GUIDANCE

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Guidance on risk assessment of the application of nanoscience and nanotechnologies in the food and feed chain: Part 1, human and animal health

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Abstract

The European Food Safety Authority has produced this Guidance on human and animal health aspects (Part 1) of the risk assessment of nanoscience and nanotechnology applications in the food and feed chain. It covers the application areas within EFSA's remit, e.g. novel foods, food contact materials, food/feed additives and pesticides. The Guidance takes account of the new developments that have taken place since publication of the previous Guidance in 2011. Potential future developments are suggested in the scientific literature for nanoencapsulated delivery systems and nanocomposites in applications such as novel foods, food/feed additives, biocides, pesticides and food contact materials. Therefore, the Guidance has taken account of relevant new scientific studies that provide more insights to physicochemical properties, exposure assessment and hazard characterisation of nanomaterials. It specifically elaborates on physicochemical characterisation of nanomaterials in terms of how to establish whether a material is a nanomaterial, the key parameters that should be measured, the methods and techniques that can be used for characterisation of nanomaterials and their determination in complex matrices. It also details the aspects relating to exposure assessment and hazard identification and characterisation. In particular, nanospecific considerations relating to *in vivo*/*in vitro* toxicological studies are discussed and a tiered framework for toxicological testing is outlined. It describes *in vitro* degradation, toxicokinetics, genotoxicity as well as general issues relating to testing of nanomaterials. Depending on the initial tier results, studies may be needed to investigate reproductive and developmental toxicity, immunotoxicity, allergenicity, neurotoxicity, effects on gut microbiome and endocrine activity. The possible use of read-across to fill data gaps as well as the potential use of integrated testing strategies and the knowledge of modes/mechanisms of action are also discussed. The Guidance proposes approaches to risk characterisation and uncertainty analysis, and provides recommendations for further research in this area.

Keywords: Nanomaterial, food, feed, guidance, nanoscience, nanotechnology, risk assessment, testing strategy

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Summary

1) Upon request of the European Food Safety Authority (EFSA), the Scientific Committee has undertaken a thorough revision of the previous Guidance on risk assessment of nanoscience and nanotechnology applications in the food and feed chain published in 2011. This Part 1 of the updated Guidance relates to human and animal health aspects of nanomaterial applications in the areas within EFSA's remit. Part 2 of the Guidance will separately address those aspects that relate to environmental risk assessment.
2) The requested revision should take into account the relevant applications areas including novel foods, food contact materials (FCMs), food/feed additives and pesticides as well as physicochemical characterisation and the other data needed for safety assessment of nanomaterials in food/feed.
3) The present Guidance therefore provides an overview on information requirements and how to perform risk assessment of nanomaterials in the food and feed area (e.g. novel food, FCMs, food/feed additives and pesticides). For example, under the new EU Regulation on Novel Food (EU) No. 2015/2283, a food consisting of **engineered nanomaterials** will be considered a novel food and as such will require authorisation. The Regulation stipulates that risk assessment of novel foods shall be carried out by EFSA, which shall also be responsible for verifying that the most up-to-date test methods have been used to assess their safety.
4) The present Guidance is aimed at providing a structured pathway for carrying out safety assessment of nanomaterials in the food and feed area. This Guidance is applicable to (see Section 1.3):
    a) a material that meets the criteria for an **engineered nanomaterial** (see Section 1.2.3) as outlined in Novel Food Regulation (EU) No 2015/2283 and Regulation (EU) No 1169/2011 on the Provision of Food Information to Consumers, i.e. nanomaterials that, amongst other criteria, have particle sizes in the defined nanoscale (1–100 nm);
    b) a **material that contains particles having a size above 100 nm** which could retain properties that are characteristic of the nanoscale (see Section 3.1), for example related to the large specific surface area of the materials or different toxicokinetic behaviour (i.e. significant changes in absorption, distribution and/or metabolism) as compared with its non-nanomaterial (see Glossary). This may be the case for materials resulting from production processes that are aimed at reducing the average diameter of materials' particles (e.g. micronisation).
    c) a material **that is not engineered as nanomaterial but contains a fraction of particles, less than 50% in the number–size distribution (as per the recommended European Commission definition), with one or more external dimensions in the size range 1–100 nm.** This is expected to be the case of manufacturing processes for powdered or particulate food chemicals that typically result in materials with a range of sizes (see Section 4.2.2);
    d) a **nanomaterial having the same elemental composition but that occurs in a different morphological shapes, sizes, crystalline forms and/or surface properties** as, for example a consequence of different production processes.
    e) a **nanoscale entity made of natural materials** that has been deliberately produced to have nano-enabled properties, or has been modified for use in the development of other nanoscale materials, e.g. for encapsulating (bioactive) compounds (see Appendix E.6).
    f) Although the European Commission recommendation on a definition for nanomaterial is currently under review, and has not yet been adopted under the relevant regulatory frameworks, the Scientific Committee advises to take this and any future reviews into consideration when assessing safety of materials consisting of particles (see section 1.2.2).
5) A decision flow scheme, developed by the NanoDefine project, is presented in this Guidance (see Section 4.1) to facilitate ascertaining whether or not a material is nanomaterial according to the European Commission recommended definition (see Section 1.2.2), and to identify relevant methods and tools for its characterisation. The European Commission has recommended a threshold of 50% of the particles in the number-based minimal external size distribution to be in the nanoscale (1–100 nm) for a material to be regarded as a nanomaterial. Although this recommendation on a definition of nanomaterial is currently

under review, and has not yet been adopted under the relevant regulatory (food) frameworks, the Scientific Committee advises to take this into consideration when assessing safety of materials containing particles.

6) Where a material has been identified as a nanomaterial, it will need to be assessed for safety and to fulfil requirements of this Guidance. It is nevertheless also important to highlight that, irrespective of the presence of a nanomaterial, the existing requirements for safety assessment according to **guidance for conventional non-nanomaterials** under relevant regulations must be followed.
7) In principle, the current risk assessment paradigm for chemicals, which is based on hazard identification/characterisation together with exposure assessment and risk characterisation, is also applicable to nanomaterials. However, as highlighted in this Guidance, reducing the size of particulate materials to the nanoscale can impart certain changes in properties and biokinetics behaviour, which may also lead to altered toxicological effects compared with corresponding non-nanomaterial. Therefore, the safety of a nanomaterial should not be automatically assumed to be similar/comparable to its corresponding non-nanomaterial or another nanomaterial. This also means that, for a specific nanomaterial, data and information **would need to be provided** on certain **nanospecific properties** (see Section 4, Table 1 + Appendix B Table B.1), in addition to the data and information generally required according to the relevant conventional regulation. Some of the currently available testing methods may also need adaptation to take account of the specific properties of nanomaterials. Therefore, safety assessment of nanomaterials must be carried out in accordance with the provisions of this Guidance.
8) As part of problem formulation, a prerequisite for risk assessment of nanomaterials is an unambiguous identification and detailed **characterisation** of the constituting components and impurities of the pristine core nanomaterial, as well as any entities on the particle surface (including coatings). Information on physicochemical parameters can also provide important pointers for potential toxicity of a nanomaterial, and thus help in deciding an appropriate testing strategy. This Guidance (see Section 4.2.1) lists the main physicochemical parameters that are considered essential for characterisation of nanomaterials, although not all are applicable to each material. It recommends that characterisation of the nanomaterial is carried out at different stages, e.g. in its the pristine state as tested and on the material as used in products and applications. The Guidance also outlines the currently available methods and tools that can be used for measuring the parameters, as well as quality control aspects that should be considered. It recommends that particle size distribution should be determined by more than one independent technique (one of which being electron microscopy).
9) It is also noteworthy that a high degradation rate, for example caused by dissolution, will render a nanomaterial into its corresponding non-nanomaterial form. Therefore, the nanospecific considerations described in this Guidance are applicable to those materials that do not **quickly degrade** (see Section 6.2.1 and Appendix D) to ions or molecules under the physiological conditions of the food production and processing processes, the food matrix and of the gastrointestinal (GI) system, and therefore have a chance of interacting with biological entities at the local or systemic levels. **Practical description of when a nanomaterial is considered to have a high degradation rate is provided in Section 6.2.**
10) Throughout various Sections, the Guidance also identifies the circumstances under which some requirements for **data generated specifically on the nanomaterial could be waived**. For example, where it can be shown that the use of a nanomaterial does not lead to local or systemic exposure (to the specific nanomaterial or degradation products in the form of a nanomaterial), or where there is no migration or transfer of a nanomaterial from a FCM into the food. Also, because a nanomaterial can be developed in several forms with different sizes, crystalline forms, shapes, surface characteristics, etc., this Guidance describes the current potential use of a **grouping/read-across** approach to avoid case-by-case testing of all variants of a given nanomaterial (see Section 6.3). It notes that, in principle, toxicological data from a nanomaterial may be used for safety assessment of another variant of the same nanomaterial, if it can be shown that there are close similarities in their physicochemical properties and toxicokinetic behaviour. Justification that a source (nano)material exhibits toxicokinetic behaviour and hazards that are more 'worst

case' than the target nanomaterial would also be possible (see Section 6.3). An up-to-date review of the published literature is also important to take account of the available information that may help in avoiding unnecessary testing.

11) The principles for **exposure assessment** of nanomaterials via food/feed are essentially the same as for non-nanomaterials and will require consideration of the likely exposure scenarios, and estimation of exposure based on consumption data and anticipated average and high intakes in various population groups (see Section 5). Probabilistic methods may also be useful in terms of determining ranges of plausible values. Where direct exposure (e.g. via novel food, flavourings, food additives), or indirect exposure (e.g. migration or transfer from FCM, carry-over from feed via animals to food or a pesticide to crop) is possible, it should be determined whether the nanomaterial or its degradation product(s) remain present as particles in the food/feed matrix to inform risk characterisation. Characteristics that may indicate a loss of nanospecific properties and thus reduce the chance of exposure to the nanomaterial include: high degradation rate in water, food/feed matrix or GI fluids; (bio)degradability to non-nanosized products; formation of larger aggregates (> 100 nm); nanoparticles being fixed or embedded in other matrices (e.g. polymer composites used as FCMs), etc. In the absence of exposure data, or where it is not possible to determine the properties and amounts of nanosized particles in complex matrices, it should be assumed as a worst-case that all nanomaterial added to a food/feed product, is present, ingested and absorbed as the nanomaterial.
12) In Chapter 6, the Guidance outlines a structured approach for testing of nanomaterials for identification and characterisation of **toxicological hazards**, and describes relevant *in vitro* and *in vivo* tests that can be used. The proposed approach is based on testing nanomaterials under 3 different steps that are preceded by an initial step (**Step 0**), at which the rate of degradation of the nanomaterial, e.g. due to dissolution, under conditions representative of the GI tract is investigated. If a nanomaterial or its degradation products in the form of a nanomaterial can be present in food/feed or food simulant, information on degradation rate under conditions relevant for the GI tract should be provided. Information on the interpretation of the degradation rate is provided in Section 6.2. Exposure to a nanomaterial can occur if the nanomaterial does not **quickly degrade**. In this case, the nanomaterial should be quantified and characterised at least by the number-based particle size distribution under digestive tract conditions and whether the particles consist of primary particles only or may also comprise aggregates and agglomerates. In cases where a high degradation rate can be demonstrated under the conditions of the human GI tract (see Section 6.2 and Appendix D), no uptake of the nanomaterial is expected, but the resulting non-nano degradation products should undergo risk assessment according to relevant EFSA guidance on conventional non-nanomaterial. However, in case of complete digestion in GI fluids, local exposure (e.g. in the upper GI tract) needs to be considered.
13) Only nanomaterials that do not quickly degrade, e.g. by dissolution, under digestive tract conditions are considered for testing under **Step 1**, which involves gathering the available information as well as data from a set of *in vitro* studies. In particular, information on carcinogenic, mutagenic, reprotoxic (CMR) properties of the nanomaterial or its components is considered at Step 1. A degradation test under simulated lysosomal conditions is also carried out (see Section 6.2.2), along with a battery of relevant *in vitro* toxicity tests including genotoxicity, in consideration of the specific properties of nanomaterials. In this regard, the bacterial reverse mutation assay (Ames test) is not considered suitable for nanomaterials owing to the inability of bacterial cells that internalise particles. The use of mammalian cell models is considered more suitable, and a suitable battery of tests is described in this Guidance (Section 6.4) to address the critical genotoxicity endpoints.
14) If the information from Step 1 indicates that the nanomaterial is non-persistent and not (geno)toxic, an argument may be made to waive further nanospecific testing in Step 2, although safety assessment for conventional (non-nano)materials will still be needed. **Step 2** involves a modified 90-day oral toxicity test in rodents (OECD TG 408 (2017a) with extended parameters from OECD TG 407 (2008)) with a satellite group for the assessment of oral absorption and tissue distribution at different time points (see Section 6.7). This should also allow for the identification of nanomaterials with the potential to accumulate and/or cause immunological, proliferative and neurotoxic effects, and effects on reproductive organs or

endocrine-mediated effects. Positive results from these tests may warrant further in-depth investigations in Step 3. Under **Step 3**, toxicokinetic studies can be designed to investigate the extent of accumulation of the nanomaterial during long-term exposure and to determine any species differences in toxicokinetic behaviour between the test animals and humans. These studies permit refinement of the risk assessment by decreasing the uncertainty. Step 3 may also include specialised and in-depth testing for neurotoxicity, immunotoxicity or endocrine-mediated effects. In view of the potential long-term exposure from food, potential effects of nanomaterials on the gut microbiome should also be considered especially where a nanomaterial has antimicrobial effects.

15) **Risk characterisation** combines all the information from hazard identification and hazard characterisation with exposure assessment and any other relevant information, e.g. from read-across. As in the risk assessment paradigm for other chemicals, a weight of evidence approach (see Section 7) is used taking into account the available information that may comprise different types of data from different sources. In general, risk characterisation of a nanomaterial would consider the same elements as for conventional chemical substances – i.e. data and information relating to physicochemical properties, exposure and toxicological effects. Where the data have been derived from appropriately conducted studies using validated methods and considering nanospecific issues where relevant, there may be no reason to use uncertainty factors for a nanomaterial that are any higher than those used for a conventional material. However, where data are either insufficient or have been derived from inadequate tests (see Sections 6.9 and 7) for nanomaterials, applying additional uncertainty factors may be considered for safety assessment of a nanomaterial.

16) The Guidance describes how to carry out and present analysis of uncertainty (see Section 8) relating to physicochemical characterisation, exposure assessment, and hazard identification and characterisation for nanomaterials. The Guidance discusses specific aspects relating to nanomaterial applications for food/feed additives, pesticides, nano-carriers, novel foods, contaminants and FCMs. The Guidance also notes ongoing developments in areas relating to alternative testing approaches, mode of action and adverse outcome pathway approaches.

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1. Introduction

This Guidance builds upon the opinion of the Scientific Committee of 2009 'The Potential Risks Arising from Nanoscience and Nanotechnologies on Food and Feed Safety' (EFSA Scientific Committee, 2009a) and more specifically Section 6 (page 34) with the title 'Guidance for risk assessment (RA) of nanomaterial in food and feed area', as well as on the subsequent 'Guidance on the risk assessment of the application of nanoscience and nanotechnologies in the food and feed chain' (EFSA Scientific Committee, 2011a). The two above-mentioned documents provided an overview of how to perform a risk assessment of nanomaterial in the food and feed area. The risk assessment paradigm is appropriate for these applications, and consequently relevant data and information for the various steps (see below) should be made available to the risk assessor to carry out a risk assessment.

There are already several EFSA guidance documents that include the concept of the 'size' of substances, e.g. from the Panel on Additives and Products or Substances used in Animal Feed (FEEDAP Panel) ('Guidance for the preparation of dossiers for sensory additives', EFSA FEEDAP Panel, 2012a) and from the Panel on Food Contact Materials (FCM), Enzymes, Flavourings and Processing aids (CEF Panel) (EFSA CEF Panel, 2016a). For polymers used in FCM, for instance, the general rule is that smaller sized additives migrate faster and at higher rates than those of larger sizes. This is also valid for nanoparticles as migrants in polymer nano-composites according to recent publications (Šimon et al., 2008; Franz and Welle, 2017) on migration modelling of nanoparticles from food contact polymers.

As a general principle, the test requirements stipulated in current EFSA guidance documents for conventional materials[1] and EU legislation for various food and feed areas should be applied to a nanomaterial according to its intended use and should be followed. However, the risk assessment of nanomaterial, in terms of testing requirements and procedures, requires additional considerations that are indicated in this Guidance. This Guidance also covers the additional information needed for physicochemical characterisation owing to the specific characteristics and properties of nanomaterial. The specific information related to the characteristics and properties of the nanomaterial, along with the information stipulated in the relevant EFSA Guidance documents for the specific intended use of the nanomaterial (e.g. novel foods, FCMs, food/feed additives and pesticides), is used for a case-by-case risk assessment.

There are substantial ongoing developments in alternatives to *in vivo* testing approaches but validated *in vitro*/*in silico* methods for specific endpoints are still limited which necessitates information from *in vivo* testing 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 in the document 'Existing approaches incorporating replacement, reduction and refinement of animal testing: applicability in food and feed risk assessment' (EFSA Scientific Committee, 2009b). It is also recommended that use of any existing data generated for other relevant regulations (e.g. REACH) should also be made to minimise/avoid animal testing. This Guidance also identifies circumstances under which some data requirements for the risk assessment could be waived (e.g. when, before ingestion, a nanomaterial is degraded in the food/feed matrix into an approved non-nanomaterial).

1.1. Background as provided by EFSA

In 2011 the Scientific Committee (SC) of EFSA published its 'Guidance on the risk assessment of the application of nanoscience and nanotechnologies in the food and feed chain' (EFSA Scientific Committee, 2011a). The approaches described therein concern mainly human exposure via the oral route and are to be implemented by applicants and risk assessors. The EFSA Panels cover nanomaterials in their assessments by cross-referring to the 2011 SC Guidance. Some food contact materials (FCM) and food/feed additives that are currently under assessment by EFSA Panels include nanomaterial, but data generated specifically on the nanomaterial. Nanomaterials may be present in FCM or additives either because nanomaterials are intentionally used or because some of the material contains nanomaterial resulting from the production processes. Both situations, however, require consideration during risk assessment (of the material under evaluation).

In 2014, to prepare for future applications, EFSA procured an inventory of nanomaterials/applications on the market or reasonably foreseen to be placed on the market (Peters et al., 2014a). In the report, 55 types of nanomaterials for agri/feed/food were identified. This literature search also resulted in the

[1] Relevant regulatory framework, administrative and EFSA scientific guidance documents per regulated product area are listed in http://www.efsa.europa.eu/sites/default/files/applications/apdeskhow.pdf

highest number of records for nanoencapsulates, silver and titanium dioxide and showed that food additives and FCM are the most frequently indicated applications. Future developments are expected in the field of nanoencapsulates and nano-composites in applications such as novel foods, food/feed additives, biocides, FCM, and especially pesticides (Kah et al., 2013; Perlatti et al., 2013; Kah and Hofmann, 2014; Kookana et al., 2014; Cano Robles and Mendoza Cantú, 2017; Chaudhry et al., 2017).

As mentioned in the conclusions of the 2011 SC Guidance, it will require updating to stay aligned with innovations and fast developments in this area. This is in line with EFSA's strategy of revision of cross-cutting guidance documents (EFSA Scientific Committee, 2015), as well as with the scientific motivation and criteria to consider in updating EFSA scientific assessments document (EFSA Scientific Committee, 2017a).

There are also legal developments that warrant the updating of the 2011 SC Guidance. Novel Food Regulation (EU) No 2015/2283,[2] for example states that EFSA will have to verify that, where a novel food consists of engineered nanomaterials, the most up-to-date analytical methods will be/are used to assess their safety and that the scientific appropriateness of the methods used are substantiated by the applicants. Art. 12 Regulation (EC) No 1333/2008 stipulated that 'When a food additive is already included in a Community list and there is a significant change in its production methods or in the starting materials used, or there is a change in particle size, for example through nanotechnology, the food additive prepared by those new methods or materials shall be considered as a different additive and a new entry in the Community lists or a change in the specifications shall be required before it can be placed on the market'.

Scientific developments and experiences from EFSA activities in this field that warrant the updating of the 2011 SC Guidance, can be classified in four areas: (1) scope extension; (2) nanomaterial characterisation needs; (3) needs for food/feed assessment; and (4) needs for environmental risk assessment. All these considerations have to be taken on board when updating the existing 2011 SC Guidance and when developing a new environmental risk assessment guidance document for nanomaterials.

1.2. Terms of Reference as provided by EFSA

The EFSA SC is requested to update the previous guidance document on human and animal risk assessment when nanoscience and nanotechnology are applied in the food and feed chain. The present Guidance on nanomaterials deals with risk assessment for three main categories of products/ applications; (i) those that are intended for consumption by humans or animals (e.g. novel foods, food/feed additives); (ii) plant protection products and (iii) nanomaterials that are incorporated into products that come into contact with food (i.e. FCM and articles).

This update should also take into account the general extensions needed to cover novel foods, food contact materials, food/feed additives and pesticides as well as an update of the physicochemical measurements and the other data needed for food/feed assessment.

In support of this work,

- EFSA is asked to set up a Working Group (WG) covering the expertise needed for the concerned EFSA Panels: PPR, NDA, ANS, CEF, FEEDAP and CONTAM, and relevant EFSA Units (in particular the EFSA Pesticides Unit), complemented with external experts for specific aspects.
- It is also asked to host experts from relevant external institutions dealing with risk assessment of nanomaterials – such as ECHA, EEA, EMA, US-FDA, US-EPA, WHO, European Commission's non-food Scientific Committees, including liaison with the Scientific Committee on Occupational Exposure Limits (SCOEL) and DG ENV – or that develop standards in this area (such as JRC, OECD Working Party on Manufactured Nanomaterials;[3] EU FP7 research projects like NanoGenotox, NANoREG and NanoDefine; and institutes for metrology or standards development like ISO/CEN, NMIs). These experts could be invited to the SC WG as observer and this cooperation will enable the coherent linkage of all these institutions' activities into this mandate, therewith avoiding duplication of work and ensuring consistency of terminology and methodologies.

[2] Regulation (EU) No 2015/2283 of the European Parliament and of the Council of 25 November 2015 on novel foods, amending Regulation (EU) No 1169/2011 of the European Parliament and of the Council and repealing Regulation (EC) No 258/97 of the European Parliament and of the Council and Commission Regulation (EC) No 1852/2001. OJ L 327, 11.12.2015, p. 1–22. See http://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:32015R2283&from=EN

[3] OECD (Organization for economic cooperation and development), online. http://www.oecd.org/department/0,3355,en_2649_37015404_1_1_1_1_1,00.html

- EFSA is also requested to formalise the input and expertise from stakeholders through consultations, e.g. with hearing experts, an EFSA discussion group or the public consultation.

1.2.1. Interpretation of the Terms of Reference

Dermal and inhalation exposure were added to cover the main routes of exposure to nanopesticides and feed additives.

Environmental risk assessment will be addressed in a separate document (Part 2) as requested in the Terms of Reference provided by EFSA.

1.2.2. Definition of nanomaterial

The International Organization for Standardization (ISO) has defined nanomaterial as a material with any external dimension on the nanoscale ('nano-object') or having an internal or surface structure in the nanoscale ('nanostructured material') (ISO, 2015). In particular, a nano-object is defined as a discrete piece of material with one, two or three external dimensions on the nanoscale. 'Nanoparticles' are nano-objects with all external dimensions on the nanoscale, where the lengths of the longest and shortest axes do not differ significantly. If the dimensions differ significantly, typically by more than a factor of three, other terms, such as 'nanofibre' (two external dimensions in the nanoscale) or 'nanoplate' (one external dimension on the nanoscale) may be preferred to the term nanoparticle. In turn, a 'nanostructured material' is defined as a material having internal or surface nanostructure, i.e. a composition of interrelated constituent parts in which one or more of those parts is a nanoscale region. 'Nanoscale' is defined as ranging from approximately 1 to 100 nm (ISO, 2015).

According to the ISO nanotechnologies vocabulary, which can be freely consulted on www.iso.org/obp, 'engineered nano-object' is defined as a nano-object designed for a specific purpose or function, 'manufactured nano-object' as a nano-object intentionally produced to have selected properties or composition and 'incidental nano-object' as a nano-object generated as an unintentional by-product of a process (ISO, 2015).

Size is the key parameter for the identification of a nanomaterial. All nanomaterials occur with a size distribution, i.e. the constituting entities do not all have the same size. Often particulate materials comprise particles with lengths both below and above 100 nm. Owing to the reactivity of nanoparticles, mainly related to their high surface free energy, larger clusters ('secondary particles') often result from agglomeration and/or aggregation of constituting primary particles. In some cases, the size distribution of manufactured nanomaterials covers a rather wide length range.

The European Commission issued a Recommendation for a definition of a nanomaterial in 2011 to provide a common basis for regulatory purposes across most areas of EU policy (currently under review[4]). The provisions of the recommended definition include a requirement for review in the light of experience and of scientific and technological developments. The European Commission is expected to conclude this review in 2018. If this recommended definition (or any update of it) were to be embedded in the food law, it would provide further information on whether or not a material should be regarded as a nanomaterial in the context of any of the food regulations**.** According to that recommended definition, 'nanomaterial' means a natural, incidental or manufactured material containing particles in an unbound state or as an aggregate or as an agglomerate and where, for 50% or more of the particles in the number–size distribution, one or more external dimensions is in the size range 1–100 nm. In specific cases and where warranted by concerns for the environment, health, safety or competitiveness the number–size distribution threshold of 50% may be replaced by a threshold between 1 and 50%.

For the purposes of the recommended definition, 'particle' means a minute piece of matter with defined physical boundaries, 'agglomerate' means a collection of weakly bound particles or aggregates where the resulting external surface area is similar to the sum of the surface areas of the individual components, and 'aggregate' means a particle comprising of strongly bound or fused particles. In addition, it is specified that fullerenes, graphene flakes and single wall carbon nanotubes should be considered as nanomaterials even though one or more external dimensions are below 1 nm.

The current recommended definition is used as the reference for determining whether a material should be considered as a 'nanomaterial' for legislative and policy purposes in the EU. According to the recommended definition the criterion is solely the size of the constituent particles of the material,

[4] Commission Recommendation 2011/696/EU of 18 October 2011 on the definition of nanomaterial. OJ L 275, 20.10.2011, p. 38–40, under revision.

without regard to hazard, toxicokinetics or risk. Although this Recommendation is currently under review, and has not yet been adopted under the relevant regulatory frameworks, the SC advises taking this and any future reviews into consideration when assessing the safety of materials consisting of small particles.

From a risk assessment perspective, it is essential to point out that size-dependent properties and biological effects that are of potential concern for human health, specifically toxicokinetic behaviour and particle–cell interactions, are not rigidly related to specific size thresholds. They depend on dose and may continue to occur even when the particles constituting the nanomaterial have a size well above 100 nm. Furthermore, whereas physical, chemical and biological properties of materials may change with size, there is no scientific justification for a single size limit associated with these changes that can be applied to all nanomaterials (SCENIHR, 2010). Therefore, potential risks arising from specific properties related to the nanoscale have to be assessed focusing on such properties and potentially related hazards, which may be independent of the proportion of particles constituting the material with a size below 100 nm. In line with the conclusions of SCENIHR (2010)[5] and the EFSA Scientific Committee (2011a), the EFSA Scientific Committee reiterates that not all nanomaterials have new hazard properties compared with larger sized counterparts and that therefore a case-by-case assessment is necessary.

This Guidance emphasises size-related properties associated with specific hazards and the need to provide relevant nanospecific information in order to perform a risk assessment. In addition to the size of the material, a number of other properties that may be associated with adverse health effects such as chemical composition, morphology (in particular, aspect ratio), surface properties, crystallinity, solubility and others) should also be taken into account.[6] Particle properties that significantly alter levels/pathways of uptake and/or affect the mobility and persistence in the body are of high relevance. Increased bioavailability of nanomaterial compared to the corresponding conventional form related to particular surface properties resulting from, e.g. use of specific coatings and encapsulation, should be flagged for risk assessment according to the present Guidance.

- The European Commission recommended that a material with 50% or more of the particles in the number size distribution in the nanoscale (1–100 nm) should be regarded a nanomaterial.
- Although this recommendation is currently under review, and has not yet been adopted under the relevant regulatory frameworks, the Scientific Committee advises to take this and any future reviews into consideration when assessing safety of materials consisting of particles.

1.2.3. Definition of engineered nanomaterial

Engineered nanomaterials are a subset of the 'nanomaterial' that is defined in the European Commission's Recommendation of 2011. As outlined in the Novel Food Regulation (EU) No 2015/2283[7] and referring to Regulation (EU) No 1169/2011[8] on the Provision of Food Information to Consumers, Engineered nanomaterial means 'any intentionally produced material that has one or more dimensions of the order of 100 nm or less or that is composed of discrete functional parts, either internally or at the surface, many of which have one or more dimensions of the order of 100 nm or less, including structures, agglomerates or aggregates, which may have a size above the order of 100 nm but retain properties that are characteristic of the nanoscale. Properties that are characteristic of the nanoscale include:

[5] 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).

[6] Measurement of these and other relevant properties is also essential for an appropriate characterisation of the material (Table 1).

[7] Regulation (EU) 2015/2283 of the European Parliament and of the Council of 25 November 2015 on novel foods, amending Regulation (EU) No 1169/2011 of the European Parliament and of the Council and repealing Regulation (EC) No 258/97 of the European Parliament and of the Council and Commission Regulation (EC) No 1852/2001 (Text with EEA relevance). OJ L 327, 11.12.2015, p. 1–22.

[8] Regulation (EU) No 1169/2011 of the European Parliament and of the Council of 25 October 2011 on the provision of food information to consumers, amending Regulations (EC) No 1924/2006 and (EC) No 1925/2006 of the European Parliament and of the Council, and repealing Commission Directive 87/250/EEC, Council Directive 90/496/EEC, Commission Directive 1999/10/EC, Directive 2000/13/EC of the European Parliament and of the Council, Commission Directives 2002/67/EC and 2008/5/EC and Commission Regulation (EC) No 608/2004 OJ L 304, 22.11.2011, p. 18–63.

i) those related to the large specific surface area of the materials considered; and/or
ii) specific physicochemical properties that are different from those of the corresponding conventional material of the same chemical composition.'

According to the Novel Food regulation, 'For consistency and coherence purposes, it is important to ensure a single definition of engineered nanomaterial in the area of food law'.

The use nanomaterial as a pesticide and the use of the term 'nanopesticide' herein is explained under the sector specific information of Appendix E.2.

1.3. Scope of this Guidance and when to apply it

This Guidance is aimed at all interested parties and, in particular, applicants and risk assessors such as EFSA Units and Panels performing risk assessment for substances considered as nanomaterials and falling under the food law. This means that this Guidance is applicable (at least partially) to the following materials. The purpose of the scope Section 1.3 is to help applicants and risk assessors to decide if the safety testing of the product requires the consideration of this guidance.

- For **engineered nanomaterials that meet the criteria of the definition (see section 1.2.3)** according to the most recent revision of the Novel Food Regulation (EU 2015/2283) and Regulation (EU) No 1169/2011, i.e. nanomaterials that, amongst other criteria, have particle sizes in the defined nanoscale (1 nm to −100 nm). The SC considers that the application of this Guidance is unconditional for EFSA and for all parties submitting applications for the use of engineered nanomaterial under the food law.
- For **materials that contain particles having a size above 100 nm which** could retain properties that are characteristic of the nanoscale (see Section 3.1), for example related to the large specific surface area of the materials or different toxicokinetic behaviour (i.e. significant changes in absorption, distribution and/or metabolism) as compared to its non-nanomaterial. This may be the case for materials resulting from production processes that are aimed at reducing the average diameter of materials' particles (e.g. micronisation). The Scientific Committee considers that on a case-by-case risk assessment judgement this Guidance may be applicable to parties submitting their assessments of such materials.
- For **materials that are not engineered as nanomaterial but contain a fraction of particles that is less than 50% in the number–size distribution (as per the recommended European Commission definition, see Section 1.2.2), with one or more external dimensions in the size range 1–100 nm**.[9] This is expected to be the case of manufacturing processes for powdered or particulate food chemicals that typically result in materials with a range of sizes (see details in Section 4.2.2). Even where the median size of the particles is generally significantly greater than 100 nm, a small fraction (< 50%) is always expected to be present with at least one dimension below 100 nm. This Guidance is applicable to parties submitting their assessments of such materials. The Scientific Committee considers that the tests as described in this Guidance have to be performed with the representative material as used in the agri/food/feed chain and as present on the market. For food additives, for example, the material used for testing should be a 'food additive EXXX' as present on the market, and that is also in compliance with European Commission specifications. The applicants must ensure that the testing strategy is selected so that the data could be relevant for the risk assessment of the fraction in the nanoscale. This may include the application of this Guidance and the test strategies included herein.
- For **nanomaterials having the same elemental composition but that occur in different morphological shapes**, sizes, crystalline forms and/or surface properties as a consequence, for example, of different production processes, this Guidance is applicable to each variant as a stand-alone case. Therefore, applicants must undertake a separate physicochemical characterisation and specific risk assessment as described in this Guidance for each distinct nanomaterial having a given elemental composition. The Applicant may also explore whether a read-across approach can be applicable in these cases (see Section 6.3).
- **Nanoscale entities made of natural materials** that have been deliberately produced to have nano-enabled/enhanced[10] properties, or that have been modified for use in the

[9] Even though the material contains a low percentage of particles (below the 50% threshold), the actual conditions of use may result in a substantial exposure (depending on the content in the final product) that may be relevant to risk assessment.
[10] See ISO/TR 18401:2017.

development of other nanoscale materials, e.g. for encapsulating (bioactive) compounds (see Appendix E.6). These materials are within the scope of this guidance for risk assessment. Other 'natural' nanoscale entities may be present in food/feed (e.g. macromolecules, colloids, micelles, such as naturally occurring nanostructures in homogenised milk) and should not be considered in the scope of this Guidance.

- Although the European Commission recommendation on a definition for nanomaterial is currently under review, and has not yet been adopted under the relevant regulatory frameworks, the SC advises to take this and any future reviews into consideration when assessing safety of materials consisting of particles (see Section 1.2.2).

When a material under the scope of this guidance is being risk assessed, its **degradation products** in the form of a nanomaterial (e.g. the core material after degradation of the coating), also have to be considered at all the relevant steps, including the determination of characteristic of the nanoscale which may affect toxicity (Section 3.1) and exposure assessment (Section 5).

- This Guidance is applicable to materials that meet the criteria of the definition of engineered nanomaterial as outlined in Regulation (EU) No 2015/2283 and Regulation (EU) No 1169/2011. Other materials consisting of particles with size range above 100 nm should also be considered, if they could retain properties characteristic of the nanoscale, for example related to the large specific surface area of the materials or different toxicokinetic behaviour.
- For materials that are not engineered as a nanomaterial but contain a fraction of the particles that is less than 50% in the number size distribution with one or more external dimensions in the size range 1–100 nm, the applicants must ensure that the testing strategy is selected so that the data could be relevant to the risk assessment of the fraction in the nanoscale. This may include the application of this Guidance and the test strategies included herein.
- Nanoscale entities made of natural materials that have been deliberately produced to have nanoenabled properties, or that have been modified for use in the development of other nanoscale materials, e.g. for encapsulating (bioactive) compounds are within the scope of this guidance for risk assessment.

1.4. How to use this guidance?

The present guidance has taken a broader overview of the existing scientific knowledge relevant for risk assessment of nanomaterials in food and feed. Guidance, as far as possible based on the current state of knowledge, is summarised in dedicated boxes at the end of sections. However, whilst highlighting the issues that require attention, it is expected that often a degree of expert judgement will be needed and therefore a broader scientific background is also provided.

In general, the applicant is responsible for the best set-up for the tests and a description of the rationale thereof. Working with a multidisciplinary team is therefore advised. This is not different as for conventional chemicals.

In order to minimize animal testing, a tiered approach including *in vitro* tests, as well as directions for read-across, have been provided.

The present guidance is cross-sectorial, and sector specific information has been provided in Appendix E.

2. Data and methodologies

Primary references of particular relevance were identified by the EG members (up to 18 April 2018). Also considered were publicly available guidance documents and reports relevant to risk assessment of nanomaterial in agri/food/feed and produced by European Commission non-food Committees, international authorities such as the FDA, WHO and JRC, ECHA and EMA. A draft of this Guidance underwent a public consultation from 12 January to 4 March 2018. The comments received were considered and have been incorporated where appropriate.

For construction of this Guidance, a problem formulation approach was followed for nanomaterials. As a result, this Guidance highlights nanospecific issues only, and will be applied in conjunction to the existing EFSA Guidances for conventional materials. Other principles of EFSA's scientific assessments, such as weight of evidence (EFSA Scientific Committee, 2017b), uncertainty (EFSA Scientific Committee, 2018) and biological relevance (EFSA Scientific Committee, 2017c), have been followed

while developing this Guidance. Also, the principle of the benchmark dose approach (EFSA Scientific Committee, 2016) applies to nanomaterial risk assessments.

3. Risk assessment of nanomaterials: general outline

The risk of a nanomaterial is determined by its chemical composition, other physicochemical properties, its interactions with tissues, and potential exposure levels. The schematic general outline for risk assessment of nanomaterials is shown in Figure 1 and developed in each of the chapters cited in the figure.

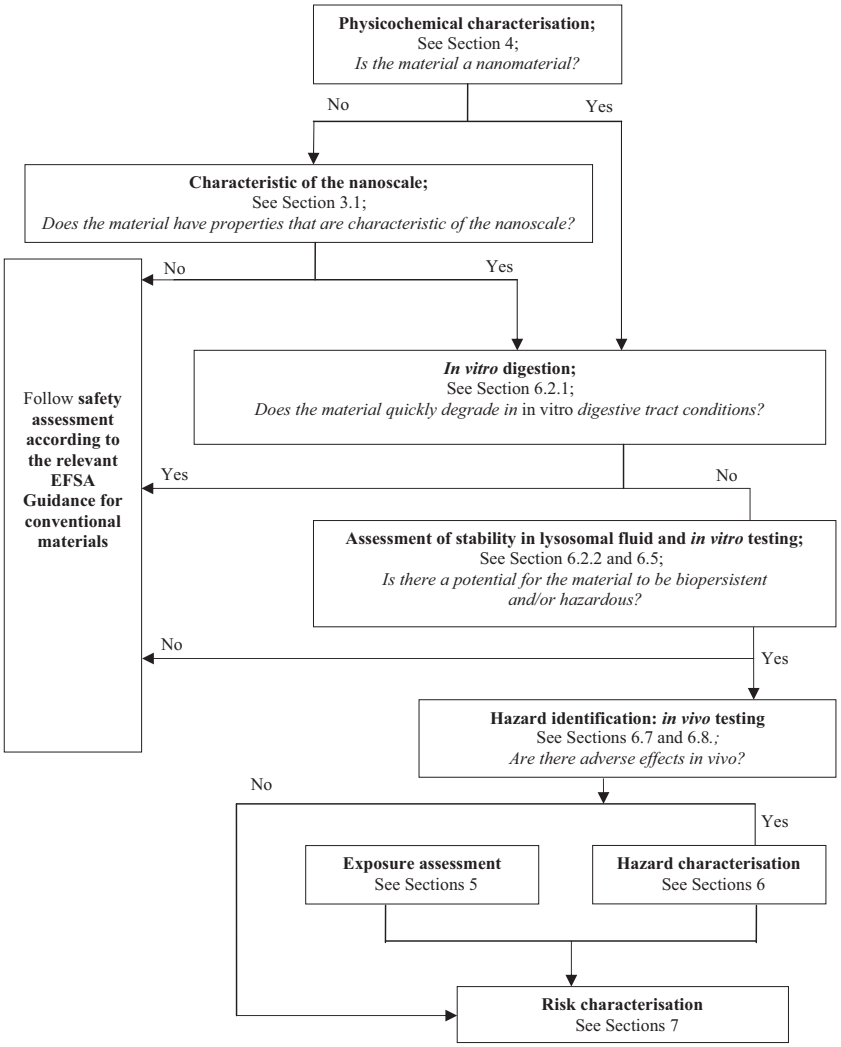


Figure 1: Schematic outline for risk assessment of ingested[11] nanomaterials for human and animal health, focussing on hazard characterisation. A complementing outline for the exposure part of the assessment is presented in Figure 2.

Physicochemical characterisation is needed to identify a material as a nanomaterial and decide whether this Guidance applies (see Section 1.3).

The results from testing of the nanomaterial will give information for hazard characterisation that, combined with the exposure assessment, will form the basis for the risk characterisation. A particular case is represented by nanomaterials incorporated in FCMs: in this case, if convincing scientific evidence and/or technically valid tests showing the absence of migration are provided, then further testing may not be needed because, in the absence of exposure, no risk to consumers can be expected.

[11] Other routes of exposure like dermal and inhalation are detailed in Appendix E for feed additives and nanopesticides.

The applicants have to follow the relevant guidance for conventional material and check for additional information requirements in this present Guidance when the evaluation concerns a nanomaterial.

There are some general aspects to consider at an initial stage before testing a nanomaterial (the problem formulation) that is proposed for use in the food/feed chain. If the available information indicates absorption and distribution of the nanomaterial leading to internal exposure, altered reactivity or biokinetics (compared with the non-nanomaterial), or persistence of the nanomaterial, these should be considered as a trigger for in-depth testing.

3.1. Characteristic of the nanoscale which may affect toxicity

As mentioned in Section 1.3, characteristics of the nanoscale which may affect the toxicity of the material, for example, relate to the large specific surface area of the materials or different toxicokinetic behaviour (i.e. significant changes in absorption, distribution and/or metabolism). Furthermore, the following non-exhaustive list of indicators of potential toxicity should be considered when deciding on an appropriate testing strategy:

1) specific morphology (e.g. rigid, long tubes or fibres, high aspect ratio nanomaterials, fullerenes, crystal structure, porosity), carrier materials with cores and shells of different biopersistence (e.g. multifunctional nanomaterials) (see e.g. Figure 1 and Section 6.2.2 for further information on biopersistence);
2) complex transformations (e.g. ageing, changes in surface properties, porosity) (see also Section 4.2.2) or metabolites or *de novo* formed particles from ionic species (see Section 6.2.1);
3) altered hydrophobicity/hydrophilicity;
4) persistence/high stability (e.g. in water, fat, or body fluids, lack of degradation/dissolution);
5) increased reactivity compared to equivalent non-nanomaterial (e.g. catalytic, chemical, biological);
6) targeted or controlled release by the nanomaterial;
7) nanomaterials having antimicrobial activity;
8) different or increased mobility of the nanomaterial *in vivo* compared to the conventional non-nanomaterial, i.e. possibility of increased bioavailability and internal exposure (e.g. transport via macrophages; transport through cell membranes, blood-brain barrier and/or placenta, delivery systems) and mobilisation potential (e.g. infiltration, sorption, complex formation);
9) interactions with biomolecules such as enzymes, DNA, receptors, potential 'Trojan horse'[12] effects (see Section 6.8.3 on immunotoxicity);
10) bioaccumulation;
11) quantum effects (e.g. altered optical, electronic, magnetic, mechanical or redox properties in nanoscale materials).

Dekkers et al. (2016) concluded that the aspects of toxicokinetics and human hazard assessment that are most likely to be influenced by the nanospecific properties of the material include: degradation/dissolution, accumulation, genotoxicity and immunotoxicity (see also draft WHO (2017) Principles and Methods to Assess the Risk of Immunotoxicity Associated with Exposure to Nanomaterials, http://www.who.int/ipcs/Immunonano/en).

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 degradations that could lead to its solubilisation, metabolic degradation/detoxification, or clearance from a biological system. The retention of a biopersistent nanomaterial or its degradation products in the form of a nanomaterial (e.g. the core material after degradation of the coating) in the body can lead to its bioaccumulation. Therefore, biopersistence and bioaccumulation of nanomaterials should be carefully considered.

The following should be considered as indicators of a potential for high external exposure:

1) high production volume for a nanomaterial for the field of application,
2) existence of several fields of application of the same material,

[12] Effects resulting from particle internalization with subsequent intracellular release of toxicants, either endogenous – e.g. constituent metal ions – or exogenous – e.g. contaminants adsorbed on particle surface.

3) high stability in products and/or persistence in the environment,
4) anticipated frequent/high volume use of the products containing the nanomaterial (see Section 5 on exposure).

Other indicators that are considered to reduce the likelihood of adverse effects of the nanomaterial, are based on specific exposure scenarios and/or on the loss of nanospecific properties. A complete loss of nanospecific properties will allow the use of data on corresponding conventional material forms in the sectorial risk assessment and the nanospecific risk assessment procedure would no longer be required.

The following parameters may indicate a loss of nanoproperties or a low exposure to nanoparticles:

1) high dissolution rate[13] (e.g. in water, food/feed matrix or body fluids as described in Section 6.2),
2) high rate of degradability (e.g. biological or photocatalytic) to non-nanosized degradation products,
3) the presence of/as aggregates rather than agglomerates (e.g. determined by conditions of production),
4) fixed, permanent bonding in matrices (e.g. stability of matrix, type of bond, end-of-life behaviour) or effective entrapment in FCMs (e.g. polymer nanocomposites).

Nanostructured modifications on surfaces, and nanostructures that do not release particles and are not reactive are generally not expected to cause adverse effects (e.g. nanopores or lotus effect structures that can be used in filters and processing equipment). In some instances, however, such applications could give rise to release of nanomaterial that should be considered (e.g. impact of functional failure[14]). In the case of particles entrapped in FCMs, mechanical release of particles by mechanical stress (bending or elongation occurring in use, surface abrasion) should be considered as well.

The considerations and concepts presented above are further developed in the following Sections. Characterisation and identification of nanomaterial are covered in Section 4. Exposure assessment is presented in Section 5. Hazard identification and hazard characterisation and toxicity testing strategies are covered in Section 6. Section 7 presents the risk characterisation. Uncertainty analysis is discussed in Section 8.

More sector specific information (e.g. for feed additives and for pesticides) is provided in Appendix E.

4. Physicochemical characterisation of nanomaterial

Clarifying the questions posed by the Terms of Reference and deciding whether they require risk assessment of applications of nanoscience and nanotechnology to the food and feed chains is part of the first stage of scientific assessment. This is often referred to as problem formulation, and is a step preceding the scientific assessment as a whole. Careful consideration will be needed early in the planning process (in problem formulation) to ensure an adequate characterisation of nanomaterial, which is essential for establishing its physicochemical identity both as a pure material and when in food and feed products. It is also essential to identify changes in the material during storage, as a result of possible interactions with the product matrix, and after ingestion. In addition, monitoring the behaviour of nanomaterial in terms of biodistribution, speciation and quantification is crucial for hazard assessment (i.e. through toxicological and toxicokinetic studies). The physicochemical characteristics of a nanomaterial are important as they can affect the outcome of the risk assessment (e.g. different sizes, shapes, crystal structure (phase) and surface properties of nanomaterials of the same chemical composition may show different toxicokinetic behaviours or toxicities). Nanosized particles of the same elemental composition may be present with different shapes, sizes, crystal structures (phases) and/or surface properties, for example as a consequence of a different production process. For each distinct nanomaterial, the applicant must undertake a separate physicochemical characterisation and risk assessment as described in this Guidance. It should also be noted that nanomaterials require specific attention with view to the representativeness of sampling and proper dispersion state (SCENIHR, 2007).

As an essential requirement, all dossiers related to nanomaterials as described in Section 1.3 have to be accompanied by thorough information on the particle size distribution and other parameters as

[13] It should nevertheless be recognized that materials can generate, while dissolving, potentially more toxic smaller particles or ions or molecules.
[14] Functional failure is a topic of good manufacturing practice and must be technically avoided.

described in Table 1 (in Section 4.2.1) of the material obtained through validated methods based on suitable analytical techniques as detailed in the present Guidance (see Appendix C).

The physicochemical characterisation of the material under investigation is relevant to the:

1) decision as to whether the material has to be considered for nanospecific risk assessment under this Guidance (see Section 4.1);
2) full determination of the physical and chemical identity of the pristine material (see Section 4.2);
3) physicochemical characterisation of the material in test media used in toxicokinetic and toxicological studies, which is needed before, during and after the studies (see Section 4.3);
4) physicochemical characterisation of the material in complex matrices e.g. product formulations, which is needed for exposure assessment (see Section 4.3).

4.1. Framework for distinguishing nanomaterials and non-nanomaterials

The first step is to consider whether a material falls under the scope of this Guidance according to Section 1.3. Therefore, it is helpful to determine if a material meets the criteria of the European Commission recommendation for a definition (under review) of nanomaterial. In many situations, this information can be deduced from the existing data from the production process and accompanying material characterisation. In other situations, it is necessary to measure the determining physical properties, **i.e. size and, where applicable, other nanospecific properties (e.g. surface area)** to decide whether a material falls within the scope of this Guidance. In such cases, it is essential to select appropriate techniques in relation to the specific material under investigation.

The EU project NanoDefine (www.nanodefine.eu[15]) has developed guidance for the selection of appropriate techniques and interpretation of results. Respective publications, technical reports and protocols are available from http://www.nanodefine.eu/index.php/nanodefine-publications. The project addresses the recommended European Commission nanomaterial definition, i.e. size and size distribution as well as volume specific surface area, where applicable. It provides a decision-flow scheme and is supported by an e-tool and methods manual. (see http://www.nanodefine.eu/index.php/nanodefiner-e-tool). The decision as to whether the material has to be considered for nanospecific risk assessment under this Guidance can be supported by using this flow scheme.

The flow scheme is based on a tiered approach. Tier 1 is based on **screening methods**, namely for determination of volume specific surface area (VSSA, by the Brunauer Emmett Teller (BET) method as described by Kreyling et al., 2010;.) for dry powders and of equivalent particle size with light scattering- and particle mobility-based methods (e.g. dynamic light scattering (DLS), centrifugal liquid sedimentation (CLS)) for dispersions. For VSSA criteria, further details are described in Wohlleben et al. (2017). Tier 2 relies on **more sophisticated particle size analysis methods**, e.g. electron microscopy. A schematic overview of the decision tree as developed by NanoDefine is provided in Appendix A. It should be noted that in an initial step the already available material information (e.g. surface area and density) is considered that may be applicable and result in a decision without further testing.

For materials with a median particle size above 100 nm, a second criterion, namely properties characteristic of the nanoscale, may be relevant for risk assessment. Based on the provided information, these materials should be assessed on a case-by-case basis as described in this Guidance. However, size remains an essential criterion and the NanoDefine decision tree and especially its implementation in the NanoDefiner e-tool therefore remain helpful for the selection of appropriate characterisation techniques expected to be used under this Guidance.

Any specific properties or effects of a nanomaterial are intrinsically linked to the stability of its nanoscale features. Where a nanomaterial loses these, e.g. due to degradation by dissolution, it will not be expected to behave any differently from its corresponding non-nanomaterial. For this reason, safety concerns over orally ingested nanomaterials are related mainly to those that are able to survive the digestive system, potentially resulting in (nano)particles being translocated to other parts of the body (see *in vitro* digestion Section 6.2.1) or exert local adverse effects in the gastrointestinal tract.

Where a material is regarded as within the scope of this Guidance, a detailed physicochemical characterisation is required, as described in the following Sections.

[15] Consortium of 28 Partners form European research institutes and universities, metrology institutes, nanomaterial producers and instrument manufacturers.

- The decision-flow scheme developed by NanoDefine project may be used to determine whether or not a material is nanomaterial according to the recommended European Commission definition, and to identify relevant methods and tools for characterisation.
- Where a material is regarded within the scope of this Guidance, detailed physicochemical characterisation must be provided as an essential element of safety assessment.

4.2. Pristine material characterisation

4.2.1. Parameters

The characterisation of the material under investigation is essential to unambiguously define its identity. Similar to conventional chemicals (e.g. food additives), names, identifiers and a number of physicochemical parameters need to be measured. In addition, a broader range of parameters needs to be addressed for nanomaterials, relating on the one hand to material identity, and to properties that may be of biological/toxicological relevance on the other.

Owing to the current gaps in knowledge relating to properties, behaviour and effects of nanomaterials, it is difficult to identify a definitive shortlist of those parameters that can adequately describe a nanomaterial in terms of both physicochemical and toxicological aspects. Different international expert committees and working groups have considered certain parameters important for safety assessment of nanomaterials. These are presented as a list of parameters to be reported in Table 1. This list is not definitive, however, and has to be changed in future to include more, less or different parameters that might be added with the advancement of scientific insights as well as legislative developments.

The parameters in Table 1 have been derived from the reports published by the Scientific Committee on Emerging and Newly Identified Health Risks (SCENIHR, 2009); the OECD Working Party on Manufactured Nanomaterials in its exploratory project on 'Safety testing of a representative set of nanomaterials' and the revised version of its 'Guidance manual for the testing of manufactured nanomaterials' (OECD WPMN, 2009a, 2010); the International Organization for Standardization; the EU's Scientific Committee on Consumer Safety (SCCS, 2012); the ProSafe[16] project (European Union H2020 project ProSafe, 2015–2017); the ECHA Guidance on the preparation of registration dossiers that cover nanoforms (ECHA, 2017b); the ECHA Appendix R.6-1 for nanomaterials applicable to the Guidance on QSARs and Grouping of Chemicals (ECHA, 2016); and a recent publication by DeLoid et al. (2017a).

In some instances**, not all of the parameters listed in Table 1 (and Table B.1 in Appendix B) may be relevant for a given material** as determined by its composition, function, purpose and/or intended use. In such cases, justification should be provided for the characteristics that are not determined or provided, or to explain why they were not deemed applicable to a particular nanomaterial.

Currently, no generally accepted systematic nomenclature exists for nanomaterials. ISO TC 229 has drafted a series on vocabulary and terminology of NMs (ISO 80004 series). The CODATA-VAMAS Working Group on the Description of Nanomaterials has published a 'Uniform Description System for Materials on the Nanoscale' (CODATA-VAMAS Working Group on the Description of Nanomaterials, 2016) that proposes in detail the information that should be supplied to describe a nanomaterial in the best possible and most unambiguous way. The SC suggests that applicants follow the schemes proposed by ISO and the CODATA-VAMAS Working Group when naming a nanomaterial.

In some instances, however, the material may be too complex to define precisely in terms of chemical composition and stoichiometry. Examples could be complex iron oxide hydroxides or polymers. Other examples include materials already authorised for use in FCMs such as a 'butadiene, ethyl acrylate, methyl methacrylate, styrene copolymer (either not crosslinked or crosslinked with divinylbenzene or 1,3-butanediol dimethacrylate) in nanosized particles, (FCM substance Nos 998, 859 and 1043)'. In simpler materials (e.g. metal oxides), the stoichiometry in the surface layer may also differ from the core of the particle. In these cases, the material should be described as exactly as

[16] The EU funded ProSafe project supported the aims of EU Member States in their EU and international efforts (OECD; http://www.oecd.org/science/nanosafety/, and EU-US CORs; http://us-eu.org/communities-of-research/) regarding risk assessment, management and governance focussing on regulatory oriented toxicology testing of nanomaterials, exposure monitoring, life cycle assessment, and disposal and treatment of waste nanomaterials.

possible. In any instance, the elemental composition must be given (e.g. the empirical formula) and additional information on the starting material, the reaction process(es) and the intended composition **should be** provided.

For nanomaterials consisting of multicomponent particles, the overall material should be described together with the individual components. In the case of a nanomaterial consisting of a **mixture** of different types of particles, each component should be described individually according to Table 1, and the ratio of all components in the mixture should be provided. The structure of the particles should also be described as exactly as possible. This includes information on the distribution of individual components in the particle, e.g. homogeneous mixture, core/shell and coatings.[17] **Coating** is a thin layer of a component that covers completely or partially the surface of a particle and is strongly bound (either chemically or physically) to the surface. Stabilisers (or dispersants) are substances that are added to a dispersion of nanomaterial to prevent agglomeration, aggregation or sedimentation. They are not seen as a part of the particle and should be reported under 'formulation'. Substances strongly bound to the particle surface for stabilisation purposes should be reported under 'Surface (chemical) composition' or as coating (when covering the entire particle).

Changes in manufacturing process(es) can not only lead to significant differences in the physicochemical and morphological characteristics of nanomaterials between different batches, but may also introduce new/different impurities and residual materials. Furthermore, for some materials, fundamentally different production processes are in place (e.g. for pyrogenic vs. precipitated silica as described in Fruijtier-Pölloth, 2012; sulfate or chloride process for converting titanium ores into TiO_2) that largely define the surface and crystallographic structure, and thus the particle properties. It is therefore important to provide a description of the manufacturing process.

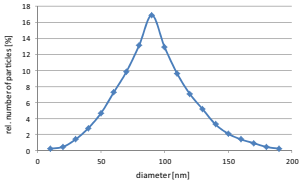
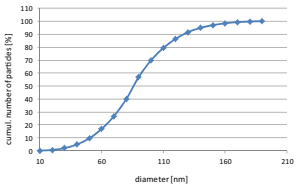
Table 1 is also meant to be applicable for multicomponent materials (e.g. core-shell or coated particles). Table 1 is therefore structured into a Section for general and ensemble information on the overall material and a Section on detailed chemical and physical information for its individual components. In the case of a monocomponent particle (e.g. uncoated TiO_2), information has to be provided for component 1 only. Examples for component 2 information are details in Table 1B of Appendix B. Some of the general parameters of Table 1 (and Table B.1 in Appendix B) might already be required under the sectorial legislations. Appendix C provides a list of corresponding techniques for each parameter.

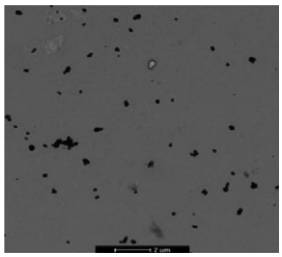
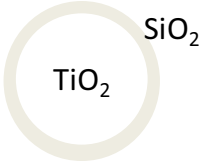
Table 1: Descriptors and parameters on what data are to be provided for characterisation of pristine material, together with hypothetical examples (not food related, not consistent, data for illustrative purposes only). For clarity, the Table is divided in the different Sections: Table 1A for Information on the overall material, Table 1B for Information on the chemical components, and Table 1C for extrinsic properties

Table 1A: Information on the overall material

Item Parameters (incl. specification ranges)	Explanation	Example
Name	The name used in the submitted application. This could for example be the name of the nanomaterial	Ti-Max
Description Short description of the material	Provide a brief description of the material	Nano grade titania coated with a protective silica layer
Intended use	Describe the foreseen use and function of the material	UV protection to be incorporated in food contact materials

[17] For the purpose of this Guidance, a material is considered as a 'coating' where it is bound or adhered to the surface of a nanomaterial in the form a continuous outside layer, or a 'shell' where it is in the form of a nanosized covering/casing in which a (nano)material may be contained.

Item Parameters (incl. specification ranges)	Explanation	Example
Material composition and purity Relative amounts of components and impurities (in mass %)	Relative amount of the constituents in mass %, as well as chemical identity of any impurities and their relative amounts in mass % should be provided	TiO_2 97.1% ± 0.3%[(a)] SiO_2 2.8% ± 0.1% purity: 99.9% impurities: Fe_2O_3 0.1% ± 0.02% Specifications composition: TiO_2 97.0% ± 0.5% SiO_2 3.0% ± 0.2% Purity ≥ 99.7%
Elemental composition Empirical formula of the complete material or relative amounts of element (in mass %)	The relative elemental composition of the particle should be provided as the simplest positive integer ratio of atoms present in the material. Alternatively, the relative mass amounts of the contained elements may be provided	$Ti_{26}SiO_{54}$ Ti 58.23% (m/m) O 40.32% (m/m) Si 1.31% (m/m)
Particle size **Agglomeration or aggregation state** Mean and median diameter [nm] graphical diagrams of size distribution	Data on primary and secondary (agglomerates and aggregates) particle size, number-based size distribution and mass-based size distribution of the material should be provided as measured by more than one independent technique, one being electron microscopy (EM) if the measurement is feasible (cfr. current publications on critical issues of EM, e.g. drying, artefacts). If EM cannot be applied, the use of a different imaging technique is suggested. Information on the used characterisation techniques and methods (e.g. which techniques, instruments, settings, SOPs, method performance characteristics, data conversion) should be provided. Data should be provided both as median particle diameter (x_{50} in nm), with an indication of the width of the distribution (e.g. standard deviation, in nm) and with an estimate of the uncertainty of the median diameter (± expanded uncertainty, confidence level 95%, in nm). Together with the size distribution information on the lower and upper cut-off limits for the calculation of the relative amount of particles has to be provided. Data obtained with a particle counting technique (such as EM) should be provided as number-based size distributions. Data obtained with other techniques (such as centrifugal liquid sedimentation (CLS) or dynamic light scattering (DLS)) should be provided in the original metrics as produced by the technique (e.g. intensity, volume- or mass-based). In these cases, a conversion to the number-based size distribution must also be provided, including information on the algorithms used for conversion and the associated uncertainty.	Primary particles: TEM data: median diameter x_{50} = 85 nm (uncertainty = 5 nm, 95% confidence level), width of distribution: SD = 15 nm mean diameter: 89 nm (uncertainty = 6 nm, 95% confidence level), width of distribution: SD = 13 nm   CLS data: median diameter x_{50} = 97 nm (uncertainty = 19 nm, 95% confidence level), width of distribution: SD = 8 nm) mass-based arithmetic mean diameter: 105 nm (uncertainty = 10 nm, 95% confidence level), width of distribution: SD = 13 nm)

Item Parameters (incl. specification ranges)	Explanation	Example
	Most light scattering based techniques (incl. CLS and DLS) provide light intensity-based distributions, which can be converted into their equivalent volume- or mass-based distributions using Mie light scattering theory. As this step can introduce considerable errors on the results due to the unknown refractive index of nanoparticles, the parameters used in the conversion must be reported in detail. Reporting of the original light intensity-based results can help to assess the reliability of the results. For each material, at least two graphs showing particle size distributions shall be shown: one with the relative number versus size (continuous graph or histogram) and one with number-weighted sum function (cumulative numbers)	[plus diagrams as above] Aggregates: [provide size data in the same manner as for primary particles, see above] Specifications size: median diameter 85 nm ± 5 nm
Particle shape Description of the shape, porosity, aspect ratio, EM image of the nanomaterial	Information should be provided on the particle shape, aspect ratio and whether or not the material is porous. This should also include appropriate EM images to support the description. For powders, information on porosity can be obtained from gas adsorption measurements	Irregular particles, aspect ratio 1 to 3, non-porous 
Structure Description of the structure, including (relative) thickness of structural elements	Spatial distribution of the components (e.g. homogeneous mixture, core–shell, surface coating) should be provided. A graphical sketch for non-homogeneous particles should be provided to demonstrate the schematic distribution if applicable. The sketch should reflect schematically the shape of the particles. Information should be provided on any surface coatings or shells in terms of coating or shell material and the proportion of the coating or shell material in relation to the mass of the nanomaterial	TiO_2 particles with a surface coating of silica. Thickness of the coating 1.8 nm (± 16% (g/g)) 
Surface chemical composition Description of the composition of the groups or coatings on the particle surface	Information on chemical characteristics of the particle surface, e.g. the components bound to the surface, the presence of functional groups (e.g. carboxy, amino, hydroxy). Information should also be provided on any surface contamination	Hydrophilic acidic silica surface, free –OH groups
Production process Name of the production process of the material	The production process used to prepare the entire nanomaterial (i.e. not of the individual components in cases of multicomponent particles) should be described as it can have a significant effect on the properties of the nanomaterial	SiO_2 precipitation on dispersion of wet-chemically synthesised TiO_2 particles
Surface area MSSA [m^2/g] VSSA [m^2/cm^3]	Where appropriate (for powder materials) data on mass and volume specific surface area of the material should be provided The conditions under which the measurements took place have to be reported	MSSA 15 m^2/g (via BET according to ISO 9277) VSSA 65 m^2/cm^3 (via BET according to ISO 9277 and assuming a density value of 4.1 g/cm^3)

Item Parameters (incl. specification ranges)	Explanation	Example
Surface charge Zeta potential [mV]	Zeta potential values along with the conditions under which measurements were made *(e.g. pH, ionic strength)* should be provided	Zeta potential: −26 mV (deionised water, pH 8), Isoelectrical point: pH 2.2, method: electrophoretic light scattering according to ISO 13099-2
Appearance Description	Describe the appearance, e.g. 'white powder'	White powder
Melting point m.p. [°C]	Provide the melting point of the nanomaterial	1,840°C
Boiling point b.p. [°C]	Provide the boiling point of the nanomaterial	2,900°C
Density Density [kg/m^3]	Information on the density (specify type of density, e.g. bulk, pour) of nanomaterial should be provided	Bulk density: 4.1 g/cm^3
Porosity fraction of the volume of voids over the total volume [%]	Information on the porosity of nanomaterial should be provided	Non-porous
Dustiness	Provide the dustiness for powder material (e.g. EN15051)	According to DIN EN 15051 B: W_r: 280 mg/kg W_i: 13,200 mg/kg
pH	The pH value of a dispersion of the nanomaterial should be provided along with description of the conditions under which the measurement was carried out	pH 5.8, 10 g/L, 20°C
Formulation Formulation medium Dispersing agents (stabilisers) Auxiliaries Concentration of nanomaterial in dispersion	Description should be provided to indicate the form in which a nanomaterial is present in a formulation, e.g. powder, dispersion. Information should also indicate other material(s) with which the nanomaterial may have been mixed/ formulated. This should include information on any dispersants/stabilisers and other auxiliaries (e.g. preservatives, processing aids, etc.) used. The concentration of the nanomaterial in the mixture should be provided, in terms of both mass (g/kg) and particle number (n/kg), as well as the mass of the material as present in its ionic form	Dry powder

(a): The measurement uncertainty should be reported as detailed in Section 4.4.2.

Table 1B: Information on the chemical components[(a)]

Item Parameters (incl. specification ranges)	Explanation	Example
Component 1		
Chemical name Systematic/IUPAC name; chemical name	Where available systematic/IUPAC name of the substance that makes up component 1 of the nanomaterial should be provided. Alternatively, the chemical name that describes the chemical composition of the component should be provided based on the best available information – e.g.'modified from XX' where XX = the nearest chemical name	Titanium dioxide Titanium (IV) oxide

Item Parameters (incl. specification ranges)	Explanation	Example
Trade name, common name, other names, synonyms **Names**	Any common names, synonyms, trade names and other names for the component should be provided	Titania
CAS number **EINECS/EC number** **E number** **other registry numbers** Registry numbers related to the constituent substance, if available	CAS number, EINECS/EC number, E number or other registry/database numbers related to the component should be provided (where available)	CAS number: 13463-67-7, 1317-80-2 (Rutile) ECHA Info card: 100.033.327 EINECS/EC number: 236-675-5 E number: E 171
Formula Molecular and structural formula (where applicable) of the constituent substance	Molecular and structural formula (where applicable) of the constituent substance should be provided	TiO_2
Relative molecular mass (molecular weight) for molecules or relative atomic mass (atomic weight) for elements [g/mol]	Molecular weight or atomic weight (for elements) (g/mol) should be provided for the component	79.866 g/mol
Elemental composition Empirical formula of this component	The relative elemental composition of the component should be provided as the simplest positive integer ratio of atoms present in the material	TiO_2
Crystal form Form and phase	Description of crystalline form (amorphous, polycrystalline, crystalline including specification of phase) should be provided, including any crystalline impurities	Crystalline, rutile phase
Purity of the component Relative amount of the constituent in mass %; and name(s) and amount(s) of any impurities in mass %	Relative amount of the constituent in mass %, as well as chemical identity of any impurities and their relative amounts in mass % should be provided	Purity 99,9% Impurities: Fe_2O_3 0,1%
Production process component Name of the production process	The production process of the component should be described as it can have a significant effect on the properties of the nanomaterial, e.g. pyrogenic or precipitated silica, sulfate or chloride process for TiO_2.	Sulfate process
Component 2		
In case of multicomponent particles: Component 2, 3, etc.	In case of multicomponent nanomaterial the same information as for component 1 should be provided for all other components individually	The full data sheet for the example including information on component 2 (SiO_2) can be found in Appendix B

(a): A material may consist of different chemical components and each component should be addressed in the physicochemical characterisation.

Table 1C: Extrinsic (more media dependent) properties of the material as it is used on the market

Item Parameters (incl. specification ranges)	Explanation	Example
Stability Stability of the nanomaterial	Provide information on the physical and chemical stability of the nanomaterial and coatings (if applicable). Conditions under which stability is tested need to be reported and justified	Report on relevant stability studies
Solubility (see glossary) Solubility (proportion of solute in solvent at room temperature) [g/L] **Degradation rate** [g/(L*h)]	Data on solubility of the nanomaterial in relevant media along with description of the media and the conditions under which the measurements were made should be provided. Note that solubility should not be confused with dispersibility of poorly soluble nanomaterials. Data on degradation rate and the conditions under which the measurements were made should be provided for slowly dissolving nanomaterials	Poorly soluble in water
Dispersibility	For poorly soluble dispersible nanomaterials, information should be provided on dispersibility in terms of a relative amount of the particles that can be dispersed in a suspending medium. The information should include stability of the dispersion in the given medium and the conditions applied (e.g. ionic strength and pH)	Best dispersibility in water at pH 8.2, max. 50 g/L, stability of dispersion of the particles (DLS) at least 48 h
Reactivity where applicable –Chemical reactivity –Catalytic activity (incl. photo-)	Information should be given on chemical reactivity of the nanomaterial as provided (including any surface coating). Information on catalytic (including photocatalytic) activity, and reactive radical formation potential of the materials should also be provided	Report on relevant reactivity studies

4.2.2. Specifications and representativeness of the test material

In view of the potential significant differences in the physicochemical characteristics of nominally the same nanomaterial **resulting from variations in the manufacturing process**, or from being produced by different manufacturers, or by ageing effects (e.g. agglomeration/aggregation, sedimentation) a detailed and comprehensive **proposed specification** for the pristine (as produced) nanomaterial intended to be used in food/feed should be provided by the applicant. The proposed specification should provide the acceptable range for each physicochemical parameter in view of **the batch-to-batch variation and ageing effects**. This information will be used by the risk assessor to decide whether or not the batch(es) used in the toxicity texting could be considered **representative for risk assessment of the use in food/feed**. More specific guidance on the number of batches and batch-to-batch variation is provided in the relevant guidance for conventional materials (e.g. EFSA ANS Panel, 2012). No more specific guidance on ageing (nor on homo/hetero agglomeration/aggregation) can be provided as little is known about complex transformation and the analytical tools are to be developed.

As mentioned in Section 1.3, this Guidance also applies to materials that are not engineered as nanomaterial but contain a fraction of particles, less than 50% in the number–size distribution, with one or more external dimensions in the size range 1–100 nm. This is expected to be the case of manufacturing processes for powdered or particulate food chemicals that typically result in materials with a range of sizes. Even where the median size of the particles is generally significantly greater than 100 nm, a small fraction is always expected to be present with at least one dimension below 100 nm. For re-evaluations of authorised materials (i.e. food and feed additives), the test requirements stipulated in current EFSA guidance documents and European Commission guidelines for the intended use in the food/feed area apply in principle to food chemicals containing a fraction of particles with at least one dimension below 100 nm and adequately conducted toxicity tests should detect hazards associated with such food chemicals, including their nanoparticulate fraction. In such cases of re-evaluation, however, it is also essential that thorough information on the size distribution of the material is provided and the assessment should consider whether the material as a whole retains

properties that require risk assessment according to this Guidance. For example, EU specifications for TiO_2 (E 171) should include a characterisation of particle size distribution present in TiO_2 (E 171) used as a food additive. The measuring methodology applied should comply with this Guidance.

4.2.3. Techniques and methods

Care should be taken in the selection of characterisation techniques, the evaluation of results and their documentation. It is known that the results obtained from different particle size measurement techniques may differ because they address slightly different physical parameters, e.g. hydrodynamic diameter vs. geometric diameter (Domingos et al., 2009). In other words, particle size analysis methods produce method-defined or procedurally defined size values. As a result, the best suited technique depends on the physical and chemical properties of the nanomaterial, as well as on the intended use of the size values. Moreover, particle size distribution data are usually reduced to an average value. There are differences in the types of averaging between methods, which can amplify the already existing differences between methods.

In a comparison of most currently available techniques, Babick et al. (2016) demonstrated that significant differences were observed in the results for a number of industrial materials. The observed differences were mostly related to the technique-specific way of size determination since all other influences on the result (e.g. data handling, sample preparation, differences in test materials) were minimised by the study design. As an example, the use of some analytical methods, such DLS, may not be optimal for measuring nanomaterials that have a low refractive index (such as nanosilica, polymer encapsulates, etc.), because that can impact the intensity with which the light is scattered. Therefore, although nanoparticles of less refractive materials would be detected by DLS, the limit of detection (LOD) in terms of particle size would be high. This means that size measurements of such materials by DLS will likely be skewed towards measuring larger sized particles, and particles and agglomerates/aggregates in the lower range of the nanoscale might not be measured accurately. For these reasons, it is required that the size parameter should always be measured by at least two independent techniques, one being electron microscopy. If electron microscopy is not applicable (e.g. for some organic nanomaterials), it is recommended to use another imaging technique instead of electron microscopy.

Keeping the existing recommended definition of nanomaterial in view, it should be straightforward to regard a particulate material as nanomaterial when it has been intentionally produced to have the particle size distribution in the nanoscale (1–100 nm). However, it may not be easy to decide the nanomaterial nature of a material if it has not been produced as such as a nanomaterial but contains a fraction of the particles in the nanoscale. A typical example of this can be a micronised material produced to have particle sizes in the micrometer range, but also contains a fraction of the particles in the nanoscale. In the absence of an upper size cut-off threshold for particles to be included in the determination of 50% in numbers in the current recommended definition, it is difficult to decide whether or not the whole material should be regarded a nanomaterial. This is where other (supporting) criteria, such the use of VSSA, or confirmatory analytical tests, have been proposed in the decision scheme developed by NanoDefine project. The scheme provides a structured way for deciding whether or not a material should be regarded as nanomaterial, and under what conditions a suitable analytical technique may be needed to confirm or exclude it as a nanomaterial. In all borderline cases, the use of imaging techniques based on electron microscopy has been recommended.

Several techniques are available for determination of the various parameters listed in Table 1. In many instances, there is more than one suitable technique available, each with advantages and disadvantages for specific materials and size ranges. In the literature, there are reviews assessing the suitability of different techniques for a range of nanomaterials (Bowen, 2002; Hassellöv et al., 2008; Domingos et al., 2009; Linsinger et al., 2012; Peters et al., 2014b; Babick et al., 2016). An overview of techniques commonly used for the characterisation of nanomaterial is given in Appendix C. **The selection of the appropriate technique** is the responsibility of the assessor/applicant in charge and depends on the parameter and the chemical nature of the material. For the size-related parameters, a technique selection support is provided by the NanoDefine e-tool and method manual (available from http://www.nanodefine.eu/index.php/downloads/nanodefine-technical-reports).

Sampling and sample preparation are often crucial steps in the overall analytical process. They usually contribute the largest uncertainty to the result. A critical issue in the sample preparation of nanomaterial is the proper dispersion of particles. This issue is addressed in detail in Section 4.3.2. General guidance for sampling also applies to the characterisation of nanomaterial. Special attention

has to be paid to sampling, e.g. minimum sample size because of the particulate nature of the analytes (Ersbøll et al., 2010)) and possible segregation and stratification effects (Brüning, 2017).

- Detailed characterisation must be provided for each nanomaterial in pristine form (as manufactured), including unambiguous description of the material's identity and relevant physicochemical properties as described in Table 1. Justification must be provided for the characteristics that are not determined or provided, or deemed not applicable to a particular nanomaterial.
- The data must be relevant to the core nanomaterial and, where applicable, other substance (s) that may have been used for surface modification/coating.
- The techniques used for characterisation must be appropriate for the type of nanomaterial (examples provided in Table 1B).
- Particle size parameters must always be measured by at least two independent methods (one being electron microscopy). Other parameters should also be preferably measured by more than one method. Special attention should be paid to protocols used for sampling, sample preparation and dispersion of particles.
- A description of the manufacturing process must be provided along with data to indicate any batch-to-batch variations, and/or due to material ageing. In cases of a significant variation, specifications should be provided for the acceptable range for each parameter.

4.3. Characterisation and quantification in matrix

Although detection and characterisation of a nanomaterial prior to use in food/feed and FCM applications (i.e. pristine material) may be relatively straightforward, it is more challenging in biological tissues and food products because of the presence of complex matrices, and the usually low concentrations of the nanomaterial. In particular, biological matrices as well as food and feed also contain a wide range of natural structures – including some in the nanoscale – that makes it difficult to separate, detect, and identify nanomaterial in these matrices.

The characterisation of nanomaterial in a matrix is relevant for various aspects of risk assessment, including:

- hazard identification and characterisation (*in vitro, in vivo, in silico* and absorption, distribution, metabolism and excretion (ADME) studies); the relevant matrices may be: water, feed, *in vitro* testing media, biological tissues and fluids;
- exposure assessment (quantification of nanomaterial in food/feed, migration from FCM); the relevant matrices may be: feed, food, food supplements, FCMs, food simulants.

Furthermore, the detection and quantification of nanomaterial in food, feed and FCM may be necessary when enforcement measures are introduced, e.g. to monitor maximum permitted levels.

Some guidance on the detection and identification of nano-objects in complex matrices is given by CEN TC 352 (2018).

4.3.1. Characterisation in agri/food/feed products

It is currently difficult to distinguish an intentionally added nanomaterial from background levels of the same materials/substances in nanosized or non-nanosized particle form that may be present in agri/food/feed products, especially when they are present at low levels. Appropriate methods (e.g. stable isotope analysis, elemental fingerprinting) can be applied to distinguish the intentionally added nanomaterial from background levels of the same or similar materials of geogenic, biogenic or anthropogenic origin.

When characterisation of nanomaterial in food/feed matrices is difficult owing to the current limited availability of analytical methods, possible food/feed matrix interactions of the nanomaterial 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 nanomaterial properties in a real food. With method development and availability, such characterisation of nanomaterial can be expected to shift from food simulants to real food/feed matrices.

- Nanomaterials must be characterised in relevant food/feed matrix.
- In cases of technical limitations in the analysis of nanomaterials in food/feed matrices, characterisation and study of matrix interactions may be carried out using food simulants.

4.3.2. Characterisation in test media for *in vitro* and *in vivo* testing and in biological matrices

For the toxicological assessment of nanomaterial, it is essential to know in which form the nanomaterial is presented to the test systems. In addition, characterisation of nanomaterial in the test system is relevant to determining the effect of the test medium/formulation (and its constituents) on the characteristics and properties of the nanomaterial so that the validity of the toxicity test outcome may be determined and to allow comparison with the nanomaterial in the food/feed matrix to which exposure takes place.

For *in vitro* testing as well as for administration of nanomaterial in *in vivo* studies it is essential that the nanomaterial is properly dispersed in the medium. Dispersion protocols have been developed and published for a number of nanomaterial and purposes (Bihari et al., 2008; Jacobsen et al., 2010; Jensen et al., 2011; Taurozzi et al., 2012a–e; Hartmann et al., 2015; Mast and De Temmerman, 2016; OECD TG318 (OECD, 2017b)). More dispersion protocols are available via the websites of international organisations (e.g. OECD – http://www.oecd.org/science/nanosafety/; European Commission-JRC – https://ec.europa.eu/jrc/en/scientific-tool/jrc-nanomaterials-repository; US-FDA – https://www.fda.gov/scienceresearch/specialtopics/nanotechnology/default.htm) and of their respective research projects (e.g. NanoGenoTox – http://www.nanogenotox.eu; Nanopartikel – http://www.nanopartikel.info; NanoDefine – http://www.nanodefine.eu/; NANoREG – www.nanoreg.eu).

In the absence of standardised dispersion protocols, the dispersion efficiency of the applied protocol and the stability of the dispersion should be tested and documented. Apart from their tendency to agglomerate and aggregate (which should be addressed by a proper dispersion protocol and use of dispersants that are compatible with the biological test system), nanomaterial may adhere to the wall of glass ware, tubing, pipette tips, vials etc. see Section 6.9.2). Appropriate analytical techniques depend on the type of nanomaterial and medium.

For *in vitro* studies, the nanomaterials may have to be characterised in the exposure medium at the start and end of the experiment to confirm actual presence in the test system and to observe potential changes that the materials may undergo (Section 6.9.1). Characterisation in these cases should include the number-based particle size distribution and concentration. Owing to the possible presence of other particulate materials in the test medium (e.g. proteins) it is mandatory to use a chemically specific method (e.g. single particle inductively coupled plasma mass spectrometry (spICP-MS) or transmission electron microscopy–energy-dispersive X-ray spectroscopy (TEM–EDX)) for these measurements. Non-specific methods such as DLS or CLS are not suited unless it can be demonstrated that the material under investigation is the only (nano)particulate material in the test medium.

ADME studies (as described in Section 6.3) require the measurement of nanomaterial in body fluids, tissues and excreta. It is not only relevant to quantify the amount of nanomaterial present, but also to specify in which form the nanomaterial is present in these compartments. This includes chemical composition, size and shape, but may also refer to surface modifications and other parameters relevant to the nanomaterial properties. Since many methods for nanomaterial analysis in biological matrices are rather complex and laborious, a tiered approach can be considered for specific cases. For inorganic nanomaterial that contains elements with very low background levels in the matrix the samples can first **be screened** by non-nanospecific methods for the total content of the respective elements, by e.g. inductively coupled plasma mass spectrometry (ICP-MS), atomic emission spectrometry (AES), atomic absorption spectroscopy (AAS), X-ray fluorescence (XRF). Only positive samples have to be measured with a nanospecific method, e.g. spICP-MS, scanning electron microscopy–energy-dispersive X-ray spectroscopy (SEM–EDX), which may reduce the effort considerably. Recent studies have shown the potential of some nanomaterials to accumulate very specifically, resulting in high concentrations in specific cell types (Sadauskas et al., 2007; Powell et al., 2010; Loeschner et al., 2011; Landsiedel et al., 2012; Kermanizadeh et al., 2015b). Homogenisation of the entire compartment (e.g. liver) may lead to a dilution of the particles below the detection limit. In these cases, mapping techniques can be applied, e.g. time-of-flight secondary ion mass spectrometry (ToF-SIMS), laser ablation inductively coupled mass spectrometry (LA-ICP-MS), chemical force microscopy (CFM), hyperspectral imaging, etc.

- Nanomaterials must be characterised in relevant biological matrices and the test media used in *in vitro* and *in vivo* testing.
- The data must indicate the form in which a nanomaterial is presented to the test system; proper dispersion of the nanomaterial in the medium; and any change in the nanomaterial characteristics due to the test medium/formulation. This should include chemical composition, size and shape, but may also refer to surface modifications and other parameters relevant to the nanomaterial properties.
- Special attention should be paid to sample preparation and selection of characterisation techniques for nanomaterials in body fluids, tissues and excreta, and when measuring very low levels (e.g. migrating nanomaterials from FCMs).

4.3.3. Solubility and degradation/dissolution rate

Information on solubility and degradation rate of the pristine material is requested as described in Table 1, Section 4.2.1. In this guidance, degradation is considered a general term for the disintegration of a nanomaterial, e.g. due to dissolution, enzymatic or chemical degradation. In addition, the degradation rate in conditions representative of the human gastrointestinal tract and lysosomal fluid is considered key information in the present nanospecific Guidance because this is where nanomaterials generally distribute to and where degradation can occur due to the acidic conditions and presence of enzymes (see Figure 2 and more details in Sections 6.2.1 and 6.2.2). It is therefore important to understand the fundamental differences between solubility and degradation/dissolution rate.

Solubility is the proportion of solute in solvent under equilibrium conditions (i.e. in a saturated state, see also glossary). It is important to note the difference between dissolution (materials are solubilised into their individual ionic or molecular species) and dispersion (colloidal suspension of particles). Solubility is determined as the concentration of the dissolved material in a saturated solution (i.e. undissolved material present as solid phase). The solubility is dependent on external parameters such as solvent, temperature, pressure and pH. Care has to be taken when the concentration of the dissolved species in the liquid phase is measured to distinguish between dissolved species and dispersed particles. A separation of those species may be achieved by suitable filtration or centrifugation techniques. Limitations for very small particles and particles of a density similar to the solvent have to be taken into account. Protocols and guidelines for the determination of the solubility of nanomaterials have been proposed (Tantra et al., 2016).

High solubility is commonly understood if more than 1 mol/L solvent is dissolved.

The degradation/dissolution rate refers to the kinetics of dissolution. Nanomaterials may degrade/dissolve faster than their bulk counterparts because of their high surface-to-volume ratio. The dissolution rate is influenced by various factors, including solvent, temperature, pH, concentration, and presence of substances interacting with the particle's surface. It can be determined by kinetic measurements such as time- dependent concentration changes (of either the nanoparticles or the dissolved species) or changes in the particle size distribution (to smaller sizes). Dissolution is addressed in detail in Section 6.2 (*In vitro* degradation tests).

4.3.4. Characterisation and quantification of nanomaterial in FCM and after transfer from FCM

Various applications of nanomaterial for use in FCM are described in published literature. In some applications, the nanomaterial is applied into surface layers (e.g. in coatings); in others they are embedded in the full FCM matrix (composites) or incorporated in active materials. Nanomaterials when incorporated into an FCM matrix may structurally differ from the pristine nanomaterial. For instance, mineral clays may exfoliate in the polymer matrix under the processing conditions. Therefore, in addition to the characterisation of the nanomaterial used for manufacture of a FCM, the need arises for characterisation of the nanomaterial when present in the FCM (on the surface, in the matrix) and possibly when migrating from the FCM.

Nanomaterials on the surface or in the host polymer matrix can be characterised by their size and shape (see Table 1), typically by using microscopy techniques (SEM, TEM). Other applied techniques for this characterisation include Fourier transform infrared spectroscopy (FTIR) and X-ray diffraction (XRD).

To assess the exposure of the consumer to a nanomaterial from FCM, it is essential to determine the potential migration of the nanomaterial from the FCM into the food matrix. This can be achieved

by direct measurements on the nanomaterial in the food matrix, or in the food simulant used in migration testing, or by migration modelling of the nanomaterial in the polymer matrix (Duncan and Pillai, 2014; Noonan et al., 2014; Franz and Welle, 2017; Stormer et al., 2017).

Additionally, consideration should be given to potential release of the nanomaterial from the FCM through mechanical stress or physical disintegration of a FCM polymer matrix. This can be achieved by abrasion testing applying appropriate FCM material stress conditions (such as bending, stretching, thermal stress) and suitable food simulants (able to disperse the nanomaterial) or abrasives (solids generating friction with the FCM surface as used in scratch or tribological tests). It should be noted that abrasion is not covered by 'conventional' migration testing or modelling and therefore case specific testing is recommended. The migration patterns of nanomaterials from biodegradable polymer nanocomposites (e.g. polylactic acid (PLA)) may be different from those in conventional (plastic) polymers when official EU food simulants such as 95% ethanol are used. In these cases, the aggressiveness of the food simulant towards the polymer may affect its integrity and the polymer chain-size distribution, and cause physical release of the nanomaterial into the food simulant. In such cases, migration modelling does not apply and testing may need to be performed. Migration/abrasion testing is also needed when nanomaterials have been applied in a coating on an FCM surface (Golja et al., 2017).

To determine the amount of nanomaterial in food simulants after migration, it is possible to use a tiered approach and first apply a total-elemental-analysis method in conjunction with migration modelling estimation (taking into account the concentrations, sizes and shapes of the nanomaterials in the FCM). In this case, an appropriately sensitive detection technique (e.g. ICP-MS) should be selected to minimise the possibility of missing (very) low levels of particles. If the test results and the models estimates indicate the possibility of migration/release of the nanomaterial, more nanospecific technique (s) should be employed to ascertain whether the migrating entities are in nanoparticle or in a solubilised (non-nanomaterial) form.

Further information specific to the evaluation of substances used to manufacture FCM are available in the EFSA CEF Panel opinion on 'Recent developments in the risk assessment of chemicals in food and their potential impact on the safety assessment of substances used in food contact materials' (EFSA CEF Panel, 2016a). In general, it is recommended to check and consider the most recent version of the EFSA Guidance(s) specific to FCM.

4.4. Quality assurance

4.4.1. Standardised methods

Preference should be given to standardised methods where available and applicable. While a number of standard methods are available for particulate materials in pure solid state (e.g. powders), there are hardly any standard methods available for the characterisation of nanomaterial in complex matrices. Appendix C provides an overview of currently available standard methods at the time of issuing this Guidance. It is recommended to search the ISO and CEN databases[18] for the most up-to-date and appropriate methods.

In cases where no standard methods are available, the applicant is responsible for providing methods for the physicochemical characterisation and quantification of the nanomaterial for which approval is sought that are appropriate both for the pristine state and in matrices. The respective methods have to have standard operation procedures (SOPs) as well as validation reports that are provided with the dossier.

4.4.2. Method validation and performance criteria

As in other analytical fields, it has to be demonstrated that the methods used for the characterisation of nanomaterials in their pristine form (as manufactured) and in commercial formulations, food/feed matrices and in toxicity test systems are fit for purpose and deliver reliable results. The ideal methods will have gone through proper validation (both intra- and inter-laboratory) following existing international guidelines (e.g. IUPAC, 2002); Commission Decision 2002/657/EC[19]), with adaptation if necessary. The use of any validation protocols differing from internationally agreed protocols would need justifying. The

[18] These are available from: https://www.iso.org/obp/ui/#home; https://www.iso.org/technical-committees.html; https://standards.cen.eu/dyn/www/f?p=CENWEB:105::RESET::::

[19] Commission Decision 2002/657/EC of 12 August 2002 implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results (Text with EEA relevance) (notified under document number C(2002) 3044). OJ L 221, 17.8.2002, p. 8–36.

validation would also include determination of the method performance parameters, such as specificity; selectivity; robustness/ruggedness; recovery/trueness; repeatability, and reproducibility; detection/quantification limits for size, number and mass concentration; and measurement uncertainties. Assay robustness for a nanomaterial could be established using similar principles for assessing assay robustness for a non-nanomaterial. It would be worthwhile to include appropriate non-nanoscale controls for the tested nanomaterials. Methods addressing the determination of particle number-weighted size distribution or external dimension of the constituent particles in the nanoscale and beyond should be assessed against general performance requirements as developed by NanoDefine (Rauscher and Mech, 2018). Guidance for the validation of methods for the detection and quantification of engineered nanoparticles in food has been published (Linsinger et al., 2013) and is also applicable to other matrices. The validation report documenting the results on these parameters should be part of the characterisation report. The performance characteristics (including detection limit) should be within reasonable limits that reflect the current state of the art and should be provided in a justification with references to similar techniques in this area. It has to be shown that the performance meets the requirement, e.g. in terms of sensitivity (detection limits) and precision.

4.4.3. Reference materials

Reference materials are essential for controlling and comparing the performance of analytical methods used for nanomaterial characterisation and in their validation. Only a few certified reference materials are currently available, however, for which certification usually covers only one property (e.g. particle size, surface area). The Federal Institute for Materials Research and Testing (BAM) online, www.nanorefmat.bam.de/en/ has inventories of the currently available nanomaterial reference materials.

More reference materials are currently under development and can be expected to become available over time. In addition to the certified reference materials, the European Commission JRC has recently made available a repository of industrial nanomaterials to be used as representative test materials for safety testing (https://ec.europa.eu/jrc/en/scientific-tool/jrc-nanomaterials-repository). These nanomaterials were used for testing by several EU-funded projects (e.g. MARINA,[20] NANoREG[21]) as well as the OECD WPMN and can be used as test materials by any research laboratories to generate comparable toxicological results (Totaro et al., 2016). In the absence of certified reference materials, self-generated and properly characterised and documented test materials may also be used. The ISO has issued a technical specification for the preparation of reference nanomaterials that should be taken into account for these cases (ISO, 2013).

- The methods used for physicochemical characterisation must be appropriate for the type of nanomaterial.
- Standardised methods should be used where available. Other fit-for-purpose methods may be used with provision of supporting documentation for validation and standard operation procedures.
- Method performance parameters must meet the requirements, e.g. in terms of sensitivity (detection limits) and precision.
- Certified reference materials should be used to control and compare the performance of the analytical method used. In the absence of certified reference materials, self-generated and properly characterised and documented test materials may also be used.

5. Oral Exposure Assessment

Anticipated uses, use levels and potential oral exposure to the nanomaterial should be outlined as demonstrated in Figure 2 and the paragraphs below. Some types of application could lead to other routes of exposure, such as dermal or inhalation. Examples of these include the use as feed additives or as pesticide. The nano specific aspects (including all relevant routes of exposure) that have to be considered in risk assessment of these types of application have been detailed in the Appendix E.

[20] FP7 MARINA project developed reference methods for managing the risk of engineered nanoparticles and engineered nanomaterials. MARINA was a project of a consortium of 47 national institutions of Member States and industries association.

[21] FP7 NANoREG project was a project with over 89 partners from the EU, Brazil and the Republic of Korea in which scientists, industry and policy makers collaborated.

When **direct exposure** of humans or animals is possible, such as from novel foods, food/feed additives, use of nanomaterial as pesticide (see Appendix E.2) it should be assessed if the nanomaterial or its dissolution/degradation products in the form of a nanomaterial remain present as particles in the food/feed matrix. If no nanomaterial remains present in food/feed, there is no exposure to nanomaterial and risk assessment should follow relevant EFSA guidance for conventional materials. If yes, it should be assessed if the nanomaterial or nanosized degradation products remain present as particles under the *in vitro* simulated conditions of the gastrointestinal tract. Figure 2 illustrates the steps of subsequent exposure assessment.

If there is no data on the quantification of the nanomaterial in the food/feed matrix (Section 4.3.3) or on the degradation under the simulated conditions of the gastrointestinal tract (Section 6.2), it has to be assumed that the exposure is to the nanomaterial **initially added** to the food/feed. It should therefore be assumed that **all** added nanomaterial is present, ingested and absorbed as the nanosized particle. This represents the worst-case scenario.

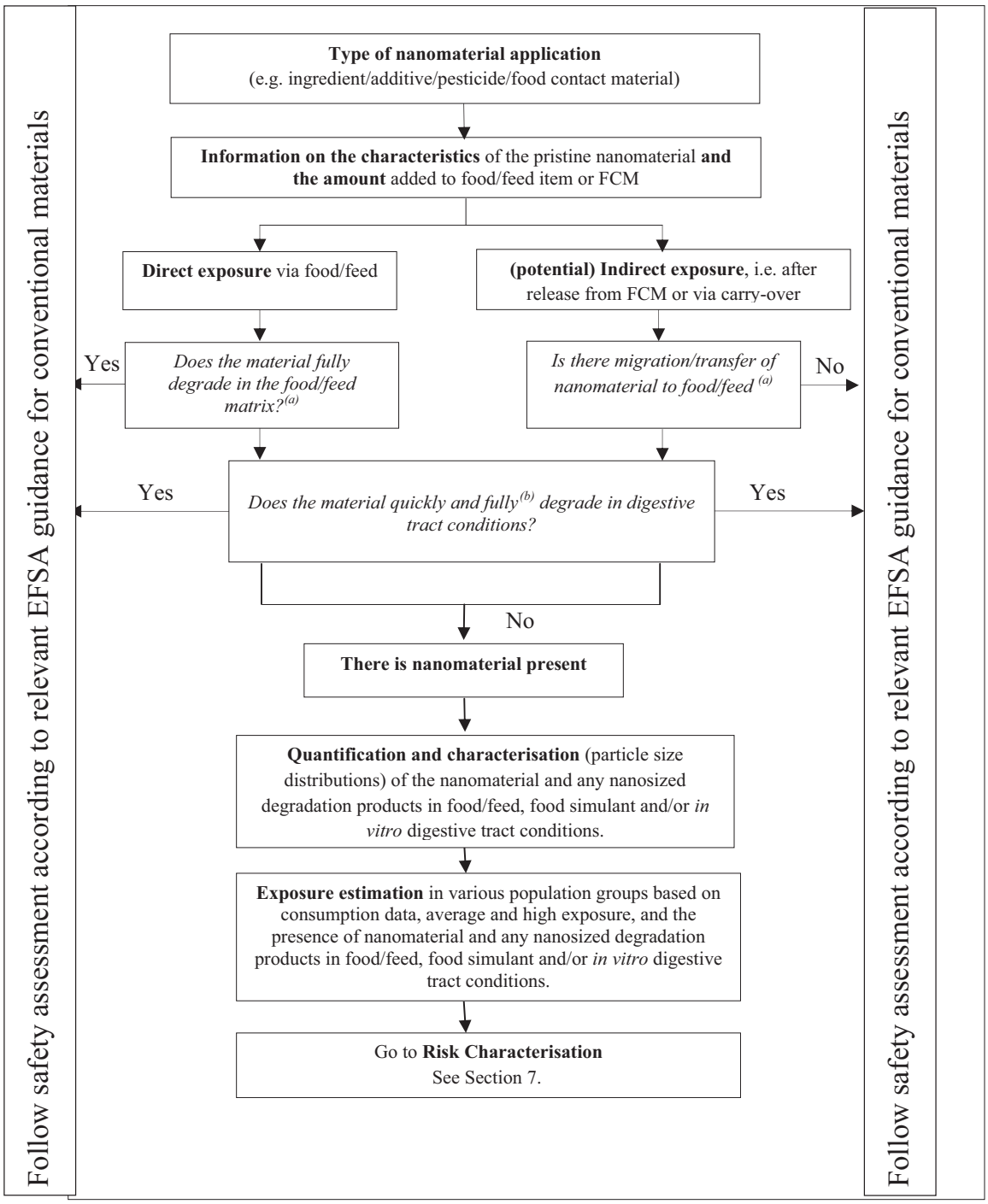
Indirect exposure includes migration or transfer from FCM and transfer by carry-over of the nanomaterial from feed, via animals to food from a pesticide to a crop or as a contaminant. It should be assessed if indirect exposure occurs via particles or solutes (ions, molecules). For FCM, this means that the elution towards food/feed or food simulant[22] should be considered. The same considerations as to whether particulate or non-particulate species are transferred need to be taken into account in the case of carry-over from feed, via animals to food, from a pesticide to crop or as contaminant. For FCMs, the extent of transfer should be measured by an appropriate technique with detection limits according to the state of the art (see Section 4.3.3), and in consideration of the particles in the relevant size distribution.[23] Occurrence of nanomaterials as contaminants may be what happens in cases of nanomaterials being persistent in the environment (see Section 3). When any transfer of nanomaterial into food/feed/food simulants can occur, the principles of this Guidance apply. Unlike non-nanomaterial (chemical) migrants from FCMs (Brüschweiler, 2014; EFSA and WHO, 2016), an acceptable threshold for the migration of nanomaterials from FCM has not yet been established owing to the paucity of data (from producers) that would be necessary to establish safe limits. In the context of this Guidance, evidence indicating no release of nanomaterial (or release exclusively as non-nanomaterial), should be sufficient to waive further nanospecific testing of food/feed products. It is equally important to note that, irrespective of the presence of a nanomaterial or nanosized degradation products in the FCM, the release of molecules/ions should be assessed in accordance with the relevant EFSA guidance for conventional FCM.

When exposure to the nanomaterial or its degradation products in the form of a nanomaterial can occur, the dietary intake should be estimated. The principles of exposure assessment of nanomaterials (via food and feed) will be the same as in exposure assessment of non-nanomaterials (Kroes et al., 2002; EFSA Scientific Committee, 2006, 2009a). Thus, guidances apply that provide specific information on the determination of consumer exposure. General issues like food/feed sampling, variability within composite samples and variation in concentrations between samples are not different from the exposure assessment for the micro/macroscale or for non-nanomaterials, and need to be addressed in the risk assessment.

The anticipated average and high exposures to nanomaterial food/feed for various population groups must be estimated based on the available consumption data. Probabilistic methods may be useful to determine ranges of plausible values rather than point estimates. If possible, particular subgroups of the population with an expected high exposure – through anticipated frequent use of the same type of food item, for example – 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 of the nanomaterial could provide additional information for the exposure assessment. Any assumptions made in the exposure assessment should be described. The exposure estimates should take into consideration the findings of the presence of nanomaterial in food/feed, food simulant and/or *in vitro* digestive tract conditions.

[22] See Table 1 of consolidated Commission Regulation (EU) No 10/2011 on plastic materials and articles intended to come into contact with food: http://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:32016R1416&from=EN

[23] Examples of nanomaterials evaluated positively on the basis of absence of a significant migration in particulate form include carbon black, titanium nitride (EFSA CEF Panel, 2012a), zinc oxide (EFSA CEF Panel, 2015a, 2016b), nanoclays (EFSA CEF Panel, 2012b, 2015b), silanated silicon dioxide (EFSA CEF Panel, 2014a), nanosized polymeric substances (EFSA CEF Panel, 2014b).



(a): If it cannot be measured whether a nanomaterial is present in e.g. food/feed matrix, food simulant or simulated digestive tract, it should be assumed it is. See Section 6.2.1 and Appendix D for details on *in vitro* gastrointestinal digestion.
(b): The assessment of degradation products that are still in the form of a nanomaterial should continue as presented in this Guidance.

Figure 2: Steps in oral exposure assessment. The arrows going out (left and right) indicate that nanospecific considerations are not needed, and risk assessment for the non-nanomaterials can follow the standard approach (i.e. the relevant EFSA Guidances for conventional materials)

- Exposure assessment should take account of the anticipated uses in line with the type of nanomaterial application (Figure 3). Particular sections of the population with an expected high exposure should be identified. Any assumptions used in the exposure assessment should be clearly described.
- A primary consideration for exposure assessment should be the presence of nanomaterial (or nanosized degradation products) in food/feed, food simulant and/or *in vitro* digestive tract conditions. Where a nanomaterial or nanosized degradation products are no longer present, risk assessment should be carried out according to the relevant EFSA guidance for conventional materials.
- For assessment of indirect exposure (e.g. migration from FCM; transfer via carry-over from animal feed or from a pesticide to crop), one should determine whether the exposure is to (nano)particles or solutes (ions, molecules).
- Unlike conventional (non-nanomaterial) migrants from FCMs, the scientific knowledge is considered to be too limited to propose a threshold for the migration of nanomaterials. An argument for safety may be made on a case-by-case basis if migration of a nanomaterial in particulate form is only in trace amounts.
- Where it is not possible to determine the nanosized particles in complex matrices, it should be assumed as a worst-case that all nanomaterial added to a food/feed product is present as the nanomaterial and is ingested and absorbed.

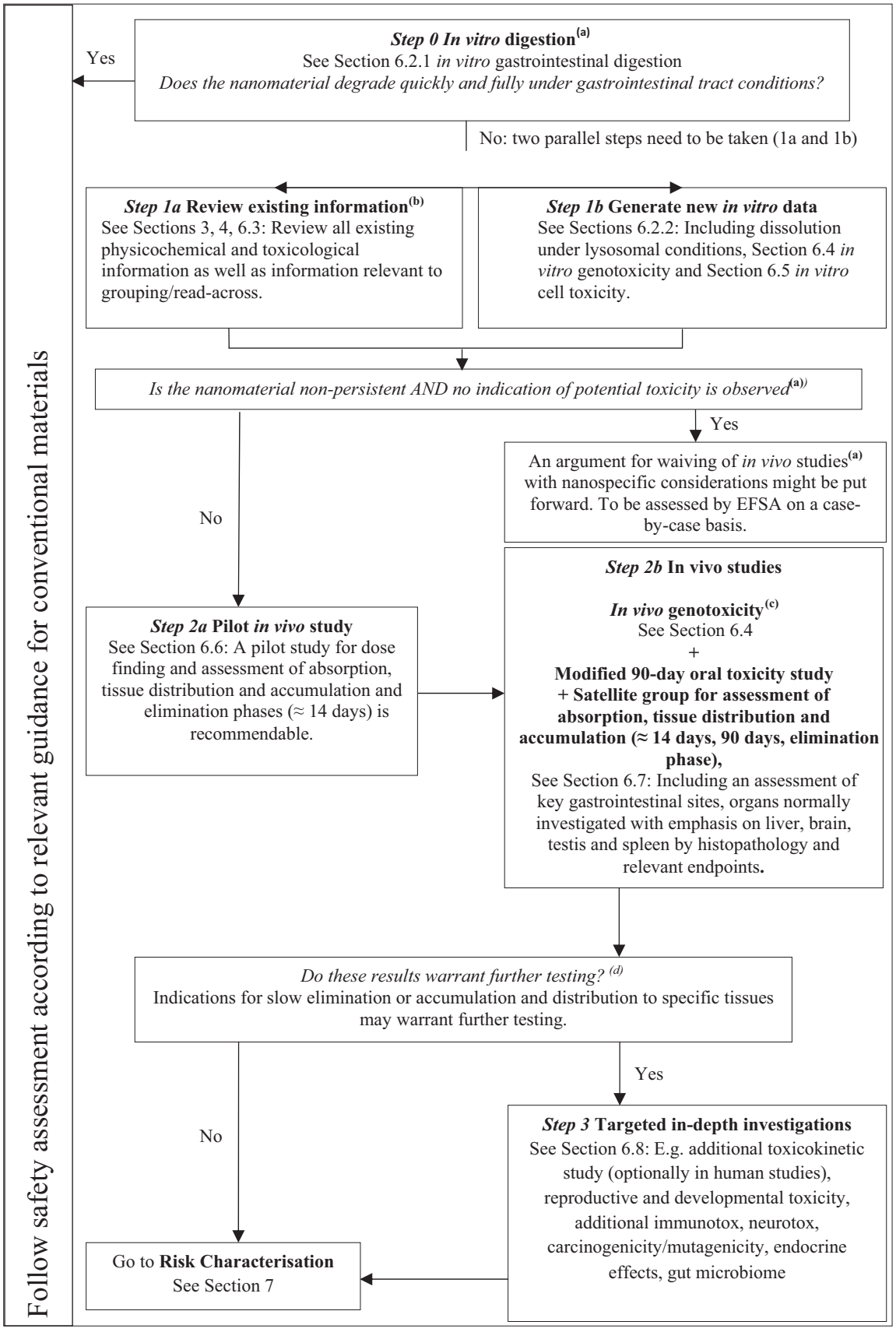
6. Hazard identification and hazard characterisation

The test requirements stipulated in current EFSA guidance documents and European Commission guidelines for different intended food/feed uses also apply in principle to nanomaterials. This Section outlines additional hazard identification and characterisation aspects to be considered that may arise because of the specific characteristics and properties of the nanomaterial or any degradation product in the form of a nanomaterial. Appropriate *in vitro* and *in vivo* studies on the nanomaterial should be undertaken to identify hazards and obtain dose-response data to characterise these hazards.

Limited data are available on toxicological hazards of nanomaterials following oral exposure (Fröhlich and Roblegg, 2012; Bouwmeester et al., 2014; Hadrup and Lam, 2014; Rossi et al., 2014; Heringa et al., 2016). The majority of the currently available information on toxicity of nanomaterial, as when EFSA's previous opinions were published (EFSA Scientific Committee, 2009a, 2011a,b), is from *in vitro* studies or *in vivo* studies using routes of exposure other than oral (e.g. inhalation). The key point for **hazard identification** is that nanomaterials may show typical'small particle' behaviour, and thereby can exhibit biological properties different from the corresponding non-nanomaterial (if applicable, see glossary). Therefore, they have to be assessed according to this Guidance. On the other hand, risk assessment for quickly degrading nanomaterial may follow the relevant existing guidance for conventional materials.

General considerations for testing nanomaterial are covered in Section 6.9 and need to be taken into account. It is important to highlight that, even around or within the nanoscale, there may be considerable fluctuation in the toxicity of a given nanomaterial due to variations in particle size. For instance, in the case of silver, 10 nm has been identified as a size threshold where a substantial increase in toxicity occurs both *in vitro* and *in vivo* compared with slightly larger nanoparticles (Ivask et al., 2014; Recordati et al., 2016). It is therefore crucial that there is **complete correlation between the material as produced and as tested**, and that the size and properties of the manufactured material used in the specific application lie within the narrow range covered by the risk assessment. In this light, batch-to-batch variation is of special concern and strict criteria should be followed to ensure the manufactured material consistently presents constant physicochemical parameters (i.e. those considered in the risk assessment).

6.1. Stepwise framework for nano-related hazard identification and characterisation in food/feed



(a): If yes, an argument can be put forward that further nanospecific testing is not necessary. However, it is anticipated that for most cases that have entered Step 1, testing under Step 2 will be required. This is because conclusive evidence for toxicity (including local effects for any relevant route of exposure) based on *in vitro* testing alone, is not expected. Hence, the decision to not perform any toxicity study has also implication for uncertainty reporting (see Section 8.3). Direct testing under Step 2 is acceptable to demonstrate if the nanomaterial represents a hazard or not. Furthermore, for many Regulatory frameworks, e.g. for food additives, there is a requirement for a 90-day test. In these cases, this study has to be designed according to the stipulations of nanospecific issues as described in this Guidance for performing the tests of Steps 2 and 3.
(b): Review of existing information should continue throughout the entire process of hazard identification and risk assessment.
(c): Step 1 genotoxcity testing is mandatory for all nanomaterial. A positive result in Step 1 requires follow-up in Step 2.
(d): Some nanomaterials have been related to inflammation, immunotoxicity, genotoxicity, reproductive organ effects and/or neurotoxicity (Dekkers et al., 2016; Bencsik et al., 2017; Higashisaka et al., 2017; Prosafe white paper, 2017). Indications for respective effects during step 1 and 2 assessment should be further investigated in Step 3.

Figure 3: Framework for step-wise hazard identification and characterisation

The SC notes that this Guidance complements the existing relevant EFSA Guidances for conventional materials (see Introduction). Hence, it is noted that some of those Guidance have a parallel tiered approach and the sequence is: Tier 1 -> Step 2, Tier 2 -> Step 3, Tier 3 -> Step 3. This is because for nanomaterials a determination of whether nanospecific properties exist (i.e. Steps 0 and 1, which do not exist in the already described tiered approach for food additives and novel foods) should be made prior to testing.

In **Step 0,** the rate of degradation of the nanomaterial to the non-nanomaterial under conditions representative of the gastrointestinal tract is investigated. Nanomaterials that quickly degrade, (i.e. have a high degradation rate; see Section 6.2) can be expected not to show nanorelated behaviours, and thus an appropriate standard risk assessment approach would be applied including read-across to the solute. If the material does not quickly degrade, one should continue to Step 1.

The aim of **Step 1a** is to gather any available information from existing literature that meets quality criteria (i.e. that has adequate characterisation data on the nanomaterial tested) and obtain information from a set of *in vitro* studies (see Section 6.5) that can identify specific issues that need to be addressed in the 90-day oral study in Step 2b, or that provide weight of evidence information for decision making in risk assessment. References and bibliographic lists must be provided in the format required by EFSA Guidances (e.g. the existing guidance for conventional materials).

This can include both existing information on the specific nanomaterial, as well as on similar materials i.e. those which only deviate to a limited extent in one or more physicochemical parameters as described in Table 1. Information on carcinogenic, mutagenic, reprotoxic (CMR) properties of one or more of the components of the material should always be considered.

The *in vitro* studies in **Step 1b** comprise degradation tests under simulated lysosomal conditions (see Section 6.2.2), *in vitro* genotoxicity tests (see Section 6.4) and a battery of tests including any relevant *in vitro* toxicity tests (see Section 6.5). Use of specific cell lines highlights further information required from investigations *in vivo* and influences the design of these studies. Where there is evidence of a lack of persistence based on degradation rate under simulated lysosomal and gastrointestinal conditions, and no indication of potential toxicity from existing information and the *in vitro* test battery, an argument may be put forward that further nanospecific testing as outlined in the present stepwise approach (e.g. continuation to Step 2) is not necessary. However, it is anticipated that for most cases that have entered Step 1, testing under Step 2 will be required. This is because conclusive evidence for toxicity (including local effects for any relevant route of exposure) based on *in vitro* testing alone, is not expected. Direct testing under Step 2 is acceptable to demonstrate if the nanomaterial represents a hazard or not.

In **Step 1b**, the *in vitro* genotoxic potential of nanomaterials will be investigated according to the tests indicated in Section 6.4. Nanomaterials that resulted negative in *in vitro* genotoxicity assays are considered non genotoxic and further *in vivo* genotoxicity test is not usually required. Nanomaterials that were positive in a least one *in vitro* genotoxicity assay have to be considered a potential hazard whose genotoxic capability requires further investigation in *in vivo* (**Step 2**). If genotoxicity cannot be tested *in vitro*, as a rule *in vivo* genotoxicity testing is necessary (see Section 6.4).

The results from the *in vitro* testing of the nanomaterial should be reviewed and other relevant information considered, such as on chemical reactivity (which might predispose to site of contact effects), bioavailability, metabolism, toxicokinetics, and any target organ specificity.

Step 2a consists of a Pilot *in vivo* study (See Section 6.6) which is recommendable for dose finding and assessment of absorption, tissue distribution and accumulation, elimination phase (≈ 14 days), for example by measurement of tissue concentrations at the end of study. Subsequently, **Step 2b** consists of a modified 90-day toxicity test (OECD TG 408 with extended parameters from OECD TG 407 (2008), e.g. for behavioural and endocrine disruptive effects) (preliminary reference: OECD TG 408 (2017a)) (see Section 6.7). For many Regulatory frameworks e.g. for food additives, there is a requirement for a 90-day study. In these cases, this study has to be designed according to the stipulations of nanospecific issues as described in this guidance for performing the tests of Steps 2 and 3. This study can be omitted only when there is robust justification (to be evaluated by EFSA) of non-absorption, and the absence of local effects for any relevant route of exposure. In the Step 2 modified 90-day study, specific attention should be paid to (indications on) effects on key gastrointestinal sites, organs normally investigated with emphasis on liver, brain, testis and spleen by histopathology and relevant endpoints. The results from this study can be used to identify a reference point (such as lower boundary of the BMD confidence interval (BMDL) or a no-observed-adverse-effect-level (NOAEL), See Section 6.7). This study should allow for the identification of nanomaterials with the potential to cause immunological, proliferative, neurotoxic, reproductive organ effects or endocrine-mediated effects that may warrant further in-depth investigation in **Step 3** as appropriate. Performing a pilot study including some toxicokinetic assessment is recommended for targeting of the hazard parameters in Step 2 and for dose ranging in Step 3 and onwards, and also to avoid the administration of highly toxic doses. *In vivo* studies should be combined where possible to minimise the use of test animals.

- A stepwise approach (Figure 3) should be adopted for hazard identification and characterisation to avoid unnecessary testing of nanomaterials.
- In the first instance (Step 0), the rate of degradation of the nanomaterial to non-nanomaterial form under conditions representative of the gastrointestinal tract should be investigated. Quickly and fully dissolving nanomaterials may be subjected to standard (non-nanomaterial) assessment, instead of further nanospecific testing.
- In Step 1, all available information should be gathered and a set of *in vitro* studies carried out to identify hazards and any need for further testing in Step 2. If the information indicates that the nanomaterial is not persistent and not (geno)toxic (investigated with the relevant tissue and a comet test in case of secondary genotoxicity due to inflammatory effects in the gut as mentioned in Section 6.4), an argument may be made to waive further nanospecific testing in Step 2. However, safety assessment for conventional (non-nano)materials will still be needed.
- In Step 2, a modified 90-day oral toxicity test (OECD TG 408 with extended parameters from OECD TG 407) should be carried out for identification of the nanomaterials with potential to cause immunological, proliferative, neurotoxic, reproductive organ or endocrine-mediated effects as appropriate.
- The results of the modified 90-day toxicity test should determine whether further in-depth investigations would be needed in Step 3 (e.g. human kinetic data from volunteer studies, additional toxicokinetic study, reproductive and developmental toxicity, additional immunotoxicity, neurotoxicity, carcinogenicity/mutagenicity, endocrine effects, gut microbiome).

6.2. *In vitro* degradation tests

6.2.1. *In vitro* gastrointestinal digestion

Assessment of the degradation rate of nanomaterials in conditions representative of the human gastrointestinal tract is considered a key first step in the stepwise approach (Figure 3). If a high degradation rate can be demonstrated, as detailed later this paragraph, the standard safety assessment procedure for conventional materials can be followed.

A suite of *in vitro* digestion models have been described in the literature that assess the release or degradation/dissolution of non-nanomaterials (Dressman et al., 1998; Krul et al., 2000; Oomen et al., 2002; Brandon et al., 2006; Minekus et al., 2014; Lichtenstein et al., 2015; Kästner et al., 2016). *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 (Versantvoort et al., 2005; Dall'Asta et al., 2010), 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 simulate the conditions of the gastrointestinal tract (including mouth, stomach and gut). The differences between these models relate to the extent to which physiology is simulated, e.g. from very simple to rather sophisticated by using static or dynamic conditions, and with or without enzymes, bile salts etc. In addition, the physiology that is simulated may vary between models: fasted versus fed conditions, baby versus adult.

An *in vitro* digestion method suitable for food under fed conditions (as opposed to fasted) has been described by Minekus et al. (2014) that is harmonised by the COST Infogest network.[24] The effects of differences in pH, mineral type, ionic strength, digestion time and, enzyme activity were also discussed. The method consists of a short simulation of mouth conditions, followed by a gastric phase at pH 3 for 2 h and an intestinal phase at pH 7 for 2 h. The composition of the digestion fluids was exactly described. While this method is not specified for nanomaterials, nor is it an officially standardised method, this it is considered a key approach also to be used for nanomaterial in food, i.e. for simulating physiological conditions in the gastrointestinal tract after food consumption.

For fasted conditions, several *in vitro* digestion methods have been described and compared by Koch et al. (2013). These methods can be explored further for applicability in this context.

Recently, some experience has been gained with the application of *in vitro* digestion models to nanomaterials. NANoREG D5.02[25] shows that the degree of aggregation/agglomeration (see Table 1) of several nanomaterials (Ag, SiO_2 and ZnO) in artificial saliva, gastric juice and intestinal juice varies over these stages. Nanoparticles were still present in the intestinal stage, although considerable degradation was observed for Ag and ZnO (up to 45% was degraded after mouth, stomach and 2 h of intestinal digestion under the specified conditions). Degradation measurement in such complex matrices was challenging, and a single, robust and rapid test method for all types of materials in all types of matrices could not be developed. Possible techniques include spICP-MS, and ICP-MS/AES based methods in combination with a separation technique like ultrafiltration (NANoREG D5.02). Furthermore, Peters et al. (2012) and Walczak et al. (2013) investigated respectively SiO_2 and Ag particle distribution in artificial mouth, gastric and intestinal conditions. Here also, the degree of aggregation/agglomeration varied between the different compartments of mouth, stomach and intestine. Sieg et al. (2017) describe that the aluminium nanoparticles remained unchanged in saliva, and strongly agglomerated in the gastric phase showing also an increased ion release. The levels of aluminium ions decrease in the intestinal fluid and particles de-agglomerated. Altogether, dissolution of nanoparticles was limited. DeLoid et al. (2017b) found that iron(III)oxide (Fe_2O_3) nanoparticles in an oil-in-water emulsion showed different size distribution in an *in vitro* digestion model with or without Fe_2O_3, in the mouth, stomach and intestinal phase. This size distribution comprised all particles, unspecified to chemical composition. There appeared to be minimal dissolution of the Fe_2O_3 particles in all three stages of digestion.

Remarkably, intestinal digestion of soluble silver ions (from $AgNO_3$) and ionic aluminium also resulted in the formation of particles (of 20–30 nm) composed of silver, sulfur and chlorine (Walczak et al., 2013) and aluminium (Sieg et al., 2017).

There is a lack of validation and standardisation of *in vitro* digestion models for nanomaterials. At present, no comparison for nanomaterials has been made between *in vitro* degradation/dissolution data from such digestion models and *in vivo* data. Lefebvre et al. (2015) concluded that *in vitro* digestion models are generally applicable to nanomaterials, as the basis of the models is mimicking the conditions of the gastrointestinal tract. Important properties affecting degradation/dissolution are expected to include physical forces, temperature, pH, presence of enzymes, salts and bile, and presence of food (Bellmann et al., 2015). Therefore, a rationale for the used *in vitro* digestion model used in the stepwise approach should be provided in view of the representativeness of the physiologic state for exposure, and whether the model is expected to represent worst-case, realistic or favourable conditions for *in vitro* degradation of the specific nanomaterial. For example, fasted conditions may be less representative of the use of nanomaterials in food products, whereas low pH conditions in the stomach – as may occur in fasted conditions – may promote the degradation of most metals and metal

[24] See https://www.cost-infogest.eu/

[25] http://rivm.nl/en/About_RIVM/International/International_Projects/Completed/NANoREG/deliverables/NANoREG_D5_02_DR_Report_on_the_development_of_a_solubility_testing_procedure.org. NanoReg was a cooperation between 71 Partners form a consortium of European RTD performers, metrology institutes and nanomaterials and instrument manufacturers.

oxides. Therefore, a careful choice between fed or fasted conditions should be made for the test system in view of anticipated conditions and the worst-case situation, depending on the characteristics of the nanomaterial and the application. In some cases, it may be relevant to investigate the degradation rate under both physiological conditions. Further scientific research on the impact of food components on the degradation of nanomaterials is recommended (Lichtenstein et al., 2015). The *in vitro* digestion model should also be critically assessed for its reliability and reproducibility. To increase the reliability of the degradation information from the model, the degradation rate should be determined by including different time points (at least four time points in duplicate at about 5, 15, 30 and 60 min) in the intestinal phase. The study should be performed at three different concentrations as this may affect the degradation, and the middle concentration should be representative of human exposure. This can be calculated by the estimated daily intake that, depending on the anticipated use, is ingested at once or throughout the day assuming that the daily volume of secretions into the gastrointestinal tract is 4–5 L. Furthermore, the particle number–size distribution and concentration should be analytically determined with a chemically specific method (i.e. verifying the chemical identity of the measured particles, e.g. spICP-MS or TEM-EDX). The concentration of the solute and, if present, other degradation products, should also be determined.

Some materials may degrade completely in the conditions of the stomach and then precipitate in the intestinal conditions as salts or nano- or microsized particles (Walczak et al., 2013). The dissolved fraction should not be separated from the rest during *in vitro* digestion as this may promote the dissolution. It has also been shown that SiO_2 particles can form large non-nanosized agglomerates in the conditions of the stomach that may disagglomerate in the intestine stage (Peters et al., 2012). This indicates that the absence of small particles in **stomach** conditions is insufficient to conclude that there will be no exposure to the nanoparticles. Therefore, only information on the dissolution rate in the intestinal conditions is considered relevant and should be provided. This means that the simulation of the stomach conditions still need to precede the intestinal conditions, but no data on the dissolution rate in the stomach phase are required.

The measured concentrations of solute, degradation products and particles should be compared with the start situation at the beginning of the *in vitro* digestion, in saliva or in the matrix as introduced into the *in vitro* digestion model. Analytical limitations such as detection limits should be taken into consideration (see Section 4.3).

A nanomaterial is considered to degrade quickly/have a high degradation rate if **the degradation rate profile in the intestinal phase shows a clear decrease** in the presence of particles over time (no plateau), and that **12% or less of the material** (mass-based[26]) – compared with the particulate concentration at the beginning of the *in vitro* digestion – is present as particles **after 30** min of intestinal digestion. This is indicative that the rest of the material should be fully degraded to non-nanomaterial (e.g. ionic) under gastrointestinal conditions (as mentioned in Figure 3). Details of the rationale and discussion of the uncertainty for this cut-off value can be found in **Appendix D**. The cut-off value assumes a first-order half-life in the **intestinal phase** of 10 min. It is considered feasible to measure this value analytically, and the time required to reach the intestinal epithelium and be taken up by cells is of the same order of magnitude. In such cases, a nanospecific risk assessment would not always be required. However, 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). When applicable to the material, a comparison to an ionic control should be included to identify *de novo* particle formation from these ions.

It should be noted that the cut-off for a high degradation rate is based a pragmatic choice based on limited science (see Appendix D) and may need modification with increasing knowledge.

If it cannot be demonstrated that the material quickly degrades, one should continue to Step 1 of Figure 3.

[26] While number based would be preferred, mass based evaluation would be accepted for pragmatic reasons.

- The use of the *in vitro* digestion model should be justified for relevance to the physiologic state (fasted or fed) for exposure, and whether it represents worst-case, realistic or favourable conditions for *in vitro* dissolution of the specific nanomaterial. It is recommended that worst case conditions (fed or fasted) should be used for the *in vitro* digestion model.
- The reliability and reproducibility of the model should be assessed and documented.
- The concentration of particles (in mass and numbers), solute, degradation products and the particle size distribution should be determined analytically (using at least an EM-technique).
- Information on the dissolution rate for each nanomaterial should be obtained from:
  - At least four time points for the intestinal phase (of up to 4 h) to allow the determination of a dissolution rate.
  - A minimum of duplicate samples at each time point should be used.
  - At least three different concentrations with a middle concentration that is calculated to be representative for human exposure should be used.
- A nanomaterial is considered to dissolve quickly/have a high dissolution rate if 12% or less of the material (mass based) is present as particles after 30 min of intestinal digestion compared to the particulate concentration at the beginning of the *in vitro* digestion.

6.2.2. Stability in lysosomal fluid

Once in the body, some nanomaterials may not be easily cleared and may accumulate over time. Assessment of the stability in lysosomal conditions is important to screen the potential of nanomaterials for biopersistence and intracellular accumulation (Utembe et al., 2015). Lysosomal conditions are considered as model as this is where nanomaterials generally distribute to and where degradation can occur due to the acidic conditions and presence of enzymes.

Release of ions due to degradation in lysosomal fluid can be an indication of toxicity due to these ions and should be considered in further testing.

Artificial lysosomal fluid simulates the inorganic environment within lysosomes (hydrolytic enzymes are typically not included) and is buffered at pH 4.5–5.0 (see e.g. Stopford et al., 2003; Stefaniak et al., 2005; Henderson et al., 2014; Pelfrêne et al., 2017).

To assess the stability in lysosomal fluid, pristine materials should be submitted to *in vitro* simulated lysosomal degradation and the degradation rate in lysosomal fluid has to be determined by considering different time points (at least four) in duplicate at three different concentrations. Time points for sampling and concentrations have to be properly selected and justified; time points are normally expected to be in the range of hours and extend up to, e.g. 72 or 96 h. Degradation and particle size of nanomaterials after lysosomal treatment should be characterised using the same approach described for *in vitro* gastrointestinal digestion, where a half-life of about 24 h is considered indicative of a high degradation rate given that degradation in lysosomal conditions should provide a predictive estimate for potential accumulation in cases of where frequent exposure is expected. This half-life would result in 12% or less of the material (mass-based) being present at 72 h compared to the particulate concentration at the beginning of the degradation test (first-order kinetics). As with *in vitro* gastrointestinal digestion, no evidence of a plateau should be visible.

- The *in vitro* dissolution rate profile for each nanomaterial in simulated lysosomal fluid should be provided. Information on the dissolution rate should be obtained in duplicate from at least four different time points up to 72–96 h and at three different concentrations tested.
- A half-life of ca. 24 h is considered indicative of high dissolution rate in lysosomal fluid. This would result in 12% or less of the material (mass based) remaining at 72 h compared to the particulate concentration at the beginning of the dissolution test.
- The information together with the dissolution rate in simulated gastrointestinal conditions would indicate the likelihood of persistence and bioaccumulation of the material.

6.3. Read-across

A particular issue identified in the risk assessment of nanomaterials is the fact that a given material can be developed in several forms with different sizes, crystalline forms, morphological shapes, and/or

surface characteristics. Adequate physicochemical and toxicological data are often not available for each individual variant to allow case-by-case assessment and generation of such data would require a considerable time and resources. This is where a scientifically based framework for grouping and read-across can facilitate risk assessment within both industry and regulatory settings. Read-across here refers to the use of data from one or more (nano)materials (the source (nano)materials) to another nanomaterial of the same chemical composition (the target nanomaterial) to fill a data gap. A number of frameworks for nanomaterials have been proposed, based on approaches already established for conventional chemicals (Arts et al., 2014, 2015, 2016; Oomen et al., 2015; ECHA, 2016; ECHA/JRC/RIVM, 2016; OECD, 2016a,b). The scientific justification for read-across should demonstrate that the physicochemical characteristics of source and target (nano)materials are similar enough to allow the prediction of the toxicological effect of the target nanomaterial. At present, however, the scientific basis for allowing substantiation based on existing data for read-across is limited. This is because, unlike conventional chemicals, the current database on physicochemical and toxicological parameters of nanomaterials is too limited.

It is, nevertheless, relevant to assess if the existing data on a nanomaterial can be useful for grouping/read-across of other variants of the nanomaterial with the same chemical composition, and to identify what would be needed to substantiate the use of existing data for a grouping/read-across approach. A scientific reference paper by ECHA, JRC and RIVM (2016) on grouping of nanomaterials has recently been developed into an ECHA Guidance in the form of an appendix to Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH) Chapter R.6 on Information Requirements and Chemical Safety Assessment (IR&CSA) on Quantitative structure–activity relationships (QSARs) and Grouping, intended to inform those preparing registration dossiers for nanomaterials (ECHA, 2016). This document aims to provide an approach on how to justify the use of hazard data between nanoforms and the non-nanoform(s) and within groups of nanoforms of the same substance. First, the target material should be similar in physicochemical characteristics to the source material(s). Subsequently, the outline proposes substantiation that would require toxicokinetic considerations (e.g. does the nanoform of a target nanomaterial differ from the source material(s) in terms of reaching the target site) and hazard considerations (e.g. does the target nanoform of a nanomaterial differ from the source material(s) in terms of hazard potential and profile). Hence, an argument should be built based on the limited number of differences in physicochemical properties between source and target materials and how they can affect exposure, toxicokinetics and hazard and substantiated with additional physicochemical, *in vitro* and/or *in vivo* data as needed. The consequences of the potential differences in exposure, toxicokinetics and hazard should be considered in view of the applicability of existing data from one or more source (nano)materials for the risk assessment of the target nanomaterial, for example by justification that the source (nano)materials exhibit toxicokinetic behaviour and hazards that are more worst case than the target nanomaterial. Owing to the current data gaps, the applicability of read-across to nanomaterials is limited and it is likely that in a majority of cases, experimental data (*in vitro*, *in vivo*) would be needed for the substantiation. In time, such data may become available that can be used as source material(s) to compare the toxicokinetic behaviour and hazard potential to substantiate the use of a grouping/read-across approach. Application of read-across would require data and information on the relationship between physicochemical properties and the toxicokinetic behaviour and hazard potential of different variants of the nanomaterial with the same chemical composition. This implies that any nanomaterials considered for grouping/read-across should firstly be well characterised regarding physicochemical parameters. It is therefore also recommended that toxicological studies be conducted in a systematic manner to decipher the relationship(s) between changes in one or a few physicochemical properties and the toxicokinetic profile and hazard potential of variants of the nanomaterial with the same chemical composition. It would facilitate the interpretation of findings if only one parameter (or at most a small number) were changed systematically allowing the critical ones to be identified.

At the moment, there is considerable uncertainty (e.g. limited usability due to lack of data) on the value of read-across for risk assessment of nanomaterials. Owing to the current data gaps, the applicability of read-across to nanomaterials is limited and it is likely that experimental data (*in vitro*, *in vivo*) for read-across substantiation would be needed in a majority of cases. With time such data, or data specifically for a specific read-across case, may become available. Once available such data can then be used to compare the toxicokinetic behaviour and hazard potential for justification of the use of a grouping/read-across approach in the setting of nanomaterials in the food and feed chain. Whether a read-across justification is acceptable for waiving further (*in vivo*) testing is to be judged by EFSA on a case-by-case basis.

- A case for read-across of data from one (nano)material to another nanomaterial to fill a data gap may be made on the basis that the two (or more) variants of the nanomaterial of the same chemical composition have comparable physicochemical and toxicokinetic profiles.
- A case for read-across may also be made on the basis that the source (nano)material exhibits a more worst case than the target nanomaterial.

6.4. *In vitro* and *in vivo* genotoxicity testing

Genotoxicity testing of nanomaterials should follow the general indications of the EFSA genotoxicity testing strategies (EFSA Scientific Committee, 2011b, 2017d) taking into account the specific properties of nanomaterials. Specific issues related to genotoxicity testing of nanomaterials have been highlighted by the OECD Expert Meeting on Genotoxicity of Manufactured Nanomaterials (OECD, 2014b) and included in the SCCS (2016) notes of guidance (SCCS/1564/15) and in ECHA (2017a). Catalán et al. (2017) described a theoretical approach for weighed assessment of the mutagenic potential of nanomaterials.

Materials, nano and non-nano, may induce genotoxic damage by direct interaction with DNA, by disturbing the process of mitosis, or by producing reactive oxygen species (ROS) (reviewed by Gonzalez et al., 2010; Magdolenova et al., 2014; Kermanizadeh et al., 2015a,b). As a consequence, various types of genetic alterations may result and a battery of tests covering different genotoxic mechanisms is needed to assay the genotoxic potential of nanomaterials. Furthermore, *in vitro* genotoxicity testing of nanomaterials should always include an assessment of cellular uptake (Magdolenova et al., 2014; Dekkers et al., 2016). Specific recommendations on how to conduct the genotoxicity tests for nanomaterials are also described by Pfuhler et al. (2013) and Doak et al. (2012).

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. The following *in vitro* tests are required for assessment of genotoxicity in the context of the present Guidance:

1) A test for induction of gene mutations – A bacterial reverse mutation (Ames) assay is usually recommended for the detection of gene mutations. However, since nanomaterials may not be able to penetrate the bacterial cell wall and because bacterial cells, unlike mammalian cells, do not have the ability to internalise (Doak et al., 2012), the OECD Expert Meeting on 'Genotoxicity of Manufactured Nanomaterials' concluded that the Ames test (OECD TG 471 (1997b)) is not a recommended method for investigating the genotoxicity of nanomaterials (OECD, 2014b). In this respect, the use of mammalian cell models is considered more suitable: both the *in vitro* mammalian cell gene mutation tests using the *Hprt* and *xprt* genes (OECD TG 476 (2016b)) and the *in vitro* mammalian cell gene mutation tests using the thymidine kinase gene (OECD TG 490 (2016e)) are appropriate. In specific circumstances (e.g. indirect genotoxic effects due to extracellular induction of reactive oxygen species), an Ames test might still be informative.
2) A test for structural and numerical chromosome damage, i.e. the *in vitro* mammalian cell micronucleus test (OECD TG 487 (2016c)) – To take into account the possibly low particle penetration into the cell nucleus and to facilitate the contact of nanomaterials with DNA after nuclear membrane dissolution during mitosis, a long-duration treatment, covering two cell cycles, is advisable (Catalán et al., 2012). If cytochalasin B is used in the test, its addition to cell cultures after nanomaterial treatment must be delayed because of its ability to inhibit endocytosis and reduce nanomaterial cell uptake (Gonzalez et al., 2011; Doak et al., 2012; Magdolenova et al., 2012; Pfuhler et al., 2013). According to the OECD TG 487 (2016c), micronucleus detection by flow cytometry is acceptable, provided the potential interference of nanomaterials with the dyes applied in the analysis is taken into account (Li et al., 2017). Several mammalian cell models have been used for nanomaterial genotoxicity assessment that show comparable or differential sensitivity (European Comission Joint Action, 2008–2013, Nanogenotox;[27] European Union Seveht Framework Program, 2007–2013, Nanoreg;[28] Cowie et al., 2015). Cell lines representative of the gastrointestinal tract

[27] Nanogenotox was a Joint Action of European Commission and a consortium of national institutions of Member States which was funded by European Commission's health programme (46%), while partners and some ministries of the participating Member States (Belgium, France, Germany, and Netherlands) provide the remaining. www.nanogenotox.eu

[28] http://www.nanoreg.eu/

or the expected target tissue should be considered as first choice. In selecting the most appropriate mammalian system for *in vitro* genotoxic hazard identification, the uptake capability should be considered as a critical feature because the internalisation of a nanomaterial is a crucial step in understanding its behaviour and toxicity (Magdolenova et al., 2014; Dekkers et al., 2016).

Most poorly soluble nanomaterials are not metabolised and the metabolic activation system (S9) may interfere with the assay reducing the nanomaterial bioavailability. Organic nanomaterials or some inorganic nanomaterials coated with organic functional groups may however exert their genotoxic effects in the presence of the metabolic activation system (Sharifi et al., 2012). The use of S9 in the tests should therefore be evaluated case by case.

The *in vitro* comet assay, though not yet validated, may provide complementary information and contribute to an understanding of the nanomaterial genotoxicity mechanisms. The modified comet assay for detection of oxidative DNA lesions can be recommended as many nanomaterials have been shown to induce oxidative stress or at least a concomitant measurement of oxidative stress (ROS, antioxidants). If *in vivo* assays are necessary, the comet assay also allows them to be better designed (Møller et al., 2015; Collins et al., 2017). Possible interference by residual intracellular nanomaterials during the assay procedure, producing artefactual positive results, must be evaluated (Ferraro et al., 2016; George et al., 2017) and explained. The *in vivo* comet assay should not be combined to repeat-dose oral studies without using satellite groups because of the need to sample at T_{max} which is not possible in the general toxicity autopsy procedures.

The interpretation of the results from the *in vitro* genotoxicity studies would be supported by an assessment of cellular uptake (and nuclear uptake, if feasible) of nanoparticles. However, an absence of observed cellular uptake does not mean that the material will have no genotoxic potential, since it can also indirectly induce secondary mechanisms of genotoxicity (without cellular uptake).

If at least one of the *in vitro* tests indicates genotoxic activity, or if it is not appropriate to test the nanomaterial *in vitro* (e.g. if the dispersion medium is not compatible with the *in vitro* system), this normally requires follow-up by ***in vivo*** testing (Eastmond et al., 2009; EFSA Scientific Committee, 2011b, 2017d), unless it can be adequately demonstrated by other means that the positive *in vitro* findings are not relevant for the *in vivo* situation. It has to be noticed that inflammatory effects induced by nanomaterials can generate reactive radical species, potentially triggering secondary genotoxicity that cannot be detected by *in vitro* systems. In this case an *in vivo* comet assay, which can also provide information on mode of action (and when combined with other tests), is recommended for inclusion in a repeated-dose oral toxicity study (Pfuhler et al., 2013).

The choice of the appropriate *in vivo* genotoxicity test(s) requires expert judgement, based on all available information. It should be related to the genotoxic endpoint(s) identified as positive *in vitro* and performed on appropriate target organ(s) or tissue(s). As outlined in the EFSA Genotoxicity testing strategies (EFSA Scientific Committee, 2011a, 2017d), *in vivo* genotoxicity testing should be performed in a step wise approach depending on the outcome of the *in vitro* tests. Accordingly, the following *in vivo* tests testing may be suitable:

- an *in vivo* micronucleus test (OECD test guideline 474). Demonstration of target tissue exposure is required following the considerations as provided in EFSA Scientific Committee, 2017d.
- an *in vivo* mammalian alkaline comet assay (OECD test guideline 489)
- a Transgenic rodent somatic and germ cell gene mutation assay (OECD test guideline 488 (OECD, 2013))

Based on expert judgement, a combination of these tests applied to the same individual animals may be advisable.

Evidence, either from the test itself or from other toxicokinetic (see Section 6.6) or repeated-dose toxicological studies (see Section 6.7), that the target tissue(s) (for instance bone marrow in the *in vivo* micronucleus test) have been exposed to the nanomaterial and/or its metabolites is essential for interpretation of negative results (EFSA Scientific Committee, 2017d).

A number of activities are currently ongoing to harmonise, update, refine and eventually validate genotoxicity tests of conventional materials for their application to nanomaterials. Such developments and any updates in the genotoxicity tests (OECD, 2016c) have to be considered before embarking on genotoxicity testing for the purposes of this guidance.

- Genotoxicity testing of nanomaterials should follow the general indications of the EFSA genotoxicity testing strategy (EFSA Scientific Committee, 2011a) taking into account the specific properties of nanomaterials.
- *In vitro* genotoxicity testing of nanomaterials should always include an assessment of cellular uptake, especially to substantiate negative test results.
- In selecting a suitable battery of *in vitro* genotoxicity tests, the three critical genotoxicity endpoints (gene mutation, structural and numerical chromosome aberrations) should be addressed.
- The bacterial reverse mutation (Ames) assay is not considered suitable for nanomaterials due to limitations in the penetration of particles through the bacterial cell wall and the lack of internalisation in bacteria.
- The use of S9 in the tests should be evaluated on a case by case basis.
- Where at least one of the *in vitro* tests indicates genotoxic activity, or if it is not appropriate to test the nanomaterial *in vitro*, a follow-up *in vivo* study should be carried out, unless it can be demonstrated by other means that the positive *in vitro* findings are not relevant for *in vivo* situation.
- Expert judgement should be used to select one or more of the available *in vivo* tests e.g. *in vivo* micronucleus test (OECD TG 474); *in vivo* mammalian alkaline comet assay (OECD TG 489); Transgenic rodent somatic and germ cell gene mutation assay (OECD TG 488)

6.5. *In vitro* toxicity testing

In vitro tests in Step 1 may also provide insights into a nanomaterial's hazard and its mode of action upon e.g. internal exposure (see Section 6.10). The *in vitro* toxicity tests may add to the weight of evidence approach (see Section 7). Although the Scientific Committee cannot provide more guidance on which assays or endpoints to use, it notes the following information.

Considering oral intake as the main *in vivo* route of administration, several *in vitro* approaches may be applied to generate additional hazard identification information (Drasler et al., 2017). *In vitro* models based on primary cells or cell lines and on monoculture or coculture systems are available to represent the gastrointestinal tract. Coculture-based systems (including 3D *in vitro* models), compared to monocultures, can provide conditions more closely mimicking *in vivo*; e.g. human colorectal epithelial cells (CaCo-2) combined with immune cells and mucus-secreting cells (Gamboa and Leong, 2013). Primary human cells, such as primary human oesophageal epithelial cells either in monoculture or (better) in coculture, may therefore be used to represent the gastrointestinal tract. Two or three different cell types need to be tested. Yet, the disadvantage of primary cells is a substantial batch to batch variations as well as they are more difficult to obtain and to culture. Besides the gastrointestinal cellular models, it is also important to test immune cells such as macrophages (e.g. primary human monocyte-derived macrophages or human monocytic cell line THP-1). As nanomaterials are prone to translocate through the gastrointestinal barrier, they might enter the circulatory system and reach, e.g. liver, spleen and kidney. It is therefore recommended to include the respective representative cell types (e.g. hepatocytes) in the testing strategy. In addition, the commercially available whole blood cytokine release kit can serve for characterisation of immunotoxic reactions, including immunostimulation and immunosuppression of immune responses (Langezaal et al., 2001, 2002) even though it is not directly representative for testing gut-associated immune responses.

Detailed cell characterisation (i.e. cell source, passage number, cell growth, morphology and differentiation before and during the test performance) and precise description of cell culture method need to be reported in order to verify the method's reliability. Exposure and post-exposure times need to be well defined and justified with respect to the individual tested parameters. For mono- or co-culture systems grown on membrane inserts, confluence and viability must be checked for the appropriate level of resistance by transepithelial electrical resistance (TEER) measurements before cytotoxicity assay. For the reporting of any non-OECD approved *in vitro* assay, it is required that the Guidance OECD 211 is followed (OECD, 2014c).

Specific endpoints can be considered to investigate the effects of nanomaterial on, e.g. impaired cell viability/cytotoxicity, oxidative stress responses, (pro-) inflammatory responses (as part of immunotoxicity), and integrity of the gastrointestinal barrier.

A number of parameters can be considered for investigation of cytotoxicity in *in vitro* models, including membrane rupture by the lactate dehydrogenase (LDH) leakage assay or impaired cellular metabolism using e.g. MTT or MTS reduction assays. Proinflammatory responses *in vitro* can be measured via enzyme-linked immunosorbent assays (ELISA) for specific proinflammatory cytokines (Elsabahy and Wooley, 2013) and/or immune markers. There are also other convenient screening methods available that allow for the simultaneous detection of multiple pro- and anti-inflammatory mediators (Multiplex analysis based cytokine profiling; Bhattacharya et al., 2017). Additionally, oral-route specific endpoints might be considered, for example the effect of nanomaterials on hepatic function by measuring metabolic activity of hepatocytes *in vitro* (e.g. via modification of the expression level or the activity of enzymes involved in the xenobiotic metabolism such as the cytochromes P450). It is also important to consider any potential interference of nanomaterials with *in vitro* test systems (see section 6.9.1) (Cornu et al., 2018).

Nanomaterial translocation through the gastrointestinal barrier *in vitro* may serve only as supporting data for further *in vivo* investigations. *In vitro* translocation models are grown on Transwell ® system membranes. These membranes may hamper translocation by adherence of nanomaterials (resulting from a possible inability to pass through the membranes or from nanomaterial attachment).

Depending on the type of application (e.g. animal feed, pesticide), *in vitro* tests may also be used to determine dermal absorption and skin sensitisation potential of the nanomaterial. These involve standard *in vitro* tests as required for bulk (non-nanomaterial) substances (see Appendix E for animal feed and pesticides) but must be carried out in consideration of the nanospecific aspects as detailed in Section 6.9.

If *in vitro* results indicate compromised epithelial barrier integrity, release of (pro-)inflammatory mediators, effects on immune cells or immune response, appropriate targeting in *in vivo* studies should be considered (see Sections 6.7 and 6.8). As mentioned in Section 6.1 for most cases that have entered Step 1, it is anticipated that testing under Step 2 will be required. In some cases, however, when the *in vitro* methods do not indicate effects and *in vitro* degradation in lysosomal and gastrointestinal conditions is fast, an argument may be made for waiving *in vivo* studies. Such an argument is to be assessed by EFSA on a case-by-case basis. Outcomes of *in vitro* tests may serve as basic evidence of a possible nanomaterial hazard and can contribute to the design and interpretation of *in vivo* studies by identifying their modes of action.

The dosimetry aspect, i.e. assessment of the dose delivered to the cells and the internalised dose is important for a sound interpretation of the *in vitro* cytotoxicity data. Besides information on particle mass per incubation volume or particle mass per cell culture dish surface, also data on incubation volume, cell culture dish size, cell number, etc., should be provided (consult also the Section 6.9.1).

- *In vitro* toxicity data may be used as additional weight of evidence and/or to target further *in vitro* testing.
  - Specific endpoints relevant for *in vitro* testing are: cytotoxicity/cell viability, induction of oxidative stress, (pro-)inflammation and gastrointestinal barrier integrity impairment.
  - Use of cocultures is often preferred over the monocultures when testing on a more complex system is desired, yet depending on the objective; standard monocultures are also informative, easier to standardize and can be useful for screening purposes.
  - Complete dosimetry information should be provided (e.g. particle mass per incubation volume or particle mass per cell culture dish surface, incubation volume, cell culture dish size, cell number).
- Where *in vitro* methods indicate lack of toxic effects, and *in vitro* dissolution of the nanomaterial in lysosomal and gastrointestinal conditions is fast, an argument can be put forward to EFSA for waiving *in vivo* studies on a case-by-case basis.

6.6. Toxicokinetics (ADME)

Toxicokinetics (ADME) is important in human health risk assessment and greater application of toxicokinetics could offer more efficiency, use fewer test animals and provide better data. For risk assessment of nanomaterials, as well as for substantiation of read-across to other materials, e.g. non-nanomaterial or a similar nanomaterial, toxicokinetic information is crucial. This is because nanomaterials may show different toxicokinetic behaviours (i.e. significant changes in absorption,

distribution and/or metabolism and excretion) compared with larger sized materials and solutes with the same chemical composition (Hagens et al., 2007; Higashisaka et al., 2017).

Their size-related properties, shape or surface characteristics can affect the toxicokinetic behaviour. For example, particle uptake by intestinal epithelial cells is size dependent (Powell et al., 2010; Fröhlich and Roblegg, 2012; Howe et al., 2014; Macierzanka et al., 2014). It seems that 20–40 nm nanomaterials are easily taken up by intestinal cells (enterocytes). M-cells in Peyer's patches can rapidly (within minutes) internalise not only a significant number of nanoparticles (20–100 nm), but also few large particles (0.5–2 μm) (Howe et al., 2014). Based on the available information, Powell et al. (2010) suggest that the mechanism of intestinal uptake is likely to be size dependent, and there may well be an optimum size for gut uptake, tentatively around 50 nm, with perhaps a range of 20–250 nm. This size dependent behaviour can also be influenced by coating.

The toxicokinetic (ADME) investigations to be followed are presented in a stepwise assessment.

In Step 1, the existing information is gathered, on the specific nanomaterials as well as similar materials (such as the non-nanomaterial). This includes existing information on the toxicokinetic behaviour. In particular, information on the absorption/bioavailability, distribution pattern and clearance is considered relevant, as physicochemical properties of nanomaterials are known to be able to affect these. In addition, *in vitro* tests with the nanomaterial are performed in Step 1 (see Figure 3). Since nanomaterials may not be easily cleared, they may accumulate over time (Geraets et al., 2014; Kermanizadeh et al., 2015a,b; Kreyling et al., 2017).

It is therefore considered important to assess the degradation of nanomaterial in lysosomal fluid, considered as model as this is where nanomaterials generally distribute to and where degradation can occur due to the acidic conditions and presence of enzymes. Information on the degradation rate under simulated lysosomal conditions in combination with the degradation rate in simulated gastrointestinal conditions (as obtained in Step 0) provides insight into the likelihood of persistence and bioaccumulation of the material. An argument may be put forward that further testing (e.g. Step 2) is not necessary only in cases of non-persistence based the degradation rate under simulated lysosomal and gastrointestinal conditions with no indication of potential toxicity based on existing information and the *in vitro* test battery.

When there is a soluble non-nanocounterpart of the material, it is important to report any differences in toxicokinetics between the nanomaterial and the corresponding soluble substance. In addition, in the case of a bulk material containing a range of particle sizes, it is recommended to do (when possible) the *in vitro* testing in Steps 0 and 1 for both the nano and non-nanomaterial to gain insight into potential differences in behaviour and nanospecific hazards that would lead to the next tier of testing. The presence of nanosized particles indicates that nanospecific considerations are relevant.

Information gathered in Step 1 should be used to fine-tune Step 2. For example, the design of the 90-day study and the satellite groups of Step 2b will benefit from pilot studies for dose finding and assessment of absorption, tissue distribution and accumulation, elimination phase (Step 2a ≈ 14 days). Demonstration of absorption of nanomaterial can be challenging in a 14-day study. In this case, the applicant goes to Step 2b directly. Also, a limited rate of degradation in gastrointestinal and lysosomal fluids suggests that the material may be persistent in tissues in particulate form and that information from longer time period may be needed to assess the clearance after the last dosing. The degradation rate may also indicate whether toxicity is due to release of ions or molecules at the sites of distribution.

Step 2b consists of a modified 90-day oral toxicity test (OECD TG 408 with extended parameters from the OECD TG 407 (2008)) (preliminary reference OECD TG 408 (2017a)). Studies on toxicokinetics in animals should be conducted using internationally agreed test guidelines, such as OECD TG 417 (2010). This guideline describes general methodologies for performing ADME studies. It provides minimum criteria for acceptance of studies but makes clear that studies should be designed on a case by case basis. Moreover, the OECD Expert Meeting on 'Toxicokinetics of manufactured nanomaterials' concluded that OECD TG 417 needs to be revised it in order to make it appropriate for nanomaterials (OECD, 2016d).

The difficulties of undertaking ADME studies on nanomaterials should not be underestimated. There may be particular difficulties in measuring the amounts of nanomaterial in blood, tissues and excreta, and in establishing the form in which they are present in the body (see Section 4.3.2). Nanomaterial surface transformations affecting, e.g. the dynamics of adherence of proteins and other biomolecules can have a profound effect on ADME.

For ADME studies, it is essential that a measurement system is available for detecting either the nanomaterial or its elemental composition in organs, tissues and other biological samples. Alternatively, labelling of the nanomaterial may be used, either directly (radioactive or stable isotopes) or indirectly

(fluorescent dyes or radiolabels). ICP-MS has the limitation that the chemical element is determined rather than the presence of the nanomaterial itself (i.e. more than the nanosized fraction may be detected), but combining it with suitable separation techniques or turning to spICP-MS and/or analytical electron microscopy (Tassinari et al., 2014) could overcome this. Radioactive isotopes have been used for certain metal nanomaterial (Geiser and Kreyling, 2010; Kreyling et al., 2017). Fluorescence labelling or labelling with radiolabelled chemicals has the disadvantage that the label may be released from the nanomaterial. In such a case, the distribution of the label may not be indicative of the presence of the nanomaterial (Geiser and Kreyling, 2010). The choice of the labelling and detection technique should be based on the composition of the nanomaterial, e.g. metal nanomaterials vs lipid-like nanomaterials. In addition, the impact of the labelling system on the properties and activity of the nanomaterial should be considered. For example, coupling certain fluorescent dyes may change the hydrophobicity/hydrophilicity of the nanomaterial.

A satellite group **should** be added to the 90-day oral toxicity study to investigate if and to what extent the nanomaterial could accumulate. In the satellite group, the tissue distribution of the nanomaterial should be determined after a short-dosing period (i.e. 2 weeks) and at the end of the 90-day study. Preferably, the tissue distribution after an elimination period should also be determined, for example for animals that were dosed for the short 2-week period. Nanomaterials are generally quickly – often within minutes – taken up by tissues (Landsiedel et al., 2012). Blood or plasma concentration–time information therefore usually has limited value, and only a small number of data points are necessary. In such cases, a rough estimate of toxicokinetic plasma parameters (t1/2, area under the curve (AUC), bioavailability, C_{max} and T_{max}) would be sufficient. Because nanomaterials are taken up by the mononuclear phagocyte system (MPS), they typically distribute to liver and spleen, and to a lesser extent to tissues such as the kidney, bone marrow and lung. Nanomaterial retention within the gut wall is also an important determinant, particularly when discriminating between retention in epithelial cells vs immune-competent M-cells in Peyer's patches. In the gastrointestinal tract, gut-associated lymph tissue (GALT), particularly in Peyer's patches and mesenteric lymph nodes, is of importance for potential nanomaterial accumulation and immune responses.

The difference in organ concentration between days 0, 14 and 90, as well as the rate of elimination should be used to assess the likelihood for accumulation, given the anticipated exposure pattern in humans. Any significant increase in tissue concentration between days 14 and 90, or slow release during the elimination period, should be discussed in this light, and triggers further assessment in Step 3.

The relationship between dose and tissue concentrations should be assessed, as oral absorption and other toxicokinetic processes may be dose dependent, e.g. as a result of aggregation of the nanomaterial at high doses. The amount distributed to tissues should be considered in estimating the absorption of the nanomaterial. Mass balance studies are recommended.

Performing a pilot study including some toxicokinetic assessment is recommended for targeting of the hazard assessment and for dose ranging to avoid the administration of highly toxic doses in Step 2 and onwards.

A negative control – a group that is not exposed – is used to assess e.g. background exposure.

For the purpose of comparison and potential use in read across, in the absence of existing data, it is advisable to include a control with the conventional non-nanomaterial.

Little is known about route-to-route extrapolation for nanomaterials. In principle, therefore, oral studies should be performed, since this will be in many cases the relevant exposure route for food/feed applications.[29]

In Step 3, there may be a need for additional toxicokinetic studies to evaluate the effect of repeated dose administration and whether this leads to steady-state conditions or accumulation of the nanomaterial. In such circumstances, it is possible that the kinetics observed in experimental animals may need to be validated in human studies. This refines the risk assessment and may also be required where there is evidence that age, physiological state, disease state, etc., could modify the toxicokinetic behaviour.

Toxicokinetic modelling can be employed to estimate/extrapolate the fate of nanomaterials in animals and humans to other exposure scenario's, e.g. other duration or dose, or to other species, e.g. animal to man.

In summary, assessment of the toxicokinetics of nanomaterials has to include the following:

[29] For dermal and inhalation routes of exposure to nanomaterial in food or feed, see Appendix E.

- Step 1 comprises assessment of existing information on the specific nanomaterial as well as similar nanomaterials and non-nanomaterials (bulk) form. This step also includes determination of the degradation rate in lysosomal conditions. Taken together with information on *in vitro* toxicity and degradation rate in simulated gastrointestinal conditions (Step 0), an argument can be put forward that further testing is not necessary in some cases. Information gathered in Step 1 should be used to fine tune Step 2.
- Step 2 consists of toxicokinetic studies linked with a 90-day oral toxicity study in rodents. Information on tissue distribution should be obtained before dosing, after a short-dosing period, i.e. 2 weeks, and at the end of the 90-day toxicity study. Preferably, tissue distribution after an elimination period would also be determined. The extent to which nanomaterials can accumulate in tissues should be investigated. Any significant increase in tissue concentration between days 14 and 90, or slow release during the elimination period triggers further assessment in Step 3.
- Step 3 toxicokinetic studies can be designed to investigate to what extent accumulation of the nanomaterial occurs with long-term exposure and determine whether there are species differences in toxicokinetic behaviour between animals and humans or because of other physiological or disease factors. These studies permit refinement of the risk assessment by decreasing the uncertainty.

- All available information must be collated along with data from *in vitro* tests from Step 1 to identify any changes in the toxicokinetic behaviour, bioavailability, and persistence and bioaccumulation of the nanomaterial compared to non-nanomaterial equivalent.
- Performing a pilot study that includes some toxicokinetic assessment is recommended for targeting of the hazard assessment, and for dose ranging to avoid administration of highly toxic doses in Step 2 and onwards.
- A satellite group should be added to the 90-day oral toxicity study to investigate if and to what extent the nanomaterial could accumulate. Tissue distribution should be included in the distribution studies.
- An appropriate measurement system should be used to detect the nanomaterial or its elemental composition in organs, tissues and other biological samples. Labelling of the nanomaterial may be used where possible.
- Distribution of the nanomaterial to specific tissues should trigger further assessment on e.g. neurotoxicity and reproductive toxicity in Step 3. If in-depth investigations in Step 3 are necessary, the kinetics observed in experimental animals may need to be validated by studies in human studies.

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

For ingested nanomaterial, the minimum requirement is the modified 90-day toxicity test (OECD TG 408 with extended parameters from the OECD TG 407 (2008)) (preliminary reference (OECD TG 408 (2017a)). The modified 90-day study should allow for the identification of nanomaterials with the **potential to cause neurotoxic, immunological, reproductive organ or endocrine-mediated effects** that either provide sufficient information for risk assessment or require further in-depth investigation. After systemic translocation (as identified in the ADME study), most nanomaterials are likely to end up in the MPS tissues, therefore, in repeated-dose studies, specific attention should be paid to cardiovascular and inflammatory parameters as well as to sites/organs involved in or part of the MPS.

Preliminary range-finding studies conducted for shorter periods can indicate target organs and help in selection of appropriate doses for 90-day studies. When range-finding studies have been conducted, the results should be submitted in the dossier. Studies of shorter duration than 90-days are generally not sufficient, by themselves, for evaluation of potential subchronic toxicity.

In the cases for which Step 2a Toxicokinetics testing indicates a lack of systemic availability, still any **local adverse effects** on the gastrointestinal tract have to be considered which will be covered by the gastrointestinal histopathology in the 90-day study (Step 2b).

The results from the 90-day toxicity study should also be used to determine whether sufficient information for risk assessment is available or whether additional testing is required (e.g. for **chronic effects, in-depth reproductive and developmental toxicity, or specific studies on immunological, neurological end points or endocrine activity**). If no triggers for additional

testing are identified, then the 90-day study can be used to identify a **reference point** for risk assessment according to the type of food chemical (e.g. food additives, feed additives, enzymes, flavourings, novel foods and nanomaterials that are incorporated into products that come into contact with food (e.g. FCMs and articles)).

- For ingested nanomaterials, the minimum requirement is the modified 90-day toxicity test (OECD TG 408 with extended parameters from the OECD TG 407 (2008).
- Specific attention should be paid in repeated-dose studies to cardiovascular and inflammatory parameters as well as to sites/organs involved in or part of the MPS.
- A satellite group should be added to the 90-day oral toxicity study to investigate toxicokinetics (Section 6.6).
- Where results indicate a lack of systemic availability, local effects on the gastrointestinal tract must be considered.
- The results from the 90-day study should be used to determine whether sufficient information for risk assessment is available or whether additional testing is required (e.g. for chronic effects, in depth reproductive and developmental toxicity, or specific studies on immunological, neurological end points or endocrine activity).
- The results from the Step 2 modified 90-day oral toxicity study can be used to identify a reference point (such as BMDL and NOAEL). The results in conjunction with evidence of absorption of the material will determine the need for proceeding to Step 3.

6.8. Higher tier toxicity testing

Evidence of absorption of the material and findings in the 90-day toxicity study are triggers for proceeding to Step 3. This step features specialised studies on the endpoints presented in the subsections below. The purpose of investigations into mechanisms and modes of action is to determine the relevance for man of effects observed in the test species as part of a mode of action framework analysis by the evaluator (Meek et al., 2013).

Information on the levels of nanomaterials present in key tissues in animals of Step 3 studies can be useful in the risk characterisation, as this provides direct information on the internal exposure. Nanomaterials may be absorbed to a very small extent from the gastrointestinal tract, whereas at the same time they may accumulate in tissues in time, making the amount of nanomaterial reaching tissues and potentially causing systemic toxicity difficult to predict. Hence, measuring the level of nanomaterials in a few relevant tissues, for example by analysis of a part of the tissue at the end of Step 3 studies, can be used for better interpretation of the study and interpretation of the study result in relation to exposure and other studies.

- Step 3 features studies on chronic toxicity and carcinogenicity in a single species, generally rat, and may also include, where triggered by the findings, further testing for reproductive and developmental toxicity, immunotoxicity and allergenicity, neurotoxicity or endocrine-mediated effects.
- Information on the levels of nanomaterials present in key tissues in animals used in step 3 studies should also be considered in risk characterisation.

6.8.1. Chronic toxicity and carcinogenicity

Chronic toxicity and carcinogenicity studies are performed in a single species, generally the rat. Either separate studies (OECD TGs 452 (2009d) and OECD TGs 451 (2009c), respectively) or preferably the combined study (OECD TG 453 (2009e)) can be carried out. Carcinogenicity study in a second species would only be triggered by the results in the preferred species (equivocal results or species specific findings) or by observations from specialised studies to investigate the mode of action or mechanism of toxicity or carcinogenicity observed.

6.8.2. Reproductive and developmental toxicity

There are indications in the literature suggesting that systemic exposure to specific nanomaterials may result in adverse effects on reproduction and development (Brohi et al., 2017). In this review,

ROS generation and inflammation, direct interaction with the male and female reproductive systems, alterations of ovarian gene expression and steroidogenesis, adverse effects on fetal morphology, organogenesis and development have been related to reproductive and developmental toxicity induced by specific nanomaterials. Some nanomaterials, depending on their physicochemical properties, were reported to be able to penetrate the blood–testis barrier and the placental barrier (Brohi et al., 2017).

The data from Step 2 subchronic toxicity testing are relevant when considering the need for reproductive and developmental testing in Step 3.

The repeated dose 90-day oral toxicity study (OECD TG 408 (2017a)) offers only limited information on reproductive toxicity and none on developmental toxicity; it can inform about effects on the reproductive organs and, if assessed, the oestrous cycle, but it does not assess fertility and the whole reproductive cycle from *in utero* exposure onwards, through sexual maturity to conception, gestation, prenatal and postnatal development. Decisions on whether tests are necessary for reproductive and developmental toxicity need to be considered in light of the toxicity data and toxicokinetics information available. **If the Step 2 toxicokinetic study shows that the test material is systemically available in the test species (normally rodents) or suspected to be systemically available in humans, Step 3 testing for reproductive and developmental toxicity is required**. For materials that do not appear to be systemically available, indications of effects on reproductive organs or parameters in the 90-day oral toxicity will also trigger Step 3 testing for reproductive and developmental toxicity. In the case where absorption appears to be very low, Step 3 reproductive and developmental toxicity studies may still be needed if the tissue distribution data from the 90-day oral toxicity study indicate that the test material is able to reach reproductive organs and distribute there.

Step 3 testing for reproductive and developmental toxicity comprises a prenatal developmental toxicity study (OECD TG 414 (2001)) and an extended one-generation reproductive toxicity study (EOGRTS) (OECD TG 443 (2012a)). Cohorts for the preliminary assessment of additional more specific endpoints should be routinely incorporated in the EOGRTS. Where it already exists, a multigeneration study, instead of an EOGRTS, would be acceptable, provided that sufficient information on possible neurotoxicity and immunotoxicity is available (for example from an extended 90-day study, OECD TG 408 (2017a)).

The EOGRTS in the rat will provide information evaluating specific life stages not covered by the other toxicity studies: fertility and reproductive function, and short to long-term developmental effects from exposure during pregnancy, lactation and prepubertal phases, as well as effects on juvenile and adult offspring will be assessed, by efficiently integrating several endpoints covering the whole reproductive cycle (from gametogenesis through to maturation of the following generation) as well as preliminary assessment of additional more-specific endpoints (i.e. developmental neurotoxicity and developmental immunotoxicity). According to OECD Guidelines (TG 443 (2012a)), the selected parameters to be measured in the EOGRTS fall into the following categories:

- reproductive endpoints
- developmental (prenatal and postnatal) endpoints
- specific endpoints (developmental neurotoxicity, immunotoxicity and endocrine disruption).

With the additional parameters evaluated in the F1 generation in the EOGRTS, it is expected that the F2, with their limited parameter assessments, would seldom affect the hazard characterisation for risk assessment (Piersma et al., 2011). When predicted human exposures are considered adequately characterised, however, this may be factored into the decision to require the assessment of an F2 generation.

In devising appropriate Step 3 additional testing, a case-by-case approach should be adopted with careful consideration given to all available data as well as animal welfare issues. Step 3 testing is triggered by results in Step 2 studies and might be comprised of additional studies for. e.g. endocrine, developmental neurotoxicity (OECD TG 426 (2007)), and mode-of-action studies that could include both guideline studies and experimental studies designed on a case-by-case basis.

- Whether tests for reproductive and developmental toxicity are necessary should be decided in the light of the toxicity data and toxicokinetics information.
- Step 3 testing for reproductive and developmental toxicity will be required if the Step 2 toxicokinetic study shows that the test material is systemically available in the test species (normally rodents) or suspected to be systemically available in humans – especially if the test material is able to reach reproductive organs.
- Testing for reproductive and developmental toxicity should comprise a prenatal developmental toxicity study (OECD TG 414) and EOGRTS (OECD TG 443) that addresses reproductive developmental (prenatal and postnatal) and other specific endpoints (developmental neurotoxicity, immunotoxicity and endocrine disruption)
- Where a multigeneration study is already available, it would be acceptable instead of an EOGRTS, provided that sufficient information on possible neurotoxicity and immunotoxicity is available from, e.g. an extended 90-day study (OECD TG 408).
- In devising appropriate Step 3 additional testing, a case-by-case approach should be adopted with careful consideration to animal welfare and all available data.

6.8.3. Immunotoxicity and Allergenicity

Immunotoxicity may manifest in the form of adverse effects on the structure and function of the immune system itself, or an adverse consequence (such as an allergic or autoimmune reaction) that may arise from the immune response. Most inorganic and small-molecule organic substances in conventional (non-nanomaterial) forms are not immunogenic, but may act as haptens and become immunogenic after binding to proteins. In addition, they may act as adjuvants for other immunogens. For example, aluminium compounds have been used as adjuvants in vaccines. Nanomaterial forms of these compounds may present different immunological behaviour compared with conventional materials.

In addition to being potentially immunogenic/antigenic themselves, nanoparticles may also act as 'Trojan horses' for other immunogens/antigens by binding them on to the particle surfaces and carrying them to immune cells (see Section 3). The state of current knowledge on the immunotoxicity of nanomaterials, and guidance on methods to evaluate this, will be described in a WHO publication, which has undergone public consultation in 2017 (DRAFT WHO, 2017 'Draft Environmental Health Criteria Document: Principles and Methods to Assess the Risk of Immunotoxicity Associated with Exposure to Nanomaterials', once the final document is published, an additional reference will be provided as a corrigendum).

The tiered approach to testing outlined in this present Guidance includes, at Step 2, a 90-day study in rats (OECD TG 408 (2017a)). This involves investigation of the effect of the nanomaterial on a number of parameters that may be indicative of an immunotoxic or immunomodulatory effect. These include: changes in spleen and thymus weights relative to body weight in the absence of overt toxicity, histopathological changes in these and other organs of the immune system (e.g. bone marrow, lymph nodes, Peyer's patches); changes in total serum protein, albumin:globulin ratio and in the haematological profile of the animals, i.e. the total and differential white blood cell counts.

The effects may be extended or, alternatively, seen for the first time in Step 3 studies, notably the EOGRTS (OECD TG 443 (2012a)), but also in chronic toxicity/carcinogenicity studies conducted according to OECD TGs 452 (2009d), OECD 451 (2009c) or OECD 453 (2009e). In the EOGRTS, a cohort of animals is specifically dedicated to assessing the potential impact of exposure on the developing immune system. In subchronic and chronic studies, haematological and clinical chemistry data are generally provided, together with phenotypic analysis of spleen cells (T-, B-, NK cells) and bone marrow cellularity. The EOGRTS provides additional information on the primary immunoglobulin M (IgM) antibody response to a T-cell-dependent antigen such as sheep red blood cells (SRBC) or keyhole limpet hemocyanin (KLH).

Evaluation of the potential of a nanomaterial to adversely affect the immune system may be based on an integrated assessment of the results obtained from these toxicity studies (Steps 2 and 3). If these results indicate that the nanomaterial has such a potential, additional Step 4 studies should be considered, on a case-by-case basis. These will normally be designed to investigate the underlying mechanisms of the effects seen, and/or their biological significance. Step 4 studies may include specialised functional, mechanistic, and disease model studies. There are no OECD guidelines for these extended specialised studies, but these should be based on IPCS.

At present, there are no data or validated studies in laboratory animals that would allow assessment of the potential of a substance to cause allergic reactions in susceptible individuals following oral exposure. Where the nanomaterial is a potential allergen (e.g. a protein or a peptide) or contains residues of proteins or other known potential allergenic molecules, the principles discussed in the EFSA Guidance on the Allergenicity of GMOs should be followed in evaluating allergenic components. These principles for the determination of allergenicity include the investigation of structural aspects of the protein or peptide, *in silico* (or bioinformatics) approaches, immunoglobulin E (IgE) binding and cell-based methods, analytical profiling techniques and animal models (EFSA GMO Panel, 2010; EFSA NDA Panel, 2014). Since no single experimental method yields decisive evidence for allergenicity and allergic responses, a weight-of-evidence approach taking into account all the information obtained from various test methods is recommended.

- Potential immunotoxicity should be investigated where data relating to the likely route(s) of exposure indicate either systemic availability of the nanoparticles, or a potential for local contact with the immune cells. This should receive particular emphasis if the nanomaterial is (entirely or partly) composed of peptides/proteins, or may have an immunogenic/antigenic moiety adsorbed/attached to the particle surface.
- The potential of nanoparticles to act as a 'Trojan horse' for carrying other immunogens/ antigens on to particle surfaces to immune cells should also be investigated.
- A thorough consideration of the manufacturing process and formulation steps is necessary because of potential changes in the secondary, tertiary or quaternary structure of proteins.
- Consideration should be given to the parameters investigated in the Step 2 90-day study that may be indicative of an immunotoxic or immunomodulatory effect. Such effects should also be focused on in Step 3 studies (EOGRTS as well as chronic toxicity/carcinogenicity studies).
- If the results indicate adverse effects on the immune system, additional Step 4 studies should be considered, on a case-by-case basis.
- Where the nanomaterial is a potential allergen (e.g. a protein or a peptide), or contains/carries residues of allergenic entities, the principles discussed in the EFSA Guidance on Allergenicity of GMOs (2017) should be followed to evaluate the allergenic components.

6.8.4. Neurotoxicity

Indications of potential neurotoxic effects of a test substance are obtained from the modified 90-day toxicity study (Step 2b). This study involves investigation of the effect of the nanomaterial on a number of parameters that may be indicative of a neurotoxic effect. These include changes in clinical signs, functional observational battery and motor activity, brain weight relative to body weight in the absence of overt toxicity, and histopathological changes in this organ. Other information, such as read-across considerations or physicochemical properties that are indicative of neurotoxic potential should also be considered. Where indications of potential neurotoxicity are seen at Step 2, further neurotoxicity testing (OECD TG 424 (1997a)) should be considered. Such testing should be carried out on a case-by-case basis and is intended to confirm or further characterise (and quantify) the potential neurotoxic response induced by the nanomaterial. Information from other studies should also be considered in designing these studies to minimise confounding effects secondary to general toxicity. Further specialised studies can also be performed to elucidate mechanisms to improve extrapolation from animals to humans when completing the risk assessment.

- Indications of the potential neurotoxic effects should be deduced from the modified 90-day study (Step 2b). Other information, such as results of *in vitro* screening tests, read-across considerations or physicochemical properties, that are indicative of neurotoxic potential, should also be considered.
- Where indications of potential neurotoxicity are noted at Step 2, further neurotoxicity testing (OECD TG 424) should be considered on a case-by-case basis.

6.8.5. Endocrine activity

Evidence exists that several types of nanoparticles may adversely affect the endocrine system, and in particular the male and female reproductive systems (Iavicoli et al., 2013; Larson et al., 2014). Hormone alterations also appear to play a role in other toxic effects of nanoparticles with sex-related patterns (Tassinari et al., 2014; Ammendolia et al., 2017). However, the role of endocrine-related modes of action in the toxic effects of nanomaterials is largely unknown and unexplored, and warrants further investigation.

The starting point for investigating endocrine disruptive properties is the design of the modified 90-day toxicity test (OECD TG 408 with extended parameters from the OECD TG 407 (2008)) (preliminary reference (OECD TG 408 (2017a)). 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.

For further testing, the EFSA Scientific Opinion of 2013 (EFSA Scientific Committee, 2013) describes scientific criteria for the identification of endocrine disruptors (EDs) and the appropriateness of existing test methods for assessing effects mediated by these substances on human health and the environment (mainly based on OECD TG 150 (2012b)). To distinguish between EDs and other groups of substances with different modes of action, it was concluded that an ED is defined by three criteria: the presence of (i) an adverse effect in an intact organism or a (sub)population; (ii) an endocrine activity; and (iii) a plausible causal relationship between the two.

- The design of the modified 90-day study (Step 2b) should provide a starting point for investigation of endocrine-disrupting properties, with additional parameters on endocrine-related endpoints, and optional assessment of oestrous cycles.
- Further testing should follow the scientific criteria provided in the EFSA opinion (2013) for the identification of endocrine disruptors and the appropriateness of existing test methods.

6.8.6. Gut microbiome

Currently, there are limited data on the interaction of nanoparticles with the gut microbiome (e.g. Frohlich and Frohlich, 2016; Bouwmeester et al., 2018), but further research is ongoing. For nanomaterials with antibacterial properties (Hadrup et al., 2012), the possibility of interactions and on the composition of the microbiome and the mucus should be considered, especially for unabsorbed nanoparticles (Fröhlich and Roblegg, 2012; Mercier-Bonin et al., 2016).

The consequences of interactions with the gut microbiota on systemic or local tissues or physiological processes (whether direct or indirect) should be identifiable in the examinations that are part of the modified 90-day toxicity study. Currently, there are neither specific tests for this nor a clear understanding of the significance of changes in composition of the microbiome or their effects on health and further research would be valuable.

- Consequences of interactions of nanomaterials with the gut microbiota on systemic or local tissues or physiological processes (whether direct or indirect) should be identified through observations made during the modified 90-day study.
- Studies on the composition of the microbiome and the mucus might be required for nanomaterial – especially for those that have antimicrobial effects.

6.9. Considerations when testing nanomaterial

Appropriate *in vitro* and *in vivo* studies may be undertaken to identify and characterise hazards. Some of the currently available testing methods may also need adapting to take account of the specific properties of nanomaterials. The following paragraphs provide general considerations for testing nanomaterials, whereas the Sections 6.9.1 and 6.9.2 provide specific information for *in vitro* or *in vivo* testing respectively.

In both *in vitro* and *in vivo* testing, it is recommended to **check the structure/properties** of the nanomaterial in the test medium (e.g. particle agglomeration/aggregation).

For hazard characterisation, mass is a convenient **metric to express the concentration/doses** used for *in vitro* and *in vivo* (oral) studies. Mass is not always the best dose metric to describe the

response but is usually the only practicable one in the laboratory. By having the number–size distribution and density, the concentration/dose can be transformed as necessary. Other metrics such as specific surface area can also be derived from the nanomaterial characterisation and might be considered.

Although, studies with abnormally **high**[30] **concentrations/doses** have been published, the SC notes that this should be avoided. The use of high concentrations or doses enhances the risk of altering the size distribution of the material, by agglomeration and, as a result, lowers absorption and toxicity. Unrealistically high dosing of particles can also lead to outcomes that may not be related to the inherent toxicity of the material, but rather to the high amount of material administered. The choice of dose levels should therefore be carefully considered and a justification of the selected doses provided. In addition, the physicochemical characteristics of the test material at the higher doses should be checked to detect whether any substantial alteration occurs (in particular, the formation of secondary particles like agglomerates).

Critical to the interpretation of studies (especially those with negative results) is **the demonstration that cells (*in vitro*) and tissues (*in vivo*) were exposed to the nanomaterial**, i.e. that the nanomaterials actually came into contact with the cells/tissues. While this provides considerable technical challenges due to the limitations of current methodologies, the lack of such evidence does represent a significant uncertainty in reaching a definitive conclusion. In addition, whenever technically feasible, it should be determined whether nanomaterial distribution occurs in specific compartments of tissues or cells, which might modulate their biological effects.

In evaluating and interpreting results from studies on nanomaterials, there should be consideration of whether a plausible **mode of action** can be envisaged. Whenever feasible, an experimental group exposed to the corresponding non-nanomaterial (if available) should be included (in both *in vivo* and *in vitro* studies).

Where possible, an experimental **control** group exposed to the corresponding **non-nanomaterial** should also be included both in *in vivo* and *in vitro* studies.

The SC is aware that **corona formation on** particle surfaces occurs in test systems. Physical and chemical interactions with proteins and/or other biomolecules (e.g. phospholipids, sugars, nucleic acids, etc.) are always present and may play a role in nanomaterial fate and/or toxicity. However, corona formation is still difficult to measure. Corona formation can affect the state of agglomeration and sedimentation as well as the overall biological identity of nanomaterials (Cedervall et al., 2007; Lundqvist et al., 2008). The formation of the corona is a dynamic process, with the composition changing over time, governed by the abundance of proteins in the blood plasma and their binding affinities. Certain components of the corona (e.g. opsonins) might activate the mononuclear phagocytic system (macrophages) and induce a subsequent nanomaterial clearance from the organism. In view of the present limitations in measurement and interpretation, information on corona formation and its characterisation is not mandatory, but may be taken into consideration.

- The test material should be checked to ensure that there is no substantial alteration in physicochemical characteristics under test conditions (e.g. particle agglomeration/ aggregation).
- Mass-based dose metric is applicable to nanomaterials, but it is advisable to also consider other metrics such as particle number and specific surface area.
- Exposure of the tests system to the test material must be demonstrated, especially for negative results to be considered valid.
- A justification on the selected doses should be provided.
- Consideration should be given to whether a plausible mode of action can be deduced.
- Where possible, an experimental group exposed to the corresponding non-nanomaterial should also be included in both *in vivo* and *in vitro* studies.

[30] Drasler et al. (2017) noted that in mechanistic studies, unrealistically high nanomaterial concentrations (used both in *in vivo* rodent and – even to a higher extent – in *in vitro* assays) are sometimes required for determination of both the effect and no-effect levels of nanomaterials. However, for assessment of potential nanomaterials hazard to humans, nanomaterial concentrations should be selected based on realistic human exposure measurements, and based on principles of toxicological study design.

6.9.1. Specific issues for *in vitro* studies

Although 'validated' *in vitro* methods specifically **for nanomaterials** are not currently available, the results of '**valid**' methods may be considered for hazard identification (See Glossary for both terms).

In vitro studies with nanomaterials require extra attention to the suitability of the test methods for the purpose, e.g. test reagents used for standardised toxicity tests might react with the nanomaterial or the read-out signal of the test.

Additional quality **controls** should include (where available), negative and positive reference nanomaterials, assay reagent controls (such as the dye) and the non-nanomaterial controls.

It also has to be shown that the target cells were exposed to the nanomaterial along with the determination of the **number-based size distribution and concentration** of nanomaterials at the start (and end if applicable) of *in vitro* testing. These should be measured in the exposure medium using an appropriate method (see Section 4.3.2). Models based on DLS and density have been proposed to estimate how much nanomaterial is reaching the cell system (DeLoid et al., 2017a). Other models exist and can also be used to assess if the cells are truly exposed (e.g. Hinderliter et al., 2010).

For nanomaterials, the nominal concentration/dose may not be representative of the concentration/dose reaching the cells. Therefore, the assessment of the **dose delivered** to the cell system (Rischitor et al., 2016) and the internalised dose (the fraction of nanomaterials internalised by the cells) is highly recommended to allow better interpretation of the results and for comparison with or extrapolation to *in vivo* situations. In *in vitro* tests a series of concentrations should be used, keeping in mind the response–concentration range, and their choice justified. Exclusive use of high concentrations that lead to extensive agglomeration/aggregation should be avoided as well as conditions leading to sedimentation of the material.

At least **two independent *in vitro* assays (see Section 6.5) per individual endpoint** need to be selected.

The possible interference of nanomaterials with *in vitro* test systems also has to be taken into account, e.g. with assay components (reagents, proteins, nutrients), and with optical read-out system (e.g. dyes) (Kroll et al., 2012; Guadagnini et al., 2015). Case-by-case background controls, e.g. cell-free medium, all the reagents and the nanomaterials, should also be included and processed in the result interpretation. These **background corrections** should also take into account any changes in the dispersion status of the nanomaterial during and after the test, because this might influence their degree of interference with a test system based on optical measurements.

Specific issues with *in vitro* digestion are addressed in Section 6.4.

- Quality controls should include (where available) negative and positive reference nanomaterials, assay reagent controls, and the non-nanomaterial controls.
- Exposure of the target cells to the nanomaterial must be demonstrated, along with the number based size distribution and concentration of nanomaterials at the start (and end if applicable) of *in vitro* testing.
- Assessment of the dose delivered to the cell system and that internalised is highly recommended to allow better interpretation of the results and for comparison or extrapolation to *in vivo* situations
- At least two independent *in vitro* methods per individual endpoint should be performed.
- It is important to consider possible nanomaterial interference with the assay reagents and to implement necessary background and reference material control experiments.

6.9.2. Specific issues for *in vivo* studies

Nanomaterials are potentially unstable when dispersed. In oral toxicity studies, the test material can be administered by adding it to the **animal feed, the drinking water or by gavage**. For proper administration the nanomaterial should be homogeneously blended into the feed matrix or stably and uniformly dispersed in the drinking water or gavage vehicle. The **stability and physicochemical characteristics of the nanomaterial in the vehicle** should always be determined (see Section 4.3). Possible interactions with the administration vehicle, either the food matrix or water, need to be determined in advance before *in vivo* administration. This may require **dispersions for testing to be prepared freshly** and used immediately after preparation. **Complete delivery** of the dose should be checked because a nanomaterial may, for example, adsorb

to the walls of the drinking vessel or the gavage syringe and therefore may no longer be available (i.e. there is no exposure) (Kreyling et al., 2017).

Application by gavage ensures that the nanomaterial is dispersed, characterised and administered under well-defined conditions. It is known that this method of administration can give a fairly precise dose of nanomaterial delivered to the animal and a well-characterised degree of dispersion (Kreyling et al., 2017).

On the other hand, application by gavage is not likely to be representative of the lower concentrations delivered over time when the nanomaterial is administered via drinking water or feed, two ways that more closely simulate human dietary exposure. Gavage provides a bolus of nanomaterial at a given time. Absorption 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. In addition, when exposure to the nanomaterial is expected to happen via solid foods, the lack of co-ingestion of dietary components (with which a nanomaterial can interact) is another limitation of gavage. However, at the current state of knowledge, bolus **gavage** administration of the nanomaterial still might be **the method of choice** for identification and characterisation of hazards associated with the nanomaterial; this is because of the certainty of the administered dose and thus the dose–response relationship for possible adverse effects. In specific cases, and especially when exposure occurs mainly through solid and liquid foods, **additional groups with dietary or drinking water administration** have to be included to determine whether hazards associated with the nanomaterial are observed under more realistic exposure scenarios.

- Oral administration may be carried out through feed, drinking water or by gavage.
- Dispersions for testing should be prepared fresh and used immediately after preparation.
- Complete delivery of the dose should be ensured by avoiding the test material sticking to the walls of the drinking vessel or in the gavage syringe.
- In specific cases, especially when exposure occurs mainly via solid and liquid foods, additional groups with dietary administration should be included.

6.10. Integrated testing strategies

The SC notes the continuing development of **integrated testing strategies (ITS)** for non-nanomaterials and welcomes the promotion of alternative methods to acquire the information required in this Guidance, at the precondition that sufficient information is provided to assess the safety the material.

Alternative methods could fulfil the goal to refine, reduce or (partly) replace (the **3Rs**) current traditional toxicological approaches (see European Partnership for Alternative Approaches to Animal Testing since 2005; National Research Council, 2007; van Leeuwen et al., 2007). ITS comprise methods that can efficiently generate toxicological data for both hazard identification and risk assessment, combining *in vitro* tests, thresholds of toxicological concern, computational methods, read-across and chemical categories and exposure assessment, hereby aiming to reduce costs and minimise the need for experimental animals.

In 2012, the OECD launched the building of a toxicological knowledge framework based on mechanistic reasoning to support chemical risk assessment: The **Adverse Outcome Pathway (AOP)** programme. The OECD's AOP knowledge-based tools, continually developed and refined, are web-based platforms bringing together available knowledge on how **chemicals** can induce adverse effects.[31] Similarly, a view on future risk assessment was provided by the EU Scientific Committees SCHER, SCENIHR and SCCS, focussing on **organic chemicals**. These committees indicate that there is a need/trend to change the basis of human health risk assessment from the one based on standard tests to one that is centred on mode of action. To enable the most effective use of resources and to limit the unnecessary use of animals, a tiered approach to the assessment of hazards from exposure to individual stressors is proposed.

For nanomaterials, the need to efficiently obtain risk assessment information is high, considering that not only chemical composition but also various physicochemical properties may affect nanomaterial

[31] See (http://www.oecd.org/chemicalsafety/testing/adverse-outcome-pathways-molecular-screening-and-toxicogenomics.htm). This is currently being developed for non-nanomaterials, but the future development for nanomaterials would be welcomed.

exposure, toxicokinetic behaviour and hazard. General outlines on testing strategies and ITS have been developed (Dekkers et al., 2016; ProSafe, 2017; Oomen et al., 2018), but a harmonised and detailed approach is not yet available.

While acknowledging that there are still many knowledge gaps in the understanding of nanomaterial toxicity, Gerloff et al. (2017) discussed the **AOP** approach in nanotoxicology. AOPs can provide a mechanistic framework to assess specific adverse outcomes. Information obtained via omics methodologies may also inform or enrich AOPs (Nymark et al., 2018). EFSA is continuing the investigate the role of omics technologies in risk assessments.

OECD now also explores **Integrated Approach to Testing and Assessment (IATAS)** and promotes the use of AOPs to build risk assessment, while assessing all the existing data.

These developments in efficient testing strategies and AOPs for nanomaterials are highly acknowledged, though they need further development and verification before incorporation into guidance documents can be considered.

Studies on the mode of action (MOA) may be used to investigate the relevance to humans of findings in animals. These studies can examine the **mode of action** for carcinogenic effects or other endpoints such as endocrine disruption, and should use the appropriate MOA frameworks when assessing the data (Boobis et al., 2006, 2008; IPCS, 2006; Meek et al., 2013).

- ITS comprise methods that can generate toxicological data for both hazard identification and risk assessment through combination of *in vitro* tests, thresholds of toxicological concern, computational methods, read-across and chemical categories and exposure assessment.
- For nanomaterials, only general outlines ITS have been proposed so far and a harmonized and detailed approach still needs to be developed and verified.

7. Risk characterisation

The risk characterisation of nanomaterials considers the same steps as for conventional non-nanomaterial. This step combines hazard characterisation with exposure assessment. Risk characterisation is essentially an iterative process and should result in **a quantitative assessment, and if not possible qualitative**.

The output from the risk characterisation is the overall assessment of the **safety** of the nanomaterial in its intended use together with the parameters under which the assessment is valid and the **uncertainties** associated with the assessment.

Several approaches to generating the information required for risk assessment are described in this Guidance. At every stage in determining whether sufficient information has been generated, **a weight of evidence process** should be applied according to the EFSA Guidance to make a decision on whether an adequate risk assessment can be undertaken. If this is not considered possible, the default presumption is that a sequence of further tests should be undertaken. For an overview of existing weight of evidence approaches and their implementation, reference is made to the Guidance Document on Weight of Evidence (EFSA Scientific Committee, 2017b).

Risk characterisation of a nanomaterial considers the same elements as for conventional chemical substances – i.e. data and information relating to physicochemical properties, exposure, and toxicological effects. Where the data have been derived from appropriately conducted studies using validated methods and considering nanospecific issues where relevant, there may be no reason to use **higher uncertainty factors** for a nanomaterial than for a conventional material. However, where data are either insufficient or have been derived from inadequate tests for nanomaterials, applying additional uncertainty factors may be considered for safety assessment.

- The output from the risk characterisation should be in the form of an overall assessment of the safety of the nanomaterial in its intended use, together with the description of the parameters under which the assessment is valid and the uncertainties associated with the assessment.
- At every stage where information is assessed, a weight of evidence process should be applied to decide whether an adequate risk assessment can be undertaken. Risk assessment can be performed at any stage where the totality of the available information is adequate, and no further testing is necessary.
- Where the data have been derived from appropriately conducted studies using validated methods and considering nanospecific issues, there should not be a reason for the use of any higher uncertainty factors for a nanomaterial than those used for a conventional material. However, where data are either insufficient, or have been derived from inadequate tests for nanomaterials, application of additional uncertainty factors may be considered for safety assessment.

8. Uncertainty analysis

To meet the general requirements for transparency, all EFSA scientific assessments must include consideration of uncertainty. The Guidance on Uncertainty in EFSA Scientific Assessment is applicable to all areas of EFSA and all types of scientific assessment (EFSA Scientific Committee, 2018). The Scientific Committee had also 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 Scientific Committee, 2009c).

That opinion makes a number of general recommendations on how to handle uncertainties in risk assessment that should also be addressed in nanomaterial risk assessment. The Scientific Committee has also adopted a Guidance related to uncertainties in dietary exposure assessment that includes practical approaches on how to handle uncertainties in risk assessment that will also be applicable in nanomaterial risk assessment (EFSA Scientific Committee, 2006).

These principles and recommendations are also applicable to risk assessment of nanomaterials.

8.1. Uncertainty in the scope

Uncertainty about the legal framework remains and can be reported. Until the European Commission recommended definition of nanomaterial is finalised in food law (e.g. specifying the size range of 1 nm to −100 nm and the threshold (of 50%)), it will remain unclear whether or not a given material is covered under a particular regulation. As a consequence, it remains unclear as to which materials shall be subject to nanospecific risk assessment as outlined in this Guidance. The Scientific Committee therefore advises that this Guidance is taken into account where a material exhibits size-related properties, even if it does not strictly fall within the defined size range or threshold.

8.2. Measurement uncertainties in the physicochemical characterisation of nanomaterial

Uncertainties in the measurement results arise within the analytical process of nanomaterial characterisation, i.e. sampling, sample preparation, instrumental analysis, data handling and evaluation of results, similar to conventional analytes. Measurement uncertainties should be reported as combined uncertainties for the entire process. They can be derived in the course of the validation process (see Section 4.4.2). The measurement uncertainty can be estimated as

$$u_c = \sqrt{\frac{s_r^2}{n} + \frac{s_d^2}{d} + u_t^2}$$

u_c is the combined uncertainty, s_r is the repeatability standard deviation, n is the number of replicates performed for the measurement, s_d is the between-day standard deviation, d is the number of days, over which the n replicates were spread, and u_t is the uncertainty of the recovery determination (Linsinger et al., 2013). Guidance for the determination and expression of measurement uncertainty is widely available, e.g. from ISO (2008).

While calculation of measurement uncertainty is the same for nanomaterial and conventional analytes the contribution of the individual sources may be different in quantity and there may be additional sources in nanomaterial characterisation. For example, the particle size distribution may not only be affected by instrument uncertainties, but also by agglomeration effects.

Accuracy of the available characterisation methods is dependent on the target nanomaterial, the matrix, sample preparation procedures and calibration of the analytical equipment against appropriate reference materials (e.g. calibration standards). The results obtained by various measurement techniques may nevertheless differ because of their method-defined nature (Domingos et al., 2009).

It is therefore essential to specify the procedures used (e.g. type of sample preparation, technique applied for size measurement) and to provide information on their analytical performance (validation study) and the combined measurement uncertainty. The specificities of individual characterisation techniques and the measurement uncertainty of the applied analytical process have to be taken into account to decide if a material is or is not regarded as a nanomaterial. The expanded measurement uncertainty U ($U = u_c \times k$) should be applied in order to avoid potential risks. A coverage factor $k = 3$ should be used that corresponds to greater than 99% confidence.

8.3. Uncertainties in exposure assessment

Exposure assessment is an integrated part of scientific assessments performed by EFSA. There are established procedures for exposure assessment in different areas of EFSA's work. Every dietary exposure assessment is affected by scientific uncertainties and it is important for assessors to characterise the extent of uncertainty so it may be taken into account by risk managers.

When it is not possible to characterise the form in which the nanomaterial substance is present in food and/or feed applications, uncertainty in exposure assessment will be increased. This uncertainty could be reduced by characterisation of the nanomaterial in the food/feed or liquid food/feed products according to intended or existing applications.

Exposure assessments should systematically examine potential sources and types of uncertainty. The assessment of uncertainties in exposure assessment should follow principles in the EFSA Guidance of the Scientific Committee related to uncertainties in dietary exposure assessment (EFSA Scientific Committee, 2006), and updated recently (EFSA Scientific Committee, 2018).

In case the tested nanomaterial is readily biodegradable, the decision to not perform any *in vivo* toxicity study specific for nanoparticles, may result in uncertainty due to the possibility of local effects. Such local effects may be deriving from an exposure to nanoparticles particularly in the mouth and in the upper gastrointestinal tracts, especially when the nanomaterial is ingested following dispersion in the food product.

8.4. Uncertainties in the hazard characterisation of the nanomaterial

Limited information is available in relation to aspects of nanomaterial toxicokinetics and toxicology, including optimal testing methods. 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 nanomaterial in currently applied standard testing protocols and test animals. There may also be additional toxic effects caused by nanomaterials that are not readily detectable with 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 for use in hazard assessments of nanomaterials.

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

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

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

8.5. Uncertainties in the risk characterisation

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

Lack of or inadequate characterisation of the nanomaterial test substance is a source of uncertainty in studies for hazard identification and characterisation in which identified NOAELs or calculated BMDLs are used to derive health-based guidance values (HBGVs) subsequently used in risk characterisation.

Uncertainty will increase when the available data on characterisation of the nanomaterial test substance used in studies for hazard identification and characterisation are insufficient to conclude that the tested nanomaterial and its form are comparable to those present in food products or commercial feed. Depending on the circumstances, the risk characterisation may under- or over-represent the risks. These uncertainties could be reduced by use of a test nanomaterial that is sufficiently characterised (see Section 4), and the form in which it is present in the feed or liquid matrices in animal studies would closely mimic the form in anticipated (as described in an application dossier) or existing food/feed products, the exposure to which has to be assessed in order to perform risk characterisation.

As with conventional risk assessment, the HBGV 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 intraspecies differences should be applied, as currently there are no indications for a need to modify these factors.

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 accordance with EFSA Guidances on weight of evidence and biological relevance (EFSA Scientific Committee, 2017b,c). Uncertainties in risk assessment should be stated and their impact on risk assessment analysed in accordance with EFSA guidance on uncertainty in risk assessment (EFSA Scientific Committee, 2018). 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 risk assessment is completed in accordance with the EFSA Guidance on transparency in the scientific aspects of risk assessment (EFSA Scientific Committee, 2009c).

- EFSA Scientific Committee (2018) provided general principles and general recommendations for the identification of uncertainties in dietary exposure assessment, and in regard to data sources, criteria for data inclusion/exclusion, confidentiality, assumptions and uncertainties. These principles and recommendations are also applicable to risk assessment of nanomaterials.
- 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.
- Uncertainties should be reported.
- Similar to conventional analytes, the uncertainties in the measurement results for nanomaterials should be described in relation to the analytical process used for characterisation, i.e. sampling, sample preparation, instrumental analysis, data handling and evaluation of results.
- It is essential to specify the procedures used and to provide information on the analytical performance and combined measurement uncertainty.
- Uncertainties relating to any limited information on toxicokinetics and toxicology, including test methods must be highlighted. Uncertainties arising from the lack of validated *in vitro* assays for nanomaterials should also be highlighted.
- Potential sources and types of uncertainty in exposure assessment should be systematically examined.
- 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 for nanomaterials.

- Any lack of data essential for the risk assessment should be indicated and the quality of the data used should be reported in accordance with EFSA guidance on weight of evidence and EFSA guidance on biological relevance.

9. Conclusions and Recommendations

- The use of a nanomaterial in food/feed and related applications will need to be assessed for safety to fulfil requirements of the relevant EU food laws, and in accordance with the provisions of this Guidance.
- Irrespective of the presence of a nanomaterial, the existing requirements for safety assessment according to EFSA Guidances for conventional non-nanomaterials under relevant regulations must be followed.
- The existing risk assessment paradigm for chemicals is also applicable to nanomaterials. However, testing of nanomaterials needs consideration of certain nanospecific aspects that have been highlighted in this Guidance.
- The Guidance proposes a structured pathway for carrying out safety assessment of nanomaterial in food/feed and related applications, and provides practical suggestions for the types of testing needed and the methods that can be used for this purpose.
- The Guidance also highlights certain gaps where further research is needed to facilitate adequate safety assessment of materials that consist of small-sized particles.
    - Although a definition of engineered nanomaterial currently exists under the Regulation on Novel Food and Regulation on Food Information to Consumers a possible revision of the existing definition in the light of the European Commission Recommendation may provide more clarity on the type of materials to be covered.
    - Currently, there is no agreed definition of the term 'nanopesticides' (see Appendix E). Clarification is needed to identify any relevant active substances and formulations that may be required to undergo nanospecific safety assessment.
    - More work is needed on analytical methods and techniques that can be used for characterisation of nanomaterials in complex matrices.
    - Further investigations are required on methods for measuring reactivity of nanomaterials.
    - More work on validation of relevant *in vitro* methods, including *in vitro* degradation, is needed to ascertain their applicability to nanomaterials.
    - More work is needed to strengthen the conceptual application of grouping and read-across and other *in silico* (computational) modelling approaches for use in risk assessment of nanomaterials.
    - The limited availability of reference materials should be addressed because reference materials are essential for ensuring quality and reliability of data.
    - For nanomaterial pesticides, specific recommended Guidance is provided in Appendix E. The Scientific Committee strongly suggests that regulatory authorities take note of this recommended Guidance. It is suggested that during the next update of the legal data requirements consideration is given to Appendix E.2.

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Abbreviations

AAS	atomic absorption spectroscopy
ADME	absorption, distribution, metabolism and excretion
AES	atomic emission spectroscopy
AFM	atomic force microscopy
ANS	Panel on Food Additives and Nutrient Sources Added to Food
AOP	adverse outcome pathway
AUC	area under the plasma concentration-time curve
BET	Brunauer Emmett Teller method
BMD	benchmark dose
BMDL	lower boundary of the BMD confidence interval
CAS	Chemical Abstracts Service
CEF	Panel on Food Contact Materials, Enzymes, Flavourings and Processing Aids
CEN	European Committee for Standardization
CFM	chemical force microscopy
CLS	centrifugal liquid sedimentation
CMR	carcinogenic, mutagenic, reprotoxic
CODATA-VAMAS	Committee on Data for Science and Technology - Versailles Project on Advanced Materials and Standards
CONTAM	Panel on Contaminants in the Food Chain
DESI	desorption electrospray ionisation
DG ENV	Directorate-General for Environment
DIN	German Institute for Standardization
DLS	dynamic light scattering
DMA/IMS	differential mobility analysis/ion mobility spectroscopy
ECHA	European Chemicals Agency

EDs	endocrine disruptors
EEA	European Economic Area
EINECS	European Inventory of Existing Commercial chemical Substances
ELISA	enzyme-linked immunosorbent assays
EM–EDX	electron microscopy–energy-dispersive X-ray spectroscopy
EM	technique electron microscopy technique
EMA	European Medicines Agency
EOGRTS	extended one-generation reproduction toxicity study
ESI	electrospray ionisation
ESZ	electrical sensing zone
EU FP7	European Union Seventh Framework Programme
FCM	food contact material/s
FEEDAP	Panel on Additives and Products or Substances used in Animal Feed
FFF	field flow fractionation
FTIR	Fourier transform infrared spectroscopy
GALT	gut-associated lymphoid tissue
GI	gastrointestinal
HBGVs	health-based guidance values
IATAS	Integrated Approach to Testing and Assessment
ICP-MS	inductively coupled plasma mass spectrometry
ICP-AES	inductively coupled plasma atomic emission spectrometry
ICR	ion cyclotron resonance
IgE	immunoglobulin E
IgM	immunoglobulin M
IPCS	International Programme on Chemical Safety
IR&CSA	Information Requirements and Chemical Safety Assessment
ISO/CEN	International Organization for Standardization/European Committee for Standardization
ITS	Integrated testing strategies
IUPAC	International Union of Pure and Applied Chemistry
JRC	Joint Research Centre
KLH	keyhole limpet hemocyanin
LA-ICP-MS	laser ablation inductively coupled mass spectrometry
LDH	lactate dehydrogenase
LOD	limit of detection
MALDI	matrix-assisted laser desorption/ionisation
MALS	multiangle light scattering
MOA	mode of action
MSSA	mass specific surface area
NDA	Panel on Dietetic Products, Nutrition and Allergies
NMIs	National Meat Inspection Service
NOAEL	no-observed-adverse-effect-level
OECD	Organisation for Economic Co-operation and Development
OES	optical emission spectroscopy
pH	potential of hydrogen
PPR	Plant Protection Products and their Residues
PLA	polylactic acid
QqQ	triple quadrupole
QSARs	quantitative structure –activity relationships
REACH	Registration, Evaluation, Authorisation and Restriction of Chemicals
RIVM	Dutch National Institute for Public Health and the Environment
RA	risk assessment
ROS	reactive oxygen species
SAXS	small angle x-ray scattering
SC	Scientific Committee
SCCS	Scientific Committee on Consumer Safety
SCENIHR	Scientific Committee on Emerging and Newly Identified Health Risks

SCHER	Scientific Committee on Health and Environmental Risks
SCOEL	Scientific Committee on Occupational Exposure Limits
SEM	scanning electron microscopy
SMPS	scanning mobility particle sizer
SOPs	Standard Operating Procedures
spICP-MS	single particle inductively coupled plasma mass spectrometry
SRBC	sheep red blood cells
SSA	specific surface area
STXM	scanning transmission X-ray microscopy
TEER	transepithelial electrical resistance
TEM	transmission electron microscopy
TEM–EDX	transmission electron microscopy–energy-dispersive X-ray spectroscopy
TG	Test guideline
ToF-SIMS	time-of-flight secondary ion mass spectrometry
US-EPA	United States Environmental Protection Agency
US-FDA	United States Food and Drug Administration
VSSA	volume specific surface area
WG	Working group
WHO	World Health Organization
WPMN	Working Party on Manufactured Nanomaterials
XPS	X-ray photoelectron spectroscopy
XRD	X-ray diffraction
XRF	X-ray fluorescence

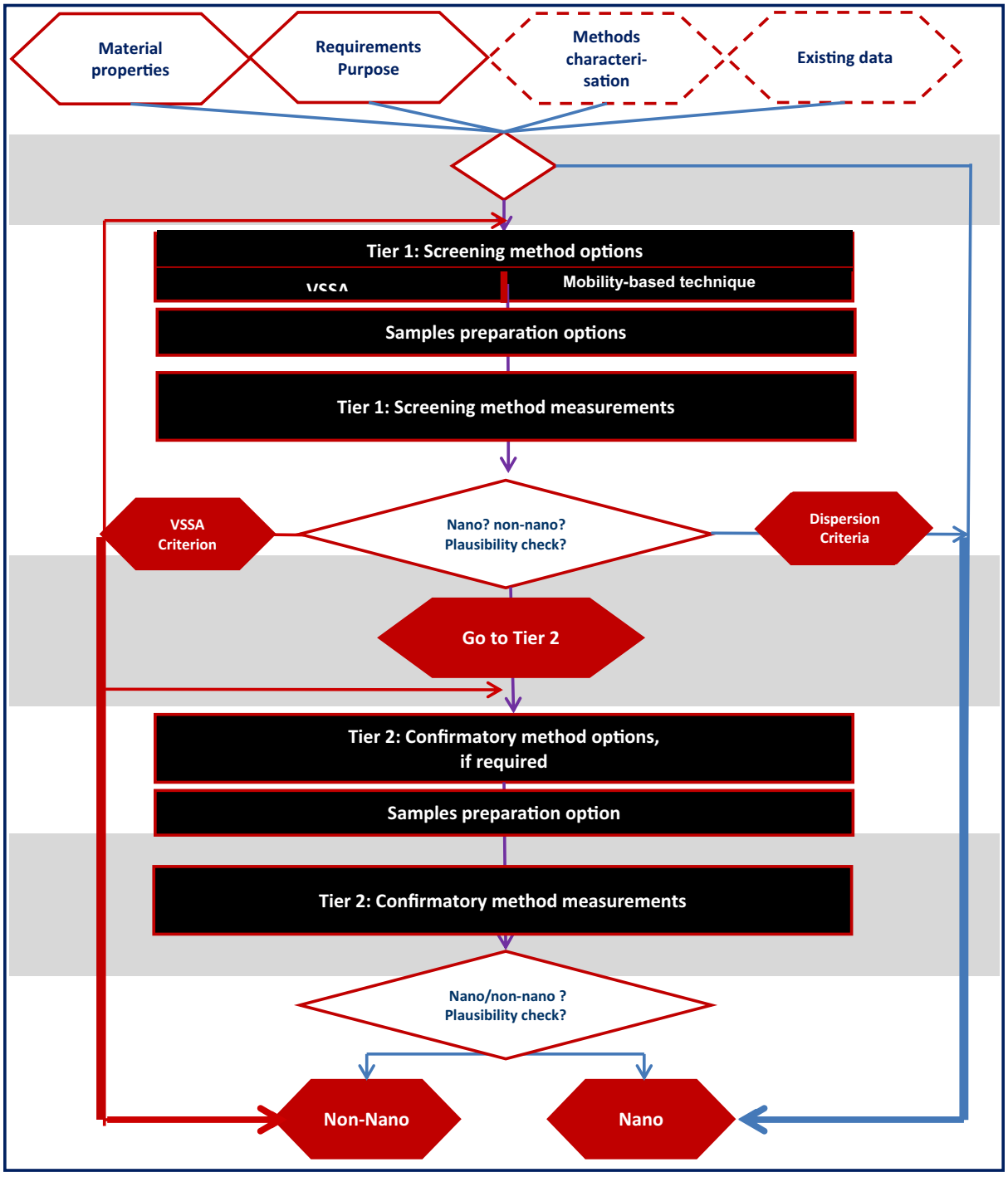
Glossary

ADME	Absorption, distribution, metabolism and excretion (elimination).
Agglomerate	'Agglomerate' refers to a collection of weakly bound particles or aggregates where the resulting external surface area is similar to the sum of the surface areas of the individual components.
Aggregate	'Aggregate' means a particle comprised of strongly bound or fused particles.
Chemically specific method	An analytical method verifying the chemical identity of the measured particles (e.g. spICP-MS or TEM-EDX).
Conventional material	See non-nanomaterial.
Degradation	Degradation as used herein is the process by which a nanomaterial is transformed to degradation products in the form of another nanomaterial (examples include photodegradation, oxidative degradation, etc.) or to solutes with the loss of nano features. A relevant example is the oxidative degradation of silver nanoparticles with the release of Ag^+ ions (i.e. dissolved form of silver).
Dissolution	Dissolution as used herein is the process by which a soluble nanomaterial in an aqueous medium or biological environment is converted to the constituent ions or molecules with the loss of nano features.
Dispersion	A system in which discrete particles are distributed in a continuous phase (e.g. a liquid) of a different composition. A poorly soluble nanomaterial introduced into a liquid forms a 'dispersion', where the liquid and the nanosized particles coexist.
Fullerene	A fullerene is a molecule composed entirely of carbon, in the form of a hollow sphere, ellipsoid, or tube. Spherical fullerenes are also called buckyballs, from buckminsterfullerene (a 60 carbon atom sphere).
High aspect ratio nanomaterials (HARN)	The aspect ratio of a shape is the ratio of its longer dimension to its shorter dimension. The length of a HARN is considerably longer than its width. Examples of HARN include materials such as carbon nanotubes (CNT) and metal nanowires.

Incidental presence	Alternative but not preferred terms are accidently present, unintentionally present, unintended presence, traces, etc.
In vivo	*In vivo* means in living organisms. For clarity, '*in situ ex vivo*' means in place, but in pathology that may also be seen as *in vivo*.
Lotus effect	A property of highly hydrophobic surfaces that creates a 'self-cleaning' effect.
Manufactured nanomaterial (ISO)	Nanomaterial intentionally produced for commercial purposes to have specific properties or a specific composition.
Micronisation	The process of reducing the average diameter of solid material particles by mechanical or other means. While the process usually aims to reduce the average particle diameters to the micrometer range, formation of particles in the nanoscale may also result.
Nanomaterial (EC)	Recommended definition is under review (see Section 1.2.2).
Nanomaterial (ISO)	Material with any external dimension on the nanoscale or having internal structure or surface structure on the nanoscale.
Nanoproperties	Examples include (but are not restricted to): size on the nanoscale, large surface area, high surface reactivity, quantum effects, possibility to translocate over biological membranes not observed in larger non-nanosized particles etc.
Nanoscale	A size measurement generally considered to refer to the size range 1–100 nm (e.g. Lövenstam et al., 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 on the nanoscale (where size- and structure-dependent properties and phenomena can emerge that are distinct from those associated with individual atoms or molecules, or with bulk materials.
Nanotechnology (ISO)	Application of scientific knowledge to manipulate and control matter on the nanoscale 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-nanomaterial	A material that is either in ionic, molecular or particulate form having a size above the nanoscale. In this guidance, the term non-nanomaterial is used for the conventional material in connection with the corresponding nanomaterial of the same chemical composition.
Nanosized degradation product	Is a degradation product in the form of a nanomaterial, meaning not in ionic or molecular form.
Pour density	A function of the degree of compaction during pelletisation.
Primary particle	For the purposes of this guidance, the term 'primary particle' is used for (i) individual particles which are not aggregates or agglomerates and (ii) constituent particles of aggregates/agglomerates which are not aggregates or agglomerates themselves. Agglomerates and aggregates are also termed secondary particles.
Solubility (OECD, ECHA)	The solubility of a substance in water is specified by the saturation mass concentration of the substance in water at a given temperature (kg/m^3 or g/L) (OECD, 1995; ECHA, 2017c), see also glossary on 'solution'. Solubility in relevant media requires description of the media and the conditions under which the measurements were made.
Solution	In a solution the solute does not exist as a solid, but is fully dissolved.
Valid methods	In toxicological testing, a method that has not necessarily gone through the complete validation process, but for which sufficient scientific data exist demonstrating its relevance and reliability (Based on Rogiers, 2003).

Validated method	A method for which the relevance and reliability are established for a particular purpose (in most cases according to the criteria established by EURL-ECVAM, taking into account that a prediction model needs to be present from the start of the validation procedure). (Based on Balls and Fentem, 1997; and Worth and Balls, 2001). These methods are taken up in Regulation (EC) No 440/2008 and/or published as OECD Technical Guidelines.

Appendix A – NanoDefine decision-flow scheme



Appendix B – Demonstration fact sheet for component 2

Table B.1: Descriptors and parameters for component 2 in Table 1 (of Section 4.2.1)

Information on the chemical component 2		
Component 1		
Chemical name Systematic/IUPAC name; chemical name	Where available systematic/IUPAC name of the substance that makes up component 1 of the nanomaterial should be provided. Alternatively, the chemical name that describes the chemical composition of the component should be provided based on the best available information – e.g. 'modified from XX' where XX = the nearest chemical name.	Silicon dioxide Silicon (IV) oxide
Trade name, common name, other names, synonyms Names	Any common names, synonyms, trade names and other names for the component should be provided	Silica, synthetic amorphous silica
CAS number **EINECS/EC number** **E number** **other registry numbers** Registry numbers related to the constituent substance, if available	CAS number, EINECS/EC number, E number or other registry/database numbers related to the component should be provided (where available).	CAS number: 7631-86-9 ECHA Info card: 100.028.678 EC number: 231-545-4 E number: E 551
Formula Molecular and structural formula (where applicable) of the constituent substance	Molecular and structural formula (where applicable) of the constituent substance should be provided	SiO_2
Molecular weight or atomic weight (for elements) [g/mol]	Molecular weight or atomic weight (for elements) [g/mol] should be provided for the component.	60.08 g/mol
Elemental composition Empirical formula of this component	The relative elemental composition of the component should be provided as the simplest positive integer ratio of atoms present in the material.	SiO_2
Crystal form Form and phase	Description of crystalline form (amorphous, polycrystalline, crystalline including specification of phase) should be provided, including any crystalline impurities	amorphous
Purity of the component Relative amount of the constituent in mass %; and name(s) and amount(s) of any impurities in mass %.	Relative amount of the constituent in mass %, as well as chemical identity of any impurities and their relative amounts in mass % should be provided.	SiO_2 97.6% $NaSO_4$ 1.4% Al_2O_3 0.3%
Production process component Name of the production process	The production process of the component should be described since it can have a significant effect on the properties of the nanomaterial, e.g. pyrogenic or precipitated silica, sulfate or chloride process for TiO_2	precipitation

Appendix C – Characterisation techniques

The techniques in Table C.1 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 a nanomaterial 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 4.4).

It should be noted that the list of techniques is not exhaustive and does not constitute a recommendation for any specific technique. The best suited technique depends largely on the material characteristics and on the specific intended use for the measured data. It is up to the responsibility of the applicant to select the appropriate measurement method. The fact that a specific technique is not listed in the table does not exclude it from being applied. The same holds for newly developed techniques. Applicants and risk assessors should refer to the most current reviews on the state of the art in characterisation techniques for nanomaterials. Rasmussen et al. (2017) provide a comprehensive overview on of current techniques and their use. Furthermore, the NanoDefine Methods Manual (Gaillard et al., 2015) also provides information on the use of techniques and outlines for which cases and materials (e.g. chemical composition, powder suspension etc.) which method is best suited.

Standardised methods should preferably be used if available and appropriate for the analytical task in question. Some examples of standard methods are given in the Table 1B. Mentioning these guidances (most of them not nanospecific) does not imply a recommendation. It is up to the applicant to check the most relevant and up to date guidance. Information on available standards is provided e.g. by ISO and CEN. ISO standards can be found via the on-line browsing platform which is searchable for the ISO definitions of terms and, also for standards on a specific subject (e.g. 'nano'): https://www.iso.org/obp/ui/#home

The work programmes and publications of the ISO Technical Committees (TCs) can also be consulted on their respective webpage, which can be found via the list of TCs. Most nanomaterial relevant standards are published in ISO/TC 229 (ISO, 2005), ISO/TC 201 (ISO, 1991) and ISO/TC 24/SC4 (ISO, 1981). https://www.iso.org/technical-committees.html

CEN TC 352 has a mandate from the EC (M/461) to develop a series of European standards and technical specifications in the area of nanotechnologies. The database search platform for CEN is available at: https://standards.cen.eu/dyn/www/f?p=CENWEB:105::RESET::::

Table C.1: Examples of characterisation techniques

Item	Suitable techniques	Examples of Guidances
Chemical composition/ identity, purity, surface chemistry, mass concentration	Elemental composition	
	Atomic absorption spectroscopy (AAS)	
	Inductively coupled plasma-optical/atomic emission spectroscopy (ICP-OES/AES)	
	Inductively coupled plasma-mass spectrometry (ICP-MS)	ISO 13278
	X-ray fluorescence spectroscopy (XRF)	
	Energy-dispersive X-ray spectroscopy (EDX)	ISO 22489
	Electron energy loss spectroscopy (EELS)	
	X-ray photoelectron spectroscopy (XPS) (surface analysis)	ISO/TR 14187, ISO 18118
	Auger electron spectroscopy (surface analysis)	ISO/TR 14187, ISO 24236
	X-ray photoelectron spectroscopy (XPS)	ISO/TR 14187, ISO 18118
	Auger electron spectroscopy	ISO/TR 14187, ISO 24236
	X-ray fluorescence spectroscopy (XRF)	

Item	Suitable techniques	Examples of Guidances
	Energy-dispersive X-ray spectroscopy (EDX)	ISO 22489
	Electron energy loss spectroscopy (EELS)	
	Molecular composition	
	Nuclear magnetic resonance spectroscopy (NMR)	
	UV/VIS absorption spectroscopy	ISO 17466
	Fourier transform infrared spectroscopy (FT-IR), Raman and other molecular spectroscopies	
	Mass spectrometry (MS) (coupled with separation methods, e.g. HPLC, GC, CE, etc.):	
	– Time of flight (ToF)	
	– Triple quadrupole (QqQ),	
	– Fourier transform ion cyclotron resonance (FT-ICR-MS, Orbitrap)	ISO/TS 14101
	– Secondary ion MS (SIMS)	ISO 13084
	using suited ionisation techniques, e.g.:	
	– Matrix-assisted laser desorption/ionisation (MALDI)	
	– Electrospray ionisation (ESI)	
	– Direct analysis in real time (DART)	
	– Desorption electrospray ionisation (DESI),	
	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.)	
Particle size and size distribution; agglomeration/ aggregation state	***Microscopy techniques***	
	– Transmission electron microscopy (TEM)	ISO/WD 21363ISO 13322-1ISO 29301
	– Scanning electron microscopy (SEM)	ISO/WD 19749ISO 13322-1ISO 16700
	– Scanning transmission electron microscopy (STEM)	
	– Atomic force microscopy (AFM)	ISO 25178 seriesIEC 62622
	– Scanning transmission X-ray microscopy (STXM)	
	Separation techniques (coupled with suitable detectors):	
	– Field flow fractionation (FFF)	ISO/TS 21362 (to be published in 2018)
	– Hydrodynamic chromatography (HDC)	
	– Size exclusion chromatography (SEC)	
	– High-performance liquid chromatography (HPLC)	
	– Differential mobility analysis/ion mobility spectroscopy (DMA/IMS)	ISO 15900ISO 28439
	Centrifugation techniques:	
	– Centrifugal liquid sedimentation (CLS)	ISO 13318 series
	– Analytical ultracentrifugation (AUC)	
	Scattering techniques	
	– X-ray diffraction (XRD) (for crystal size, crystallite size)	ISO 22309
	– Small angle x-ray scattering (SAXS)	ISO17867
	– Laser diffraction methods	ISO 13320
	– Dynamic Light scattering (DLS)	ISO 22412
	– Multiangle light scattering (MALS)	ISO 18196

Item	Suitable techniques	Examples of Guidances
	– Light scattering airborne and liquid-borne particle counters	ISO 21501 series
	– Particle tracking analysis (PTA)	ISO 19430
	other techniques	
	– Single particle ICP-MS	ISO/TS 19590
	– Condensation particle counter (CPC)	ISO 27891
	– Acoustic methods	ISO 20998 series
	– Electrical sensing zone (ESZ)	ISO 13319
	– Resonant mass technique (Archimedes)	
Shape	Microscopy techniques – TEM – SEM – STXM – AFM	ISO 16700 ISO 25178, ISO/TS 11888, ISO 9276-6
	Diffraction	
Crystal form and phase	XRD	EN 13925-1, -2, -3
Particle concentration	Light scattering airborne and liquid-borne particle counters	ISO 21501 series
	Single particle ICP-MS	ISO/TS 19590
	Scanning mobility particle sizer (SMPS)	
	CPC	ISO 27891, CEN EN 16897
Surface area (volume, mass specific)	Adsorption isotherms methods, e.g. Brunauer Emmett Teller method (BET)	ISO 9277, ISO 15901-2/-3, ISO 18757
	Liquid porosimetry	ISO 15901-1
Surface charge	Electrophoretic light scattering (ELS)/zeta potential	ISO 13099 series
	Capillary electrophoresis (CE)	
	Electroosmosis	
	Electric sonic amplitude	
	Colloidal vibration current	
Degradation/ Dissolution/ Solubility	Standard tests for water solubility Degradation rate constants	e.g. OECD TG 105
Chemical reactivity	Kinetic measurements of the chemical, biochemical reactions	
Catalytic activity	Kinetic measurements of the catalysed reactions, including photocatalytic activity (where applicable)	
Density - Apparent (Bulk) powder density)	Gravitational sedimentation; centrifugal sedimentation (for suspensions for submicrometre and nanoparticles).	OECD TG 109 DIN ISO 697, EN/ISO 60
Density - Effective (hydrodynamic) particle density	Gravitational sedimentation; centrifugal sedimentation (for submicrometre and nanoparticles)	ISO 18747 series
Dustiness	Standard methods	EN 15051:2006, DIN 33897-2
Viscosity	Standard methods	OECD TG 114

Appendix D – Uncertainty Analysis of high degradation rate

This Section provides the rationale for the cut-off value of the degradation rate that is used to decide whether a nanomaterial quickly degrades (i.e. has a high degradation rate) in the gastrointestinal tract and can therefore follow the safety assessment according to relevant EFSA guidance on non-nanomaterials (see Figure 2). Transparency on the rationale for the proposed cut-off value is important as this value is partly based on pragmatism. Further scientific knowledge may be used by the EFSA Scientific Committee to revise the cut-off value.

The time nanoparticles take to cross the mucus layer adhering to the gastrointestinal tract epithelium can be short, i.e. within minutes. For some particles, the mucus does not seem to inhibit the diffusion of particles smaller than 100 nm, whereas 500 nm particles display limited diffusion (Ensign et al., 2012; Bajka et al., 2015). This is assumed to be due to the pore size in net-like mucin sheets that was found to be about 200 nm by Bajka et al. (2015), and is considered to be about 100 nm by Fröhlich and Roblegg (2012). As an example, Szentkuri (1997) showed the ability of 14 nm latex particles to cross the mucus layer within 2 min.

The time required for particles to be taken up by intestinal cells also seems to be short, i.e. within minutes. For example, the accumulation of nanoparticles in lymphatic tissue began 5 min after administration into the small intestine (Hazzard et al., 1996; Fröhlich and Roblegg, 2012).

Based on these observations, the time needed for nanomaterials to cross the gastrointestinal mucus layer and be taken up by intestinal cells is short (within minutes) and thus cannot be considered a rate-limiting step compared with degradation under gastrointestinal conditions.

A cut-off value for a degradation rate based on a half-life of 10 min is therefore proposed to differentiate the quickly dissolving nanomaterials that can follow a safety assessment according to relevant EFSA guidance on non-nanomaterials. Such a time frame is considered analytically feasible, and the time required to reach the intestinal epithelium and be taken up by cells is of the same order of magnitude. It is considered important that information on the degradation in time is obtained by measuring at different time points. It is proposed that the material is considered to degrade quickly, if the degradation in the intestinal compartment shows a clear decrease in time (no plateau) and no more than 12 mass % of the material (compared with the particulate concentration at the beginning of the *in vitro* digestion) is present as particles after 30 min of intestinal digestion.

Studies by NANoREG (Deliverable 5.02; available via the NANoREG result repository) show that silver particles such as nanomaterial-300K < 20 nm and silver particles of 60 nm show low degradation (< 5 mass %) after 2 h digestion in the intestinal phase. The overall dissolution rate was higher in the earlier gastric phase, but still incomplete after 2 h. In the same series of studies, zinc oxide particle (nanomaterial-110, which is rather heterogeneous in size and shape) appeared to degrade completely in gastric conditions and 25–65 mass% was degraded at the end of the intestinal phase. There are indications that not all silver and zinc oxide was in fact dissolved because ion-salt/protein complexes may have formed in the digestive juices and precipitated, resulting in an underestimation of the degraded fraction. This issue complicated the measurement of the actual degradation rate and needs to be considered in further testing. Furthermore, these studies indicate that silver and zinc oxide nanomaterials cannot be assigned as quickly dissolving materials.

The NANoREG results (Deliverable 5.02; available via the NANoREG result repository) indicate that the degradation rate can be concentration dependent. Therefore, at least three different concentrations should be studied, with the middle concentration being representative of the human exposure level. The concentration with the lowest degradation rate should be used for further assessment.

Taken together, there is some scientific evidence that the time required to cross the gastrointestinal mucus layer and be taken up by intestinal cells is of the same order of magnitude as the proposed cut-off value for quick degradation. The time taken to reach intestinal cells would preferably be the rate limiting step. For reasons of pragmatism and feasibility, a half-life of 10 min was considered suitable. As a sub-argument, it is also assumed that even if a fraction of such quickly degrading materials is absorbed as particles, it is expected that further degradation will occur under e.g. lysosomal conditions and that they are unlikely to remain as particles for a long time.

This uncertainty in the assessment of quickly dissolving nanomaterials under gastrointestinal tract conditions needs to be considered, and the cut-off value may need revision in the future.

Appendix E – Sector Specific Information

Risk assessment for nanomaterials and the data requested can be different depending on their origin and intended use. While the general Guidance is for a typical case of a novel food or food additive more sector-specific information is given below.

E.1. Feed Additives

Feed additives are substances, microorganisms or preparations other than feed materials and premixtures that are intentionally added to feed or water to perform one or more functions[32] mentioned in Article 5.3 of Regulation (EC) No 1831/2003[33,34] governing the Community authorisation of additives for use in animal nutrition. Regulation (EC) No 429/2008[35] provides detailed rules for the implementation of Regulation (EC) No 1831/2003 as regards the preparation and presentation of applications and the assessment and authorisation of feed additives.

The Panel on Additives and Products or Substances used in Animal Feed (FEEDAP Panel) has adopted a series of guidance documents that aim at complementing Regulation (EC) No 429/2008 to support applicants in the preparation and submission of technical dossiers for the authorisation of additives for use in animal nutrition according to Regulation (EC) No 1831/2003.[36]

According to Article 8 of Regulation (EC) No 1831/2003, EFSA shall undertake an assessment to determine whether the feed additive is safe (for the target animals, consumer, user and the environment) and efficacious, when the proposed conditions of use are followed.

To allow EFSA to perform an assessment of a feed additive, its condition of use should be specified (at which dose range it is used and for which target species) and the additive and active substance should be characterised (including details on the impurities and manufacturing process); data on stability (shelf-life, stability in premixtures and feedstuffs) and homogeneity are also assessed. The above mentioned Regulations and guidance documents were not developed specifically for nanomaterial feed additives, but the ongoing revision of these documents anticipates the provision of data on particle size for those feed additives whose nature allows the presence of nanoparticles and the potential for the feed additive to be classified as an engineered nanomaterial as defined by European legislation (See Section 1.2.3).

Although nanomaterial forms of different feed additives have been reported to enhance absorption of nutrients and supplements and improve health of the livestock (Hill and Li, 2017), up to now, no application for feed additives as nanomaterial has been received in the EU. For future applications the present Guidance should be followed regarding the general considerations for risk assessment of nanomaterial (Section 3) and physicochemical characterisation (Section 4), in particularly Section 4.3 on the characterisation in matrix.

For safety assessment of nanomaterial-containing feeds, direct exposure of target animals by ingestion of the nanomaterial should be assessed following the general approach given in Figures 3 and 4 (Sections 5 and 6) and the FEEDAP guidance (EFSA FEEDAP Panel, 2011).

As described in Section 6.2, a justification of the validity of an *in vitro* system to check if the material under assessment **quickly degrades in digestive tract conditions** has to be provided by the applicant and supported by sound scientific arguments to demonstrate the suitability of the model proposed for a particular animal species. If a sound argument cannot be provided, then testing should be performed *in vivo*. For instance, an *in vitro* digestion model has already been developed for pigs (Boisen and Eggum, 1991; Boisen and Fernández, 1997), although a comparison with *in vivo* data for degradation or release of substances or materials from its matrix has not been performed. If a nanomaterial feed additive is intended to be used in food-producing animals, the exposure of consumers

[32] http://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:32003R1831&from=en

[33] Regulation (EC) No 1831/2003 of the European Parliament and of the Council of 22 September 2003 on additives for use in animal nutrition (Text with EEA relevance) OJ L 268, 18.10.2003, p. 29–43.

[34] The feed additive shall: (a) favourably affect the characteristics of feed, (b) favourably affect the characteristics of animal products, (c) favourably affect the colour of ornamental fish and birds, (d) satisfy the nutritional needs of animals, (e) favourably affect the environmental consequences of animal production, (f) favourably affect animal production, performance or welfare, particularly by affecting the gastro-intestinal flora or digestibility of feedingstuffs, or (g) have a coccidiostatic or histomonostatic effect.

[35] Commission Regulation (EC) No 429/2008 of 25 April 2008 on detailed rules for the implementation of Regulation (EC) No 1831/2003 of the European Parliament and of the Council as regards the preparation and the presentation of applications and the assessment and the authorisation of feed additives (Text with EEA relevance) OJ L 133, 22.5.2008, p. 1–65. Available online: http://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:32008R0429&from=EN

[36] http://www.efsa.europa.eu/en/publications/advanced-search?page=0&subject=62281&type=guidance&results_per_page=5

to nanomaterials present in animal food products (indirect exposure, **carry-over)** should be assessed. To this end, data should be provided on nanomaterial residues in tissues/products from target animals receiving the nanomaterial feed additive under the conditions of the use requested (see FEEDAP guidance on the safety for the consumer) (EFSA FEEDAP Panel, 2012b). Assessment of carry-over for consumer safety is particularly relevant when there is a concern that the nanomaterial is persistent and bioaccumulative. If the same nanomaterial is also intended to be used as a food additive, there needs to be also an assessment of the nanomaterial for food use. In such cases, carry-over of the feed additive can also be supported by the food use evaluation (e.g. by the safe intake level).

The hazard identification and characterisation of the nanomaterial feed additive should follow the principles in the current EFSA guidance documents and European Commission guidelines for feed additives (see above) taking into account the additional aspects to be considered for nanomaterials (Section 6.9 of the present guidance).

It should be noted for instance that toxicological data derived from laboratory species may not be directly applicable for nanomaterial foreseen to be administered in feed to target animals. For example, when evaluating the nanomaterial as feed additive, the risk assessor will have to consider if the results from a modified 90-day study are sufficient to extrapolate to a target farm animal species or if additional testing in a specific farm animal species is necessary, e.g. tolerance tests for the target species might be needed.

It should be noted that sheep, dogs, rabbit and cow have been reported to have two types of Payer's Patches that differ in cellular composition, location, structure and function, and this differs from human and rodents where no such differences have been reported (Gebert et al., 1996). Such species differences must be taken into account when considering regional differences or similarities in terms of mechanisms and structures involved in particulate uptake in the large intestine.

As part of the safety assessment, **inhalation** of nanomaterial feed additives contained in feed should be considered as an important route of exposure. This is because of the likelihood that, while consuming the (dry) feed, animals will also inhale certain quantity of the particulate materials.

The inhalation and deposition of particles (including nanoparticles) in the lung is known to be dependent on a number of factors, such as particle size, shape, breathing rate of the animal, etc. (Sarangapani, 2000; Geiser and Kreyling, 2010). Although clearance mechanisms are similar in humans and most mammals, it is known that clearance rates may differ significantly between species (Elder et al., 2005; Kreyling et al., 2013).

Where the inhaled particles are solubilised in the lung, they are likely to be quickly removed from the lung through absorption. The poorly soluble particles on the other hand are cleared by different mechanisms depending on the region where they are deposited (Bakand et al., 2012; Kreyling et al., 2013). Large particles ($\geq$ 5 μm) generally only reach the extrathorasic (mouth and throat) and/or trachea-bronchial regions where they are cleared mechanically via coughing and are largely swallowed into the gastrointestinal tract. Small particles, particularly nanoparticles, can reach and deposit in the alveolar region (deep lung), where they can be retained for much longer periods before being cleared via phagocytosis by the alveolar macrophages. It is important to note that, contrary to clearance from the tracheo-bronchial region, clearing of particles from the alveolar region is much slower and may take weeks to years (Möller et al., 2008) as nanoparticles deposited in the lung may escape both mucociliary clearance and alveolar macrophages (El-Sherbiny et al., 2015).

A small proportion of the inhaled particles may pass through the pulmonary epithelial barrier and reach systemic circulation. Although penetration through the endothelial cell layer has been shown for particles of different chemical identities, this seems to be restricted to 'small' nanoparticles (Geiser and Kreyling, 2010; Kreyling et al., 2013). A trace fraction of nanoparticles may also reach the brain directly via the olfactory bulb (Oberdörster et al., 2009). It is therefore important that potential adverse effects of animal exposure to nanoparticles through feed are carefully investigated considering both the oral as well as the inhalation routes.

Safety for the user

Users/workers are defined as the persons who may be exposed to the additive while handling it, when incorporating it into premixtures or feedstuffs or using feedstuffs supplemented with the additive. The safety of the user of nanomaterial feed additives should be assessed following the general approach given in Regulation (EC) No 429/2008 and the specific FEEDAP guidance (EFSA FEEDAP Panel, 2008).

Risks to users/workers should be assessed in a series of studies using the additive in all forms of the final product for which the application has been submitted. Any other available toxicological data should be used to assess the potential systemic toxicity of the additive.

The requirements to assess the effect on the respiratory system, **skin** and eye irritation and skin sensitisation potential indicated in the FEEDAP guidance (EFSA FEEDAP Panel, 2011), should be followed.

- The present Guidance should be followed when evaluating a nanomaterial as a feed additive. The direct exposure of target animals to the nanomaterial should be assessed following the general approach given in Figures 3 and 4 of this Guidance and the FEEDAP guidance to assess the safety for the target species (EFSA FEEDAP Panel, 2011).
- The risk assessor must consider if the results from an extended 90-day study (Step 2) are sufficient to conclude on the safety of target animal species, or if testing in a specific target species is necessary.
- As part of the safety assessment of nanomaterial-containing feeds, inhalation of the nanomaterial feed additives should also be considered as an important route of exposure for animals feeding on a nanomaterial-containing feed.
- Risks to users/workers should be assessed in a series of studies using the nanomaterial additive in all forms of the final product for which the application has been submitted.
- All available toxicological data should be used to assess the potential systemic toxicity of the additive.

E.2. Recommended guidance for nanomaterial pesticides

The developments in the field of nanosized active ingredients and formulations have also opened up new avenues for enhancing the delivery and efficacy of pesticides and other agrochemicals (not necessarily in the remit of EFSA). The expected worldwide use of nanopesticides in the future may contribute to a reduction in overall pesticide use through enhanced efficacy and better control of applications in the field as well as better stability of the dispersions, and slow- or controlled-release of the active ingredients (Perlatti et al., 2013; Kah and Hofmann, 2014; Cano Robles and Mendoza Cantú, 2017).

The term nanopesticides in this Guidance is used as a synonym for plant protection products (PPP) in a broad way. In addition to active substances, PPPs may also contain other materials such as solvents, carriers, inert material, wetting agents referred to as co-formulants that can also form nanoparticles. Therefore, the recommended Guidance for nanomaterial pesticides covers nano plant protection product active substances, its co-formulants and formulations (i.e. the plant protection product). In general, pesticides is a broader term that also covers products as biocides, but biocides are not falling under the scope of this Guidance document Plant protection products (PPPs) may also contain other materials such as solvents, carriers, inert material and, wetting agents, referred to as co-formulants that can also form nanoparticles. The term 'nanopesticide' as used herein therefore covers nano plant protection product active substances, its co-formulants and the formulations (i.e. the plant protection product). However, it is acknowledged that there are many similarities between plant protection products and biocides products since same active substances can be used for both purposes.

Several examples of nanopesticides have been quoted in published literature (Kah et al., 2013; Perlatti et al., 2013; Kah and Hofmann, 2014; Kookana et al., 2014; Cano Robles and Mendoza Cantú, 2017). However, a clear and agreed definition of nanopesticide is currently not available,[37] and many of the publications have regarded products containing particles ranging from the typical nanoscale (between 1 and 100 nm) to much larger sizes (up to 1,000 nm) as nanopesticides. Most publications did not differentiate between pesticides and biocides and have categorised both as nanopesticides. Commercial sensitivities over nanopesticides pose further difficulties in identifying the scale of industrial activity in this area. With these constraints in view, the available information suggests that developments in this area are largely under R&D and as such there is little evidence of an apparent

[37] There is currently no definition for 'nanopesticides'. A (possible) definition based only the size in the nanoscale (1–100 nm) could exclude many recent formulations that are larger (e.g. 'nanoemulsions formulation'). On the other hand, some products (e.g. 'microemulsion formulation') maybe contain fractions in the 1–100 nm range and that have been on the market for decades without previously being classified as 'nano'.

example of a nanopesticide that is currently available on the market**.** It has, however, been reported that microemulsions of some already available pesticides may contain droplets in the nanoscale range (Kah et al., 2013).

Under Biocides Regulation (EU) No 528/2012, nanomaterial means 'a natural or manufactured active or non-active substance containing particles, in an unbound state or as an aggregate or as an agglomerate and where, for 50% or more of the particles in the number–size distribution, one or more external dimensions is in the size range 1 nm to –100 nm'. When test methods are applied to nanomaterials under the Biocides Regulation, an explanation shall be provided of their scientific appropriateness for nanomaterials, and where applicable, of the technical adaptations/adjustments that have been made in order to respond to the specific characteristics of these materials (according to the legal requirement for biocides under Reg. (EU) No 528/2012). An example of an approved nanomaterial for use in biocidal products in Europe is that of synthetic amorphous silica, which is approved under the Biocides Regulation (EU) No 528/2012[38] as an active substance for use in biocidal products used for product type 18, insecticides, acaricides and products to control other arthropods, as defined in the Annex to the relevant Regulation.[39] The approval covers synthetic amorphous silicon dioxide (CAS No 112926-00-8) as a nanomaterial in the form of stable aggregated particles of size > 1 μm, with primary particles in the nanosize scale (< 25 nm). It is also noteworthy that there is a searchable database available for active substances approved in the EU under Regulation (EC) No 1107/2009 for use in plant protection products[40]), which confirms that currently there are no nanomaterial-based pesticides. However, there is no such database for formulations, and it is not clear if there are any nanomaterials already approved in the EU to be used as a pesticide formulation.

The currently available information on R&D relating to active substances in the form of nanopesticides indicates that they are most likely to fall under one of following types of formulations where the **active substance** is:

a) in the form of a **nanoparticle** as such or surface modified, or is contained in a nanoparticle carrier - such as porous nanosilica;
b) in the form of nanosized **droplets** in an emulsion, or in solid lipid particles;
c) nano**encapsulated** in a natural or synthetic (usually degradable) polymer shell.

From a risk assessment perspective, but deviating from the current legal requirements,[41] the Scientific Committee notes that safety considerations for a nanopesticide used in agriculture will be necessary in the future. From the current perspective, these considerations cover two aspects: (1) safety of the individual components (the active substances, co-formulants or other adjuvants), and (2) safety of all the components that together form the nanopesticide entity.

To ensure a high level of consumer protection, it is necessary to develop a uniform definition for nanomaterials and to specify that the approval of an active substance does not include the nanomaterial form unless explicitly mentioned, which is comparable to the biocide legislation. It can then be considered as a new entity. Safety of the individual components of a nanopesticide may not represent safety of all the components put together to form a nanosized pesticide active substance and/or formulation (Kookana et al., 2014). As discussed before, because nanosizing of substances may impart certain changes in properties, behaviour and effects compared with the corresponding conventional forms, an explanation of their scientific appropriateness for nanomaterials shall be provided for all test methods applied to nanomaterials. Where applicable, an explanation of the technical adaptations/ adjustments that have been made in response to the specific characteristics of these materials shall be provided. For example, nanodimensions may enable a nanopesticide to penetrate different biological membrane barriers and thus manifest a different ADME profile in the exposed organism compared with its conventional form. A change in physicochemical properties and/or bio-kinetic behaviour may also lead

[38] Regulation (EU) No 528/2012 of the European Parliament and of the Council of 22 May 2012 concerning the making available on the market and use of biocidal products Text with EEA relevance, OJ L 167, 27.6.2012, p. 1–123.
[39] Commission Implementing Regulation (EU) No 408/2014 of 23 April 2014 approving synthetic amorphous silicon dioxide as an existing active substance for use in biocidal products for product-type 18, Official Journal of the European Union, L 121, 24 April 2014.
[40] http://ec.europa.eu/food/plant/pesticides/eu-pesticides-database/public/?event=homepage&language=EN
[41] In the current legal framework the co-formulants used in the pesticide products are regulated under REACH and Pesticides Regulation. The European Commission is currently working on a Regulation setting out rules for the implementation of article 27 of the Regulation (EC) No 1107/2009 for the identification of non-acceptable co-formulants. Based on the information available, Member States shall consider taking action according to other Regulations than Regulation (EC) No 1107/2009. EFSA will be requested to peer-review the assessment of co-formulants when the Pesticides Regulation appears to be the relevant legal framework.

to altered toxicological effects. **Therefore, the properties, behaviour and effects of a nanopesticide should not be automatically assumed to be similar to its conventional form, even when the individual components of the nanopesticide are considered to be safe on their own.** This means that in addition to the data and information generally considered in risk assessment of the same pesticide in a conventional form certain additional nanospecific aspects would need to be considered for a nanopesticide.

Therefore, and since requested by the Network Representatives of Member States to give guidance (see Minutes of the 17th meeting of the Network on Pesticide Steering point 5.6.4[42]), the Scientific Committee recommends National Authorities to request from the applicants that for authorisation purposes it should be declared in the dossier if a PPP does not include any nanomaterial form or to explicitly mention it and then follow this guidance when a nanomaterial is used in a PPP, e.g. as active substance, co-formulant or synergist. Furthermore, in line with spirit of the overall protection goal for human and animal health as outlined in Regulation (EC) No 1107/2009 Art.4.(2.a) for active substances, when National Authorities are assessing a formulation (i.e. the active substance together with a co-formulant), it is also advisable to follow the approach outlined in this Guidance not only for food safety, but also for application safety (for operators, workers, bystanders and residents).[43]

The current level of knowledge relating to the human health effects and environmental fate, behaviour and impacts of nanopesticides is still nascent, and therefore, this Guidance has only highlighted the main aspects that need considering in regard to hazard identification and hazard characterisation of nanopesticide active substances and formulations.

Data requirements

According to the EFSA PPR Panel Scientific Opinion of 2009, the PPR Panel could not give at that time a definitive statement on whether or not the data requirements given in Annex II and III of the Biocidal Products Regulation (BPR, Regulation (EU) 528/2012) are sufficient to gauge the risks of nanopesticides owing to the emerging nature of this new technology. Here below, the Scientific Committee provides an update of the situation based on the currently available knowledge. However, the Scientific Committee notes that a comparable request to Annexes II (Information requirements for active substances) and III (Information requirements for biocidal products) would also be very helpful for PPP.

'When test methods are applied to nanomaterials, an explanation shall be provided of their scientific appropriateness for nanomaterials, and where applicable, of the technical adaptations/adjustments that have been made in order to respond to the specific characteristics of these materials.' This should be completed by the request from Annex IV (Common principles for the evaluation of dossiers for biocidal products):' In the case of biocidal products containing nanomaterials, the principles set out in this Annex will also need to be adapted and elaborated in technical guidance to take account of the latest scientific information. For pesticides, this Guidance of the EFSA Scientific Committee cannot be regarded as legal data requirements. For legal data requirements in the area of pesticides, EFSA Guidance Documents should be taken note in the PAFF meeting in order to be implemented. This is not the case for other Units in EFSA or fields of application.

Data requirements – Physical, chemical and technical parameters

According to Commission Regulations (EU) No 283/2013[44] and (EU) No 284/2013[45] setting up the data requirements for active substances and for PPPs, in accordance with Regulation (EC) No 1107/2009,[46] there is a request for the submission of data on the identity of active substance, the identity and content of additives and impurities.

Because of the above mentioned safety considerations, the Scientific Committee is of the opinion that detailed physicochemical characterisation of a nanopesticide active substance as well as other

[42] http://www.efsa.europa.eu/sites/default/files/event/141111a-m.pdf

[43] For the environment (non-target organisms, ecotoxicology as well as fate and behaviour) will be addressed in Part 2 (see Section 1.2.1).

[44] Commission Regulation (EU) No 283/2013 of 1 March 2013 setting out the data requirements for active substances, in accordance with Regulation (EC) No 1107/2009 of the European Parliament and of the Council concerning the placing of plant protection products on the market Text with EEA relevance. OJ L 93, 3.4.2013, p. 1–84.

[45] Commission Regulation (EU) No 284/2013 of 1 March 2013 setting out the data requirements for plant protection products, in accordance with Regulation (EC) No 1107/2009 of the European Parliament and of the Council concerning the placing of plant protection products on the market Text with EEA relevance. OJ L 93, 3.4.2013, p. 85–152.

[46] Regulation (EC) No 1107/2009 of the European Parliament and of the Council of 21 October 2009 concerning the placing of plant protection products on the market and repealing Council Directives 79/117/EEC and 91/414/EEC. OJ L 309, 24.11.2009, p. 1–50.

co-formulants in a formulation is an essential prerequisite for risk assessment. This is currently not expected for co-formulants. The parameters listed in Table 1 that are relevant to nanopesticides would therefore need to be measured by methods that are suitable for nanomaterials. It is also important that other technical parameters for dispersions/formulations such as stability, susceptibility, wettability, etc., are also determined for nanopesticides.

Data on particle size distribution are very important in this regard, because it is the nanosize dimensions that are likely to bring about changes in the properties, behaviour and effects of a nanopesticide.

From the active substance evaluation process for conventional (non-nano) pesticides, two main formulation types could be considered relevant to nanopesticides. These are capsule suspension or micro encapsulated particles (CS), and micro encapsulated emulsions (ME). There is already a data requirement for particle size distribution under Art. 2.8 of Reg. (EU) No 284/2013 setting up the data requirements for plant protection products. A test on particle size distribution might be asked for a pesticide CS of microencapsulated active substances in an aqueous continuous phase intended for dilution with water before use. Such a test is not requested for ME in the regulation. Particle size range is requested, however, if the representative formulation for a conventional (non-nano) pesticide is a multiple phase formulation, to restrict the sizes of suspended particulates to a sufficiently narrow range to ensure optimum efficacy and/or safety of the product. The analytical method used for measurement of size distribution is CIPAC method MT 187, which is based on ISO 13320-1:1999(E) (ISO, 1999) (revised by ISO 13320:2009) (ISO, 2009) particle size analysis – Laser diffraction methods, and the particle size distribution is calculated using a model (e.g. Fraunhofer model (ISO 13320:2009; Commission communication 2013/C 95/01[47])).

Also, as discussed before, unless a valid justification can be provided, **each formulation should be assessed for any change(s) in the properties and behaviour of the nanopesticide**. This is because different formulations may alter the degree of particle dispersion, agglomeration and aggregation. Thus, data on physicochemical parameters, including particle size distribution, will be required both for an active substance(s) and the formulation(s) intended for use. Any significant changes in the physicochemical properties of a nanopesticide, either as such or in a formulation, would make it difficult to justify the use of toxicological data on conventional equivalents in risk assessment.

Toxicity assessment of active substances and co-formulants

According to Commission Regulations (EU) No 283/2013[44] and (EU) No 284/2013[45] setting up the data requirements for active substances and for PPPs, in accordance with Regulation (EC) No 1107/2009,[46] the submission of data on the toxicity of active substance and PPP is requested. The toxicological data currently required for the safety assessment of an active substance include studies on ADME (both intravenous and oral), acute toxicity (oral, dermal, inhalation), skin and eye irritation, skin sensitisation, short-term toxicity (90-day study in two species), genotoxicity (*in vitro* and *in vivo*), carcinogenicity and, reproductive toxicity as well as other endpoints, such as neurotoxicity and immunotoxicity studies. For co-formulants, such data requirements (currently) do not exist. For formulations, the data requirements are currently limited to acute toxicity (oral, dermal, inhalation), skin and eye irritation, skin sensitisation, dermal absorption and exposure assessment for operators, workers, bystanders and residents (Regulation EU 284/2013; EFSA, 2014).

In a scenario of data rich substances, like pesticide active substances, for nanoformulations of such pesticides a read-across hypothesis could be allowed on the condition that toxicokinetic behaviour has been addressed (e.g. a described in this Guidance in Step 2a). The allowance of read-across hypothesis as well as the comparative assessments are described in the Guidance for the Residue Definition (EFSA PPR Panel, 2016). If a full read read-across case cannot be built, as a further study an enhanced 28-day (OECD 407) for comparative assessment would be accepted.

As mentioned before, nanosize may bring about certain changes in the properties and behaviour of a pesticide, and may alter its toxicological effects. Any data relating to the toxicity and exposure of a conventional (non-nanomaterial) pesticide would therefore also be applicable to its nanopesticides if it can be justified that physicochemical properties and toxicokinetic behaviour of the active substance (as such, or in a formulation) have not significantly changed at the nanoscale (also see Section 6.3 on read-across). This means that data on physicochemical properties and ADME profile of nanopesticides

[47] Commission communication in the framework of the implementation of Commission Regulation (EU) No 284/2013 of 1 March 2013 setting out the data requirements for plant protection products, in accordance with Regulation (EC) No 1107/2009 of the European Parliament and of the Council concerning the placing of plant protection products on the market.

are crucial elements needed to decide whether any new toxicological data would be needed on the pesticide nanoformulation. A significant departure in the properties and/or behaviour of a nanopesticide compared with non-nano equivalents should trigger the need for new toxicological studies according to this Guidance in consideration of the relevant routes of exposure. It is noted that this proposal from the Scientific Committee is compatible to the current practice for active substances, for example by taking nanospecific aspects into account during the standard 90-day oral toxicity study that is to be provided. However, such studies are not provided under the current framework for pesticides formulations (meaning PPP).

In general, the toxicity data requirements for a nanopesticide will be similar to those for a conventional (non-nano) PPP and the toxicological testing methods used for conventional (non-nano) PPP will also be applicable to nanopesticides. However, the tests need to be carried out using the nanopesticide and considering of the nanospecific aspects (e.g. dispersion, agglomeration/aggregation) in accordance with this Guidance. It should also be noted that some of the currently available testing methods may need certain adaptations to take account of the special nanoscale features of nanopesticides (Rocks et al., 2008; SCENIHR, 2009; OECD, 2009a). For example, most of the currently available *in vitro* methods have been developed and validated for substances that can be solubilised, whereas nanopesticides are likely to comprise poorly soluble nanomaterials, either as such, or in a suspension, dispersion, or formulation. It is also known that, owing to high surface energies, nanoparticles generally tend to stick together to form larger sized agglomerates and aggregates (Šimon and Joner, 2008). The testing protocols should therefore take account of the potential agglomeration, sedimentation, binding with other moieties in the medium, or sticking of the particles to glass/plasticware used in handling/testing, because this could change the concentration of the material during a test (Alger et al., 2014; Ong et al., 2014; DeLoid et al., 2017a). Data on stability of a nanodispersion/formulation are therefore important to ensure that an applied concentration is maintained and the target cells are exposed during the test to avoid false negative results from *in vitro* tests. As an example, any negative results from *in vitro* genotoxicity studies also need to be provided with an assessment of the cellular and nuclear uptake of the nanomaterial to demonstrate target exposure (see Section 6.4).

Nanomaterials are also known to bind various moieties on the particle surfaces and may thus transport other (potentially harmful) substances to various organs and into cells. Detailed characterisation of the actual nanopesticide active substances and formulations used in a toxicological test would therefore be essential. It is also important to use appropriate dispersions/formulations in the toxicity tests because different co-formulants and dispersion methods may differently affect the degree of particle aggregation/agglomeration, which may in turn influence the results of a toxicological test.

According to Commission Regulation (EU) No 283/2013[44] setting up the data requirements for active substances and Regulation (EU) No 284/2013[45] for PPPs, the submission of data on the active substance is requested. For conventional pesticides, the same applies to (at least) one representative formulation, which should be assessed as part of the active substance evaluation process. At present, little is known about the effects of different dispersions/formulations on the properties, behaviour and toxicological effects of nanopesticides, and it may not be appropriate to regard one or a few selected formulations as representative for safety evaluation of all other formulations without a valid scientific justification. It is therefore requested under this Guidance that **all** the nanopesticide formulations that are intended for final use are always tested in toxicological studies. In view of the ability of nanomaterials to penetrate different membrane barriers, and the potential for altered biokinetics in the test organism, the toxicological studies should also consider new/unexpected target sites when testing a nanopesticide.

Exposure assessment of active substances and co-formulants

The EFSA PPR Panel has published guidance on the assessment of exposure of operators, workers, residents and bystanders in risk assessment for plant protection products (EFSA, 2014), that in general should also be applied to nanopesticides. Like other PPPs, the potential for consumer exposure to a nanopesticide would be dependent on the concentration of the active substance, the type of formulation, the mode of application, as well as the persistence of the nanopesticide and the level of its residues in foodstuffs. Information on the persistence of a nanopesticide should be provided in the registration dossier, whereas determining the level of residues is subject to post-market monitoring/surveillance of specific food/feedstuffs by the competent authorities of the European Member States.

The likely scenarios for direct human and animal exposure to a nanopesticide can be envisaged from accidents and (mis)handling during manufacture, transportation and storage, but most importantly during preparation and application at the farm. While intentional (suicidal) or accidental ingestion cannot

be ruled out, oral exposure from normal use of a nanopesticide is unlikely. On the other hand, dermal and/or inhalation exposure is possible for the operators as well as farm animals, workers, bystanders and residents in the vicinity during manual handling, mixing/loading, and (spray) application of a nanopesticide. Other potential exposure scenarios may include in and around the home and gardens, seed treatment facilities, etc. pending confirmation. The relevant Regulation (EU) No 284/2013 requires estimation of acute and chronic exposure to operators, workers, residents and bystanders considering each relevant type of application. The exposure estimation for operators and workers is first carried out assuming that they are not using personal protective equipment, followed, where appropriate, by further estimation on the assumption they are using effective and readily obtainable protective equipment. For bystanders and residents, exposure estimation should assume that they do not use any personal protective equipment.

Dermal exposure/toxicity

Under Regulation (EU) No 284/2013 setting out the data requirements for plant protection products (the formulations), dermal absorption studies would be required where dermal exposure is a significant exposure route, and no acceptable risk is estimated using a default absorption value. The EFSA PPR Panel has published guidance on dermal absorption (EFSA, 2017), that should in general also be applied to nanopesticides. These studies should provide a measurement of the absorption through the skin of the active substances.

Dermal absorption studies can be performed using *in vivo* (OECD TG 427 (2004a)) or *in vitro* (OECD TG 428 (2004b)) methods. As Regulation (EU) No 284/2013 stipulates, the dermal absorption data should preferably be derived from studies on human skin *in vitro*. In this regard, EFSA PPR Panel (2011) has published a scientific opinion on the science underpinning the assessment of dermal absorption of PPPs and a detailed Guidance on dermal absorption (EFSA PPR Panel, 2017). These should be referred to when conducting dermal absorption studies on nanopesticides with additional consideration of the relevant nano-aspects. It is also important for dermal absorption studies to consider whether a formulation can affect bioavailability of the active substances and/or other toxicologically relevant compounds in a PPP.

For nanopesticide active substances and formulations, the likelihood and extent of the absorption through skin, lung, and gastrointestinal tract (if relevant) should be determined whilst mimicking the potential exposure scenarios, with due considerations to the nanoaspects. Dermal absorption is generally determined by chemical analysis of the receptor fluid (*in vitro* tests), or blood/tissues (*in vivo* studies). However, most analytical methods can indicate the chemical nature but not the particle nature of the absorbed substances. Thus, where chemical analysis of skin sections, tape-strippings, and/or receptor fluid has indicated dermal absorption of a nanopesticide, further investigations should be carried out to ascertain whether the absorbed substance(s) are still nanomaterial or have degraded. From a risk assessment point of view, this is important because the loss of nanostructure due to degradation (dissolution, enzymatic or chemical breakdown), would render a nanomaterial to the corresponding non-nanomaterial. Certain analytical methods – e.g. electron microscopy based imaging, fluorescence labelling, and single-particle ICP-MS, etc., have been used to establish the particle nature of the substances absorbed in or through the skin/lung (Vogt et al., 2014; Lin et al., 2015).

Where the absorption of a nanopesticide cannot be ruled out either by experimental data or on the basis of information on degradation, a default value of 100% absorption as a nanomaterial should be applied in risk assessment, unless data become available that prove otherwise and trigger a revision of this default value. Also, irrespective of the presence of a nanomaterial pesticide active substance/formulation, requirements under the existing regulations for safety assessment must be followed. As described in Section 4, detailed characterisation data on the identity, chemical composition and purity/impurity profile of the nanopesticide active substances and formulations must be provided.

Inhalation exposure/toxicity

Regulation (EU) No 284/2013 setting out the data requirements for PPPs (formulations) requires acute inhalation toxicity studies. For this purpose, head/nose exposure shall be used, unless body exposure can be justified. The studies are carried out following the OECD Guidelines Tests 403 (OECD, 200b).

In regard to a nanopesticide (especially nanoformulations), it is important to note that particle size and the mode of application (e.g. dusting or spraying) will determine the extent of the exposure in terms of whether the particles/droplets can be inhaled and which part of the respiratory tract they can reach.

- Although an agreed definition of nanopesticide is currently not available, the definition provided in Biocides Regulation (EU) No 528/2012 may be used as a guide.
- It is advisable that a pesticide active substance or formulation should be considered within the scope of this Guidance if it is:
  - in the form of a nanoparticle as such or surface modified, or is contained in a nanoparticle carrier;
  - in the form of nanosized droplets in an emulsion, or in solid lipid particles;
  - nanoencapsulated in a natural or synthetic polymer shell.
- Risk assessment of a nanopesticide should consider both the individual components (the active substances and co-formulants), as well as all the components together that form the nanopesticide entity.
- The approach outlined in this Guidance should be followed for food and, application safety (for operators, workers, bystanders and residents) as well as for the environment (non-target organisms, ecotoxicology fate and behaviour). The EFSA PPR Panel has published guidance on the assessment of exposure of operators, workers, residents and bystanders in risk assessment for plant protection products (EFSA, 2014), that should be in general also applied to nanopesticides.
- Detailed physicochemical characterisation of a nanopesticide active substance and other co-formulants in a formulation must be carried out considering relevant parameters listed in Table 1, along with additional parameters for dispersions/formulations such as stability, susceptibility, wettability, etc.
- Toxicity data requirements for a nanopesticide are similar to that for a conventional (non-nano) PPP and testing methods used for conventional (non-nano) PPP will also be applicable to nanopesticides. However, the tests need to be carried out using the nanopesticide and cover the nanospecific aspects (e.g. dispersion, agglomeration/aggregation) in accordance with this Guidance.
- Under Regulation (EU) No 284/2013 setting out the data requirements for PPPs (the formulations), dermal absorption studies would be required where dermal exposure is significant route, and no acceptable risk is estimated using default absorption value. The EFSA PPR Panel has published guidance on dermal absorption (EFSA, 2017) that should be in general also be applicable to nanopesticides.
- It is also important for dermal absorption studies to consider whether a formulation can affect bioavailability of the active substances and/or other toxicologically relevant compounds in a PPP.
- For nanopesticide active substances and formulations, the likelihood and extent of the absorption through skin, lung, and gastrointestinal tract (if relevant) should be determined whilst mimicking the potential exposure scenarios, giving due considerations to the nanoaspects.
- Where studies indicate dermal absorption of a nanopesticide, further investigations should be carried out to ascertain whether the absorbed substance(s) are in nanoform, or a degraded or dissolved form. Where the absorption of a nanopesticide cannot be ruled out either by experimental data or on the basis of information on dissolution/degradation, a default value of 100% absorption in nanoform should be applied in risk assessment.
- Regulation (EU) No 284/2013 setting out the data requirements for PPPs (formulations) requires acute inhalation toxicity studies. For this purpose, head/nose exposure should be assessed, unless body exposure can be justified. The studies should be carried out following OECD Guidelines Test 403.

The human respiratory tract is divided in three Sections: the nasopharyngeal, tracheobronchial and pulmonary regions. Particle fractions reaching these regions are designated inhalable (size > 30 μm), thoracic (size 10–30 μm), and respirable fractions (size < 10 μm). The particle fraction in the size range < 10 μm (including nanoparticles) is generally considered respirable, i.e. particles can potentially reach the alveolar region of the lung and this may lead to local or systemic effects in/through the respiratory system. In view of this, data on the particle/droplet size alone will not be considered sufficient for estimation of inhalation exposure of a sprayable nanopesticide emulsion/dispersion, and

data on dried particles will also be required for risk assessment. This is because, depending on the dispersion medium, larger air-borne droplets may dry out quickly and become small enough to reach the alveolar region of the lung. Currently, this cannot be simulated in any of the available computational models, and the applicant for a nanopesticide should provide measurement data on the size range of both the spray droplets as well as the dried particles.

E.3. FCM

The development of new FCMs is a major area of current nanomaterial applications (Smolander and Chaudhry, 2010; Bradley et al., 2011; Duncan, 2011; Wyser et al., 2016). In these cases, exposure to nanomaterial can principally occur indirectly because of migration or transfer of the nanomaterial from FCM, into food. EFSA has published a few opinions on the application of nanomaterials such as carbon black, inorganic substances like TiN and metal oxides, and nanoclays in food contact polymers (EFSA CEF Panel, 2012a,b, 2014a,c, 2015a,b, 2016b). In all cases, it was concluded that no significant migration or transfer of the nanoparticles was expected under the defined conditions of use. Recently, a critical review of the published literature on the migration potential of nanomaterials from food contact polymers has been published by Stormer et al. (2017). One important conclusion is that analytical observations reporting migration of nanomaterials in many cases did not demonstrate that the measured migrants were in nanoparticulate form. This reemphasises that the amount migrated or transferred, particularly its particle properties, should be determined. More information about the migration of engineered nanomaterial from polymer-based food-contact materials has been reviewed by Jokar et al. (2017).

Guidance summary for FCM

- Detailed physicochemical characterisation of the nanomaterial used as an additive or applied as a surface coating on a food contact material must be provided.
- Exposure to a nanomaterial must be assessed based on the experimental data on migration or transfer from a FCM to food. Potential release of the nanomaterial from the FCM due to mechanical stress or physical disintegration of a FCM polymer matrix should also be considered.
- Appropriate techniques should be used to both quantitatively and qualitatively determine the migrating species, and to establish whether they are in nanoparticulate or solubilised/ degraded form.
- A case may be made for exemption from carrying out toxicological investigations where it can be shown that either the migrating species are not in nanomaterial form (in that case standard risk assessment should apply), or migration of the nanomaterial is only in trace amounts.

E.4. Novel food

The Novel food legislation is not prescriptive, but EFSA needs to check if the best test protocols are being used. This novel legislation stipulates that vitamins, minerals or other substances that contain or consists of a engineered nanomaterial be considered as novel foods. It remains to be clarified whether the wording 'contains or consists' would also warrant an assessment of these nutrients or other substances if they are **encapsulated** or in other forms of carrier nanomaterials as mentioned in this Guidance. Testing of whole food is the ultimate alternative for testing after the nanomaterial specific testing *in vitro* and *in vivo*. For the testing of a novel food material, it depends on the case if the applicant needs to test the whole food and/or parts of it.

E.5. Nano(plastic) contaminants

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

It is known that waste nanoplastics are generated and that exposure of humans and animals occurs through the food chain (Chae and An, 2017). However, this topic is considered outside of the scope of this working group and is not addressed in this Guidance.

E.6. Nanocarriers

Currently the European Commission recommended definition of nanomaterial might be modified towards 'solid particles'. As explained in Rauscher et al. (2015) 'Solid' is one of the four fundamental states of matter (the others being liquid, gas, and plasma). It is characterised by structural rigidity and resistance to changes of shape or volume. This excludes emulsions (liquid particles in liquid media) and micelles. A rationale for this is the fact that for these materials the external dimensions generally depend more on chemical and physical (mechanical) forces from their surroundings than those of solid particles. For micelles, also the high frequency of molecules leaving and entering the structure makes their structure highly dynamic'.

Also previous guidance on nanomaterials has concentrated on manufactured particulate nanomaterials since many of the observed biological effects reported occurred with micro- and nanoparticles (such as exhaust particulates). However, it is recognised that uses of nanotechnology in food and feed (and other areas) is wider than manufactured nanoparticles per se.

As mentioned in the scope Section 1.3, organic nanomaterial, such as encapsulates are considered subject to this Guidance. Such nanoencapsulates can function as a delivery system for nutrient sources and to incorporate food additives into products (such as lipophilic colours in hydrophilic beverages).

Nanoencapsulation is an extension of drug delivery systems based on liposomes and (bio)polymers that have existed for 30–40 years and were designed to increase the bioavailability of pharmaceuticals. These generally consist of an amphiphilic compound (such as a phospholipid) which can be organised into bilayer structures such as spheres so that one surface is hydrophilic and the other lipophilic. These can be structured with either the hydrophilic or lipophilic surface on the interior and the other on the exterior depending on the intended use. A compound of relevant 'philicity' is contained within the interior surface. In general, the components of the shell are either normal constituents of the body or approved food additives such as emulsifiers. The amounts of the shell components derived from food materials for use in delivery systems are generally far lower than their normal intake from dietary sources or other approved uses. As such there would be little concern over the shell components, unless these were neither normal constituents of the body or approved food additives. If nanoencapsulates function as intended, however, there will **be increased bioavailability (systemic exposure) of the encapsulated material**. This represents a potential concern since health based guidance values are currently set based on the external rather than the internal dose and may no longer provide an appropriate level of protection to the consumer.

Nanomaterials used as carrier systems for other food components (e.g. vitamins) may increase their bioavailability. The, effects of the increased bioavailability need to be considered in terms of toxicity (if these encapsulation materials are not disintegrated in the gastrointestinal tract) for (1) the active ingredient per se, (2) the encapsulating material, and (3) the encapsulate/nanocarrier as a whole. The exposure assessment of a nanoscale delivery system should include assessment of the amount of encapsulated bioactive compound (in addition to the assessment of the nanocarrier system itself) and the amount present in free form in the food. For this, the analytical isolation, detection and characterisation procedures need to meet such requirements. It might also be necessary, when appropriate and possible, to analyse the relevant chemical components of a nanocarrier system as such.

Guidance summary for nanocarriers

- For nanomaterials used as carrier systems for other substances, the implications of any significant alteration (increase) in bioavailability to potential harmful effects must be considered – especially when a nanocarrier is not disintegrated in the gastrointestinal tract.
- The safety assessment should consider the active ingredient per se, the encapsulating material, and the encapsulate/nanocarrier as a whole.