of information, evidences that point to a likely technological development, or a future application of a new material. Weak signals are then elaborated by experts to identify regulatory-relevant data and issues. Sometimes, suggestions about regulatory actions are given.

Foresight application to nanotechnology as such is not so common, being sometimes investigated as part of a wider sector (e.g. health, food/feed). The first part of this report will review applications of horizon scanning (HS), foresight, and future technology assessment applied to nanotechnology, as published in the open literature, thus excluding the market research reports. Market research reports are foresight exercises, and there are some nanotechnology focused reports (e.g. BCC Reports[9]), but the content is economic-focused, with little or nothing about safety issues. Also, market reports are expensive and not easily accessible.

The scope of this chapter is to analyse the available literature, and identify, if possible, future trends in nanotechnology applications, identifying potential issues in terms of safety from the regulatory point of view. Some of the questions that will guide the information collection are:

- What innovations are expected in different nanotechnology sector?
- When is the innovation expected to reach the market?
- What are the implications for safety in regulatory terms?
- What should be addressed in the White Paper?

The review of the foresight exercises is covering two main aspects:

1. Role of Foresight for governance, as a contribution to define the role of regulators in the SbD concept
2. Literature review about technology-based case studies and national strategies, to give the widest perspective on nanotechnology foresight state-of-the-art

2.2.2 *Foresight in governance of nanotechnology*

Concerning foresight for innovation technology, and specifically nanotechnology, Schaper-Rinkel published in 2013[10] a review of future-oriented technology analysis (FTA) (i.e. part of foresight activity, sometimes a synonym) in the US and in Germany. FTA is described as a governance tool covering both anticipating and realizing future opportunities and identifying and reacting to potential risks.

The US approach to nanotechnology developed over time from a science-centric vision to one that addressed the whole innovation chain, including R&D, innovation, infrastructures as well as education, and risk governance. Initially the future of nanotechnology focussed on molecular manufacturing (Drexler,

[9] http://www.bccresearch.com/market-research/nanotechnology/nanotechnology-market-assessment-report-nan031f.html

[10] Petra Schaper-Rinkel (2013). The role of future-oriented technology analysis in the governance of emerging technologies: The example of nanotechnology. *Technological Forecasting and Social Change*, 80(3):444–452

1987[11]), as clarified in the US National Nanotechnology Initiative (NNI) report "Nanotechnology — Shaping the World Atom by Atom".

However, from 2004, public perceptions on nanotechnology benefits and risks were also taken into account, incorporating components of research on Ethical, Legal and Social Implications into nanotechnology R&D programs. The new vision report of 2010 was written after engaging a wider expert base (i.e. from industry, NGOs, physical and biological sciences, engineering, medicine, social sciences, economics, and philosophy). The report emphasized governance and concepts to involve and mobilize an increasing variety of stakeholders. The report stressed two main concepts:

(i) the concept of "anticipatory governance of nanotechnology", meaning having participatory FTA be taken up into on-going sociotechnical processes to shape their eventual outcomes at all levels; and

(ii) the concept of "real time technology assessment", related to the integration of natural science and engineering investigations with social science and policy research from the outset.

In Germany, nanotechnology as policy issues started to be discussed in early 1990 by the government. In '80s the German Engineer Association produced the so called technology analyses mainly focused on economic impacts of nanotechnology. The goal of further studies carried out in earlier 1990s, was to "identify new and promising fields for research funding, to deliver a sound and broad information basis for funding decisions in these research fields and to prepare these issues for funding activities". The goal of these activities was mainly to increase the competitiveness of specific industrial sectors with nanotechnology research. Other agencies, more concerned with the safety of nanotechnology applications, entered the field later on, when the funding strategies where already established. According to Schaper-Rinkel[1], "*Germany lacks an organizational structure that brings together the expertise of the broad variety of ministries, agencies, stakeholders, and research to pool the distributed strategic knowledge gained from different activities such as technology intelligence, parliamentary technology assessment, technology monitoring and dialogue processes*". The main conclusion of the review by Schaper-Rinkel (2013) was that "*looking ahead to the next decades, an inter-organizational governance framework is crucial to uptake the knowledge as well as the requirements derived from various stakeholders*".

Rafols et al. (2011)[12] investigated the governance of nanotechnology and argued that the possibility of regulators to influence the innovation at early R&D phases is limited. However, the authors also argued that to effectively govern nanotechnology innovation, it is necessary to further move the regulator's attention further down the value chain, by providing policies for sectors close to the consumers, assuring that the innovation will produce societal advantages. One example is the nanotechnology used to overcome environmental problems (e.g. city air pollution); another can be the energy sector. Foresight is instrumental for regulators in this context, because identifying early-on technological developments with impact on consumers and society can provide precious information to formulate appropriate policies to achieve safe and societal-acceptable outcomes.

2.2.3 *Literature review: horizon scanning results*

This section reports the results of a literature review on previous foresight activities applied to MNs and nanotechnologies. The papers are ordered in terms of topics and year of publication. The main sources of information as identified in the literature are patents, published papers, and experts. Papers are sometimes generic, and sometimes specific to some sector of application or technology.

2.2.3.1 Nanotech development through patenting analysis

Alencar et al[13] examined in 2007 the patents related to nanotechnology from 1994 to 2005, evaluating the patenting patterns in USA, Japan, and Germany, along three life cycle stages: raw MN, nano-intermediates, and nano products. Although patenting analysis is limited due to discrepancies between patents and product (not all patents are finally developed into products), patenting can provide a good overview of the technology development.

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[11] Eric Dexler (1987). Engines of Creation: The Coming Era of Nanotechnology. Published by Doubleday. ISBN: 0-385-19973-2

[12] Rafols I, van Zwanenberg P, Morgan M, Nightingale P, Smith A, 2011. Missing links in nanomaterials governance: bringing industrial dynamics and downstream policies into view. J Technol Transf 36:624–639.

[13] Alencar MSM, Porter AL, Antunes AMS, 2007. Nanopatenting patterns in relation to product life cycle. Technological Forecasting & Social Change 74:1661–1680.

On the basis of 46 terms nominated by experts, the search gave around 20,000 single hits, with the main categories represented by semiconductors and non-metallic components (more than 2,000 hits), and medical and cosmetic applications (1,500 – 2,000 hits).

In particular in Germany, specific patents on coating, plastic, textiles, ceramics, ink, and glass were issued by large industrial groups (e.g. Bayer, Degussa, BASF), while large research groups (e.g. Max Planck, Fraunhofer) were more focused on catalyst, sensors, and cosmetics. The patent analysis is a good tool to identify trends and potential future developments. It is necessary to update the research terms to include new generation MN, and to update the search to 2015.

Patenting analysis can also give indications about the time scale to be considered in the foresight assessment. In 2007, Daim et al.[14] performed an assessment of the time lag between research funding, patenting, and research publishing (conference presentations right after the funding were granted, journal articles 2-3 years after funding, and patenting 5-6 years after initial funding). These results and methodology can be helpful to identify timescales of nanotech innovation along innovation chain, and to identify when a MN is likely to reach the market.

2.2.3.2 Technology assessment

De Miranda Santo et al.[15] reported in 2006 the work done by the Brazilian Center for Management and Strategic Studies to inform the federal government R&D strategy for nanotechnology development. In particular, this report provided results of text mining concerning nanotech publications. The main results were illustrated as tables, with frequency of keywords found in papers from 1994 to 2004. A table shows keywords in key countries (i.e. US, Japan, Germany, France, UK, Switzerland, Spain, Sweden, and Canada) and in competitor countries for Brazil (i.e. China, South Korea, India, Taiwan, Israel, Australia, Singapore, Mexico, South Africa, and Malaysia), which includes terms such as nanocrystals, quantum dots, carbon nanotubes, fullerenes, and nanowires. However, these terms are not useful for identifying thematic areas. More interesting is the analysis of the general worldwide view of nanotech publications. The terms in this table include sectors and techniques, such as nanolithography, nano-electronics, nanofabrication (between 500-700 occurrences), and with much less counts (less than 50) terms like nanomedicine, nanodrugs, nanophotonics, nanofilters, and nanocatalyst. These results can give an indication of growing research fields, and an update of the results by using the same (or similar) keywords can be carried out. An update of the research, taking into account the current more growing nanotech sectors, a sort of trend can be drawn (e.g. growth of interest in nanomedicine since 2004) even if no projection to the future is possible.

2.2.3.3 Sectorial analysis

In this paragraph, specific sectorial results are reported.

2.2.3.3.1 Sensors and nano-bio-sensors

In 2014, Bowles et al.[16] assessed the potential use of nanodevices to manage the supply chain. The paper paid particular attention to nanosensors for:

i) supply chain management system (e.g. tracking logistic) performed through customized MNs;

ii) innovative packaging and labelling to detect leaks and microbiological conditions;

iii) detection of environmental conditions along the supply chain (i.e. temperature, humidity, gas and hazardous substances);

iv) food chain safety (e.g. detection of type and source of spoilage DNA chips to detect pathogens); and

v) tracking (e.g. mobile and distributed self-powered sensors).

[14] Daim T, Brown N, Mitali M, Dash P. 2007. Time lag assessment between research funding and output in emerging technologies. Foresight 9(4):33-44.

[15] de Miranda Santo M, Coelho Massari G, dos Santos DM, Filho Fellows L, 2006. Text mining as a valuable tool in foresight exercises: A study on nanotechnology. Technological Forecasting & Social Change 73:1013–1027.

[16] Bowles M, Jianjun L, 2014. Removing the blinders: A literature review on the potential of nanoscale technologies for the management of supply chain. Technological Forecasting & Social Change 82: 190–198.

However, according to the authors, potential health and environmental problems, privacy issues, and occupational downturns, require policymakers to develop new laws to manage nanotechnology's potential risks.

On the same line, Robinson et al.[17] published in 2013 the results of the application of the Forecasting Innovation Pathways approach to nanobiosensors. The procedure, including four stages ((1) Understand, (2) Profile and Link, (3) Project and Assess and (4) Report), produced several useful outputs, concerning four application domains: healthcare, environmental monitoring, agri-foods, homeland security and defence. The first output is about the main R&D areas, which includes mainly chemistry, biomedical science, and military science. Also, main applications of nanobiosensors were identified on the basis of MN structure and functions, in fields such as toxicity identification, disease diagnosis, microorganism identification, and explosive sensing. With the support of experts and researchers, two main innovation pathways were identified:

Pathway 1. Enhancing biorecognition/bioconjugation using nanostructured materials in biosensors. In general, this path uses MNs passively, that is it focuses on surface properties, such as surface to volume ratio, surface affinity, and selectivity to biomolecules and cells.

Pathway 2. Enhancing signal transduction or creating new transduction mechanisms using MNs in biosensors. In general, this pathway seeks to utilize MNs in a more active way, taking advantage of unique properties of materials with nanoscale dimensions, such as quantum effects, piezoelectric effect, etc.

2.2.3.3.2 Construction sector

The construction sector is a subject of different reviews found in open literature. For example, van Broekhuizen et al. published in 2011[18] a paper about the use of MNs in the construction sector, also analysing some occupational safety aspects in 4 hypothetical situations. A survey carried out in 2009, which was revised and updated by consulting a panel of experts and companies, identified a set of representative applications in the construction sector. In 2009, the main MNs were TiO_2, ZnO, aluminium oxide, Ag, and SiO_2. The main uses were coatings (68% of the market), while concrete and insulation type products covered only the 7 and 12% of the market, respectively. TiO_2 and ZnO are mainly used in coatings. SiO_2 is mainly used in cement and insulation; however, SiO_2 cement covers only the 5% of the whole concrete market.

Another paper by Khitab and Arshad, published in 2014[19], reviews uses of MN in construction sector. The findings reported in the paper shows that SiO_2 is used in cements to obtain ultra-high performance products; TiO_2 is used in paints and in self-cleaning concrete; CNT is used in scratch-resistant paints, while CNT use in cement was limited to experimental studies; carbon nanofibers are proposed to be used in self-de-icing surfaces, but no actual applications were reported.

Arora et al. (2014)[20] published a paper concerning the nanotechnology applications in the building construction sector. Table 1 summarises the main expected development, as reported in 2006.

Table 1. Nanotechnology uses in construction sector, as foreseen in 2006. Arora et al. (2014)[12].

Applications	Nano enabled property	Enhanced functionality	Timescale
Steel coating	Nano-polymer bonds to material surface, eliminates oxidation	Steel coated with nano-polymer has higher resistance to corrosion	2007-2016
Glass coating	Titanium dioxide film affixed to surface of glass	Decomposes organic materials upon contact which self-cleans glass surface	2007-2012
Ceramics	Carbon nano-tubes or other nano-tube based	Improved resistance to stress; increased	2012-2026

[17] Robinson DKR, Huang L, Guo Y, Porter AL, 2013. Forecasting Innovation Pathways (FIP) for new and emerging science and technologies. Technological Forecasting & Social Change 80:267–285.
[18] van Broekhuizen P, van Broekhuizen F, Cornelissen R, Reijnders L, 2011. Use of nanomaterials in the European construction industry and some occupational health aspects thereof. *J Nanopart Res* 3:447–462.
[19] Khitab A and Arshad MT, 2014. Nano Construction Materials: Review. Rev.Adv. Mater. Sci. 38:181-189.
[20] Arora SK, Foley RW, Youtie J, Saphira P, Wiek A, 2014. Drivers of technology adoption — the case of nanomaterials in building construction. Technological Forecasting & Social Change 87:232–244.

	materials are grown through bottom up approach to form nano-structured ceramics	strength and flexibility; reduced deterioration; less volume and weight; surfaces can conduct electricity	
Concrete	Carbon nano-tubes are mixed into the concrete replacing steel rebar	Improved strengths and reduced thickness; less volume and weight vs. strength	2012-2026
Insulation	Nano-pores of air or nitrogen are created within gels or polymers	Efficiency increased due to high surface-to-volume ratio; reduced toxics and non-renewables	2007-2016

The analysis carried out by the authors about the innovation preparedness and intention to use MN in building construction among building companies (i.e. a sample of 19 stakeholders was interviewed) showed that even if there are already beneficial nanotechnology solutions (measured by assessing the number of patents), the awareness is moderate, and there is a low level of assessment and use, due to risk-averse nature of the building construction sector.

The analysis identified a set of technologies, including specific MN, such as: carbon nanotubes, nano-silver, Cryogel™, nanopolymers, organic LED in paint, lanthanum hexaboride, bio-active agents in concrete, alumina foam (insulation), Pyrogel®. All these technologies can be seen as relevant examples to identify potential promising MNs.

Finally, in 2015, Hincapié et al.[21] published a study of the use of MN in constructions in Switzerland, with considerations about their presence in construction and demolition waste. A survey of business representatives of Swiss companies found, as previous reviews, that MN are mainly used in paints and cement. Also, the most frequently used MNs were found to be TiO_2, SiO_2, ZnO, and Ag. The qualitative study showed that 14t/y of TiO_2, 12 t/y SiO_2, 5 t/y ZnO, and 0.2 t/y Ag are used in paints. The main potential of release into environment and technical compartments was considered to be during recycling phase.

2.2.3.3.3 Other specific applications

Glenn in 2006[22] reported some examples of nanotechnology military applications, where military is one of the largest forces behind nanotech R&D. For the scope of this report, they considered MNs limited to inorganic MN (showing that organic MN in 2006 were not on the horizon yet), and nanosystems. Through a Delphi model, applications at short term (to 2010) and in long term (to 2025) were identified. Some examples of applications at short term were:

i) creams and bioweapon shields;

ii) MN in equipment to make it stronger, harder, and so on;

iii) as fuel additive;

iv) nanosensors;

v) filtering membranes;

vi) MN in weapons;

vii) body-implantable RFID;

viii) batteries; and

ix) implantable prosthetics.

[21] Hincapié I, Caballero-Guzman A, Hiltbrunner D, Nowack B, 2015. Use of engineered nanomaterials in the construction industry with specific emphasis on paints and their flows in construction and demolition waste in Switzerland. Waste Management 43:398–406.

[22] Glenn J.C., 2006. Nanotechnology: Future military environmental health considerations. Technological Forecasting & Social Change 73:128–137.

The following long term applications were identified:

i) artificial blood cells;

ii) smart weapons;

iii) smart receptor enhancers;

iv) permanent nano-sensors and nano-medicine in general;

v) targeted bio weapons;

vi) contaminated sites clean-up MN;

vii) ubiquitous surveillance and control systems;

viii) nanoscale ATP-driven motors to enhance human performance;

ix) tele-operated soldiers; and

x) remote controlled weapons.

Also, a list of R&D potentially benefiting the reduction of hazard posed by MN used in military is reported in the paper. Some of these indications can be helpful for SbD implementation.

Avila-Robinson and Miyazaky, in 2013[23] published a paper concerning a methodological approach to technological foresight. As a case study, the authors identified several MEMS/NEMS (micro/nano electromechanical systems technologies) applications. The basic set included accelerometers, Bio-MEMs, gyroscopes, microfluidics, MOEMS display, Micro-tips (AFM), Power MEMS, Pressure sensors, Printheads, RF-MEMS, ZnO nanosensors, CNT nanotubes. On the basis of research integrating bibliometric, social network analysis and multivariate statistical methods on scientific publications, the authors identified that in comparison to other conventional technologies, nano-based applications shows the highest accelerating rates of knowledge diffusion (average of the 'slopes of the slopes' of the cumulative proportion curve of publications and citations). Some other conventional technologies, such as ink jet printing heads shows higher dynamism due to nanotechnology implementation.

Also, the authors performed an assessment of the knowledge structure, where for the ZnO-based sensors as clustered around Fabrication/Analysis (devices), with material production to a lesser degree, and very few about material characterization. The main areas of work are gas and chemical sensors, bio-sensors, and nano-generators. However, the sectors are still very broad, without a finer specialization.

Hussein et al. published in 2015[24] a comprehensive review of nanotechnology used in renewable energy applications. The paper is organized in tables, reporting applications at both experimental and theoretical stages for solar energy, hydrogen energy, wind energy, and geothermal energy. Mentioned technologies include:

i) nanowire arrays,

ii) nanofluids,

iii) TiO_2 nanowires coated with Au or Ag ENP,

iv) graphite, carbon black, silver, and Al_2O_3 MN in nanofluids,

v) MgO MN for solar heating systems,

vi) Al particles and Al nanofluid in diesel fuel,

vii) TiO_2/SnO_2 particles in fuel cells,

viii) Ag/TiO_2 nanocomposite films,

ix) CNT, and cobalt oxide/graphene nanocomposite in fuel cells,

x) KF/CaO nanocatalyst in biofuel production,

[23] Avila-Robinson A, Miyazaky K. 2013. Dynamics of scientific knowledge bases as proxies for discerning technological emergence — The case of MEMS/NEMS technologies. *Technological Forecasting & Social Change* 80:1071–1084.

[24] Hussein AK, 2015. Applications of nanotechnology in renewable energies—A comprehensive overview and understanding. Renewable and Sustainable Energy Reviews 42:460–476.

xi) nano-magnetic solid-base catalyst, and

xii) nano-colloidal boron nitride additive as component of wear protective coating.

2.2.3.4 National strategies

A subset of foresight activities involved the definition of national strategies for nanotech development. There are initiatives in different countries, focusing on specific domains/sectors, or on wide spectra of applications. This section is a summary description of the content of such documents, which are often known as road maps.

The Asia-Pacific Economic Cooperation (APEC), with the Industrial Science and Technology Working Group, published in 2002 a report titled "Nanotechnology: The Technology for the 21st Century"[25]. The goal of the foresight activity in this report was to define alternative scenarios for nanotechnology development till 2015. The study identified ten issues for nanotechnology development:

i) definition of nanotechnology,

ii) opportunities,

iii) scientific and technological inputs,

iv) education and training,

v) R&D funding,

vi) regional collaboration and networks,

vii) commercialization,

viii) metrology,

ix) implication for small economies, and

x) societal implications.

The scenarios developed on the basis of these issues focused on the benefits of nanotechnology, with safety as a secondary concern, and the policy definition aimed at increasing the acceptability of nanotechnology. The main nanotech opportunities identified in the study were molecular engineering inspired by biotechnology (e.g. implantable biosensors), electronic and photonic technology based on conventional and novel semiconductor materials (fabrication of electronic structures on the nanometre scale based on entirely new processes), and other devices and processes based on new materials (including IT peripherals, medical and biomedical applications, automotive and industrial equipment, communications, process control, environmental monitoring and household products). The report includes also the results of an expert which foresaw the development (availability):

- **by 2005**, of:

  i) Selective bio nano-sensors;

  ii) Specific drug delivery systems;

  iii) Nano-electronics based on miniaturised silicon devices;

  iv) Novel devices based on magnetic spin electronics;

  v) Nanostructured materials as industrial catalysts; and

  vi) Self-cleaning surfaces based on MNs.

- **by 2012** of:

  i) Advanced medical diagnostics;

  ii) Targeted human cells for organ repair;

  iii) Single electron devices;

  iv) Optical computing;

[25] The APEC Center for Technology Foresight National Science and Technology Development Agency, Bangkok, Thailand January 2002.

v) Portable fuel cell and advanced battery; and

vi) Artificial photosynthesis.

In some cases, the estimations were too optimistic. Also, the same experts identified the following uncertainties:

1. Technical Uncertainties,
   - Nanotechnology fails to deliver,
   - Inability to solve standards issues,
   - Breakthroughs in current technical paradigms – devices and materials;
2. Environmental / Economic Uncertainties,
   - Major financial crisis,
   - Kyoto Protocol ratified by all economies,
   - Major disruption of energy supplies;
3. Political / Societal Uncertainties,
   - Lack of public acceptance of nanotechnology,
   - Nanotechnology facilitates major advances in bio-health,
   - Terrorism and national security;
4. Global Uncertainties,
   - World War III,
   - Widespread epidemic.

The European Foresight Monitoring Network published a report by Birgitte Rasmussen and Per Dannemand Andersen (2004)[26], developed by Risø National Laboratory and organized by The Danish Ministry of Science, Technology and Innovation. The goal of the work was to deliver the National action plan for Danish nano-science and nano-technology, including research, innovation, and education policy. Important building blocks in the Danish Nano-foresight process were hypotheses and statements about future research, industrial possibilities and consequences both beneficial and adverse of nano-science and nano-technology. A basic concept in the process was the formulation of statements about the scientific and commercial potentials of nanotechnology within a time horizon of 20 years, and then to allow a critical scientific discussion about them. The main nanotech domains investigated in the foresight exercise were nanobio-systems, nano-electronics & nano-optics, and MNs.

Another example of foresight reference is “Foresight for the semiconductor industry in Taiwan”, published in 2006 by Yuan et al.[27]. The scope of the study was to explore the possible future business environment, industrial structure, technological transformation, and market for the semiconductor industry in Taiwan. The results were technological and economical in nature, and no safety issues were addressed.

Ghazinoory et al. published the national strategy for Iran innovation in 2009[28], with a case study for nanotechnology. A first step of the strategy development was to identify areas of interest for Iran community on the basis of the capability–attractiveness matrix. A list was drawn taking into account “attractiveness”, defined on the basis of national policy, and the following indicators:

- Ease of access to required natural and mineral resources;
- Having competitive advantage in production and exports;

[26] Rasmussen B., Andersen Dannemand P, 2004. Danish Nano-science and Nano-technology for 2025.

[27] Benjamin J.C. Yuan John Chih-Hung Hsieh Champion Wang, 2006. Foresight for the semiconductor industry in Taiwan. Foresight 8(5):45 – 55.

[28] Ghazinoory S, Divsalar A, Soofi AS. 2009. A new definition and framework for the development of a national technology strategy: The case of nanotechnology for Iran. *Technological Forecasting & Social Change* 76:835–848.

- Quality improvement and customer satisfaction;
- Public perceptions of technological outcomes and their use by the general public;
- Coordination with government financial budget allocations for technology development;
- The time required to acquire technology and gain proficiency in using it;
- The comparative position of the new technology vis-à-vis the competitive technologies;
- The significance of national security issues in acquiring the technology.

Experts defined the following ranked list: Nano particles, Nano tubes, Nano organic structure, Nano composites, Nano electronic and optical systems, Nano electro mechanic systems, Nano wires, Fullerenes, Nano fibres, Nano capsules, Nano crystals, Nano porous materials, Nano fluids.

A similar analysis was carried out in Australia, and published by Tegart (2009)[29] which presented the results of 4 workshops held in 2007 to assess the potential applications of nanotechnology to the energy sector. Three temporal horizons were identified, with in the short term (2012) emphasis on energy conservation, environmental management, catalysts for combustion, photovoltaic cells. In the mid-term (2012-2022), the focus shifts towards catalysts for conversion of biomass, gas and coal, fuel cells, advanced photovoltaic systems using engineered MNs. Finally, in the long term (> 2022) hydrogen production, storage and use become important. Some examples of technologies for each horizon were reported in the paper. In the short term, low weight materials for mobility, better building insulation, and LED are seen as good examples of nanotechnology contribution to energy conservation. In the mid-term, an example is the use of better gas catalyst off-shore to process the gas to liquids easily transported inland. In the long term, an example is defined as fuel cells. All these applications can be considered interesting domains, and MN used in these applications are of interest.

Another paper, published in 2010 by Su et al.[30], reported an assessment of the foresight national survey based on Delphi method carried out in 2007 in Taiwan. Three domains were selected:

1. nano material;
2. nano electronic and semiconductor; and
3. nano bio medicine.

The three fields are further divided into 13 sub fields containing 75 technique topics, 26 in Nano Material, 24 in Nano Electronic and Semiconductor, and 25 in Nano Bio Medicine. According to the experts, R&D maturation time will fall in the period 2010-2015 for both ‘‘Nano material’’ and also ‘‘Nano electronic and semiconductor’’ domains, but most answers fall into the period of 2007-2010 for ‘‘Nano bio medicine’’ domain. Taking into account the criteria “degree of industrial application”, the main technologies foreseen in 2020 are:

1. “Nanomaterial”:
    a. metallic nanoparticles coating material and devices,
    b. polymeric particles manufacturing,
    c. polymeric nanocomposites,
    d. polymeric optical display,
    e. thin film application processes, and
    f. nano organic-inorganic composites
2. “Nano electronic”:
    a. epitaxy technique and measurements,
    b. nano coating, and

[29] Tegart G, 2009. Energy and nanotechnologies: Priority areas for Australia's future. Technological Forecasting & Social Change 76:1240–1246.
[30] Su HN, Lee PC, Yuan BJC, 2010. Foresight on Taiwan nanotechnology industry in 2020. Foresight 12(5):58-79.

c. nano storage

3. "Nano bio medicine":
    a. antibacterial TiO_2 technique,
    b. bio chip and protein chip manufacturing, and
    c. preparation and detection of metal and fluorescent nanoparticles.

Finally, in 2015, Karaca and Öner[31] published a foresight study about the nanotechnology development in Turkey. The study was aimed to identify the willingness of the public to buy nano-enabled products in a set of sectors, namely:

1) Nano-medicine and drug delivery,
2) Biocompatible materials,
3) Nano-sensors and nano-fluidics,
4) Plastic electronics,
5) Nano-optics and nano-photonics,
6) Nano-catalysis, hydrogen technology, etc.,
7) Nano-materials with new functional properties.

The survey, involving more than 300 subjects, evaluated 9 criteria for 7 scenarios, from "nano-averse" to "go nano", going through "incapable to nano", in two different times: 2009 and 2029. The main results shows that "*all the different contexts determined by clusters, converge to a final setting where there is high development potential, neutral public awareness, almost evenly distributed consumer demand with the lead of environmentally friendly nano-products, more laws and regulations, high profit potential and public investments and a medium risk assessment by the public*".

2.2.4 *Summary*

The foresight activity for nanotechnology covers almost all topics, from methodological developments (e.g. how to apply a specific method to nanotechnology) to foresight studies related to specific sectors, such as military applications, as well as energy and building applications. However, in general, the assessment is rarely linked to very specific MNs, and the level of assessment is often limited to the domains and sub-domains. The nanotech domains mostly investigated or considered in the literature are energy and medicine. Specific technologies identified in different papers include nano-bio-sensors, nano-electro mechanic devices, super capacitors and energy storage devices in general, and nano drug-delivery systems.

However, information on specific and promising MN, such as for example new generation MNs, is lacking in foresight literature. Only in few cases (e.g. see MN in building construction and in energy) examples were reported such as i) KF/CaO nanocatalyst, ii) MgO, graphite, carbon black, and silver in nano-fluids, iii) lanthanum hexaboride, iv) alumina foam.

Given the limitations of the available data, it is difficult to identify specific trends and timeline of appearance on the market of new products and applications. Some information can be inferred from some documents, such as national registries and national assessments. For example, the most recent French Registry Report[32] describing the results of the 2014 campaign shows that there is an increase in declared uses in agriculture, fisheries and forestry, with 71% of the declarations within this category. At the same time, the category "fabrication of food" represents only 2% of all the declarations. This result could suggest that in the mid-term (5-10 years) several nano-products will potentially enter the food value chain in the first phases (e.g. primary production, food processing), with potential repercussions on EFSA work, and an increased need of data of environmental behaviour of MNs. Another interesting data is the use of chemicals, where the first three categories are phytopharmaceuticals, cosmetics, and varnishes/solvents.

However, these data are based on registrations that are reflecting the current situation. In order to identify actual trends, and to achieve a better understanding of the new MN development, it is necessary to perform

[31] Karaca F, Öner MA, 2015. Scenarios of nanotechnology development and usage in Turkey. Technological Forecasting & Social Change 91:327–340.
[32] Ministère de l'Environnement, de l'Énergie et de la Mer. Éléments issus des déclarations des substances à l'état nanoparticulaire. RAPPORT D'ETUDE 2015.

a focused search by using different sources (not only published papers), taking into account a limited set of technologies or a single sector. Such detailed analyses were not within the scope of the PROSAFE project as it requires specialised knowledge and expertise. However, in Task 2.3 a Delphi poll is carried out to determine the current understanding of the available knowledge on risk assessment and management for MNs as well as what the main gaps are.

2.3 Equipment needs

2.3.1 *Introduction*

This part of the report describes addresses the equipment needs for implementing appropriate risk assessment and management methodologies. Put simply, the question may be framed as "What types of equipment are needed to enable industry to effectively manage the risks of MNs of NM through a SbD process?"

The scope of this part of the report has been agreed as follows;

- This document is intended to inform the stakeholder process of the PROSAFE project about insufficiencies and gaps in equipment to support risk management of MN uses (i.e., for sampling, analysis and monitoring of exposure and hazard characteristics of MNs being used in commerce).
- The focus is on equipment needs. Other types of tools (e.g. models, protocols) may be identified but not assessed in this document.
- In some cases equipment needs are suggested by what may come from developments in emerging areas of functional assays, environmental fate and biological effects modelling, and systems biology or Tox21 style screening approaches. These sorts of needs must be considered in light of the developing science in an iterative fashion. For example, specific *in vitro* assays calibrated for MNs in high throughput screening (HTS) are beginning to be used in development of predictive models. The predictive value of the models for regulatory decisions and the equipment to perform the assays are interrelated in ways that are difficult to assess at this time, in part because the biological basis for toxicity varies widely across MNs and in some cases is not yet clearly understood. However, it is clear that the models and the equipment to develop the data supporting them will be important for MN evaluation.
- Furthermore, integrative approaches across toxicology, physical chemical characterization, and exposure that incorporate "big data" analytic and modelling methods to utilize the kinds of information that can come from HTS, lab on a chip, organ on a chip, and automated image analysis are also considered as a kind of meta-equipment under development by some laboratories.
- This is intended to be a high level review and summary highlighting the main issues to stimulate the foresight process
- As part of a coordination and support activity rather than a primary research activity, the primary sources of information will be existing FP6, FP7, and H2020 project reports and reviews (in NANOREG and elsewhere). Reference will be made to the underlying science where appropriate; however, we will not make an independent evaluation of the scientific methodologies.
- The review will focus on the NANOREG priority materials and will consider how these materials may be used or transformed in the value chains
- The review will use responses to the regulatory questions from NANOREG [33] as the main driver for identifying what are the equipment and methodology issues, considering different points on the value chain
- Ultimately, this report will attempt to address the question of where there are equipment road blocks specific to anticipated risk management decision needs and how these barriers can be resolved.

2.3.2 *Approach*

The work in this task was carried out according to the steps identified below.

- Relevant literature and other information sources were identified, drawing largely on the PROSAFE process to identify and gather the relevant key strategic and oversight documents published internationally over the last 10 years. This was supplemented by information available from completed and running European FP6, FP7 and H2020 projects. In this respect particular reference was made to the available (final or draft) deliverables of the NANOREG project. Additional information was drawn as required from the wider literature.
- An initial assessment was made of the measurement needs identified or implied by the 16 Regulatory Questions developed and elaborated in the NANOREG project[34]. This included analysis

[33] NANOREG Deliverable D1.2 – Results of GAP analysis: Regulatory data gaps and research needs

to identify for each question the types of data/parameters needed and the types of tools necessary to provide that data. A further elaboration of the measurement context was developed for each question. Based on the analysis and grouping of measurement types was developed and mapping made between the regulatory question and the measurement type.

- Summary descriptions were made for each of the measurement method and the available options for possible types of equipment. This included information on types of equipment under development.
- An initial assessment of each of the measurement methods was carried out and an assessment of the adequacy and fitness for purpose of the equipment. Major gaps were identified, where there are prospects for these to be filled and where it seems that work is not likely to fill this gap.

2.3.3 *Analysis of the NANOREG regulatory questions*

2.3.3.1 Question 1 - Measurement and characterization - Identification: How can MNs be identified according to the EC recommendation for a definition of MNs

2.3.3.1.1 Question statement

Measurement and characterization - Identification: How can MNs be identified according to the EC recommendation for a definition of MNs and for regulatory purposes (i.e. the implementation of the EC definition in e.g. REACH, CLP, cosmetics, novel food, etc.), including other jurisdictions (global harmonisation)? Can we develop robust measurement protocols which enable assessment of whether a NM falls under, or not, the EC definition? Are there robust measurement protocols available (and for which matrices) that enable identification?

2.3.3.1.2 Data needed and types of tools implied

Equipment for the measurement of particle size distribution, number size distribution, and/or particle surface area for dry powders or for particles in suspension (along with associated and validated protocols).

2.3.3.1.3 Measurement context

Commission Recommendation 2011/696/EU[35] defined 'nanomaterial' as follows:

1) "Nanomaterial" means a natural, incidental or manufactured material containing particles, in an unbound state or as an aggregate or as an agglomerate and where, for 50 % or more of the particles in the number size distribution, one or more external dimensions is in the size range 1 nm - 100 nm.

2) In specific cases and where warranted by concerns for the environment, health, safety or competitiveness the number size distribution threshold of 50 % may be replaced by a threshold between 1 and 50 %.

By derogation from point 1, fullerenes, graphene flakes and single wall carbon nanotubes with one or more external dimensions below 1 nm should be considered as nanomaterials.

Where technically feasible and requested in specific legislation, compliance with the definition in point (1) may be determined on the basis of the specific surface area by volume. A material should be considered as falling under the definition in point (1) where the specific surface area by volume of the material is greater than 60 m^2/cm^3. However, a material which, based on its number size distribution, is a nanomaterial should be considered as complying with the definition in point (1) even if the material has a specific surface area lower than 60 m^2/cm^3.

To identify nanomaterials according to the EC definition, the main characteristics to be tested are the (primary) particle size distribution or volume-specific surface area (VSSA). Although, there are several methods available to measure these characteristics, for most materials a combination of different methods is needed to determine if they fulfil the EC criteria. It is difficult to determine if aggregated materials fall under the EC definition, because it is usually not possible to measure the size distribution of their constituent primary particles. However, measuring the external surface area is at the moment only straight forward for powders. There are several methods to measure most of these characteristics for MNs in the form they are produced (e.g. powders or liquid dispersions). However, these methods are not always suitable to

[34] NANOREG Deliverable D1.2 – Results of GAP analysis: Regulatory data gaps and research needs
[35] European Commission, 2011. Commission Recommendation of 18 October 2011 on the definition of nanomaterial (2011/696/EU). Official Journal of the European Union 275: 38-40.

characterize MNs in different matrices (such as products, environmental compartments, test media and biological tissues) and after transformation, agglomeration and/or aggregation of the MNs within these matrices[36,37,38]. In addition some of these methods, such as electron microscopy, are not readily accessible for everybody, because they require expensive equipment, highly trained personnel and a large amount of time. Accessible, standardized methods to characterize MNs in different media are needed to identify, quantify and characterize the MNs in all stages of their life cycle and within exposure and toxicity testing.

2.3.3.2 Question 2 - Measurement and characterization: Intelligent testing strategy for characterisation?

2.3.3.2.1 Question statement

Could an "intelligent characterisation strategy" be defined? What is a minimal set of physical (and/or chemical) characteristics that should be available for risk assessors within the context of regulatory toxicology? What are the relevant features to characterise MNs, e.g. size, form, aspect ratio, rigidity, flexibility and coating? What methods (SOPs) should be developed / used to determine the physical chemical characteristics of MNs throughout their different life cycle stages within the context of regulatory toxicology?

These questions (closely related to Q1) refer to developing cost effective standard methods, detailed protocols and reference materials both for calibration and analysis of both pristine materials and materials in relevant media or complex matrices throughout the complete life cycle of the MN. They also refer to whether different categories of characterisation methods (varying e.g. in precision and accuracy) can be defined.

2.3.3.2.2 Data needed and types of tools implied

Equipment for the measurement of particle size, particle surface area, form, aspect ratio, rigidity, flexibility and coating for dry powders or in suspension with associated protocols.

High throughput data analytics and decision tools to integrate multi-parameter data sources using, for example, functional assays, high throughput *in vitro* screening and automated image analysis, are considered in this report as “integrative equipment” and will be discussed separately as “high throughput data/analytics” or HTD/A. Integrative approaches across toxicology, physical chemical characterization, and exposure are considered in Tox21 and systems biology approaches for “intelligent characterization” of chemicals. Their use for MNs is also being considered and developed by some laboratories.

2.3.3.2.3 Measurement context

The question is more related to development of a strategy in which the measurements can be combined in a in an intelligent way. The purpose of this is not explicitly stated in the question but is likely to be towards identification of whether or not a material is an MN (i.e. Q1) and if so, whether it can be grouped with other similar MNs based on the physico-chemical properties in such a way as to predict the hazard or to associate the MNs to a tailored testing strategy, in itself a major challenge.

Equipment needs are identified necessary for the measurement of particle size, particle surface area, form, aspect ratio, rigidity, flexibility and coating for dry powders or in suspension. Associated protocols are also required.

- Particle size measurement

Measurement context and gaps for particle size measurement has been extensively covered in the Question 1. Major difficulties were identified in

[36] Committee to Develop a Research Strategy for Environmental, Health, and Safety Aspects of Engineered Nanomaterials; Board on Environmental Studies and Toxicology; Board on Chemical Sciences and Technology; Division on Earth and Life Studies; National Materials and Manufacturing Board; Division on Engineering and Physical Sciences; National Research Council, 2012. A Research Strategy for Environmental, Health, and Safety Aspects of Engineered Nanomaterials.

[37] OECD, 2012. Series on the Safety of Manufactured Nanomaterials No. 36 - Guidance on sample preparation and dosimetry for the safety testing of manufactured nanomaterials.

[38] Kermanizadeh A, Vranic S, Boland S, Moreau K, Baeza-Squiban A, Gaiser BK, Andrzejczuk LA and Stone V, 2013. An in vitro assessment of panel of engineered nanomaterials using a human renal cell line: cytotoxicity, pro-inflammatory response, oxidative stress and genotoxicity. BMC Nephrol 14:96.

i. Dealing with agglomerates/aggregates: no method is available that can reliably distinguish whether a large particle is an agglomerate, aggregate or a single particle, and at the same time measure the size of large numbers of individual constituent particles;

ii. Working range: no single method alone can cover, in a single measurement, for all materials the complete size range from lower than 1 nm to well above 100 nm; and

iii. Method validation: none of the mentioned measurement methods have been specifically validated for their use in the implementation of the nanomaterial definition.

- Particle surface area

The most frequently used method to determine the surface area of dry powders is the BET (Brunauer-Emmett-Teller) method. This method measures how much gas (usually nitrogen) is adsorbed at a specific temperature and pressure. The BET method measures particle surface area of a sample. Dividing the absolute surface area by the sample mass gives the mass-specific surface area, commonly reported in the unit square metre per gram (m^2/g). This is not the same unit as used in the EC definition, which refers to the volume- specific surface area (VSSA) with the unit m^2/cm^3 or m^2/m^3. Therefore, the calculation of the VSSA from the common result of a BET measurement requires the knowledge of the particle density which may or may not be the same as density of the bulk material. The method works only for dry powders. The result corresponds to the surface area of the aggregate or agglomerate, not of the constituent particles. The method will give the total surface area accessible to the gas used, which includes inner surface such as pores. The BET method is relatively simple and straight forward to use. The method has been a standard method for many years. No significant further changes are to be expected in the near future.

2.3.3.3 Question 3 – Characterization and transformation

2.3.3.3.1 Question statement

What testing should be performed to identify surface modifications that occur once a MN has been released into the environment or taken up into the body? How can transformation, including agglomeration surface modification, dissolution and incineration, be determined and considered in the exposure and hazard assessment and how do they change the intrinsic toxic properties and biodistribution. Do we need to know the details of such surface modifications or of what is bound, or do we need some simple test systems that actually determine the behaviour and transformation of MN in relevant media throughout all life cycle stages? Is a nano-derived material still nano when it becomes agglomerated?

2.3.3.3.2 Data needed and types of tools implied

Equipment and protocols for

- Size distribution,
- Chemical composition,
- Shape,
- Surface area and charge,
- Agglomeration / aggregation – methods for distinguishing between primary particles and aggregates / agglomerates are essential,
- Solubility / dissolution and dissolution rate,
- Biocorona/protein corona formation/surface chemistry, and
- HTD/A methods.

2.3.3.3.3 Measurement context

- Aggregation/agglomeration

Aggregation or agglomeration is important process in the transport and fate behaviour of MN in different environments and biological compartments. Numerous mechanisms of agglomeration may play a role in MN from the beginning. The recommended EC definition of MN is intended to enable identification of a MN using the 3D size-dimensions of nano-objects (and VSSA) as an inclusion criterion for reporting MN materials. In the environment and biological compartments a materials is a MN until it has transformed to the point that it no longer satisfies the size-(and VSSA)-criterion. Consequently, the aggregates and agglomerates are MN

as long as it is possible to identify their constituting nano-objects. Hence the review of the methods for Q1 is relevant here.

- Solubility/dissolution

In general chemical terms solubility is the property of a solid, liquid, or gaseous chemical substance called solute to dissolve in a solid, liquid, or gaseous solvent to form a solution of the solute in the solvent. The solubility of a substance fundamentally depends on the physical and chemical properties of the solute and solvent as well as on temperature, pressure and the pH of the solution. The extent of the solubility of a substance in a specific solvent is measured as the saturation concentration, where adding more solute does not increase the concentration of the solution and begins to precipitate the excess amount of solute.

The question of solubility / dissolution and dissolution rate is important in relation to toxicity. Dissolution of MN will result in size-reduction, modification of surface chemistry and shape, and ultimately transforms the entire MN to its molecular or ionic constituents after which it is no longer a MN. "If nanomaterials are dissolved into the molecular or ionic form of their chemical components, then they are of course also no longer nanomaterials and the behaviour and related toxicity follows that of the molecular form of the chemical components". [39,40]

BAuA (2013)[41] has suggested a threshold value for nanomaterials of 100 mg/L, i.e. those nanomaterials whose water solubility exceeds this level are defined as soluble.

The solubility of a substance is an entirely different property from the rate of solution, which is how fast it dissolves. The dissolution rate is a measure of the actual release rate of the compound at the given particle size etc. in an aqueous media. It often varies considerably with solid form, e.g. particle size and shape. Particle dissolution rate is an important parameter in pharmacology and may be also be of interest in a risk context, but is much less mentioned in this context.

2.3.3.4 Question 4 - Metrology and dose metrics: Which metrics (metrology) should be used for MNs in regulatory toxicology?

2.3.3.4.1 Question statement

As recommended by several committees and guidance, notwithstanding e.g. the OECD GSPD, NANOREG should use mass, particle numbers and surface area (as far as possible) to characterise dose. The data generated within the project will contribute to the development of a body of comparative data (e.g. shape and aspect ratio should be examined when appropriate for the MN). Using this comparative data, NANOREG should examine which metrics are the most appropriate depending on the different types of materials and media involved, as well as the (eco)toxicological effects and exposure to be assessed in the Risk Assessment process.

2.3.3.4.2 Data needed and types of tools implied

Equipment and protocols for;

- Measurement methods for particle number, mass and surface area concentrations (and size distribution) in air,
- Measurement methods for particle number, mass and surface area (and size distribution) for dry powders or in suspension (various media), and
- HTD/A methods.

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[39] EFSA Scientific Committee, 2011. Guidance on the risk assessment of the application of nanoscience and nanotechnologies in the food and feed chain. EFSA Journ 9: 2140.
[40] Wijnhoven et al., 2009
[41] BAuA, 2013. Bundesanstalt für Arbeitsschutz und Arbeitsmedizin. Bekanntmachung zu Gefahrstoffen. Hergestellte Nanomatierialien. – German Federal Institute for Occupational Safety and Health. Announcement regarding hazardous substances. Manufactured nanomaterials. BekGS 527. BMBl 2013, 498–511 (Nr. 25). Available at: http://www.baua.de/de/Themen-von-A-Z/Gefahrstoffe/TRGS/Bekanntmachung-527.html.

2.3.3.4.3 Measurement context

The risk of MNs is influenced by many characteristics and properties, which means that information on the administered weight (mass) alone is usually not sufficient to describe the dose that determines a particular response in a biological system. In order to identify which dose metrics should be used for MNs in regulatory risk assessment, one should first identify in which parts of the regulatory frameworks quantitative information on the amount of MNs are used. Within REACH, for example, the first quantitative information that is used is the production volume of the substance. This production volume determines if registration is needed and which data requirements are applicable. Within the Classification, Labelling and Packaging (CLP) regulation, dose levels at which toxicity effects are observed within different toxicity tests and/or the elimination rate from and relative concentrations in organisms, determine the classification and labelling of the substance. Within most regulatory frameworks, dose levels at which effects are observed in experimental tests are used to determine exposure limits (DNELs, PNECs, OELs, ADIs, etc.) which can be compared to the estimated exposure levels to estimate the risk.

Which is the most appropriate dose metric remains an open question. Most quantitative information used in regulatory frameworks is used to distinguish substances with a relatively low potential risk from those with a relatively high potential risk. The dose metrics that is most appropriate to compare the risks of MNs is probably not the same for each situation, but is likely to depend on the type of MN, the route of exposure, the kinetics and/or the toxicological endpoint. For example, the dose response curves for two sizes of otherwise identical titanium dioxide are markedly different from each other when the deposited mass in the lungs are used. However, when the mass is converted in the total surface area of the deposited particles, there is a remarkable overlap in the dose response curves[42].

The decision need and problem formulation of a supporting risk assessment will determine which dose metrics should be used in which situation. In some cases more knowledge on the key characteristics/properties that influence the exposure (release and fate in the environment), kinetics (internal dose at the target tissue) and subsequent toxicity of MNs will be needed. In other cases, simply ruling out exposure or the possibility of sufficient potency may be all that is needed. Furthermore, knowledge on the implications of using different dose metrics in the different parts of the regulatory frameworks is needed. It is not practical to use all relevant characteristics in the dose description of MNs in all parts of the regulatory frameworks. This would, for example, mean that different exposure limits would need to be derived with respect to each MN with (slightly) different characteristics, such as size or surface chemistry, etc. A more pragmatic way would be to use a reduced dose metric, in which, for example, the dose of MNs consisting of the same chemical composition is characterized with fewer parameters. This approach can be justified if, for example, the role of some characteristics in the induced response is negligible compared to that of others. Alternatively, certain particle properties influencing the response may be uniquely related, such as particle size with surface area, reactivity and solubility, in such a way that only one parameter combining these properties needs to be included in the dose metric of all MNs of the same chemical composition. Justification of the use of such a reduced dose metric should first be established by experimental study.[43] However, the implications of using different dose metrics for different types of MNs, routes of exposure and/or toxicological endpoints within the regulatory toxicology should also be considered. It may be difficult, for example, to compare the acute toxicity of MNs and bulk materials if different dose metrics are used for the classification. The same holds true with respect to comparing different exposure routes or toxicological endpoints. Moreover, to be able to compare exposure and hazard the estimated exposure should be expressed in same dose metrics as the relevant exposure limit (e.g. OEL, ADI or PNEC).

Short term research needs to fill the data gaps with respect to the most appropriate dose metric are the development and use of standardized protocols for sample preparation and the characterization of MNs within exposure and toxicity studies (including sampling strategy, data handling and the characterization of the MNs that the cells or organisms are actually exposed to and the interaction of the materials with culture media, biological matrices, proteins, tissues and cells). These standardized protocols for sample preparation and characterization are needed to obtain a clear picture of the characteristics of the different nanoparticles (including their stability, homogeneity and aging) in realistic exposure situations and different stages of

[42] Oberdörster G, Oberdörster E, Oberdörster J, 2007. Concepts of Nanoparticle Dose Metric and Response Metric. Environ Health Perspect. 115: A290.
[43] (Park, et al., 2012).

toxicity tests and their impact on the risk. Several initiatives and projects have been working on standardized protocols for sample preparation[44].

Long term remaining research needs are further identification, verification and validation of the key MN characteristics that influence the exposure, behaviour (fate and kinetics), effects (hazards) and subsequent risks and the identification of the most appropriate metrics for each type of MNs within each specific route of exposure and toxicological endpoint. In addition, implementation of these most appropriate metrics and the approach to determine these metrics within the risk assessment approaches and regulatory frameworks is needed.

2.3.3.5 Question 5 – Extrapolation and grouping

2.3.3.5.1 Question statement

Extrapolation and grouping: What guidance can be provided on how to decide when information from different forms of manufactured nanomaterials (MNs) (or from the bulk material) can be "re-used" in the sense of read-across, categorisation and grouping? Should / could guidance be based exclusively on physico-chemical properties or could exposure related (eco)toxicological and mechanistic information (as Mode of Action) be used as well and how?

2.3.3.5.2 Data needed and types of tools implied

Potentially a very long list as many differ properties are being considered as potentially being part of a grouping scheme

- Data on physicochemical properties, e.g.
    - Size,
    - Shape,
    - Solubility,
    - Rigidity, and
    - Surface chemistry;
- Data relating to adverse outcome pathways (AOP) which could include early (potentially reversible) effects such as inflammation long term effects such as genotoxicity or the pathology itself e.g.
    - cytotoxicity,
    - inflammation,
    - oxidative stress, and
    - genotoxicity;
- The uptake, biodistribution, and biopersistence (biokinetics) of a MN in an organism;
- HTD/A methods.

2.3.3.5.3 Measurement context

So-called 'grouping of substances' (or category approach) is an important means to avoid unnecessary new testing. In this approach, closely related chemicals are considered as a group, or category, rather than as individual chemicals so that not every chemical needs to be tested for every endpoint.

There have been many initiatives and suggestions for grouping of MNs but as yet no agreed framework. These include the material properties and biophysical interactions, specific types of use and exposure, uptake and kinetics, and possible early and apical biological effects. This question is being actively

[44] OECD, 2012. Series on the Safety of Manufactured Nanomaterials No. 36 - Guidance on sample preparation and dosimetry for the safety testing of manufactured nanomaterials.

considered within NANOREG and in MARINA. As discussed by van Tongeren et al 2014[45], more than a dozen material properties and biophysical interactions of MNs have been identified that could potentially contribute to hazardous effects. These are summarised as

- Their production, use and release (throughout the life cycle);
- The physico-chemical characteristics of a MN, which can be different in different life cycle stages (e.g. release and external exposure of organisms);
- The uptake, biodistribution, and biopersistence (biokinetics) of a MN in an organism and the physico-chemical characteristics of a MN inside the organism at different target sites; and
- The early and apical biological effects

For an effective grouping, a group needs to be well-defined to determine which MNs belong or do not belong within a certain group. Groups are generally defined by benchmarks, i.e. a certain value for a specific physico-chemical property that sets a boundary of the group. For MNs, such benchmarks will often need to be a combination of several different physico-chemical properties. Especially for MNs, the need for setting multidimensional groups with several criteria is acknowledged by several institutions and working groups, but lack of data often hampers setting benchmarks for grouping approaches. With the exception of some (Q)SARs, scientific justification of those approaches that include benchmarks is often hampered by limited amount of high quality scientific data[46].

SOPs for high-throughput test systems that have high potential in providing data to support read-across and grouping approaches are being developed and optimised.

Current knowledge on grouping does not clearly address the question which properties determine the similarity between MNs and their behaviours to allow read-across. It is unclear how much uncertainty within a group-based safety assessment of MNs would be accepted under REACH.

2.3.3.6 Question 6 - Fate, persistence and long-term effects

2.3.3.6.1 Question statement

Fate, persistence and long-term effects: Can effective in vitro and alternative models to understand long-term effects be developed? Will MNs accumulate in humans, the environment, environmental species and the food chain and what are the driving forces? Is this mechanistically different from bulk materials? Will nanomaterials present long-term and/or cause deferred effects? How will coatings or surface modifications or the bio-based nature of the MN affect biopersistence / biodegradability rates?

2.3.3.6.2 Data needed and types of tools implied

- Data on kinetics
    - Distribution and accumulation measurement (*in vivo* systems) through inhalation/ ingestion/ injection,
    - Measurement of absorption (estimated by *in vitro* systems);
- Data on physicochemical properties, e.g.
    - Size,
    - Shape,
    - Solubility, including dissolution testing (solubility) in biological systems,
    - Surface chemistry,
- HTD/A methods.

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[45] van Tongeren, M., Oomen, A., et al 2013 M5 Report on the MARINA Workshop to Implement Exposure and Hazard in Risk Assessment Strategy for Nanomaterials, NL-Hoofddorp http://www.marina-fp7.eu/publications/

[46] NANOREG D1.3

2.3.3.6.3 Measurement context

From a regulatory perspective, applicants with new MNs have to report on the toxicity of their materials with the help of standardized test guidelines. Regulators provide validated guidelines and if possible, an integrative testing strategy. *In vitro* models can play an important role. Nanospecificity has to be guaranteed and hence, certain existing test guidelines need amendments, and in few cases, might be developed from scratch.

- Absorption and accumulation potential is relevant for likelihood for long-term toxicity and thereby relevance for long-term toxicity testing
- Inhalatory absorption and intestinal absorption are estimated by *in vitro* systems but it is still questionable whether the test systems are tested for their validity rather than for testing the absorption of the respective MNs.
- Both for estimation of absorption and accumulation information on dissolution in e.g. lung lining fluid, intestinal fluids and macrophages fluid is pivotal. Dissolution testing in biological media like macrophages fluid or other relevant fluids is an important source for estimating kinetics.
- In dissolution testing especially dissolution rate is important.
- In NANOREG, SOPs for dissolution testing in biologically relevant fluids is under development. In schemes on potentials or indicators for risk take dissolution testing and accumulation into account, thereby linking information on kinetics and initial assessment of risk of a MN.
- The *in vivo* studies merely seem to give input to insight on kinetics of specific particles rather than coming to conclusions on the validity of *in vivo* test protocols for kinetics.

2.3.3.7 Question 7 - Kinetics and fate, determination

2.3.3.7.1 Question statement

Kinetics and fate, determination: How and when should information on absorption from the various routes of exposure, on deposition (e.g. lung burden), on biodistribution, on potential persistence and bioaccumulation, and on internal exposure (taking into account dose, duration, coating and interaction with biological systems) be generated and used? Relate the information with, for instance, the following objectives:

- *To perform more accurate risk assessment,*
- *To decrease uncertainty (safety factors)*
- *To select, if needed, a second route for acute toxicity testing,*
- *To design additional tests – that are 'affordable' – or to relate to studies that involve exposed workers, such as in the silica industry,*
- *To decide on a strategy for further testing (carcinogenicity, reproductive toxicity etc.)*

2.3.3.7.2 Data needed and types of tools implied

As for Question 6

2.3.3.7.3 Measurement context

Depending on the regulatory regime MN falls into (e.g. cosmetics, food, plant protection agents, and chemicals) the determination of fate and kinetics may look different. We need tailored guidelines to assess the fate and kinetics of the MNs in animals and different environmental media. Testing strategies are necessary to decide when and for what MNs such tests are needed.

2.3.3.8 Question 8 - Kinetics and fate, extrapolation

2.3.3.8.1 Question statement

Kinetics and fate, extrapolation: How and when can information on kinetics and fate be used to justify grouping / read across or testing triggering / waiving and for building knowledge on the relationship between physical- chemical properties and toxicity? In other words: to what extent are the kinetics and fate of MNs (e.g. environmental distribution or deposition and biodistribution in the lung) different from the bulk material?

Are there ways to extrapolate this information from the bulk material or from several forms (size, shape, coating, etc.) of the same chemical and how should this extrapolation be made?

2.3.3.8.2 Data needed and types of tools implied

As for Question 6.

2.3.3.8.3 Measurement context

The regulatory question/context can be constructed as to: How and which kinetic information can support the development of read across and grouping approaches/tools? As such this question overlaps with other questions addressing read across and grouping, but also the questions 6 and 7 addressing differences in kinetics between bulk (molecular) and MNs. This requires information on when kinetics can be considered as similar or not given a variety of forms like size, size distribution, coatings, aggregation/agglomeration status etc. for MNs of the same chemical entity.

2.3.3.9 Question 9 – Mode of action

2.3.3.9.1 Question statement

What are the physical and chemical properties driving exposure and (eco) toxicity of MNs at all stages of their life cycle? How is MN interaction with biological systems affected? What are critical characteristics of MNs that need to be considered and included / excluded when developing MNs to ensure they are safe and which materials have a known increased toxicity in the nanoform vs. the bulk form, and why? How will this facilitate the regulatory safety assessment of new nanomaterials?

2.3.3.9.2 Data needed and types of tools implied

As for Question 5

- Data on physicochemical properties, e.g.
    - Size,
    - Shape,
    - Solubility,
    - Rigidity,
    - Surface chemistry;
- Data relating to adverse outcome pathways (AOP) which could include early (potentially reversible) effects such as inflammation long term effects such as genotoxicity or the pathology itself e.g.
    - cytotoxicity,
    - inflammation,
    - oxidative stress,
    - genotoxicity;
- The uptake, biodistribution, and biopersistence (biokinetics) of a MN in an organism;
- HTD/A methods.

2.3.3.9.3 Measurement context

Studies dealing with Mode of Action (MOA) for MN face two questions that need to address simultaneously:

- What are the specific physical-chemical properties of a given MN at a specific stage of the life cycle; and
- Which physical-chemical properties influence biological properties and processes including;
    - Uptake, storage and excretion of MN compared to bulk materials, taking into account both cellular and tissue perspectives,
    - Specific roles exerted by barriers (such as the blood-brain-barrier, intestinal and respiratory epithelia, mucous membranes, the placenta),

- The role of dissolved chemical species, modifications of MN by environmental matrices and biological components, MN aggregation/agglomeration.

2.3.3.10 Question 10 - Hazard: Which methods should be used to assess the human and environmental toxicity?

2.3.3.10.1 Question statement

Hazard: Which methods should be used to assess the human and environmental toxicity? What is the applicability of conventional testing methods for nanomaterials? Is adaptation of the conventional methods needed, for example by including nano-specific endpoints or additional guidance on sample preparation? What testing is relevant at all stages of the nanomaterial?

2.3.3.10.2 Data needed and types of tools implied

A number of appropriate and validated testing guidelines for hazard assessment of chemicals have been published by organizations such as OECD and regulatory bodies.

- Data on physicochemical properties, e.g.
  - Size,
  - Shape,
  - Solubility,
  - Rigidity,
  - Surface chemistry;
- Data relating to adverse outcome pathways (AOP) which could include early (potentially reversible) effects such as inflammation long term effects such as genotoxicity or the pathology itself e.g.
  - cytotoxicity,
  - inflammation,
  - oxidative stress,
  - genotoxicity;
- The uptake, biodistribution, and biopersistence (biokinetics) of a MN in an organism;
- HTD/A methods.

2.3.3.10.3 Measurement context

Usually, OECD/EU (REACH) test guidelines are used to evaluate the hazard potential of chemicals. Some of the tests are not specific enough to measure nanoparticle toxicology. Therefore, nano-specific adaptation may be needed to the test guidelines. A nano-specific test strategy for the hazard assessment ideally relies on a small but sufficient subset of such tests, which account for conventional and nano-specific effects. The results are evaluated by the regulators, according to clear rules and criteria.

The MN-specific issues regarding such tests currently being considered include

- MN-related appropriateness
- Need for MN-specific adaptations/modifications?
- Need for novel tests designed to detect “novel” end-points?
- Usefulness of high-content and high-throughput testing approaches?

2.3.3.11 Question 11 – Exposure determinants

2.3.3.11.1 Question statement

Exposure: What are the main determinants for occupational and consumer exposure to MN and what are the duration and type of exposure? What factors determine the level and types of exposures to workers and consumers for MN and are they different from those for other materials?

2.3.3.11.2 Data needed and types of tools implied

- Determinants of exposure include material characteristics (quantities, phase, matrix, and dustiness), process characteristics (type, confinement, ventilation, duration) and worker and consumer characteristics (tasks, activities). In general these parameters are relatively easily measured.
- Exposure measurements (occupational, consumer). In order to understand and answer this question better exposure data are required.
- HTD/A methods

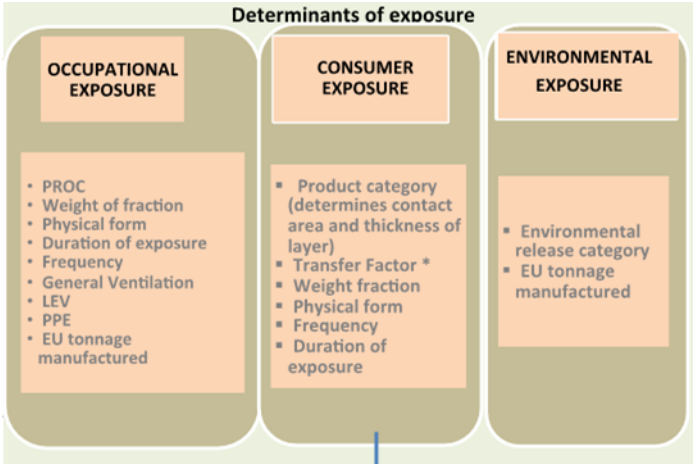
2.3.3.11.3 Measurement context

Despite more studies now being published there is a critical absence of data regarding exposure, in particular relating to consumer data. Where data have been collected, in many cases associated contextual data are not collected at least in a format which would allow proper understanding of determinants. In the frame of REACH, a determinant is a defined set of information which describes conditions of use in exposure scenarios. Specifically determinants of exposure are operational conditions (OC) and risk management measures (RMM). Operational conditions in REACH are defined as the physical form of the substance, duration and frequency of exposure, applied amount of a chemical, temperature, capacity of the surroundings (size of room, ventilation) and containment.

Information is lacking on the effectiveness of various RMMs and on the values of OCs for MN processes and how there contribute to emission and exposure.

Figure 1 lists the main determinants of exposure for occupational, consumer and environmental exposure.[47]

Figure 1. Determinants of exposure



2.3.3.11.4 Assessment

For this topic there is much more of a data gathering need than an equipment need. In general these parameters are relatively easily measured. In order to understand and answer this question better exposure data are also required.

Equipment need in relation to measurement of exposures is discussed in detail in the assessment of Question 12.

2.3.3.12 Question 12 - How should human and environmental exposure be assessed in practice?

2.3.3.12.1 Question statement

[47] NANOREG Deliverable (public) D 3.1 Gap analysis report, identifying the critical exposure scenarios within the key value chains

How should human and environmental exposure be assessed in practice (determining exposure scenario, quantify input data for models, assumptions and use of proxy indicators, background and uncertainty estimation)? Consider both measuring and specific modelling for nanomaterials and evaluate the needs for standardisation and validation.

2.3.3.12.2 Data needed and types of tools implied

The primary focus for measurement of human exposure is through the airborne route, usually expressed as a concentration;

- Particle number, mass and surface area concentrations in air,
- Direct reading instruments (portable), sample collection for off-line analysis,
- Different measurement methods may be needed for high aspect ratio nanomaterials (HARNs) (e.g. CNT), and
- HTD/A methods.

For environmental exposure, airborne concentration is also relevant, but data are also required for particle detection in other media, in particular number and mass concentrations in water.

2.3.3.12.3 Measurement context

Inhalation is considered to be the primary route by which particles suspended in air can enter the bodies of workers. Once inhaled, particles will deposit in all regions of the respiratory tract. There are three main metrics, all of which could have some utility in measuring exposure to nanoparticles. These are: i) mass concentration (units mg/m^3); ii) number concentration (units m^{-3}) and; iii) surface area concentration units (m^2/m^3). A case may be made for the use of any of these metrics under certain circumstances. Currently, mass concentration is most commonly used in occupational settings.

Exposure assessment is a critical part of the regulatory processes. Independent of the matrix, MN should be measurable and characterisable. This would allow a robust exposure assessment. If modelling leads to similar results with less effort, exposure modelling can replace lengthy experiments.

Multiple methods are available to assess the concentration of particle number, mass and surface area in air (and size distribution)[48]. Particle size (MMAD) is also critical. Methods include real-time instruments and off-line analysis methods. In all cases, however, there are limitations to the applicability of these instruments and the interpretation of the data which they produce.

2.3.3.13 Question 13 – Exposure and life cycle analysis

2.3.3.13.1 Question statement

Exposure and life cycle analysis: Which scenarios could denote potential exposure and what information do we have on them? Can we develop standardized and efficient testing procedures for estimating release of nanoparticles (NP) from powders and NPs in matrices? What are situations in which MN exposure is expected to be negligible / high? Are the amount and the nature of releases of MN similar to regular chemicals, when common recycling and end-of-pipe techniques are used? How to minimise and structure LCA to avoid ending up with a '1:1 model of the world'? In other words: what is the exposure probability throughout the different life cycle stages of the MN: production process of the MN itself, releases during the production process of products in which MN are used, waste treatment, consumer articles, wearing, abrasion, etc.? Do waste treatment / recycling processes lead to exposure to MNs that can be hazardous to health and environment? If so, are additional risk management measures required? Do the recycled product / residues lose some value /usefulness due to undesired characteristics?

2.3.3.13.2 Data needed and types of tools implied

The primary focus is for measurement of human exposure through the airborne route, usually expressed as a concentration;

[48] Specific advice on fulfilling information requirements for nanomaterials under REACH (RIP-oN-2) – Final Project Report.

- Particle number, mass and surface area concentrations in air (including as a function of particle size (MMAD),
- Direct reading instruments (portable), sample collection for off-line analysis,
- Different measurement methods may be needed for HARNs (e.g. CNT), and
- HTD/A methods.

For environmental exposure, airborne concentration is also relevant. Data are also required for particle detection in other media, in particular number and mass concentrations in water.

2.3.3.13.3 Measurement context

Inhalation is considered to be the primary route by which particles suspended in air can enter the bodies of workers. Once inhaled, particles will deposit in all regions of the respiratory tract. There are three main metrics, all of which could have some utility in measuring exposure to nanoparticles. These are: i) mass concentration (units mg/m^3); ii) number concentration (units m^{-3}) and; iii) surface area concentration units (m^2/m^3). A case may be made for the use of any of these metrics under certain circumstances. Currently, mass concentration is most commonly used in occupational settings.

Multiple methods are available to assess the concentration of particle number, mass and surface area in air (and size distribution). Methods include real-time instruments and off-line analysis methods. In all cases, however, there are limitations to the applicability of these instruments and the interpretation of the data which they produce.

2.3.3.14 Question 14 - Risk assessment: What are the no-adverse-effect or benchmark dose levels of long-term (low dose) exposures?

2.3.3.14.1 Question statement

What are the no-adverse-effect or benchmark dose levels of long-term (low dose) exposures and can they be derived from short-term exposures (acute and sub-acute)? If not, what kind of information should be generated?

2.3.3.14.2 Data needed and types of tools implied

- Data on physicochemical properties, e.g.
    - Size,
    - Shape,
    - Solubility,
    - Rigidity,
    - Surface chemistry;
- Data relating to adverse outcome pathways (AOP) which could include early (potentially reversible) effects such as inflammation long term effects such as genotoxicity or the pathology itself e.g.
    - cytotoxicity,
    - inflammation,
    - oxidative stress,
    - genotoxicity;
- The uptake, biodistribution, and biopersistence (biokinetics) of a MN in an organism.

2.3.3.15 Question 15 – Risk management, how can exposure to MNs be minimized?

2.3.3.15.1 Question statement

How can exposure to MNs be minimized / eliminated? Are risk management measures (RMM), in particular existing personal protective equipment, effective and sufficient when hazards and/or risks are high, uncertain or unknown? Should the RMM be different from bulk powders? Are currently available control banding tools

appropriate for NPs or will these need to be further evaluated, improved (related to exposure assessment, too)?

2.3.3.15.2 Data needed and types of tools implied

Managing or minimizing exposure is critical element of risk management and there are several tools and methods which can be used to achieve this. These tools form two main categories:

- Specific control methods used to control/mitigate the level of exposure, including equipment. In a regulatory context, these are sometimes referred to as *Risk Management Measures (RMM)*
- *Decision-making tools* to decide on the appropriate level of control to be implemented.

In addition measurements of exposure and release are needed so as to validate the performance of the control method implying a need for;

- Particle number, mass and surface area concentrations in air
- Direct reading instruments (portable), sample collection for off-line analysis
- Different measurement methods may be needed for HARNs (e.g. CNT)
- HTD/A methods

2.3.3.15.3 Measurement context

RMMs encompass a wide range equipment, processes and activities according to what is commonly referred to as the hierarchy of control. This provides a way of prioritising (in terms of considered effectiveness) different types of control approaches. The hierarchy of control is shown in Table 2.

Table 2. Hierarchy of control

Type of Control	Examples
Elimination	removal of the material
Substitution	substitution of the (nano) material by another substance
engineering control	enclosure of process, fume hoods, local exhaust ventilation
administrative means	management measures, segregation, signage
personal protective equipment	dust masks, respirators, gloves

Multiple methods are available to assess the concentration of particle number, mass and surface area in air (and size distribution). Methods include real-time instruments and off-line analysis methods. In all cases, however, there are limitations to the applicability of these instruments and the interpretation of the data which they produce.

2.3.3.16 Question 16 - Health surveillance

2.3.3.16.1 Question statement

Health surveillance: *What are the triggers to indicate that biological monitoring or health surveillance of (occupational) exposed individuals is needed? Can an 'intelligent strategy' be developed?*

2.3.3.16.2 Data needed and types of tools implied

- Particle number, mass and surface area concentrations in air
- Direct reading instruments (portable), sample collection for off-line analysis
- Different measurement methods may be needed for HARNs (e.g. CNT).

2.3.3.16.3 Measurement context

The published scientific literature concerning health effects that may result from exposure of workers to engineered MNs was reviewed by Schulte et al. (2008) in order to determine possible options for the health surveillance of workers. Various options for occupational health surveillance were identified. The options ranged from no action targeted to nanotechnology workers to an approach that includes documentation of the presence of engineered nanoparticles, identification of potentially exposed workers, and general and

targeted medical testing. Whilst they concluded that additional efforts to monitor employee health may be warranted no clear example approaches were identified. The first priority should be to implement appropriate primary measures to prevent exposure (Schulte et al., 2008)[49].

The NIOSH report "Interim Guidance for the Medical Screening of Workers Potentially Exposed to Engineered Nanoparticles" indicated that there is insufficient medical and scientific evidence to recommend 'specific medical screening' for workers who are exposed to engineered nanoparticles (NIOSH, 2007). The authors also recommend that medical surveillance methods are established in order to help assess whether or not implemented control measures are effective and to identify new or unrecognised problems and health effects (NIOSH, 2007)[50].

One starting point would be to begin to develop exposure registries. This would serve as a baseline activity and could at a minimal level just include collection of information about the types and quantities of MN being used or handled by workers in different companies or institutions. If more sophisticated exposure information was also deemed to be important, the methods indicated above would provide the means to collect this.

Although preliminary medical surveillance activities such as documentation of the presence of engineered nanoparticles and identification of potentially exposed workers are likely to be beneficial in the long term, no clear guidance at this time can be given as to specific medical endpoints which should be tested for. There is no current consensus on the applicability of medical surveillance for MNs which would provide the basis for inclusion in or adaptation of the REACH guidance.

2.3.3.17 Summary assessment

Mapping of NANOREG questions to Equipment and other tools is in Table 3. This illustrates both the extent of the different types of measurement systems that are necessary and also the grouping of the needs according to the type of question.

The next section will describe the equipment requirements for each data need listed in Table 3.

[49] (Schulte P, Geraci C, Zumwalde R, Hoover M, Kuempel E, 2008. Occupational risk management of engineered nanoparticles. J Occup Environ Hyg 5: 239-249.

[50] NIOSH, 2007. Current Intelligence Bulletin 60: Interim Guidance for Medical Screening and Hazard Surveillance for Workers Potentially Exposed to Engineered Nanoparticles.

Table 3; Questions and Tools matrix

Measurement type/equipment need			Measurement and characterisation - identification	Measurement and characterisation	Characterisation / transformation	Metrology and dose metrics	Extrapolation and grouping	Fate, persistence and long term effects	Kinetics and fate, determination	Kinetics and fate, extrapolation	Mode of action	Hazard assessment	Exposure determinants	Exposure assessment	Exposure and life cycle analysis	Risk assessment	Risk Management	Health surveillance
			1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Physicochemical characteristics																		
Particle size distribution			X	X	X	X	X	X	X	X	X	X		X		X		
Volume specific surface area (VSSA)			X	X	X	X												
Aspect ratio (shape)				X	X		X	X	X	X	X	X				X		
Rigdity				X														
Solubility or dissolution rate					X		X	X	X	X	X	X				X		
Chemical composition (inc surface)					X		X	X	X	X	X	X				X		
Detection and quantification of concentration and exposure																		
Number concentration in air						X							X	X			X	X
Mass concentration in air						X							X	X			X	X
Surface area concentration						X							X	X			X	X
Toxicity																		
Cytotoxicty							X	X	X	X	X	X				X		
Inflamation							X	X	X	X	X	X				X		
Oxidative stress							X	X	X	X	X	X				X		
Genotoxicity							X	X	X	X	X	X				X		
HTS							X	X	X	X	X	X				X		
Omics methods							X	X	X	X	X	X				X		
Kinetics																		
Absorption							X	X	X	X	X	X				X		
Distribution /accumulation							X	X	X	X	X	X				X		
Microscopy methods							X	X	X	X	X	X				X		
Other "Equipment"																		
HTD/A				X	X		X	X	X	X	X	X	X	X	X			
Exposure control																	X	
Exposure models														X				
Release systems															X			

2.3.4 *Physicochemical characteristics*

2.3.4.1 Particle size distribution (powder or dispersion)

2.3.4.1.1 Relevant questions

Q1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
X	X	X	X	X	X	X	X	X	X		X		X		

Data on particle size distribution is an almost universal requirement in relation to all of the regulatory questions as indicated above. Particle size distribution is also relevant to Q11, 13, 15 and 16 (not indicated above) but in these cases the required distribution is for particles suspend in air in which case the methods and equipment used are different. Therefore, this is dealt with in a separate section.

A major review of equipment needs in relation to the EU definition (Q1) was carried out by Linsinger et al., (2012)[51]. They identified generic issues for reliable particle size determination as;

- Sampling: The number of particles counted in a measurement is generally extremely small in comparison with the number of particles in the material under investigation. Care must be taken to obtain a representative sample for analysis.
- Sample preparation: Most size measurement methods necessitate a sample preparation procedure that breaks up agglomerates and aggregates, if the size of their constituent particles must be measured. This and other sample preparation can influence the measured sizes. In practice, strongly-bound aggregates cannot be dispersed into their constituent particles and are therefore often indistinguishable from large particles.
- Size distributions: Measured particle size distributions are weighted according to the number of particles per size group, the surface area of particles per size group, the volume of particles per size group, or the light-scattering intensity of particles per size group, to name only the most common weighting methods. Most methods produce size distributions that need to be mathematically converted to the number-based size distribution required in the definition. This conversion is based on various assumptions, and becomes increasingly prone to error, difficult or impossible, if the mass fraction of nanoscale particles is not sufficiently large.
- Method-defined size values: Most measurement methods provide a method-defined, apparent value for the selected external dimension. Therefore, different size measurement methods may result in significantly different size values. It is impossible to rank different methods according to trueness or reliability for all possible applications, so any definition of a universal reference method would be arbitrary. On the contrary, the method-dependence of size measurements allows selecting the most appropriate method for the specific application or concern. As results of size measurements are method-defined, standardisation of measurement methods is needed to ensure comparability between different laboratories.

Linsinger et al[51] identified candidate measurement methods as follows;

- Ensemble methods (methods that measure large numbers of particles simultaneously), like dynamic light scattering (DLS) or small-angle X-ray scattering (SAXS), report intensity-weighted particle sizes. Conversion to number-based size distributions is reliable only for nearly monodisperse, spherical particles, and when sufficient shape information about the particles is available. Other ensemble methods, such as X-ray diffraction (XRD) only measure an average size value and do not provide useful information about size distribution.
- Counting methods like particle tracking analysis (PTA) study particle by particle, and assume that the particles have a specific shape. Imaging methods, such as electron microscopy (EM) and atomic force microscopy (AFM), are also counting methods and can deal with non-spherical particles on a surface. However, a high number of particles making up a representative sample must be measured to obtain reliable size distributions for industrial, polydisperse materials.

[51] Linsinger T., Roebben G., Gilliland D., Calzolai L., Rossi F., Gibson N., Klein C. (2012) Requirements on measurements for the implementation of the European Commission definition of the term 'nanomaterial' JRC Reference Report EUR 25404 EN

- Fractionation methods like centrifugal liquid sedimentation (CLS), field-flow fractionation (FFF), size-exclusion chromatography (SEC) or hydrodynamic chromatography (HDC) separate the sample into monodisperse fractions prior to quantifying the particles. This eliminates several of the problems associated with measurements on polydisperse samples. Some of these fractionation methods can be coupled on-line or off-line with ensemble or counting methods, and are expected to become a crucial component of size measurement approaches to be developed for the implementation of the definition.

The capabilities for each of the measurement methods is summarised in Table 4 (extracted from Linsinger et al., 2012).[51]

Table 4. Summarised measurement methods

			Ability to deal with particular types of nanomaterials (scale ++, +, o, -, - -)				
Method	Measurement range and medium (limiting factors)	Types of size distribution	Poly dispersity	Non-spherical particles	Low density materials	Aggre-gates	Standards available
Electron microscopy (EM)	1 nm and higher; dry (dynamic range)	number-based	+	Long: + Flat: -	-	-	Y
Dynamic light scattering (DLS)	5 nm to 500 nm; suspension (sedimentation, scattering intensity)	(no distribution, or scattering-intensity–based)	- -	- -	+	- -	Y
Centrifugal liquid sedimentation (CLS)	20 nm and higher; suspension (particle density)	extinction-intensity–based	+	- -	-	- -	Y
Small-angle X-ray scattering (SAXS)	5 nm and higher; suspension (dynamic range)	scattering-intensity–based	o	-	o	- -	Y
Field flow fractionation (FFF)	1 nm to 200 nm; suspension (dynamic range)	(depends on detector)	+	-	+	- -	N
Particle tracking analysis (PTA)	25 nm and higher; suspension (scattering intensity)	number-based	+	- -	o	- -	N
Atomic force microscopy (AFM)	1 nm and higher; dry (dynamic range)	number-based	+	Long: + Flat: +	o	-	Y
X-ray diffraction (XRD)	1 nm and higher; dry (only for crystalline materials)	(no distribution measured)	- -	- -	-	+	Y

2.3.4.1.2 Assessment

There are various scientific-technical challenges related to the measurement of materials in the implementation of the recommended nanomaterial definition (Q1).

- Dealing with agglomerates/aggregates: A particular challenge is the requirement of measuring the constituent particles inside aggregates. No method is available that can reliably distinguish whether a large particle is an agglomerate, aggregate or a single particle, and at the same time measure the size of large numbers of individual constituent particles. For some materials a distinction is possible

using EM, but DLS, CLS, SAXS, AFM and PTA regard each agglomerate/aggregate as a single, large particle.

- Working range: Most current methods have a detection limit higher than 1 nm or a lower sensitivity for smaller particles. No single method alone can cover, in a single measurement, for all materials the complete size range from lower than 1 nm to well above 100 nm, as would be required for a universal assessment according to the definition. In particular, it is expected that working ranges, when expressed in terms of the measurable particle number fractions (which must include the 50 % or median value), will strongly depend on particle mass fractions.
- Method validation: None of the mentioned measurement methods have been specifically validated for their use in the implementation of the nanomaterial definition.

Summarising the current technical limitations, none of the currently available methods can determine for all kinds of potential MNs whether they fulfil the definition or not. Therefore, a range of measurement methods is required to investigate whether MNs fulfil the regulatory definition. Implementation of the definition via measurements poses significant difficulties for polydisperse materials and is currently usually not possible for aggregated materials if the size distribution of their constituent primary particles must be determined, unless the aggregates themselves fulfil the nanomaterial definition.

Short-term research needs to fill the data gaps with respect to standardized methods are the identification or development of reasonably priced, accessible, standardized and validated methods and procedures to quickly identify and quantify MNs in different media according to the EC definition. In addition, standardized and validated methods to determine the most important characteristics of MNs in different media are also needed to characterize the key properties of MNs within the most critical stages of their life cycle and within the toxicity testing.

The future improvement of measurement technology, development of analytical methods and standardised sample preparation protocols may partly resolve the mentioned limitations. If rapid implementation of the definition through measurements is needed, dedicated guidance documents will have to be provided for specific materials and sectors, with clear and justified indication of the relevant particle size measurement methods and test conditions. A combination of several methods, ideally supported by information on the manufacturing process of the material under investigation, will have to be employed for robust assessments. The reliability of each of the measurement methods used in such combined, tiered processes will need to be thoroughly checked in dedicated method validation and inter-laboratory comparison studies. Such technical developments and experiences should be taken into account for a future revision of the definition stipulated by the recommendation.

2.3.4.2 Volume specific surface area (VSSA)

2.3.4.2.1 Relevant questions

Q1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
X	X	X	X												

Assessment of specific surface area (VSSA) is a specific requirement in Q1 but this information is also relevant to questions 2, 3 and 4.

In relation to the EU Definition (Q1), measurement of the specific surface area (SSA) by the BET (Brunauer, Emmett and Teller) method is recommended. This method, measures how much gas (usually nitrogen) is adsorbed at a specific temperature and pressure.

The BET method measures particle surface area. Dividing the absolute surface area by the sample mass gives the so-called mass-specific surface area, commonly reported in m^2/g. This is not the same unit as used in the definition, which refers to the volume-specific surface area (VSSA) with the unit m^2/cm^3 or m^2/m^3. Therefore, the calculation of the VSSA from the result of a BET measurement requires the knowledge of the particle density. The method works only for dry powders. The result corresponds to the surface area of the aggregate or agglomerate, not of the constituent particles. The method will give the total surface area accessible to the gas used, which includes inner surface such as pores, and so the results of the measurements will depend on the molecular size of the test gas. Many porous materials have been developed that by far exceed the limit of 60 m^2/cm^3 due to their porosity, although their particle size may be as high as 1 mm.

Linsinger et al (2012) identified similar, but less mature methods to measure the surface area of suspended particles. These included Hydrodynamic chromatography (HDC) and size-exclusion chromatography (SEC), Gas-Phase Electrophoretic Molecular Mobility Analysis (GEMMA), Single-particle inductively coupled plasma-mass spectrometry (ICP-MS) and Specific surface area measurements via nuclear magnetic resonance (NMR). These methods are still under development and applied by only a few research laboratories.

2.3.4.2.2 Assessment

The BET method is relatively simple and straight forward to use. The method has been a standard method for many years. No significant further changes are to be expected in the near future.

2.3.4.3 Aspect ratio (shape)

2.3.4.3.1 Relevant questions

Q1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
	X	X		X	X	X	X	X	X						

Aspect ratio is considered to be an important parameter because of well-known associations between fibre-length for materials such as asbestos and diseases such as mesothelioma and cancer. This is clearly an important issue in relation to HARNs such as carbon nanotubes and graphene. Aspect ratio is the ratio of fibre length to diameter and so requires both of these parameters. Currently there are no real-time systems which are able to measure these parameters so the only options are for the imaging systems electron microscopy (EM) and atomic force microscopy (AFM). EM is a counting method: it produces a size value for each of the particles selected for analysis on the obtained images. Transmission electron microscopy (TEM) has the required resolution to determine the smallest external dimension of nanoparticles. Scanning electron microscopy (SEM) is limited to particles above some nm. Table 5 summarises the methods for measuring HARNs.

Table 5; Methods for high aspect ratio nanomaterials

			Ability to deal with particular types of nanomaterials (scale ++, +, o, -, - -)				
Method	Measurement range and medium (limiting factors)	Types of size distribution	Poly dispersity	Non-spherical particles	Low density materials	Aggre-gates	Standards available
Electron microscopy (EM)	1 nm and higher; dry (dynamic range)	number-based	+	Long: + Flat: -	-	-	Y

2.3.4.3.2 Assessment

Sample preparation and dispersion is key. For well dispersed materials, the EM measurement process can be automated but this presents special challenges for HARN. For all other materials, EM is a slow technique. Operator intervention is especially required for aggregated materials, if the analysis of the constituent particles in these materials is possible at all. Counting of HARNs presents particular problems.

2.3.4.4 Rigidity

2.3.4.4.1 Relevant questions

Q1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
	X			X				X	X						

Rigidity is sometimes mentioned as a property which is important in relation to the toxicity of HARNs and appears in a number of grouping schemes. For example a categorization of a rigid, bio-persistent fibre is one type of grouping mentioned by BSI (2006) in relation to CNT and other type of HARNs.

Assessment of the rigidity is a research tool in which an individual particle is manipulated in an electron microscope or atomic force microscope. For example Enomoto et al (2006)[52] reported on the development of a method for quantifying the nanomechanics of MNs using a nanoprobe manipulator fitted into a transmission electron microscope. Apparent Young's moduli of various carbon nanotubes (CNTs) were measured using this method. As a single particle method which initially requires manipulation and alignment, such methods are very time consuming, labour intensive and hence expensive.

2.3.4.4.2 Assessment

These methods have seldom been used in a toxicology context. Arts et al (2015)[53] suggested that, for the purpose of grouping, fibre diameter could be used as a proxy but this does not seem to have been evidence based. This does indicate a gap in the equipment base to which some effort should be directed towards solving. However, until there is further understanding in the role of rigidity in toxicity it is difficult to make a case that it is an important one.

2.3.4.5 Solubility or dissolution rate

2.3.4.5.1 Relevant questions

Q1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
		X		X	X	X	X	X	X						

The solubility of a substance (usually expressed as a mass per volume) is an entirely different property from the rate of solution, which is how fast it dissolves (expressed as mass per unit time). The dissolution rate is a measure of the actual release rate of the compound at the given particle size etc. in an aqueous media. It often varies considerably with solid form, e.g. particle size and shape. Particle dissolution rate is an important parameter in pharmacology and may be also be of interest in a risk context, although it is much less mentioned in this context.

2.3.4.5.2 Assessment

Measuring MN solubility is not straightforward because the corresponding dissolution process can be highly dynamic. Several factors can affect the solubility (Misra et al., 2012)[54], which include:

a) pH

b) presence of dissolved ligands, synthetic stabilizing agents (surfactants, capping agents, surface coatings, etc.) and natural organic matter such as humic substances or proteins

c) surface tension, as governed by the Ostwald–Freundlich equation

d) MN particle size and surface composition,

e) mass of the particles exposed, aggregate/agglomerate state and surface area

f) temperature

g) redox potential of the MN within the liquid dispersion and oxygen concentration

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[52] Enomoto K, Kitakata S, Yasuhara T, Ohtake N, Kuzumaki T, Mitsuda Y, 2006. Measurement of Young's modulus of carbon nanotubes by nanoprobe manipulation in a transmission electron microscope. Appl Phys Lett 88:153115.

[53] Arts JH, Hadi M, Irfan MA, Keene AM, Kreiling R, Lyon D, Maier 7, Michel K, Petry T, Sauer UG, Warheit D, Wiench K, Wohlleben W, Landsiedel R, 2015. A decision-making framework for the grouping and testing of nanomaterials (DF4nanoGrouping). Regul Toxicol Pharmacol 71(2 Suppl):S1-27.

[54] Misra SK, Dybowska A, Berhanu D, Luoma SN, Valsami-Jones E. 2012. The complexity of nanoparticle dissolution and its importance in nanotoxicological studies. Sci Total Environ 438:225–32.

h) hydrodynamic conditions (e.g. stagnant water vs. mechanical agitation) determining the mass transport mechanisms, which affect dissolution kinetics, except in those cases where surface chemical reaction is the rate-limiting step

i) presence of salts in the test medium, such as chlorides, phosphates and carbonates, which can lead to concomitant formation of solid precipitates

Water solubility is an existing information requirement under REACH[55]. In REACH the solubility of a substance in water is specified by the saturation mass concentration of the substance in water at a given temperature. The solubility in water is specified in units of mass per volume of solution. The SI unit is kg/m3. Three approaches are mentioned in REACH, the column elution method, the flask method and, OECD series on Testing and Assessment Number 29 - Guidance Document on Transformation/Dissolution of Metals and Metal Compounds in Aqueous media. This last approach is described as being applicable to sparingly soluble inorganic metal compounds. In the OECD method[56], this Test Guidance is intended to be a standard laboratory transformation/ dissolution protocol based on a simple experimental procedure of agitating various quantities of the test substance in a pH buffered aqueous medium, and sampling and analysing the solutions at specific time intervals to determine the concentrations of dissolved metal ions in the water. The two methods are: column and flask methods. The column method involves packing a column with a ‘‘support material’’ and test substance, after which the column is connected to a series of pumps (allowing liquid to flow through). A piece of glass wool inserted at the end of the column acts as a plug to ensure that any liquid flowing through is free from particulate matter. In the case of the flask method, the test substance (in a liquid medium) is placed inside a flask and shaken for a certain time period. The solution is then filtered separately in order to remove particulates. In both cases, the sample, which assumed to be free from particulate matter, can then be subsequently analysed using an appropriate detection/quantification technique. In principle these relative simple approaches should be applicable to MNs but protocols will need to be adapted and verified.

Possible methods for assessment of solubility for MNs have been extensively reviewed as part of the NANOREG project[57] and are summarised in Table 6. As was noted, an important factor when measuring solubility of MN is the ability to differentiate signal arising from the dissolved species as opposed to particulate species. Therefore methods essentially comprise a separation method (separating particles from dissolved molecules/ions) and a chemical analysis method.

In NANOREG it is reported that “Different suitable methods to evaluate the solubility for a given MN have been identified, but no conclusions have been reached so far. Furthermore, the methods are not being validated”.

A special case is for fibres where various studies[58] have used an approach comprising PC measurements of durability combined with *in vitro* toxicology testing. This is based on previous approach for testing biopersistence of fibres including asbestos. Durability is assessed by suspending the material in simulated biological fluid (Gamble’s solution) over a period of several days. Dissolution may be determined by assessing the weight recovery after filtration, or by assessing changes in the fibres themselves, e.g. by changes in fibre length distribution (as assessed by microscopy (optical or SEM) where a shorter fibre would suggest dissolution. This is quite far from being a standard method however.

[55] Guidance on Information Requirements and Chemical Safety Assessment Chapter R.7a: Endpoint specific guidance (Version 4.1 – October 2015) R 7.1.7 Water Solubility

[56] OECD, 2009. OECD Series on Testing and Assessment Number 29: Guidance document on transformation/dissolution of metals and metal compounds in aqueous media.

[57] Tantra R, Bouwmeester H, Bolea E, Rey-Castro C, David CA, Dogné JM, Jarman J, Laborda F, Laloy J, Robinson KN, Undas AK, van der Zande M, 2016. Suitability of analytical methods to measure solubility for the purpose of nanoregulation. Nanotoxicology 10:173-84.

[58] Osmond-McLeod MJ, Poland CA, Murphy F, et al. Durability and inflammogenic impact of carbon nanotubes compared with asbestos fibres. Part Fibre Toxicol. 2011..

Table 6; Methods for solubility

Separation methods	
OECD Guideline, filtration and centrifugation	Extremely affordable and accessible. However, these methods are deemed to be unreliable as particulates in the nanoscale can easily end up in the sample for analysis
Dialysis and ultrafiltration	Intended to improve the reliability of the separation process, Dialysis relies on the diffusion across a semi-permeable membrane, UF is a pressure-driven process (through the use of a vacuum/centrifugal force), which leads to the separation across a semi-permeable membrane. When using such methods, an assumption is being made that particulates cannot have size smaller than that of the pores of the membranes, which may not always be the case (even though pore sizes as low smaller than 1 nm are currently available)
High-performance liquid chromatography (HPLC)	HPLC offers better reliability of the separation process area chromatography column (stationary phase) is used to separate different components in a sample
Ion exchange technology (IET)	IET provides a promising separation performance with the most common being based on ion exchange resins. This method is mainly applicable in the separation of free metal ions and involves the equilibration of a known mass of resin column with a given volume of sample. Only a few studies have been carried out in relation to the measurement of MN dissolution/solubility
Capillary zone electrophoresis (CZE)	In CZE, high voltages are applied in order to separate molecules when moving in an electric field. The use of electrophoretic separation techniques have been developed and refined over decades and are now widely used for the separation of different analytes in complex mixtures. In relation to MN characterisation, electrophoresis has been shown to be able to separate MN of different, size, shape and compositions
Field flow fractionation (FFF)	FFF separates analytes with different physicochemical properties. The separation principle is based on the differential movement of analytes. Separation is not directly caused by the flow itself, but by a generated field perpendicular to the direction of this flow e.g. gravitation, electrical or magnetic forces
Detection techniques	
Electrochemical	Simplest and cheapest is by measuring electrical conductivity. One limitation in conductivity measurement is that it is unspecific and without the addition of a separation technique, the conductivity signal can be easily masked by background ions. Ion selective electrodes (ISE) offer better selectivity. The ISE is a membrane-based electrochemical sensor that responds specifically to the activity of a particular ionic species. To date, the vast majority of studies surrounding the use of ISE to measure dissolution/solubility has been associated with ecotoxicology investigations using different MNs. Voltammetric methods have primarily been used for the measurement of free ions. Promising voltammetric methods are those based on some kind of pre-concentration step, such as: anodic stripping, adsorptive cathodic stripping voltammetry (AdCSV) and absence of gradients and Nernstian equilibrium stripping (AGNES). Stripping-based methods can potentially have remarkably high sensitivities, mostly due to the large pre-concentration factors (of the order of 10^3) achieved prior to stripping. These methods are usually able to reach sub-ppb detection limits
Colorimetric and fluorometric assays	These methods rely on the interaction of the metal with the complexing agent to result in a coloured complex, which can be monitored using appropriate spectrometers, e.g. through measuring a change in absorbance or fluorescence signal. As with electrochemical-based methods, these methods are inexpensive and do not require extensive sample preparation. It is important to ensure that the presence of the MN do not interfere with the analysis.
Inductively coupled plasmas (ICP)	In the case of ICP-Optical Emission Spectrometry (OES), the plasma works as an excitation source for atoms and ions, whereas in ICP-Mass Spectrometry (MS) it is a source of ions. Samples are introduced as solutions or suspensions through a nebulisation system, consisting of a nebuliser and a spray chamber, which produces an aerosol of droplets. Once the droplets are in the plasma, solvent evaporates, forming solid particles, which in turn are vaporised and their elements atomised and ionised.
Atomic absorption spectrometry (AAS)	AAS is a quantitative technique for the determination of the total element content in a sample. The technique involves the atomisation of the analyte, to get atoms (in the ground state), which are promoted to a higher excitation state by absorption of radiation at specific wavelengths.

2.3.4.5.3 Assessment

A range of chemical and analytical equipment is required as specified. Methods essentially comprise a separation method (separating particles from dissolved molecules/ions) and a chemical analysis method which in the main are relatively "standard" chemical analysis methods and equipment. Specific equipment required depends on the method chosen. A critical element is the development of protocols for sample preparation which will differ depending on the materials and circumstances.

Water solubility is an existing information requirement under REACH and the methods indicated there do not imply a particularly complex equipment need. Solubility is not specifically mentioned in the EC definition of a nanomaterial but the term insoluble particle is used in the Cosmetics Regulations, where a nanomaterial is defined as an ''insoluble or biopersistent and intentionally manufactured material with one or more external dimensions, or an internal structure, on the scale from 1 to 100 nm'' (EU Cosmetics Regulation, 2009)[59]. No boundary condition is attached to that however i.e. at what level does a sparing soluble material become insoluble. In practice though from a risk assessment point of view, solubility is a property that has been identified as being important. If a fraction (or the totality) of a MN is dissolved then this amount of material will no longer be considered as ''nano'' and thus can be treated much in the same way as conventional chemicals. But until such time as clear specifications emerge concerning the need to provide measurements of solubility at a specific level of confidence it is difficult to draw the conclusion that the current measurement approaches and equipment specifications are inadequate for regulatory purposes.

2.3.4.6 Chemical composition

2.3.4.6.1 Relevant questions

Q1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
		X		X	X	X	X	X	X				X		

This is considered to be chemical information and crystal structure of the entire sample of nano-objects including: (a) composition, (b) crystalline structure including lattice parameters and space group, and (c) impurities, if any. Chemical composition and crystalline structure are well established as significant toxicological determinants at the molecular level. The discrimination between surface chemistry and composition becomes blurred as the size of nano-objects approaches the lower range of the nanoscale. It is possible in some cases that the molecules attached to the surface could be considered under composition; however, the preference is to characterize surface chemistry separately (see 4.7).[60]

2.3.4.6.2 Assessment

There is an enormous range of general chemical analytical techniques which are beyond the scope of this document to cover (e.g. inductively coupled plasma mass spectroscopy (ICP-MS), atomic absorption mass spectroscopy (AA-MS)). Here we focus on single particle chemical analysis of MN particles. Methods for single particle chemical analysis have been reviewed by Bzdek et al (2012)[61] from which much of the following is drawn. Several methods currently exist for the chemical analysis of individual nanoparticles in ambient aerosol which are summarised in the Table 7. These include both off-line and on-line (real time) methods

[59] European Commission, 2009. Regulation (EC) NO 1223/2009 of the European Parliament and of the Council of 30 November 2009 on Cosmetic Products.
[60] ISO/TR 13014:2012. Nanotechnologies -- Guidance on physico-chemical characterization of engineered nanoscale materials for toxicologic assessment.
[61] Bzdek BR, Pennington MR, Johnston MV, 2012. Single particle chemical analysis of ambient ultrafine aerosol: A review. J Aerosol Sci 52: 109-120.

Table 7. Methods for single particle chemical analysis of MN particles

Method	On-line/off-line	Size range (nm)	Distinguishing features	References
Transmission Electron Microscopy-Energy Dispersive X-ray Spectroscopy (TEM-EDS)	Off-line	~5-(100)	Physical size and morphology; metals and some non-metals	Makela et al. (2002)
Rapid Single Particle Mass Spectrometer (RSMS)	On-line	30-(100)	Aerodynamic sizing; metals and some molecular information	Mallina et al. (2000)
Ultrafine Aerosol Time-of-Flight Mass Spectrometer (UF-ATOFMS)	On-line	50-(100)	Optical aerodynamic sizing; metals and some molecular information	Su et al. (2004)
Single Particle Laser Ablation Time-of-Flight Mass Spectrometer (SPLAT)	On-line	50-(100)	Optical aerodynamic sizing; metals and some molecular information	Zelenyuk and Imre (2005)
Nano Aerosol Mass Spectrometer (NAMS)	on-line	10-30	Electrodynamic sizing; quantitative elemental analysis	Wang et al. (2006)

2.3.4.6.3 Assessment

In off-line analysis, aerosols are collected on a filter or grid and then the morphology and composition of individual nanoparticles is probed. The most common off-line method is Transmission Electron Microscopy (TEM) coupled with Energy Dispersive X-Ray Spectroscopy (EDS) in order to give both morphological and chemical information about the particle.

The main benefits of the off-line methods are determination of

1. structural information, such as crystallographic structure, coatings, embedding, or aggregation, and
2. heavy metal content, both of which are not well- characterized by the currently available on-line single particle techniques.

The main disadvantages are

1. longer sampling and analysis times are required, leading to smaller single particle datasets than typically obtained with on-line techniques, and
2. long delay in sample analysis (samples must be taken to a remote laboratory).

Nanoparticle analysis is challenging because the amount of material is limited and measurement signal intensities are low. Several instruments are able to probe particle composition in the ultrafine size range. All of these instruments are mass spectrometry based (aerosol mass spectrometers). The main benefits of these on-line methods are

1. acquisition of particle chemical composition information in real time, and
2. ability to obtain large datasets.

The main disadvantages are

1. morphological features such as coatings and mixing states must be inferred, and
2. potential biases in ionization yields can depend on matrix effects and particle size.

One of the earlier methods for ultrafine aerosol analysis is the Ultrafine Aerosol Time-of-Flight Mass Spectrometer (UF-ATOFMS) (Su et al., 2004)[62]. Particles are drawn into the instrument through an aerodynamic lens assembly and are detected and aerodynamically sized using light scattered as the particle passes through two continuous-wave laser beams. The scattered radiation from each particle also provides a means of synchronizing the ablation laser pulse with the arrival of the particle in the mass spectrometer source region. The UF-ATOFMS incorporates an inlet with superior focusing properties in the ultrafine size range and upgraded optics and electronics to improve the sensitivity of particle detection by light scattering.

A completely different approach to ambient ultrafine aerosol analysis is the Nano Aerosol Mass Spectrometer (NAMS) (Wang & Johnston, 2006).[63] This instrument uses electrodynamics rather than aerodynamics to focus and size particles for subsequent chemical analysis. Particles are charged with a unipolar charger prior to entering the instrument. Inside the instrument, aerodynamic and electrodynamic focusing elements transmit particles into an ion trap. Particles are size-selectively captured in the trap and subsequently analysed by laser ionization. The advantage of this configuration is the ability to size and analyse particles in the 10–30 nm range.

Chemical characterization of sub-100 nm diameter particles remains a challenging analytical problem. Currently, a combination of off-line and on-line approaches are necessary to obtain detailed morphological and chemical composition information about individual nanoparticles. Mass spectrometry is likely to dominate future on-line studies in this size range because of the small sample size of the particles involved and the necessity of obtaining sufficiently large ambient datasets. Single particle analysis by the more conventional mass spectrometric methods of light scattering and laser desorption ionization becomes difficult as particle diameter decreases below 100 nm. As a result, relatively few mass spectrometric studies have focused on particles in the 30–70 nm size range. Additionally, little is known about the prevalence of metals or the molecular composition of individual nanoparticles smaller than 30 nm diameter. Finally, there exists no instrumentation that currently can perform on-line single particle chemical analysis below 10 nm diameter, which is crucial in order to fully understand the mechanisms of ambient new particle formation. Addressing these knowledge gaps will require the development of alternative ionization methods and improved measurement sensitivity.

2.3.4.7 Surface chemistry

2.3.4.7.1 Relevant questions

Q1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
		X		X	X	X	X	X	X				X		

2.3.4.7.2 Assessment

Surface chemistry may be thought of as chemical nature, including composition, of the outermost layers of a single MN particle. It is widely hypothesized that surface chemistry will play one of the key roles in determining the ultimate risk of any given MN. In some cases, surface chemistry is controlled by a single atomic species as, for example, in inorganic fullerene-like materials (e.g. MoS2, can form nested spheres where the outer atomic layer is typically sulphur), or where specific chemical moieties have been deliberately attached to the surface with complete coverage (e.g. coated TiO2). Protein adsorption onto the surface of particles can also occur and alter the surface chemistry.

The various functional groups attached to the surface are likely to play a key role in determining

(1) entry into and distribution inside organisms;

(2) fate in natural aqueous systems;

(3) colloid stability; and

(4) exposure to target cells or tissues.

[62] Su, Y.X., Sipin, M.F., Furutani, H., & Prather, K.A. (2004). Development and characterization of an aerosol time-of-flight mass spectrometer with increased detection efficiency. Analytical Chemistry, 76, 712–719.
[63] Wang SY., Johnston MV, 2006. Airborne nanoparticle characterization with a digital ion trap-reflectron time of flight mass spectrometer. Int. J. Mass Spectrom 258: 50-57.

For a given functional group, this in turn will affect other physico-chemical properties, such as agglomeration, dustiness, zeta potential, specific surface area, and water solubility.

2.3.5 *Detection and quantification of concentration and exposure*

2.3.5.1 Relevant questions

Q1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
			X						X	X	X			X	X

2.3.5.2 Methods

Multiple methods are available to assess the concentration of particle number, mass and surface area in air (and size distribution)[64]. Methods include real-time instruments and off-line analysis methods. In all cases, however, there are limitations to the applicability of these instruments and the interpretation of the data which they produce.

- *Number*: Measurement of particle number concentration is relatively straightforward with condensation particle counter (CPC) devices. Typically hand-held devices, CPCs operate by condensing vapour onto particles in a sampled air-stream to grow them to a size range that can be detected optically. The detection range is typically 3 nm to 20 nm at the lower end to 1 000 nm at the upper end, depending on the instrument used. Optical particle counters (OPCs) measuring particle number concentration can be useful for identifying larger agglomerates/aggregates of size between 300 nm and 10 µm.
- *Mass:* For mass measurements, the simplest approach is to use a filter-based personal sampler comprising some form of inertial particle pre-selector, together with off-line analysis of the sample using gravimetric or chemical techniques. Most of the filters used for occupational aerosol sampling will collect nano-objects. Optical aerosol monitors (photometers) are widely used to measure aerosol mass concentration but detection efficiency falls off rapidly below approximately 500 nm (useful only in measuring exposure to aggregated or agglomerated nano-objects).
- *Surface area:* For surface area concentration, several devices are available that measure specific surface area, based on the principle of diffusion charging.
- *Size distribution:* Several devices allow examination of the size distribution of any aerosol measured. These instruments are typically larger, more complex and more expensive. The most commonly used instrument of this type is the SMPS, of which there are a number of variants. These devices are capable of measuring aerosol size distribution from approximately 3 nm to 800 nm, although not simultaneously over the complete range. The size distribution is expressed in terms of particle mobility diameter. SMPS instruments comprise two parts: a stepped (in which the classifier voltage is stepped between discrete voltage levels) or scanning (in which the voltage is varied continuously) differential mobility analyser (DMA), which sequentially separates sampled aerosol into size intervals according to their differential mobility, and a CPC which then counts the particles in sequence. The electrical low pressure impactor (ELPI, Dekati, Finland) is a static aerosol sampler capable of measuring particle size distribution and concentration in the size range 7 nm to 10 µm. Particles sampled in the ELPI are charged and then passed into a low-pressure cascade impactor with a series of thirteen electrically isolated collection stages.

Some of the main instruments and their capabilities are summarised in Table 8.

[64] Specific advice on fulfilling information requirements for nanomaterials under REACH (RIP-oN-2) – Final Project Report.

Table 8. Available instruments and techniques for monitoring MN exposure adapted from BSI 6699-3[65]

Devices	M	N	SA	SD	Remarks
Size-selective personal sampler	X				No current devices offer a cut point of 100 nm. Off-line gravimetric or chemical detection is necessary. Mass may also be derived from size distribution measurements (see below).
CPC		X			CPCs provide real-time number concentration measurements between their particle diameter detection limits. Without a nanoparticle preseparator, they are not specific to the nanometre size range (no suitable preseparators are currently available).
TEOM®	X				Sensitive real-time monitors such as the Tapered Element Oscillating Microbalance (TEOM®) may be useable to measure nano-aerosol mass concentration on-line with a suitable size-selective inlet.
SMPS	X	X	X	X	Real-time size-selective (mobility diameter) detection of number concentration (size distribution) Data may be interpreted in terms of aerosol mass concentration, only if particle shape and density are known or assumed. Data may be interpreted in terms of aerosol surface area under certain circumstances. For instance, the mobility diameter of open agglomerates has been shown to correlate well with projected surface area.
ELPI	X	X	X	X	Real-time size-selective (aerodynamic diameter) detection of active surface-area concentration. Data may be interpreted in terms of number concentration. Data may be interpreted in terms of mass concentration if particle charge and density are assumed or known. Size-selected samples may be further analysed off-line.
EM	X	X	X	X	Off-line analysis of electron microscope samples can provide information on size-specific aerosol number concentration. Off-line analysis of electron microscope samples can provide information on particle surface area with respect to size. TEM analysis provides direct information on the projected area of collected particles, which may be related to the geometric area for some particle shapes.
NSAM			X		Diffusion charger instrument. Real-time measurement of aerosol active surface area. Active surface area does not scale directly with geometric surface area above 100 nm. Note that not all commercially available diffusion chargers have a response that scales with the particle active surface area below 100 nm. Diffusion chargers are only specific to nanoparticles if used with an appropriate
DiSCmini		X	X	X	One of a number of small personal instruments that have been developed in recent years based on the principle of unipolar charging[66] Handheld diffusion size classifier for nanoparticle measurement. Wide detection range both in terms of particle concentration and particle diameter

M: mass concentration, N: number concentration, SA: surface area concentration, SD: size distribution.

This is not an exhaustive list. Many of these instruments and methods are now quite mature. DiSCmini is an example of a relatively new instrument with some unique features, but as yet has not been widely used and so the possibilities and limitations are not fully understood. The EU FP7 project NANODEVICE[67] developed a series of prototype instruments but none have as yet been fully commercialised. No current FP7 or H2020 projects are active in this instrument development area.

[65] BSI, 2010. PD 6699-3:2010 Nanotechnologies. Guide to assessing airborne exposure in occupational settings relevant to nanomaterials.

[66] Fierz M, Meier D, Steigmeier P, Burtscher H, 2015. Miniature nanoparticle sensors for exposure measurement
and TEM sampling. J. Phys.: Conf. Ser. 617: 012034.

[67] http://www.nano-device.eu/index.php?id=225

2.3.5.3 Assessment

Amongst the challenges which continue to be raised are

- What metric should be used for measuring nanoparticle exposure?
- What is the maximum size we should measure?
- How to deal with high spatial and temporal variability?
- How to discriminate nanoparticles and other nano-objects from background aerosol?
- Can HARNs such as CNT or nanowires be measured using methods for, derived from, or analogous to current methods for fibres?

The utility of image analysis in assessing exposure to MNs is clear, particularly so in the case of CNT where analogous to asbestos and other fibres, number concentration has been suggested as an appropriate metric. None of the current particle real-time instrumentation is able to discriminate between CNT and other (background) particles which could be present in an aerosol. Ultimately, this will rely on sample collection and high-resolution microscopy techniques. However the utility of these has not been validated.

For CNT, NIOSH is now recommending[68] using elemental carbon (EC) (measured by NIOSH Method 5040[69]) as a means to determine personal exposure to CNT and CNF. NIOSH Method 5040 comprises sampling and off-line thermal-optical analysis; flame ionization detector (FID). They quote an analytical limit of quantification of 1 $\mu g/m^3$ for this method, however this is not specific to CNT (or CNF).

In summary;

- Multiple methods are available including direct reading and off-line approaches,
- Many of these have been available for quite some time and are well established,
- Many are large and not very portable,
- Diffusion classifiers provide the basis of a new set of surface area related metrics and are small enough to be portable or personal devices,
- They also may be used to measure (indirectly) other metrics however, their utility in this respect has yet to be established, and
- Measurements of CNT remain challenging and the development of a real-time monitor to assess airborne fibre concentration would be a major step forward. However there are no current candidate methods for this.

2.3.6 *Toxicity*

2.3.6.1 Relevant questions

Q1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
				X	X	X	X	X	X						

2.3.6.2 High-throughput screening (HTS)

Discussion of HTS is included within the toxicology section in this report due to the frequent use of *in vitro* biological effects assays in HTS arrays. However, HTS methods can include functional (e.g., beneficial effects as employed by the pharmaceutical industry, or use-related data), chemical, and physical analysis methods that are addressed elsewhere in the report. HTS is also included under the more general category of high throughput data/analysis (HTD/A).

2.3.6.2.1 HTS general applicability for toxicity testing

HTS assays are being increasingly used in toxicity testing. HTS assays can simultaneously test many chemicals or materials, including MNs but have seen limited use in the regulatory arena, in part because of

[68] NIOSH, 2013. Current Intelligence Bulletin 65: Occupational Exposure to Carbon Nanotubes and Nanofibers.
[69] NIOSH, 2003. Elemental Carbon (Diesel Particulate): Method 5040.

the need to undergo rigorous, time-consuming formal validation. High-throughput assays are efficiently designed experiments that can be automated and rapidly performed to measure the effect of substances on a biologic process of interest. These assays can evaluate hundreds to many thousands of chemicals over a wide concentration range to identify chemical actions on gene, pathway, and cell function. HTS brings together expertise in liquid handling and robotic automation, multi-platform plate readers and more recently high content imaging. This combinatorial approach means that complex, content rich data set can be produced over a relatively short space of time. The advantage of miniaturisation and automated liquid handling allows rapid, inexpensive and effective up-scaling of small scale bench top assays to large scale assay formats.

This shift towards HTS is largely because:

1. The recognition that current testing methods, which are costly, time consuming, and often use large numbers of animals without always providing correspondingly large benefits, are not adequate to manage the increasing backlog of largely untested chemicals; and
2. The frequent inability of current *in vivo* tests to provide clear mechanistic insight into toxicity pathways[70].

Currently, there are hundreds of *in vitro* HTS assays. Before these assays can be used for making regulatory decisions there needs to be a process to appropriately evaluate their reliability, relevance and fitness for purpose. Judson et al. (2013) in their assessment of the potential utility of these assays identified 5 key issues.

1. HTS assays provide a new capability for simultaneously testing the ability of thousands of chemicals to trigger intermediate biological or biochemical key events (KEs), as opposed to observable or apical endpoints, associated with toxicity pathways that can lead to adverse health outcomes.
2. The data from these assays can be used to prioritize which chemicals out of large sets of previously untested ones should be subject to further study sooner rather than later.
3. Before using these assays, even for prioritization, their relevance, reliability and fitness for purpose should be established and documented. In the present context, relevance is related to the ability to detect KE's with documented links to adverse outcomes, and the ability to reproduce data and to respond appropriately to carefully selected reference compounds, either in a qualitative (e.g. positive/ negative for effect) or quantitative (e.g. relative potency) manner. Fitness for purpose is more subjective since it is use-case dependent, but is typically established by characterizing the ability of an HTS assay to predict the outcome of guideline tests for which prioritization scores are being generated.
4. It should be possible to develop a streamlined validation process to evaluate the relevance, reliability and fitness for purpose for HTS assays. This is largely because the data from the HTS assays generally provide quantitative, reproducible read-outs with a focused and mechanistically simple interpretation. These attributes should make evaluation of the performance of the HTS assays, and hence peer review and decisions on acceptance for use by regulatory bodies based on the scientific evidence, relatively straightforward.
5. It is unlikely that any single *in vitro* assay will ever yield the "perfect" result. Even mechanistically similar assays are expected to yield some degree of discordance due to the complexities of biological processes and assay-specific interference by some test chemicals. Hence multiple assays for critical targets and a weight of evidence approach are likely to be needed. Additionally, many environmental chemicals are likely to be of low potency, and hence subject to variation in hit calling from assay to assay.

Judson et al proposed an approach for streamlining the validation process, specifically for prioritisation applications in which HTS assays are used to identify a high-concern subset of a collection of chemicals. The high-concern chemicals could then be tested sooner rather than later in standard guideline bioassays. The streamlined validation process would continue to ensure the reliability and relevance of assays for this application. They suggested the following practical guidelines:

[70] Judson R, Kavlock R, Martin M, Reif D, Houck K, Knudsen T, Richard A, Tice RR, Whelan M, Xia M, Huang R, Austin C, Daston G, Hartung T, Fowle JR 3rd, Wooge W, Tong W, Dix D, 2013. Perspectives on validation of high-throughput assays supporting 21st century toxicity testing. ALTEX 30: 51-6.

1. follow current validation practice to the extent possible and practical;
2. make increased use of reference compounds to better demonstrate assay reliability and relevance;
3. deemphasize the need for cross-laboratory testing, and
4. implement a web-based, transparent and expedited peer review process.

2.3.6.2.2 HTS applicability for MN

There has been widespread consideration and investigation of the use of HTS methods for screening and prioritisation hazard assessment of MN. However, in general use of these approaches is still at a research level. The available approaches can be broken down into

- Classical plate reader-based assays using readouts such as luminescence, fluorescence, absorption, time-resolved fluorescence and fluorescence polarization; or
- High content screening (HCS) approaches. Because traditionally plate reader assays have been used for small molecule safety assessment, they have yielded multiple platforms and read-out modes that can be used for toxicological assessment. These platforms include the use of luminescence, fluorescence or absorption approaches to screen for mutagenicity or genotoxicity in bacteria, yeast or mammalian backgrounds.

As noted by Nel et al. (2013)[71] transitioning from animal models to newly developed HTS analyses would increase not only the number of MN that can be assessed simultaneously, but also speed data acquisition (testing, data generation), which can be used to explore the effectiveness of computational data analyses further. In addition, these new approaches hold the potential promise of greater accuracy in the prediction of human health effects versus currently performed descriptive animal studies. This strategy will enable comparative analyses of large numbers of existing and newly introduced MNs. Such approaches could be implemented during product development to understand better the structural and functional determinants of toxicity that could be applied in lead-molecule selection to result in the development of safer and more sustainable products. These approaches could also be applied for the comparative assessment of MNs that could be used to support materials grouping for purposes of establishing and supporting read- across for hazard characterization and risk assessment. Although ATS has the potential to eliminate animal testing in the distant future, focused animal testing, including for toxicity and bio-distribution (dosimetry) is currently still required to validate, to verify or to bridge the *in vitro* testing and to develop exposure-dose-response extrapolation for hazard analysis.

Methodological approach to MN toxicity testing through HTS requires the following 4 steps.

- Conceptualization of assay development,
- Proof of principle experiments,
- Validation on the automated platform, and
- Biological/ pharmacological validation.

Examples of plausible toxicity paradigms, possible analytes, readout modes and potential problems when using various readouts for MN toxicity screening are shown in Table 9[72].

HTS is a powerful method to acquire large data sets but it has to be supplemented with equally powerful data analysis tools to establish property-activity relationships. The development of a computational framework to assess the toxicological effects of MNs requires a novel integrative approach that combines information technologies that allows data management through advanced algorithms and modelling approaches. The three main elements of the data analysis framework are

(a) an MN database system,

[71] Nel A, Xia T, Meng H, Wang X, Lin S, Ji Z, Zhang H, 2013. Nanomaterial toxicity testing in the 21st century: use of a predictive toxicological approach and high-throughput screening. Acc Chem Res 46: 607-21.

[72] Damoiseaux R, George S, Li M, Pokhrel S, Ji Z, France B, Xia T, Suarez E, Rallo R, Mädler L, Cohen Y, Hoek EM, Nel A, 2011. No time to lose--high throughput screening to assess nanomaterial safety. Nanoscale 3: 1345-60.

(b) modelling, and

(c) hazard-ranking.

HTS approaches are important for testing of large number of MN types but these kinds of approaches are only to a limited extent used within NANOREG.

Table 9. Summary of HTS approaches

Toxicity Type or paradigm	Analyte	Probe	Readout Mode	Utility	Potential problem
Cytotoxicity	Cell number/proliferation	Hoechst 33342/DAPI	Fluorimetry/High content assay	Cell quantification, nuclear content	Background signal from NPs with blue fluorescence
	Membrane leakage	Propidium Iodide/Syto 9	Fluorimetry/High content assay	Compromised cell membrane integrity	Background signal from NPs with red fluorescence (e.g. QDs)
	Membrane integrity	LDH assay	Absorbance at 490 nm	Cell viability	NPs may inhibit enzyme and/or absorb at 490 nm (CNTs, Ag)
	ATP	ATPlite™	Luminescence	Mitochondrial activity and viability status of cells	Not appropriate for NPs that may inhibit enzyme and/or absorb light e.g. CNTs
	Mitochondrial membrane potential	JCI/TMRM/chloromethy-X-rodhamine	Fluorimetry/High content assay	Loss of MMP	Background signal from NPs with red or green fluorescence
	Metabolic activity	MTT, WST-1, XTT	Absorbance	Mitochondrial activity and Viability status of cells	NPs may inhibit enzyme and/or absorb light or substrates
	Intracellular calcium flux	Fluo-4/Fura 2-AM/Rho 2-AM	Fluorometry/High content assay	Increased Intra-cellular calcium level	Background signal from NPs with green fluorescence
	Apoptosis	Calcein-AM	Fluorometry/High content assay	Mitochondrial membrane permeability transition (MPT)	Background signal from NPs with green fluorescence
Genotoxicity	DNA cleavage	Micro-nuclei assay (HCS), BrDU incorporation	High content screening	Chromosome damage	Background signal from NPs with blue fluorescence
Inflammation	IL-1, IL-8, TNF-α	Antibody based ELISA or TR-FRET	Luminescence/TR-FRET	Expression level of inflammatory markers	Not appropriate for NPs that interfere with TR-FRET (e.g. absorb protein) or luminescence reactions
	NF-kB and AP-1 activation	Reporter genes	Luminescence	Activation of inflammatory pathways	Not appropriate for NPs that may inhibit enzyme and/or absorb light
Oxidative stress	GSH, ROS	Absorbance/HCS using fluorescence probe	Fluorometry/High content assay	Free radical generation, Glutathione depletion	Not appropriate for NPs that may interfere with fluorescence output
Fibrogenesis	TGF-1b, Collagen 1&3, MMPs	Fluorescence probe coupled antibody	Fluorometry/High content assay	Induction of fibrogenesis	Not applicable for NPs that may interfere with fluorescence output

2.3.6.2.3 Assessment

The purpose of a screening test is to provide an indicator of potential adverse outcomes and effects on human health or the environment. When used in a tiered testing strategy, HTS methods have the potential to eliminate further *in vivo* testing or to identify hazardous materials for targeted *in vitro* or *in vivo* investigation, thus streamlining the hazard identification process. Toxicological screening is part of most early tiers in weight-of-evidence-based, intelligent testing strategies (ITS). ITS take into account the data available for the MN of interest and provide a rational testing strategy to understand the hazard properties of that MN without resorting. By their nature, screening tests are intended for hazard identification and for range-finding when preparing for the more involved, exacting test protocols.

New *in vivo* model systems such as the embryonic zebrafish, can be easily and rapidly interrogated at the cellular and molecular levels, in addition to the whole animal level, to rapidly advance our understanding of the biological consequences of MN exposure.

2.3.6.3 Organ-on-a-chip

The usefulness of Organ-on-a-Chip (OC) models has recently been reviewed in the NANOREG project[73] from which the following section is taken. By smart combinations of microfluidics and cell cultures, dedicated OC models have been developed that show a more accurate representation of human organs compared to current *in vitro* systems. An OC is a microfluidic device containing miniaturized bioreactors in which living cells are cultured in a precise spatial arrangement and under a continuous flow of culture fluid in order to simulate the physiology of an entire living organ. The device may allow high-resolution, real-time imaging of the cultured cells as if they were in a living organ. The term lab-on-a-chip is sometimes used for such systems as well, but here lab-on-a-chip is used to demarcate and refer to a device containing a chemical analysis instrument (such as a gas chromatograph) on a microchip, without containing biological materials such as cells. Therefore, the term organ-on-a-chip (OC) is used for the devices containing cells. The goal of such a device is not to construct an entire organ but rather to fabricate the minimal functional units that can perform the functions of the organ at the level of tissue, if not an organ. All the devices contain cells that are cultured in microchannels or microchambers that are continuously perfused with a stream of culture medium. The OC architecture is usually composed of polymeric or glass microchannels. Microfabrication techniques such as replica moulding and micro-contact printing can create micro-scale structures and patterns that enable the control of the organ features and fluidic flow.

The simplest OC system is a single microfluidic chamber containing only one type of cell from a certain organ (e.g. hepatocytes, epithelial cells of the renal tubule, endothelial cells from the intestine, etc.). More complex systems include two or more microchannels connected by porous membranes whose opposite sides are plated with different cells so as to recreate the interface between different tissues (lung alveolus, blood-brain barrier). Indeed, the possibility of integrating porous materials to separate two microchannels enables the analysis of the barrier function of some tissues (such as the blood-brain barrier), of the trans-cellular transport and of the mechanisms of cellular absorption and secretion. This is important because it allows the creation of an experimental model that can simulate the interactions of the vascular endothelium and the parenchymal tissue that define virtually all organs.

OCs can be applied as a screening tool to get an impression of the substance's effects. This information can then be used for prioritizing further testing and/or identifying the market suitability of different MNs. For the purpose of in house testing during a development phase, the OCs would not have to be validated and accepted by regulators. Thus for this purpose, the OCs could be introduced relatively easily, enabling the generation of data with these OCs that could then be used to further evaluate and verify their potential value.

2.3.6.3.1 Assessment

OC approaches provide a number of advantages compared with traditional in-vitro assays (Table 10).

[73] NANOREG Deliverable D 6.2 - Inventory of safety assessment issues and new approaches to research and governance

Table 10. Overview of advantages of OC devices compared to traditional *in vitro* assays.

Shear stress	Shear stress is the stress on a surface (of e.g. a cell) caused by the physical force of the movement of liquid along this surface. It is established that cell behaviour is different in the presence of fluidic shear stress.
Defined concentrations gradients	Through the fluid flow of the culture medium, complex concentration grades are achievable. Repeated flow stream lamination can for example promote cell chemotaxis, i.e. the migration of cells (e.g. macrophages) in response to a stimulus.
Homogenous chemical distribution in medium	Only a small portion of the administered dose reaches the cell membrane. Colloidal behaviour, particle sedimentation and diffusion have to be taken into consideration when correcting for the initial dose at the start of the experiment. With microfluidics a homogenous suspension of chemicals in culture medium can be applied to the cell membrane under continuous perfusion, thereby creating a more physiological relevant situation.
Micropatterning possible	Micropatterning allows for improved control over homo- and heterotypic cell-cell interactions and easier 3D culturing. These micropatterns can be used to control the geometry of adhesion and therefore the orientation of the cell division axis and is needed for the maintenance of stem cells or the exact placement of cell on a sensor.
Sensor integration	With the integration of biosensors in the device itself, a more reliable and quantitative monitoring of cell behaviour can be obtained (e.g. electrical activity, cytotoxicity measurements, optical sensors, cell based bio-sensors, microscale patch clamp devices).
Mechanical strains	(Cyclic) mechanical strain can accentuate toxic and inflammatory effects and enhance the transport of particles over organ barriers
High throughput screening	Small size will eventually allow multiple tests in one plate and allow for robotic plating and reading. In addition, due to a highly controlled environment, it is possible to reduce the otherwise labour-intensive micromanipulations that can be required in experimental assays.
Expanded cell viability	High optimal control over environmental conditions for cell-based assays, including waste removal by medium flow, increases the cell-viability and culture time.

To use OCs for regulatory testing, a full validation of the tests would be necessary, complete with an OECD test guideline (TG). This would be more challenging to achieve, especially since the regulatory frameworks for MN are spread across several sectors in the EU (e.g. chemicals, cosmetics, drugs). Each framework involves different requirements for information that need to be submitted in the dossier for market acceptance, with guidelines or guidance documents usually describing which tests are acceptable for providing this information. For studies on systemic toxicity (e.g. acute toxicity, repeated dose toxicity and reproduction toxicity), no accepted alternatives to animal testing are available yet.

2.3.7 *Kinetics*

2.3.7.1 Relevant questions

Q1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
				X	X	X	X	X	X				X		

2.3.7.2 Methods

Nanoparticle biokinetics is important in hazard identification and characterization of inhaled, ingested or injected particles. Aspects range from inhaled particle deposition probability and retention in the respiratory tract to biokinetics and clearance of particles out of the respiratory tract, transport into the blood circulation (translocation), towards secondary target organs and tissues (accumulation), and out of the body (clearance). The macroscopically assessed amount of particles in the respiratory tract and secondary target

organs provides dose estimates for toxicological studies on the level of the whole organism. Complementary, microscopic analyses at the individual particle level provide detailed information about which cells and subcellular components are the target of inhaled particles. Kinetic studies contribute to shed light on mechanisms and modes of action eventually leading to adverse health effects by inhaled nanoparticles.

A comprehensive review of methods for macroscopic and microscopic analyses of particle deposition, retention and clearance was carried out by Geiser & Kreyling (2010).[74]

2.3.7.2.1 Methods for macroscopic studies

Quantitative biokinetics can be performed after nano- particle administration by any route: by inhalation or intratracheal instillation to the respiratory tract, by gavage to the gastro-intestinal tract, by intravenous or intra-arterial injection to the blood circulation and by dermal applications to the skin. The concept is simple, aiming to estimate the total amount of nanoparticles in the entire body at a certain time point after exposure and in all excretions until this time point. Hence, not only the distribution of nanoparticles in organs and tissues of interest is assessed, but nanoparticles in the remaining carcass and those excreted are also included. With this, a 100% balance to the administered nanoparticles is achieved and a complete, yet detailed, quantitative analysis of their biokinetics is obtained.

Analytical chemistry e.g. ICP-MS or atomic absorption mass spectroscopy (AA-MS) provide suitable methods to quantify particles in the collected specimens.

Radio-spectroscopy may provide an elegant alternative, allowing direct analysis of native organs and tissues without any pre-treatment. In this case, the radio-label needs to be firmly integrated into the nanoparticle matrix without any leaching. This is challenging but can be fulfilled when a radio-isotope is blended into the nanoparticles during production, stable labelling is obtained, when the radio-isotope belongs to the same chemical element as the nanoparticle matrix. A well-known example is gold that is neutron-activated in a nuclear research reactor such that the gold nanoparticles are labelled with the 198Au radio-isotope[75].

2.3.7.2.2 Methods for microscopic studies

Analysis of nanoparticles at the individual particle level by electron microscopy requires

(i) adequate organ preservation,

(ii) representative tissue sampling, and

(iii) unambiguous identification of the nanoparticles in ultra- thin tissue sections.

Important measurement aspects include morphological characterization and elemental microanalysis of nanoparticles and systematic uniform random tissue sampling and particle quantification.

2.3.7.2.3 In vivo fluorescence imaging methods

Fluorescence sampling of cellular function is widely used in all aspects of biology, allowing the visualisation of cellular and sub-cellular biological processes with spatial resolutions in the range from nanometres up to centimetres. In the last decade, full-body pre-clinical imaging systems have emerged with a wide range of utilities and niche application areas. The range of fluorescent probes that can be excited in the visible to near-infrared part of the electromagnetic spectrum continues to expand, with the most value for *in vivo* use being beyond the 630 nm wavelength, because the absorption of light sharply decreases. Information derived from *in vivo* fluorescence imaging systems can be regarded as an important complement to microscopy studies performed on cell cultures and tissue slices because it provides information about specific biological processes in fully integrated living systems. The state of the art was has been reviewed by Leblond et al (2010)[76] from which much of the following is extracted.

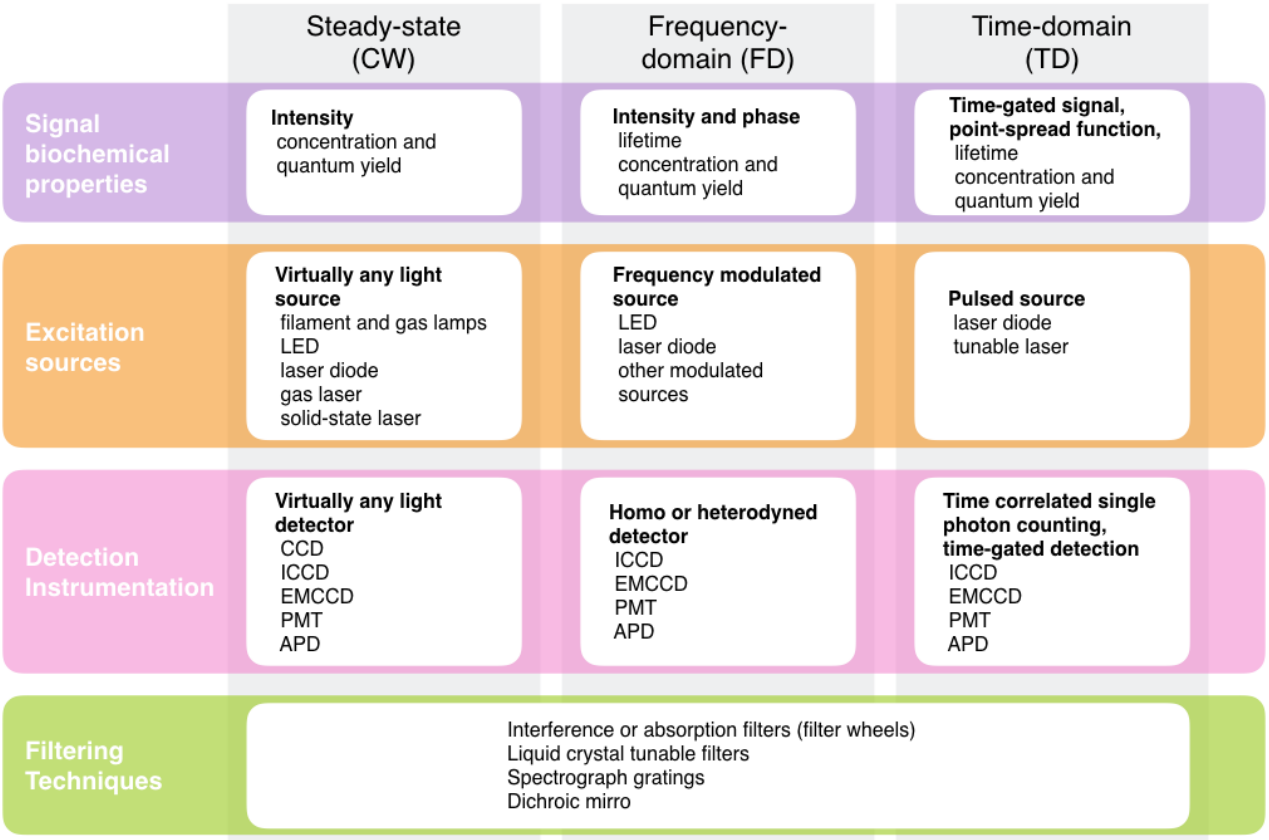
The basic principle behind *in vivo* fluorescence imaging is similar to that used in fluorescence microscopy techniques (e.g., conventional fluorescence microscopy, confocal microscopy, multiphoton microscopy, optical coherence tomography). However, when whole-animals are interrogated, the desired information is typically associated with biochemical events occurring deep within the tissue. This implies that photons being

[74] Geiser M, Kreyling WG, 2010. Deposition and biokinetics of inhaled nanoparticles. Part Fibre Toxicol 7:2.
[75] Geiser M, Kreyling WG, 2010. Deposition and biokinetics of inhaled nanoparticles. Part Fibre Toxicol 7:2.
[76] Leblond F, Davis SC, Valdés PA, Pogue BW, 2010. Pre-clinical whole-body fluorescence imaging: Review of instruments, methods and applications. J Photochem Photobiol B 98: 77-94.

part of the detected signal have undergone multiple scattering events in the process of irradiance of the excitation light into the body and radiance.

In vivo fluorescence imaging methods comprise 4 main elements, biomarker fluorescent signal, an excitation source, detection instrumentation and filtering techniques. Figure 2 provides an overview of the hardware required for different types of imaging data. Excitation can be achieved by a wide variety of light sources including filtered filament or gas discharge lamps (tungsten and xenon), light-emitting-diodes (LED), or any laser system, including gas, crystal, and diode lasers. Versatile imaging instruments, in terms of number of available wavelengths, can be designed using expansive systems such as tuneable lasers but relatively inexpensive broad band lamps can be used to provide a flexible platform since filters can be used to select different excitation wavelengths. Available detectors vary widely and include charged coupled device arrays (CCD's), intensified CCD's, electron multiplication CCD's (EMCCD), avalanche photodiodes (APD's), and photomultiplier tubes (PMT's). All *in vivo* fluorescence imaging systems are designed around the idea that a portion of the fluorescence emission spectrum of the molecule of interest can be separated spectrally from the excitation and/or background light signals. This is achieved with filtering strategies, such as dielectric interference filters, liquid crystal tuneable filters, and spectrograph gratings. Ultimately the filtering capability of a system is a major fundamental limitation which will dictate lower limits of detection of fluorophores.

Figure 2. Overview of imaging methods and equipment needs



2.3.7.2.4 Commercial systems

Table 11 summarizes the main technical features of the imagers that are commercially available.

Table 11; Technical features of commercially available imaging equipment

Instrument (company)		**Optix MX3 (ART)**	**NightOWL II-LB 983 NC 100 (Berthold Tech.)**	**IVIS Spectrum (Caliper Life Sciences)**	**Maestro (CRi)**	**KODAK *in vivo* imaging system (Carestream Health)**	**Pearl Imager (Li-COR Biosciences)**	**iBox (UVP)**	**FMT 2500 LX (VisEn Medical)**
Application	Planar mode	Epi-illumination	Epi-illumination	Epi-illumination & trans-illumination	Epi-illumination	Epi-illumination	Epi-illumination	Epi-illumination	Epi-illumination
	Multiplexing	Yes (limited by scan time)	Yes (>2 wavelengths)	Yes (>2 wavelengths)	Yes (>2 wavelengths)	Yes (>2 wavelengths)	Yes (2 wavelengths)	Yes (>2 wavelengths)	Yes (4 wavelengths)
	Multi-fluorophore separation	Lifetime imaging	-	Spectral un-mixing	Spectral un-mixing	Spectral un-mixing	-	Spectral un-mixing	-
	3D	Topography (depth calculation)	-	Tomography	-	-	-	-	Tomography
	Surface Rendering	Yes	No	Yes	No	No	No	No	Yes
Illumination	Light source	Pulsed laser diodes (300-800 µW) external CW tunable laser	Tungsten lamp (75W)	Tungsten lamp (150W)	Xenon-halogen lamp (300W)	Haloen (150W) or xenon lamp (175W)	Laser diodes	Halogen lamp (150W)	Laser diodes (LEDs)
	Wavelengths	470, 532, 635, 670, 735, 785 nm (up to 4 internal lasers)	340-1100 nm (selectable, 4 filter positions)	430-840 nm (selectable 28 filter positions)	455-780 nm (selectable, 9 filter positions)	385-770 nm (selectable, 29 filter positions)	680, 780 nm	365-750 nm (selectable, 8 filter positions)	635, 670, 745, 785 nm
	Method	Epi-illumination (raster-scanning)	Epi-illumination (broad beam)	Epi-illumination (broad beam) trans-illumination (raster-scanning)	Epi-illumination (broad beam)	Epi-illumination (broad beam)	Epi-illumination (NA)	Epi-illumination (broad beam)	Epi-illumination (broad beam, LEDs) trans-illumination (laser scanning)
Detection	Technology	PMT TCSPC	CCD cooled -80 to -90^{0}C 1.0 MP (13 µm) 16-bit digitizer	CCD cooled -90^{0}C 4.2 MP (13.5 µm) 16-bit digitizer	CCD cooled 0^{0}C or +8^{0}C 1.4 MP (6.5 µm) 12-bit digitizer	CCD cooled -29^{0}C 4.2 MP (10 µm) 16-bit digitizer	CCD (details NA)	CCD cooled -28^{0}C 4.2 MP (7.4 µm) 16-bit digitizer	CCD cooled 16-bit digitizer
	Wavelengths (filtering)	450-900 nm (selectable, 24 filter positions)	300-1050 nm (selectable, 4 filter positions)	300 – 1100 nm (selectable, 28 filter positions)	500-950 nm (continuous, liquid crystal tunable filter, 20 or 40 nm resolution, 1 nm steps)	440-830 nm (selectable, 4 filter positions)	700, 800 nm (selectable, 2 filter positions)	450-850 nm (5 filter positions)	700, 800 nm (selectable, 8 filter positions)

Instrument (company)		Optix MX3 (ART)	NightOWL II-LB 983 NC 100 (Berthold Tech.)	IVIS Spectrum (Caliper Life Sciences)	Maestro (CRi)	KODAK *in vivo* imaging system (Carestream Health)	Pearl Imager (Li-COR Biosciences)	iBox (UVP)	FMT 2500 LX (VisEn Medical)
	FOV (optics)	20 × 8.4 cm^2 (raster-scan detection – 0.5-3 mm resolution)	1 × 1 – 25 × 25 cm^2 (motorized camera, lens setting for magnification, focus adjustment)	4 × 4 – 25 × 25 cm^2 (lens settings for magnification, focus adjustment)	11.2 × 8.4 cm^2 (max) (lens settings for magnification, focus adjustment)	2 × 2 to 20 × 20 cm^2 (lens settings for magnification, focus adjustment)	12 × 9 cm^2 (NA)	8 × 8 – 16 × 16 cm^2 or 14 × 14 – 30 × 30 cm^2 (lens settings for magnification, focus adjustment)	8 × 8 cm^2 (epi-illumination) 5 × 5 cm^2 (trans-illumination)
Multi-modal imaging	While light image	Yes	Yes	Yes	Yes	-	Yes	Yes	Yes
	Bioluminescence	Yes	Yes	Yes	-	-	-	-	-
	Multi-modal	CT-coupling stage	CT, MRI, PET/SPECT, US	CT, MRI, digital mouse atlas	-	X-ray, radioisotopic detection (planar)	-	-	Adapters for CT, MRI, PET/SPECT
	Co-registration software	Yes (to CT imaging)	Yes	Yes	-	Yes)automated to planar X-ray images)	-	-	Yes
Animal Handling	Anaesthesia hook-up	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
	Temperature control	Yes (heated stage)	Yes (heated stage)	Yes (heated stage)	Yes (heated stage)	Yes (heated chamber)	Yes (heated stage)	Yes (heated stage)	Yes (heated chamber)
	Number per image	Up to 5	Up to 2	Up to 5	Up to 3	NA	1	Up to 3	1

2.3.7.2.5 Emerging approaches - 'State-of-the-art' fluorescence tomography instruments and methods

Realisation of full 3D optical capabilities has been a technological challenge that many researchers have investigated, with varying levels of success. The fluorescence tomography problem is a difficult one to resolve because of its hyper-sensitivity to various sources of noise such as modelling errors and statistical noise. In part, this is caused by the fact that the diffuse optical tomography problem is intrinsically ill-posed; meaning that for a given fluorescence dataset there exists a large number of equivalent solutions. The challenge of researchers working in this field consists of devising approaches that minimize this effect, ensuring that the images that are obtained are as close as possible to reality.

2.3.7.3 Assessment

In considering the potential use of *in vivo* fluorescence imaging in biological studies, there are intrinsic limitations researchers should consider. In part, these limitations relate to the interaction of light with microscopic components of tissue. Also, consideration must be given to difficulties ensuring that biomarkers of interest are associated with a detectable level of optical contrast and that the origin of the latter is specific enough to deliver useful objective information.

The full potential of *in vivo* fluorescence imaging has not been fully exploited yet. The wide variety of available commercial systems partly illustrates this fact demonstrating that a clear consensus may never emerge as to the optimal approach for *in vivo* small animal imaging. This is because the choice of any one individual system is associated with one offering a balance between sufficient biological information at a price compatible with the scale of the available funding or related to the cost-effectiveness motive of the user.

An important aspect that has to be considered in assessing the state of the technology is that convergence to a universally adopted imaging method is prevented by the wide variety of biological markers and fluorescent probe combinations that are being used and will be developed. Each biological application typically involves fluorophores with different spectral signatures, anatomical accumulation sites and contrast levels. What we need from kinetics study is the time course of the burden of NP in different organs. Imaging techniques are useful but does not lend itself easily to toxicology because the organ dose (burden) is not quantitated.

2.3.7.4 Systems biology and omics

2.3.7.4.1 What are systems biology and omics approaches?

Systems biology is the computational and mathematical modelling of complex biological systems. It involves the iterative and integrative study of perturbations by chemicals and other stressors of gene and protein expression that that may be linked to toxicological outcomes. Systems biology constitutes a powerful approach to describing and understanding the fundamental mechanisms by which biologic systems operate[77]. Specifically, systems biology focuses on the elucidation of biologic components and how they work together to give rise to biologic function. Frequently it is it referred to in the context of Omics technologies. 'Omics' technologies are primarily aimed at the universal detection of genes (genomics), mRNA (transcriptomics), proteins (proteomics) and metabolites (metabolomics) in a specific biological sample[78]. The development of Omics research has progressed due to advances in microarray technology. Data analysis is complex as a huge amount of data is generated and statistician and bioinformatician involvement in the process is essential.

Developing systems biology approaches was identified as one of the key priorities for hazard assessment in the Nanosafety cluster roadmap[79] although the target date of 2015 was clearly optimistic.

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[77] Toxicity Testing in the 21st Century: A Vision and a Strategy, Committee on Toxicity Testing and Assessment of Environmental Agents, National Research Council, (2007) http://nap.edu/11970

[78] http://onlinelibrary.wiley.com/doi/10.1576/toag.13.3.189.27672/full

[79] Nanosafety Cluster Roadmap - Nanosafety in Europe 2015-2025: Towards Safe and Sustainable Nanomaterials and Nanotechnology Innovations (2013) http://www.ttl.fi/en/publications/Electronic_publications/Nanosafety_in_europe_2015-2025/Documents/nanosafety_2015-2025.pdf

2.3.7.4.2 How can they be used in hazard assessment?

Omics approaches can contribute to a better understanding of NP activated (or depressed) pathways and allow an improved prediction of NP effects. A systems approach can be used to describe the fundamental biologic events involved in toxicity pathways and to provide evolving biologic modelling tools that describe cellular circuits and their perturbations by environmental agents. 'Omics' profiling can generate massive data and an unbiased view of cellular processes. These approaches have been used to discover mechanistically similar compounds by using what is called a connectivity mapping approach. Connectivity mapping, is similar to read-across in concept, can be equally applied to toxicogenomics data and offers great potential for more widespread applicability in risk assessment.[80]

Omics approaches contribute to a better understanding of NP activated (or depressed) pathways and allow an improved prediction of NP effects. The amount of data from appropriately designed omics experiments can cover most parts of cell metabolism and toxic phenotypical effects. So, *in vivo* responses can in principle be extrapolated from *in vitro* experiments, long term effects from short term experiments – e.g. cell division in lung epithelium, and low dose exposure experiments may give additional information about effects of high doses. Classification of NP toxicology according to the amount and kind of pathways activated and extrapolation of those to new designed NPs contribute strongly to the Safe- by-Design approach of NP development[81].

Omic approaches allow researchers not only to look at the expression of a single or a few entities of interest but up to a few hundred or thousands. Due to the improvement of high throughput analysis methods like DNA and RNA sequencing, microarrays, mass spectroscopy (MS) or chromatography (HPLC, MALDI-TOF) methods biologists can gain information about a few hundred up to ten thousands of data points per sample. This allows a much more detailed picture of the metabolic processes to be constructed.

In most cases, -omics analyses result in a list of up and down regulated genes. To handle the massive data created analytics should include association and visualization methods. Association tools help e.g. to find the connections of genes/metabolites with pathways or phenotypes. Visualization makes analysis and interpretation easier or even possible especially when it comes to highly interconnected data points. Gene ontology and pathway analysis reduce the complexity from thousands of genes to a smaller set of biological processes and functions allowing a quicker and more detailed analysis.

Critical to this activity is data sharing. Data needs to be efficiently organized, safely stored, and on-line. Data bases store information about biological entities like genes, proteins or metabolites and makes them available for manual and automated information request. Some examples include Ensembl (http://www.ensembl.org) and NCBI Gene (http://www.ncbi.nlm.nih.gov/gene) which provide information about genomes, genes and transcripts. UniProt (www.uniprot.org) contains information about proteins and ChEMBL (https://www.ebi.ac.uk/chembl) is a database for metabolites.

2.3.7.4.3 Challenges and way forward

Currently Omics approaches offer great potential but do not form part of the NP regulatory toolkit and application of such in this context seems rather distant..

A central element is a shift away from traditional toxicity testing that focuses on demonstrating adverse health effects in experimental animals toward a deeper understanding of biologic perturbations in key toxicity pathways that lead to adverse health outcomes. There are many challenges to be overcome before these approaches can be utilised in this frame. Much of this relates to the appropriateness and interpretation of the science. Questions include

- Toxicity-Pathway Identification—What are the key pathways whose perturbations result in toxicity?
- Adversity—What adverse effects are linked to specific toxicity-pathway perturbations? What patterns and magnitudes of perturbations are predictive of adverse health outcomes?

[80] Grafström RC, Nymark P, Hongisto V, Spjuth O, Ceder R, Willighagen E, Hardy B, Kaski S, Kohonen P. Toward the Replacement of Animal Experiments through the Bioinformatics-driven Analysis of 'Omics' Data from Human Cell Cultures. Altern Lab Anim. 2015 Nov;43(5):325-32.

[81] Ehrhart F, Evelo C, Willighagen E Current systems biology approaches in hazard assessment of nanoparticles bioRxiv (09 October 2015), 028811, doi:10.1101/028811 by

- Assays for Large-Scale Application—Which assays best capture the elucidated pathways and best reflect *in vivo* conditions?
- Suite of Assays—What mix of pathway-based high- and medium- throughput assays and targeted tests will provide adequate coverage?

Relevant to the issues of equipment needs the following recommendations apply:

1. Improved micro array technologies - Microarray technologies have allowed the development of the field of toxicogenomics, which evaluates changes in genetic response to environmental agents or toxicants. These technologies permit genome wide assessments of changes in gene expression associated with exposure to environmental agents. Protein microarrays potentially offer the ability to evaluate all expressed proteins in cells or tissues. However, whole-cell or tissue profiling of expressed proteins is still in the developmental stage. These techniques remain expensive, and the technology is in flux.

2. Standardization of Research Assays and Results - With the development of data-management systems, processes for standardizing platforms would have to be developed. Currently, there is little standardization of microarrays.

3 Data-Storage, Data-Access, and Data-Management Systems need to be developed and standardized. Those efforts could be stymied without easy and wide public access to databases of results from a broad array of research studies: high-throughput assays, quantitative-SAR model development, protein and DNA microarrays, pharmacokinetic and metabolomic experiments, *in vivo* apical tests, and human biomonitoring, clinical, and population-based studies. Central repositories for -omics data are under development and exist to a small extent for some *in vivo* toxicity data. The scale of data storage and access required is much larger. The data-management system must also be able to accommodate confidential data but allow for data-sharing of confidential components of the database among parties that agree to the terms of confidentiality. The data-management system would also provide procedures and guidelines for adequate quality control.

2.3.8 *Other "Equipment"*

2.3.8.1 High throughput data/analytics HTD/A

2.3.8.1.1 Relevant questions

Q1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
	X	X		X	X	X	X	X	X	X	X	X		X	

The ability to use multiple measures of variations in MN form from HTS arrays or organ on a chip approaches to routinely measure many MN for multiple routes of exposure, toxicokinetic modelling points, and exposure sampling points is a relatively recent advancement that may figure prominently in support of risk management for MNs. Similarly, the ability to collect and manage the data from large numbers of spectral/image measurements at high resolution for similar variations in time, spatial, and media sampling points may also play a critical role in connecting exposure, dose, and effect information from real-world uses of MN. These approaches can provide data that can bridge *in vivo* toxicity testing to grouping, modelling, and prioritization or optimization strategies that are particularly important for SbD methods.

2.3.8.1.2 Assessment

Applications of HTD/A are likely to be developed initially to provide information that is supportive of mode of action for toxic or beneficial health effects, transformations in environmental or biological media, or grouping/prioritization rather than information that is itself determinative for decision support for risk management or regulatory purposes. This likely path for development is due to the complexity of the methods and due to the current reliance of chemical and MN risk assessment on standardized chronic duration *in vivo* bioassays. For example, HTD/A methods used for chemicals (e.g., US EPA ToxCast[82]) have not yet reached the level of replacing standard bioassays. As such, the standardization and validation needs for HTD/A methods would be expected to face a lower bar for utility than would be expected for determinative studies.

[82] https://www.epa.gov/chemical-research/toxicity-forecaster-toxcasttm-data

Nonetheless, validation and standardization will need to be done so that utility of HTD/A methods are sufficient to justify the expense of employing the methods. Missed benefits from over-culling and poor screening resulting in failed candidates as full risk analysis is done are the primary costs to avoid. The pharmaceutical industry has had experience in applying similar methods to screen candidates for development and so the types of validation and standardization that may be needed could be modelled after experiences from pharmaceutical development.[83]

2.3.8.2 Exposure control

2.3.8.2.1 Relevant questions

Q1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
												X		X	

In Europe in a regulatory context the CEFIC RMM Library[84] is a tool which specifically designed to provide default values for the efficiency of RMMs in their usage to control exposures. Use of this tool is specifically recommended in the REACH guidance document R13 "Risk management measures and operational conditions". An extensive review of the efficacy of RMM for nano was carried out as part of the RiPON activity (RiPON 2011)[85] with a specific purpose of recommending whether changes in the default values were needed. Amongst the conclusions from this review were:

- Substitution: no evidence for the substitution or modification of MNs being undertaken for the purposes of EHS management. Modification of the hazard potential of certain types on MNs appeared to have some, as yet not fully explored possibilities (SbD approach).
- Engineering controls – Enclosure: In most of the studies where an enclosed or sealed process was used, containment was effective as long as it was maintained however this was not universal. An enclosed system is not sufficient in itself to guarantee that there is no release of MNs into the workplace air. This would imply that such systems should be tested directly to demonstrate effective containment. In almost all cases, elevated level were measured associated with opening of the containment for recovery of testing of the product.
- Engineering controls – Ventilation: Almost no information about the quantification degree of effectiveness provided i.e. what level of effectiveness was given by the LEV. In some cases the LEV was effective, in some cases not.
- Engineering controls – Filters: Filtration theory indicates that filtration will be effective for particles in the nm size range. The evidence available appears to support this.
- Administrative controls: There is no specific evidence to suggest that administrative controls which are used for conventional materials will not be appropriate for MNs.
- Personal protective Equipment (PPE): Evidence suggests that the performance of respiratory protective equipment (RPE) will be effective against particles in the nanoscale size range. Particle theory suggests that the maximum penetrating particle size is of the order of 300mn and that collection efficiency improves below that particle size due to capture by diffusion. This has been confirmed in several studies. Further work is required to investigate human factors such as leakage around the face seal.

In relation to dermal exposure the use of gloves and airtight fabric clothing has been examined. It has been suggested that some kinds of skin protective equipment (SPE) might have limited effectiveness. For gloves manufacturing design and material thickness are major issues in determining whether or not nanoparticles penetrate. In some cases two layers are recommended. More work is required.

2.3.8.2.2 Assessment

[83] Hughes, J., Rees, S., Kalindjian, S., & Philpott, K. (2011). Principles of early drug discovery. *British Journal of Pharmacology*, *162*(6), 1239–1249. http://doi.org/10.1111/j.1476-5381.2010.01127.x

[84] CEFIC RMM Library http://www.cefic.org/Industry-support/Implementing-reach/Guidances-and-Tools1/

[85] Specific advice on fulfilling information requirements for nanomaterials under REACH (RIP-oN-2) – Final Project Report.

There are no significant equipment needs or gaps in RMMs. RMMs used for MNs are in general the same as are used for traditional chemicals. No new nano-specific control approaches have been or need to be developed. On the evidence available, a change to the default values for these types of RMM could not be justified. However, data of the efficacy of these control approaches (with the exception of PPE – Respirators) when used to control risks associated with exposure to MNs is largely lacking.

2.3.8.3 Decision making tools (Exposure models)

2.3.8.3.1 Relevant questions

Q1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
											X				

The predominant type of decision-making tool is control banding (CB). CB combines available hazard information about a substance with a qualitative estimate of potential for inhalation exposure associated with a particular task or activity. Scores are estimated for both hazard and exposure and the combination of these provides a risk score. Generally, CB tools are developed to help employers and employees with limited occupational health and safety experience to prioritize the risks in exposure situations and so it is the intention that the information needed for the use of the tool should be accessible and understandable by the user.

Information used to assess that hazard is typically drawn from safety data sheets, including physico-chemical characteristics, presence of risk phrases of specific statements about the hazard level. Factors which modify expose estimates can combine physico chemical characteristics such as the dustiness (friability) of the material and aspects of the usage scenario such quantity of the materials used, type of handling, localized controls, segregation, dilution/dispersion, personal behaviour, separation (personal enclosure), surface contamination, and respiratory protective equipment.

Hazard and exposure are scored and the combination of these two provides an overall risk score typically assessed in a 3 x 3 or 5 x 5 matrix. At that level it is a risk assessment tool. In some such schemes once risk levels are evaluated, users are then provided with options on control strategies which they may choose to apply. Based on the application of these control measures of the risk assessment can be modified.

Several CB schemes have been developed for nano, either as variants on existing schemes or developed from new. Among the most useful/well known are;

Stoffenmanager Nano[86]

ANCES CB tool[87]

ISO/TS 12901-2:2014[88]

Swiss Precautionary Matrix

Advanced REACH Tool (ART)[89]

Key aspects of these tools have been review as part of the FP7 project MARINA[90] and will be further assessed and validated within the NANOREG project.

Stoffenmanager Nano is publically available web based tool developed out of Stoffenmanager, an existing risk management software. A relatively high level of expertise is required to use this effectively. Stoffenmanager Nano provides control banding, risk banding and risk assessment (qualitative). It concerns single particles as well as agglomerates or aggregates. Possible worker exposure situations in the life cycle of nano products are distinguished in four general source domains, fugitive emissions during synthesis,

[86] Stoffenmanager Nano https://nano.stoffenmanager.nl/default.aspx
[87] Riediker M, Ostiguy C, Triolet J,Troisfontaine P, Vernez D, Bourdel G, Thieriet N, Cadene. A Development of a Control Banding Tool for Nanomaterials. Hindawi Publishing Corporation Journal of Nanomaterials Volume 2012
[88] ISO/TS 12901-2:2014 Nanotechnologies -- Occupational risk management applied to engineered nanomaterials -- Part 2: Use of the control banding approach
[89] https://www.advancedreachtool.com/
[90] Marina Deliverable D 6.2 Evaluation of existing exposure models (2014)

handling and transfer of powder, dispersion/spraying, fracturing and abrasion. Parameters used for hazard banding approaches are particle diameter and length, morphology, (water) solubility, degree of agglomeration, bioavailability, (surface) reactivity, catalytic activity, and composition. In addition, characteristics may be based on its parental material, such as its classification and labelling. Certain types of MNs are specifically allocated to hazard bands, dependent on particle size. As the scientific basis on which the Stoffenmanager Nano is developed is still evolving, the estimates of potential risks have a high degree of uncertainty, much larger than those in risk assessments involving traditional chemicals. It covers only respiratory exposure. There is no published validation of this tool.

ANCES CB banding tool is targeted at OSH practitioner level and assumes a 'central support' that overlooks the risk management strategy and provides risk assessment expertise. Model covers all stages of the value chain and includes powder handling, spraying, and abrasion and synthesis activities. The allocation to an emission potential band is defined according to the physical form of the MN, whether raw or included in a matrix and can then the band can be modified by the process.

The control banding tool in ISO/TS 12901-2:2014 is closely linked to the ANCES CB banding tool and essentially the same parameters and logic to allocate to exposure and hazard bands. Currently there is no web based implementation of this tool.

The Advanced REACH Tool (ART) version 1.5 incorporates a mechanistic model of inhalation exposure and a statistical facility to update the estimates with measurements selected from an in-built exposure database or the user's own data. This combination of model estimates and data produces more refined estimates of exposure and reduced uncertainty. However, the ART is not specifically designed or calibrated for nanoparticle exposure.

The Swiss precautionary matrix is a questionnaire based tool which leads to a score lead with two possible answers, (i) The need for action can be rated as low, or (ii). Nano-specific action is needed. However, the tool does not provide a recommendation as to what the necessary control action is.

In NANOREG there is a specific task which is assessing and validating these tools. All of the models listed above will be reviewed and a comprehensive validation exercise will be performed using existing scenarios and data from the MARINA database (as a first phase) and data collected in the NANOREG project (2nd phase) including data generated as part of a large scale multi-instrument experiment assessing determinants, dispersion in a chamber and side by side comparison of instruments (NANOREG D3.4). The model validation exercise will compare predicted with actual exposures, consider ranking and will include an inter-user study. The outcomes will be validated models, recommendations for developers (on how the models could be improved) and guidelines for users of each model which will identify assumptions, limitations, range of application and adjustment factors (NANOREG D3.8).

2.3.8.3.2 Assessment

CB schemes are relatively easy to use and are well understood in risk management context but there are a number of challenges with their use for risk management. None of these schemes are well validated with respect to the validity of the assumptions which they use, the levels of the exposure which they predict or the effectiveness of the control methods which they produce. All require significantly greater knowledge than similarly schemes used for traditional chemicals. In many cases, the information required may not be readily or at all available to the user and in some cases the information which the user has available (for example from Safety Data Sheets) may simply be erroneous and that may not be evident to the user. These difficulties are likely to remain while the underlying knowledge about MN risks continues to develop. However, when used by someone with a sufficient level of knowledge, including some knowledge of MN risks these tools may provide a good starting point in the development of an effective control approach for working with MNs.

2.3.8.4 Release systems including dustiness testing

2.3.8.4.1 Relevant questions

Q1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
												X			

Dustiness, defined as the tendency of a powder material to generate airborne particles under an external energy input, has been tested by different systems to simulate powder handling processes in occupational settings. These experiments, which characterize airborne particle concentrations and size distributions,

facilitate possible scenario predictions in exposure assessments. European Standard 15051, for measuring the dustiness of bulk materials, describes two reference testing procedures: the rotating drum method (EN 15051, part 2) and the continuous drop method (EN 15051, part 3). However, these systems required large amounts of test materials that are not suitable for MNs due to their costs and potential risks. Alternate methods are being developed which use smaller quantities and are more appropriate to MN. A downscaled, modified test system has been developed combining continuous drop and a significantly smaller rotating drum, and this permits the use of smaller quantities of test materials (Schneider and Jensen 2008). Boundy et al. (2006) established an air jet dispersion method for testing the dustiness of pharmaceutical powders. The basic principle involves injecting powder through an orifice into a glass jar for subsequent characterization.

Ding et al (2015)[91] developed and evaluated four experimental setups delivering different aerosolization energies were used to test the resultant aerosols of two distinct MNs (hydrophobic and hydrophilic TiO2). The reproducibility of results within each system was good. However, the number concentrations and size distributions of the aerosols created varied across the four systems; for number concentrations, e.g., from 103 to 106 particles/cm^3. Moreover, distinct differences were also observed between the two materials with different surface coatings.

2.3.9 *Overall assessment of equipment priorities*

In this deliverable we have reviewed the equipment in place and under development which can and will provide support for improved regulatory oversight for nanotechnology, MN risk issues. The assessment of regulatory relevance of the identified equipment was based largely on the needs framework identified in the 16 regulatory questions developed as part of the NANOREG project. The regulatory questions are broad in scope and cover the full spectrum of the risk assessment paradigm from characterisation, toxicology, exposure assessment and risk management. There has been no prioritisation between these questions, all are considered equally important. Consequently, the types of equipment necessary to answer these questions are also broad including characterisation techniques, methods for the detection and quantification of exposure, new and improved methods of toxicity and kinetics assessment.

At a first level of assessment, the regulatory questions were analysed to determine for each question which types of equipment were necessary to provide improved answers to that question. The analysis developed for each question, the data need the tools implied, and the measurement context. This enabled a matrix to be developed in which the type of measurement and therefore equipment needed was mapped to the questions. It was clear that each question required multiple measurement types in order to provide an answer to the question and that each measurement type would support the development of answers for multiple questions. It was also possible through this process to group the measurement types into 5 groups, namely, Physicochemical characteristics, Detection and quantification of concentration and exposure, Toxicity, Kinetics and a catchall “Others” category. Other types of grouping may have been possible.

In the final stages of the analysis we assessed the state of the art for the equipment options for each measurement type required. This did not produce a clear outcome in terms of the level of maturity of each of the methods.

Given the fact that the specific methods needs are often specific to individual MN types, it would be difficult and perhaps counter-productive to develop a general prioritisation across the highly diverse equipment needs implied to answer the set of NANOREG regulatory questions. For example, saying that one kind of particle sizing or biological effects assay is more important than another would depend entirely on the MN type (e.g., carbon nanotube or metal oxide) and the decision being made (e.g., screening analysis of product development candidates for construction materials by industry or a safety evaluation of a food additive by a regulatory authority). However, based on qualitative review of the breadth of utility (i.e., number of NANOREG questions potentially addressed in Table 3) and assessment of need (i.e., support of decisions for many MN types), the categories of HTD/A and particle size distribution are most generally called out as supportive of many MN types and decision types. Therefore, resources applied to the development of methods under these categories may affect the greatest number of decisions.

Furthermore, based on application specifically to SbD the argument can be made that selecting safer alternatives among multiple possibilities with similar utility in a particular product would be difficult if not impossible without high throughput data acquisition and data analysis methods capable of comparing many versions of MN and many biological and environmental contexts for those versions. Knowing what the final MN form is to which exposure occurs would also be important in SbD, and so methods that allow evaluation

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of transformations (e.g., multiple forms from multiple release scenarios and pathways) for similar utility would also be needed.

To gain further insight into the relative needs for equipment and methods development to support SbD we have engaged with the wider PROSAFE project, specifically in relation to the PROSAFE Delphi poll and have developed a short set of questions to be included in the second round of the poll. These questions are intended to gather a wider opinion on the relative importance of the identified measurement types and equipment needs. The questions are shown below.

The responses to these questions will be analysed and presented in the overall output of the Delphi Poll in Deliverable 2.3 which will serve as input into the white paper.

SECTION 3: MORE DETAIL ABOUT METHODS NEEDS

1) Improvements to which of the following overall nanomaterial measurement systems are most urgently needed to support risk management?

There are many possible improvements to nanomaterial measurement systems suggested by research; however, it is not clear which improvements are most urgently needed to support risk management.

Please rank from 1 = most urgent to 7 = least important, or not needed

If you include the 'other' option in your ranking, please identify this measurement system in the text field below

	Assessment of Physicochemical Characteristics	Detection and quantification of Exposure	Methods for Toxicity Testing	Methods for Assessment of Toxicokinetics	High Throughput Testing Systems	Lab on a Chip approaches to support risk assessment	Other
1	O	O	O	O	O	O	O
2	O	O	O	O	O	O	O
3	O	O	O	O	O	O	O
4	O	O	O	O	O	O	O
5	O	O	O	O	O	O	O
6	O	O	O	O	O	O	O
7	O	O	O	O	O	O	O

Please explain your answer(s)

2) For Physicochemical Characterization, which measurement types most urgently need methods development to support risk management for current nanomaterial uses?

Please rank from 1 = most urgent to 7 = least important, or not needed

If you include the 'other' option in your ranking, please identify this measurement system in the text field below

	Particle Size Distribution	Volume Specific Surface Area	Aspect Ratio (shape)	Particle Rigidity	Solubility or Dissolution Rate	Chemical Composition (including surface)	Other
1	O	O	O	O	O	O	O
2	O	O	O	O	O	O	O
3	O	O	O	O	O	O	O
4	O	O	O	O	O	O	O
5	O	O	O	O	O	O	O
6	O	O	O	O	O	O	O
7	O	O	O	O	O	O	O

Please explain your answer(s)

3) For Detection and Quantification of Exposure, which measurement types most urgently need methods development to support risk management for current nanomaterial uses?

Please rank from 1 = most urgent to 5 = least important, or not needed

If you include the 'other' option in your ranking, please identify this measurement system in the text field below

	Number Concentration	Number Size Distribution Concentration	Mass Concentration	Surface Area Concentration	Other
1	O	O	O	O	O
2	O	O	O	O	O
3	O	O	O	O	O
4	O	O	O	O	O
5	O	O	O	O	O

Please explain your answer(s)

4) For Toxicity and Toxicokinetics, which measurement types most urgently need methods development to support risk management for current nanomaterial uses?

Please rank from 1 = most urgent to 7 = least important, or not needed

If you include the 'other' option in your ranking, please identify this measurement system in the text field below

	Cytotoxicty	Inflammation	Oxidative stress	Genotoxicity	Absorption	Distribution /accumulation	Other
1	O	O	O	O	O	O	O
2	O	O	O	O	O	O	O
3	O	O	O	O	O	O	O
4	O	O	O	O	O	O	O
5	O	O	O	O	O	O	O
6	O	O	O	O	O	O	O
7	O	O	O	O	O	O	O

Please explain your answer(s)

5) Please rank the following sectors of use with regard to need for development of High Throughput Test Systems to support risk management decisions.

Please rank from 1 = most urgent to 4 = least important, or not needed

If you include the 'other' option in your ranking, please identify the use in the text field below

	Physicochemical characterisation	Toxicology testing	Ecotoxicology testing	Other
1				
2				
3				
4				

Please explain your answer(s)

3 Deviations from the work plan

The deliverable was delayed partly due to delivery of contributions from partners, and partly due to linking the report to the next phase of the Delphi poll. In addition, further comments from the MC needed to be addressed.