SCIENTIFIC OPINION

Guidance on the risk assessment of the application of nanoscience and nanotechnologies in the food and feed chain[1]

EFSA Scientific Committee[2, 3]

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ABSTRACT

The European Food Safety Authority has developed a practical approach for assessing potential risks arising from applications of nanoscience and nanotechnologies in the food and feed chain. Guidance is provided on: (i) the physico-chemical characterisation requirements of engineered nanomaterials used e.g. as food additives, enzymes, flavourings, food contact materials, novel foods, feed additives and pesticides and; (ii) testing approaches to identify and characterise hazards arising from the nanoproperties which, in general, should include information from *in vitro* genotoxicity, absorption, distribution, metabolism and excretion and repeated-dose 90-day oral toxicity studies in rodents. The guidance allows for reduced information to be provided when no exposure to the engineered nanomaterial is verified by data indicating no migration from food contact materials or when complete degradation/dissolution is demonstrated with no absorption of engineered nanomaterials as such. The guidance indicates uncertainties that should be considered to perform a risk assessment. As this sector is under fast development, this guidance document will be revised as appropriate. ©European Food Safety Authority 2011.

KEY WORDS

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

SUMMARY

Following a request from the European Commission the Scientific Committee was asked to prepare a guidance document for the safety assessment of applications involving the application of nanoscience and nanotechnology to food and feed. This scientific opinion offers practical guidance for the risk assessment of applications involving the use of Engineered Nanomaterials (referred to as the ENM Guidance) in the food and feed chain (including food additives, enzymes, flavourings, food contact materials, novel foods, feed additives and pesticides).

The risk assessment paradigm (hazard identification and hazard characterisation followed by exposure assessment and risk characterisation) is appropriate for these applications. Consequently relevant 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 and under testing conditions. The physico-chemical parameters may change in various environments and the characterisation of ENM should ideally be determined in five stages, i.e. as manufactured (pristine state), as 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 interactions with tissues, and potential exposure levels. The physico-chemical characterisation is needed to identify an ENM and decide whether the ENM Guidance is appropriate. 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 the basis for the risk characterisation. 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).

Prior to commencing the detailed risk assessment of the nanomaterial, anticipated exposure scenarios from the proposed uses should be outlined. 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.

Six cases are presented which outline different toxicity testing approaches. Where convincing evidence is provided indicating that ENM use does not result in presence of the ENM or its degradation/solubilisation products in the food/feed then there is no need for any additional testing. When transformation of the ENM into a non-nanoform in the food/feed matrix is judged to be complete before ingestion, then EFSA guidance for non-nanoforms for the specific intended use should be applied. When it can be demonstrated that an ENM completely dissolves/degrades in the gastro-intestinal tract without absorption of the ENM, the hazard identification and hazard characterisation can rely on data for the non-nanoform substance (if available). When information on a non-nanoform of the same substance is available and where some or all of the ENM persists in the food/feed matrix and in gastrointestinal fluids, a testing approach is recommended which is based on comparing information on ADME, toxicity and genotoxicity of the non-nanoform with ADME , repeated-dose 90-day oral toxicity study and genotoxicity information of the ENM. When information on a non-nanoform is not available and where some or all of the ENM persists in the food/feed matrix and in gastrointestinal fluids, the approach for toxicity tests on the ENM should follow the relevant EFSA guidance for the intended use with the modifications in the present ENM Guidance to take into account the nanoproperties.

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 amount of the ENM present in the food/feed matrix. 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. 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 added ENM, is present, ingested and absorbed as the nanoform, although the structure/properties of the ENM remain undetermined and difficult to relate to the structure/properties of the ENM used in the toxicity studies.

There are currently uncertainties related to the identification, characterisation and detection of ENM that 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 as appropriate.

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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, EMA, 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].

(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.

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[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

(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.

GUIDANCE

1.   Introduction

This guidance on engineered nanomaterials (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). The ENM Guidance encompasses applications of nanoscience and nanotechnologies in the whole food and feed chain that fall under the remit of EFSA (including food additives, enzymes, flavourings, food contact materials, novel foods, feed additives and pesticides).

This ENM Guidance builds upon the opinion of the Scientific Committee of 2009 "The Potential Risks Arising from Nanoscience and Nanotechnologies on Food and Feed Safety" (EFSA, 2009a) 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 of how to perform a risk assessment of ENM in the food and feed area. The risk assessment paradigm (hazard identification and hazard characterisation followed by exposure assessment and risk characterisation) is appropriate for these applications, and consequently relevant data and information for the various steps should be made available to the risk assessor to carry out a risk assessment.

As a general principle, the test requirements stipulated in current EFSA guidance documents and EC guidelines for various food and feed areas should be applied for ENM according to its intended use and should be followed. The risk assessment of ENM, in terms of testing requirements and procedures, requires additional considerations that are indicated in this ENM Guidance. This ENM Guidance also aims to cover the additional risk assessment information needs for the physico-chemical characterisation that may arise due to the specific characteristics and properties of ENM. The specific information related to the characteristics and properties of the nanomaterial with the information stipulated in the relevant EFSA Guidance documents for the specific intended use of the ENM (e.g. as a pesticide, food contact material, flavouring etc.) is used for a case-by-case risk assessment. EFSA Guidance documents are found at www.efsa.europa.eu. A compilation of Guidance documents can be found in the 2010 technical report of EFSA www.efsa.europa.eu/en/scdocs/doc/1518.pdf (EFSA, 2010a).

This ENM Guidance is aimed at all interested parties, e.g. applicants and risk assessors. 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.

There are already a few EFSA guidance documents which include the concept "size" of substances e.g. from the FEEDAP Panel (Guidance for the preparation of dossiers for sensory additives) and 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) (EFSA, 2008a, 2010b).

The ENM Guidance also identifies circumstances under which some data requirements for the risk assessment could be waived (e.g. when an ENM is degraded in the food/feed matrix into an approved non-nanoform before ingestion).

There are substantial ongoing developments in alternatives to *in vivo* testing approaches but validated *in vitro* methods for specific endpoints are still limited which necessitates information from *in vivo* testing to be used for risk assessment purposes. The use of animals for risk assessment should be considered thoroughly during the design of experimental studies and applicants are advised to consult the Scientific Committee opinion on the Existing approaches incorporating replacement, reduction and refinement of animal testing: applicability in food and feed risk assessment (EFSA, 2009b).

It should be recognized that additional assessments (e.g. efficacy assessments) not outlined in this ENM Guidance may be prescribed under other specific Guidance documents or required by legislation.

There is the possibility that certain ENM enter the food and feed chain as contaminants through traditional processes of waste disposal, from anthropogenic or natural sources. In principle, the data resulting from toxicity testing of ENM as recommended in this ENM Guidance, can also be used for assessing the human health risk from ENM as contaminants of food/feed.

Environmental considerations and worker exposure are not addressed in this ENM Guidance as this was not requested by the terms of reference provided by the European Commission.

A draft of this document underwent a public consultation from the 14th of January to 25th of February 2011. The comments received were considered and have been incorporated where appropriate.

1.1.   The term engineered nanomaterial

The term engineered nanomaterial (ENM) as used in this guidance refers to a nanomaterial produced either intentionally or unintentionally (due to the production process) to be used in the food and feed area. It is generally accepted in nanosciences that a nanomaterial refers to a material with at least one size measurement between approximately 1 and 100 nm (ISO, 2008; Lövenstam et al., 2010; SCENHIR, 2010). 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)

It is recognized that in the EU there are ongoing legislative discussions aiming to define nanomaterials. This ENM Guidance has been written independently of a regulatory definition. However, it is possible that the term ENM as used in the ENM Guidance may need to be revised once a legal definition has been agreed. It is not the intention of this ENM Guidance to provide any definitions.

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

It should be noted that 'nanomaterial' is a categorisation of a material by the size of its constituent parts. It neither implies a specific risk, nor does it necessarily mean that this material actually has new hazard properties compared to its constituent parts or larger sized counterparts (SCENIHR, 2010).

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

2.   General considerations for assessing ENM

This ENM Guidance applies an approach, evaluating at each stage the information and data needed to accomplish the risk assessment (outlined in figure 1). Decisions on which tests to conduct depend on the amount and quality of any pre-existing information available, and the validity of tests used to generate data. If the totality of the available information is considered sufficient then a risk assessment can be performed, and no further testing would be required. However, if the information is considered insufficient, further testing is necessary.

The schematic outline for risk assessment of ENM in figure 1 addresses situations characterised by differences in the properties and the data availability for the ENM and the non-nanoform from which the ENM has been manufactured (if relevant).
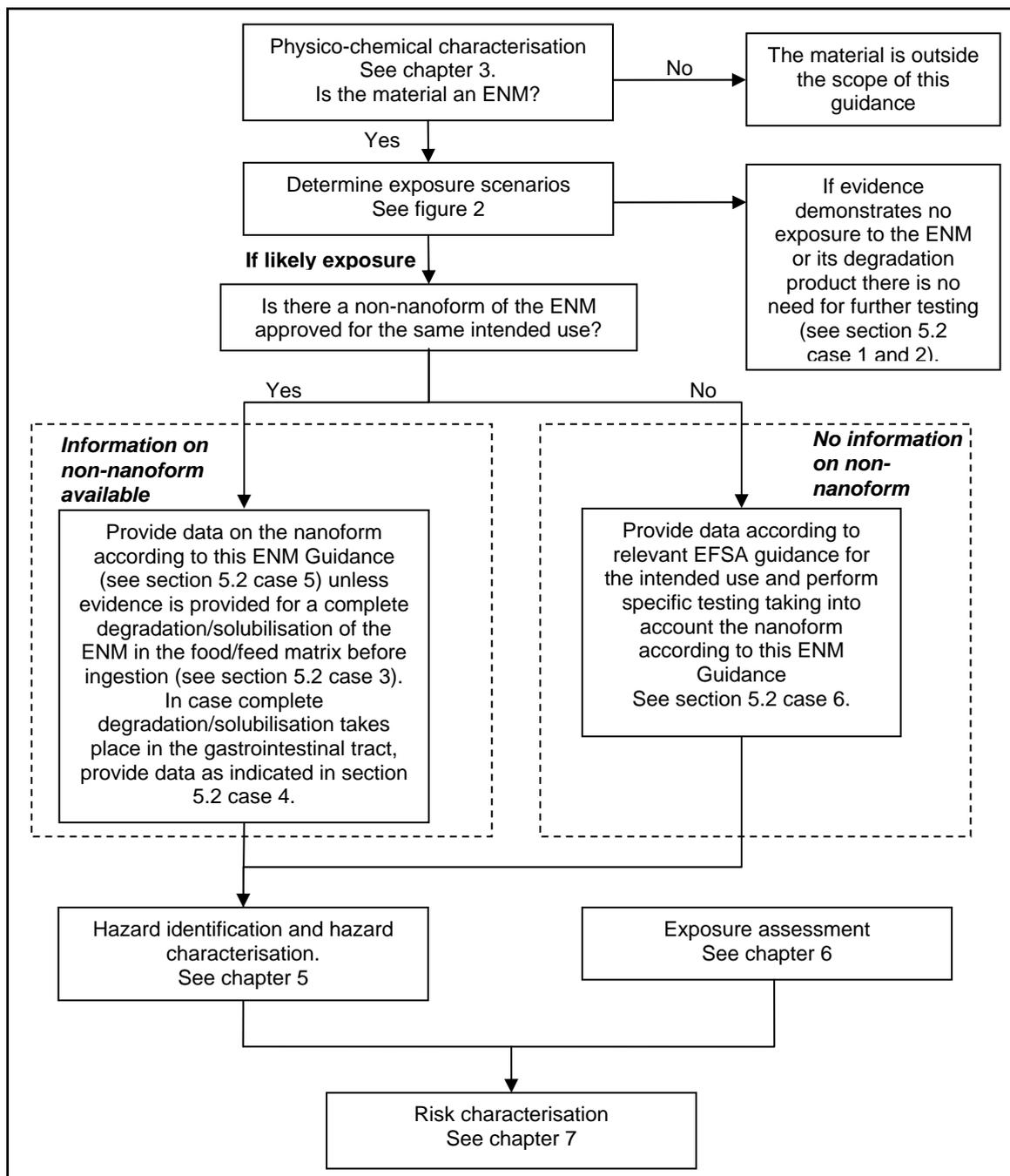
Figure 1: Schematic outline for testing and risk assessment of ENM

The risk of an ENM will be determined by its chemical composition, physico-chemical properties, its interactions with tissues, and potential exposure levels. The physico-chemical characterisation is needed to identify an ENM and decide whether the ENM Guidance applies. 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 the basis for the risk characterisation. The absorption, distribution, metabolism and excretion (ADME) parameters are also 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).

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

the ENM and its intended use. In this situation a test strategy may be developed which focuses on the identified differences between the ENM and the approved non-nanoform. The supplementary information for the ENM can then be compared with the available information of the non-nanoform. A subcategory to this situation arises when an ENM has been assessed earlier but for a different intended use.

In the situation of a new ENM without an approved non-nanoform, the data submitted will need to include the tests set out in the relevant EFSA Guidance for the specific intended use, supplemented by additional data on physico-chemical characterisation of the nanoform and tests as indicated in this ENM Guidance.

There are some general aspects to consider at an initial stage before testing ENM that is proposed for use in applications in the food/feed chain. Absorption and distribution leading to internal exposure, and a high level of ENM reactivity or internal mobility as well as persistence of the ENM are general indicators for in-depth testing.

The following are indicators of potential toxicity that should be considered when deciding on an appropriate testing strategy:

- 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 (dynamic corona))
- ENM intended to be used as antimicrobials (e.g. unintended consequences on the gut flora)

The following are indicators of a potential for high exposure:

- High production volume for the field of application
- High mobility of the nanoform in organisms (probability of internal exposure) (e.g. transport via macrophages; transport through cell membranes, blood-brain barrier and/or placenta; drug delivery systems) and mobilization potential (e.g. infiltration, sorption, complex formation)
- Targeted or controlled release
- Persistence/stability (e.g. in water, fat, and body fluids, lack of solubility/degradation)
- Bioaccumulation

There are indicators that are considered to reduce the likelihood of adverse effects of the ENM that are based on the specific exposure scenario under consideration and/or on loss of nano-properties. A complete loss of nano-properties will allow for reliance on a conventional risk assessment and the nano-specific risk assessment procedure would no longer be required.

The following parameters may indicate a loss of nanoproperties:

- Increased rate of dissolution[6] (e.g. in water, food/feed matrix or body fluids)
- Increased rate of degradability (e.g. biological or photocatalytic) to non-nanoform degradation products
- 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)

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

Nanostructured modifications on surfaces, and nanostructures that do not release particles and are not reactive are generally considered to reduce the likelihood of adverse effects (e.g. nanopores or lotus effect structures which can be used in filters and processing equipment). However, in some instances such applications could give rise to issues that should be considered (e.g. impact of functional failure).

The metabolism and excretion parameters are important indicators of biopersistence. Persistence of a substance/material is 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 considered.

The 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. Uncertainty analysis is discussed in chapter 8 .

3.   Characterisation of ENM

In addition to the small size, which is the main physical 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; Simon and Joner, 2008; Tiede, 2008; Nel et al., 2009; EFSA, 2009a; SCENIHR, 2010; JRC, 2010).

Adequate characterisation of ENM is essential for establishing its identity and the physico-chemical forms in food/feed products and under testing conditions. The information is needed to assess whether the material tested is representative and relevant for the exposure from the intended use. It is also essential for comparing materials tested (including for toxicity) in different products, between different manufacturers, and between similar tests of different duration. Such information will contribute to the knowledge base which in the future can be used for extrapolation or read-across procedures. The specifications of an ENM are important as they will influence the outcome of the risk assessment (e.g. different sizes and shapes of ENMs of the same chemical composition may have different toxicities). An ENM should be tested with properties and characteristics falling within the specifications provided for that ENM since subsequent approval will be based on the outcome of the risk assessment.

Early in the risk assessment it should be considered whether the ENM structure/properties are likely to be affected following addition to the food/feed matrix. In the case where the conformation of the ENM changes significantly in the food/feed matrix from what was added or manufactured, it will be important to carry out tests on the ENM in test environments with the structure of the ENM as acquired in the food/feed matrix.

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 spherical-shape deformable structures (e.g. encapsulates and micelles).

As for conventional chemicals, 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, 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 could be considered in five stages, i.e. as manufactured (pristine state), as 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 determination of physico-chemical characteristics is important for the subsequent experimental design and for the exposure assessment. The *in situ* characterisation of the ENM in the food/feed matrix and as used in the toxicity testing are considered to be most relevant to ensure the relevance of the toxicity data. Moreover, the characterisation of the ENM as present in biological fluids and tissues is important particularly for the ADME studies. However, it is noted that characterisation may not be feasible in all situations.

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 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 and provided, 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 (Domingos et al., 2009). The parameters in Table 1 include those listed by the OECD's Working Party on Manufactured Nanomaterials 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, 2010a).

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 EFSA Guidance document for the area of intended use, supplemented with nano-specific information as required in Table 1.

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 and number size distribution (indicating batch to batch variation – if any). The same information would be needed for secondary particles (e.g. agglomerates and aggregates) if present. .
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 particle 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.
Solubility and partition properties[a]	Essential	Information on solubility of the ENM in relevant solvents and their partitioning between aqueous and organic phase (e.g. as log Kow if appropriate).
pH	Essential for liquid dispersions	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 relevant chemical reactivity or catalytic activity of the ENM and of any surface coating of the ENM.
Photocatalytic activity	Essential for photocatalytic materials	Information on photocatalytic activity of relevant materials used in food packaging, coatings, and printing inks and internal reactions.

a) 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 ENM is dissolved (and thus not present) (see OECD ENV/JM/MONO(2010)25)

b) If an ENM has catalytic properties, it may catalyse a redox or other reaction that 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 that uses up the substrate, ENM reaction centres may perpetuate catalytic reactions.

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 (a non exhaustive list); 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 may be 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 nanoscale size range, 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, sulphydryl groups) should be taken into account as this may lead to potential binding with biopolymers such as proteins, lipids, polysaccharides, nucleic acids, etc. In general, ENM will not be present in food/feed products in a free form but will bind to the food/feed components. The interaction with proteins may form a dynamic "corona" surrounding the ENM which may affect the behaviour of the protein, and the protein that of the ENMs (EFSA, 2009a). It may therefore be necessary to use a combination of methods for detection and characterisation of ENM in food/feed matrices. 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 to allow for analysis of the ENM. For example, a method for separation (e.g. Field Flow Fractionation) of the nano-fraction may be needed prior to the use of a detection/characterisation method. However, such a separation step may result in alterations of the ENM structure/properties that should be carefully considered.

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

3.1.3.   Characterisation of ENM for toxicological testing

For the toxicological assessment of ENM, it is essential to know in which form the ENM are presented to the test systems. In addition, 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 and to allow for comparison with the ENM in the food/feed matrix to which exposure takes place.

The current available information indicates that special consideration is needed to address potential batch-to-batch variations and aging effects (e.g. agglomeration/aggregation, sedimentation).

3.1.4.   Characterisation of ENM as present in biological fluids and tissues

In ADME studies there may be particular difficulties in measuring the amounts of ENM in blood, tissues and excreta, and in establishing the form in which ENM 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 in organs, tissues and other biological samples (see also section 5.4.2.).

3.2. Performance criteria for characterisation methods

Methods used for the characterisation of ENM in their pristine form (as manufactured), 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 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 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)); Commission Decision 2002/657/EC[7]) 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. Applicants are reminded that it may be expected that regulatory authorities will systematically require routine methods for monitoring compliance with the specification of the ENM.

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 which are validated for size measurements only. Silica nanoparticles size reference materials (IRMM-304 and ERM-FD100) are available from the Joint Research Centre, Institute of Reference Materials and Measurements and gold nanoparticles (NIST RM 8011, 8012 and 8013) from the National Institute of Standards and Technology (NIST). Additional reference materials are expected to become available with time, and should be used as appropriate. In addition to reference materials, the European Commission Joint Research Centre has recently made available a repository composed of 25 nanomaterials. These nanomaterials can be used as standardised research tools/reference points by laboratories to verify that results are comparable to those of other laboratories. It is acknowledged that where methods of analysis are still relatively weak, there has to be a greater reliance on standard materials. In the absence of certified reference materials, self-generated and properly documented standards may be used.

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 for the risk assessment.

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, it is possible to 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 completely solubilised in the food/feed matrix, no human exposure is expected apart from exposure to resulting degradation products (non-nanoform fraction). In case of complete digestion in gastrointestinal fluids, localised exposure (e.g. in the upper gastrointestinal tract) needs to be considered (Holpuch et al., 2010).

In contrast, when ENM are present in an indirect way, e.g. due to migration or transfer of non-nanoform degradation products of the ENM , including possible carry-over from feed, via animals to food, its type and amount should be determined. The EFSA guidance for food contact materials gives information for testing migration (EFSA, 2009c). The extent of migration or transfer into food/feed will determine whether and to what extent information on hazard characterisation and absorption,

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[7] Commission Decision (EC) 2002/657 of 12 August 2002 implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results. OJ L 221, 17.08.2002 p.8-36.

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 migrates into the food or feed an exposure assessment should be performed.

If identification of the ENM in the food/feed matrix is not possible or in case there is indication of an alteration of the ENM, no conclusion can be reached on the ENM exposure apart from what was initially added to the food/feed, with the understanding that the nature of the ENM to which exposure takes place is unknown or altered (see chapter 6).
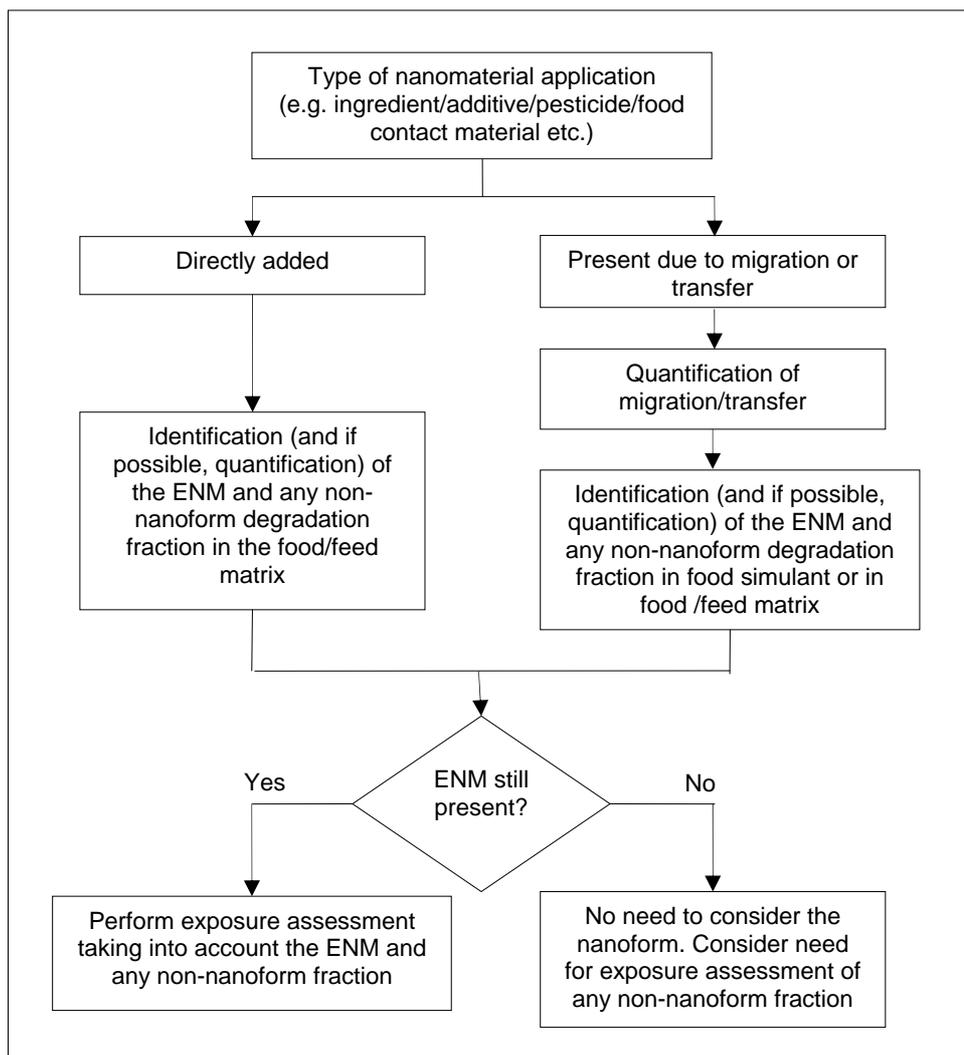
Figure 2: Exposure scenarios

5. Hazard identification and hazard characterisation

5.1. General considerations

It is reiterated from chapter 1 that the test requirements stipulated in current EFSA guidance documents and EC guidelines for the intended use in the food/feed area apply in principle also to ENM. This chapter outlines additional aspects that should be considered for the ENM hazard identification and characterisation that may arise due to the specific characteristics and properties of

ENM. 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.

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, as at the time of EFSA's previous opinion (EFSA, 2009a), is from *in vitro* studies or from *in vivo* studies using routes of exposure other than the oral one (e.g. inhalation).

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/degradable ENM, which tend to have effects more similar to the non-nanoform (Auffan et al., 2009; EFSA, 2009a).

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 could 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 and to allow for comparison also with non-nanoforms, if relevant.

Studies have been published that 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 meet these requirements. It might be necessary, when appropriate and possible, to analyse the relevant chemical components as such.

5.2. Toxicity testing outline

The toxicity testing strategy is determined by the presence of ENM in the food/feed matrix and if appropriate, by available information on a non-nanoform of the same substance. This toxicity testing strategy is illustrated by six general cases and the toxicity tests are indicated in table 2 and figure 3. For ENM applications where exposure to humans or animals are not anticipated (e.g. for certain types of pesticides) the testing strategy described in the relevant EFSA guidance should be applied with adaptations of tests as appropriate taking into account the considerations and modifications outlined in this guidance.

> *Case 1– No persistence of ENM in preparations/formulations as marketed*
> For nanotechnology applications where convincing evidence is provided demonstrating, by appropriate analytical methods, that the ENM is completely degraded/solubilised to non-nanoform, then EFSA Guidance for non-nanoforms for the specific intended use should apply, and this ENM Guidance would no longer apply.

Case 2 – No migration from food contact materials (i.e. no exposure)
Where evidence is provided convincingly demonstrating, by appropriate analytical methods, that there is no migration, the risk assessment could be based on the information that there is no exposure to the ENM via food and therefore there is no toxicological concern.

Case 3 – Complete ENM transformation in the food/feed matrix before ingestion
When evidence is provided convincingly demonstrating, by appropriate analytical methods, that transformation of the ENM into a non-nanoform in the food/feed matrix is judged to be complete (i.e. non-nanoform degradation products are present) before ingestion, then EFSA Guidance for non-nanoforms for the specific intended use should apply, and this present ENM Guidance would no longer apply.

Case 4 – Transformation during digestion
When evidence is provided convincingly demonstrating, by appropriate analytical methods, that that an ENM completely dissolves/degrades in the gastro-intestinal tract, the hazard identification and hazard characterisation can rely on data for the non-nanoform substance (if available) as long as the possibility of ENM absorption before the dissolution/degradation stage can be excluded. When evidence is provided convincingly demonstrating that no ENM absorption takes place a limited set of tests in general consisting of *in vitro* genotoxicity, *in vivo* local effects and/or other appropriate *in vivo* testing may be deemed as sufficient.

The systemic toxicity profile of a dissolved ENM is likely to be similar to the soluble (ionic or molecular) form. If this is demonstrated, further testing on the ENM is not necessary. In cases where data on the non-nanoform are not available, testing of the non-nanoform is required according to the relevant EFSA Guidance for the intended use.

Case 5 – Information on non-nanoform available
When information on a non-nanoform of the same substance is available and where some or all of the ENM persists in the food/feed matrix and in gastrointestinal fluids, a testing approach is recommended which is based on comparison of information on ADME, toxicity and genotoxicity of the non-nanoform with, in first instance, ADME, 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 the differences observed indicate increased hazard, then more toxicity testing will be required on the ENM, beyond ADME, 90-day and genotoxicity tests.
- If the differences observed indicate less hazard then any request to waive further testing should be scientifically justified.

Case 6 – No information on non-nanoform available
When information on a non-nanoform is not available and where some or all of the ENM persists in the food/feed matrix and in gastrointestinal fluids, the approach for toxicity tests on the ENM should follow the relevant EFSA guidance for the intended use with the modifications in the present opinion to take into account the nanoproperties. The ENM toxicity testing strategy provided for hazard identification and hazard characterisation takes into account the nanoproperties (see section 5.3 and 5.4).

Table 2: ENM toxicity testing strategy

| Type of test	Information
In vitro genotoxicity tests	Usually necessary for case 4. Necessary for cases 5 and 6 (see section 5.3.2.)
ADME	Usually necessary for case 4. Necessary for cases 5 and 6 (see section 5.4.1 and 5.4.2.)
Repeated-dose 90-day oral toxicity study in rodents	Usually necessary for case 4. Necessary for cases 5 and 6 (see section 5.4.3.)
In vitro digestion studies	Usually necessary for cases 3, 4, 5 and 6 (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.).

5.3. *In vitro* studies

There are ongoing developments in *in vitro* methods but currently there are no *in vitro* methods validated to be used for hazard assessment of ENM (Park et al., 2009). However, *in vitro* tests may provide information on hazards (e.g. genotoxicity), give indication of potential toxicity of an ENM and may be used to elucidate possible mode of action. The primary aim of *in vitro* testing is for ENM toxicity screening and the understanding of biological responses and underlying mechanisms. Information on the mode of action of the ENM may be helpful, e.g. if reactive oxygen species are generated then genotoxicity and other toxic effects can be anticipated.

For *in vitro* testing attention should be given to the suitability of the test system and to possible interactions of ENM with *in vitro* culture medium components (e.g. growth factors, proteins and nutrients) resulting into alterations of the ENM structure and properties, and possible influence of culture medium components on cellular uptake of ENM, and to the possibility that treatment times may need to be extended to allow uptake of ENM into cells (Doak et al., 2009; Stone et al., 2009; Donaldson et al., 2010). The possibility to use biological *in vitro* model fluids (e.g. saliva, gastrointestinal fluids, mucous, plasma or lymph) should be considered. Consideration also needs to be given to what should be used as negative and positive controls. There may also be a need to consider whether impurities may be present in the ENM that are known to be toxic (see chapter 3). Possible interference of the ENM with the read out system of the *in vitro* assay should be investigated.
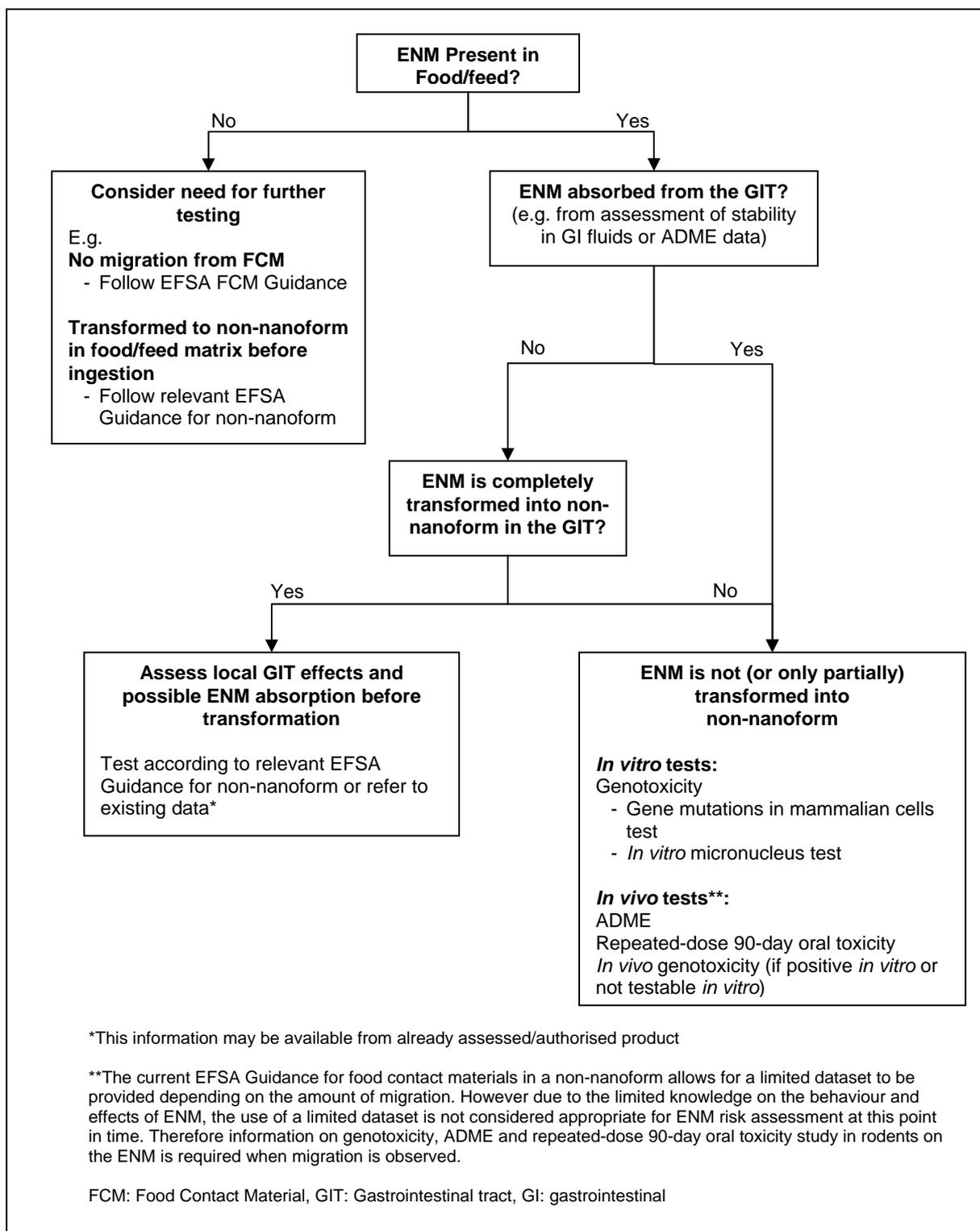
Figure 3:   Decision tree for toxicity testing

5.3.1.   *In vitro* digestion studies

In vitro digestion can be used to demonstrate dissolution/degradation of the ENM. In these cases only limited or no further testing might be needed. Various models are available, most have been designed

to assess the release or dissolution of non-nanomaterials (Oomen et al., 2002; Dressman et al., 1998; Krul et al., 2000; Brandon et al., 2006). With an *in vitro* digestion model, the conditions of the human gastrointestinal tract can be simulated, i.e. temperature, mixing, transit time, composition of salt, enzymes and other constituents such as bile. *In vitro* digestion models have been applied to determine the release of various orally ingested compounds e.g. contaminants from soil (Oomen et al., 2003; Van de Wiele et al., 2007), food contaminants (Dall'Asta et al., 2010; Versantvoort et al., 2005), food mutagens (Krul et al., 2000), food components (Blanquet-Diot et al., 2009; Tydeman et al., 2010), contaminants in toys (Brandon et al., 2006) and drugs (Dressman et al., 1998; Kostewicz et al., 2002; Blanquet et al., 2004). These models vary in the degree in which they simulate human gastro intestinal tract conditions from very simple to rather sophisticated. To which extent the different *in vitro* models lead to different conclusions regarding dissolution and degradation of nanomaterials has not yet been studied.

5.3.2. *In vitro* genotoxicity testing

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.

A bacterial reverse mutation assay is usually recommended for the detection of gene mutations, as also included in the work of the Scientific Committee on genotoxicity testing strategies[8]. However, since ENM may not be able to penetrate the bacterial cell wall (Landsiedel et al., 2009) and because bacterial cells, unlike mammalian cells, do not have the ability to phagocytose particles, the use of a bacterial reverse mutation test for detection of genotoxicity of ENM is not considered to be appropriate.

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* micronucleus assay (OECD test guideline 487)

There may be circumstances under which it may be justified to deviate from the above-mentioned core set (e.g. when there is a need to test the ENM in a matrix that cannot be added *in vitro*). In such cases a scientific justification should be provided and additional types of considerations or *in vivo* studies may be needed. In certain instances (e.g. with induction of reactive oxygen species, soluble ENM, very small ENM) a bacterial reverse mutation test might still be informative.

If at least one of the *in vitro* tests indicates positive results or if it is impossible to test the ENM *in vitro*, *in vivo* genotoxicity testing is required (see section 5.4.5). If both *in vitro* tests are negative, but there are indications that reactive radical species are generated then also *in vivo* genotoxicity testing should be considered (see section 5.4.5).

5.3.3. Other *in vitro* studies

In vitro tests may provide additional insights into e.g. internal exposure, toxicity, and mode of action of the ENM (e.g. on cytotoxicity, oxidative stress, potential for inflammation and immunotoxicity). Considering oral intake as the *in vivo* route of administration, several *in vitro* approaches may be applied to generate additional hazard identification information.

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[8] The Scientific Committee anticipates it will adopt an opinion later in 2011 on genotoxicity testing strategies. A draft of the opinion published for public consultation is available at:
http://www.efsa.europa.eu/en/consultations/call/scaf110420.pdf (April 2011).

Studies can be performed to investigate the effects of ENM on e.g. the integrity of the gastrointestinal barrier, inflammatory responses to assess gut maintenance, immune cells and immune responses.

In vitro models to assess barrier integrity/permeability may be based on several types of cells, e.g. primary human oesophageal epithelial cells, M-cells (modified enterocytes present throughout the epithelial lining) and differentiated CaCo-2 cells. A number of parameters can be considered for investigation in such *in vitro* models including e.g. LDH leakage or MTT reduction, trans-epithelial electrical resistance, paracellular flux, inflammatory mediators and generation of reactive radical species.

In vitro models to assess the systemic effects on immune cells and immune responses include e.g. the whole blood cytokine release model. Cytotoxicity, inflammatory and immunological parameters may be measured in this model. 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). The whole blood assay has been validated for the evaluation of pyrogenic contaminations (e.g. endotoxins) or aspecific immune cell activation (Hoffmann et al., 2005; Schindler et al., 2006). For this model, discrimination between endotoxin effects and cellular immune reactivity should be considered. For ENM that become systemically available *in vitro* models to investigate complement activation may also be considered. However, the whole blood assay is not representative for testing gut–associated immune responses. *In vitro* models may also be applied to investigate ENM removal from blood/tissues via uptake by the mononuclear phagocyte system (MPS) (composed primarily of macrophages).

If *in vitro* results indicate increased epithelial permeability, release of inflammatory mediators, effects on immune cells or immune response, appropriate *in vivo* studies should be considered (see section 5.4) or scientific justification should be provided for waiving *in vivo* methods.

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 of the ENM and if necessary to follow up results from *in vitro* genotoxicity studies. Tissue distribution, accumulation/persistency and elimination from tissues is considered to be more relevant than blood plasma levels. Special attention should be paid to the typical target organs that have been shown to have an increased capacity for uptake of particles (e.g. liver, spleen, and lungs).

5.4.1.   Administration of ENM for *in vivo* 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. In case the ENM structure/properties are affected by the food/feed matrix, and can not be mimicked by simulants, administration of the whole food/feed[9] matrix with the ENM to the test animal should be considered.

For administration 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 not be uniformly mixed within the food matrix.

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[9] The Scientific Committee anticipates it will adopt an opinion later in 2011 discussing modification of the OECD test guideline 408 for testing of whole food/feed which should be considered for the experimental design in cases where the ENM has to be given in the whole food/feed matrix.

It is recommended that coherence between the structure/properties of the ENM in the toxicity test medium and the structure/properties of the ENM in the food /feed matrix is checked before undertaking any toxicity test and that, wherever possible, the same vehicle is used in all the toxicological tests (i.e. ADME, *in vitro* toxicity, genotoxicity and *in vivo* studies etc.). The administration of the test material requires 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 becomes therefore no longer available (i.e. there will be no exposure). Possible interactions with the administration vehicle, either the food matrix or water, need 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, 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 that may or may not mix with the gastrointestinal fluids, which might result in a higher local concentration and increased 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 gavage 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, which is one of the reasons for *in vivo* testing. Consideration of the potential for time dependent dissolution/degradation is essential, as well as physico-chemical ENM modifications such as 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 in organs, tissues and other biological samples. 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), but combining with suitable separation techniques could overcome this. 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 labelling and detection technique should be based on the composition of the ENM, e.g.

metal nanomaterials or lipid-like nanomaterials. In addition, the impact of the labelling system on the properties and activity of the ENM should be considered.

Many types of ENM exhibit inherent polydispersity (large size distribution) due to their complex composition. 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 mononuclear phagocyte system (MPS) especially in spleen and liver, there may be a need for extended toxicokinetic studies depending on the biopersistence of the ENM. The toxicokinetic studies 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 MPS 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 biological persistence of ENM may correlate with long-term toxicity.

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.

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 e.g. liver and spleen should be investigated.

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

For the main ADME 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**[10]

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 test guideline 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 in repeated-dose studies should be paid to cardiovascular and inflammatory parameters as well as to the mononuclear phagocyte system (MPS), as after systemic translocation, most ENM are likely to end up in the MPS tissues. The results from the repeated-dose 90-day oral toxicity study can be used to

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[10] The Scientific Committee anticipates it will adopt an opinion later in 2011 discussing modification of the OECD test guideline 408 for testing of whole food/feed which should be considered for the experimental design in cases where the ENM has to be given in the whole food/feed matrix.

identify a Benchmark Dose lower confidence limit (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 toxic effects and/or accumulation of ENM (or degradation products/metabolites) 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 offers only limited information on reproductive toxicity and no information on developmental toxicity; it can inform about effects on the reproductive organs and, if assessed, the oestrous cycle, but it does 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 of chemicals 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[11]

If at least one of the *in vitro* tests indicates genotoxic activity, or if it is impossible to test the ENM *in vitro*, 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. Before embarking on any necessary follow-up, the results from the *in vitro* testing should be reviewed and other relevant data on the substance, such as information about chemical reactivity (which might predispose to site of contact effects), bioavailability, metabolism, toxicokinetics, and any target organ specificity should be considered.

In vivo genotoxicity tests should relate to the genotoxic endpoint(s) identified as positive *in vitro* and to appropriate target organs or tissues. Evidence, either from the test itself or from other toxicokinetic or repeated-dose toxicological studies, that the target tissue(s) have been exposed to the test substance and/or its metabolites is essential for interpretation of negative results.

The choice of the appropriate *in vivo* genotoxicity test(s) requires expert judgement based on all available information, to be applied case-by-case. Any of the following *in vivo* tests may be suitable

- an *in vivo* micronucleus test (OECD test guideline 474)

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[11] The Scientific Committee anticipates it will adopt an opinion later in 2011 on genotoxicity testing strategies. A draft of the opinion published for public consultation is available at:
http://www.efsa.europa.eu/en/consultations/call/scaf110420.pdf (April 2011).

- an *in vivo* Comet assay (no OECD test guideline at present; internationally agreed protocols available, e.g. see hptt://cometassay.com)

- a transgenic rodent gene mutation assay (draft OECD test guideline)

If both *in vitro* tests are negative, but there are indications that reactive radical species are generated, or in case it is impossible to test the ENM *in vitro*, then an *in vivo* Comet assay, which can also provide information on mode of action, is recommended to be included in the ADME or in the repeated-dose 90-day oral toxicity study (Karlsson, 2010).

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, 2009d). 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 that 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, ENM should be analysed at an early stage of the food chain, and effects of processing and storage and the stability of the ENM 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, which could be determined by measurement of the ENM in relevant animal tissue or products (EFSA, 2008b).

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 added ENM is present, ingested and absorbed in the nanoform, although the structure/properties of the ENM remain undetermined and therefore difficult to relate to the structure/properties of the ENM used in the toxicity studies. In this case, the toxicity testing could be performed by administering the ENM in the food/feed matrix.

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.

Several approaches for generating information required for risk assessment are 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.

8. Uncertainty analysis

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, 2009d). 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.

8.1. Uncertainties in the physico-chemical characterisation of ENM

It may be difficult to characterise, detect and measure ENM in food/feed and in biological matrices. It is important to note that currently there are no standard methods available for physico-chemical characterisation of all the various ENM structures/properties. However, 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.

Reproducibility and accuracy 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 various measurement techniques may nevertheless differ because of differences in the physical principles applied for the measurement (e.g. variations in size measurements (Domingos et al., 2009)). 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 analytical methods. Different results obtained by various methods could influence the assessment and decision on whether a material should be regarded as a nanomaterial or not.

It is currently difficult to distinguish an ENM from background levels of the same materials/substances in nano- or non-nanoform that may be present in food/feed products. Appropriate methods (e.g. stable isotope analysis, elemental fingerprinting) can be applied to distinguish the intentionally added 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, possible food/feed matrix interactions of the ENM may be determined using food simulants (e.g. water, oil, ethanol, acetic acid, 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.

8.2. Uncertainties in the hazard characterisation of the ENM

Limited information is available in relation to aspects of ENM toxicokinetics and toxicology, including optimal methods for testing ENM. Existing toxicity testing methods (e.g. OECD test guidelines) 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 and test animals. There may also be additional toxic effects caused by ENM that are not readily detectable by current standard protocols. Additional endpoints (e.g. cardiovascular or immune function endpoints) not routinely addressed may need to be considered in addition to traditional endpoints. Currently there are no *in vitro* methods validated to be used for hazard assessment of ENM.

It is still not fully 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 the generation of such knowledge, the reactivity of a given ENM will be better understood and potential effects may be predicted.

Assays for the allergy testing of food components are currently not available. For ENM a comparison with existing allergic proteins does not seem appropriate. However, the identification of proteins of the food matrix adhering/bound to the ENM might give some insight on potential of ENM for promoting allergy induction. Also post marketing monitoring may provide useful information.

Information emerging from studies on ENM in the future may point to other modifications in test protocols.

8.3. Uncertainties in exposure assessment

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. However, these uncertainties could be reduced by *in vivo* testing of the ENM in the relevant food/feed matrix.

8.4. Uncertainties in the risk characterisation

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. In the absence of essential data, the risk assessor will not be able to conclude on the risk assessment. It should be clear from the assessment how the available 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 food and animal feed intakes by the 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

The risk assessment paradigm (hazard identification and hazard characterisation followed by exposure assessment and risk characterisation) is appropriate for these applications. Consequently relevant 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 and under testing conditions. The physico-chemical parameters may change in various environments and the characterisation of ENM should ideally be determined in five stages, i.e. as manufactured (pristine state), as 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 interactions with tissues, and potential exposure levels. The physico-chemical characterisation is needed to identify an ENM and decide whether the ENM Guidance is appropriate. 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 the basis for the risk characterisation. 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).

Prior to commencing the detailed risk assessment of the nanomaterial, anticipated exposure scenarios from the proposed uses should be outlined. 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.

Six cases are presented which outline different toxicity testing approaches. Where convincing evidence is provided indicating that ENM use does not result in presence of the ENM or its degradation/solubilisation products in the food/feed, then there is no need for any additional testing. When transformation of the ENM into a non-nanoform in the food/feed matrix is judged to be complete before ingestion, then EFSA guidance for non-nanoforms for the specific intended use should be applied. When it can be demonstrated that an ENM completely dissolves/degrades in the gastro-intestinal tract without absorption of the ENM, the hazard identification and hazard characterisation can rely on data for the non-nanoform substance (if available). When information on a non-nanoform of the same substance is available and where some or all of the ENM persist in the food/feed matrix and in gastrointestinal fluids, a testing approach is recommended which is based on

comparing information on ADME and toxicity of the non-nanoform with ADME and repeated-dose 90-day oral toxicity study and genotoxicity information of the ENM. When information on a non-nanoform is not available and where some or all of the ENM persist in the food/feed matrix and in gastrointestinal fluids, the approach for toxicity tests on the ENM should follow the relevant EFSA guidance for the intended use with the modifications in the present opinion to take into account the nanoproperties.

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 amount of the ENM present in the food/feed matrix. 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. 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 added ENM, is present, ingested and absorbed as the nanoform, although the structure/properties of the ENM remain undetermined and difficult to relate to the structure/properties of the ENM used in the toxicity studies.

There are currently uncertainties related to the identification, characterisation and detection of ENM that 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 as appropriate.

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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).

Table with examples of characterisation methods

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 TG 105), and log k_{ow} (OECD TG 107, 117) can be used. Dissolution rate constants.
Viscosity	Methods such as OECD TG 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 chemical, biochemical and/or catalysed reactions
Photocatalytic activity	Kinetic measurements of the chemical, biochemical and/or 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 related to table with characterisation methods

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
MALS – Microwave absorption line-spectra
NMR – Nuclear magnetic resonance spectroscopy
NTA – Nanosecond Transient Absorption
OES – Optical emission spectroscopy
PCCS – Photo Cross Correlation Spectroscopy
QqQ – Triple quadrupole mass spectrometer
SAXS – Small-angle X-ray scattering
SEC – Size exclusion chromatography
SedFFF – Sedimentation field flow fractionation
SEM – Scanning Electron Microscopy
SLS – Static Light Scattering 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

GLOSSARY AND ABBREVIATIONS

Term	Explanation
ADME	Adsorption, Distribution, Metabolism and Excretion (elimination)
Agglomerate	A group of particles held together by weak forces such as van der Waals forces, some electrostatic forces and/or surface tension.
Aggregate	A group of particles held together by strong forces such as those associated with covalent or metallic bonds.
Dispersion	Particles are dispersed in a continuous phase of a different composition.
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).
ENM Guidance	Shorter name of this EFSA Guidance on the risk assessment of the application of nanoscience and nanotechnologies in the food and feed chain
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.
Lotus effect	A property of highly hydrophobic surfaces which creates a "self cleaning" effect.
Manufactured nanomaterial (ISO)	Nanomaterial intentionally produced for commercial purposes to have specific properties or specific composition
Nanomaterial (ISO)	Material with any external dimension in the nanoscale or having internal structure or surface structure in the nanoscale
Nanoproperties	Examples include (but are not restricted to); size in the nanoscale, large surface area, high surface reactivity, quantum effects, possibility to translocate over biological membranes not observed in larger non-nanoforms etc.
Nanoscale	A size measurement generally considered to refer to the size range 1-100 nm (e.g. Lövenstam , 2010; SCENIHR 2010). From a metric interpretation nanoscale encompasses the range from 1-999 nm. The size range below 1 nm is measured in picometers, and the size range above 999 nm is measured in micrometers.
Nanoscience (ISO)	Study, discovery and understanding of matter in the nanoscale (where size- and structure-dependent properties and phenomena, as distinct from those associated with individual atoms or molecules or with bulk materials, can emerge)
Nanotechnology (ISO)	Application of scientific knowledge to manipulate and control matter in the nanoscale in order to make use of size- and structure-dependent properties and phenomena, as distinct from those associated with individual atoms or molecules or with bulk materials
Non-nanoform	A material that 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).
Pour density	A function of the degree of compaction during pelletisation.
Solubility	The property of a substance to dissolve in a solution and for a homogenous solution of the solute in the solvent.
Solution	In a solution the solute does not exists as a solid but is fully dissolved.