A common European approach to the regulatory testing of nanomaterials

NANoREG

Grant Agreement Number 310584

Deliverable D6.04

Inventory of existing regulatory accepted toxicity tests

applicable for safety screening of MNMs

Due date of deliverable: *2015/10/31*

Actual submission date: *2016/04/25*

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Work package/task:	WP6 / Task 6.2
Document status:	draft / final
Confidentiality:	confidential / restricted / public
Key words:	Safe-by-design, safety screening, risk potentials

This project has received funding from the European Union Seventh Framework Programme (FP7/2007-2013) under grant agreement no 310584

Lead beneficiary for this deliverable: National Institute for Public Health and the Environment, RIVM, partner number 5

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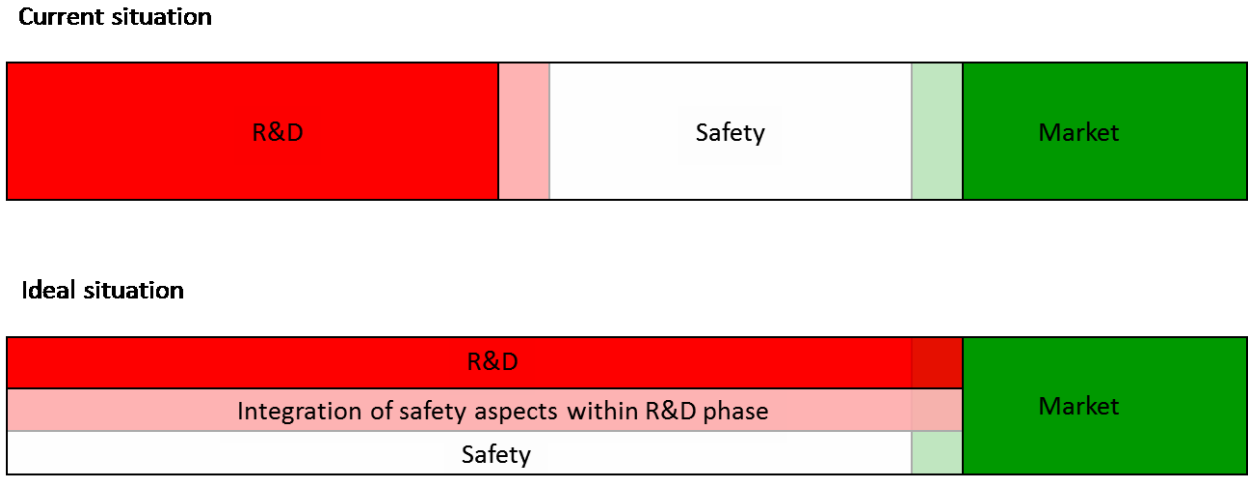
1 Description of task

1.1 Keeping pace with innovation

Innovation through nanotechnology is seen as a potentially powerful booster of economy. However, innovation requires responsible risk management by companies to prevent unnecessary unacceptable risks and costs. For that reason many companies prefer to reduce uncertainties by leveraging a stage-gate innovation process. Stage gates provide a discipline and a structure for identifying problems, and allow keeping track on the evolving business case. The discussion on safety of nanomaterials, however, demonstrates that the evaluation criteria typically used at each gate need to be elaborated. The evaluation criteria merely focus on getting the technology ready, on time to market and return on investments. Insufficient knowledge on health risks for workers, users and environment in early stages of innovation leads to discussions about safety at the end of the innovation process when the nanomaterial or nanomaterial containing product is ready for commercialization.

'Safe-by-design' is a concept that has been developed and applied in the engineering and construction sectors and is now gathering momentum amongst scientists, regulators, and industry for use in the development of manufactured MNMs. Applying the concept of Safe-by-design to the development of MNMs has been encouraged by some of the significant challenges facing regulation as a means of guaranteeing safety in this field. For example, a report by the UK Royal Commission on Environmental Pollution (2008) concluded that there is a fundamental problem with relying solely on regulatory instruments because there is often a considerable time lag between innovation, the products that result from it, and the subsequent case for amendment or development of appropriate regulations. This has also created a growing awareness amongst stakeholders that responsible innovation is required. That is, innovation that is directly oriented towards social and environmental needs, actively seeking to anticipate potential future impacts, carefully considering social and ethical issues and adapting innovation trajectories where necessary. In many countries, responsible innovation is supported by state-led initiatives in a quest to ensure the safety, desirability and usefulness of emerging innovations for both society as a whole and the environment on which we depend. While regulation plays an ongoing important role for ensuring product safety, within the approach of responsible innovation, considerations of safety questions are also moved further up in the innovation chain. Here, they become integrated into the research and development process itself, e.g. through the operationalization of concepts such as Safe-by-design. Figure 1 contrasts the targeted transition from the current situation where regulation is the primary approach to guaranteeing safety and occurs pre-market readiness of a product, to a new order where principles such as those of 'Safe-by-design' are integrated into the development and manufacture of new MNMs, a key aim of NANoREG.

Figure 1. The changing role of safety in innovation



1.2 Beyond the State of the Art

Within NANoREG, WP6 seeks to explore possible routes of filling the increasing gap between innovation and risk analysis, by:

a) *Being prepared*: The development of more effective foresight of the potential impact of new manufactured MNM (MNM) applications on human and environmental health by coupling horizon scanning to risk analysis. This will have strong potential to ensure the earliest identification of uncertainties and associated concerns for emerging innovations and will contribute to better availability of safety data before marketing.

b) *Safe-by-design*: The Safe-by-design concept aims at creating an integrated research strategy, which enables the consideration of safety aspects for humans and the environment throughout the product/material design phase. Such an approach maximizes resource use and expedites the development of products containing MNMs and new MNMs that are Safe-by-design. Two building blocks will be addressed in this activity. One building block is based on better exploiting existing knowledge and tools, and where possible applications of those tools already accepted for regulatory testing. The second building block will focus on how and with which toxicity information the Safe-by-design approach will best fit the requirements.

c) *Turning risks into business opportunities*: The requirements placed on industry to comply with future legislation might provide new business opportunities for standardisation and testing laboratories, as well as high tech industries who can translate the issues raised to investigate (eco)toxicity or exposure into efficient tools. Eventually the principles of Safe-by-design will evolve into practical measures for product and material design that can also offer new business opportunities.

1.3 Aim of deliverable

The Safe-by-design concept has gained interest over recent years as it aims at reduction of hazard in order to reduce potential health and environmental risks at an early phase in the innovation process. The aim of this deliverable (D6.4) is to propose a strategy to efficiently screen for potentials for toxicity during early stages of an innovation process. In addition, existing test methods are evaluated for their applicability.

2 Description of work & main achievements

2.1 Summary

Safe-by-design has to take place in early stages of innovation. In present everyday practice, innovation is focused on technology readiness rather than investigating potential for health risks. In this report, an innovative and efficient screening strategy is proposed to identify potential risks of MNMs at early stages of innovation. The basis for the screening strategy are six key risk potentials: solubility/dissolution rate, stability of coating, accumulation, genotoxicity, inflammation and ecotoxicity. The second layer of the screening strategy addresses the testing strategy: which parameters are key to describe the risk potentials at the first stages of the innovation process. The final layer of the screening strategy addresses the methods: which tests are available to measure the parameters? This screening strategy is a concrete method within the Safe-by-Design concept, useful for both industry and regulators at the first stages of the innovation process.

2.2 Introduction

2.2.1 Definition of a MNM

A clear definition to discriminate MNMs from other materials is a prerequisite to include provisions for MNMs in legislation. In October 2011, the European Commission published the 'Recommendation on the definition of a MNM'. The European Commission based its recommended definition mainly on a reference report by the European Commission Joint Research Centre (JRC) (Lövestam et al., 2010) and a scientific opinion by the SCENIHR (SCENIHR, 2010). Inevitably, the final wording, and especially the thresholds, comprise political compromises as well. In its Recommendation (EU, 2011) the European Commission states that:

'MNM' 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.

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%.

Alternatively, it is stated that a material should be considered as falling under the definition where the specific surface area by volume of the material is greater than 60 m^2/cm^3. The Recommendation also includes definitions for 'particle', 'agglomerate' and 'aggregate'. The

Commission foresees a review of the definition, particularly focusing on the appropriateness of the 50% limit.

The recommendation contains a rather broad description of the term MNM. It potentially includes all kinds of MNMs/nanoparticles irrespective of their origin. The definition thus also covers natural nanoparticles, such as volcanic ashes, as well as incidental MNMs like nanoparticles originating from human activity such as exhaust of combustion processes, and the intentionally manufactured MNMs/nanoparticles by industry, which may be used in various industrial processes and consumer products. With the definition the Commission solely aims to identify substances within a specific size range and explicitly does not aim to classify MNMs as intrinsically hazardous. For this purpose it is appropriate to use a broad description, especially since no distinction can be made on the origin of the particle when only size is measured. However, as with other materials, distinctions between natural, incidental or manufactured materials need to be made in the specific areas of legislation since the need for such a distinction will be related to the purpose of that legislation.

The Commission states that the definition should be used as a reference for identifying MNMs for legislative and policy purposes in the European Union. However, whether defining a material as MNM has regulatory consequences should be decided on in the specific regulatory frameworks. It is also indicated that for certain regulatory frameworks deviations may in some cases be necessary, either to exclude materials that fall within the definition or to include materials that are beyond the definition. For this reason, the option is given by the Commission to adjust the number size distribution threshold to a value between 1% and 50% (Bleeker et al., 2013).

Furthermore, the JRC Science and Policy report series have recently published three reports, where information on scientific-technical issues is collected that should be considered when reviewing the current EC MNM definition. The first report discusses how the different MNMs definitions are used and how they diverge. The second report also puts the EC MNM definition in perspective by comparing it with other existing MNM definitions, thereby identifying the most prominent characteristics of the EC definition.
Based on the feedback received regarding the current definition, compiled in the first report of the series, and its assessment, presented in the second report, the JRC has provided a new a set of indications on how the definition could be modified to improve its clarity, effectiveness and implementability. These recommendations were included in a final report (Part 3 of the series), which was released in 2015. Within NANoREG, the definition recommended by the Commission will be used for identifying MNMs.

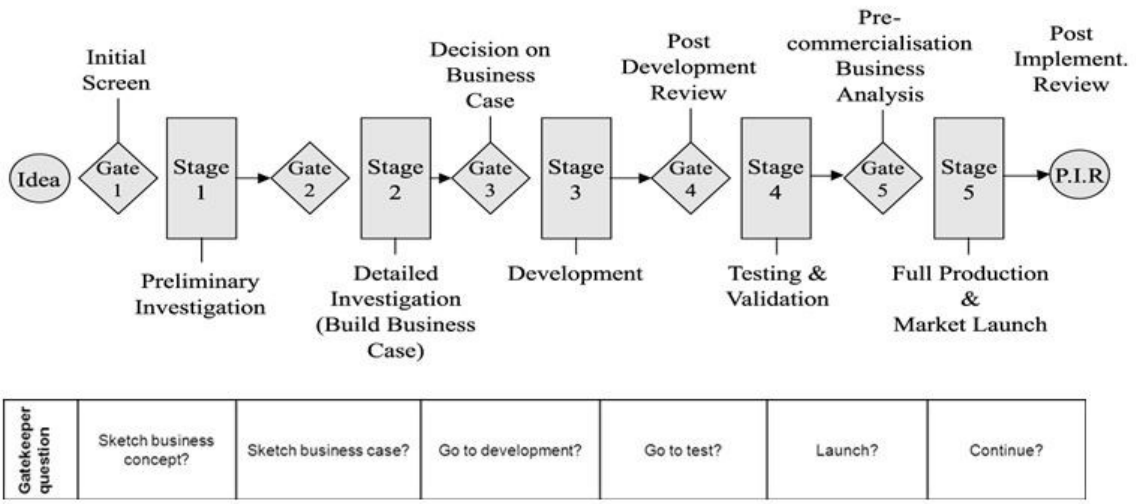
2.2.2 Safe-by-design concept

Safety by design or Safe-by-design is originally a concept that was developed and utilized by engineers, particularly those working within the construction industry. The basic idea is that in the

design and development of products, it is important to consider and incorporate safety considerations. Within the Safe-by-design concept, the functionality of a material and its toxicity are considered in an integrated way. Safe-by-design has traditionally been about incorporating safety considerations into the design, construction and maintenance of engineered products and workplaces. It can be formally understood as the integration of hazard identification and risk assessment methods early in the design process to eliminate or minimise the risks of injury throughout the life of a product or structure being designed, including construction, use, maintenance and destruction. It encompasses all design factors including facilities, hardware, systems, equipment, products, tooling, materials, energy, controls, layout and configuration. Safe-by-design can also be addressed as 'Prevention by Design' with the goal of designing out occupational hazards by focusing on hazard elimination and substitution (NIOSH, 2014). Safe-by-design in this WP is about incorporating considerations of potential health (workers and envisaged users) and environmental safety concerns into the research and development phase of an innovation process and where necessary adapting the process and/or product design so as to create safer outcomes.

An innovation process model is needed to define and structure a Safe-by-design approach. A widely implemented product innovation process model is the Stage-Gate idea-to-launch model (see Figure 2). The Stage-Gate product innovation system is a conceptual and operational map for moving new product projects from idea to launch and beyond, a blueprint for managing the new product development process to improve effectiveness and efficiency (Cooper, 2008).

Figure 2. Stage-Gate product innovation process



During the stages the proper work is carried out: ideation, development, tests, up-scaling etc. In each gate so-called gatekeepers decide on the fate of an innovation project: proceed, alter (proceed through gate but minor alterations in the next phase), recycle (repeat the stage with major alterations), on-hold (wait for other projects, technologies, licenses, regulations etc.), or terminate.

The main questions addressed by the gatekeepers are included in Figure 2. The decision is always based on balancing costs, benefits and risks (not health risks!). Whether, how and to which extent a stage gate process is run depends on the scope of an innovation project:

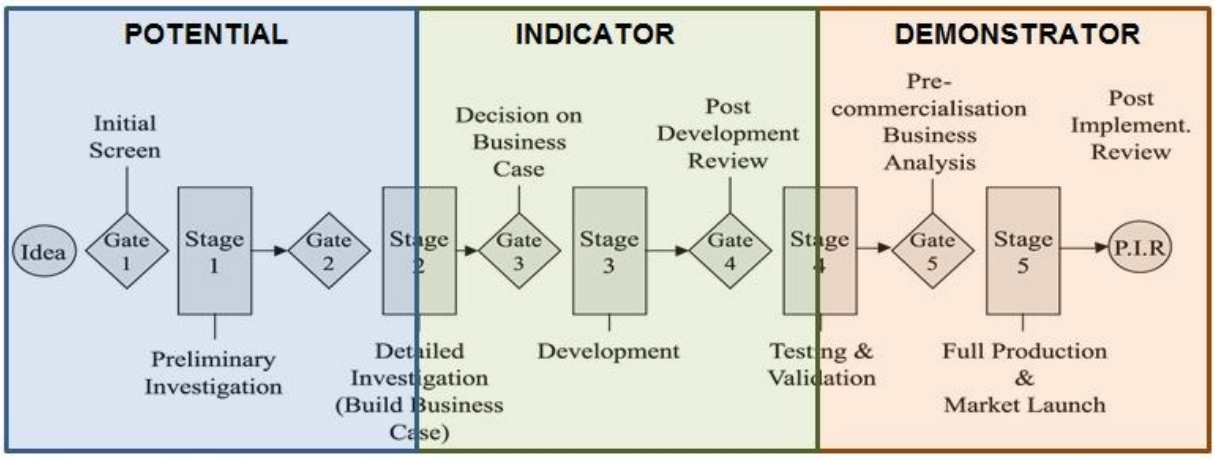
- For smaller projects stages 1 and 2 and/or stages 3 and 4 can be merged; with only 1 idea for a smaller project gate 1 may be merged with gates 2 and 3.
- The stage gate process can be run in two or more sequences and these also in parallel: E.g. During the first stage gate process /innovation project a technology is developed, during a second a product platform using this technology (there might be other platforms and products developed in yet other stage gate processes) and in the third every geographical business unit develops a product for its market's requirements (i.e. several daughter projects run parallel).
- The stage gate process can even contain built in loops within a stage e.g. if certain criteria are failed.

This stage-gate innovation model can give guidance to develop the Safe-by-design concept within NANoREG. The structure of this innovation process will be used in this deliverable to indicate the applicability of the safety screening method for MNMs.

2.3 Safety screening of MNMs

MNMs of the same chemical composition can vary in many ways (*e.g.* size, shape, charge). In essence, current regulatory requirements would require testing all different variations or forms. To perform a risk assessment for all different variations of MNMs in a fast and efficient way, innovation within the risk assessment and regulatory process of MNMs is advisable. Moreover, insight in potential hazard or risks is needed during the early stages of innovation without performing an extensive testing strategy. Here, we propose a first screening strategy to identify potential risks of MNMs at early stages of innovation. When innovations progress towards market application, information has to be lined up in the direction of regulatory requirements. We therefore suggest for the first stages of innovation to identify the *potential* for health risks, followed by *indicators* for risk at mid stages of innovation, and to be finalized by *demonstrators* for health risks as laid down in regulatory requirements (see Figure 3). This approach aims at improved insights into health risks, both environmental as human, before marketing of innovative MNM or nanoproducts. Moreover, it supports decisions at the various stages of development e.g. for further investments, thereby supporting to build a strong business case.

Figure 3. Stage-Gate innovation process including risk terminology

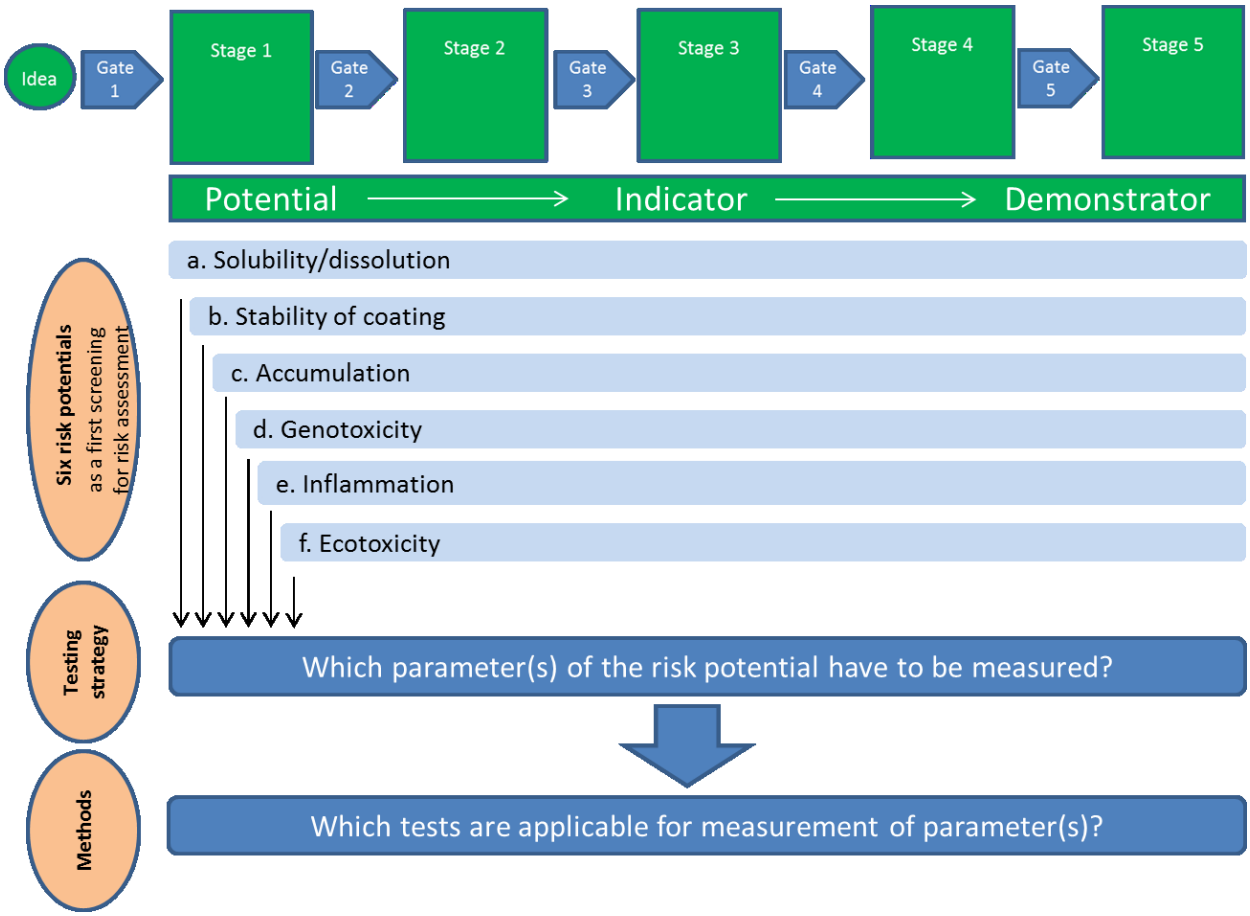


This deliverable is focused on the first two stages where the potential for a risk of a MNM is potentially assessed in a preliminary way by desk assessment and/or basic experimental datasets (Figure 3, blue part).

2.3.1 Description of the screening strategy

An innovative screening strategy is proposed to identify potential risks of MNMs along the innovation process in a fast and efficient way (see Figure 4). The basis for the screening strategy is formed by six topics: solubility/dissolution rate, stability of coating, accumulation, genotoxicity, inflammation and environmental toxicity. The next step is defining a testing strategy: which parameters are key to describe the risk potentials at the stage of 'potential', 'indicator' and 'demonstrator'. Subsequently appropriate methods need to be described to measure the parameters. Moreover, lessons learned in the Dutch NanoNextNL (www.nanonextnl.nl) programme indicate that innovators are in need for assistance to interpret the results. At this moment, we prefer the development of a kind of manual rather than defining bench mark values which we envisage as a source for debate. The latter would distract attention from working towards safe designs.

Figure 4. Schematic view of the screening strategy for risk assessment of MNM in relation to the testing strategy and methods



2.3.2 Who can use this screening strategy?

The screening strategy could be of use for all actors along the innovation chain but mainly for academics and industry, since the screening strategy presented here focusses on the first stages of the innovation chain. In the first stages of the innovation chain, safety information may be gathered to gain insight in potential risks of a MNM and to make a balanced decision in the gates about the fate of an innovation. In general, the strategy can be applied throughout the whole life cycle of a MNM; from the development of the MNM (including Safe-by-design) until its disposal. However, it seems likely that different types of data (tests) and different choices will be made, depending on the stage of development and availability on the market.

2.3.3 What is the argumentation for this selection of risk potentials?

When looking for *nanospecific* potentials for health risks the following aspects were considered:

1) Aspects specific for MNMs like solubility (if a particle readily dissolves into its molecular or dissociated chemically form, then it no longer needs to be treated as a MNM) or stability of the coating of a particle (if the coating readily dissolves of the core then one needs to know to consider whether the coated particle and the uncoated particle may exhibit the same kinetics and toxicity)

2) Markers for increased probability of health risks like accumulation in tissue and organs or in environmental compartments

3) Unacceptable toxicity like genotoxicity as a first marker for carcinogenicity or inflammation as a general marker for many (chronic) pathological conditions

Along the incremental stages and entering the regulatory arena more quantitative data suitable for quantitative risk assessment need to be gathered. The dossier requirements as demanded by the regulations in place need to be met. In NANoREG, this is merely given in by the REACH requirements. The SbD concept tries to tune the potentials for risk as much as possible to the REACH requirements in order to develop a most efficient approach.

The argumentation behind the selection of each of the six risk potentials is described below.

Solubility: Solubility and dissolution rate are characteristics which may influence the properties of the MNM and determine its fate in organisms and the environment. By the time a MNM is fully dissolved, it need no longer be regarded for as an MNM, but it its molecular form needs to be regarded.

If a MNM immediately falls apart into its molecular form or dissociate, no nano-specific risk assessment is needed and one can refer to the risk assessment of the molecular form. However, it is very important under which circumstances this is measured (in water, in cell medium, in physiological solutions, such as macrophage fluid or lung lining fluid, in the organism as tested in an in vivo experiment, etc.), and whether the MNMs fall apart into the molecular form or ions and where as well as how fast this process takes place. Furthermore, the term solubility should be described very precisely, as it is sometimes confused with dissolution or dissolution rate.

Stability: The stability of the coating of a MNM is very important for the behaviour and effects in humans and the environment. This risk potential should answer the question if the surface coating or modification of a MNM will maintain or will be removed from the MNMs in its different life cycle stages. Kinetics and toxicity depend on the stability of the coating of a MNM and this may differ from the core. Therefore, information is needed about the form and coating of a MNM in order to gather the relevant kinetic and toxicity data.

Accumulation: Accumulation of MNMs in the human body or environment is a marker for an increased likelihood of long term effects. Therefore, accumulation is included as a risk potential. MNMs will probably only seldom cause acute toxicity. However, MNMs tend to accumulate, which may cause long term effects after chronic exposure.

Inflammation/immunotoxicity: Inflammation or immunotoxicity is an important marker for chronic pathological conditions, such as lung cancer, cardiovascular disease or neurological diseases. MNMs are likely to trigger the immune response due to a high degree of surface reactivity and the

cell membrane permeability. Depending on the material, size, and ligands, particles themselves can induce the immune response or lead to other health effects.

Genotoxicity: Genotoxicity is the ability of substances to damage DNA within organisms. Genotoxic agents can give rise to mutations. Because mutations can lead to cancer, genotoxicity evaluation has been utilized widely to evaluate the carcinogenic potential of chemical and physical exposures. Although both positive and negative results have been reported on the genotoxicity of MNMs in various cell and animal test models, some data indicate that MNMs may be genotoxic. Since MNMs may be genotoxic, genotoxicity is included as a risk potential.

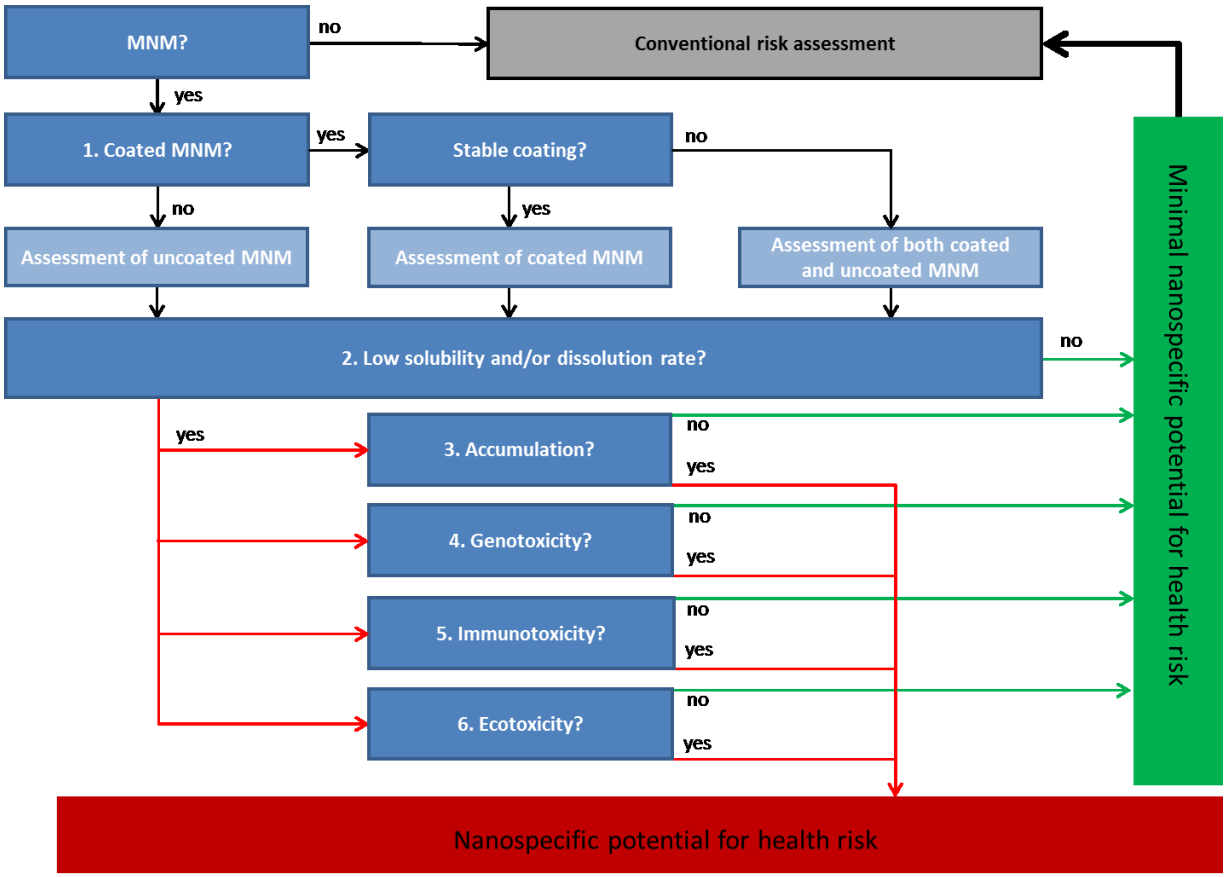
Ecotoxicity: In parallel to immunotoxicity and genotoxicity for human health, toxicity for the environment should be regarded. The potential for ecotoxicity is described here as a kind of bulk potential but is explored for further refinement in this report. Up till now several publications have indicated there is a ground for assuming ecotoxicity caused by MNM (Wang et al., 2012, Van Hoecke et al., 2009, Tong et al., 2015, Garner et al., 2015).

2.4 From potentials to a screening strategy

The arguments for the suggested potentials are based on a logical reasoning thereby presuming a certain order of addressing the potentials. Figure 5 visualizes this order and demonstrates that it is important to first address the question that relate to whether a material is to be considered as a MNM. This is the starting point of the flow chart, followed by addressing the risk potentials in case of a MNM.

Arguments for concluding no *nanospecific* potential for health risk can roughly be divided into two types: 1) the material is not to be regarded as a (nano)particle, or 2) the material is regarded as a (nano)particle but no indications for causing toxicity were observed. The first types of arguments can be addressed by physical-chemical properties, whereas the second types need to be addressed by simple toxicity tests.

Figure 5. Flow chart for assessing potential for health risk of MNMs



2.4.1 *MNM characterisation*

The key question in the first step of the flow chart is: Is it a MNM? (This is the first question in the flow chart, however, this is not a risk potential)

If this question is answered with **no**: no nanospecific potential for health risk is expected and risk assessment can be performed in the conventional way.

If this question is answered with **yes**: nanospecific data/testing is required (see testing strategy)

2.4.1.1 Testing strategy for MNM characterisation

The following identifiers/parameters (physical-chemical characteristics) are proposed to address the question whether you are dealing with a MNM:

1. Particle size (distribution)
2. Chemical composition and impurities
3. Shape
4. Surface area
5. Surface charge
6. Agglomeration/aggregation

1. Particle size (distribution)

To determine whether a substance is a MNM, the EC definition of a MNM is used, which is based on size (EC, 2011). The EC definition for a MNM is as follows: *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.*
(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 %.)
However, here we deviate from this definition by not including the size distribution threshold of 50% as not to exclude materials that contain only a small fraction in the nanoscale. It should be kept in mind however that if the definition is applied without a cut-off entirely, all substances will likely be defined as MNMs as they may contain a small fraction in the nanoscale range (ICCA, 2010). However, during R&D phases it is recommended to consider all nanofractions regardless any given threshold percentage. Knowing the particle size distribution can also be an important determinant of its behaviour. Furthermore, it is important to specify that MNMs are materials that were specifically engineered. As such, naturally occurring and unintentionally produced substances are not included here.

Once it is established that a substance contains a nanofraction relevant to be studied, it is regarded highly relevant to determine the physical and chemical properties as described under 2 to 6. As described in more detail below, several of these parameters are inter-dependent, which means that changing one property may result in a change to several others. For example, in the dissolution media an agglomerate of nanoparticles may dissolve into singular nanoparticles with different properties (including, size, shape, surface chemistry) than the agglomerate.

2. Chemical composition

The chemical composition (i.e. carbon based, nanocomposites, metals & alloys, nano-polymers, -glasses or -ceramics) of the MNM is an important discriminator parameter as it determines in large part its behaviour and toxic properties. The MNM chemical composition also includes the degree of purity, the nature of the impurities and the percentages of the main impurities (Bleeker et al., 2013). Impurities could also determine/be responsible for the hazardous properties of the MNM. Chemical composition may change as the MNM for example is dissolved, incorporated into products or breaks down. The chemical composition of a substance fundamentally determines its fate and toxicity effects.

3. Shape

MNMs of the same chemical composition but with different shapes may have very different properties. MNMs can have one (i.e. nanowires, rods, tubular, straw), two (i.e. sheets, flakes, surfaces) or three (i.e. spherical, elliptical, cubical, tetrahedral objects) external dimensions in the nanorange; the resulting MNMs may have different electronic, optical, chemical reactivity etc, even if the chemical composition is the same. The shape of the MNM can also influence how it interacts with other particles (leading to agglomeration, for example), its dispersion in various media or the environment and how they interact within organisms or the environment (DuPont, 2007). The term “aspect ratio” refers to the ratio between a particle’s length and width. This parameter can be relevant to the toxicity of carbon nanotubes, nanowires, and other “needle shaped” particles.

4. Surface area

Relative surface area is related to particle size, shape, and porosity. As the size of a particle decreases, the ratio of surface area to volume increases or, in other words, the proportion of the atoms on the surface of the particle increases. This characteristic is important with respect to rate of reaction, dissolution, and adsorption. Specific surface area appears to be relevant for a number of parameters for toxicological and ecological risk assessment. It will dictate the surface charge density in cases where MNMs are surface functionalized, which has direct consequences on: (a) MNM interaction (i.e., agglomeration) with other naturally occurring particulate matter (i.e., contaminant vectors); (b) route of exposure as a function of surface ligand-biological interface (i.e., bioaccumulation pathway, bioavailability); and (c) mechanisms of toxicity (Sellers et al., 2015).

5. Surface charge

Surface charge influences the interaction of a particle with its surroundings (including agglomeration/aggregation behaviour, its stability and interaction with other with other materials), but also depends on the environment of the MNM (for example pH). Surface charge, as represented by zeta potential, influences the fate and transport of nanoparticles. Any surface

charge on nanoparticles causes electrostatic repulsion between particles or charge that can counter the tendency to agglomerate. Zeta (ζ) potential is an abbreviation for the electrokinetic potential in colloidal systems and can be related to the stability of colloidal dispersions. The zeta potential indicates the degree of repulsion between adjacent, similarly-charged particles in dispersion. For molecules and particles that are small enough, a high zeta potential will confer stability, i.e. the solution or dispersion will resist agglomeration. When the potential is low, attraction exceeds repulsion and the dispersion will break and flocculate. In nanotoxicology, zeta potential (surface charge) plays a key role in determining (1) the degree of colloidal interaction, which is itself a function of the pH and ionic strength of the bulk solution, and (2) the bioavailability of a compound when considering mass transport through charged membranes as related to exposure (Sellers et al., 2015).

6. Agglomeration/aggregation

Information on how easily aggregates or agglomerates are formed, and if they are formed at all, is important for assessing the toxic behaviour of the MNM in organisms or the environment. Moreover, its propensity to agglomerate can also influence its solubility, stability and its behaviour in the environment. On the other hand, agglomeration behaviour can differ, depending on the surrounding matrix in which the MNM is used (*e.g.* pH, ionic strength, medium used). Finally, the aggregation behaviour of the MNM also influences the morphology of the formed aggregates.

2.4.1.2 Methods: Which tests are available for MNM characterization?

1. Particle size (distribution)

The size distribution of a material should be presented as size distribution based on the number concentration (i.e. the particle number) and not on the mass concentration of a MNM product as a small mass concentration may contain the largest number fraction (Recommendation of European Commission, last updated 18 October 2011). Table 1 lists the various analytical methods to measure particle size (distribution) of nanomaterials. The Joint Research Centre's Institute for Health and Consumer Protection (JRC) evaluated a number of analytical methods of measuring particle size distributions as required for classification with respect to the EC Recommendation for the definition on nanomaterials[1]. Three techniques, i.e. Laser Diffraction (LD), Dynamic Light Scattering (DLS), and Centrifugal Liquid Sedimentation (CLS) were benchmarked against Electron Microscopy (EM). Eight nanomaterials which exhibit a wide diversity of physical-chemical properties (i.e. different primary particle sizes and shapes, different levels of aggregation/agglomeration, both inorganic and organic substances, and both uncoated and

[1] Douglas Gilliland, Neil Gibson, Uwe Hempelmann (2014).JRC technical reports; Basic comparison of particle size distribution measurement of pigments and fillers using commonly available industrial methods.

uncoated pigments) were tested. From the results of this study it was concluded that EM is the method which comes closest to being able to provide information about the particle size distribution as required by the EC definition, with none of the others being considered suitable for the task. However, there are many issues related to sample preparation and counting protocols which need to be resolved and standardized before EM could provide representative results and reasonable comparability. In addition, it was concluded that expertise and knowledge of the specific substances in question is necessary to obtain reliable results. Product-specific information supplied by manufacturers will be valuable in this respect. Hoehener et al. 2015 also state that direct imaging (Scanning Electron Microscopy (SEM) and Transmission Electron Microscopy (TEM)) allow to measure the number based size distribution (and structure, aggregates, shape) but that this technique is time consuming (manual process and a lot of images to process) and that the lack of an universal technique is hampering comparability between the results[2]. TEM has a higher resolution and therefore provides more information than the other microscopy techniques, SEM, and Atomic Force Microscopy (AFM). However, TEM is also more time consuming and costly. AFM is less costly and time consuming than SEM/TEM but sample preparation and choice of substrate have more requirements. The use of X-ray diffraction (XRD) is often compared to the microscopy techniques. XRD avoids issues of representative samples. However XRD can be time consuming and requires a large volume of sample. Nanoparticle Tracking Analysis (NTA) provides information of particle size, size distribution and a real time view of the nanoparticles in the sample. The sample must be a suspension for which a wide range of solvents can be used. NTA could be combined with techniques such as DLS to maximize the information obtained about a sample and check accuracy. Aerosol Time of Flight Mass Spectroscopy (ATOF-MS) can provide information about the particle size distribution but ATOF-MS is less efficient for smaller particles and therefore for nanoparticles the technique may still require further development. Direct reading techniques like, Differential mobility Analyzer (DMA) and Scanning mobility particle Sizer (SMPS), are easy to use, quick, and provide real time the particle size distribution, i.e. every x minutes. However, these instruments are calibrated for more or less spherical particles and therefore less appropriate for non-spherical particles like carbon nanotubes, fibres or non-spherical agglomerates/aggregates. In addition, the size and weight of these instruments makes them unsuitable for personal exposure measurements.

Table 1. Analytical methods suitable for size/size distribution measurements (Obtained from Hoehener and Hoeck, 2015)

Name/Acronym/Spatial resolution or LOD	Comment	Referenced documents

[2] Hoehener K, Hoeck J (2015) ERA-NET SIINN Safe Implementation of Innovative Nanoscience and Nanotechnology Deliverable D2.6: Consolidated Framework for EHS of Manufactured MNMs

Atomic force microscopy / **AFM /** ~0.1 nm		ISO/15900:2009 ISO 28439:2011 ISO/21501:2009 EN 13925-1:2005 ISO/13318-1:2001 ISO/13322-1:2014 ISO/TS 13762:2001 ISO/13320:2009
Differential mobility Analyzer / **DMA** / 3 nm to µm particles		
Field flow fractionation / **FFF** / Flow FFF 1 nm - 1µm; Sed FFF 50 nm - 1µm		ISO/22412:2008 ISO/13321:1996 ASTM E2490-09 ISO/20998-1:2006 ISO/21501-1:2009 BS EN 13925-1:2005 ISO/AWI TS 10797: 2012 ISO/NP TS 10868:2011 ISO/AWI TR 13014:2012 ISO/DTR 10929: 2012 ISO/CD 12025:2012 ISO/TS 13762:2001 JRC (Lövestam, 2012) (SCENIHR, 2010) (ECHA, 2008)
Hydrodynamic Chromatograph / **HDC**		
Scanning electron microscopy / **SEM** / 1 nm to 1 µm		
Scanning mobility particle Sizer / **SMPS** (5 - 1000 nm)		
Scanning transmission electron microscopy / **STEM** / < 0.1 nm		
Single particle mass Spectrometer / **SPMS**		
Size exclusion Chromatograph / **SEC**		
Transmission electron Microscopy / **TEM** / > 0.1 nm		
X-ray diffraction / **XRD** / 1-3 wt%		
Dynamic light scattering (photon correlation spectroscopy or quasi elastic light scattering) / **DLS (PCS, QELS)** / 3 nm - µm		
Fluorescence correlation spectroscopy (Confocal microscopy)/ **FCS** / ~200nm		
Nanoparticle Tracking Analysis (10 nm – 2 µm)		Filipe, 2010; Sanchis, 2015
Disc Centrifuge (5 nm – 75 µm)		Atherton, 2008; Tantra, 2015
Centrifugal Liquid Sedimentation/ **CLS**		ISO/13318-1:2001
Resonant mass measurement / 50nm – 5µm		Malvern
Laser diffraction/ **LD**/<100nm to >2mm		ISO/13320: 2009
The aerosol time-of-flight mass spectrometry / **ATOF-MS**	Is less efficient for smaller particles and therefore for nanoparticles the technique may still require further development.	University of Essex for Nanocap

2. Chemical composition

The chemical composition, in terms of elemental composition and chemical structure, is an intrinsic property of all materials and it is consequently an important parameter influencing the behaviour of nanoparticles. Nanoparticles can have very different chemical compositions, from completely inorganic, e.g. metals (iron, nickel, zinc, titanium, gold, silver, palladium, iridium, and platinum), and metal oxides (titanium oxide, zinc oxide, silica, iron oxide, etc.), to entirely organic (fullerenes, CNT, nanopolymers, biomolecules). Various analytical methods can be used to identify the chemical composition of nanomaterials, e.g. electron microscopy (e.g. TEM, SEM, AFM, AES), X-ray spectroscopy (e.g. XRD, XPS, PIXE, EDX, XRF,), mass spectrometry (e.g. ATOF-MS, ICP-MS) etc. (Table 2). A combination of energy dispersive X-ray spectroscopy with electron

microscopy (EM/XRD) is often used to quantitatively investigate the electronic structure and chemical composition of the nanomaterials,

Table 2. Analytical methods mentioned as suitable for chemical composition measurements (Obtained from Hoehener and Hoeck, 2015)

Name/Acronym/Spatial resolution or LOD	**Referenced documents**
X-ray diffraction / **XRD** / 1-3 wt%	(OECD, 2010) (OECD, 2012) (ENRHES, 2009) BS EN 13925-3:2005 ISO/29081:2010 ISO/16129:2012 (Hankin S.M., 2011) RIP-oN 2
Analytical electron microscopy / **AEM** / H 0.5nm	
Nuclear magnetic resonance spectroscopy and Pulsed field gradient / **NMR**	
X-ray photoelectron spectroscopy / **XPS** / H1 mm	
Auger Electron Spectroscopy / **AES** / H1–2nm	
Atomic Absorption Spectrometry Analysis / **AASA**	
Particle Induced X-Ray Emission Analysis / **PIXE**	
Energy Dispersive X-Ray Fluorescence Analysis/ **XRF**	
Inductively-coupled plasma combined with the selectivity and sensitivity of optical emission spectrophotometry or mass spectrometry /**ICP-OES /ICP-MS /** Detailed analysis of the main components, as well as trace impurities	
Chemical force microscopy / **CFM**	
The aerosol time-of-flight mass spectrometry / **ATOF-MS**	
Electron paramagnetic resonance and electron spin resonance spectroscopies / **EPR** / **ESR**	
Fourier transform infrared spectroscopy / **FTIR**	
Raman Spectroscopy / **RS**	
Transmission/Scanning electron microscopy / **SEM** / **TEM-EDS** (energy dispersive spectrometry)	
Gas chromatography (**GC**)	ISO 6974-1:2012
Electron probe X-ray microanalysis /**EPMA**)	ISO 23833:2013
Laser-induced breakdown spectroscopy /**LIBS**)	Applied spectra
High-performance liquid chromatography/ **HPLC**	

3. Shape

A detailed description of the physical shape of the MNM should be provided using terms such as spheres, fibres, tubes or plates (Guidance for Notifiers Handbook REQUIREMENTS FOR NOTIFICATION OF NEW INDUSTRIAL MNMS: Guidance on new chemical requirements for notification of industrial MNMs). Shape is important as it is the variation of the hydrodynamic radius between spherical particles and oblong ones (larger for the latter) with the same mass, which triggers a variation in their mobility and diffusion in both gas and liquid phases. The second effect is that the shape influences the deposition and ad-sorption kinetics in biological media. Therefore it is argued that physico-chemical or other properties may change when deviating from the more spherical shape, i.e. increased aspect ratio.

Some of the methods used to characterize the size of nanoparticles can also be used to measure the particle shape/aspect ratio of nanoparticles discussed above in section ‘particle size (distribution)’ (Table 3). Compared to size measurement, smaller numbers of samples and images

are generally sufficient to identify the particle shape(s), because they usually do not vary as much as the particles size[3].

Table 3. Analytical methods suitable for shape/aspect ratio measurements (Obtained from Hoehener and Hoeck, 2015)

Name/Acronym/Spatial resolution or LOD	Referenced documents
Atomic force microscopy / **AFM /** ~0.1 nm	ISO/13322-1:2014 ISO/NP TS 10868:2011 ISO/AWI TS 10797: 2012 ISO/AWI TS 10798:2011 ISO/AWI TR 13014:2012 ISO/DTR 10929: 2012 ISO/DTS 11888:2011 (ENRHES, 2009) JRC (Lövestam, 2012) (SCENIHR, 2010) (Hankin S.M., 2011) RIP-oN 2
Electron microscopy / **SEM** / 1 nm to 1µm	
Scanning transmission electron microscopy / **STEM** / < 0.1 nm	
Transmission electron microscopy / **TEM** / > 0.1 nm	
X-ray diffraction / **XRD** / 1-3 wt%	
Scanning tunneling microscopy / **STM** / resolution of ~1 nm or better.	
Dynamic light scattering / **DLS/** 3 nm - µm	
Static Light Scattering **SLS**	
Field Flow Fractionation **FFF /** Flow FFF: 1nm -1 µm; Sed FFF: 50nm-1 µm	
FFF-ICP-MS **FFF-Confocal Microscopy** **FIFFF-SLS** **SedFFF-DLS**	

4. Surface area

Surface area is the measure of how much exposed area a solid object has, expressed in square units. The reduction in size to the nanoscale is accompanied by an inherent increase in the surface-to-volume ratio, and therefore a greater proportion of entities at the surface compared to the bulk (non-nanoscale) material. Increase in surface area increases reactivity and sorption behaviour. Porosity or void fraction is a measure of the void (i.e., "empty") spaces in a material, and is a fraction of the volume of voids over the total volume, between 0–1, or as a percentage between 0–100%. The method generally used to measure specific surface area is the Brunauer, Emmett and Teller (BET) method[4]. With BET the external surface area can be measured and also the surface area of the internal porous structurer of porous nanomaterials and agglomerates/aggregates. The critical step of BET is the sample preparation, i.e. placing substrate in the analysis vessel. Since it is not possible to use BET to measure nanomaterials in a dispersion, the sample must be dried which has the risk of introducing artefacts in the measurement process e.g. particle aggregation[5]. A second method to measure surface area is the Epiphaniometer. The drawback of this method is the use of a radio-active source and complexity of use which requires experts to operate the instrument. In addition, monitors, i.e. NSAM, DiSCmini, Nanocheck, can be used to measure the lung deposited surface area. These instruments are

[3] JRC; RIP-oN 1 (2011)
[4] JRC; RIP-oN 2 (2011)
[5] JRC; RIP-oN 1 (2011)

portable and easy to use. A disadvantage of these monitors is the assumption that particles are spherically shaped which make their usefulness for powders containing particles whose morphology is different questionable.

Table 4. Analytical methods suitable for Surface area (&porosity) measurements (Obtained from Hoehener and Hoeck, 2015)

Name/Acronym/Spatial resolution or LOD	Comment	Referenced documents
Brunauer Emmett Teller / **BET**	The BET-method allows surface area or porosity measurements within pores or other nanostructures as small as about 1 nm. The density of only the material without the empty spaces in between is required. Thousands of m2 g−1 A limitation of the BET-method is that it is only applicable to powders and/or dry solid materials and not to nano-materials embedded in solids and suspensions.	(OECD, 2009)
Epiphaniometer	Monitoring environmental aerosols However, it has not been used widely in assessing aerosol exposure, possibly due to its use of a radioactive source, and its complexity of use.	ISO/9277:2010 ISO/18757:2005
Monitors for lung deposable particle surface areas (NSAM, Discmini, Partector,Nanocheck)	The effect of initial aerosol charge, the composition of the material, presence of aggregates and the effect of particle shape have to be considered. Sampled particles are charged, collected in an electrically isolated filter and the charge rate measured. Monitor measure the surface area of particles (reported as mm2 cm-3) deposited in the lung.	(TSI, 2012)

5. Surface charge

Surface charge is the electric charge present at an interface. There are many different processes which can lead to a surface being charged, including ad-sorption of ions, protonation/deprotonation, and the application of an external electric field. Surface charge causes a particle to emit an electric field, which causes particle repulsions and attractions, and is responsible for many colloidal properties. The surface charge of particles can be measured with capillary electrophoresis (CE) which separates the particles based on their charge. Alternatively, the zeta potential can be determined as an indicator of the surface charge. The higher the zeta potential, the stronger the repulsion, the more stable the particle is. Zeta potential is not measurable directly but it can be calculated. Various instruments are available for determining the zeta potential of dispersed nanomaterials, i.e. DLS and NTA (Table 5). It has been suggested by Zuin et al. (2010)[1] that it is essential to determine the zeta potential as a function of pH, as this allows the determination of the point of zero charge where a dispersion of engineered nanomaterials exhibits the highest propensity to aggregate.

The following relationship between zeta potential and stability are identified[6]:

[6] Zuin, S., Micheletti, C., Critto, A., Pojana, G., Johnston, H., Stone, V., Tran, L., Marcomini, A. 2010, "Weight of evidence approach for the relative ranking of nanomaterials", Nanotoxicology, 2011 Sep;5(3):445-58. doi: 10.3109/17435390.2010.512986 .

- Nanoparticles with zeta potential at pH 7 of > +30 mV or < -30 mV have a high water stability (i.e. no aggregation over time);
- Nanoparticles with zeta potential at pH 7 of between -30 mV and + 30 mV have a low water stability (i.e. tendency to aggregate over time).

Table 5. Analytical methods suitable for surface charge/zeta potential measurements (Obtained from Hoehener and Hoeck, 2015)

Name/Acronym/Spatial resolution or LOD	Referenced documents
Capillary electrophoresis/**CE**	ISO/CD 13099-1 ISO/CD 13099-2 ISO/AWI TR 13014:2012 (Hankin S.M., 2011) RIP-oN 2
Zeta potential Indicates the degree of repulsion between adjacent, similarly charged particles in a dispersion	
Zeta potential measurement, combined with Dynamic Light Scattering (**DLS**)	
Zeta Potential Nanoparticle Tracking Analysis (Z-NTA) adds measurements of electrostatic potential to simultaneous reporting of nanoparticle size, light scattering intensity, fluorescence and count, and does so particle-by-particle.	-

6. Agglomeration/aggregation

The terms agglomeration and aggregation are often used interchangeably to describe the attractions that hold together a collection of particles. However, it has been suggested that it is more appropriate to consider nanoparticle aggregation and agglomeration as distinct phenomena (c.f. *Recommendation of European Commission. Last updated 18 October 2011*) with agglomerates formed by clusters of particles that are held together by electrostatic interactions, whereas aggregates are formed from covalently fused or sintered particles that are not easily separated (Oberdörster, 2007). Aggregation and agglomeration can occur due to a number of deliberate and accidental mechanisms (Schneider, 2008). When hierarchical assemblies, aggregates and agglomerates are included in the determination of the size, their presence induces a shift to larger sizes. The extent of agglomeration/aggregation depends on the properties of the MNMs investigated and on the composition/properties of the medium. It is for instance known that agglomeration is promoted by increasing ionic strength of the medium, and by changes of the pH. When interpreting results of analytical assessment of agglomeration, it is needed to take account of the analytical conditions and the composition of the medium used for the assessment. The methods displayed in Table 6 suffer from the problem that the analytical conditions commonly are not representative of the conditions that typical in either the media used for (eco)toxicity assessment or for the actual environment. Methods used to characterize the shape and size of nanoparticles can also be used to determine the aggregation degree of nanomaterial powders (Table 6). See paragraph ‘particle size (distribution)’ of this chapter for more details of the analytical techniques. In addition, for determining the agglomeration state and dispersion stability

of nanoparticles in solution, zeta potential measurements are often employed. However, it is concluded that zeta potential measurements alone are not sufficient for predicting the stability of nanoparticles in physiological suspensions, recommending that measurements of the particle size distribution are also undertaken. It has been suggested that a method for measuring the strength of the agglomerates is needed[7].

Table 6. Analytical methods suitable for aggregation/agglomeration measurement (Obtained from Hoehener and Hoeck, 2015)

Name/Acronym/Spatial resolution or LOD	**Referenced documents**
Atomic force microscopy / **AFM /** ~0.1 nm	ISO/20998-1:2006 ISO/13322-1:2014 ISO/TS 13762:2001 ISO/AWI TS 10797:2012 ISO/AWI TS 10798:2011 ISO/AWI TR 13014:2012 (Hankin S.M., 2011) RIP-oN 2 (ENRHES, 2009) JRC (Lövestam, 2012) (SCENIHR, 2010)
Electron microscopy / **SEM** / 1 nm to 1µm	
Scanning transmission electron microscopy / **STEM** / < 0.1 nm	
Transmission electron microscopy / **TEM** / > 0.1 nm	
X-ray diffraction / **XRD** / 1-3 wt%	
Scanning tunneling microscopy / **STM** / resolution of ~1 nm or better.	
Small angle neutron scattering / **SANS**	
Dynamic Light Scattering/ **DLS**	ISO/22412: 2008
Nanoparticle Tracking Analysis/ **NTA**	Filipe, 2010; Sanchis, 2015

2.4.2 Stability of the coating

The key question addressing the first risk potential (descriptor of substance) of the flow chart is:

Does the MNM have a coating?

If this question is answered with **no**: additional data/testing of the uncoated MNM is required (see testing strategy).

If this question is answered with **yes**: this leads to the subquestion: **Is the coating stable?**

[7] JRC; RIP-oN 2 (2011)

If this question is answered with **no**: additional data/testing of both the coated and the uncoated MNM is required (see testing strategy).

If this question is answered with **yes**: additional data/testing of the coated MNM is required (see testing strategy).

NB This is the only question in the flow chart that does not lead to the green or red box. The question gives guidance on what part of the material has to be assessed.

2.4.2.1 Testing strategy for stability of MNM coating

The following parameters are proposed to address the question about the (stability of the) coating of a MNM:

1. Particle size (distribution)
2. Chemical composition and impurities
3. Shape
4. Surface area
5. Surface charge
6. Agglomeration/aggregation

Addressed in paragraph 2.4.1

7. Surface coating
8. Degree of coating
9. Coating stability (if relevant: ratio of coated/uncoated MNMs)

Thus, the same set of parameters set out in question 1 (MNM characteristics) needs to be considered when more information is required regarding the properties of a coated MNM. Additionally, information is required on the chemical composition of the coating, the degree of coating (i.e. is the MNM entirely covered, are there multiple layers, etc.) and stability of the coating.

7. Surface coating

The surface coating on a nanoparticle may affect the solubility, behaviour of a nanoparticle and its (eco)toxicity. The role of a coating and the coating material can affect the properties of the MNM. A coating can increase particle stability in solutions or improve the wettability. On the other hand, a coating could prevent dissolution of the particle core, and reduce or prevent aggregation. It could also improve the physical and chemical functions, or help with biocompatibility and functionality of the MNM (Sperling and Parak, 2010).

8. Degree of coating

Surface coatings of MNMs are generally applied to selectively change or influence distinct particle properties. MNMs (the core) can be covered with a wide variety of substances that can generate single- or multi-layers (the shell). These can cover the core completely, but not necessarily. If this coating is stable, it is the shell rather than the core that determines the final properties of the coated MNM. In that case, testing can be confined to the coated MNM. As such, the coating should be considered as an integral component of the MNM. However, if the coating is unstable, both the coating and the MNM itself should be submitted to testing. A coating can also be acquired after preparation through contact with materials in the environment. Surface coating is not necessarily uniform; the degree of coating can vary from particle to particle within a batch of manufactured MNMs or between batches of MNMs (Sellers et al., 2015).

9. Coating stability

MNMs may be transformed during their life cycle if dispersed in solution or decomposed in their constituent materials if embedded in a matrix as a composite. The stability of their original appearance (including the coating, corona, agglomeration and aggregation) are very important for their behaviour and effects in humans and the environment. If relevant, the ratio of uncoated and coated MNMs should be measured as well.

2.4.2.2 Methods: Which tests are available for surface coating measurements?

Surface modification of a MNM can either be done by coating, functionalisation or other means, which may be chemical (organic, inorganic or both) or physical (e.g. irradiation, surface attrition). Purposely applied and environmentally acquired coatings can have a major impact on MNM interaction with biological systems.

The term surface chemistry is often used in the context of surface chemical composition, and is somewhat a broad and non-specific term which does not predispose itself to 'quantitative' characterisation according to a single comparable metric or measurand. Surface chemistry includes elements of solubility equilibrium, catalytic properties, surface charge, and surface adsorption and de-sorption of molecules from solution, amongst others. Most of these properties are functions of the atomic or molecular composition of the surface and the physical surface structure. Chemical purity, functionalisation and surface coating are also important aspects to take into account. The nature of surface functionalization and coatings can be measured with the same analytical methods as for the determination of the chemical composition (Table 7). AFM and TEM can provide 2D or 3D imaging of nanoparticles on a flat surface allowing accessing the surface texture and roughness. It has been found that X-ray diffraction (XRD), and high-resolution methods, such as transmission electron microscopy (TEM), combined with surface methods, including XPS, are important for detailed characterization of particles.[8,9] The impact of natural organic coatings formed on the particles in solution has been studied. XPS, as well as optical methods, again have been useful for characterizing of these coatings[10]. In addition, EM combined with XPS has shown to provide important information about the presence and nature of molecules attached to the surface[11,12].

[8] Baer D, Tratnyek P, Qiang Y, Amonette JE, Linehan JC, Sarathy V, Nurmi JT, Wang CM, Antony J. Synthesis, Characterization and Properties of Zero Valent Iron Nanoparticles. In: Fryxell G, Cao G, editors. Environmental Applications of Nanomaterials: Synthesis, Sorbents, and Sensors Imperial College Press; London: 2007

[9] Nurmi JT, Tratnyek PG, Sarathy V, Baer DR, Amonette JE, Pecher K, Wang CM, Linehan JC, Matson DW, Penn RL, Driessen MD. Characterization and properties of metallic iron nanoparticles: Spectroscopy, electrochemistry, and kinetics. Environmental Science & Technology. 2005;39:1221–1230.

[10] Johnson RL, Johnson GO, Nurmi JT, Tratnyek PG. Natural Organic Matter Enhanced Mobility of Nano Zerovalent Iron Environmental Science & Technology. 2009;43:5455–5460

[11] Chen RJ, Zhang YG, Wang DW, Dai HJ. Noncovalent sidewall functionalization of single-walled carbon nanotubes for protein immobilization. Journal of the American Chemical Society. 2001;123:3838–3839

[12] Dyke CA, Stewart MP, Maya F, Tour JM. Diazonium-based functionalization of carbon nanotubes: XPS and GC-MS analysis and mechanistic implications. Synlett. 2004:155–160.

Table 7. Analytical methods available for surface chemistry measurements (Obtained from Hoehener and Hoeck, 2015)

Name/Acronym/Spatial resolution or LOD		**Referenced documents**
Analytical electron microscopy / **AEM** / Spatial resolution H 0.5nm		(Hankin S.M., 2011) RIP-oN 2
Chemical force microscopy / **CFM /** Identifying the nature of individual atoms		
X-ray photoelectron spectroscopy / **XPS** / Spatial resolution H1 mm / Atomic composition of layers from 1–10nm		
Auger Electron Spectroscopy / **AES** / H1–2nm		
Secondary ion mass spectrometry / **SIMS /** Atomic composition of layers from 1–3nm		
Electron microscopy / **SEM or TEM** / **TEM-EDS** (energy dispersive spectrometry		
Infrared / **IR**		
UV/Visible spectrometry/ UV-VIS		
Nuclear magnetic resonance spectroscopy and Pulsed field gradient / **NMR**		
Raman Spectroscopy / **RS**		
Time-of-Flight Secondary Ion Mass Spectrometry/ **TOF-SIMS**		ISO/TR 14187
Low energy ion scattering/ **LEIS**		
Scanning probe microscopy/ **SPM**		
Scanning probe microscopy/ **SPM**	Scanning tunneling microscopy/**STM**	
	Atomic force microscopy/ **AFM**	

2.4.3 Solubility / Dissolution rate

The key question addressing the second risk potential of the flow chart is: Does the MNM have low solubility and/or dissolution rate?

If this question is answered with **no**: no nanospecific potential for health risk is expected and risk assessment can be performed in the conventional way.

If this question is answered with **yes**: nanospecific data/testing is required (see testing strategy)

2.4.3.1 Testing strategy for solubility/dissolution

The following parameters are proposed to obtain solubility information:

1. Particle size (distribution)
2. Chemical composition and impurities
3. Shape
4. Surface area
5. Surface charge
6. Agglomeration/aggregation

Addressed in paragraph 2.4.1

7. Surface coating
8. Degree of coating

Addressed in paragraph 2.4.2

9. Coating stability
10. Solubility/dissolution
11. Hydrolytic stability
12. Acid dissociation

10. Solubility/Dissolution/Dispersibility

Solubility is a significant parameter, especially for environmental assessments, as the mobility of a test substance is largely determined by its solubility in water. Also, water solubility can affect adsorption and desorption on soils and volatility from aquatic systems. The knowledge of the water solubility is a prerequisite for setting up test conditions for e.g. aquatic toxicity, bioaccumulation (ECHA 2011). Solubility is the degree to which a material (the solute) can be dissolved in another material (the solvent) such that a single, homogeneous, temporally stable phase (a suspension down to the molecular level) results, and is relevant to solids, liquids and gases. Dissolution rate is the rate at which a particulate material is dissolved in the solvent. Dispersibility is the degree to which a particulate material can be uniformly distributed in another material (the dispersing medium or continuous phase). In the case of colloidal suspensions, which are uniform or not uniform (in term of size) dispersion of nanoparticles in a liquid, a distinct definition of solubility/dispensability can be difficult. Moreover, the water or media conditions to which the MNM is exposed must also be considered and standardized as both parameters solubility and dispersibility may vary and be affected by pH, ionic strength, presence of inorganic/organic fractions, types of media considered (OECD, EN/JM/MONO (2009) 20/REV).

The toxicological relevance to quantity solubility/dispersability in water and in relevant exposure matrices is because the biological and/or environmental distribution, identity and fate of a MNM will be different whether it will exist as soluble or insoluble form. In the first case, if an MNM is entirely solubilized it will be present totally in a molecular or ionic form thus possibly eliciting the same response in an in vitro/in vivo system as the corresponding chemical forms of the material. The solubility rate at a precise temperature and pH will be then indicative of MNM interaction with cells thus allowing in theory to comparison of toxicological information for two MNMs with similar structure, composition and solubility. Solubility property is also strictly connected to dissolution of MNM as solubility sometimes corresponds to a gradual release of primary constituents or secondary species (free ions, ion-biomolecules chelates, ion-ion chelates, etc.) that may in turn interact with the in vitro/in vivo systems as independent and different molecular species. Their identification by analytical characterization is relevant for different purposes, which include read across information (silver ions released by silver NPs may have the same behaviour of a correspondent ion solutions, etc.). Not only, fast soluble MNMs are often also fast dissolving MNMs. This however cannot be considered a fixed rule as aggregation/agglomeration phenomena may occur thus altering the initial thermodynamic equilibrium in the solution leading to a complex mixture of nanostructured and molecular species in the solution.

Water solubility of MNM is typically measured by using a known unit of particles in a standardised solution and temperature, to measure the amount of mass that diminishes after a precise set of time or the time necessary to reach the mass below a X% amount. Solubility is addressed by OECD TG105 Water Solubility as possible method (Sabella et al. Nanoscale 2014).

11. Hydrolytic stability/Acid Dissociation

The three properties, solubility, hydrolytic stability and acid dissociation constant (pKa) are interrelated. It is not possible to measure any of these without some knowledge of the other two (ECHA 2012). Solubility cannot be measured without considering the stability of MNM in water and the interactions of active functional chemical groups with water in a hydrolysis process. Moreover the isoelectric point and pKa of MNM are strictly connected to their surface chemistry properties and may vary depending on the environmental conditions considered. The characterization of pKa and dissolution rate may be firstly conducted in water at fixed values of Temperature and pHs by different techniques. The trend and the extent of changes of dissolution rate of MNM should be ideally checked in relevant exposure matrices where molecule composition may strongly affect such parameters due to complexation and hydrolysis phenomena. For instance, ZnO NPs are considered fast dissolving and highly soluble nanoparticles but their solubility changes as function of pHs. At strong acidic conditions, ZnO MNMs quickly dissociate in Zn2+ ions and water whereas in basic conditions the formation of different oxidized soluble hydro-chelates occurs (e.g. $Zn(OH)_n^{n+}$). The acid dissociation is also connected to the redox potential property of the material as well as the capacity to induce free radicals when interacting with oxygen species and organisms in relevant exposure matrices. For the case of MNM, dissolution rate and solubility cannot be strictly considered as physical properties but they are also properties strongly linked to concepts such as stability and transformation of MNM in exposure matrices (fate). This is also the reason that prompted the European Chemicals Agency (ECHA) to suggest in a recent guidelines the need to quantity not only the dissolution rate in relevant exposure matrices but also provide information on speciation in solutions (the characterization and identification of all species in a solution) (European Chemicals Agency (ECHA) Appendix R7-1, Chapter R7a)

There are many methods currently under evaluation for the determination of solubility, dissolution rate of MNM, however although each presents some advantages none of them may be considered universal for all types of MNMs (listed below), Chemical composition of MNM dictates the first choice among the analytical techniques available (for instance, MNM characterized by net and typical plasmonic peak in the UV-VIS regions may be characterized by UV-Vis methods, other MNM whose ions do not chelates with matrices may be characterized by colorimetric sensors, etc.)

Methods suggested for the measurement of Hydrolytic stability/Acid Dissociation/Dissolution**:**

- Shake flask method (conventional, it needs to be adapted to MNMs)
- Column Elution method (conventional, it needs to be adapted to MNMs)
- Acid-base titration curve (to be specifically addressed for MNMs)
- Electrochemical and potentiometer techniques
- Measurement of ROS formation or oxygen based free radicals formation (DCFH-DA assay for evaluating nanoparticle-induced intracellular reactive oxygen species (ROS) production, under evaluation in ISO TC 229; Electron spin resonance (ESR) as a method for measuring reactive oxygen species (ROS) generated by metal oxide MNMs, under evaluation in

ISOTC 229; hydro chemical reactivity (both pH and O2) by sensor dish reader is under evaluation by CEN)

Methods for dissolution rate and speciation of dissolved ions

- Atomic Spectroscopy (AAS) couples to Ultrafiltration (UF)
- UV-Vis
- UF-ICP-OES
- UF-ICP-MS
- spICP-MS (this techniques work without pre-separation techniques)
- Flow FFF-ICP-MS
- Other methods are based on separation techniques such as HPLC and CE equipped with different detectors such as ISE (ion selective electrodes), UV-Vis, Voltametric techniques (AGNES)

Moreover, colorimetric techniques which are selectively sensitive to ions are under development.

2.4.3.2 Methods: Which tests are available for solubility/dissolution rate?

The solubility of a chemical in water may be defined as the maximum amount of the chemical that will dissolve in pure water at a specified temperature. Above this concentration, two phases will exist if the organic chemical is a solid or a liquid at the system temperature: a saturated aqueous solution and a solid or liquid organic phase. Aqueous concentrations are usually stated in terms of weight per weight (ppm, ppb, g/kg, etc.) or weight per volume (mg/L, moles/L, etc.) - [Lyman, 1990].

• The property of water solubility could be relevant and applicable to MNMs. It has been suggested that the measurand of interest (beginning with a pre-determined unit of particles in a standardised solution and temperature) is to measure the mass proportion of MNMs which are held in solution, and whether this mass diminishes after a set period of time, or; determine the amount of time required for mass to diminish by X% (*ENV/JM/MONO(2009)20/REV*);

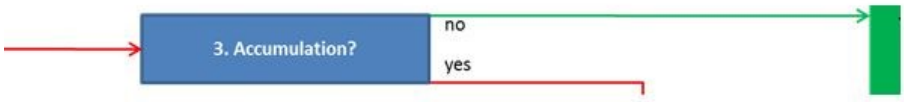
• Distinguish between solubilisation and dispersion. Water solubility has the potential to increase in the nano-size range. For MNMs, it can be difficult to distinguish between when a substance is dispersed and when it is dissolved due to its small particle size. It is important to recognise that solubility and dispersibility are different and distinct phenomena, with different implications on testing and characterisation, and it is important to differentiate between them. It should also be ensured that no undissolved material contributes to what is being measured. Update to guidance was proposed regarding the difference between solubilisation and dispersion and a recommendation to elucidate between the two for MNMs. However, as highlighted in *ENV/JM/MONO(2009)20/REV*, specific methods to determine dispersion stability remain to be determined.

Tests/Guidance documents

- OECD TG105 (water solubility) (New test guideline needed for MNMs) (OECD 1995).
- Dissolution tests must be conducted under different conditions (pH 4-7-9, with and without organisms, with or without natural organic matter/proteins, filtration (Sellers et al., 2015).

2.4.4 Accumulation

The key question addressing the third risk potential (parallel to genotoxicity, immunotoxicity and ecotoxicity) of the flow chart is: Is accumulation to be expected?



If this question is answered with **no**: no nanospecific potential for health risk is expected and risk assessment can be performed in the conventional way.

If this question is answered with **yes**: nanospecific data/testing is required (see testing strategy).

Accumulation represents the relationship between the absorption and the rate of elimination from a tissue or organ. Accumulation occurs when the influx of MNM into a tissue or organ over time is relatively high in comparison to the elimination rate. MNMs can be eliminated either as particle or by dissolution into its molecular or ion form.

2.4.4.1 Testing strategy for accumulation

The following parameters are proposed to describe accumulation:

1. Particle size (distribution)
2. Chemical composition and impurities
3. Shape
4. Surface area
5. Surface charge
6. Agglomeration/aggregation

Addressed in paragraph 2.4.1

7. Surface coating
8. Degree of coating
9. Coating stability

Addressed in paragraph 2.4.2

10. Solubility/dissolution
11. Hydrolytic stability
12. Acid dissociation

Addressed in paragraph 2.4.3

13. Exposure route
14. Dose
15. Protein binding
16. Clearance

13. Exposure route

The safety issues derived from MNMs routes of entry and their potential distribution are governed by surface area, shape, agglomeration, aggregation solubility and size with protein (opsonisation) interactions within the host (Poland et al., 2008). The size fractions in the nanoscale range have greater lung deposition and rapid systemic translocation having various inflammatory, oxidative

and cytotoxic effects on experimental animals than larger particles (Andrew et al., 2004; Obordorster et al., 2005a). The exposure route may lead to an estimation of the exposure; this information is needed to indicate whether accumulation can be expected.

The main routes of entry are through the skin, lungs or intestinal tract causing adverse biological effects (Peter et al., 2004; Warheit et al., 2004; Oberdorster et al., 2005; Davoren et al., 2007; Li et al., 2007). Other potential routes of NPs in the case of biomedical applications include parental administration such as intravenous, intradermal and peritoneal exposures (Stern and McNeil, 2008; De Jong et al., 2008). The toxicity of MNMs depend on their persistence or clearance from the different organs due to the immune response of the host (Jeffrey et al., 2008)[13].

14. Dose

In view of the active functionalisation and the possible interaction of nanoparticles with bio-molecular structures, it is important to consider the dose and dose rate of the MNM, its ability to spread within the body, the decay of number concentration and the erosion of individual particles. Many nanoparticles will have considerable solubility. For these materials the interaction with living systems remains close enough to the bulk chemical agent to justify the use of well-established toxicological testing procedures and approaches. For biodegradable particles, the particle composition and degradation products will influence their biological effects. On the other hand, materials with very low solubility or degradability, could accumulate within biological systems and persist there for long durations. It is with nanoparticles of this character that the greatest concerns must arise, and attention will have to be paid to the comparison of the persistence of the particles and the time constants of the metabolic and cellular activities within the target host.

The dose and frequency determine the influx rate in the body or organ/tissue, which will give direction whether accumulation can be expected.

15. Protein binding

Within the body, nanoparticles may contact biological fluids, proteins, phospholipids and nucleic acids (Wang et al., 2013). Interactions with these biological components can alter the size, aggregation state and interfacial properties of the MNM. Various proteins may bind to the MNM, forming a protein corona. Protein coronas can affect the biodistribution and translocation of MNMs, as well as their excretion (Wang et al., 2013). The protein binding can alter the agglomeration status, dissolution kinetics, surface charge and surface chemistry. The composition of the protein corona may change over time due to continuous protein association and dissociation (Kettinger et al., 2013). The specific surface properties of the MNM impact the composition of the protein

[13] Yah et al. 2012 Nanoparticles Toxicity and Their Routes of Exposures, Pak. J. Pharm. Sci., 25, 477-491

corona. Hydrophobic MNMs more easily adsorb proteins, whereas hydrophilic ones are less prone to protein binding (Kettinger et al., 2013). In addition, positively charged MNMs adsorb a different set of proteins on their surface than negatively charged ones, which may influence the mode of cell entry, biodistribution and biocompatibility (Kettinger et al., 2013). Saptarshi et al. (2013) found that size may impact protein binding in some instances, while in other studies size was not a factor. Saptarshi et al. (2013) also suggested that shape may influence protein binding. While shape and size may have some influence on protein binding, hydrophobicity and surface charge generally have the greatest influence on protein binding (Landsiedel et al., 2012).

16. Clearance

Rapid clearance of MNMs is a critical issue and has made it necessary to understand the factors affecting particle biodistribution and blood circulation half-life. Many factors can influence nanoparticle blood residence time and organ specific accumulation. These factors include interactions with biological barriers and MNM parameters, such as composition, size, core properties and surface modifications. All these factors have been shown to substantially affect the biodistribution and blood circulation half-life of circulating nanoparticles by reducing the level of nonspecific uptake, delaying opsonization, and increasing the extent of tissue specific accumulation.

2.4.4.2 Methods: Which tests are applicable for accumulation?

Since there are no generally accepted methods for determining the biological persistence of MNMs, solubility/dissolution is used as a surrogate for persistence in biological media (e.g. biopersistence). Furthermore, exposure route, dose and clearance can give an indication whether accumulation can be expected.

Key guidance resources for the exposure of MNMs are summarised below.

- **BSI (2007) Guide to safe handling and disposal of manufactured MNMs**

This Published Document gives guidance on assessing risks and recognising uncertainties in the development, manufacture and use of MNMs, and on developing and implementing an effective strategy to address and control the risks. It also provides information about measurement instruments and how they can be used.

- **BSI (2010) Guide to assessing airborne exposure in occupational settings relevant to MNMs**

This Published Document describes a structured, step-by-step approach to exposure assessment which takes into account the purpose, the type of information needed, and the usefulness and limitations of the various approaches for exposure assessment for different types of MNMs, and provides a rationale for method selection.

- **NIOSH (2009) Nanoparticle emission assessment technique for the identification & measurement of potential inhalation exposure to engineered MNMs**

This paper describes the nanoparticle emission assessment technique (NEAT), developed by researchers at the National Institute for Occupational Safety and Health (NIOSH), which uses a combination of measurement techniques and instruments to assess potential inhalation exposures in facilities that handle or produce engineered MNMs. Results from using the NEAT at 12 facilities are presented in a companion article (Part B).

- **NIOSH (2013) Occupational exposure to carbon nanotubes and nanofibres**

This NIOSH document (CIB 65) provides occupational safety and health guidance for carbon nanotubes (CNT) and carbon nanofibres (CNF). The document, which is available to download from the NIOSH website, i) reviews the animal and other toxicological data relevant to assessing the potential non-malignant adverse respiratory effects of CNT and CNF; ii) provides a quantitative risk assessment based on animal dose-response data; iii) proposes a recommended exposure limit (REL) of 1 µg/m3 elemental carbon as a respirable mass 8-hour time-weighted average (TWA) concentration, and iv) describes strategies for controlling workplace exposures and implementing a medical surveillance program. The NIOSH REL is expected to reduce the risk for pulmonary inflammation and fibrosis. However, because of some residual risk at the REL and uncertainty concerning chronic health effects, including whether some types of CNTs may be carcinogenic, continued efforts should be made to reduce exposures as much as possible.

- **OECD (2009) Emission assessment for the identification of sources and release of airborne manufactured MNMs in the workplace: compilation of existing guidance**

This guidance document describes a simple semi-quantitative determination of MNM release and may be used whether release of MNMs occurs in the workplace. It provides details of the instruments to use, the approach to follow and how to interpret the results.

Table 8. Overview of analytical strategies to monitor MNM–protein interactions (Li et al., 2010)

Functional purpose	**Analyses strategy**	**Advantages**	**Limitations**
Binding affinity and ratio	UV-vis	Faster, more flexible, less complicated	Absorption spectrum show different characters for varied NPs
	Fluorescence spectroscopy	Quantitative, sensitive	NPs or proteins should have intrinsic or labeled fluorescence
	Dynamic light scattering	Size distribution of NPs	Not suitable to non-spherical nanoparticles
	Atomic force microscopy	3-D surface profile	Limited scanning area
Conformational changes of NP-bound proteins	Circular dichroism	Secondary structures of proteins in the absence or presence of NPs	No residue-specific information
	Fourier transform infrared spectroscopy	NP-bound protein amide bands	Objects have to be dried down
	Raman spectroscopy	NP-bound protein amide bands in aqueous solution	Fluorescence and Rayleigh scattering noise
	X-Ray crystallography	3-D structure of nanoparticle–protein complex	Objects have to be crystallized
	Nuclear magnetic resonance	NP-bound protein binding site mapping	Line broadening in spectrum when proteins bind to NPs

Isolation and separation of NP-bound proteins	Chromatography	Quantitative	Limited application range
	Capillary electrophoresis	Quantitative, no need of desorption procedure	Limited detection sensitivity
	1-D electrophoresis	Simpler, faster	Less effectively separation
	2-D electrophoresis	Higher ability of separating proteins	More complex, less repeatable
Identification of NP-bound proteins	Mass spectroscopy	Efficient, low quantity of protein samples	Less quantitative
	N-terminal microsequencing	Obtains directly amino acid sequences of proteins	Less quantitative, restrictiveness of N-terminal amino acids
Kinetics	Quartz crystal microbalance	Real-time, label-free, sensitive, quantitative	Immobilization of one party on the chip
	Surface plasmon resonance	Real-time, label-free, sensitive and quantitative	Immobilization of one party on the chip

2.4.5 Inflammation/immunotoxicity

The key question addressing the fifth risk potential (parallel to accumulation, genotoxicity and ecotoxicity) of the flow chart is: Does the MNM induce an immunotoxic response?

If this question is answered with **no**: no nanospecific potential for health risk is expected and risk assessment can be performed in the conventional way.

If this question is answered with **yes**: nanospecific data/testing is required (see testing strategy).

2.4.5.1 Testing strategy for inflammation/immunotoxicity

Immunotoxic response of a MNM can be induced by generating ROS and releasing pro-inflammatory cytokines (TNF-α, IL-1, IL-6, IL-8) and/or releasing chemokines and immunoregulatory cytokines (IFN-γ- IL-2, IL-8). In general, MNMs have the potential to

1. interact with immune cells, such as macrophages, monocytes, dendritic cells, stroma and lymphocytes
2. trigger non-specific inflammatory responses via generation of ROS (oxidative burst) and release of pro-inflammatory cytokines such as TNF-α, IL-1, IL-6, IL-8, etc.
3. activate the complement cascade, and platelet aggregations.
4. facilitate antigen-specific hypersensitivity reactions via interactions with T-lymphocytes and release of chemokines and immunoregulatory cytokines (IFN-γ- IL-2, IL-8, GM-CSF (Granulocyte-macrophage colony-stimulating factor), etc.)

However, some MNMs can also suppress inflammatory responses and exert no immune modulatory properties. Therefore, it must be kept in mind that an immunological effect is not necessarily an immunotoxicological effect.

The interaction between MNMs and immune system is a complex one and parameters such as size, charge, morphology and most importantly, surface chemistry dictates the toxicity and immune response induced by MNMs (For an overview of the complexity of the immune system, see Appendix 6.2). Hydrophilicity, lipophilicity, catalytic activity, composition, electronic structure, capacity to bind or coat surface species and solubility, the dimension, and consequently the surface area, also seem to be the important factors that contribute to the interactions of MNMs with biological tissues and immune system in particular. Certain MNMs accumulate to regional lymph nodes, where they can be taken up and processed by dendritic cells, interact with self-proteins and, hence, modify their antigenicity and elicit altered immune responses and even autoimmunity. The following parameters are needed to describe inflammation/immunotoxicity:

1. Particle size (distribution)
2. Chemical composition and impurities
3. Shape
4. Surface area
5. Surface charge
6. Agglomeration/aggregation

Addressed in paragraph 2.4.1

7. Surface coating
8. Degree of coating
9. Coating stability

Addressed in paragraph 2.4.2

10. Solubility/dissolution
11. Hydrolytic stability
12. Acid dissociation

Addressed in paragraph 2.4.3

13. Hydrophobicity/lipophilicity
14. Cytokine induction
15. ROS generation

13. Hydrophobicity

Lower hydrophobicity is associated with lower immunotoxicity. For instance, immune response to poly(ε-caprolactone) MNMs following both intramuscular and intranasal administration was increased, as compared to PLGA, due to the lower hydrophobicity of the latter (Singh, Pandit et al. 2006). The same trend (greater immune response for MNMs with higher hydrophobicity) was observed with other particles of varying degrees of hydrophobicities (Kreuter, Liehl et al. 1988).

14. Cytokine induction

Evaluation of the immunotoxicity of MNMs early in the development process by measuring, for instance, the levels of cytokines or other immune-indicators, is of particular importance for ensuring the safety of MNMs. Measuring the levels of proinflammatory cytokines and other inflammatory mediators and monitoring the balance of TH1/TH2 cytokines can be useful tools in evaluating MNM

immunotoxicity. Moreover, screening of a single reporter is inadequate; combinations of cytokine levels should be measured, due to the cytokine network influence on the immune system. Monitoring the levels of cytokines, in particular the proinflammatory ones, following administration of MNMs, appear to be an important tool for partially screening their immunomodulatory effects. High levels of cytokines, as compared to the untreated controls, are considered as biomarkers of nanoparticle immunotoxicity (Elsabahy and Wooley 2013).

15. ROS generation

The generation of ROS is seen as one of the primary mechanisms for toxicity induced by MNMs. MNMs may generate ROS by different processes. The first is the reactivity of the MNM itself. The relatively large surface area and increased reactivity of many MNMs can enhance their formation of ROS (Soenen 2011). Surface bound radicals such as SiO· and SiO2· present on quartz particles are responsible for the formation of ROS such as OH· and O2·. The second factor is the body's natural defence system. Under stress, certain cells can produce chemically-active oxygen-containing molecules that can "defend" the cell by oxidizing the foreign substance. Prolonged generation of ROS, however, can damage the cell itself (Manke A 2013). It is important to note that the mechanism for ROS generation may differ depending on the specific MNM (Manke A 2013).

2.4.5.2 Methods: Which tests are available for immunotoxicity?

Key test guidelines and tests for immunotoxicity are listed below.

- Cytotoxicity (*in vitro*):
  - MTT test (measures cell viability through mitochondrial activity)
  - Neutral Red test (measures cell viability through intact lysosomes)
  - LDH Release test (measures necrosis)
  - Annexin V / Propidium Iodide test (measures apoptosis / necrosis)
  - Caspase-3 test (measures apoptosis)
- Stress Response (*in vitro*):

  A number of methods have been identified for detecting ROS generation from nanomaterials, under both abiotic conditions and in cells (Table 9).

Table 9 Analytical methods suitable for ROS generation measurements (Obtained from Hoehener and Hoeck, 2015)

Name/ Acronym
Electron spin resonance / ESR (spin trap / spin probe based approaches)
XTT (XTT is a tetrazolium derivative) assay
Electron paramagnetic resonance / EPR (spin trap / spin probe

based approaches)
Spectrofluorimetry
Singlet Oxygen Sensor Green / SOSG
Dithiothreitol (DTT) assay / DTT
Furfuryl alcohol assay / FFA assay
High-performance liquid chromatography /HPLC combined with electrochemical or UV detectors, pulse radiolysis, fluorometric methods, ESR, spectrophotometric detection, capillary electrophoresis (CE), and CL based determination methods
Fluorogenic probes / DCFH and dihydrorho-damine-123 (DHR-123),
ROS responsive nanosensor, based on PEBBLE (Probes Encapsulated By Biologically Localised Embedding) technology

- Cytokine release:

  As part of its Programme of Work, the Working Party on Manufactured Nanomaterials (WPMN) established in 2006 a project entitled "Manufactured Nanomaterials and Test Guidelines" to review the adopted Test Guidelines and assess whether or not they are suitable for manufactured nanomaterials safety testing[14]. This review of Test Guidelines related to testing for human health concluded that, in general the OECD guidelines are applicable for investigating the health effects of nanomaterials with the important proviso that additional consideration needs to be given to the physicochemical characteristics of the material tested, including such characteristics of the nanomaterial in the test media. In some cases, there will be a need for further modifications to OECD guidelines (OECD, 2009). The OECD TG 439 (*In Vitro* Skin Irritation: Reconstructed Human *Epidermis* Test Method) (OECD 2013) lists the following techniques to test cytokine release:

  - Human skin models can be obtained commercially (e.g. EpiDerm™, EPISKIN™, SkinEthic™ RHE LabCyte EPI-MODEL24 SIT models).
  - EpiDerm™ System consists of normal, human-derived epidermal keratinocytes (NHEK)
  - Episkin™ is an *in vitro* reconstructed human epidermis from normal human keratinocytes cultured on a collagen matrix at the air-liquid interface. This model is histologically similar to the *in vivo* human epidermis.

[14] OECD (2009), Preliminary Review of OECD Test Guidelines for their Applicability to Manufactured Nanomaterials

- SkinEthic™: The *in vitro* Reconstructed Human Epidermis consists of normal human keratinocytes cultured on an inert polycarbonate filter at the air-liquid interface, in a chemically defined medium.
- LabCyte EPI-MODEL is a 3-dimensional reconstructed human epidermis model that is produced by culturing normal human epidermal cells to become multilayered.
- Keratinocytes produce and release inflammatory cytokines interleukins [IL-1α, tumor necrosis factor α (TNF-a)], chemotactic cytokines [IL-8, interferon, e.g. induced protein 10 (IP-10)], growth-promoting factor [IL-6, IL-7, IL-15, granulocyte/macrophage colony-stimulating factor GM-CSF)], transforming growth factor [TGF], cytokines regulating humoral versus cellular immunity [IL-10, IL-12] and other signalling factors, which rapidly generate cutaneous inflammation, suggesting that measurement of such keratinocyte responses may allow the evaluation of toxicological properties of chemicals in order to identify irritants.

- TG 406: Skin Sensisitization (OECD 1992).
- Detection of cytokine release by ELISA (inflammatory response)
- Immunophenotyping (using flow cytometry or immunohistochemistry)
- Modular immune *in vitro* construct system (Test series comprising Peripheral Tissue Equivalent and Lymphoid Tissue Equivalent modules)
- T-cell dependent antibody response
- Natural Killer (NK) cell activity assay
- Host resistance study (*in vivo* challenging of rats or mice)
- Macrophage and neutrophil function assays (*in vitro, in vivo* or *ex vivo)*

Table 10 Test, indicators and models for the evaluation of immune responses (FDA 1999)*

Immune responses	**Functional assays**	**Soluble mediators**	**Phenotyping**	**Other****
Histopathology	NA	NA	Cell surface markers	Morphology
Humoral responses	Immunoassays (e.g. ELISA) for antibody response to antigen plus adjuvant* Plaque-forming cells Lymphocyte proliferation Antibody-dependent cell-mediated cytotoxicity Passive cutaneous anaphylaxis Direct anaphylaxis	Complement (incl. C3a and C5a anaphylatoxins)*, Immune complexes	Cell surface markers	
T-Cells	Guinea pig maximization test*	Cytokine patterns	Cell surface	

		Mouse local lymph node assay* Mouse ear swelling test Lymphocyte proliferation Mixed lymphocyte reaction	indicative of T cell subsets (e.g. Th1 and Th2)	markers (helper and cytotoxic T-cells)	
	Natural killer cells	Tumor cytotoxicity	NA	Cell surface markers	
	Macrophages	Phagocytosis* Antigen presentation	Cytokines (IL-1, TNFα, IL-6, TGFβ)	MHC markers	
	Granulocyte***	Degranulation Phagocytosis	Chemokines, Bioactive amines, Inflammatory cytokines, Enzymes	NA	Cytochemistry
Hot resistance		Resistance to bacteria, viruses and tumors	NA	NA	
Signs of illness		NA	NA	NA	Allergy, Skin rash, Urticaria, Edema, Lymphadenopathy

NA = Not Applicable or Not Needed
*Indicates most commonly used tests. Functional assays are generally more important than tests for soluble mediators or phenotyping. References at the end of this guidance provide detailed testing protocols.
**Animal models of some human autoimmune diseases are available (see references at the end of guidance). However, routine testing for induction of autoimmune disease by materials/devices is not recommended.
***Basophils, Eosinophils and/or Neutrophils

Table 11. List of parameters recommended for observation in the screening phase according to EMEA and FDA guidelines on immunotoxicity (Dujmovic 2005)

	EMEA	**FDA**
Initial screening phase	Haematology Lymphoid organ weights (thymus, spleen, draining and distant lymph nodes) Microscopy of lymphoid tissues (thymus, spleen, draining and distant lymph nodes, Peyers patches) Bone marrow cellularity Distribution of lymphocyte subsets NK-cell activity If later two are unavailable: primary antibody response to a T-cell dependent antigen (e.g. sheep red blood cells) should be done	Haematology Lymphoid organ weights (thymus, spleen, draining and distant lymph nodes) Microscopy of lymphoid tissues (thymus, spleen, draining and distant lymph nodes, Peyers patches) Bone marrow cellularity Globulin levels

2.4.6 Genotoxicity

The key question addressing the fourth risk potential (parallel to accumulation, inflammation/immunotoxicity and ecotoxicity) of the flow chart is: Does the MNM induce/cause DNA damage?

If this question is answered with **no**: no nanospecific potential for health risk is expected and risk assessment can be performed in the conventional way.

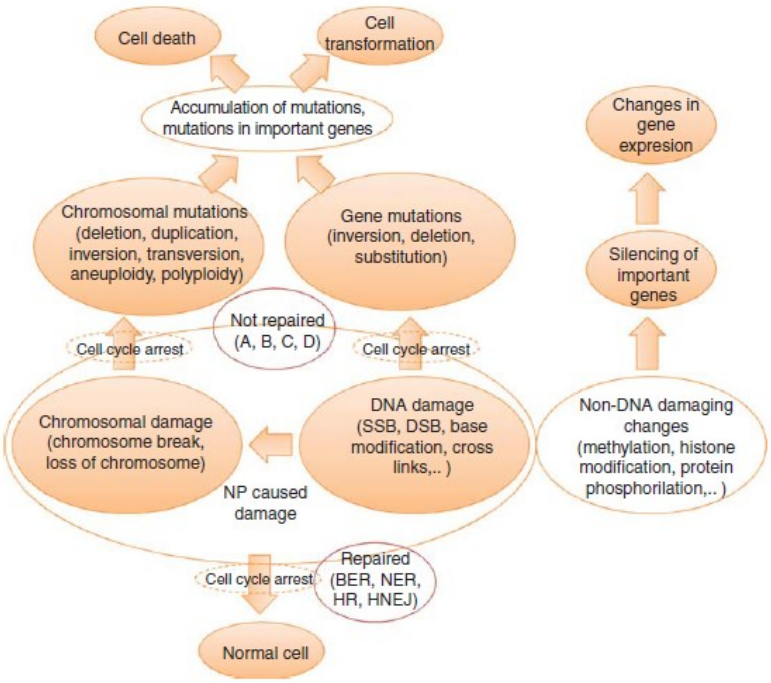
If this question is answered with **yes**: nanospecific data/testing is required (see testing strategy).

2.4.6.1 Testing strategy for genotoxicity

DNA damage can be induced d**irectly** by interacting with DNA and/or i**ndirectly** by:

- Interaction with nuclear proteins
- Interaction with mitotic spindle apparatus
- Disturbance cell cycle checkpoints
- Induction of DNA damage indirectly via
    - ROS production
        - from MNM surface
        - generated by cell components (mitochondria)
        - generated by inflammatory cells (cytokines)
    - Interaction with transition metal from MNM surface
    - Interaction with antioxidants (GSH depletion)?

Figure 6. Possible consequences for NP-induced genotoxicity (Magdolenova, Collins et al. 2014)



The following parameters are proposed for genotoxicity:

1. Particle size (distribution)
2. Chemical composition and impurities
3. Shape
4. Surface area
5. Surface charge
6. Agglomeration/aggregation

Addressed in paragraph 2.4.1

7. Surface coating
8. Degree of coating
9. Coating stability

Addressed in paragraph 2.4.2

10. Solubility/dissolution
11. Hydrolytic stability
12. Acid dissociation

Addressed in paragraph 2.4.3

13. Cell uptake
14. Cytotoxicity
15. ROS generation

Detailed information about the parameter selection for genotoxicity is presented in Appendix 6.3.

13. Cell uptake

Cellular uptake by nanoparticles is a fundamental determinant of toxicity. Nanoparticle size is an important factor dictating nanoparticle uptake. Nanoparticles with a diameter of 50 nm are more efficiently internalized by cells than smaller particle sizes (approximately 15–30 nm) or larger

particles (approximately 70–240 nm); nanoparticles with a diameter of 30–50 nm interact with membrane receptors and are subsequently taken up by receptor-mediated endocytosis (Kettiger, Schipanski et al. 2013). Nanoparticles of 20-50 nm are taken up more rapidly by cells than smaller or larger particles (Iversen T-G 2011). Size seems to be the most important physical property of a MNM in determining cellular uptake (Zhu 2009, Iversen T-G 2011).

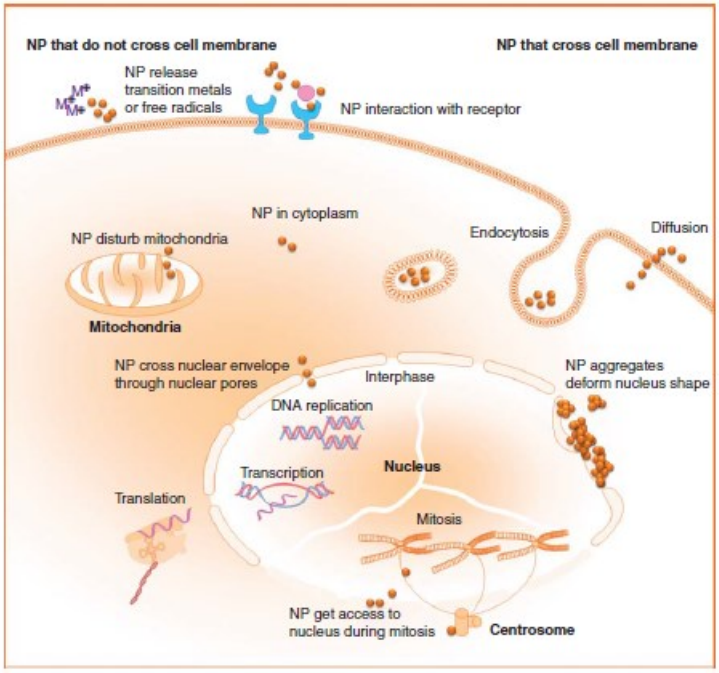


Figure 7. Cellular uptake of MNMs and access to nucleus (Magdolenova, Collins et al. 2014)

The cellular membrane integrity can be affected by the size, surface charge, and surface chemistry of nanoparticles (Wu, Putcha et al. 2013).Positively charged nanoparticles appear to cause membrane damage either directly or by detachment of adsorbed polymers (e.g., polyethyleminine), whereas anionic particles cause intracellular damage (Frohlich 2012). Positively charged particles interact strongly with the anionic membrane and may disrupt membrane integrity (Kettiger, Schipanski et al. 2013). In addition, positively charged particles may induce a more fluid state for easier penetration but negatively charged nanoparticles induce gelation of the membrane (Wu, Putcha et al. 2013). A cationic surface charge correlates with higher cellular uptake and greater cytotoxicity in nonphagocytic cells, whereas cationic nanoparticles appear to cause plasma-membrane disruption to a greater extent than anionic nanoparticles. However, anionic nanoparticles are taken up and are more cytotoxic in phagocytic cells (Frohlich 2012). The size of nanoparticles may also contribute to the membrane toxicity. Nanoparticles in the size range of 1.2 to 22 nm were found to induce holes in lipid membranes, where as those nanoparticles less than 1.2 nm or greater than 22 nm did not have a similar effect (Wu, Putcha et al. 2013). In one study, gold MNMs (approximately 6 nm in diameter) that had the same chemical composition but different

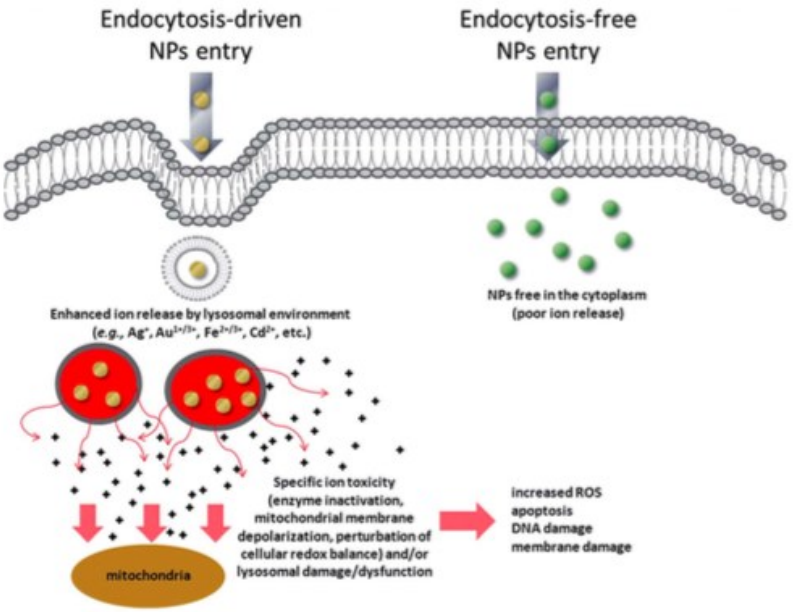
surface ligand organization (sub-nanometer striations of alternating anionic and hydrophobic groups or same moieties but in random distribution) showed dramatic differences in cell membrane response (Wu, Putcha et al. 2013).

14. Cytotoxicity

Cytotoxicity is an important parameter because through cytotoxicity tests, important mechanistic information on toxicity is an indication of the number of cells which are dead or alive after exposure to test chemicals is obtained. Results of cytotoxicity tests are vital in the interpretation of *in vitro* genotoxicity tests. Generally, a positive genotoxicity response under cytotoxic conditions is indicative of a secondary mechanism of genotoxicity (e.g. generation of ROS).

15. ROS generation

The generation of ROS is one of the primary mechanisms of MNM toxicity. MNMs may generate ROS by different processes. The first is the reactivity of the MNM itself. The relatively large surface area and increased reactivity of many MNMs can enhance their formation of ROS (Soenen S 2011). Surface bound radicals such as SiO· and SiO2· present on quartz particles are responsible for the formation of ROS such as OH· and O2·. The second factor is the body's natural defence system. Under stress, certain cells can produce chemically-active oxygen-containing molecules that can "defend" the cell by oxidizing the foreign substance. Prolonged generation of ROS, however, can damage the cell itself (Manke A 2013). It is important to note that the mechanism for ROS generation may differ depending on the specific MNM (Manke A 2013), Generation of ROS is a major indirect mechanism for genotoxicity. Moreover, recently some general mechanism of toxicity for metal oxide nanoparticles have been suggested. It has been demonstrated that NPs when enter cells by active internalization mechanisms, compared to endocytosis-free NPs, are rapidly confined in vesicular structures, endosomes, and finally in lysosomes. The acidic lysosomal pH triggers a lysosome-enhanced Trojan horse effect (LETH effect) that combines the abundant



cellular internalization of the NPs via active processes with the consequent enhanced release of the relatively toxic ions (e.g., Ag+, Cd2+, Fe2+/3+, Au1+/3+ ions). The significant amount of intracellularly leaked ions may then exert ion- specific toxicity (e.g., enzyme depletion/inactivation, protein denaturation, etc.) against some cellular targets (e.g., mitochondria, RER) and/or lysosomal damage/dysfunction. This finally results in increased ROS levels, apoptosis, DNA and membrane damage.

Figure 8. Cellular uptake of MNMs and intracellular leaked ions. They may exert ion- specific toxicity (e.g., enzyme depletion/inactivation, protein denaturation, etc.) against the cellular targets (e.g., mitochondria, RER) and/or lysosomal damage/dysfunction resulting in increased ROS levels, apoptosis, DNA and membrane damage. (Sabella et al. Nanoscale 2014)

2.4.6.2 Methods: Which tests are available for genotoxicity?

Key test guidelines and tests for genotoxicity are listed below.

Test Guidelines or Tests for Cell uptake

The commonly used methods for assessment of uptake of NPs in the cells are:

- TEM,
- confocal and fluorescence microscopy,
- reflection-based imaging and
- flow cytometry

Test Guidelines or Tests for Cytotoxicity

- Cell viability (indicator of state of cultured cells *in vitro*)
  - MTT test (measures cell viability through mitochondrial activity)
  - Neutral Red test (measures cell viability through intact lysosomes)
  - Lactate dehydrogenase (LDH) Release test (measures necrosis)
  - Annexin V / Propidium Iodide test (measures apoptosis / necrosis)
  - Caspase-3 test (measures apoptosis)

Test Guidelines or Tests for Stress Response (*in vitro*)

- ROS determination test using H2DCF-DA
- The Total Reactive Oxygen Species (ROS) Assay Kit 520 nm (Affymetrix 2014)
- ROS determination test using GSH (intracellular ROS generation)

The preparation of the dispersion is key, as it is expected to affect agglomerate size, which in turn affects sedimentation, cell exposure, cytotoxicity, dose selection and genotoxicity (OECD 2014).

There are several nanoparticle properties that may affect or interfere with *in vitro* assays (Kroll, Pillukat et al. 2009):

- High adsorption capacity. Due to their large surface per unit mass nanoparticles display an increased adsorption capacity and biological reactivity as compared to the bulk material
- Optical properties. If light absorption or fluorescence detection is used to evaluate particle toxicity, it has to be considered that many nanoparticles display optical properties potentially interfering with the detection systems.
- Catalytic activity. The high surface/mass relationship of nanosized materials results in an excess surface energy enhancing any catalytic activity.
- Acidity/alkalinity. The pH of nanoparticles in solutions is important since the most widely used in vitro assays are pH-dependent.
- Magnetic properties. Some metal oxide nanoparticles like Fe2O3 are super paramagnetic and generate strong, local magnetic fields which lead to the

production of free radicals that in turn may interfere with cytotoxicity methods based on redox reactions.

- Dissolution. The release of metal ions or trace metals into biological media may interfere with cytotoxicity tests.

Test Guidelines or Tests for Genotoxicity

OECD guidelines exist for several *in vitro* and *in vivo* genotoxicity assays:

- OECD 473 for the in vitro CHA test,
- OECD 474 and 475 for the mammalian erythrocyte and bone marrow CHA tests,
- OECD 476 for the in vitro mammalian cell gene mutation test and
- OECD 487 draft for the in vitro MN test. OECD guidelines are under preparation for the *in vivo* comet assay test (http://www.jacvam.jp/en_effort/en_oecd.html).

*OECD 471 for the bacterial reverse mutation test [**not appropriate** for MNM],* Refer to Section 8.1 for a discussion on the gaps, uncertainties and recommendations associated with the use of the current genotoxicity tests.

2.4.7 Ecotoxicity

The key question addressing the sixth risk potential (parallel to accumulation, inflammation/immunotoxicity and genotoxicity) of the flow chart is: Is ecotoxicity expected?

If this question is answered with **no**: no nanospecific potential for health risk is expected and risk assessment can be performed in the conventional way.

If this question is answered with **yes**: nanospecific data/testing is required (see testing strategy).

2.4.7.1 Testing strategy for ecotoxicity

The most critical parameters in determining nanoparticle ecotoxicity are 1) parameters of the test medium itself and 2) the following nanoparticle characteristics: size/surface area, shape (especially for algae and fish embryo), reactivity, photoactivity, the presence of functional groups/coatings and surface charge. Each set of parameters, related to the medium or to the nanoparticle, is discussed below.

Test Medium

Firstly, the key nanoparticle characteristics should be determined (physico-chemical properties). As stressed in the OECD (2014a) expert meeting report on ecotoxicology and environmental fate of manufactured MNMs test guidelines, the physico-chemical properties of a nanoparticle are not steady and those properties can change with sample preparation, choice of testing media, dispersant use, the presence of environmental ligands and other factors. The OECD Guidance on sample preparation and dosimetry for the safety testing of manufactured MNMs (2012a) provides tentative guidance.

The way in which nanoparticles are added/dispersed in the aqueous test medium will also influence the fate/behaviour of the nanoparticle and influence the ecotoxicity effects. OECD (2014a) has stated that dispersion methods can lead to a change in MNM properties and therefore the nature of the MNM in the dispersion must be characterised in order to quantify exposure. In summary, the dispersion methods and the composition of the aqueous media could all determine ecotoxicity endpoints.

Test box. Test medium

Dispersion of MNMs into aqueous medium

The OECD guidance on the sample preparation of nanoparticles (2012a) describes the possible methods of suspension (e.g. stirring, sonication, grinding, use of solvents). But there is no consensus on the best approaches for preparing MNM samples. Handy et al. (2012) give an overview of the advantages and disadvantages of dispersion methods: sonication, use of (natural) dispersants, stirring/mixing and no dispersion. The use of natural dispersants such as humic acids could influence the toxicity of metal-containing MNMs and could lead to analytical problems for carbon-based MNMs. The use of synthetic dispersants could have an influence on the organisms and render the ecotoxicity data less ecological

relevant. The dispersant could also interact with the coating and thus change the nanoparticle characteristics. Furthermore, sonication and mixing/stirring also seems less ecologically relevant and can fragment multiwall carbon nanotubes, increase the production of ROS, remove coatings and hydroxylate surfaces (Handy et al., 2012, OECD, 2012a). No protocol has been developed to standardise dispersion protocols. The OECD expert meeting report (2014a) notes that the discussion group on ecotoxicity testing examined the practicality of creating a technical guidance (TG) and considered the following points: type of dosimetry, the degree of monodispersiveness of the stock suspensions, the renewal of the test media and the use of stabilisers. However, no consensus was reached on sample preparation. The workgroup decided to update the OECD guidance on the sample preparation of MNMs (2012a) and to develop a flow chart(s) that would guide the user to a decision on the dispersion protocol based on nanoparticle characteristics. Gaps could also be identified based on such a flow chart(s). Critical variables include the type or category of material and the presence of a coating. Work on the flow charts would be based on data-rich substances such as nanosilver and titanium dioxide.

The discussion on the fate and behaviour of MNMs at this OECD expert meeting (2014a) also addressed a tiered approach for the testing of MNMs. Participants concluded that the first step should be to describe the dissolution and dispersion of MNMs and the second step should be to determine the agglomeration state and dispersion stability. The third step would include testing biodegradation and the fourth step would include abiotic degradation. For the first three steps, a new technical guidance must be developed. Regarding the TG 305, the expert group also concluded that the use of BCF (bioconcentration factors) is not applicable for tests with MNMs. The octanol-water partition coefficient (K_{OW}) value is not suitable for predicting bioaccumulation and is not an appropriate endpoint for physico-chemical characterisation of MNMs. Water solubility/dispersibility and dissolution seem to be the main parameters affecting fate and behaviour in the environment and therefore have to be tested before testing ecotoxicity. To represent the main environmental conditions, these tests must be conducted under different conditions, considering four variables: 1) pH (i.e. pH 4-7-9); 2) With and without organisms; 3) With or without NOM (natural organic matter/proteins); and 4) Using filtration to isolate certain particle size ranges, e.g. to eliminate agglomerates. In a second step, dispersion stability and aggregation state should be analysed if the material is dispersible and not soluble (OECD, 2014a). These suggestions from the expert meeting report must be further developed to create practical testing protocols. For example, no OECD test guideline exists to measure the dispersion of primary or agglomerated nanoparticles. Some methods are available or under development (OECD, 2014a).

Aquatic media characteristics

The behaviour of the MNMs in the aquatic environment (and in the aquatic media tested) will strongly depend on environmental conditions, such as pH, organic matter and ionic strength (Ma and Lin, 2013). OECD (2012a) has observed that the media quality should be sufficiently addressed during intervals during a test. At the OECD expert meeting (2014a), however, the parameters that influence the MNM characteristics could not be fully addressed. Yet, as described in several papers, the above-mentioned parameters could influence nanoparticle ecotoxicity and should therefore be taken into account when applying a read-across strategy.

Nanoparticle characteristics that determine ecotoxicity effects

The nanoparticle characteristics that determine ecotoxicity effects are summarised in Table 12 and briefly described below according to trophic level (i.e. algae, daphnia and fish).

Table 12 Parameters critical to ecological endpoints (Adapted from Sellers et al., 2015)

Property	Summary of Relevance
Chemical identity	
Chemical composition	Chemical composition can fundamentally determine the effects of exposure.

Crystalline structure	Crystalline structure may influence reactivity for some materials in a way that affects toxicity.
Surface characteristics	Surface characteristics will influence sorption to environmental or biological media and the reactivity of a nanomaterial.
Impurities	Impurities can substantially contribute to ecotoxicity.
Particle characteristics	
Particle size/range	The size of the nanoparticle impacts other physico-chemical properties, and can determine whether it can be internalised into an organism. Not a static parameter; may change during the course of ecotoxicity testing.
Shape	Particle shape can enhance the internalisation of a nanoparticle and potentially its ecotoxicity.
Surface area	The increase in relative surface area with decreasing particle size can increase the reactivity per unit mass of the nanoparticle.
Coating	In environmental media the coating of MNMs can change. E.g. in presence of natural organic matter can affect ecotoxicity based on a changed exposure due to adsorbing to the nanomaterials, similar to a protein corona.
Fundamental behaviour	
Water solubility • Rate of dissolution • Equilibrium solubility	Fundamentally affects the bioavailability of substances in the aquatic environment.
Hamaker constant	Parameter can influence the degree of agglomeration and sorption, but is not typically characterised in ecotoxicity studies.
Zeta potential	Parameter can influence the degree of agglomeration and sorption, but is not typically characterised in ecotoxicity studies.
Dispersiveness	Parameter can influence the degree of exposure but is often not characterised in ecotoxicity studies.
Activity and reactivity	
Physical hazards	Parameter may be relevant to the risk of injury in occupational exposures, but is not a primary variable in ecotoxicity studies.
Reactivity	The reactivity of a nanomaterial – particularly relative to the non-nanoform of the substance – can impact the generation of ROS, induce inflammation and elicit cellular toxicity.
Photoreactivity	Increased photoreactivity with decreasing particle size may affect ecotoxicity.

Chemical Composition

The expression *surface chemistry* (generally speaking, the chemical nature and composition of the outermost layers of the MNM) may need to be considered in greater detail or perhaps in a hierarchical manner, including coatings, functional groups and capping agents; these may be involved in surface reactions in different media (e.g. redox reactions, coordination chemistry, catalysis) (OECD, 2012c).

Coatings can also influence agglomeration processes (next to other environmental characteristics such as pH, ionic strength) (OECD, 2012a). The physico-chemical properties of the coating will also affect nanoparticle dispersion in an aqueous medium. When organic-coated, engineered MNMs are tested, the standard biodegradation TG is not applicable due to the low concentration of carbon used for the coating. It was concluded in the OECD expert meeting (2014a), therefore, that the TG dealing with biodegradation is not directly applicable for engineered MNMs and it was

decided that a specific TG for the biodegradation of engineered MNMs or different groups of engineered MNMs is needed.

Surface charge is also important; this property can depend on the nature of the nanoparticle and/or its coating. A study from Rodea Palomares et al. (2011) indicated that the tendency to form nanoparticle aggregates strongly depends on the surface charge. The surface charge of a given particle may depend both on pH and solution composition, and may be measured as zeta potential. Clearly, ecotoxicologists conducting research in this area will need to ensure that the surface charge is measured and that the exact measurement conditions are given within the bounds of the fluid properties likely to occur in the medium of interest (OECD, 2012a).

Particle characteristics

The size of a nanoparticle will determine whether it can be internalised in an organism. The cell wall of algae is an efficient barrier that prevents most MNMs internalisation via endocytosis. However, cell wall pores with diameters ranging from 5–20 nm can be a potential uptake port for small MNMs (von Moos and Slaveykova, 2014). Smaller particles can cross the gut lumen of the daphnids. Studies from Ebert et al. 2005 suggested that particles of less than 50 µm are more easily ingested by daphnids. As particles aggregate into masses, there is a decrease in ingestion and toxicity (Zhu et al., 2009).

Particle size can change during the course of a test. Introducing the nanoparticle into an aqueous media can lead to the formation of agglomerates or aggregates, which could also then cause shading or influence the photosynthetic capacity of algae and may affect uptake at higher trophic levels. A number of media parameters or environmental parameters will influence the agglomeration behaviour (and thus size) of the nanoparticle, e.g. ionic strength, media composition (OECD, 2014a), pH, solvent, the presence of proteins, sonication (OECD, 2012a).

The National Institute of Standards and Technology (NIST) has commented on the appropriate procedures for characterising dispersed MNMs. NIST advises that experimenters should ensure that particle size distribution is stable from the point that treatment to sustain the dispersion (e.g. sonication) is suspended to the point of measurement, and throughout the duration of relevant tests to be conducted with the material (Taurozzi et al., 2012).

The shape of a nanoparticle may enhance the internalisation of a nanoparticle. Needle-like nanoparticles may perforate cell membranes or cell walls, or damage the gut or gill epithelium (e.g. Handy et al., 2011; Ivask et al., 2013; Jackson et al., 2013; Ma and Lin, 2013). Furthermore, rod-shaped or fibre particles can have a greater contact area with the cell membrane, can more easily get through capillaries, adhere to blood vessels, stimulate platelet aggregation and block potassium ion channels, compared with spherical carbon nanoparticles such as fullerenes. Carbon nanotubes in fibrous structures may be difficult to engulf by macrophages. Longer carbon nanotubes may show a higher inflammatory response. The shape of nanometal can have an effect on fish embryos. A study with spherical nickel nanoparticles of different sizes and dendritic

structures consisting of aggregated 60 nm particles indicated that dendritic clusters were more toxic than the soluble nickel and the nanoparticles of the different sizes. In addition, it seemed that the toxicity of the spherical nanoparticles manifested as organ defects (Shaw and Handy, 2011).
The surface area of a nanoparticle relates to its size and porosity. Smaller particles have a larger surface area and there a larger surface energy relative to the volume of the particle, compared with the corresponding ratio for larger particles. The surface area of a particle determines the particle reactivity and affects the generation of ROS and radical activity (von Moos and Slaveykova, 2014). This indicates that the smaller the size (the larger the surface area), the higher the relative potential for oxidative stress. And, as been noted by a study with silver MNMs and algae, the larger the surface area, the larger the number of reaction sites for UV (ultraviolet) adsorption.
The extent of the influence of aggregation/agglomeration on available surface area is still unclear (OECD, 2014a).

Fundamental behaviour

"Nanosizing" a substance may increase the rate of dissolution of a soluble material; it may also increase the equilibrium solubility concentration of certain substances, although that effect may not be seen within the duration of most standard ecotoxicity tests. The dissolution/solubility rate of the metal ions from metal-containing nanoparticles is crucial. Studies with fish indicate poorly soluble metal oxide nanoparticles may have low toxicity (Shaw and Handy, 2011). Studies have shown, for example, that nanosilver was less toxic than silver to adult zebrafish. The opposite was found for a test with nanocopper and dissolved copper with respect to juvenile zebrafish (the reverse was seen with adult fish) (Shaw and Handy, 2011).
The rate of dissolution is a key factor affecting environmental behaviour and test performance (OECD, 2014a). If a nanoparticle can be dissolved in the test media within a given timeframe, nano-specific testing should not be considered and testing methodologies for traditional chemicals can be applied. However, TG 105 (Water Solubility) is considered not to be appropriate for MNMs and the development of a new TG has been suggested (OECD, 2014a).

Activity and Reactivity

Functional groups and the charge reactivity and/or photoactivity of the nanoparticle can play a role in toxicity. The surface area of a particle determines the particle reactivity and the generation of oxidants and radical activity (von Moos and Slaveykova, 2014). This indicates that the smaller the size (the larger the surface area), the higher the relative potential for oxidative stress. Photoactivity may also be influenced by other particle properties (defect sites, structural disorder). A study involving titanium dioxide and *Daphnia magna* indicated that the effects observed in a test under UVA light are likely to be due to ROS, compared with a test performed in the dark (Amiano et al., 2012).

2.4.7.2 Methods: Which tests are applicable for ecotoxicity?

Key test guidelines,methods or protocols for ecotoxicity are presented in Table 13.

Table 13. Parameters and methods/protocols applicable for ecotoxicity

Property	Methods and protocols
Chemical identity	
Chemical composition	See 2.4.1.2
Crystalline structure	See 2.4.1.2.
Surface characteristics	Zeta potential can be measured on surface charge in 2.4.1.2
Impurities	See 2.4.1.2.
Particle characteristics	
Particle size/range	See 2.4.2.1, additional note: In ecotoxicity testing in addition to characterizing the nanomaterials, the possible agglomeration and aggregation needs to measured, see below under Fundamental behaviour for methods to measure the agglomeration or aggregation rate.
Shape	See 2.4.1.2
Surface area	See 2.4.1.2.
Coating	Changes in coating can be quantified using the zeta potential which can be measured using electrophoretic mobility.methods (Li, 2009 #1619), see also section 2.4.1.2 on surface charge. Atomic Force Microscopy (Schön, 2007) Reflectrometry (Dijt, 1994)
Fundamental behaviour	
Dissolution rate	The rate of dissolution can be measured using a measure of quantifying the dissolved fraction of the chemical components of a nanomaterial in time. This can be done using: Ion selective electrode ICP-MS, ICP-OES, AAS after a separation step. Often using (ultra)- filtration or centrifugation. An OECD test guideline is under development (Kuhnel, 2014). Several studies have reported and used methods to measure dissolution, e.g. (Wang, 2012; Waalewijn-Kool, 2013)
Agglomeration or aggregation rate	A good measure is the hydrodynamic diameter in relevant media suing DLS, NTA or DC. A standard protocol is being developed by the OECD (Kuhnel, 2014). Several methods reported in literature based on: Measuring the changein particles size in time (Liu, 2013; Chen, 2007). Measuring the change in concentration of freely dispersed ENMs in time (Ottofuelling, 2011; Quik, 2012).
Hamaker constant	Not measured as part of ecotoxicity experiments. Possible include calorimetry and AFM (Médout-Marère, 2000; Soma, 2007).
Zeta potential	See 2.4.1.2, surface charge.
Dispersiveness	See Agglomeration or aggregation rate above. The OECD test guideline on agglomeration should be a valid method when available.
Activity and reactivity	
Physical hazards	
Reactivity	No direct measurements, although several studies have shown relationships between the redox potential of metal oxides and reactivity. But no direct measurement method is available.
Photoreactivity	No direct measurement method is available. There are studies that have included this effect by comparing experiments with and without UV radiation (Pathakoti, 2014;Jovanovic, 2015).

Ecotoxicity testing

Regulatory testing is essential nowadays in order to preserve human and environmental health from the deleterious effects of chemicals and MNMs. The Organization of Economic Cooperation and Development (OECD) in its function as a regulatory agency is committing significant resource in order to determine the applicability of its test guidelines (TG) initially designed for chemicals, to NMs. The OECD test guidelines on the ecotoxicological testing of chemical substances were evaluated, and considered to be principally suitable also for the testing of MNMs (OECD, 2009), although some adaptations were found to be necessary. There is already guidance on sample preparation and dosimetry in ecotoxicological testing (OECD, 2012). However, the various subjects are described in general terms, hence a more detailed description is required. To achieve progress, a workshop was launched by the OECD and several topics and test guidelines were comprehensively discussed (OECD, 2014). Nevertheless, currently no compilation of proposed nano-specific test modifications on OECD TGs on ecotoxicity is available.

In this context, significant progress on the adaptation of generally applied OECD TGs in regulatory testing was achieved in the context of the European FP7 project MARINA (http://www.marina-fp7.eu/). The OECD test guidelines that are most commonly used for toxicity testing of chemicals, are listed in Table 14.

Table 14. OECD test guidelines on ecotoxicological testing of chemicals that, after MNM-specific modification, are deemed suitable for ecotoxicity testing of nanoparticles.

OECD TG [1]	**Media**	**Test organism**	**Test species used in the project**	**Endpoint**
201	Water	Algae	*Ps. subcapitata*	Growth
202	Water	Daphnids	*D. magna*	Immobilisation
210	Water	Fish	*D. rerio*	Survival, hatching, morphometrics
216	Soil	Terrestrial Microorganisms	Soil microflora	Nitrogen transformation
217				Carbon transformation
220	Soil	Worms	*E. crypticus*	Reproduction
222	Soil		*E.fetida*	Reproduction
225	Sediment	Sediment worms	*L. variegatus*	Emergence

[1] Number of OECD test guideline

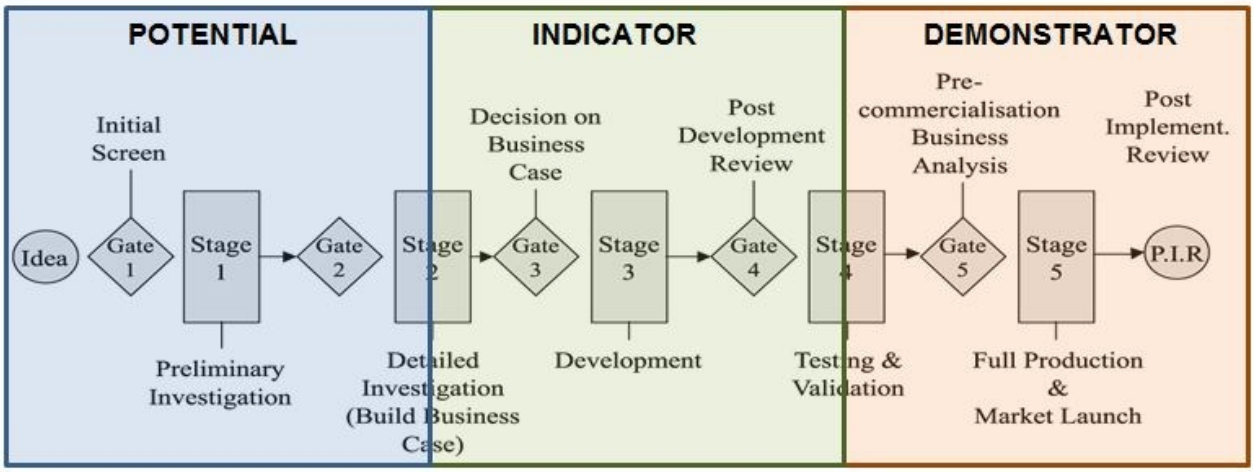
A proposal for the MNM-specific adaptation of the ecotoxicological test systems originally developed for soluble chemicals was recently prepared by Hund-Rinke et al. within the context of the European FP7-project MARINA (www.marina.eu). In the MARINA project, OECD test guidelines on aquatic, sediment and soil test species were adapted for the testing of nanoparticular metals and metal oxides. Concrete proposals on the modifications with their justification are now available for the testing of the green algae *Ps. subcapitata* (OECD TG 201), the daphnid *D. magna* (OECD TG 202), the fish *D. rerio* (OECD TG 210), the sediment organism *L. variegatus* (OECD TG 225), soil microflora (OECD TGs 216, 217) and terrestrial invertebrates (*E. crypticus, E. fetida*,

OECD TGs 220, 222). Tests on root elongation of hydroponic plants in hydroponic systems and on fish cells can provide additional information on the toxicity of the NMs to further organisms. Test descriptions are also available.

2.5 Summary and Discussion

In this deliverable, we propose a first screening strategy to identify potential risks of MNMs at early stage of innovation. This deliverable focusses on the first two stages where the potential of a MNM to give rise to human or environmental health risks can be deduced on the basis of a limited set of information (Figure 8, blue part).

Figure 8. Stage-Gate innovation process including risk terminology



The basis for the screening strategy is formed by six key risk potentials for MNMs: solubility/dissolution rate, stability of coating, accumulation, genotoxicity, inflammation and ecotoxicity. The aim of this screening strategy is to bring information on potential health risks in line with the stage of innovation.

To develop this screening strategy, the risk potentials were identified and discussed within an expert workshop and an overview of literature was compiled on relevant parameters for these risk potentials and their relevance for the behaviour, fate and toxicity of MNMs in organisms and the environment. The overview of parameters including the methods or tools for measurement was based on current knowledge on the behaviour and toxicity of MNMs. It resulted in a minimal base set of parameters that are regarded essential to describe each risk potential for safety screening. Table 15 presents the key parameters per risk potential for safety screening of MNMs.

This proposed screening strategy focusses only on the first stages of the innovation process. To implement a safety screening useful for the entire innovation process, it is recommended to develop a safety strategy for stages 2 till 5 as well (“Indicator” and “demonstrator” in Figure 8).

Moreover, it is recommended that this proposed screening strategy has to be tested in several case studies to demonstrate the efficiency of it in practice. Based on real-life experiences, the screening strategy should evaluated for its feasibility. One need to be aware that this strategy forms no part of a regulatory arena and therefore it is key that the proposed strategy is regarded by innovators as a feasible strategy, both from a testing point of view as well as from a business point

of view. Furthermore, the approach of performing experiments could be improved based on this safety screening.

It is recommended to investigate whether multiple parameters could be measured within combined test methods. The safety screening strategy for MNMs will be more efficient when the methods and the experimental approach are in line with this screening strategy. In addition, it is recommended to link this proposed safety screening strategy with the results of other WPs (e.g. SOPs) and the toolbox of NANoREG.

Table 15. Overview of the key parameters per risk potential for safety screening of MNMs

RISK POTENTIAL KEY PARAMETERS	MNM characteristics	Stability of coating	Solubility Dissolution	Accumulation	Genotoxicity	Inflammation	Ecotoxicity
Particle size (distribution)	X	X	X	X	X	X	X
Composition and impurities	X	X	X	X	X	X	X
Shape	X	X	X	X	X	X	X
Surface area	X	X	X	X	X	X	X
Surface charge	X	X	X	X	X	X	X
Agglomeration Aggregation	X	X	X	X	X	X	X
Surface coating		X	X	X	X	X	X
Degree of coating		X	X	X	X	X	X
Coating stability		X	X	X	X	X	X
Solubility/dissolution			X	X	X	X	X
Hydrolytic stability			X	X	X	X	X
Acid dissociation			X	X	X	X	X
Exposure route				X			
Dose				X			
Protein binding				X			

This project has received funding from the European Union Seventh Framework Programme (FP7/2007-2013) under grant agreement no 310584

Clearance				X			
Cell uptake					X		
Cytotoxicity					X		
ROS generation					X	X	
Hydrophobicity **Lipophilicity**						X	
Cytokine induction						X	
Test medium							X
Crystalline structure							X
Reactivity							X
Photoreactivity							X

3 Deviations from the work plan

The Safe-by-Design concept was not intended to be discussed in this deliverable. However, during the NANoREG project, RIVM has developed new ideas on a Safe Innovations Approach (RIVM-SIA) that includes a Safe-by-Design concept. It was thought that this Safe-by-design concept could be relevant and useful for the development of a safety screening strategy of MNMs. Therefore, the WP-leader and task-leader have decided, in consultation with WP-partners, to include the Safe-by-Design concept in this deliverable.

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This project has received funding from the European Union Seventh Framework Programme (FP7/2007-2013) under grant agreement no 310584

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5 Annexes

5.1 The Immune System

Organs of the immune system: *Bone marrow, thymus, spleen, lymph nodes and mucosa-associated lymphoid tissues*. During hematopoiesis, bone marrow-derived stem cells differentiate into either mature cells or into precursors of cells that migrate out of the bone marrow to continue their maturation elsewhere, for example, maturation of T lymphocytes occurs in the thymus. The spleen acts as an immunologic filter of the blood, whereas the nodes filter the tissue fluids (*i.e.* lymph). The mucosa-associated lymphoid tissues are aggregates of lymphoid tissues near the mucosal surfaces.

Cells of the immune system: Leukocytes (white blood cells) consist of: **(a)** Polymorphonuclear granulocytes (PMN): Neutrophils, eosinophils and basophils; **(b)** Monocytes: can be differentiated into macrophages; **(c)** Natural killer cells; **(d)** B lymphocytes: produce antibodies and **(e)** T lymphocytes: T helper cells, suppressor T cells and cytotoxic T cells. Lymphocytes and mononuclear phagocytes play a central role in the immune response.

Immune response: Antigen presenting cells (APCs) are cells that capable of processing an antigen and presenting part of it onto the MHC II where it can interact with the appropriate immune cell receptors. Dendritic cells, macrophages and B cells are the main APCs for T cells, whereas follicular dendritic cells are the main APCs for B cells. APCs and B or T cells communicate together, either directly or *via* cytokines, to initiate an immune response. Antigen processing and presentation signal these cells to proliferate and secrete antibodies (B cells), cytokines (CD4+), or become activated to kill cells expressing the antigen presented by the APC (CD8+). The antibodies secreted by B cells can directly bind to that antigen, which accelerate the clearance by the PMN or macrophages. The antibodies may also initiate the complement destruction cascade by attracting the serum protein to bind to the immobilized antibodies that are bound to the antigen, and thereby aiding in the phagocytosis process and elimination of the immune complex.

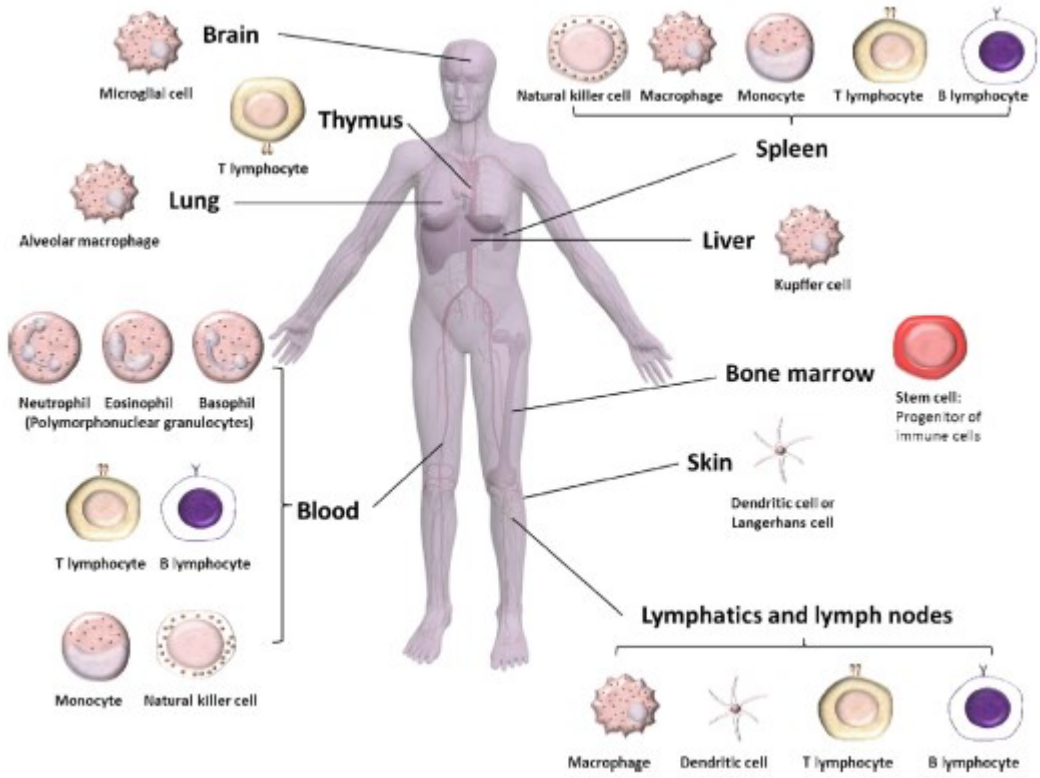

Figure 6.2.1: Human body showing the various organs of the immune system and the distribution of the various immune cells into the immune organs and other organs that are rich in macrophages (Elsabahy and Wooley 2013).

Mononuclear phagocytic system (MPS): The original term used to refer to the phagocytic system is reticulo-endothelial system (RES). It refers to the mononuclear cells of mysenchymal origin that reside in the reticular organs including the liver, spleen and lymph nodes and other organs, and possess the collective property of rapidly engulfing colloids and particulate materials. Since not all endothelial and reticular cells are phagocytic, the term RES was recently replaced with MPS, however in the literature, RES and MPS are often used interchangeably. MPS is distributed throughout the body and are mainly responsible for the phagocytosis, clearance and initiation of the immune response due to the introduction of the MNMs, foreign particles or antigens in the body, and it also engulfs and clears aged cells in the blood and tissues. Phagocytic cells include PMN, blood monocytes and tissue macrophages (Kupffer cells in the liver, alveolar macrophages in the lung, splenic macrophages, peritoneal cells in the peritoneal fluids, microglial cells in the central nervous tissues, histiocytes of the connective tissues, and dendritic cells). These cells have a number of surface receptors that allow them to bind carbohydrates, complement protein and immunoglobulins. If the MHC II is expressed on the surface of the phagocytes, it enables them to function as APCs.

Opsonins (“prepare food for”) are antibodies, complement proteins and other serum components that upon binding to foreign particles make them easier targets for phagocytes.
Complement system: It is a part of the immune system comprised of a biochemical proteolytic cascade that aids (complement) the antibodies and phagocytic cells to eliminate pathogens from the body (Elsabahy and Wooley 2013).

MNMs and the immune system

Since MNMs can interact with proteins, and proteins, including antibodies, are often used to target MNMs to specific cells and tissues, understanding the use of cytokines as biomarkers of undesirable immunostimulation associated with engineered MNMs is emerging as an essential component of MNM safety testing. Evaluation of the immunotoxicity of MNMs by measuring the levels of cytokines, in particular the proinflammatory cytokines can be useful tools in evaluating MNM immunotoxicity. High levels of cytokines upon treatment with MNMs are usually associated with toxicity, adverse reactions and low therapeutic efficacy, as will be discussed later. Hence, cytokines might be utilized to partially predict the MNM immunotoxicity (Elsabahy and Wooley 2013).

The ability of MNMs to induce an immune response (immunogenicity) is a product of the MNMs’ physicochemical properties (size, charge, hydrophobicity, *synthesis*). It is also influenced by other factors such as the surface targeting moieties and therapeutic payload, the animal model, and the route of administration. MNMs can reach the systemic circulation *via* different sites, where the route of administration plays a pivotal role in dictating the fate of the MNMs and their immunotoxicity. The immune system may recognize many of the components of MNMs (*e.g.* shell, core, surface decorating moieties, and cargoes) as foreign, and initiates an immune response through a complex process. There are several markers and measures, which can be utilized to study and predict the immune response of biomaterials, such as, lymphocyte proliferation, cell surface markers, morphological and histopathological examination. Soluble mediators, such as, antibodies (*e.g.* IgG, IgE, IgM), complement proteins (*e.g.* C3a, C5a), and cytokines (*e.g.* TH1 and TH2 cytokines) have also been exploited to evaluate the immunogenicity of various therapeutics (Elsabahy and Wooley 2013). However, there is still a fundamental lack of biomarker specific for the safety screening of nanoproducts (Bergamaschi et al, NANOTODAY 2015).
Proper design of MNMs has been always a subject of interest to improve their *in vitro* and *in vivo* characteristics, mainly to impart greater stability to their cargoes and to prolong blood circulation times to allow the accumulation at target sites, as well as greater safety. However, little motivation has been directed towards designing MNMs of low immunogenicity. When designing MNMs of low toxicity and immunogenicity, every component in the MNM should be considered and systematic

studies should be carried out to evaluate the effect of structural modifications on immunotoxicities. It is equally important to consider both the MNM-forming material and its innovative functionality (Elsabahy and Wooley 2013).

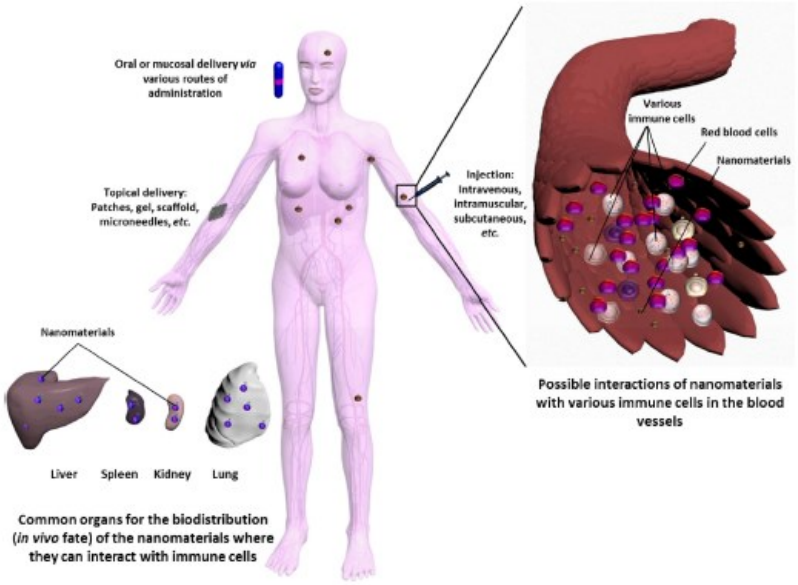

Figure 6.2.2: The possible interactions of MNMs with the various components of the immune system after entering the body *via* various routes of administration (oral, mucosal, systemic or topical) (Elsabahy and Wooley 2013).

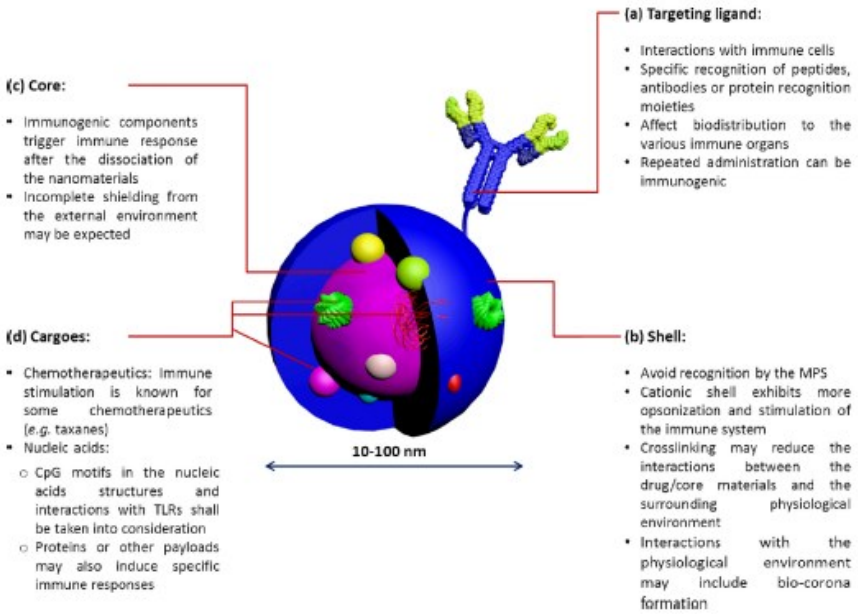

Figure 6.2.3: The general composition of a multifunctional MNM for biomedical deliver applications is illustrated with highlighting some important considerations for the design of MNMs of low immunogenicity. The size of MNMs usually ranges from 10– 100 nm (MPS: Mononuclear phagocytic system; TLR: Toll-like receptors) (Elsabahy and Wooley 2013).

The size of MNMs can determine the cellular uptake, intracellular trafficking pathways, biodistribution and *in vivo* fate. The general consensus is that nanometer sized particles are more toxic than micrometer sized particles, probably due to the increased surface to volume ratio. Hussain *et al.* (2009) investigated the role of surface area and oxidative stress in the cellular effects of two chemically distinct MNMs, carbon black (CB, average size 13, 21 and 95 nm) and titanium dioxide (TiO2 average size 15 and 25–75 nm), on the bronchial epithelial cell line (16HBE14o-). CB and TiO2 MNMs were taken up by 16HBE cells in a dose-dependent manner and were localized within the endosomes or free in the cytoplasm. Oxidative stress produced inside the cell by MNMs was well correlated to the Brunauer, Emmett and Teller (BET) surface area and endocytosis of MNMs. Inflammatory effects of MNMs were dependent on the surface area and were mediated through oxidative stress as they were inhibited by catalase. It can be concluded that MNM induced oxidative stress and pro-inflammatory responses are well correlated not only with the BET surface of the individual MNMs but also with the internalized amount of MNMs. Differences of even few nanometers in primary particle size lead to significant changes in inflammatory and oxidative stress responses (Hussain, Boland et al. 2009).

The size of a MNM affects both the type and the strength of the immune response to an antigen. nanometer and micrometer (220, 500 and 1200 nm) RNA/protamine particles were examined and results showed that smaller particles elicited viral-like responses by triggering the release of IFN-α, whereas the larger particles developed bacterial-like immune responses and induced the release of TNF-α, whereas neither of the MNM components (RNA or protamine) could increase the level of any of the tested cytokines (IFN-α and TNF-α) in human peripheral blood mononuclear cells. It was suggested that the immune system distinguishes the size of the particles associated with the antigen (*i.e.* single stranded RNA) to trigger antiviral and antibacterial/antifungal immune responses for the nano- and micro-sized particles, respectively (Rettig, Haen et al. 2010). Other studies, have shown that only small MNMs (about 50 nm) conjugated to a model antigen could induce TH1 response, whereas antigen conjugates to larger particles tend to promote a TH2 response (Fifis, Gamvrellis et al. 2004, Minigo, Scholzen et al. 2007, Mottram, Leong et al. 2007).

Depending on the cell line, the shape of the MNMs can affect the immunotoxicity of the MNMs. The effect of shape of zinc oxide MNMs (spherical *vs.* sheet) of approximately similar specific surface area was studied. Although the higher cellular association of the spherical particles, there were no significant differences in the generation of the ROS and in stimulating the secretion of proinflammatory cytokines (IL-6 and TNF-α) (Kreuter, Liehl et al. 1988). The secretion of TNF-α was higher in RAW 264.7 mouse macrophages treated with spherical zinc oxide MNMs, as compared to the sheet zinc oxide particles. In contrast, the opposite pattern was observed in

mouse primary dendritic cells, where the sheet-like MNMs induced higher release of TNF-α than the spherical ones. Hence, the cell type is an important factor in dictating the immune response to MNMs (Elsabahy and Wooley 2013). The critical role of shape in immunotoxicity was also demonstrated when increased markers of inflammation were detected in bronchoalveolar lavage of anatase nanobelt aspiration-treated mice compared with those determined in animals treated with titanium dioxide nanospheres (Iavicoli, Leso et al. 2012).

Lower charge density, lower zeta-potenial and lower cellular binding/uptake are associated with lower immunotoxcity. Surface modification of the particles with carboxyl groups reduced the activation of MAPKs and subsequently the inflammatory responses both *in vitro* and *in vivo*. Partial modification (15%) of cationic shell cross-linked needle-like MNMs with histamine (instead of primary amines) was found to significantly reduce the toxicity and immunotoxicity of the MNMs, probably due to the lower charge density (lower primary amine content) which was confirmed by the lower zeta-potential value and lower cellular binding/uptake (Shrestha, Elsabahy et al. 2012). The immunotoxicities of the 0%- and 15%- Histamine modified particles were studied by measuring the levels of 23 cytokines upon treatment of RAW 264.7 mouse macrophages with the MNMs for 24 h. Generally, lower secretion of the cytokines was observed from cells treated with the histaminemodified MNMs. A similar trend was observed for most of the tested cytokines, although the differences between the levels of the secreted cytokines were significant for 12 cytokines, IL-3, IL-6, IL-9, IL-10, IL-12(p40), IL-13, Eotaxin, RANTES, monocyte chemotactic protein (MCP)-1, MIP-1β, KC and TNF-α (Elsabahy and Wooley 2013).

Environment or medium

Several characteristics of MNMs change depending on the medium and environment. The surface of NPs in a biological environment is modified by the adsorption of biomolecules such as proteins, polysaccharides and lipids. These interact with the NP surface forming a relatively stable ’biomolecular corona’ (Monopoli, Walczyk et al. 2011). Thus, the same NPs in different experimental environments can give different outcomes (Maiorano et al. ACS Nano 2010). It is therefore important to test NPs under conditions similar to those of potential human exposure (Magdolenova, Collins et al. 2014).

5.2 Genotoxicity

Size

The general consensus is that nanometer sized particles are more toxic than micrometer sized particles, probably due to the increased surface to volume ratio. Several *in vitro* and *in vivo* studies consistently show that transition from micro to nano-scale particle size increases genotoxicity (Gurr, Wang et al. 2005, Chan 2006, Papageorgiou, Brown et al. 2007, Kang, Lee et al. 2011, Oesch and Landsiedel 2012, Xu, Li et al. 2012).

The increased surface area of MNMs provide the potential for much greater activity which may potentially increase the probability of interactions and interference with genotoxicity assay components. *In vitro* assay components that may potential interact with MNMs include serum content in culture media, as well as cytochalasin B (cyto B) used in the cytokinesis-block micronucleus assay (OECD 2014).

Table 6.3.1. Positive versus negative genotoxicity results according to particle size (Oesch and Landsiedel 2012)

Smaller material (<20 nm) positive, same material in larger size (>200 nm) negative:
3 publications (TiO_2, Carbon Black)
Smaller material (<30 nm) more active than same material in larger size (>2,900 nm):
1 publication (cobalt chrome alloy)
Larger material ("bulk sized") positive, same material in smaller size ("nanosized") negative:
1 publication (V_2O_5)
Material of the same chemical composition but different particle size tested for potential genotoxicity by the same assays in the same laboratory (reports up to 2008)

Shape

The shape of the MNM has been postulated to being an important determinant for genotoxicity (Yang, Liu et al. 2009). For instance, silica nano-rods (100 nm) were able to disrupt the filaments of the cytoskeleton, whereas the silica NPs (100 nm) did not (Wu, Putcha et al. 2013). Another study showed that rod-shaped carbon nantotubules (CNTs) (length less than 5μm and diameter of 8 nm) and crystalline ZnO nanoparticles (19.6 nm) had similar oxidative damage potency but CNTs were more genotoxic (Yang, Liu et al. 2009). This difference in genotoxic potency was attributed to mechanical injury whereby CNTs penetrate the cell nucleus via nucleopores to damage the DNA double helix (Pantarotto, Briand et al. 2004). The most known example is the role of asbestos nano-fibers in genotoxicity and mesothelioma induction (Sanchez, Pietruska et al. 2009, Barlow, Lievense et al. 2013).

Surface area

The large surface area in MNMs results in an increased number of free radicals and transient metal ions arising from the NP surface. This in turn can increase the opportunity for their possible interaction with cells. A direct relationship between surface area and ROS formation has been observed where ROS formation and DNA damage were size- and surface area-dependent (Li, Sun et al. 2011).

Surface coating

Because variability in surface coating can result in changes in behaviour in solution, it is an important determinant of genotoxicity. For instance, polysaccharide coated Ag NPs exhibited more severe genotoxic damage than uncoated Ag NPs. This study suggests that polysaccharide coated particles are more individually distributed while agglomeration of the uncoated particles limits the surface area availability and access to membrane bound organelles (Ahamed, Karns et al. 2008). The genotoxicity of ZnO NPs in three-dimensional (3D) mini organ cultures of human nasal mucosa differed depending on coating. ZnO coated with with poly-methacrylic acid was more genotoxic, compared to uncoated ZnO (Yin, Casey et al. 2010).

Surface charge

The surface charge determines whether MNMs can be dissolved in medium or whether they form aggregates; it can also influence their biocompatibility and ability to traverse biological barriers (McNeil 2005).

Agglomeration

The agglomeration potential of MNMs is an important feature which may influence their behaviour. Generally highly agglomerated MNMs cannot enter the nucleus and mitochondria while NPs that

do not agglomerate can be distributed all over the cell (Ahamed, Karns et al. 2008). TiO2 NPs were found to be internalised into human skin epidermal cells or to adhere to the cell membrane, depending on their size. NPs of 30–100 nm were found in the cytoplasm, vesicles and nucleus, while larger particles (>500 nm) remained outside the cells. In medium, NPs can be dissolved or tend to form agglomerates/aggregates, depending on their surface charge (hydrophilic or hydrophobic) and interactions with medium (medium pH, salinity, protein content, etc.) (Shukla, Sharma et al. 2011).

Cell uptake

Importance of size in cellular uptake - The size of MNMs can determine the cellular uptake, intracellular trafficking pathways, biodistribution and *in vivo* fate (Chan 2006). Size is particularly important when cellular uptake occurs through porins which depend on particle size (Arcadis 2014). Organisms with a cell wall, such as bacteria and algae, are quite resistant to internalization, whereas *direct* uptake (i.e., at the external nano-bio-interface) is much more likely in animal organisms (no rigid cell wall) due to the occurrence of endocytosis. Internalization via endocytosis is known to occur in mammalian cells, with the internalization efficiency dependent on particle ***size*** (highest efficiency for particles with a diameter of ca. 40-50 nm). Endocytosis also occurs in other animal organisms (including protozoa), however, other intake mechanisms (e.g., dietary) may be more important in organisms such as aquatic invertebrates and fish (Ma and Lin 2013, Arcadis 2014, Ivask, Juganson et al. 2014). In any case, *direct* internalization pathways other than endocytosis, such as via ion transporters or paracellular diffusion, are less likely than endocytosis, mainly due to size limitations.

The size of a nanoparticle will determine if it can be internalized into an organism. The cell wall of algae is an efficient barrier that prevents most engineered MNMs (ENM) internalization via endocytosis. However, the cell wall pores with diameters ranging from 5-20 nm can be a potential uptake port for small MNM (von Moos and Slaveykova 2014). Smaller particles can cross the gut lumen of the daphnids with particles less than 50 microns being more easily ingested by daphnids (Ebert 2005). As particles aggregate into masses there is a decrease in ingestion and toxicity (Zhu 2009, Arcadis 2014).

Importance of coating in cellular uptake: *Coatings* also determine whether or not internalization is likely to occur. For instance, the presence of polyvinyl alcohol coatings can increase membrane permeability in bacteria, as alkaline compounds dissolve the external part of the cell membrane, which is the major cellular protective barrier (Arcadis 2014, Ivask, Juganson et al. 2014). Polymer

coatings can enhance toxicity compared to the uncoated form in some tests (Perrault et al., 2012) but not in others (Allen et al., 2010).

Importance of shape in cellular uptake: *Shape* is an important factor determining internalization in non-mammalian organisms because it enhance internalization through perforation (e.g., in fish embryos, (Handy, Al-Bairuty et al. 2011, Arcadis 2014)).

The shape of a nanoparticle may enhance the internalization of a nanoparticle. Needle-like nanoparticles may perforate cell membranes or cell walls, or damage the gut or gill epithelium (e.g.: (Handy, Al-Bairuty et al. 2011, Jackson, Jacobsen et al. 2013, Ma and Lin 2013, Ivask, Juganson et al. 2014)). Further, rod-shaped or fiber particles can have more contact area with the cell membrane, can more easily get through capillaries, adhere to blood vessels, stimulate platelet aggregation, and block potassium ion channels, compared to spherical carbon nanoparticles such as fullerenes. CNT in fibrous structures may be difficult to engulf by macrophages. Longer CNT may show a higher inflammatory response. The shape of nanometal can have an effect on fish embryos. A study with spherical Ni-nanoparticles of different size and dendritic structure consisting of aggregated 60 nm particles indicated that dendritic clusters were more toxic than the soluble Ni and the nanoparticles of the different sizes. In addition it seemed that the toxicity of the spherical nanoparticles manifested as organ defects (Shaw and Handy 2011, Arcadis 2014).

Importance of surface charge in cellular uptake: *Surface charge* and functional groups also play a role in cellular uptake (Kettiger, Schipanski et al. 2013). Negatively-charged gold nanoparticles appear to enter cells through the endocytic pathway resulting in higher cytotoxicity compared to positively-charged silver nanoparticles of similar size (Reidy B 2013). It is also important to note that the specific cell type is an important factor in whether positively or negatively charged particles are more likely to be taken up (Iversen T-G 2011).

Importance of surface area in cellular uptake: The *surface area* of a nanoparticle relates to its size and porosity. Smaller particles have a larger surface area and thus a larger surface energy relative to the volume of the particle, compared to the corresponding ratio for larger particles. The surface area of a particle determines the particle reactivity and affects the generation of ROS and radical activity (von Moos and Slaveykova 2014). This indicates that the smaller the size (the larger the surface area), the higher the relative potential for oxidative stress. And furthermore, as been noted by a study with Ag engineered MNMs and algae, the larger the surface area, the larger number of reaction sites for UV (Ultra Violet) adsorption.

Importance of agglomeration in cellular uptake: The *agglomeration* potential of MNMs is an important feature which may influence their behaviour. Generally highly agglomerated MNMs cannot enter the nucleus and mitochondria while NPs that do not agglomerate can be distributed all over the cell (Ahamed, Karns et al. 2008). TiO2 NPs were found to be internalised into human skin epidermal cells or to adhere to the cell membrane, depending on their size. NPs of 30–100 nm were found in the cytoplasm, vesicles and nucleus, while larger particles (>500 nm) remained outside the cells. In medium, NPs can be dissolved or tend to form agglomerates/aggregates, depending on their surface charge (hydrophilic or hydrophobic) and interactions with medium (medium pH, salinity, protein content, etc.) (Shukla, Sharma et al. 2011).

Importance of environment or medium in cellular uptake: Several characteristics of MNMs change depending on the medium and environment. The surface of NPs in a biological environment is modified by the adsorption of biomolecules such as proteins, polysaccharides and lipids. These interact with the NP surface forming a relatively stable 'biomolecular corona' (Monopoli, Walczyk et al. 2011). Thus, the same NPs in different experimental environments can give different outcomes. It is therefore important to test NPs under conditions similar to those of potential human exposure (Magdolenova, Collins et al. 2014). Environmental conditions affecting the nano-bio interactions are reported to be *pH, ionic strength, presence of other toxicants, presence of natural organic matter, temperature, and light* (Ma and Lin 2013, Arcadis 2014).

The fate of the NPs largely depends on behaviour, bioavailability and their interaction with the surrounding medium. Proteins are usually adsorbed on the surface of the NPs by different electrostatic, hydrogen bonding and hydrophobic interactions and affect the dispersity, uptake and bioavailability of the particles (Magdolenova, Bilanicova et al. 2012).

5.3 Gaps, uncertainties and recommendations associated with the use of the current genotoxicity tests

The Ames test (OECD TG 471) is an essential component of the genotoxicity testing battery, but it may not be suitable for detecting genotoxicity induced by MNMs. This is because the bacterial cells used lack the ability to perform endocytosis and because MNM diffusion across the bacterial cell wall may be limited, both of which limit MNM uptake; as well, some MNMs have antibacterial properties. As an alternative *in vitro* gene mutation assay, the *in vitro* mammalian cell gene mutation assay (OECD TG 476) was recommended, where no reports have yet identified specific limitations when testing MNMs. When considering the existing literature in assessing best practices for testing MNMs for genotoxicity, a number of limitations have reduced the utility of the results. These include:

- the use of cell lines with high background levels of DNA damage and high genetic instability,
- the absence of positive or negative control data that hinders assessment of data quality,
- the absence of data on cellular or target tissue uptake,
- no data provided to support dose selection,
- limited physical-chemical characterisation,
- insufficient cell numbers assessed, or
- no description of dispersion protocols used (OECD 2014).

Additional research is necessary to clarify the unresolved questions, including:

- Determining what *in vitro* assays are most informative, least likely to produce misleading positive results and best correlate with their *in vivo* counterparts.
- Determine which cell lines should be used for *in vitro* assays.
- Better characterise the extent and impact of MNM degradation within *in vitro* systems.
- Develop better approaches to consider the consequences of chronic exposures
- Develop assays better able to identify and characterise DNA damage induced by secondary mechanisms, such as oxidative stress caused by chronic inflammation.
- Develop a more detailed understanding of structure-activity and property-activity relationships to better predict genotoxicity based on physical-chemical features (OECD 2014).

In 2013, the MNMs Working Group was formed under the Health and Environmental Sciences Institute (HESI) Genetic Toxicology Technical Committee to address some of the issues identified. There were seven consensus statements agreed during the meeting:

1. The use of the Ames test (TG 471) is ***not*** a recommended test method for the investigation of the genotoxicity of MNMs. The test guidelines programme should consider modifying the applicability domain within this test guideline accordingly.

2. Measures of cytotoxicity based on cell proliferation that are described in the test guidelines are appropriate for determining the top concentration to be applied for *in vitro* tests of MNMs. It is appropriate in some cases to consider wider concentration spacing than the standard √10 in order to ensure the concentration-response relationship is well characterized, and at concentrations not associated with cytotoxicity.

3. Characterisation of the materials should be undertaken in the cell culture medium used both at the beginning of treatment and, where methodologies exist, after treatment. The intent when applying MNMs to a cell culture medium is to create conditions that are comparable, to the extent possible, with the biological and physiological conditions within the in vivo system.

4. The extent of cellular uptake is a critical factor to consider when interpreting test results. In some circumstances, a lack of uptake in a mammalian cell may indicate a low intrinsic hazard from a direct genotoxicity perspective.

5. The test guidelines program should consider modification of the *in vitro* micronucleus assay to recommend, where cyto B is used, its addition using a post-treatment or delayed co-treatment protocol, in order to ensure a period of exposure of the cell culture system to the MNM in the absence of cyto B.

6. Prior to conducting an *in vivo* genotoxicity study, there is a need to conduct some toxicokinetic investigations to determine if the MNM reaches the target tissue, where the target issue is not the site of contact. In the absence of data to the contrary, the test is not applicable for detecting primary genotoxicity if the MNM does not reach the target tissue.

7. There are insufficient data to recommend one route of administration over another. The basis for selecting the route of administration for testing should be to consider the route most applicable to human exposure(s).

In addition, workshop participants acknowledged that there remained a number of ***knowledge gaps*** that will need to be addressed in order to resolve many of the outstanding issues. Examples of these knowledge gaps include:

5. What are appropriate nano-specific positive and negative controls? The NANOGENOTOX Joint Action (JA) assessed nano-TiO2 as a potential universal positive control; however, it was found not to be suitable in all cases.
6. Are there (or could there be developed) *in vitro* test methods suitable for detecting secondary genotoxicity?
7. Can we identify appropriate metrics, complementary to existing toxicity/cytotoxicity measures, to allow better definition of the dose/concentration range to be tested?
8. What are the most suitable cell lines to use?
9. What is the influence of dispersants (e.g., BSA) on test outcomes?
10. What is the effect of exogenous metabolic activation (S9)?
11. Can we identify the biological mechanisms underlying the genotoxicity of MNMs

5.4 Standards and standards under development by the referenced committees

Committee	Reference	Title	Current state
ASTM International	ASTM E2490-09.	Standard Guide for Measurement of Particle Size Distribution of Nanomaterials in Suspension by Photon Correlation Spectroscopy (PCS) http://www.astm.org/Standards/E2490.htm	April 2009 published
BSI - British Standards Institution	BS EN 13925-3:2005	Non-destructive testing. X-ray diffraction from polycrystalline and amorphous materials. – Part 3: Instruments http://shop.bsigroup.com/ProductDetail/?pid=000000000030071999	July 2005 Published
International Standards Organisation (ISO)	ISO 6974-1: 2012	Natural gas -- Determination of composition and associated uncertainty by gas chromatography -- Part 1: General guidelines and calculation of composition http://www.iso.org/iso/home/store/catalogue_tc/catalogue_detail.htm?csnumber=55839	TC 193/SC 1 Published
International Standards Organisation (ISO)	ISO/13321:1996	Particle size analysis -- Photon correlation spectroscopy http://www.iso.org/iso/iso_catalogue/catalogue_tc/catalogue_detail.htm?csnumber=21707	TC 24/SC 4 Published
International Standards Organisation (ISO)	ISO/13318-1: 2001	Determination of particle size distribution by centrifugal liquid sedimentation methods -- Part 1: General principles and guidelines http://www.iso.org/iso/iso_catalogue/catalogue_tc/catalogue_detail.htm?csnumber=21704	TC 24/SC 4 Published
International Standards Organisation (ISO)	ISO/TS 13762: 2001	Particle size analysis -- Small angle X-ray scattering method http://www.iso.org/iso/iso_catalogue/catalogue_tc/catalogue_detail.htm?csnumber=22376	TC 24/SC 4 Withdrawn
International Standards Organisation (ISO)	ISO/13322-1: 2014	Particle size analysis -- Image analysis methods -- Part 1: Static image analysis methods http://www.iso.org/iso/home/store/catalogue_ics/catalogue_detail_ics.htm?csnumber=51257	TC 24/SC 4 Published
International Standards Organisation (ISO)	BS EN ISO 18757: 2005	Fine ceramics (advanced ceramics, advanced technical ceramics). Determination of specific surface area of ceramic powders by gas adsorption using the BET method http://shop.bsigroup.com/ProductDetail/?pid=000000000030117333	Interna-tional Equivalent: EN ISO 18757:2005, ISO 18757:2003Dec 2006 Published
International Standards Organisation (ISO)	ISO/20998-1: 2006	Measurement and characterization of particles by acoustic methods -- Part 1: Concepts and procedures in ultrasonic attenuation spectroscopy http://www.iso.org/iso/iso_catalogue/catalogue_tc/catalogue_detail.htm?csnumber=39869	TC 24/SC 4 Published

International Standards Organisation (ISO)	ISO/22412: 2008	Particle size analysis -- Dynamic light scattering (DLS) http://www.iso.org/iso/iso_catalogue/catalogue_tc/catalogue_detail.htm?csnumber=40942	TC 24/SC 4 Published
International Standards Organisation (ISO)	ISO/13320: 2009	Particle size analysis -- Laser diffraction methods http://www.iso.org/iso/iso_catalogue/catalogue_tc/catalogue_detail.htm?csnumber=44929	TC 24/SC 4 Published
International Standards Organisation (ISO)	ISO/15900: 2009.	Determination of particle size distribution -- Differential electrical mobility analysis for aerosol particles http://www.iso.org/iso/catalogue_detail.htm?csnumber=39573	TC 24/SC 4 Published
International Standards Organisation (ISO)	ISO/21501-1: 2009	Determination of particle size distribution -- Single particle light interaction methods -- Part 1: Light scattering aerosol spectrometer http://www.iso.org/iso/iso_catalogue/catalogue_tc/catalogue_detail.htm?csnumber=42728	TC 24/SC 4 Published
International Standards Organisation (ISO)	ISO 9277: 2010	Determination of the specific surface area of solids by gas adsorption -- BET method http://www.iso.org/iso/iso_catalogue/catalogue_tc/catalogue_detail.htm?csnumber=44941	TC 24/SC 4 Published
International Standards Organisation (ISO)	ISO/TR 14187	Surface chemical analysis -- Characterization of nanostructured materials http://www.iso.org/iso/iso_catalogue/catalogue_tc/catalogue_detail.htm?csnumber=54487	TC 201/SC 7 Published
International Standards Organisation (ISO)	ISO 28439: 2011	Workplace atmospheres -- Characterization of ultrafine aerosols/nanoaerosols -- Determination of the size distribution and number concentration using differential electrical mobility analysing systems http://www.iso.org/iso/iso_catalogue/catalogue_tc/catalogue_detail.htm?csnumber=44697	TC 146/SC 2 Published
International Standards Organisation (ISO)	ISO/ TS 10797: 2012	Nanotechnologies – Characterization of single-wall carbon nanotubes using transmission electron microscopy http://www.iso.org/iso/catalogue_detail?csnumber=46127	TC 229 Published
International Standards Organisation (ISO)	ISO/AWI TS 10798.	Nanotechnologies – Characterization of single-wall carbon nanotubes using scanning electron micros-copy and energy dispersive X-ray spectrometry analysis http://www.iso.org/iso/iso_catalogue/catalogue_tc/catalogue_detail.htm?csnumber=46128	TC 229 Published
International Standards Organisation (ISO)	ISO/TR 10929: 2012	Nanotechnologies -- Characterization of multiwall carbon nanotube samples http://www.iso.org/iso/iso_catalogue/catalogue_tc/catalogue_detail.htm?csnumber=46424	TC 229 Published

International Standards Organisation (ISO)	ISO/TS 12025: 2012	Nano-materials -- Quantification of nano-object release from powders by generation of aerosols http://www.iso.org/iso/home/store/catalogue_tc/catalogue_detail.htm?csnumber=62368	TC 229 Published
International Standards Organisation (ISO)	ISO 13099-1: 2012	Colloidal systems -- Methods for zeta-potential determination -- Part 1: Electroacoustic and electrokinetic phenomena http://www.iso.org/iso/iso_catalogue/catalogue_tc/catalogue_detail.htm?csnumber=52807	TC 24/SC 4 Published
International Standards Organisation (ISO)	ISO 13099-2: 2012	Colloidal systems -- Methods for zeta-potential determination -- Part 2: Optical methods http://www.iso.org/iso/iso_catalogue/catalogue_tc/catalogue_detail.htm?csnumber=52832	TC 24/SC 4 Published
International Standards Organisation (ISO)	ISO/TS 10868: 2011	Nanotechnologies -- Characterization of single-wall carbon nanotubes using ultraviolet-visible-near infrared (UV-Vis-NIR) absorption spectroscopy http://www.iso.org/iso/iso_catalogue/catalogue_tc/catalogue_detail.htm?csnumber=46247	ISO/TC229 Published
International Standards Organisation (ISO)	ISO/TS 23833: 2013	Microbeam analysis — Electron probe microanalysis (EPMA) — Vocabulary https://www.iso.org/obp/ui/#iso:std:61781:en	TC 202/SC 1 Published
International Standards Organisation (ISO)	ISO/ TR 13014: 2012.	Nanotechnologies - Guidance on physico-chemical characterization of engineered nanoscale materials for toxicologic assessment http://www.iso.org/iso/iso_catalogue/catalogue_tc/catalogue_detail.htm?csnumber=52334	ISO/TC 229 Published
International Standards Organisation (ISO)	ISO/TS 11888: 2011	Nanotechnologies -- Characterization of multiwall carbon nanotubes -- Mesoscopic shape factors http://www.iso.org/iso/iso_catalogue/catalogue_tc/catalogue_detail.htm?csnumber=50969	ISO/TC 229 Published