EFSA GUIDANCE ON TECHNICAL REQUIREMENTS FOR REGULATED FOOD AND FEED PRODUCT APPLICATIONS TO ESTABLISH THE PRESENCE OF SMALL PARTICLES INCLUDING NANOPARTICLES

EFSA Scientific Committee
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Abstract
Following a mandate from the European Commission, the European Food Safety Authority has developed a Guidance on Technical Requirements (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. These requirements apply to particles requiring specific assessment at the nanoscale 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 outlines appraisal criteria grouped in three sections, 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 physicochemical 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 to demonstrate that the fraction of small particles, including particles at the nanoscale, has been properly evaluated. In addition, in order to guide the appraisal of existing safety studies, recommendations for fulfilling the data gaps while minimising the need for conducting
new animal studies are provided. This Guidance on Particle-TR complements the Guidance on Nanoscience and Nanotechnology adopted by the EFSA Scientific Committee in 2018. Applicants are advised to consult both guidance documents before conducting new studies.

**Keywords**

Nanoparticles, nanoscale, novel foods, food additives, feed additives, food flavourings, vitamins, minerals, food supplements, solubility, dissolution rate, sample dispersion protocol, particles, particle size distribution, electron microscopy.

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Following a mandate from the European Commission, the European Food Safety Authority (EFSA) has developed this Guidance on Technical Requirements (Guidance on Particle-TR), setting out the 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, including particles requiring specific assessment at the nanoscale, in conventional materials which do not meet the definition of engineered nanomaterial set out in the Novel Food Regulation (EU) 2015/2283.

This document complements the EFSA Scientific Committee Guidance on Nanoscience and Nanotechnology mentioned above and should be considered by the applicant when preparing the application and dossier, and then by the EFSA Panels and Units when considering the information submitted. Applicants are advised to consult both guidance documents before conducting new studies.

The present Guidance on Particle-TR provides mandatory information requirements for novel food applications submitted in accordance to Regulation (EU) 2015/2283, and for those regulated food products to which the engineered nanomaterial definition is also directly or indirectly applicable: food flavourings, food additives, feed additives, vitamins and minerals used in food in accordance with Regulation (EC) No 1925/2006 and/or in food supplements in accordance with Directive 2002/46/EC as well as vitamins, minerals or other substances used in food for specific groups in accordance with Regulation (EU) No 609/2013. Certain aspects of this Guidance may also be of value in assessing particles at the nanoscale in other domains,
including in applications in areas not covered by the engineered nanomaterial definition in food and feed (e.g. food contact materials and pesticide active substances), as well as assessments that do not involve an application. In those cases, the applicable specific guidance (e.g. the note for guidance on food contact materials) which may refer to specific aspects set out in the present Guidance, should be followed.

The document guides the applicant on different ways for confirming when a conventional risk assessment is sufficient. 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. The guidance also provides information related to best practices for reporting and assessing existing safety studies, and recommendations for generating additional information.

This Guidance on Particle-TR is applicable to all chemical materials, marketed or to be marketed as substances or mixtures, to be assessed by EFSA, including mixtures and products marketed as liquid formulations unless the information confirms that they are true liquids and do not contain small particles in suspension. The characterisation of the fraction of small particles, including the particle size distribution, is needed in all cases unless the applicant demonstrates that the material will be fully dissolved under the intended use conditions and consumers will not be exposed to particles. For multi-constituent substances and mixtures, the information to be submitted should cover each single constituent or each component in the mixture, as well as the multi-component material. 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 should 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, e.g. through hazard identification and characterisation methods that take into account the properties of the material.

The general principles and the summary of appraisal routes that applicants may follow to confirm that a fraction of small particles is either not present or covered by the conventional risk assessment are presented in Section 2. Solubility and dissolution rate can be used to demonstrate that under the anticipated use conditions the material will be fully dissolved in the food matrix or, following ingestion, during the gastrointestinal tract processes, and that consumers will not be exposed to particles. Under these circumstances the conventional safety assessment is appropriate and no further characterisation of the fraction of small particles is required.

Section 3 describes the information requirements and methods that should be used when characterisation of the particle size distribution is needed. Screening methods are sufficient when the applicant is able to demonstrate that the particle size is larger than 500 nm and that the material contains less than 10% of particles (number-based) smaller than 500 nm; the level of detection of the method(s) used for this assessment should provide convincing
Evidence that particles smaller than 500 nm are adequately counted. As a proper sample dispersion is essential for the characterisation of the size distribution, a dispersion protocol to be followed by the applicants is included in Section 3.2.

Quantitative methods for characterising the particle size distribution are required for materials containing a fraction of small particles (particles ≤500 nm). Electron microscopy is the preferred method and Appendix I provides information on how this characterisation should be reported in the application. A pragmatic trigger for materials consisting of or containing a fraction of small particles is applied when the large majority of the material is not at the nanoscale, but a tiny tail within the particle size distribution at the nanoscale may be present due to the manufacturing process. The trigger is that less than 10% (number-based) of the particles below 500 nm are smaller than 250 nm and implies that the total mass of particles at the nanoscale is very low; in these cases, a conventional risk assessment is sufficient and no further assessment for properties at the nanoscale is needed. In addition to the numerical criteria, a description of the fraction of small particles (i.e. particles smaller than 500 nm) should confirm that the particles at the nanoscale are just part of the size distribution tail resulting from a manufacturing process of the powder, and that all particles have similar characteristics except for size.

Section 4 presents the information to be provided on existing safety studies on conventional materials, not originally designed to specifically consider the presence of a fraction of small particles. There are two essential conditions to address that the possible adverse effects linked to the fraction of small particles, including nanoparticles, are covered by the study: a) the available information should demonstrate that the test material included the fraction of small particles, and b) the suitability of the study selection, study design and the level of dispersion of the test material for assessing the hazard of small particles including nanoparticles.

Regarding the first condition, the applicant should provide at least the characterisation of the fraction of small particles of the marketed material and the variability between production processes and batches. Ideally, a comparison of the characterisation of the fraction of particles in the material as marketed and in the material used for the safety study should be provided. In other cases, the comparison should focus on the manufacturing process in order to demonstrate that the test material was produced by the same manufacturing process and current production conditions, and that the test material was in compliance with the current material specifications. If the manufacturing process has been modified, information addressing the changes regarding the fraction of small particles, should be provided. In case of broad-range technical specifications of the bulk material that is placed on the market, the full range of small particles or at least the conditions representing the worst case, have to be covered by the test materials used in the existing studies.

The criteria for checking the adequacy of the study selection, study design, and level of dispersion are presented in Section 4.2. Information on the design and execution of any existing toxicity study is required to ensure that the study had addressed specific aspects related to particles in the nanoscale; including information on preparation of the test material to understand whether or not it would have minimised aggregation/agglomeration of the
particles, the matrix/vehicle used, and the mode of administration. Specific recommendations are provided for the appraisal of *in vivo* and *in vitro* studies, and Appendix II describes best practices for reporting the information. Additional considerations are required for genotoxicity and toxicokinetic studies.

A decision process for assessing data gaps, determining if new additional studies are needed, and establishing the testing strategy are presented in Section 4.3; recommendations for conducting new studies are provided in Section 5. Applicants should consult these sections before conducting new studies, and follow the recommendations in order to avoid unnecessary duplication of animal studies.
1. Introduction

1.1 Background and Terms of Reference as provided by the European Commission.

The engineered nanomaterial definition set out in Regulation (EU) 2015/2283¹ (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 1925/2006² and/or in food supplements in accordance with Directive 2002/46/EC³ as well as vitamins, minerals or other substances used in food for specific groups in accordance with Regulation (EU) No 609/2013⁴), and in food information to consumers, and implies significant requirements in terms of the premarket safety assessment and the post market labelling requirements for foods containing engineered nanomaterials.

In the context of regulated food and feed products that are subject to the engineered nanomaterial definition set out in Regulation (EU) 2015/2283⁵, we are currently facing two situations on nanomaterials that link their regulatory status with their safety assessment:

(1) When an application concerns a material that meets the definition of engineered nanomaterial set out in the Novel Food Regulation (EU) 2015/2283, the application must contain all the information needed, e.g. description of physical and chemical properties, technical, biological and toxicological data on the material as required by the specific legislation, in order to allow for its safety assessment by EFSA, leading to its eventual authorisation. In terms of the safety assessment, EFSA is conducting its assessment in line with its recently updated guidance on risk assessment of the application of nanoscience and nanotechnologies in the food and feed chain.

(2) A potentially more complex situation arises when an application concerns a conventional material which does not meet the definition of engineered nanomaterial but may contain small particles including particles at the nanoscale (< 100 nm in one or more dimensions). Particles at the nanoscale can be formed naturally or as by-products in the production process or during handling and processing of the foods.

To proactively ensure that materials that fall in case (2) above, are properly assessed for safety by the applicants themselves when they conduct the safety tests required by the applicable legislation and by EFSA when the application is evaluated, technical guidance for applicants is necessary. It is therefore appropriate to request that EFSA develops technical guidance setting out the technical information that will need to be part of a new regulated food or feed application, to demonstrate whether a conventional material, which is not covered by the definition of engineered nanomaterial set out in the Novel Food Regulation (EU)

⁴ OJ L 181, 29.6.2013, p. 35
2015/2283, consists of or contains a fraction of small particles, including particles at the nanoscale.

TERMS OF REFERENCE

In accordance with Art 29 (1) of Regulation (EC) No 178/2002\(^6\), the European Commission asks the European Food Safety Authority to develop technical guidance setting out the information requirements for applications in the regulated food and feed product areas of conventional materials which do not meet the definition of engineered nanomaterial set out in the Novel Food Regulation (EU) 2015/2283\(^7\), in order to demonstrate whether a portion or the whole of the material is in the nanoscale.

Furthermore, for those materials which have been determined to contain a fraction of small particles, including particles at the nanoscale, the European Commission asks the European Food Safety Authority to include in the guidance the information requirements demonstrating that the nanoscale fraction of the material was properly evaluated in the safety studies of the material.

The guidance should in particular address the following parameters and include any other technical or scientific elements considered appropriate to be included:

a. The physicochemical criteria that will help identify/signal materials that are likely to consist of small particles, including nanoparticles, or contain a fraction of small particles, including nanoparticles, in a given regulated food or feed product.

b. The technical and scientific information (e.g. particle size distribution including necessary meta-information) that the applicant will need to include in the application if a material meets the above criteria that will document the presence or absence of small particles, or a fraction of small particles, including nanoparticles, in the material and/or in the final regulated food and feed products.

c. When applicable, the evidence requirements, in line with the EFSA guidance on risk assessment of the application of nanosciences and nanotechnologies in the food and feed chain that will demonstrate that the safety studies conducted included the fraction of small particles, including nanoparticles, so that EFSA can carry out the proper safety assessment of the material.

1.2 Interpretation of the Terms of Reference

In line with the Terms of Reference, EFSA has produced this EFSA Guidance on Technical Requirements addressing the presence small particles (hereafter “Guidance on Particle-TR”) describing the information to be submitted by applicants in the food and feed areas to allow the safety assessment of the materials with a fraction of small particles, including particles at the nanoscale.

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1.3 Implementation of this Guidance on Technical Requirements in EFSA regulatory assessments

This Guidance on Particle-TR provides mandatory information requirements for novel food applications submitted in accordance to Regulation (EU) 2015/2283 (the Novel Food Regulation), and for those regulated food products where the engineered nanomaterial definition is also directly or indirectly applicable: food flavourings, food additives, feed additives, vitamins and minerals used in food in accordance with Regulation (EC) No 1925/2006 and/or in food supplements in accordance with Directive 2002/46/EC as well as vitamins, minerals or other substances used in food for specific groups in accordance with Regulation (EU) No 609/2013.

Certain aspects of this Guidance on Particle-TR may also be of value in assessing particles at the nanoscale in other domains, including in applications in areas not covered by the engineered nanomaterial definition in food and feed (e.g. food contact materials and active substances in plant protection products), as well as assessments that do not involve an application. In those cases, the applicable specific guidance, e.g. the note for guidance on food contact materials (EFSA CEP Panel, 2018), which may refer to specific aspects set out in the present Guidance, should be followed.

This Guidance on Particle-TR should be considered by the applicants when preparing the application/dossier, and then by the EFSA Panels and Units when assessing the information submitted. The document guides the process to decide whether or not the material, or a fraction of it, does require specific assessment of properties at the nanoscale. This Guidance on Particle-TR provides ways for confirming that a conventional risk assessment is sufficient. 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 that this fraction is covered by the conventional risk assessment and relevant sectoral guidance documents, and does not require a separate assessment for the nanoscale. When preparing the application/dossier, if a particular appraisal route does not provide adequate justification regarding points a) and b) above, the applicant may consider other appraisal routes, nevertheless all information should be presented in the application/dossier. Some pieces of information, such as verifiable quantitative assessment of solubility or dissolution rates, may be essential. The Guidance also provides information related to best practices for reporting and assessing existing studies, and recommendations for generating additional information.

This Guidance on Particle-TR complements the EFSA Scientific Committee Guidance on risk assessment of the application of nanoscience and nanotechnologies in the food and feed chain: Part 1, human and animal health on Nanomaterial and Nanotechnology (EFSA SC, 2018; hereafter “Guidance on Nano-RA”). Applicants and other readers are referred to that guidance regarding the specific scientific elements to be considered when assessing the risk of particles at the nanoscale. It should be considered that the Guidance on Nano-RA is currently being updated. Figure 1 presents the connectivity between this Guidance on Particle-TR, the
Guidance on Nano-RA and the sectoral guidance documents; clarifying under which conditions the material requires assessment at the nanoscale following the Guidance on Nano-RA.

Figure 1. Decision process for selecting the applicable guidance document(s) to be used for the risk assessment of the material regarding the assessment of small particles.

According to this decision process, the applicants or other risk assessors should apply this Guidance on Particle-TR when assessing whether the material contains small particles. A full assessment addressing the properties at the nanoscale, in line with the Guidance on Nano-RA, is required if 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 according to the provisions under Commission Regulation (EU) 2018/1881 modifying certain Annexes of the REACH Regulation to introduce nano-specific clarifications, or
- consists of or contains a fraction of small particles as outlined in this Guidance on Particles-TR, unless according to the principles in this Guidance on Particle-TR, the information confirms that the conventional assessment is sufficient to address the risk for consumers.

This Guidance on Particle-TR intends to highlight that safety assessment of food/feed ingredients and additives composed or comprised of small particles may not be complete if only chemical aspects are considered without taking into account certain particle-size related
physical aspects as well. It should be noted that this EFSA Guidance on Particle-TR only addresses the risk for consumers exposed via food. Other assessments to be performed by applicants, in particular human occupational exposure and environmental risk assessments are not addressed. However, some general principles may be relevant.

This Guidance on Particle-TR is intended to assist applicants and interested parties intending to submit application dossiers or other comparable forms of submissions.

2. Materials requiring assessment according to this Guidance on Technical Requirements and general principles

2.1 Introduction

This Guidance on Particle-TR is applicable to chemical materials either as substances or mixtures to be assessed by EFSA. This Guidance on Particle-TR is also applicable to mixtures and products marketed as liquid formulations (e.g. emulsions and suspensions) unless the methods described below confirm that do not contain small particles in suspension (true liquids or fully solubilised solids). Substances that are true liquids or fully solubilised at normal conditions of oral ingestion (37 °C and 1 atmospheric pressure, see Section 2.3.3) are sufficiently covered by the sectoral guidance.

The characterisation of the fraction of small particles, including the particle size distribution, is needed in all cases unless the applicant demonstrates that the material will be fully dissolved and consumers will not be exposed to particles. This Guidance on Particle-TR focusses on the implications for the safety assessment. The decision whether or not a material is an engineered nanomaterial according to the definition in the Novel Food Regulation is a regulatory decision outside EFSA’s remit and is not addressed in this Guidance on Particle-TR.

For multi-constituent substances and mixtures, the information to be submitted according to this Guidance on Particle-TR should cover each single constituent or each component in the mixture, as well as the multi-component material. 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 should 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, e.g. through hazard identification and characterisation methods that take into account the properties of the material.

2.2. General principles and list of appraisal routes for confirming that a fraction of small particles is either not present or covered by the conventional risk assessment

This Guidance on Particle-TR presents several appraisal routes for confirming that a) the material does not contain a fraction of small particles, or b) a fraction of small particles is present but properly covered by the conventional risk assessment. In both cases, an additional assessment related to the presence of particles at the nanoscale is not needed. The appraisal
routes are listed according to simplicity for generating the information, but the list should not
be considered as a tiered approach and the applicant may select an appraisal route or routes
appropriate for each material.

The applicant may select one or several appraisal routes to demonstrate the absence of a
fraction of small particles, or that the fraction is present but well-covered by the studies
submitted by the applicant for the conventional risk assessment.

If the applicant confirms that a fraction of small particles is present and cannot demonstrate
that the hazard assessment of this fraction of small particles is well-covered, the applicant
should present the necessary information to complement the risk assessment, which includes
the fraction of small particles with nanoscale properties in line with the Guidance on Nano-RA
(2018); indications are provided in Section 4.3.

Table 1 complements Figure 1 summarising the different appraisal routes, describing the
parameters or options to be considered for each appraisal route, and the associated decision
criteria for confirming that the conventional risk assessment is sufficient, and methodology for
obtaining the information.
Table 1. List of appraisal routes and corresponding decision criteria for demonstrating that the material does not require specific assessment at the nanoscale (i.e. assessment according to Guidance on Nano-RA).

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<th>Parameters/Options</th>
<th>Decision criteria</th>
<th>Methodology</th>
<th>Comments</th>
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<tbody>
<tr>
<td><strong>Appraisal routes using solubility and dissolution rate to demonstrate that consumers will not be exposed to particles (Section 2.3)</strong></td>
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<tr>
<td>Solubility in water (Section 2.3.1)</td>
<td>Equal to or higher than 33.3 g/L</td>
<td>According to OECD TG 105 with specific considerations for small particles</td>
<td>For multi-constituent substances and mixtures, the decision criterion has to be fulfilled for each constituent/component</td>
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<tr>
<td>Dissolution/transformation rate in water (Section 2.3.2)</td>
<td>Half-life of 10 min or less corresponding to dissolved fraction equal to or higher than 88% in 30 min</td>
<td>Single concentration corresponding to exposure at the maximum use level in water</td>
<td>For multi-constituent substances and mixtures, the decision criterion has to be fulfilled for each constituent/component. If solubility is pH dependent, the criteria should be confirmed at pH=3 and/or pH=7</td>
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<td>Solubility or dissolution rate in other relevant media (e.g. oils) simulating or representing the application matrix of the product (Section 2.3.4)</td>
<td>The criteria for water should be used as reference</td>
<td>solubility or dissolution rate tests in a relevant medium according to OECD TG 116 or equivalent method with specific considerations for small particles</td>
<td>Results should confirm that under the intended use conditions (e.g. food matrix) the material will be solubilised in the products ingested by consumers</td>
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**Appraisal routes related to the particle size distribution (Section 3), proper dispersion of the material is essential in each case**
<table>
<thead>
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<th>Parameters/Options</th>
<th>Decision criteria¹</th>
<th>Methodology</th>
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| Particle size distribution of the material              | Particles larger than 500 nm  
The level of detection of the method(s) used for this assessment should provide convincing evidence that the material contains less than 10% of particles (number-based) smaller than 500 nm | Centrifugal Liquid Sedimentation (CLS)  
Particle tracking analysis  
Descriptive EM  
Filtration complemented with chemical analysis  
The method selection should be justified, and level of detection should be reported | Proper dispersion of the material should be ensured (Section 3.2) |
| For liquid formulations/products (Sections 2.3.3 and 3.3) | Results should confirm the absence of particles in suspension | Ultrafiltration of the liquid product  
The level of detection should be reported | To be tested for the full formulation as marketed |
| Particle size distribution of fraction of small particles (Section 3.4) | Less than 10% particles (number-based) of the fraction below 500 nm are smaller than 250 nm | Quantitative EM or a different method with justification | Applies to the fraction of small particles of the full material (also for multi-constituent substances and mixtures)  
When the criterion is not met, this information is also required for assessing if the fraction of small particles is covered by the existing safety |
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<th>Decision criteria&lt;sup&gt;1&lt;/sup&gt;</th>
<th>Methodology</th>
<th>Comments</th>
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<td>studies following the criteria described in Section 4</td>
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<tr>
<td><strong>Appraisal routes demonstrating that the fraction of small particles is properly covered by the existing safety studies (Section 4)</strong></td>
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<tr>
<td>The studies address properly the potential hazards of the fraction of small particles (Sections 4.1. and 4.2)</td>
<td>The test material included the fraction of small particles, and The test design, and the level of dispersion was sufficient for addressing the fraction of small particles</td>
<td>Characterisation of the test material (particle characteristics and particle size distribution), and comparison with the marketed material, and Specific consideration for genotoxicity and toxicokinetic assessments, and Demonstration of a sufficient level of dispersion based on extraction of information from study protocol or additional information (See Appendix II)</td>
<td>For existing studies see details in Section 4. Before conducting new safety studies for materials containing a fraction of small particles, see the recommendations of the Guidance on Nano-RA, Section 6</td>
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</table>
The submitted risk assessment covers the fraction of small particles (Section 4.3)

The gaps observed in the safety studies are covered (or are of overall low relevance) and do not trigger additional concerns

The lines of evidence are combined in a weight of evidence approach

See examples under Table 4, Section 4.3

1 Fulfilling one decision criterion is sufficient for demonstrating that the assessment according to the sectoral guidance is sufficient

The technical feasibility for applying the criteria above may be problematic for complex materials of biological origin that are not fully characterised or are of variable composition, such as some novel foods, botanicals, enzymes and other proteins or extracts. These materials may have a fraction of small particles of natural origin similar to that of foods considered safe for consumption. In these cases, the applicant may justify that the fraction of small particles is similar to that present in comparable foods, and covered by the available studies and does not require additional assessment. A scientifically sound case-by-case justification supported by the available evidence should be presented.

2.3. Confirmatory appraisal routes related to solubility and dissolution rate

The assessment of particles at the nanoscale refers to entities in solid form, including particles in suspension/dispersion but excluding liquid drops in emulsion (Rauscher et al., 2019b). From the risk assessment perspective, as indicated in the Guidance on Nano-RA, if the material is expected to be fully solubilised in the solid or liquid food or in the gastrointestinal tract (GIT), an assessment at the nanoscale may not be needed.

The terms relating to solubility, dispersibility and dissolution rate have been explained in the Guidance on Nano-RA. Briefly, solubility is the proportion of a solute in a solvent under equilibrium conditions (i.e. in a saturated state), whereas dissolution rate refers to the kinetics of dissolution. For nanomaterials, dissolution means that the material is solubilised into individual ionic or molecular species, and this needs to be differentiated from dispersion, which is a colloidal suspension of the particles. In addition, it is also important to consider the form in which a material may be solubilised. For example, some materials may not be solubilised as such, but they may do so as a result of a chemical transformation - such as hydrolysis or oxidation – if permitted by the conditions in a solvent.

A Guidance on safety assessment of nanomaterials in cosmetics has been published by the Scientific Committee on Consumer Safety. Alike the Guidance on Nano-RA, it emphasises the need to distinguish between solubility and dispersion of nanomaterials, and describes solubility as disintegration of a nanomaterial in an aqueous medium or biological environment into molecular components with the loss of nano features. It also highlights the consideration of
the time required for dissolution, because that will determine the consideration for risk assessment to be based on either particle risk or solubilised substance risk (SCCS, 2019).

Following the ingestion of small particles, the material may dissolve in the GIT, if the dissolution kinetics is rapid enough to achieve full solubilisation in the stomach or in the intestine before gastrointestinal uptake of the particles, a conventional risk assessment is sufficient. The principles of the Guidance on Nano-RA have been adapted to offer a simplified dissolution rate assay in water. If solubility is pH-dependent and the criterion is not achieved at pH=7, it would be sufficient to demonstrate that the dissolution rate at pH=3, representing the stomach conditions, is sufficiently rapid to ensure full dissolution in the stomach.

2.3.1 Solubility in water

2.3.1.1 Decision criteria

If the solubility of the substance in water is equal to or higher than 33.3 g/L, no additional assessment for the fraction of small particles is needed.

This criterion can also be applied to multi-constituent substances and mixtures. For mixtures, each substance in the mixture should be tested separately and achieve the solubility criterion of equal to or higher than 33.3 g/L. If some components in the mixture do not achieve the solubility criterion the other appraisal routes should focus on those components.

2.3.1.2 Information required and methodology

A solubility test according to OECD TG 105 or equivalent is required. If the measured solubility is less than 33.3 g/L, it is important to remove any suspended particles from the suspension through ultrafiltration before carrying out chemical analyses to avoid overestimation of the truly solubilised amounts of the material.

2.3.1.3. Principles

The criterion on solubility in water is based on the SCCS Guidance (2019) and specifically on the criteria for measuring the solubility of nanoparticles. According to the SCCS (2019), nano-specific risk assessment may be waived for a material composed/comprised of small particles that has a high solubility (i.e. solubility of 33.3 g/L or more).

A substance is commonly understood to have high solubility if it is soluble at more than 1 mol/L solvent. As a guide, the solubility of a substance should be seen in the context of the categories for degrees of solubility proposed by JECFA and the European and US Pharmacopoeias (Table 2).

Table 2: Solubility Terms as defined by JECFA (2006) and European and US Pharmacopeias (European Pharmacopoeia 10th Edition 2019; USP38 and USP38 NF33)

<table>
<thead>
<tr>
<th>Term</th>
<th>Parts of solvent required for 1 part of solute</th>
<th>Solubility defined in g/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Very soluble</td>
<td>Less than 1 part</td>
<td>&gt;1000</td>
</tr>
<tr>
<td>Freely soluble</td>
<td>1 to 10 parts</td>
<td>1000-100</td>
</tr>
<tr>
<td>Soluble</td>
<td>10 to 30 parts</td>
<td>100-33.3</td>
</tr>
<tr>
<td>Sparingly soluble</td>
<td>30 to 100 parts</td>
<td>33.3-10</td>
</tr>
<tr>
<td>Slightly soluble</td>
<td>100 to 1000 parts</td>
<td>10-1</td>
</tr>
</tbody>
</table>
Very slightly soluble 1000 to 10000 parts 1-0.1
Insoluble* >10000 parts <0.1

* The European Pharmacopeia terms it as ‘practically insoluble’

Even if the criterion is not met, a proper measurement of solubility is essential for applying other appraisal routes. When testing insoluble or partially-soluble substances, it must be kept in view that they will be present in a test medium as a nano-dispersion rather than a solution.

Special attention should be paid to the agglomeration/aggregation behaviour, and the insoluble/partially-soluble nature of small particles in the nanoscale (SCENIHR, 2009; OECD, 2009; Chaudhry et al., 2010; Gottardo et al., 2017).

Apart from the chemical nature, solubility of a material is dependent on a number of other factors, such as type of solvent, temperature and pH. The OECD TG 105 provides instructions for testing solubility of substances, but is not considered suitable for nanomaterials (OECD, 2014). Protocols and guidelines for the determination of the solubility of nanomaterials have been proposed, e.g. by Tantra et al. (2016). Insoluble and partially soluble nanomaterials form suspensions of nanoparticles in the solvent media, and it is important to remove any suspended particles from the suspension through ultrafiltration before carrying out chemical analyses to avoid overestimation of the truly solubilised amounts of the nanomaterial (instructions about how to perform ultrafiltration can be found in Section 2.3.2). The SCCS Guidance (2019) has also emphasised that, depending on the amount added, a final formulation may still contain (nano)particles if the nanomaterial is only partially solubilised.

For example, a ‘sparingly soluble’ nanomaterial will, by definition, have virtually most of the material in insoluble particle form.

2.3.2 Dissolution rate in water

2.3.2.1 Decision criteria

If at a concentration corresponding to exposure at maximum use level (as detailed in Section 2.3.2.3), the dissolution rate of the material in water (or the dissolution rate of each constituent in case of a multi-constituent substance) shows a half-life of 10 minutes or faster dissolution (mass-based), no additional assessment for the fraction of small particles is needed. This dissolution rate corresponds to 12% or less of the material (mass-based) remaining present as particles after 30 min. Multiple time points should be determined to establish the dissolution rate. No indication for incomplete dissolution should be observed.

This criterion is also applicable to mixtures. Each substance in the mixture should be tested separately and achieve the dissolution rate mentioned here. If some components in the mixture do not achieve this dissolution rate, the other appraisal routes should focus on those components.

2.3.2.2 Information required and methodology

A dissolution rate test as described below is required. The dissolution rate test should measure the dissolution of the test item at a single concentration, representing the maximum level of consumer exposure, at different time-points.
2.3.2.3. Principles

Size-specific properties or effects of a material composed of small particles, including nanoparticles, are intrinsically linked to the stability of its small particle/nanoscale features. When the material loses these properties, e.g. due to dissolution, it will not be expected to behave differently from its corresponding conventional chemical form (i.e. the soluble substance having the same chemical composition).

The dissolution rate test is aimed to investigate whether a particulate material that is not completely soluble in water (i.e. with solubility < 33.3 g/L) shows a dissolution rate in water indicating that, at the exposure levels expected for consumers, the particles will dissolve, and therefore consumers will not be exposed to small particles even if present in the material as marketed. It is a screening test in water, at room temperature, meant to address point 3 (‘Degradation rate [g/(L*h)]’) in Table 1C of the Guidance on Nano-RA. If the test criterion is met, conventional risk assessment is sufficient, whereas if the criterion is not met, other appraisal routes should be considered.

The general outline of the test strategy is as follows.

The starting point is the identification of the intended maximum use level and the concentration at which the dissolution rate is to be tested. This process includes two steps. The first step is to estimate the maximum daily amount to be ingested by the consumers. This should be estimated according to the intended use patterns and the exposure assessment methods described in the relevant guidance documents covering the risk assessment of regulated products in the different areas. It should be based on estimations of the maximum concentration in foods and maximum expected consumption of the food conducted according to the relevant sectoral guidance; when the material may be present in several food commodities, the estimation should include the maximum levels per day for the relevant population groups and diets. This step is a standard part of the exposure assessment. The second step is to estimate the concentration at which the dissolution rate is to be tested. This estimation is done by assuming that the amount ingested per day is diluted in the daily total volume of secretions in the gastrointestinal tract, which for adults is 4–5 L (EFSA SC, 2018). The volume should be adapted in case of assessments for infants and children or when the use pattern suggests that the ingestion will not be distributed over the day. In case of doubts on the exposure assessment, applicants may test somewhat higher worst-case exposure levels for the dissolution rate than those included in the application.

Following the estimation of the concentration to be tested, the applicants should use the protocol proposed below. Deviations should be justified.

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8 Corresponding to the definition of ‘sparingly soluble’, or ‘slightly soluble’, or ‘very slightly soluble’, or ‘insoluble’, as given by JECFA and US and European Pharmacopoeias.

9 The degradation/dissolution rate refers to the kinetics of dissolution and degradation. Particulate materials composed of small particles may degrade/dissolve faster than their bulk counterparts because of their high surface-to-volume ratio. The dissolution rate is influenced by various factors, including solvent, temperature, pH, concentration, and presence of substances interacting with the particle’s surface. It can be determined by kinetic measurements such as time-dependent concentration changes (of either the nanoparticles or the dissolved species) or changes in the particle size distribution (to smaller sizes).
Solid materials

- Disperse the test item at room temperature in water containing 85 mM NaHCO₃ and 40 mM NaCl, at the concentration selected as described above. Allow the soluble components to dissolve under stirring conditions (in general 300 rpm should be sufficient). If there are insoluble particles these will exist in the medium forming a dispersion. An appropriate dispersion method (see Section 3.2) has to be applied to deagglomerate potential agglomerates. Attention should be paid to employing sonication techniques delivering sufficient energy (i.e. probe sonication instead of bath sonication). The analytical report should indicate the equipment used and the operating conditions (time, power output) and the measured pH.

- Analyse this dispersion (t=0) for the chemical substances constituting the particles, e.g. for inorganic materials measure the associated characteristic elements (by techniques such as ICP-OES or ICP-MS) after microwave-assisted digestion.

- Then perform an ultrafiltration of the dispersion (t=0) using a membrane filter with pore size in the range 3-100 kDa (10 kDa membranes are appropriate for most materials). The membrane will retain the particles and the filtrate will contain only solubilised components. In some cases, filtration through a 0.22 µm filter may be needed beforehand to remove larger particles (including potential agglomerates and aggregates) and prevent obstruction of the 10 kDa membranes in the subsequent ultrafiltration step.

- Submit aliquots of the dispersion at multiple, appropriate time points to the same separation treatment and the filtrate to the same chemical analysis as described above. The filtrate should be free of small particles/nanoparticles, which are retained on the membrane surface (if present).

- The materials is considered to degrade/dissolve quickly (i.e. to have a high degradation/dissolution rate with a half-life of 10 min or less) if the degradation rate profile shows a clear decrease in the presence of particles over time (no plateau) and 12% or less of the material (mass-based) – compared with the concentration at the beginning of the test – remains as particles after 30 min. This is indicative that no (or a very limited number of) particles will remain to be taken up as such by consumers.

- At least four time points should be tested for the determination of the degradation/dissolution rate. For instance, the ultrafiltration and subsequent analysis should be repeated; in addition to t=0, also at for example t=5 min, t=10 min, t=30 min

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10 Estimated according to: Gilliland 2016, NanoDefine TR D2.3.
11 Only ultra-small particles, smaller than the pore size, may pass through the membrane: such potential presence does not affect significantly the test results.
12 For inorganic elements, microwave assisted digestion may be omitted at this stage.
13 The particulate fraction is measured by comparison, at each time point, of the concentration in the filtrate versus the concentration before ultrafiltration.
14 I.e. before ultrafiltration.
and t=60 min. The degradation/dissolution curve has to be documented and the degradation/dissolution rate at t=30 min calculated.

- If degradation/dissolution is pH-dependent, the dissolution test should be carried out at different points covering the pH range of physiological relevance (pH=3\textsuperscript{15} to pH=7). If 88% dissolution or more is achieved in 30 min at pH=7 and/or pH=3\textsuperscript{16} the confirmatory criterion is achieved.

- The applicant may also consider the recommendations for degradation/dissolution rate of nanomaterials in the GIT described in the Guidance on Nano-RA, including the characterisation of the degradation products.

2.3.3 Confirmation of absence of particles for liquid materials

For materials marketed in liquid form it should be sufficient to demonstrate by ultrafiltration that the material is a true liquid and not a dispersion containing small particles.

Mass balance or verification of the absence of small particles including nanoparticles by electron microscopy need to be provided.

2.3.4 Solubility or dissolution rate in lipophilic media

2.3.4.1 Decision criteria

This appraisal route is only applicable to substances marketed in a non-aqueous matrix or when all intended uses require a dispersion in a lipophilic matrix (e.g. oils) before consumption. The information should confirm that the solubility and/or dissolution rate of the substance in the non-aqueous matrix achieves the criteria mentioned under Sections 2.3.1. and 2.3.2, respectively.

The approach related to solubility and dissolution rate can also be applied to mixtures. Each substance in the mixture should be tested separately and achieve the solubility or dissolution rate criteria. If some components in the mixture do not achieve the solubility or dissolution rate criteria, the other appraisal routes should focus on these components.

2.3.4.2 Information required and methodology

A solubility test or dissolution rate test is required similar to those mentioned under Sections 2.3.1. and 2.3.2. respectively but using a solvent relevant for the intended uses. The OECD Test No. Guideline 116: Fat Solubility of Solid and Liquid Substances, with specific considerations for small particles, should be used as reference.

\textsuperscript{15} Upper bound of the physiological pH in the stomach.

\textsuperscript{16} If solubility is pH dependent and the criterion is not achieved at pH=7, it would be sufficient to demonstrate that the criterion is met at pH=3.
2.3.4.3. Principles

The approach related to solubility and dissolution rate extrapolates the principles of Sections 2.3.1 and 2.3.2 to substances marketed in a non-aqueous matrix or when all intended uses require a dispersion in a non-aqueous matrix (e.g. oil or fat) before consumption. If the criterion is achieved, the material will be fully dissolved in the matrix and, therefore, a conventional risk assessment is sufficient and further assessment of nanoscale properties is not needed.

3. Characterisation of the size distribution of the material including the fraction of small particles

3.1 Introduction

This Section includes the information on the particle size distribution that should be included in the application/dossier when the criteria on solubility and dissolution rate dealt with in Section 2 are not met. It may also be used as an appraisal route without details on solubility or dissolution rate.

For practical reasons and in line with current methods for measuring particle size distribution, the information requirements are divided in two steps: the characterisation of the particle size distribution of the full material and the characterisation of the fraction of small particles (≤ 500 nm). This characterisation is also needed for applying the appraisal routes described in Section 4.

Particle size is a distribution and should be described accordingly (e.g. see Section 3.4.2.3 for describing the particle size distribution for materials contains a fraction of small particles).

It should also be noted that the fraction of small particles, including nanoparticles, require specific attention with regard to the representativeness of sampling and proper dispersion state. All measurements of particle size distribution should ensure a proper dispersion of the sample in line with the recommendations presented in Section 3.1. These recommendations should be also considered for preparing the samples for conducting safety studies and when interpreting results of the studies. Also, possibilities for deagglomeration of the small particles under different testing and physiological conditions should be considered in risk assessment (OECD, 2012; Peters et al. 2012 (ACS NANO 2012)).

3.2. Dispersion protocol for sample preparation

Dispersion of materials composed of small particles (usually powders) in liquids is an important step in the sample preparation for most common particle size measurement methods. The dispersion procedure used may influence the particle size distribution measurements. For material characterisation, the final liquid dispersion of the material should result in a particle size distribution that consists of the smallest dispersible particles (primary or constituent particles). 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 application in in vitro or in vivo toxicological tests.

Due to the likelihood of high physical and chemical variability amongst most materials, a generally applicable standard operation procedure (SOP) is currently not available for
dispersion of a dry powder in liquids. The following general steps should be considered when developing a dispersion procedure (Mech et al., 2020c):

i. Choice of media, pre-dispersion and wetting can be the main limitations for an instrumental method used later; also inertness of the particles towards the media, i.e. particles do not dissolve or swell in the selected media.

ii. Choice of the method for deagglomeration of the material; the input energy used – i.e. is it sufficient to disaggregate without changing the particle morphology. A general choice for most materials with a fraction of small particles is ultrasonic treatment using either a probe or vial sonicator.

iii. Stabilisation is the final and complex step in the process, therefore consideration of the choice of stabiliser is needed, along with other possible variables such as compatibility of the stabilisation method with the measurement method. Effectiveness of the stabilisation including the timescale for which stability must be retained.

Concerning step (ii), it has to be noted that sonication may introduce a significant variation in the dispersing process due to the differences amongst the variety of sonication instruments that are available on the market. In order to reduce this variability, a ‘Generic SOP for calorimetric calibration of an ultrasonic probe sonication’ has been developed (Mech et al., 2020c). This SOP allows harmonisation of the different sonication instruments and methods (probe versus vial) as well as determination and definition of the actual energy effectively transferred to the test sample.

For the purposes of this Guidance on Particle-TR, a dispersion protocol can be considered effective if it yields samples which consist as much as possible of non-agglomerated/non-aggregated particles. To monitor the effectiveness of a protocol, analytical methods, which can reliably distinguish constituent particles from agglomerates and aggregates, are required. Suitable methods are those based on electron microscopy (EM) techniques, such as SEM or TEM. As EM methods require dry samples, care must be taken to optimise the specimen preparation to minimise re-agglomeration of particulates during specimen drying. If it is not possible to verify agglomeration and aggregation state with EM, efforts should be made to empirically verify that the protocol has been optimised to produce the minimum mean particle size possible with the facilities available for dispersion. To do this, the dispersion steps should be applied systematically using different sonication times and fixed power settings with the mean particle size being measured by methods such as CLS or DLS. The most suitable sonication time and power should be chosen as the time when further incremental increases in sonication time and/or power do not result in significant further changes in mean size (Rauscher et al., 2019a).

As mentioned above, there is no universally applicable test protocol for preparing stable dispersions of materials with a fraction of small particles. On the other hand, in the scientific literature numerous specific methods for certain types of particles are reported. A more systematic approach has been proposed in the NanoDefine EU-project, where a specific optimised dispersion protocol was developed for a number of priority nanomaterials and laid down in the form of SOPs (Mech et al., 2020c). Examples of these substances are CaCO$_3$ (fine grade), BaSO$_4$ (fine and ultrafine grade), kaolin, coated TiO$_2$ (pigment grade), zeolite powder or MWCNT (highly tangled fibrous carbonaceous material). For each of these materials specific dispersion requirements need to be met, along with corresponding types of
dispersants, stabilisers and sonication conditions. Typical probe sonication dispersion conditions involve applying energies between 2.3 kJ to 25 kJ into sample volumes of 6 ml to 10 ml. This equates to applying energy densities from 600 J/ml to 2500 J/ml sample volume. These SOPs can serve as a starting point or guide templates for developing protocols for other materials on the basis of physicochemical similarities with one of the standard nanomaterials used in developing the SOPs.

As elaborated in a JRC report (Rauscher et al., 2019a) the effective stability (resistance to re-agglomeration and/or selective sedimentation) of a prepared sample for the time between sample preparation and the end of the measurement should be verified. In order to check the stability of dispersions, periodic verifications should be made of the apparent particle size distribution from stock dispersions using at least one technique which is sensitive to changes in particle size and quantity. CLS can be used to very sensitively reveal any change in size distribution, and therefore CLS can be regarded as well suitable for such verification - assuming the expected particle size range and density are compatible with the lower size limit of the CLS instrument being used. Re-agglomeration changes the particle size distribution and often leads to sedimentation. However, certain dispersions may change over time due to gravitational sedimentation only (without re-agglomeration); this can be easily reversed and therefore would not be regarded as instability of the dispersion. Before verifying size distributions with CLS or other appropriate alternative the sample dispersion should be re-homogenised by a short period (few minutes) of bath sonication.

After dispersion particles may re-agglomerate. Therefore, it is important to ensure that suspensions are prepared with the intention of achieving sufficient dispersion stability to allow appropriate measurement of the needed parameters. Hence, further to optimising the dispersion energy mentioned above, additional steps should be taken to avoid re-agglomeration of dispersed particles for the applied measurement techniques. In the case of aqueous dispersions this may be achieved by natural electrostatic stabilisation and appropriate modification of the pH value. In many cases it may be necessary to add a dispersing agent or a surfactant. The choice of, e.g., surfactants depends on the intrinsic properties such as hydrophobicity, surface charge and chemical functionality of the specific material under consideration. Commercially available chemical surfactants e.g. phosphate, sulfonate or polyethylene glycol and others have been successfully applied for the above-mentioned priority nanomaterials. Where applicable, measurements of the zeta potential measurements can be useful to check whether a dispersion is expected to be stable for a sufficiently long period of time. An absolute value higher than 25 mV would be indicative for sufficient stability. (Dukhin AS and Xu R, 2020). As a guide, a minimum required stability time of 30 minutes has been suggested for these priority nanomaterials for the various applied measurement techniques as described by Mech et al., 2020c. Other protocols for the preparation of nanomaterial dispersions by sonification and guidance for optimisation of the tests conditions and process parameters have also been published elsewhere (Taurozzi et al., 2012a,b).

Concerning characterisation of the dispersion stability, ISO Guidelines (ISO, 2013) and an OECD test approach for nanomaterials in simulated environmental media (OECD, 2017) have addressed the key aspects and described a method based on dispersion and re-agglomeration characteristics of nanomaterials.
It is recommended that applicants follow the ISO and OECD guidelines mentioned above to adopt appropriate methods for preparing stable dispersions of their materials. In case of a broad particle size distribution with a high content of large particles, it may be necessary to remove the larger sized particle fraction by means of filtration or centrifugation, and then apply the guidelines to the remaining smaller particle fraction.

3.3. Characterisation of the particle size distribution

The characterisation of the particle size distribution is needed for all materials for which the solubility or dissolution rate cannot exclude the likelihood of exposure of consumers to undissolved particles. Considering currently available methods, a practical threshold of 500 nm as the upper limit for the range of “small particles” is proposed. When a fraction of small particles is detected, the information provided in this section on the characteristics of this fraction is also needed for the appraisal routes described in Section 4, and consequently should be provided by the applicant even when the appraisal criteria are not met.

3.3.1 Particle size distribution of the material

3.3.1.1 Decision criteria

When the material is not expected to have a fraction at the nanoscale, the applicant should demonstrate that the particles are larger than 500 nm after a proper dispersion of the test material. The level of detection of the method(s) used for this assessment should provide convincing evidence that the material contains less than 10% particles (number-based) smaller than 500 nm.

3.3.1.2 Information required and methods

Different methods can be used for characterising the particle size distribution of the material. For the assessment of the fraction of small particles, these methods should be suitable for detecting particles smaller than 500 nm.

Methods allowing the detection of small particles include:

- Centrifugal Liquid Sedimentation (CLS)
- Particle tracking analysis
- Descriptive Electron Microscopy
- Filtration complemented with chemical analysis

The applicant should select the method(s) according to the characteristics of the material and justify the selection. For each method, a description of the test conditions, including the sample preparation and dispersion protocols, the range of particle sizes covered by the method, and the level of detection should be reported. The selected method or methods should provide convincing evidence that the material contains less than 10% of particles (number-based) smaller than 500 nm. A combination of different methods may be needed in some cases.

3.3.1.3. Principles

This appraisal route offers the applicant the possibility to confirm that the particles are larger than 500 nm using screening methods. Considering the currently available methods and their
capability in terms of the particle size range and limit of quantification of the methods, a pragmatic threshold for ensuring the capability of the selected method(s) for detecting small particles is proposed. The value of 10% number-based for particles smaller than 500 nm implies that the total mass of particles at the nanoscale would be very low, and a conventional risk assessment is sufficient and no further assessment of material properties at the nanoscale is needed.

3.3.1.4. Centrifugal Liquid Sedimentation (CLS)

A relatively quick and economic method of ascertaining the presence of small particles in a dispersion of particulate material is through analysis by Centrifugal Liquid Sedimentation (CLS). CLS is a scaled-down version of analytical centrifugation that is designed in the form of a rotating disc, which contains a thin layer of liquid sucrose gradient. When a sample of particle dispersion is applied at the centre of the disc, which is rotating at around 24,000 rpm (30,000 G-force), the centrifugal force leads the particles to sediment and travel outwards at velocities dependent on their particle size and morphology. Using X-ray diffraction or optical detection, a typical equipment can measure the velocity of particle sedimentation to calculate particle size distribution against standards of known particle density and size. The equipment is generally also accompanied with proprietary software that can convert mass-based particle size distribution into number-based values on the basis of particle morphology and density. The method is regarded as quick, highly-reproducible, and with a good sensitivity that can range from 3 nm to several μm (e.g. see validation study by Braun et al., 2011). Analysis of low-density materials can require much longer times, but special discs are also available that can reduce the run time.

There is an ISO standard 13318 for the method, which is also mentioned in the REACH guidance R7.1 for the characterisation of nanomaterials. Standard operating procedures for CLS have been developed by the NanoReg project for particle size determination17.

3.3.1.5. Particle tracking analysis

The evaluation of the number-based particle size distribution in liquid dispersions can be effectively carried out using the particle tracking analysis (PTA) method for diffusion velocity measurements. PTA, also known as nanoparticle tracking analysis in some instrumental versions, is a single particle technique where scattered laser light is recorded with a microscope and a digital camera. The Brownian motion of the particles, as recorded in a video, is processed by the software to determine the diffusion coefficient for each individual particle and from it calculate the hydrodynamic diameter.

By means of PTA the sizing and number concentration measurement of particles in the size range of one or few tenths of nm (depending on the chemical nature of the material) up to 1 μm approximately can be performed in aqueous suspensions. PTA measures the hydrodynamic diameter using the spherical model as DLS does but suffers less perturbation from large particles in polydisperse samples compared to DLS. However, the measured hydrodynamic diameter can be affected by the salt concentration of the suspending medium, pH and concentration of the dispersant. Therefore, the effects of these parameters should be investigated or controlled. For instance, when performing sample dilutions to obtain the

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17 NANoREG D2.08 SOP 03, available via the NANoREG Results Repository.
optimal concentration for PTA measurement – which is in the range $10^6$-$10^9$ particles/mL – it is important to ensure that the ionic strength, stabilizer concentration and pH are kept constant. All these parameters should be documented and reported. Reduction of dispersant concentration and changes in pH can lead to particle agglomeration and increase in polydispersity of the sample.

There is an ISO standard for the PTA method (ISO 19430:2016), whereas other sources are available which describe method validations (Kestens et al., 2017), interlaboratory comparisons (Hole et al., 2013; Maguire et al., 2017), and provide SOPs (EUNCL, 2018).

### 3.3.1.6. Descriptive Electron Microscopy (dEM)

To assess the presence or absence of nano-objects, and before more advanced quantitative analyses are initiated, a detailed description of the material is a first and essential step to determine the basic properties of the examined nano-objects and to assess the quality of the sample and specimen preparation.

This method may be used to confirm that the fraction of small particles is just a tail resulting from a manufacturing process of a powder and that all particles have similar characteristics except for size.

Based on a series of representative electron micrographs recorded at magnifications typically ranging from 100 to 100,000 times, and covering the entire specimen, an overview of the specimen, and the properties of the nano-objects of interest such as size, shape, surface and inner morphology, and crystallographic structure, can be visualised by the electron microscope. A descriptive EM analysis includes, at least, representative and calibrated micrographs, and a description of the following parameters: (i) the roughly estimated size (distribution) of the primary and aggregated/agglomerated particles; (ii) the agglomeration and aggregation status; (iii) the general morphology; (iv) the surface topology; (vi) the structure (crystalline, amorphous, etc.); and (vii) the presence of contaminants and aberrant particles. If contaminants are observed, such as salt crystals, organic matter, protein agglomerates, lipids, other type of particles (e.g. catalysts), they should be described and reported; in addition, selected micrographs can highlight unusual or rare features, such as impurities and crystal defects.

When nano-objects are observed, a descriptive EM analysis should further report the specimen quality describing possible impurities and artefacts, the number of particles in the EM specimen, their contrast with the background and how even they are distributed on the EM specimen. On the basis of these parameters, the relevance and suitability of a quantitative EM analysis of the examined specimen, and the need for optimization of the sample and specimen preparation should be assessed.

The characteristics of the nano-objects of interest such as aggregates and agglomerates, and primary particles, are described in Appendix I Table AI-1.

For each sample, the report of a descriptive EM analysis must include a detailed description in text form of the above-mentioned seven parameters. To categorize and describe the shape of aggregates and agglomerates (Appendix I, Figure AI-1A), the system of Lopez-de-Uralde et al. (2010) is proposed. To categorize and describe the surface topology (Appendix I, Figure AI-1AB), and the shape of constituent particles (Appendix I, Figure AI-2), the systems of Krumbein and Sloss (1963), and Munoz-Marmol et al. (2015), respectively, are proposed.
Further, calibrated micrographs must be provided giving an overview of the sample, as well as calibrated micrographs showing the (smallest) constituent particles. The magnification of the latter can be determined based on the criterion of Merkus (2009).

For convenience, a tabulated summary of this description, as exemplified in Appendix I, Table AI-1, is proposed for efficient reporting of the descriptive EM results assuring uniformity in reporting, reducing misunderstanding by suggesting a set of possible descriptions.

3.3.1.7. Filtration complemented with chemical analysis

The approach described in Section 2.3.2, based on filtration and analytical determination of the substance(s) constituting the particles before and after filtration, can be used to gain an insight into the mass-based particle size distribution of a material and, specifically, to assess the fraction of small particles (<500 nm).

A sequential, stepwise approach should be used to characterize the mass fraction of (i) particles ≥500 nm, (ii) particles <500 nm, and (iii) dissolved species.

As far as the mass fraction of particles ≥500 nm is concerned, several membrane filters with pore sizes ≥0.5 µm should be used and the results appropriately described and interpreted. Membranes made of different materials should be compared and data which are worst case (i.e. indicating the lowest amount of particles ≥500 nm) should be selected for the size characterisation. This is due to the fact that filtration overestimates the amount of larger particles because a fraction of particles smaller than the pore sizes are always retained on the membrane due to the build-up of material on the membrane surface and physicochemical interactions of the particles with the membrane material. In some cases, filtration through membranes with (considerably) larger pore sizes may be needed beforehand to remove very large particles (including potential agglomerates and aggregates) and prevent obstruction of the 0.5 µm membrane.

For the characterisation of the small particles (<500 nm), the filtrate ('worst-case') has to be submitted to ultrafiltration using a membrane filter with pore sizes in the range 3-100 kDa (10 kDa membranes are appropriate for most materials). The membrane will retain the small particles and the filtrate will contain only soluble components.

The mass-fraction corresponding to the small particles (corrected for the soluble fraction, if any) has to be compared to the one of the particles ≥500 nm. The particle size distribution data can be converted from mass- to number-based by using assumptions (e.g. spherical particle shape) and appropriate algorithms. Range of plausible values should be obtained using upper- and lower-bound values for the number-based particle size distribution.

The measurement process should be clearly documented and quality control demonstrated by using an adequate number of (certified) reference materials (or, if not available, size control samples) matching as close as possible the chemical composition and the physicochemical properties of the material to be characterised. The experimental design and the results should be discussed in a transparent way and the conclusions justified.

3.4. Characterisation of the fraction of small particles

If a fraction of small particles (particles ≤500 nm) is expected or identified, the applicant should provide additional information on this fraction, starting with a description of the
particles, e.g. using descriptive EM as presented in Section 3.2.1.6, followed by a quantitative
assessment of the size distribution of the fraction of small particles. A proper dispersion of the sample according to the recommendations in Section 3.1 is essential.

3.4.1 Identification of a fraction at the nanoscale

In line with the Guidance on Nano-RA, no specific concern regarding the nanoscale is needed for particles larger than 250 nm.

3.4.1.1 Information required and methods

Electron microscopy is the recommended method for the quantification of the size distribution of the fraction of small particles. The applicant should justify the use of other methods and provide sufficient evidence on the limit of particle size detection and the level of dispersion avoiding the aggregation/agglomeration of the particles.

3.4.1.2 Decision criterion

When the fraction of the material with a particle size below 500 nm contains less than 10% particles (number-based) smaller than 250 nm, the material is adequately covered by the conventional safety assessment and does not require an additional assessment for the fraction of small particles. This criterion can also be applied to mixtures. Each substance in the mixture should be tested separately and meet the criterium. If some components in the mixture do not meet this criterion, additional information on the fraction of small particles of those components should be presented.

3.4.1.3. Principles

This consideration implements a pragmatic trigger for materials consisting of or containing a significant fraction of small particles, to be applied when the large majority of the material is not at the nanoscale but a tail of particle size distribution at the nanoscale may be present due to the manufacturing process. The value of 10% number-based for particles smaller than 250 nm implies that the total mass of particles at the nanoscale would be very low, and a conventional risk assessment is sufficient and no further assessment for properties at the nanoscale is needed.

In addition to the numerical criteria, a description of the fraction of small particles (i.e. particles smaller than 500 nm) should confirm that the particles at the nanoscale are just part of the size distribution tail resulting from a manufacturing process of the powder and that all particles have similar characteristics except for size.

3.4.1.4. Specimen preparation for EM analysis

Specimen preparation for EM analysis aims at depositing on a carrier suitable for EM analysis a fraction of particles which optimally represents the particles in the sample and which allows detecting and measuring the constituent particles individually. Critical parameters that need to be controlled and reported are the level of dispersion of the particles, the type of carrier,
the compatibility of the particle charge with the charge of the carrier and the particle concentration.

The quality of EM particle size and shape measurement results strongly depends on sample and specimen preparation. Standard methods for depositing (nano)particles on measurement substrates are described, for example, in ISO/DIS 19749 for SEM and ISO/PRF 21363 for TEM. There is, however, no single best preparation method for all particles, rather suitability of the methods is determined by the characteristics of nanoparticles and the measurand, the quantity or property to be measured. It is indispensable to ensure that the EM specimens are representative and relevant to the measurand. The knowledge about and the number of methods of nanoparticle deposition is growing rapidly, and it is advisable to explore and use the most suitable methods from the available literature that are specific to the nanoparticle measurand and the material type (Vladár et al., 2020).

Suspensions of particles in their most dispersed state, prepared as described in Section 3.2 "Sample preparation (liquid dispersion) for particle size measurement" are suitable to prepare specimens for both descriptive and quantitative EM analyses.

Detailed guidelines to select suitable carriers for TEM analysis, approaches to tailor the EM grids to the particles and to deposit and concentrate particles to the EM grids are proposed in Mast et al., 2015, and Mast et al., 2020 and in CEN/TS 17273. CEN/TS 17273 provides specific guidance on the preparation of EM specimens for detection and identification of nano-objects in complex matrices using electron microscopy. A detailed standard operating procedure on EM specimen preparation: “Preparation of EM-grids containing a representative sample of a dispersed nanomaterial” describing how to bring a dispersed nanomaterial in contact with an EM-grid, and to select the appropriate concentration of the nanomaterial, and the type and charge of the grid is provided by NANoREG D2.10 SOP 0118.

Specific guidelines for SEM sample specimen are described by Vladár et al., 2020.

For TEM and STEM analysis, EM grids are typically applied as sample carriers. For SEM analyses, various types of carriers, including SEM stubs, are applied. All TEM specimens are, however, suitable for SEM imaging, so nanoparticle preparation methods published for TEM imaging and measurement also apply to sample preparation for SEM. The use of TEM grids is recommended as a carrier for all types of EM analysis (TEM, STEM and SEM), to optimally control specimen preparation conditions and obtain best limits of detection (CEN/TS 17273:2018). Approaches to prepare EM specimens from dry powders, often applied to prepare conventional SEM specimens (references SEM), are only suitable if particle properties are monodispersed or if particles are well separated and not (strongly) agglomerated.

A sufficiently high number of particles has to be deposited, ideally as single particles, to assure efficient analysis of a representative fraction of particles. At the same time however, care has to be taken not to overload the carrier with particles because overlapping particles will limit particle detection and the applicability of image analyses methods to accurately measure the particles. Overlay of particles, certainly in multiple layers and of polydisperse materials, must be avoided, because it results in larger particles blocking imaging of smaller particles and consequently in biased measurements. As a rule of thumb, a coverage with nanoparticles of a few percent of the TEM grid surface is well suited for descriptive and subsequent quantitative

18 NANoREG D2.10 SOP 01, available via the NANoREG Results Repository.
EM analysis with (automated) image analyses. In case samples are very polydisperse in size, simultaneously containing micrometer- and nanometer-sized particles, omission, e.g. by sieving, of the largest particles that can cover large parts of the EM-grid, might be necessary to make detection of the fraction of nanosized particles possible.

3.4.2 Characterisation of the fraction of small particles by quantitative Electron Microscopy

When the decision criteria described under Sections 2 and 3 above are not met, the applicant should provide a characterisation of the fraction of small particles using electron microscopy. Electron microscopy allows to visualise and identify individual nano-objects of interest based on specific criteria such as size, shape, crystallographic structure, and elemental composition. It is one of the few methods that, to some extent, allows to identify constituent particles in aggregates and agglomerates. Due to its high spatial resolution in two dimensions, EM analysis covers a size range of 1 nm to 1000 nm, depending on the type and configuration of the EM. Possibilities and limitations of quantitative EM to characterise the fraction of nano-sized particles are reviewed in Mast et al., 2015 and Mast et al., 2020.

By combining EM imaging with image analysis, the properties of individual nano-objects, such as the size, shape and surface texture, can be estimated from their two-dimensional projection. Combining these measurements, the material can be characterised quantitatively as number-based distributions and corresponding descriptive statistics of selected measurands.

Guidance on the determination and reporting of size and shape distributions based on SEM and TEM images of both nanoscale and larger than nanoscale particles is provided in ISO 19749 and ISO 21363. Guidance on sample preparation, particle detection, imaging and identification of nano-objects in complex matrices is provided in CEN/TS 17273. This guideline is also useful for EM analysis of relatively pure materials and materials in simple matrices.

3.4.2.1 EM imaging

EM imaging for quantitative EM analysis aims to record a set of calibrated electron micrographs showing objects that are representative of the objects deposited on the EM carrier, typically an EM-grid. The conditions for TEM, SEM and STEM imaging suitable for quantitative particle analysis can be determined from the following general guidelines.

Before image analysis is initiated, particular attention has to be given to the optimisation of the dispersion and specimen preparation protocols because poor separation and overlap tend to bias particle identification and measurement. Image analysis is much easier and gives more accurate and precise measurements when particles are well separated and not overlapping (Dudkiewicz et al., 2019; Verleysen et al., 2020).

Before analysis, the electron microscope must be suitably aligned to reach lens conditions optimal for EM imaging, and calibrated using appropriate (certified) reference materials to assure precise and accurate measurements related to the international system of units (SI). Guidelines for EM alignment and calibration are provided in the magnification calibration guidance document ISO 29301.
The magnification for EM imaging should be selected such that the useful working range covers the size distribution of the analysed particles. This useful range is determined by the lower and upper size quantification limits, as defined in CEN/TS 17273:2018. Briefly, the magnification should be selected in such a way that the size of the smallest particle to be measured, which is estimated during the descriptive TEM analysis (Section 3.3.1.6) complies with the criterion of Merkus (2009). This criterion requires that the area of equiaxial particles consists of at least one hundred pixels ensuring that large systematic deviations in size measurements are avoided. To measure the dimensions of non-equiaxial particles, as a rule of thumb, the minimal external particle dimension should consist of at least 10 pixels (CEN/TS 17273:2018). The upper size quantification limit is set to the size of pixels making up one-tenth of the image size in one dimension, according to ISO 13322-1. Post analysis, the appropriateness of the chosen magnification can be evaluated based on the percentages of particles smaller and larger than, respectively, the lower and upper limits of quantification. These percentages should be reported.

The applicant must assure that the recorded micrographs are representative for the specimen, avoiding selectivity during imaging, for example, by applying a random and systematic imaging scheme. De Temmerman et al. (2012) and Verleysen et al. (2014) addressed this by recording images at several positions, pre-defined by the microscope stage, that were distributed over the entire grid.

### 3.4.2.2. Image analysis

Particles visualised in electron micrographs can be detected and measured using manual and (semi) automated approaches provided that individual nano-objects of interest can be detected and identified based on criteria such as mass-thickness contrast, elemental composition, size, morphology, and crystallographic structure. Manual particle measurement is still applied for complex samples and particle types (Hougaard et al., 2013) and to validate new, automated approaches (De Temmerman et al., 2014). It is however prone to operator bias, measures only a limited number and types of measurands, and is relatively time-consuming and labour-intensive. In many cases, (semi-)automatic approaches are more efficient and accurate. ISO 13322 provides a standardised description of the technique used to analyse particles, including nano-objects, in static images, such as electron micrographs.

Specific guidance for TEM-based particle size and shape measurement is provided in ISO/PRF 21363 and CEN/TS 17273:2018. Many of these guidelines can be applied for SEM and STEM analyses too. A typical image analysis consists of an image preparation step, the setting and adjusting of the threshold value for particle detection, the definition of the detection area, the setting of the detection parameters and the detection of nano-objects and the selection of measurands and measurement of nano-objects (CEN/TS 17273:2018).

Approaches for image preparation, algorithms to suppress the background signal and to identify constituent particles in aggregates and agglomerates are rapidly evolving. They should be optimised for types of particles and overlap of particles (Wagner, 2016).

A range of size and shape measurands (descriptors) can be measured according to ISO 9276:2008 in function of the purpose of the analyses, but reported measurands should include, at least, an estimate of the minimal external particle dimension, such as the Feret Min diameter, and the aspect ratio, quantifying the particles shape.
3.4.2.3. Representation of measurement results

ISO standards provide detailed guidelines for the representation of the results of analyses of particle size (ISO 9276:2014 and ISO 92765:2005), particle shape and morphology (ISO 92766:2008), the fitting of distributions (ISO 92763:2008) and the classification of particles (ISO 92764:2001). Each report should present at least the number-based size and shape distributions (Feret Min and aspect ratio), and the corresponding descriptive statistics (Median, Q25, Q75, % <100 nm, % <250 nm).

The results should, as for any other quantitative analytical methods, be reported with an estimate of the measurement uncertainty obtained in a validation study. Examples of EM method validation studies are published by Dudkiewicz et al., 2015, Waegeneers et al., 2019, Verleysen et al., 2019, and Verleysen et al., 2020. One of the factors that determine the robustness of the measurements is the number of analysed particles. By the numerical method of Masuda and Gotoh (1999) described in ISO 13322-1:2014 which assumes a log-normal distribution of the relevant measurand, and by determining the relation between the number of measured particles and the measurement uncertainty for the relevant measurand (De Temmerman et al., 2013; De Temmerman et al., 2014; Verleysen et al., 2015), the number of particles required to estimate a measurand with a known uncertainty can be objectified.

3.4.3 Considerations for materials with broad specifications

For the characterisation of the fraction of small particles of the marketed material as described in Section 3.4.2, the following elements should be considered when materials with different characteristics are marketed under the same specifications.

The specifications should include acceptable ranges for each physicochemical parameter according to the Guidance on Nano-RA, and the applicant should provide safety data taking into account and closely matching those ranges. Manufacturing of a material by different processes, or by the same process but under different conditions or by different manufacturers, can potentially alter the physicochemical characteristics of the material. Where data on different batches indicate a significant difference in the physicochemical characteristics between batches, it is important that detailed specifications of the material produced by different processes or conditions are provided by the applicant. The applicant should also provide information on the characteristics of the test material used in the safety studies. In case of incomplete characterisation of the test material, detailed information on the production process and conditions should be provided. The applicant should also provide information on the effect of ageing on the materials including particle agglomeration, aggregation, and sedimentation behaviour.

The applicant should indicate the physicochemical characteristics considered worst case for the safety assessment. Such a rationale should comprise considerations for toxicokinetics (e.g. absorption, distribution, accumulation) as well as hazard. This exercise is similar to that of read-across, as elaborated in Section 6.3 in the Guidance on Nano-RA. If such a rationale cannot be provided, multiple representative materials would need to be investigated that cover all physicochemical properties within the provided ranges. This may, for example, be the case, when different shapes and surface coatings, coating thickness or surface properties are present in the material according to the specifications provided by the applicant.
On the basis of this information, a risk assessor can decide whether the batch(es) used in toxicity testing cover(s) the worst-case situation and therefore, can be used in risk assessment of the material. For example, it can be checked whether a material that represents the size distribution with the smallest particles sizes of the possible range has been tested, since these materials can be assumed to be orally absorbed to a greater extent than materials composed of larger particles and are typically more reactive due to their larger surface area.

For mixtures and formulations, a description should be provided to indicate the form in which the material is present in a mixture or formulation, e.g. powder, dispersion. The information should cover all components, as well as dispersants/stabilisers and other auxiliaries (e.g. preservatives, processing aids, etc.) used.

4. Evidence to be submitted on safety studies conducted without documented consideration of the properties of small particles

This Section focuses on the information to be provided on safety studies originally designed for addressing conventional materials, in order to ensure that the possible adverse effects linked to the fraction of small particles, including nanoparticles, can be identified by the study. These studies are referred here as “existing safety studies”.

There are two essential conditions to be followed by the applicant, interested parties, and risk assessors, regarding existing safety studies that were not originally designed to specifically consider the presence of a fraction of small particles, including guideline studies and scientific publications retrieved from literature reviews:

1. The available information should demonstrate that the test material includes the fraction of small particles. In case of broad-range technical specifications of the bulk material that is placed on the market, the full range of small particles or at least the conditions representing the worst case, has to be covered by the test materials used in the existing studies.

2. The study selection, study design and the level of dispersion of the test material, should be appropriate for assessing the hazard of small particles including nanoparticles.

These elements are detailed in the Sections below. In addition, some recommendations are provided for deciding how to gather additional information when the available studies do not cover the possible hazards associated to the fraction of small particles.

4.1. Information to demonstrate that the tested materials used in the existing safety studies included the fraction of small particles

A comparison of the characterisation of the particles (see Section 3) in the material as marketed and in the material used for the safety studies should be presented for each study. In line with the Guidance on Nano-RA and ECHA (2019), the information should include: chemical composition (confirming identity or presenting the differences with that of the bulk material), size, crystallinity, shape, purity, and surface modifications, if relevant. If this information is lacking in the study report but the batch used as test material is still available.
and the stability of the material, including its particle size distribution, can be confirmed, this information should be generated.

If the test material is no longer available, the comparison should focus on the manufacturing process in order to demonstrate that the test material was produced by the same manufacturing process and current production conditions, and that the test material was in compliance with the current material specifications. Information regarding the characteristics of the fraction of small particles in the manufactured material, should include variability between the batches from the same production process, and between batches from different processes when applicable. If the manufacturing process has been modified, information addressing the changes regarding the fraction of small particles, focusing on chemical composition (confirming similarity or presenting the differences with that of the bulk material), particle size distribution, crystallinity, particle shape, purity, and any particle surface modifications should be provided.

For materials with considerable variability in particle size distribution, or in case of variations between the tested material and the current specifications, the applicant should provide justification that the tested material covers “worst case conditions” regarding the fraction of small particles e.g. the test material contains a larger proportion of particles in the nanoscale and/or higher level of dispersion of aggregates and agglomerates than the material intended for the market. When comparing the characteristics of the test material with the specifications of the material currently marketed, applicants may consider the principles for grouping and read-across of different nanoforms of the same substance to support their conclusions.

The applicant should provide at least the characterisation of the marketed material including particle size distribution (see Section 3) and variability between production processes and batches, and a comparison with the description available for the test material.

When the available information is insufficient for confirming the presence of the fraction of small particles in the test material, a case-by-case judgement is required in order to decide if a new toxicity study should be provided. Recommendations are presented under Section 4.3.

In addition to the studies conducted with the full material, safety studies conducted with the fraction of small particles, or with a specific set of nanoparticles, are relevant for the risk assessment, provided that the characteristics of the tested particles are as much as possible similar to those of the equivalent fraction present in the marketed material. The possibility for read-across may be considered when the characteristics of the tested particles deviate, in terms of particle size or other features, from those reported for the particles present in the marketed material.

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19 ECHA (2017a,b,c; 2019) and several research projects, including GRACIOUS EU H2020, have proposed frameworks for the read-across of nanoforms and nanomaterials that could be adapted and used for comparing the characteristics of the fraction of small particles in marketed and tested materials.
4.2 Adequacy of the study for assessing the hazards of the fraction of small particles: selection, design and dispersion

In general, the safety studies, conducted according to the EFSA guidance documents and OECD test guidelines for assessing the toxicity of conventional materials, are considered to sufficiently cover the hazard assessment of small particles present in conventional materials provided that the study duration is adequate and some specific precautions are implemented to ensure sufficient dispersion of the nanoparticles, as described in Section 4.2.3. Additional considerations are required for genotoxicity and toxicokinetics.

For repeated oral toxicity studies, the duration of exposure should be sufficient to address the potential hazard of small particles. The sectoral guidances for food additives (EFSA ANS Panel, 2012), feed additives (EFSA FEEDAP Panel, 2017) and novel foods (EFSA NDA Panel, 2018), consider a 90-day animal study (OECD TG 408) as the first tier for the assessment of toxicity. The Guidance on Nano-RA considers that OECD TG 408 has sufficient duration for the safety assessment of particles at the nanoscale; the appraisal of the capacity of OECD TG 408 guideline studies for addressing potential hazards related to the fraction of small particles should focus on appropriate exposure, which is achieved by good dispersion as detailed in Section 4.2.3.

The capacity of small particles to produce local effects, such as inflammation, at the site of first contact should be considered. In case of oral exposures, this requires sufficient information on the histopathological assessment of the GIT demonstrating for instance that the epithelia of the different GIT parts and Peyer patches were examined. The OECD TG 408 describes the tissues to be covered for the histopathological assessment of the GIT; the area covered by the assessment should be large enough, considering that possible local effects of particles are expected to be less homogeneously distributed than those produced by the soluble fraction. If inflammatory responses or other local lesions in the GIT are observed, the possible role of the fraction of material present as small particles should be investigated. This may require additional mechanistic studies to elucidate the mode of action of small particles.

4.2.1 Genotoxicity testing for materials containing a fraction of small particles

The applicant should submit results of two different in vitro assays on mammalian cells, one covering induction of gene mutation and the other covering structural and numerical chromosome damage, in line with Section 6.4 of the Guidance on Nano-RA (2018 revision). In case of equivocal results the applicant should refer to EFSA guidance on genotoxicity (EFSA SC, 2011; EFSA SC, 2017).

The bacterial AMES test is not a suitable method for assessing the genotoxicity potential of the fraction of small particles; the mammalian cell mutagenicity test (OECD TG476) is an appropriate alternative (see Section 6.4 of the Guidance on Nano-RA (2018)). It has been shown that small particles are not taken up by the cellular system of the AMES test and this may lead to a false negative result. However, in case of an already available AMES test, a positive result should not be disregarded, as this may be linked to indirect genotoxic effects.
due to extracellular induction of reactive oxygen species or to the genotoxic potential of the dissolved fraction or degradation products.

**4.2.2. Toxicokinetic studies for materials containing a fraction of small particles**

It should be considered that the dissolved and particulate fractions of the conventional material may have different toxicokinetic behaviours. In particular, the potential uptake of particles in the nanoscale by size-specific pathways in the GIT epithelia and accumulation in tissues should be investigated (e.g. see Section 6.6 in Guidance on Nano-RA). In addition to the general consideration on a sufficient level of dispersion (see Section 4.2.3), specific assessment is needed regarding the study duration and the sensitivity of the analytical methods used for determining the content of test material in tissues, e.g. ICP-MS (Aureli et al., 2020), as the pathways for particle uptake require more time and provide lower absorption rates than those for dissolved chemicals. Otherwise, the absorption may have remained undetected. The presence of small particles/nanosized particles may be studied quantitatively by single particle ICP-MS (Tassinari et al., 2014; Talamini et al., 2019; Peters et al., 2020), whereas ultrastructural localisation in tissues requires visual methods, e.g. electron microscopy, enhanced darkfield microscopy (Modrzynska et al., 2018) or enhanced darkfield hyperspectral microscopy (Campbell et al., 2017). The two latter methods can be applied on hematoxylin and eosin-stained tissue samples, which are used for microscopic diagnostic of changes in toxicity studies.

The applicant should evaluate and provide the available experimental data, e.g. ADME information from administration of different doses or comparison of concentrations in organs, such as liver or spleen, observed after exposure to several doses, to get an insight into whether the absorption of the material is dose-dependent.

**4.2.3. Adequacy of the study design of existing studies for covering the hazard assessment of the fraction of small particles**

In addition to ensuring that the fraction of small particles was present in the test material, information on the design and performance of any existing toxicity study is required to ensure that the study had addressed the specific characteristics of particles in the nanoscale. These should include information on preparation of the test material to understand whether or not it would have minimised aggregation/agglomeration of the particles, the matrix/vehicle used, and the method of administration.

As indicated in the Guidance on Nano-RA for *in vivo* oral studies, gavage or administration with food and through drinking water may be used and the method selection should be in line with the expected route of oral exposure. The applicant who submits an existing study should therefore justify the administration route used, describe the sample preparation and dispersion of the tested material, focusing on the potential for aggregation and agglomeration of the fraction of small particles and provide results of analyses at the achieved concentrations, and particle size distribution in the matrix/vehicle.
The information on solubility and dissolution rate of the test material, to be provided in line with the requirements in Section 2 of this Guidance, and the information for each study to be reported according to OECD test guidelines, are the starting points for assessing whether or not a sufficient level of dispersion was achieved. Appendix II summarises the information to be extracted and reported by the applicant for each existing study based on the current OECD test guidelines. Studies that were conducted under other guidelines or non-guideline protocols should be assessed on a case-by-case basis for analogy with the OECD test guideline requirements prior to being used for submission.

It needs to be kept in view that higher doses may result in a higher degree of particle agglomeration compared to lower doses. On the other hand, for materials that show some degree of solubility, the fraction of particles that is dissolved compared to that that maintain particulate nature depends on the concentration of the material dispersed in the testing medium to achieve the intended dose. Consequently, where complete dissolution is not expected from the doses used in an existing study, the assessment of solubility/dissolution rate, dispersion, aggregation and agglomeration, should be conducted separately for each dose.

The following principles should be considered by the applicant for evaluating the suitability of existing in vivo studies:

1. The lack of turbidity or a precipitate should not be taken as proof of full dissolution because particles in the nanoscale may not be detected as turbidity or as a precipitate visible by eye or with the aid of an inverted microscope.

2. If solutions/suspensions had been prepared during sample preparation for an existing study, the presence of small particles in the dispersion should be assumed for all concentrations exceeding the measured solubility (see Section 2).

3. Further dissolution in the GIT of the experimental animals could be considered when using the existing study. A pH value of 3 in the stomach and pH value of 7 for the intestine may be considered for material which solubility is pH-dependent (Ward and Coates, 1987). The water content in the gastrointestinal tract has been estimated in the order of 1 mL for the mouse and between 8 (fed) and 3 (fasted) mL for the rat (McConnell et al., 2008).

4. For multi-constituent substances and mixtures, the solubility of each constituent/component should be assessed independently when interpreting the adequacy of the existing studies and drawing conclusions from them.

5. For materials that show some degree of solubility, when the material was only partially solubilised (i.e. remained in part as particles) but was properly dispersed, the results are relevant for the safety assessment. The use of dispersion agents is generally expected to increase the level of particle dispersion and reduce agglomeration.

6. If the test material is still available and the stability is ensured, the applicant may consider repeating the dose preparation method used in the existing study and check for the presence of particles and the level of dispersion achieved at each dose, using EM or other suitable methods (see Section 3 for details).
For *in vitro* studies, if the level of dispersion, investigation of cellular uptake, and other considerations ensuring a proper coverage of the fraction of small particles, cannot be verified, the study should be repeated.

Following the considerations of the issues described above, the applicant should provide, for each relevant existing study, a detailed assessment of the capacity of the study for detecting and covering the fraction of small particles with properties in the nanoscale.

### 4.3. Criteria for requiring new studies and setting the assessment and testing strategy

When the applicant cannot demonstrate that the existing safety studies offer a proper coverage for assessing the hazards and risks of the fraction of small particles, additional considerations during the risk assessment are needed in order to decide if additional tests in line with the Guidance on Nano-RA (EFSA SC 2018, under update) are needed, particularly when the decision requires the replication of an existing *in vivo* study.

The need for new studies may be triggered by different reasons. These include the quality (i.e. reliability, relevance and level of reporting) of the existing study results, as well as concerns about the representativeness of the test material relative to the marketed or intended to be marketed material. In addition, updates of the guideline protocols, e.g. the 2018 update of the OECD TG 408 for addressing concerns on endocrine disruption, should be used in the weight of evidence for deciding if a new study is necessary.

When the existing toxicity studies are considered reliable and sufficient for assessing the safety of the bulk material, but the information on the presence of the fraction of small particles is lacking or only partially available, the applicant should:

1. Summarise the available information, in particular:
   1.1. the results of the safety assessment of the conventional material;
   1.2. the characteristics of the fraction of small particles (Section 3); and
   1.3. the areas of the safety assessment (e.g. genotoxicity, toxicokinetics, repeated oral toxicity, etc.) covered by studies with identified gaps for addressing the fraction of small particles (Section 4);
2. Identify, in a weight of evidence approach, the areas where safety can be demonstrated for the conventional material but there are still concerns regarding the fraction of small particles in the nanoscale;
3. For those areas of the safety assessment with insufficient coverage of the fraction of small particles:
   3.1. develop an Integrated Testing and Assessment Strategy (IATA) ([OECD, EC, Sakuratani et al., 2018](#)) to address the gaps and integrating the assessment of the fraction of small particles in the conventional assessment; or
   3.2. conduct a complementary assessment for the fraction of small particles at the nanoscale in line with the Guidance on Nano-RA.
The assessment under point 2 above requires the integration of a large number of lines of evidence, thus it is not feasible to address all possible combinations of such lines of evidence in this Guidance on Particle-TR. Some general recommendations and a non-exhaustive set of examples on the combination of different pieces of evidence are provided below, covering some typical cases.

The first consideration is related to the homogeneity of the material. The characterisation under Section 3, may provide evidence that the marketed material (a substance or a mixture) has a small particle fraction that differs from the rest of the material in terms of particle size distribution, particle shape, crystal structure, chemical composition, or a combination of more than one of these features. In these cases, the existing 90-days oral studies should always be complemented with the required provisions (Section 4) for addressing the fraction of small particles; and if needed, with new safety studies addressing the effects of small particles according to the Guidance on Nano-RA are needed.

When size is the only difference between the fraction of small particles and larger particles, the applicant may consider the available information on chemical composition, toxicity and toxicokinetics, as exemplified for some typical cases in Table 3.

When no toxicity or unclear indications are observed in the safety studies, the applicant may consider complementary information in a weight of evidence approach to address some concerns.

### Table 3. Non-exhaustive set of examples of observations and additional information for combining the different pieces of evidence regarding the coverage of the fraction of small particles by the conventional risk assessment

<table>
<thead>
<tr>
<th>Observations from existing toxicity studies</th>
<th>Observations from existing toxicokinetic studies</th>
<th>Additional information</th>
<th>Conclusion from the pieces of evidence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Existing 90 d oral studies cannot be complemented with the required provisions (Section 4) for addressing the fraction of small particles in the nanoscale</td>
<td>The marketed material (a substance or a mixture) has a small particle fraction that differs from the rest of the material in terms of particle size distribution, particle shape, crystal structure, chemical composition, or a combination of more than one of these features.</td>
<td>Safety studies addressing the effects of small particles according to the Guidance on Nano-RA are needed</td>
<td></td>
</tr>
</tbody>
</table>
Local toxicity in the GIT observed | Chemical composition indicates that the soluble/degradation products are of low toxicity | Effects likely associated to small particles, specific assessment needed according to SG

Systemic toxicity with dose-response observed in oral studies | Significant absorption at toxic doses | Chemical composition of the fraction of small particles similar to bulk material | Conventional risk assessment offers sufficient coverage for the fraction of small particles

Some systemic toxicity observed in oral studies but no clear dose-response | Low or negligible absorption in standard toxicokinetic studies | Safety studies addressing the effects of small particles according to the Guidance on Nano-RA are needed

No toxicity observed in oral 90 d studies | Significant absorption in the GIT | Chemical composition of the fraction of small particles similar to bulk material | Conventional risk assessment offers sufficient coverage for the fraction of small particles

No toxicity observed in oral 90 d studies | Negligible absorption but no information on accumulation of particles in toxicokinetic studies | Chemical composition of the fraction of small particles similar to bulk material | Toxicokinetic studies according to SG are needed for confirming the negligible absorption and absence of accumulation of the small particles

No toxicity observed in oral 90 d studies | Low toxicity confirmed following repeated dose parenteral (e.g. intravenous) administration | Conventional risk assessment offers sufficient coverage for the fraction of small particles, except for local effects in the GIT

In some cases, and before conducting a full assessment for the fraction of small particles in line with Guidance on Nano-RA, the applicant may develop an Integrated Testing and Assessment Strategy (IATA) for addressing the identified gaps and integrating the assessment of the fraction of small particles in the conventional assessment. The opportunity for addressing the gaps through New Approach Methodologies (NAM), including *in vitro* or other alternative methods, avoiding or minimising the use of experimental animals, should be
considered. For some processes and sectoral legislation the applicants may consider support from EFSA in line with the EFSA Catalogue of services (EFSA, 2016) or the new provisions for pre-submission advice under the Transparency Regulation.

Where the applicant cannot demonstrate that the conventional safety assessment addresses properly the fraction of nanoparticles, an assessment focusing on the fraction of small particles with nanoscale characteristics should be conducted in line with Guidance on Nano-RA (2018) and submitted within the dossier.

When differences in the toxicokinetic and/or toxicodynamic properties are known between the fraction of particles at the nanoscale and the rest of the material, both whole mixture approaches and component-based approaches can be applied, adapting the general principles for assessing mixture toxicity described in the EFSA Scientific Committee Guidance on harmonised methodologies for human health, animal health and ecological risk assessment of combined exposure to multiple chemicals (EFSA SC, 2019). The component-based approaches can also be used when differences in the toxicokinetic and/or toxicodynamic properties, due to particle size, crystallinity, or other properties, are observed between different fractions of particles at the nanoscale, contained in the marketed material. When the component-based approach is used, the dose addition method should be the default assumption.

5. Recommendations for conducting new studies on materials consisting on or containing a fraction of small particles

When conducting new studies, applicants and other interested parties, including contract laboratories, should check the presence of a fraction of small particles in the material. A solubility test according to the provisions in Section 2.3.1 should be conducted, considering the special provisions for materials with solubility lower than 33.3 g/L. If the criterion for solubility is not met, a dissolution rate study according to the provisions in Section 2.3.2 should be considered.

The presence of a fraction of small particles in the nanoscale should be considered for all concentrations above the material’s own solubility limit. If the material does not meet the decision criteria presented in Sections 2 and 3 of this Guidance on Particle-TR, the recommendations of the Guidance on Nano-RA for the genotoxicity testing, the assessment of toxicokinetic characteristics and the design of in vivo studies should be followed with regard to the characterisation, dispersion and assessment of any potential accumulation of the fraction of small particles.

The characterisation of the material as marketed, including the particle size distribution and the variability between batches, and the characterisation of the sample used in the study should be included in the study report (see Section 3 and Appendix I for details on how to report quantitative EM); the adaptations of the study design for ensuring its applicability for assessing materials containing small particles should be presented in the protocol and the study report. The need for electron microscopy examination of samples with the aim to demonstrate the presence of small particles in tissues should be considered in the study protocol.
References


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Tassinari R, Cubadda F, Moracci G, Aureli F, D’Amato M, Valeri M, De Berardis B, Raggi A,


Abbreviations

ADME absorption, distribution, metabolism and excretion
ANS EFSA Panel on Food Additives and Nutrient Sources Added to Food
CEN European Committee for Standardization
CEN TC European Committee for Standardization Technical Committee
CLS centrifugal liquid sedimentation
dEM descriptive electron microscopy
DLS dynamic light scattering
ECHA European Chemicals Agency
EM electron microscopy
FCM food contact material
FEEDAP EFSA Panel on Additives and Products or Substances used in Animal Feed
GIT gastrointestinal tract
IATA integrated approach to testing and assessment
ICP-MS inductively coupled plasma mass spectrometry
ICP-OES inductively coupled plasma optical emission spectroscopy
ISO International Organization for Standardization
JRC Joint Research Centre
NAM new approach methodology
NDAA EFSA Panel on Nutrition, Novel Foods and Food Allergens
OECD Organisation for Economic Co-operation and Development
pH minus (-) logarithm of the concentration of H+ ions.
PTA particle tracking analysis
REACH Registration, Evaluation, Authorisation and Restriction of Chemicals
SC Scientific Committee
SCCS Scientific Committee on Consumer Safety
SCENIHR Scientific Committee on Emerging and Newly Identified Health Risks
SEM scanning electron microscopy
SG scientific guidance
SOPs standard operating procedures
TEM transmission electron microscopy
TG (OECD) test guideline
**APPENDIX I. Particle size distribution of the constituent particles**

When the solubility and dissolution rate studies confirm that consumers will be exposed to particles, the applicant will need to document the presence or absence of small particles, or of a fraction of small particles, including nanoparticles, in the material and/or in the final regulated food and feed products, and to characterise this fraction. Particle size distribution of the constituent particles of the material has to be provided, including the meta-information necessary to evaluate these data. This information may have legal consequences and should therefore be based on robust and precise data and be supported by applying scientifically sound criteria to the outcomes of the analysis.

The methods and measurement techniques applied to measure particle size distributions can be based on considerably different measurement principles, and the level of detail that they provide and their region of applicability may also differ considerably. These aspects are reviewed in Mech et al., 2020a. Likewise, there is an overwhelming diversity of materials that potentially contain a fraction of small- or nano-sized particles, with a wide spectrum of physicochemical properties. For most materials, methods are available that allow to determine the presence or absence of small particles, including nanoparticles. The suitability of different methods is reviewed in Rauscher et al. 2019a and Mech et al. 2020a,b and examples of standard operating procedures are provided in Mech et al. 2020c.

There are a number of issues that should be considered when assessing the appropriateness of the analysis method and the outcome. When selecting and implementing a method suitable to experimentally assess the presence of small particles the following requirements need to be met:

**Sufficient physicochemical information on the material needs to be available**

Basic physicochemical information on the material to be assessed should be obtained and considered before a measurement of the particle size distribution is performed, including chemical composition, dispersibility, particle shape, expected size range, aggregation and agglomerations status, and other properties that could affect the choice of size measurement techniques. Guidance to describe the physicochemical properties based on descriptive EM analysis are described in 3.3.1.6. Guidance on other methods is provided in Mech et al. 2020c.

**The selected technique is compatible with the material**

The measurement technique(s) to be applied must be compatible with the material. For example, if the measurement or preparation conditions induce changes in the particle size distribution of the material, the method should not be used to assess the particle size. The physicochemical properties of a material should therefore be matched with the capabilities of the techniques and the measurement conditions. Reference to guidances, papers, examples.

**The selected technique should provide confirmatory information allowing conclusive assessment**
Although screening methods may provide a more affordable, cost-efficient indication that the material contains a fraction of small particles, a conclusive assessment cannot be achieved by screening methods only and requires a more elaborate analysis using confirmatory method such as quantitative EM (3.4) (Rauscher et al., 2019a; Mech et al. 2020b).

The applied methods should be validated in line with (ISO). Methods for which (international) documentary standards exist, or those that are internationally recognized are preferred. A non-exhaustive overview of relevant (international) documentary standards is given in Mech et al. (2020b). The applied methods should be validated according to the guidance of ISO 5725-1:1994 and ISO 5725-2:2019. The user should ensure the robustness and reliability (trueness) of the method by applying good measurement practices. This should be provided along with estimate of the uncertainty of the measurement results. Examples of the validation of particle sizing methods are provided in Linsinger et al., 2013, De Temmerman et al., 2013 and Verleysen et al., 2019.

The number of analysed particles should be sufficiently high to obtain precise results. Approaches to determine a suitable particle number are described in (CEN/TS 17273) The outcome of the analysis has to be accompanied by all meta-data necessary for assessing the reliability of the data. The analysis outcome needs to be supported with the information on how the analysis was performed. Precise and detailed information on meta-data are pivotal to ensure the proper assessment of the robustness and reliability of the generated data and is necessary to guarantee reproducibility and comparability of the measurement outcomes. For instance, for the purpose of data comparability, the exact details of all steps involved in a dispersion protocol, the equipment and measurement procedure applied should be reported.

The type of raw data produced by the selected method should allow a true and precise estimation of the constituent particle size distribution. Depending on the measurement principle of a method different types of raw data are produced. When choosing a method, the analyst must be aware of the type of size distribution of raw (primary) data a technique yields, i.e., whether the technique provides a number-, mass- or scattered light intensity-based distribution or any other type of quantity, and whether the technique counts individual particles or measures ensembles of particles instead.

If the technique does not provide a number-based size distribution as raw data, the analyst must be aware of the potential uncertainties and bias that are involved when converting another type of distribution into a number-based size distribution.

The analysed samples have to be representative for the original material. The outcome of the sample analysis should be applicable to the whole material. Nearly all particle measurement techniques involve a degree of subsampling to perform the analysis. It is therefore essential to ensure appropriate subsampling of the material from the bigger batch so the analysed sample can be considered representative. The sampling method, as well as the sample preparation and the analysis methods need to be selected such that the fraction
of small particles is representatively sampled. Specific guidance regarding sampling is provided in ISO 14488:2007.

The applied sample preparation has to be appropriate for the intended method. Sample preparation is an essential step in the process of making measurements of the particle size distribution which must be efficient, reproducible and deliver a particle size distribution which is as close as possible to the true distribution of the constituent particles. Different materials often require different sample preparation conditions that can have an impact on the observed particle size and particle concentration. Therefore, the effectiveness of the employed sample preparation method for the intended method and stability of the dispersion should be verified.

The useful (size) range of the performed analysis method needs to be suitable for the analysed (sub)sample. The determined particle size distribution should be a reliable representation of the true distribution of the size of the constituent particles of the material. This means that the measurements should be performed in a size range in which all involved particles can be analysed. Thus, the upper and lower limits in which given sample should be analysed are determined by the presence of its biggest and the smallest constituent particles. For example, when considering an electron microscopy technique, if the variation of particle size in the analysed sample is very large (i.e. polydispersed materials), then the results of images taken at different magnifications may need to be combined to assure correct determination of the median of the particle size distribution. Guidelines specific for

Bias related due to the selection of a sampling, sample preparation and analysis method should be avoided. Therefore, in certain cases, such as for materials with a high size polydispersity, different sampling, sample preparation and analysis methods are required to characterize (sub)fractions of differently sized particles of a material. Approaches to represent and evaluate the resulting different size distributions are discussed in ISO 9276-2:2014, ISO 9276-3:2008, ISO 9276-4:2001, ISO 9276-6:2008, ISO 9276-5:2005 Table AI. 3 includes a tabulated summary for efficient reporting of the descriptive EM results assuring uniformity in reporting, reducing misunderstandings by suggesting a set of possible descriptions.
Table AI-1. Example of a scheme for systematic reporting of descriptive EM analyses (Adapted from Mast et al., 2020)

1) Description of the material, batch and sample
   a) Provide information on the material (chemical composition, batch, molecular formula, molecular weight, CAS No., stability, purity, and physicochemical properties)
   b) Provide information on the selection of the sample and the preparation of the specimen for the EM analysis
   c) Confirm that a quantitative EM analysis is feasible and provide evidence on the following aspects:
      i) the EM specimen is representative for the sample, and,
      ii) the particles are evenly distributed over the grid, and,
      iii) the particles can be distinguished from the background and matrix.

2) Presence of impurities
   a) Describe the level of purity
      i) the sample is pure, no impurities are found
      ii) occasionally an impurity is observed
      iii) nanoparticles are embedded in a matrix
      iv) between the impurities the NP are visible
      v) micrographs only contain impurities, no nanoparticles are observed
   b) If impurities are observed, include a description of the impurities

3) Describe the findings regarding the level and characteristics of aggregation/agglomeration
   a) State
      i) The particles are individual particles
      ii) The particles are agglomerated/aggregated and contain X to Y particles per agglomerate
   b) Size
      i) Estimated size:
         (1) approximately X nm
         (2) ranging from X nm to Y nm
      ii) The minimal aggregate/agglomerate size is ...
   c) Shape: describe shape according to Figure AI-1A
      i) Spheroidal
      ii) Ellipsoidal
      iii) Linear
      iv) branched/dendritic

4) Describe the characteristics of the primary particles
   a) Type of polydispersity:
      i) Monomodal distribution
ii) Bimodal distribution
1920 iii) Trimodal distribution
1921 iv) Polymodal or broad distribution
1922
1923 b) Estimation of size
1924 i) approximately XX nm
1925 ii) smaller than XX nm
1926 iii) ranging from XX nm to XX nm
1927 iv) XX ± XX nm for N = XX
1928 v) The minimal constituent particle size is ...
1929
1930 c) Crystal structure
1931 i) Particles show diffraction contrast suggesting a crystalline structure
1932 ii) Particles show no diffraction contrast suggesting an amorphous structure
1933
1934 d) Describe 2D shape according to the taxonomic hierarchy of 2D shapes in Figure AI-2
1935
1936 e) Describe 3D shape according to the taxonomic hierarchy of 3D shapes in Figure AI-2
1937
1938 f) Surface topology: describe surface topology according to Figure AI-1B
1939
1940
1941
1942
Figure AI-1: Description of the shape of aggregates/agglomerates (A) according to López-de-Uralde, and of the surface topology of constituent particles (B; sphericity and shape factor) according to Krumbein and Sloss. These figures correspond to Figures 3 and 5, respectively, of the NANoREG Deliverable 2.10 available at http://portal.s2nano.org:8282/files/TEM_protocol_NANoREG.pdf
Figure AI-2: Description of the shape of constituent particles in 2D and 3D according to Muñoz-Mármol et al. 2015. These figures corresponds to Figures S1 and S2 in the Supplementary data of https://doi.org/10.1016/j.nano.2014.07.006 and are protected by copyright, the inclusion is pending on the ongoing authorisation process by the copyright owner.
APPENDIX II. Summary of the information to be provided for each safety study according to the requirements presented under Section 4.

Table AII-1. Best practices for extracting and reporting information from existing safety studies. Similar steps can be used and adapted for presenting information from *in vitro* studies.

<table>
<thead>
<tr>
<th>Elements to be extracted from the study report of <em>in vivo</em> studies</th>
<th>Related info to be reported by applicants</th>
</tr>
</thead>
<tbody>
<tr>
<td>Test material¹</td>
<td>Chemical identity, purity, form used in the study (powder, liquid, dispersion, etc)</td>
</tr>
<tr>
<td>Test material purity, batch, physicochemical properties including solubility</td>
<td>Solubility, dissolution rate² of the marketed material</td>
</tr>
<tr>
<td>Test material</td>
<td>Particle size distribution³ and characterisation of the fraction of small particles in the marketed material</td>
</tr>
<tr>
<td>Test material used in the study</td>
<td>Particle size distribution³ and characterisation of the fraction of small particles for the test material used in existing studies if the sample is still available</td>
</tr>
<tr>
<td></td>
<td>If the material is not available, provide information on the similarity of the test and marketed materials</td>
</tr>
<tr>
<td>Test material preparation</td>
<td>Comparison of the concentrations at different steps of sample preparation with the limits of solubility of the material²</td>
</tr>
<tr>
<td>Details on the test material preparation, indicating if the material was expected to be fully solubilised or dispersed</td>
<td>Assessment of the level of dispersion for preparations above the solubility limits.</td>
</tr>
<tr>
<td>Justification for the choice of any solvents or vehicles used</td>
<td></td>
</tr>
<tr>
<td>Info on methods used for ensuring solubility or proper dispersion, stability and homogeneity of the test material in the media and possible signs of precipitation if noticed</td>
<td></td>
</tr>
<tr>
<td>Preparation of doses</td>
<td>Comparison of the concentrations for each final dose level with the limit of solubility²</td>
</tr>
<tr>
<td>Rationale for dose level selection³</td>
<td>Assessment of the level of dispersion at each dose for doses above solubility limits.</td>
</tr>
<tr>
<td>Details on the preparation of doses indicating if the material was expected to be fully solubilised or dispersed at each dose and dilution</td>
<td>For doses not fully dispersed, consider to correct the doses for the solubilised proportion to take account of the potential bioavailability of the particles</td>
</tr>
<tr>
<td>Justification for the choice of any solvents or vehicles used</td>
<td>If testing material is still available, consider replication of the dose preparation to check the level of</td>
</tr>
<tr>
<td>Info on the methods used for ensuring solubility or proper dispersion, stability and homogeneity of the test material (and of the test material in the solvent if</td>
<td></td>
</tr>
<tr>
<td>Test material administration</td>
<td>dispersion by EM or other suitable method</td>
</tr>
<tr>
<td>-------------------------------</td>
<td>------------------------------------------</td>
</tr>
<tr>
<td>• Rationale for the selection of the administration method (e.g. by gavage, with diet or drinking water) and relevance to the intended uses</td>
<td></td>
</tr>
<tr>
<td>• Details on the administration of the test material</td>
<td></td>
</tr>
</tbody>
</table>

- Assess expected level of solubilisation for each dose considering potential further dissolution in GIT conditions of the experimental animals and results of the dissolution rate study
- Report dose-response relationship for the nominal and corrected dose levels

1. For multi-constituent substances and mixtures, each constituent/component should be assessed independently.
2. Info provided should be in line with Section 2 of the Guidance on Particle-TR.
3. Info provided should be in line with Section 3 of the Guidance on Particle-TR.
4. Note that full dispersion cannot be observed by visual examination or with the aid of an inverted microscope.
5. Note that higher doses may show a higher degree of agglomeration as compared to lower doses.