METHODOLOGY AND SCIENTIFIC SUPPORT UNIT

Frequently Asked Questions

Stakeholder workshop on small particles and nanoparticles in food | 31 March – 1 April 2022

EFSA organised a Stakeholder workshop on small particles and nanoparticles in food to engage with its Stakeholders on an open discussion on the implementation of the 'Guidance on technical requirements for regulated food and feed product applications to establish the presence of small particles including nanoparticles' (Guidance on Particles – Technical Requirements[1]) and the 'Guidance on risk assessment of nanomaterials to be applied in the food and feed chain: human and animal health' (Guidance on Nano – Risk Assessment[2]).

During the event, participants had the opportunity to pose questions for clarification and technical difficulties identified during the implementation of the Nano Guidances to real cases. This document presents the main questions collected during the Workshop and the related responses provided by the experts of the cross-cutting Working Group on Nanotechnologies. Questions have been grouped by topic, and in some case generalised to address multiple questions received on similar subjects. Applicants who have specific questions that have not been covered during the meeting or by this FAQ Document, are invited to submit their requests through the EFSA 'Ask a Question' portal (https://connect.efsa.europa.eu/RM/s/new-ask-efsa-request).

[1] https://efsa.onlinelibrary.wiley.com/doi/10.2903/j.efsa.2021.6769
[2] https://efsa.onlinelibrary.wiley.com/doi/10.2903/j.efsa.2021.6768

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Submissions

1 Will there be a mechanism for an EU centralised submission approach that would encompass the country specific nanomaterial declaration such as exist in France, Belgium, etc avoiding individual submissions?

The Nano Guidances are cross-cutting documents, covering common technical aspects related to characterisation and risk assessments. The approaches for submission of applications and dossiers to be assessed by EFSA are provided in the specific sectoral guidance documents.

Scope and applicability of Nano Guidances

2 Is the Guidance on Particle – Technical Requirement applicable to all materials? Referring only to sectoral guidance documents (e.g. on feed additives, food contact materials, food additives and flavourings) is not enough?

The Guidance on Particle – Technical Requirements was produced in response to a mandate received from the European Commission and should be considered applicable to all applications/dossiers submitted for EFSA assessment. As described by the explanatory figures and related text included in both Nano Guidances, the user should consider these Guidances as complementary to relevant sectoral guidance documents.

3 Should the Nano Guidances be applied to products already placed on the market or only for future marketed products?

The Guidance is applicable to all new EFSA assessments, including new applications as well as renewals and re-evaluations of materials already placed on the marked. In addition, the European Commission, Member States or the European Parliament may mandate EFSA to conduct new assessments, or to review previous assessments, including those related to marketed products.

4 Based on the provisions described by the Guidance on Particle – Technical Requirements, how should mixtures or multi-constituent substances be considered?

As defined in the Guidance on Particle – Technical Requirements, for multi-constituent substances and mixtures, the information to be submitted should cover each single constituent or component in the mixture, as well as the full material. Details are provided in the Guidance on Particle – Technical Requirements for each appraisal route.

In the case of (a) botanicals and other complex materials of biological origin with unknown or variable composition, (b) macromolecules of biological origin (e.g. enzymes and other proteins), or (c) other similar cases, the applicant could provide a rationale demonstrating that an assessment of the fraction of small particles including nanoparticles is not needed, or that is already covered in the safety assessment process.

It should be noted that the terminology and definitions for 'substance', 'multi-constituent substance' and 'mixture' used in both Nano Guidances have been harmonised with the European Chemicals Agency (ECHA) to be in line with those under the REACH and CLP Regulations.

5 Although nanomaterials or conventional materials containing a fraction of small particles may need to be checked in detail in the individual case for potential risks, it should be kept in mind that "…'nanomaterial' is a categorization 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…".

Whether nano-specific or not, in risk assessment a risk refers to the likelihood that the hazard occurs. The challenge for smaller particles is that their behaviour may be different than the respective non-nanoforms. This Guidance is aimed at characterising the possible risks arising from the presence of nanoparticles (i.e. determining what is the hazard and how likely is that it is leading to risks). These Guidances cover the risk assessment considerations, and are not connected to the definition of nanomaterials.

6 How do the Nano Guidances deal with organic lipophilic compounds? Many natural compounds used in food are present in plants or animals in nano form, and on the other hand, normal cell physiology and biochemistry is in the nano range (e.g. ribosomes).

A difference should be made between materials that are manufactured to be in particulate form, versus materials that are naturally present in our body, to which our physiology is used to. The first case, which is also the one relevant for these Nano Guidances, includes materials that due to their particulate form and related nano-specific biokinetic properties could circumvent body homeostatic regulations and lead to adverse effects.

Particles' terminology and status

7 What is the difference between aggregation and agglomeration? Why is this key for the assessment of nanoparticles?

The difference between aggregation and agglomeration is that in the aggregation status, particles are held with strong forces and bonds, and are not likely to de-aggregate, except with the use of specific techniques. Particles in agglomerates are on the other hand bound together by weak forces, and are likely to deagglomerate under certain conditions (e.g. pH, salt concentration, physical forces). In order to ensure consumer safety, the risk assessment should always cover the worst-case scenario, which is considered to be the exposure to particles in deagglomeration form.

8 What are the concepts and definitions of constituent particles, primary and secondary particles? Does EFSA keep to the ISO definition, which says that aggregates are constituent particles?

All the terminology used by the Nano Guidances is extensively explained in a Glossary. Regarding the concept of 'Constituent particles', the difference between the ISO definition and that stated by the European Commission in the recommendation on a definition of nanomaterial is the point of reference (i.e. constituent of what): in the ISO definition, the point of reference refers to aggregates and agglomerates, whereas in the other case the point of reference is the entire material. Further clarifications on this difference will be mentioned in a OECD Test Guidance on the measurement of particles which will be published soon.

Solubility and Dissolution Rate

9 What is the correct methodology to assess solubility? Is a nano-adapted OECD TG 105 available? In relation to ultrafiltration, if "small particles" remain on the filter, what is their significance?

As described in the Guidance on Particle – Technical Requirements, modification of OECD TG 105 is needed because particles in suspension should be removed using an appropriate methodology. The conceptual basis of the solubility test requires the identification and separation of two phases, a solid and a liquid phase. Solubility is the maximum concentration where at equilibrium only the liquid phase is present. When the solid phase starts to separate, the solubility value can be identified. If small particles are present, they will be floating in the liquid phase. As this would lead to an overestimation of solubility, this artefact should be avoided using ultrafiltration as recommended methodology.

10 When applied to food ingredients, the threshold for solubility is too strict and does not consider the diversity of the different food ingredients. For example, a number of polysaccharides such as xanthan gum or gellan gum are known to be well soluble in water but, due to their physicochemical properties (thickening/gelling agents), it is unlikely technically possible to determine their solubility at 33.3 g/L.

Two cases must be distinguished. A first case concerns truly water-soluble gelling agent. This type of material does not form suspensions but true solutions. Demonstration that a solution (and not a suspension) is present may require case by case considerations; in case of technical issues, alternative methods or appraisal routes should be preferred. The second case concerns materials where gelling properties are related to the formation of networks of solid entities (e.g. fibres) in which water molecules are entrapped. The solid entities are not dissolved and maintain their particulate nature. In this case, other appraisal routes should be used as the resulting suspension confirms that particles are not dissolved in the medium.

11 A marked discrepancy between the new "general" threshold of 33.3 g/L and the definition of solubility of daily intake in 1-2 L of stomach fluid is apparent. Will one criterion rule out the other one? Which criterion described by the Guidance on Particle - Technical Requirements is planned to supersede? Would EFSA's request for testing all particulate materials on the market according to the new guidance mean that also all food and food additive materials in powder form with a solubility less than 33.3 g/L have to be tested against the Guidance on Nano - Risk Assessment?

It should be noted that, as described in the Guidance of Particle – Technical Requirements, the applicant is encouraged to use the best appraisal route or combination of them based on the material under investigation to exclude the need for nano-specific risk assessment. Solubility and dissolution rate should be considered as the simplest appraisal routes in terms of cost and complexity and are aimed at demonstrating that the material will be fully dissolved in the food matrix or in the gastrointestinal tract. If at least one of the proposed appraisal routes is met, conventional risk assessment is sufficient and no further nano-specific risk assessment (i.e. according to the Guidance on Nano – Risk Assessment) is needed.

The Guidance is applicable to all new EFSA assessments. In addition, as consumer safety is a common goal and responsibility, applicants and other stakeholders my decide by own initiative to check materials on the market in order to identify the need for including nanoscale considerations in the safety assessment. Sectoral obligations should be considered.

12 Regarding the appraisal route related to dissolution in the gastrointestinal tract, does the Guidance on Particle – Technical Requirements provide pH recommendation for infants?

The cross-cutting Working Group (WG) on Nanotechnologies is working to produce additional guidance for substances that only meet the dissolution rate threshold under acidic conditions, including specific recommendations for infants according to the different physiological conditions. A dedicated Annex is expected to be published by the end of 2022. The activities of the WG can be monitored consulting the public minutes[3].

13 How can a particle dissolve in a food matrix and how can this be measured?

A specific approach should be followed based on the type of food matrix. For example, in a liquid food matrix, the time to obtain dissolution should be considered. For semisolid materials, dissolution may be more difficult to be achieved. However, guidance is given in Section 2.3.4. of the Guidance on Particle – Technical requirements on how to address these cases.

Particle size distribution

14 Based on the provisions described by the Guidance on Particle - Technical Requirements, is it required to determine the particle size distribution for each substance applied for? If this is needed, how can the reporting for particle size characterisation be done?

Particle size distribution of the materials is only needed for substances where simpler appraisal routes (e.g. solubility and dissolution rate) are not met. The 500nm threshold should be considered as a practical threshold and further guidance on how to report the characterisation of particle size is described in the Nano Guidances.

15 Why was a size definition chosen (250-500 nm) that deviates from the Commission's Recommendation and also the definition in Novel Food regulation (100 nm)?

The mandate from the European Commission referred to the need to address the presence of nanoparticles for materials which do not cover the definition of nanomaterials. Therefore, EFSA established thresholds for safety assessment of those conventional materials that could contain a fraction of small particles requiring nano-scale considerations. It should be noted that, when it comes to safety, risk assessment considerations are needed for materials containing particles up to a size rage of 250-500nm.

16 On which biological bases was the 250 nm limit for the crossing of gut barrier set?

There is still limited knowledge on the uptake and translocation of small particles by intestinal cells but, based on the current knowledge, an association with size was made. Detailed information and specific references are reported in Section 7.6. of the Guidance on Nano – Risk Assessment.

[3] https://www.efsa.europa.eu/sites/default/files/wgs/cross-cutting-science/wg-nanotechnologies.pdf

17 Regarding the particle size distribution screening, if a material consists only of aggregates > 500 nm but the constituent particles are all in the nano size range (e.g. 20 nm), couldn't such a material accidentally pass the "screening appraisal route"(e.g. PTA or CLS are used), or is it only the aggregate size that matters in such a case?

Such material is a structured nanomaterial and falls under the applicability domain of the Guidance on Nano – Risk Assessment. Therefore, in this specific case, the user is directed to follow the requirements prescribed by this Guidance for the assessment of nano-specific features.

18 How to proceed if the material to be investigated is a biological substance? This makes screening by Electron Microscopy impractical and unreliable.

For biological samples, Electron Microscopy (EM) should be adapted using proper methodology such as a suitable staining method for ensuring better visualisation.

19 Sonication with 600 – 2500 J/ml may lead to a very "artificial" material. In the food industry there are usually no processes where an ingredient is exposed to such high energies. Moreover, samples may be contaminated by debris from the sonication finger. The longer the sonication time, the higher the possibility of release of material from the sonication probe.

Sonication-based assays should be developed testing different energy values ensuring they are not influencing the constituent particle size. Furthermore, probes should be cleaned, changed in time, and checked with appropriate controls.

20 Is it possible to clarify the reason behind requiring "properly dispersed samples"? Does a dispersion always need to be dispersed as a worst-case scenario? For instance, when a material is dry handled and no chemical forces will be applied, is it still needed to make a dispersion as a worst-case scenario? Some other nanomaterials show such high agglomeration tendency and such high adhesion forces to each other, that the "highest possible" degree of dispersion does not at all reflect the true particle size of the marketed product and the product as used by the consumer.

The dispersion as used for testing should cover all possible exposure conditions. The need for properly dispersed samples as a worst-case scenario is due to the fact that agglomeration is a very dynamic status of particles, influenced by biological and physical factors, especially in the gastrointestinal tract. Proper dispersion should be ensured for particle size distribution analysis as well as during toxicological studies. For example, the presence of large agglomerates in *in vitro* and *in vivo* studies may hamper the uptake of nanoparticles, and thereby obscure effects. Lastly, it should be considered that the production processes of nanoparticles/small particles are often designed to prevent the formation of large agglomerates, as the presence of large agglomerates may affect the functionality of the material.

Toxicological testing

21 For many long-established materials containing a fraction of small particles high value toxicological studies are available since a long time. However, they have been conducted according to the standards valid back then (e.g dispersion protocols have been less well defined). Repeating these studies on the other hand would conflict with animal welfare regulations. How can this conflict be solved?

Existing studies can be used when it is possible to demonstrate that nanoscale considerations are covered. If the test material is still available, this could be also done comparing its characterisation with the one of the material available in the market. Both Guidances provide recommendations for using New Approach Methodologies (NAM) and Integrated Approaches to Testing and Assessment (IATA) in order to cover the information gaps, avoiding or at least minimising the need for new animal studies.

22 How does the necessity for full mammalian all gene mutation test and rejection of Ames tests comply with the clearly stated EU target of animal test reduction?

The non-suitability of the Ames test for nanoparticles do not cause *per se* an increase of animal studies, as it should be replaced by a second *in vitro* test evaluating the same genotoxicity endpoint (e.g. mammalian gene mutation test). Additional animal studies for the assessment of nanoscale considerations may be needed but only in case possible safety concern are identified and they are not covered by the available information.

23 Are there any recommendations for *in vivo* genotoxicity testing concerning the organs to consider and the duration of treatment?

Toxicokinetic information, e.g. on tissue distribution and accumulation, can be used to identify target tissues for which *in vivo* genotoxicity testing is most relevant. *In vivo* studies should preferably have a duration that covers steady state conditions, rather than a duration in which tissue concentrations are still rising.

24 How to assure the particles are in nano size when performing pathological examination during toxicological testing?

The use of an appropriate protocol for dispersion of nanoparticles and adequate reporting are needed to ensure the representativeness of the test material and to ensure the reliability assessment of the results.

25 How to justify why new extensive studies need to be done on materials where toxicity has already been extensively studied in the past, especially when no effect is expected from the outset?

Existing data should always be considered before performing new studies. Available information should be assessed to define whether nanoscale considerations were taken into account to demonstrate in a weight of evidence whether nanoscale considerations are covered (e.g. physicochemical characterisation of the material, proper dispersion).

26 If it is known from the continued essentially unchanged production process that an old material used in animal studies has the same characteristics as materials marketed today, but in these old studies characterization of presence of small particles was not performed because it was not the standard back then, how can it still be made use of the old studies to avoid unnecessary repetitions?

Information on the production process as well as on the comparison between test material used to conduct experimental studies with the marketed material can be used in a weight of evidence approach to avoid producing additional testing. The Guidances provide recommendations for using New Approach Methodologies (NAM) to cover information gaps, and Integrated Approaches to Testing and Assessment (IATA) for integrating existing information with the newly generated data.

27 How could the link between the nanotechnology risk assessment guidance and endocrine disruptors risk management be made?

The risk assessment for nanomaterials does not differ from the risk assessment of conventional materials with regards to the assessment of endocrine active substances. A dedicated subsection in the Guidance on Nano – Risk Assessment reports additional information.

28 Is there a food allergen assessment expected considering that protein nano materials could present new epitopes tested by Elisa/PCR or by HPLC MS for qualitative confirmation?

If increased or changed sensitivity to allergens is expected, this should be mentioned and if possible, be underpinned with analyses. This issue is not addressed in the Nano Guidances.

29 Determining the fate of nanoparticles (NPs) in the body might present other challenges. In order to decide whether particles can be internalised/absorbed, it must be selected a model that represents the main route of contact (oral, skin, inhalation). How can be calculated the initial amount of NPs to be introduced into the model to know if there is a real risk of absorption?

These Guidances focus on dietary oral exposure. Some EFSA assessments in the pesticides and feed additives areas also include scenarios for dermal and inhalation exposure, and specific considerations are provided in the relevant sections under Appendix D of the Guidance on Nano – Risk Assessment.

In the case of Food Contact Materials (FCMs), a physicochemical characterisation and a migration test should be performed as a first step. The measured or estimated mass-based migration value can be used to calculate the number of migrated particles based on a worst-case scenario. If further information is available, specific considerations to refine this approach can be used, as described in Appendix D.3 of the Guidance on Nano – Risk Assessment.

In the case of food applications, the Guidance on Nano – Risk Assessment provides clear instruction on how to define (mass-based) concentration/doses to be used in the different experimental *in vitro* and *in vivo* studies. Mass-based concentration can always be converted into particle-based concentration with appropriate calculations. It should be noted that the need to identify use levels is prescribed by the relevant sectoral Guidances, which should be considered as complementary to the Nano Guidances, and use levels inform the choice of the doses to be tested.

Coating

30 May nanocoatings (nanolayers) be a concern? Or just if they disintegrate into nanoparticles?

The potential disintegration of nanocoatings (nanolayers) to generate nanoparticles should be considered as part of risk assessment.

31 There are products that are fully solubilized in a solvent and then spraydried with a carrier. How can the small particles assessment be done? Is it based on the final product?

Each individual component of a nanostructured material, as well as the nano-entity as a whole (including the carrier), should be considered for nano-specific risk assessment.

Specific regulated products

32 Many nanofertilisers (n, P, Zn) are now recommended in agriculture. Zn also catalyses many metabolic pathways. What is the stand of EFSA on these nano fertilisers? Many vegetables are consumed raw and possible residues should be considered.

EFSA is not generally involved in the assessment of fertilisers, but may receive specific mandates from the European Commission. In case mandates are received, the methods and recommendations provided by these Guidances will be applied, with specific adaptations if relevant.

33 The workshop does not address the specifics for feed ingredients, and particularly target animal safety. Does safety extrapolation from laboratory animal studies (according to sectorial guidance) apply also to nanomaterials or conventional materials with nanofractions? And, in this case, is a safety factor of 100 still relevant to extrapolate between species (in case nano-specific effects are identified in laboratory animals)?

The risk assessment paradigm should be considered the same for nanomaterials and conventional materials. An Appendix is provided in the Guidance on Nano – Risk Assessment to detail specific information on feed additives and how to assess the safety for target animal and humans exposed via food or occupational exposure.

34 There are many publications about certain Food Contact Materials (FCMs) releasing micro and nanoplastics (e.g. nanoparticles from plastics, inks, adhesives, coatings, rubers...) that have not been intentionally added into food or drinks. Should industries producing such FCMs start nanotesting? Should companies using multilayer multi material (e.g. plastic layer, adhesive layer, microcellulose fibre layer) food contact laminates start investigating the (potential) presence of nanocellulose in the microcellulose? Industries producing FCMs are sometimes using products that (may) contain nanomaterials without their suppliers having informed them. Should suppliers inform them?

According to Art. 3 of EU Regulation 1935/2004 FCMs should be safe. FCM producers should consider this topic to be part of their FCM safety-in-use evaluation with regard to microplastic released into

packed foods. It is important to identify the source material and process step(s) responsible of the production and release of microplastics. It should be noted that risk assessment of micro plastics is still in its infancy and not yet well substantiated either by robust analytical methods (to measure exposure) or by toxicological standard tests (to identify the hazard). For further reading on this topic the following references may be visited:

- Welle F. and Franz R.; Microplastic in bottled natural mineral water – literature review and considerations on exposure and risk assessment. Food Addit Contam, Part A, 2018, Vol 35 (12), 2482–2492. https://doi.org/10.1080/19440049.2018.1543957
- Koelmans, A.A., Redondo-Hasselerharm, P.E., Nor, N.H.M. et al. Risk assessment of micro-plastic particles. Nat. Rev Mater 7, 138 - 152 (2022). https://doi.org/10.1038/s41578-021-00411-y

According to EU Regulations 1935/2004 and 10/2011, suppliers to FCM producers should inform their customers about the constituents and the compliance of the materials they deliver in the supply chain, and so should inform on the presence of nano- and micro-cellulose in the manufactured materials.

35 Did you experience applications on nanopesticides?

The area of pesticides is characterised by a biphasic system: the assessment of active substance is performed by EFSA, while the plant protection products are assessed at national level. For the moment, no applications on nano-enabled active substances were experienced so far. It is expected that most of these applications will concern nano formulations, therefore covered by EU Member States.

36 Novel Foods consisting of, isolated from or produced from cell culture or tissue culture derived from animals, plants, fungi or algae: the culture media used for the food production should undergo nanomaterial screening?

Cells are not particles and as such they do not deserve to be assessed regarding these Guidances. But the culture conditions/production process can introduce small particles, and these may be taken up by the novel food. The applicant should investigate if this is the case.

Other questions

37 Is the NanoDefiner e-tool still suitable and in line with the current guidelines? If yes, is there a workshop planned for this tool in the future?

The NanoDefiner e-tool was developed to help the user to decide whether a material complied the definition of nanomaterial according to the European Commission definition. The e-tool will be soon updated to align with the newly published definition.

38 How many substances have been identified as posing an additional risk due to nanoparticles through the application of nano-specific studies? This looks not a risk assessment, but an hazard search.

The aim of EFSA Nano Guidances is to ensure that the safety assessment covers the possible fraction of nanoparticles in conventional materials and that consumers are protected from possible nano-related risks. For conventional materials the assessment starts with appraisal routes to identify if consumers may be exposed to particles, in these cases, the risk assessment should cover nanoscale considerations.

39 How to safely dispose of the nanomaterial for research and/or other testing?

Please refer to this website[4] and specific Guidance[5] for more information on this topic.

40 Do EFSA and EFSA Nano Guidances consider ongoing international efforts to adapt and/or produce new guidelines for the assessment of nanomaterials (e.g. at OECD level, EU Projects)?

EFSA is contributing to OECD developments in this area and following several EU research projects. In addition, the EFSA Nanonetwork provides opportunities for collaboration and information exchange with organisations in the Member States and internationally. The Guidance on Nano – Risk Assessment recommends applicants and interested parties to consider new OECD developments, such as new or updated test guidelines and guidance documents not available at the time of publication of the EFSA Guidances, when planning new studies or preparing applications/dossiers.

41 How does EFSA consider non-validated test methods for the assessment of nanomaterials and nanoparticles within application dossiers?

As a general rule, all information relevant and with sufficient scientific quality, that can be verified by the risk assessors (i.e., with access to the raw data and to details on the internal quality assurance system), can be considered in the risk assessment. Some sectoral mandatory requirements highlight the need for using specific guidelines unless proper justifications can be provided; but this requirement does not exclude the consideration of additional information from other studies. The Guidance on Nano – Risk Assessment distinguishes 'valid' and 'validated' methods, as defined in the Glossary. Due to the current state of the art, non-validated methods may be the only option in some cases, in particular for assessments maximising the use of NAMs and NAM-based IATA. In addition, even OECD Test Guidelines may require adaptations to cover nano-scale considerations.

[4] https://osha.europa.eu/en/legislation/guidelines/guidance-protection-health-and-safety-workers-potential-risks-related-nanomaterials-work
[5] https://ec.europa.eu/social/BlobServlet?docId=13087&langId=en