ENDORSED FOR PUBLIC CONSULTATION

DRAFT SCIENTIFIC OPINION

Guidance on risk assessment concerning potential risks arising from applications of nanoscience and nanotechnologies to food and feed[1]

EFSA Scientific Committee[2, 3]

European Food Safety Authority (EFSA), Parma, Italy

SUMMARY

Following a request from the European Commission the Scientific Committee was asked to deliver guidance on risk assessment concerning potential risks arising from applications of nanoscience and nanotechnologies to food, feed and pesticides.

This engineered nanomaterial (ENM) Guidance offers practical guidance for the risk assessment of applications involving the use of nanoscience and nanotechnology in the area of food and feed (including food additives, enzymes, flavourings, food contact materials, novel foods, feed additives and pesticides).

The general risk assessment paradigm (hazard identification and hazard characterisation followed by exposure assessment and risk characterisation) is applicable for these applications, and consequently appropriate data and information for the various steps should be made available to the risk assessor to carry out a risk assessment.

Adequate characterisation of ENM is essential for establishing its identity and physico-chemical forms in food/feed products. The physico-chemical parameters change in various environments and the characterisation of ENM has to be considered in various stages, i.e. as manufactured (pristine state), in formulations delivered for use in food/feed products, as present in the food/feed matrix, as used in toxicity testing, and as present in biological fluids and tissues.

The risk of an ENM will be determined by its chemical composition, physico-chemical properties, its hazard characterisation and potential exposure. The physico-chemical characterisation is needed to understand the properties of the nanomaterial and decide if the ENM guidance is applicable. The absorption, distribution, metabolism and excretion (ADME) parameters are likely to be influenced by both the chemical composition of the ENM as well as its physico-chemical properties (e.g. size, shape,

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3 Acknowledgement: The Scientific Committee wishes to thank the members of the Working Group on Nanotechnologies – Guidance for the preparatory work for this scientific opinion: Mona-Lise Binderup, Qasim Chaudhry, Wim De Jong, Corrado Galli, David Gott, Rolf Hertel, Akihiko Hirose, Wolfgang Kreyling, Hermann Stamm and Stefan Weigel, and EFSA's staff member David Carlander for the support provided to this EFSA scientific output.

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solubility, surface charge, surface reactivity etc). Absorption and distribution leading to internal exposure, a high level of ENM reactivity or mobility as well as persistence of the ENM are general indicators for in depth testing. A loss of nano-specific properties will move the risk assessment into a conventional risk assessment and the nano-specific risk assessment procedure will no longer apply.

In cases in which transformation of the ENM into a non-nanoform in the food/feed matrix or in gastrointestinal fluids is judged to be complete, then EFSA guidance for non-nanoforms for the specific intended use should apply. However, for ENM transformation the timing and location of the dissolution/degradation are crucial as until that moment the nanoform nature of the ENM may influence the biological behaviour, including kinetics and local effects.

The ENM covered by this ENM Guidance fall into two categories — the first is when a nanoform of an already approved non-nanoform with the same intended use in food/feed is produced and the second is when a new ENM without a corresponding approved non-nanoform is produced.

In the situation where there is an approved non-nanoform of a substance with the same intended use in food/feed, the aim of the ENM Guidance is to indicate the supplementary and specific information required on the potential additional hazards and risks that may arise from the nanoform. For such an ENM, *in vitro* genotoxicity tests, ADME and a repeated dose 90-day oral toxicity study in rodents according to this ENM guidance should be provided. Depending on the outcome of these studies and on the comparison with data on the non-nanoform other *in vivo* studies may be needed.

In the situation where the ENM persists in the food/feed matrix and in gastrointestinal fluids and has no approved non-nanoform application, toxicity tests on the ENM should follow the relevant EFSA guidance for its intended use with some modifications in the testing due to the nanoproperties as described in this ENM Guidance.

Prior to commencing the detailed risk assessment of the ENM, anticipated exposure scenarios from the proposed uses should be outlined. These exposure scenarios will contribute to decisions on the extent of the hazard characterisation required and will provide parameters for the exposure assessment required for the risk assessment.

Appropriate *in vitro* and *in vivo* studies on the ENM should be undertaken to identify hazards and obtain dose-response data to characterise the hazards. Some test models and standard testing protocols used for non-nanoform substances may not necessarily be appropriate or optimal for the testing of ENM, and ongoing efforts in the research community are currently addressing these issues.

The starting point for determining the amount of ENM for the exposure assessment currently has to rely on information on the material added to food/feed or that is in contact with food/feed. The initial characteristics of the added ENM can be used as an assumption in the exposure assessment, but it is preferable to determine the amounts present in the nanoform in the food/feed matrix. Currently it is not possible routinely to determine ENM *in situ* in the food or feed matrix, which increases the uncertainty in the exposure assessment. If it is not possible to determine the nanoform in the food/feed matrix or the form in which it is absorbed, an assumption should be made that all ENM that is added is present, ingested and absorbed in the nanoform.

There are currently several uncertainties related to the identification, characterisation and detection of ENM which are related to the lack of suitable and validated test methods to cover all possible applications, aspects and properties of ENM. Similarly, there are a number of uncertainties related to the applicability of current standard biological and toxicological testing methods to ENM. For these reasons, this ENM Guidance will need to be updated based on experience and acquired knowledge. It is acknowledged that the field is under fast development, and consequently this guidance document will be revised following appropriate developments.

KEY WORDS

Food, Feed, Guidance, Engineered Nanomaterials, Nanoscience, Nanotechnology, Nanotechnologies, Risk Assessment,

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BACKGROUND AS PROVIDED BY THE EUROPEAN COMMISSION

On 10 February 2009, EFSA adopted a scientific opinion on "The Potential Risks Arising from Nanoscience and Nanotechnologies on Food and Feed Safety"[4] in response to question number: EFSA-Q-2007-124a. Specifically, the opinion states that "current guidance documents in the food and feed area do not address engineered nanomaterials (ENM)."

The Panel on food contact materials, enzymes, flavourings, and processing aids (CEF) and the Panel on food additives and nutrient sources added to food (ANS) have already started reflections on the update of guidance documents on food additives, food contact materials, flavourings and enzymes in view of potential risks from nanomaterials.

The Panel on additives and products or substances used in animal feed (FEEDAP) already includes particle sizes and their effects in its evaluations of feed additives. Therefore, applications for new feed additives contain a chapter on particle size.

The present state of knowledge still contains many gaps preventing risk assessors from establishing the safety, according to standard procedures, for many of the possible food related applications of nanotechnology and thus ensuring that the safety aspects of engineered nanomaterials and nanotechnology bases processes are addressed in a coherent and comprehensive manner.

The purpose of this request is to obtain guidance on risk assessment thus providing the necessary transparency for stakeholders and regulators in order to develop an appropriate approach for the assessment and authorisation of engineered nanomaterials and other nanotechnologies.

However, even with the current state of knowledge, use scenarios probably exist for which different risk assessment approaches could be considered. These include, for example, applications where it could be established that consumer exposure would not arise (e.g. food contact materials with no nanomaterial migration) or that nanomaterials are soluble or biodegradable or when a delivery system for bulk substance is in nanoscale (e.g. micelles, nanoemulsions or other encapsulation).

TERMS OF REFERENCE AS PROVIDED BY THE EUROPEAN COMMISSION

(1) EFSA is requested to prepare a guidance document for the safety assessment of applications involving the application of nanoscience and nanotechnology to food and feed (including food additives, enzymes, flavourings, food contact materials, novel foods, feed additives and pesticides). This document should provide practical recommendations for the risk assessment of food related applications of nanotechnology to the extent possible with current knowledge. In the cases where knowledge is insufficient, it should indicate the endpoints and/or parameters that would have to be known in order to carry out a risk assessment. The guidelines should indicate where necessary, the additional requirements in terms of endpoints, tests, and data that would have to be fulfilled to be able to perform conclusive risk assessments.

In support of this work, the EFSA should consider any relevant documents developed for risk assessment in the context of nanotechnologies by scientific advisory bodies at European level (SCENIHR, SCCS, EMEA, ECHA, ECDC, SCOEL, OSHA etc.), EU Member States, third countries and international organisations including documents produced by the OECD Working Party on Manufactured Nanomaterials[5].

[4] http://www.efsa.europa.eu/EFSA/efsa_locale-1178620753812_1211902361968.htm
[5] http://www.oecd.org/department/0,3355,en_2649_37015404_1_1_1_1_1,00.html

(2) Consultation with stakeholders: The proposed guidance document should be subject to public consultation and if deemed appropriate discussed with stakeholders in a dedicated meeting prior to its adoption.

(3) Follow-up: Subsequent to these opinions, the Commission invites EFSA to monitor scientific advances and keep the Commission informed on relevant developments and, when appropriate, to revise the document.

ASSESSMENT

1. Introduction

This guidance builds upon the opinion of the Scientific Committee of 2009 "The Potential Risks Arising from Nanoscience and Nanotechnologies on Food and Feed Safety" (The EFSA Journal (2009)958, 1-39) (EFSA 2009) and more specifically chapter 6 (page 23) with the title "Guidance for risk assessment (RA) of ENM (Engineered Nanomaterials) in food and feed area". That chapter provided a general overview how to perform a risk assessment of nanomaterials in the food and feed area.

This guidance (referred to as the ENM Guidance) deals with risk assessment of three main categories of products/applications; those that are intended for consumption (by humans or animals), agrochemicals used in plant production (e.g. pesticides) and nanomaterials that are incorporated into products which come into contact with food/feed (e.g. packaging materials).

This ENM Guidance aims to provide guidance on the necessary information required and how to generate this information to perform a risk assessment of applications of nanotechnologies and nanomaterials in the food and feed area (including food additives, enzymes, flavourings, food contact materials, novel foods, feed additives and pesticides). The ENM Guidance gives information on the necessary data required for a comprehensive risk assessment in the food and feed area to the extent possible with current knowledge. This ENM Guidance is aimed at all interested parties, e.g. applicants and risk assessors.

As a general principle, the test requirements stipulated in current EFSA guidance documents and EC guidelines for various food and feed areas are applicable and should be followed also for nanomaterials. EFSA Guidance documents are found at www.efsa.europa.eu and a compilation of guidance can be found in the 2010 technical report of EFSA www.efsa.europa.eu/en/scdocs/doc/1518.pdf (EFSA Journal 2010:8(3):1518). However, the risk assessment of nanomaterials requires additional considerations that are indicated in this ENM Guidance and should be followed. This ENM Guidance aims to cover the additional information needs for risk assessment that may arise due to the specific characteristics and properties of ENM.

There are already a few EFSA guidance documents which includes the concept "size" of substances e.g. from the CEF Panel (Guidelines on submission of a dossier for safety evaluation by the EFSA of active or intelligent substances present in active and intelligent materials and articles intended to come into contact with food (The EFSA Journal (2009)1208) and from the FEEDAP Panel (Guidance for the preparation of dossiers for sensory additives (The EFSA Journal (2008)799)).

Food and feed may contain components that have internal structures that individually could be present at the nanoscale, e.g. naturally occurring molecules, micelles or crystals. However, "natural" components are considered within the context of this ENM Guidance only if they have been deliberately used or engineered to have nanoscale properties, or used e.g. to encapsulate bioactive compounds.

For the purpose of this ENM Guidance, ENM in feed will in general be treated in a similar way as those in food, since the impact on animals is likely to be similar to that on humans.

Environmental considerations and worker exposure are not addressed in this ENM Guidance. There is the possibility that certain ENM enter the food and feed chain as contaminants through traditional processes of waste disposal, or from other anthropogenic (e.g. from fertilizers or veterinary medicines) or natural sources, which is also outside the scope of this guidance.

Terms used in the ENM Guidance

In relation to risk assessment (RA) of engineered nanomaterials (ENM), the actual characteristics and properties of the ENM in question are the determining factors, rather than the terms used for its description. This ENM Guidance does not provide any definitions.

A proposal for a definition in the food and feed area is presented in the 7 September 2009 Common Position with a view to adopting a Regulation of the European Parliament and of the Council on novel foods, amending Regulation (EC) No 1331/2008 and repealing Regulation (EC) No 258/97 and Commission Regulation (EC) No 1852/2001 (http://register.consilium.europa.eu/pdf/en/09/st11/st11261.en09.pdf). Article 3.2.c in the Common Position has the following proposed definition:

c) "engineered nanomaterial" means any intentionally produced material that has one or more dimensions of the order of 100 nm or less or that is composed of discrete functional parts, either internally or at the surface, many of which have one or more dimensions of the order of 100 nm or less, including structures, agglomerates or aggregates, which may have a size above the order of 100 nm but retain properties that are characteristic of the nanoscale.

Properties that are characteristic of the nanoscale include:
(i) those related to the large specific surface area of the materials considered; and/or
(ii) specific physico-chemical properties that are different from those of the non-nanoform of the same material

It is noted that the European Commission in October 2010 released a proposal for a definition which was under public consultation until November 2010. This proposal provides a general over-arching definition for a nanomaterial in Article 2 as follows:

1. Nanomaterial: means a material[6] that meets at least one of the following criteria:
– consists of particles, with one or more external dimensions in the size range 1 nm - 100 nm for more than 1 % of their number size distribution;
– has internal or surface structures in one or more dimensions in the size range 1 nm - 100 nm;
– has a specific surface area by volume greater than 60 m2/cm3, excluding materials consisting of particles with a size lower than 1 nm.

2. Particle: means a minute piece of matter with defined physical boundaries (ISO 146446:2007)

It is however, noted that the proposed overarching EC definition is only intended for the consultation process and it is not adjusted for the specific legal context of nanomaterials in the food and feed area.

In addition, Scientific Committee on Emerging and Newly Identified Health Risks (SCENIHR) points out that at the lower limit of the definition of nanomaterials, the size of 1 nm, there is ambivalence between molecules, nanoclusters and nanoparticles (SCENIHR, 2010; (http://ec.europa.eu/health/scientific_committees/emerging/docs/scenihr_o_032.pdf)

The Scientific Committee notes that the deliberations on a definition suitable for use in the food and feed area are still ongoing. The term used in this ENM Guidance, engineered nanomaterial (ENM) is not defined in this guidance but refers to the concept of a nanomaterial that is deliberately produced to be used in the food and feed area. It is possible that the use of the term in this ENM Guidance will need to be revised once a legal definition have been agreed.

Within the context of this ENM Guidance, the term ”engineered” is equivalent to the term ”manufactured” and/or “processed” as used in other reports (e.g. SCENIHR, 2009, 2010).

Non-nanoform material refers to a material which in this ENM Guidance is either in ionic, molecular (i.e. generally smaller than the nanoform) or bulk form (i.e. larger size than the nanoform which can include aggregated nanomaterials).

Further, in this ENM Guidance, the terms and definitions suggested by the SCENIHR are used, as they are considered relevant for risk assessment (SCENIHR, 2007, 2010).

[6] The term “material” is replaceable with other terms for an object used in the specific legal context

2. General considerations for assessing ENM

This ENM Guidance is intended to cover two general situations of ENM in the food/feed area and a schematic presentation outlining the risk assessment of ENM is presented in figure 1. The first situation is when a nanoform of an already approved substance in food/feed is engineered for the same intended use**,** and the second situation is when a new ENM without a corresponding approved non-nanoform is produced.

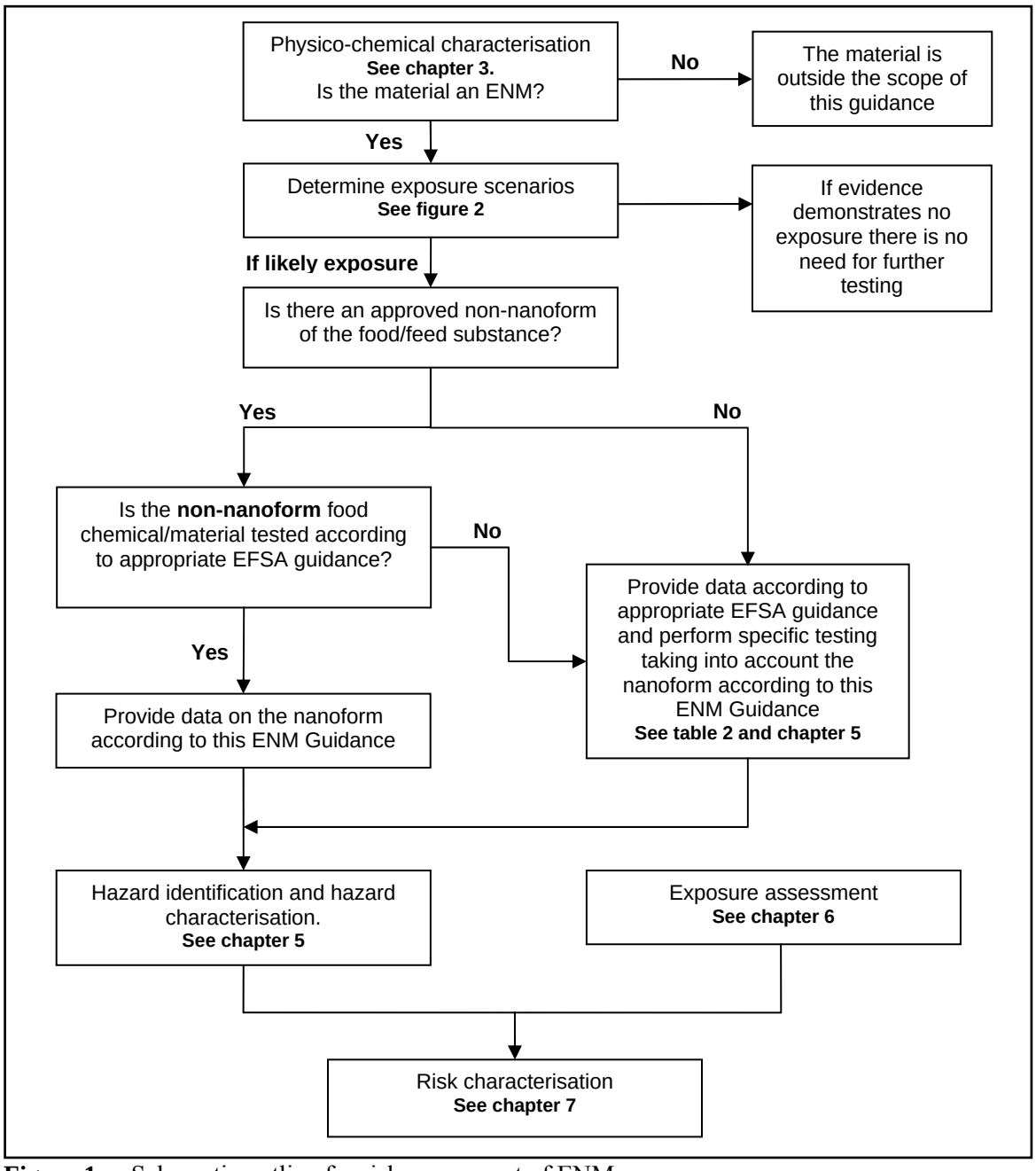

Figure 1: Schematic outline for risk assessment of ENM

In the situation where there is an approved non-nanoform of a substance with the same intended use in food/feed the aim of the ENM Guidance is to outline the data needed on the potential additional hazard and risks that may arise from the nanoform. The supplementary information required for the ENM can then be compared to the available information of the non-nanoform.

In the situation of a new ENM without an approved non-nanoform, the data submitted will need to include the toxicity tests set out in the current EFSA guidance documents on the relevant non-

nanoforms, supplemented by additional data on chemical characterisation of the nanoform and where appropriate modification of the toxicity tests as indicated in this ENM Guidance.

This ENM Guidance applies an approach, evaluating at each step, what additional information and data are needed to accomplish the risk assessment. Decisions on which tests to conduct depend on the amount and quality of the information available, and the validity of tests originally used to generate data. If the totality of the available information is considered sufficient at a particular stage, then a risk assessment can be performed, and no further testing would be required. However, if the information is considered insufficient, then the default presumption is that the next stage of the scheme should be applied with a sequence of further testing.

The risk of an ENM will be determined by its chemical composition, physico-chemical properties, its hazard characterisation and potential exposure. The physico-chemical characterisation is needed to understand the properties of the ENM and decide if the ENM Guidance is applicable. If the ENM guidance is applicable, the results from the testing will give information to assess the hazard which, combined with the exposure assessment, will form basis for the risk characterisation.

There are some general aspects to consider at an initial stage for ENM proposed for use in food/feed applications. Absorption and distribution leading to internal exposure, a high level of ENM reactivity or mobility as well as persistence of the ENM are general indicators for in-depth testing. The following are indicators of potential effects that should be considered when a decision on appropriate testing strategy has to be taken:

- High level of reactivity (e.g. catalytic, chemical, biological)
- Complex morphology (e.g. rigid, long tubes or fibres, high aspect ratio nanomaterials, fullerenes, crystal structure, porosity). ENM with cores and shells of different biopersistence (e.g. multifunctional ENM)
- Interactions with biomolecules such as enzymes, DNA, receptors, “Trojan horse” effect
- Complex transformations (e.g. aging, changes of surface properties, porosity) or metabolites (e.g. changes to or loss of coating)

The following are indicators of a potential for high exposure:

- Production volume and/or degree of purity used for the field of application
- High mobility of the nanoform in organisms (probability of internal exposure) (e.g. macrophage mobility; transport through cell membranes, blood-brain barrier and/or placenta; drug delivery systems) and mobilization potential (e.g. infiltration, sorption, complex formation)
- Targeted release
- Persistence/stability (e.g. in water, fat, and body fluids, lack of solubility/degradation), quasi-persistence of non-persistent nanomaterials due to permanent exposure
- Bioaccumulation

The following indicators are considered to reduce the likelihood of adverse effects of the ENM and are based on the specific exposure scenario under consideration and/or on loss of nano-properties. A loss of nano-properties will then move the risk assessment into a conventional risk assessment and the nano-specific risk assessment procedure will no longer apply.

- Good solubility[7] (e.g. in water, food/feed matrix or body fluids)
- Rapid degradability (e.g. biological or photocatalytic) to non-nanoform degradation products

[7] A soluble nanomaterial is dissolved to a non-nanoform (i.e. to its molecular or ionic form) (OECD ENV/CHEM/NANO(2009)7/Rev3)

- Presence of strongly bound aggregates (e.g. determined by conditions of production), fixed, permanent bonding in matrices (e.g. stability of matrix, type of bond, end-of-life behaviour)
- Nanostructured modifications on surfaces, and nanostructures that do not release particles and are not reactive (e.g. nanopores or lotus effect structures which can be used in filters and processing equipment)

The absorption, distribution, metabolism and excretion (ADME) parameters are likely to be influenced by both the chemical composition of the ENM as well as its physico-chemical properties (e.g. size, shape, solubility, surface charge and surface reactivity).

The metabolism and excretion parameters are important indicators of biopersistence. Persistence of a substance/material can be defined as its ability to continue to remain in the body or the environment. Biopersistence means that a substance/material is able to withstand those transformations that could lead to its solubilisation, metabolic degradation/detoxification, or clearance from a biological system. The retention of a biopersistent nanomaterial in the body can lead to its bioaccumulation. Therefore, biopersistence and bioaccumulation of ENM should be taken into account.

These general considerations and concepts presented above are further developed in the following chapters. Characterisation and identification of ENM are covered in chapter 3. Exposure scenarios are presented in chapter 4 and exposure assessment in chapter 6. Hazard identification and hazard characterisation and toxicity testing strategies are covered in chapter 5. Chapter 7 presents the risk characterisation. Sections on uncertainties are included throughout the document.

3. Characterisation of ENM

In addition to the small size, which is the main characteristic of nanomaterials, a number of other physico-chemical parameters are important in determining the properties and potential biological effects of ENM (e.g. shape, solubility, surface charge and surface reactivity) (Nel et al. 2006; SCENIHR 2007; Šimon and Joner 2008, Nel et al. 2009, EFSA, 2009; SCENIHR, 2010; JRC, 2010).

Adequate characterisation of ENM is essential for establishing its identity and physico-chemical forms in food/feed products. It is also essential for comparing materials tested (including for toxicity) in different products, between different manufacturers, and between similar tests of different duration carried out on the same material/product. Such information will contribute to the knowledge base which in the future can be used for extrapolation or read-across procedures.

The selection of physico-chemical parameters and characterisation methods will depend on the nature, functionalities, and intended uses of the ENM. Current knowledge gaps make it difficult to identify a shortlist of priority parameters for characterisation of ENM. For example, if a particular shape of an ENM raises a toxicological concern (e.g. a rigid needle shape) the determination of shape will become a mandatory parameter for measurement. However, this may not be so crucial in other cases, e.g. for micelle-shape structures.

The selection of an optimal method for measurement of a physico-chemical parameter will be dependent on the type of ENM, and the measurement environment (e.g. if in liquid dispersion, food matrix, food packaging). For example, chemical characterisation of a metal ENM will need a different analytical method compared to an organic encapsulate. Thus the choice of parameters/methods will need to be made on a case-by-case basis.

The physico-chemical parameters change in various environments and the characterisation of ENM has to be considered in various stages, i.e. as manufactured (pristine state), in formulations delivered for use in food/feed products, as present in the food/feed matrix, as used in toxicity testing, and as present in biological fluids and tissues.

Applicants should provide appropriate methods of analysis of the specific ENM in its intended uses including detailed methodology and the achieved method performance characteristics (see section 3.2.).

A selection of currently available methods which may be applied for providing information on the parameters for identification and characterisation of manufactured ENM are described in Appendix A. Methods for identification and characterisation of ENM in complex matrices are under development.

3.1. Requirements for identification, detection and characterisation of ENM

The most prominent characteristics of the ENM, as determined by its function, purpose and intended use, should be described and relevant parameters must be determined, according to Table 1. Justification should be provided for characteristics that are not determined or provided.

The size parameter should always be measured by at least two independent methods (one being electron microscopy) as the results obtained from different measurement techniques may differ because of the physical principles applied in the measurement method. The parameters in Table 1 include those measured by the OECD's Working Party on Manufactured Nanomaterials (WPMN) in its exploratory project on 'Safety testing of a representative set of nanomaterials'. OECD recently issued a revised version of its 'Guidance manual for the testing of manufactured nanomaterials: OECD's sponsorship programme; First revision (2 June 2010) ENV/JM/MONO92009)20/REV'.

3.1.1. Characterisation of ENM prior to use in food/feed related applications

Information related to characterisation of the ENM prior to its use in food/feed applications should be provided following the relevant guidance document for the area of use supplemented with nano-specific information as required in Table 1. The ENM should fall within the specifications provided for the identity of the material.

Examples of information from non-nanoform guidance that could be included are the following; name (generic or proprietary), CAS Number (if available), method of production (e.g. precipitation, gas phase), details on the intended uses, and the reasons for use in food/feed related applications, batch to batch variation and stability/shelf life.

3.1.2. Characterisation of ENM in food/feed related applications

Whilst detection and characterisation of the ENM prior to its use in the food/feed application may be relatively straightforward, it is more problematic in final food/feed products because of the presence of complex matrices, and usually low concentrations of ENM. Food/feed also contain a wide range of natural structures – including some in the nano size scale, which make it difficult to separate, detect, and identify an intentionally-added ENM.

The reactivity of ENM surfaces towards main functional groups of organic (macro)molecules in the food/feed matrix (such as carboxyl, hydroxyl, amino, sulphhydryl groups) should be taken into account as this may lead to potential binding with biopolymers such as proteins, lipids, polysaccharides, nucleic acids, etc. It can be envisaged that many ENM will not be present in food/feed products in a free form but will bind with the food/feed components. It may therefore be necessary to use a combination of methods for detection and characterisation of ENM in food/feed matrices. For example, a method for separation of the nano-fraction may be needed prior to the use of a detection/characterisation method. If the food matrix causes interference in the analysis of ENM, it may be degraded or separated from the ENM by appropriate biochemical, physical, or chemical methods. However, it should be considered that such a separation step may affect the ENM properties.

412 **Table 1:** Parameters for characterisation and identification of ENM (see appendix A for methods)

Parameter	Requirements	Description
Chemical composition/ identity	Essential	Information on chemical composition of the ENM – including purity, nature of any impurities, coatings or surface moieties, encapsulating materials, processing chemicals, dispersing agents and/or other formulants e.g. stabilisers.
Particle size (Primary/ Secondary)	Essential (two methods, one being electron microscopy)	Information on primary particle size, size range (indicating batch to batch variation – if any). Additional information on secondary size, size range, and number size distribution if the ENM is in an agglomerated/aggregated form.
Physical form and morphology	Essential	Information on the physical form and crystalline phase/shape. The information should indicate whether the ENM is present in a particle-, tube-, rod-/shape, crystal or amorphous form, and whether it is in free particulate form or in an agglomerated/aggregated state as well as whether the preparation is in the form of a powder, solution, suspension or dispersion.
Particle and mass concentration	Essential for dispersions and dry powders	Information on concentration in terms of particle number and mass per volume when in dispersion and per mass when as dry powder.
Specific surface area	Essential for dry powders	Information on specific surface area of the ENM.
Surface chemistry	Essential (for ENM with surface modifications)	Information on ENM surface – including any chemical/ biochemical modifications that could modify the surface reactivity, or add a new functionality.
Surface charge	Essential	Information on zeta potential of the ENM.
Redox potential	Essential for inorganic ENMs	Information on redox potential. Conditions under which redox potential was measured need to be documented.
Dissolution/ Solubility[a]	Essential	Information on water solubility and dissolution kinetics of all ENMs, and octanol-water partition coefficient (log k_{OW}) for organic particles.
pH	Essential	pH of aqueous suspension.
Viscosity	Essential for liquid dispersions	Information on viscosity of liquid dispersions.
Density and pour density	Essential for granular materials	Information on density/porosity of unformulated ENM and pour density.
Dustiness	Essential for dry powders	Information on dustiness of powder products – such as spices, creamers and soup powders.
Chemical reactivity/catalytic activity[b]	Essential	Information on chemical reactivity of the ENM and of any surface coating.
Photocatalytic activity	Essential for photocatalytic materials	Information on photocatalytic activity of relevant materials used in food packaging, coatings, and printing inks and internal reactions.

413 a) Dispersion, solution, dissolved: An insoluble ENM introduced to a liquid form a 'dispersion' where the liquid and the
414 ENM coexist. In a true solution the ENM is dissolved (see OECD ENV/CHEM/NANO(2009)7/Rev3)
415 b) If an ENM has catalytic properties, it may catalyse a redox or other reaction which may perpetuate resulting in a much
416 larger biological response even with small amounts of the catalytically active ENM. Thus, compared to a conventional
417 biochemical reaction which uses up the substrate, ENM reaction centres may perpetuate catalytic reactions.

418 Any catalytic activity of ENM needs to be measured and reported as it may trigger unexpected
419 reactions in the food/feed, as well as in the body after ingestion. Examples of such reactions may be
420 the generation of reactive radical species, photoreactions in food/feed, interactions with biological
421 processes in the body, etc.

Until more precise and validated analytical methods become available the ENM in a food/feed product is considered to be present in the form, and at the concentration, that was added to the food/feed product throughout the shelf-life of the product for the purpose of exposure assessment (see chapters 4 and 6).

3.1.3. Characterisation of ENM for toxicological testing

For the toxicological assessment of ENM, it is essential to know in which form the tested ENM are present in the test systems. Therefore, characterisation of ENM in the test system is relevant to determine the effect of the test medium/formulation (and its constituents) on the characteristics and properties of the ENM, in order to determine the validity of the toxicity test outcome.

The current available information indicates that special consideration is needed to address potential batch-to-batch and aging variations and that relevant characteristics of an ENM have to be verified/confirmed before addition to test systems to identify any changes.

3.1.4. Uncertainties in characterisation of ENM

It is important to note that currently there are no 'gold standard' methods available for characterisation of various ENM properties. However, a careful choice and use of appropriate methods, and properly documented results should provide adequate data for the purpose of identification and characterisation of the ENM.

It is also important to note that reproducibility and accuracy of any of the available characterisation methods will be dependent on the target ENM, sample preparation procedures, and calibration of the analytical equipment against appropriate standards. The results obtained by different measurement techniques may, nevertheless, still differ because of the different physical principles applied in different measurement methods (e.g. variations in size measurements as reported by Domingos et al. 2009).

In addition, differences may also arise due to aggregation/agglomeration behaviour of ENM, sample handling/preparation procedures, and other factors such as dilutions or dispersions required for different methods. It is, therefore, crucial that sample preparation is carried out in a consistent manner between tests to allow reproducibility of results from a given method, and/or a meaningful comparison of results from different methods. Different results measured by different methods could possibly influence the assessment and decision on whether a material will be defined as a nanomaterial or not.

It is also currently difficult to distinguish an ENM from background levels of the same materials/substances in non-nanoforms that may be present in food/feed products. Appropriate methods (e.g. stable isotope analysis, elemental fingerprinting) can be applied to distinguish the purposely-introduced ENM from background levels of the same or similar materials from geogenic, biogenic or anthropogenic origin.

As characterisation of ENM in food/feed matrices may be insufficient due to the current limited availability of analytical methods, it is suggested that possible food/feed matrix interactions of the ENM may be determined using food simulants (e.g. water, oil, alcohol, or simulants representing the characteristic composition of the target food, e.g. starch for carbohydrate-rich foods). However, the use of a simulant creates an uncertainty, as extrapolation from the results obtained with the simulant may not fully reflect the ENM properties in a real food. Following method development and availability, characterisation of ENM can be refined so that analysis can shift from food simulants to real food/feed matrices.

3.2. Performance criteria for characterisation methods

Methods used for the characterisation of ENM in their pristine form, commercial formulations, in food/feed matrix and in toxicity test systems should adhere to recognized criteria for method performance. Especially in the early phase of the introduction of new methods/methods for new target analytes the measurement uncertainty may often be (unacceptably) high. Therefore, it is deemed essential to demonstrate in this new analytical field that the applied methods are fit for purpose and deliver reliable results.

Applicants are requested to provide appropriate descriptions and documentation of the methods applied and method performance. Method performance parameters to be determined and documented would include various criteria (e.g. specificity, selectivity, recovery/trueness, repeatability, reproducibility, detection/quantification limits etc). Where possible, existing guidelines (e.g. IUPAC (Harmonized guidelines for single-laboratory validation of methods of analysis, Pure and Applied Chemistry 74 (5), 835 - 855 (2002)), 2002/EC/657) should be taken into account or adopted. The most up-to-date edition of any method performance test guideline should be followed. Use of any methods differing from internationally agreed protocols should be justified.

Reference materials are essential to control and compare the performance of analytical methods. However, in the field of ENM there are currently only silica and gold reference materials available that are validated only for size measurements. The silica nanoparticles size reference material (IRMM-304) is available from the Joint Research Centre, Institute of Reference Materials and Measurements and the gold nanoparticles (NIST RM 8011, 8012 and 8013) from the National Institute of Standards and Technology (NIST).

4. Exposure scenarios

Prior to commencing the detailed risk assessment of the nanomaterial, anticipated exposure scenarios from the proposed uses should be outlined (see figure 2). These exposure scenarios will contribute to decisions on the extent of the hazard characterisation and will provide parameters for the exposure assessment required in risk assessment. Once it has been determined that an ENM is present, exposure scenarios can be used to decide on further testing requirements.

Where ENM are directly added to food/feed, it should be ascertained whether the type and quantity of ENM added are known. If these are known, proceed directly to an exposure scenario. In other circumstances it is necessary to identify and quantify the ENM in food/feed. In cases where it can be demonstrated that the ENM are solubilised in the food/feed matrix, or digested in gastrointestinal fluids, no specific testing for the nanoform is required, but there may be a need to assess the resulting substances (see section 5.3.1).

In contrast, when ENM are present in an indirect way, e.g. due to migration or transfer of residues of the ENM, including possible carry-over from feed to food, its type and amount should be determined. The EFSA guidance for food contact materials gives information for testing migration. The extent of migration or transfer will determine whether and to what extent information on hazard characterisation and absorption, distribution, metabolism and excretion (ADME) of the ENM are required. The characteristics of the analytical methods used should follow the guidance given in chapter 3. If there is any migration then the nanomaterial should be physico-chemically characterised additionally in the food simulant or within the food/feed matrix. If ENM are present in the food or feed, exposure scenarios for risk assessment of ENM should be developed.

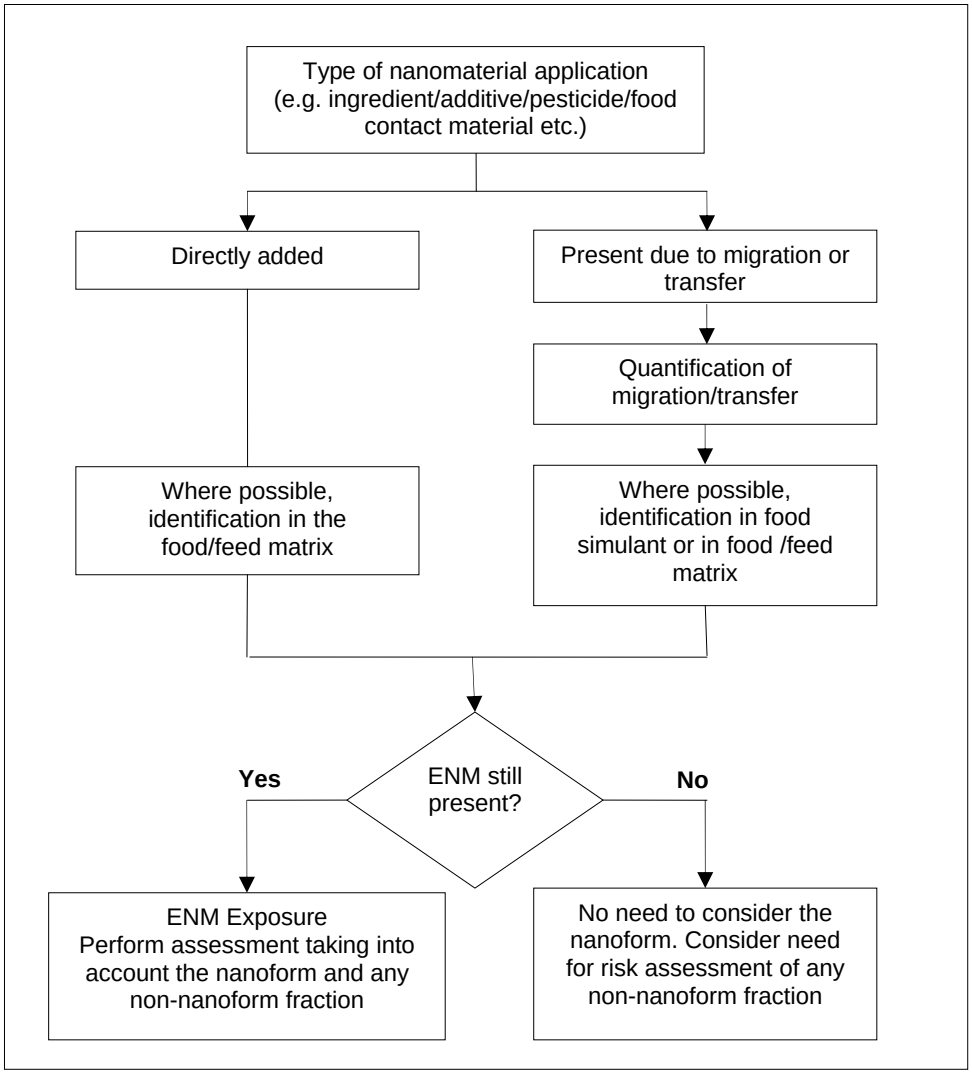

Figure 2: Exposure scenarios

The presence of ENM in food/feed could be due to either direct addition (e.g. as an ingredient) or indirectly present (e.g. migration from a food contact material or carry-over from feed to animal products). If the ENM is present in the food/feed an exposure to the ENM is assumed and a risk assessment taking into account the nanoform should be performed.

5. Hazard identification and hazard characterisation

5.1. General considerations

The currently available data on oral exposure to ENM, their absorption, distribution, metabolism and excretion (ADME) and any consequent toxicity are extremely limited; the majority of the available information on toxicity of ENM is from *in vitro* studies or from *in vivo* studies using other routes of exposure.

The evidence currently suggests that non-soluble/non-degradable ENM are more likely to exhibit different biological properties to ionic, molecular or bulk forms (i.e. non-nanoform) unlike soluble[8]/degradable ENM, which tend to have effects more similar to the non-nanoform.

[8] A soluble nanomaterial is dissolved to a non-nanoform (i.e. to its molecular or ionic form) (OECD ENV/CHEM/NANO(2009)7/Rev3)

Appropriate *in vitro* and *in vivo* studies on the ENM should be undertaken to identify hazards and obtain dose-response data to characterise the hazard. Some test models and standard testing protocols used for non-nanoform substances may not necessarily be appropriate or optimal for the testing of ENM, and ongoing efforts in the research community are currently addressing these issues. Therefore the recommendations for approaches to toxicity testing in this ENM Guidance will be updated as necessary in the light of future, emerging information.

For hazard characterisation, the relationship of any toxicity to the various dose metrics that may be used is currently being discussed in the scientific community and several dose metrics may need to be explored in addition to mass, e.g. number concentration and total surface area. Mass is a convenient metric, but information on the characterisation of the ENM should provide information allowing for conversion of the mass dose to other metrics, e.g. surface area and/or number of particles

Studies have been published that have used very high doses for the testing. Unrealistic high dosing can lead to outcomes that may not be related to the inherent toxicity of the material but to the high amount of the material administered. The choice of dose levels should therefore be carefully considered and a justification on the selected doses should be provided.

ENM used as carrier systems for other food components (e.g. vitamins) may increase the bioavailability of these food components, and the effects of the increase in bioavailability in terms of toxicity may need to be considered. The exposure assessment of a nanoscale delivery system should in addition to the assessment of the nanocarrier system itself include assessment of the amount of encapsulated bioactive compound as well as the amount present in free form in the food. For this, the analytical isolation, detection and characterisation procedures need to be designed to meet these requirements. It might be necessary, when appropriate and possible, to analyse the relevant chemical components as such.

5.2. Testing outline

The toxicity testing strategy is determined by the presence of ENM in the food/feed matrix and if applicable, information on a non-nanoform of the same substance. This strategy is illustrated by three general cases and is presented in table 2.

1. In cases in which transformation of the ENM into a non-nanoform in the food/feed matrix or in gastrointestinal fluids is judged to be complete, then EFSA guidance for non-nanoforms for the specific intended use should apply, and this ENM Guidance would no longer apply. However, it should be noted that for ENM transformation, the timing and location of the dissolution/degradation are crucial as, until that moment, the nanoform nature of the ENM may influence the initial biological behaviour, including kinetics and local effects.

2. In cases where, some, or all of, the ENM persists in the food/feed matrix and in gastrointestinal fluids and information on the non-nanoform of the same substance is also available, a testing approach is recommended which is based on comparison of information on ADME and toxicity of the non-nanoform with, in first instance, ADME and repeated-dose 90-day oral toxicity study in rodents and genotoxicity information of the ENM (see section 5.3 and 5.4). The purpose of comparing ADME and toxicity data from the two forms is to identify any major differences between the behaviour of the non-nanoform and that of the ENM. If there are differences, e.g. in distribution, or effects from repeated dose testing, then more toxicity testing will be required on the ENM, beyond ADME, 90-day and genotoxicity tests.

3. In cases where the ENM persists in the food/feed matrix and in gastrointestinal fluids and has no approved non-nanoform application, toxicity tests on the ENM should follow the relevant EFSA guidance for its intended use with some modifications in the testing due to the nanoproperties. The ENM toxicity testing strategy provided below for hazard identification and hazard characterisation takes into account the nanoproperties (see section 5.3 and 5.4).

570 **Table 2:** ENM toxicity testing strategy

Type of test	Information
In vitro genotoxicity tests	Necessary (see section 5.3.2.)
ADME	Necessary (see section 5.4.1 and 5.4.2.)
Repeated-dose 90-day oral toxicity study in rodents	Necessary (see section 5.4.3.)
In vitro digestion studies	Might be necessary (see section 5.3.1.)
Other *in vitro* tests	Might be necessary for screening and mechanistic information (see section 5.3.3.)
Reproduction study	Might be necessary, or required by specific sector regulations or by EFSA guidance (see section 5.4.4)
Developmental toxicity study	Might be necessary, or required by specific sector regulations or by EFSA guidance (see section 5.4.4)
In vivo genotoxicity tests	Might be necessary, or required by specific sector regulations or by EFSA guidance (see section 5.4.5.)
Chronic toxicity/carcinogenicity study	Might be necessary, or required by specific sector regulations or by EFSA guidance (see section 5.4.4.)
Specific toxicity tests	Might be necessary, or required by specific sector regulations or by EFSA guidance (see section 5.4.4.)

571 The Scientific Committee is aware that current EFSA guidance for food contact materials in a non-
572 nanoform, allows for a limited dataset to be provided depending on the amount of migration. However
573 due to the limited knowledge on the behaviour and effects of ENM, the use of a limited dataset is not
574 considered appropriate for ENM risk assessment at this point in time. Therefore information on
575 genotoxicity, ADME and repeated-dose 90-day oral toxicity study in rodents on the ENM is required
576 independent of the amount of migration.

577 ## 5.3. *In vitro* studies

578 The primary aim of *in vitro* testing is for ENM toxicity screening and the understanding of biological
579 responses and underlying mechanisms. *In vitro* tests may provide information on potential hazards and
580 a first indication of potential toxicity of an ENM and may be used to elucidate possible mode of action
581 and local site of contact effects. Information on the mode of action of the ENM may be helpful, e.g. if
582 reactive oxygen species are generated then genotoxicity and other toxic effects can be anticipated.

583 For *in vitro* testing attention should be given to the suitability of the test system and to possible
584 interactions of ENM with *in vitro* culture medium components, such as growth factors, proteins and
585 nutrients, to the influence of culture medium components on cellular uptake of ENM, and to the
586 possibility that treatment times may need to be extended to allow adequate uptake of ENM into cells
587 (Doak et al., 2009; Stone et al., 2009; Donaldson et al., 2010). Consideration also needs to be given to
588 what should be used as negative and positive controls. There may also be a need to consider whether
589 impurities may be present in the ENM that are known to be toxic.

5.3.1. *In vitro* digestion studies

In cases where the ENM persists in the food/feed matrix and before starting *in vivo* toxicity studies the transformation and stability of the ENM in gastric fluids (i.e. simulated gastrointestinal fluids) should be investigated. If it can be clearly demonstrated that an ENM dissolves/degrades quickly (e.g. in the gastric environment) the hazard identification and hazard characterisation can rely on data for the non-nanoform substance. The systemic toxicity profile of a dissolved ENM is likely to be similar to the soluble (ionic or molecular) form and further testing on the ENM is not necessary except when site of contact effects, before dissolution/solubilisation may be an issue, in which case studies, as indicated in section 5.3.3.1 and *in vitro* genotoxicity studies on the ENM should be conducted (see section 5.3.2).

5.3.2. *In vitro* genotoxicity testing[9]

In selecting a suitable battery of *in vitro* genotoxicity tests the three critical genotoxicity endpoints (gene mutation, structural and numerical chromosome aberrations) should be considered.

In most guidelines for genotoxicity testing a bacterial reverse mutation assay is included for the detection of gene mutations. However, since nano particles may not be able to penetrate the cell wall (Landsiedel, 2009) and because bacterial cells do not have the ability to phagocytose particles like mammalian cells, the use of a bacterial reverse mutation test for detection of genotoxicity of ENM may not be appropriate. However, in certain instances (e.g. with ROS induction, soluble ENM, very small ENM) a bacterial reverse mutation test might still be informative.

Information on the mode of action of the ENM may be helpful, e.g. if reactive oxygen species are generated then genotoxic effects can be anticipated, which can be detected in the comet assay (Karlsson, 2010).

The following *in vitro* tests are required for ENM added to, or migrating into food:

1. A test for induction of gene mutations in mammalian cells (preferably the mouse lymphoma *tk* assay with colony sizing) (OECD test guideline 476)

2. An *in vitro* chromosomal aberration test (OECD test guideline 437) or *in vitro* micronucleus assay (OECD test guideline 487)

There may be circumstances under which it may be justified to deviate from the above-mentioned core set. In such cases a scientific justification should be provided and additional types of considerations or mechanistic studies may be needed. If at least one of the *in vitro* tests indicates positive results, *in vivo* genotoxicity testing is required (see section 5.4.5).

5.3.3. *Other in vitro* studies

In vitro tests may provide additional insights into toxicity and mode of action of the ENM (e.g. on cytotoxicity, oxidative stress and potential for inflammation). Considering oral intake as the *in vivo* route of administration, the following *in vitro* approaches may be applied in a tiered fashion to generate additional hazard identification information. The first tier aims to investigate the effects of ENM on the integrity of the gastrointestinal barrier and enterocyte inflammatory responses to assess gut maintenance and defence. The second tier aims to investigate the effects of ENM on immune cells. If epithelial permeability increases and inflammatory mediators are released, ENM are likely to be systemically absorbed and immune activation should be evaluated.

[9] The composition of the test battery may be revised following the outcome of ongoing discussion on genotoxicity test strategies in the EFSA Scientific Committee.

5.3.3.1. Gastrointestinal barrier integrity and inflammatory response

The effect of nanoparticles on the integrity of the gastrointestinal barrier may be investigated using e.g. differentiated CaCo-2 cells as a model of the intestinal barrier. CaCo-2 cells, a human colon adenocarcinoma cell line, are probably the most suitable *in vitro* model of human enterocytes currently available. CaCo-2 cells can differentiate into enterocytes, with the separation of the apical from the basolateral compartment, reproducing the *in vivo* organization of the intestinal mucosa. Differentiated CaCo-2 cells express functional tight junctions, brush border characteristics and biotransformation enzymes (Pinto et al., 1982). In cases where local site of contact effects may be an issue, the parameters below should be assessed.

- Cytotoxicity, as assessed by LDH leakage or MTT reduction or equivalent assays, to identify the non-cytotoxic concentration range (viability > 80%) to be used in further experiments.
- Barrier integrity, as assessed by the trans-epithelial electrical resistance (TEER) measured with a volt-ohm meter and the paracellular flux of the extracellular marker phenol red. Twenty percent ethanol can be used as a positive control.
- Release of inflammatory mediators, i.e. IL-6, IL-8, PGE-2, NO, etc.
- Reactive oxygen species (e.g. DCFH assay), lipid peroxidation (thiobarbituric acid method), glutathione content, etc.

For such studies a Transwell® system with cells on one side of the barrier might be a suitable approach. Cells can be exposed on the apical side of the barrier to the ENM, while the presence of the ENM or their degradation products can be determined on the basolateral side of the barrier together with the release of inflammatory mediators. Cell extracts can be used to assess oxidative stress parameters or gene expression or additional parameters considered relevant (Puerto et al., 2010; Van De Walle et al., 2010).

5.3.3.2. Effect on immune cells

If the ENM can be absorbed or show passage in the *in vitro* barrier GI-model or activate an inflammatory response, the effect of the ENM on immune cells should be assessed. A possible *in vitro* assay relevant to humans is the whole blood assay. Whole-blood cytokine release models are broadly used for pharmacological *in vitro* and *ex vivo* studies. The whole blood assay has been internationally validated for the evaluation of pyrogenic contaminations or aspecific immune cell activation (Hoffmann et al., 2005; Schindler et al., 2006).

The following parameters may be assessed:

- Cytotoxicity, as assessed by LDH leakage or MTT reduction or equivalent assays, to identify the non-cytotoxic concentration range (viability > 80%) to be used in further experiments;
- Release of inflammatory and immunological mediators in the presence or absence of co-stimulatory molecules, i.e. lipopolysaccharide for monocyte activation, staphylococcal enterotoxin B or antiCD3 plus anti CD28 antibodies for lymphocyte activation. Following 24-48 h treatment, a plethora of cytokines including IL-1beta, IL-4, IL-6, IL-8, IL-10, TNF-alpha, γ-interferon, etc. can be measured by ELISA.

Using blood from healthy donors, as in the *in vitro* pyrogen test (IPT), the ENM is incubated with diluted fresh human whole blood, and the release of the proinflammatory cytokine, i.e. interleukin-1beta (IL-1beta), is detected by enzyme-linked immunosorbent assay (ELISA) 24-48 h after treatment. The whole blood assay allows the characterisation of immunotoxic reactions, including immunostimulation (inflammatory processes, pyrogenicity, priming, idiosyncratic reactions) and immunosuppression of immune responses (Langezaal et al., 2001; 2002).

5.4. *In vivo* studies

In vivo testing is performed to identify any adverse responses and to determine dose-response relationships. *In vivo* studies are also essential to generate ADME information for determining the toxicokinetic profile and the tissue distribution of the ENM and if necessary to follow up results from *in vitro* genotoxicity studies.

5.4.1. Administration of ENM for ADME and toxicity studies

The administration of test material in the *in vivo* oral toxicity studies could be by adding the ENM to the animal feed, to the drinking water, or by gavage. For administration via the feed or drinking water, the ENM should ideally be homogeneously blended into the feed matrix or stably and uniformly dispersed in the drinking water or gavage vehicle. The stability and physico-chemical characteristics of the ENM in the vehicle should be determined (see chapter 3). There may be limitations on the amounts of ENM that can be administered because the ENM may agglomerate in the drinking water or gavage vehicle, or they will already be blended as agglomerated powder into the feed, which in addition may then not be uniformly mixed within the food matrix. It is recommended that, wherever possible, the use of an aqueous solution/suspension is considered first, in an attempt to use the same vehicle in all the toxicological tests (i.e. ADME, *in vitro* toxicity, genotoxicity and *in vivo* studies etc.). Therefore, these applications require careful control and dynamic characterisation of ENM in either the liquid or the feed matrix. For example, an ENM in liquid may adsorb to the walls of the drinking vessel and is therefore no longer available (i.e. there will be no exposure). Possible interactions with the administration vehicle, either the food matrix or water, needs to be determined in advance before *in vivo* administration.

To overcome some of the obstacles mentioned above, ENM can be applied by gavage, aiming for the ENM to be dispersed, well-characterised and administered under well-defined conditions. This method of administration can give a fairly precise dose of ENM delivered to the animal and a well characterised degree of dispersion. However, application by gavage is not likely to be representative of the lower concentrations delivered over time from ENM administered via feed. Gavage provides a bolus of ENM at a given time which may or may not mix with the gastrointestinal fluids, likely resulting in a higher quantity of absorbed material due to the ENM being in the form of a single, large dose and the lack of co-ingestion of dietary components to which ENM can easily bind.

Whilst kinetics following bolus administration differs from kinetics following continuous administration leading to a greater likelihood of effects associated with the peak concentration rather than total exposure, use of multiple doses in ADME studies and use of these results to appropriately design repeated-dose 90-day oral toxicity studies can correct for this possibility. At the current state of knowledge, overall the uncertainties will be minimised by using bolus gavage administration of ENM. The limitations of the bolus administration for ADME studies may be accepted in view of the certainty obtained on the administered dose and thus the dose-response relationship of possible adverse effects.

In any of the oral administrations mentioned above one has to consider that the passage through the acid environment of the stomach and mixing with the chyme in the gut may affect the ENM. Consideration of the potential for time dependent dissolution/degradation is essential as well as physico-chemical ENM modifications like agglomeration and ENM surface modifications by proteins and biomolecules.

5.4.2. ADME studies

Absorption, distribution, metabolism and excretion (ADME) studies are essential for the safety evaluation of ENM as the nature of nanomaterials can result in altered and specific toxicokinetics and tissue distribution when compared to non-nanoforms. However, the difficulties of undertaking ADME studies on ENM should not be underestimated. In addition to the issues involved in administration of

ENM to test animals discussed above, in ADME studies there may also be particular difficulties in measuring the amounts of ENM in blood, tissues and excreta, and in establishing the form in which they are present in the body. ENM surface transformations e.g. the dynamics of adherence of proteins and other biomolecules can have a profound effect on the ADME.

For ADME studies it is essential that a measuring system is available either detecting the nanomaterial or its elemental composition. Alternatively, a labelling system may be used, either directly (radioactive isotopes) or indirectly (fluorescent dyes or radiolabel). ICP-MS has the limitation that the chemical element is determined and not the presence of the nanomaterial itself (i.e. not only the nanoform may be detected). Radioactive isotopes may be used for certain metal ENM (Geiser and Kreyling, 2010). Fluorescence labelling or labelling with radio-labelled chemicals have the disadvantage that the label may be released from the ENM. In such cases the distribution of the label can be determined, but not, with any certainty, that of the ENM (Geiser and Kreyling, 2010). The choice of the detection technique should be based on the composition of the ENM, e.g. metal nanomaterials or lipid like nanomaterials.

Many types of ENM exhibit inherent polydispersity (large size distribution) due to their complex composition. The term "bioavailability" could be used for either the ENM carrier or the encapsulated active ingredients (where applicable). In order to account for ENM absorption in the body, comprehensive mass balance studies are suggested. Repeated administration may alter the toxicokinetics of the ENM, therefore an appropriate study design should be chosen to address this issue. Because ENM are taken up by the reticuloendothelial system (RES) especially in spleen and liver, there may be a need for extended toxicokinetic studies depending on the biopersistence of the ENM. These will provide information on the timing and extent of ENM accumulation in organs and tissues and clearance from these tissues. ENM retention within the gut wall is also an important determinant, particularly when discriminating between retention in epithelial cells versus immune-competent M-cells in Payers patches. Additional studies could be conducted to investigate the localization of ENM in RES organs, which have a high content of macrophages and other immunocompetent cells. In the GI tract, GALT (gut associated lymph tissue), such as Peyer's patches and mesenteric lymph nodes, are of importance for potential ENM accumulation and potential effects on immune responses.

The design of toxicokinetic studies for chemicals is described in OECD test guideline 417. This guideline describes general methodologies with multiple measurements and endpoints for performing ADME studies.

5.4.2.1. ADME pilot study

The use of a pilot study is recommended for selection of the experimental parameters and for dose ranging to avoid the administration of highly toxic doses. The dose in the pilot study should be sufficient to allow for identification of the ENM in excreta and when appropriate in blood or plasma. Blood samples should be taken at regular intervals initially up to 24 hours after administration of the ENM. In addition, ENM retention in the gut epithelium and in secondary organs and tissues of expected risk such as liver, spleen and kidneys should be investigated.

In order to ensure delivery of the desired dose, oral gavage can be used. However, this has the disadvantage that possible interaction with the gastric contents is limited (see section 5.4.1).

5.4.2.2. ADME main study

For the main study, a minimum of two ENM dose levels should be used since this information may aid in dose setting in other toxicity studies (OECD test guideline 417). Repeated ENM administration may provide information on possible accumulation.

5.4.3. *In vivo* repeated-dose 90-day oral toxicity study

For ingested ENM, the minimum requirement is a repeated-dose 90-day oral toxicity study in rodents (OECD test guideline 408), modified to include assessment of some additional parameters described in the more recent guideline on repeated-dose 28-day oral toxicity study in rodents (OECD guideline test 407). The additional parameters place more emphasis on endocrine-related endpoints, (e.g. determination of thyroid hormones, gross necropsy and histopathology of tissues that are indicators of endocrine-related effects, and (as an option) assessment of oestrous cycles). Specific attention should be paid to the RES (reticuloendothelial system) in repeated dose studies, as after systemic translocation, most ENM are likely to end up in the RES tissues. The results from the repeated-dose 90-day oral toxicity can be used to identify a Benchmark Dose lower confidence bound (BMDL) or a No-Observed-Adverse-Effect-Level (NOAEL).

It should be noted that toxicological data derived from laboratory species may not be directly applicable for ENM foreseen to be administered in feed to target animals, and that additional tests, e.g. tolerance tests for the target species might be needed.

5.4.4. Other *in vivo* toxicity tests

In cases where there are appropriate toxicity and ADME data available on a non-nanoform (i.e. the same chemical substance in a bulk, molecular or ionic form), a repeated-dose 90-day oral toxicity study in rodents together with the outcome of genotoxicity and ADME studies on the ENM can provide a comparative basis for deciding whether long-term toxicity testing of the ENM may be needed. If there is evidence of accumulation of ENM in organs and tissues, then chronic toxicity testing may be appropriate in order to reveal progressive toxic effects or delayed toxicity, and to identify a BMDL or a NOAEL.

The repeated-dose 90-day oral toxicity study offer only limited information on reproductive toxicity and no information on developmental toxicity; they can inform about effects on the reproductive organs and, if assessed, the oestrous cycle, but they do not assess the whole reproductive cycle from *in utero* exposure onwards, through sexual maturity to conception, gestation, prenatal and postnatal development. Thus decisions on whether tests on the ENM are necessary for reproductive and developmental toxicity will need to be considered in the light of the toxicity data available on these aspects for the non-nanoform comparator and on comparative ADME information. For a decision on whether a developmental toxicity study on an ENM will be necessary, consideration also needs to be given as to whether the nanoform of the substance may cross the placenta and thereafter behave in a different way from the non-nanoform, due to nano-specific characteristics. Such information may not be readily available, since ADME studies do not routinely include pregnant animals. The study design for reproduction and developmental studies are described in OECD test guidelines 414, 415 and 416. Chronic toxicity and carcinogenicity study is described in OECD test guideline 453.

5.4.5. *In vivo* genotoxicity testing[10]

If at least one of the *in vitro* tests indicate genotoxic activity this normally requires follow-up by *in vivo* testing (Eastmond et al., 2009), unless it can be adequately demonstrated by other means that the positive *in vitro* findings are not relevant for the *in vivo* situation. *In vivo* assays should cover similar endpoints to those found positive in the *in vitro* assays. *In vivo* genotoxicity testing may also be considered where there is evidence for a prolonged inflammatory response from *in vivo* studies.

[10] The composition of the test battery may be revised following the outcome of ongoing discussion on genotoxicity test strategies in the EFSA Scientific Committee.

The choice of the appropriate *in vivo* genotoxicity test(s) requires expert judgement based on all available information, to be applied case-by-case. Guidance for the follow-up of positive results from *in vitro* assays could be taken from guidance document issued by e.g. European Chemicals Agency (ECHA, 2008) which recommends that any of the following tests may be conducted:

- a rodent bone marrow or mouse peripheral blood micronucleus test (OECD test guideline 474) or a rodent bone marrow clastogenicity study (OECD test guideline 475)
- a comet (single cell gel electrophoresis) assay
- a test for gene mutations in a transgenic rodent model, *e.g.* using *lacI*, *lacZ* or *cII* as reporter gene
- an unscheduled DNA synthesis test with mammalian liver cells *in vivo* (OECD test guideline 486)

A combination of the *in vivo* micronucleus assay and the Comet assay in a single study may also be acceptable (Pfuhler et al. 2009; Rothfuss et al., 2010; EFSA Panel of Food Contact Materials, Enzymes Flavourings and Processing Aids, 2010). Other studies (e.g. DNA adduct studies) may also be relevant in order to clarify the mechanism of genotoxicity.

5.5. Uncertainties in toxicity testing of ENM

As mentioned before, it may be difficult to characterise, detect and measure ENM in food/feed and in biological matrices and limited information is available in relation to aspects of ENM toxicokinetics and toxicology, including optimal methods for testing ENM. Current toxicity testing approaches used for conventional materials are recommended as a suitable starting point for case-by-case risk assessment of ENM. Toxicity testing methods may need methodological modifications (e.g. regarding sample preparation and characterisation). Specific uncertainties arise due to limited experience of testing ENM in currently applied standard testing protocols. There may also be additional toxic effects caused by ENM that are not readily detectable by current standard protocols. Additional endpoints not routinely addressed may need to be considered in addition to traditional endpoints.

6. Exposure assessment

Basically, the principles of exposure assessment of ENM (via food and feed) will be the same as in exposure assessment of non-nanoform materials (Kroes et al., 2002; EFSA 2006). Issues like food/feed sampling and variability within composite samples and variation in concentrations between samples are not different from the exposure assessment of micro/macroscale or dissolved chemicals. On the basis of the available consumption data, the anticipated average and high intakes in various population groups of the ENM food/feed must be estimated. Probabilistic methods may be useful to determine ranges of plausible values rather than point estimates. If possible, particular sections of the population with an expected high exposure should be identified and this should be considered in the risk assessment. There is limited information on the consumption (amounts and frequency) of food supplements. Data on import and production quantities could provide additional information for the exposure assessment. Any assumptions made in the exposure assessment should be described.

A central aspect of exposure assessment is the determination of the amount and characterisation of the ENM present in the food or feed as consumed. In most cases, the starting point for determining the amount of ENM currently has to rely on information on the material added or that is in contact with food/feed. The initial characteristics of the added ENM can be assessed and used as an assumption in the exposure assessment, however, currently it is not possible to routinely determine ENM *in situ* in the food or feed matrix which increases the uncertainty in the exposure assessment (see chapter 3).

The structure of the ENM in food/feed may be changed in the food/feed production chain during processing or storage because of their interactions with proteins, lipids and other substances present in the food/feed matrices. Hence, if ENM are analysed at an early stage of the food chain, effects of

processing and storage should be considered in the exposure assessment. Also, effects of digestion or other causes of degradation of the matrix on ENM characteristics need to be considered.

For ENM added to feed, the potential carry over to food should be considered for human exposure.

In the absence of exposure data, and where it is not possible to determine the nanoform in the food/feed matrix, it should be assumed that all ENM that is added, is present, ingested and absorbed in the nanoform.

7. Risk characterisation

The risk characterisation step is the point at which all the information from the hazard identification and hazard characterisation is combined with that from the exposure assessment and other relevant information from read-across of other ENM or non-nanoforms (i.e. bulk, molecular and ionic forms). Although it is essentially an iterative process throughout the assessment, the final risk characterisation should result in informed qualitative, and if possible quantitative, guidance to risk managers. The output from the risk characterisation is the overall assessment of the safety of the ENM in its intended use together with the parameters under which the assessment is valid and the uncertainties associated with the assessment. It should explain clearly what assumptions have been made during the risk assessment, and what is the nature and magnitude of any uncertainties.

A tiered approach for generating information required for risk assessment is described in this ENM Guidance. At every stage where information is assessed, a weight-of-evidence process should be applied to make a decision on whether a risk assessment can be undertaken. The weight-of-evidence approach takes into account all available sources of information and types of data. At each evaluation step, decisions depend on the amount and quality of the information available at that particular stage and the validity of the tests used to generate the data. The identification/characterisation of the assessed ENM is essential to demonstrate that the data generated are obtained with the ENM that will be used in food/feed applications. If the totality of the available information is considered suitable at a particular stage, then a risk assessment can be performed, and no further testing would be required. However, if this is not considered possible, then the default presumption is that a sequence of further testing should be undertaken.

7.1. Uncertainties in ENM risk characterisation

The Scientific Committee adopted a Scientific Opinion in 2009 that deals with general principles to be applied in the identification of data sources, criteria for inclusion/exclusion of data, confidentiality of data, assumptions and uncertainties (EFSA, 2009). That opinion makes a number of general recommendations on how to handle uncertainties in risk assessment which should be addressed also in the ENM risk assessment. The Scientific Committee has also adopted a Guidance related to uncertainties in dietary exposure assessment which include practical approaches on how to handle uncertainties in risk assessment that will also be applicable in ENM risk assessment (EFSA, 2006).

The terms for the expression of risks and associated uncertainties should be as precise, understandable and transparent as possible. Any uncertainties inherent in the different risk assessment steps should be highlighted and quantified as appropriate. Distinction should be made between various types of uncertainties that reflect natural variations in biological parameters (including variations in susceptibility in populations), and possible differences in responses between species. Estimation of uncertainties in experimental data should be handled by proper statistical analysis, while quantification of uncertainties in assumptions (e.g. extrapolation of data from animals to humans, extrapolation from laboratory studies to complex systems) may be more difficult, but should be highlighted and discussed.

When it is not possible to characterise the form in which the ENM test substance is present in the test system and compare this with what would be present in food/feed then uncertainty will be increased; depending on the circumstances, the risk characterisation may under- or over-represent the risks. The specific properties of ENM may introduce additional uncertainties. There may be difficulties in determining the dose administered which may add to the uncertainty.

At present, specific protocols for toxicity tests for ENM are lacking. Existing standard protocols may, nevertheless, be suitable, and should be used after consideration of the modifications recommended in chapter 5 of this ENM Guidance. However, it has to be recognised that information emerging from studies on ENM in the future may point to other modifications in test protocols.

A major uncertainty is the fact that it is still not understood how and to what extent biochemical reactions occur at the molecular level of the ENM surface with biological fluids, cell membranes and cell compartments, e.g. which and how many of the atomic/molecular clusters on the ENM surface area are causing what kind of biochemical or catalytic reactions, such as electron exchange, etc. With generation of such knowledge, the reactivity of a given ENM will be better understood and potential effects may be predicted.

As for conventional non-nanoforms of substances in food/feed, risk assessment should preferably be quantitative, but at present, in some circumstances, only a qualitative ENM risk assessment may be possible.

The absence of data essential for the risk assessment should be indicated and the quality of the existing data and that provided should be reported. It should be clear from the assessment how this body of information has been taken into account when the final risk assessment is determined.

As with conventional risk assessment, the NOAELs or BMDLs derived from the hazard characterisation can be used to estimate safe human intakes by application of uncertainty factors. These uncertainty factors allow for inter- and intra-species differences in toxicokinetics and toxicodynamics. If not indicated otherwise by consideration of the data, the conventional default uncertainty factors of 10 for inter- and 10 for intra-species differences should be applied as currently there are no indications for a need to modify these factors.

CONCLUSIONS

This ENM Guidance offers practical guidance for the risk assessment of applications involving the use of nanoscience and nanotechnology in the area of food and feed (including food additives, enzymes, flavourings, food contact materials, novel foods, feed additives and pesticides).

The general risk assessment paradigm (hazard identification and hazard characterisation followed by exposure assessment and risk characterisation) is applicable for these applications, and consequently appropriate data and information for the various steps should be made available to the risk assessor to carry out a risk assessment.

Adequate characterisation of ENM is essential for establishing its identity and physico-chemical forms in food/feed products. The physico-chemical parameters change in various environments and the characterisation of ENM has to be considered in various stages, i.e. as manufactured (pristine state), in formulations delivered for use in food/feed products, as present in the food/feed matrix, as used in toxicity testing, and as present in biological fluids and tissues.

The risk of an ENM will be determined by its chemical composition, physico-chemical properties, its hazard characterisation and potential exposure. The physico-chemical characterisation is needed to understand the properties of the nanomaterial and decide if the ENM guidance is applicable. The absorption, distribution, metabolism and excretion (ADME) parameters are likely to be influenced by

both the chemical composition of the ENM as well as its physico-chemical properties (e.g. size, shape, solubility, surface charge, surface reactivity etc). Absorption and distribution leading to internal exposure, a high level of ENM reactivity or mobility as well as persistence of the ENM are general indicators for in depth testing. A loss of nano-specific properties will move the risk assessment into a conventional risk assessment and the nano-specific risk assessment procedure will no longer apply.

In cases in which transformation of the ENM into a non-nanoform in the food/feed matrix or in gastrointestinal fluids is judged to be complete, then EFSA guidance for non-nanoforms for the specific intended use should apply. However, for ENM transformation the timing and location of the dissolution/degradation are crucial as until that moment the nanoform nature of the ENM may influence the biological behaviour, including kinetics and local effects.

The ENM covered by this ENM Guidance fall into two categories — the first is when a nanoform of an already approved non-nanoform with the same intended use in food/feed is produced and the second is when a new ENM without a corresponding approved non-nanoform is produced.

In the situation where there is an approved non-nanoform of a substance with the same intended use in food/feed, the aim of the ENM Guidance is to indicate the supplementary and specific information required on the potential additional hazards and risks that may arise from the nanoform. For such an ENM, *in vitro* genotoxicity tests, ADME and a repeated-dose 90-day oral toxicity study in rodents according to this ENM guidance should be provided. Depending on the outcome of these studies and on the comparison with data on the non-nanoform other *in vivo* studies may be needed.

In the situation where the ENM persists in the food/feed matrix and in gastrointestinal fluids and has no approved non-nanoform application, toxicity tests on the ENM should follow the relevant EFSA guidance for its intended use with some modifications in the testing due to the nanoproperties as described in this ENM Guidance.

Prior to commencing the detailed risk assessment of the ENM, anticipated exposure scenarios from the proposed uses should be outlined. These exposure scenarios will contribute to decisions on the extent of the hazard characterisation required and will provide parameters for the exposure assessment required for the risk assessment.

Appropriate *in vitro* and *in vivo* studies on the ENM should be undertaken to identify hazards and obtain dose-response data to characterise the hazards. Some test models and standard testing protocols used for non-nanoform substances may not necessarily be appropriate or optimal for the testing of ENM, and ongoing efforts in the research community are currently addressing these issues.

The starting point for determining the amount of ENM for the exposure assessment currently has to rely on information on the material added to food/feed or that is in contact with food/feed. The initial characteristics of the added ENM can be used as an assumption in the exposure assessment, but it is preferable to determine the amounts present in the nanoform in the food/feed matrix. Currently it is not possible routinely to determine ENM *in situ* in the food or feed matrix, which increases the uncertainty in the exposure assessment. If it is not possible to determine the nanoform in the food/feed matrix or the form in which it is absorbed, an assumption should be made that all ENM that is added is present, ingested and absorbed in the nanoform.

There are currently several uncertainties related to the identification, characterisation and detection of ENM which are related to the lack of suitable and validated test methods to cover all possible applications, aspects and properties of ENM. Similarly, there are a number of uncertainties related to the applicability of current standard biological and toxicological testing methods to ENM. For these reasons, this ENM Guidance will need to be updated based on experience and acquired knowledge. It is acknowledged that the field is under fast development, and consequently this guidance document will be revised following appropriate developments.

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APPENDIX A – CURRENTLY USED CHARACTERISATION METHODS

The methods in the table below are based on light scattering, microscopy, spectrometry, chromatography and other size separation methods such as electrophoresis and centrifugation, surface characterisation methods, and their different variants and combinations. Adequate characterisation of an ENM will generally require multiple methodologies to measure various characteristics, the use of which should be justified and documented with a detailed description of the protocols used. Method performance characteristics should also be provided (see section 3.2).

Parameter	**Currently available methods**[a]
Chemical composition/ identity	Elemental analysis: OES, AAS, XPS, EDX, NMR, Mass Spectrometry (MS) in particular ICP-MS, TXFX, etc. Molecular composition: Mass spectrometry (ToF, QqQ) using suited ionisation techniques (e.g. MALDI, ESI), coupled with separation methods (e.g. HPLC, GC, CE etc), NMR, FT-IR Shell/core composition (for encapsulates, micelles): by a suitable method given above, after disintegration of the particles and separation of the components by a suitable method (e.g. HPLC, SEC, CE, HDC etc)
Physical form and morphology	Microscopy methods (TEM, SEM, STXM, AFM), X-ray diffraction
Particle size (Primary/ Secondary)	Microscopy methods[b] - e.g. TEM, SEM, STEM, AFM, STXM. Separation methods: Flow separation, chromatography methods – e.g. FFF, HDC, SEC, RP/NP-HPLC; DMA/IMS (ultra)Centrifugation methods. Spectroscopy methods – e.g. XRD (for crystal size, crystallite size) Light (laser) scattering methods[c] – e.g. DLS, MALS, SLS;; PCCS, NTA
Crystalline phase	XRD
Particle concentration	Mainly light scattering methods[c] (for dispersions). Particle concentration (in pure dry powders) may also be calculated from particle size, mass concentration and density of the material.
Mass concentration and density	Suited methods from those listed under chemical composition e.g. mass spectrometry (ICP-MS) AEM, CFM; Gravimetric methods; centrifugal sedimentation (for suspensions). A possible method for measurement of density is provided by OECD TG 109.
Specific surface area[d]	BET method
Surface chemistry	Any of the suitable chemical characterisations methods listed above
Surface charge	Electrophoresis, e.g. CE, LDE (Laser Doppler Electrophoresis)[e]
Redox potential	Potentiometric methods
Dissolution/Solubility[f]	Standard tests for water solubility (e.g. OECD 105), and log k_{ow} (OECD 107, 117) can be used. Dissolution rate constants.
Viscosity	Methods such as OECD Guideline 114.
Pour density	DIN ISO 697, EN/ISO 60
Dustiness	Methods such as EN 15051:2006, DIN 33897-2.
Chemical reactivity/ catalytic activity[g]	Kinetic measurements of the catalysed reactions
Photocatalytic activity	Kinetic measurements of the catalysed reactions

a) Many of the currently available methods have not yet been validated for ENMs, and certainly not for complex matrices. It is, therefore, not possible to recommend a method of choice for the measurement of a given parameter. However, the use of well recognised mainstream analytical methods should provide adequate data for identification and characterisation of an ENM. It may be necessary in some cases to use more than one method to generate sufficient reliable data for this purpose (see chapter 3).

b) Electron microscopy methods (SEM, TEM) are useful in visualising nanoparticles as well as determining their size, aggregation state, structure, shape etc. TEM requires very thin specimens for the electrons to pass through. TEM also requires vacuum conditions, and therefore can not handle liquid samples. To overcome this, cryogenic-TEM has been used that can handle frozen samples. The use of Wet-SEM has also been reported (Tiede et al., 2008), which can

handle liquid samples in a specially designed capsule that allows characterisation of nanoparticles in liquid samples. Scanning probe microscopy tools, such as AFM, can also be used to examine liquid samples. High throughput use of microscopy methods are currently limited due to the length to time required for manual processing of images.

c) Light scattering methods are commonly used to measure size and distribution of particles as well as agglomerates and aggregates. However, accuracy of light scattering methods is dependent on sample preparation and monodispersity, and may be limited to raw materials rather than ENMs in final products.

d) The specific surface area measurement can be used to calculate Volume Specific Surface Area (VSSA) according to the method described by Kreyling et al., 2010.

e) Zeta potential of ENM is calculated from electrophoretic mobility. Preferably this should be measured in water to avoid discrepancies between tests in different solvents and pH/ ionic conditions.

f) Dispersion, solution, dissolved: An insoluble ENM introduced to a liquid form a 'dispersion' where the liquid and the ENM coexist. In a true solution the material is dissolved (see OECD ENV/CHEM/NANO(2009)7/Rev3)

g) If an ENM has catalytic properties, it may catalyse a redox or other reaction which may perpetuate resulting in a much larger biological response even with small amounts of the catalytically active ENM. Thus, compared to a conventional biochemical reaction which uses up the substrate, ENM reaction centres may perpetuate catalytic reactions.

Abbreviations

AAS – Atomic absorption spectroscopy
AEM – Analytical Electron Microscopy (a combination of analytical tools, such as spectroscopy, and electron microscopy for composition analysis).
AFM – Atomic Force Microscopy
BET – Brunauer Emmett Teller method (based on nitrogen absorption)
CE – Capillary electrophoresis
CFM – chemical force microscopy (a recent development in scanning probe microscopy that can enable identification of chemical nature of materials, Tiede et al., 2008)
DLS – Dynamic light scattering
DMA – Differential mobility analysis
EDX – Energy Dispersive X-ray spectroscopy
FFF – Field Flow Fractionation
FT-IR – Fourier Transform Infrared spectroscopy
GC-MS – Gas chromatography – mass spectrometry
HDC – Hydrodynamic chromatography
HPLC – High performance liquid chromatography
ICP-MS – Inductively coupled plasma mass spectrometry
IMS – Ion mobility spectrometry
LDE – Laser Doppler Electrophoresis
MALDI-ToF-MS – Matrix-assisted laser desorption/ionization – time of flight mass spectrometry
NMR – Nuclear magnetic resonance spectroscopy
OES – Optical emission spectroscopy
PCCS – Photo Cross Correlation Spectroscopy
SAXS – Small-angle X-ray scattering
SEC – Size exclusion chromatography
SedFFF – Sedimentation field flow fractionation
SEM – Scanning Electron Microscopy
SMPS – Scanning Mobility Particle Sizing
SPMS – Single Particle Mass Spectrometry
STEM – Scanning Transmission Electron Microscopy
STM – Scanning Tunnelling Microscopy
STXM – Scanning Transmission X-ray Microscopy
TEM – Transmission Electron Microscopy
XPS – X-ray Photoelectron Spectroscopy
XRD – X-ray diffraction

1152 **GLOSSARY**

Term	Explanation
ENM	Engineered nanomaterial(s)
Fullerene	A fullerene is a molecule composed entirely of carbon, in the form of a hollow sphere, ellipsoid, or tube. Spherical fullerenes are also called buckyballs, from buckminsterfullerene (a 60 carbon atom sphere).
High aspect ratio nanomaterials (HARN)	The aspect ratio of a shape is the ratio of its longer dimension to its shorter dimension. The length of a HARN is considerably longer than its width. Examples of HARN include materials such as carbon nanotubes (CNT) and metal nanowires.
Non-nanoform	A material that in this guidance is either in ionic, molecular (i.e. generally smaller than the nanoform) or bulk form (i.e. larger size than the nanoform which can include aggregated nanomaterials).
Pour density	A function of the degree of compaction during pelletisation.

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