EFSA Journal

GUIDANCE

ADOPTED: 30 June 2021

doi: 10.2903/j.efsa.2021.6768

Guidance on risk assessment of nanomaterials to be applied in the food and feed chain: human and animal health

EFSA Scientific Committee,
Simon More, Vasileios Bampidis, Diane Benford, Claude Bragard, Thorhallur Halldorsson, Antonio Hernández-Jerez, Susanne Hougaard Bennekou, Kostas Koutsoumanis, Claude Lambré, Kyriaki Machera, Hanspeter Naegeli, Søren Nielsen, Josef Schlatter, Dieter Schrenk, Vittorio Silano (deceased), Dominique Turck, Maged Younes, Jacqueline Castenmiller, Qasim Chaudhry, Francesco Cubadda, Roland Franz, David Gott, Jan Mast, Alicja Mortensen, Agnes G. Oomen, Stefan Weigel, Eric Barthelemy, Ana Rincon, José Tarazona and Reinhilde Schoonjans

Abstract

The EFSA has updated the Guidance on risk assessment of the application of nanoscience and nanotechnologies in the food and feed chain, human and animal health. It covers the application areas within EFSA's remit, including novel foods, food contact materials, food/feed additives and pesticides. The updated guidance, now Scientific Committee Guidance on nano risk assessment (SC Guidance on Nano-RA), has taken account of relevant scientific studies that provide insights to physico-chemical properties, exposure assessment and hazard characterisation of nanomaterials and areas of applicability. Together with the accompanying Guidance on Technical requirements for regulated food and feed product applications to establish the presence of small particles including nanoparticles (Guidance on Particle-TR), the SC Guidance on Nano-RA specifically elaborates on physico-chemical characterisation, key parameters that should be measured, methods and techniques that can be used for characterisation of nanomaterials and their determination in complex matrices. The SC Guidance on Nano-RA also details aspects relating to exposure assessment and hazard identification and characterisation. In particular, nanospecific considerations relating to *in vitro/in vivo* toxicological studies are discussed and a tiered framework for toxicological testing is outlined. Furthermore, *in vitro* degradation, toxicokinetics, genotoxicity, local and systemic toxicity as well as general issues relating to testing of nanomaterials are described. Depending on the initial tier results, additional studies may be needed to investigate reproductive and developmental toxicity, chronic toxicity and carcinogenicity, immunotoxicity and 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 or mechanisms of action are also discussed. The Guidance proposes approaches to risk characterisation and uncertainty analysis.

Keywords: nanoparticle, physico-chemical characterisation, dietary exposure, nanotoxicology, safety assessment, testing strategy

Requestor: EFSA

Question number: EFSA-Q-2020-00269

Correspondence: sc.secretariat@efsa.europa.eu

Panel members: Simon More, Vasileios Bampidis, Diane Benford, Claude Bragard, Thorhallur Halldorsson, Antonio Hernández-Jerez, Susanne Hougaard Bennekou, Kostas Koutsoumanis, Claude Lambré, Kyriaki Machera, Hanspeter Naegeli, Søren Nielsen, Josef Schlatter, Dieter Schrenk, Vittorio Silano (deceased), Dominique Turck and Maged Younes.

Waiver: R. Franz declared that Fraunhofer institute at which he was employed provides advisory services to private business operators active in the sector on food contact materials. In line with EFSA's Policy on Independence (https://www.efsa.europa.eu/sites/default/files/corporate_publications/files/policy_independence.pdf) and the Decision of the Executive Director on Competing Interest Management (https://www.efsa.europa.eu/sites/default/files/corporate_publications/files/competing_interest_management_17.pdf), a waiver wasgranted to R. Franz regarding his participation to the EFSA's Working Group on Nanoscience and Nanotechnologies in Food and Feed in accordance with Article 21 of the Decision of the Executive Director on Competing Interest Management. Pursuant to Article 21(6) of the above-mentioned Decision, the involvement of R. Franz is authorised as member in the cross-cutting Working Group (ccWG) on Nanotechnologies, allowing him to take part in the discussions and in the drafting phase of the scientific output, but he is not allowed to be, or act as, a chairman, a vice-chairman or rapporteur of the working group.

Note: Mandate nr M-2016-0082. This document replaces the previous versions of this guidance that are now obsolete and shall be marked as such: (1) The old 2011 document is still on the website as such and should become obsolete: https://www.efsa.europa.eu/en/efsajournal/pub/2140. (2) The 2018 guidance that was used for testing should also become obsolete: https://www.efsa.europa.eu/en/efsajournal/pub/5327.

Declarations of interest: The declarations of interest of all scientific experts active in EFSA's work are available at https://ess.efsa.europa.eu/doi/doiweb/doisearch.

Acknowledgments: The Scientific Committee and the cross-cutting Working Group on Nanotechnologies wish to thank the experts: Jonathan Powell (until March 2017), Andrea Zijno (April-June 2017), Barbara Drasler (June 2017-March 2018), Shareen Doak (as from 2020) and EFSA staff members: Hermine Reich, Renata Leuschner, Maria Vittoria Vettori, Melpo Karamitrou (in 2018), Berrak Eryasa (in 2019), Irene Cattaneo, and Maria Chiara Astuto for the support provided to this scientific output. The Scientific Committee and the cross-cutting Working Group on Nanotechnologies wish to acknowledge JRC, ECHA and the Member States delegates of the EFSA Scientific Network of risk assessment of nanotechnologies in food and feed that provided feedback for this scientific output.

Suggested citation: EFSA Scientific Committee, More S, Bampidis V, Benford D, Bragard C, Halldorsson T, Hernández-Jerez A, Hougaard Bennekou S, Koutsoumanis K, Lambré C, Machera K, Naegeli H, Nielsen S, Schlatter J, Schrenk D, Silano V, Turck D, Younes M, Castenmiller J, Chaudhry Q, Cubadda F, Franz R, Gott D, Mast J, Mortensen A, Oomen AG, Weigel S, Barthelemy E, Rincon A, Tarazona J and Schoonjans R 2021. Guidance on risk assessment of nanomaterials to be applied in the food and feed chain: human and animal health. EFSA Journal 2021;19(8):6768, 111 pp. https://doi.org/10.2903/j.efsa.2021.6768

ISSN: 1831-4732

The EFSA Journal is a publication of the European Food Safety Authority, a European agency funded by the European Union.

Summary

Upon request of the European Food Safety Authority (EFSA), the Scientific Committee has undertaken a revision of the Guidance on risk assessment of the application of nanoscience and nanotechnologies in the food and feed chain, human and animal health, published in 2018. Following a mandate from the European Commission, an accompanying Guidance on Technical Requirements for regulated food and feed product applications is developed to establish the presence of small particles including nanoparticles (hereafter Guidance on Particle-TR). This Guidance on Particle-TR helps to identify materials that do not meet the definition of engineered nanomaterial or nanoform but consist of, or contain a fraction of, small particles requiring nanospecific assessment according to this Guidance (hereafter SC Guidance on Nano-RA). Both guidances are applicable from the moment of their publication and are to be followed to establish the safety of engineered nanomaterials as defined in Regulation (EU) 2015/2283 or of nanomaterials that do not meet the legal definition of nanomaterials but consist of, or contain a fraction of, small particles requiring nanospecific assessment. Irrespective of the presence of a nanomaterial, the existing requirements for safety assessment according to EFSA guidances for conventional materials under relevant regulations must be followed (see Figure 1). The present Guidance provides an overview of additional or complementary information requirements and how to perform risk assessments of nanomaterials in the food and feed area.

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, testing of nanomaterials needs consideration of certain nanospecific aspects that have been highlighted in this SC Guidance on Nano-RA and the accompanying Guidance on Particle-TR. Nanomaterials can have specific morphological and chemical characteristics that may alter their biokinetic behaviour and/or toxicological responses. A full assessment addressing properties at the nanoscale is required when the applicant or the risk assessor concludes that the material:

- fulfils the definition of engineered nanomaterial according to the Novel Food Regulation (Regulation (EU) 2015/2283),
- contains a nanoform as defined in the provisions under Commission Regulations (EU) 2018/1881 and (EU) 2020/878 amending the Annexes I, II, III, VI, VII, VIII, IX, X, XI and XII of the REACH Regulation to introduce nanospecific clarifications, or
- consists of or contains a fraction of small particles as outlined in the Guidance on Particle-TR.

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. When materials under the scope of this Guidance have the same elemental composition but occur in different morphological characteristics (e.g. shapes, sizes, crystalline forms and/or surface properties as a consequence, e.g. of different production processes), this Guidance is applicable to each nanoform as a stand-alone case. Applicants must undertake a separate physico-chemical characterisation and specific risk assessment for each distinct nanomaterial having a given chemical composition. The applicant may explore whether a read-across approach is applicable.

This SC Guidance on Nano-RA is complementary to the EFSA sectoral guidances that will remain the frameworks of the risk assessment procedure (see Figure 1). This means that applicants and risk assessors should follow the relevant sectoral guidance for the uses to be covered by the application, but also have to consider additional requirements (e.g. specific information on the material, new studies, or adaptation of the safety testing) and incorporate the specific elements described in this Guidance when the evaluation concerns an (engineered) nanomaterial or a material that consists of nanoparticles or has nanospecific characteristics (See Section 4.3).

The present Guidance proposes a structured pathway for carrying out safety assessment of nanomaterials in food/feed and related applications (see Figure 2) and provides practical suggestions for the types of testing needed and the methods that can be used. The Guidance includes an 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 key issues that require attention, it is expected that often a degree of expert judgement will be needed and, therefore, a broader scientific background is provided in this document.

The applicant is responsible for the best set-up of the tests and a description of the rationale thereof. It is recommended to consider multidisciplinary teams to cover in-depth scientific

understanding of all the risk assessment steps, as well as proficient technical skills to collect the necessary data and information using the most appropriate instrumentation and techniques.

For a specific nanomaterial, data and information would need to be provided on certain nanospecific properties (see Section 5, Table 1 and Appendix A), in addition to the data and information generally required according to the relevant sectoral regulation. Some of the currently available testing methods should be adapted, according to the indications provided in this Guidance, to take account of the specific properties of nanomaterials. Therefore, safety assessment of nanomaterials should be carried out in accordance with the provisions of this Guidance.

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 physico-chemical 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 5.1.2) lists the main physico-chemical parameters that are considered essential for characterisation of nanomaterials, although not all are applicable to each material. Characterisation of the nanomaterial must be carried out at different stages, e.g. the pristine state as manufactured, the material as prepared for testing and on the material as present in products and applications. The Guidance outlines the currently available methods and tools that can be used for measuring the nanomaterials characteristics, as well as quality control aspects that should be considered. Particle size distribution should be determined by electron microscopy, or another suitable technique when scientifically justified.

A high dissolution/degradation rate will convert a nanomaterial to its corresponding non-nanomaterial form. Therefore, the nanospecific considerations described in this Guidance are only applicable to those materials that do not **quickly dissolve or degrade** (into ions or molecules) under the physiological conditions of the gastrointestinal system, and therefore may interact with biological entities at the local or systemic levels. Other characteristics that may indicate a reduction in the chance of exposure to the nanomaterial include high dissolution/degradation rate in food/feed matrix or nanoparticles being fixed or effectively embedded in matrices. Throughout various Chapters, the Guidance identifies the circumstances under which some requirements for **data to be generated specifically on the nanomaterial can be waived**.

Because a nanomaterial can be developed in several forms with different particle 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 7.9). In principle, toxicological data from a nanomaterial may be used for safety assessment of another form of the same nanomaterial, if it can be shown that there are close similarities or representation of a worse case in relation to their physico-chemical properties and toxicokinetic behaviour.

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 Chapter 6). Exposure scenarios covered by this Guidance are for instance use as novel food, addition of the nanomaterial to the food (e.g. flavourings, food additives), transfer of substances from FCM to food or carry-over of residues of feed additives or pesticides to food. When exposure is possible, and to inform risk characterisation, it should be determined whether the nanomaterial or its degradation product(s) remain present as particles in the food/feed matrix. In the absence of exposure data, or when it is not possible to determine the properties and amounts of nanoparticles in complex matrices, it should be assumed as a worst case that all material added to a food/feed product, is present, ingested and absorbed as nanoparticles.

In Section 7, 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 in four different steps.

- In Step 1, 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 conventional (non-nanomaterial) assessment, instead of further nanospecific testing.

- In Step 2, 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 3. Specific endpoints relevant for *in vitro* testing are: cytotoxicity/cell viability, induction of oxidative stress, (pro-)inflammation and gastrointestinal barrier integrity impairment. When *in vitro* methods indicate a lack of toxic effects, and *in vitro* dissolution of the nanomaterial in lysosomal and gastrointestinal conditions is fast, an argument can be put forward for waiving *in vivo* studies (specifically designed for the nanoscale aspects) on a case-by-case basis, provided that a sub-chronic (90-day) *in vivo* toxicity study is not mandatory under the sectoral legal framework for the substance. **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 as outlined in this Guidance. *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. Furthermore, the bacterial reverse mutation (Ames) assay is not considered suitable for nanomaterials since particles do not penetrate the bacterial cell wall resulting in lack of internalisation in bacteria. When 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 the *in vivo* situation. Expert judgement should be used to select and justify one or more of the available *in vivo* tests, e.g. *in vivo* mammalian erythrocyte micronucleus test (OECDTG 474 (OECD, 2016a)); *in vivo* mammalian alkaline comet assay (OECD TG 489 (OECD, 2016d)); transgenic rodent somatic and germ cell gene mutation assay (OECD TG 488 (OECD 2013)).
- In Step 3, following an adapted dose-range finding and pilot toxicokinetic study, a 90-day oral toxicity study (OECD TG 408 adapted for the assessment of nanomaterials) should be carried out for identification of potential immunological, proliferative, neurotoxic, reproductive organ or endocrine-mediated effects. The extent to which nanomaterials can accumulate in tissues should be investigated. A significant increase in tissue concentration between days 14 and 90, or slow release during the elimination period, will necessitate further assessment as described in Step 4. In the test design, a satellite group should be added to the 90-day oral toxicity study to investigate if and to what extent and in which tissues the nanomaterial accumulates. Specific attention should be paid in the 90-day repeated-dose study to cardiovascular and inflammatory parameters as well as to sites/organs involved in or part of the mononuclear phagocyte system (MPS). The results from the 90-day oral toxicity study can be used to identify a reference point (such as BMDL or NOAEL).
- In Step 4, if the results of the 90-day toxicity study demonstrated accumulation of the nanomaterial in tissues, further in-depth studies can be identified (e.g. human kinetic data, additional toxicokinetic studies, reproductive and developmental toxicity, additional immunotoxicity, neurotoxicity, mutagenicity, carcinogenicity, endocrine effects, effects on gut microbiome). 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. Overall, these studies provide additional information on the toxicokinetic and toxicodynamic nanoscale considerations, and permit refinement of the risk assessment by decreasing the uncertainty.

Risk characterisation combines all the information from hazard characterisation with exposure assessment and any other relevant information. The output from the risk characterisation should be in the form of an overall assessment of the safety of the nanomaterial for its intended use, together with a description of the parameters under which the assessment is valid and the uncertainties associated with the assessment (see Section 8). When the data have been derived from appropriately conducted studies using validated methods that have considered nanospecific issues (i.e. OECD guidelines or equivalent protocols adapted according to the recommendations provided in this Guidance), there may be no reason to use uncertainty factors that are higher than those used for a conventional material. However, when data are either insufficient or have been derived from inadequate tests for nanomaterials, applying additional uncertainty factors may be considered for safety assessment of a nanomaterial (see Section 9).

Furthermore, the Guidance discusses specific aspects relating to nanomaterial applications for food/feed additives, pesticides, food contact materials, novel foods and nanofibres, nanocarriers and fertilisers. The Guidance also notes ongoing developments in areas relating to alternative testing approaches, mode of action and adverse outcome pathway approaches. The Guidance also highlights certain gaps where further research is needed to facilitate adequate safety assessment of materials that consist of small-sized particles.

Table of contents

1. Introduction

This Scientific Guidance on risk assessment of the application of nanoscience and nanotechnologies in the food and feed chain (SC Guidance on Nano-RA) builds upon the opinion of the Scientific Committee of 2009 on 'The Potential Risks Arising from Nanoscience and Nanotechnologies on Food and Feed Safety' (EFSA Scientific Committee, 2009a) and more specifically Section 6 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). This first part of the Guidance was published in July 2018 and was implemented in a pilot phase until the end of 2020. The 2018 meeting of the EFSA Network on Risk assessment of nanotechnologies in food and feed was dedicated to the discussion of the implementation of the Guidance and Member States presented their experiences or research linked to aspects of the EFSA Guidance. Further feedback on the Guidance was received from stakeholders during a workshop held on 1–2 April 2019. The present revision of the Guidance has addressed all feedback received and updates or revises relevant aspects. The scope has been adapted to account for another complementary guidance, the 'Guidance on Technical Requirements for regulated food and feed product applications to establish the presence of small particles including nanoparticles' (Guidance on Particle-TR), developed by EFSA following a mandate from the European Commission (EFSA Scientific Committee, 2021).

As a general principle, the test requirements stipulated in EFSA guidance documents for conventional materials[1] and EU legislation for various food and feed areas should be applied to and be followed for a nanomaterial according to its intended use. However, the risk assessment of nanomaterials, 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 the physico-chemical characterisation of the nanomaterials. The specific information related to the characteristics and properties of nanomaterials and 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) should be used for a (case-by-case) risk assessment.

There are substantial new developments of alternatives to *in vivo* testing approaches. The availability of validated *in vitro* and/or *in silico* methods for specific endpoints is still limited, but the situation may change in the future. Information from *in vivo* testing is still often required 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 alternative methods validated by OECD or EURL ECVAM and other options for fulfilling the information requirements and data gaps are considered and existing data generated for other relevant regulations (e.g. REACH) should also be used to minimise or 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).

Together with the accompanying Guidance on Particle-TR, this Guidance specifically elaborates on physico-chemical characterisation, including key parameters that should be measured, methods and techniques that can be used for characterisation of nanomaterials and their determination in complex matrices, and specific requirements for their risk assessment.

The **Guidance on Particle-TR** sets out information requirements for applications in the regulated food and feed product areas, and establishes criteria for assessing the presence of a fraction of small particles. These requirements apply to particles requiring nanospecific assessment in conventional materials that do not meet the definition of engineered nanomaterial as set out in the Novel Food Regulation (EU) 2015/2283. The Guidance on Particle-TR outlines three groups of appraisal criteria to confirm whether or not the conventional risk assessment should be complemented with nanospecific considerations. The first group addresses solubility and dissolution rate as key physico-chemical properties to assess whether consumers will be exposed to particles. The second group establishes the information requirements for assessing whether the conventional material contains a fraction or consists of small particles, and its characterisation. The third group describes the information to be presented for existing safety studies conducted with the conventional material under consideration to demonstrate that the fraction of small particles, including particles at the

[1] Relevant regulatory framework, administrative and EFSA scientific guidance documents per regulated product area are listed in http://www.efsa.europa.eu/en/applications/regulatedproducts. See also http://www.efsa.europa.eu/en/applications

nanoscale, has been evaluated properly. The applicants may select, according to their knowledge and available information, the best appraisal route or combination of appraisal routes to justify: (a) the absence of a fraction of small particles, or (b) that the material contains a fraction of small particles but it is covered by the conventional risk assessment and does not require a separate assessment regarding nanoscale properties.

In summary, but see the Guidance on Particle-TR for more information, the appraisal routes and corresponding decision criteria are as follows.

Demonstrating non-exposure of consumers:

- Solubility and dissolution rate: consumers will not be exposed to particles when equal to or higher than 33.3 g/L;
- Dissolution/degradation rate in water: half-life of 10 min or less corresponding to dissolved fraction equal to or higher than 88% in 30 min;
- Solubility/dissolution in the marketed product or in food: at the expected maximum levels: the substance is fully dissolved in an aqueous or a non-aqueous matrix; or residues in food are below the reported/ relevant solubility limit.

Information on particle size distribution:

- Particle size distribution of the material: particles with at least one external dimension equal to or larger than 500 nm; the material contains less than 10% of particles (number-based) have at least one external dimension smaller than 500 nm;
- For liquid formulations/products: absence of particles in suspension;
- Particle size distribution of a fraction of small particles: less than 10% of the particles (number-based) of the sub-500 nm fraction with at least one external dimension smaller than 250 nm.

Demonstrating that the fraction of small particles is properly covered by the existing safety studies:

- The studies address properly the potential hazards of the fraction of small particles: the test material included the fraction of small particles and the test design, and the level of dispersion/degree of agglomeration was sufficient to address the fraction of small particles;
- The submitted risk assessment covers the fraction of small particles.

The appraisal routes related to the particle size distribution are not applicable to nanostructured materials.

1.1. Background as provided by EFSA in 2016 (M-2016-0082)[2]

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 are not provided. 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 process. 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 and nano-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 a large 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 nanocomposites 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 Cantu, 2017; Chaudhry et al., 2017).

As mentioned in the conclusions of the 2011 SC Guidance, the Guidance 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

[2] The text is kept as the original text from the mandate in 2016. Legal and scientific updates are covered in Section 1.3.

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. For example, Novel Food Regulation (EU) No 2015/2283[3] states that EFSA will have to verify that, when a novel food consists of engineered nanomaterials, the most up-to-date analytical methods are used to assess safety and that the scientific appropriateness of the methods used are substantiated by the applicants. Article 12 of Regulation (EC) No 1333/2008 stipulates 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, e.g. 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 developing a new environmental risk assessment guidance document for nanomaterials.

1.2. Terms of Reference as provided by EFSA in 2016 (M-2016-0082)

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 or 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 (FCM).

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 physico-chemical measurements and the other data needed for food/feed risk 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,[4] FEEDAP and CONTAM and relevant EFSA Units (in particular the EFSA Pesticides Unit), complemented with external experts for specific aspects.
- EFSA 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[5]; EU FP7 research projects including NanoGenotox, NANoREG and NanoDefine; and institutes for metrology or standards development such as 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.
- 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

Part one of the Guidance was published in 2018, and has been updated following a pilot phase. In 2019, EFSA received a complementary mandate from the European Commission for preparing an accompanying Guidance on Technical requirements for regulated food and feed product applications of

[3] 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

[4] Since the mandate, the Panel on ANS is renamed Panel on FAF (Panel on Food Additives and Flavourings); Panel on CEF is renamed Panel on CEP (Panel on Food Contact Materials, Enzymes and Processing Aids).

[5] OECD (Organization for Economic Cooperation and Development), online: https://www.safenano.org/knowledgebase/standards/working-party-on-manufactured-nanomaterials/

materials that do not meet the legal definition of nanomaterials but may contain a fraction of small particles to establish the presence of small particles including nanoparticles (Guidance on Particle-TR). The two guidance documents should be considered as complementary and they include a number of cross-references. The scope of this Guidance has been adapted accordingly, including also recent updates of regulatory frameworks, e.g. REACH, to specifically address nanomaterials.

The focus of this Guidance is on the dietary risk assessment of nanomaterials, covering all materials under EFSA's remit, such as novel foods, food and feed additives, food contact materials and pesticides. EFSA is also responsible for assessing non-dietary exposures in the case of feed additives and active substances in plant protection products. The related Appendices present a set of overall recommendations. Additional information on non-dietary risk assessment for nanomaterials has been produced by ECHA and by the EU's Scientific Committee on Consumer Safety (SCCS).

For a description of the terms used in this Guidance, see the glossary.

Contaminants in the food chain are considered outside the Terms of Reference and are not addressed in this Guidance. Nonetheless for contaminants in (nano)particle form, the principles for testing as recommended in this Guidance apply. Data resulting from appropriate toxicity testing as set out for nanomaterials herein can be used for assessing the human health risk from nanoparticles as contaminants of food/feed. Nanoplastics, which are thought to be primarily generated through traditional waste disposal and fragmentation of microplastic debris originating from plastic pollution in the environment, may lead through trophic transfer to human and animal exposure via ingestion (EFSA CONTAM Panel, 2016). Their impact on human health is presently subject to extensive research (Lehner et al., 2019; Rubio et al., 2020; Allan et al., 2020).

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

1.3. Scope of and when to apply this Guidance

This Guidance is aimed at all interested parties and, in particular, applicants and risk assessors such as principal investigators with good technical understanding of nanomaterial characterisation and adequate toxicity testing, EFSA units and Panels performing risk assessment for nanomaterials or conventional substances that contain particles in the nanoscale.

A full assessment addressing the properties at the nanoscale, in line with this SC Guidance on Nano-RA, is required if the applicant or the risk assessor concludes that the material:

a) meets the criteria of the definition of **engineered nanomaterials**[6] of the Novel Food Regulation (EU) No 2015/2283,
b) **is a substance to be used to manufacture FCMs,** which is in **nanoform** in accordance with Article 9(2) of Commission Regulation (EU) 10/2011[7], or deliberately engineered to particle size which **exhibit functional physical and chemical properties** that significantly differ from those at a larger scale in accordance to Article 5(2)(c)(ii) of Commission Regulation (EC) No 450/2009[8].

[6] The definition of 'engineered nanomaterial' in the Novel Food Regulation has an implicit component of intentionality linking the manufacturing of the material with an intended nano-enable function. Hence, in practice the implementation of the definition relies on the discretion of food business operators to declare such materials in the products as analytically it may not be possible to do so. According to the Novel Food Regulation (EU 2015/2283) '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.

[7] Commission Regulation (EU) No 10/2011 of 14 January 2011 on plastic materials and articles intended to come into contact with food (Text with EEA relevance).

[8] Commission Regulation (EC) No 450/2009 of 29 May 2009 on active and intelligent materials and articles intended to come into contact with food (Text with EEA relevance)

c) **is an active substance in PPPs,** consisting of or containing **nanoforms** according to the provisions of Commission Regulations (EU) 2018/1881[9,10] and (EU) 2020/878[11], amending the Annexes I, II, III, VI, VII, VIII, IX, X, XI and XII of the REACH Regulation to introduce nanospecific clarifications, or is a PPP with co-formulants in nanoform;
d) **does not meet the above mentioned legal definitions (a, b, c) but consists of or contains a fraction of small particles** requiring assessment in the nanoscale, identified according to the Guidance on Particle-TR, setting out information requirements for applications in the regulated food and feed product areas, and establishing criteria for assessing the presence of a fraction of small particles,[12]
e) is a nanostructured material or a material, including materials formulated in the form of nanocarriers (see Appendix D.5), which could retain properties that are characteristic of the nanoscale, e.g. 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.

The term nanomaterial is used in this Guidance, however the provisions apply to all descriptions and definitions given above; see also the glossary.

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) will also have to be considered at all the relevant steps, including the determination of nanospecific characteristics (Section 4.3), which may affect toxicity and exposure assessment (Section 6).

When materials under the scope of this Guidance consists of **nanoparticles** with **the same chemical composition but different shapes, sizes, crystalline forms and/or surface properties** as a consequence of, e.g. different production processes, the risk assessment to be conducted under this Guidance should cover each different type of nanoparticle. For nanomaterials and nanostructures, applicants must undertake a separate physico-chemical characterisation and specific risk assessment as described in this Guidance for each distinct nanomaterial having a given chemical composition. The applicant may also explore whether a read-across approach is applicable (see Section 7.9). The concept of 'set of similar nanoforms' described in Regulation (EU) 2018/1881 is applicable. Clearly defined boundaries in the (number-based) particle size distribution of constituent particles, surface functionalisation or treatment; shape, crystallinity and other morphological characteristics; and surface area, should be provided for the individual nanoforms within the set and the information should allow to conclude that the hazard assessment, exposure assessment and risk assessment of these nanoforms can be performed jointly.

Conventional materials with a fraction of small particles requiring nanospecific assessment are identified according to the provisions of the Guidance on Particle-TR. Additional information according to this Guidance, may be required to assess possible differences in the hazard assessment, exposure assessment and risk assessment of similar nanoparticles in the set or to assess if the risk assessment covers worst-case conditions.

For future types of materials (e.g. advanced materials), applicants may also have to address the question 'Does the material have characteristics of the nanoscale' (see Section 4.3) and in consequence may also have to apply this Guidance.

[9] Commission Regulation (EU) 2018/1881 of 3 December 2018 amending Regulation (EC) No 1907/2006 of the European Parliament and of the Council on the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH) as regards Annexes I, III, VI, VII, VIII, IX, X, XI and XII to address nanoforms of substances (Text with EEA relevance).

[10] Coverage of REACH: Additives and the other substances covered by the Novel Food Regulation for engineered nanomaterials are excluded from REACH (except Title III Data sharing and avoidance of unnecessary testing and Title VIII Restrictions on the manufacturing, placing on the market and use of certain dangerous substances and preparations) according to Art 2(5) and Art 2(6). FCM are not excluded, and the only difference is that the risk assessment does not need to consider human safety (means that the focus is environmental risk assessment) if assessed as FCM (Art 14(5). Pesticide active substances are also not excluded. The difference here is that after approval, active substances in PPP are considered registered under REACH according to Art 15(1).

[11] Commission Regulation (EU) 2020/878 of 18 June 2020 amending Annex II to Regulation (EC) No 1907/2006 of the European Parliament and of the Council concerning the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH) (Text with EEA relevance).

[12] This Guidance is accompanied by a Guidance on Particle-TR: Guidance on technical requirements for regulated food and feed product applications to establish the presence of small particles including nanoparticles. The Guidance on Particle-TR provides information to decide whether the present Guidance is applicable to materials that contain small particles.

1.4. Definitions

The term 'nanomaterial' as used herein, encompasses materials covered by the legal definitions for engineered nanomaterials and nanoforms relevant in the EFSA remit; the specific provisions regarding the risk assessment of nanomaterials also apply to other materials requiring assessment at the nanoscale.

As outlined in the Novel Food Regulation (EU) No 2015/2283[13], point (f) of Article 3(2), engineered nanomaterial means 'any intentionally produced material that has one or more dimensions of the order of 100 nm or less or that is composed of discrete functional parts, either internally or at the surface, many of which have one or more dimensions of the order of 100 nm or less, including structures, agglomerates or aggregates, which may have a size above the order of 100 nm but retain properties that are characteristic of the nanoscale. Properties that are characteristic of the nanoscale include: (i) those related to the large specific surface area of the materials considered; and/or (ii) specific physico-chemical properties that are different from those of the non-nanoform of the same material.'

The engineered nanomaterial definition set out in Regulation (EU) No 2015/2283 (the Novel Food Regulation) is also directly or indirectly applicable to other EU legislation concerning regulated food products (food flavourings, food additives, feed additives, vitamins and minerals used in food in accordance with Regulation (EC) No 1925/2006[14] and/or in food supplements in accordance with Directive 2002/46/EC[15] as well as vitamins, minerals or other substances used in food for specific groups in accordance with Regulation (EU) No 609/2013[16]).

For substances in food contact materials assessed under Regulation (EC) No 1935/2004, the provisions cover substances in nanoform in accordance with Article 9(2) of Commission Regulation (EU) 10/2011, and those deliberately engineered to particle size which exhibit functional physical and chemical properties that significantly differ from those at a larger scale in accordance to Article 5(2)(c)(ii) of Commission Regulation (EC) No 450/2009.

For active substances in Plant Protection Products (PPPs) assessed under Regulation (EC) No 1107/2009, the definitions of nanoforms and particles are those established by Commission Regulation (EU) 2018/1881:

- a nanoform is a form of a natural or manufactured substance containing particles, in an unbound state or as an aggregate or as an agglomerate and when, 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, including also by derogation fullerenes, graphene flakes and single wall carbon nanotubes with one or more external dimensions below 1 nm; and
- 'particle' means a minute piece of matter with defined physical boundaries; 'agglomerate' means a collection of weakly bound particles or aggregates when 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.

The term 'conventional material' is used herein to cover all chemical substances and mixtures that do not fulfil the legal definitions for engineered nanomaterials or nanoforms.

The term 'nanoparticle' is used to cover all particles exhibiting characteristics at the nanoscale. This covers all particles with any external dimension on the nanoscale and includes '**nanofibres'** (two external dimensions in the nanoscale) and '**nanoplates'** (one external dimension in the nanoscale).

A 'nanostructured material' is defined as a material having an internal or surface nanostructure. For conventional materials, the term 'fraction of small particles' is used herein to describe the fraction of

[13] 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.

[14] Regulation (EC) No 1925/2006 of the European Parliament and of the Council of 20 December 2006 on the addition of vitamins and minerals and of certain other substances to foods. OJ L 404, 30.12.2006, p. 26.

[15] Directive 2002/46/EC of the European Parliament and of the Council of 10 June 2002 on the approximation of the laws of the Member States relating to food supplements (Text with EEA relevance). OJ L 183, 12.7.2002, p. 51.

[16] Regulation (EU) No 609/2013 of the European Parliament and of the Council of 12 June 2013 on food intended for infants and young children, food for special medical purposes and total diet replacement for weight control and repealing Council Directive 92/52/EEC, Commission Directives 96/8/EC, 1999/21/EC, 2006/125/EC and 2006/141/EC, Directive 2009/39/EC of the European Parliament and of the Council and Commission Regulations (EC) No 41/2009 and (EC) No 953/2009 (Text with EEA relevance).

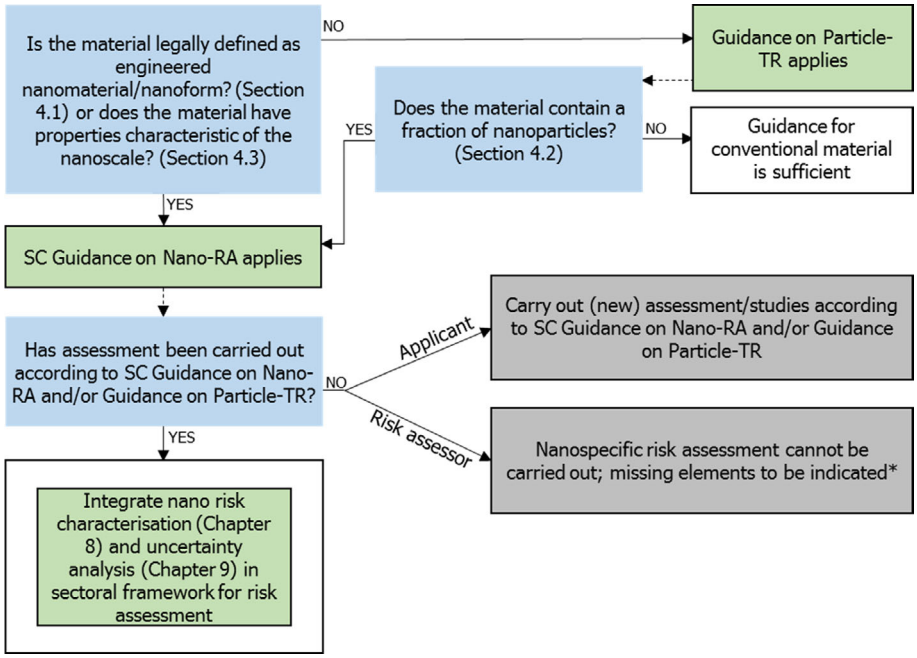
the materials composed by particles with at least one external dimension that measures 500 nm or less.

The physical property 'particle size' can only be defined unambiguously, i.e. with a single descriptor, for particles with a regular shape (e.g. spheres, cubes). More precise in this context is the term 'external dimension'. Industrial materials often consist of irregularly shaped particles with many possible external dimensions. The size reported for such particles thus strongly depends on the external dimension measured and reported. Many particle size analysis techniques produce equivalent spherical particle diameters and tend to overestimate the minimum external dimensions. The minimum external dimensions of particles with irregular shape can be better assessed by the minimum Feret diameter and/or the maximum inscribed circle diameter. For a detailed discussion of the measurement principles, measurands and how to interpret the results of different sizing techniques, users should consult the NanoDefine Methods Manual (Mech et al., 2020a; Mech et al., 2020b; Mech et al., 2020c). In all cases, users should report and justify the measurand used to determine the minimum external dimension.

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 also 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 nanospecific properties have to be assessed focussing on such properties and potentially related hazards, which may be independent of the fraction of particles of the material with a size below 100 nm. In line with the conclusions of SCENIHR (2010)[17] and EFSA Scientific Committee (2011a), the EFSA Scientific Committee reiterates that nanomaterials may not exhibit additional hazards compared with larger sized counterparts and that therefore a case-by-case assessment is necessary. As indicated in Section 7.6, the mechanism of intestinal uptake is likely to be size-dependent and uptake may be expected for particles with minimum external dimensions up to 250 nm.

[17] 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).

1.5. How to use this Guidance in relation to sectoral EFSA guidances



Legend: in green: nanospecific considerations for risk assessment; in blue: question to be answered; in white: risk assessment follows sectoral guidance. Dashed lines indicate unconditional workflows (no yes/no answer).
*: Sectoral legislation options to be considered for getting the missing elements or if not possible could lead to an inconclusive opinion.

Figure 1: Relationship among EFSA guidances for risk assessment (novel foods, food/feed additives, pesticides or FCM): the application of the SC Guidance on Nano-RA should be integrated into the risk assessment of relevant sectoral frameworks, including questions and steps to enter the guidances. For conventional materials, the Guidance on Particle-TR should be consulted to answer the first questions

The SC Guidance on Nano-RA is complementary to the EFSA sectoral guidances that will remain the frameworks of the risk assessment procedure as depicted in Figure 1. This means that applicants and risk assessors should follow the relevant guidance for the uses to be covered by the application, but should also apply and consider additional requirements as described in this Guidance when the evaluation concerns an (engineered) nanomaterial/nanoform or a material that contains nanoparticles, is nanostructured or has characteristics of the nanoscale (see Section 4). The risk characterisation needs to be performed according to the relevant sectoral guidance, taking into account the outcome of the assessment of the nanomaterial as described in this Guidance. In other words, an applicant or risk assessor should consider whether the risk assessment data adequately covers aspects highlighted by the current Guidance.

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

An applicant should answer the following questions: does the dossier contain an engineered nanomaterial/nanoform or a material with properties characteristic of the nanoscale. If confirmative, the present SC Guidance on Nano-RA should be taken into consideration when generating the necessary data. If not confirmative, as well as in the case of conventional materials, the applicant should generate and assess the information according to the Guidance on Particle-TR to establish if there is presence of a fraction of small particles requiring risk assessment at the nanoscale. The applicant is responsible for the best set-up of the tests and a description of the rationale thereof. It is

recommended that multidisciplinary teams cover in-depth scientific understanding of all the risk assessment steps, and all proficient technical skills to collect the necessary data and information using the most appropriate instrumentation and techniques.

A risk assessor assesses the risk of a substance submitted in a dossier or answers to a question, e.g. a mandate of the European Commission (EC) or a self-task of EFSA. The risk assessor evaluating a dossier will check whether the questions 'does the dossier contain an engineered nanomaterial or a material with characteristics of the nanoscale' and 'is there presence of a fraction of small particles requiring risk assessment?' are answered or can be answered convincingly. If so, the risk of the presence of the nanomaterial or material with small particles should be assessed, followed by a risk characterisation according to the relevant sectoral guidance, taken into account the outcome of the assessment of the nanomaterial.

The general outline of risk assessment of nanomaterials is described in Section 3, followed by characterisation and identification of nanomaterials in Sections 4 and 5. Exposure assessment is presented in Section 6. Hazard identification, hazard characterisation and toxicity testing strategies are described in Section 7. Section 8 presents the risk characterisation and uncertainty analysis is discussed in Section 9.

The present Guidance is cross-sectoral; sector-specific information (e.g. for feed additives and pesticides) is provided in Appendix D.

- This Guidance is applicable to materials used in food and feed that meet the criteria of the definition of engineered nanomaterials as outlined in Regulation (EU) No 2015/2283, FCM substances in nanoform as outlined in Commission Regulation (EU) 10/2011, or deliberately engineered to particle size which exhibit functional physical and chemical properties that significantly differ from those at a larger scale as outlined in Commission Regulation (EC) No 450/2009; and pesticide active substances and PPPs consisting of or containing nanoforms as outlined in Regulation (EU) 2018/1881.
- This Guidance should be used also for the assessment of conventional materials requiring assessment in the nanoscale, identified according to the Guidance on Particle-TR, setting out information requirements for applications in the regulated food and feed product areas, and establishing criteria for assessing the presence of a fraction of small particles.
- Furthermore, the Guidance should be used for nanostructured materials and materials which could retain properties that are characteristic of the nanoscale, e.g. related to the large specific surface area of nanomaterials or different toxicokinetic behaviour (i.e. significant changes in absorption, distribution and/or metabolism) as compared to its corresponding conventional material.
- When a material falls under the scope of this Guidance, its degradation products in the form of a nanomaterial and materials with the same elemental composition but different particle morphology, e.g. shapes, sizes, crystalline forms and/or surface properties also must be assessed.
- This Guidance is complementary to the EFSA Guidance documents on conventional materials. This means that applicants and risk assessors have to follow the relevant guidance for conventional material and to apply also the additional information requirements in this present Guidance when the evaluation concerns a nanomaterial. When an applicant can demonstrate that the conventional assessment has covered the fraction of nanoparticles sufficiently (see also Guidance on Particle-TR), then this Guidance does not need to be applied in that particular dossier.

2. Data and methodologies

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

The first part of the Guidance, on human and animal health, was published in July 2018. The 2018 meeting of the EFSA cross-cutting Network for Risk Assessment of Nanotechnologies in Food and Feed was dedicated to the discussion on the implementation of this Guidance and Member States presented their experiences or research linked to aspects of the Guidance. Further feedback on the Guidance was received from stakeholders during a workshop held on 1–2 April 2019. The pilot phase of the Guidance continued until the end of 2020. The Nano Network was consulted on the updated version in February

2021. The present revision of the Guidance has taken all the feedback into account to update or revise some of the aspects where necessary. The scope has been adapted to account for the complementary guidance, the 'Guidance on Technical requirements for regulated food and feed product applications to establish the presence of small particles including nanoparticles' (Guidance on Particle-TR), developed by EFSA following a mandate from the European Commission.

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, 2017f) applies to nanomaterial risk assessments.

3. Risk assessment of nanomaterials: general outline

The risk of a nanomaterial is determined by its chemical composition, physico-chemical properties, its interactions with tissues and exposure levels. There are some general aspects (the problem formulation) to consider at an initial stage before testing a nanomaterial that is proposed for use in the food/feed chain. Physico-chemical characterisation is needed to identify a material as a nanomaterial.

Any specific properties or effects of a nanomaterial are intrinsically linked to the stability of its nanoscale features. When a nanomaterial loses these, e.g. due to degradation or dissolution, it will not be expected to behave any differently from its corresponding non-nanomaterial form, if existing. For this reason, safety concerns over orally ingested nanomaterials are related mainly to those that are able to bypass the digestive system, potentially resulting in (nano)particles being translocated to organs and other tissues (see *in vitro* degradation tests, Section 7.2) or exert local adverse effects in the gastrointestinal tract.

The schematic general outline for risk assessment of nanomaterials is shown in Figure 2. The scheme follows a tiered approach. Furthermore, a number of exit points based on scientific evidence are provided. **An exit point implies that (only) the relevant sectoral guidance on conventional materials will be sufficient**. Each step is described in the following Sections.

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 nanospecific testing.

A **loss of nanospecific properties** will allow the use of data on corresponding conventional materials in the sectorial risk assessment and the nanospecific risk assessment would no longer be required.

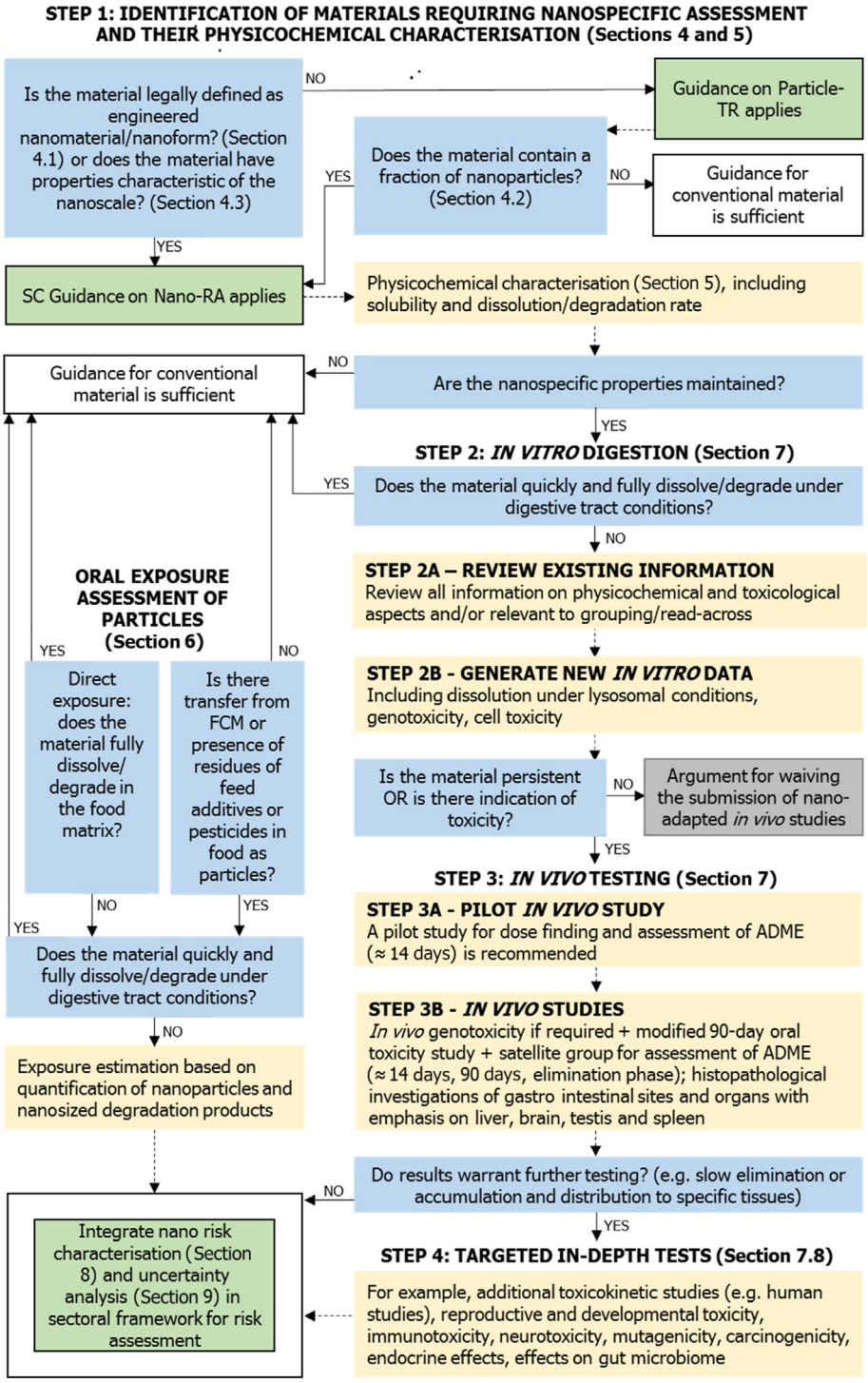
The following parameters may indicate a loss of nanospecific properties or a low exposure to nanoparticles.

- High/sufficient solubility and/or high dissolution/degradation rate[18] in water, food simulant, food or feed matrix or body fluids such as synthetic gastric fluids (see Section 5.2 and 7.2);
- 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).

The results from testing of the nanomaterial will give information for hazard characterisation that, combined with the oral[19] exposure assessment, will input risk characterisation. A particular case is represented by nanomaterials incorporated in FCMs. If scientific and/or experimental evidence shows absence of transfer of nanoparticles from FCM, then further toxicological testing may not be needed, because, in the absence of exposure, no risk to consumers can be expected.

[18] It should nevertheless be recognized that materials can generate, while dissolving, potentially toxic particles, ions or molecules.

[19] Other routes of exposure such as dermal and inhalation are detailed in Appendix D for feed additives and nanopesticides.



Legend: in green: nanospecific considerations for risk assessment; in blue: question to be answered; in white: risk assessment follows sectoral guidance; in yellow: nanospecific risk assessment steps. Dashed lines indicate unconditional workflows (no yes/no answer).

Figure 2: Schematic outline for the implementation of the SC Guidance on Nano-RA

4. Materials to be Assessed under this Guidance

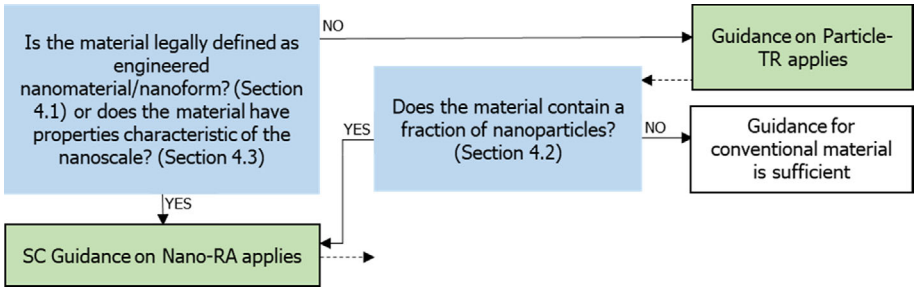


Figure 3: Step 1 includes the identification of materials requiring assessment according to the SC Guidance on Nano-RA (detail from Figure 2)

The first step in the risk assessment is to identify whether the material under consideration is a nanomaterial, has properties characteristic of the nanoscale or contains a fraction of nanoparticles. The Guidance on Particle-TR may be helpful to decide whether the material contains a fraction of nanoparticles. If a material is legally defined as being a nanomaterial (see Section 1.3), has properties of the nanoscale or contains a relevant fraction of nanoparticles, then the present Guidance applies for risk assessment of the material.

4.1. Is the material legally defined as engineered nanomaterial or nanoform?

An applicant has to consider whether the material intended to be placed on the market meets the criteria of the definitions as mentioned in Section 1.3 under a, b and c.

For engineered nanomaterials subject to the definition of Regulation (EU) 2015/2283 (Section 1.3 bullet point a), and for FCM substances in nanoform as outlined in Commission Regulation (EU) 10/2011 or deliberately engineered to particle size which exhibit functional physical and chemical properties that significantly differ from those at a larger scale as outlined in Commission Regulation (EC) No 450/2009 (Section 1.3 bullet point b); the applicant should include information in the submission dossier on the particle size distribution that demonstrates compliance with the definition.

To declare whether the material is an engineered nanomaterial/nanoform or not, according to Section 1.3 (bullet points a and b) the applicant should consider the relevant legislation. Currently, there are no specific guidance documents under such specific legislation but the applicant may use the strategies recommended by JRC and consult the documents listed hereunder to underpin the quality of results reported in submission of dossiers (in particular for particle size distribution).

Two reports by JRC (Rauscher et al., 2019a; Rauscher et al., 2019b) provide guidance on concepts and terms used in the European Commission (EC) Recommendation 2011/696/EU[20] of 18 October 2011 on the Definition of Nanomaterial. They also provide guidance on the identification of nanomaterials according to the EC's recommended definition through measurements. Furthermore, the EU-funded project NanoDefine (www.nanodefine.eu) addressed many challenges in the implementation of the EC's definition of nanomaterial. Key results and deliverables of the project address the selection of appropriate particle size measurement techniques and their evaluation, strategies for material dispersion, interpretation of results, standard operating procedures and approaches towards validation, as well as the resulting best practices can be found in the NanoDefine Methods Manual (in three parts) published by JRC (Mech et al., 2020a, 2020b, 2020c). The NanoDefine decision flow scheme and its implementation in the NanoDefiner e-tool (Brüngel et al., 2019 and https://labs.inf.fh-dortmund.de/NanoDefiner) can assist the applicant for the selection of appropriate characterisation techniques expected to be used for materials that fall under this Guidance (see Section 1.3) when needed.

In case of active substances in PPPs (Section 1.3, bullet point c) consisting of or containing nanoforms as outlined in Commission Regulation (EU) 2018/1881, the applicant should consider the

[20] European Commission (EC). Recommendation 2011/696/EU of 18 October 2011 on the Definition of Nanomaterial; OJ L 275, 20.10.2011; 2011; pp. 38–40.

relevant ECHA guidance on Registration and substance identification and in particular the specific Annex on nanoforms (ECHA, 2019).

4.2. Does the material contain a fraction of nanoparticles?

When an application concerns a conventional material which does not meet the applicable sectoral legal definition of engineered nanomaterial/nanoform (Section 1.3), it may still contain small particles including particles with external dimensions at the nanoscale that may be relevant for a nanospecific risk assessment. A typical example of this can be a material produced to have particle sizes in the micrometre range but also containing a fraction of nanoparticles.

The Guidance on Particle-TR (see the Introduction for a summary of the Guidance) is setting out the technical information expected to be submitted in new regulated food or feed applications, to demonstrate whether a conventional material (see glossary) consists of or contains a fraction of small particles, thus requiring nano-specific risk assessment. The Guidance on Particle-TR (EFSA Scientific Committee, 2021) has to be followed for all conventional particulate materials (available either as powder or in suspension). When the Guidance on Particle-TR leads to the conclusion that a nano-specific risk assessment is needed, the principles and approaches under this SC Guidance on Nano-RA apply for testing and assessing the material.

4.3. Does the material have properties characteristic of the nanoscale?

This Guidance applies to materials with properties that are characteristic of the nanoscale, irrespective of being nanomaterials, conventional materials containing a fraction of small particles, or other materials requiring nanospecific assessment such as nanostructured (see Section 1.3 point e) and advanced materials (see Section 1.3). Characteristics of the nanoscale result from quantum effects and other physical properties, e.g. a large specific surface area. The characteristics concern physical, chemical, mechanical and optical properties and include higher chemical and catalytic reactivities, plasmonic effects and altered electric surface charge. For the purpose of this Guidance, 'characteristics of the nanoscale' shall also include properties that are not in a strict sense a result of nanospecific effects, but result from processes that are typically applied to small particles to obtain specific characteristics that are different from the analogous conventional material, e.g. surface coating, surface functionalisation, core/shell structure or encapsulation. Furthermore, materials also have to be considered, when their chemical composition, morphology (in particular aspect ratio) and crystal phase differ from those of the conventional material for which data is available. Some characteristics of the nanoscale may affect the toxicity and the toxicokinetic behaviour of a material.

The following non-exhaustive list of indicators that may affect toxicity should be considered, in comparison to the equivalent conventional material when applicable, by an applicant when deciding if nanoscale properties have to be assumed and hence, an appropriate testing strategy according to this guidance has to be applied.

- Specific morphology (e.g. rigid long tubes or fibres and other high aspect ratio materials, high porosity) and crystal phase;
- Core/shell structure, including multifunctional materials and materials with cores and shells of different biopersistence;
- Altered hydrophobicity or hydrophilicity;
- Increased reactivity (e.g. catalytic, chemical, biological) compared to equivalent conventional material;
- Materials with functionalities such as targeted or controlled release;
- Materials with enhanced antimicrobial activity;
- Quantum effects (e.g. altered optical, electronic, magnetic, mechanical or redox properties).

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 cases, however, such applications can release nanomaterial that should be considered (e.g. impact of functional failure[21]). In the case of particles embedded in FCMs, release of particles by mechanical stress (bending or elongating occurring in use, surface abrasion) should be considered as well.

[21] Functional failure is a topic of good manufacturing practice and must be technically avoided.

4.4. Issues of concern for safety of nanomaterials

There are several issues around nanomaterials, fractions of nanoparticles and materials with characteristics of the nanoscale that may be indicative of a potential risk to the consumer. The Scientific Committee on Consumer Safety (SCCS) has published a Scientific Advice (SCCS, 2019), highlighting the general aspects of nanomaterials that should raise a safety concern, so that they could be subjected to risk assessment. Acknowledging the lack of any hard rules for working out the overall level of safety concern for a given nanomaterial, the Advice identified certain attributes, each of which should add a degree of concern to the safety of a nanomaterial. This includes the first alert over the presence of small particles (especially with external dimensions at the nanoscale), and when:

- the nanomaterial has constituent particles that have minimum external dimensions in the lower range of the nanoscale;
- the nanomaterial is insoluble, or only partially-soluble;
- the chemical composition of the nanomaterial suggests a toxicological hazard, e.g. due to the release of toxic ions or molecules;
- the nanomaterial has certain morphological characteristics (e.g. needle shape, rigid long fibres) that point to potential for harmful effects (e.g. plasma membrane perturbation);
- the nanomaterial has surface reactivity, potential for radical formation, or other surface properties (e.g. that can enhance cellular uptake, or allergenicity due to proteinaceous surface);
- the nanomaterial has a different ADME/biokinetic behaviour compared to the conventional equivalent, e.g. due to surface modification/coating;
- the nanomaterial is used as a vehicle to carry other substances that have not been assessed for safety as individual components, or together in the nanoscale entity;
- there is a likelihood of systemic exposure of the consumer to nanoparticles through the use of final products;
- the frequency of use, and/or the amounts of the consumer product are relatively high;
- there is evidence of persistence/accumulation of nanoparticles in the body;
- nanoparticles have other distinctive properties not present in conventional form of the same material, or a new activity/function (e.g. a smart/functional nanomaterial);
- the nanomaterial does not have a conventional comparator to allow assessment of changes in properties, behaviour or effects;
- the nanomaterial is used in a product that is inhalable (taken up by inhalation into the respiratory tract), and the particles are respirable (can reach respiratory epithelium i.e. alveoli);
- the assessment of genotoxicity is inadequate, e.g. *in vitro* studies are without information on stability of the test suspension, or evidence of cell exposure (internalisation).

The Advice also used a scoring system proposed by Brand et al. (2019), in conjunction with expert judgement, for assigning an indicative score for risk potential of nanomaterials. While not all of the points of attention listed above apply to food applications, they give a good indication on issues of concerns arising from the materials. In addition, the applicants are also asked to consider the following indicators of a potential for exposure:

- high production volume for a nanomaterial for the field of application (Brand et al., 2019),
- existence of several fields of application of the same material,
- high stability in products and/or persistence in the environment,
- anticipated frequent/high volume use of the products containing the nanomaterial (see Section 6 on exposure).

Dekkers et al. (2016) concluded that the aspects of toxicokinetics and human hazard assessment that are most likely influenced by nanospecific properties of the material include: degradation/ dissolution, accumulation, genotoxicity and immunotoxicity. These aspects should therefore be leading in hazard assessment of nanomaterials. See also the WHO's Draft Environmental Health Criteria Document: Principles and Methods to Assess the Risk of Immunotoxicity Associated with Exposure to Nanomaterials, 2017, http://www.who.int/ipcs/Immunonano/en).

The metabolism and excretion parameters are important indicators of biopersistence. Persistence of a substance or material is its ability to continue to remain in the body or the environment.

Biopersistence means that a substance or material is able to withstand those transformations that could lead to its solubilisation, metabolic degradation, detoxification or clearance from a biological system. The retention of a biopersistent nanomaterial or its transformation 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.

- Applicants should consider the relevant legal definition (i.e. engineered nanomaterial, substance in nanoform) for their application.
- The NanoDefine decision support flow scheme and its implementation in the NanoDefiner e-tool are helpful for the selection of appropriate characterisation techniques.
- For active substances in PPPs, the ECHA guidance document on Registration and substance identification and in particular the specific Annex on nanoforms (ECHA, 2019) should be consulted.
- The Guidance on Particle-TR is setting out the required technical information for new regulated food or feed applications, to demonstrate whether a conventional material, which is not covered by the definition of engineered nanomaterial set out in the Novel Food Regulation (EU) No 2015/2283, contains a fraction of small particles, including particles at the nanoscale.
- If a material has properties that are characteristic of the nanoscale, then this Guidance applies for risk assessment.
- In addition to the particle size of the material, a number of other properties such as chemical composition, morphology (in particular aspect ratio), surface properties (e.g. coating), crystallinity or solubility may be associated with adverse health effects. These properties may also alter the biokinetic behaviour and/or toxicological responses. A (non-exhaustive) list of indicators that may affect toxicity is provided.

5. Physico-chemical characterisation of nanomaterial

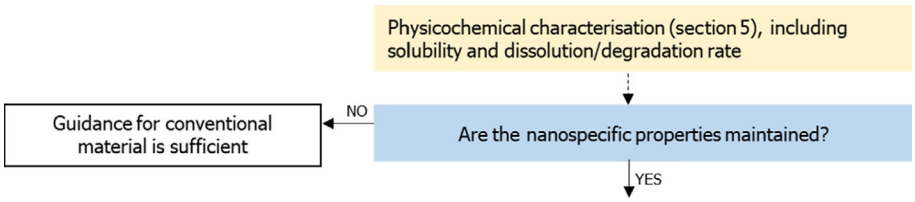


Figure 4: Step 1: Physico-chemical characterisation (detail from Figure 2)

The step preceding risk assessment is problem formulation and deciding whether the risk assessment includes applications of nanoscience and nanotechnology. Therefore, careful consideration will be needed early in the process to ensure an adequate characterisation of the material, which is essential for establishing its physico-chemical identity both as a pristine material and in food and feed products. It is also essential to identify changes in the material or products during storage and use, and after ingestion. In addition, monitoring the behaviour of a material in terms of biodistribution, speciation and quantification is crucial for hazard assessment (i.e. through toxicological and toxicokinetic studies). When a material is regarded as within the scope of this Guidance, a detailed physico-chemical characterisation is required, as described in this Section.

Physico-chemical characteristics of a nanomaterial are important as they can affect the outcome of the risk assessment. Nanoparticles of the same elemental composition may be present with different shapes, sizes, crystal structures (phases) or surface properties, e.g. as a result of a different production process, and may show different toxicokinetic behaviour of toxicities. For each distinct nanomaterial, nanoform, or set of similar nanoforms, the applicant must undertake a separate physico-chemical characterisation and risk assessment as described in this Guidance. It should also be noted that nanomaterials require specific attention regarding the representativeness of sampling and proper dispersion state (SCENIHR, 2007).

The physico-chemical characterisation of the material under investigation is relevant to the:

- full determination of the physical and chemical identity of the pristine material (see Section 5.1);
- physico-chemical characterisation of the material in test media used in *in vitro* studies, which is needed before, during and after experimental studies (see Section 5.3.2);

- physico-chemical characterisation of the material in the preparation to be administered *in vivo* (e.g. feed, drinking water) and in biological tissues and fluids from toxicokinetic and toxicological studies (see Section 5.3.2);
- physico-chemical characterisation of the material in complex matrices such as product formulations (see Section 5.3.1) and biological matrices (see Section 5.3.2), which is needed for exposure assessment (see Section 5.3.1).

5.1. Pristine material characterisation (as manufactured)

5.1.1. Dispersion of the material

Proper dispersion of particulate materials prior to characterisation and application in toxicological tests is essential to achieve valid and comparable results. A dispersion protocol can be considered effective if it yields samples which consist as much as possible of non-agglomerated/non-aggregated particles. The dispersion procedure used is often a critical step for proper particle size distribution measurements. For material characterisation, the final liquid dispersion of the material should result in particles in their most dispersed state. The dispersion also needs to be sufficiently stable, i.e. show a constant size distribution pattern or minimal re-agglomeration over the time necessary to carry out particle size measurements or for use in *in vitro* or *in vivo* toxicological tests.

A generally applicable dispersion protocol is not available due to the differences in physicochemical properties among materials. General steps for the development and validation of dispersion procedures are described in the literature (Mech et al., 2020c).

Specific dispersion protocols have been developed and published for a number of nanomaterials and purposes (Bihari et al., 2008; Jacobsen et al., 2010; Jensen et al., 2011; Guiot and Spalla, 2013; Taurozzi et al., 2012a–e; Hartmann et al., 2015; Mast and De Temmerman, 2016; OECD, 2017a (OECD TG 318); Verleysen et al., 2020). More dispersion protocols are available via the websites of international organisations (e.g. OECD – http://www.obce.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 – https://www.rivm.nl/en/about-rivm/mission-and-strategy/international-affairs/international-projects/nanoreg). In the absence of standardised dispersion protocols, the dispersion efficiency of the applied protocol and the stability of the dispersion (e.g. DLS, CLS) should be tested and documented.

More detailed considerations on the practical aspects of dispersion are given in the Guidance on Particle-TR.

5.1.2. Parameters for material characterisation

The characterisation of the material under investigation is essential to unambiguously define its identity. Similar to conventional materials, names and identifiers have to be provided and a number of physicochemical parameters need to be measured as detailed below.

Owing to the current gaps in knowledge relating to properties, behaviour and effects of nanomaterials, it is difficult to identify a definitive shortlist of parameters. Different international expert committees and working groups have considered the 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 might be changed in the 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, 2010); the International Organization for Standardization; the EU's Scientific Committee on Consumer Safety (SCCS, 2012); the ProSafe[22] project (European Union H2020

[22] 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.

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 quantitative structure–activity relationships (QSARs) and Grouping of Chemicals (ECHA, 2016); and a publication by DeLoid et al. (2017).

Not all the parameters listed in Table 1 (and Table A.1 in Appendix A) 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.

All measurement uncertainties should be reported as detailed in Section 9.1.

Currently, no generally accepted systematic nomenclature exists for nanomaterials. ISO TC 229 has drafted a series on vocabulary and terminology of nanomaterials (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 recommends 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 in terms of chemical composition and stoichiometry only. Examples could be complex iron oxide hydroxides or polymers. Other examples include substances already authorised for use in FCMs such as a butadiene, ethyl acrylate, methyl methacrylate, styrene copolymer (either not cross-linked or cross-linked with divinylbenzene or 1,3-butanediol dimethacrylate) in nanoparticles (FCM substance Nos 998, 859 and 1043). In less complex 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 precisely as possible. Thus, to properly characterise the materials subject to an application, in all instances the elemental composition should be provided (e.g. the empirical formula) together with additional information on the starting material(s), the reaction process(es) as well as the intended composition.

For nanomaterials consisting of **multi-component 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 precisely as possible. This includes information on the distribution of individual components in the particle, e.g. homogeneous mixture, core/shell and coatings.

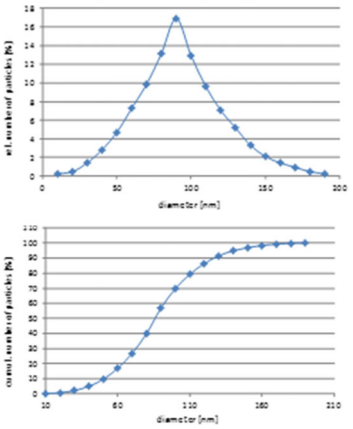
For the purpose of this Guidance, a material is considered as a 'coating' when it is bound or adhered to the surface of a nanomaterial in the form of a continuous outside layer, or a 'shell' when it is in the form of a nanosized covering/casing in which a material may be contained. **Coating** is a thin layer of a component that covers the surface of a particle completely or partially 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 particle).

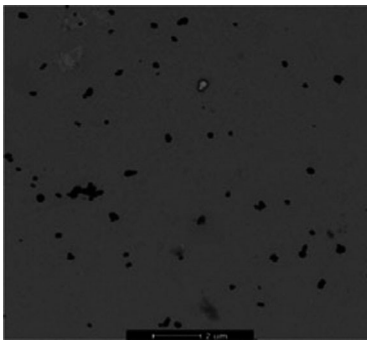
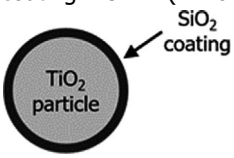
Changes in manufacturing process(es) can not only lead to significant differences in the physicochemical and morphological characteristics of nanomaterials between different batches but may also introduce new/different impurities and residual materials. Furthermore, for some materials, fundamentally 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 detailed description of the manufacturing process.

Table 1 is also meant to be applicable for materials consisting of multi-component particles (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 mono-component particle (e.g. uncoated TiO_2), information has to be provided for component 1 only. Examples for component 2 information are detailed in Table A.1 of Appendix A. Some of the general parameters of Table 1 (and Table A.1 in Appendix A) may already be required under the sectorial legislations. Appendix B provides a list of corresponding techniques for each parameter.

Table 1: Descriptors and parameters on data to be provided for characterisation of a pristine material, together with hypothetical examples (not food-related, not consistent, data for illustrative purposes only). For clarity, the Table is divided in 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 to be provided on the overall material, including details relevant for setting the specifications, i.e. ranges for the parameter, when applicable

Parameters	Explanation	Example (hypothetical)
Name	The name of the (nano)material used in the submitted application.	Ti-Max
Description	Brief description of the material.	Nanograde titania coated with a protective silica layer
Intended use	Foreseen use(s) and function of the material.	UV protection to be incorporated in food contact materials
Material composition and purity	Relative amount of the constituents in mass % as well as chemical identity of any impurities and their relative amounts in mass %.	TiO_2 97.1 ± 0.3% 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 elements	Relative elemental composition as the simplest positive integer ratio of atoms present in the material. Alternatively, the relative mass amounts of the contained elements (in mass %).	$Ti_{26}SiO_{54}$ Ti 58.23% (m/m) O 40.32% (m/m) Si 1.31% (m/m)
Constituent particle size Mean and median minimum external dimension with its number-based distribution	Data on the minimum external dimension of the constituent particles should be provided as measured by electron microscopy (EM). In case EM measurement is not feasible, the use of a different imaging technique is suggested. (See Appendix B) Justification and detailed information on the used specimen preparation and characterisation methods (e.g. instruments, settings, SOPs, calibration, method performance characteristics, data conversion and measurands) should be provided. Data should contain, at least, the median particle size (x50 in nm), with an estimate of the uncertainty of the median diameter (± expanded uncertainty, confidence level 95%, in nm), indicating the width of the distribution (e.g. standard deviation, in nm), information on the lower and upper limits of quantification. Data should further be presented as a number-based size distributions of the minimum external dimension of the particles. Measurands that target minimum external dimensions of particles are, for instance, minimum Feret diameter and maximum inscribed diameter. For each material, at least two graphs showing particle size distributions are to be shown, one with the relative number versus	TEM data on constituent particles: minimum external dimension estimated as the minimum Feret 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 

Parameters	Explanation	Example (hypothetical)
	size (continuous graph or histogram) and one with the number-weighted sum function (cumulative numbers). More detailed considerations on the practical aspects of EM and the reporting are given in the Guidance on Particle-TR.	
Particle shape Description of the shape, porosity, aspect ratio, EM image of the nanomaterial	Information on the particle shape, aspect ratio and whether or not the material is porous. This should also include representative 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 through He pycnometry results 
Structure Description of the structure, including (relative) thickness of structural elements	Spatial distribution of the components (e.g. homogeneous mixture, core–shell, surface coating). A graphical sketch for non-homogeneous particles to demonstrate the shape and schematic distribution. Information 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 25 nm in diameter 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 on any surface contamination.	Hydrophilic acidic silica surface, free –OH groups
Production process	Description of the production process used to prepare the entire nanomaterial (i.e. not of the individual components in cases of multicomponent particles) 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	For powder materials, data on mass specific surface area – MSSA (m^2/g) – and volume specific surface area – VSSA (m^2/cm^3) – of the material should be provided along with the conditions under which the measurements took place.	MSSA 15 m2/g (nitrogen absorption via BET method according to ISO 9277), VSSA 65 m^2/cm^3 (nitrogen absorption via BET method according to ISO 9277 and assuming a skeletal density value of 4.1 g/cm^3)
Appearance	Description of the appearance.	White powder, free flowing and non-cohesive
Melting point	Melting point of the nanomaterial (m.p., °C).	1,840°C
Boiling point	Boiling point of the nanomaterial (b.p., °C).	2,900°C

Parameters	Explanation	Example (hypothetical)
Density	Information on the density (g/m^3) of the nanomaterial (specify type of density, e.g. bulk, pour).	Bulk density: 4.1 g/cm^3
Porosity	Information on the porosity of nanomaterial (fraction of the volume of voids over the total volume, %).	Non-porous through He pycnometry
Dustiness	Dustiness for powder material (e.g. EN15051).	According to DIN EN 15051 B: W_r: 280 mg/kg Wi: 13,200 mg/kg
Formulation Formulation medium Dispersing agents (stabilisers) Auxiliaries Concentration of nanomaterial in dispersion	Description of the form in which a nanomaterial is present in a formulation, e.g. powder, dispersion. Information on other material(s) with which the nanomaterial may have been mixed/ formulated or any dispersants/stabilisers and other auxiliaries (e.g. preservatives, processing aids, etc.) used. The concentration of the nanomaterial in the mixture, in terms of both mass (g/kg) and particle number per kg, as well as the mass of the material as present in its ionic form.	Dry powder

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

Parameters (incl. specification ranges)	Explanation	Example (hypothetical)
Component 1		
Chemical name	When available systematic/IUPAC name of the substance that makes up component 1 of the nanomaterial. Alternatively, the chemical name that describes the chemical composition of the component based on the best available information, e.g. 'modified from XX' where XX = the nearest chemical name.	Titanium dioxide Titanium(IV)oxide
Trade name, common name, other names, synonyms	Any common names, synonyms, trade names and other names for the component.	Titania
Registry numbers	CAS number, EINECS/EC number, E number or other registry/database numbers related to the component (when available).	CAS number: 13463-67-7 ECHA Info card: 100.033.327 EINECS/EC number: 236-675-5
Formula	Molecular and structural formula (when applicable) of the constituent substance.	TiO_2
Molecular mass or atomic mass	Relative molecular mass (molecular weight) for molecules or relative atomic mass (atomic weight) for elements (g/mol).	79.866 g/mol
Elemental composition Empirical formula of this component	Relative elemental composition of the component 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), including any crystalline impurities.	Crystalline, rutile phase

Parameters (incl. specification ranges)	Explanation	Example (hypothetical)
Purity of the component	Relative amount of the constituent in mass %, as well as chemical identity of any impurities and their relative amounts in mass %.	Purity 99.9% Impurities: Fe_2O_3 0.1%
Production process component	The production process of the component 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 for all other components individually.	The full data sheet for the example including information on component 2 (SiO_2) can be found in Appendix A

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

Table 1C: Extrinsic (media dependent) properties of the material as in the final product

Parameters (incl. specification ranges)	Explanation	Example (hypothetical)
Stability	Information on the physical and chemical stability of the nanomaterial and coatings (if applicable). Conditions under which stability is tested.	Report on relevant stability studies
pH	pH value of a suspension of the nanomaterial, along with a description of the conditions under which the measurement was carried out.	pH 5.8, 10 g/L, 20°C
Solubility (see glossary)	Data on solubility of the nanomaterial in water (i.e. proportion of solute in solvent at room temperature, g/L) according to OECD TG 105, and solubility in other media if relevant. Note that solubility should not be confused with dispersibility of poorly soluble nanomaterials	Less than 0.1 mg/L, in water, 20°
Dissolution/ degradation rate	For nanomaterials with solubility in water less than 33.3 g/L, data on dissolution/degradation rate (g/(L*h)) and the conditions under which the measurements were carried out.	x g/(L × h) for Si and y g/(L × h) Ti, in water at pH = 7, 20°C.
Dispersibility	Information on dispersibility in terms of a relative amount of the particles that can be dispersed in a suspending medium, the stability of the dispersion in the given medium and the conditions applied (e.g. ionic strength and pH).	Optimal dispersibility in water at pH 8.2, max. 50 g/L, stability of dispersion of the particles (DLS) at least 48 h
Surface charge	Zeta potential (mV) values along with the conditions under which measurements were made (e.g. pH, ionic strength).	Zeta potential: −26 mV (deionised water, pH 8), Isoelectrical point: pH 2.2, method: electrophoretic light scattering according to ISO 13099-2
Agglomeration and/or aggregation state and size Mean and median diameter graphical	The agglomeration and/or aggregation state should be indicated and data on the size of the agglomerates/aggregates. The level of agglomeration/aggregation depends on the characteristics of the media and the protocol used for producing the dispersion. This level should be reported for the relevant condition(s), e.g. those	CLS data of the stock solution used for *in vivo* studies prepared according to the Nanogenotox dispersion protocol: median diameter x50 = 97 nm (uncertainty = 19 nm, 95% confidence level), width of distribution: SD=8 nm) mass-based arithmetic mean diameter:

Parameters (incl. specification ranges)	Explanation	Example (hypothetical)
diagrams of size distribution	used for the physico-chemical characterisation, stock and final doses used for *in vivo* testing, stock and culture media used for *in vitro* testing. Justification and detailed information on the used environmental conditions (medium, pH, etc.), characterisation techniques and methods (e.g. which techniques, instruments, settings, SOPs, method performance characteristics, data conversion and measurands) should be provided. Data should include median particle diameter (x50 in nm), with an estimate of the uncertainty of the median diameter (± expanded uncertainty, confidence level 95%, in nm) and with an indication of the width of the distribution (e.g. standard deviation, in nm), information on the lower and upper cut-off limits for the calculation of the relative amount of particles. Data obtained with a particle counting technique (such as EM) should be presented as number-based size distributions. Data obtained with other techniques (such as centrifugal liquid sedimentation (CLS) or dynamic light scattering (DLS)) in the original metrics as produced by the technique (e.g. light intensity, volume- or mass-based). Conversion to the number-based size distribution must also be provided, including information on the algorithms used for conversion and the associated uncertainty. 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 complex 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 are to be included, one with the relative number versus size (continuous graph or histogram) and one with number-weighted sum function (cumulative numbers).	105 nm (uncertainty=10 nm, 95% confidence level), width of distribution: SD = 13 nm) (plus diagrams as above] Aggregates: provide size data in the same manner as for constituent particles, see above Specifications size: median diameter 85 nm ± 5 nm
Reactivity when applicable	Information on chemical reactivity of the nanomaterial (including any surface coating). Information on catalytic (including photocatalytic) activity and reactive radical formation potential of the materials.	Report on relevant reactivity studies

5.1.3. Specifications and representativeness of the test material

In view of the potential significant differences in the physico-chemical characteristics of nominally the same nanomaterials **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

manufactured) nanomaterial intended to be used in food/feed should be provided by the applicant. The proposed specification should provide the acceptable range for each physico-chemical 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 testing could be considered **representative for risk assessment of use in food/feed**. 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). When it is not possible to manufacture a material within a narrow range of physico-chemical parameters, the applicant should develop specifications for its materials based on the data from a representative set of batches. An applicant should then provide safety data that cover different worst cases for the key parameters (such as size, coatings, crystalline shape) by separately testing each worst case, e.g. a worst case for particle size with other parameters kept constant.

5.1.4. Techniques and methods

Care should be taken in the selection of characterisation techniques, the evaluation of results and their documentation. It is, e.g. known that the results obtained from different particle size measurement techniques may differ because they address different measurands, e.g. hydrodynamic diameter vs. geometric diameter (Domingos et al., 2009). In other words, particle size analysis methods produce method-defined or procedure-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 sizes. Furthermore, particle size data are often presented as average values (e.g. arithmetic mean, harmonic mean, mode, median) rather than distribution data (e.g. percentiles). There are differences in the types of averaging between methods, which can amplify the already existing differences between methods.

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 B. **The selection of the appropriate technique** is the responsibility of the applicant 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 (Brüngel et al., 2019 and http://www.nanodefine.eu/index.php/nanodefiner-e-tool) and by the Methods Manual published by JRC (Mech et al., 2020a,b,c).

Sampling and sample preparation are often crucial steps in the overall analytical process and can significantly contribute to the measurement uncertainty. A critical issue in the sample preparation of nanomaterial is the proper dispersion of particles. This issue is addressed in detail in Section 5.1.1. General guidance for sampling also applies to the analysis of nanomaterials. Additional reporting recommendations for EM observations are provided in the Guidance on Particle-TR. 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 data must be provided for each nanomaterial in its pristine form (as manufactured), including an unambiguous description of the material's identity and relevant physico-chemical 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.
- For coated materials, the data must be relevant for the core nanomaterial and 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, the measured parameters and the measurands. The minimum external dimension of the constituent particles should, e.g. be measured with electron microscopy (EM). In case EM measurement is not feasible, a different, suitable technique should be applied and justified. 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.

5.2. Solubility and dissolution/degradation rate

Information on solubility and dissolution/degradation rate of the pristine material in water, and other relevant solvents if appropriate, is requested as described in Table 1, Section 5.1.2. In this Guidance, dissolution/degradation is considered a general term for the disintegration of a nanomaterial, e.g. due to physical, enzymatic or chemical processes. In addition, the dissolution/degradation rate in conditions representative of the human gastrointestinal tract and lysosomal fluid is considered key information in the present Guidance because this is where nanomaterials generally distribute to and where degradation can occur due to the acidic conditions and presence of enzymes (see more details in Section 7.2). It is therefore important to understand the fundamental differences between solubility and dissolution/degradation rate.

Solubility is the mass proportion of solute in solvent under equilibrium conditions (i.e. in a saturated state, see also glossary) and is determined as the concentration of the dissolved material in a saturated solution (i.e. undissolved material present as solid phase). It is important to note the difference between dissolution (materials are solubilised into their individual ionic and/or molecular species) and dispersibility (colloidal suspension of particles). The solubility is dependent on various factors 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. Simple and efficient separation of those species may be achieved by suitable filtration techniques, e.g. ultrafiltration, tangential flow filtration. Centrifugation techniques such as ultracentrifugation may be experimentally more difficult for very small particles and particles of a density similar to the solvent, but they are considered very useful for small particles in general. Protocols and guidelines for the determination of the solubility of nanomaterials have been proposed (OECD, 2020; Tantra et al., 2016). More practical guidance and analytical details can be found in the Guidance on Particle-TR.

The extent of solubility as well as the rate of dissolution/degradation are crucial parameters which determine the level of exposure to a nanomaterial. Sufficiently high levels of solubility and dissolution rates in foods will convert solid materials in dissolved species already before ingestion of food. A material composed/comprised of small particles that, on a mass basis, has a solubility equal to or more than 33.3 g/L in water is considered high so that nanospecific risk assessment may be waived for this material (SCCS, 2019).

The dissolution/degradation rate refers to the kinetics of the dissolution/chemical transformation process. Nanomaterials may dissolve/degrade faster than their bulk counterparts because of their high surface-to-volume ratio. The dissolution/degradation rate is influenced by various factors, including solvent, temperature, pH, concentration and presence of substances interacting with the particle's surface. The rate 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 as dissolution/degradation progresses). When under the standard conditions (for more details see Guidance on Particle-TR) the decrease of the solid material shows a half-life of 10 min or less corresponding to a dissolved fraction of 88% or higher in 30 min, no additional assessment for the fraction of small particles may be needed. For more details it is recommended to refer to the Guidance on Particle-TR.

5.3. Characterisation and quantification in a 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 can be more challenging in biological tissues and feed and food products because of the presence of complex matrices, and the low concentrations of the nanomaterial. In particular, biological matrices as well as food and feed products 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 silico, in vitro, in vivo* and absorption, distribution, metabolism and excretion (ADME) studies); the relevant matrices may be: water, food or feed, *in vitro* testing media, biological tissues and fluids;

- exposure assessment (characterisation and quantification) of nanomaterial in food/feed, transfer from FCM; the relevant matrices may be: feed, food, FCMs, food simulants.

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

Some guidance on the detection and identification of nanoparticles in complex matrices is given by CEN/TS 17273:2018 (CEN, 2018).

5.3.1. Characterisation in 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 particulate form that may be naturally occurring or otherwise present in 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 in food/feed products of the same or similar materials of geogenic, biogenic or anthropogenic origin.

When the characterisation of nanomaterial in food/feed matrices is difficult owing to the current limited availability of analytical methods, possible food/feed matrix interactions and interferences with 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 be representative for the nanomaterial properties in the actual food. With method development and availability, such characterisation of a nanomaterial should shift from food simulants to actual food/feed matrices.

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

For the toxicological assessment of a nanomaterial, it is essential to know in which form the nanomaterial is presented to the test systems. In addition, characterisation of a nanomaterial in the test system is relevant to determine the effect of the test medium/formulation (and its constituents) on the characteristics and properties of the nanomaterial. This will allow determination of the validity of the toxicity test outcome and will 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 a nanomaterial in *in vivo* studies it is essential that the nanomaterial is properly dispersed in the medium (see general information on dispersion in Section 5.1.1).

Apart from the nanomaterial particles' tendency to agglomerate and aggregate (which should be addressed by an appropriate dispersion protocol and use of dispersants that are compatible with the biological test system), the nanomaterial may adhere to the surface of glassware, tubing, pipette tips, syringes, vials etc. Appropriate analytical procedures (i.e. recovery tests) should be in place to quantify such loss of nanomaterial.

For *in vitro* studies, the nanomaterials have to be characterised in the exposure medium before, during and after the experiment to confirm actual presence in the test system and to observe potential changes that the materials may undergo (Section 7.5). 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 electron microscopy–energy-dispersive X-ray spectrometry (EM–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 detected in the test medium.

ADME studies (as described in Section 7.6) require the measurement of a nanomaterial in body fluids, tissues and excreta. It is relevant to quantify the amount of nanomaterial present, as well as to specify in which form the nanomaterial is present in these compartments. This includes chemical composition, particle 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 nanomaterials that contain elements with very low background levels in the matrix, the

samples could 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 spectrometry (AAS), X-ray fluorescence (XRF). Only samples containing the relevant elements have to be measured with a nanospecific method, e.g. spICP-MS, SEM–EDX. 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 and dilution of the entire compartment (e.g. liver) may lead to a concentration 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.

5.3.3. Characterisation and quantification in and released from FCM

This section applies to FCM substances in nanoform in accordance with Article 9(2) of Commission Regulation (EU) 10/2011, or which are deliberately engineered to particle size which exhibit functional physical and chemical properties that significantly differ from those at a larger scale in accordance to Article 5.2(c)(ii) of Commission Regulation (EC) No 450/2009; as well as to conventional FCM substances with a fraction of small particles requiring assessment at the nanoscale according to the Guidance on Particle-TR, and nanostructured or other substances used in FCM that may release nanoparticles.

Various applications of substances in nanoform or materials containing fractions of small particles with characteristics of the nanoscale for use in FCM are described in the literature or can be found on the market. Typically, these materials are used in applications where they are fully embedded in a host FCM polymer matrix, i.e. as polymer-nanoparticle composite (cfr. Commission Regulation (EU) 10/2011) or are incorporated as parts of active or intelligent materials (cfr. Commission Regulation (EC) No 450/2009 on active and intelligent materials). Polymer-nanomaterial composites can also be applied in coatings as part of a FCM. When incorporated into a FCM matrix, nanomaterials may structurally differ from the pristine nanomaterial. For instance, nanostructured mineral clays may exfoliate in the polymer matrix under the processing conditions and this can change the particle size distribution. Therefore, in addition to the characterisation of the nanomaterial used for manufacture of a FCM, the need arises for characterisation of the nanomaterial as present in the FCM (on the surface, in the matrix) and possibly when being released from the FCM.

Nanomaterials in the host polymer matrix should be characterised by their size and shape according to the parameters as presented in Table 1. This can be achieved 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 actual or potential migration of the nanomaterial from the FCM into the food matrix. This can be achieved by direct measurement of the nanomaterial in the food matrix, in the appropriate food simulant used in migration testing or by migration modelling of the nanomaterial in- and from 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 (i) swelling effect and (ii) mechanical stress or physical disintegration of a FCM polymer matrix.

- Swelling effects are due to strong interactions between a liquid food matrix or food simulant and the FCM polymer matrix. Swelling may facilitate nanoparticle release. In such cases, migration modelling using simple diffusion parameters may not be reliable and testing may need to be performed.
- Mechanical stress or physical disintegration can result from applying FCM material stress conditions (such as bending, stretching, thermal stress) and followed by testing using 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.

If the test results indicate the possibility of transfer of the nanomaterial to food/simulant, one or more nanospecific techniques should be employed to ascertain whether the migrating entities are in a nanoparticle form or in a solubilised (non-nanomaterial) form. A critical review of the published literature on the migration potential of nanomaterials from food contact polymers concluded that analytical observations reporting migration of nanomaterials were in most cases based on chemical analyses and did not demonstrate that the measured migrants were in nanoparticulate form or rule out the formation of observed nanoparticles as experimental artefacts (Stoermer et al., 2017). This reemphasises the need that in case of any measured transfer from a nanomaterial containing FCM, the particle nature of the measured migrants must be determined.

Comprehensive guidance for testing the potential transfer of nanoparticles from FCM, taking the above considerations into account, can be found (Franz et al., 2020). 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 consult always the most recent version of the EFSA Guidance(s) specific to FCM.

Appendix D/Section 3 gives a schematic outline and workflow for risk assessment with FCM substances in nanoform or with small particle sizes. Also guiding practical information is provided there on two examples of FCM applications from recent EFSA CEP Panel opinions on the assessment of montmorillonite clay modified with hexadecyl trimethyl ammonium bromide (EFSA CEP Panel, 2019a) and titanium dioxide, surface treated with fluoride-modified alumina (EFSA CEP Panel, 2019b). Finally, it includes some background information and gives assistance on testing of biodegradable polymers.

5.4. Quality assurance for physico-chemical characterisation

5.4.1. Standardised methods

Standardised methods (also known as documentary standards) should be used when available and applicable. Other fit-for-purpose methods (literature or in-house methods) may be used by the applicant with provision of supporting documentation for validation and standard operation procedures. Standard methods for physico-chemical characterisation are foremost delivered by standardisation bodies.

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 in-situ characterisation of nanomaterial in complex matrices. Appendix B provides a non-exhaustive overview of standard methods available at the time of issuing this Guidance. Applicants are recommended to consult the ISO and CEN databases[23] for the most up-to-date version and to look for applicable standard methods that may have been issued after the publication of this Guidance, and consider the ongoing work from OECD methods on nanomaterials.[24]

When no standard methods are available, **the applicant is responsible** for providing methods for the physico-chemical characterisation and quantification of the nanomaterial for which approval is sought. The respective methods must include standard operation procedures and must be validated (see Section 5.4.2).

5.4.2. Method validation and performance parameters

The applicant has to demonstrate that the methods used for the characterisation of nanomaterials in their pristine form (as manufactured), in commercial formulations, food/feed matrices and as used in toxicity tests are fit for purpose and **deliver reliable results**.

The methods should be validated intra- laboratory and preferably also inter-laboratory. Such validation process should follow existing international guidelines, e.g. IUPAC, 2002; Commission Decision 2002/657/EC[25]; Eurachem Guide from Magnusson and Örnemark, 2014, with adaptation if necessary. The use of validation protocols differing from internationally agreed ones would need justification. The validation study should establish the pertinent method **performance parameters**,

[23] 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:::

[24] https://www.oecd.org/env/ehs/testing/oecdguidelinesforthetestingofchemicals.htm

[25] 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.

such as specificity, selectivity, robustness/ruggedness, recovery/trueness, repeatability, intermediate precision (day-to-day variation) and reproducibility, detection/quantification limits for particle size, number and mass concentration. In addition, measurement uncertainties should be reported. 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.

Assay **robustness** for a nanomaterial could be established using similar principles as for assessing assay robustness for a non-nanomaterial. The robustness study should, in addition to the given nanomaterials, also include appropriate non-nanoscale controls materials. Methods addressing the determination of particle **number-weighted size distribution** or **external dimensions** of the constituent particles in the nanoscale and beyond should be assessed against general **performance requirements** as developed by NanoDefine (Rauscher and Mech, 2018).

When the results of a validation study meet the **predefined criteria of the specific performance characteristics**, then a method can be considered fit for its intended purpose.

The validation report documenting the results on these parameters should be part of the characterisation report. The performance characteristics (e.g. in terms of sensitivity (detection limits), trueness and precision) 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 demonstrating that the performance meets the requirements.

5.4.3. Reference materials

Reference materials are essential metrology tools for calibrating, validating, controlling and comparing the performance of analytical methods. Only few fit-for-purpose (certified) reference materials are currently available for nanomaterial characterisation. The German Federal Institute for Materials Research and Testing (BAM) (https://www.bam.de/Navigation/EN/Home/home.html) has established the COMAR Database which includes inventories of nanomaterial reference materials that are currently available from different global suppliers, such as JRC,[26] NIST,[27] BAM,[28] LGC[29] and others.

New types of reference materials are currently under development and can be expected to become available over time. In addition to its range of certified reference materials, the European Commission's JRC has also established a repository of industrial nanomaterials available to be used as representative test materials for safety testing[30]. These nanomaterials were used for testing by several EU-funded projects (e.g. MARINA,[31] NANoREG[32]) as well as the OECD WPMN and can be used as representative test materials by any research laboratories to generate comparable toxicological results (Roebben et al., 2013; Totaro et al., 2016). In the absence of suitable certified reference materials, self-generated and properly characterised and documented test materials may be used alternatively. 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 physico-chemical characterisation must be appropriate for the type of nanomaterial.
- Standardised methods should be used where available and applicable. Other fit-for-purpose methods may be used with provision of supporting documentation for validation and standard operation procedures.
- Method performance, as evaluated during validation studies, must meet the requirements for different performance characteristics, e.g. in terms of sensitivity (detection limits), trueness and precision.
- Certified reference materials should be used to calibrate, validate, control and compare the performance of the analytical methods used. In the absence of suitable certified reference materials, self-generated and properly characterised and documented test materials may be used alternatively.

[26] https://crm.jrc.ec.europa.eu/
[27] https://www.nist.gov/srm
[28] https://www.bam.de/RRR/Navigation/EN/
[29] https://www.lgcstandards.com/GB/en/
[30] https://ec.europa.eu/jrc/en/scientific-tool/jrc-nanomaterials-repository
[31] 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.
[32] 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.

- Nanomaterials must be characterised in a relevant food/feed matrix and in relevant test media.
- In case of technical limitations in the analysis of nanomaterials in food/feed matrices, characterisation and study of matrix interactions should be carried out using food simulants.
- 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. nanomaterials transferred from FCMs).
- To assess the exposure of the consumer to a nanomaterial from FCM, it is essential to determine the actual or potential transfer of the nanomaterial from the FCM into the food matrix.

6. Oral exposure assessment of nanomaterial

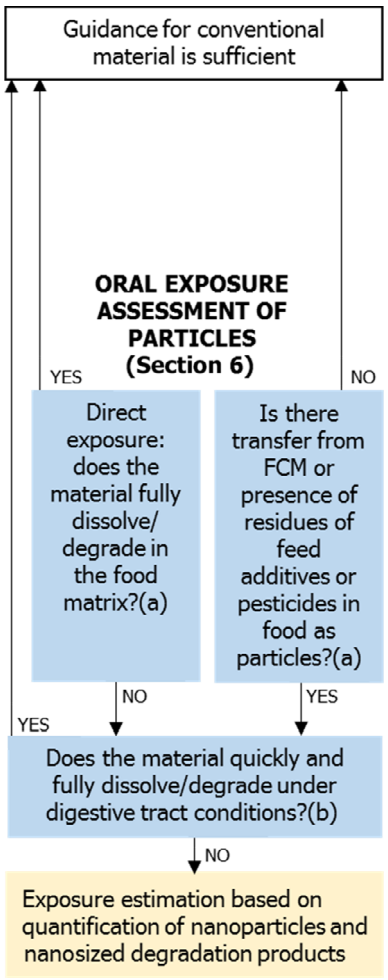


Figure 5: Steps in oral exposure assessment (detail from Figure 2).
(a) If it cannot be determined whether a nanomaterial is present in the food/feed matrix or food simulant it should be assumed that nanomaterial is present.
(b) The assessment of degradation products that are still in the form of nanoparticles should continue as presented in this Guidance. See Section 7.2 and Appendix C for details on *in vitro* gastrointestinal digestion. If it cannot be determined whether a nanomaterial is present in simulated digestive tract conditions, it should be assumed that nanomaterial is present

Exposure scenarios covered by this Guidance are for instance use as novel food, addition of the nanomaterial to the food (e.g. flavourings, food additives), transfer of substances from FCM to food, or carry-over of residues of feed additives or pesticides to food. Anticipated uses, use levels and potential oral exposure to the nanomaterial should be investigated as shown in Figure 5 and the paragraphs below. Some types of application could lead to other routes of exposure, such as dermal or inhalation exposure (e.g. use as feed additives or pesticides during handling). The nanospecific aspects (including all relevant routes of exposure) that have to be considered in risk assessment of these types of applications are detailed in the Appendix D.

Firstly, the intended use of the nanomaterial should be described in the application (e.g. novel food/additive/pesticide/food contact material). Secondly, information has to be provided on the characteristics of the pristine nanomaterial (see Section 5.1) and the amount added to food or released from FCM into the food, including in which form (dissolved or solid and when solid with information on the particle size distribution). Feed additives require information on the levels in feed and animal exposure, as well as on the carry-over of residues to food of animal origin.

When direct **exposure** of humans or animals is possible, such as from novel foods or food/feed additives, it should be assessed whether the nanomaterial or its dissolution/degradation products in the form of nanomaterials remain present as particles in the food/feed matrix. If no nanomaterial remains present in food/feed products (see Section 5.3.1), there is no exposure to nanomaterial and risk assessment should follow the relevant EFSA guidance for conventional material. If (some of) the nanomaterial is still present, then the presence of the nanomaterial or of its nanosized degradation products remaining present as particles under the *in vitro* simulated conditions of the gastrointestinal tract should be assessed (Section 7.2).

If there are no data on the quantification of the nanomaterial in the food/feed matrix or on the degradation under simulated conditions of the gastrointestinal tract, it has to be assumed that the exposure will be equal to the amount of nanomaterial **initially added** to the food/feed and that **all** added nanomaterial is present, ingested and absorbed as nanoparticles. This represents the worst-case scenario.

Exposure assessments in the EFSA remit should also cover the transfer by migration or physical release from FCM, as well as the presence of residues from nanomaterials used as feed additives or active substances in PPP. The first step in these cases is to assess if consumers will be exposed to particles or only to non-particulate species (ions, molecules). Verifiable evidence confirming that consumers will not be exposed to nanoparticles may be sufficient for concluding that a conventional risk assessment according to sectoral guidances is sufficient.

For **substances in FCM**, the type of transfer into food or food simulant[33] should be considered, including the potential solubilisation of any released nanomaterial in the food. The nature and extent of transfer of the substance in nanoform from the FCM should be measured by an appropriate technique with detection limits according to state of the art (see Sections 5.1.4 and 5.3.3), and considering relevant size distribution of the constituent particles.[34]

In the context of this Guidance, evidence (see Section 5.3.3) indicating essentially either no release of the substance in nanoform, no release of other nanoparticles, or any release being non-nanomaterial in nature, should be sufficient to waive further nano-specific testing for FCM substances.

Furthermore, a generic migration limit of 0.06 g/L (60 mg/L) has been prescribed for chemical substances from FCMs (Art. 12 Regulation (EU) 10/2011[35]). Keeping in mind this limit, it is important to note that if the solubility of a FCM substance in food or food simulant is greater than 60 mg/L then the migrating amount of this substance up to this limit is likely to be solubilised and will not be in nanoform before ingestion of the food. In such a case, a conventional risk assessment may be sufficient. In a case where migration of a FCM substance may exceed the prescribed generic limit of 60 mg/L, then from the risk assessment perspective it will be irrelevant whether it is in solubilised or in particle form because it will not comply with legislation.

[33] 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

[34] 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).

[35] Plastic materials and articles shall not transfer their constituents to food simulants in quantities exceeding 10 milligrams of total constituents released per dm^2 of food contact surface or 60 mg/6 dm^2. This corresponds for a packaging surface of 6 dm^2 in contact with 1 kg food to a migration of 0.06 g/L food simulant.

It is also equally important to note that, irrespective of the presence of a nanomaterial or nanosized degradation products in a substance used in FCM, the potential release of molecules/ions from the FCM should be assessed in any case in accordance with the relevant EFSA guidance on conventional FCM substances.

For **feed additives**, the ADME and residue studies should investigate if the residues are in molecular or ionic form or present as nanoparticles. For substances with solubility levels in water or lipids higher than their maximum level of residues, the absence of consumer exposure to nanoparticles can be assumed and a conventional risk assessment is sufficient. The confirmation of exposure to nanoparticles would trigger assessment according to this guidance.

For **pesticide active substances** in nanoform, if consumer exposure is confirmed, the residue definition should indicate whether the residues in food are in molecular/ionic form, present as nanoparticles, or a combination of both. If there is evidence that the residues are not as nanoparticles, a conventional risk assessment is sufficient. In case of nanoformulated PPPs with active substances in molecular form (i.e. coformulants in nanoform encapsulating or carrying a conventional active substance), the exposure assessment should consider that the nanoformulation may affect the fate in the crop as well as in animals. The available information should be sufficient for verifying the expected fate of the nanoformulated PPP in the crop following the application, and subsequent exposure and metabolism in livestock when relevant. In particular, it is essential to assess whether or not consumers may be exposed to residues in nanoform, and in particular to particles of the nanoformulated PPP, in the diet. The confirmation of exposure to nanoparticles would trigger assessment according to this guidance; while if all residues are in molecular form, a conventional risk assessment for consumers is sufficient. See Appendix D.2 regarding specific considerations for the risk assessment of applicants, workers, bystanders and residents related to non-oral exposure routes.

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 and following the sectorial methodology/scenarios to assess exposure. Thus, guidances apply that provide specific information on how to determine consumer exposure. General issues such as food/feed sampling, variability within composite samples and variation in concentrations between or among samples are not different from the exposure assessment for the micro- or macroscale or for non-nanomaterials and need to be addressed in the risk assessment.

The anticipated average and high exposures to nanomaterial in or as food/feed for various population groups must be estimated based on the available occurrence and food consumption data. Sectoral guidance documents provide the principles and approaches used for exposure estimations in the different areas. Assumptions made in the exposure assessment should be described as well as its related uncertainties. 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.

- Exposure assessment should take account of the anticipated uses in line with the type of nanomaterial application. 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.
- When a nanomaterial or nanosized degradation products is/are no longer present, risk assessment should be carried out according to the relevant EFSA guidance for conventional materials.
- For assessment of exposure through the transfer via migration or physical release from FCM; transfer via carry-over from animal feed or from a pesticide to crop), one should determine whether there is transfer and if the exposure is to (nano)particles or solutes (ions, molecules). Convincing scientific evidence showing the absence of transfer of nanoparticles indicates that the conventional risk assessment may be sufficient.
- Specific considerations are required when establishing the residue definition for pesticides, to inform whether the residues in food are in molecular form or present as nanoparticles.
- When it is not possible to determine the nanoparticles 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 as such.

7. Hazard identification and hazard characterisation of nanomaterial

The test requirements stipulated in current EFSA and EC guidance documents for different intended food/feed uses also apply in principle to nanomaterials. This Chapter outlines additional considerations on 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 assess these hazards.

The key point for **hazard identification** is that nanomaterials may show typical 'small particle' behaviour, and thereby can elicit a biological reaction different from the corresponding non-nanomaterial (if applicable, see glossary). Therefore, nanomaterials have to be assessed according to this Guidance. On the other hand, the risk assessment for quickly degrading nanomaterials may follow the relevant existing guidance for conventional materials.

The general considerations regarding **quality criteria** for toxicity testing (e.g. performed according to OECD Test Guidelines and Guidance documents as well as Good Laboratory Practice) are applicable for testing nanomaterials, and should be complemented with the specific requirements mentioned in this Guidance. General considerations for testing nanomaterial are covered in Section 7.3. Even around or within the nanoscale, there may be considerable differences in the toxicity of a given nanomaterial due to variations in particle size. For instance, in the case of silver, a particle diameter of 10 nm has been identified as a size threshold where a substantial increase in toxicity occurs both *in vitro* and *in vivo* compared to slightly larger nanoparticles (Ivask et al., 2014; Recordati et al., 2016). It is therefore crucial that **the material as produced is the same material as used for testing (see Section 5.1.3)**, and that the size and properties of the manufactured material used in the specific application lie within the range covered by the risk assessment. Accordingly, the applicant has to demonstrate, by providing analytical data, that batch-to-batch variation is within the range in the specifications proposed by the applicant. For conventional materials with broad specifications the Guidance on Particle-TR provides specific advice.

7.1. Stepwise framework for *in vitro* and *in vivo* testing: overview

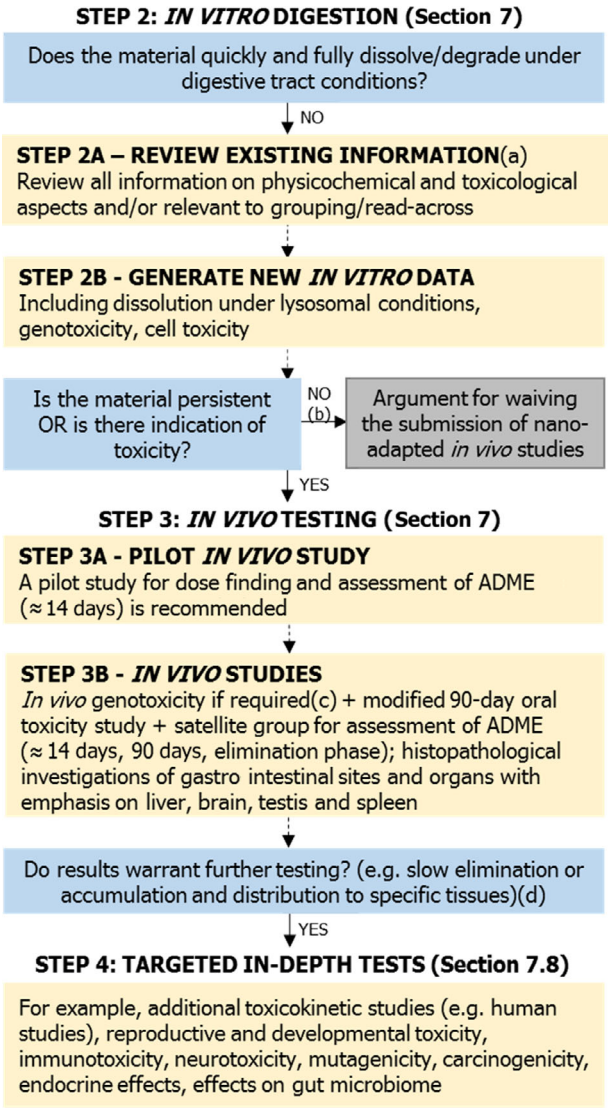


Figure 6: Steps in testing (detail from Figure 2)

a) Review of existing information should continue on each step throughout the entire process of risk assessment.

b) If NO, an argument can be put forward that further nanospecific testing is not necessary. It is anticipated that for most cases that have entered Step 2, conclusive evidence covering all endpoints required for a safety assessment (including local effects for any relevant route of exposure) based on *in vitro* testing would require the integration, in a weight of evidence (see Chapter 8), of information from existing *in vivo* studies conducted with related (nano or non-nano) materials (see Section 7.9 on read-across). Direct testing under Step 3 may be required to demonstrate whether the nanomaterial represents a hazard or not. Furthermore, for many regulatory frameworks, e.g. for food additives, there is a requirement of testing for systemic toxicity and for local effects in the gastrointestinal tract by means of a 90-day oral toxicity study. This study has to be designed according to the stipulations of nanospecific issues as described in this Guidance for performing the tests of Steps 3 and 4.

c) *In vivo* genotoxicity testing is required when at least one of the *in vitro* tests indicates genotoxic activity, or if it is not appropriate to test the nanomaterial *in vitro*.

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 Steps 2 and 3 assessment should be further investigated in Step 4.

It should be noted that OECD Test Guidelines and test designs must be adapted for assessing nanomaterials (see Section 7.3).

Step 1, the identification of materials requiring nanospecific assessment and their physico-chemical characterisation, is described in Sections 4 and 5.

In **Step 2,** the rate of degradation of the nanomaterial to the non-nanomaterial under conditions representative of the gastrointestinal tract is investigated. Nanomaterials that quickly dissolve or degrade, (i.e. have a high dissolution/degradation rate; see Section 7.2) can be expected not to show nano-related behaviours, and thus, a 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 2A.

Step 2A is aimed at gathering available information from existing literature that meets quality criteria (i.e. that has adequate characterisation and toxicity data on the nanomaterial tested), that can identify specific issues that need to be addressed for a proper conduction of *in vitro* studies (see Section 7.5) and *in vivo* studies (ADME) and the 90-day oral study in Step 3B (Section 7.7), or that provides weight of evidence information for decision-making in risk assessment. Therefore, a comprehensive and systematic review of scientific literature covering, but not limited to, safety and efficacy, should be provided by the applicants as integral part of a safety dossier. References and bibliographic lists should be provided in the format required by EFSA guidances (e.g. the existing guidance for conventional materials). The search terms used for the reviewing process, the number of relevant publications found and the reasons for selecting or rejecting publications should also be provided. In particular, scientific reasoning should be provided for not considering any contradicting results.

Step 2A can include existing information on the specific nanomaterial as well as on similar materials, i.e. materials that only deviate to a limited extent in one or more physico-chemical parameters as described in Table 1 (Section 5.1.2). 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 2B** comprise degradation tests under simulated lysosomal conditions, representing conditions after phagocytosis of the nanomaterial by macrophages (see Section 7.2), *in vitro* genotoxicity tests (see Section 7.4) and a battery of tests including relevant *in vitro* toxicity tests (see Section 7.5). The use of specific cell lines *in vitro* may further inform investigations *in vivo* and may support the design of the studies. When there is evidence of a lack of persistence based on dissolution/degradation rate under simulated lysosomal and gastrointestinal conditions, and no indication of potential toxicity from existing literature information or 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 3) is not necessary. However, it is anticipated that for most cases that have entered Step 2, testing under Step 3 will be required. Testing under Step 3 is considered necessary to demonstrate if the nanomaterial represents a hazard or not.

In **Step 2B**, the *in vitro* genotoxic potential of nanomaterials is investigated according to the tests indicated in Section 7.4. Nanomaterials that tested negative in *in vitro* genotoxicity assays are considered non-genotoxic and further *in vivo* genotoxicity test is usually not required. Nanomaterials that tested positive in at least one *in vitro* genotoxicity assay have to be considered to be potentially hazardous and their genotoxic capability requires further investigation in *in vivo* testing (**Step 3**). If genotoxicity cannot be tested *in vitro*, as a rule *in vivo* genotoxicity testing is necessary (see Section 7.4).

To decide on whether to proceed to **Step 3**, the results from the *in vitro* testing of the nanomaterial should be considered as well as other relevant information, e.g. on chemical reactivity (which might predispose to site of contact effects), bioavailability, metabolism, toxicokinetics and target organ specificity.

Step 3A consists of a pilot *in vivo* study (≈ 14 days; see Section 7.6), which is recommended for dose-finding and assessment of absorption, tissue distribution and accumulation and elimination, e.g. by measuring tissue concentrations at the end of study. Subsequently, **Step 3B** consists of a modified 90-day toxicity test (OECD TG 408) (OECD, 2018a) (see Section 7.7). This study has to be designed according to the stipulations of nanospecific issues as described in this Guidance for performing the tests of Steps 3 and 4. A waiver for the study may be presented when there is scientific justification of non-absorption and the absence of local effects for any relevant route of exposure. In the 90-day study of Step 3, specific attention should be paid to (indications of) local effects in the gastrointestinal tract and organs routinely investigated by histopathology, with emphasis on liver, spleen, brain and gonads. 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 7.7). 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 4**.

- A stepwise approach (Figure 6) should be adopted for hazard identification and hazard characterisation to avoid unnecessary testing of nanomaterials.
- In Step 2, the rate of degradation of the nanomaterial to a 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 2A, all available information should be gathered and a set of *in vitro* studies carried out to identify hazards and the need for further testing in Step 3.
- In Step 2B, the *in vitro* genotoxic potential of nanomaterials must be investigated according to the tests indicated in Section 7.4. Nanomaterials that tested positive in at least one *in vitro* genotoxicity assay have to be considered a potential hazard and their genotoxic capability requires further investigation in *in vivo* testing (Step 3).
- Step 3A consists of a pilot *in vivo* study (≈ 14 days) for dose-finding and assessment of absorption, tissue distribution and accumulation and elimination.
- Step 3B consists of a modified 90-day toxicity test (OECD TG 408 (OECD, 2018a) with the adaptations mentioned under Section 7.3 and inclusion of a satellite group for assessing uptake and tissue distribution/accumulation, as mentioned in Section 7.6.2. In the 90-day study specific attention should be paid to (indications of) local effects in the gastrointestinal tract and organs normally investigated by histopathology, with emphasis on liver, spleen, brain and gonads). 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 4.

7.2. *In vitro* degradation tests

7.2.1. *In vitro* gastrointestinal digestion

Assessment of the dissolution/degradation rate of nanomaterials in conditions representative of the human gastrointestinal tract is considered a key first step in the stepwise approach (Figure 6). If a high dissolution/degradation rate can be demonstrated, as detailed later this Section, the standard safety assessment procedure for conventional materials can be followed.

A suite of *in vitro* digestion models has been described in the literature that assesses the release or dissolution/degradation 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 sophisticated models by using static or dynamic conditions, and with or without the application of enzymes, bile salts etc. In addition, the physiology that is simulated may vary between models: fasted versus fed conditions, infant versus adult.

An *in vitro* digestion method suitable for food under fed conditions (as opposed to fasted conditions) harmonised by the COST Infogest network[36] was described by Minekus et al. (2014), with updates in Brodkorb et al. (2019). The effects of differences in pH, mineral type, ionic strength, digestion time and enzyme activity are discussed. The method consists of a simulation of mouth conditions, followed by a gastric phase at pH 2 to 3 for 2 hours and an intestinal phase at pH 7 for 2 hours. The composition of the digestion fluids is fully described. Although this method is not specific for nanomaterials and it is not an officially standardised method, it is considered a key approach also

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

to be used for nanomaterial in food, i.e. for simulating physiological conditions in the gastrointestinal tract after food consumption.

In the case of substances in foods for infants below 16 weeks of age, the design of the dissolution/ degradation rate test requires adaptations for this age group, including those adaptations related to gastrointestinal tract volume and gastric pH (see also EFSA Guidance on the risk assessment of substances present in food intended for infants below 16 weeks of age (EFSA Scientific Committee, 2017e)). For information on the physiological conditions in gastrointestinal tract of infants and *in vitro* digestion of infant formula, see Nguyen et al. (2015).

For conditions mimicking the fasted state, several *in vitro* digestion methods have been described and compared by Koch et al. (2013). These methods can be explored further for applicability in the area of nanomaterials.

Some experience has been gained with the application of *in vitro* digestion models for nanomaterials. NANoREG D5.0[37] showed 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, varied. 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 hours of intestinal digestion under the specified conditions). Degradation measurement in such complex matrices is 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 single particle inductively coupled plasma mass spectrometry (spICP-MS) and inductively coupled plasma mass spectrometry/atomic emission spectroscopy (ICP-MS/AES)-based methods in combination with a separation technique such as 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 among the different compartments: mouth, stomach and intestine. Sieg et al. (2017) reported that the aluminium nanoparticles remained unchanged in saliva and strongly agglomerated in the gastric phase showing an increased ion release. The levels of aluminium ions decreased in the intestinal fluid and particles deagglomerated. Altogether, dissolution of nanoparticles was limited. These size distributions comprised all particles, irrespective of their chemical composition. There appeared to be minimal dissolution of the Fe_2O_3 particles in all three stages of digestion. Sohal et al. (2018) investigated the dissolution behaviour of TiO_2, SiO_2, ZnO and two Fe_2O_3 nanomaterials by measuring both the particle and ion concentrations in the mouth (1 hour), stomach (8 hours) and intestinal phase (8 hours). TiO_2 was found to be most persistent with a maximum of 0.4% dissolution in simulated gastrointestinal fluids. Also the iron oxides were highly persistent: 0.7% maximum dissolution for a rod-like form and 2.3% for an acicular one. SiO_2 and ZnO were dissolved for 65.5% and 100% in the gastric phase, respectively. In the intestinal phase Si ions reprecipitated while ZnO remained completely dissolved.

Of note, intestinal digestion of soluble silver ions (from $AgNO_3$) and ionic aluminium 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).

Validation and standardisation of *in vitro* digestion models for nanomaterials are lacking. No comparison for nanomaterials has been made between *in vitro* degradation/dissolution data from 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 factors 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 *in vitro* digestion model used in the stepwise approach should be provided indicating whether the model chosen is applicable for fed or fasted conditions, and whether the model is expected to represent worst-case, realistic or favourable conditions for *in vitro* degradation of the nanomaterial. For example, fasted conditions may be less representative for effects of nanomaterials in foods, whereas low pH conditions in the stomach – as may occur in fasted conditions – may promote the degradation of most metals and metal 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 dissolution/degradation rate under fed

[37] NanoReg was a cooperation between 71 Partners form a consortium of European RTD performers, metrology institutes and nanomaterials and instrument manufacturers. NANoREG Deliverable D 5.03 is available at: https://www.rivm.nl/en/documenten/nanoreg-d503-dr-in-vitro-screening-methodology-for-absorption-or-crossing-of-other

and fasted conditions. Furthermore, as previously suggested (Lichtenstein et al., 2015), scientific research on the impact of food components on the degradation of nanomaterials should be considered.

The *in vitro* digestion model used should be critically assessed for its reliability and reproducibility. To increase the reliability of the degradation information from the model, the dissolution/degradation rate should be determined by including different time points (at least four time points in duplicate at approximately 5, 15, 30 and 60 min) in the intestinal phase. The study should be performed with three different concentrations as this may affect the degradation; the middle concentration should be representative of human exposure. This can be calculated by the estimated daily intake and the appropriate volume of GIT secretions. This volume depends on the anticipated use and whether the material is ingested at one instant or throughout the day. Considering the daily volume of secretions into the gastrointestinal tract (e.g. Kiela and Ghishan, 2016; Ogobuiro et al., 2020), the proposed values are 2 L for adults and 1 L for infants and children if the ingestion is related to one instant (e.g. one meal), and 4L for adults if the ingestion is distributed over the day. 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. using spICP-MS or Transmission Electron Microscopy-Energy Dispersive X-Ray Spectroscopy (TEM-EDX)). The concentration of the solute and, if present, other degradation products, should also be determined under the intestinal conditions.

Some materials may degrade completely in the conditions of the stomach and then precipitate in the intestinal conditions as salts or nanoparticles or microparticles (Walczak et al., 2013). The dissolved fraction should not be separated from the rest during *in vitro* digestion as this may promote dissolution. It has been shown that SiO_2 particles can form large non-nanosized agglomerates in the conditions of the stomach that may deagglomerate in the intestine (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 under **the intestinal conditions** is considered relevant and should be provided. Simulation of the mouth and stomach conditions still needs to precede simulation of the intestinal conditions, but data on the dissolution rate in the mouth or stomach phase are not strictly required.

The measured concentrations of solute, degradation products and particles should be compared with the 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 Sections 5.1.3 and 5.4.2).

A nanomaterial is considered to degrade quickly/have a high dissolution/degradation rate if **the dissolution/degradation rate profile in the intestinal phase shows a clear decrease** in the presence of particles over time (no plateau), and when **12% or less of the material** (mass-based) – 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 is fully degraded to non-nanomaterial (e.g. ionic) under gastrointestinal conditions. Details of the rationale and discussion of the uncertainty for this cut-off value can be found in Appendix C. 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. In such cases, a nanospecific risk assessment would not always be required. However, in case of complete digestion in gastrointestinal fluids, potential local effects (e.g. in the upper gastrointestinal tract) need to be considered (Holpuch et al., 2010).

For materials that dissolve under acid conditions, precipitation may occur with the change in pH in the intestine. If *de novo* precipitation in the intestine occurs, it would occur independently of the physical state (solid or liquid) and particle size of the administered material, and consequently, the hazard of the *de novo* precipitate will be covered by the risk assessment process of the material independent of the particle size at marketed conditions.

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

In the absence of data on degradation, the applicant has to assume that 100% of the ingested material remains in particulate form. Furthermore, if it cannot be demonstrated that the material quickly degrades, one should continue to Step 2 (Figure 2 or 6).

7.2.2. Stability in lysosomal fluid

Once in the body, some nanomaterials may not be cleared easily and may accumulate over time. Macrophages often take up the nanomaterials as these cells engulf and digest cellular debris, foreign substances, microbes, etcetera, in a process called phagocytosis (Gray and Botelho, 2017). Macrophages are found in many tissues and a large amount of macrophages are stationed at strategic points where microbial invasion or accumulation of foreign particles is likely to occur, e.g. in Peyer's patches and the liver. Liver macrophages are also referred to as Kupffer cells. After a macrophage engulfs a nanomaterial, lysosomes of the macrophage fuse with the phagocytosed nanomaterial. 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 a suitable model as lysosomal fluid 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 induce toxicity 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). Tests are generally carried out in static (i.e. batch) conditions, but the use of dynamic flow-through methods (see OECD (2020)) might be more relevant in specific cases and need to be justified.

To assess the stability in lysosomal fluid, pristine materials should be submitted to *in vitro* simulated lysosomal degradation and the dissolution/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 can extend up to 72 or 96 hours. Degradation and particle sizes 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 hours is considered indicative of a high dissolution/degradation rate given that degradation in lysosomal conditions provides 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 hours 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.

- If a high dissolution/degradation rate can be demonstrated, the standard safety assessment procedure for conventional materials can be followed.
- 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. In the case of substances in foods for infants below 16 weeks of age, the design of the dissolution/degradation rate test requires adaptations for this age group, including those adaptations related to gastrointestinal tract volume and gastric pH.
- The *in vitro* digestion model used should be critically assessed for its reliability and reproducibility. To increase the reliability of the degradation information, the dissolution/degradation rate should be determined by including at least four time points in duplicate in the intestinal phase (5, 15, 30 and 60 min). The study should be performed with three different concentrations: the middle concentration should be representative of human exposure. The particle number–size distribution and concentration of the solute and possible degradation products should be analytically determined, using at least an EM technique.
- A nanomaterial is considered to degrade quickly/have a high dissolution/degradation rate if **the dissolution/degradation rate profile in the intestinal phase shows a clear decrease** in the presence of particles over time (no plateau), and when **12% or less of the material** (mass-based[38]) – 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 is fully degraded to non-nanomaterial (e.g. ionic) under gastrointestinal conditions.

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

- In the absence of data on degradation, the applicant has to assume that 100% of the ingested material remains in particulate form.
- To assess the stability in lysosomal fluid, pristine materials should be submitted to *in vitro* simulated lysosomal degradation and the dissolution/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 are normally expected to be in the range of hours and can extend up to 72 or 96 hours. Degradation and particle sizes of nanomaterials after lysosomal treatment should be characterised.
- A half-life of about 24 hours is considered indicative of a high dissolution rate in lysosomal fluid. This would result in 12% or less of the material (mass-based) being present at 72 hours compared to the particulate concentration at the beginning of the degradation test.

7.3. Adaptation of Test Guidelines and test designs for toxicity testing of nanomaterial

As indicated by the OECD (2012), when testing nanomaterials for toxicity, the test design should consider their potential for aggregation/disaggregation and agglomeration/deagglomeration as well as stability in different media. OECD recommends the adaptation of the OECD Test Guidelines takes into account the specific properties of manufactured nanomaterials (OECD, 2017b). Special considerations are also needed for testing nanostructured materials and materials containing a fraction of particles at the nanoscale. In the last case, both options, testing the full material with special nano-considerations or specific testing for the fraction of nanoparticles are acceptable, as long as the combined information covers the safety assessment of the full material. In case of nanomaterials consisting of different nanoforms or materials with broad specifications regarding particle size and properties, the information should cover the full range of properties or at least the worst-case conditions.

The following paragraphs provide general considerations for testing nanomaterials and the Sections 7.3.1 and 7.3.2 provide specific information for *in vitro* or *in vivo* testing, respectively.

For hazard characterisation, mass is a convenient **metric to express the concentration/dose** used for *in vitro* and *in vivo* (oral) studies. Mass is not always the best dose metric to describe the response but is currently a practical one for hazard characterisation. By having the number–size distribution and density, the concentration/dose can be derived as necessary. Other metrics such as specific surface area can also be derived from the nanomaterial characterisation and might be considered.

In both *in vitro* and *in vivo* testing, it is essential to check the **physical status/properties** of the nanomaterial in the test medium including possible changes in the surface or structure of the nanoparticles, and specifically particle agglomeration and the stability of the dispersion during the exposure period.

The use of an appropriate protocol for dispersion of nanomaterials and stability monitoring, and proper reporting of the level of **dispersion/degree of agglomeration**, are needed for allowing the reliability assessment of the results. The atoms or molecules at the surface of a solid particle do not have adjoining atoms/molecules above the surface. This leads to the atoms/ molecules at the surface being under an inward force in the form of extra potential energy at the particle surface – termed as surface free energy. Compared to conventional (bulk) form of materials, particles at the nanoscale have vastly greater surface areas, and therefore, nanoparticles possess a relatively huge surface free energy. For this reason, whilst particles of all sizes tend to stick together to some degree to form agglomerates, the tendency to agglomerate is far more pronounced when the particles are in the nanosize scale (Simon and Joner, 2008).

It is important to note that, in the agglomerated form, constituent (nano)particles are not bonded together chemically, but are only loosely-held together by weak van der Waals forces or electrostatic interactions. Agglomerates may therefore deagglomerate to release nanoparticles under certain conditions, e.g. due to a physical force or a change in pH or ionic condition. Therefore, nanoparticle agglomeration/deagglomeration can be a dynamic process under different physical and biological conditions. This interchangeable nature of the process poses a moving target for characterising a nanomaterial's agglomeration/deagglomeration status at different times and under different conditions.

The level of agglomeration is also dependent on particle concentration, and can be expected to increase with the concentration/dose. Thus testing a nanomaterial at high doses may lead to a reduction in the actual exposure levels to constituent nanoparticles. For risk assessment of nanomaterials, it is important to consider the worst-case scenario in relation to the potential risk. Nanomaterial can be conceptualised to pose the highest risk when they are in the form of dispersed

constituent nanoparticles. Therefore, for risk assessment, the testing a nano-sized material needs to be carried out when in fully dispersed form – irrespective of how it is intended for use in a food/feed product.

The Guidance on Particle-TR offers a generic dispersion protocol for conventional materials containing a fraction of small particles that can be used and adapted to specific characteristics of the nanomaterial to be tested. Sections 5.1.1 and 5.1.2 describe dispersion and characterisation in a test medium.

It is also important to note that the phenomenon of agglomeration is different from aggregation, which generally results from manufacturing processes that involve the use of high energy. This leads the constituent particle to fuse together through strong bonds, e.g. metallic bond or covalent bond. Thus, unlike the loosely-held agglomerates, particles in an aggregate are not likely to disaggregate under normal conditions. Therefore, in case of aggregated materials, the toxicity testing should be conducted with a material with the lowest level of aggregation to which consumers will be exposed following normal use conditions, and the dispersion protocol should be designed for deagglomerating the aggregates.

The selection of the concentrations/doses to be used in the *in vitro* and *in vivo* safety studies is usually a challenge. On the one hand, the tested concentrations/doses should be high enough to cover the maximum actual or expected human exposure levels plus the uncertainty factors (i.e. *in vitro* to *in vivo* extrapolation when relevant, interspecies and intraspecies variability, and additional uncertainties in the data set). On the other hand, agglomeration is expected to increase with the concentration/dose, leading to a reduction in actual exposure levels to individual non-agglomerated nanoparticles at high doses. As a consequence, the degree of agglomeration should be assessed for each dose or concentration, unless there is verifiable confirmation from a validated SOP indicating that a high and similar level of dispersion in the administered material or test concentration will be achieved through the full dose/concentration range. Similarly, the colloidal stability of the dispersion should be measured and reported, or ensured through a validated SOP, for the full period of administration to the animals or the full exposure period for *in vitro* studies. When the level of agglomeration changes with the dose/concentration and/or time, a quantitative or at least a semiquantitative assessment of these changes should be reported and considered for the interpretation of the results. Studies conducted at high doses (for *in vitro* tests all doses above 100 μg/mL; for *in vivo* studies, all doses above 50 mg/kg body weight (for liquid forms) or 100 mg/kg body weight (when incorporated in the food matrix)), without information on dispersion and stability or confirmation of cellular/tissue exposure are insufficient for the hazard identification and characterisation of nanoparticles, as well as for the hazard identification and characterisation of the fraction of nanoparticles of conventional materials.

Getting **actual confirmation on exposure of cells/tissues to the nanoparticles** increases the reliability of the results and is highly recommended. For *in vivo* studies, the combination of the toxicokinetic and toxicity assessment proposed in this Guidance will provide actual exposure estimations and facilitate the evaluation of the results. For *in vitro* studies, the direct confirmation of the **cellular uptake** of nanoparticles is generally feasible and highly recommended. In case of nanomaterials with partial dissolution, a combination of methods may be required for assessing both the presence of particles and dissolved material in the tissues/cells.

It should be noted that studies conducted at high doses or high level of agglomeration, although inadequate to provide insight into particle-related deposition and toxicity, may nevertheless provide information on the hazard associated to the dissolved fraction and non-nano degradation products. The Guidance on Particle-TR provides specific proposals for assessing existing safety studies and recommendations for filling information gaps related to the level of coverage of the nanoscale hazards.

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).

A detailed level of reporting covering the study design and the results is essential for the use of literature studies in the regulatory context. In addition to the general recommendations, Faria et al. (2018) have proposed a 'minimum information standard' for reporting the characterisation of the material, the biological system and the experimental protocol in scientific publications.

The SC is aware that biomolecular **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 fate and/or toxicity of a nanomaterial. However, corona formation is difficult to measure. Corona formation can affect the state of agglomeration and sedimentation behaviour as well as the overall biological identity of nanomaterials

(Cedervall et al., 2007; Lundqvist et al., 2008; SCCS, 2019). 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 possible binding affinities. Certain components of the corona (e.g. opsonins) may activate the mononuclear phagocytic system (macrophages) and induce a subsequent nanomaterial clearance from the organs. In view of the present limitations in measurement and interpretation, information on corona formation and its characterisation may be taken into consideration.

7.3.1. Specific adaptations for *in vitro* studies

There is a large number of research projects, in the EU and beyond, that focus on developing *in vitro* methods for the safety assessment of nanomaterials, as well as several activities at OECD and ECVAM[39] related to the validation of these methods. Until '**validated**' ***in vitro*** methods specifically **for nanomaterials** become 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 or interfere with the nanomaterial resulting into inconsistent test data.

Additional **quality controls** should include (when available) negative and positive reference nanomaterials, assay reagent controls (e.g. the dye) and 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 Sections 5.1.1 and 5.3.2). Models based on DLS and centrifugation have been proposed to estimate how much nanomaterial is reaching the cell system (DeLoid et al., 2017). 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 may not be representative of the concentration 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 should be justified. Use of concentrations that lead to extensive agglomeration should be avoided as well as conditions leading to sedimentation of the material. Concentration-response relationships showing a plateau or even a change in the response direction at higher doses may be indicative of concentration-dependent agglomeration; confirmation of internal exposure is essential for a proper assessment of these effects.

In vitro studies may provide mechanistic information on the toxicokinetics of the nanomaterials, e.g. selective uptake of particles and/or agglomerates with specific sizes, shapes or surface properties, as well as on the cellular distribution and fate of the nanoparticles following internalisation. In addition, these studies may facilitate the mechanistic understanding of the toxicodynamics of the nanomaterials and its comparison with the toxicodynamics of the non-nanomaterial forms (see Section 7.10 on Integrated approaches to testing and assessment).

At least **two independent *in vitro* assays (see Section 7.5) 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 systems (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 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 on ***in vitro*** **digestion** are addressed in Section 7.5.

[39] EURL ECVAM is an integral part of the Joint Research Centre (JRC), the science and knowledge service of the European Commission and is located at the JRC site in Ispra, Italy.

7.3.2. Specific issues for *in vivo* studies

Nanomaterials are potentially unstable when dispersed. In oral toxicity studies, the test material can be administered via **animal feed or drinking water or by gavage**. For proper administration, the nanomaterial should be stably and uniformly dispersed in the food/feed, drinking water or gavage vehicle by using an effective dispersion protocol. A dispersion protocol can be considered *effective* if it yields samples which consist as much as possible of non-agglomerated/non-aggregated particles (see Section 5.1.1). Using such a dispersion protocol in toxicity testing would ensure the highest level of gastrointestinal absorption of nanoparticles (worst-case exposure conditions).

Toxicological studies in which the exposure of laboratory animals mimics the consumers' exposure to nanoparticles are of relevance for risk assessment. Nanomaterials and conventional materials containing a fraction of nanoparticles used in food are frequently subjected to technological processes to maximise their nanoscale properties. In case of administration via the animal diet, the process for mixing the nanomaterial with the feed should mimic the conditions for consumer exposure to the nanoparticles, reflecting the expected level of agglomeration. Unless consumers are directly exposed to the nanomaterial in powder form, the nanomaterial should be first suspended in a liquid medium by using an appropriate dispersion protocol, and then, the resulting suspension should be thoroughly blended into the feed matrix to ensure a homogeneous mixture. A direct admixing of nanomaterials in **powder** form to the animal diet, without ensuring that the preparation is representative for the technological use patterns in foods, may lead to exposure conditions different from those of consumers' exposure due to a high level of particle agglomeration.

The **stability and physico-chemical characteristics of the nanomaterial in the vehicle** should always be determined (see Section 5.2). 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 freshly prepared** and used immediately after preparation. **Complete delivery** of the dose should be checked because a nanomaterial may, e.g. adsorb to the walls of the drinking vessel, gavage syringe or tube and therefore may no longer be available, reducing actual exposure (Kreyling et al., 2017).

Application by gavage provides conditions for ensuring the administration of an accurate dose of a dispersed material, also allowing to measure and report the degree of dispersion prior dosing (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. 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 is **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 food or drinking water administration** have to be included to determine whether hazards associated with the nanomaterial are observed under realistic exposure scenarios.

In addition to the reporting of issues for a test material as indicated in respective OECD Test Guidelines, addition of nanospecific issues is necessary in the reports of the guideline studies and in scientific publications. In particular the characteristics of the test material, a detailed description of the adaptations of the study design for testing nanomaterials according to the provision of this Section, detailed information on the dispersion protocol, and achieved levels of dispersion/agglomeration, and stability of the dispersion should be reported. The Guidance on Particle-TR contains Annexes with recommendations on how to report these nanospecific issues.

- OECD Test Guidelines and other testing protocols require specific adaptations for testing nanomaterials. These adaptations must also be considered for testing materials containing a fraction of small particles at the nanoscale.
- 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.

- The test material should be checked to ensure that there is no substantial alteration in physico-chemical characteristics under test conditions, including possible changes in the surface or structure of the nanoparticles (e.g. particle agglomeration and the stability of the dispersion during the exposure period). Validated SOP for dispersion are available for some nanomaterials. The Guidance on Particle-TR includes a generic dispersion protocol that can be used and adapted to the characteristics of the nanomaterial.
- 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/concentrations should be provided. Studies conducted at high doses – for *in vitro* tests all doses above 100 μg/mL; for *in vivo* tests all doses above 50 mg/kg body weight (for liquid forms) or 100 mg/kg body weight (when incorporated in the food matrix) – without further information on dispersion and stability or confirmation of cellular/tissue exposure are insufficient for the hazard identification and hazard characterisation of nanoparticles, as well as for the hazard identification of the fraction of nanoparticles of conventional materials.
- Consideration should be given to whether a plausible mode of action can be deduced.
- When possible, an experimental group exposed to the corresponding non-nanomaterial should also be included in *in vivo* and *in vitro* studies.
- *In vitro* studies:
  - *In vitro* studies may provide mechanistic information on the toxicokinetics and toxicodynamics of the nanomaterials. In addition to 'validated' methods, scientifically 'valid' methods providing sufficient information for their regulatory use may be used in the hazard assessment.
  - Quality controls should include 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 of 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* assays 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.
- *In vivo* studies:
  - Toxicological studies in which the exposure of laboratory animals mimics the consumers' exposure to nanoparticles are of relevance for risk assessment.
  - Oral administration may be carried out through feed, drinking water or by gavage. When gavage is the method of choice, the study may be complemented with additional groups; this is especially needed when exposure occurs mainly via foods.
  - Dispersions for testing should be prepared freshly and used immediately after preparation. Information should be provided regarding the stability and degree of agglomeration.
  - The confirmation of systemic exposure is essential, and whenever possible, the quantification of internal levels in relevant tissues, is highly recommended.
  - Complete delivery of the dose should be ensured by avoiding the test material adsorbing to the walls of the drinking vessel or the gavage syringe and tube.
  - In specific cases, especially when exposure occurs mainly via solid and liquid foods, additional groups with dietary administration should be included in the study applying administration by gavage.
  - The reporting recommended in OECD TGs should be supplemented with the detailed description of the nanospecific issues as indicated in this Section.

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

Genotoxicity testing of nanomaterials should follow the outline 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, 2014a) and included in the SCCS (SCCS, 2016; 2019) notes of guidance (SCCS/1564/15; SCCS/1611/19) and ECHA (2017a; (2020), and recognised by risk managers in the update of the information requirements described in Annex VII of the REACH Regulation (Commission Regulation (EU) 2018/1881). Catalán et al. (2017) described a theoretical approach for a weighted assessment of the mutagenic potential of nanomaterials.

Nano- and non-nanomaterials may induce genotoxic damage, e.g. 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 determine 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 described by Pfuhler et al. (2013) and Doak et al. (2012). Specific recommendations on best practices, assays and methods for the assessment of nanomaterials genotoxicity have been published (Elespuru et al., 2018).

In selecting a suitable battery of ***in vitro*** genotoxicity tests, the three critical genotoxicity endpoints (gene mutation and 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 (OECD, 1997)) is not a recommended method for investigating the genotoxicity of nanomaterials (OECD, 2014a). 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 (OECD, 2016b)) and the *in vitro* mammalian cell gene mutation tests using the thymidine kinase gene (OECD TG 490 (OECD, 2016e)) are appropriate. REACH Annex VII has been adapted accordingly '8.4.1. In vitro gene mutation study in bacteria. The study does not need to be conducted for nanoforms when it is not appropriate. In this case other studies involving one or more in vitro mutagenicity study(ies) in mammalian cells (Annex VIII, Sections 8.4.2. and 8.4.3 or other internationally recognised in vitro methods) shall be provided.'
2) A test for structural and numerical chromosome damage, i.e. the *in vitro* mammalian cell micronucleus test (OECDTG 487 (OECD, 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 at least one cell cycle, is advisable (Elespuru et al., 2018). 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). Manual scoring of micronuclei is time-consuming and thus there have been developments in high-throughput micronucleus detection methods, including automated imaging systems and flow cytometry (Bryce et al., 2007; Shibai-Ogata et al., 2011; Seager et al., 2014). Care must however be taken when applying micronucleus detection by flow cytometry as optically active nanomaterials can interfere with the flow scoring analysis (Li et al., 2017; Nelson et al., 2016). Furthermore, nanoparticle agglomerates can be erroneously scored as micronuclei. Use of high-throughput imaging systems for analysis (e.g. Metafer Scanning and Imaging Platform, MetaSystems) can overcome the nanomaterial interference issues associated with flow cytometric scoring for micronuclei (Li et al., 2017; Manshian et al., 2015). Several mammalian cell models have been used for nanomaterial genotoxicity assessment that show comparable or differential sensitivity (European Commission Joint Action, 2008–2013, Nanogenotox[40]; European Union Seventh Framework Programme, 2007–2013; NANoREG[41]; Cowie et al., 2015). Cell lines representative of the gastrointestinal tract or the expected target tissue should be considered as first choice. However, it is important to ensure that the background level of micronuclei is equal to or lower than 2% to be appropriate for use with the *in vitro* mammalian cell micronucleus test. 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

[40] 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 the Netherlands) provide the remaining. www.nanogenotox.eu

[41] https://www.rivm.nl/en/about-rivm/mission-and-strategy/international-affairs/international-projects/nanoreg

nanomaterial is a crucial step in understanding its behaviour and toxicity (Magdolenova et al., 2014; Dekkers et al., 2016).

In line with the use of NAMs (see Section 7.10), additional *in vitro* tests that provide mechanistic understanding may be taken into consideration in a weight of evidence approach, e.g. Pig-a test, toxicogenomics, recombinant cell models, γH2AX, high content analysis.

Most poorly soluble nanomaterials are not metabolised and the extracellular metabolic activation system (S9-mix) 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-mix 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 with lesion-specific enzymes for detection of oxidised DNA bases can be recommended as many nanomaterials have been shown to induce oxidative stress or at least a concomitant surrogate measure of oxidative stress (ROS, antioxidants). The comet assay also facilitates the design of *in vivo* assays when those are necessary (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. This mechanism of DNA damage is typically observed *in vivo* as it is the result of a chronic immune response causing genotoxicity in epithelial tissues through the release of inflammatory mediators (Evans et al., 2019; Pfuhler et al., 2017). Secondary genotoxicity mechanisms can only be detected *in vitro* if co-culture models are used, consisting of both immune and epithelial cells (Evans et al., 2019).

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. The choice of the appropriate *in vivo* genotoxicity test(s) requires expert judgement, based on all available information. The mode of action should be investigated *in vitro* in order to select the most appropriate *in vivo* follow-up (EFSA Scientific Committee, 2011b), which 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 may be suitable:

- an *in vivo* mammalian erythrocyte micronucleus test (OECD TG 474 (OECD, 2016a)). 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 TG 489 (OECD, 2016d)),
- a transgenic rodent somatic and germ cell gene mutation assay (OECD TG 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 7.6) or repeated-dose toxicological studies (see Section 7.7), 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, 2011b, 2017d) taking into account the specific properties of nanomaterials as outlined in this Guidance.
- *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.
- When 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 the *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 (OECD, 2016a)); *in vivo* mammalian alkaline comet assay (OECD TG 489 (OECD, 2016d)); transgenic rodent somatic and germ cell gene mutation assay (OECD TG 488 (OECD 2013).

7.5. *In vitro* toxicity testing

In vitro tests in Step 2 may also provide insights into a nanomaterial's hazard and its mode of action upon e.g. internal exposure (see Section 7.10). Although currently the number of successfully validated *in vitro* toxicity methods is limited, the situation will change in the future as the consequence of ongoing activities covering chemical safety assessment in general and the assessment of nanomaterials in particular. In addition to a number of EU-funded projects under the Horizon 2020 programme, in collaboration with the OECD Programme on Manufactured Nanomaterials and the OECD Test Guidelines Programme, in May 2020, the NANOMET project was launched that aims to standardise test methods to characterise the properties that are specific to nanomaterials and to investigate the adaptations needed to existing testing and assessment methodologies. As this is a field under exponential development (e.g. Romeo et al., 2020) in the food and feed area (e.g. Xavier et al., 2021), the Scientific Committee recommends applicants and interested parties to explore the availability of 'validated' and so-called 'valid' *in vitro* methods covering the different endpoints before embarking on *in vivo* testing. It should be noted that although *in vitro* methods do not offer a full replacement of *in vivo* studies for safety testing in the food and feed area, they are very useful for obtaining mechanistic information. See also OECD Guidance Document on Good *In Vitro* Method Practices (OECD, 2018d). In certain cases, *in vitro* methods for a nanomaterial or fraction of small particles may be combined with available safety studies on the non-nanomaterial and provide sufficient evidence and avoid carrying out additional *in vivo* studies.

In the area of nanomaterials, *in vitro* toxicity tests have an advantage, because, when properly designed, it is usually possible to monitor directly the cellular internalisation and subsequent fate of the nanoparticles. This may provide a mechanistic understanding on the toxicodynamics of the nanomaterial, compared to the non-nanomaterial, informing the weight of evidence approach (see Section 8).

Taking into account that the oral intake is the *in vivo* route of administration in the scope of this Guidance, there are several *in vitro* approaches available to generate additional hazard information (Drasler et al., 2017). *In vitro* models based on primary cells or cell lines and on monoculture or co-culture systems are available to represent the gastrointestinal tract. Co-culture-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). These systems are suitable for investigating both, the uptake of particles and possible local effects. Primary human cells, such as primary human oesophageal epithelial cells either in monoculture or (better) in co-culture, may 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 variation and they are 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 can translocate through the gastrointestinal barrier, they can enter the circulatory system and reach liver, spleen, kidney and other organs. It is, therefore, recommended to include the respective representative cell types for these organs (e.g. hepatocytes) in the testing strategy. In addition, the commercially available whole blood cytokine release kit can serve to characterise immunotoxic reactions, including immunostimulation and immunosuppression of immune responses (Langezaal et al., 2001, 2002), although this is not (fully) 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 a precise description of the cell culture method need to be reported in order to determine the method's reliability. Exposure and post-exposure times need to be well-defined and justified with respect to the individually tested parameters. For monoculture 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 results, it is required that OECD Guidance 211 is followed (OECD, 2014b).

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. tetrazolium reduction assays (MTT or MTS). 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 proinflammatory and anti-inflammatory mediators (multiplex analysis-based cytokine profiling; Bhattacharya et al., 2017). Additionally, oral route specific endpoints may be considered, e.g. 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 potential interference of nanomaterials with media components of *in vitro* test systems (see Section 7.3.1) (Cornu et al., 2018).

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.

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 cell culture inserts. These inserts may hamper translocation by adherence of nanomaterials (resulting from a possible inability to pass through the membranes or from nanomaterial attachment) to the membrane.

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. This involves standard *in vitro* tests as required for bulk (non-nanomaterial) substances (see Appendix D sector-specific information) but must be carried out in consideration of the nanospecific aspects as detailed in Section 7.3.

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 7.7 and 7.8). As mentioned in Section 7.1 for most cases that have entered Step 2, it is anticipated that testing under Step 3 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. It is anticipated that in the future the combination of knowledge about toxicity and understanding of mechanism or mode of action and *in vitro* methods may be sufficient for the safety assessment of nanomaterials, reducing the need for animal testing. Meanwhile, for most cases that have entered Step 2, conclusive evidence for a safety assessment (including local effects for a relevant route of exposure) would require the combination of *in vitro* testing and information from existing *in vivo* studies conducted with related nanomaterials or the non-nanomaterial (see Section 7.9). Furthermore, for many regulatory frameworks, e.g. for food additives or novel foods, there is a requirement of testing for systemic toxicity in case of absorption and for local effects in the gastrointestinal tract by means of a 90-day oral toxicity study. A 90-day study is also useful for the detection of local effects (even of the dissolved fraction). Nevertheless, the outcomes of *in vitro* tests may provide the mechanistic understanding and serve as basic evidence of a

possible nanomaterial hazard and can contribute to the design and interpretation of *in vivo* studies by identifying 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 (see also Section 7.3.1).

- In vitro toxicity data may be used as additional weight of evidence and/or to target further in vitro testing.
- Before embarking on in vivo testing, applicants and interested parties are recommended to explore the availability of validated and valid in vitro methods covering the different endpoints to gain more mechanistic information.
- Specific endpoints relevant for in vitro testing are: cytotoxicity/cell viability, induction of oxidative stress, (pro-)inflammation and gastrointestinal barrier integrity impairment. Toxicokinetic considerations, including gastrointestinal uptake and cellular internalisation may be combined with toxicity endpoints. Confirmation of cellular exposure increases the relevance and reliability of in vitro toxicity results.
- Use of cocultures is often preferred over monocultures when testing on a more complex system is desired (depending on the objective of the study); standard monocultures are informative, easier to standardise and can be used 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).

When *in vitro* methods indicate a 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 (specifically designed for the nanoscale aspects) on a case-by-case basis, provided that an (90-day*) in vivo* study is not mandatory under the sectoral legal framework for the substance that will have to be followed. Such a 90-day study is also considered useful to detect local effects (even of the dissolved fraction).

7.6. *In vitro* and *in vivo* toxicokinetics testing (ADME)

This Section provides guidance for applicants on how to apply a strategy regarding testing for Absorption, Distribution, Metabolism and Excretion (ADME) to cover nanospecific aspects. However, that all ADME requirements under the sectoral legal frameworks/guidances (i.e. studies to define toxicokinetic parameters following single and repeated exposure) remain to be addressed within the context of the sectoral risk assessment of the substance.

Toxicokinetic information is crucial for risk assessment of nanomaterials, as well as for substantiation of read-across scenarios on the basis of other materials, e.g. a similar nanomaterial or a non-nanomaterial. 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; ISO, 2019).

As mentioned in Section 4.3, the size, shape or surface properties of particles can affect the toxicokinetic behaviour of nanomaterials or materials containing small particles with nano properties. For example, one prominent finding in recent research is that 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). Nanomaterials with particle sizes of 20–40 nm in diameter can be taken up easily by intestinal cells (enterocytes). M cells in Peyer's patches can internalise rapidly (within minutes) not only a significant number of nanoparticles (20–100 nm), but also some larger particles (0.5–2 μm) (Howe et al., 2014). The intestinal epithelium is covered with a layer of mucus (continually secreted by the Globet cells). Any particles reaching the intestinal epithelium have to cross the mucus layer first. It has been suggested that their passage through the mucus layer is restricted to particles not larger than 50–55 nm. However, this is probably true only for neutral, uncoated nanoparticles, because their passage through the mucus layer is also dependent on surface charge and coating. For example, it has been shown that cationic (positively charged) nanoparticles become trapped within the negatively charged mucus, whereas anionic (negatively charged) nanoparticles can pass through the mucus layer. Also, larger particles (up to 200 nm) can pass through the mucus layer when they are coated with certain materials, e.g. with polyethylene glycol (PEG), which is used widely to coat particulate medicines to prevent their uptake by phagocytic immune cells. This is explained in more details in the review of Chaudhry et al. (2008), pages 248–250. Based on the available information

(e.g. Powell et al., 2010), the mechanism of intestinal **uptake is likely to be size dependent and there may well be an optimum size for uptake, tentatively around 50 nm, with a potential uptake for particles up to 250 nm**. This size-dependent behaviour can also be influenced by surface properties (e.g. coating).

7.6.1. Existing ADME information and *in vitro* testing

Toxicokinetic (ADME) studies are to be presented by applicants following a stepwise assessment. In **Step 2**, existing information is to be gathered on the specific nanomaterial under assessment as well as similar materials (such as the non-nanomaterial counterpart). This step 2 should include existing information on toxicokinetic behaviour. In particular, information on the absorption and bioavailability, distribution pattern and clearance is relevant and expected.

In addition, *in vitro* tests with the nanomaterial are to be performed in **Step 2** (see Figure 6), particularly the lysosomal degradation test as described in Section 7.2.2. Nanomaterials may not be easily excreted and can accumulate over time (Geraets et al., 2014; Kermanizadeh et al., 2015a; Kermanizadeh et al., 2015b; Kreyling et al., 2017). It is, therefore, considered important to assess the degradation of nanomaterial in lysosomal fluid. Artificial lysosomal fluid is a suitable model because nanomaterials generally distribute in lysosomes in which degradation can occur due to the acidic conditions and presence of enzymes. Information on the dissolution/degradation rate under simulated lysosomal conditions in combination with the dissolution/degradation rate in simulated gastrointestinal conditions (as obtained in Step 1) provides insight into the likelihood of persistence and bioaccumulation of the material. When the material is non-persistent (based on dissolution/degradation rate under simulated lysosomal and gastrointestinal conditions) and there are no indications of toxicity observed (based on existing information and the *in vitro* test battery), an argument may be put forward that further testing is not necessary.

When there is a soluble non-nano counterpart of the material, it is important to report differences in toxicokinetics between the nanomaterial and the soluble non-nano substance.

In vitro methods are proposed as particularly suitable for investigating the mechanism of gastrointestinal absorption. OECD is developing an integrated *in vitro* approach for intestinal fate of orally ingested nanomaterials based on a coculture of three different cell lines, and EFSA has launched a grant for covering the *in vitro* uptake of nanofibres. Until validated methods become available, current developments allow the investigation of the uptake mechanisms and the estimation of relative absorption rates. Still under development, microphysiological systems, conventionally named as gut-on-a-chip, reproduce peristaltic movements and can include the interaction of the nanoparticles with the gut microbiome (Steinway et al., 2020); these systems could become a suitable alternative to *in vivo* systems in the future. Information gathered in **Step 2** should be used to fine-tune **Step 3**. For example, the design of the 90-day study and the satellite group of Step 3B will benefit from pilot studies for dose-finding and assessment of absorption, tissue distribution and accumulation and elimination phase.

7.6.2. *In vivo* ADME testing

Little is known about route-to-route extrapolation for nanomaterials. There are some indications that a different exposure route may result in a different tissue distribution pattern (Kreyling et al., 2017; ISO TR 22019). In principle, oral studies should be performed, since this will be in many cases the relevant exposure route for food/feed applications.[42]

For new nanomaterials, for which no information on ADME can be retrieved from scientific literature, ADME studies should be performed in order to generate data and toxicokinetic parameters such as elimination half-life, should be characterised. Studies on toxicokinetics in animals should be conducted using internationally agreed test guidelines, such as OECD TG 417 (OECD, 2010) with the adaptations mentioned in Section 7.3. This OECD Test Guideline describes general methodologies for performing ADME studies. It provides minimum criteria for acceptance of studies but indicates that studies should be designed on a case-by-case basis. Moreover, following the recommendations from the OECD Expert Meeting (OECD, 2016d), initiatives are ongoing on the development of an OECD Test Guideline on toxicokinetics to accommodate testing of (nano)particles.

The difficulties of undertaking ADME studies of nanomaterials should not be underestimated (ISO, 2019). There may be particular difficulties in measuring the concentrations of nanomaterial in blood,

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

tissues and excreta, and in establishing the form in which they are present in the body (see Section 5.3). Transformations of the surface of nanomaterial can affect, e.g. the dynamics of adherence of proteins and other biomolecules and can have a profound effect on ADME. Therefore, 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 nanomaterials (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. In addition, the impact of the labelling system on the properties and activity of the nanomaterial should be considered. For example, coupling with certain fluorescent dyes may change the hydrophobicity/hydrophilicity of the nanomaterial.

Toxicokinetic modelling can be used to estimate or 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.

Performing a pilot study including some toxicokinetic assessments is recommended for targeted hazard identification and for deciding on dose ranging to avoid the administration of highly toxic doses in Step 3 and Step 4. In **Step 3A**, a pilot *in vivo* study for dose finding and assessment of absorption, tissue distribution and accumulation and elimination phases (≈ 14 days) is recommended (Section 7.6). Demonstration of absorption of nanomaterial can be challenging in a 14-day study. If so, the applicant can proceed directly to *in vivo* studies, **Step 3B**. This could be the case when a limited rate of degradation in gastrointestinal and lysosomal fluids suggests that the material may be persistent in tissues in particulate form and information from a longer time period may be needed to gain insight into how fast a material is eliminated from the body (e.g. clearance) after the last dosing. The dissolution/degradation, e.g. in lysosomes, may also indicate whether ions or molecules can be released after cellular uptake, i.e. from macrophages. The local release of potentially toxic ions or molecules should be taken into account.

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 agglomeration of the nanomaterial at high doses. The actual number of free particles at the nanoscale is likely to be higher at low exposure levels and may decrease due to the formation of agglomerates at higher doses. The agglomeration of nanoparticles may lead to non-proportional internal exposure.

The amount distributed to tissues should be considered in estimating the absorption of the nanomaterial. Mass balance studies are recommended.

A satellite group should be added to the 90-day oral toxicity study in rodents (OECD TG 408, 2018) to investigate if and to what extent the nanomaterial accumulates (**Step 3B**). 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 (e.g. 4 weeks), e.g. in animals that were dosed for the short 2-week period. Note that further guidance on the duration of the elimination period for toxicokinetic assessment will be developed by OECD, and may depend on the dissolution potential of the material. Nanomaterials are generally quickly – often within minutes – taken up by tissues (Landsiedel et al., 2012) and, therefore, information on concentrations in blood may have a limited value. In such cases, a rough estimate of toxicokinetic plasma parameters (half-life, 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 kidney, bone marrow, lung, brain and heart (Landsiedel et al., 2012; ISO, 2019; ECHA, 2017a). Nanomaterial retention within the gut wall is also an important determinant, particularly when discriminating between retention in epithelial cells and immune-competent M-cells in Peyer's patches. In the gastrointestinal tract, gut-associated lymphoid tissue (GALT), particularly in Peyer's patches and mesenteric lymph nodes, is of importance and related to potential nanomaterial accumulation and immune responses.

A negative control group – a group that is not exposed – is used to assess, e.g. background exposure. For the purpose of comparison and potential use of read-across, in the absence of existing data, it is advisable to include a control with the conventional (non-nano) material.

The difference in organ concentrations between days 0, 14 and 90, and after the elimination period (e.g. four weeks), as well as the rate of elimination should be used to assess the likelihood of 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 and triggers further assessment in **Step 4**.

7.6.3. Targeted in-depth ADME testing

In Step 4, 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 by decreasing the uncertainty and may also be required when there is evidence that age, physiological state, disease state, etc., could have an effect on the toxicokinetic behaviour of the nanomaterial.

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

- An appropriate analytical technique/method 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.
- Step 2 comprises assessment of existing information on the specific nanomaterial as well as similar nanomaterials and non-nanomaterials (in bulk form). This step also includes determination of the dissolution/degradation rate in lysosomal conditions, as well as other information on or relevant for toxicokinetic behaviour. In Step 3 information on the toxicokinetics of the nanomaterial is obtained, with a focus on absorption, distribution pattern and potential for accumulation/elimination. Information gathered in Step 2 should be used for Step 3.
- Performing a pilot study that includes some toxicokinetic assessments (Step 3A), is recommended to identify the hazards and for selecting dose ranges to avoid administration of highly toxic doses in steps to follow.
- Step 3B consists of toxicokinetic studies linked to a 90-day oral toxicity study in rodents, e.g. as a satellite group. 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 a post-treatment period should also be determined. The extent to which nanomaterials can accumulate in tissues should be investigated. A significant increase in tissue concentration between days 14 and 90, or a slow release during the elimination period, triggers further assessment in Step 4.
- The toxicokinetic studies of Step 4 should 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. These studies permit refinement of the risk assessment by decreasing the uncertainty.

7.7. *In vivo* local and systemic toxicity testing: adapted repeated-dose 90-day oral toxicity study

In Step 3B, the genotoxicity and toxicokinetic assessments are complemented with studies addressing local and systemic toxicity. The sectoral guidances for food additives (EFSA ANS Panel, 2012), feed additives (EFSA FEEDAP Panel, 2017c) and novel foods (EFSA NDA Panel, 2016), consider a 90-day animal study (OECD TG 408) as the first tier for the assessment of toxicity. The OECD TG 408 has sufficient duration for the safety assessment of particles at the nanoscale, but requires a set of specific adaptations as described below (and also detailed in Sections 7.3 and 7.6.2).

For an ingested nanomaterial, the minimum requirement for *in vivo* toxicity testing is the modified 90-day toxicity test (OECD TG 408 (OECD, 2018a) with the adaptations mentioned under Section 7.3 and inclusion of a satellite group for assessing uptake and tissue distribution/accumulation, as mentioned in Section 7.6.2. The adapted 90-day study should allow for the identification of nanomaterials with the **potential to cause neurotoxic, immunological, reproductive or endocrine-mediated effects**. The results will 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 tissues of the mononuclear phagocyte

system (MPS). Therefore, in repeated-dose studies, specific attention should be paid to the hematopoietic system and parameters linked to inflammatory response and sites/organs involved in or part of the MPS. Specific attention should be paid to local effects in the gastrointestinal tract and organs routinely investigated (with emphasis on liver, spleen, brain and gonads. Whenever feasible, the histopathological examination should be combined with appropriate techniques for detecting the presence of the nanomaterial in tissues and its (intracellular) distribution. Ideally, the measurements should provide quantitative or at least semiquantitative information on the presence of nanoparticles and its distribution within the tissue and within the cells, exploring in particular possible accumulation in specific cell types. Histochemistry and/or a combination of microscopy and chemical analysis may be required for this quantification. A justification should be included in the study report when this information is not provided.

Dose-finding studies conducted for short periods of time can indicate target organs and help in selecting appropriate doses for 90-day studies. However, such studies of shorter duration than 90 days are generally not sufficient for the evaluation of potential subchronic toxicity.

Even if in Step 3A toxicokinetics testing indicates a lack of systemic availability of the nanomaterial, **local adverse effects** on the gastrointestinal tract have to be considered by gastrointestinal histopathology in the 90-day study (Step 3B).

The results from the 90-day toxicity study should also be used to determine whether sufficient information for a 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 and neurological end points or endocrine activity**). If the ADME studies indicate that there is absorption and no other triggers for additional testing are identified, then the 90-day study can be used to identify a **reference point** for risk assessment, unless the sectoral guidance requires further testing. A reference point e.g. can be the lower boundary of the BMD confidence interval (BMDL) or a no observed adverse effect level (NOAEL).

- For ingested nanomaterials, the minimum requirement for *in vivo* toxicity testing is the 90-day toxicity study (OECD, TG 408, 2018) adapted for assessing nanomaterials (according to Section 7.3). These adaptations are also required for assessing conventional materials with a fraction of small particles requiring assessment at the nanoscale.
- Specific attention should be paid in the 90-day repeated-dose study to cardiovascular and inflammatory parameters and 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 7.6).
- Even when results indicate a lack of systemic availability, local effects on the gastrointestinal tract must be considered.
- The results from the 90-day study are 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 and neurological end points or endocrine activity).
- The results from the adapted 90-day oral toxicity study can be used to identify a reference point (such as BMDL or NOAEL).

7.8. Higher tier local and systemic toxicity testing

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, this should be considered as a trigger for in-depth testing.

Evidence of absorption of the material and findings in the 90-day toxicity study are triggers for proceeding to Step 4. This step features 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 humans of effects observed in the animal test species as part of a mode of action framework analysis by the risk assessor (Meek et al., 2013).

Information on the levels of nanomaterials present in key tissues in animals of Step 4 studies are used for 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, but they may accumulate in tissues over time. The amount of nanomaterial reaching tissues and potentially causing systemic toxicity is difficult to predict. Hence, measuring the concentrations of nanomaterials in a few

relevant tissues, e.g. by analysing some of the tissues obtained from Step 4 studies, can be used for better interpretation of study results.

In the following Sections, only those aspects of toxicity studies that contain nanospecific aspects are described.

7.8.1. Chronic toxicity and carcinogenicity

Chronic toxicity and carcinogenicity studies are performed in a single species, usually the rat. Either separate studies (OECD TG 452 (OECD, 2009c) and OECDTG 451 (OECD, 2009b)) or, preferably, the combined study (OECD TG 453 (OECD, 2009d)) can be carried out, with the adaptations mentioned in Section 7.3. A carcinogenicity study in a second animal species would be triggered by the results in the preferred species (equivocal results or species-specific findings) or by observations from studies to investigate the mode of action or mechanism of toxicity or carcinogenicity observed.

7.8.2. Reproductive and developmental toxicity

There are indications in the scientific literature that suggest that systemic exposure to specific nanomaterials may result in adverse effects on reproductive and developmental health of animals (Brohi et al., 2017). In this review, generation of reactive oxygen species (ROS) and inflammation, direct interaction with the male and female reproductive systems, alterations of ovarian gene expression and steroidogenesis, adverse effects on fetal morphology and organogenesis and development are related to reproductive and developmental toxicity induced by specific nanomaterials. Some nanomaterials, depending on their physico-chemical properties, were reported to be able to penetrate the blood-testis barrier and the placental barrier (Brohi et al., 2017).

The data from the subchronic toxicity testing (Step 3) are relevant when considering the need for reproductive and developmental testing in Step 4.

The repeated dose 90-day oral toxicity study (OECD TG 408 (OECD, 2018a)) 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 effects on 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. The following situations may require reproductive and development toxicity testing in Step 4.

If the toxicokinetic study (Step 3) shows that the test material is systemically available in laboratory animals or suspected to be systemically available in humans, testing for reproductive and developmental toxicity in Step 4 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 study will also trigger Step 4 testing for reproductive and developmental toxicity.

When absorption is very low, Step 4 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 (see Section 5.3.2).

Testing for reproductive and developmental toxicity (Step 4) can be addressed by a prenatal developmental toxicity study (OECD TG 414 (OECD, 2001)) or an extended one-generation reproductive toxicity study (EOGRTS) (OECD TG 443 (OECD, 2012b)), both with the adaptations mentioned in Section 7.3. Cohorts for the preliminary assessment of additional more specific endpoints should be routinely incorporated in the EOGRTS. A multigeneration study, instead of an EOGRTS, would be acceptable, provided that sufficient information on possible neurotoxicity and immunotoxicity is available (e.g. from an extended 90-day study, OECD TG 408 (OECD, 2018a) with the adaptations mentioned in Section 7.3.

An EOGRTS in rats will provide information on specific life stages not covered by other toxicity studies: fertility and reproductive function, short-term to long-term developmental effects from exposure during pregnancy, lactation and prepubertal phases and effects on juvenile and adult offspring. By efficiently integrating several endpoints covering the whole reproductive cycle (from gametogenesis to maturation of the following generation) as well as preliminary assessment of additional more-specific endpoints (i.e. developmental neurotoxicity and developmental immunotoxicity), effects on various life stages can be assessed. According to OECD Test Guideline (TG

443 (OECD, 2012b)), 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 limited parameter assessments, would rarely 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 Step 4 additional testing, a case-by-case approach should be adopted with careful consideration given to all available data as well as animal welfare issues.

7.8.3. Immunotoxicity and allergenicity

Immunotoxicity may manifest in the form of adverse effects on the structure and function of the immune system, or an adverse allergic or autoimmune reaction. Most inorganic and small-molecule organic substances in conventional (non-nano)material 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. Aluminium nanomaterial may present different immunological behaviour compared with non-nano aluminium material.

In addition to being potentially immunogenic or antigenic, nanoparticles may also act as 'Trojan horses' for other immunogens/antigens by binding them to the particle surfaces and carrying them into immune cells. The state of current knowledge on the immunotoxicity of nanomaterials and guidance on methods to evaluate this, is described in a WHO publication (WHO, 2019).

The tiered approach to testing outlined in the present Guidance includes, at Step 3, a 90-day study in rats (OECD TG 408 (OECD, 2018a)). This involves investigating 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 the haematological profile of the animals, i.e. the total and differential white blood cell counts.

The effects may be extended or seen for the first time in Step 4 studies, notably the EOGRTS (OECD TG 443 (OECD, 2012b)), but also in chronic toxicity/carcinogenicity studies conducted according to OECD TGs 452 (OECD, 2009e), 451 (OECD, 2009b) or 453 (OECD, 2009d). In the EOGRTS, a cohort of animals is specifically dedicated to assess the potential impact of exposure on the developing immune system. In subchronic and chronic studies, haematological and clinical chemistry data are generated, together with a 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 haemocyanin (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 toxicity studies (Steps 3 and 4). If these results indicate that the nanomaterial can affect the immune system, additional Step 4 studies should be considered, on a case-by-case basis. Then these studies will be designed to investigate the underlying mechanisms of the effects and/or their biological significance. Additional 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 principles as published by IPCS (International Programme on Chemical Safety).

At present, there are no data or validated studies in laboratory animals that would allow assessment of a substance to potentially cause allergic reactions in susceptible individuals following oral exposure. When 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.

7.8.4. Neurotoxicity

Indications of potential neurotoxic effects of a test substance are obtained from the modified 90-day toxicity study (Step 3B). 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. Other information, such as read-across considerations or physico-chemical properties that are indicative of neurotoxic potential should also be considered. When indications of potential neurotoxicity are seen at Step 3, further neurotoxicity testing (OECD TG 424, OECD, 1997; OECDTG 426 (OECD, 2007a)) with the adaptations mentioned in Section 7.3, must be considered. Such testing is intended to confirm or further characterise (and quantify) the potential neurotoxic response induced by the nanomaterial. Further studies may be required to elucidate mechanisms to improve extrapolation from animals to humans.

7.8.5. 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, OECD, 2018a). The ECHA/EFSA Guidance for the identification of endocrine disruptors (ECHA/EFSA, 2018) and the Guidance Document on Standardised test guidelines for evaluating chemicals for endocrine disruption (OECD, 2018b) should also be considered. The additional parameters place more emphasis on endocrine-related endpoints (e.g. determination of hormone levels 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 and the appropriateness of existing test methods for assessing effects mediated by these substances on human health and the environment (mainly based on OECD Guidance Document 150 (OECD, 2018b)). To distinguish between endocrine disruptors and other groups of substances with different modes of action, it was concluded that an endocrine disruptor 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 (i) and (ii).

7.8.6. Effects on gut microbiome

The number of studies using animal models to determine the impact of nanoparticles on the gut microbiota and *in vitro* studies exploring the impact of nanoparticles on human microbiota is increasing (see review by Lamas et al., 2020). These studies demonstrate that nanomaterials can affect microbial composition in the gut. The underlying mechanisms (e.g. the role of intestinal epithelial cells) and the health implications for humans remain unclear (Bouwmeester et al., 2018).

It is not essential (nor required) to check the effects on the gut microbiota composition and function (microbiome) for every nanomaterial, but it should be considered for an insoluble/persistent nanomaterial, especially when having antimicrobial effects (e.g. some metals or metal oxides). For nanomaterials with antimicrobial properties (Hadrup et al., 2012), the possibility of interactions and effects on the composition and function of the microbiota, the mucus and immunity (see Lamas et al., 2020) should be considered, especially with regard to unabsorbed nanoparticles (Fröhlich and Roblegg, 2012; Mercier-Bonin et al., 2018).

In a tiered approach, one could start with *in vitro* intestinal (not only colonic) models, followed by conventional animal models, leaving germ-free models as a final step, when needed.

- Validated *in vitro* gastrointestinal simulators inoculated with human gut microbiota, can be used as preliminary screening methods for nanomaterials to study microbial metabolisation and effects on gut microbiota composition.
- When appropriate, pilot studies and 90-day *in vivo* studies can be used to assess the impact of nanomaterials on gut homeostasis, including effects on the gut microbiota (composition and functionality) and immune system, as well as to identify potential adverse effects at systemic level or physiological processes, which could be related to microbial imbalances. The consequences of interactions with the gut microbiota on systemic or local tissues or physiological processes (direct or indirect) should be identifiable in the examinations that are part of the modified 90-day toxicity study.
- Rodents inoculated with human microbiota (e.g. germ-free rodents, rodents with depleted endogenous microbiota or gnotobiotic rodents with known microbial composition) and inoculated with nanoparticles-disrupted microbiota are a valuable tool to confirm previous findings and establish causality between a nanoparticle-induced gut microbiota signature and associated diseases.

However, a clear understanding of the significance of changes in structure and functionality of the microbiome or their effects on health is not yet elucidated, and further research is ongoing (e.g. Zhang et al., 2020).

- Step 4 includes targeted in-depth studies. Studies on chronic toxicity and carcinogenicity are carried out in a single species, usually rat, and may include, when triggered by the findings, testing for reproductive and developmental toxicity, immunotoxicity and allergenicity, neurotoxicity, endocrine-mediated effects on gut microbiome.
- Information on the concentrations of nanomaterials present in key tissues of animals and obtained from Step 4 studies should be considered in risk characterisation.
- Step 4 testing for reproductive and developmental toxicity will be required if the Step 3 toxicokinetic study shows that the test material is systemically available in the test species (usually rat) or suspected to be systemically available in humans or 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). The test design should be adapted for the assessment of nanomaterials (see Section 7.3).
- A multigeneration study can be an acceptable alternative for 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 Step 3 additional testing, a case-by-case approach should be adopted with careful consideration to animal welfare and all available data.
- Immunotoxicity and allergenicity:
  - Potential immunotoxicity should be investigated when 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' and carry other immunogens/antigens on to particle surfaces of immune cells should be investigated.
  - A thorough consideration of the manufacturing process and formulation steps is necessary because of potential changes in the structure of proteins.
  - Consideration should be given to the parameters investigated in the Step 3 90-day study that may be indicative of an immunotoxic or immunomodulatory effect. Such effects should 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.
  - If the nanomaterial is a potential allergen (e.g. a protein or a peptide), or contains residues of allergenic entities, the principles discussed in the EFSA Guidance on Allergenicity of GMOs (2017) should be followed to evaluate the allergenic components.

- Neurotoxicity: Indications of potential neurotoxic effects should be deduced from the modified 90-day study (Step 3B). Other information, such as results of *in vitro* screening tests, read-across considerations or physico-chemical properties, that are indicative of neurotoxic potential, should be evaluated. When there are indications of potential neurotoxicity, further neurotoxicity testing should be considered on a case-by-case basis.
- Endocrine activity: The design of the modified 90-day study (Step 2B) should provide a starting point for investigating endocrine-disrupting properties and 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.
- Effects on gut microbiome: Consequences of interactions of nanomaterials with the gut microbiota on systemic or local tissues or physiological processes (direct or indirect) should be identified through observations during the modified 90-day study. Studies on the composition and functionality of the microbiome and the mucus might be required when the nanomaterial has antimicrobial effects.

7.9. Read-across

A particular issue identified in the risk assessment of nanomaterials is the fact that a given nanomaterial can be developed in several variant forms in terms of e.g. different particle sizes, crystalline forms, particle shapes and/or surface characteristics. Often, adequate physico-chemical and toxicological data are not available for each individual variant thus excluding case-by-case assessment of each variant, and generation of such data would require a considerable amount of time and resources. Hence, 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 that have been established for conventional chemicals (Arts et al., 2014, 2015, 2016; Oomen et al., 2015; ECHA, 2016; ECHA/JRC/RIVM, 2016; OECD, 2016f, 2016g; Stone et al., 2020). The scientific justification for read-across, however, needs to demonstrate that the source and target (nano)materials are sufficiently similar to allow prediction of the toxicological effect of the target nanomaterial. This justification must include detailed information on physico-chemical characteristics, as well as aspects related to toxicokinetic behaviour and toxicological hazard. It should be noted that currently the scientific basis for allowing substantiation based on existing data for read-across for nanomaterials is limited, as, unlike for conventional chemicals, the current database on physico-chemical and toxicological parameters of nanomaterials is limited.

Nevertheless, it may be feasible to assess if the existing data on a nanomaterial can be used 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 (ECHA, JRC and RIVM, 2016) on grouping of nanomaterials has 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 for nanoforms and the non-nanoform(s) and within groups of nanoforms of the same substance. First, the target material needs to be similar in physico-chemical 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). An argument should be built based on the limited number of differences in physico-chemical properties between source and target materials and how they can affect exposure, toxicokinetics and hazard and substantiated with additional physico-chemical, *in vitro* and/or *in vivo* data. 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, e.g. by justifying that the source (nano)materials exhibit toxicokinetic behaviour and hazards that are more worst-case than the target nanomaterial.

The ECHA Guidance has been taken into account for grouping and read-across of nanomaterials in the development of the GRACIOUS framework (Stone et al., 2020) by the H2020 GRACIOUS project that aims to facilitate the practical application of grouping and read-across of nanomaterials. A number of hypotheses have been described that could be used to justify grouping and read-across (see www.h2020gracious.eu). Through an Integrated Approach to Testing and Assessment (IATA), the information is gathered to accept or reject a hypothesis in a targeted manner. If accepted, the information can be used for justification of grouping or data read-across from a source nanomaterial to other target materials.

Owing to 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*) will be needed for substantiation. Over time, more data may become available on source material(s) to allow comparison of the toxicokinetic behaviour and hazard potential, and thus substantiation of the use of a grouping/read-across approach.

Application of read-across will require data and information on the relationship between physico-chemical properties and toxicokinetic behaviour and hazard potential of different variants of the nanomaterial with the same chemical composition. This implies that nanomaterials considered for grouping/read-across should be well characterised regarding physico-chemical 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 physico-chemical properties and the toxicokinetic profile and hazard potential of a nanomaterial with the same chemical composition. It would facilitate the interpretation of findings if only one parameter, or at most a small number of parameters, were changed systematically allowing the critical ones to be identified.

Whether a read-across justification is acceptable for waiving further (*in vivo*) testing will be assessed 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 two (or more) variants of the nanomaterial of the same chemical composition have comparable physico-chemical, toxicokinetic and hazard profiles.
- A case for read-across may also be made when the source (nano)material exhibits worse toxicokinetic behaviour and hazards than the target nanomaterial.

7.10. Integrated approaches to testing and assessment

The Scientific Committee notes the continuing development of **Integrated Approach to Testing and Assessment (IATA)** and promotes the use of AOPs to carry out risk assessments. The developments for conventional materials can also be applied to nanomaterials. EFSA 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 of the material.

New Approach Methodologies (NAMs) explore advances in toxicology and exposure science to support the global transition towards 21st century approaches to chemical risk assessment. NAMs 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 https://ec.europa.eu/growth/sectors/chemicals/epaa_en; National Research Council, 2007; van Leeuwen et al., 2007), and in addition provide the basis for the methodological understanding of the interaction of chemicals and nanoparticles with biological systems.

In 2012, 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 AOP knowledge-based tools, which are continually developed and refined, are web-based platforms bringing together available knowledge on how chemicals can induce adverse effects.[43] Similarly, a view on future risk assessment was provided by the EU Scientific Committees SCHER, SCENIHR and SCCS, focussing on **organic chemicals**. These committees indicated that there is a need and trend to change the basis of human health risk assessment from being based on standard (animal) tests to being centred on mode of action. To enable the most effective use of resources and limit unnecessary use of animals, a tiered approach for the assessment of hazards from exposure to individual stressors is proposed.

[43] 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.

For nanomaterials, the need to efficiently obtain risk assessment information is high, considering that not only chemical composition but also various physico-chemical properties may affect nanomaterial 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 AOPs (Nymark et al., 2018). EFSA is continuing to investigate the role of omics-technologies in risk assessment.

These developments in efficient testing strategies and AOPs for nanomaterials are acknowledged, but 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).

In the case of engineered nanomaterials (ENM), the specific intention for having the material at nanoscale and the information generated for confirming the intended biological interactions (i.e. mode of action when relevant) of the nanomaterial, can be useful for constructing an IATA, using AOP-like approaches for identifying and exploring possible side effects. This is exemplified below for a nutrient engineered as nanomaterial to facilitate intestinal uptake, reduce toxicity and improve its systemic bioavailability.

The elucidation for formulating a hypothesis-driven IATA would require generating information to answer questions such as the following.

- Is the ENM absorbed by the intestinal epithelia as nanoparticles or is the nutrient released in the gastrointestinal lumen and then absorbed by the intestinal cells in ionic or molecular form?
- If absorbed as nanoparticles, what is the behaviour of the nanoparticles in the enterocytes? Is the nutrient released and then systemically transferred in molecular form? Is the nutrient incorporated in the nutrient metabolic pathways or follows different pathways for systemic distribution? Do nanoparticles accumulate in the enterocytes?
- Is the nutrient transferred to the systemic circulation and target organs in the form of nanoparticles by paracellular absorption? What is the role of the mononuclear phagocyte system (MPS) regarding uptake and systemic distribution?
- Which homeostatic processes control the uptake, accumulation and elimination of the nutrient and what is the level of control of each process regarding the ENM? Are there alterations compared to the (non-engineered) nutrient?
- Which events (e.g. inflammation, oxidative stress) lead to an adverse outcome?

The applicant is expected to generate experimental data to elucidate the above mentioned mechanistic information. For answering some questions, *in vitro* studies are better suited than *in vivo* studies to obtain mechanistic information. If properly designed, these studies may also cover mechanistic pathways for adverse effects, informing the risk assessment, facilitating the weight-of-evidence approach and minimising further experimental studies.

A hypothetical example on the use of IATA and AOPs is presented below. For example, if after transit through the stomach the ENM dissolves partially in the intestine, it could be relevant to investigate the internalisation of the remaining ENM by intestinal epithelial cells, checking if despite dissolution, a fraction of the ENM may be absorbed as nanoparticles. Although *in vitro* intestinal cells may not be suitable yet to assess this process quantitatively, both static co-culture and micro-physiological systems (gut-on-a-chip), may provide mechanistic understanding to facilitate the design and interpretation of *in vivo* studies, and there are ongoing efforts to validate some models for regulatory assessments. Similarly, if the expected pathway is that the material will be internalised as nanoparticles, and then, the nanoparticles undergo dissolution/degradation by the lysosomes resulting in a nutrient in molecular form, it could be relevant to account for this in the hazard assessment. Therefore, the hazard assessment should investigate the possible accumulation of a fraction of nanoparticles in the intestinal epithelia, and alternative mechanisms that, even to a minor degree, may result in the transfer of nanoparticles at systemic level, such as exocytosis, paracellular transport or the mononuclear phagocyte system-mediating systemic distribution. The toxicokinetics of nutrients is

regulated by homeostatic processes. The presence of a nutrient in nanoform may affect the physiological processes and feedback mechanisms regulating the uptake, distribution, accumulation and elimination of a nutrient. The experimental design should investigate the different pathways that regulate the kinetics of a nutrient according to the nutrient status in the body, such as a possible hormonal control or alternative mechanisms regulating its uptake, distribution, storage and excretion. The information on main and alternative pathways could be used in an AOP approach for identifying and investigating possible adverse effects, including targeted accumulation of nanoparticles in specific cell types and tissues.

The use of IATA and AOPs could facilitate targeting subsequent testing or building of a grouping or read-across approach.

- Integrated Approach to Testing and Assessment (IATA) uses hypothesis-driven tools for combining *in vitro* tests, thresholds of toxicological concern, computational methods, read-across of chemical categories and exposure assessment for risk assessment, and is applicable to nanomaterials.
- New Approach Methodologies (NAMs) explore advances in toxicology and exposure science and could fulfil the goal to refine, reduce or (partly) replace (the 3Rs) current traditional toxicological approaches.
- 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 are web-based platforms bringing together available knowledge on how chemicals can induce adverse effects. AOPs can provide a mechanistic framework to assess specific adverse outcomes.
- In the case of engineered nanomaterials (ENM), the specific intention for having the material at nanoscale, and the information generated for confirming the intended biological interactions (i.e. mode of action when relevant) of the nanomaterial, can be useful for constructing an IATA, using AOP-like approaches for identifying and exploring possible side effects.

8. Risk characterisation of nanomaterial

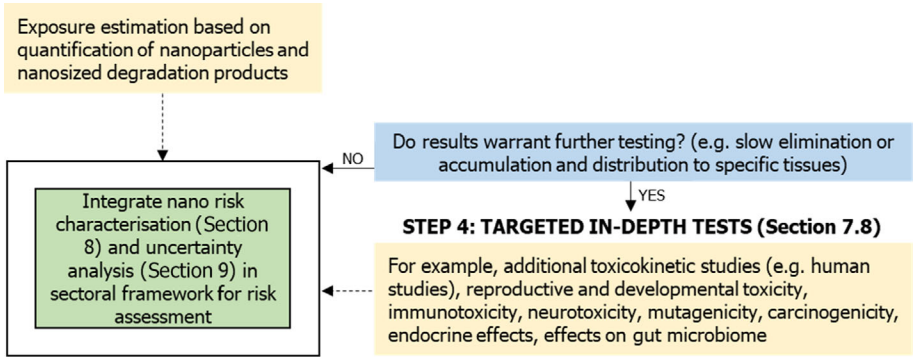


Figure 7: Risk characterisation (detail from Figure 2)

Conceptually, the risk characterisation of nanomaterials does not differ *per se* from that of a conventional material and needs to be integrated in the risk characterisation under the sectoral legislation. However, there are specific considerations when integrating the hazard and exposure assessments of nanomaterials, as follows.

1) For engineered nanomaterials, the integration step should be achieved by combining the provisions under this Guidance (for physico-chemical characterisation, hazard characterisation and exposure assessment) into the conventional risk characterisation under the sectoral legislation.
2) For conventional materials containing a fraction of small particles requiring nanospecific assessment, the risk characterisation should consider both the material as marketed and the fraction with nanoscale properties (see the mixture toxicity considerations for designing the test strategy below).

Risk characterisation is essentially an iterative process and should result in **a quantitative assessment or, if not possible, in a qualitative assessment**.

When materials under the assessment of this Guidance consist of **nanoparticles** with **the same elemental composition but different shapes, sizes, crystalline forms and/or surface properties** as a consequence of, e.g. different production processes, the risk characterisation should describe for each type of nanoparticles the confirmed properties they have and that have been assessed sufficiently. This can then be reflected in the specifications for market authorisation.

The output from the risk characterisation gives a basis to conclude on the **safety** of the nanomaterial under the condition of manufacturing as described by the applicant in the technical part of the dossier under the intended uses and use levels (the parameters for which the assessment is valid). The **uncertainties** associated with the assessment must be described.

Several approaches to generate the information required for a risk assessment are described in this Guidance. A **weight-of-evidence process** should be applied according to the EFSA Guidance on the use of the weight-of-evidence approach in scientific assessments to determine whether sufficient information has been generated and if a risk assessment can be performed. 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 (EFSA Scientific Committee, 2017b). The quality of the existing data and data provided should be reported in accordance with the EFSA Guidance on the use of the weight-of-evidence approach in scientific assessments and the Guidance on the assessment of the biological relevance of data in scientific assessments (EFSA Scientific Committee, 2017b,c).

The toxicokinetic and toxicodynamic information should inform the **hazard** identification processes. For materials containing nanoparticles or particles with external dimensions at both nanoscale and microscale, or particles undergoing partial dissolution or transformation in the gastrointestinal tract, the first step of the process is to identify possible toxicokinetic and toxicodynamic differences between the particulate and solubilised material as well as among different particle types (e.g. coated and uncoated particles when coating is partially removed after ingestion). In these cases, the assessment of nanomaterials may require a case-by-case mixture toxicity approach. In line with the EFSA Guidance on harmonised methodologies for human health, animal health and ecological risk assessment of combined exposure to multiple chemicals mixtures (EFSA Scientific Committee, 2019), for hazard assessment mixture toxicity may be addressed through direct **testing** of the whole mixture or independent assessments of each component, and both approaches are acceptable as long the principles mentioned in the present Guidance are applied. The application of these methods to nanomaterials and conventional materials containing a fraction of nanoparticles requires nanospecific considerations. In some cases the assessment may be straight forward, e.g. when the uptake from the gastrointestinal tract is limited to the nanoscale fraction, while in others, e.g. when there is partial degradation and consumers are exposed to several types of particles and solubilised material, the risk assessment is complex and requires a proper description and evaluation of the uncertainties. The **exposure** assessment and risk characterisation should be adapted to the results of the **hazard** assessment, addressing the exposure to particles, the exposure to solubilised material and the overall exposure. As further dissolution of the fraction absorbed as particles may occur after cell internalisation and systemic distribution, a detailed toxicokinetic assessment covering ADME for the nanoparticles and for the dissolved chemical species is essential. In absence of sufficient information conservative approaches should be used in the risk characterisation.

- The output from the risk characterisation should be in the form of an overall assessment of the safety of the nanomaterial for its intended use, together with a description of the parameters under which the assessment is valid and the uncertainties associated with the assessment.
- For engineered nanomaterials, the risk assessment should integrate the provisions described in this Guidance (for physico-chemical characterisation, hazard characterisation and exposure assessment) into conventional risk characterisation under relevant sectoral legislation.
- For conventional materials containing a fraction of small particles requiring nanospecific assessment, the risk characterisation should consider both the material as marketed and the fraction with nanoscale properties
- Mixture toxicity considerations may be required for this assessment. Mixture toxicity may be addressed through direct testing of the whole mixture or independent assessments of each component; both approaches are acceptable.
- The application of mixture toxicity approaches to nanomaterials and conventional materials containing a fraction of small particles including nanoparticles requires nanospecific considerations. In some cases the assessment may be straight forward, e.g. when the uptake from the gastrointestinal tract is limited to the

fraction of nanoparticles, while in others, e.g. when there is partial degradation and consumers are exposed to several types of particles and solubilised material, the risk assessment is complex and requires an full description of the uncertainties.
- The quality of the data used should be reported in accordance with the EFSA Guidance on the use of weight-of-evidence approach in scientific assessments and the EFSA Guidance on the assessment of the biological relevance of data in scientific assessments.

9. Uncertainty analysis of nanomaterial risk assessment

To meet the general requirements for transparency, all EFSA scientific assessments must include considerations of uncertainty. The Scientific Committee adopted a Scientific Opinion in 2009 that deals with general principles to be applied in the identification of data sources, criteria for inclusion and exclusion of data, confidentiality of data, assumptions and uncertainties (EFSA Scientific Committee, 2009b). In 2018, a Guidance on Uncertainty analysis in scientific assessments (EFSA Scientific Committee, 2018a) and the principles and methods behind EFSA Guidance on Uncertainty analysis in scientific assessment (EFSA Scientific Committee, 2018b) were published. EFSA requires the quantification of uncertainties. The principles and recommendations in these documents should also be used in the risk assessment of nanomaterials. For specific types of uncertainties related to exposure, the EFSA Scientific Committee adopted in 2006 a guidance related to uncertainties in dietary exposure assessment that includes practical approaches on the various types on uncertainties that can be identified for the exposure assessment (EFSA Scientific Committee, 2006).

Furthermore, in 2019, EFSA published a Guidance on Communication of uncertainty in scientific assessments (EFSA, 2019).

9.1. Measurement uncertainties in the physico-chemical 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. Measurement uncertainties should be reported as combined uncertainties for the entire process. They can be derived from data obtained during a validation study; examples are available for measurements based on TEM (De Temmerman et al., 2014), DLS (Braun et al., 2011), PTA (Kestens et al., 2017; Ramaye et al., 2021), CLS (Antunez-Dominguez et al., 2020) or ELS (Ramaye et al., 2021). The measurement uncertainty associated to measurement results obtained during routine application of the validation method 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 (at a confidence level of 68%), 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 are spread and u_t is the uncertainty of the recovery or trueness determination (Linsinger et al., 2013). Alternatively, measurement uncertainty may also be estimated using a bottom-up approach during which all significant uncertainty sources are identified, quantified and combined to yield the combined uncertainty; the proposal for reporting metrological traceability for Line-start incremental centrifugal liquid sedimentation (disc-CLS) may be used as example (Kestens et al., 2019). Guidance for the determination and expression of measurement uncertainty is widely available, e.g. from ISO (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. When validation studies are conducted on reference materials, or in the absence of reference materials on representative test materials, that have physico-chemical properties similar to those of the routine test materials, then the uncertainties due to sample preparation are expected to be covered by the uncertainties for precision and recovery/trueness. When measurement uncertainties are estimated

using a bottom-up approach, then uncertainties for sample preparation should be considered separately.

Accuracy of the available characterisation methods is dependent on the tested 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; Kestens et al., 2016); key issues of measurand definition and estimation of measurement uncertainty are presented by Kestens et al. (2016).

It is therefore essential to specify the procedures used (e.g. type of sample preparation and conditions, technique applied for size measurement), to provide unambiguous information on the reported measurands, the analytical performance (validation study) and, finally, on the overall 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 an engineered nanomaterial/nanoform. The expanded measurement uncertainty U ($U = u_c \times k$), estimated in a realistic manner, will allow both the applicant and the risk assessor to evaluate and judge the fit for purpose of the measurement result. A coverage factor $k = 3$ should be used that corresponds to greater than 99% confidence.

9.2. Uncertainties in exposure assessment of the nanomaterial

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. Oral exposure assessment is affected by scientific uncertainties and it is important for assessors to characterise the extent of uncertainty in order to be taken into account by risk managers.

The exposure assessment should include a systematic examination of potential sources and types of uncertainty. The assessment of uncertainties in exposure assessment should follow the principles in the EFSA Guidances.

When it is not possible to characterise the form in which the nanomaterial is present in food and/or feed applications, uncertainty in exposure assessment will be increased. This uncertainty can be reduced by characterisation of the nanomaterial in the food/feed or liquid food/feed products according to intended or existing applications. This information should be available from solubility and degradation tests (Section 5.2) and *in vitro* gastrointestinal digestion and stability in lysosomal fluid (Sections 7.2.1 and 7.2.2) and included in the dossier.

9.3. Uncertainties in the hazard characterisation of the nanomaterial

Often limited information is available in relation to aspects of nanomaterial toxicokinetics and toxicology; some limitations are linked to the use of test methods not specifically designed for nanomaterials. As highlighted in Chapter 7, existing toxicity testing methods (e.g. OECD test guidelines) require methodological adapatations when the test material is a nanomaterial or contains a fraction of nanoparticles. Specific uncertainties arise as the standard testing protocols have not been adapted yet for testing nanomaterials, although work is in progress and additional OECD test guidelines are expected for the coming years. 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.

It is 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 or 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 not available. For nanomaterials, a comparison with existing allergic proteins does not seem appropriate. However, the identification of proteins of the food matrix adhering or bound to the nanomaterial surface may 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 may point to other modifications in test protocols.

9.4. Uncertainties in the risk characterisation

Lack of or inadequate characterisation of the nanomaterial test substance may be a source of uncertainty in studies for hazard identification and hazard characterisation. Uncertainty will increase when the available data on characterisation of the nanomaterial test substance used in studies for hazard identification and hazard characterisation are insufficient to conclude that the tested nanomaterial and its form are comparable to that present in food or feed. Depending on the circumstances, the risk characterisation may under- or over-represent the risks. These uncertainties could be reduced by using a test nanomaterial that is sufficiently characterised (see Section 5) 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.

- Uncertainties should be reported and quantified to the highest possible degree. 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.
- The uncertainties in the measurement results of 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, measurand and expanded measurement uncertainty.
- Uncertainties relating to any limited information on toxicokinetics and toxicology, including test methods must be indicated. Uncertainties arising from the lack of validated *in vitro* assays for nanomaterials should also be indicated.
- Potential sources and types of uncertainty in exposure assessment to nanomaterial should be systematically examined.
- Other uncertainties, e.g. in risk characterisation from a weight-of-evidence approach or related to biological relevance of data in scientific assessments, should be listed.

References

Allan J, Sokull-Kluettgen B and Patri AK, 2020. Global Summit on Regulatory Science 2019 Nanotechnology and Nanoplastics, EUR 30195 EN. Publications Office of the European Union, Luxembourg. ISBN 978-92-76-18435-5. https://doi.org/10.2760/517689

Ammendolia MG, Iosi F, Maranghi F, Tassinari R, Cubadda F, Aureli F, Raggi A, Superti F, Mantovani A and De Berardis B, 2017. Short-term oral exposure to low doses of nano-sized TiO_2 and potential modulatory effects on intestinal cells. Food and Chemical Toxicology, 102, 63–75. https://doi.org/10.1016/j.fct.2017.01.031

Antúnez Domínguez JM, Ramaye Y, Dabrio M and Kestens V, 2020. Validation of a homogeneous incremental centrifugal liquid sedimentation method for size analysis of silica (nano)particles. Materials, 13, 3806. https://doi.org/10.3390/ma13173806

Arts JHE, Hadi M, Keene AM, Kreiling R, Lyon D, Maier M, Michel K, Petry T, Sauer UG, Warheit D, Wiench K and Landsiedel R, 2014. A critical appraisal of existing concepts for the grouping of nanomaterials. Regulatory Toxicology and Pharmacology, 70, 492–506. https://doi.org/10.1016/j.yrtph.2014.07.025

Arts JHE, Hadi M, Irfan M-A, Keene AM, Kreiling R, Lyon D, Maier M, Michel K, Petry T, Sauer UG, Warheit D, Wiench K, Wohlleben W and Landsiedel R, 2015. A decision-making framework for the grouping and testing of nanomaterials (DF4nanoGrouping). Regulatory Toxicology and Pharmacology, 71(2 Suppl), S1–S27. https://doi.org/10.1016/j.yrtph.2015.03.007

Arts JHE, Irfan M-A, Keene AM, Kreiling R, Lyon D, Maier M, Michel K, Neubauer N, Petry T, Sauer UG, Warheit D, Wiench K, Wohlleben W and Landsiedel R, 2016. Case studies putting the decision-making framework for the grouping and testing of nanomaterials (DF4nanoGrouping) into practice. Regulatory Toxicology and Pharmacology, 76, 234261. https://doi.org/10.1016/j.yrtph.2015.11.020

Avella M, De Vlieger JJ, Errico ME, Fischer S, Vacca P and Volpe MG, 2005. Biodegradable starch/clay nanocomposite films for food packaging applications. Food Chemistry, 93, 467–474. https://doi.org/10.1016/j.foodchem.2004.10.024

Babick F, Mielke J, Wohlleben W, Weigel S and Hodoroaba VD 2016. How reliably can a material be classified as a nanomaterial? Available particle-sizing techniques at work. Journal of Nanoparticle Research, 18, 158. https://doi.org/10.1007/s11051-016-3461-7

Bajka BH, Rigby NM, Cross KL, Macierzanka A and Mackie AR, 2015. The influence of small intestinal mucus structure on particle transport ex vivo. Colloids and Surfaces B, Biointerfaces, 135, 73–80. https://doi.org/10.1016/j.colsurfb.2015.07.038

Balls M and Fentem JH, 1997. Progress toward the validation of alternative tests. Alternatives to Laboratory Animals, 25, 33–43.

Bellmann S, Carlander D, Fasano A, Momcilovic D, Scimeca JA, Waldman WJ, Gombau L, Tsytsikova L, Canady R, Pereira DI and Lefebvre DE, 2015. Mammalian gastrointestinal tract parameters modulating the integrity, surface properties, and absorption of food-relevant nanomaterials. Wiley Interdisciplinary Reviews: Nanomedicine and Nanobiotechnology, 7, 609–622. https://doi.org/10.1002/wnan.1333

Bencsik A, Lestaevel P and Guseva Canu I 2017. Nano- and neurotoxicology: an emerging discipline. Progress in Neurobiology, 2017;S0301–0082, 30002–30003. https://doi.org/10.1016/j.pneurobio.2017.10.003

Bhattacharya K, Kilic G, Costa PM and Fadeel B, 2017. Cytotoxicity screening and cytokine profiling of nineteen nanomaterials enables hazard ranking and grouping based on inflammogenic potential. Nanotoxicology, 11, 809–826. https://doi.org/10.1080/17435390.2017.1363309

Bihari P, Vippola M, Schultes S, Praetner M, Khandoga A, Reichel C, Coester C, Tuomi T, Rehberg M and Krombach F, 2008. Optimized dispersion of nanoparticles for biological *in vitro* and *in vivo* studies. Particle and Fibre Toxicology, 5, 14. https://doi.org/10.1186/1743-8977-5-14

Blanquet S, Zeijdner E, Beyssac E, Meunier JP, Denis S, Havenaar R and Alric M, 2004. A dynamic artificial gastrointestinal system studying the behavior of orally administered drug dosage forms under various physiological conditions. Pharmaceutical Research, 21, 585–591. https://doi.org/10.1023/B:PHAM.0000022404.70478.4b

Blanquet-Diot S, Soufi M, Rambeau M, Rock E and Alric M, 2009. Digestive stability of xanthophylls exceeds that of carotenes as studied in a dynamic *in vitro* gastrointestinal system. Journal of Nutrition, 139, 876–883. https://doi.org/10.3945/jn.108.103655

Boisen S and Eggum BO, 1991. Critical evaluation of *in vitro* methods for estimating digestibility in simple-stomach animals. Nutrition Research Reviews, 4, 141–162. https://doi.org/10.1079/NRR19910012

Boisen S and Fernnández JA, 1997. Prediction of the total tract digestibility of energy in feedstuffs and pig diets by *in vitro* analyses. Animal Feed Science Technology, 68, 277–286. https://doi.org/10.1016/S0377-8401(97)00058-8

Boobis AR, Cohen SM, Dellarco V, McGregor D, Meek ME, Vickers C, Willcocks D and Farland W, 2006. IPCS framework for analyzing the relevance of a cancer mode of action for humans. Critical Reviews in Toxicology, 36, 781–792. https://doi.org/10.1080/10408440600977677

Boobis AR, Doe JE, Heinrich-Hirsch B, Meek ME, Munn S, Ruchirawat M, Schlatter J, Seed J and Vickers C, 2008. IPCS framework for analyzing the relevance of a noncancer mode of action for humans. Critical Reviews in Toxicology, 38, 87–96. https://doi.org/10.1080/10408440701749421

Bouwmeester H, van der Zande M and Jepson MA, 2018. Effects of food-borne nanomaterials on gastrointestinal tissues and microbiota. WIREs Nanomedicine and Nanobiotechnology, e1481. https://doi.org/10.1002/wnan.1481

Bowen P, 2002. Particle size distribution measurement from millimeters to nanometers and from rods to platelets. Journal of Dispersion Science and Technology, 23, 631–662. https://doi.org/10.1081/DIS-120015368

Brand W, van Kesteren PCE and Oomen AG, 2019. Potential health risks of nanomaterials in food: a methodology to identify signals and prioritise risks. RIVM letter report 2019–0191. Available at: www.rivm.nl/bibliotheek/rapporten/2019-0191.pdf.RIVM

Brandon EF, Oomen AG, Rompelberg CJ, Versantvoort CH, van Engelen JG and Sips AJ, 2006. Consumer product *in vitro* digestion model: bioaccessibility of contaminants and its application in risk assessment. Regulatory Toxicology and Pharmacology, 44, 161–171. https://doi.org/10.1016/j.yrtph.2005.10.002

Braun A, Couteau O, Franks K, Kestens V, Roebben G, Lamberty A and Linsinger TPJ, 2011. Validation of dynamic light scattering and centrifugal liquid sedimentation methods for nanoparticle characterization. Advanced Powder Technology, 22, 766–770. Available online: https://www.sciencedirect.com/science/article/pii/S0921883110002153

Brodkorb A, Egger L, Alminger M, Paula A, Ricardo A, Simon B, Torsten B and Claire BL, 2019. INFOGEST static in vitro simulation of gastrointestinal food digestion. Nature Protocols, 14, 991–1014. https://doi.org/10.1038/s41596-018-0119-1

Brohi RD, Wang L, Talpur HS, Wu D, Khan FA, Bhattarai D, Rehman ZU, Farmanullah F and Huo LJ, 2017. Toxicity of nanoparticles on the reproductive system in animal models: a Review. Frontiers in Pharmacology, 8, 606. https://doi.org/10.3389/fphar.2017.00606.eCollection.2017

Brüngel R, Rückert J, Wohlleben W, Babick F, Ghanem A, Gaillard C, Mech A, Rauscher H, Hodoroaba V-D, Weigel S and Friedrich CM, 2019. NanoDefiner e-Tool: an implemented decision support framework for nanomaterial identification. Materials, 12, 3247. Available online: http://www.nanodefine.eu/index.php/nanodefiner-e-tool

Bryce SM, Bemis JC, Avlasevich SL and Dertinger SD, 2007. In vitro micronucleus assay scored by flow cytometry provides a comprehensive evaluation of cytogenetic damage and cytotoxicity. Mutation Research, 630, 78–91. https://doi.org/10.1016/j.mrgentox.2007.03.002

Brüning P, 2017. Review and practical evaluation of sampling guidelines, NanoDefine Technical Report D2.7. NanoDefine Consortium, Wageningen. Available online: http://www.nanodefine.eu/publications/reports/NanoDefine_TechnicalReport_D2.7.pdf

Campos EVR, 2021. 23 - Commercial nanoproducts available in world market and its economic viability. In: Jogaiah S, Singh HB, Fraceto LF and Lima Rd (eds.). Advances in Nano-Fertilizers and Nano-Pesticides in Agriculture. Woodhead Publishing, pp. 561–593.

Cano Robles FK and Mendoza Cantù A, 2017. Nanopesticides, a real breakthrough for agriculture? Revista Bio Ciencias, 24, 164–178. https://doi.org/10.15741/revbio.04.03.03

Catalán J, Stockmann-Juvala H and Norppa H, 2017. A theoretical approach for a weighted assessment of the mutagenic potential of nanomaterials. Nanotoxicology, 11, 964–977. https://doi.org/10.1080/17435390.2017.1382601

Cedervall T, Lynch I, Foy M, Berggård T, Donnelly SC, Cagney G, Linse S and Dawson KA, 2007. Detailed identification of plasma proteins adsorbed on copolymer nanoparticles. Angewandte Chemie International Edition, 46, 5754–5756. https://doi.org/10.1002/anie.200700465

CEN (European Committee for Standarisation), 2018. Nanotechnologies - Guidance on detection and identification of nano-objects in complex matrices. CEN/TC 17273:2018. Available online: https://standards.cen.eu/dyn/www/f?p=204:110:0::FSP_PROJECT,FSP_ORG_ID:37705,508478&cs=1B363D2465349533EBD3CABA96180879D

Chaudhry Q, Castle L and Watkins R (eds.). 2017. Nanotechnologies in Food, 2nd Edition. Royal Society of Chemistry, London, UK. ISBN 978-1-78262-171-3.

Chaudhry Q, Scotter M, Blackburn J, Ross B, Boxall A, Castle L, Aitken R and Watkins R, 2008. Applications and implications of nanotechnologies for the food sector. Food Additives & Contaminants: Part A, 25, 241–258. http://doi.org/10.1080/02652030701744538

CODATA-VAMAS Working Group on the Description of Nanomaterials, 2016. Uniform description system for materials on the nanoscale, version 2.0. Available online: www.codata.org/nanomaterials

Collins A, El Yamani N and Dusinska M, 2017. Sensitive detection of DNA oxidation damage induced by nanomaterials. Free Radical Biology and Medicine, 107, 69–76. https://doi.org/10.1016/j.freeradbiomed.2017.02.001

Cornu R, Rougier N, Pellequer Y, Lamprecht A, Hamon P, Li R, Beduneau A and Martin H, 2018. Interspecies differences in the cytochrome P450 activity of hepatocytes exposed to PLGA and silica nanoparticles: an *in vitro* and *in vivo* investigation. Nanoscale, 10, 5171–5181. https://doi.org/10.1039/C8NR00226F

Cowie H, Magdolenova Z, Saunders M, Drlickova M, Correia Carreira S, Halamoda Kenzaoi B, Gombau L, Guadagnini R, Lorenzo Y, Walker L, Fjellsbø LM, Huk A, Rinna A, Tran L, Volkovova K, Boland S, Juillerat-Jeanneret L, Marano F, Collins A and Dusinska M, 2015. Suitability of human and mammalian cells of different origin for the assessment of genotoxicity of metal and polymeric engineered nanoparticles. Nanotoxicology, 9 (Suppl 1), 57–65. https://doi.org/10.3109/17435390.2014.940407

Dall'Asta C, Falavigna C, Galaverna G, Dossena A and Marchelli R, 2010. In vitro digestion assay for determination of hidden fumonisins in maize. Journal of Agricultural and Food Chemistry, 58, 12042–12047. https://doi.org/10.1021/jf103799q

De Temmerman PJ, Lammertyn J, De Ketelaere B, Kestens V, Roebben G, Verleysen E and Mast J, 2014. Measurement uncertainties of size, shape, and surface measurements using transmission electron microscopy of near-monodisperse, near-spherical nanoparticles. Journal of Nanoparticle Research, 16, 2177. https://doi.org/10.1007/s11051-013-2177-1

Dekkers S, Oomen AG, Bleeker EAJ, Vandebriel RJ, Micheletti C, Cabellos J, Janer G, Fuentes N, Vazquez-Campos S, Borges T, Silva MJ, Prina-Mello A, Movia D, Nesslany F, Ribeiro AR, Leite PE, Groenewold M, Cassee FR, Sips AJAM, Dijkzeul A, Teunenbroek T and Wijnhoven SWP, 2016. Towards a nanospecific approach for risk assessment. Regulatory Toxicology and Pharmacology, 80, 46–59. https://doi.org/10.1016/j.yrtph.2016.05.037

DeLoid GM, Cohen JM, Pyrgiotakis G and Demokritou P, 2017. Preparation, characterization, and *in vitro* dosimetry of dispersed, engineered nanomaterials. Nature Protocols, 12, 355–371. https://doi.org/10.1038/nprot.2016.172

Doak SH, Manshian B, Jenkins GJ and Singh N, 2012. *In vitro* genotoxicity testing strategy for nanomaterials and the adaptation of current OECD guidelines. Mutation Research/Genetic Toxicology and Environmental Mutagenesis, 745, 104–111. https://doi.org/10.1016/j.mrgentox.2011.09.013

Domingos RF, Baalousha MA, Ju-Nam Y, Reid MM, Tufenkji N, Lead JR, Leppard GG and Wilkinson KJ, 2009. Characterizing manufactured nanoparticles in the environment: multimethod determination of particle sizes. Environmental Science & Technology, 43, 7277–7284. https://doi.org/10.1021/es900249m

Drasler B, Sayre Ph, Steinhäuser KG, Petri-Fink A and Rothen-Rutishauser B, 2017. *In vitro* approaches to assess the hazard of nanomaterials. NanoImpact, 8, 99–116. https://doi.org/10.1016/j.impact.2017.08.002

Dressman JB, Amidon GL, Reppas C and Shah VP, 1998. Dissolution testing as a prognostic tool for oral drug absorption: immediate release dosage forms. Pharmaceutical Research, 15, 11–22. https://doi.org/10.1023/A:1011984216775

Duncan TV and Pillai K, 2014. Release of engineered nanomaterials from polymer nanocomposites: diffusion, dissolution, and desorption. ACS Applied Materials & Interfaces, 7, 1–19.

Eastmond DA, Hartwig A, Anderson D, Anwar WA, Cimino MC, Dovrev I, Douglas GG, Nohmi T, Philips DH and Vickers C, 2009. Mutagenicity testing for chemical risk assessment: update of the WHO/IPCS Harmonized Scheme. Mutagenesis, 24, 341–349. https://doi.org/10.1093/mutage/gep014

ECHA (European Chemicals Agency), 2016. Appendix R6–1: Recommendations for nanomaterials applicable to the Guidance on QSARs and Grouping of Chemicals. Guidance for the implementation of REACH. Draft (Public) Version 1.0., Helsinki, Finland. Available online: https://echa.europa.eu/documents/10162/13564/appendix_r6-1_nano_draft_for_committees_en.pdf/cb821783-f534-38cd-0772-87192799b958

ECHA (European Chemicals Agency), 2017a. Guidance on information requirements and chemical safety assessment: Appendix R7-1 for nanomaterials applicable to chapter R7a endpoint specific guidance. Version 2.0. Helsinki, Finland. Available online: https://echa.europa.eu/documents/10162/13632/appendix_r7a_nanomaterials_en.pdf

ECHA (European Chemicals Agency), 2017b. How to prepare registration dossiers that cover nanoforms: best practices. Version 1.0. Helsinki, Finland. Available online: https://echa.europa.eu/documents/10162/13655/how_to_register_nano_en.pdf/f8c046ec-f60b-4349-492b-e915fd9e3ca0

ECHA (European Chemicals Agency), 2017d. Guidance for identification and naming of substances under REACH and CLP. Version 2.1. Helsinki, Finland.

ECHA (European Chemicals Agency), 2019. Appendix for nanoforms applicable to the Guidance on Registration and substance identification. Version 1.0- December 2019. Available online: https://echa.europa.eu/documents/10162/13655/how_to_register_nano_en.pdf/f8c046ec-f60b-4349-492b-e915fd9e3ca0

ECHA (European Chemicals Agency), 2020. Guidance on information requirements and chemical safety assessment, Appendix R7-1 for nanoforms applicable to Chapter R7a and R7c Endpoint specific guidance, Draft (Public) Version 3.0.

ECHA/EFSA (European Chemicals Agency and European Food Safety Authority) with the technical support of the Joint Research Centre (JRC), Andersson N, Arena M, Auteri D, Barmaz S, Grignard E, Kienzler A, Lepper P, Lostia AM, Munn S, Parra Morte JM, Pellizzato F, Tarazona J, Terron A and Van der Linden S, 2018. Guidance for the identification of endocrine disruptors in the context of Regulations (EU) No 528/2012 and (EC) No 1107/2009. EFSA Journal 2018;16(6):5311, 135 pp. https://doi.org/10.2903/j.efsa.2018.5311. ECHA-18-G-01-EN.

ECHA/JRC/RIVM (European Chemicals Agency, Joint Research Centre and Dutch National Institute for Public Health and the Environment), 2016. Usage of (eco)toxicological data for bridging data gaps between and grouping of nanoforms of the same substance – Elements to consider. Helsinki, Finland. ISBN 978-92-9247. Available online: https://echa.europa.eu/documents/10162/13630/eco_toxicological_for_bridging_grouping_nanoforms_en.pdf

EFSA (European Food Safety Authority), 2014. Guidance on the assessment of exposure of operators, workers, residents and bystanders in risk assessment for plant protection products. EFSA Journal 2014;12(10):3874, 55 pp. https://doi.org/10.2903/j.efsa.2014.3874

EFSA (European Food Safety Authority), Buist H, Craig P, Dewhurst I, Hougaard Bennekou S, Kneuer C, Machera K, Pieper C, Court Marques D, Guillot G, Ruffo F and Chiusolo A, 2017. Guidance on dermal absorption. EFSA Journal 2017;15(6):4873, 60 pp. https://doi.org/10.2903/j.efsa.2017.4873

EFSA (European Food Safety Authority), Hart A, Maxim L, Siegrist M, Von Goetz N, da Cruz C, Merten C, Mosbach-Schulz O, Lahaniatis M, Smith A and Hardy A, 2019. Guidance on Communication of Uncertainty in Scientific Assessments. EFSA Journal 2019;17(1):5520, 73 pp. https://doi.org/10.2903/j.efsa.2019.5520

EFSA ANS Panel (Panel on Food Additives and Nutrient Sources Added to Food), 2012. Guidance for submission for food additive evaluations. EFSA Journal 2012;10(7):2760, 60 pp. https://doi.org/10.2903/j.efsa.2012.2760

EFSA CEF Panel (EFSA Panel on Food Contact Materials, Enzymes, Flavourings and Processing Aids), 2012a. Scientific Opinion on the safety evaluation of the substance, titanium nitride, nanoparticles, for use in food contact materials. EFSA Journal 202;10(3):2641, 8 pp. https://doi.org/10.2903/j.efsa.2012.2641

EFSA CEF Panel (EFSA Panel on Food Contact Materials, Enzymes, Flavourings and Processing Aids), 2012b. Scientific Opinion on the safety evaluation of the active substance iron (II) modified bentonite as oxygen absorber for use in active food contact material. EFSA Journal 2012;10(10):2906, 11 pp. https://doi.org/10.2903/j.efsa.2012.2906

EFSA CEF Panel (EFSA Panel on Food Contact Materials, Enzymes, Flavourings and Processing Aids), 2014a. Statement on the safety assessment of the substance silicon dioxide, silanated, FCM Substance No 87 for use in food contact materials. EFSA Journal 2014;12(6):3712, 7 pp. https://doi.org/10.2903/j.efsa.2014.3712

EFSA CEF Panel (EFSA Panel on Food Contact Materials, Enzymes, Flavourings and Processing Aids), 2014b. Scientific Opinion on the safety assessment of the substances (butadiene, ethyl acrylate, methyl methacrylate, styrene) copolymer either not crosslinked or crosslinked with divinylbenzene or 1,3-butanediol dimethacrylate, in nanoform, for use in food contact materials. EFSA Journal 2014;12(4):3635, 8 pp. https://doi.org/10.2903/j.efsa.2014.3635

EFSA CEF Panel (EFSA Panel on Food Contact Materials, Enzymes, Flavourings and Processing Aids), 2014c. Scientific Opinion on the safety assessment of the substances, kaolin and polyacrylic acid, sodium salt, for use in food contact materials. EFSA Journal 2014;12(4):3637, 8 pp. https://doi.org/10.2903/j.efsa.2014.3637

EFSA CEF Panel (EFSA Panel on Food Contact Materials, Enzymes, Flavourings and Processing Aids), 2015a. Scientific Opinion on the safety evaluation of the substance zinc oxide, nanoparticles, uncoated and coated with [3-(methacryloxy)propyl] trimethoxysilane, for use in food contact materials. EFSA Journal2015;13(4):4063, 9 pp. https://doi.org/10.2903/j.efsa.2015.4063

EFSA CEF Panel (EFSA panel on Food Contact Materials, Enzymes, Flavourings and Processing Aids), 2015b. Scientific Opinion on the safety assessment of the substance montmorillonite clay modified by dimethyldialkyl (C16–C18)ammonium chloride for use in food contact materials. EFSA Journal 2015;13(11):4285, 10 pp. https://doi.org/10.2903/j.efsa.2015.4285

EFSA CEF Panel (EFSA Panel on Food Contact Materials, Enzymes, Flavourings and Processing Aids), 2016b. Scientific opinion on the safety assessment of the substance zinc oxide, nanoparticles, for use in food contact materials. EFSA Journal 2016;14(3):4408, 8 pp. https://doi.org/10.2903/j.efsa.2016.4408

EFSA CEF Panel (Panel on Food Contact Materials, Enzymes, Flavourings and Processing Aids), 2016a. Scientific 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 Journal 2016;14(1):4357, 28 pp. https://doi.org/10.2903/j.efsa.2016.4357

EFSA CEP Panel (EFSA Panel on Food Contact Materials, Enzymes and Processing Aids), Silano V, Barat Baviera JM, Bolognesi C, Brüschweiler BJ, Chesson A, Cocconcelli PS, Crebelli R, Gott DM, Grob K, Lampi E, Mortensen A, Steffensen I-L, Tlustos C, Van Loveren H, Vernis L, Zorn H, Castle L, Cravedi J-P, Kolf-Clauw M, Milana MR, Pfaff K, Tavares Poças MF, Svensson K, Wölfle D, Barthélémy E and Rivière G, 2019a. Scientific Opinion on the safety assessment of the substance, montmorillonite clay modified with hexadecyltrimethylammonium bromide, for use in food contact materials. EFSA Journal 2019;17(1):5552, 8 pp. https://doi.org/10.2903/j.efsa.2019.5552

EFSA CEP Panel (EFSA Panel on Food Contact Materials, Enzymes and Processing Aids), Silano V, Barat Baviera JM, Bolognesi C, Brüschweiler BJ, Chesson A, Cocconcelli PS, Crebelli R, Gott DM, Grob K, Lampi E, Mortensen A, Steffensen I-L, Tlustos C, Van Loveren H, Vernis L, Zorn H, Castle L, Cravedi J-P, Kolf-Clauw M, Milana MR, Pfaff K, Tavares Poças MF, Svensson K, Wölfle D, Barthélémy E and Rivière G, 2019b. Scientific Opinion on the safety assessment of the substance, titanium dioxide surface treated with fluoride-modified alumina, for use in food contact materials. EFSA Journal Journal 2019;17(6):5737, 11 pp. https://doi.org/10.2903/j.efsa.2019.5737

EFSA CONTAM Panel (EFSA Panel on Contaminants in the Food Chain), 2016. Statement on the presence of microplastics and nanoplastics in food, with particular focus on seafood. EFSA Journal 2016;14(6):4501, 30 pp. https://doi.org/10.2903/j.efsa.2016.4501

EFSA FEEDAP Panel (EFSA Panel on Additives Products or Substances used in Animal Feed), 2012. Guidance on studies concerning the safety of use of the additive for users/workers. EFSA Journal 2012;10(1):2539, 52 pp. https://doi.org/10.2903/j.efsa.2012.2539

EFSA FEEDAP Panel (EFSA Panel on Additives Products or Substances used in Animal Feed), Rychen G, Aquilina G, Azimonti G, Bampidis V, Bastos MdL, Bories G, Chesson A, Cocconcelli PS, Flachowsky G, Gropp J, Kolar B, Kouba M, López-Alonso M, López Puente S, Mantovani A, Mayo B, Ramos F, Saarela M, Villa RE, Wallace RJ, Wester P, Anguita M, Dujardin B, Galobart J and Innocenti ML, 2017a. Guidance on the assessment of the safety of feed additives for the consumer. EFSA Journal 2017;15(10):5022, 52 pp. https://doi.org/10.2903/j.efsa.2017.5022

EFSA FEEDAP Panel (EFSA Panel on Additives Products or Substances used in Animal Feed), Rychen G, Aquilina G, Azimonti G, Bampidis V, Bastos MdL, Bories G, Chesson A, Cocconcelli PS, Flachowsky G, Gropp J, Kolar B, Kouba M, López-Alonso M, López Puente S, Mantovani A, Mayo B, Ramos F, Saarela M, Villa RE, Wallace RJ, Wester P, Anguita M, Galobart J, Innocenti ML and Martino L, 2017b. Guidance on the assessment of the safety of feed additives for the target species. EFSA Journal 2017;15(10):5021, 34 pp. https://doi.org/10.2903/j.efsa.2017.5021

EFSA FEEDAP Panel (EFSA Panel on Additives Products or Substances used in Animal Feed), Rychen G, Aquilina G, Azimonti G, Bampidis V, Bastos MdL, Bories G, Chesson A, Cocconcelli PS, Flachowsky G, Gropp J, Kolar B, Kouba M, López-Alonso M, López Puente S, Mantovani A, Mayo B, Ramos F, Saarela M, Villa RE, Wallace RJ, Wester P, Anguita M, Galobart J and Innocenti ML, 2017c. Guidance on the identity, characterisation and conditions of use of feed additives. EFSA Journal 2017;15(10):5023, 75 pp. https://doi.org/10.2903/j.efsa.2017.5023

EFSA GMO Panel (EFSA Panel on Genetically Modified Organisms), 2010. Scientific opinion on the assessment of allergenicity of GM plants and microorganisms and derived food and feed. EFSA Journal 2010;8(7):1700, 168 pp. https://doi.org/10.2903/j.efsa.2010.1700

EFSA NDA Panel (Panel on Dietetic Products Nutrition and Allergies), 2014. Scientific Opinion on the evaluation of allergenic foods and food ingredients for labelling purpose. EFSA Journal 2014;12(11):3894, 286 pp. https://doi.org/10.2903/j.efsa.2014.3894

EFSA NDA Panel (EFSA Panel on Dietetic Products, Nutrition and Allergies), Turck D, Bresson J-L, Burlingame B, Dean T, Fairweather-Tait S, Heinonen M, Hirsch-Ernst KI, Mangelsdorf I, McArdle H, Naska A, Neuhauser-Berthold M, Nowicka G, Pentieva K, Sanz Y, Siani A, Sjodin A, Stern M, Tome D, Vinceti M, Willatts P, Engel K-H, Marchelli R, Poting A, Poulsen M, Salminen S, Schlatter J, Arcella D, Gelbmann W, de Sesmaisons-Lecarre A, Verhagen H and vanLoveren H, 2016. Guidance on the preparation and presentation of an application for authorisation of a novel food in the context of Regulation (EU) 2015/2283. EFSA Journal 2016;14(11):4594, 24 pp. https://doi.org/10.2903/j.efsa.2016.4594

EFSA PPR Panel (EFSA Panel on Plant Protection Products and their Residues), 2011. Scientific opinion on the science behind the revision of the guidance document on dermal absorption. EFSA Journal 2011;9(7):2294, 73 pp. https://doi.org/10.2903/j.efsa.2011.2294

EFSA PPR Panel (EFSA Panel on Plant Protection Products and their Residues), 2016. Guidance on the establishment of the residue definition for dietary risk assessment. EFSA Journal 2016;14(12):4549, 129 pp. https://doi.org/10.2903/j.efsa.2016.4549

EFSA PPR Panel (EFSA Panel on Plant Protection Products and their Residues), 2017. Guidance on dermal absorption. EFSA Journal 2017;15(6):4873, 60 pp. https://doi.org/10.2903/j.efsa.2017.4873

EFSA Scientific Committee, 2006. Guidance of the Scientific Committee on a request from EFSA related to Uncertainties in Dietary Exposure Assessment. EFSA Journal 2006;438, 54 pp. https://efsa.onlinelibrary.wiley.com/doi/epdf/10.2903/j.efsa.2007.438

EFSA Scientific Committee, 2009a. Scientific Opinion on the potential risks arising from nanoscience and nanotechnologies on food and feed safety. EFSA Journal 2009;7(3):958, 39 pp. https://doi.org/10.2903/j.efsa.2009.958

EFSA Scientific Committee, 2009b. Scientific Opinion of the Scientific Committee on existing approaches incorporating replacement, reduction and refinement of animal testing: applicability in food and feed risk assessment. EFSA Journal 2009;7(6):1052, 77 pp. https://doi.org/10.2903/j.efsa.2009.1052

EFSA Scientific Committee, 2009c. Guidance of the scientific committee on transparency in the scientific aspects of risk assessments carried out by EFSA. Part 2: general principles. EFSA Journal 2009;7(5):1051, 22 pp. https://doi.org/10.2903/j.efsa.2009.1051

EFSA Scientific Committee, 2011a. Guidance on the risk assessment of the application of nanoscience and nanotechnologies in the food and feed chain. EFSA Journal 2011;9(5):2140, 36 pp. https://doi.org/10.2903/j.efsa.2011.2140

EFSA Scientific Committee, 2011b. Scientific Opinion on genotoxicity testing strategies. EFSA Journal 2011;9(9):2379, 69 pp. https://doi.org/10.2903/j.efsa.2011.2379

EFSA Scientific Committee, 2013. Scientific Opinion on the hazard assessment of endocrine disruptors: scientific criteria for identification of endocrine disruptors and appropriateness of existing test methods for assessing effects mediated by these substances on human health and the environment. EFSA Journal 2012;11(3):3132, 84 pp. https://doi.org/10.2903/j.efsa.2013.3132

EFSA Scientific Committee, 2015. Guidance on the review, revision and development of EFSA's cross-cutting guidance documents. EFSA Journal 2015;13(4):4080, 11 pp. https://doi.org/10.2903/j.efsa.2015.4080

EFSA Scientific Committee, 2017a. Scientific motivations and criteria to consider updating EFSA scientific assessments. EFSA Journal 2017;15(3):4737, 11 pp. https://doi.org/10.2903/j.efsa.2017.4737

EFSA Scientific Committee, 2017b. Guidance on the use of the weight of evidence approach in scientific assessments. EFSA Journal 2017;15(8):4971, 69 pp. https://doi.org/10.2903/j.efsa.2017.4971

EFSA Scientific Committee, 2017c. Guidance on the assessment of the biological relevance of data in scientific assessments. EFSA Journal 2017;15(8):4970, 73 pp. https://doi.org/10.2903/j.efsa.2017.4970

EFSA Scientific Committee, 2017d. Scientific Opinion on the clarification of some aspects related to genotoxicity assessment. EFSA Journal 2017;15(12):5113, 25 pp. https://doi.org/10.2903/j.efsa.2017.5113

EFSA Scientific Committee, 2017e. Guidance on the risk assessment of substances present in food intended for infants below 16 weeks of age. EFSA Journal 2017;15(5):4849, 58 pp. https://doi.org/10.2903/j.efsa.2017.4849

EFSA Scientific Committee 2017f. Update: Guidance on the use of the benchmark dose approach in risk assessment. EFSA Journal 2017;15(1):4658, 41 pp. https://doi.org/10.2903/j.efsa.2017.4658

EFSA Scientific Committee, 2018a. Guidance on uncertainty analysis in scientific assessments. EFSA Journal 2018;16(1):5123, 39 pp. https://doi.org/10.2903/j.efsa.2018.5123

EFSA Scientific Committee, 2018b. Scientific Opinion on the principles and methods behind EFSA's Guidance on Uncertainty Analysis in Scientific Assessment. EFSA Journal 2018;16(1):5122, 235 pp. https://doi.org/10.2903/j.efsa.2018.5122

EFSA Scientific Committee, 2019. Guidance on harmonised methodologies for human health, animal health and ecological risk assessment of combined exposure to multiple chemicals. EFSA Journal 2019;17(3):5634, 77 pp. https://doi.org/10.2903/j.efsa.2019.5634

EFSA Scientific Committee, 2021. Guidance on Technical requirements for regulated food and feed product applications to establish the presence of small particles including nanoparticles (Guidance on Particle-TR).

Elespuru R, Pfuhler S, Aardema MJ, Chen T, Doak SH, Doherty A, Farabaugh CS, Kenny J, Manjanatha M, Mahadevan B, Moore MM, Ouédraogo G, Stankowski Jr LF and Tanir JY, 2018. Genotoxicity assessment of nanomaterials: recommendations on best practices, assays, and methods. Toxicological Sciences, 164, 391–416. https://doi.org/10.1093/toxsci/kfy100

Elsabahy M and Wooley KL, 2013. Cytokines as biomarkers of nanoparticle immunotoxicity. Chemical Society Reviews, 42, 5552–5576. https://doi.org/10.1039/C3CS60064E

El-Sherbiny IM, El-Baz NM and Yacoub MH, 2015. Inhaled nano- and microparticles for drug delivery. Global Cardiology Science & Practice, 2. https://doi.org/10.5339/gcsp.2015.2

Ensign LM, Cone R and Hanes J, 2012. Oral drug delivery with polymeric nanoparticles: the gastrointestinal mucus barriers. Advanced Drug Delivery Reviews, 64, 557–570. https://doi.org/10.1016/j.addr.2011.12.009

Ersbøll BK, Andersen V, Loschner K and Larsen EH, 2010. NanoLyse: deliverable 3.1: sampling protocols for inorganic ENP from at least 3 matrices. Expected to be published on https://www.openaire.eu/

European Commission Joint Action, 2008–2013. No 20092101 under EU Health Programme. Safety evaluation of manufactured nanomaterials by characterisation of their potential genotoxic hazard (NANOGENOTOX). Available online: http://ec.europa.eu/chafea/projects/database/database_new.inc.data.20092101.pdf

European Union H2020 project ProSafe, 2015–2017. Promoting the Implementation of Safe by Design. Grant agreement 646325. Available online: http://www.h2020-prosafe.eu/prosafe/?cat=32

European Union Seventh Framework Programme (FP7), 2007–2013. NANoREG – A common European approach to the regulatory testing of manufactured nanomaterials. Available online: http://www.nanoreg.eu/

Evans SJ, Clift MJD, Singh N, Wills JW, Hondow N, Wilkinson TS, Burgum MJ, Brown AP, Jenkins GJ and Doak SH, 2019. In vitro detection of in vitro secondary mechanisms of genotoxicity induced by engineered nanomaterials. Particle and Fibre Toxicology, 16, 8. https://doi.org/10.1186/s12989-019-0291-7

Faria M, Björnmalm M, Thurecht KJ, 2018. Minimum information reporting in bio–nano experimental literature. Nature Nanotech, 13, 777–785. https://doi.org/10.1038/s41565-018-0246-4

Ferraro D, Anselmi-Tamburini U, Tredici IG, Ricci V and Sommi P, 2016. Overestimation of nanoparticles-induced DNA damage determined by the comet assay. Nanotoxicology, 10, 861–870. https://doi.org/10.3109/17435390.2015.1130274

Franz R, Bott J and Störmer A 2020. Considerations for and guidance to testing and evaluating migration/release of nanoparticles from polymer based nanocomposites. Nanomaterials, 10, 1113. https://doi.org/10.3390/nano10061113

Franz R and Welle F, 2017. Mathematic modelling of migration of nanoparticles from food contact polymers. In: Veraart R (ed). The Use of Nanomaterials in Food Contact Materials. DEStech Publications Inc, Lancaster, PA, USA. pp. 345–383.

Fruijtier-Pölloth C, 2012. The toxicological mode of action and the safety of synthetic amorphous silica—a nanostructured material. Toxicology, 294, 61–79. https://doi.org/10.1016/j.tox.2012.02.001

Fröhlich E and Roblegg E 2012. Models for oral uptake of nanoparticles in consumer products. Toxicology, 291, 10–17. https://doi.org/10.1016/j.tox.2011.11.004

Gamboa JM and Leong KW, 2013. *In vitro* and *in vivo* models for the study of oral delivery of nanoparticles. Advanced Drug Delivery Reviews, 65, 800–810. https://doi.org/10.1016/j.addr.2013.01.003

Gebert A, Rothkötter HJ and Pabst R, 1996. M cells in Peyer's patches of the intestine. International Review of Cytology, 167, 91–159.

Geiser M and Kreyling WG, 2010. Deposition and biokinetics of inhaled nanoparticles. Particle and Fibre Toxicology, 7, 2. https://doi.org/10.1186/1743-8977-7-2

George JM, Magogotya M, Vetten M, Buys A and Gulumian M, 2017. An Investigation of the genotoxicity and interference of gold nanoparticles in commonly used *in vitro* mutagenicity and genotoxicity assays. Toxicological Sciences, 156, 149–166. https://doi.org/10.1093/toxsci/kfw247

Geraets L, Oomen AG, Krystek P, Jacobsen NR, Wallin H, Laurentie M, Verharen HW, Brandon EF and de Jong WH, 2014. Tissue distribution and elimination after oral and intravenous administration of different titanium dioxide nanoparticles in rats. Particle and Fibre Toxicology, 11, 30. https://doi.org/10.1186/1743-8977-11-30

Gerloff K, Landesmann B, Worth A, Munn S, Palosaari T and Whelan M, 2017. The Adverse Outcome Pathway approach in nanotoxicology. Computational Toxicology, 1, 3–11. https://doi.org/10.1016/j.comtox.2016.07.001

Gimeno-Benito I, Giusti A, Dekkers S, Haase A and Janer G, 2021. A review to support the derivation of a worst-case dermal penetration value for nanoparticles. Regulatory Toxicology and Pharmacology, 119, 104836. https://doi.org/10.1016/j.yrtph.2020.104836

Gonzalez L, Decordier I and Kirsch-Volders M, 2010. Induction of chromosome malsegregation by nanomaterials. Biochemical Society Transaction, 38, 1691–1697.

Gonzalez L, Sanderson BJ and Kirsch-Volders M, 2011. Adaptations of the *in vitro* MN assay for the genotoxicity assessment of nanomaterials. Mutagenesis, 26, 185–191. https://doi.org/10.1093/mutage/geq088

Gray M and Botelho RJ, 2017. Phagocytosis: Hungry, Hungry Cells. In: Botelho R (ed). Phagocytosis and Phagosomes. Methods in Molecular Biology, vol 1519. Humana Press, New York, NY.

Grillo R, Fraceto LF, Amorim MJB, Scott-Fordsmand JJ, Schoonjans R and Chaudhry Q, 2021. Ecotoxicological and regulatory aspects of environmental sustainability of nanopesticides. Journal of Hazardous Materials, 404, 124148. https://doi.org/10.1016/j.jhazmat.2020.124148

Guadagnini R, Halamoda Kenzaoui B, Walker L, Pojana G, Magdolenova Z, Bilanicova D, Saunders M, Juillerat-Jeanneret L, Marcomini A, Huk A, Dusinska M, Fjellsbø LM, Marano F and Boland S, 2015. Toxicity screenings of nanomaterials: challenges due to interference with assay processes and components of classic in vitro tests. Nanotoxicology, 9(S1), 13–24. https://doi.org/10.3109/17435390.2013.829590

Guiot C and Spalla O, 2013. Stabilization of TiO2 Nanoparticles in Complex Medium through a pH Adjustment Protocol. Environmental Science & Technology, 47, 1057–1064. https://doi.org/10.1021/es3040736

Hadrup N, Loeschner K, Bergström A, Wilcks A, Gao X, Vogel U, Frandsen HL, Larsen EH, Lam HR and Mortensen A, 2012. Subacute oral toxicity investigation of nanoparticulate and ionic silver in rats. Archives of Toxicology, 86, 543–551. https://doi.org/10.1007/s00204-011-0759-1

Hagens WI, Oomen AG, de Jong WH, Cassee FR and Sips AJ, 2007. What do we (need to) know about the kinetic properties of nanoparticles in the body? Regulatory Toxicology and Pharmacology, 49, 217–229.

Hartmann NB, Jensen KA, Baun A, Rasmussen K, Rauscher H, Tantra R, Cupi D, Gilliland D, Pianella F and Riego Sintes JM, 2015. Techniques and protocols for dispersing nanoparticle powders in aqueous media—is there a rationale for harmonization? Journal of Toxicology and Environmental Health, Part B, 18, 299–326. https://doi.org/10.1080/10937404.2015.1074969

Hassellöv M, Readman JW, Ranville JF and Tiede K, 2008. Nanoparticle analysis and characterization methodologies in environmental risk assessment of engineered nanoparticles. Ecotoxicology, 17, 344–361. https://doi.org/10.1007/s10646-008-0225-x

Hazzard RA, Hodges GM, Scott JD, McGuinness CB and Carr KE, 1996. Early intestinal microparticle uptake in the rat. Journal of Anatomy, 189(Pt 2), 265–271.

Henderson RG, Verougstraete V, Anderson K, Arbildua JJ, Brock TO, Brouwers T, Cappellini D, Delbeke K, Herting G, Hixon G, Wallinder IO, Rodriguez PH, Van Assche F, Wilrich P and Oller AR, 2014. Inter-laboratory validation of bioaccessibility testing for metals. Regulatory Toxicology and Pharmacology, 70, 170–181. https://doi.org/10.1016/j.yrtph.2014.06.021

Higashisaka K, Nagano K, Yoshioka Y and Tsutsumia Y 2017. Nano-safety research: examining the Associations Among The Biological Effects Of Nanoparticles And Their Physicochemical Properties And Kinetics. Biological and Pharmaceutical Bulletin, 40, 243–248.

Hill EK and Li J, 2017. Current and future prospects for nanotechnology in animal production. Journal of Animal Science and Biotechnology, 8, 26. https://doi.org/10.1186/s40104-017-0157-5

Hinderliter PM, Minard KR, Orr G, Chrisler WB, Thrall BD, Pounds JG and Teeguarden JG, 2010. ISDD: a computational model of particle sedimentation, diffusion and target cell dosimetry for *in vitro* toxicity studies. Particle and Fibre Toxicology, 7, 36. https://doi.org/10.1186/1743-8977-7-36

Holpuch S, Hummel G, Tong M, Seghi G, Pei P, Ma P, Mumper R and Mallery S, 2010. Nanoparticles for local drug delivery to the oral mucosa: proof of principle studies. Pharmaceutical Research, 27, 1224–1236. https://doi.org/10.1007/s11095-010-0121-y

Hopf NB, Champmartin C, Schenk L, Berthet A, Chedik L, Du Plessis JL, Franken A, Frasch F, Gaskin S, Johanson G, Julander A, Kasting G, Kilo S, Larese Filon F, Marquet F, Midander K, Reale E and Bunge AL, 2020. Reflections on the OECD guidelines for in vitro skin absorption studies. Regulatory Toxicology and Pharmacology, 117, 104752. https://doi.org/10.1016/j.yrtph.2020.104752

Howe SE, Lickteig DJ, Plunkett KN, Ryerse JS and Konjufca V, 2014. The uptake of soluble and particulate antigens by epithelial cells in the mouse small intestine. PLoS One, 9, e86656. https://doi.org/10.1371/journal.pone.0086656

Iavicoli I, Fontana L, Leso V and Bergamaschi A, 2013. The Effects of Nanomaterials as Endocrine Disruptors. International Journal of Molecular Sciences, 148, 16732–16801.

IPCS (International Program on Chemical Safety), 2006. Harmonisation project document No. 4: Parts 1 and 2. IPCS framework for analysing the relevance of cancer and non-cancer modes of action for humans and case studies. Available online: http://www.who.int/ipcs/methods/harmonization/areas/cancer_mode.pdf

ISO (International Organization for Standardization), 1999. ISO 13320–1:1999(E): Particle size analysis. Laser diffraction methods, General principles.

ISO (International Organization for Standardization), 2005. ISO/TC 229. Nanotechnologies.

ISO (International Organization for Standardization), 2008. ISO/IEC Guide 98–3:2008 Uncertainty of measurement – Part 3: Guide to the expression of uncertainty in measurement (GUM:1995), International Organization for Standardization, Geneva, Switzerland.

ISO (International Organization for Standardization), 2009. ISO 13320:2009: Particle size analysis. Laser diffraction methods, General principles, 51 pp.

ISO (International Organization for Standardization), 2013. ISO/TS 16195:2013: Nanotechnologies - Generic requirements for reference materials for development of methods for characteristic testing, performance testing and safety testing of nano-particle and nano-fiber powders. 8 pp.

ISO (International Organization for Standardization), 2019. ISO/TR 22019:2019. Nanotechnologies – Considerations for performing toxicokinetic studies with nanomaterials. 2019-05, edition 1, 54 pp. Available online: https://www.iso.org/standard/72381.html

ISO (International Organization for Standardization), 1981. ISO/TC 24/SC 4. Particle characterization.

ISO (International Organization for Standardization), 1991. ISO/TC 201. Surface chemical analysis.

ISO/IEC, 2010. ISO/IEC GUIDE 99:2007: International vocabulary of metrology — Basic and general concepts and associated terms (VIM). Publication date: 2007-12, Corrected version (en): 2010-09. 92 pp. ICS:17.020. Available online: https://www.iso.org/standard/45324.html

IUPAC (International Union of Pure and Applied Chemistry), 2002. Harmonized guidelines for single-laboratory validation of methods of analysis. Pure and Applied Chemistry, 74, 835–855. Available online: http://citeseerx.ist.psu.edu/viewdoc/download?doi=10.1.1.137.3802&rep=rep1&type=pdf

Ivask A, Kurvet I, Kasemets K, Blinova I, Aruoja V, Suppi S, Vija H, Ka€kinen A, Titma T, Heinlaan M, Visnapuu M, Koller D, Kisand V and Kahru A, 2014. Size-dependent toxicity of silver nanoparticles to bacteria, yeast, algae, crustaceans and mammalian cells *in vitro*. PLoS One, 9. e102108. https://doi.org/10.1371/journal.pone.0102108

Jacobsen NR, Pojan G, Wallin H and Jensen KA, 2010. Nanomaterial dispersion protocol for toxicological studies in ENPRA. Internal ENPRA Project Report. Copenhagen, Denmark: National Research Centre for the Working Environment.

Jensen KA, Kembouche Y, Christiansen E, Jacobsen NR, Wallin H, Guiot C, Spalla O and Witschger O, 2011. Final protocol for producing suitable manufactured nanomaterial exposure media. In: Jensen KA and Thieret N (eds). Report. The generic NANOGENOTOX dispersion protocol. Standard operation procedure (SOP) and background documentation. 32 pp. Available online: https://www.anses.fr/en/system/files/nanogenotox_deliverable_5.pdf

Jogaiah S, Singh HB, Fraceto LF and Lima R, 2021. Advances in Nano-Fertilizers and Nano-Pesticides in Agriculture, 1st Edition. A Smart Delivery System for Crop Improvement. Woodhead Publishing, 9th December 2020, 650 pp. https://doi.org/10.1016/C2019-0-01205-8

Kah M, Beulke S, Tiede K and Hofmann T, 2013. Nanopesticides: state of knowledge, environmental fate, and exposure modeling. Critical Reviews in Environmental Science and Technology, 43, 1823–1867. https://doi.org/10.1080/10643389.2012.671750

Kah M and Hofmann T, 2014. Nanopesticide research: current trends and future priorities. Environment International, 63, 224–235. https://doi.org/10.1016/j.envint.2013.11.015

Kästner C, Lichtenstein D, Lampen A and Thünemann AF, 2016. Monitoring the fate of small silver nanoparticles during artificial digestion. Colloids and Surfaces A: Physicochemical and Engineering Aspects, 526, 76–81. https://doi.org/10.1016/j.colsurfa.2016.08.013

Kermanizadeh A, Balharry D, Wallin H, Loft S and Møller P, 2015b. Nanomaterial translocation–the biokinetics, tissue accumulation, toxicity and fate of materials in secondary organs–a review. Critical Reviews in Toxicology, 45, 837–872. https://doi.org/10.3109/10408444.2015.1058747

Kermanizadeh A, Chauché C, Brown DM, Loft S and Møller P, 2015a. The role of intracellular redox imbalance in nanomaterial induced cellular damage and genotoxicity: a review. Environmental and Molecular Mutagenesis, 56, 111–124. https://doi.org/10.1002/em.21926

Kestens V, Bozatzidis V, De Temmerman PJ, Ramaye Y and Roebben G, 2017. Validation of a particle tracking analysis method for the size determination of nano- and microparticles. Journal of Nanoparticle Research, 19, 271. https://doi.org/10.1007/s11051-017-3966-8

Kestens V, Roebben G, Herrmann J, Jämting A, Coleman V, Minelli C, Clifford C, De Temmerman P-J, Mast J, Junjie L, Babick F, Cölfen H and Hendrik E, 2016. Challenges in the size analysis of a silica nanoparticle mixture as candidate certified reference material. Journal of Nanoparticle Research, 18, 171. https://doi.org/10.1007/s11051-016-3474-2

Kestens V, Coleman A, Herrmann J, Minelli C, Shard AG and Roebben G, 2019. Establishing SI-traceability of nanoparticle size values measured with line-start incremental centrifugal liquid sedimentation. Separations, 6, 15. https://doi.org/10.3390/separations6010015

Kiela PR and Ghishan FK, 2016. Physiology of intestinal absorption and secretion. Best Practice & Research Clinical Gastroenterology, 30, 145–159. https://doi.org/10.1016/j.bpg.2016.02.007

Koch I, Reimer KJ, Bakker MI, Basta NT, Cave MR, Denys S, Dodd M, Hale BA, Irwin R, Lowney YW, Moore MM, Paquin V, Rasmussen PE, Repaso-Subang T, Stephenson GL, Siciliano SD, Wragg J and Zagury GJ, 2013. Variability of bioaccessibility results using seventeen different methods on a standard reference material, NIST 2710. Journal of Environmental Science and Health. Part A, Toxic/Hazardous Substances & Environmental Engineering, 48, 641–655. https://doi.org/10.1080/10934529.2013.731817

Kookana RS, Boxall ABA, Reeves PT, Ashauer R, Beulke S, Chaudhry Q, Cornelis G, Fernandes TF, Gan J, Kah M, Lynch I, Ranville J, Sinclair C, Spurgeon D, Tiede K and Van den Brink PJ, 2014. Nanopesticides: guiding principles for regulatory evaluation of environmental risks. Journal of Agricultural and Food Chemistry, 62, 4227–4240. https://doi.org/10.1021/jf500232f

Kostewicz ES, Brauns U, Becker R and Dressman JB, 2002. Forecasting the oral absorption behaviour of poorly soluble weak bases using solubility and dissolution studies in biorelevant media. Pharmaceutical Research, 19, 345–349. https://doi.org/10.1023/A:1014407421366

Kreyling WG, Semmler-Behnke M, Takenaka S and Möller W, 2013. Differences in the biokinetics of inhaled nano- versus micrometer-sized particles. Accounts of Chemical Research, 46, 714–722. https://doi.org/10.1021/ar300043r

Kreyling WG, Holzwarth U, Schleh C, Kozempel J, Wenk A, Haberl N, Hirn S, Schaℓffler M, Lipka J, Semmler-Behnke M and Gibson N, 2017. Quantitative biokinetics of titanium dioxide nanoparticles after oral application in rats: part 2. Nanotoxicology, 11, 443–453. https://doi.org/10.1080/17435390.2017.1306893

Kroll A, Pillukat MH, Hahn D and Schnekenburger J, 2012. Interference of engineered nanoparticles with *in vitro* toxicity assays. Archives of Toxicology, 86, 1123–1136. https://doi.org/10.1007/s00204-012-0837-z

Krul C, Luiten-Schuite A, Baandagger R, Verhagen H, Mohn G, Veron F and Havenaar R, 2000. Application of a dynamic *in vitro* gastrointestinal tract model to study the availability of food mutagens, using heterocyclic aromatic amines as model compounds. Food and Chemical Toxicology, 38, 783–792. https://doi.org/10.1016/S0278-6915(00)00071-5

Lamas B, Martins Breyner N and Houdeau E, 2020. Impacts of foodborne inorganic nanoparticles on the gut microbiota-immune axis: potential consequences for host health. Particle and Fibre Toxicology, 17, 19. https://doi.org/10.1186/s12989-020-00349-z

Landsiedel R, Fabian E, Ma-Hock L, van Ravenzwaay B, Wohlleben W, Wiench K and Oesch F, 2012. Toxico-/biokinetics of nanomaterials, 2012 biokinetics of nanomaterials. Archives of Toxicology, 86, 1021–1060. https://doi.org/10.1007/s00204-012-0858-7

Langezaal I, Coecke S and Hartung T, 2001. Whole blood cytokine response as a measure of immunotoxicity. Toxicology in Vitro, 15, 313–318. https://doi.org/10.1016/S0887-2333(01)00028-5

Langezaal I, Hoffmann S, Hartung T and Coecke S, 2002. Evaluation and prevalidation of an immunotoxicity test based on human whole-blood cytokine release. Alternatives to Laboratory Animals, 30, 581–595.

Larson JK, Carvan III MJ and Hutz RJ, 2014. Engineered nanomaterials: an emerging class of novel endocrine disruptors. Biology of Reproduction, 91, 20. https://doi.org/10.1095/biolreprod.113.116244

van Leeuwen C, Patlewicz G and Worth A, 2007. Intelligent Testing Strategies. In: Leeuwen C and Vermeire T (eds.). Risk Assessment of Chemicals, Dordrecht, Springer. pp. 467–509. https://doi.org/10.1007/978-1-4020-6102-8_11

van Leeuwen CJ, Patlewicz GY and Worth AP, 2007. Intelligent testing strategies. In: van Leeuwen CJ and Vermeire TG (eds.). Risk Assessment of Chemicals: An introduction, 2nd Edition, Springer, The Netherlands. pp. 467–509.

Lefebvre DE, Venema K, Gombau L, Valerio Jr LG, Raju J, Bondy GS, Bouwmeester H, Singh RP, Clippinger AJ, Collnot EM, Mehta R and Stone V, 2015. Utility of models of the gastrointestinal tract for assessment of the digestion and absorption of engineered nanomaterials released from food matrices. Nanotoxicology, 9, 523–542. https://doi.org/10.3109/17435390.2014.948091

Lehner R, Weder C, Petri-Fink A and Rothen-Ritishauser B, 2019. Emergence of nanoplastic in the environment and possible impact on human health. Environmental Science & Technology, 53, 1748–1765. https://doi.org/10.1021/acs.est.8b05512

Li Y, Doak SH, Yan J, Chen DH, Zhou M, Mittelstaedt RA, Chen Y, Li C and Chen T, 2017. Factors affecting the in vitro micronucleus assay for evaluation of nanomaterials. Mutagenesis, 32, 151–159. https://doi.org/10.1093/mutage/gew040

Lichtenstein D, Ebmeyer J, Knappe P, Juling S, Böhmert L, Selve S, Niemann B, Braeuning A, Thünemann AF and Lampen A, 2015. Impact of food components during *in vitro* digestion of silver nanoparticles on cellular uptake and cytotoxicity in intestinal cells. Biological Chemistry, 396, 1255–1264. https://doi.org/10.1515/hsz-2015-0145

Lin LL, Nufer KL, Tomihara S and Prow TW, 2015. Non-invasive nanoparticle imaging technologies for cosmetic and skin care products. Cosmetics, 2, 196–210. https://doi.org/10.3390/cosmetics2030196

Linsinger TPJ, Roebben G, Gilliland D, Calzolai L, Rossi F, Gibson N and Klein C, 2012. Requirements on measurements for the implementation of the European Commission definition of the term nanomaterial, JRC Reference Report, EUR 25404 EN. European Union, Luxembourg. ISBN 978-92-79-25603-5.

Linsinger TPJ, Chaudhry Q, Dehalu V, Delahaut P, Dudkiewicz A, Grombe R, Kammer F, Larsen EH, Legros S, Loeschner K, Peters R, Ramsch R, Roebben G, Tiede K and Weigel S, 2013. Validation of methods for the detection and quantification of engineered nanoparticles in food. Food Chemistry, 138, 1959–1966. https://doi.org/10.1016/j.foodchem.2012.11.074

Liu L and Kong F, 2021. The behavior of nanocellulose in gastrointestinal tract and its influence on food digestion. Journal of Food Engineering, 292, 110346. https://doi.org/10.1016/j.jfoodeng.2020.110346

Loeschner K, Hadrup N, Qvortrup K, Larsen A, Gao X, Vogel U, Mortensen A, Lam HR and Larsen EH, 2011. Distribution of silver in rats following 28 days of repeated oral exposure to silver nanoparticles or silver acetate. Particle and Fibre Toxicology, 8, 18. https://doi.org/10.1186/1743-8977-8-18

Lundqvist M, Stigler J, Elia G, Lynch I, Cedervall T and Dawson KA, 2008. Nanoparticle size and surface properties determine the protein corona with possible implications for biological impacts. Proceedings of the National Academy of Sciences of the United States of America, 105, 14265–14270. https://doi.org/10.1073/pnas.0805135105

Lövenstam G, Rauscher H, Roebben G, Sokull Klütten B, Gibson N, Putaud JP and Stamm H, 2010. Considerations on a definition of nanomaterial for regulatory purposes. JRC (Joint Research Centre) Reference Report, EUR 24403 EN. https://doi.org/10.2788/98686

Macierzanka A, Mackie AR, Bajka BH, Rigby NM, Nau F and Dupont D, 2014. Transport of particles in intestinal mucus under simulated infant and adult physiological conditions: impact of mucus structure and extracellular DNA. PLoS One, 9, e95274. https://doi.org/10.1371/journal.pone.0095274

Magdolenova Z, Collins A, Kumar A, Dhawan A, Stone V and Dusinska M, 2014. Mechanisms of genotoxicity. A review of in vitro and in vivo studies with engineered nanoparticles. Nanotoxicology, 8, 233–278. https://doi.org/10.3109/17435390.2013.773464

Magdolenova Z, Lorenzo Y, Collins A and Dusinska M, 2012. Can standard genotoxicity tests be applied to nanoparticles? Journal of Toxicology and Environmental Health, Part A, 75, 800–806. https://doi.org/10.1080/15287394.2012.690326

Magnusson B and Örnemark U (eds.), 2014. Eurachem Guide: The Fitness for Purpose of Analytical Methods – A Laboratory Guide to Method Validation and Related Topics, 2nd Edition. ISBN 978-91-87461-59-0. Available online: www.eurachem.org

Manshian BB, Soenen SJ, Al-Ali A, Brown A, Hondow N, Wills J, Jenkins GJS and Doak SH, 2015. Cell type-dependent changes in CdSe/ZnS quantum dot uptake and toxic endpoints. Toxicological Sciences, 144, 246–258. https://doi.org/10.1093/toxsci/kfv002

Mast J and De Temmerman P-J, 2016. Protocol(s) for size-distribution analysis of primary NM particles in air, powders, and liquids. Available online: https://www.rivm.nl/en/About_RIVM/Mission_and_strategy/International_Affairs/International_Projects/Completed/NANoREG/deliverables:s_ezJOgJTEaL0lCqO1cAJQ/NANoREG_D2_05_DR_Protocol_for_characterization_and_categorization_of_MNM_in_powders_and_liquid_dispersions:0LTEw1N2R5W8K54VJfPy7Q.org

Mech A, Rauscher H, Babick F, Hodoroaba V-D, Ghanem A, Wohlleben W, Marvin H, Weigel S, Brüngel R and Friedrich CM, 2020a. The NanoDefine Methods Manual. Part 1: The NanoDefiner Framework and Tools, EUR 29876 EN, Publications Office of the European Union, Luxembourg.

Mech A, Rauscher H, Rasmussen K, Babick F, Hodoroaba V-D, Ghanem A, Wohlleben W, Marvin H, Brüngel R and Friedrich CM, 2020b. The NanoDefine Methods Manual. Part 2: Evaluation of methods, EUR 29876 EN, Publications Office of the European Union. Luxembourg. ISBN 978-92-76-11953-1, https://doi.org/10.2760/071877, JRC117501.

Mech A, Rauscher H, Rasmussen K, Gilliland D, Babick F, Hodoroaba VD, Ghanem A, Wohlleben W, Marvin H, Löschner K, Brüngel R and Friedrich CM, 2020c. The NanoDefine Methods Manual. Part 3: Standard Operating Procedures (SOPs), EUR 29876 EN. Publications Office of the European Union, Luxembourg.

Meek ME, Boobis A, Cote I, Dellarco V, Fotakis G, Munn S, Seed J and Vickers C, 2013. New developments in the evolution and application of the WHO/IPCS framework on mode of action/species concordance analysis. Journal of Applied Toxicology, 34, 1–18. https://doi.org/10.1002/jat.2949

Mercier-Bonin M, Despax B, Raynaud P, Houdeau E and Thomas M, 2018. Mucus and microbiota as emerging players in gut nanotoxicology: The example of dietary silver and titanium dioxide nanoparticles. Critical Reviews in Food Science and Nutrition, 58, 1023–1032. https://doi.org/10.1080/10408398.2016.1243088

Minekus M, Alminger M, Alvito P, Ballance S, Bohn T, Bourlieu C, Carriere F, Boutrou R, Corredig M, Dupont D, Dufour C, Egger L, Golding M, Karakaya S, Kirkhus B, Le Feunteun S, Lesmes U, Macierzanka A, Mackie A, Marze S, McClements DJ, Menard O, Recio I, Santos CN, Singh RP, Vegarud GE, Wickham MSJ, Weitschies W and Brodkorb A, 2014. A standardised static *in vitro* digestion method suitable for food – an international consensus. Food and Function, 5, 1113–1124. https://doi.org/10.1039/c3fo60702j

Møller P, Jensen DM, Christophersen DV, Kermanizadeh A, Jacobsen NR, Hemmingsen JG, Danielsen PH, Karottki DG, Roursgaard M, Cao Y, Jantzen K, Klingberg H, Hersoug LG and Loft S, 2015. Measurement of oxidative damage to DNA in nanomaterial exposed cells and animals. Environmental and Molecular Mutagenesis, 56, 97–110. https://doi.org/10.1002/em.21899

Mujtaba M, Sharif R, Ali Q, Rehman R and Khawar KM, 2021. Biopolymer based nanofertilizers applications in abiotic stress (drought and salinity) control. In: Jogaiah S, Singh HB, Fraceto LF and Lima Rd (eds). Advances in Nano-Fertilizers and Nano-Pesticides in Agriculture, Woodhead Publishing. pp. 85–110.

National Research Council Committee on Toxicity Testing and Assessment of Environmental Agents, 2007. Toxicity testing in the 21st century: a Vision and a strategy. Available online: https://www.nap.edu/resource/11970/Toxicity_Testing_final.pdf

Nelson BC, Wright CW, Ibuki Y, Moreno-Villanueva M, Karlsson HL, Hendriks G, Sims CM, Singh N and Doak SH, 2016. Emerging metrology for high-throughput nanomaterial genotoxicology. Mutagenesis, 32, 215–232. https://doi.org/10.1093/mutage/gew037

Nguyen TTP, Bhandari B, Cichero J and Prakash S, 2015. A comprehensive review on in vitro digestion of infant formula. Food Research Internationa, 76(part 3), 373–386. https://doi.org/10.1016/j.foodres.2015.07.016

Noonan GO, Whelton AJ, Carlander D and Duncan TV, 2014. Measurement methods to evaluate engineered nanomaterial release from food contact materials. Comprehensive Reviews in Food Science and Food Safety, 13, 679–692. https://doi.org/10.1111/1541-4337.12079

Nymark P, Rieswijk L, Ehrhart F, Jeliazkova N, Tsiliki G, Sarimveis H, Evelo CT, Hongisto V, Kohonen P, Willighagen E and Grafström R, 2018. A data fusion pipeline for generating and enriching adverse outcome pathway descriptions. Toxicological Sciences, 162, 264–275. https://doi.org/10.1093/toxsci/kfx252

OECD (Organisation for Economic Co-operation and Development), 1997. OECD Guidelines for the Testing of Chemicals. Test No. 424: Neurotoxicity Study in Rodents. OECD Publishing, Paris, France. https://doi.org/10.1787/9789264071025-en
OECD (Organisation for Economic Co-operation and Development), 2001. OECD Guidelines for the Testing of Chemicals. Test No. 414: Prenatal development toxicity study. OECD Publishing, Paris, France, 11 pp. https://doi.org/10.1787/9789264070820-en
OECD (Organisation for Economic Co-operation and Development), 2004a. OECD Guidelines for the Testing of Chemicals. Test No. 427: Skin absorption: in vivo method. OECD Publishing, Paris, France, 8 pp. https://doi.org/https://doi.org/10.1787/9789264071063-en
OECD (Organisation for Economic Co-operation and Development), 2004b. OECD Guidelines for the Testing of Chemicals. Test No. 428: Skin absorption: in vitro method. OECD Publishing, Paris, France, 8 pp. https://doi.org/10.1787/9789264071087-en
OECD (Organisation for Economic Co-operation and Development), 2007a. OECD Guidelines for the Testing of Chemicals. Test No. 426: Developmental neurotoxicity study. OECD Publishing, Paris, France, 26 pp. https://doi.org/10.1787/9789264067394-en
OECD (Organisation for Economic Co-operation and Development), 2007b. OECD Guidelines for the Testing of Chemicals. Test No. 501: Metabolism in Crops. OECD Guidelines for the Testing of Chemicals. OECD Publishing, Paris. https://doi.org/10.1787/9789264061835-en
OECD (Organisation for Economic Co-operation and Development), 2007c. OECD Guidelines for the Testing of Chemicals. Test No. 502: Metabolism in Rotational Crops. OECD Publishing, Paris. https://doi.org/10.1787/9789264061859-en
OECD (Organisation for Economic Co-operation and Development), 2007d. OECD Guidelines for the Testing of Chemicals. Test No. 503: Metabolism in Livestock. OECD Publishing, Paris. https://doi.org/10.1787/9789264061873-en
OECD (Organisation for Economic Co-operation and Development), 2007e. OECD Guidelines for the Testing of Chemicals. Test No. 504: Residues in Rotational Crops (Limited Field Studies). OECD Publishing, Paris. https://doi.org/10.1787/9789264013384-en
OECD (Organisation for Economic Co-operation and Development), 2007f. OECD Guidelines for the Testing of Chemicals. Test No. 505: Residues in Livestock. OECD Publishing, Paris. https://doi.org/10.1787/9789264061903-en
OECD (Organisation for Economic Co-operation and Development), 2007g. OECD Guidelines for the Testing of Chemicals. Test No 506: Stability of Pesticide Residues in Stored Commodities. OECD Publishing, Paris. https://doi.org/10.1787/9789264061927-en
OECD (Organisation for Economic Co-operation and Development), 2007h. OECD Guidelines for the Testing of Chemicals. Test No. 507: Nature of the Pesticide Residues in Processed Commodities - High Temperature Hydrolysis. OECD Publishing, Paris. https://doi.org/10.1787/9789264067431-en
OECD (Organisation for Economic Co-operation and Development), 2008a. OECD Guidelines for the Testing of Chemicals. Test No. 407: Repeated dose 28-day oral toxicity study in rodents. OECD Publishing, Paris, France, 13 pp. https://doi.org/10.1787/9789264070684-en
OECD (Organisation for Economic Co-operation and Development), 2008b. OECD Guidelines for the Testing of Chemicals. Test No. 508: Magnitude of the Pesticide Residues in Processed Commodities. OECD Publishing, Paris. https://doi.org/10.1787/9789264067622-en
OECD (Organisation for Economic Co-operation and Development), 2009a. OECD Guidelines for the Testing of Chemicals. Test No. 403: Acute inhalation toxicity. OECD Publishing, Paris, France, 19 pp. https://doi.org/10.1787/9789264070608-en
OECD (Organisation for Economic Co-operation and Development), 2009b. OECD Guidelines for the Testing of Chemicals. Test No. 451: Carcinogenicity studies. OECD Publishing, Paris, France, 15 pp. https://doi.org/10.1787/9789264071186-en
OECD (Organisation for Economic Co-operation and Development), 2009c. OECD Guidelines for the Testing of Chemicals. Test No. 452: Chronic toxicity studies. OECD Publishing, Paris, France, 16 pp. https://doi.org/10.1787/9789264071209-en
OECD (Organisation for Economic Co-operation and Development), 2009d. OECD Guidelines for the Testing of Chemicals. Test No. 453: Combined chronic toxicity/carcinogenicity studies. OECD Publishing, Paris, France, 20 pp. https://doi.org/10.1787/9789264071223-en
OECD (Organisation for Economic Co-operation and Development), 2009e. OECD Guidelines for the Testing of Chemicals. Test No. 509: Crop Field Trial. OECD Publishing, Paris. https://doi.org/10.1787/9789264076457-en
OECD (Organisation for Economic Co-operation and Development), 2010. OECD Guidelines for the Testing of Chemicals. Test No. 417: Toxicokinetics. OECD Publishing, Paris, France, 20 pp. https://doi.org/10.1787/9789264070882-en
OECD (Organisation for Economic Co-operation and Development), 2012a. Series on the Safety of Manufactured Nanomaterials, No 33, Important issues on risk assessment of manufactured nanomaterials. ENV/JM/MONO (2012)8.

OECD (Organisation for Economic Co-operation and Development), 2012b. OECD Guidelines for the Testing of Chemicals. Test No. 443: Extended one-generation reproductive toxicity study. OECD Publishing, Paris, France, 25 pp. https://doi.org/10.1787/9789264185371-en
OECD (Organisation for Economic Co-operation and Development), 2013. OECD Guidelines for the Testing of Chemicals. Test No. 488: Transgenic rodent somatic and germ cell gene mutation assays. OECD Publishing, Paris, France, 15 pp. https://doi.org/10.1787/9789264203907-en
OECD (Organisation for Economic Co-operation and Development), 2014a. Genotoxicity of manufactured nanomaterials: report of the OECD Expert Meeting. Available online: http://www.oecd.org/officialdocuments/publicdisplaydocumentpdf/?cote1/4env/jm/mono(2014)34&doclanguage1/4en
OECD (Organisation for Economic Co-operation and Development), 2014b. Guidance document for describing non-guideline in vitro test methods. ENV/JM/MONO(2014)35. Available online: http://www.oecd.org/officialdocuments/publicdisplaydocumentpdf/?cote=ENV/JM/MONO(2014)35&doclanguage=en
OECD (Organisation for Economic Co-operation and Development), 2016a. OECD Guidelines for the Testing of Chemicals. Test No. 474: Mammalian erythrocyte micronucleus test. OECD Publishing, Paris, France, 21 pp. https://doi.org/10.1787/9789264264762-en
OECD (Organisation for Economic Co-operation and Development), 2016b. OECD Guidelines for the Testing of Chemicals. Test No. 476: In vitro mammalian cell gene mutation tests using the Hprt and xprt genes. OECD Publishing, Paris, France, 18 pp. https://doi.org/10.1787/9789264264809-en
OECD (Organisation for Economic Co-operation and Development), 2016c. OECD Guidelines for the Testing of Chemicals. Test No. 487: In vitro mammalian cell micronucleus test, OECD Publishing, Paris, France, 29 pp. https://doi.org/10.1787/9789264264861-en
OECD (Organisation for Economic Co-operation and Development), 2016d. OECD Guidelines for the Testing of Chemicals. Test No. 489: In vivo mammalian alkaline comet assay. OECD Publishing, Paris, France, 27 pp. https://doi.org/10.1787/9789264264885-en
OECD (Organisation for Economic Co-operation and Development), 2016e. OECD Guidelines for the Testing of Chemicals. Test No. 490: In vitro mammalian cell gene mutation tests using the thymidine kinase gene. OECD Publishing, Paris, France, 24 pp. https://doi.org/10.1787/9789264264908-en
OECD (Organisation for Economic Co-operation and Development), 2016f. Categorization of manufactured nanomaterials. Series on the Safety of Manufactured Nanomaterials No. 66, ENV/JM/MONO(2016). Available online: http://www.oecd.org/officialdocuments/publicdisplaydocumentpdf/?cote=env/jm/mono%282016%299&doclanguage=en
OECD (Organisation for Economic Co-operation and Development), 2016g. Grouping and read-across for the hazard assessment of manufactured nanomaterials. Report from the expert meeting. In Series on the Safety of Manufactured Nanomaterials No. 76, ENV/JM/MONO(2016)59. Available online: http://www.oecd.org/officialdocuments/publicdisplaydocumentpdf/?cote=env/jm/mono(2016)59&doclanguage=en
OECD (Organisation for Economic Co-operation and Development), 2017a. OECD Guidelines for the Testing of Chemicals. Test No. 318: Dispersion stability of nanomaterials in simulated environmental media. OECD Publishing, Paris, France, 32 pp. Available online: https://www.oecd-ilibrary.org/docserver/9789264284142-en.pdf?expires=1525785799&id=id&accname=guest&checksum=4C55DFB0F6151E2EAA4871732687F506age=bi
OECD (Organisation for Economic Co-operation and Development), 2017b. Recommendation of the Council on the Safety Testing and Assessment of Manufactured Nanomaterials. OECD/LEGAL/0400. Available online: https://legalinstruments.oecd.org/en/instruments/OECD-LEGAL-0400
OECD (Organisation for Economic Co-operation and Development), 2018a. Test No. 408: Repeated Dose 90-Day Oral Toxicity Study in Rodents. OECD Guidelines for the Testing of Chemicals, Section 4. OECD Publishing, Paris. https://doi.org/10.1787/9789264070707-en
OECD (Organisation for Economic Co-operation and Development), 2018b. OECD Series on Testing and Assessment: Revised Guidance Document 150 on Standardised Test Guidelines for evaluating chemicals for endocrine disruption. No. 150, OECD Publishing, Paris, https://doi.org/10.1787/9789264304741-en
OECD (Organisation for Economic Co-operation and Development), 2018c. OECD Series on Testing and Assessment: Guidance document on inhalation toxicity studies. No. 39, OECD Publishing, Paris.
OECD (Organisation for Economic Co-operation and Development), 2018d. OECD Series on Testing and Assessment: Document on Good In Vitro Method Practices (GIVIMP). No. 286, ENV/JM/MONO(2018)19. Available onnline: www.oecd.org/officialdocuments/publicdisplaydocumentpdf/?cote=ENV/JM/MONO(2018)19&doclanguage=en
OECD (Organisation for Economic Co-operation and Development), 2019. Guidance notes on dermal absorption, Series on Testing and Assessment No. 156. Draft Second Edition.
OECD (Organisation for Economic Co-operation and Development), 2020. Guidance document for the testing of dissolution and dispersion stability of nanomaterials and the use of the data for further environmental testing and assessment strategies. Series on Testing and Assessment, No. 318. OECD Publishing, Paris. Available online: http://www.oecd.org/officialdocuments/publicdisplaydocumentpdf/?cote=env/jm/mono(2020)9&doclanguage=en

OECD WPMN (Organisation for Economic Co-operation and Development Working Party on Manufactured Nanomaterials), 2010. Preliminary guidance notes on sample preparation and dosimetry for the safety testing of manufactured nanomaterials. Available online: www.oecd.org/officialdocuments/displaydocumentpdf/?cote=env/jm/mono(2010)25&doclanguage=en

Ogobuiro I, Gonzales J and Tuma F, 2020. Gastrointestinal. [Updated 2020 Oct 27]. In: StatPearls [Internet]. Treasure Island (FL): StatPearls Publishing; 2021 Jan. Available online: https://www.ncbi.nlm.nih.gov/books/NBK537103/#!po=94.4444

Oomen AG, Hack A, Minekus M, Zeijdner E, Cornelis C, Schoeters G, Verstraete W, Van de Wiele T, Wragg J, Rompelberg CJM, Sips AJAM and Van Wijnen JH, 2002. Comparison of five in vitro digestion models to study the bioaccessibility of soil contaminants. Environmental Science and Technology, 36, 3326–3334. https://doi.org/10.1021/es010204v

Oomen AG, Bleeker EAJ, Bos PMJ, van Broekhuizen F, Gottardo S, Groenewold M, Hristozov D, Hund-Rinke K, Irfan M-A, Marcomini A, Peijnenburg WJGM, Rasmussen K, Jiménez AS, Scott-Fordsmand JJ, van Tongeren M, Wiench K, Wohlleben W and Landsiedel R, 2015. Grouping and read-across approaches for risk assessment of nanomaterials. International Journal of Environmental Research and Public Health, 12, 13415–13434. https://doi.org/10.3390/ijerph121013415

Oomen AG, Rompelberg CJM, Bruil MA, Dobbe CJG, Pereboom DPKH and Sips AJAM, 2003. Development of an *in vitro* digestion model for estimating the bioaccessibility of soil contaminants. Archives of Environmental Contamination and Toxicology, 44, 281–287. https://doi.org/10.1007/s00244-002-1278-0

Oomen AG, Steinhauser KG, Bleeker EAJ, vanBroekhuizen F, Sips A, Dekkers S, Wijnhoven SWP and Sayre PG, 2018. Risk assessment frameworks for nanomaterials: scope, link to regulations, applicability, and outline for future directions in view of needed increase in efficiency. NanoImpact, 9, 1–13. https://doi.org/10.1016/j.impact.2017.09.001

Pelfrêne A, Cave MR, Wragg J and Douay F, 2017. *In vitro* investigations of human bioaccessibility from reference materials using simulated lung fluids. International Journal of Environmental Research and Public Health, 14, 112. https://doi.org/10.3390/ijerph14020112

Perlatti B, deSouza Bergo PL, Fernandes da Silva MFG, Fernandes JB and Forim MR, 2013. Polymeric nanoparticle-based insecticides: a controlled release purpose for agrochemicals. In: Stanislav T (ed.). Insecticides - Development of Safer and More Effective Technologies. Intech. ISBN 978-953-51-0958-7. https://doi.org/10.5772/53355

Peters R, Kramer E, Oomen AG, Rivera ZEH, Oegema G, Tromp PC, Fokkink R, Rietveld A, Marvin HJP, Weigel S, Peijnenburg ACM and Bouwmeester H, 2012. Presence of nano-sized silica during *in vitro* digestion of foods containing silica as a food additive. ACS Nano, 6, 2441–2451. https://doi.org/10.1021/nn204728k

Peters R, Brandhoff P, Weigel S, Marvin H, Bouwmeester H, Aschberger K, Rauscher H, Amenta M, Moniz FB, Gottardo S and Mech A, 2014a. Inventory of nanotechnology applications in the agricultural, feed and food sector. EFSA supporting publication 2014:11(7):EN-621, 125 pp. https://doi.org/10.2903/sp.efsa.2014.EN-621

Peters RJB, Herrera Rivera Z, Bouwmeester H, Weigel S and Marvin HJP, 2014b. Advanced analytical techniques for the measurement of nanomaterials in complex samples: a comparison. Quality Assurance and Safety of Crops & Foods, 6, 281–290. https://doi.org/10.3920/QAS2014.0410

Pfuhler S, Downs TR, Allemang AJ, Shan Y and Crosby ME, 2017. Weak silica nanomaterial-induced genotoxicity can be explained by indirect DNA damage as shown by the OGG1-modified comet assay and genomic analysis. Mutagenesis, 32, 5-12. https://doi.org/10.1093/mutage/gew064. Epub 2016 Nov 17. PMID: 27864332.

Pfuhler S, Elespuru R, Aardema MJ, Doak SH, Maria Donner E, Honma M, Kirsch-Volders M, Landsiedel R, Manjanatha M, Singer T and Kim JH, 2013. Genotoxicity of nanomaterials: refining strategies and tests for hazard identification. Environmental and Molecular Mutagenesis, 54, 229–239. https://doi.org/10.1002/em.21770

Piersma AH, Rorije E, Beekhuijzen ME, Cooper R, Dix DJ, Heinrich-Hirsch B, Martin MT, Mendez E, Muller A, Paparella M, Ramsingh D, Reaves E, Ridgway P, Schenk E, Stachiw L, Ulbrich B and Hakkert BC, 2011. Combined retrospective analysis of 498 rat multi-generation reproductive toxicity studies: on the impact of parameters related to F1 mating and F2 offspring. Reproductive Toxicology, 31, 392–401. https://doi.org/10.1016/j.reprotox.2010.11.013

Powell JJ, Faria N, Thomas-McKay E and Pele LC, 2010. Origin and fate of dietary nanoparticles and microparticles in the gastrointestinal tract. Journal of Autoimmunity, 34, J226–J233. https://doi.org/10.1016/j.jaut.2009.11.006

ProSafe, 2017. The ProSafe White Paper: towards a more effective and efficient governance and regulation of nanomaterials. Available online: https://www.rivm.nl/sites/default/files/2018-11/ProSafe%20White%20Paper%20updated%20version%2020170922.pdf

Raliya R, Saharan V, Dimkpa C and Biswas P, 2018. Nanofertilizer for precision and sustainable agriculture: current state and future perspectives. Journal of Agricultural and Food Chemistry, 66, 6487–6503. https://doi.org/10.1021/acs.jafc.7b02178

Ramaye Y, Dabrio M, Roebben G and Kestens V, 2021. Development and Validation of Optical Methods for Zeta Potential Determination of Silica and Polystyrene Particles in Aqueous Suspensions. Materials, 14, 290. https://doi.org/10.3390/ma14020290

Rasmussen K, Rauscher H and Mech A, 2017. Physicochemical characterisation. In: Fadeel B, Pietroiusti A and Shvedova A (eds.). Adverse Effects of Engineered Nanomaterials, 2nd Edition. Academic Press, London, UK. (p. 15 ff.). ISBN: 9780128091999.

Rauscher H, Roebben G, Boix Sanfeliu A, Emons H, Gibson P, Koeber R, Linsinger T, Rasmussen K, Riego Sintes J, Sokull Kluettgen B and Stamm H, 2015. Towards a review of the EC Recommendation for a definition of the term 'nanomaterial' - Part 3: Scientific-technical evaluation of options to clarify the definition and to facilitate its implementation. Edited by Rauscher H. and Roebben G. Luxembourg: Publications Office of the European Union. https://doi.org/10.2788/678452

Rauscher H and Mech A, 2018. NanoDefine Technical report D7.9: General performance requirements for methods to be used in the regulatory context of the definition. Expected to be published on. https://www.openaire.eu/

Rauscher H, Roebben G, Mech A, Gibson N, Kestens V, Linsinger TPJ and Riego Sintes J, 2019a. An overview of concepts and terms used in the European Commission's definition of nanomaterial. EUR 29647 EN. European Commission, JRC, Ispra. ISBN 978-92-79-99660-3, https://doi.org/10.2760/459136, JRC113469.

Rauscher H, Roebben G, Mech A, Gibson N, Kestens V, Linsinger TPJ and Riego Sintes J, 2019b. Identification of nanomaterials through measurements. EUR 29942 EN. Publications Office of the European Union. Luxembourg. ISBN 978-92-76-10371-4. https://doi.org/10.2760/053982, JRC118158

Recordati C, De Maglie M, Bianchessi S, Argentiere S, Cella C, Mattiello S, Cubadda F, Aureli F, D'Amato M, Raggi A, Lenardi C, Milani P and Scanziani E, 2016. Tissue distribution and acute toxicity of silver after single intravenous administration in mice: nano-specific and size-dependent effects. Particle and Fibre Toxicology, 13, 12. https://doi.org/10.1186/s12989-016-0124-x

Ricci M, Tilbury L, Daridon B and Sukalac K, 2019. General principles to justify plant biostimulant claims. Frontiers in Plant Science, 16 April 2019. https://doi.org/10.3389/fpls.2019.00494

Rischitor G, Parracino M, La Spina R, Urban P, Ojea-Jimenez I, Bellido E, Valsesia A, Gioria S, Capomaccio R, Kinsner-Ovaskainen A, Gilliland D, Rossi F and Colpo P, 2016. Quantification of the cellular dose and characterization of nanoparticle transport during in vitro testing. Particle and Fibre Toxicology, 13, 47. https://doi.org/10.1186/s12989-016-0157-1

Roebben G, Kirsten R, Kestens V, Linsinger TPJ, Rauscher H, Emons H and Stamm H, 2013. Reference materials and representative test materials: the nanotechnology case. Journal of Nanoparticle Research, 15, 1455. https://doi.org/10.1007/s11051-013-1455-2

Rogiers V, 2003. 'Validated' and'valid' alternative methods available today for testing of cosmetic products and their ingredients. In Safety Assessment of Cosmetics. EU Training Course, Vrije Universiteit, Brussels, Belgium. 1 pp. 7–12 April 2003, Part 2.

Romeo D, Salieri B, Hischier R, Nowack B and Wick P, 2020. An integrated pathway based on in vitro data for the human hazard assessment of nanomaterials. Environment International, 137, 105505. https://doi.org/10.1016/j.envint.2020.105505

Rossi M, Cubadda F, Dini L, Terranova ML, Aureli F, Sorbo A and Passeri D, 2014. Scientific basis of nanotechnology, implications for the food sector and future trends. Trends in Food Science & Technology, 40, 127–148. https://doi.org/10.1016/j.tifs.2014.09.004

Rubio L, Marcos R and Hernández A, 2020. Potential adverse health effects of ingested micro- and nanoplastics on humans. Lessons learned from in vivo and in vitro mammalian models. Journal of Toxicology and Environmental Health, Part B, 23, 51–68. https://doi.org/10.1080/10937404.2019.1700598

Sadauskas E, Wallin H, Stoltenberg M, Vogel U, Doering P, Larsen A and Danscher G, 2007. Kupffer cells are central in the removal of nanoparticles from the organism. Particle and Fibre Toxicology, 4. https://doi.org/10.1186/1743-8977-4-10

Sarangapani R, 2000. Modeling particle deposition in extrathoracic airways. Aerosol Science and Technology, 32, 72–89. https://doi.org/10.1080/027868200303948

Scarfato P, Di Maio L, Milana MR, Giamberardini S, Denaro M and Incarnato L, 2017. Performance properties, lactic acid specific migration and swelling by simulant of biodegradable poly(lactic acid)/nanoclay multilayer films for food packaging. Food Additives & Contaminants. Part A, Chemistry, Analysis, Control, Exposure & Risk Assessment, 34, 1730–1742. https://doi.org/10.1080/19440049.2017.1321786

SCCS (Scientific Committee on Consumer Safety), 2012. Guidance on the safety assessment of nanomaterials in cosmetics. SCCS/1484/12, 62 pp. https://doi.org/10.2772/82675

SCCS (Scientific Committee on Consumer Safety), 2016. SCCS notes of guidance for the testing of cosmetic ingredients and their safety evaluation, 9th revision. SCCS/1564/15, 151 pp. Available online: http://ec.europa.eu/health/scientific_committees/consumer_safety/docs/sccs_o_190.pdf

SCCS (Scientific Committee on Consumer Safety), 2019. Guidance on the Safety Assessment of Nanomaterials in Cosmetics. 30-31 October 2019, SCCS/1611/19.

SCENIHR (Scientific Committee on Emerging and Newly Identified Health Risks), 2009. Risk assessment of products of nanotechnologies. 71 pp. Available online: http://ec.europa.eu/health/ph_risk/committees/04_scenihr/docs/SCENIHR._o_023.pdf

SCENIHR (Scientific Committee on Emerging and Newly Identified Health Risks), 2010. Scientific basis for the definition of the term "nanomaterial". 46 pp. Available online: http://ec.europa.eu/health/scientific_committees/emerging/docs/SCENIHR._o_032.pdf

SCENIHR (Scientific Committee on Emerging and Newly-Identified Health Risks), 2007. The appropriateness of the risk assessment methodology in accordance with the Technical Guidance Documents for new and existing substances for assessing the risks of nanomaterials, 21-22 June 2007. Available online: http://ec.europa.eu/health/archive/ph_risk/committees/04_SCENIHR./docs/SCENIHR._o_010.pdf

Seager AL, Shah U-K, Brüsehafer K, Wills J, Manshian B, Chapman KE, Thomas AD, Scott AD, Doherty AT, Doak SH, Johnson GE and Jenkins GJS, 2014. Recommendations, evaluation and validation of a semi-automated, fluorescent-based scoring protocol for micronucleus testing in human cells. Mutagenesis, 29, 155–164. https://doi.org/10.1093/mutage/geu008

Sharifi S, Behzadi S, Laurent S, Forrest ML, Stroeve P and Mahmoudi M, 2012. Toxicity of nanomaterials. Chemical Society Reviews, 41, 2323–2343. https://doi.org/10.1039/C1CS15188F

Shibai-Ogata A, Kakinuma C, Hioki T and Kasahara T, 2011. Evaluation of high-throughput screening for in vitro micronucleus test using fluorescence-based cell imaging. Mutagenesis, 26, 709–719. https://doi.org/10.1093/mutage/ger037

Sieg H, Kästner C, Krause B, Meyer T, Burel A, Böhmert L, Lichtenstein D, Jungnickel H, Tentschert J, Laux P, Braeuning A, Estrela-Lopis I, Gauffre F, Fessard V, Meijer J, Luch A, Thünemann AF and Lampen A, 2017. Impact of an artificial digestion procedure on aluminum-containing nanomaterials. Langmuir, 33, 10726–10735.

Šimon P and Joner E 2008. Conceivable interactions of biopersistent nanoparticles with food matrix and living systems following from their physicochemical properties. Journal of Food and Nutrition Research, 47, 51–59.

Sohal IS, Kwan Cho Y, O'Fallon KS, Gaines P, Demokritou P and Bello D, 2018. Dissolution behavior and biodurability of ingested engineered nanomaterials in the gastrointestinal environment. ACS Nano, 12, 8115–8128. https://doi.org/10.1021/acsnano.8b02978

Souza VGL and Fernando AL, 2016. Nanoparticles in food packaging: biodegradability and potential migration to food—a review. Food Packaging and Shelf Life, 8, 63–70. https://doi.org/10.1016/j.fpsl.2016.04.001

Stefaniak AB, Guilmette RA, Day GA, Hoover MD, Breysse PN and Scripsick RC, 2005. Characterization of phagolysosomal simulant fluid for study of beryllium aerosol particle dissolution. Toxicology in Vitro, 19, 123–134. https://doi.org/10.1016/j.tiv.2004.08.001

Steinway SN, Saleh J, Koo BK, Delacour D and Kim DH, 2020. Human microphysiological models of intestinal tissue and gut microbiome. Front Bioeng Biotechnol, 8, 725. https://doi.org/10.3389/fbioe.2020.00725

Stone V, Gottardo S, Bleeker EAJ, Braakhuis H, Dekkers S, Fernandes T, Haase A, Hunt N, Hristozov D, Jantunen P, Jeliazkova N, Johnston H, Lamon L, Murphy F, Rasmussen K, Rauscher H, Jiménez AS, Svendsen C, Spurgeon D, Vázquez-Campos S, Wohlleben W and Oomen AG, 2020. A framework for grouping and read-across of nanomaterials- supporting innovation and risk assessment. Nano Today, 35, 100941. https://doi.org/10.1016/j.nantod.2020.100941

Stopford W, Turner J, Cappellini D and Brock T, 2003. Bioaccessibility testing of cobalt compounds. Journal of Environmental Monitoring, 5, 675–680. https://doi.org/10.1039/b302257a

Störmer A, Bott J, Kemmer D and Franz R, 2017. Critical review of the migration potential of nanoparticles in food contact plastics. Trends in Food Science & Technology, 63, 39–50. https://doi.org/10.1016/j.tifs.2017.01.011

Szentkuri L, 1997. Light microscopic observation on luminally administered dyes, dextrans, nanospheres and microspheres in the pre-epithelial mucus gel layer of the rat distal colon. Journal of Controlled Release, 46, 233–242. https://doi.org/10.1016/S0168-3659(96)01600-8

Tantra R, Bouwmeester H, Bolea E, Rey-Castro C, David CA, Dogné J-M, Jarman J, Laborda F, Laloy J, Robinson KN, Undas AK and van der Zande M, 2016. Suitability of analytical methods to measure solubility for the purpose of nanoregulation. Nanotoxicology, 10, 173–184. https://doi.org/10.3109/17435390.2015.1038661

Tassinari R, Cubadda F, Moracci G, Aureli F, D'Amato M, Valeri M, De Berardis B, Raggi A, Mantovani A, Passeri D, Rossi M and Maranghi F, 2014. Oral, short-term exposure to titanium dioxide nanoparticles in Sprague-Dawley rat: focus on reproductive and endocrine systems and spleen. Nanotoxicology, 8, 654–662. https://doi.org/10.3109/17435390.2013.822114

Taurozzi JS, Hackley VA and Wiesner MR, 2012a. NIST Special Publication 1200–1: reporting guidelines for the preparation of aqueous nanoparticle dispersions from dry materials. Version 1.1. https://doi.org/10.6028/NIST.SP.1200-1

Taurozzi JS, Hackley VA and Wiesner MR, 2012b. NIST Special Publication 1200–2: preparation of nanoparticle dispersions from powdered material using ultrasonic disruption. Version 1.1. https://doi.org/10.6028/NIST.SP.1200-2

Taurozzi JS, Hackley VA and Wiesner MR, 2012c. NIST Special Publication 1200–3: preparation of a nanoscale TiO2 aqueous dispersion for toxicological or environmental testing. Version 1.2. https://doi.org/10.6028/NIST.SP.1200-3

Taurozzi JS, Hackley VA and Wiesner MR, 2012d. NIST Special Publication 1200–4: preparation of nanoscale TiO2 dispersion in biological test media for toxicological assessment. Version 1.1. 1. https://doi.org/10.6028/NIST.SP.1200-4

Taurozzi JS, Hackley VA and Wiesner MR, 2012e. NIST Special Publication 1200–5: preparation of nanoscale TiO2 dispersion in an environmental matrix for eco-toxicological assessment. Version 1.1. https://doi.org/10.6028/NIST.SP.1200-5

Totaro S, Cotogno G, Rasmussen K, Pianella F, Roncaglia M, Olsson H, Riego Sintes JM and Crutzen HP, 2016. The JRC Nanomaterials Repository: a unique facility providing representative test materials for nanoEHS research. Regulatory Toxicology and Pharmacology, 81, 334–340. https://doi.org/10.1016/j.yrtph.2016.08.008

Tydeman EA, Parker ML, Wickham MS, Rich GT, Faulks RM, Gidley MJ, Fillery-Travis A and Waldron KW, 2010. Effect of carrot (Daucus carota) microstructure on carotene accessibility in the upper gastrointestinal tract. 1. In vitro simulations of carrot digestion. Journal of Agricultural and Food Chemistry, 58, 9847–9854. https://doi.org/10.1021/jf101034a

Utembe W, Potgieter K, Stefaniak AB and Gulumian M, 2015. Dissolution and biodurability: important parameters needed for risk assessment of nanomaterials. Particle and Fibre Toxicology, 12, 11. https://doi.org/10.1186/s12989-015-0088-2

Van de Wiele TR, Oomen AG, Wragg J, Cave M, Minekus M, Hack A, Cornelis C, Rompelberg CJ, De Zwart LL, Klinck B, Van Wijnen J, Verstraete W and Sips AJ, 2007. Comparison of five *in vitro* digestion models to *in vivo* experimental results: lead bioaccessibility in the human gastrointestinal tract. Journal of Environmental Science and Health, 42, 1203–1211. https://doi.org/10.1080/10934520701434919

Verleysen E, Waegeneers N, Brassinne F, De Vos S, Jimenez IO, Mathioudaki S and Mast J, 2020. Physicochemical characterization of the pristine E171 food additive by standardized and validated methods. Nanomaterials, 10, 592.

Versantvoort CHM, Oomen AG, Van de Kamp E, Rompelberg CJM and Sips AJAM, 2005. Applicability of an *in vitro* digestion model in assessing the bioaccessibility of mycotoxins from food. Food and Chemical Toxicology, 43, 31–40. https://doi.org/10.1016/j.fct.2004.08.007

Vogt A, Rancan F, Ahlberg S, Berouz Nazemi Choe CS, Darvin ME, Hadam S, Blume-Peytavi U, Loza K, Diendorf J, Epple M, Graf C, Rühl E, Meinke MC and Lademann J, 2014. Interaction of dermatologically relevant nanoparticles with skin cells and skin. Beilstein. Journal of Nanotechnology, 5, 2363–2373. https://doi.org/10.3762/bjnano.5.245

Walczak AP, Fokkink R, Peters R, Tromp P, Rivera ZEH, Rietjens IMCM, Hendriksen PJM and Bouwmeester H, 2013. Behaviour of silver nanoparticles and silver ions in an *in vitro* human gastrointestinal digestion model. Nanotoxicology, 7, 1198–1210. https://doi.org/10.3109/17435390.2012.726382

WHO and IPCS (World Health Organization and International Programme on Chemical Safety), 2019. Principles and methods to assess the risk of immunotoxicity associated with exposure to nanomaterials. Environmental health criteria, 244. World Health Organization, 380 pp. Available online: https://apps.who.int/iris/handle/10665/331744. License: CC BY-NC-SA 3.0 IGO.

Worth AP and Balls M, 2001. The importance of the prediction model in the validation of alternative tests. Alternatives to Laboratory Animals, 29, 135–144.

Xavier M, Parente IA, Rodrigues PM, Cerqueira MA, Pastrana L and Gonçalves C, 2021. Safety and fate of nanomaterials in food: The role of in vitro tests. Trends in Food Science & Technology, 109, 593–607. https://doi.org/10.1016/j.tifs.2021.01.050

Zhang S, Jiang X, Cheng S, Fan J, Qin X, Wang T, Zhang Y, Zhang J, Qiu Y, Qiu J, Zou Z and Chen C, 2020. Titanium dioxide nanoparticles via oral exposure leads to adverse disturbance of gut microecology and locomotor activity in adult mice. Archives of Toxicology, 94, 1173–1190. https://doi.org/10.1007/s00204-020-02698-2

Abbreviations

AAS	atomic absorption spectroscopy
ADME	absorption, distribution, metabolism and excretion
AES	atomic emission spectroscopy
AFM	atomic force microscopy
ANS	EFSA Panel on Food Additives and Nutrient Sources Added to Food
AOP	adverse outcome pathway
AUC	area under the plasma concentration-time curve
BAM	Federal Institute for Materials Research and Testing
BET	Brunauer Emmett Teller method
BMD	benchmark dose
BMDL	lower boundary of the BMD confidence interval
CaCo-2	human colorectal epithelial cells
CAS	Chemical Abstracts Service
CEF	EFSA Panel on Food Contact Materials, Enzymes, Flavourings and Processing Aids
CEN	European Committee for Standardization
CEN/TC	European Committee for Standardization/Technical Committee
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	EFSA 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
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	electron microscopy
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
FEEDAP	EFSA 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
GIT	gastrointestinal tract
GMO	Genetically Modified Organisms
IATA	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	International Organization for Standardization
ISO TC	International Organization for Standardization Technical Committee
IPCS	International Programme on Chemical Safety
IUPAC	International Union of Pure and Applied Chemistry
JRC	Joint Research Centre
KLH	keyhole limpet haemocyanin
LA-ICP-MS	laser ablation inductively coupled mass spectrometry
LDH	lactate dehydrogenase
MALDI	matrix-assisted laser desorption/ionisation
MALS	multiangle light scattering
MoA	mode of action
MPS	mononuclear phagocyte system
MSSA	mass specific surface area
NAM	new approach methodology
NDA	EFSA Panel on Nutrition, Novel Foods and Food Allergens
NK	natural killer cells
NOAEL	no-observed-adverse-effect-level
OECD	Organisation for Economic Co-operation and Development
OES	optical emission spectroscopy
pH	potential of hydrogen
PLA	polylactic acid
PPP	plant protection product

PPR	EFSA Panel on Plant Protection Products and their Residues
QqQ	triple quadrupole
QSAR	quantitative structure-activity relationship
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
S9	metabolic activation system
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
SEM–EDX	scanning electron microscopy–energy-dispersive X-ray spectroscopy
SMPS	scanning mobility particle sizer
SOPs	Standard Operating Procedures
spICP-MS	single particle inductively coupled plasma mass spectrometry
SRBC	sheep red blood cells
STEM	scanning transmission electron microscopy
STEM-EDX	scanning transmission electron microscopy-energy-dispersive X-ray spectroscopy
STXM	scanning transmission X-ray microscopy
TEER	transepithelial electrical resistance
TEM	transmission electron microscopy
TEM–EDX	transmission electron microscopy–energy-dispersive X-ray spectroscopy
THP-1	human monocytic cell line
TG	(OECD) 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

In brackets, the source used for the description, EFSA: taken from EFSA glossary (https://www.efsa.europa.eu/en/glossary-taxonomy-terms). When no source is provided, the text represents the working description used for this Guidance.

ADME (EFSA)	An abbreviation for absorption, distribution, metabolism and excretion, the four key processes which describe how drugs and chemicals get into the body, what happens to them while they are there, and how they are eliminated.
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).

Coating	For the purpose of this Guidance, a material is considered as a 'coating' when it is bound or adhered to the surface of a nanomaterial in the form of a continuous outside layer, or a 'shell' when it is in the form of a nanosized covering/casing in which a material may be contained.
Constituent particle	Constituent particles are the (morphologically) identifiable particles, including those inside an aggregate or agglomerate. In agglomerates the constituent particles are only weakly bound. In aggregates the constituent particles are strongly bound. Mobility-based techniques cannot be used to measure the size of constituent particles in aggregates and agglomerates (from Rauscher et al., 2019a)
Conventional material	Material not covered by the legal definition for engineered nanomaterials or nanoforms.
Degradation	Degradation 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). The degradation/dissolution rate includes degradation into soluble chemical species in addition to dissolution, while excludes the degradation into other nanoparticles.
Dissolution	The process by which a nanomaterial in a liquid medium dissolves into its respective ions or molecules, resulting in the loss of its nanospecific features.
Dissolution rate	Dissolution rate is the amount of substance dissolved (solute) into a solvent over time (OECD).
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 nanoparticles coexist.
Dispersibility	Is the condition of particular material of being dispersible or a measure to which extent it is dispersible in a dispersing medium or continuous phase. Dispersion stability refers to the ability of a dispersion to resist change in its properties over time (OECD TG 318 (OECD, 2017a)).
Engineered nanomaterial	(Novel Food Regulation (EU) No 2015/2283, point (f) of Article 3(2): intentionally produced material that has one or more dimensions of the order of 100 nm or less or that is composed of discrete functional parts, either internally or at the surface, many of which have one or more dimensions of the order of 100 nm or less, including structures, agglomerates or aggregates, which may have a size above the order of 100 nm but retain properties that are characteristic of the nanoscale. Properties that are characteristic of the nanoscale include: (i) those related to the large specific surface area of the materials considered; and/or (ii) specific physico-chemical properties that are different from those of the non-nanoform of the same material.
Fraction of small particles	For conventional materials, the term 'fraction of small particles' is used in this document to describe the fraction of the materials composed by particles with at least one external dimension lower than 500 nm.
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 silico (EFSA)	Research theoretical method, particularly involving computer models, to predict the likely toxicological, or other, effects of substances.
In vitro (EFSA)	Research method which involves testing cells or tissues extracted from living organisms.
In vivo (EFSA)	Research method which involves testing individual live animals or populations of live animals.
In situ ex vivo	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.
Material	A general term to be used in this Guidance independently from a regulation and be adaptable to different sectors. It is a generic term for what is covered and regulated afterwards by sector-specific legislation. Hence, 'material' can, e.g. be an ingredient, a mixture, a preparation or be intended as test 'material'.
Measurand	Quantity intended to be measured (ISO/IEC, 2010).
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 micrometre range, formation of particles in the nanoscale may also result.
Mixture	Mixture or solution composed of two or more substances (REACH, Art. 3(2)). It should be noted that the surface treatment of a substance is a 'two dimensional' modification of macroscopic particles which implies a chemical reaction between the functional groups only on the surface of a macroscopic particle. A chemically surface treated substance cannot be regarded as a mixture of the core substance with the surface treating substance.
Multi-constituent substance	A substance, defined by its quantitative composition, in which more than one main constituent is present in a concentration $\geq$ 10% (w/w) and $<$ 80% (w/w). A multi-constituent substance is the result of a manufacturing process (ECHA, 2017d). These thresholds do not apply to the cover surface treatment. The presence of coating (synonym of surface functionalisation treatment) should be considered a characteristic of the substance/nanoform and requires proper characterisation.
Multi-component particle	Particles composed by two or several bound or fused components with different chemical or morphological properties. Includes coated or core shell particles and also engineered particles ensembled from more than one component, as in some advanced materials.
Nanocarrier	Nanomaterial used as carrier system, e.g. able to transport a substance either on its surface, within its bulk structure or within an internal cavity.
Nanofertiliser	Fertiliser in particulate form (including encapsulated forms and nanocarriers) with one or more dimensions in the nanoscale. Fertiliser encapsulated/coated with nanomaterial.
Nanofibre	Refers to nanoparticles characterized by significantly different lengths of the longest and shortest axes.
Nanoform	Form of a natural or manufactured 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 1–100 nm, including also by derogation fullerenes, graphene flakes and single wall carbon nanotubes with one or more external dimensions below 1 nm (REACH, Annex VI).
Nanomaterial	Nanomaterial encompasses materials covered by the legal definitions. For the purposes of this guidance the provisions for the risk assessment of nanomaterials are also applicable to the fraction of nanoparticles, present in

	conventional materials and any other material or fraction exhibiting characteristics of the nanoscale and, consequently, requiring risk assessment with specific considerations for addressing these characteristics.
Nanoparticle	Covers all particles exhibiting characteristics at the nanoscale. This covers particles with any external dimension on the nanoscale including 'nanofibres' (two external dimensions in the nanoscale) and 'nanoplates' (one external dimension on the nanoscale).
Nanopesticide	The term nanopesticide in this Guidance is used to cover both active substances in nanoform and nanoformulations of plant protection products (PPPs). In addition to active substances, PPPs may also contain other materials such as solvents, carriers, inert material, wetting agents referred to as co-formulants. Co-formulants may be manufactured as nanoforms and used as nanocarriers of the active substance in nanoformulated PPPs.
Nanoproperty	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-nanoparticles etc.
Nanoscale	The size range 1–100 nm (e.g. Lövenstam et al., 2010; SCENIHR, 2010). From a metric interpretation, nanoscale encompasses the range from 1 to 999 nm. The size range below 1 nm is measured in picometers, and the size range above 999 nm is measured in micrometres.
Nanoscience (EFSA)	The study of nanomaterials.
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.
Nanosized degradation product	Is a degradation product in the form of a nanomaterial, meaning not in ionic or molecular form.
Nanostructured material (ISO)	A material having internal or surface nanostructure, this includes a composition of interrelated constituent parts in which one or more of those parts is at the nanoscale.
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.
Particle	It refers to a minute piece of matter with defined physical boundaries, and of any shape.
Particulate material	A material consisting of solid particles.
Pesticide	The term pesticide in this Guidance is used to cover both active substances and plant protection products (PPP).
Pour density (ISO)	Apparent mass per unit volume of bulk powder, pellets or other discontinuous solid material (ISO 1382:2020).
Pristine material	Original, pure material (before it is processed).
Solid particle	According to the European Commission's position (JRC113469), the EC recommended definition of a nanomaterial covers only particles that are solid at normal temperature and pressure. 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.
Solubility (OECD)	The quantity of solute that dissolves in a given quantity of solvent to form a saturated solution (OECD). Solubility is specified by the saturation mass concentration of the substance in water or another solvent at a given temperature (kg/m^3 or g/L). Solubility in relevant media requires description of the media and the conditions under which the measurements were made.
Substance (REACH, Art. 3(1))	A chemical element and its compounds in the natural state or obtained by any manufacturing process, including any additive necessary to preserve its stability and any impurity deriving from the process used, but excluding any solvent which may be separated without affecting the stability of the substance or changing its composition.
Transfer of FCM substances	For substances used in Food Contact Materials, the term transfer includes both migration and physical release (abrasion) of the substance into food.
Ultrafiltration	Ultrafiltration is technique that employs centrifugation of liquid samples through a semi-permeable ultrafiltration membrane, which separates smaller sized substances from the larger ones, depending on the size cut-off the membrane. In the context of testing solubility of nanomaterials, the use of ultrafiltration is recommended to first separate out the solubilised fraction of a nanomaterial from any (undissolved) nanoparticles that are retained by the ultrafiltration membrane. Subsequent analysis of the filtered media then reflects the truly solubilised proportion of the nanomaterial.
Valid method	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 – Demonstration fact sheet for component 2

Table 1 provided in Section 5.1.2 lists the parameters to be addressed in the physico-chemical characterisation of the materials under assessment and gives examples how to report the requested data for a hypothetical example. The chemical information on the individual chemical components of multi-component particles has to be reported individually for each component. The example in Table 1 is a bi-component particle (titanium dioxide coated with silicon dioxide). While the chemical information for component 1 (titanium dioxide) is provided in Table 1B, the information on component 2 (silicon dioxide) is provide in Table A.1 below.

Table A.1: Descriptors and parameters for component 2 in Table 1 (Section 5.1.2)
Information on the chemical component 2

Parameters (incl. specification ranges)	**Explanation**	**Example (hypothetical)**
Chemical name Systematic/IUPAC name; chemical name	When 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
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 (when 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 (when applicable) of the constituent substance should be provided	SiO_2
Molecular mass or atomic mass (for elements) (g/mol)	Molecular mass (weight) or atomic mass (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 B – Characterisation techniques

Table B.1 provides an overview of techniques currently used for the characterisation of nanomaterials. They 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 5.3).

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 of current techniques and their use. Furthermore, the NanoDefine Methods Manual 2020 (Mech et al., 2020; 3 parts[44]) also provides information on the use of techniques and outlines which method is best suited for certain cases and materials (e.g. chemical composition, powder, suspension etc.)

Standardised methods should preferably be used if available and appropriate for the analytical task in question. Examples of guidance documents for some characterisation techniques are given in the Table B.1. It should be noted that some standards describe only the design and use of a specific instrument while other standards describe a process of operations (i.e. a measurement procedure or method). 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 Online 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/.

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 B.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/TS 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

[44] https://ec.europa.eu/jrc/en/publication/nanodefine-methods-manual

Item	Suitable techniques	Examples of guidances
	X-ray photoelectron spectroscopy (XPS)	ISO/TR 14187, ISO 18118
	Auger electron spectroscopy	ISO/TR 14187, ISO 24236
	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 21363, ISO 13322-1, ISO 29301,
	– Scanning electron microscopy (SEM)	ISO 19749, ISO 13322-1, ISO 16700
	– Scanning transmission electron microscopy (STEM)	
	– Atomic force microscopy (AFM)	ISO 25178 series IEC/TS 62622
	– Scanning transmission X-ray microscopy (STXM)	
	Separation techniques (coupled with suitable detectors):	
	– Field flow fractionation (FFF)	ISO/TS 21362
	– Hydrodynamic chromatography (HDC)	
	– Size exclusion chromatography (SEC)	
	– High-performance liquid chromatography (HPLC)	
	– Differential mobility analysis/ion mobility spectroscopy (DMA/IMS)	ISO 15900, ISO 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)	ISO 17867
	– Laser diffraction methods	ISO 13320
	– Dynamic Light scattering (DLS)	ISO 22412, ISO/TR 22814
	– Multiangle light scattering (MALS)	

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 series (under development)
	– Resonant mass technique (Archimedes)	
Shape	Microscopy techniques – TEM – SEM – STXM – AFM	ISO 16700 ISO 25178, ISO/TS 11888, ISO 9276-6
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, ISO/TR 19997
	Capillary electrophoresis (CE)	
	Electroosmosis	
	Electric sonic amplitude	
	Colloidal vibration current	
Degradation/ Dissolution/ Solubility	Standard tests for water solubility, dissolution/ degradation rate constants	e.g. OECD TG 105 Further?
Chemical reactivity	Kinetic measurements of the chemical, biochemical reactions	
Catalytic activity	Kinetic measurements of the catalysed reactions, including photocatalytic activity (when 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 C – Uncertainty analysis of high dissolution/degradation rate

This Section provides the rationale for the cut-off value of the dissolution/degradation rate that is used to decide whether a nanomaterial quickly degrades (i.e. has a high dissolution/degradation rate) in the gastrointestinal tract and can therefore follow the safety assessment according to relevant EFSA guidance on non-nanomaterials. 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 with diameters 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 diameter latex particles to cross the mucus layer within 2 min.

The time required for particles to be taken up by intestinal cells is also 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 dissolution/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.

The NANoREG results (Deliverable 5.02; available via the NANoREG result repository[45]) indicate that the dissolution/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 dissolution/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.

[45] https://www.rivm.nl/en/documenten/nanoreg-d5-02-dr-report-on-development-of-solubility-testing-procedure

Appendix D – Additional information on specific regulated products

Risk assessment for nanomaterials and the data requested can be different depending on their origin and intended use.

D.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[46] mentioned in Article 5.3 of Regulation (EC) No 1831/2003[47,48] governing the Community authorisation of additives for use in animal nutrition. Regulation (EC) No 429/2008[49] 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[50].

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 (the dose range used and 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, however, in the Guidance on the identity, characterisation and conditions of use of feed additives (EFSA FEEDAP Panel, 2017c) 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, is considered (see Section 1.3).

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 physico-chemical characterisation (Sections 4 and 5), in particularly Section 5.3 on the characterisation in a 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 5 and 6 (Sections 6 and 7) in addition to the requirements of the FEEDAP Guidance on the assessment of the safety of feed additives for the target species (EFSA FEEDAP Panel, 2017b). It should be noted for instance that toxicological data derived from laboratory species may not be directly applicable for nanomaterial intended 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, rabbits and cows have been reported to have two types of Peyer'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

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

[47] 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.

[48] 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.

[49] 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

[50] http://www.efsa.europa.eu/en/applications/feedadditives/regulationsandguidance

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 described in Section 7.2, 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 to residues of the nanomaterials present in animal food products (**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 assessment of the safety of feed additives for the consumer) (EFSA FEEDAP Panel, 2017a). 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 also needs to be 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).

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, 2012). 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, 2012), should be followed. As part of the safety assessment, inhalation of nanomaterial feed additives contained in feed should be considered as an important route of exposure. Small particles, particularly nanoparticles, can reach and be deposited 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). The OECD Guidance Document on inhalation toxicity studies ENV/JM/MONO(2009)28/REV1 has been updated in 2018 for addressing the additional consideration required for testing nanomaterials (OECD, 2018c); and should be considered.

- 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 5 and 6 of this Guidance and the FEEDAP Guidance to assess the safety for the target species (EFSA FEEDAP Panel, 2017b).
- The risk assessor must consider if the results from an extended 90-day study (Step 3B) are sufficient to conclude on the safety of target animal species, or if testing in a specific target species is necessary.
- 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. The identification of residues in form of nanoparticles in consumer products would trigger the need for considering nanoscale consideration in the safety studies and consumers risk assessment.

D.2. Pesticides[51]

The developments in the field of nanosized active ingredients and formulations have also opened up new avenues for enhancing the delivery and efficacy of plant protection products (PPP) and related substances (not necessarily in the remit of EFSA), such as biocides and plant biostimulants. 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, slow- or controlled-release of the active ingredients and lower environmental footprint of pesticide active ingredients (Perlatti et al., 2013; Kah and Hofmann, 2014; Cano Robles and Mendoza Cantú, 2017).

A clear and agreed definition of nanopesticide is currently not available[52]. Several examples of nanopesticides have been described since almost a decade (Kah et al., 2013; Perlatti et al., 2013; Kah and Hofmann, 2014; Kookana et al., 2014; Cano Robles and Mendoza Cantú, 2017; Grillo et al., 2021). 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 PPP and biocides and have categorised both as nanopesticides. Commercial interests limit the information publicly available regarding prospective and premarketing research, posing further difficulties in identifying the scale of industrial premarketing activities in this area. With these constraints in view, the available information suggests that there are continued developments in this area that are largely under R&D and as such there is limited evidence of example of nanopesticides that are currently available on the (EU) market.

The term nanopesticides in this Guidance is used to cover:

- active substances in nanoform
- active substances containing a fraction of small particles including nanoparticles identified according to the Guidance on Particle -TR
- nanoformulations of PPP, including nanoencapsulates and nanocarriers with co-formulants in nanoform, and nanocarriers as defined in Appendix D.5 even if the co-formulants do not meet the legal definition for nanoforms.

Nanopesticide entity including all components of formulations

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

a) in the form of a **nanoparticle**, 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, independently of the applicable current legal requirements,[53] the Scientific Committee notes that safety considerations for a nanopesticide used in agriculture are necessary to 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

[51] The terms 'pesticides' and 'nanopesticides' are used in this Guidance to cover plant protection products and their active substances. In other contexts, it is used as a broader term that also covers products such as biocides, which are intended for non-plant uses and do not fall within the remit of EFSA and hence are outside the scope of this Guidance.

[52] There is currently no definition for 'nanopesticides'. A (possible) definition based only on 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) may contain fractions in the 1–100 nm range and have been on the market for decades without previously being classified as 'nano'.

[53] In the current legal framework, active substances in PPPs are approved under Regulation (EC) No 1107/2009, through a process that includes an EFSA assessment, and once approved are considered as registered under the REACH Regulation. PPPs are authorised under Regulation (EC) No 1107/2009, through a process that includes (zonal) assessments by the EU MSs. Co-formulants used in the pesticide products are regulated under the REACH Regulation, with additional considerations under Regulation (EC) No 1107/2009 for the identification of non-acceptable co-formulants in PPPs. 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 Regulations other than Regulation (EC) No 1107/2009. EFSA may be requested to peer-review the assessment of non-acceptable co-formulants in PPPs.

nanopesticide entity. It should be noted that co-formulants are included under the REACH legislation that has been updated with specific provisions for substances in nanoform.

EFSA remit includes the assessment of active substances, including the representative formulations that are part of the active substances dossiers; to develop guidance for the risk assessment of the active substances and for the risk assessment of PPP formulations that may support the zonal/Member States (MS) evaluations; when necessary, to support the assessment and develop guidance regarding co-formulants of potential concern.

Necessity to assess safety of all components in a nanopesticide

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. When applicable, an explanation of the technical adaptations/adjustments that have been made in response to the specific characteristics of these materials shall be provided by the applicant. 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 physico-chemical properties and/or bio-kinetic behaviour may also lead 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**. 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[54]), the Scientific Committee recommends National Authorities to request from applicants that for authorisation purposes it should be declared in the dossier if a PPP does not include any nanoform or nanostructured material 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 the 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).[55]

The current level of knowledge relating to the human health effects and environmental fate, behaviour and impact of nanopesticides is increasing. This Guidance has only highlighted the main aspects that need to be considered in regard to hazard identification and hazard characterisation of nanopesticide active substances and formulations.

Data requirements – physical, chemical and technical parameters

According to Commission Regulations (EU) No 283/2013[56] and (EU) No 284/2013[57] setting up the data requirements for active substances and PPPs, respectively, in accordance with Regulation (EC) No 1107/2009[58], there is a request for the submission of data on the identity of active substance, the identity and content of additives and impurities.

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

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

[56] 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.

[57] 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.

[58] 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.

Because of the above mentioned safety considerations, the Scientific Committee is of the opinion that detailed physico-chemical characterisation of a nanopesticide active substance as well as other co-formulants in a formulation is an essential prerequisite for risk assessment. The update of the REACH Regulation regarding the consideration of substances in nanoform is applicable to co-formulants. The ECHA guidance implementing the regulatory requirements should be considered by applicants for identifying and reporting the presence of co-formulants in nanoform. The parameters listed in Table 1 that are relevant for 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.

For active substances, data on particle size distribution and shape (morphology) are very important in this regard, because these nanosize characteristics are likely to bring about changes in the properties, behaviour and effects of a nanopesticide. For nanoformulated PPPs, in addition to particle size, the specific morphology of the particles and the linkage between the active substance and the co-formulants (e.g. characteristics of the nanocapsules or nanocarriers as well as those related to the physico-chemical bound and interaction between the active substance and the other components of the nanoformulated PPP) are essential elements to be provided by the applicants and to be considered in the safety assessment.

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 of micro-encapsulated particles (CS), and micro-encapsulated emulsions (ME). There is already a data requirement for particle size distribution under Art. 2.8 of Regulation (EU) No 284/2013 establishing the data requirements for plant protection products. A test on particle size distribution might be asked for a pesticide CS or 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; the aim of this request is not connected to the identification of a fraction requiring assessment for nanocharacteristics, but 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 the 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[59]). In addition to the above described current requirements, the Guidance on Particle-TR should be followed for identifying the presence of a fraction of small particles, including nanoparticles, requiring assessment at the nanoscale.

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 physico-chemical 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 physico-chemical 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.

Adaptation of oral toxicity and consumer risk assessment for active substances and co-formulants in nanoform

The provision of this guidance, including the adaptation of the toxicokinetic and oral toxicity studies, must be implemented for active substances in nanoform, and for those containing a fraction of small particles requiring an assessment at the nanoscale identified according to the provision of the Guidance on Particle-TR. This is necessary for ensuring the safety for consumers. The provisions in this Guidance are also relevant for identifying co-formulants in nanoform of potential concern.

According to Commission Regulations (EU) No 283/2013[43] and (EU) No 284/2013[44] setting up the data requirements for active substances and for PPPs, in accordance with Regulation (EC) No 1107/ 2009[45], the

[59] 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.

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, if applicable, other endpoints, such as neurotoxicity and immunotoxicity studies. For co-formulants, such data requirements (currently) do not exist under Regulation (EC) No 1107/ 2009; however, the provisions for nanoforms under the updated REACH Regulation are applicable.

The human health risk assessment for pesticides focuses on the hazards associated to the active substances and their residues in food; while the characteristics of the formulation are mostly used to adjust the exposure assessment (magnitude of plant residues and dermal absorption). In a scenario of data-rich substances, such as pesticide active substances, for active substances in nanoform, a read-across hypothesis should be considered before conducting additional animal studies (see Section 7.9 of this Guidance). A proper characterisation of the toxicokinetic behaviour (e.g. as described in this Guidance in Section 7.6) may be sufficient for supporting a read-across to the conventional active substance. The allowance of a 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-across case cannot be built, as a further study an enhanced 28-day study (OECD TG 407, OECD, 2008a) for comparative assessment would be acceptable.

As mentioned before, nanosize/characteristics 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 physico-chemical properties and toxicokinetic behaviour of the active substance (as such, or in a formulation) have not significantly changed at the nanoscale 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 with the current practice for active substances, e.g. by taking nanospecific aspects into account during the standard 90-day oral toxicity study that is to be provided.

In general, the toxicity data requirements for an active substance in nanoform will be similar to those for a conventional (non-nano) substance. However, the toxicological testing methods should be adapted for considering the nanospecific aspects (e.g. dispersion, agglomeration/aggregation) in accordance with this Guidance Section 7.3.

According to Commission Regulation (EU) No 283/2013 setting up the data requirements for active substances and Regulation (EU) No 284/2013 for PPPs, the submission of data on the active substance and (at least) one representative formulation, is requested. At present, little is known about the effects of different dispersions/formulations on the properties, behaviour and toxicological effects of active substances in nanoform 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 formulations containing active substances in nanoform that are intended for final use are always tested in toxicological studies or covered by appropriate read-across justifications. 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.

The provisions of this Guidance are also applicable to the identification of nanoformulated co-formulants of potential concern. According to the tiered process recommended in this Guidance, the first steps are the assessment of dissolution/degradation, including those relevant for the application pattern and those simulating digestive tract conditions and lysosomal degradation. If consumers may be exposed to nanoparticles toxicokinetic studies are required, the results may be sufficient for developing a read-across approach when valid information is available on the conventional form of the co-formulant.

Consumer risk assessment of conventional active substances in nanoformulated PPPs

The expected 'nano' benefits (e.g. in terms of efficacy and reduction in application rate) require the PPP to reach the target organisms in nanoform. This implies that the crop will in most cases be also exposed to nanomaterials. 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 foods.

The information provided in the dossier should include details on the expected behaviour of the nanoparticles and the active substance in the crop following application.

When consumers are expected to be exposed to particles of the nanoformulated PPP, a full assessment according to the provisions of this Guidance should be conducted. According to the recommended tiered process, the first step is the assessment of dissolution/degradation under simulating digestive tract conditions. Studies on cellular internalisation and lysosomal degradation may require design adaptations, as the key question in this case is not the persistency of the nanoparticles itself, but the potential for a nanoscale distribution profile of the PPP, with release of the active substances in tissues and organs different from those identified following the administration of the solubilised active substance or at different amounts. This may trigger specific toxicity studies with the nanoformulated PPP, that should be designed according to the recommendations of this Guidance in order to the address nanoscale characteristics.

When consumers are not exposed to particles a conventional assessment should be sufficient. As the nanoformulation of the PPP may affect the toxicokinetics of the active substance, the possible implications for setting the residue definition, according to the Guidance for the Residue Definition (EFSA PPR Panel, 2016) should be considered.

Non-oral exposure assessment of active substances and co-formulants

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 EFSA PPR Panel has published guidance on the assessment of exposure of operators, workers, residents and bystanders in risk assessment for PPPs (EFSA, 2014), that in general should also be applied to active substances in nanoform and nanoformulated PPPs.

The exposure estimation for operators and workers is first carried out assuming that they are not using personal protective equipment, followed, when 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 and toxicity

Under Regulation (EU) No 284/2013 setting out the data requirements for PPPs (the formulations), dermal absorption studies would be required when dermal exposure is a significant exposure route and no acceptable risk is estimated using a default absorption value. EFSA 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 (OECD, 2004a)) or *in vitro* (OECD TG 428 (OECD, 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, the 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 The OECD Guidance document GN156 (OECD, 2019) is currently under review but not finalised, the available draft from 2019 and related publications do not include nanospecific considerations (Hopf et al., 2020); although some recent reviews have addressed the nanospecific elements (Gimeno-Benito et al., 2021). In addition to consider whether the nanoform or nanoformulation affects bioavailability of the active substance, and/or other toxicologically relevant compounds, it should be important to investigate the uptake of particles as such, and possible local long-term effects in case of accumulation of particles in the cellular layers of the (epi)dermis.

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, when 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) is (are) still nanomaterial(s) or have degraded. From a risk assessment point of view, this is important because the loss of the nanostructure due to dissolution or degradation (enzymatic or chemical breakdown) of the active substance in nanoform or, in case of nanoformulated PPPs, the release of the active substance in molecular form from the nanocarrier or nanocapsules, would indicate that a conventional risk assessment according to sectoral guidances is sufficient. 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).

When 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 5, detailed characterisation data on the identity, chemical composition and purity/impurity profile of the nanopesticide active substances and formulations must be provided.

Inhalation exposure and 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 OECD Test Guideline 403 (OECD, 2009a).

The human respiratory tract is divided in three sections: the nasopharyngeal, tracheobronchial and alveolar regions. Particle fractions that potentially reach and adversely affect specific regions of the respiratory tract 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. Soluble particles trapped in the airways will simply dissolve, while insoluble particles are transported up the conducting airways by the ciliated epithelium and swallowed or expelled from the body by coughing or nose blowing.

The OECD Guidance Document on inhalation toxicity studies ENV/JM/MONO(2009)28/REV1 has been updated in 2018 for addressing the additional consideration required for testing nanomaterials (OECD, 2018c); and should be considered when assessing both active substances in nanoform and nanoformulated PPPs.

- Nanospecific considerations are required for the risk assessment of:
  - active substances in nanoform
  - active substances containing a fraction of small particles including nanoparticles identified according to the Guidance on Particle-TR
  - nanoformulations of plant protection products (PPP), including nanoencapsulates and nanocarriers with co-formulants in nanoform, and nanocarriers as defined in Appendix D.5 even if the co-formulants do not meet the legal definition for nanoforms.
- The risk assessment should address the individual components in nanoform (the active substances or co-formulants) and the nanoformulated PPPs.
- The approach outlined in this Guidance should be followed for consumers risk assessment unless it is verifiable information indicating that consumers will not be exposed to particles.
- The Guidance on the assessment of exposure of operators, workers, residents and bystanders in risk assessment for plant protection products (EFSA, 2014), should be adapted when considering non-oral exposure assessments.
- Detailed physico-chemical characterisation of active substances in nanoform must be carried out according to Table 1. For nanoformulated PPP, the relevant parameters listed in Table 1, along with additional parameters for dispersions/formulations such as stability, susceptibility, wettability, etc., should be reported.
- 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. The Guidance on dermal absorption (EFSA, 2017) should be expanded with nanospecific considerations. The OECD Guidance Document on inhalation toxicity studies ENV/JM/MONO(2009)28/REV1 updated in 2018 to cover nanomaterials should also be considered.
- 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 Test Guideline 403.

D.3. Substances used in Food Contact Materials (FCM)

Exposure to a nanomaterial can occur as a result of transfer (either by diffusion or mechanical release) of small particles into food from substances used in FCM. An evaluation is needed for substances in nanoform in accordance with Article 9(2) of Commission Regulation (EU) 10/2011, substances deliberately engineered to particle size which exhibit functional physical and chemical properties that significantly differ from those at a larger scale in accordance to Article 5.2.(c).(ii) of Commission Regulation (EC) No 450/2009, conventional materials with a fraction of small particles requiring assessment at the nanoscale according to the Guidance on Particle-TR and nanostructured or other substances used in FCM that may release nanoparticles. Figure D.1 gives an overview on how to follow this Guidance for evaluating the use of FCM substances.

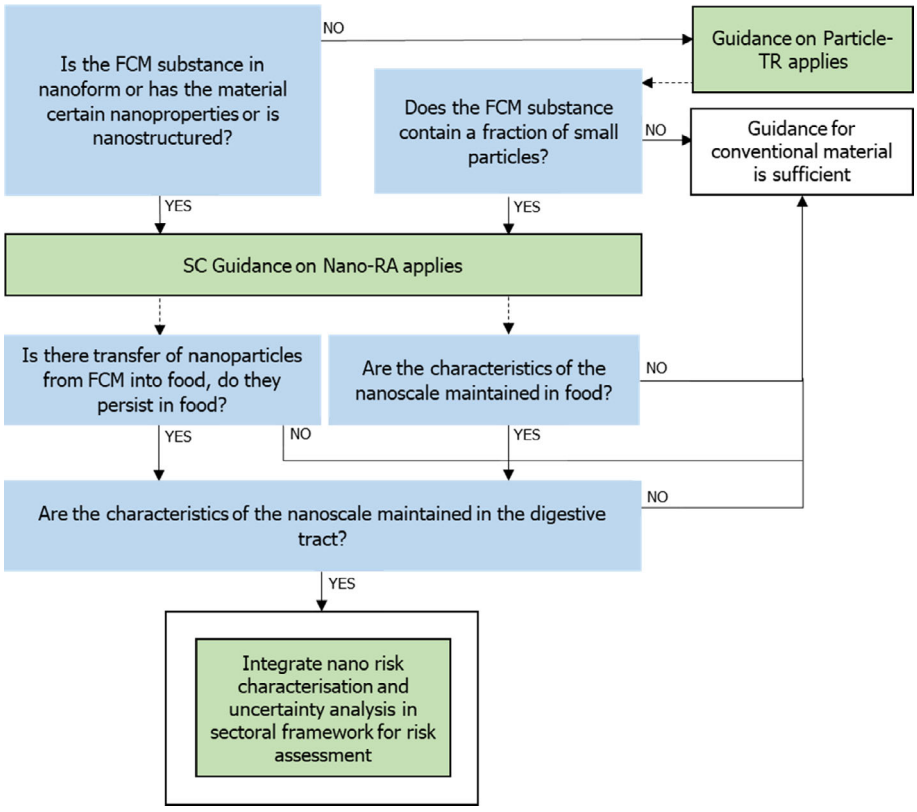


Figure D.1: Schematic outline and overview of workflow for the nanospecific risk assessment of FCM substances. In green: nanospecific risk assessment (RA); in blue: question to be answered; in white: risk assessment follows sectoral guidance

EFSA has published several opinions on the use of nanomaterials in FCMs, such as carbon black, titanium nitride, metal oxides and nanoclays (EFSA CEF Panel, 2012a,b, 2014a,c, 2015a,b, 2016b, 2019a,b). In all cases, it was concluded that no significant migration or transfer of the nanoparticles was expected under the defined conditions of use. It is recommended to consult the most recent opinions (EFSA CEP Panel 2019a,b) as they provide practical examples of the methodology used to assess the potential transfer of a nanostructured clay (cf. Section 1.3 case d) and a surface-treated metal oxide (cf. Section 1.3 case c) from plastics. Those assessments also provide examples on how the possibility of swelling and abrasion were addressed, as well as the analytical techniques used.

Potential migration of the substance (at max use level in 'worse-case' plastic(s)) is typically assessed through a set of considerations and experimental approaches including (when relevant):

- theoretical considerations, migration modelling
- specific migration from plastic(s) into simulants using a constituent element as indicator
- migration test using a surfactant solution to stabilise NPs followed by MALLS and AF4-ICP-MS
- surface analysis of plastic before and after exposure to a potentially swelling simulant/solvent
- an abrasion test of a plastic followed by analysis for released NPs

Examples on the implementation are available in opinions by the CEP Panel such as (a, b and d) for a modified clay (EFSA CEP Panel 2019a) and (a-e) for a modified TiO_2 (EFSA CEP Panel 2019b).

A comprehensive guidance for testing the potential transfer of nanoparticles from polymer nanocomposites has been published (Franz et al., 2020).

The available information so far regarding the (lack of) migration of nanomaterials from FCMs largely relates to synthetic plastics. A few available studies (Avella et al., 2005; Souza and Fernando 2016; Scarfato et al., 2017) indicate that migration patterns of nanomaterials from biodegradable polymer nanocomposites, e.g. starch or polylactic acid (PLA), may be different from those in the conventional (plastic) polymers. Biodegradable plastics are intended to breakdown in the environment and/or during composting. This being so, they can be especially sensitive to the conditions of use and may have only niche applications and/or a limited service life in food contact. Special care is needed when migration testing uses water or other official EU food simulants such as 3% acetic acid or 95% ethanol. This is because biopolymers may interact with water differently than plastic polymers and the aggressiveness of the food simulants towards the polymer may affect its integrity and the polymer chain-size distribution and cause physical release of the nanomaterial. Therefore, realistic migration/ release testing need to be used when testing biopolymer-nanomaterial composite based FCMs, i.e. under conditions that mimic their practical use without damaging the polymer performance properties – unless that would also occur for the food contact applications that are intended.

- Detailed physico-chemical characterisation of the substance used as an additive for the manufacture of food contact materials must be provided.
- In addition to the characterisation of the substance used for manufacture of a FCM, the need arises for characterisation of the nanoparticles as present in the FCM (on the surface, in the matrix) and when being released from the FCM.
- Potential transfer of nanoparticles from the FCM due to swelling effects, 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.
- Convincing scientific evidence showing the absence of transfer of nanoparticles indicates that the conventional risk assessment may be sufficient.

D.4. Nanofibres

Nanofibres include both dietary fibre of different types produced at the nanoscale (e.g. nanocellulose, nanochitin) and organic or inorganic nanosized fibres of any other nature. Some dietary fibres are inert to digestive enzymes in the upper GIT and thus, if nanosized, may reach the small intestine retaining its nanoscale features essentially intact. Therefore, potential nanofibre uptake and local effects in the small intestine have to be addressed. The range in physico-chemical properties of nanofibres can influence both local effects, gastrointestinal uptake and potential consequent effects. For instance, nanocellulose is produced in different forms (bacterial nanocellulose, nanofibrillated cellulose, cellulose nanocrystals) that can, e.g. be surface modified. This results in a wide variations in the physico-chemical properties among and within forms (Liu and Kong, 2021).

Once in the colon, the possible digestion or degradation of nanocellulose and other nanofibres by the microbiota, potentially leading to smaller fibres, has to be addressed as well. With this regard, the *in vitro* digestion and ADME studies require special attention as far as their design is concerned. For instance, the variability in digestibility between species (e.g. rodents versus humans) may be high for certain fibres and needs to be taken into account.

Tracking the fate of carbon-based nanofibres poses exceptional detection challenges. Studies on the uptake and crossing of the human intestinal epithelium are complicated by the lack of suitable analytical techniques for quantitative measurements, whereas different systems for tagging may offer some detection potential. Therefore, development of a proper visualisation technique to clarify what is being absorbed and in what form is crucial. This is particularly relevant when there is the potential for degradation to smaller fibres. For nanofibres, the assessment of possible changes in the characteristics of the nanomaterial in the gastrointestinal tract through *in vitro* digestion studies and ADME considerations on the substances produced after degradation are key elements to support risk assessment. The outcome of *in vitro* digestion studies (including the fate in the large intestine) and ADME studies can be useful for the selection of the proper material for *in vitro* toxicity testing, which may be of particular relevance for informing the design of any in vivo study. This is especially true in

the case of nanofibres with different shapes (or that can be degraded to smaller fibres), taking into account the possibility of selective absorption of particular fibre lengths or shapes.

D.5. Nanocarriers

As mentioned in the scope, Section 1.3, nanocarriers such as organic nanomaterial encapsulates are considered subject to this Guidance. Such nanoencapsulates can function as delivery systems, e.g. nutrient sources or to fulfil a technological function (e.g. incorporate food additives into products, such as lipophilic colours in hydrophilic beverages). Nanocarriers may exist in the form of solid particles (e.g. mesoporous silica), polymer, protein or lipid-based nanodelivery systems (e.g. micelles or liposomes, see Rossi et al. 2014). The biopolymers, proteins and lipid-based carriers are often referred to as 'soft' nanomaterials.

Nanoencapsulation is an extension of microencapsulation-based drug delivery systems comprised of liposomes and (bio)polymers 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.

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, in particular if the encapsulation materials are not fully disintegrated in the gastrointestinal tract, for (1) the active ingredient per se, (2) the encapsulating material and (3) the encapsulate/nanocarrier as a whole. This may require specific adaptations in the hazard characterisation. 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.

If there is potential for the nanocarrier to be taken up as such by intestinal cells, evidence of the mechanism of uptake and translocation should be provided to support the risk assessment.

Section D.2 provides specific provisions for pesticide nanocarriers.

- For nanomaterials used as carrier systems for other substances, a significant alteration (increase) in bioavailability may lead to potential harmful effects and 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.

D.6. Fertilisers

The development of nanoscale fertilisers advances rapidly, and may require specific considerations.

A recently published book by Jogaiah et al. (2021) provides an account of the developments in regard to nanofertlisers and nanopesticides, highlighting numerous studies that have shown the potential of nanofertilisers for delivering nutrients and to alleviate abiotic stress (drought and salinity) in plants.

The small particle size and large surface area of nanofertilisers can increase the uptake and utilisation of nutrients by plants compared to conventional forms. The slow release formulations of nanofertilisers are thought to increase the duration of nutrient availability in plants and formulations with hydrophilic polymers to provide additional benefits under conditions of drought and salinity due to the greater water holding capacity of the polymers (Mujtaba et al., 2021). Examples of study trials have been quoted to indicate the potential improvement of crops after treatment with urea-coated hydroxyapatite nanoparticles for slow-release and targeted delivery of nitrogen, seed treatment with phosphorus-nanofertiliser and foliar spray or soil applications of nanoparticles of zinc oxide, iron oxide, calcium oxide, etc.

Quoting the StatNano database (https://product.statnano.com/), Campos (2021) has listed various nanofertilisers and plant growth regulators that are currently on the global market. The author

has noted that the nanomaterials used in many of the nanofertilisers have not been specified, while some have been reported to contain nanoparticles or nano-encapsulated forms of silver, zeolites, boron, molybdenum, magnesium, phosphorus, zinc/zinc oxide, potassium, iron, silicon dioxide, calcium and manganese and titanium dioxide.

In addition to a broader reflection as to the provisions to regulate and assess them under REACH, the new Fertilisers Regulation[60] establishes the possibility for consultations with EFSA, the European Chemicals Agency (ECHA) and DG JRC that may trigger additional need for guidance. The use of nanofertilisers may lead to residues of nanoforms and degradations products in plants and consequently food commodities. Two main situations should be considered from a consumer's risk assessment perspective. First is the use of nanoforms as carriers of nutrients (e.g. Raliya et al., 2018). As for fertilisers in general, the presence of the nutrient in the plant is considered part of the natural process, and covered by the overall assessment of nutrient requirements in the diet. Consequently, the risk assessment should focus on the residues of the nanocarrier and the possible presence at harvesting of fertilisers in nanoform not present under natural conditions. The second situation, covered by the new EU regulation on fertilisers, is for biostimulants, meaning products stimulating plant nutrition processes independently of the product's nutrient content (Ricci et al., 2019). In this case, the use may lead to chemical residues in the plants and consequently in food commodities. The assessment of the environmental processes leading to these residues is very complex and difficult to model with the current scientific knowledge. The possible options are low-tier unrealistic worst-case estimations, based on mass balance or conducting field residue trials, such as those proposed in the OECD Guideline 509 (OECD, 2009e), and complementary guidelines for assessing the metabolism and presence of pesticide residues in crops and food (OECD Guidelines 501 to 508) (OECD, 2007b–h, 2008b). These guidelines should be adapted to identify the presence and properties of the nanoforms. Once the presence of nanoparticles in food commodities is quantified, the provisions of this EFSA SC Guidance on Nano-RA should be considered for the identification and characterisation of the particles and for assessing the risk to consumers, especially for the oral routes of exposure. In conclusion, for any substance at the nanoscale evaluated under REACH and when intake through food is likely, the EFSA Scientific Committee recommends that this Guidance is being applied.

- Nanofertilisers have the potential to deliver nutrients and to alleviate abiotic stress (drought and salinity) in plants.
- The risk assessment should focus on the residues of the nanocarrier and the possible presence of fertilisers at harvesting present under natural conditions.
- The use of biostimulants, products stimulating plant nutrition processes independently of the product's nutrient content, may lead to chemical residues in the plants and consequently in food commodities. The assessment of the environmental processes leading to these residues is very complex and difficult to model.

[60] Regulation (EU) 2019/1009 of the European Parliament and of the Council of 5 June 2019 laying down rules on the making available on the market of EU fertilising products and amending Regulations (EC) No 1069/2009 and (EC) No 1107/2009 and repealing Regulation (EC) No 2003/2003.